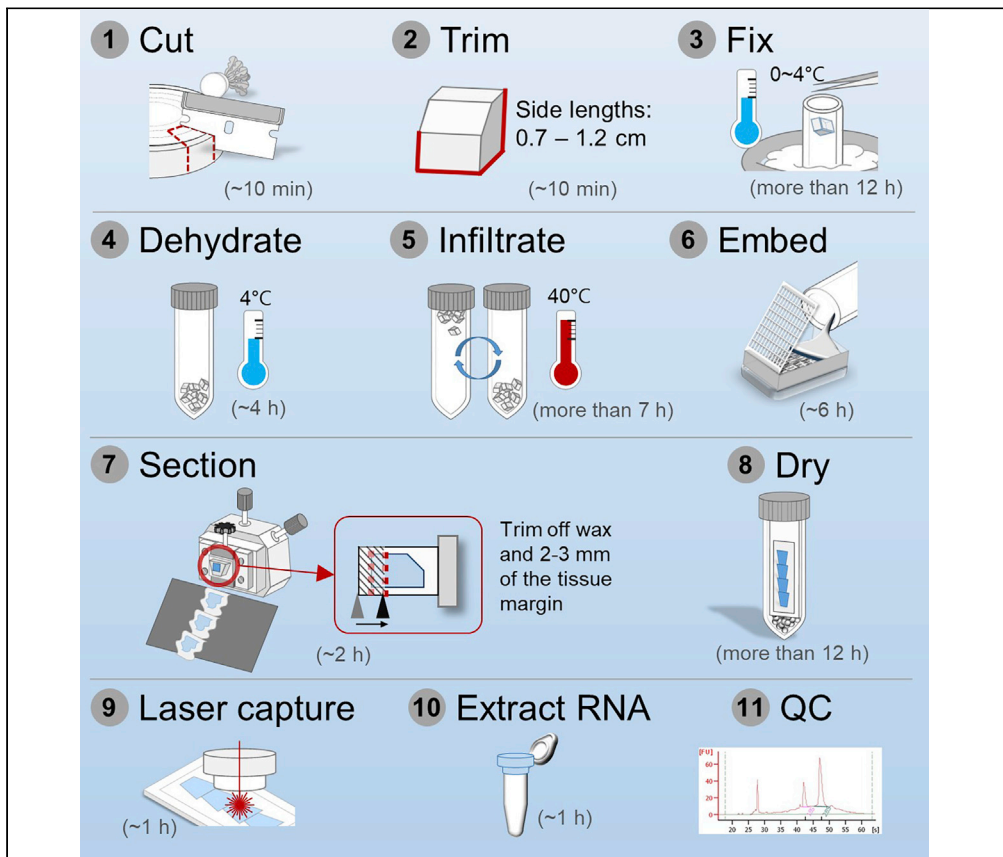


Protocol

An Optimized Protocol of Laser Capture Microdissection for Tissue-Specific RNA Profiling in a Radish Tap Root



Choe, Hoang, and Lee describe the stepwise protocol for laser capture microdissections of tissues in radish tap roots, focusing on how to prepare target tissues for dissection while keeping RNAs intact for next-generation sequencing. This method enabled identifying both novel and annotated protein-coding genes from de-novo assembled transcripts as described in [Hoang et al. \(2020\)](#).

Goh Choe, Nam V.
Hoang, Ji-Young
Lee

gohchoe@kaist.ac.kr
(G.C.)
jl924@snu.ac.kr (J.-Y.L.)

HIGHLIGHTS

Laser capture microdissection (LCM) for capturing tissues in radish tap roots

Steedman's wax embedding method for samples incompatible with cryo-sectioning

High-quality tissue-specific RNAs suitable for next-generation sequencing analysis

Choe et al., STAR Protocols 1,
100110
December 18, 2020 © 2020
The Author(s).
<https://doi.org/10.1016/j.xpro.2020.100110>



Protocol

An Optimized Protocol of Laser Capture Microdissection for Tissue-Specific RNA Profiling in a Radish Tap Root

Goh Choe,^{1,2,3,*} Nam V. Hoang,¹ and Ji-Young Lee^{1,4,*}¹School of Biological Sciences, Seoul National University, Seoul 08826, Korea²Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 34141, Korea³Technical Contact⁴Lead Contact*Correspondence: gohchoe@kaist.ac.kr (G.C.), jl924@snu.ac.kr (J.-Y.L.)
<https://doi.org/10.1016/j.xpro.2020.100110>

SUMMARY

Laser capture microdissection (LCM) coupled with transcriptome profiling is a powerful technique that allows for tissue-specific gene expression analysis in a complex system. One major challenge in using this technique is to obtain RNA without compromising its integrity. Here, we present a protocol optimized for radish root tissue sections using Steedman's wax embedding to obtain high-quality RNA suitable for next-generation sequencing analysis.

For complete details on the use and execution of this protocol, please refer to Hoang et al. (2020).

BEFORE YOU BEGIN

LCM technique on plant tissues was first used in a study of gene expression in the rice phloem (Asano et al., 2002). Since then, LCM coupled with RNA sequencing (RNA-seq) has been applied successfully in transcript profiling of diverse cell or tissue types from multiple species including several herbaceous and woody plants (Blokchina et al., 2016, Roux et al., 2014, Jardinaud et al., 2016). However, for different tissue types, LCM requires optimizations to obtain high-quality RNAs that are suitable for subsequent sequencing. As a standard metric for RNA quality assessment, RNA Integrity Number (RIN) devised by Agilent Technologies is widely used and the minimum recommended RIN threshold for RNA-seq is 7.0 (Espina et al., 2006, Schroeder et al., 2006, Jaffe et al., 2017).

This protocol, an adaptation of LCM protocol used for developing barley seeds (Thiel et al., 2011), describes a detailed method for isolating high-quality RNA from wax-embedded and laser dissected tissues from radish tap roots. Sectioning of wax-embedded tissue blocks was chosen over cryo-sectioning because high water contents (~95%) of radish tap roots make the frozen tissue blocks too hard to be cut with cryo-sectioning. To obtain high-quality RNA samples from wax-embedded tissues, we tested several existing tissue processing protocols, which include (1) testing different fixatives and sample preservatives such as acetone, methanol, formaldehyde, and RNAlater; (2) testing different embedding media including polyethylene glycol (PEG) and paraplast; and (3) testing different tissue sampling depth in a tissue block and different sizes of tissue blocks. Using tissue blocks prepared with Farmer's fixative solution and Steedman's wax, we could obtain high-quality RNAs with RINs higher than seven and successfully construct high-quality tissue-specific transcriptome data for radish tap roots at different developmental stages.



△ **CRITICAL:** Always work in an RNase-free environment. Use RNaseZap or other commercially available cleaner solution. The use of RNase-free disposable plasticware and filter-tips is recommended. Wear gloves, clean with RNaseZap, and change them frequently.

Note: This protocol is written for sample volume appropriate for the use of a single 50 mL conical tube.

Refer to “[Materials and Equipment](#)” to prepare solutions, materials, and tools prior to the procedure.

Note: In this protocol, we will focus on tissue preparation process more in detail, and briefly describe LCM and RNA extraction processes. For the latter two, follow the manufacturer’s instructions and be familiarized with the required processes before starting the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Glacial acetic acid	Merck	Cat# 1.00063
1-Hexadecanol	Merck	Cat# 258741
Ethanol	Merck	Cat# 1.00983
Diethyl pyrocarbonate (DEPC)	Merck	Cat# D5758-100ML
Polyethylene glycol (PEG) distearate	Merck	Cat# 305413
RNaseZap	Thermo Fisher Scientific	Cat# AM9780
RNase-Free DNase	Qiagen	Cat# 79254
Critical Commercial Assays		
PicoPure RNA Isolation Kit	Thermo Fisher Scientific	Cat# KIT0204
RNA 6000 Pico chip	Agilent Technologies	Cat# 5067-1513
Other		
CapSure Macro LCM Caps	Thermo Fisher Scientific	Cat# LCM0211
0.5 mL microcentrifuge tubes	Thermo Fisher Scientific	Cat# N8010611
PEN Membrane Glass Slides	Thermo Fisher Scientific	Cat# LCM0522
Heating oven (40°C)	N/A	N/A
Heating oven (60°C)	N/A	N/A
Heating plate	N/A	N/A
Arcturus Veritas LCM microdissection system	Thermo Fisher Scientific	N/A
Disposable embedding cassette	Simport Scientific	Cat# M512
Disposable base mold	Simport Scientific	Cat# M475-10
Rotary microtome	Leica	Cat# RM2255
2100 Bioanalyzer system	Agilent Technologies	N/A
Centrifuge	N/A	N/A
Falcon 50 mL conical tube	BD Biosciences	Cat# 352070
Experimental Models: Organisms/Strains		
Radish (<i>Raphanus sativus</i>)	National Institute of Horticultural and Herbal Science (NIHHS), Republic of Korea	N/A

MATERIALS AND EQUIPMENT

Alternatives: This protocol uses an Arcturus Veritas LCM microdissection system with CapSure Macro LCM caps for collecting dissected tissues. Other popular types of laser microdissection equipment are Zeiss PALM MicroBeam and Leica LMD systems. These systems also use membrane slides for sampling tissue sections. Briefly, tissue areas of interests are dissected by UV laser, then different technologies are employed to collect dissected tissue segments. Arcturus Veritas system uses an adhesive lid to capture tissue samples. Zeiss PALM MicroBeam system utilizes laser light to catapult dissected tissues, while Leica LMD simply drops tissue sections by gravity (Espina et al., 2006). Therefore, our tissue preparation protocol can be applied to these systems with minor adaptations if required. If you need to use LCM machine different from Arcturus Veritas system, prepare tissue sections using this protocol, then perform LCM following the manufacturer's instruction of the system you have.

Note: Currently, Arcturus Veritas instrument used in this protocol has been discontinued and replaced by Arcturus XT system.

Note: For embedding cassette and mold, you can use products from different suppliers with appropriate sizes for your samples. When we perform embedding, if needed, we cut the middle plastic frame to make more room for tissue blocks.

Ethanol-Acetic Acid (EAA) Farmer's Fixative Solution

⌚ Timing: ~30 min

Reagent	Final Concentration	Volume (mL)
Absolute ethanol	75% (v/v)	75
Glacial acetic acid	25% (v/v)	25
Total	N/A	100

Prepare this solution fresh right before use. Make a 45 mL aliquot, and chill on ice for 20 min. Remaining EAA fixative can be stored either on ice or at 4°C until use.

DEPC-Treated Water (0.1%)

⌚ Timing: 4–5 h

Reagent	Final Concentration	Volume (mL)
DEPC	0.1% (v/v)	1
DW	99.9% (v/v)	999
Total	N/A	1,000

Mix well, incubate at 22°C–25°C in a fume hood for at least 2 h, autoclave, and cool down. DEPC-treated water can be stored at 22°C–25°C.

Diluted Ethanol Series Solution

Solution	Reagents	Final Volume (mL)
75% ethanol in DEPC-treated water	Mix 150 mL of absolute ethanol and 50 mL of DEPC-treated water	200
90% ethanol in DEPC-treated water	Mix 45 mL of absolute ethanol and 5 mL of DEPC-treated water	50

Store the prepared ethanol series at 4°C.

Steedman's Wax

Reagent	Final Concentration	Weight (g)
Polyethylene glycol (PEG) distearate	90% (w/w)	450
1-Hexadecanol	10% (w/w)	50
Total	N/A	500

Melt PEG and 1-hexadecanol separately at 60°C and mix them well by stirring at 60°C for about 3 h. Prepared mixture can be used immediately or can be stored at 22°C–25°C until use. There was no significant difference in the result when we used Steedman's wax stored up to 4 weeks. However, we do not recommend prolonged storage of Steedman's wax at high temperature as molten solution or multiple cycles of re-melting.

Note: In the original methods (Norenburg and Barrett, 1987, Steedman, 1957), Steedman's wax was prepared using PEG 400 distearate and 1-hexadecanol. In this protocol, PEG distearate (mw ~930) from Sigma-Aldrich (cat. 305413) was used.

Diluted Steedman's Wax Series

Heat ethanol to 40°C in an oven set at 40°C before combining it with Steedman's wax as shown below.

⚠ **CRITICAL:** Heating ethanol in the oven set beyond 70°C can cause fire.

Solution	Reagents	Final Volume (mL)
25% Steedman's wax in ethanol	Mix 12.5 mL of Steedman's wax and 37.5 mL of absolute ethanol	50
50% Steedman's wax in ethanol	Mix 25 mL of Steedman's wax and 25 mL of absolute ethanol	50
75% Steedman's wax in ethanol	Mix 37.5 mL of Steedman's wax and 12.5 mL of absolute ethanol	50

Store the prepared Steedman's wax series at 40°C until use.

RNase-free Materials

Bake metal forceps, spatulas, knives and glassware at 180°C–200°C for 3–4 h. Autoclave Kimwipes and put them in a drying oven. Treat plastic mold cassettes and base molds with RNaseZap, rinse with DEPC-treated water three times, dry, and store until use.

RNase-free Membrane Glass Slides

To prepare RNase-free membrane slides, treat membrane glass slides with RNaseZap, wash three times with DEPC-treated water, dry at 22°C–25°C, and store in an RNase-free container at 22°C–25°C until use

- △ **CRITICAL:** If you use forceps to move membrane slides, be careful not to damage the coated membrane over the slide.

STEP-BY-STEP METHOD DETAILS

Tissue Fixation and Processing – Day 1

⌚ **Timing:** ~3.5 h

This section describes the fixation step to preserve tissue architecture and cellular contents including macromolecules. LCM depends on histological features for sample preparation and requires multiple steps which could degrade RNA molecules very easily. Therefore, it is important to fix sample properly and quickly to minimize morphological distortion and sample deterioration.

1. Harvest radish roots from the soil, wash them with tap water, and dry them off with paper towel.
2. Cut root into a large piece using a kitchen knife, and trim into tissue blocks of approximately 1.2×1.2×1.2 cm³ in size using a razor blade (Figures 1A–1C).

- △ **CRITICAL:** We recommend preparing for tissue blocks longer than 7 mm and less than 1.2 cm on each side. This is because ~2 mm of the block height should be trimmed off before collecting sections for LCM. In our hands, tissue sections exposed to the outside gave poor quality of RNA with a RIN less than 7.0. Trimming off the outer part and using the inner sections for LCM greatly enhanced RNA quality. Even though the block size can depend on the target tissue area or the radish root size used for sampling, we recommend not to make a tissue block too big (i.e., >1.2 cm each side). Making a tissue block too big results in poor RNA quality due to incomplete fixation and wax infiltration.

Note: We suggest trimming off one side of the opposite phase of the LCM to mark a block orientation. It helps positioning a tissue block in the mold in a right orientation.

3. Place dissected root tissue blocks immediately into ice-cold Farmer's fixative (Figure 1D).
4. Incubate samples at 4°C for 3 h. During the incubation, mix samples by inverting the tube about five times every hour.
5. Replace with fresh cold Farmer's fixative and incubate at 4°C for at least 12 h.

Note: Make sure that the fixative is chilled completely before use. A 20-min ice incubation is usually enough for a small volume (e.g., 45 mL) of fixative, but incubation time can be extended depending on the amount of fixative prepared.

- △ **CRITICAL:** Preparing for tissue blocks should be performed quickly to minimize RNA degradation. Transfer each trimmed tissue block into cold fixative immediately rather than waiting for others to be fixed altogether.

- △ **CRITICAL:** The volume of fixative should be at least ten times the volume of the tissue prepared. For instance, for an amount of 45 mL of fixative, fill tissue blocks up to 5 mL mark of a 50 mL tube.

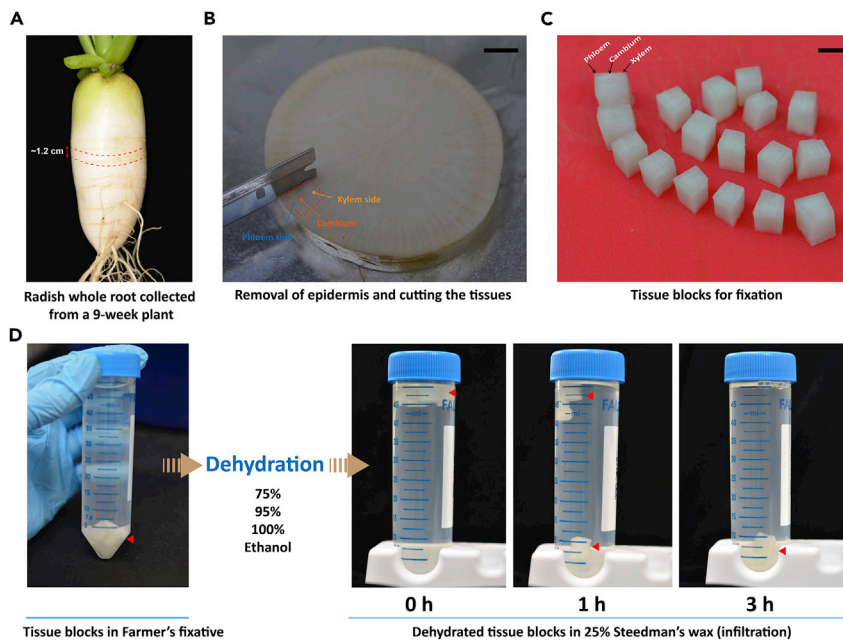


Figure 1. Radish Root Tissue Block Preparation, Sample Fixation, Dehydration and Infiltration

(A) A whole tap root from a 9-week old radish plant was collected and washed, and then a ~1.2 cm thick cross-section was cut out for the next steps.

(B and C) (B) The root epidermis was peeled and tissue blocks of maximum $1.2 \times 1.2 \times 1.2 \text{ cm}^3$ were excised and shown in (C). Each tissue block included cambium layer and surrounding tissues (cortex on the phloem side and parenchyma on the xylem side). Dashed rectangle denotes a tissue block. Scale bars in (B) and (C), 1 cm.

(D) The tissue blocks were immediately transferred to chilled Farmer's fixative solution in a 50 mL conical tube, dehydrated with ethanol, and infiltrated with Steedman's wax. For visualization, tissue blocks infiltrated in 25% Steedman's wax after 0 h, 1 h, and 3 h were shown. Red arrows in (D) point to tissue blocks.

See also [Methods Videos S1](#) and [S2](#).

Dehydration and Infiltration – Day 2

⌚ Timing: ~7 h

Steps 6 to 8 describe how to remove water from tissue, and fill the tissue spaces with embedding medium to provide a firm support during the process of microtome sectioning.

6. Dehydrating tissue blocks (~4 h).
 - a. Replace Farmer's fixative with 45 mL of 75% ice-cold ethanol, mix by inverting the tube about five times, and incubate on ice for 15 min.
 - b. Repeat **step 6a** additional three times.
 - c. Replace with 45 mL of ice-cold 90% ethanol, mix well by inverting the tube about five times, and incubate at 4°C for 1 h.
 - d. Replace with 45 mL of ice-cold absolute ethanol, mix well by inverting the tube about five times, and place the tube at 4°C for 1 h.
 - e. Replace with 45 mL of 22°C–25°C-stored absolute ethanol, mix well by inverting the tube about five times, and place the tube at 40°C for 1 h to gradually raise the sample temperature to 40°C.
7. Infiltrating Steedman's wax (~3 h).
 - a. Replace absolute ethanol with 45 mL of 25% Steedman's wax, mix well by inverting the tube about five times, incubate at 40°C for 3 h (tissue blocks should drop to the bottom) ([Figure 1D](#)).

- b. Replace with 45 mL of 50% Steedman's wax, mix well by inverting the tube several times, and incubate at 40°C for at least 12 h.

Note: You can continue and finish **step 8** on Day 2. In that case, incubate samples in 50% Steedman's wax in **step 7b** for 2 h or until the tissue blocks drop to the bottom of the tube.

△ **CRITICAL:** The time required for infiltration is dependent on the block size and the tissue characteristics such as density and size of constituting cells. Check the speed of the fall of tissue blocks in the embedding medium when the tube is turned upside down. Well-infiltrated samples drop immediately to the bottom of the tube when flipped ([Methods Video S1](#)).

Infiltration (Continued) – Day 3

⌚ **Timing:** ~4 h

8. Infiltrating Steedman's wax continued (~4 h).
 - a. Replace with 45 mL of 75% Steedman's wax, mix well by inverting the tube several times, and incubate at 40°C for minimum 2 h or until the tissue blocks drop to the bottom of the tube.
 - b. Replace with 45 mL of 100% Steedman's wax, mix well by inverting the tube several times, and incubate at 40°C for minimum 2 h or until the tissue blocks drop to the bottom of the tube.
 - c. Replace with 45 mL of 100% Steedman's wax, mix well by inverting the tube several times, and incubate at 40°C for at least 12 h.

Note: The higher concentration the Steedman's wax is, the longer time it takes for a tissue block to be well-infiltrated.

Note: In a high concentration of Steedman's wax, the falling speed of a well-infiltrated tissue block is slower than that in a lower concentration. For examples, refer to [Methods Videos S1](#) and [S2](#) (in Steedman's wax 25% and 100%, respectively)

Embedding – Day 4

⌚ **Timing:** ~6 h

Here, we describe how to embed and solidify the infiltrated tissue blocks (obtained from **step 8**) using Steedman's wax for sectioning.

9. Replace with 45 mL of 100% Steedman's wax one more time, mix well by inverting the tube several times, and incubate at 40°C for another 3 h.
10. Embed tissue blocks and solidify for sectioning ([Figure 2A](#)).
 - a. Place and arrange the orientation of tissue blocks in a mold.
 - b. Fill up the mold with 100% Steedman's wax.
 - c. Solidify at 22°C–25°C for about 3 h.
 - d. Store at 4°C in an air-tight container for at least 12 h.

⏸ **Pause Point:** The embedded tissue blocks can be stored at 4°C for up to a week.

Optional: It is not comfortable to handle tissue blocks in a conical tube. Set heating plate to 50°C prior to use. Samples can be transferred to a wide-mouth container such as an aluminum foil boat, and place on the heating plate for easy access. You still need to work very quickly not to let the tissue block be solidified.

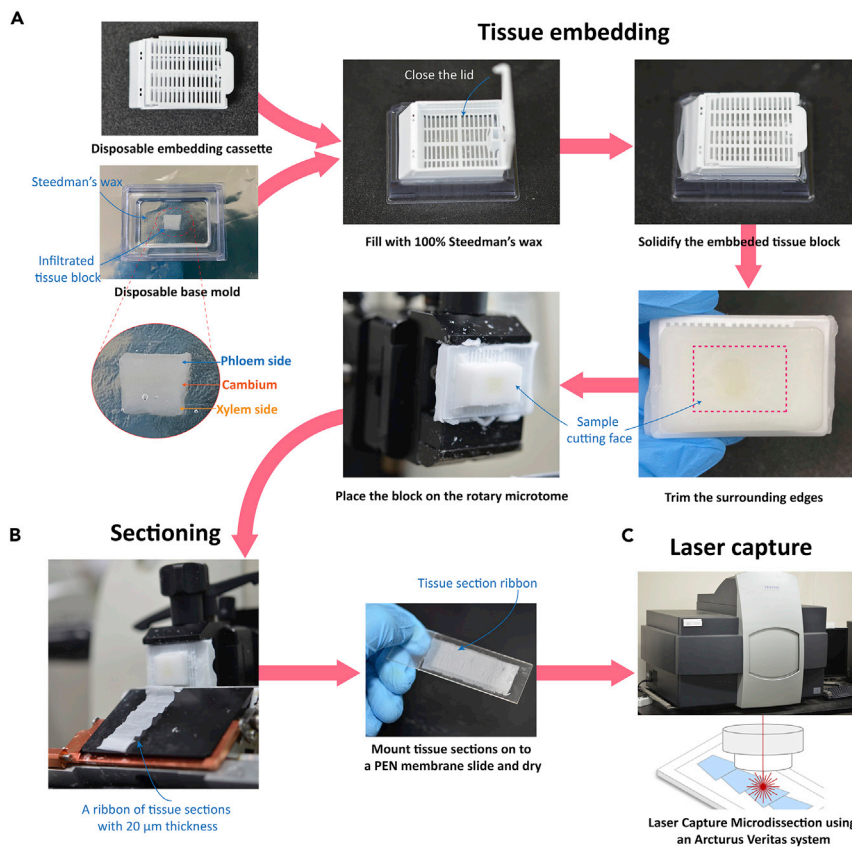


Figure 2. Embedding, Sectioning and Laser Capture Microdissection (LCM) of Radish Root Tissue Samples

(A) An infiltrated tissue block in a mold filled with 100% Steedman's wax. The sample orientation is shown in the bottom of the image. The image was taken from the side of sample which was subsequently attached to the embedding cassette. Once, the embedded tissue block was solidified and the surrounding wax was trimmed, the tissue block was placed on a rotary microtome for sectioning.

(B) A ribbon of tissue with 20 µm thickness was sectioned and mounted on a PEN membrane slide and dried in a 50 mL conical tube.

(C) Laser Capture Microdissection using an Arcturus Veritas system. A UV laser was used to define the tissue areas corresponding to cambium, phloem cortex, and xylem parenchyma, which were subsequently microdissected (see Figure 3).

Note: When multiple tissue blocks are placed in a single mold, arrange them parallel in the same orientation. CapSure Cap used for the LCM step has a limited space (circular, 5 mm in diameter). If the region of interest of your sample has a specific orientation, that will help to line up multiple cell files within a small capture area. It will also maximize RNA yield.

⚠ **CRITICAL:** You must work quickly, placing the samples in the mold before the wax starts to solidify.

Tissue Sectioning – Day 5

⌚ **Timing:** ~2 h

In this step, an embedded tissue block is cut into thin sections on a microtome and mounted on PEN membrane slides.

11. Equilibrate a tissue block at 22°C–25°C for 1 h.

12. Sectioning the tissue block (Figure 2B).
 - a. Trim the surrounding edges using a razor blade.
 - b. Place the block on the rotary microtome, set thickness setting at 20 μm , and trim the block until you reach and get a full face of the tissue.
 - c. Trim off additional 100–150 sections.
 - d. Make a ribbon of tissue sections with 20 μm thickness.

△ **CRITICAL:** Trim off 2–3 mm of the outer part of the tissue block. In our experience, RNA extracted from the outer margin of a tissue block mostly has severe degradation.

Note: Sample section thickness can be adjusted differently. Thin section results in low RNA yield. On the other hand, a thick section may not be properly captured on the LCM cap. LCM is commonly performed with section thickness of 2–15 μm . 20 μm sections used for radish tap root had been successively captured using the settings on step 16. For other section thicknesses, UV cutting and IR wetting need to be optimized accordingly. Thick sections may also require multiple UV cutting with a high power setting. RNA degradation may occur with repeated exposure to UV laser in the region close to the cutting paths.

Note: When you mount tissue sections on the PEN membrane slide, place them in the middle membrane coated part of the slide. Tissues on the edges of the slide cannot be captured. If you look at the membrane slide by slightly turning up and down, you can easily tell a rectangular area in the middle where the angle of light reflection is different from the other part of the slide.

13. Mounting embedded tissue on a slide (Figure 2C).
 - a. Place a suitable length of ribbon sections on a membrane slide.
 - b. Place DEPC-treated water underneath the sample ribbon to stretch and straighten the sample sections.
 - c. Wick away excess water using autoclaved filter paper or Kimwipes.

Note: Remove excess water carefully not to disturb sample arrangement.

Optional: You could use regular glass slides for mounting dissected tissues and perform LCM using IR laser. However, membrane slides allow dissecting and capturing large area at once using UV and IR laser, which can save tissue processing time and increase capture efficiency greatly.

14. Place the slide in an air-tight container containing silica balls and dry them for at least 12 h.

△ **CRITICAL:** Wick away excess water from the sample slide as much as you can before placing it into a container with silica balls. It is critical to completely dry the tissue samples on the membrane slide.

△ **CRITICAL:** Silica balls and debris should not touch the sample area. They stick to sample easily and interfere sample transfer to CapSure LCM cap. Use silica balls without dust particles or any small debris. If you use a 50 mL conical tube, fill up with silica balls carefully to a lower level than the conical part (i.e., below the 5 mL mark). Membrane glass slide fits only in the cylinder part of a 50 mL tube.

Note: We recommend for LCM without delay. Although dried tissue sections might preserve RNAs in an air-tight container, RNAs in thin sections are more prone to degradation by RNases in the air or spontaneous oxidation than those in tissue blocks.

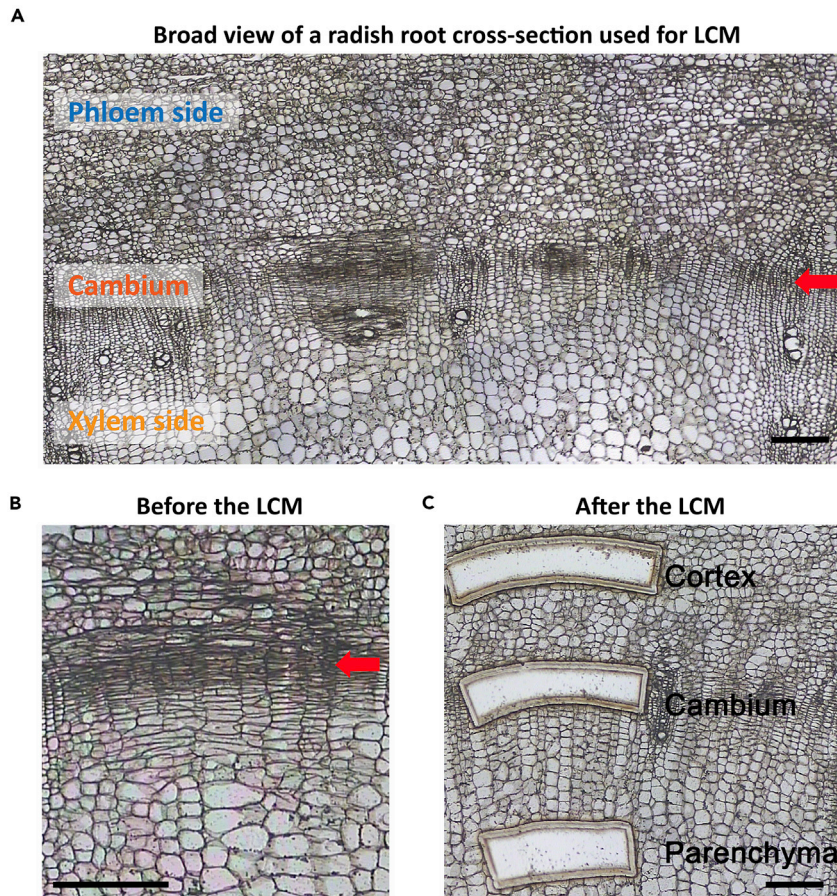


Figure 3. Microscope Views of Radish Root Cross-Sections Used for Laser Capture Microdissection (LCM)

(A and B) Broad and magnified views of radish root cross-sections captured before the LCM. Red arrows point to cambium cell layers.

(C) Radish root cross-section captured after the LCM with tissue regions of interest marked. These include phloem cortex, cambium, and xylem parenchyma. Scale bars, 200 μm .

LCM and RNA Extraction – Day 6

⌚ Timing: ~2.5 h

This section describes how to perform LCM to isolate a tissue region of interest, and subsequently extract RNA from the captured tissue (Figure 2C). Figure 3 shows tissue images of a radish root section, captured before and after the LCM.

15. Removing wax from the tissue sections (~25 min).

⚠ **CRITICAL:** If necessary, turn on the LCM instrument and warm up the system to make the system ready for operation. For Arcturus Veritas LCM, turn on the instrument before starting this step.

- a. Put your sample slide into a 50 mL conical tube filled with 45 mL or more absolute ethanol and incubate for 10 min.
- b. Transfer the slide to a new 50 mL conical tube filled with fresh absolute ethanol and incubate for 10 min.

c. Dry out the slide by leaving it at 22°C–25°C for about 3 min.

Note: If required, mix gently by tilting the tube 2–3 times while incubating.

△ **CRITICAL:** Slide should be dried completely. Wet tissue samples would not be captured on the Capsure LCM caps.

16. Collecting tissue samples using LCM (~1 h).

- Load CapSure LCM caps and sample slides on the slide station.
- Setup parameters of the laser (see note below).
- Highlight regions of interest using drawing tools.
- Fill the sampling area in the CapSure LCM cap by cutting and capturing the highlighted area (Figure 3C).
- Unload the CapSure LCM caps after tissue capture and move on to the next step immediately.

△ **CRITICAL:** Finish tissue capturing within 2 h. We normally finish sampling within 1 h. A small amount of nuclease in the air would degrade RNA in your sample.

Note: We used IR laser setting (power: 97 mv, pulse: 7,500 μ s, No of hit: 3, intensity 200 mV) and UV laser power 87.00. The settings may need to be optimized for each sample slide. Try test firing of the IR and UV lasers to check the laser focus and wetting in a blank area. To ensure capturing large areas (e.g., larger than about 1 mm), we added more than three points of IR capture laser marks in multiple positions in addition to the points marked automatically.

Note: CapSure Macro LCM cap provides a circular sampling space with 5 mm in diameter. If possible, dissect the areas of interests similar in shape to maximize the amount of sample collected and the yield of RNA.

Note: Detailed instructions for the LCM and software operation are available in the instrument user guide provided by the manufacturer.

17. Extracting RNA using PicoPure RNA isolation kit (~1 h).

- Insert CapSure LCM cap on a 0.5 mL tube filled with 50 μ L of Extraction Buffer and incubate the tube in inverted position at 42°C for 30 min.
- Add 50 μ L of 70% ethanol and mix well.
- Pass the cell extract mixture through an activated RNA purification column from the kit.
- Wash the spin column with 100 μ L of Wash Buffer 1 by centrifuging at 8,000 \times g for 1 min.
- Add DNase I solution mixture directly onto the spin column membrane and incubate for 15 min.
- Wash the spin column twice with Wash Buffer 2 by centrifuging at 16,000 \times g for 2 min.
- Elute with 11 μ L of Elution Buffer.
- Aliquot 2 μ L for bioanalyzer analysis and store the remaining RNA at –80°C until use. [Troubleshooting 1](#) and [2](#).

Note: This is very succinct outline of RNA preparation procedure. For detailed procedure for extracting RNA, follow the manufacturer's instructions.

EXPECTED OUTCOMES

Representative quality assessments of RNA samples extracted from three different tissue regions of radish root sections analyzed by an Agilent 2100 bioanalyzer are presented in [Figures 4A–4C](#). All

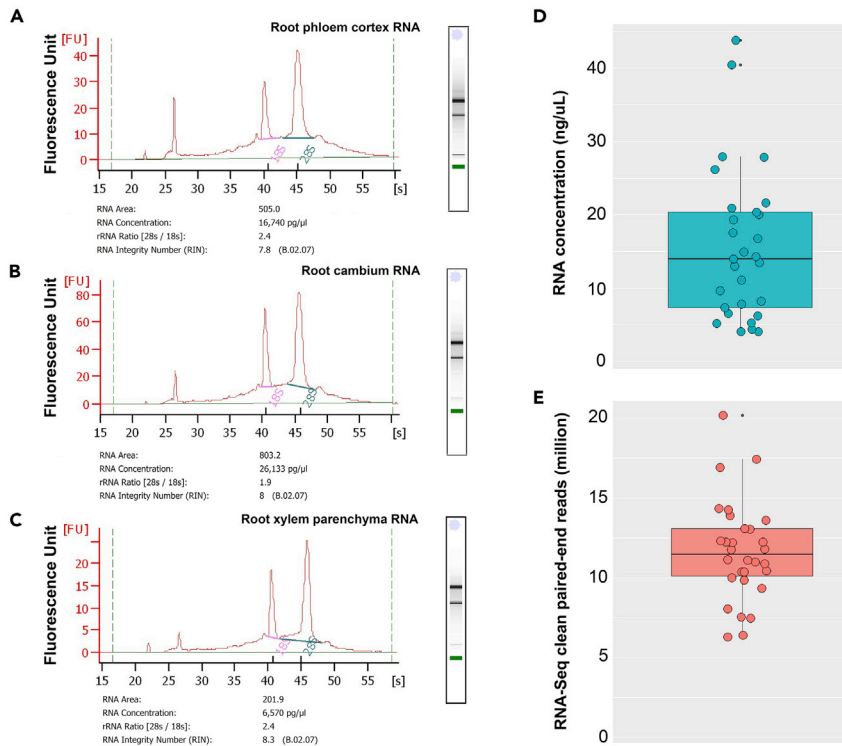


Figure 4. Quality Assessment of RNA Isolated from Laser Capture Microdissected Radish Root Samples

(A–C) Electropherograms and RNA Integrity Number (RIN) of three representative samples from phloem cortex, cambium, and xylem parenchyma tissues analyzed by an Agilent 2100 bioanalyzer. The plot displays fluorescence intensity versus migration time. RNA yield and integrity measured by RIN value are shown below each plot. (D and E) Statistical summary of RNA extraction and sequencing results based on a total of 33 LCM-derived RNA samples reported in Hoang et al. (2020). Each dot represents one sample. For visualization, samples with high concentration (above 45 ng/μL) in (D) and read numbers above 25 million in (E) are not shown.

three samples show electropherogram profiles of high-quality RNA with a RIN value over 7.0. The yield of RNA was greatly dependent on the number of cells captured in each CapSure LCM cap. We obtained an average of ~15 ng/μL of RNA per sample (Figure 4D) and an average total RNA yield of ~150 ng (more than 60% of the sampling space of individual cap had been filled by captured cells). In this, we found that phloem cortex and xylem parenchyma cells, which are larger than cambial cells (Figure 3), tend to generate lower RNA yields than that of cambia, for the same tissue amounts captured by LCM.

For our RNA-seq experiment reported in Hoang et al. (2020), we started with a minimum of 10 ng total RNA for library construction and subsequently sequenced with an Illumina HiSeq 4000 instrument. For a total of 33 samples used, we obtained an average number of clean paired-end reads of 16,265,639 (minimum 6,314,138 and maximum 66,143,828 reads) (Figure 4E).

LIMITATIONS

Although our protocol produces relatively high-quality RNA samples acceptable for ordinary RNA-seq analysis, a broader background fluorescence peak around the two very sharp ribosomal RNA peaks is detected in the bioanalyzer electropherogram (Figures 4A–4C), which could indicate that partial degradation is still present in the extracted RNA samples. In addition, although RIN value has been used as a standard for RNA integrity assessment, there are some reports arguing that RIN value does not properly reflect RNA integrity (Hasegawa et al., 2018, Jaffe et al., 2017, Wang et al., 2016). Therefore, it is possible that there are some sensitive assays which would require

RNA sample with different quality standards. Collectively, there is still room for improvement in the protocol.

TROUBLESHOOTING

Problem 1

The RNA is degraded and RIN value is low (step 17).

Potential Solution

RNA may be degraded by external sources of RNase contamination. Inspect your working environment, change gloves frequently, and decontaminate plasticware and lab appliances with RNaseZap.

RNA may be degraded by internal sources of RNase. Sample processing time could be a critical factor. You can check your sample quality in the critical steps. Check earlier steps first when there are more than one step that need to be checked.

Problem 2

The RNA yield is low (step 17).

Potential Solution

Increase your section thickness, but be aware that thick tissues are more difficult to transfer to the CapSure LCM caps.

CapSure LCM cap-based laser capture instrument has limitations for collecting large amount of tissue sections. Use different laser dissection system. For example, Leica LMD collects dissected tissue samples by gravity, and allows to pool many samples for a single experiment.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ji-Young Lee (ji924@snu.ac.kr).

Materials Availability

This study did not generate any unique materials or reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xpro.2020.100110>.

ACKNOWLEDGMENTS

The authors would like to thank all Lee lab members, past and present, for assisting with this work at various stages. This work is supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry (IPET) through Golden Seed Project (213006-05-4-SBK30), funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA), Ministry of Oceans and Fisheries (MOF), Rural Development Administration (RDA) and Korea Forest Services (KFS) to J.-Y.L. G.C. and N.V.H. were supported by Brain Korea 21 Plus Program.

AUTHOR CONTRIBUTIONS

Conceptualization, G.C. and J.-Y.L.; Investigation, G.C., N.V.H., and J.-Y.L.; Writing – Original Draft, G.C.; Writing – Review & Editing, G.C., N.V.H., and J.-Y.L.; Visualization, G.C., N.V.H., and J.-Y.L.; Funding Acquisition and Supervision, J.-Y.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Asano, T., Masumura, T., Kusano, H., Kikuchi, S., Kurita, A., Shimada, H., and Kadowaki, K. (2002). Construction of a specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the genes expressed in the rice phloem. *Plant J.* *32*, 401–408.
- Blokhina, O., Valerio, C., Sokolowska, K., Zhao, L., Karkonen, A., Niittyta, T., and Fagerstedt, K. (2016). Laser capture microdissection protocol for xylem tissues of woody plants. *Front. Plant Sci.* *7*, 1965.
- Espina, V., Wulfskuhle, J.D., Calvert, V.S., Vanmeter, A., Zhou, W., Coukos, G., Geho, D.H., Petricoin, E.F., 3rd, and Liotta, L.A. (2006). Laser-capture microdissection. *Nat. Protoc.* *1*, 586–603.
- Hasegawa, T., Takahashi, J., and Iwahashi, H. (2018). RNA quality control using external standard RNA. *Pol. J. Microbiol.* *67*, 347–353.
- Hoang, N.V., Choe, G., Zheng, Y., Aliaga Fandino, A.C., Sung, I., Hur, J., Kamran, M., Park, C., Kim, H., Ahn, H., et al. (2020). Identification of conserved gene-regulatory networks that integrate environmental sensing and growth in the root cambium. *Curr. Biol.* *30*, 2887–2900.
- Jaffe, A.E., Tao, R., Norris, A.L., Kealhofer, M., Nellore, A., Shin, J.H., Kim, D., Jia, Y., Hyde, T.M., Kleinman, J.E., et al. (2017). qSVA framework for RNA quality correction in differential expression analysis. *Proc. Natl. Acad. Sci. U S A* *114*, 7130–7135.
- Jardinaud, M.F., Boivin, S., Rodde, N., Catrice, O., Kisiala, A., Lepage, A., Moreau, S., Roux, B., Cottret, L., Sallet, E., et al. (2016). A laser dissection-RNAseq analysis highlights the activation of cytokinin pathways by nod factors in the medicago truncatula root epidermis. *Plant Physiol.* *171*, 2256–2276.
- Norenburg, J.L., and Barrett, J.M. (1987). Steedman's polyester wax embedment and de-embedment for combined light and scanning electron microscopy. *J. Electr. Microsc. Tech.* *6*, 35–41.
- Roux, B., Rodde, N., Jardinaud, M.F., Timmers, T., Sauviac, L., Cottret, L., Carrere, S., Sallet, E., Courcelle, E., Moreau, S., et al. (2014). An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J.* *77*, 817–837.
- Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., and Ragg, T. (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol. Biol.* *7*, 3.
- Steedman, H.F. (1957). Polyester wax; a new ribboning embedding medium for histology. *Nature* *179*, 1345.
- Thiel, J., Weier, D., and Weschke, W. (2011). Laser-capture microdissection of developing barley seeds and cDNA array analysis of selected tissues. *Methods Mol. Biol.* *755*, 461–475.
- Wang, L., Nie, J., Sicotte, H., Li, Y., Eckel-Passow, J.E., Dasari, S., Vedell, P.T., Barman, P., Wang, L., Weinshiboum, R., et al. (2016). Measure transcript integrity using RNA-seq data. *BMC Bioinformatics* *17*, 58.