Journal of Visualized Experiments

A Simple Dry Sectioning Method for Obtaining Whole-seed-sized Resin Section and Its Applications

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE61822R2
Full Title:	A Simple Dry Sectioning Method for Obtaining Whole-seed-sized Resin Section and Its Applications
Corresponding Author:	Cunxu Wei CHINA
Corresponding Author's Institution:	
Corresponding Author E-Mail:	cxwei@yzu.edu.cn
Order of Authors:	Jiajing Qiu
	Yinhui Ren
	Lingxiao Zhao
	Biao Zhang
	Cunxu Wei
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Biology
Please provide any comments to the journal here.	

TITLE:

A Simple Dry Sectioning Method for Obtaining Whole-Seed-Sized Resin Section and Its Applications

AUTHORS AND AFFILIATIONS:

Jiajing Qiu[1,2#], Yinhui Ren[1,2#], Lingxiao Zhao[1,2], Biao Zhang[1,2], Cunxu Wei[1,2*]

[1]Key Laboratory of Crop Genetics and Physiology of Jiangsu Province/Jiangsu Key Laboratory of Crop Genomics and Molecular Breeding, Yangzhou University, Yangzhou, China

[2]Co-Innovation Center for Modern Production Technology of Grain Crops of Jiangsu Province/Joint International Research Laboratory of Agriculture & Agri-Product Safety of the Ministry of Education, Yangzhou University, Yangzhou, China

[#]These authors contributed equally.

Email Addresses of Co-Authors:

Jiajing Qiu (1539916373@qq.com)
Yinhui Ren (2236173661@qq.com)
Lingxiao Zhao (1010354750@qq.com)
Biao Zhang (zhangbiao@yzu.edu.cn)
Cunxu Wei (cxwei@yzu.edu.cn)

*Corresponding Author:

Cunxu Wei (cxwei@yzu.edu.cn)

KEYWORDS:

whole-seed-sized section, dry sectioning method, morphology, cell, starch granule, protein body

SUMMARY:

This technique allows for the fast and simple preparation of whole-seed-sized resin section for the observation and analysis of cells, starch granules, and protein bodies in different regions of the seed.

ABSTRACT:

The morphology, size and quantity of cells, starch granules, and protein bodies in seed determine the weight and quality of seed. They are significantly different among different regions of seed. In order to view the morphologies of cells, starch granules and protein bodies clearly, and quantitatively analyze their morphology parameters accurately, the whole-seed-sized section is needed. Though the whole-seed-sized paraffin section can investigate the accumulation of storage materials in seeds, it is very difficult to quantitatively analyze the morphology parameters of cells and storage materials due to the low resolution of the thick section. The thin resin section has high resolution, but the routine resin sectioning method is not suitable to prepare the whole-seed-sized section of mature seeds with a large volume and

high starch content. In this study, we present a simple dry sectioning method for preparing the whole-seed-sized resin section. The technique can prepare the cross and longitudinal whole-seed-sized sections of developing, mature, germinated, and cooked seeds embedded in LR White resin, even for large seeds with high starch content. The whole-seed-sized section can be stained with fluorescent brightener 28, iodine, and Coomassie brilliant blue R250 to specifically exhibit the morphology of cells, starch granules, and protein bodies clearly, respectively. The image obtained can also be analyzed quantitatively to show the morphology parameters of cells, starch granules, and protein bodies in different regions of seed.

INTRODUCTION:

Plant seeds contain storage materials such as starch and protein and provide energy and nutrition for people. The shape, size, and quantity of cell and storage materials determine the weight and quality of seed. The cells and storage materials in different regions of seed have significantly different morphologies, especially for some high-amylose cereal crops with inhibition of starch branching enzyme IIb[1–3]. Therefore, it is very important to investigate the morphologies of cells and storage materials in different regions of seed.

Paraffin sectioning is a good method to prepare the whole-seed-sized section and can exhibit the tissue structure of seed and the accumulation of storage material in different regions of seed[4–6]. However, the paraffin sections usually have 6–8 μm thickness with low resolution; thus, it is very difficult to clearly observe and quantitatively analyze the morphology of cell and storage materials. The resin sections usually have 1–2 μm thickness and high resolution and are very suitable to observe and analyze the morphology of cell and storage materials[7]. However, the routine resin sectioning method has difficulty in preparing the whole-seed-sized section, especially for seeds with a large volume and high starch content; thus, there is no way to observe and analyze the morphology of cells and storage materials in different regions of the seed. LR White resin is an acrylic resin and exhibits low viscosity and strong permeability, leading to its good applications in preparing the resin section of seeds, especially for cereal mature kernels with large volume and high starch content. In addition, the sample embedded in LR White resin can be stained easily with many chemical dyes to clearly exhibit the morphology of cells and storage materials under light or fluorescent microscope[7]. In our previous paper, we have reported a dry sectioning method for preparing the whole-seed-sized sections of mature cereal kernels embedded in LR White resin. The method can also prepare the whole-seed-sized section of developing, germinated and cooked cereal kernel[8]. The obtained whole-seed-sized section has many applications in micromorphology observation and analysis, especially for clearly viewing and quantitatively analyzing the morphology differences of cell and storage materials in different regions of seed[8,9].

This technique is appropriate for researchers who want to observe the microstructure of tissue and the shape and size of cells, starch granules, and protein bodies in different regions of seed using light microscope. The images of whole-seed-sized sections stained specifically for exhibiting cells, starch granules, and protein bodies can be analyzed by morphology analysis software to quantitatively measure the morphology parameters of cells, starch granules, and protein bodies in different regions of seed. In order to demonstrate the technical applicability

and whole-seed-sized section applications, we have investigated the mature seeds of maize and oilseed rape and the developing, germinated, and cooked kernels of rice in this study. The protocol contains four processes. Here, we use mature maize kernel, which is the most difficult in preparing the whole-seed-sized sections due to the large volume and high starch content, as a sample to exhibit the processes step by step.

PROTOCOL:

1. Preparation of resin-embedded seed (Figure 1)

1.1. Fix six maize mature kernels in 10 mL of 2.5% phosphate-buffered glutaraldehyde (0.1 M, pH7.2) at 4 °C for 48 h. The researchers can choose other fixative mixtures, fixative concentrations, and fixation conditions according to their research objectives and tissue types.

1.2. Take out the kernels and slice them longitudinally or transversally to 2–3 mm thickness using a sharp double-sided blade, and fix them in 10 mL of 2.5% phosphate-buffered glutaraldehyde (0.1 M, pH 7.2) again for 48 h.

1.3. Wash the samples three times with 10 mL of 0.1 M phosphate buffer (pH 7.2) for 30 min every time.

1.4. Dehydrate the samples in increasing grades of ethanol aqueous solution (10 mL) from 30% to 50%, 70%, 90% once, and 100% three times for 30 min every time.

1.5. Infiltrate the samples in 10 mL increasing grades of LR White resin solution diluted with ethanol from 25% to 50%, 75% once, and 100% twice at 4 °C for 12 h every time.

1.6. Prepare the pedestals for samples before embedding. Add 0.25 mL of 100% LR White resin into a 2-mL centrifuge tube, and polymerize it at 60 °C for 48 h.

1.7. Successively add the pure LR White resin (0.5 mL) and the infiltrated sample into the centrifuge tube with a pedestal. Straighten the samples with the anatomical needle, and polymerize them at 60 °C in an oven for 48 h.

2. Dry sectioning for preparing whole-seed-sized section (Figure 1)

2.1. Take out the embedded kernels from the centrifuge tube and cut out the excess resin around the sample using a sharp blade.

2.2. Clamp the resin block in the sample holder of ultramicrotome (EM UC7), and trim off the superfluous resin on the surface of the sample and around the sample with a blade.

2.3. Polish the surface of the sample finely with a glass knife until a complete section can be formed.

2.4. Put a small copper hook about 2 mm above the blade edge before cutting and cut the sample into a 2 µm section. The role of the hook is to avoid the curling upward of the section.

2.5. Put the hook under the section to support it when the section becomes long.

2.6. Add 100 µL of water on an unpretreated slide, and carefully transfer the complete and unbroken section to the water with the tweezers.

2.7. In order to smooth the wrinkled section, heat and dry the sample on the flattening table at 50 °C overnight.

2.7.1. If the section crumbles or tears, extend the time for each resin infiltration of the sample from 12 h to 24 h or 48 h.

2.7.2. If the section has some lines paralleled to the knife, clamp the sample block tightly. If the section has some lines vertical to the knife, please use a new knife.

3. Staining and observation of the section

NOTE: In order to observe the tissue structure and morphology of cells, starch granules, and protein bodies, stain the sections with specific stains according to the purpose of the research. Here, we use the fluorescent brightener 28, iodine solution, and Coomassie brilliant blue R250 to stain the cell walls, starch granules, and protein bodies, respectively.

3.1. For observing the morphology of cells, stain the section with 40 mL of 0.1% (w/v) fluorescent brightener 28 aqueous solution in a 70 mL compact glass staining jar at 45 °C for 10 min, and then rinse it with running water for 5 min. Observe and photograph the section under a fluorescence microscope equipped with a CCD camera.

3.2. For observing the morphology of starch granules, stain the section with 40 µL of iodine solution (0.07% (w/v) I_2 and 0.14% (w/v) KI in 25% (v/v) glycerol) for 1 min, and cover the sample containing iodine solution with a coverslip. View and photograph the sample under a light microscope equipped with a CCD camera.

3.3. For observing the morphology of protein bodies, immerse the section with 40 mL of 10% (v/v) acetic acid in a 70 mL compact glass staining jar for 10 min at 45 °C, and then stain it in 40 mL of 1% (w/v) Coomassie brilliant blue R250 in 25% (v/v) isopropanol and 10% (v/v) acetic acid for 15 min at 45 °C. Wash the stained sections with running water for 5 min, and dry it. Observe and photograph the section under a light microscope equipped with a CCD camera.

4. Quantitative analysis of morphology parameters

4.1. Process and quantitatively analyze the photographed images for area, long/short axis,

and roundness of cells, starch granules, and protein bodies in different regions of seed using morphology analysis software (Image-Pro Plus 6.0 software) following the procedures of Zhao et al.[9] exactly.

REPRESENTATIVE RESULTS:

Simple dry sectioning method for obtaining a whole-seed-sized section

We establish a simple dry sectioning method for preparing a whole-seed-sized section of seed embedded in LR-white resin (**Figure 1**). The method can prepare transversal and longitudinal whole-seed-sized sections with thickness of 2 μm (**Figure 2–5, Supplementary Figure 1–4**). For examples, the mature seed of oilseed rape can be sectioned transversally and longitudinally (**Figure 2**). For cereal crops, their mature kernels are full of starch granules, leading to that it is very difficult in preparing the whole-seed-sized section. Using the present technique, the transversal and longitudinal whole-seed-sized sections of mature maize with large volume could also be prepared (**Figure 4, Supplementary Figure 1**). In addition, the developing kernel (**Supplementary Figure 2**), germinated kernel (**Supplementary Figure 3**), and cooked kernel (**Supplementary Figure 4**) of rice can be investigated using the method.

Applications of the whole-seed-sized section

Observation of tissue structure of seed

The whole-seed-sized section can be used to observe the tissue structure of seeds. For examples, the embryo of oilseed rape consists of radicle, hypocotyl, plumule, and two cotyledons. The inner and outer cotyledons are bent in half, wrapping the hypocotyl and radicle and making the embryo spherical (**Figure 2A,C**). The longitudinal and transversal whole-embryo-sized sections stained with safranin clearly exhibited the radicle, hypocotyl, inner cotyledon, and outer cotyledon (**Figure 2B,D**). The longitudinal whole-embryo-sized section of oilseed rape is prepared more difficultly than the transversal section. Therefore, the transversal sections of embryos are widely used to investigate the micromorphology of embryos in references[5,10].

Morphology and analysis of cells in different regions of seed

The whole-seed-sized section can be used to observe and analyze the morphology of cells in different regions of seed. For example, the transversal whole-embryo-sized sections of oilseed rape were stained with fluorescent brightener 28, and the cell walls were stained specifically (**Figure 3A**). The micromorphology of cells in any regions of embryo could be clearly displayed at high magnification (**Figure 3B,C**). The radicle consists of epiderm, cortex, and vascular tissues. The epidermal cells located in the outermost layer of radicle were rectangular and radially arranged. The cortical parenchyma cells were round in shape and large in size. Some distinct spaces were observed between cortical cells. The cortical cells were arranged in layers from the inside to the outside (**Figure 3B**). The epidermal cells of cotyledon were square and had small volume. There were no significant differences in shape and size of epidermal cells among outer and inner surfaces of inner and outer cotyledons. Some vascular cylinders were scattered in the middle of mesophyll tissues of inner and outer cotyledons. The mesophyll parenchyma cells were significantly larger than the epidermal cells and vascular cylinder cells in the cotyledon.

The mesophyll parenchyma cells showed a typical palisading arrangement in the inner region of outer cotyledon and the outer region of inner cotyledon (**Figure 3C**). The parenchyma cells had significantly different morphologies in different regions of embryo. In order to reveal their differences in morphology, regions 1, 2, 3, 4, and 5 were chosen in the radicle cortical tissue, inner region of inner cotyledon, outer region of inner cotyledon, inner region of outer cotyledon, and outer region of outer cotyledon, respectively (**Figure 3B,C**). The morphology parameters of the parenchyma cells in the above 5 regions were quantitatively analyzed using morphology analysis software (**Supplementary Table 1**). The area, long axis length, short axis length, and roundness of parenchyma cells showed some differences in different regions of embryos.

The cells in endosperm were full of starch and storage protein. Using the whole-seed-sized resin section, it is easy in observing and analyzing the cells in different regions of endosperm. For example, the morphology of cells in any regions of maize endosperm could be viewed clearly after the transversal whole-seed-sized sections were stained with fluorescent brightener 28. The peripheral, middle, and central endosperms in the same kernel exhibited significantly different shapes and sizes of cells (**Supplementary Figure 1**). In order to quantitatively analyze the morphology parameters of cells in different regions of endosperm, the images of regions were analyzed using morphology analysis software; the morphology parameters of cells are presented in **Supplementary Table 2**. The endosperm cells in region 1 had the smallest area among four regions, those in region 2 were larger than those in region 3, but smaller than those in region 4.

Morphology and analysis of starch granules in different regions of seed

The mature seeds from most plant resources, especially for cereal crops, contain high starch content. The granule morphology and size of starch have important effects on starch properties and play a role in the quality of seed. The resin section of seed can be stained with iodine solution to exhibit the morphology of starch granules in different regions of seed. For example, the transversal and longitudinal whole-seed-sized sections of maize were prepared successfully. The sections stained with iodine exhibited the morphology of starch (**Figure 4**). In order to show the morphology of starch granule in different regions of endosperm, the four regions and nine regions were chosen in the transversal and longitudinal whole-seed-sized sections, respectively (**Figure 4**). The starch granules in different regions showed significantly different morphology, size and quantity in endosperm cells. For transversal section, region 1 had spherical starch granules, region 2 had polygonal granules, and starch granules in both regions 3 and 4 were spherical. For longitudinal section, starch granules with polygonal shape in regions 1, 4, 5, and 8 were larger than those with spherical shape in regions 3, 7, and 9, and some compound starch granules were observed in regions 2 and 6.

The quantitative analysis of morphological parameters of starch granules in four regions of transversal section was shown in **Supplementary Table 3**. Starch granules in region 1 had the smallest size, those in region 2 had the largest size, and those in region 3 were larger than in region 4.

Micromorphology and analysis of protein bodies in different regions of seed

The whole-seed-sized section with high storage protein can be used to obverse and analyze the morphology of protein bodies in different regions of seed. For example, the transversal section of embryo of oilseed rape was stained with Coomassie brilliant blue R250, and the storage protein was stained blue (**Figure 5**). The spatial distribution of storage protein in the embryo could be clearly observed at the low magnification (**Figure 5A**). Storage protein is present in protein bodies. At high magnification, the protein body exhibited a heterogeneous matrix with some black granules and some unstained transparent structure (**Figure 5B**). The protein bodies in seed have three types: the first type consists of a homogeneous protein matrix and has no inclusions, the second type contains globular crystals, and the third type contains globular crystals and pseudocrystals[11]. The globular crystals in the protein body are composed of phytate and other inorganic salts, which are not stained. These globular crystals are black due to that the light cannot pass through them under microscope. In addition, the spherical crystal is fragile and difficult to be penetrated by the fixative and the embedding agent. When making the section, the spherical crystals sometimes burst out, resulting in a transparent cavity inside the protein body[11]. The protein body of oilseed rape embryo contained spheroidal crystals according to its micromorphology (**Figure 5B**). In order to investigate the spatial distribution of protein bodies in the embryo, five regions in the whole-embryo-sized section were chosen to represent the radicle cortical tissue, inner region of inner cotyledon, outer region of inner cotyledon, inner region of outer cotyledon, and outer region of outer cotyledon (**Figure 5A,C–G**). The protein bodies in all regions of embryo were spherical, ellipsoidal, and irregular in shape (**Figure 5C–G**).

Quantitative analysis of protein bodies in the first and second lay parenchyma cells close to the epidermis in the above chosen five regions are presented in **Supplementary Table 4**. The area of protein body had slight difference among the chosen five regions. The roundness of protein body was significantly lower in the outer region of outer cotyledon than in the other four regions, indicating the protein body in outer cotyledon was close to sphere. The number and area index of protein body in cell were significantly higher in the radicle parenchyma cell than in the cotyledon parenchyma cell (**Supplementary Table 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of whole-seed-sized resin semithin section using dry sectioning method.

Figure 2: Tissue structure of embryo in mature seed of oilseed rape variety Huashuang 5. (**A**) Morphology of embryo. (**B**) Tissue structure of longitudinal whole-embryo-sized section. (**C**) Morphology of transversal whole-embryo-sized section. (**D**) Tissue structure of transversal whole-embryo-sized section. The sections were stained with safranin. H, hypocotyl; IC, inner cotyledon; OC, outer cotyledon; R, radicle. Scale bar = 1 mm.

Figure 3: Morphology of cells in embryo of oilseed rape variety Huashuang 5. (**A**) Transversal whole-embryo-sized section stained with fluorescent brightener 28. (**B**) Amplification of region B in (**A**), showing the cell morphology and tissue structure of radicle. (**C**) Amplification of region

C in (**A**), showing the cell morphology and tissue structure of inner and outer cotyledon. Scale bar = 500 μm for (**A**) and 100 μm for (**B,C**).

Figure 4: Morphology of starch granules in mature kernel of maize variety Zheng 58. The transversal (**A**) and longitudinal (**B**) whole-seed-sized sections were stained with iodine solution, and their regional amplifications exhibit the morphology of starch granules in different regions of endosperm. Scale bar = 1 mm for whole-seed-sized section and 20 μm for regional amplifications.

Figure 5: Morphology of protein bodies in embryo of oilseed rape variety Huashuang 5. (**A**) Transversal whole-embryo-sized section stained with Coomassie brilliant blue R250. (**B**) Amplification of protein bodies, showing their microstructure. (**C–G**) Amplification of region C–G in (**A**), showing the morphology of protein body in radicle (**C**), inner region of inner cotyledon (**D**), outer region of inner cotyledon (**E**), inner region of outer cotyledon (**F**), outer region of inner cotyledon (**G**). Scale bar = 500 μm for (**A**), 5 μm for (**B**) and 50 μm for (**C–G**).

Supplementary Figure 1: Morphology of cells in mature kernel of maize variety Zheng 58. The transversal whole-seed-sized section was stained with fluorescent brightener 28, and its regional amplifications (1–4) exhibit the morphology of endosperm cells in different regions of endosperm. Scale bar = 1 mm for whole-seed-sized section and 100 μm for regional amplifications.

Supplementary Figure 2: Morphology of developing kernel of rice variety 9311. The transversal whole-seed-sized sections at different days after flowering (DAF) were counterstained with safranin O and iodine solution. Scale bar = 0.5 mm.

Supplementary Figure 3: Morphology of germinated kernel of rice variety Te-qing. The longitudinal whole-seed-sized section at 8 days after imbibition was counterstained with periodic acid-Schiff's and toluidine blue O, and its regional amplifications exhibit the morphology changes of endosperm in different regions of seed. Scale bar = 20 μm.

Supplementary Figure 4: The morphology of cooked kernel of rice variety Te-qing. The transversal whole-seed-sized section was stained with iodine solution, and its outer, middle, and inner region amplifications exhibit the morphology changes of starch granules in seed during cooking process for 0, 10, 20, and 30 min. Scale bar = 20 μm.

Supplementary Table 1: Morphology parameters of cells in different regions of oilseed rape embryo[a]

[a]The data is means ± standard deviations (n = 3), and the values in the same column with different letters are significantly different ($p < 0.05$).

[b]The regions are shown in **Figure 3B,C**.

[c]LAL: long axis length; SAL: short axis length; Roundness: (perimeter ^{2})/(4×π×area).

Supplementary Table 2: Morphology parameters of cells in different regions of maize endosperm[a]

[a]Data is means ± standard deviations (n = 3). Values in the same column with different letters are significantly different ($p < 0.05$).

[b]The regions are shown in transversal section of maize kernel in **Supplementary Figure 1**.

[c]LAL: long axis length; SAL: short axis length; Roundness: (perimeter ^{2})/(4×π×area).

Supplementary Table 3: Morphology parameters of starch granules in different regions of maize endosperm[a]

[a]Data is means ± standard deviations (n = 3). Values in the same column with different letters are significantly different ($p < 0.05$).

[b]The regions are shown in transversal section of maize kernel in **Figure 4A**.

[c]LAL: long axis length; SAL: short axis length; Roundness: (perimeter ^{2})/(4×π×area).

Supplementary Table 4: Morphology parameters of protein bodies in different regions of oilseed rape embryo[a]

[a]The data is means ± standard deviations (n = 3), and the values in the same column with different letters are significantly different ($p < 0.05$).

[b]The regions are shown in **Figure 5**.

[c]Roundness: (perimeter ^{2})/(4×π×area); Area index is the area ratio of protein body to cell.

DISCUSSION:

The seeds are the most important renewable resource for food, fodder, and industrial raw material, and are rich in storage materials such as starch and protein. The morphology and quantity of cells and the content and configuration of storage materials affect the weight and quality of seeds[7,12]. Though the stereology and image analysis technology can measure the size and quantity of cells in a tissue region, they are lacking in many laboratories. The paraffin and resin sections give a two-dimensional (2D) picture, leading to no way in analyzing the true size and quantity of cells. However, the cells are cut randomly at their any planes, the mean size of many cells (over 100) from at least three different sections of tissue region can reflect the 2D morphology parameters (length, width, and area) of cells, and the ratio of the chosen region area to mean cell area can reflect the quantity of cells. Therefore, it is very important for in situ viewing and analyzing the morphology of cells and storage materials in different regions of seed. The paraffin section is the most suitable for preparing the whole-seed-sized section, especially for large sized seeds[7]. However, the cells are full of storage materials with seed development, leading to that it is very difficult in obtaining the good whole-seed-sized section from late developing seeds and mature seeds. In addition, the paraffin section is too thick to exhibit the morphology clearly, and is only suitable for investigating the tissue structure of seed[7].

The resin section is thin, and can exhibit the morphology of cells, starch granules, and protein bodies clearly[7]. However, the routine resin is not suitable for whole-seed-sized section. The technique presented here represents a fast, simple, and keen approach toward preparing transversal and longitudinal whole-seed-sized sections of mature seeds embedded in resin for viewing the morphology of cells, starch granules, and protein bodies in different regions of seed

using light microscopy (**Figure 2–5**, **Supplementary Figure 1**). In addition, the technique can also prepare the section of developing, germinated, and cooked seeds to in situ investigate the morphology changes of cell, starch, and protein bodies in different regions of seed.

Another distinct advantage that this technique provides is the application of whole-seed-sized sections. In the new era of phenomics and metabolomics, it is important to quantitatively measure the morphology parameters of cells, starch granules, and protein bodies in different regions of seeds. The new technique, in conjunction with morphology analysis software, allows the researcher to quantitatively analyze the morphology parameters of cells, starch granules, and protein bodies in different regions of seed (**Supplementary Table 1S4**).

Though the present dry sectioning method can successfully prepare the whole-seed-sized resin section, it has some limitations and shortcomings. For the paraffin section, the paraffin can be removed easily from the section; but for the resin section, the resin cannot be removed from the section, leading to the plant sample embedded in resin. Therefore, compared with the paraffin section, the present whole-seed-sized resin section is not suitable to carry out the histochemistry and immunohistochemistry. In addition, the routine resin sectioning method can cut samples into 0.5–2 μm smooth sections due to the sample block with small volume. But the present dry sectioning method is difficult to prepare the smooth sections with thickness less than 2 μm, especially for mature seeds with large volume and high starch content.

ACKNOWLEDGMENTS:

Funding was provided by the Talent Project of Yangzhou University and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Cai, C. et al. Heterogeneous structure and spatial distribution in endosperm of high-amylose rice starch granules with different morphologies. *Journal of Agricultural and Food Chemistry*. **62** (41), 10143–10152 (2014).
2. He, W. et al. The defective effect of starch branching enzyme IIb from weak to strong induces the formation of biphasic starch granules in amylose-extender maize endosperm. *Plant Molecular Biology*. **103** (3), 355–371 (2020).
3. Wang, J. et al. Gradually decreasing starch branching enzyme expression is responsible for the formation of heterogeneous starch granules. *Plant Physiol*. **176** (1), 582–595 (2018).
4. Chen, X. et al. Dek35 encodes a PPR protein that affects cis-splicing of mitochondrial nad4 intron 1 and seed development in maize. *Molecular Plant*. **10** (3), 427–441 (2017).
5. Hu, Z. Y. et al. Seed structure characteristics to form ultrahigh oil content in rapeseed. *PLoS One*. **8** (4), e62099 (2013).
6. Huang, Y. et al. Maize VKS1 regulates mitosis and cytokinesis during early endosperm development. *Plant Cell*. **31** (6), 1238–1256 (2019).

7. Xu, A., Wei, C. Comprehensive comparison and applications of different sections in investigating the microstructure and histochemistry of cereal kernels. *Plant Methods*. **16**, 8. (2020).
8. Zhao, L., Pan, T., Cai, C., Wang, J., Wei, C. Application of whole sections of mature cereal seeds to visualize the morphology of endosperm cell and starch and the distribution of storage protein. *Journal of Cereal Science*. **71**, 19–27 (2016).
9. Zhao, L., Cai, C., Wei, C. An image processing method for investigating the morphology of cereal endosperm cells. *Biotech & Histochemistry*. **95** (4), 249–261 (2020).
10. Borisjuk, L. et al. Seed architecture shapes embryo metabolism in oilseed rape. *The Plant Cell*. **25** (5), 1625–1640 (2013).
11. Lott, J. N. A. Protein bodies in seeds. *Nordic Journal of Botany*. **1**, 421–432 (1981).
12. Jing, Y. P. et al. Development of endosperm cells and starch granules in common wheat. *Cereal Research Communications*. **42** (3), 514–524 (2014).

Figure 1

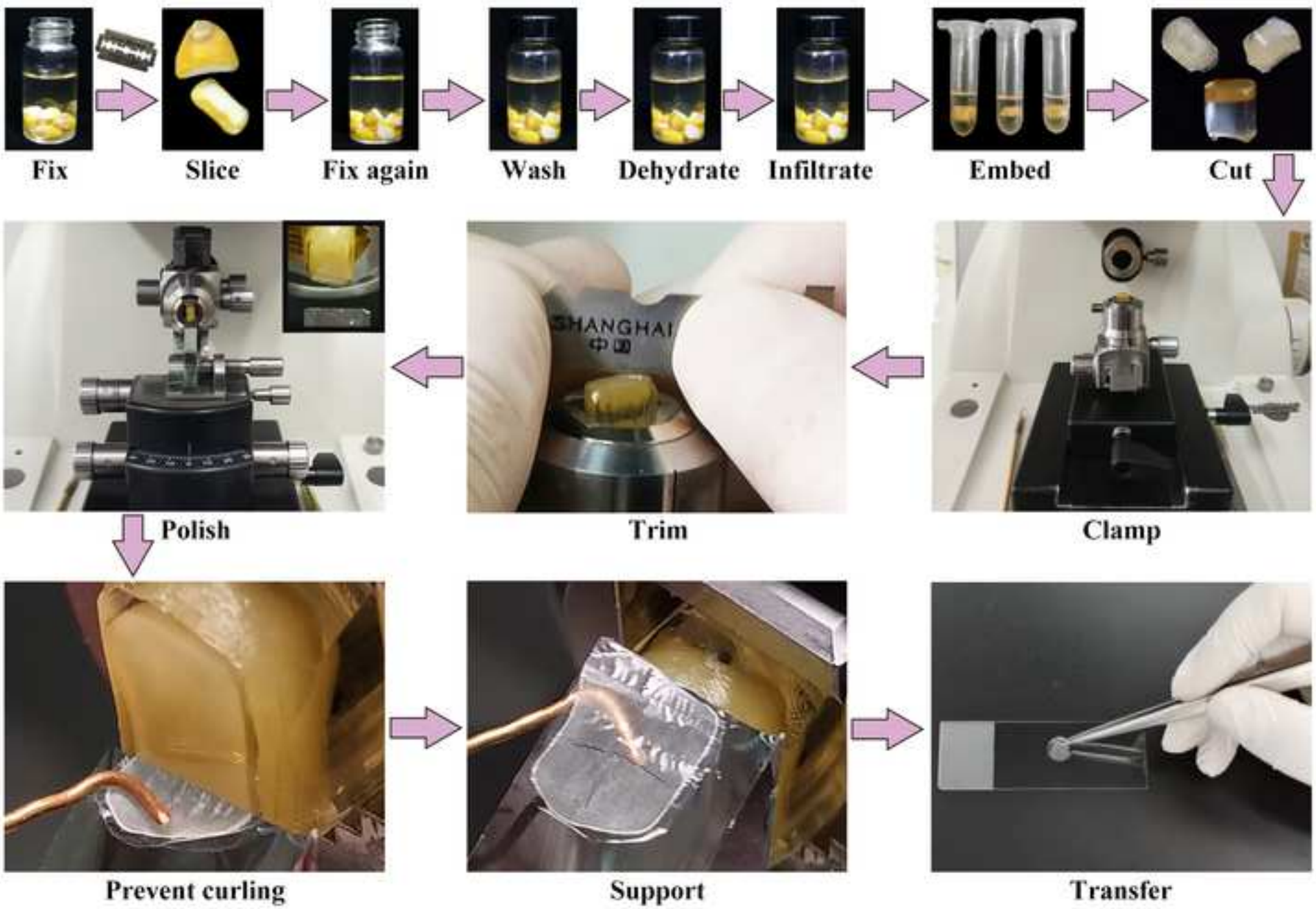


Figure 2

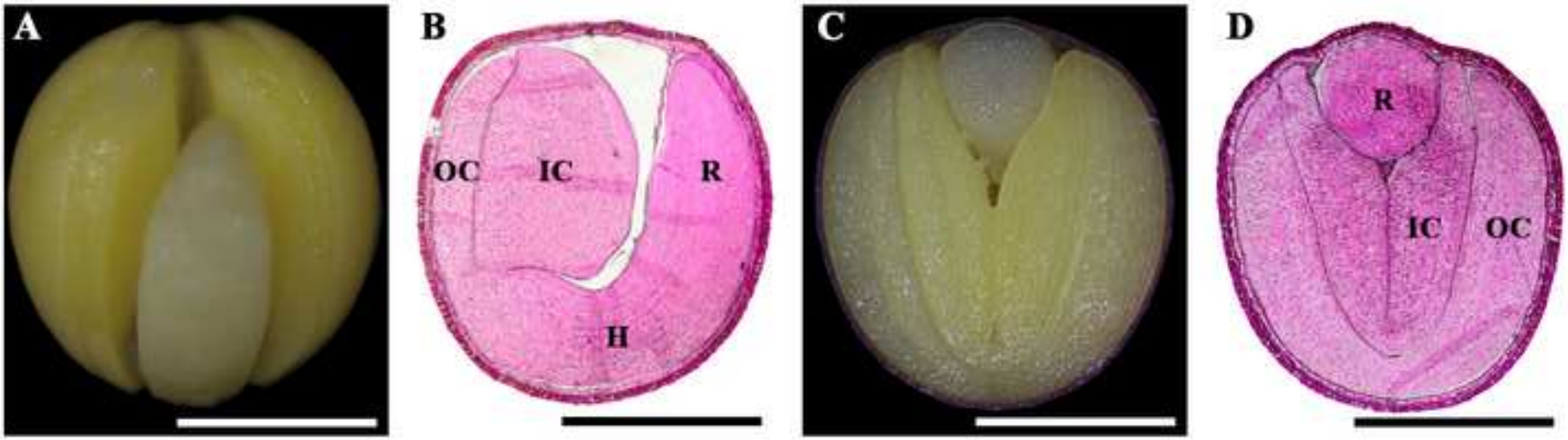


Figure 3

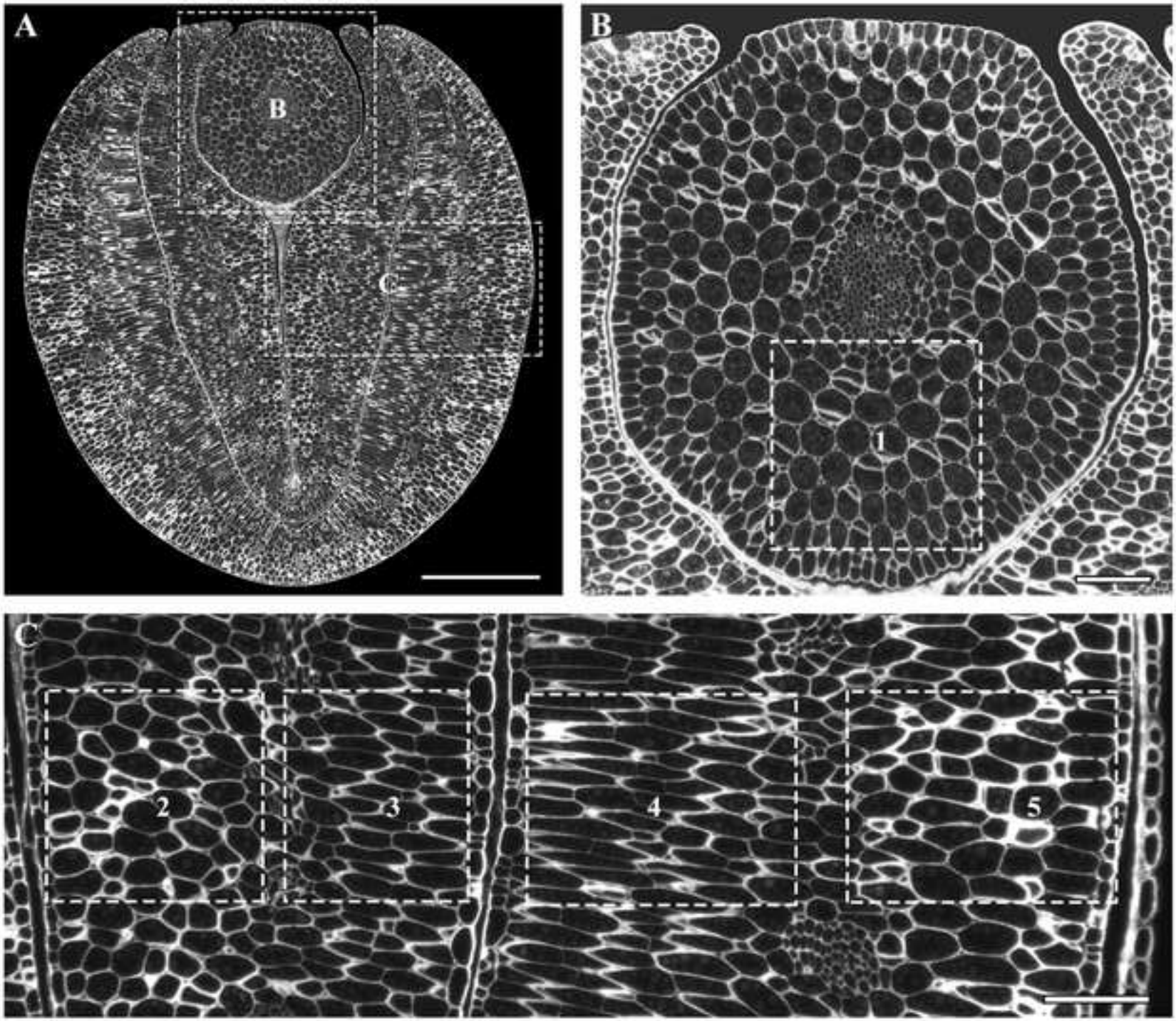


Figure 4

Click here to access/download;Figure;Figure 4.tif

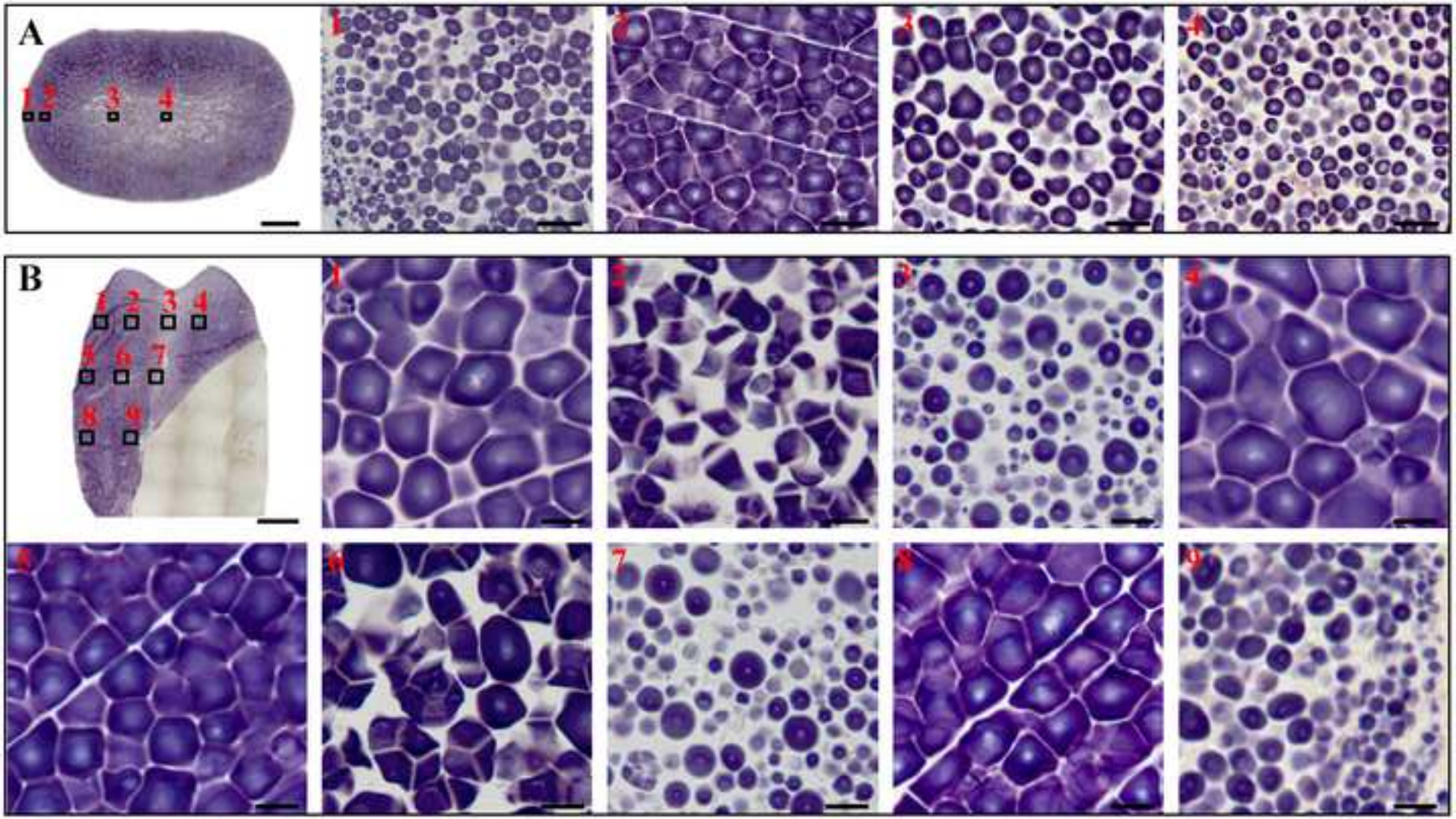


Figure 5

Click here to access/download;Figure;Figure 5.tif

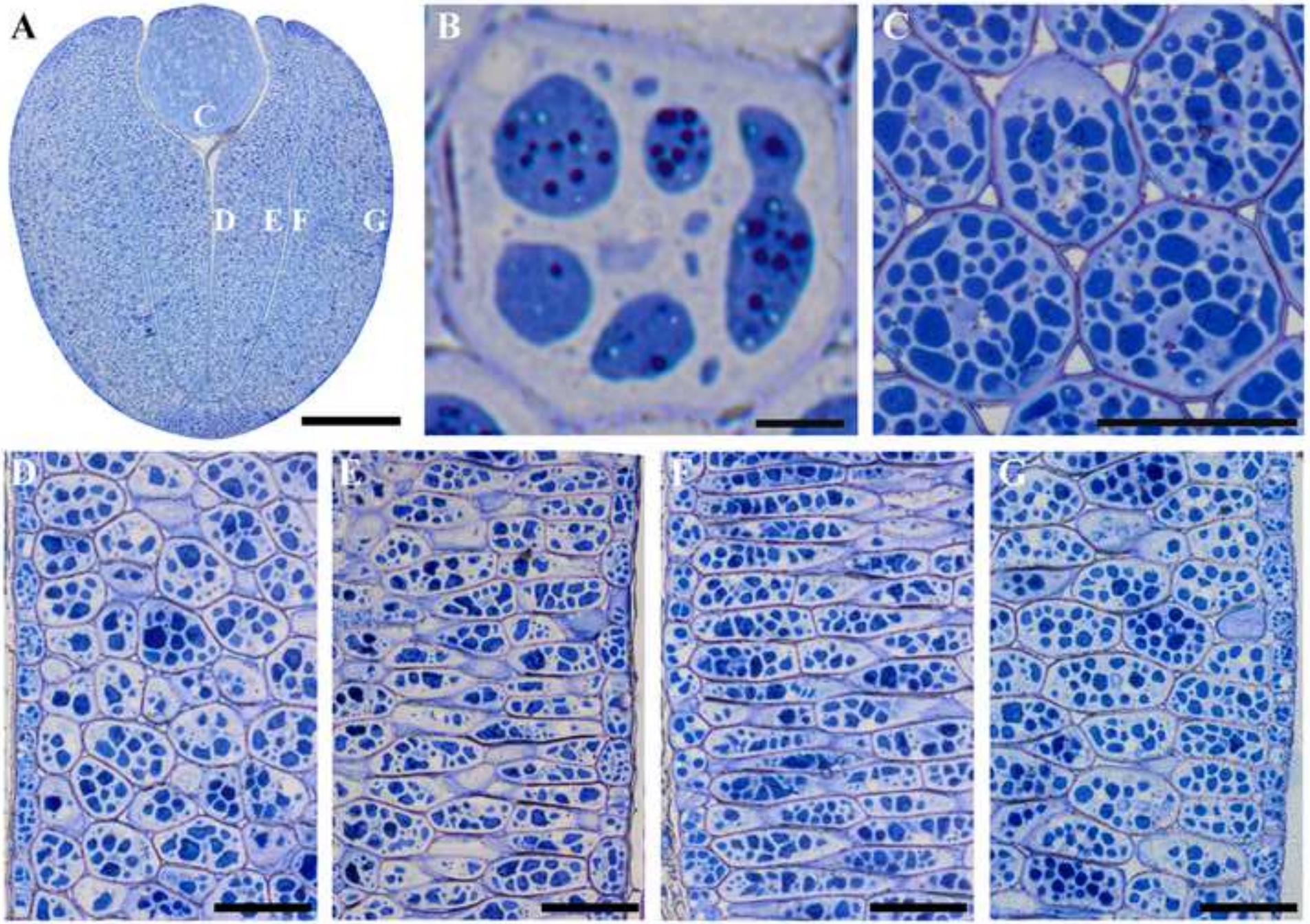


Figure S1

Click here to access/download;Figure;Figure S1.tif

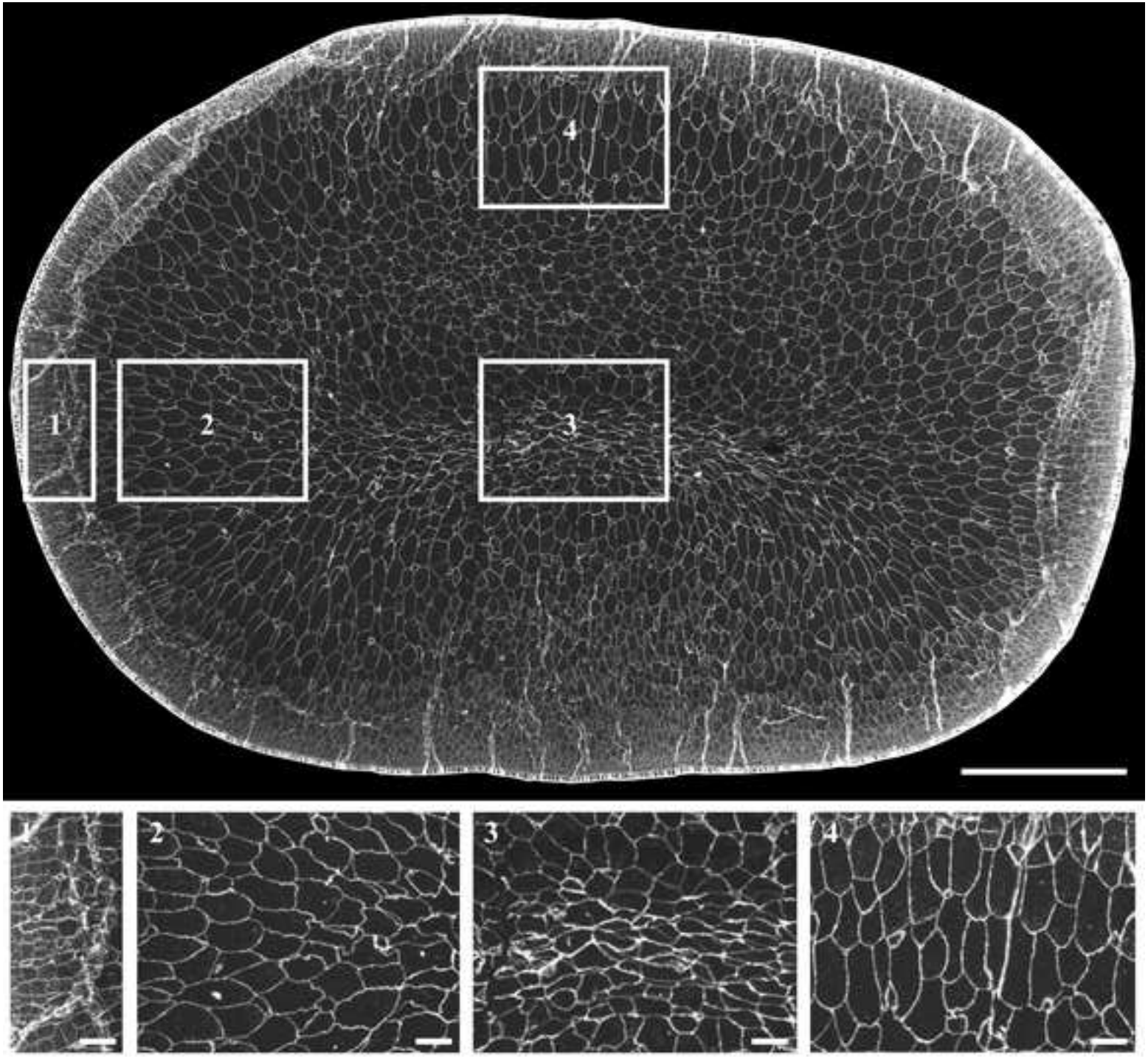


Figure S2

Click here to access/download;Figure;Figure S2.tif

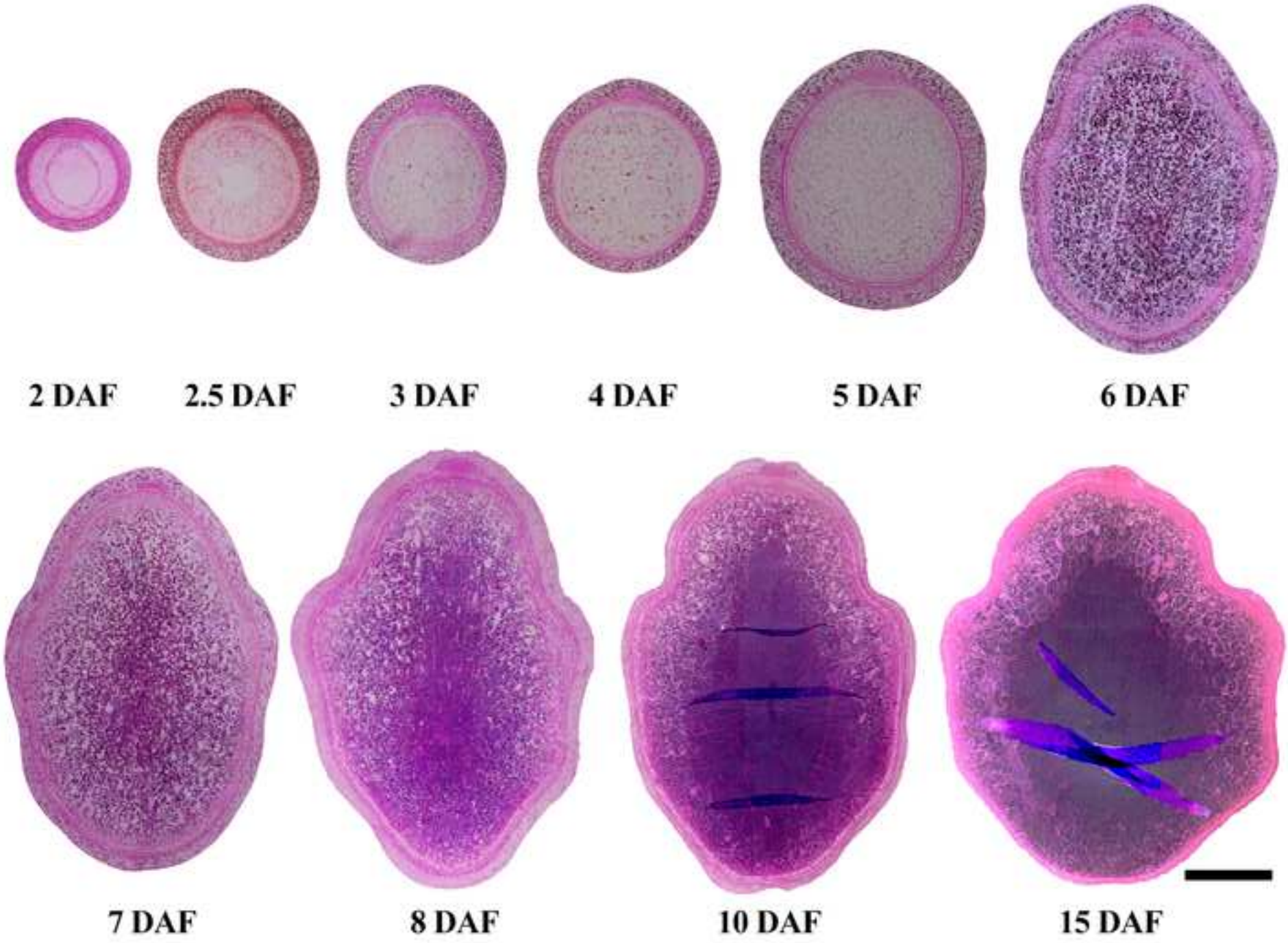


Figure S3

Click here to access/download;Figure;Figure S3.tif

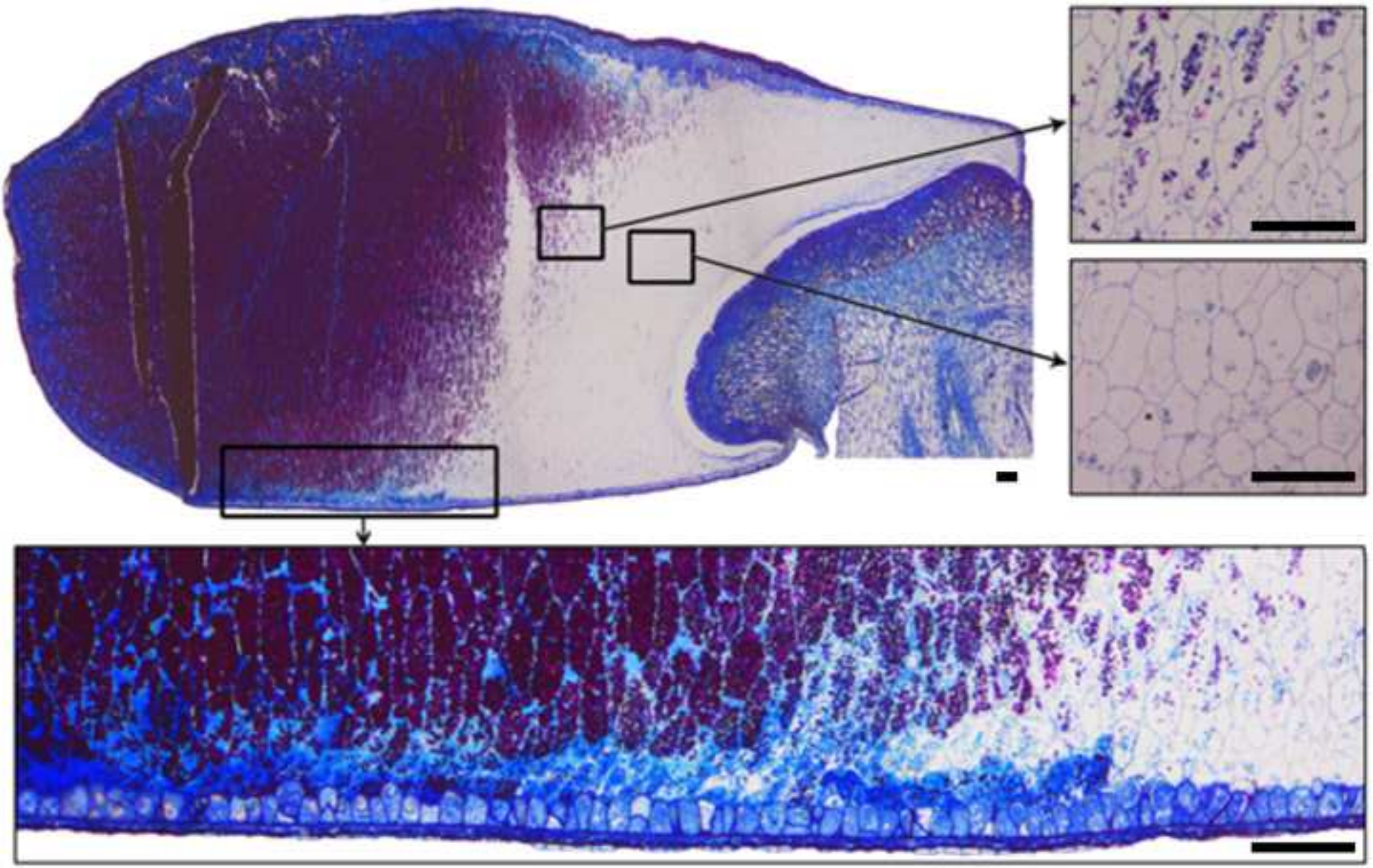
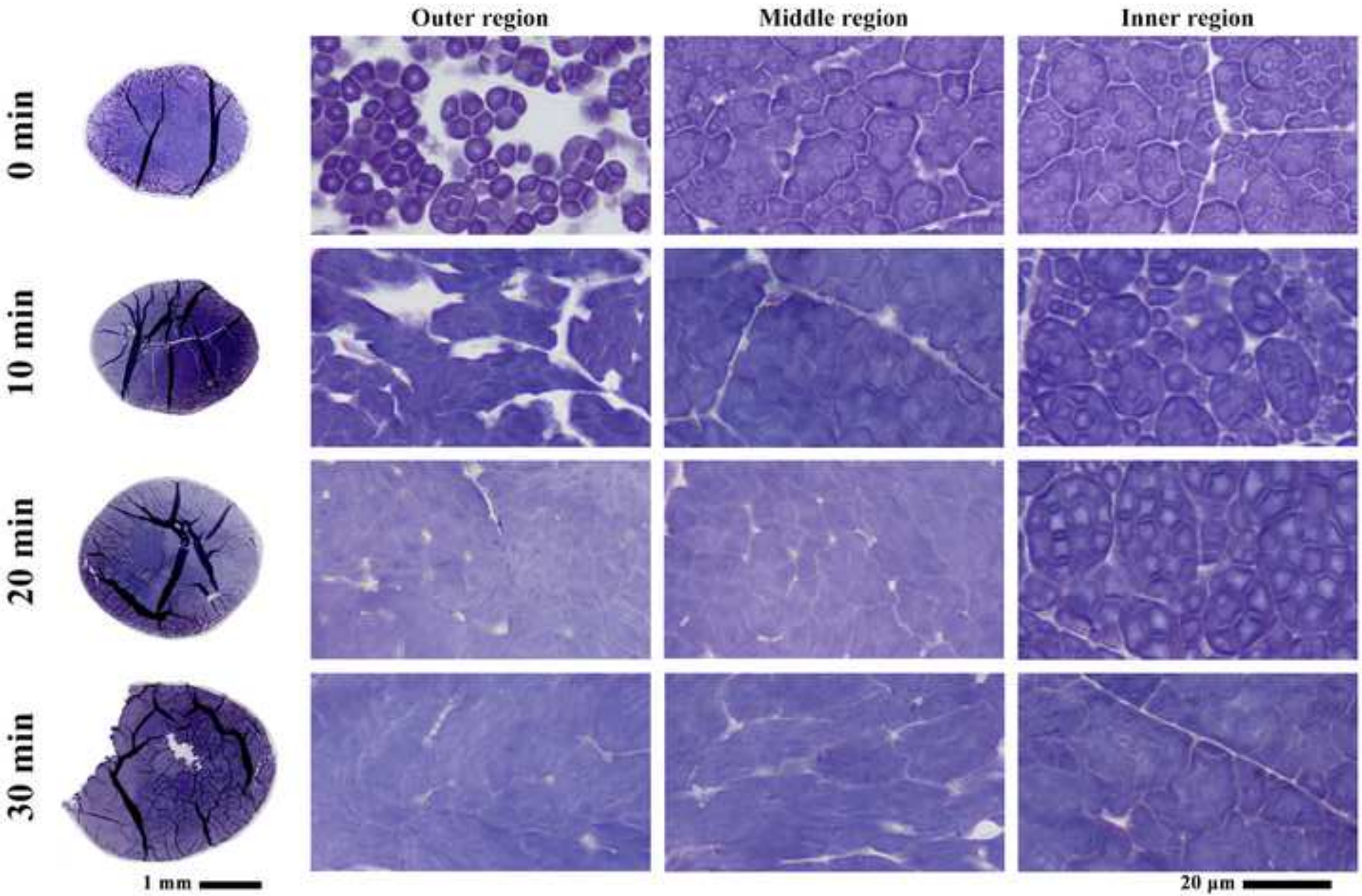


Figure S4

Click here to access/download;Figure;Figure S4.tif



Name of Material/Equipment	Company	Catalog Number
Acetic acid	Sangon Biotech (Shanghai) Co., Ltd.	A501931
Compact glass staining jar (5-Place)	Sangon Biotech (Shanghai) Co., Ltd.	E678013
Coomassie brilliant blue R-250	Sangon Biotech (Shanghai) Co., Ltd.	A100472
Coverslip	Sangon Biotech (Shanghai) Co., Ltd.	F518211
Double-sided blade	Gillette Shanghai Co., Ltd.	74-S
Ethanol absolute	Sangon Biotech (Shanghai) Co., Ltd.	A500737
Flattening table	Leica	HI1220
Fluorescence microscope	Olympus	BX60
Fluorescent brightener 28	Sigma-Aldrich	910090
Glass strips	Leica	840031
Glutaraldehyde 50% solution in water	Sangon Biotech (Shanghai) Co., Ltd.	A600875
Glycerol	Sangon Biotech (Shanghai) Co., Ltd.	A600232
Iodine	Sangon Biotech (Shanghai) Co., Ltd.	A500538
Isopropanol	Sangon Biotech (Shanghai) Co., Ltd.	A507048
Light microscope	Olympus	BX53
LR White resin	Agar Scientific	AGR1281A
Oven	hanghai Jing Hong Laboratory Instrument Co.,Ltd	9023A
Potassium iodide	Sangon Biotech (Shanghai) Co., Ltd.	A100512
Slide	Sangon Biotech (Shanghai) Co., Ltd.	F518101
Tweezers	Sangon Biotech (Shanghai) Co., Ltd.	F519022
Sodium phosphate dibasic dodecahydrate	Sangon Biotech (Shanghai) Co., Ltd.	A607793
Sodium phosphate monobasic dihydrate	Sangon Biotech (Shanghai) Co., Ltd.	A502805
Ultramicrotome	Leica	EM UC7

Dec 10, 2020

Editor Nam Nguyen

JoVE

Manuscript ID: JoVE61822R1

Dear Editor Nguyen,

We would like to thank you and reviewers very much for your careful review, professional comments, and valuable corrections and suggestions to our manuscript.

Based on the comments, corrections, and suggestions of you and reviewers, we have made a major revision. The major revisions have been highlighted in RED in the revised manuscript. The enclosed is the point-to-point responses to comments of Editorial, Production, and Reviewers.

If you have any question, please do not hesitate to let us know.

Again, many thanks for your efforts to make our manuscript much better!

Sincerely yours,

Prof. Cunxu Wei

Responses to comments of Editorial and Production

1. Changes to be made by the Author(s) regarding the written manuscript

1.1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

> Thanks for your comment and suggestion. In the revised manuscript, we have tried to proofread and revise our manuscript.

1.2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 196-201

> Thanks for your comment and suggestion. In the revised manuscript, we have rewritten the sentences as the below.

(L210-213) For example, the morphology of cells in any regions of maize endosperm could be viewed clearly after the transversal whole-seed-sized sections were stained with fluorescent brightener 28. The peripheral, middle, and central endosperms in the same kernel exhibited significantly different shapes and sizes of cells (Figure S1).

1.3. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

> Thanks for your professional comment and suggestion. In the revised manuscript, we have added the information in Table of materials as the below.

	A	B	C
1	**Name of Material/Equipment**	**Company**	**Catalog Number**
2	Acetic acid	Sangon Biotech (Shanghai) Co., Ltd.	A501931
3	Compact glass staining jar (5-Place)	Sangon Biotech (Shanghai) Co., Ltd.	E678013
4	Coomassie brilliant blue R-250	Sangon Biotech (Shanghai) Co., Ltd.	A100472
5	Coverslip	Sangon Biotech (Shanghai) Co., Ltd.	F518211
6	Double-sided blade	Gillette Shanghai Co., Ltd.	74-S
7	Ethanol absolute	Sangon Biotech (Shanghai) Co., Ltd.	A500737
8	Flattening table	Leica	HI1220
9	Fluorescence microscope	Olympus	BX60
10	Fluorescent brightener 28	Sigma-Aldrich	910090
11	Glass strips	Leica	840031
12	Glutaraldehyde 50% solution in water	Sangon Biotech (Shanghai) Co., Ltd.	A600875
13	Glycerol	Sangon Biotech (Shanghai) Co., Ltd.	A600232
14	Iodine	Sangon Biotech (Shanghai) Co., Ltd.	A500538
15	Isopropanol	Sangon Biotech (Shanghai) Co., Ltd.	A507048
16	Light microscope	Olympus	BX53
17	LR White resin	Agar Scientific	AGR1281A
18	Oven	Shanghai Jing Hong Laboratory Instrument Co.,Ltd.	9023A
19	Potassium iodide	Sangon Biotech (Shanghai) Co., Ltd.	A100512
20	Slide	Sangon Biotech (Shanghai) Co., Ltd.	F518101
21	Tweezers	Sangon Biotech (Shanghai) Co., Ltd.	F519022
22	Sodium phosphate dibasic dodecahydrate	Sangon Biotech (Shanghai) Co., Ltd.	A607793
23	Sodium phosphate monobasic dihydrate	Sangon Biotech (Shanghai) Co., Ltd.	A502805
24	Ultramicrotome	Leica	EM UC7

1.4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

> Thanks for your professional comment and suggestion. In the revised manuscript, we have rewritten the Section of Protocol in the imperative tense. For detail, please see the revised manuscript.

1.5. How many kernels are prepared?

> Thanks for your comment. In the revised manuscript, we have added the information as the below.

(L98) Fix 6 maize mature kernels in 10 mL of 2.5% phosphate-buffered glutaraldehyde (0.1 M, pH7.2) at 4 °C for 48 h.

1.6. Please specify all volumes used throughout.

> Thanks for your comment. In the revised manuscript, we have added the information. For detail, please see the revised manuscript.

1.7. Please discuss limitations of the technique in the discussion.

> Thanks for your suggestion. In the revised manuscript, we have added the discussion as the below.

(L362-370) Though the present dry sectioning method can successfully prepare the whole-seed-sized resin section, it has some limitations and shortcomings. For paraffin section, the paraffin can be removed easy from section; but for resin section, the resin cannot be removed from section, leading to plant sample embedded in resin. Therefore, compared with paraffin section, the present whole-seed-sized resin section is not suitable to carry out the histochemistry and immunohistochemistry. In addition, the routine resin sectioning method can cut samples into 0.5~2 μm smooth sections due to the sample block with small volume. But the present dry sectioning method is difficult to prepare the smooth sections with thickness less than 2 μm, especially for mature seeds with large volume and high starch content.

2. Changes to be made by the Author(s) regarding the video

2.1. Furthermore, please revise the narration to be more homogenous with the written manuscript after revising the written protocol into the imperative tense. Ideally, the narration is a word for word reading of the written protocol.

> Thanks for your comment. We have revised the narration according to the revised protocol. Please see the revised video.

2.2. The editing is very fast and jumps around with no transitions, it makes the video difficult to follow and disorienting. See 1:25 and 2:23 as examples. Some cross dissolve transitions

can help smooth things out.

> Thanks for your comment. We have revised the video.

2.3. There are beeps that I am assuming are from equipment in the lab that you can hear in the VO at 0:59, 2:04

> Thanks for your comment. We have revised the video, and avoid the beeps.

2.4. No name lower thirds (name captions) in the conclusion section, should be in the intro and conclusion. They could also stay up on screen for another second

> Thanks for your comment. In the revised video, we have added the content captions and the speaker name in the Sections of Introduction and Conclusion.

2.5. The first word by the speaker at 0:06 starts too late, the beginning of the word is cut off

> Thanks for your comment. We have revised the video.

2.6. The last word in the VO in the results section is cut off at 8:48

> Thanks for your comment. We have revised the video.

Responses to comments of reviewer 1

Comment: Manuscript Summary: Review of JoVE61822_R1 "Simple Dry Sectioning of Whole Kernel Embedded in Resin and Its Applications". Title is appropriate. The topic is very informative and useful for plant histology applications. The method is well presented, but I would like to see some more detailed information, as written below. I support publication of the protocol with minor modification. The text should be copyedited and proofed. Specific comments are listed as the below:

> Thank you very much for your careful review, professional comment, and valuable correction and suggestion. We have made a major revision and uploaded it with major changes in RED.

1. Introduction

1.1. comment on why should one choose LR White resin - because it's hydrophilic and such sections can be used in traditional histological staining protocols?

> Thanks for your comment. In the revised manuscript, we have added the reason in Section of Introduction in Manuscript and Video as the below.

(L71-75) LR White resin is an acrylic resin and exhibits low viscosity and strong permeability, leading to its good applications in preparing the resin section of seeds, especially for cereal mature kernels with large volume and high starch content. In addition, the sample embedded in LR White resin can be stained easily with many chemical dyes to clearly exhibit the morphology of cells and storage materials under light or fluorescent microscope[7].

1.2. using word "section" is not clear: do you mean histological section, cross section, longitudinal section. Perhaps just explain what types or orientations of sections can be used.

> Thanks for your professional comment and suggestion. In the revised manuscript, we have added the information for section according to your suggestion. For detail, please see the revised manuscript.

1.3. authors write about size and quantity of cells, but appropriate quantitative methods (eg. stereology) are not mentioned. Quantitative image analysis methods should be discussed if speaking about sizes and numbers.

> Thanks for your professional comment. We totally agree with you. In the revised manuscript, we have discussed the analysis of cell size and quantity as the below.

(L335-341) Though the stereology and image analysis technology can measure the size and quantity of cells in a tissue region, they are lack in many laboratories. The paraffin and resin sections give a two-dimensional picture, leading to no way in analysing the true size and quantity of cells. However, the cells are cut randomly at their any planes, the mean size of many cells (over 100) from at least three different sections of tissue region can reflect the two-dimensional morphology parameters (length, width and area) of cells, and the ratio of chose region area to mean cell area can reflect the quantity of cells.

1.4. for quantitative analysis of cell size and number it is important how to sample. Do you have some tips how many cross-sections and longitudinal sections should one prepare?

> Thanks for your professional comment and suggestion. For quantitative analysis of cell size and number, over 100 cells from at least 3 different sections of tissue region need to be analysed. In the revised manuscript, we have added the information. Please see the above response to your comment (1.3).

1.5. in my opinion the word "kernel" should be used only for grasses, for others species, eg. oilseed rape I suggest to use the word "seed"

> Thanks for your professional comment and suggestion. We totally agree with you. For oilseed rape, the “seed” is right, not kernel. In the revised manuscript, we have changed the “kernel” to “seed”.

2. Protocol

2.1. you use 2.5% GA fixation. Did you try other fixative mixtures or concentrations and you can give some tips how to choose an optimal fixative?

> Thanks for your comment and suggestion. We try other fixatives such as Carnoy's fixative (3:1, ethanol : glacial acetic acid), and they are all OK for the sample preparation. In the revised manuscript, we have added some informaiton as the below.

(L99-100) The researchers can choose other fixative mixtures, fixative concentrations, and

fixation conditions according to their research objectives and tissue types.

2.2. do you have any experience in microwave processing for fixation and embedding? It can substantially shorten the protocol, so if you have some experience it would be very valuable.

> Thanks for your professional comment. We know the microwave processing for fixation and embedding, but have not used the method in our study. Therefore, we do not discuss the method in the present manuscript. We will try it in our coming sample preparation. Thanks for your comment and suggestion, which will help us to improve our researches.

3. Results

3.1. The tables with numeric data are not needed and should be left out. They are not relevant, since the purpose of the paper is not to solve a biological question, but only to demonstrate the usefulness of protocol. I think that the presented micrographic images in figures serve that purpose excellently. The quantitative methods are only briefly mentioned, so the quantitative results are not needed.

> Thanks for your comment and suggestion. We totally agree with you. In the revised manuscript, we have put the numeric data in Supplemental Tables for references of readers.

4. Table of materials

4.1. Table of materials at the end should include also smaller equipment (eg. flasks, tweezers) and chemicals (fixatives, solvents, dyes), this is what researchers would need the most.

> Thanks for your professional comment and suggestion. In the revised manuscript, we have added the information in Table of materials as the below.

	A	B	C
1	**Name of Material/Equipment**	**Company**	**Catalog Number**
2	Acetic acid	Sangon Biotech (Shanghai) Co., Ltd.	A501931
3	Compact glass staining jar (5-Place)	Sangon Biotech (Shanghai) Co., Ltd.	E678013
4	Coomassie brilliant blue R-250	Sangon Biotech (Shanghai) Co., Ltd.	A100472
5	Coverslip	Sangon Biotech (Shanghai) Co., Ltd.	F518211
6	Double-sided blade	Gillette Shanghai Co., Ltd.	74-S
7	Ethanol absolute	Sangon Biotech (Shanghai) Co., Ltd.	A500737
8	Flattening table	Leica	HI1220
9	Fluorescence microscope	Olympus	BX60
10	Fluorescent brightener 28	Sigma-Aldrich	910090
11	Glass strips	Leica	840031
12	Glutaraldehyde 50% solution in water	Sangon Biotech (Shanghai) Co., Ltd.	A600875
13	Glycerol	Sangon Biotech (Shanghai) Co., Ltd.	A600232
14	Iodine	Sangon Biotech (Shanghai) Co., Ltd.	A500538
15	Isopropanol	Sangon Biotech (Shanghai) Co., Ltd.	A507048
16	Light microscope	Olympus	BX53
17	LR White resin	Agar Scientific	AGR1281A
18	Oven	Shanghai Jing Hong Laboratory Instrument Co.,Ltd.	9023A
19	Potassium iodide	Sangon Biotech (Shanghai) Co., Ltd.	A100512
20	Slide	Sangon Biotech (Shanghai) Co., Ltd.	F518101
21	Tweezers	Sangon Biotech (Shanghai) Co., Ltd.	F519022
22	Sodium phosphate dibasic dodecahydrate	Sangon Biotech (Shanghai) Co., Ltd.	A607793
23	Sodium phosphate monobasic dihydrate	Sangon Biotech (Shanghai) Co., Ltd.	A502805
24	Ultramicrotome	Leica	EM UC7

5. Suggestions for improvement of video

5.1. please comment on why should one choose LR White resin

> Thanks for your professional comment and suggestion. In the revised manuscript and video, we have highlighted the reason for chose LR White resin as the below.

(L71-75) LR White resin is an acrylic resin and exhibits low viscosity and strong permeability, leading to its good applications in preparing the resin section of seeds, especially for cereal mature kernels with large volume and high starch content. In addition, the sample embedded in LR White resin can be stained easily with many chemical dyes to clearly exhibit the morphology of cells and storage materials under light or fluorescent microscope[7].

5.2. how to solve difficulties in sectioning, for example what to do if the sections crumble or tear while sectioning on the microtome

> Thanks for your professional comment and suggestion. Following the method exactly, the sample can be cut into 2 μm sections successfully. But not every section is complete and unbroken. We only choose the good sections. In the revised manuscript and video, we have added the information as the below.

(L126-127) 2.6. Add 100 μL water on an unpretreated slide, and carefully transfer the complete and unbroken section to the water with the tweezers.

(L128-129) 2.7. In order to smooth the wrinkled section, heat and dry the sample on the flattening table at 50 °C overnight.

(L130-133) Tips need attention: (1) If the section crumbles or tears, extend the time for each resin infiltration of the sample from 12 h to 24 h or 48 h. (2) If the section has some lines paralleled to the knife, clamp the sample block tightly. If the section has some lines vertical to the knife, please use a new knife.

5.3. do you use pretreated objective slides?

> Thanks for your comment. The slides are not treated before use in our study. In the revised manuscript and video, we have added the information as the below.

(L126-127) 2.6. Add 100 μL water on an unpretreated slide, and carefully transfer the complete and unbroken section to the water with the tweezers.

5.4. is it possible in the final video to write out the chemical names and concentrations during the embedding protocol, and dye names besides microscopic images?

> Thanks for your professional comment and suggestion. In the revised video, we have added the information according to your suggestion. For detail, please see the revised video.

Responses to comments of reviewer 2

Comment: Manuscript Summary: In summary, the reported technique is of value, and worth being published. But, the manuscript is not well written so that it marginalized the significance of the procedure. One could not see clearly where the improvement or specific merit of the reported technique as compared with many other resin methods in use. If the language is the issue, the authors should seek professional help! The manuscript needs revision with proper and logical arguments.

> Thank you very much for your careful review, professional comment, and valuable correction and suggestion. We have made a major revision and uploaded it with major changes in RED.

1. Major Concerns

The reported technique is definitely worth being published in JoVE. However, the manuscript needs to be almost completely re-written to reach the publication quality and conform to the JoVE standards, although it is already a revision. The current version did not even describe the technique correctly!

> Thanks for your comment and suggestion. In the revised manuscript, we have rewritten some Sections, especially for Section of Abstract, Introduction and Protocol. For detail, please see the revised manuscript.

2. The following are some specific issues.

2.1. The technique is not about "sectioning of whole kernel", as the kernel was sliced longitudinally and transversely into large pieces (Procedure 1.2) for complete infiltration. The technique, which should also what the authors meant, is about obtaining whole-kernel-sized sections (or slices). For this purpose, there's no problem to do the longitudinal and transverse partition for proper infiltration, which would still allow obtaining the whole-kernel-sized sections. An example of a proper title should be " a simple method for obtaining whole-kernel-sized sections and its applications". The authors need to be careful using the "sectioning of whole kernel" in all the text. The technique did not, nor needs to, section an intact whole kernel. Rather it did the whole-kernel-sized sectioning of kernel pieces.

> Thanks for your professional comment and suggestion. We totally agree with you. We prepare the whole-seed-sized sections, not sections of whole seed. In the revised manuscript, we have changed the wrong expression. The title of manuscript has been changed as the below.

Title: A Simple Dry Sectioning Method for Obtaining Whole-seed-sized Resin Section and Its Applications

2.2. The abstract is lengthy, but didn't clearly spell out the most important, i.e. what this technique is different from current ones and why it is important. This is also the problem

in the introduction. It made it as if the authors themselves don't have a clue why it is good, and worth being published.

> Thanks for your professional comment. In the revised manuscript, we have written the Section of Abstract and Introduction to highlight the difference and importance of the present method. For detail, please see the revised manuscript.

2.3. The authors mixed up the anatomical parts of some cereal crops in using the expression "kernels from cereal crops such as rice, wheat, barley and maize and some dicotyledon plants such as oilseed rape". One may use "grains" for rice, wheat and barley, but can in no way use "kernel" for oilseed rape. For an anatomical technique, this doesn't look good!

> Thanks for your professional comment. We totally agree with you. For oilseed rape, the "seed" is right, not kernel. In the revised manuscript, we have changed the "kernel" to "seed". For detail changes, please see the revised manuscript.

2.4. In the introduction, the two sentences (line 61-63) are logically contradictory in addition to a distorted citation (The authors should at least cite faithfully what others meant). The paraffin embedding and sectioning could obtain only thicker sections of relatively good integrity for the size of a whole kernel. This is the drawback of the paraffin embedding, and the reason why the reported simple method is needed for thin and intact whole-kernel-sized sections. The authors missed the most important point. In addition, as the sectioning technique is relatively mature one, there're many excellent ones for each type, paraffin or resin-embedded. If one wants to convince people of the excellence or usefulness of their technique, piling-up of own published works is not going to make a good argument! The authors need to do a much better job for the introduction.

> Thanks for your professional comment and suggestion. In the revised manuscript, we have rewritten the Section of Introduction as the below.

(L62-71) The paraffin section is a good method to prepare the whole-seed-sized section, and can exhibit the tissue structure of seed and the accumulation of storage material in different regions of seed[4-6]. However, the paraffin sections usually have 6~8 μm thickness with low resolution, leading to that it is very difficult to clearly obverse and quantitatively analyze the morphology of cell and storage materials. The resin sections usually have 1~2 μm thickness and high resolution, and are very suitable to observe and analyze the morphology of cell and storage materials[7]. However, the routine resin sectioning method has difficulty in preparing the whole-seed-sized section, especially for some seeds with large volume and high starch content, leading to that it is no way to observe and analyze the morphology of cells and storage materials in different regions of seed.

2.5. The authors' claim about the shortcoming of other resin-based method was that "the whole kernel is sliced into small sample blocks for routine resin sample preparation". This argument is futile, to be frank, as the authors themselves did slice the kernel longitudinally and transversely in order to get a complete infiltration, albeit perhaps larger chunks than

others. The authors have to come up with a logical argument why their resin method is superior or simpler than those currently in wide uses! If not, it is not qualified for a full publication on JoVE.

> Thanks for your professional comments. We agree with you. In the revised manuscript, we have rewritten the Section of Abstract and Introduction. For detail, please see the revised manuscript.

2.6. The protocol is written in complete passive voice for all the steps, which is not to the standards of JoVe. Given an example, the 1.1 section may be better written as follows: " Fix a mature kernel in sufficient 2.5% phosphate-buffered glutaraldehyde (pH 7.2) at 4 °C for 48 h.

> Thanks for your professional comment. In the revised manuscript, we have rewritten the Section of Protocol using imperative tense, and avoid usage of passive voice. For detail, please see the revised manuscript.

Supplementary Table 1

Region [b]	Area (μm^2)	LAL (μm) [c]	SAL (μm) [c]	Roundness [c]
Region 1	1621.2±109.8d	51.2±2.2a	41.0±1.4c	1.24±0.01a
Region 2	827.8±45.3ab	40.9±2.3a	26.6±0.6b	1.39±0.04a
Region 3	686.9±28.4a	48.0±1.3a	18.8±1.2a	1.79±0.12b
Region 4	1076.2±186.2c	72.4±10.4b	18.8±0.7a	2.27±0.19c
Region 5	963.6±14.5bc	47.1±1.9a	27.5±1.2b	1.46±0.09a

Supplementary Table 2

Region [b]	Area (μm^2)	LAL (μm) [c]	SAL (μm) [c]	Roundness [c]
Region 1	1593.4±135.1a	54.8±3.8a	38.2±1.0a	1.44±0.07a
Region 2	6275.2±814.3c	157.4±11.1c	57.8±0.9b	2.30±0.06b
Region 3	4517.6±266.7b	120.7±0.3b	57.6±4.0b	2.17±0.08b
Region 4	10827.3±51.8d	207.3±7.7d	72.1±0.4c	2.26±0.24b

Supplementary Table 3

Region [b]	Area (µm^2)	LAL (µm) [c]	SAL (µm) [c]	Roundness [c]
Region 1	27.5±4.2a	6.2±0.5a	5.3±0.5a	1.19±0.01a
Region 2	121.5±8.4c	14.3±0.3c	11.4±0.5c	1.33±0.03b
Region 3	80.8±9.0b	11.2±0.4b	9.1±0.7b	1.21±0.00a
Region 4	26.9±5.4a	6.4±0.7a	5.3±0.5a	1.21±0.04a

Supplementary Table 4

Region [b]	Area (μm^2)	Roundness [c]	Number (/cell)	Area index [c]
Region C	20.4±0.9a	1.27±0.04b	27.5±3.3b	0.35±0.03b
Region D	25.4±1.4a	1.24±0.02b	8.4±1.7a	0.26±0.03a
Region E	24.1±4.2a	1.26±0.01b	8.0±0.9a	0.28±0.01a
Region F	23.5±2.8a	1.24±0.04b	12.2±2.5a	0.27±0.03a
Region G	25.8±3.1a	1.17±0.01a	11.5±0.2a	0.31±0.03ab