Development of a film-assisted honeybee egg collection system (FECS)

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Abstract – Despite the huge potential of genome editing in honeybee research and breeding programs, only a few successful studies have been reported, implying the presence of several obstacles. One such obstacle is the difficulty in obtaining a large quantity of young eggs. To facilitate the overall procedures for egg collection and microinjection, we developed the film-assisted honeybee egg collection system (FECS), in which a removable transparent film is used as the platform for consecutive collection and injection of embryos. The FECS provides a significantly higher (~2-fold) collection efficiency and usability compared with the conventional plug-based queen rearing system while maintaining a high hatching rate. The system can be readily utilized for various studies that require a large number of honeybee eggs, including honeybee genome editing.

honeybee / *Apis mellifera* / embryo / egg collection / genome editing

1. INTRODUCTION

The western honeybee, *Apis mellifera*, plays a pivotal role in the pollination of many crop plants, thereby being one of the key elements of modern agriculture, and produces honey and other useful hive products (Klein et al. 2007). Despite economic importance in agriculture and value as a unique model with unusual biological characteristics, research on honeybee biology has been limited by lack of several classical genetic tools (Robinson et al. 2000). Although transgenesis and genome editing, in particular, have huge potential to be utilized in both honeybee studies and breed-

ing programs, they were not available to honeybee researchers until the first report of a transgenic honeybee by Schulte et al. in 2014 (Schulte et al. 2014).

Despite various attempts for germline transformation in honeybees (Robinson et al. 2000; Pen 2018), microinjection into young embryos is the only successful method for delivery of manipulated genetic materials, leaving egg collection as the first prerequisite (Schulte et al. 2014; Kohno et al. 2016; Kohno and Kubo 2018). To date, various methods have been proposed for honeybee egg collection either in small (Taber 1961; Milne Jr et al. 1988; Collins 2002) or large (Omholt et al. 1995; Evans et al. 2010) quantities. Among these, plug-based queen rearing kits have been commonly employed for successful transgenesis and genome editing studies (Schulte et al. 2014; Kohno et al. 2016; Kohno and Kubo 2018) as they enable collection of intact eggs with minimal damage. It appears that collection of intact

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eggs is crucial because embryos in early stages are particularly fragile (Collins 2002).

In the plug-based system, plugs are inserted one by one into the back of a plastic honeycomb, and queens are allowed to lay eggs onto the plugs. Harvesting the plugs without any direct contact with eggs ensures collection of high-quality eggs. In this method, however, egg collection efficiency is limited because only 1/3 of honeycomb cells can be plugged. Also, repeated removal and re-confinement of the queen and worker bees are required whenever a plug is installed or retrieved. There is increased probability of accidental physical damage to eggs during such complex procedures. Furthermore, the concave structure of a plug bottom hinders approach of an injection needle to the posterior end of embryos, thereby impeding efficient injection.

To overcome the drawbacks of the plug-based collection system, we developed a new system optimized for typical honeybee research protocols. Here, we report the film-assisted honeybee egg collection system (FECS) that provides simplified procedures and significantly enhanced egg collection efficiency. Acquisition of a large number of high-quality honeybee eggs of any embryonic stage via FECS will greatly facilitate transgenesis or genome editing of honeybees.

2. MATERIAL AND METHODS

2.1. Honeybees

Bees were maintained at an apiary located on the rooftop of an eight-story building in Seoul National University, South Korea. Queens were Italian hybrids with some potential Carniolan backgrounds. Two 5-month-old sibling queens were used for comparison testing. The numbers of bees in each hive were adjusted to a similar level, both being maintained in two-story hives, each with one super, 10 frames, and roughly 20,000 workers. Both colonies were fed sufficiently with pollen patties throughout the experiment, although sugar syrup was not provided.

2.2. Design of FECS components, 3D printing, and post-processing

Prototypes and final versions of FECS components (queen excluder and comb box) were designed using the Fusion 360 program (Autodesk Inc., San Rafael, CA, USA). For high-resolution printing of the comb box, a SLA (stereolithography apparatus) type 3D printer (Form2; Formlabs, Somerville, MA, USA) was used. Supports were generated using the Preform software (Formlabs), and a support-added comb box model was printed with a z-axis resolution of 50 μm . Printed boxes were soaked in 90% isopropyl alcohol (IPA) for 15 min, and supports were removed using cutting pliers. The printed comb boxes were cured by UV radiation (395 nm) to the back and front sides of boxes for 10 min each. The backside of the cured comb boxes was coated with silicone rubber using aquarium sealant (Marineland® 31003; Spectrum Brands Pet, Blacksburg, VA, USA). The front surface of each comb box was rubbed with beeswax in advance to promote comb building.

Queen excluders were printed with a FDM (fused deposition modeling)-type 3D printer (DP200; Sindoh, Seoul, Korea), which has better accessibility with less printing cost compared with the SLA-type 3D printer. Supports were generated using 3D Wox software (Sindoh), and prints were directly used after removing supports and adding a film door (Fig. S1b).

Transparent polyester film, conventionally used with overhead projectors (OHP), was purchased from a local stationary store and used as the platform onto which queens were guided to lay eggs.

2.3. Use of plug-based system

Description of the plug-based system is based on the Royal queen rearing comb box, which is a part of the Royal queen rearing kit (Yasaeng Inc., Gwangju, Korea). The kit was fixed to a Langstroth deep frame as shown in Fig. S2b. According to the manufacturer's instructions, the frame was installed in advance to let worker bees build wax comb on the plastic foundation.

2.4. Comparison of collection efficiency and hatching rate

For egg collection by FECS, queens were placed into the FECS unit by relocating them directly from the brood frame. Queens were encouraged to move toward the comb box by themselves instead of transferring by hand to reduce stress. To minimize any putative effects of residual queen pheromones on the behavior of worker bees, a designated FECS or Royal queen rearing kit was provided to each queen. Queens were confined to the comb box for 120–130 min, and the number of harvested eggs per 120 min was calculated. Films were collected at the apiary and brought into the laboratory using a plastic chamber filled with 30°C water saturated with K_2SO_4 to maintain ~95% relative humidity during transport (Schmehl et al. 2016). The number of eggs on the film was counted, and films were stored at 34 °C and 95% humidity up to 80 h.

For egg collection using the plug-based system, queens were confined to the Royal queen rearing comb box for 24 h before the experiment to pre-fill the plugless cells with eggs. Then, the queen bee was transferred to the queen cage, the plugs were replaced, and the experiment was initiated by placing the queen and retinue bees back into the comb box. To harvest eggs from royal queen rearing comb box, bees were removed by gentle shaking and brushing. Frames with a comb box were brought into the laboratory, and plugs were removed at room temperature (24 °C). Plugs with egg were inserted into a plastic pipette tip box (Fig. S3) within 15 min of removal from the frame and stored at 34 °C and 95% humidity for 80 h, when the number of hatched larvae was counted.

The frame housing the FECS (Fig. S2a) or Royal queen rearing kit (Fig. S2b) was inserted in between the first and third frames of supers where eggs were laid by the queen bee. All egg collection experiments, either using the FECS or Royal queen rearing kits, were conducted in parallel during the same time of year (late September).

Four repeats (two repeats from each queen) of collection were performed for both Royal queen rearing kit and FECS. Unpaired *t* tests were performed to calculate significance in mean difference between two groups.

3. RESULTS

3.1. Specification of FECS components

The FECS unit was composed of three major components: plastic comb box, queen excluder, and film (Figure 1a). The standard comb box consisted of 256 cells (16×16), and the diameters of inscribed and circumscribed circles of each hexagonal cell are 4.85 mm and 5.37 mm, respectively (Fig. S1a, b). The cell wall was 0.67-mm thick, which allowed reliable 3D printing with a SLA 3D printer. The wall height from the backside was 5.18 mm (Fig. S1c). The opening of the queen excluder was 4.1 mm, and the gap between comb box and the queen excluder was 3.66 mm (Fig. S1d). The space between the queen excluder and the comb surface was 13.44 mm (Fig. S1d). The backside of the comb box was designed to have a large surface area for tighter film attachment. The circular opening on the backside had a 3.93-mm diameter (Fig. S1b). The queens laid eggs normally into the fabricated cell units without any extra comb built by worker bees.

3.2. Feature of FECS

The major feature of the FECS distinct from conventional egg collection systems was that the removable OHP film was used as the platform for egg laying (Figure 1a and c) and could be subsequently detached for egg collection and consecutive in situ injection. The film was attached to the backside of the comb box. The flexible nature of the film enabled the easy attachment/detachment processes (online resource 1, 2) and tight contact with the backside of the comb box coated with silicone (Figure 1b). The simple detachment process allowed mass collection of eggs at any embryonic stage. The transparent nature of film enabled easier observation of oviposition without removing the queen excluder and brushing out the bees. Unlike the cumbersome procedures of plug collection and reinstallation, which necessitate removing the queen and workers from the comb box, as well as transferring the comb box from the apiary, the film could be readily harvested and reinstalled in the apiary within 1 min, without repeated removal and confinement of the

Figure 1. Overview of the film-assisted honeybee egg collection system (FECS). **a** Transparent film is attached to the back side of the comb box. **b** A silicone coating offers tight and easy attachment of the film. **c** Eggs are deposited on the film surface in the original alignment established by the queen.

queen (Figure 2). As eggs were attached on the flat film surface with the original alignment maintained, they were ready for consecutive microinjection without additional alignment or disturbance by the surrounding structure (Figure 3a, b).

3.3. Comparison of collection efficiency and hatching rate

To investigate the overall performance of FECS and the Royal queen rearing comb box (plug type), we compared the collection efficiency and hatching rate between these two egg collection systems. Within 2 h after confinement of the queen for egg laying, 62.3 eggs on average were collected by FECS, which is more than twice the number (30.8 eggs) collected by the Royal queen rearing comb box (unpaired *t* test, $p = 0.019$, Figure 3c), demonstrating the significantly enhanced egg collection efficiency of FECS. Although no significant differences were observed in hatching rate, eggs collected by FECS showed a slightly higher hatching rate (95.5%) than those of the Royal queen rearing comb box (93.2%) (unpaired *t* test, $p = 0.39$, Figure 3d).

4. DISCUSSION

We developed a novel honeybee egg collection system that is optimized for microinjection of embryos, which should facilitate downstream processes in transgenesis or genome editing. The use of transparent film and silicone coating enhanced collection efficiency significantly and simplified the overall collection protocol. Before completing the current version of the FECS, various types of egg-collecting materials, including integrated plugs, beeswax, parafilm, and parchment paper, were tested. Among those, only the OHP film satisfied all criteria of removability, flexibility, and durability. The silicone coating was necessary to ensure the performance of FECS, as long-term use of a printed comb box usually resulted in slight distortion that eventually formed a gap between the box and film. Queens were hesitant to lay eggs in such comb boxes until workers filled the gaps with beeswax. The silicone coating alleviated this distortion problem by ensuring tight adhesion of the film to the backside of the comb box.

In our collection efficiency comparison, the plug-type comb box showed a significantly lower collection efficiency than FECS even though the plugless cells were pre-filled with eggs before the experiment. This finding suggests that if empty

Figure 2. Workflow of egg collection and microinjection using the FECS. Eggs can be harvested continuously without repeated queen confinement. Collected eggs can be readily applied to experiments, such as microinjection.

cells are scattered around the honeycomb, the queen wastes time finding them.

The full egg collection capacity of FECS estimated during the spring season (120–180 eggs/2 h), which was almost identical to the intrinsic egg

laying capacity of a queen bee, is much larger than the currently reported egg collection efficiency (i.e., 62 eggs/2 h) that was estimated during the fall season. Therefore, the collection efficiency of FECS is likely more dependent upon the egg laying

Figure 3. Comparison of the FECS and Royal queen rearing kit (RQ). **a** Microinjection to the posterior end is not hindered during injection of the embryos collected on a film. **b** Microinjection to the posterior end is hindered by the concave structure of the plug. **c** FECS showed significantly higher egg yield (62.3 eggs/2 h) than the Royal queen rearing kit (30.8 eggs/2 h). The asterisk highlights statistical significance according to unpaired *t* test ($p < 0.05$). **d** A high hatching rate was recorded with FECS (95.5%) as in the Royal queen rearing kit (93.2%).

capacity of a queen bee, which changes due to various factors, including seasonal temperature fluctuation, worker bee number, and available food resource (Dunham 1930; Fine et al. 2018).

By employing FECS in our genome editing experiment (unpublished data), a total of 1900 eggs were routinely collected in 20 days by a single person (J. Lee) while carrying out other laboratory works (e.g., beekeeping, microinjection, and grafting). For any researcher with average experience in beekeeping and microinjection, the number of eggs collected by FECS would be enough for any subsequent procedures and at least would not be a limiting factor in successful honeybee genome editing. Recently, Roth et al. (2019) have shown one-step honeybee genome editing protocol which allows phenotype observation without subsequent screening and mating (Roth et al. 2019). However, there are many phenotypes that can only be measured from substantial numbers of mutants such as social behaviors and resistance to stress factors. The high egg collection efficiency of FECS would enable to obtain sufficient numbers of genome-edited honeybee mutants in a more convenient way.

Aside from use in honeybee genome editing, the FECS can be applied to other studies as well. For instance, when applied to toxicity assessment, it can be used for real-time evaluation of negative impacts of pesticides on embryonic development and their ovicidal activity. The FECS can be also employed in honeybee developmental biology. In honeybees, the location and timing of germ cell development are still unclear, as are the exact mitotic cycles of embryos (Dearden 2006; Pires et al. 2016; Cridge et al. 2017). As the transparent nature of the film enables real-time observation of egg laying from the backside of the comb box, researchers can easily record or mark eggs, thereby allowing collection of a large quantity of eggs with known ages. The queen-monitoring cage (QMC) recently reported by Fine et al. (2018) showed that 3D printed artificial comb can be used to monitor egg laying activity of the queen bee and they used the device to evaluate the effect of pollen nutrition on egg laying (Fine et al. 2018). Although both FECS and QMC share several common features, QMC is more specialized for monitoring of egg laying under controlled

laboratory system whereas FECS is more specialized for mass egg collection from field colonies. Complementary use based on each system's features would improve the performances of these devices in egg collection and monitoring and further serve as basic platforms to invent new tools for modified experimental purposes.

AUTHORS' CONTRIBUTIONS

Both J Lee and SH Lee designed the experiments and wrote the paper. J Lee conducted all experiments.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests The authors declare that they have no conflict of interest.

Développement d'un système de collecte basé sur un film pour les œufs d'abeilles (FECS)

abeille mellifère / *Apis mellifera* / embryon / collecte d'œufs / édition du génome

Entwicklung eines Folien-basierten Sammel-systems für Honigbieneneier (FECS)

Honigbiene / *Apis mellifera* / Embryo / Sammeln von Eiern / Genomeditierung

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