Submission ID #: 61517
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18762733

Title: Laser-Capture Microdissection RNA-Sequencing for Spatial and Temporal Tissue-Specific Gene Expression Analysis in Plants

Authors and Affiliations: Lim Chee Liew[1,2], Yan Wang[1,2], Marta Peirats-Llobet[1], Oliver Berkowitz[1,2,3], James Whelan[1,2,3], and Mathew G. Lewsey[1,3]

[1]Department of Animal, Plant and Soil Science, AgriBio Building, La Trobe University
[2]Australian Research Council Centre of Excellence in Plant Energy Biology, La Trobe University
[3]Australian Research Council Research Hub for Medicinal Agriculture, AgriBio Building, La Trobe University

Corresponding Author:
Mathew G. Lewsey
M.Lewsey@latrobe.edu.au

Co-authors:
L.Liew@latrobe.edu.au
Yan.Wang@latrobe.edu.au
M.Peirats-Llobet@latrobe.edu.au
O.Berkowitz@latrobe.edu.au
J.Whelan@latrobe.edu.au

Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **46**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Lim Chee Liew**: This protocol uses laser-capture microdissection coupled with RNA-sequencing to obtain spatial and temporal transcriptomes from specific cells in plants of interest using small quantities of biological materials **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Yan Wang**: The main advantage of this technique is that it facilitates the direct visualization of cells within their normal tissue context, allowing discrete cells to be precisely isolated in a contact-free manner **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Marta Peirats-Llobet**: Although this protocol is optimized for the isolation of plant cells, it can be applied to most cells that can be histologically identified **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

OPTIONAL:

1.4. **Yan Wang**: : Good sample preparation is critical. Therefore, when performing this technique for the first time, proper optimization of the tissue fixation and embedding is important before laser-capture microdissection **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Tissue Fixation

2.1. Before collecting the tissue sample, prepare a fixative appropriate to the species and tissue type to be harvested **[1-TXT]**.

2.1.1. WIDE: Talent mixing fixative, with ethanol and glacial acetic acid containers visible in frame **TEXT: *e.g.*, for barley seed, use Farmer's fixative**

2.2. For barley seed, cut the seed samples in half **[1]** before submerging the samples into an at least 10x volume of ice-cold fixative **[2]**.

2.2.1. Seed being cut in half

2.2.2. Seed hal(ves) being submerged in fixative, with fixative container visible in frame

2.3. Use vacuum infiltration to accelerate the penetration of the fixative. The tissues should sink by the end of the infiltration **[1-TXT]**.

2.3.1. Sample/fixative being infiltrated **TEXT: 30 min for barley seed**
~~2.3.2. Shot of sinking seed/seed at bottom of container~~ NOTE: Seeds already sunk to the bottom of the container due to their weight.

2.4. Then refresh the fixative for an overnight incubation **[1]** and transfer the samples into cassettes for tissue processing **[2]**.

2.4.1. Talent adding fixative to seed, with fixative container visible in frame
2.4.2. Talent adding sample to cassette

3. Tissue Processing

3.1. Place the tissue-loaded cassettes into the metal basket of the automatic processor **[1]** and attach the metal basket to its holder above chamber 1 **[2]**.

3.1.1. WIDE: Talent placing cassette into basket
3.1.2. Talent attaching basket to holder

3.2. Set the program on the control panels of the tissue processor **[1-TXT]** and press **Start** to begin the automatic processing program **[2]**.

3.2.1. Talent setting program **TEXT: See text for processing parameter details** NOTE: 3.2.1 and 3.2.2 combined
3.2.2. Talent pressing Start

3.3. The system will dehydrate the samples by dipping the cassette into a gradient series of ethanol for 90 minutes per concentration as indicated **[1-TXT]**.

3.3.1. Basket dipping into ethanol **TEXT: 75% -> 85% -> 100% x3**

3.4. After the last ethanol immersion, the system will submerge the samples in xylene gradients for 90 minutes per solution as indicated **[1-TXT]** followed by two, 90-minute in immersions in molten paraffin at 55-60 degrees Celsius **[2]**.

3.4.1. Basket dipping into xylene **TEXT: 75:25 -> 50:50 -> 25:75 ethanol:xylene -> 100% xylene x2**

3.5. The next morning, remove the paraffin infiltrated cassettes from the tissue processor and proceed to the paraffin embedding **[1]**.

3.5.1. Talent removing cassette

4. Paraffin Embedding

4.1. The next morning, use fine forceps to transfer the processed samples into suitably sized molds **[1]** and add molten paraffin over each sample **[2]**.

4.1.1. WIDE: Talent placing sample into mold NOTE: 4.1.1 and 4.1.2 combined
4.1.2. Paraffin being added to mold

4.2. For barley seeds, orient the samples in the paraffin longitudinally to the cutting direction to facilitate the acquisition of longitudinal sections **[1]**.

4.2.1. Seed being oriented

4.3. Place a clean cassette onto the mold **[1]** and ensure that the entire cassette is fully covered with a sufficient volume of paraffin to secure the sample to the cassette **[2]**.

4.3.1. Cassette being placed *Videographer: Important step*
4.3.2. Shot of sufficient paraffin *Videographer: Important step*

4.4. Then place the mold onto a cold plate for 10-20 minutes until the paraffin is set **[1]** before releasing the block from mold **[2-TXT]**.

4.4.1. Talent placing mold onto cold plate
4.4.2. Shot of set paraffin, then block being released **TEXT: Optional: Store embedded blocks at 4 °C ≤3 mo**

5. Polyethylene Naphthalate (PEN) Membrane Slide Preparation

5.1. To prepare PEN (pen) membrane slides, submerge the slides in RNase-deactivating solution for 3 seconds **[1]** followed by two brief washes in DEPC (D-E-P-C)-treated water **[2]**.

5.1.1. WIDE: Talent adding slides to RNase deactivating solution, with deactivating solution container visible in frame
5.1.2. Talent adding DEPC-treated water to slides, with DEPC container visible in frame

5.2. Then dry the slides in a 37-degree Celsius incubator to remove any leftover solution **[1]** and UV-treat the slides in a laminar flow cabinet for 30 minutes to enhance their paraffin adhesiveness **[2]**.

5.2.1. Talent placing slide(s) into incubator
5.2.2. Shot of slides in hood, then UV light going on over slides

6. Sectioning

6.1. For sample tissue sectioning, place the paraffin blocks on the cold plate **[1-TXT]** and fill the water bath with 42-degree Celsius-warmed DEPC-treated water **[2]**.

6.1.1. WIDE: Talent placing block(s) onto cold plate **TEXT: Re-cool softening blocks on plate during sectioning as necessary**
6.1.2. Talent filling bath with DEPC-treated water

6.2. Trim blocks to the depth of the region of interest **[1]** and section the paraffin blocks to a 6-10-micron thickness. A well-sectioned block will form a 'ribbon' at the edge of the blade **[2-TXT]**.

6.2.1. Block being trimmed *Videographer: Important step*
6.2.2. Block being sectioned *Videographer: Important step* **TEXT: Acquire 8-micron-thick barley sections**

6.3. Use a fine pain brush to gently transfer ribbons from the microtome to the water bath **[1]**, taking care that the ribbon lays flat on the surface of the water **[2]**.

6.3.1. Section being collected with brush *Videographer: Important step*
6.3.2. Section being flattened on water surface *Videographer: Important step*

6.4. To collect the sections, holding a slide at a 45-degree angle, use an upward motion to lift a ribbon out of the water onto the slide **[1]** and use a lint-free tissue to carefully remove the excess water **[2]**.

6.4.1. Slide being held at 45-degree angle, then tissue being lifted out of water
6.4.2. Water being removed

6.5. When all of the sections have been collected, wash the slides with three, 20-second washes in xylene **[1]** followed by two, 30-seconds washes in 100% ethanol **[2]** and two, 30-seconds washes in 70% ethanol **[3]**.

6.5.1. Talent placing slides in xylene, with xylene container visible in frame
6.5.2. Talent placing slide(s) in 100% ethanol, with ethanol container visible in frame
6.5.3. Talent placing slide(s) in 70% ethanol, with 70% ethanol container visible in frame

7. Laser-Capture Microdissection (LCM)

7.1. To microdissect cells of interest from the deparaffinized and dried tissue sections, load slides on the three LCM (L-C-M) microscope slots **[1]** and load collection tube into the available slots **[2-TXT]**.

7.1.1. WIDE: Talent loading slide(s) onto slot(s)
7.1.2. Talent loading tube(s) **TEXT: Samples will be collected into adhesive caps**

7.2. Move the stage to locate the region of the sample that needs to be cut **[1]** and cut a blank segment free of tissue on the membrane slide to optimize the cutting speed and the cutting and laser pressure catapulting energy and focus **[2-TXT]**.

7.2.1. Stage being moved *Videographer: Important/difficult step*
7.2.2. Segment being cut *Videographer: Important/difficult step* **TEXT: Barley seed cutting speed 18, CutEnergy = 52, CutFocus = 63, LPCEnergy = 78, LPC focus = 61** NOTE: SCREEN shot also available on project page

7.3. Use the **Drawing tools** to outline the area of interest in the tissue **[1]** and use the optimized parameters and the **RoboLPC function** to catapult cells into the adhesive caps **[2]**.

7.3.1. SCREEN: 7.3.1: 00:03-00:18

7.3.2. SCREEN: 7.3.1: 00:22-00:27

7.4. Select **Flag** from the elements list to use the flag tool to mark the regions of interest **[1]**.

7.4.1. SCREEN: 7.4.1: 00:02-00:08

7.5. Check the **CapCheck** button to inspect the adhesive cap to confirm that the samples have been captured. Typically, 10-15 sections per cap are required for RNA extraction **[1-TXT]**.

7.5.1. SCREEN: 7.5.1: 00:03-00:28 *Video Editor: please speed up* **TEXT: Place samples on ice upon acquisition**

7.6. Immediately after all of the samples have been acquired, use an automated electrophoresis system according to the manufacturer's instructions to quantify and qualify the antisense RNA to avoid RNA degradation **[2]**.

7.6.1. Talent placing sample on ice
7.6.2. Talent adding sample to automated electrophoresis system

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
4.2., 6.2., 6.3., 7.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
7.2., 7.3.

Results

8. Results: Representative LCM Sample Acquisition and RNA Quantification

8.1. In this study, LCM RNA-sequencing **[1]** was applied to a small number of cells from three embryo organs **[2]** every 8 hours over a 48-hour time course during germination **[3]**.

8.1.1. LAB MEDIA: Figure 2A
8.1.2. LAB MEDIA: Figure 2 *please sequentially add/emphasize Scutellum, Plumule, and Radicle text and lines in Figure 2A*

8.2. It is important to adjust the cutting parameters correctly to allow the tissue of interest **[1]** to be precisely cut and dislodged from the surrounding tissue without burning the edge of the selected area **[2]**.

8.2.1. LAB MEDIA: Figures 3A and 3B *Video Editor: emphasize Figure 3A*
8.2.2. LAB MEDIA: Figures 3A and 3B *Video Editor: emphasize Figure 3B*

8.3. Quantification of the total RNA before RNA amplification **[1]** allows the detection of distinct electrophoretic bands and fluorescent peaks of 18- and 28S ribosomal subunits in good quality RNA samples **[2]**.

8.3.1. LAB MEDIA: Figure 3D
8.3.2. LAB MEDA: Figure 3D *Video Editor: please emphasize 18S and 28S peaks and/or texts*

8.4. Successfully synthesized antisense RNA **[1]** will exhibit a unimodal, symmetrical size distribution from 100 to 1000 nucleotides **[2]** with a peak around 300 nucleotides after two rounds of amplification **[3]**.

8.4.1. LAB MEDIA: Figure 3E
8.4.2. LAB MEDIA: Figure 3E *Video Editor: please emphasize x-axis from 100 to 1000*
8.4.3. LAB MEDIA: Figure 3E *Video Editor: please emphasize peak*

8.5. Multidimensional scale plotting of genes expressed in the different tissues over 48 hour of germination **[1]** illustrates a greater similarity between samples of a single tissue than between samples from the same time point but different tissues **[2]**.

8.5.1. LAB MEDIA: Figure 4A

8.5.2. LAB MEDIA: Figure 4A *Video Editor: please individually emphasize blue, red, and orange/yellow/green clusters*

8.6. The number of differentially expressed genes increases progressively over the course of germination in each tissue **[1]** relative to the 0-hour timepoint of the tissue **[2]**.

8.6.1. LAB MEDIA: Figure 4B
8.6.2. LAB MEDIA: Figure 4B *Video Editor: please sequentially add diagonal arrow over each set of tissue data bars to indicate progressive increase*

8.7. In this representative analysis **[1]**, 25% of plumule **[2]**, 34% of radicle **[3]**, and 41% of scutellum differentially expressed genes were found to be exclusively expressed within each tissue **[4]**.

8.7.1. LAB MEDIA: Figure 4C
8.7.2. LAB MEDIA: Figure 4C *Video Editor: please emphasize Plumule circle*
8.7.3. LAB MEDIA: Figure 4C *Video Editor: please emphasize Radicle circle*
8.7.4. LAB MEDIA: Figure 4C *Video Editor: please emphasize Scutellum circle*

Conclusion

9. Conclusion Interview Statements

9.1. **Lim Chee Liew**: It is important to correctly adjust the cutting parameters for a precise excision and dislodging of the selected area from the surrounding tissue without burning the edge of the selected region **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (7.2.,7.3.)

9.2. **Marta Peirats-Llobet**: With improved chromatin and protein purification methods and enhanced mass spectrometry instruments, LCM can be used for cell-type-specific epigenomic and proteomic studies in plants **[1]**.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can skip for time*