Chapter 23

Laser Capture Microdissection on Frozen Sections for Extraction of High-Quality Nucleic Acids

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Abstract

Many cancers harbor a large fraction of nonmalignant stromal cells intermixed with neoplastic tumor cells. While single-cell transcriptional profiling methods have begun to address the need to distinguish biological programs in different cell types, such methods do not enable the analysis of spatial information available through histopathological examination. Laser capture microdissection offers a means to separate cellular samples based on morphological criteria. We present here an optimized method to retrieve intact RNA from laser capture microdissected tissue samples, using pancreatic ductal adenocarcinoma as an example, in order to separately profile tumor epithelial and stromal compartments. This method may also be applied to nonmalignant tissues to isolate cellular samples from any morphologically identifiable structure.

Key words Laser capture microdissection, RNA sequencing, DNA sequencing, Pancreatic ductal adenocarcinoma

1 Introduction

Next-generation sequencing analysis of bulk tumor specimens has transformed cancer research over the last decade and provided detailed catalogues describing the genetic basis of virtually every common tumor entity [1]. However, the assessment of epigenetic, transcriptional, and proteomic profiles of malignant tissues is complicated by the fact that tumors comprise both transformed malignant and nontransformed cells from many lineages [2]. In extreme cases, such as in pancreatic ductal adenocarcinoma (PDA), nontransformed stroma commonly represents as much as 70–95% of the cells in a tumor mass [3], and thus confounds the biological interpretation of results from bulk tissues. For example, epithelial–-mesenchymal gene expression signatures may originate from true epigenetic and transcriptomic changes in tumor cells, a higher proportion of cancer-associated fibroblasts in the tumor microenvironment, or both. Furthermore, the admixture of highly specialized cell types from the parenchyma of the tissue of origin

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may mimic more well-differentiated molecular tumor subtypes or even lead to the proposal of erroneous class labels altogether [4, 5]. While DNA assays can use computational techniques to subtract out the contribution of known normal genomes, the interpretation of most molecular assays performed on bulk tumor tissues will be weakened in direct proportion to the degree of cellular heterogeneity within the tumor. To overcome this problem, tissue enrichment techniques have become critical to the study of tumor biology [6].

Several techniques may be employed to isolate cellular subsets from bulk tissue including magnetic bead separation and fluorescence-activated cell sorting (FACS), which rely on population–specific antibodies to separate a suspension of cells following disruption of the tumor. While these approaches often yield substantial amounts of nucleic acid for certain abundant cell types, they cannot prevent the contamination with nonpertinent cell types expressing the same antigen as the target cell, for example normal, atrophic, preneoplastic, or metaplastic epithelial cells in EPCAM+ populations. Furthermore, neither one of these techniques allows for the purification of rare cell types that do not express specific antigens which is true for most precursor lesions including pancreatic intraepithelial neoplasia (PanIN).

To this day, precancer and cancer diagnoses both continue to be made by pathologists examining histological/cytological evidence while accounting for further clinical cues. Laser capture microdissection (LCM) operates along those same principles and combines the power of morphological diagnosis with the ability to isolate highly purified cell populations of interest from tissues with an intact architecture.

Here we describe how to apply LCM to frozen tissue sections to extract high-quality nucleic acids suitable for next-generation sequencing.

2 Materials

2.1 Equipment

1. Cryotome.
2. Styrofoam box with dry ice.
3. Standard slide boxes.
4. P20 micropipette.
5. Ice bucket.
6. Laser capture microdissection microscope such as Zeiss PALM MicroBeam.

2.2 Reagents

1. Cresyl Violet acetate—pure and certified (Acros Organics).
2. PEN Membrane Glass Slides (Arcturus).

3. AdhesiveCap 200 opaque, Zeiss.
4. Tris–HCl 1 M, pH 8.5—molecular biology grade.
5. Ethanol—molecular biology grade.
6. RNAse-free water (e.g., Invitrogen).
7. Cell lysis buffer (e.g., RLT plus buffer for RNA and ATL buffer for DNA—QIAGEN).
8. Proteinase K (e.g., QIAGEN) for DNA extraction.

2.3 Consumables

1. 15 and 50 mL Falcon tubes.
2. Pipette tips (RNAse-free).
3. Petri dish with clear lid.

3 Methods

3.1 Cryosectioning

Ensure that all surfaces that make contact with the tissue of interest are clean. Double check cutting angle and sharpness of the blade (also *see* **Note 1**).

1. Prepare two boxes with dry ice:
   (a) One fitting a small (e.g., 25 slides) slide box—place box on dry ice just before starting.
   (b) One fitting all tissue blocks of interest—place blocks on dry ice.
2. Cut one block at a time, leaving it in the cryotome (~−20 to −25 °C) for a few minutes before mounting.
3. Cut 8–9 μm sections and pick them up using room temperature PEN membrane slides (*see* **Note 2**). Immediately place slides into the slide box on dry ice. Proceed to staining or transfer slide box to −80 °C if staining is carried out another time (*see* **Note 3**).

3.2 Staining

3.2.1 Cresyl Violet Stock Solution

1. Dissolve solid cresyl violet acetate at a concentration of 3% (w/v) in 100% Ethanol at room temperature.
2. Stir/agitate for several hours to overnight.
3. Filter the resulting suspension before use using a 0.22 μm filter to remove unsolubilized powder.
4. Keep at room temperature protected from light for up to several weeks.

3.2.2 Cresyl Violet Working Solution

1. On the day of staining, prepare a Cresyl violet working solution in a 15 mL Falcon tube, by mixing:
   (a) 580 μL cresyl violet stock solution.
   (b) 500 μL 100% ethanol.

(c) 385 µL Tris–HCl 1 M, pH 8.5.

(d) 200 µL RNAse-free water.

2. Place in an ice water bath (*see* **Note 4**).

3.2.3 Cresyl Violet Staining

1. Prepare one 95%, two 70% and 100% ethanol solutions in 50 mL falcons, respectively, using RNAse-free water. Place them in an ice water bath.
2. Retrieve PEN membrane slides with frozen sections from −80 or prepare fresh slides the same day. Keep them on dry ice, preferably in a "sandwich" with dry ice underneath and a container with dry ice on top.
3. Transfer slide(s) to 95% ethanol using and fix for 2 min (*see* **Note 5**).
4. Transfer slide(s) to bottom of a fresh petri dish and add 200 µL cresyl violet working solution. Stain for 30 s to 1 min while ensuring an even distribution by gently swirling the petri dish. Exact time will depend on desired staining intensity.
5. Transfer to 70% ethanol.
   (a) Hold slides with forceps and dip rapidly to remove both staining solution and OCT (*see* **Note 6**).
6. Repeat **step 5** with fresh 70% ethanol solution.
7. Transfer to 100% ethanol for 2 min.
8. Air-dry for about 1–2 min (*see* **Note 7**).

3.3 Laser Capture Microdissection

1. Perform laser capture microdissection using a system such as PALM MicroBeam, as per manufacturer directions (Fig. 1).
2. Use AdhesiveCap *opaque* tubes, or similar, for collection of microdissected tissues (*see* **Note 8**).

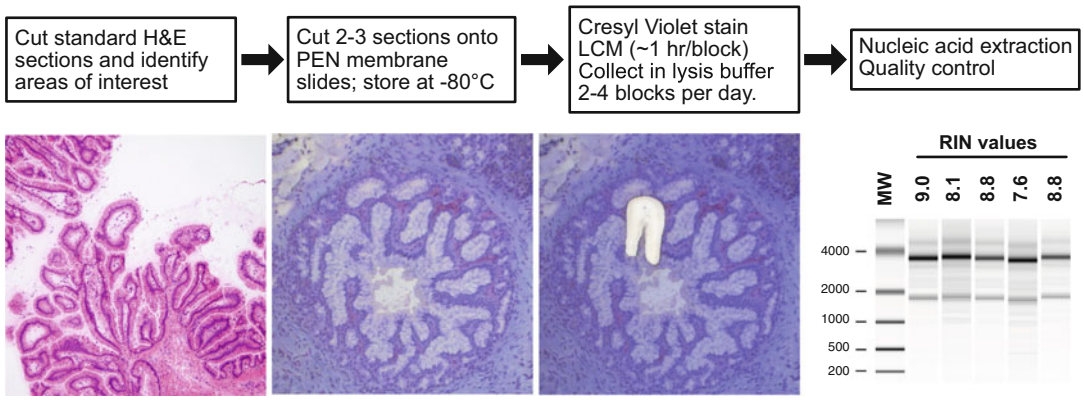

Fig. 1 Overall workflow of LCM-RNA-seq method. Images depict a human intraductal papillary mucinous neoplasm stained with H&E or cresyl violet, followed by Bioanalyzer results for five tumor samples microdissected according to this protocol

3. Collect tissue microdissected fragments from areas of interest into the cap (*see* **Note 9**).
4. Transfer tissue pieces:
   (a) Add 20 μL of lysis buffer to the cap, pipet up and down several times and transfer the 20 μL back to the separate tube containing lysis buffer.
   (b) Repeat once or twice.
5. Let cells lyse according to nucleic acid type and extract nucleic acids (*see* **Note 10**).
6. Assess nucleic acid yield and quality (*see* **Note 11**).

4 Notes

1. Insufficient yields can best be addressed by scaling up. This will require putting several sections on one PEN membrane slide. We recommend cutting multiple frozen sections on one PEN membrane slide, particularly if the number of target cells is low (e.g., precursor lesions).
2. With regard to section thickness, there is an obvious trade-off between yield and morphology. We have found 8–9 μm to be a good balance. Also, we routinely UV-irradiate PEN membrane slides for at least 30 min before use. This does not impact nucleic acid yield or quality, but we have observed that microdissected fragments are more easily cut and catapulted from PEN membrane slides following irradiation.
3. After tissues sections are sectioned onto a PEN-membrane slide, RNA and DNA are generally stable for at least 2 weeks.
4. For human tissues, we recommend using an ice water bath during the staining procedure as warmer temperatures have been associated with reduced RNA quality. Murine frozen sections appear to be less labile and may yield intact RNA when stained at room temperature with cresyl violet acetate working solution or other ethanol solutions.
5. It is possible to stain two PEN membrane slides back-to-back in one run, but take care that the correct sides of the slides are facing outward. When staining several tumors a day, the 95% ethanol solution—which receives the −80 °C PEN membrane slides—can become very cold (well below 0°, ice will form on the outside of the falcon tube). On occasion, "bubble" may be apparent on the PEN membranes, particularly at the end facing the bottom of the tube. This does ***not*** affect the success of procedure.
6. Removal of OCT is ***very*** important. OCT is water soluble and it is conceivable to use higher ethanol dilutions (e.g., 50%) to

enhance removal. However, there is a trade-off between water content and potential reactivation of endogenous RNAses in more aqueous staining conditions. In our experience, 70% ethanol provides the best balance for OCT removal, but it requires mechanical support (i.e., fast dipping) for OCT removal.

7. If the tissue is kept in a dry environment, RNA should be stable for at least 6 h after staining. Nevertheless, LCM should be performed quickly after staining for best results.
8. The AdhesiveCap's white/opaque background clearly enhances tissue morphology during LCM and can yield a microscopic view comparable to that of a coverslipped tissue section.
9. We only use the collection tubes for their caps, i.e., we keep the lysis buffer (e.g., RLT plus for RNA or Proteinase K containing buffers for DNA) in a separate tube. The PALM MicroBeam platform usually allows us to gather thousands of cells for a given lesion within 1 h.
10. For LCM sample nucleic acid extraction, we use the RNeasy Micro Plus Kit (QIAGEN) for RNA and the QIAamp DNA Micro Kit (QIAGEN) for DNA. For RNA samples, we lyse the cells at room temperature for 30 min under agitation after their transfer into RLT plus lysis buffer. For DNA samples, we do not use the LCM protocol specified in the manual, but the regular protocol with 180 µL ATL buffer and 20 µL Proteinase K solution. We have observed best yields after digesting in this mixture *overnight*.
11. For RNA, we suggest using a Bioanalyzer (Agilent) with a RNA 6000 Pico chip to check both yield and quality. For DNA, we suggest using a Qubit (Invitrogen) fluorometer to get precise estimates of yield. In our experience, Nanodrop instruments lacked sufficient precision for this application. A useful positive control is to extract RNA/DNA from a full bulk section of adjacent tissue in the same block under identical condition to assess the overall integrity of macromolecules within the tissue block.

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