Article:
Cummings, M, Mappa, G and Orsi, N (2018) Laser Capture Microdissection and Isolation of High-Quality RNA from Frozen Endometrial Tissue. Methods of Molecular Biology, 1723. pp. 155-166. ISSN 1064-3745

https://doi.org/10.1007/978-1-4939-7558-7_8

Title: Laser capture microdissection and isolation of high quality RNA from frozen endometrial tissue

Running head: RNA isolation from LCM frozen tissue

Michele Cummings, Georgia Mappa & Nicolas M. Orsi

Leeds Institute of Cancer and Pathology, Wellcome *Trust Brenner Building, St. James's* University Hospital, Beckett St., Leeds LS9 7TF, UK

Corresponding author: Nicolas M. Orsi, Email: n.m.orsi@leeds.ac.uk

i. Abstract

Laser capture microdissection (LCM) allows expression profiling of specific cell populations within tissues. However, isolation of high quality RNA from laser capture microdissected frozen tissue is beset by problems arising from intrinsic tissue RNase activity. Herein, we describe an optimised staining/LCM/RNA extraction protocol developed for the isolation of epithelial RNA from frozen tissue sections using human endometrial cancer as a model tissue. This method combines excellent, reproducible visualisation of tissue morphology with the isolation of high integrity RNA suitable for downstream applications such as expression microarray analysis. We present quantitative and qualitative RNA data obtained from >200 endometrial epithelial samples (normal, hyperplastic and cancerous), where 92% of samples had RIN values of 7 and above and highlight common pitfalls faced by investigators. This method should also be broadly applicable to a range of other tissue types.

ii. Key words

Frozen tissue, laser capture microdissection, cresyl violet, RNA integrity, RIN, endometrium, epithelium.

1. Introduction

Laser capture microdissection enables the visualisation and isolation of homogeneous cell populations from tissue sections for subsequent downstream molecular analyses. However, isolation of high quality RNA suitable for whole genome expression profiling via microarray [1] or RNA sequencing [2] technologies presents a particular challenge due to the ubiquity of RNases. These enzymes are present in varying amounts in different tissues [3] and degrade RNA during ex vivo autolytic processes. Most problematic for LCM is the fact that RNases are released from cells upon freeze-thawing of tissue and cause rapid RNA degradation. Thus, the necessity for the adequate visualisation of tissue morphology for accurate LCM must be balanced against the need to inactivate tissue RNases. Unfortunately, since storage in tissue RNA stabilisers (e.g. RNAlater) fails to maintain adequate tissue morphology [4], one is inevitably tied to using fresh frozen tissue. Whilst commercially developed stains developed for LCM/RNA isolation (e.g. HistoGene) work well with certain tissues such as brain [5], we [6] and others [7, 8] have found that use of this stain is associated with significant RNA degradation. In order to minimise RNase activation during tissue staining and LCM, various approaches have been adopted, such as the inclusion of RNase inhibitors in aqueous stains [8], or the use of alcohol-based stains incorporating cresyl violet [9] which limit tissue hydration and hence RNase activation. In our hands, alcohol-based cresyl violet gave the best results in terms of tissue morphology and allowed the isolation of high integrity RNA from LCM of epithelium [6]. We also found that both the staining intensity and reproducibility achieved using alcohol-based cresyl violet solutions were improved by adjusting the pH of the stain to pH 8.0 prior to use (Fig.1). Our protocol also incorporates the addition of an appropriate RNase protection reagent immediately after RNA isolation in order to inactivate any residual co-purified tissue RNase.

2. Materials

Staining solutions: Use a commercial RNase-free water (not DEPC-treated) and ACS grade absolute ethanol to prepare all fixing/staining solutions. Glassware/non-disposable plasticware should be sprayed with RNase-ZAP (Fisher Scientific, Loughborough, UK), rinsed thoroughly in Milli-Q water and allowed to dry prior to use. Anhydrous ethanol (for the final slide dehydration steps) is made by adding 15 g of 3-Å molecular sieve beads to 500 ml ethanol (prepare this the day before so as to allow the beads to settle fully and store bottle tightly closed thereafter). Alcohol-based cresyl violet stock is made by dissolving 1 g cresyl violet acetate (Acros Organics) per 100 ml 75% (v/v) ethanol overnight on a magnetic stirrer and protected from light. The solution is then filtered through Whatman Grade 1 filter paper and stored at room temperature, again protected from light (this keeps for months). Just prior to staining (see **note 1**), take the required amount of cresyl violet stock and adjust the pH by adding 1 M Tris-HCl (pH 8.0) so that the final concentration of Tris is 20 mM (i.e. add 20 µl Tris-HCl per 1 ml cresyl violet stock).

Specialist equipment/materials: Cryostat (CM3050S; Leica Microsystems; Wetzlar, Germany), Laser Capture Microscope (PALM MicroBeam UV; Carl Zeiss, Herts., UK), polyethylene naphthalate (PEN) membrane coated glass slides (Carl Zeiss or Arcturus), adhesive capped collection tubes (Carl Zeiss), RNeasy plus micro kit (Qiagen, Crawley, UK), RNase inhibitor: RNAsecure or SUPERase-In (Ambion; Thermo Fisher Scientific), Bioanalyser 2100 (Agilent technologies), RNA 6000 Nano kit (Agilent Technologies), SpeedVac with a cold trap (Savant SC110 or similar; if required).

3. Methods

3.1.Tissue collection and storage

Tissue should be transferred to ice and snap frozen as quickly as possible in order to minimise autolytic RNA degradation or changes in RNA expression profiles due to warm ischaemia [10]. For the human endometrial samples used in this study (ethical approval Ref: 05/Q1107/41), hysterectomy specimens were immediately collected from theatre and endometrial tissue sampled by a histopathologist and placed into labelled tubes. Samples were transported to the laboratory on wet ice, wrapped in foil and snap frozen in liquid nitrogen (see **note 2**) within 1 h. Tissues were stored long term at -80°C in tightly sealed tubes to prevent desiccation. Alternatively, tissue can be embedded in optimal cutting compound (OCT) if preferred (see **note 2**). However, a modification of the staining procedure is required to remove residual OCT from tissue sections (see **note 3**). We have used this latter approach to obtain high integrity RNA from laser capture microdissected murine endometrial stroma and epithelia [11].

3.2. Cryosectioning

Tissue should only be handled on dry ice with previously cooled forceps/tweezers. At no point should tissue be allowed to thaw even slightly. Either an anti-roll plate or a natural fibre artist's paintbrush can be used to stop sections rolling. Clean the cryostat stage, brushes, tweezers and anti-roll plate with ethanol before use. Change the microtome blade before use and move along to an unused area when sectioning a new specimen. Make sure the handwheel of the cryostat is locked at all times except when sectioning.

1) Set the chamber and chuck temperature in the cryostat to -16°C (this is ideal for endometrial tissue but may need optimising for different tissues). Once the desired

temperature is reached, place the tissue (wrapped in foil) in the cryostat chamber and allow to equilibrate for 15 min – 1 h depending upon the size of the specimen.

2) Set the section thickness on the cryostat to 8 or 12 µm (see **note 4**). Set the trim thickness to 30 µm.
3) Remove the tissue from the foil with tweezers and mount on the chuck (pre-chilled inside the cryostat) using RNase free water which will freeze it in place (see **note 5**). Alternatively, use OCT to mount OCT embedded tissue.
4) Secure the chuck in the chuck stage.
5) Unlock the handwheel and check the distance of the specimen from the blade and bring the specimen very close to the blade.
6) Set to "trim" and use the handwheel to trim tissue until a suitable "face" is reached. Be careful not to over-trim so as to minimise tissue wastage.
7) To take sections, brush away tissue trimmings, turn the trim function off and cut a few sections (the first few will typically be thicker than 8 µm). If not using an anti-roll plate, cut slowly and tease the tissue slowly away from the blade using a paintbrush to hold the edge.
8) Mount sections on either uncharged glass slides or PEN slides (for LCM) stored at room temperature. Depending on the section size, more than one section may be mounted on a PEN slide, which is more economical. Arrange tissue section(s) on the cryostat stage using a paintbrush. Label slide, hold at the labelled end and rest the opposite edge on the stage below the tissue section. Pivot to lower the slide quickly and gently (membrane side down) onto the section(s); the section(s) will adhere to the slide as it thaws very briefly. Quickly place the slide face-up on the cryobar within the cryostat and allow to freeze for a few seconds. Wrap the slide(s) loosely in foil within

the cryostat chamber and transfer quickly to dry ice for transport to the staining bench.

9) To check that a suitable area of tissue is selected prior to mounting on PEN slides, sections can be mounted on uncharged glass slides and visualised by cresyl violet quick stain (see **note 6**). Glass slides of tissue sections should also be prepared for standard haematoxylin and eosin (H&E) staining as a permanent record and for histopathological assessment since the nature of the tissue (e.g. cancer vs. background normal tissue) can be presumptive based on its macroscopic assessment at the time of collection.
10) Once cryosectioning is complete, carefully remove the mounted tissue from the chuck using a razor blade to cut through the ice and transfer the tissue quickly to dry ice prior to storage.

3.3. Staining for LCM

This protocol is for non-OCT embedded tissue sections; for OCT embedded sections the protocol is modified slightly (see **note 3**).

i) Fill coplin jars as follows: A, D (95% ethanol) B, C (75% ethanol), E, F, G (anhydrous ethanol) – see Materials.
ii) Calculate the amount of cresyl violet stain required for the experiment (based on needing 250 µl per slide) and adjust the pH to 8.0 with Tris-HCl (see Materials).
iii) Cryosectioned tissue slides should be stored face up on dry ice, wrapped loosely in foil, until use. Slides should be stained sequentially rather than in batches and up to 30 slides can be stained before changing the ethanol

solutions. Dab off excess liquid from the slide in between each step by placing the edge of the slide on absorbent tissue paper. Stain as follows:

1) Immerse slide up to frosted edge in jar A (95% ethanol) for 30 seconds.
2) Immerse slide as above in jar B (75% ethanol) for 30 seconds.
3) Lay slide flat and face up on absorbent tissue. Using a hydrophobic barrier pen, draw 2 lines either side of the PEN membrane parallel to the frosted edge of the slide.
4) Pipette 250 µl cresyl violet stain (prepared in ii) evenly over the tissue. Wait 30 seconds then remove excess stain by dabbing the long edge of the slide on a tissue.
5) Immerse slide in jar C (75% ethanol) for 30 seconds.
6) Immerse slide in jar D (95% ethanol) for 30 seconds.
7) Immerse slide in jar E (anhydrous ethanol) for 30 seconds.
8) Immerse slide in jar F (anhydrous ethanol) for 30 seconds.
9) Immerse slide in jar G (anhydrous ethanol) for 5 min (one can commence staining the next slide at this point).
10) Blot off excess ethanol from edge of slide and lay face side up to air dry. Either proceed directly to LCM or store the slide at -80°C as described in 3.4.

3.4. Slide storage

Place slides individually in 50 ml Falcon tubes containing ~5 ml pre-dried silica desiccant beads. Close tightly, seal with Parafilm and store at -80°C. Slides can be stored at least 2 months in this way without any deterioration in RNA integrity. Important: always allow tubes to equilibrate to room temperature completely before opening in order to prevent condensation forming on the slides, which would activate tissue RNases.

3.5. Laser capture microdissection

Laser capture microdissection was carried out using a PALM MicroBeam UV LCM microscope, using the Palm Robo software, version 3.2. Gloves must be worn throughout.

1. Before starting, check the relative humidity level in the laboratory, which should ideally be 45% or less (see **note 7**).
2. Remove the selected slide from the -80°C freezer (section 3.4), and allow to equilibrate to room temperature before removing it from the storage tube.
3. Switch on the LCM microscope and allow to warm up (the indicator light turns from orange to green when the laser can be switched on). Open the Palm Robo software and load the PEN slide and the adhesive-capped collection tube as per the manufacturer's instructions.
4. Ensure energy, focus and laser alignment are suitable on an area of PEN membrane free of tissue: Use the freehand draw tool to draw a line, select "cut" and realign laser with the crosshairs on the screen if necessary using the "Position UV laser" option.
5. Position the diffusor above the slide to enable better tissue visualisation and draw around selected areas (at 10 – 20 x magnification).
6. When ready to cut, position the collection tube above the slide; select the drawn areas (which can be viewed on the "elements list") and harvest using the Robo LPC function with your pre-optimised settings (see **notes 8 & 9**).
7. Once enough material has been collected (or if all the available material on a particular slide has been collected), add an appropriate volume (see **note 10**) of RLT plus buffer (Qiagen RNeasy plus microkit), to which β-mercaptoethanol has been freshly added to the collection tube. Vortex thoroughly to solubilise the LCM material (invert several times to ensure removal of material from the lid) and either extract immediately or store at -80°C until ready to perform RNA isolation (see **note 11**).

3.6. RNA isolation, QC and quantitation

For RNA extraction and handling, use a dedicated set of equipment (pipettes and microfuge), RNase-free plasticware and barrier tips throughout. RNA isolation from LCM material is carried out on the LCM lysate using the Qiagen RNeasy plus micro kit (see **note 12**). Manufacturer's instructions are followed but with the following modifications: a) an additional 80% ethanol wash step is performed to remove any traces of guanidinium salts and b) an RNA protection reagent (RNAsecure; see **note 13**) is added to the RNA immediately on purification as follows: RNAsecure reagent (25 x; 0.5 µl) is added to the bottom of the eluate collection tube prior to eluting RNA from the column with 14 µl RNAse-free water (12 µl RNA is eluted). Samples are then incubated for 10 min at 60°C to inactivate RNases and subsequently evaporated to dryness using a SpeedVac with a cold trap at ambient temperature and resuspended in 5 µl RNase-free water. A sample (1 µl) is run on a RNA 6000 Nano LabChip (see **notes 14 & 15**) for both RNA quantitation and RIN measurements (Fig. 2) and the remainder of the samples stored at -80°C.

For comparison, RNA was extracted from whole frozen sections (8 µm thick, about 1 – 3 cm^2 surface area) of endometrial cancer specimens. Tissue was mounted on glass slides and stained with alcohol-based cresyl violet as described for the LCM specimens. Tissue was then scraped from the slide into RLT plus buffer (Qiagen) pipetted onto the section, vortexed thoroughly and passed through a Qiashredder column (Qiagen) before extracting with RNeasy plus micro kit as described (omitting the SpeedVac concentration step).

Results of 203 separate LCM/RNA extractions from normal, hyperplastic and cancerous human endometrial epithelia from a total of 131 study participants are shown in Fig. 3. There was no significant difference in RIN values between normal, hyperplastic or cancerous LCM

RNA samples (Kruskall-Wallis test) but a significant inverse correlation (Pearson's product moment correlation $P<0.001$) between time taken to LCM and RIN value was identified, although the estimated effect of this was modest (0.24 RIN unit decrease per hour, a figure which agrees well with our previous experimentally derived estimate of 0.1 – 0.3 RIN unit per hour) [6]. Taken as a whole, the vast majority of LCM RNA samples (91.6%) had RIN values of 7 and above, and only 1.5% of samples had RIN values <5. No significant correlation was observed between RIN values and time taken from collection to freezing (data not shown), although this variable was kept to a minimum. No significant differences were observed in RNA yields between the different types of epithelia (normal, hyperplasia, cancer) were observed. The median RNA yield for all samples was 23 ng/10^6 μm^2 (interquartile range 17 – 27).

4. Notes

1. Adjusting the pH of the cresyl violet stock with Tris-HCl pH 8.0 was found to give more intense and reproducible tissue staining. However, it does cause slight precipitation of cresyl violet, although this occurs slowly and the stain is good to use over the course of an hour. For this reason, the required amount of cresyl violet stock for the staining experiment should be pH-adjusted just prior to starting.
2. Snap freezing in isopentane slurry cooled on liquid nitrogen is an alternative freezing method and can give better preservation of morphology in some tissues since the tissue freezes more quickly as it is not insulated by a layer of nitrogen gas. This method is compatible with nucleic acid isolation but may not be compatible with other potential applications such as lipid analysis. Similarly, the use of OCT enables greater ease in frozen sectioning but OCT may interfere with

potential downstream applications, although it is compatible with RNA isolation (see **note 3**). Snap freezing directly in liquid nitrogen without OCT affords the greatest versatility for downstream analyses (important for clinical samples), and in our experience endometrial tissue morphology is largely unaffected using this approach.

3. To remove residual OCT the staining protocol described in 3.3 is modified by the inclusion of an additional 30 second incubation in 50% ethanol both immediately before and immediately after the cresyl violet staining step.
4. Sections of 12 µm thickness are advantageous compared to 8 µm since more material is harvested per unit area during LCM. However, in our experience, cutting 8 µm sections allows better consistency in section quality.
5. Mounting tissue onto the cryostat chuck using water requires good timing. The trick is to place a small blob of water (50 – 150 µl, depending on the size of tissue) on the chuck using a plastic pastette, allow the water to freeze just enough (so it starts to become slightly opaque) before placing the tissue on the water mound, where it will adhere as it freezes. If the water freezes for too long prior to placing the tissue it will not adhere properly. However, not allowing the water to freeze sufficiently will result in the tissue sitting in a puddle of water which will risk tissue thawing and seriously compromise RNA integrity.
6. To visualise tissue sections quickly whilst sectioning, we found it convenient to use a quick cresyl violet staining protocol: remove the glass slide from dry ice and immerse in 95% ethanol for 30 seconds to fix, blot and stain with buffered cresyl violet stain (see Materials) for 30 seconds, blot and immerse in 2 x absolute ethanol (not anhydrous) washes for 30 seconds each. Proceed to collecting

sections for mounting on PEN slides for LCM and glass slides for H&E if satisfied with the area of tissue selected.

7. Other researchers [5] have noted that humidity levels can affect RNA quality from LCM specimens, presumably due to tissue rehydration and activation of RNases. As a precautionary measure, we used a portable dehumidifier (MEACO DD8L Junior) to avoid humidity levels exceeding 45%.
8. Optimised cutting settings for our purposes were at 10 x magnification, set to a speed of 55% maximum with cutting and LPC (laser pulse catapult) energies of 49% and 90% of maximum, respectively, although the cutting energy required did need to be increased for some of the normal endometrial specimens.
9. Although tempting, avoid harvesting too many areas at once as the collection cap becomes "saturated" with pieces of tissue. Periodically inspect the collection tube cap at 5 x magnification and adjust its x and y coordinates to align an empty area above the slide. Pieces that do not adhere can be identified on the slide at 5 x magnification and catapulted onto the cap using the LPC function.
10. In this study, ~4 – 8 x 10^6 μm^2 (preferably ~6 μm^2) of LCM epithelia was harvested to obtain the yields stated. If necessary, material from different slides can be harvested separately and combined before extracting, ensuring the final volume of RLT plus buffer lysate is 350 µl.
11. LCM tissue lysates in RLT plus buffer with β-mercaptothanol can be stored for months at -80°C before extracting with no apparent loss of RNA integrity.
12. We used the standard manufacturer's protocol which purifies RNA species >200 nucleotides. A modified manufacturer's protocol is available for the co-purification of small RNA species if desired, although we have not tested this. We have not compared this kit with others but suggest that approaches using

phenol/chloroform be avoided for limited material owing to RNA losses at the aqueous/organic interface.

13. We found RNAsecure to be an ideal RNase inhibitor as it irreversibly inactivates any residual tissue RNase in purified RNA from tissue samples. RNA preserved in this way (25 ng) was used successfully in microarray hybridisation experiments using Agilent SurePrint G3 Human GE 8×60k microarrays, where cRNA probes generated using the Agilent Low Input Quick Amp One Color labelling kit had specific activities and yields that met the recommended levels for array hybridisation and subsequent microarray data passed all Agilent QC metrics. However, we recommend that users check compatibility of RNAsecure with their downstream applications. Specifically, RNAsecure is active above 45°C and may inhibit certain enzymes during incubations above this temperature. As an alternative, SUPERase-In (a broad spectrum protein-based RNase inhibitor) may be added to purified RNA, although in this case RNase inactivation is potentially reversible.
14. Note that RNA concentration was necessary for the purposes of our downstream analysis and to bring the RNA within the measurable range of the RNA 6000 Nano LabChip. The Agilent RNA 6000 Pico LabChip could be used as alternative, depending on RNA concentration requirements. As Pico LabChips are very sensitive to the presence of ions, check whether runs are affected by any RNA protectants used.
15. Note also that addition of either RNAsecure or SUPERase-In render UV spectrophotometric readings (nanodrop) unreliable but do not affect quantitation methods based on fluorescence (e.g. Bioanalyser concentration readings).

References

1. Thompson KL, Pine PS, Rosenzweig BA, Turpaz Y, Retief J (2007) Characterization of the effect of sample quality on high density oligonucleotide microarray data using progressively degraded rat liver RNA. BMC Biotechnol 7:57
2. Gallego Romero I, Pai AA, Tung J, Gilad Y (2014) RNA-seq: impact of RNA degradation on transcript quantification. BMC Biol 12:42
3. http://www.thermofisher.com/uk/en/home/references/ambion-tech-support/nuclease-enzymes/tech-notes/rnase-activity-in-mouse-tissue.html
4. Staff S, Kujala P, Karhu R, Rökman A, Ilvesaro J, Kares S, Isola J (2013) Preservation of nucleic acids and tissue morphology in paraffin-embedded clinical samples: comparison of five molecular fixatives. J Clin Pathol 66:807-810
5. Ordway GA, Szebeni A, Duffourc MM, Dessus-Babus S, Szebeni K (2009) Gene expression analyses of neurons, astrocytes, and oligodendrocytes isolated by laser capture microdissection from human brain: detrimental effects of laboratory humidity. J Neurosci Res 87:2430-2438
6. Cummings M, McGinley CV, Wilkinson N, Field SL, Duffy SR, Orsi NM (2011) A robust RNA integrity-preserving staining protocol for laser capture microdissection of endometrial cancer tissue. Anal Biochem 416:123-125
7. McGinley JN, Zhu Z, Jiang W, Thompson HJ (2010) Collection of epithelial cells from rodent mammary gland via laser capture microdissection yielding high-quality RNA suitable for microarray analysis. Biol Proced Online 12:9026
8. Kube DM, Savci-Heijink CD, Lamblin AF, Kosari F, Vasmatzis G, Cheville JC, Connelly DP, Klee GG (2007) Optimization of laser capture microdissection and RNA amplification for gene expression profiling of prostate cancer. BMC Mol Biol 8:25

9. Clément-Ziza M, Munnich A, Lyonnet S, Jaubert F, Besmond C (2008) Stabilization of RNA during laser capture microdissection by performing experiments under argon atmosphere or using ethanol as a solvent in staining solutions. RNA 14:2698-2704
10. Ma Y, Dai H, Kong X. (2012) Impact of warm ischemia on gene expression analysis in surgically removed biosamples. Anal Biochem 423:229-235
11. Field SL, Cummings M, Orsi NM (2015) Epithelial and stromal-specific immune pathway activation in the murine endometrium post-coitum. Reproduction 150:127-138

Figure legends

Fig. 1. Duplicate 8 µm frozen sections of endometrial adenocarcinoma were mounted on glass slides and stained using the protocol described in Methods (3.3). Alcohol-based cresyl violet stain was buffered with 20 mM (final concentration) Tris-HCl pH 7.0 (**a**), and pH 8.0 (**b**).

Fig. 2. (**a, b**) An endometrial adenocarcinoma frozen tissue section mounted on a PEN membrane slide and stained with alcohol-based cresyl violet (buffered to pH 8.0) according to the protocol described in Methods (3.3). The section is depicted before (**a**) and after **(b**) LCM of epithelial nests. (**c**) A typical Bioanalyser pseudogel image obtained after running LCM endometrial epithelial RNA on an RNA 6000 Nano LabChip (N, H, C correspond to RNA extracted from normal, hyperplastic and cancerous endometrial epithelia, respectively). Examples of Bioanalyser traces from samples 9 and 11 with corresponding RIN and RNA concentrations are depicted in (**d**); 28S and 18S ribosomal RNA peaks are indicated, as well as the internal marker (M).

Fig. 3. (**a**) RIN values (median and interquartile range) of RNA extracted from normal (N), hyperplastic (H) and cancerous (C) human endometrial epithelia (n=203) isolated by LCM from 8 µm thick frozen sections using the optimised protocol described herein. RIN values for RNA extracted from whole frozen sections of endometrial cancer tissues from different patients (WS; n=10) are also shown for comparison. RNA yields per unit surface area (median and interquartile range) obtained from the same LCM samples are shown in (**b**). RIN values for all LCM RNA samples are summarised as a table in (**c**) and the relationship between LCM time and RNA quality is shown in graph (**d**), where the slope of the linear regression curve is -0.24±0.06 RIN units/h; the Pearson correlation coefficient of LCM time vs RIN is -0.34, $P<0.001$.