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Laser Capture Microdissection of Epithelium from a Wound Healing Model for MicroRNA Analysis

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Abstract

MicroRNAs are ~22 nucleotide-long noncoding RNAs influencing many cellular processes (including wound healing) by their regulatory functions on gene expression. The ability to analyze microRNA in different cells at the wound site is essential for understanding the critical role(s) of microRNA during various phases of wound healing. Laser capture micro-dissection (LCM) is an effective method to distinguish between relevant and non-relevant cells or tissues and enables the researcher to obtain homogeneous, ultra-pure samples from heterogeneous starting material. We present here our protocol for procuring epithelial cells from a mouse wound healing model using a Leica LMD7000 Laser Microdissection system, as well as the RNA isolation and downstream microRNA analysis. Using this method, researchers can selectively and routinely analyze regions of interest down to single cells to obtain results that are relevant, reproducible, and specific.

Keywords

LCM; MicroRNA; Wound healing; RNA isolation; Epithelial cells; Mouse; Frozen sample

1 Introduction

Laser Capture Microdissection (LCM) was developed in 1996 at the National Cancer Institute [1]. It is an automated sample preparation technique that enables isolation of specific cells from a mixed population under microscopic visualization. This technique is characterized by the use of a laser coupled to an inverted light microscope to selectively extract a pure cell population of interest from a heterogeneous mixture of cells in a typical tissue section [2]. There are two general classes of LCM: The infrared (IR) laser capture system and the ultraviolet (UV) laser-cutting system. In the IR system, while viewing the target cells, an IR, low power laser is pulsed through the top of the cap, hitting the transfer film, which then melts and bonds to the identified cells. Cells are then removed selectively from the tissue section when the cap is lifted [3]. In the UV-system, the tissue sections are attached to a specific membrane slide. The laser energy melts the demarked membrane area, allowing the selected cells to fall into a collection device by gravity [3].

LCM allows for more efficient and accurate results with downstream applications, including microgenomic analysis of microRNAs using next-generation sequencing, microarrays, and quantitative PCR (qPCR) techniques. However, common challenges observed during LCM collection are related to low yield and suboptimal RNA quality. The quantity and quality of LCM-derived RNA are affected by many factors, including the quality of starting material,

tissue fixation procedure, time to preservation before microdissection, type of preservation, tissue handling during sectioning, efficiency of microdissection, and the post-LCM RNA isolation procedure [4, 5]. Proper optimization of these procedures will substantially improve the effectiveness of LCM and provide greater utility for investigating critical questions in complex biological systems, such as the role of microRNA in wound healing. In this chapter, we demonstrate LCM-epithelial cell collection from tissue harvested from a wound healing model; these samples were collected for future analysis of microRNAs.

MicroRNAs, non-protein-coding small endogenous RNAs, have been shown to regulate many developmental, physiological, and disease processes, including wound healing [6, 7]. The mechanism underlying epithelial tissue healing and pathologies, such as failure to repair, are not fully understood. This is an important topic in basic biomedical research since it involves a fundamental principle of survival [8]. An oral mucosal wound healing model was chosen because it nearly always shows superior wound healing when compared with other tissues. Thus oral mucosa wound healing is an optimal model for investigating the mechanism underlying the highly orchestrated healing process.

2 Materials and Equipment

An RNase-free environment is essential when working with RNA samples. It is important to designate a special area for RNA work only. All the surfaces and the tools (including glassware and plastic ware) that will be in contact with the samples need to be cleaned with RNaseZap (ThermoFisher Scientific) or another RNase decontamination solution and then rinsed with DEPC-treated nuclease-free water. Prepare all the solutions using DEPC-treated nuclease-free water and analytical grade reagents. If not indicated, the reagents are prepared and stored at room temperature. Always wear sterile disposable gloves when handling reagents and RNA samples and discard all biohazard material in appropriate containers. Change gloves frequently as the protocol progresses from crude extract to more purified material.

2.1 Sample Harvest and Section

1. Surgical instruments, such as incisors, forceps, scalpel, etc.
2. Disposable base molds (15 × 15 × 5 mm).
3. Optimal Cutting Temperature (OCT) Compound (Fisher HealthCare). OCT is a ready-to-use embedding medium for embedding tissue samples prior to frozen sectioning on a microtome-cryostat.
4. Permanent marker to label samples.
5. Dry ice (avoid direct skin contact).
6. −80 °C freezer.
7. Cryostat for the preparation of frozen tissue sections. The cryochamber temperature should be at −20 °C.
8. Glass slides.

9. Microscope.
10. Polyethylene naphthalate (PEN) membrane glass slides (ThermoScientific, catalog #: LCM0522). For LCM, special membrane mounted slides should be used to facilitate the photo ablation and sample collection. PEN membrane slides are suitable for both the IR laser capture system and the UV laser-cutting system.
11. 50 mL centrifuge sterile tubes to store individual slides.

2.2 Staining and LCM

1. 100% ethanol (ethyl alcohol, absolute, 200 proof for molecular biology).
2. Freshly prepared 50% (vol/vol) ethanol in DEPC-treated Nuclease-free water. Add 250 mL of ACS grade 100% ethanol and 250 mL of DEPC-treated Nuclease-free water to a bottle labeled “50% Ethanol.” Mix well.
3. Freshly prepared 75% (vol/vol) ethanol in DEPC-treated Nuclease-free water. Add 375 mL of ACS grade 100% ethanol and 125 mL of DEPC-treated Nuclease-free water to a bottle labeled “75% Ethanol.” Mix well.
4. Freshly prepared 95% (vol/vol) ethanol in DEPC-treated Nuclease-free water. Add 475 mL of ACS grade 100% ethanol and 25 mL of DEPC-treated Nuclease-free water to a bottle labeled “95% Ethanol.” Mix well.
5. Cresyl violet stain (contained in LCM Staining Kit, Ambion; stored at 4 °C).
6. Absorbent paper, timer, pipette, and tips.
7. Xylene.
8. Slide chambers, slide tubes, and slide holder.
9. Hydrophobic barrier pen (Thermo Fisher Scientific), designed for use with frozen or paraffin-embedded tissue sections mounted on glass slides.
10. Forceps to insert and remove slides from solutions.
11. Collection tubes with caps (0.5 mL).
12. Special collection devices for caps and microcentrifugation tubes.
13. QIAzol Lysis reagent, included in miRNeasy Mini Kit (Qiagen).
14. Mini centrifuge.

2.3 RNA Isolation

1. QIAzol Lysis reagent, included in miRNeasy Mini Kit (Qiagen).
2. Chloroform.
3. Collection tubes (1.5 and 2 mL), included in miRNeasy Mini Kit (Qiagen).
4. 100% ethanol (ethyl alcohol, absolute, 200 proof for molecular biology).
5. RNeasy Mini Spin Columns, included in miRNeasy Mini Kit (Qiagen).

6. RNase-free water.
7. RNase-free DNase Set (Qiagen). This kit contains DNase I (lyophilized), RNase free water (to dissolve the DNase), and Buffer RDD.
8. Buffer RWT, included in miRNeasy Mini Kit (Qiagen). This buffer is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96–100%) to prepare a working solution.
9. Buffer RPE, included in miRNeasy Mini Kit (Qiagen). This buffer is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to prepare a working solution.

3 Methods

3.1 Sample Harvest and Section

The mouse oral mucosal wound healing protocol was followed and the tissue specimens were harvested as previously described [9, 10]. In brief, 8-week-old female Balb/c mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) and a palate incision was made (1.0 mm width and 4 mm length, approximately) using a scalpel blade surgical #15. At 24 h post-wounding, the animals were sacrificed and specimens were harvested. The animal study was approved by the Institutional Animal Care and Use Committee at University of Illinois at Chicago.

The quality of starting material is essential for the RNA yield and quality. For best results, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at -80°C . This will minimize degradation of crude RNA by limiting the activity of endogenous RNases.

1. Clean all contacting surface with RNaseZap.
2. Add OCT Compound to the cryomold (about 2/3 full).
3. Immediately after tissue collection, embed tissue directly in the OCT Compound. Use forceps to orient the tissue (if necessary) and add more OCT Compound until the tissue is completely covered (Fig. 1a, b). Place OCT-embedded tissue upon a flat surface of dry ice (*see* notes regarding the specimen orientation inside the embedded medium).
4. Once frozen, store the samples at -80°C until the sections will be prepared.
5. Precool the cryostat to -20°C .
6. Clean the knife holder with 100% ethanol and treat the brushes with RNaseZap to eliminate RNase contamination.
7. Mark the base of the sample with a permanent marker for correct orientation. Next, remove the block from the cryomold and attach it to the stainless-steel tissue holder using OCT compound. Place the tissue holder on the metal grid of the cryochamber.

8. Trim the excess frozen OCT until you start to see the wound tissue on the microscope (use a glass slide to collect the sections until the wound is observed).
9. Set the cutting thickness to 10 μm (Fig. 1c).
10. Cut at least eight frozen sections per slide, avoiding the top and bottom thirds of the slide (Fig. 1d). Sections should be placed upon a labeled PEN membrane slide.
11. Place the slide inside a sterile slide tube previously cooled at $-20\text{ }^{\circ}\text{C}$. Keep all the slides at $-20\text{ }^{\circ}\text{C}$ until they can be stored at $-80\text{ }^{\circ}\text{C}$. (Frozen sections for RNA isolation can be stored at $-80\text{ }^{\circ}\text{C}$ for up to 1 month [5]).

3.2 Staining of the LCM Slides

This step is required to visualize the cells of interest under the microscope for the LCM procedure. The steps described below are modified from a LCM Staining Kit protocol (Ambion). Other commercially available kits such as the Arcturus HistoGene LCM Frozen Section Staining Kit or a simple Toluidine Blue Staining can also be used.

1. Remove the slides from $-80\text{ }^{\circ}\text{C}$ (using dry ice) and place them at $-20\text{ }^{\circ}\text{C}$.
2. Dip the slide in a tube with 95% ethanol solution for 35 s.
3. Dip the slide in a tube with 75% ethanol solution for 35 s.
4. Dip the slide in a tube with 50% ethanol solution for 30 s. Cap and invert the tube gently during this step to help dissolve the OCT.
5. Lay the slide on a flat surface. Mark the location of the tissue section on the edges of the slide with a hydrophobic barrier pen.
6. Add 300 μL of Cresyl Violet to the tissue section using a pipette and wait 40 s (Note: The exact time of each step of the staining procedure may be different depending on the tissue type).
7. Drain off stain in a plastic tube and dip the slide in a tube with 50% ethanol solution for 30 s.
8. Transfer the slide to a tube with 75% ethanol solution for 30 s.
9. Transfer the slide to a tube with 95% ethanol solution for 35 s.
10. Place the slide in the first 100% ethanol solution for 35 s.
11. Transfer the slide to a tube with the second 100% ethanol solution for 35 s.
12. Rinse the slide in a tube with a solution of Xylene (under fume hood)
13. Place the slide in a tube with a second Xylene solution for 5 min (under fume hood).
14. Immediately after the final Xylene step, place the slide on ice. The samples are now ready for the LCM procedure. (The slides can be kept in $-80\text{ }^{\circ}\text{C}$ for short-term storage. For best RNA quality and yields, perform LCM procedure on the same day).

3.3 Laser Capture Microdissection (LCM)

The LCM procedure described here is based on a Leica LMD7000 Laser Microdissection system.

1. Clean the LCM staging area and contacting surface with RNaseZap. Turn on the microscope, laser, and computer.
2. Access the LCM software program and load the collection tubes and LCM slides on their respective holder assemblies. The cap of the collection tube is used for collecting the LCM-captured cells, and it is secured in the holder. The LCM slide is clamped in the specimen holder with the membrane to the bottom.
3. The collection tube holder and the LCM slide holder can be selected through LCM software. Movement and focusing, for example, can be adjusted with a joystick controller.
4. Calibrate the laser in a region far away of the specimen (“Laser”, “Calibrate”).
5. Use a 1.25× objective to localize the section.
6. Change to objective 10×.
7. Select the respective cap (the location of the collector to receive the LCM harvested cells).
8. Select “Line,” “Close Lines” (Draw shapes), and “Draw + Cut” (Cut shapes) to draw a line around the interested area. Several pieces can be marked and cut consecutively (“Multiple Shapes”).
9. After the selection, set the power, aperture, and speed of laser to 50, 20, and 10, respectively.
10. Select “Start Cut” to start laser cut (Fig. 1e, f). The laser cutting can be monitored on the computer screen in real time, and the selectively dissected tissue pieces should drop to the cap of the collection tube by gravity.
11. If the sample does not drop, click “Move + Cut” on the “Cut Shape” toolbar and move the laser manually to cut the bridges of tissues (Figs. 1g and 2a–d).
12. Move to another section and repeat the procedure.
13. To remove the slide and the collection tube, click “Unload.” Detach the collection tube carefully and put the tube onto the cap to close.
14. Immediately after LCM collection the samples will be centrifuged ($10,000 \times g$ for 1 min).
15. Add 700 μ L QIAzol Lysis Reagent to the samples. Homogenize the samples by vortexing for 1 min. Homogenized cell lysate can be stored at -80°C for several months.

3.4 RNA Isolation and Downstream MicroRNA Analysis

The total RNA (including small RNA) isolation protocol presented here is based on a miRNeasy Mini Kit from Qiagen. A number of other commercially available kits can be used for this step (with proper modification), including the mirVana miRNA Isolation Kit (Ambion) and the Direct-zol RNA MiniPrep (Zymo Research). RecoverAll Total Nucleic Acid Isolation Kit (Ambion) can be used for extracting total nucleic acids (RNA, miRNA, and DNA) from LCM captured cells from formalin-fixed paraffin-embedded (FFPE) tissues.

1. Incubate the homogenate at Room Temperature (RT) for 5 min.
2. Add 140 μL chloroform to samples and cap the tube securely. Shake vigorously for 20 s.
3. Incubate at RT for 2–3 min.
4. Centrifuge for 15 min at $12,000 \times g$ at 4 °C.
5. Transfer the upper aqueous phase to a new collection tube. Avoid transferring any interphase.
6. Add 1.5 volume (usually 525 μL) of 100% ethanol and mix thoroughly by pipetting.
7. Pipet up to 700 μL of the sample, including any precipitate, into a RNeasy Mini column in a 2 mL collection tube. Close the lid and centrifuge at $10,000 \times g$ for 30 s at RT. Discard the flow-through.
8. Repeat **step 7** for the remainder of the samples.
9. Pipet 350 μL Buffer RWT into the RNeasy Mini Spin Column and centrifuge for 30 s at $10,000 \times g$ to wash. Discard the flow-through.
10. Add 10 μL DNase I stock solution to 70 μL Buffer RDD. Mix by gently inverting the tube. Do not vortex.
11. Pipet the DNase I incubation mix (80 μL) directly onto the RNeasy Mini Spin Column membrane and place on the bench-top at 20–30 °C for 15 min (make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane).
12. Pipet 500 μL Buffer RWT into the RNeasy Mini Spin Column and centrifuge for 30 s at $10,000 \times g$. Keep the flow-through.
13. Apply the flow-through to the same RNeasy Mini Spin Column and centrifuge for 30 s at $10,000 \times g$. Discard the flow-through.
14. Add 500 μL Buffer RPE to the same RNeasy Mini Column. Close the lid and centrifuge for 2 min at $10,000 \times g$.
15. Place the RNeasy Mini column into a new 2 mL collection tube. Centrifuge at full speed for 1 min to further dry the membrane.
16. Transfer the RNeasy Mini column to a new 1.5 mL collection tube. Pipette 30 μL RNase-free water directly onto the RNeasy Mini column membrane. Close the lid, and centrifuge for 1 min at $10,000 \times g$ to elute the RNA.

17. Use the flow-through to analyze the RNA quantity and quality (Fig. 1f). RNA concentration is typically quantified based on OD absorbance using a spectrophotometer (e.g., NanoDrop from Thermo Fisher Scientific). However, due to the relatively low RNA yield from the LCM-captured cells, an alternative quantification method may be used in addition to (or instead of) the OD method, such as the Qubit RNA HS Assay Kit from ThermoFisher Scientific. Typical RNA quality control methods include electrophoresis-based analysis using instruments such as TapeStation System from Agilent Technologies. The TapeStation System offers the RIN algorithm (RNA Integrity Number) to determine RNA quality using a numerical system which represents the integrity of RNA. With high-quality starting material, optimized procedure, and proper technique, an RIN value as high as 7.0 can be achieved for RNA isolated from LCM-captured cells.
18. If sufficient RNA quality and quantity are achieved, downstream microgenomic analysis based on next-generation sequencing, microarray, and qPCR technologies can be performed to assess the levels of specific microRNAs (Figs. 1g and 3). A representative result from TaqMan-based qPCR analysis is shown in Fig. 3, demonstrating a decrease in the miR-100 level in epithelial cells harvested from a wounded area as compared to epithelial cells from an unwounded area (control sample). This is in agreement with our previously published findings [11, 12].

4 Notes

1. Pay attention to the orientation of the sample when embedding it. The wound should be perpendicular to the blade. Mark the direction of the cut that should be performed on the disposable base mold (cryomold).
2. Avoid bubbles in the OCT Compound when embedding the sample.
3. The staining process is crucial for the tissue layer visualization as it permits accurate selection of the desired areas of the tissue.
4. To preserve RNA integrity during sample fixation, storage, and staining, as well as during the LCM procedure and isolation process, perform all the procedures as quickly as possible and avoid storing the sections for too long between the procedures.
5. During the sectioning process, the microscope slide should be kept in the cryochamber while the sections are placed onto it to avoid RNA degradation.
6. If you are cutting different tissues, you should use a different blade area for each one to avoid contamination between them.
7. Use a new blade to perform the sections.
8. Do not allow the slides to thaw before starting the staining steps.

9. During the staining steps, in between transferring the slides from one solution to another, blot the slides gently on absorbent paper to remove excess solution.
10. For best staining results, change the wash solutions (the four solutions after using Cresyl Violet dye) every four slides and the Xylene solutions every six slides.
11. Some of the stained tissue mounted LCM slides (especially those retrieved from -80°C storage) may be too wet to proceed to LCM immediately. These slides can be dried on a 42°C hot plate for short time. However, this drying step should be kept to a minimum because long-term exposure to elevated temperature will lead to RNA degradation.
12. When performing LCM, be sure that you insert the slide in the specimen holder with the tissue facing downward. Otherwise, the desired area will be dissected but not dropped on the cap.
13. PEN Membrane Glass Slides (ThermoFisher Scientific) can be used for both IR laser capture and UV laser cutting. PEN Membrane Frame Slides (ThermoFisher Scientific) provide additional flexibility by allowing microdissection of non-dehydrated samples.
14. Depending on the cells of interest, pooling LCM-captured samples from multiple slides may be necessary to obtain sufficient amounts of RNA. For example, to obtain sufficient RNA for microRNA analysis on epithelial cells from the wound edges of our oral mucosa wound healing model, we typically pool samples from six slides (with at least eight sections on each slide).
15. During LCM, the sections must be sufficiently dehydrated to drop into the cap of the collection tube after laser ablation.
16. After the initial LCM cut, occasionally some target tissues (especially large pieces) may not fall into the collection cap. Further cutting these tissue pieces in half may help them to fall by the force of gravity.
17. When removing the collection tube with the microdissected samples, you should detach the tube very carefully because if the cap hits the holder, the samples may be lost.
18. The homogenization step before the RNA isolation is critical for disrupting the cell membranes. Incomplete homogenization may lead to significantly reduced RNA yields.
19. The On-Column DNase Digestion step with the RNase-Free DNase Set (Qiagen) is optional based on the downstream application. For example, removal of genomic DNA contamination is highly recommended for qPCR and small-RNA-seq analysis. An additional DNA removal procedure can be carried out after the RNA isolation using kits such as RNA Clean-up & Concentration from Zymo Research.

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Fig. 1.

Overall procedure. This cartoon demonstrates an approach regarding LCM-mediated epithelial cell collection from a mouse oral mucosa wound healing model. (a) Mouse oral mucosa tissue obtained from wound healing model. (b) Tissue specimen is embedded in Optimal Cutting Temperature (OCT) Compound. (c) Specimen is sectioned and (d) placed on PEN membrane glass slide. (e) After staining and dehydration, LCM was performed and the cells of interest were selectively harvested and fall into a collection device by gravity. (f) RNA isolated and RNA quality and quantity analyses. (g) MicroRNA expression analysis using the RNA samples isolated from LCM-captured epithelial cells from the wound healing model

Fig. 2. LCM-mediated epithelial cell collection from a wound healing model. Representative images of LCM mediated isolation of epithelial cells from the wound edge (**a** and **b**) and unwounded (**c** and **d**) areas from a mouse oral mucosal wound healing model. **a** and **c**: before LCM. **b** and **d**: after laser ablation. Magnification 200×

Fig. 3. QPCR-based microRNA analysis on LCM-captured epithelial cells from a wound healing model. Representative qPCR analysis of RNA samples isolated from LCM-captured epithelial cells from wounded and unwounded (control) areas. TaqMan microRNA assays for miR-100 and U6 small nuclear RNA (Applied Biosystems) were used for the qPCR analysis using a 7900HT Fast Real-Time PCR system (Applied Biosystems). The qPCR amplification curves were shown in (a). The relative miR-100 levels were computed using the $2^{-\Delta\Delta C_t}$ method, where U6 was used as an internal reference (b)