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Fabrication of microcage arrays to fix plant ovules for long-term live imaging and observation

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ABSTRACT

The long-term observation of plant ovules is important to investigate embryogenesis and the development occurring inside. However, it has been difficult to observe the ovule consistently in a specific position because ovules are randomly dispersed on a substrate with conventional methods. In this work, we fabricated PDMS microcage arrays to fix plant ovules from *Arabidopsis thaliana* and observed them over a long period. Microcages were designed based on experimental data of ovule growth so that ovules could be positioned inside the cage and observed in real time. First, we performed the sieving test to investigate the relationship between ovule size and different microcage widths. Next, we performed ovule culture for a week using microcage arrays developed based on the results of sieving tests. We successfully positioned and fixed ovules inside the microcages and confirmed that the ovules did not move from their original positions even though the culture dish was frequently moved for observation and incubation. Moreover, we observed that the ovules grew inside the cages without being affected due to the flexibility of PDMS. Thus, we confirmed the validity of microcage arrays as the useful tool to fix and culture plant ovules for long-term observation. We expect that microcage arrays could be widely used as the essential device for pursuing scientific findings in further researches of plant embryogenesis and reproduction.

1. Introduction

The development and differentiation of higher plants begins with embryogenesis, which includes the formation and development of a fertilized egg [1,2]. The zygote (a fertilized egg) undergoes complex morphological and cellular changes that result in its development into a mature embryo during embryogenesis [3]. Subsequently, the events during embryonic development establish the organization of the plant and lead to germination of the embryo.

Ovules, which develop into seeds after fertilization, are the key organs for plant reproduction [4]. They play an important role as the site for reproductive processes such as megagametophyte formation, fertilization, embryogenesis, and the formation of seeds [5]. Thus, numerous studies on the ovules of higher plants have been performed to understand the embryogenesis, including molecular genetic analyses from the beginning of 20th century [6–8]. *Arabidopsis thaliana* is a model plant and has been used widely for genomic researches because it has several advantages such as a short generation time, small size, relatively small genome, and large numbers of offspring [9,10]. These advantages have also promoted researches on embryogenesis as well as genomic analysis.

Most researchers have observed the ovules in an ovary through dissection, fixation, and whole-mount analysis [11]. However, it is impossible to observe ovules over time by these conventional methods because the ovules have to be chemically fixed. As it has become necessary to analyze samples over time in a controlled environment to elucidate embryogenesis, live imaging of embryogenesis has been researched using isolated embryos [12,13]. However, such embryo culture system allows only short-term live imaging during late embryogenesis [13]. Since Sauer and Friml developed a system for *in vitro* culture of the embryo within the ovule of *A. thaliana*, the use of isolated ovules has enabled long-term live imaging of embryogenesis [14].

Currently, the embryogenesis of *A. thaliana* is observed by the conventional process that includes extracting ovules from an ovary, dispersing them on a culture dish, and observing them with an inverted microscope. Recently, the use of two-photon excitation microscope has enabled the observation of double fertilization as well as the details of embryogenesis [13,15]. However, the

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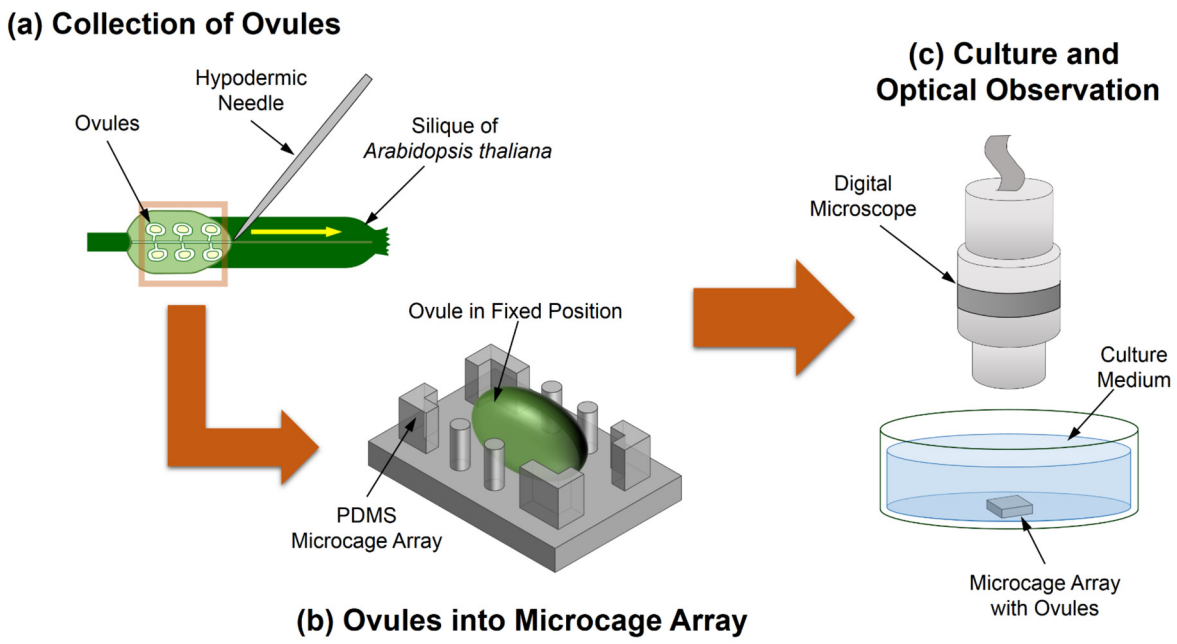

(a) Ovules were extracted from siliques of *Arabidopsis thaliana*. (b) Collected ovules were introduced into PDMS microcage arrays. (c) Long-term culture of microcaged ovules was performed. Also, the ovule growth was observed using a digital microscope periodically.

Fig. 1. Schematic diagram of the experimental process in this research.

observation of ovules is mostly limited to a specific direction because they have asymmetric structures, forming an elliptical shape with two different axes. As a result, most observations have been performed only with ovules that are positioned flat on a substrate, with the minor axis of the ovule parallel to the horizontal plane. Thus, it is necessary to adjust the ovules' orientations so that the minor axis is perpendicular to the horizontal plane to acquire more information on embryo development.

In addition, it is important to position ovules in a specific location without relocation for long-term observations. However, it is difficult to place ovules in a fixed position with conventional methods because they are dispersed randomly when first introduced to a substrate. Thus, it has become necessary to develop the new method to position and fix ovules in a specific location for the realization of long-term live imaging.

Microfabrication has been researched and developed in various fields since its inception. Along with the development of microfabrication, MEMS technology, which originated from semiconductor fabrication technologies, is now widely used to fabricate microchips for biological applications and MicroTAS (Micro Total Analysis System). Among microfabrication techniques, soft lithography has gained much attention and been widely used for various applications because it uses a flexible polydimethylsiloxane (PDMS) elastomer fabricated from micromolds and allows rapid prototyping and low-cost fabrication [16–18]. Furthermore, the biocompatibility of PDMS-based microdevices has contributed to their rapid development for biomedical or biological applications, for example, in microfluidic systems for biological assays [19,20], DNA [21], RNA [22] and cell-related research [23], electrophoresis [24,25], and plant reproduction research [26]. Several reports using chamber-type PDMS devices have been published for research requiring the confinement of animal cells or biomaterials such as protein and DNA. These have mainly focused only on animal cell-related experiments such as cell trapping [27], culture [28], and biochemical assays [29,30]. However, microchamber devices specialized for the positioning or fixing of plant ovules and related research have not yet been developed.

In this work, we developed PDMS microcage arrays to fix the position and direction of ovules to enable long-term observation in real time (Fig. 1). The arrays were designed so that an individual ovule could be confined, cultured, and observed in an individual cage. Such microcage arrays help researchers to find and designate a specific ovule of interest during observation. We used conventional photolithography and PDMS casting to fabricate the microcage array. Ovules of *A. thaliana* were then collected and introduced into the device with culture medium. First, we investigated the appropriate width of microcages by sieving tests with four different widths. Next, we cultured ovules inside the devices and performed optical observations over a long period to confirm the validity of the fabricated devices. Finally, we discuss the feasibility and prospects of the fabricated devices for plant biology researches.

2. Materials and methods

2.1. Plant material

A. thaliana (ecotype Col-0) seeds were grown on plates in a growth chamber (MLR-351, Panasonic Co., Japan) and then transferred to soil in a growth room at 22 °C under continuous light as described in the previous literature [12].

2.2. Fabrication of PDMS microcage array devices

We first fabricated an SU-8 mold for a PDMS cage-type micro device. A 3-inch silicon wafer was cleaned by a conventional wafer cleaning method. SU-8 photoresist (SU-8 3050, MicroChem Co., USA) was then spincoated at 1000 rpm for 30 s. The SU-8 spincoating and pre-baking processes were performed in two cycles in total to achieve a high aspect ratio SU-8 layer over 300 μm. We designed microcage arrays with four different widths, 150, 200, 250, and