Chapter 15

Using Giant Scarlet Runner Bean (*Phaseolus coccineus*) Embryos to Dissect the Early Events in Plant Embryogenesis

Min Chen, Anhthu Q. Bui, and Robert B. Goldberg

Abstract

The giant embryo of the scarlet runner bean (*Phaseolus coccineus*) has been used historically to investigate the molecular and developmental processes that control the early events of plant embryo development. In more recent years, our laboratory has been using scarlet runner bean embryos to uncover the genes and regulatory events that control embryo proper and suspensor region differentiation shortly after fertilization. In this chapter we describe methods that we have developed to isolate scarlet runner bean embryos at the globular stage of development, and capture embryo proper and suspensor regions by either hand dissection or laser capture microdissection (LCM) for use in downstream genomic analysis. These methods are also applicable for use in investigating the early events of common bean (*Phaseolus vulgaris*) embryo development, a close relative of scarlet runner bean, which also has a giant embryo in addition to a sequenced genome.

Key words Scarlet runner bean, Pollination, Seed development, Embryo, Globular stage, Embryo proper, Suspensor, Embryo isolation, Microscopy, Laser capture microdissection

1 Introduction

One of the major challenges in plant biology is to uncover the mechanisms that program embryo differentiation into specialized regions, tissues, and cell types shortly after fertilization. This is a difficult task because the earliest events in embryo development occur deep within the flower and are difficult to access and investigate using molecular and genomic techniques. In recent years, the development of novel methods such as laser capture microdissection (LCM) [1] and isolation of nuclei tagged in specific cell types (INTACT) [2] have overcome some of this difficulty by making it possible to capture either cells or nuclei from postfertilization embryo proper and suspensor regions in order to carry out high-throughput transcriptome analysis. Each of these methods, however, has their strengths and limitations. For example, LCM requires expensive, highly specialized microscopy equipment, and

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a significant amount of time and effort is required to capture adequate amounts of embryonic regions for genomic studies—especially from tiny *Arabidopsis* embryos. In addition, methods for isolating chromatin from paraffin-embedded tissues on slides are just beginning to be developed [3]. On the other hand, LCM can be applied universally to all plants regardless of whether the plant can be transformed or not. INTACT, by contrast, requires generating transgenic plants with chimeric cell-specific genes but permits the capture of nuclei from a variety of embryo regions and cell types for genomic studies—as long as cell-and region-specific promoters are available. Thus, it cannot be used with plants that do not have established transformation protocols. Nevertheless, applying both LCM and INTACT techniques to the investigation of postfertilization plant embryos has uncovered novel insights into the genes and processes controlling early plant embryo development [2, 4–6].

The scarlet runner bean (*Phaseolus coccineus*) (Fig. 1) provides a novel opportunity for dissecting the molecular processes controlling plant embryo development. At the globular stage, the scarlet runner bean embryo is ~100 times larger than that of *Arabidopsis*, contains a suspensor with 200 cells that is highly polyploid, and can be isolated directly from developing seeds within the flower [7–11]. Because of its large embryonic size, both embryo proper and suspensor regions can be separated from each other manually and used directly for biochemical and molecular studies [11]. Almost 50 years ago, the late Ian Sussex and his collaborators pioneered the use of giant scarlet runner bean embryos and provided the first insights into the molecular processes controlling early embryogenesis; for example, the suspensor produces signals that are required for embryo proper development [7, 8, 12–15]. During this same period, others demonstrated that hormones, such as gibberellic acid, are synthesized within the giant scarlet runner bean suspensor and contribute to embryo proper formation [16]. The ability to manually isolate large numbers of

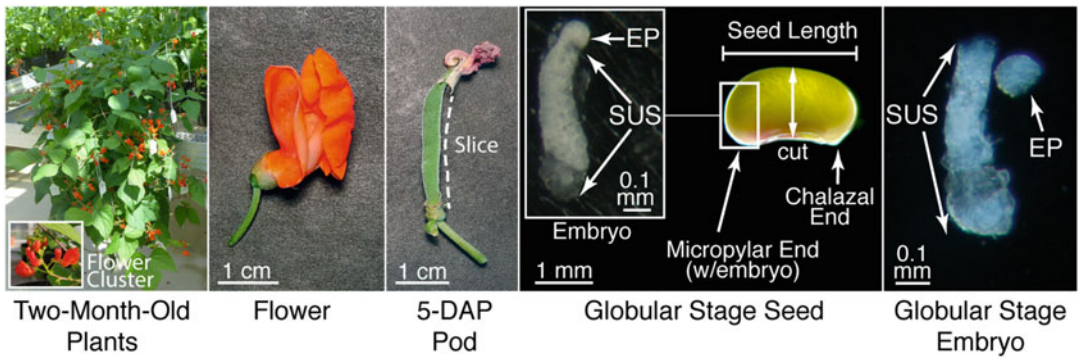

Fig. 1 Scarlet runner bean plant, flower cluster, open flower, 5-DAP (days after pollination) pod, globular stage seed, and embryo

globular stage embryo proper and suspensor regions, in addition to using state-of-the-art LCM techniques, provides a unique opportunity to use the scarlet runner bean to gain entry into the earliest events in plant embryogenesis—complementing the elegant studies that can be carried out with *Arabidopsis* [2, 4–6] and maize [17].

Our laboratory has resurrected the use of scarlet runner bean for the study of early plant embryo development. We have sequenced thousands of expressed sequence tags (ESTs) from embryo proper and suspensor regions (GenBank Accessions CA896559-916678; GD289845-GD660862) [11], used in situ hybridization [11, 18, 19] and RNA-Seq (GenBank Accession GSE57537) to identify embryo proper- and suspensor-specific mRNAs, uncovered a suspensor cis-regulatory module that activates region-specific transcription of genes within the suspensor shortly after fertilization [19–21], and generated a rough draft of the scarlet runner bean genome (GenBank Accession QBDZ00000000). In this chapter, we outline the methods that we have developed to use giant scarlet runner bean embryos to identify processes controlling the differentiation and functions of the embryo proper and suspensor regions at the globular stage of development. We specifically focus on methods used to grow scarlet runner bean plants, harvest embryos, and manually dissect embryo proper and suspensor regions for direct use in genomic studies, as well as techniques required to fix and embed young seed tissues for LCM and in situ hybridization experiments.

2 Materials

2.1 Planting Scarlet Runner Bean Seeds

1. Scarlet runner bean cultivar "Hammond's Dwarf Red Flower" (Vermont Bean Seed Company).
2. Soil mix (equal parts of ground Canadian sphagnum, horticultural grade sand, white pumice, and redwood compost).
3. Water-soluble 20-20-20 Nitrogen-Phosphorus-Potassium fertilizer.
4. 1-gallon pot.

2.2 Hand-Pollinating Scarlet Runner Bean Flowers

1. Fine-tipped watercolor brush.
2. White merchandise tag with white strings (7/8 in. × 1¼ in.).
3. Permanent marker.

2.3 Collecting Globular Stage Scarlet Runner Bean Seeds

1. Dissecting microscope.
2. Aluminum foil.
3. Ice bucket.
4. Ruler.

5. Plain glass microscope slide.
6. Disposable scalpel with No. 11 blade.

2.4 Dissecting Whole-Mount Globular Stage Scarlet Runner Bean Embryos

1. Dissecting microscope.
2. Concavity slide with depressed well (0.6–0.8 mm deep).
3. Concavity slide with depressed well (1.5 mm deep).
4. Plain glass microscope slide.
5. Disposable scalpel with No. 11 blade.
6. 1 ml syringe with BD PrecisionGlide needle (No. 30.5 gauge).
7. 1.5 ml microcentrifuge tube (Nuclease-free).
8. Tie wire.
9. Dewar flask.
10. Permanent marker.
11. Liquid nitrogen.
12. Micropipette (1 ml).
13. 1 ml pipette tips (Nuclease-free).
14. Fine-tipped forceps.
15. Nuclease-free water.

2.5 Fixing Globular Stage Scarlet Runner Bean Seeds for LCM or in situ Hybridization

1. Ethanol/acetic acid fixative solution for LCM [(ethanol/acetic acid, 3:1 (v/v)]: 15 ml 100% ethanol; 5 ml acetic acid. Prepare freshly and keep on ice.
2. 1% glutaraldehyde fixative solution for in situ hybridization (1% glutaraldehyde, 0.1 M sodium phosphate buffer, 0.1% Triton X-100): 0.8 ml 25% glutaraldehyde solution; 0.2 ml 100% Triton X-100; 10 ml 0.2 M sodium phosphate buffer, pH 7.0; add nuclease-free water to 20 ml. Prepare freshly and keep on ice.
3. Scintillation glass vial.
4. Dissecting microscope.
5. Plain glass microscope slide.
6. Disposable scalpel with No. 11 blade.
7. Forceps.
8. Vacuum oven.
9. Rotator.

2.6 Dehydration for LCM

1. A series of ethanol concentrations in nuclease-free water for dehydration: 75%, 85%, 95%, and 100%.
2. Rotator.

2.7 Dehydration for in situ Hybridization

1. 0.1 M sodium phosphate buffer, pH 7.0.
2. A series of ethanol concentrations in nuclease-free water for dehydration: 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%.
3. Rotator.

2.8 Infiltration and Embedding for LCM or in situ Hybridization

1. Xylene.
2. 100% ethanol.
3. Rotator.
4. Paraplast X-tra Tissue Embedding Medium (VWR, cat. no. 15159-486).
5. 1-l beaker.
6. Incubator (42 °C and 58 °C).
7. Disposable aluminum crinkle dishes with tabs (e.g., VWR, cat. no. 25433-008).
8. Hot plate with divided hot/cold sections.
9. Spatula.
10. Alcohol lamp.

2.9 Sectioning for LCM or in situ Hybridization

1. Microtome.
2. Standard razor blade.
3. Plastic block holder.
4. Aluminum foil.
5. Slide warmer.
6. Fine-tipped watercolor brushes.
7. Nuclease-free water.
8. Micropipettes (1 ml and 100 μl).
9. 1 ml and 100 μl filtered pipette tips (Nuclease-free).
10. Ice bucket.
11. Polyethylene naphthalate (PEN) membrane slides for LCM (Leica, cat. no. 11505158 for DNA work or no. 11505189 for RNA work).
12. Superfrost Plus Slides for in situ hybridization.

2.10 LCM

1. Leica LMD 6000 system or similar LCM microscope system.
2. 0.5 ml microcentrifuge tubes (Nuclease-free).
3. Extraction buffer: Buffer XB from PicoPure RNA isolation kit (Thermo Fisher, cat no. KIT0204) can be used for RNA isolation, and Buffer ATL from QIAamp DNA FFPE Tissue Kit (QIAGEN, cat. no. 56404) can be used for DNA isolation.

3 Methods

3.1 Planting Scarlet Runner Bean Seeds

1. Sterilize the soil mix by autoclaving at 121 °C for 90 min. Let the soil cool before planting seeds.
2. Wash pots with soapy water, rinse, and dry fully.
3. Fill a 1-gallon pot with sterilized soil mix up to 2 in. from the top, and water the soil mix completely.
4. Spread 10 scarlet runner bean seeds evenly on the wet soil mix and lightly press the seeds into the soil.
5. Cover the seeds with a thin layer (~ 1 in.) sterilized soil mix.
6. Place pots with scarlet runner bean seeds in a greenhouse, or growth chamber, with 16 h-light–8 h-dark cycle at 22 °C (*see* **Note 1**).
7. Transfer seedlings to a second 1-gallon pot 10 days after sowing (two seedlings per pot).
8. Water as needed with water containing 250 ppm of water-soluble 20-20-20 Nitrogen-Phosphorus-Potassium fertilizer.

3.2 Hand-Pollination of Scarlet Runner Bean Flowers

1. Take a flower that opened in the morning, such as flowers #3 and #4 (Fig. 2). Push the standard and wing petals (Fig. 2) away from the coiled keel that contains the style, stigma, and anthers to expose dehiscent anthers (*see* **Note 2**).
2. Collect pollen by gently brushing against the anthers using a fine-tipped watercolor brush and make sure the pollen grains, like greyish yellow powder, are visible on the brush tip.

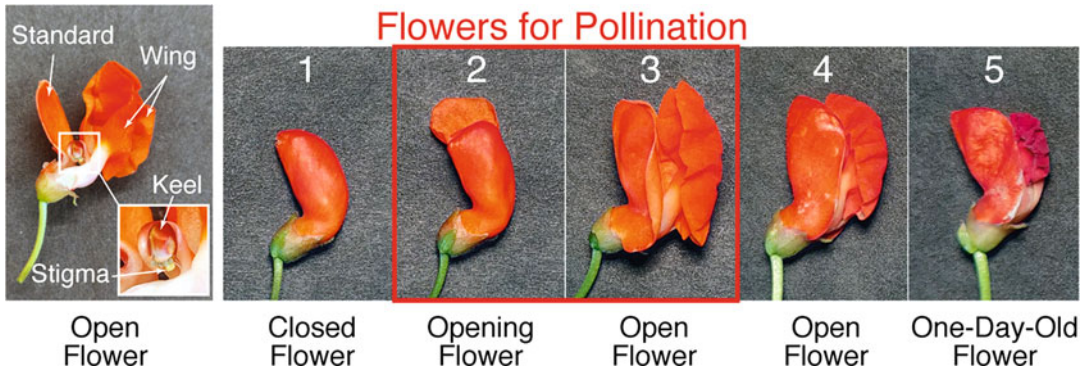

Fig. 2 Scarlet runner bean flowers. All flowers were collected at the same time. In flower #1, the pollen has not been released and the stigma is dull. In flower #2, the anther is dehiscing and the stigma is shiny and sticky. In flower #3, the anther is fully dehisced and stigma is shiny and sticky. In flower #4, the pollen is completely released, but the stigma is senescing and withered. In addition, the wing petal has started to wither. In flower #5, the petal is withered and the pollen is not viable. Flowers #3 and #4 are good for collecting pollen. Flowers #2 and #3 are excellent for pollination

3. Take a second flower that opened in the morning, such as flowers #2 and #3 (Fig. 2). Push the standard and the wing petals away from the coiled keel until the stigma is exposed.
4. Touch the pollen-covered brush to receptive stigma. Pollen grains will be visible sticking to the stigma if the pollen was successfully transferred.
5. Label the pollinated flower with a white merchandise tag attached to a string.
6. Repeat **steps 1–5** and pollinate more flowers (*see* **Note 3**).

3.3 Collecting Scarlet Runner Bean Seeds Containing Embryos at the Globular Stage

1. Collect pods 5–7 days after pollination (DAP) and wrap pods in aluminum foil. Keep collected pods on ice at all times (*see* **Note 4**). The stage of the embryo development within the expanding pods depends upon both the pollination effectiveness and time of the year. Therefore, pod length and days after pollination provide only a rough estimate of embryonic stage. Precise staging should be done just before the real experiment starts by hand-dissecting embryos from seeds within young expanding pods and examining under the microscope to calibrate the timing of embryo development.
2. Under the dissecting microscope, use the blade to slice open the pod, remove the seeds, and place seeds on a plain microscope slide (Fig. 1).
3. Measure the seed length (Fig. 1). The length of scarlet runner bean seeds containing globular stage embryos is ~2.0–2.5 mm [18].
4. If an entire whole-mount embryo, or a specific embryonic region (e.g., embryo proper or suspensor), is needed for the experiment [11], follow steps in Subheading 3.4 immediately to isolate and dissect whole-mount embryos. If LCM [1, 4], or in situ hybridization [18, 19, 22], is planned, immediately follow steps in Subheading 3.5 to fix the seeds and perform the downstream procedures.

3.4 Dissecting Scarlet Runner Bean Globular Stage Whole-Mount Embryos

1. Pour liquid nitrogen into a Dewar flask before opening the pods.
2. Label a 1.5 ml microcentrifuge tube and loop around with the tie wire. Shape the extra piece of tie wire into a hook and hang the 1.5 ml tube on the edge of the Dewar flask. It is important that the 1.5 ml tube is suspended in the liquid nitrogen.
3. Fill the 1.5 ml tube with liquid nitrogen.
4. On a plain microscope slide, using the disposable scalpel with a No. 11 blade, cut the seed in half transversely (Fig. 1). Place the micropylar half of the seed on the plain microscope slide

upright on its cut side, and carefully slice the seed coat from the left and right sides of the standing micropylar-half seed to expose the embryo.

5. Trim the tissues around the embryo as much as possible.
6. Move the trimmed micropylar-half seed onto the concavity slide with the 1.5 mm deep well.
7. Add ~100 μl nuclease-free water into the well.
8. Using the beveled edge of the needle, carefully remove the remaining seed coat and endosperm and isolate the globular stage embryo (*see* **Note 5**).
9. After removing all seed tissues surrounding the dissected embryo, the cleaned embryo is transferred into another clean concavity slide with a small amount of nuclease-free water using a micropipette with a cut off 1 ml tip.
10. If you need to collect different embryo regions, separate the embryo proper from the suspensor using a new needle (Fig. 1) (*see* **Note 6**).
11. Using a fine point forceps, transfer the embryo, embryo proper, or suspensor directly into a microfuge tube suspended within liquid nitrogen. Inspect the forceps under a dissecting microscope to ensure that the embryo, or specific embryo region, was removed successfully.
12. We are able to dissect globular stage embryos at the rate of ~10 per hour. Be patient, as this step takes practice. Store the collected embryos and embryo regions at −70 °C until use in downstream experiments. Each collection should last no more than 3 h to ensure that the DNA and RNA remain intact because the pods will be on ice during this entire period.
13. Based on our experience, an average of 5 ng and 80 ng of total RNA can be isolated from one globular-stage embryo proper and suspensor, respectively (*see* **Note 7**).

3.5 Fixing Seeds with Globular-Stage Embryos for LCM or in situ Hybridization

1. Based on Subheading 2.5, prepare fresh fixative solution in a glass scintillation vial right before collecting pods (*see* **Note 8**) and keep the fixative on ice at all times.
2. Using the disposable scalpel with a No. 11 blade, cut the seed in half transversely on a plain glass slide and transfer the micropylar-half seed into the fixative solution.
3. Once all seeds have been collected, vacuum-infiltrate the seeds for 30 min in a vacuum oven at maximum vacuum (~25 in. Hg) with no heat to obtain complete fixation. The seeds should be immersed in the solution.
4. Transfer the vial containing seeds to 4 °C and store overnight.

3.6 Seed Dehydration for LCM

1. Perform dehydration steps in the glass vial with serial ethanol concentrations in the following order: 75%, 85%, 95%, 100%, 100%, and 100%. At each step, the glass vial should be on the rotator at room temperature for 2–3 h.
2. Seeds can be stored in 100% ethanol at 4 °C overnight (*see* **Note 9**).

3.7 Seed Dehydration for in situ Hybridization

1. Replace the fixative solution with 0.1 M Sodium Phosphate Buffer (pH 7.0), and incubate for 30 min on a rotator at room temperature.
2. Perform dehydration steps in the glass vial with serial ethanol concentrations in the following order: 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 100%, and 100%. At each step, the glass vial should be on the rotator at room temperature for 30 min.
3. Seeds can be stored in 100% ethanol at 4 °C overnight.

3.8 Infiltration and Embedding of Seeds for LCM or in situ Hybridization

1. Prepare liquid Paraplast by filling a 1-l beaker with solid Paraplast chips, and place the Paraplast chip-filled beaker in a 58 °C incubator until all chips have melted (*see* **Note 10**). Keep the liquid Paraplast in the 58 °C incubator.
2. Perform seed clearing steps in a glass vial with serial xylene solutions in ethanol using the following order: 25%, 50%, 75%, 100%, and 100% (*see* **Note 11**). At each step, the glass vial should be on the rotator at room temperature for 2–3 h. At the last step, fill the glass vial only half way with 100% xylene because the solid Paraplast chips will gradually be added directly into the vial in the following steps and the solid Paraplast chips will take up space within the vial.
3. Perform seed infiltration steps by adding 10 solid Paraplast chips to the cleared seeds in 100% xylene within the glass vial and incubate at room temperature for 1 h (*see* **Note 12**).
4. Repeat **step 3** three times, adding 10 more chips each time.
5. Add 10 more solid Paraplast chips to the cleared seeds within the glass vial and incubate at 42 °C for 1 h (*see* **Note 13**).
6. Repeat **step 5** two times, adding 10 more chips each time.
7. Discard the xylene and paraffin mixed solution carefully, making sure to retain the infiltrated seeds within the vial. Add the liquid Paraplast prepared earlier to the glass vial with the cleared seeds, and incubate at 58 °C for 2–3 h.
8. Change the liquid Paraplast solution at 2–3 h intervals until the xylene is removed and xylene odor is gone. Usually six to eight changes are needed to accomplish this prior to embedding the seeds.

9. Turn on the hot plate. Place an aluminum crinkle dish on the hottest part of the plate.
10. Pour the infiltrated seeds within the liquid Paraplast into the aluminum dish.
11. Top off the dish with liquid Paraplast.
12. Heat up one end of the spatula under the alcohol lamp, and use the heated spatula to arrange the seeds within the dish until the seeds are at least 5 mm away from each other (*see* **Note 14**).
13. Carefully move the dish to the coolest part of the hot plate without disturbing the seeds.
14. Label each dish with a marked tag.
15. Let the liquid Paraplast containing fixed seeds within the dish harden completely to form a block. Store the seed-containing paraffin block at 4 °C for least 4 h or until ready to section (*see* **Note 15**).

3.9 Sectioning Paraffin-Embedded Seeds for LCM or in situ Hybridization

1. Turn on slide warmer and set at 42 °C.
2. Cut the paraffin block from the aluminum dish into small blocks containing only one seed.
3. Heat up one end of the spatula under the alcohol lamp. Apply the heated end of the spatula to the bottom of the paraffin block in order to melt the paraffin, and quickly mount the paraffin block onto the plastic block holder. Add extra paraffin to the side of the plastic block holder, if necessary, to secure the paraffin block on the holder. Place the block holder at 4 °C to harden the paraffin for at least 4 h.
4. Before sectioning, place the mounted paraffin blocks on ice.
5. Trim the tissue block such that the face of the tissue block is shaped as a trapezoid (*see* **Note 16**).
6. Insert the plastic holder with the trimmed block into the microtome stage. Orient the parallel sides of the trapezoid parallel to the blade's edge.
7. Bring the stage closer to the blade and determine how to angle the block in relation to the blade. Adjust the block as necessary (*see* **Note 17**).
8. Once the block is at the correct angle, begin cutting at 10 μm until the blade reaches the seed.
9. Set the section thickness (usually 5–10 μm). Maximize the thickness, while still allowing for precise microscopic cell recognition. In our experience, the optimal thickness for scarlet runner bean globular stage embryo longitudinal sections is 6 μm.

10. Slowly begin to turn the wheel of the microtome. When the paraffin sections start to form a ribbon, gently pick up the end of the ribbon with a watercolor brush and lift the ribbon from the blade.
11. When a 10-section ribbon is generated, lock the microtome. Use the watercolor brush to transfer the ribbon from the microtome to aluminum foil covering the top of the lab bench until enough ribbons have accumulated to make a slide (*see* **Note 18**).
12. Place a labeled (e.g., date, person sectioning) slide on the slide warmer. Use PEN membrane slides for LCM and Superfrost Plus slides for in situ hybridization.
13. Put 1.5 ml nuclease-free water on the slide. Gently place the ribbons with the desired length onto the water, and arrange ribbons containing serial seed sections on the slide in order of sectioning with a watercolor brush (*see* **Note 19**).
14. Leave the slide on the slide warmer for 15–30 min until the sections become transparent and flatten out.
15. Drain excess water off the slide and let the slide dry for ~1 h. Save the slide in a slide box and store at room temperature.
16. A day before in situ hybridization or capturing embryo regions using LCM, deparaffinize the slides by dipping slides in 100% xylene for 2 min twice. Let the slides air dry in a fume hood overnight. The deparaffinized slides are stored in a slide box at room temperature until used for LCM (*see* Subheading 3.10) or in situ hybridization (*see* **Note 20**).

3.10 LCM of Scarlet Runner Bean Globular Stage Embryos

1. LCM usually is performed in a 2–3 h session (*see* **Note 21**).
2. We use a Leica LMD 6000 system [4, 23], but other microscopes capable of carrying out LCM can be used (e.g., Pix-Cell II [1]). It is best to read the relevant operation manual before proceeding with LCM.
3. Turn the Leica LMD 6000 power on in the following order: CTR-MIC control box, computer, and laser. During laser warm up, one green light (left) will be displayed. When the second green light (right) turns on, the laser is ready for use.
4. Start the LMD application software when the microscope is fully initialized.
5. Click the "LOAD" slide button from the software menu. The slide holder stage will move forward. Slide the slide holder with the PEN membrane slide containing mounted seed sections onto the slide holder stage until it snaps into place. Make sure the sections on PEN slide are facing down. Return the slide holder stage back by clicking the "Continue" button on the dialog box.

6. Click the "LOAD" collector button from the software menu. The tube holder stage will move forward. Place the tube tray with loaded PCR tubes into tube holder stage. The tube caps should face up. Add 30 μl extraction buffer into the caps of PCR tubes. The captured embryo regions will fall into the caps after cutting. Depending on the type of experiment, extraction buffer XB from the PicoPure RNA isolation kit can be used for RNA isolation, whereas Buffer ATL from the QIAamp DNA FFPE Tissue Kit can be used for DNA isolation. Return the tube holder stage back by clicking the "OK" button on the dialog box.
7. Test different laser settings (power, aperture, and speed) to obtain the optimized cutting condition for each embryo region (e.g., embryo proper and suspensor) and/or seed part (e.g., seed coat) that you are interested in capturing. The ideal condition is one with relatively quick cutting speed using the lowest power possible, which will allow the cut region to fall easily into the PCR cap (*see* **Note 22**).
8. Select "Draw and Cut" mode from the software interface. Use the touch screen pen to outline the specific embryo region of interest for capture directly on the touchscreen. Click the "Start Cut" button to perform the laser cutting. If the embryo or seed region is not completely released after cutting, manually move the laser to cut the attached part by clicking the "Move and Cut" button to release the cut section. Because the extraction buffer in the PCR cap will slowly evaporate due to the heat generated by the microscope lamp, we recommend adding 10 μl nuclease-free water to the cap halfway through the session if the session lasts more than 1 h.
9. When capturing is complete, take out the PCR tube, briefly spin it in a microfuge, then store it at −70 °C until RNA or DNA isolation. RNA can be isolated from the captured seed regions for transcriptome analysis [4] or DNA for methylome analysis [24] (*see* **Note 23**).
10. Close LMD software and turn off power in the following order: laser, CTR MIC control box, and computer.
11. We have used this LCM procedure successfully for both transcriptome [4] and methylome [24] analysis of specific seed regions and subregions.

4 Notes

1. Do not water between sowing and transferring seedlings. The water in the soil mix is enough for germination. The common greenhouse insect pests that we observe are two-spotted spider mites and thrips. Two-spotted spider mites can be treated with

Forbid 4F Ornamental Insecticide/Miticide (1 ml per gallon water). Thrips can be treated with Hachi-Hachi SC Insecticide (7.5 ml per gallon water).

2. Scarlet runner bean plants start flowering 30–40 days after sowing, and produce clusters of typical legume red flowers (Fig. 1). The two lowermost petals combine to form the "keel," the uppermost petal is modified into the hood-like "standard," and side petals spread into the "wings" (Fig. 2). Flowers open at sunrise and close at sunset. Usually, there are two open flowers in a flower cluster (Fig. 1). The cultivar that we use is a dwarf variety, which grows to about 18 in. in height. Because scarlet runner bean is an open pollinator that utilizes bees for pollination under natural field conditions [25, 26], hand-pollination is required in the greenhouse. In our experience, this takes practice and patience in order to be successful and obtain large numbers of developing pods and seeds. Pollinate flowers early in the morning because the heat of the day can affect pollen viability, and the stigma remains receptive to pollen for 1 day only [25]. Peeling, or cutting back the petals of the flowers carefully, makes it easier to identify flower parts at the beginning of hand-pollination. The receptive stigma is shiny and sticky. The flower with the receptive stigma has bright red petals (flowers #2 and #3 in Fig. 2). Flowers that are open for more than 1 day have dark red petals and withered stigmas, and cannot be used for pollen collection or pollination (flower #5 in Fig. 2). Flowers similar to those like flowers #3 and #4 in Fig. 2 are a good source of viable pollen; however, flowers similar to flower #4 in Fig. 2 cannot be used for pollination because their stigmas are withered and senescing. Unopened flowers such as flower #1 in Fig. 2 cannot be used for either collecting pollen or pollination because pollen has not been released from the anthers and their stigmas are dull and unreceptive to pollination. Common bean (*Phaseolus vulgaris*) can be a useful alternative for studying early embryo development because (1) it has a large embryo and giant suspensor morphologically similar to those of scarlet runner bean [15]; (2) it self-pollinates, making hand-pollination unnecessary [27]; and (3) an annotated complete genome sequence is available [28]. We typically allow a subset of pods on both scarlet runner bean and common bean to develop to maturity, collect the dry seeds, and establish our own seed stocks for both bean varieties.
3. Pollen can be collected from the flowers of the same plant or from different plants. Hand-pollination using different plants will encourage a higher pollination rate. In our laboratory, the pollination rate varies from 10% to 80% depending upon the experience of the person pollinating.

4. If RNA is to be isolated from whole-mount embryos, or from LCM captured embryo regions, maintaining an RNase-free environment is critical. Clean work areas and equipment extensively—including aluminum foil, gloves, dissecting microscope, scalpels, forceps, ice bucket, pipettes, microtome, slide warmer, brushes, razor blades, plastic blocks, spatulas, and aluminum dishes, among others—with freshly made 0.1% DEPC water or commercially available cleaning reagents, such as RNase Zap.
5. Because a globular stage embryo is embedded within the seed coat, it is a little tricky to remove surrounding seed coat tissues. We do this by stirring the water to move the embryo around so that tissue debris attached to the embryo falls off during the water movement.
6. To avoid cross-contamination of embryo proper and suspensor regions, use different forceps to transfer each embryo region into separate tubes containing liquid nitrogen.
7. Isolated RNA can be used for classical EST sequencing [10, 11] or transcriptome analysis [4]. Because scarlet runner bean suspensor cells are highly polyploid [7], a significant amount of genomic DNA should be able to be isolated for use in chromatin immunoprecipitation (ChIP) sequencing and chromatin studies.
8. Depending on your sample, the fixation time should be adjusted. In our experience, the fixation time for scarlet runner bean seeds is ~12–16 h. Therefore, always collect pods in the afternoon to avoid excessive fixation during the night prior to processing the sample through a series of increasing alcohol concentrations for sample dehydration.
9. Fixed seeds can be stored in 100% ethanol for several months, and both RNA and DNA remain intact.
10. This step will take at least 5 h. The liquid Paraplast will be used in filtration and embedding and should be ready before infiltration and embedding. Therefore, this step needs to be done ahead of time.
11. Xylene is toxic. Handle with care in the fume hood.
12. Wax infiltration must be done gradually in order to preserve seed internal structures. Thus, solid Paraplast chips are dissolved in the xylene-containing vial with the seeds, and more chips are added and dissolved in xylene sequentially. This allows the concentration of Paraplast in xylene to gradually increase within the seed-containing vial.
13. At room temperature, solid Paraplast cannot be dissolved in xylene when the concentration of Paraplast within the xylene reaches a critical point. In order to complete the infiltration steps it is necessary to increase the temperature.

14. Think in advance how you want to position seeds in the Paraplast so that you can cut the Paraplast into small blocks containing one seed. Usually, approximately 10 globular stage seeds can be placed in one aluminum dish. Seeds should lay flat on the bottom of the aluminum dish so that the seed can be sectioned longitudinally on the microtome.
15. The total tissue preparation time for LCM or in situ hybridization up to this step takes at least a week. We have also used the rapid microwave paraffin method for LCM experiments, which cut the total preparation time to 5 h and reduced RNA degradation [29].
16. The face of the tissue block should be as small as possible so that as many sections as possible can be placed onto one slide. The edges of the trapezoid should be as parallel as possible; otherwise the ribbon will be curved and will take up too much space on the slide. Do not trim too close to the seed, as that will prevent the sections from forming a ribbon.
17. In a longitudinal seed section, the serial sections that contain embryo proper and suspensor within the same section are called "middle" or "medial" sections (Fig. 3). These are the

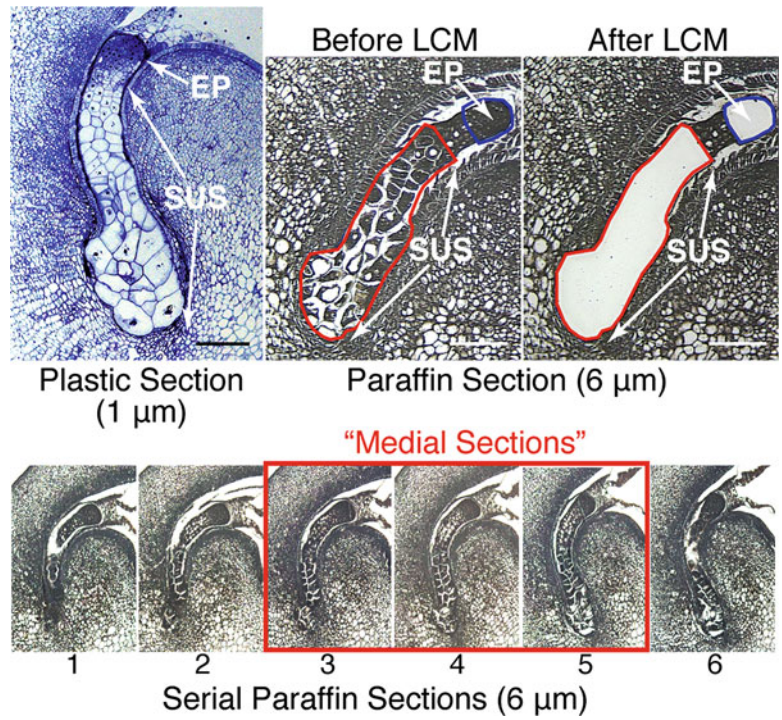

Fig. 3 Scarlet runner bean seed sections captured using LCM. The bottom row shows medial sections, which contain complete embryo proper and suspensor regions within the same section. Sections #1, #2, and #5 do not contain a complete suspensor

only serial sections we use to collect embryo proper and suspensor regions using LCM because they (1) contain morphologically intact embryo proper and suspensor regions and (2) minimize contamination with surrounding seed tissue (e.g., endosperm and seed coat). To maximize the number of middle or medial sections, it is important to orient the face of the tissue block parallel to the blade. This does not mean that you will always get medial sections because it depends on the orientation of the embedded seed and embryo within the seed. However, most of the time this step will help to ensure that you have as many medial sections as possible for use in downstream experiments. In our experience, two to four medial sections (6 μm) out of ~16–20 serial sections through a globular stage embryo can be expected per globular stage seed.

18. Usually only the sections with an embryo will be mounted on the slides. The first 20–30 sections will not contain any part of the embryo. With experience, it will become obvious during sectioning when you will begin to obtain sections with the embryo.
19. Think in advance how many sections you want to put on one slide. Because the ribbon will expand by ~25%, make sure you leave enough space around the ribbon.
20. For in situ hybridization using scarlet runner bean seeds, we follow procedures developed extensively in our laboratory [18, 19, 22]. Our detailed in situ hybridization protocol using radioactive probes is contained within refs. 18 and 22. Our recent method using nonradioactive probes is contained within ref. 19, although the fixing and sectioning steps are the same for both types of probes.
21. To capture as many embryo regions as possible in one LCM session, examine your slides in advance, and take a picture of the slides using the "overview function" of the LMD 6000 system. Print out the slide "overviews" and mark all of the medial sections. This will allow you to "target" specific sections on each slide for embryo region capture. To avoid contamination of embryo regions with seed coat and endosperm tissues, only capture the embryo proper and suspensor from medial sections (Fig. 3). To prevent cross-contamination between embryo proper and suspensor regions (1) the junction of the embryo proper and the suspensor should not be captured, and (2) only one embryo region should be captured during the same LCM session.
22. The settings that we use to capture globular stage embryo proper and suspensor regions with the Leica LMD 6000 system are as follows: magnification = 20×; power = 31; aperture = 12; speed = 12.

23. The number of captures required depends on the amount of RNA or DNA needed for downstream experiments. Based on our experience, an average of 0.3 ng and 0.8 ng RNA can be obtained per scarlet runner bean embryo proper and suspensor capture, respectively. To maximize RNA or DNA quality, we recommend that all captures should be done within 1 month after fixation for RNA, and 3 months after fixation for DNA. Using an embedding station can speed up the infiltration and embedding steps, which could maximize RNA or DNA quality. Total RNA from LCM captured embryo regions can be isolated using the PicoPure RNA Isolation Kit (Thermo Scientific) according to the manufacturer's instructions. RNA-Seq libraries can be prepared using as little as 5 ng of total RNA from LCM-captured embryo regions. Double-stranded cDNA can be synthesized and then amplified using Ovation RNA-Seq System V1 (NuGen) according to the manufacturer's instructions. RNA-Seq libraries can be generated from the double-stranded cDNA using any commercially available kits. DNA from LCM-captured embryo regions can be isolated using FFPE DNA isolation kit (QIAGEN), and isolated DNA can be used to construct Bisulfite-Seq libraries for methylome studies [24].

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