Published in final edited form as:

Methods Mol Biol. 2018 ; 1723: 385–396. doi:10.1007/978-1-4939-7558-7_22.

Laser-Capture Microdissection and RNA Extraction from Perfusion-Fixed Cartilage and Bone Tissue from Mice Implanted with Human iPSC-Derived MSCs in a Calvarial Defect Model

Xiaonan Xin, Xi Jiang, Alexander Lichtler, Mark Kronenberg, David Rowe, Joel S. Pachter

Abstract

Laser-capture microdissection (LCM) coupled to downstream RNA analysis poses unique difficulties for the evaluation of mineralized tissues. A rapid protocol was thus developed to enable sufficient integrity of bone and cartilage tissue for reliable sectioning, while minimizing RNA loss associated with prolonged decalcification and purification steps. Specifically, the protocol involves pump-assisted, cardiac perfusionfixation with paraformaldehyde, and moderate digestion of LCM-acquired tissue with proteinase K followed by DNase treatment and separation of RNA using magnetic beads. Reverse transcription and cDNA synthesis are performed immediately after RNA purification, without need for further protein removal.

Keywords

Calvarial defect model; Cartilage; Mesenchymal stem cell; Pluripotent stem cell

1 Introduction

Laser-capture microdissection (LCM) allows for selective acquisition of distinct subpopulations of cells from a heterogeneous background [1–3]. While LCM can be used to retrieve designated whole cell targets from ex vivo culture preparations, it has found its widest application to the removal of cell “profiles”; i.e., slices of cells, from their in situ environments within tissue sections. And though most tissues are readily amenable to LCM—e.g., use of fresh-frozen samples and routine glass slides—mineralized ones such as bone present particular challenges due in large part to difficulties in sectioning and adherence to slides. This has largely restricted the use of LCM to the analysis of bony tissue at early developmental stages, when mineralization is limited [4].

To overcome these obstacles, adult bone is often decalcified prior to sectioning, but this too is not without consequences and caveats. Decalcification protocols are often long in duration, e.g., 3–15 days, which in turn significantly increases risk to RNA integrity [5]. Cross-linking fixatives have been introduced to mitigate RNA loss as well as to improve morphology, but can themselves compromise RNA quality [6, 7]. Immersion fixation, in particular, further suffers from not being able to deliver fixative to all regions of tissue at the same rate [8]. For example, optimal preservation by formaldehyde solutions requires an

excess of 20 times the volume of tissue [6]. And digestion conditions to reverse cross-linking can fracture RNA and pose impediments to delicate enzymatic reactions employed in downstream platforms [9]. Lengthy RNA purification procedures to eliminate contaminants can further depress nucleic acid yield and be cumbersome when dealing with numerous, small volume samples.

A favorable LCM protocol for bone is thus one that will enable efficient sectioning in the absence of prolonged decalcification, while maximizing RNA integrity. Accordingly, we have developed a facilitated protocol for LCM of adult bone tissue using pump-assisted, cardiac perfusion-fixation with 3.2% buffered paraformaldehyde, coupled to post-LCM in situ tissue digestion, followed by magnetic bead separation of RNA and reverse transcription.

This LCM protocol has most recently been applied to a novel, bone model system established by this group [10]. Specifically, the model system involves the following steps: (1) deriving-induced pluripotent stem cells (iPSC) from patients, control individuals, and potentially from individuals with identified genetic traits that are associated with a skeletal phenotype such as high or low bone mass; (2) differentiating these cells into a mesenchymal stem cell-like (MSC) population that has the potential to produce cartilage and bone, and (3) implanting these cells into a mouse calvarial defect model. In this model system, our MSCs produce proliferating stage cartilage, hypertrophic and mineralized cartilage, and bone. Although significant amounts of these cells types are reproducibly produced, the structures of the skeletal elements generated are not consistent enough to dissect out specific tissues on a macro scale for analysis and, thus, LCM affords unique opportunities for analysis. Here, we present our most recent LCM protocol for adult bony tissue that could potentially yield RNA of sufficient quantity and quality for amplification and deep sequencing using Illumina technology.

2 Materials

2.1 MSCs

Human iPSCs were differentiated into MSCs and implanted into 3.5 mm diameter calvarial defect in a HEALOS scaffold (*Xin et al., manuscript in preparation*). If implants are allowed to develop for 6 weeks before sacrifice, the implants contain large amounts of human cartilage. If the implants are allowed to develop longer, the cartilage progressively becomes hypertrophic, mineralizes and is replaced by bone.

2.2 Perfusion-Fixation

1. Stock paraformaldehyde 32% solution, EM grade (methanol-free), Electron Microscopy Sciences.
2. 10× Phosphate-buffered saline (PBS), pH 7.4, Bio-Rad.
3. 1 volume 10× PBS: 8 volumes double distilled water.
4. Masterflex L/S peristaltic pump with 96410–13 tubing speed setting 5.

2.3 Tissue Sectioning

1. Cryostat, CM 3050, Leica.
2. Shandon Cryomatrix, Thermo Scientific, or O.C.T. Compound, Tissue-Tek, Sakura.
3. Disposable base molds, Fisherbrand, 24 × 24 × 5 mm, Thermo Fisher.
4. CryoJane ECU Tape Transfer System, Leica, which is installed in the Cryostat.
5. Cryojane Cryostat Frozen Sectioning Aid Adhesive Coated Slides (CFSA) CS 4×, Leica.
6. CryoJane AdhesiveTapeWindows, Leica.

2.4 LCM

1. ArcturusXT LCM System, with or without epifluorescence optics, Thermo Fisher.
2. CapSure® Macro LCM Caps (48 caps), Life Technologies.

2.5 RNA Extraction

1. Oven set to 55 °C (an Autoblot® microhybridization oven [Bellco Glass, Inc.] is used by this group). Inside the oven is a block wrapped in plastic tape, sticky side up, onto which a microfuge tube, containing a Capsure® Macro LCM Cap, can be placed so that LCM-acquired tissue can be fully exposed to Modified Lysis Buffer for efficient RNA extraction (Fig. 1).

2. TempBlock or water bath set to 75 °C.
3. Direct cDNA Cell Lysis buffer, Signosis.
4. Proteinase K, AmericanBio.
5. Modified Lysis Buffer (MLB).

Preparation of Proteinase K stock solution: Dissolve Proteinase K in 10 mM Tris-HCl, pH 7.5, 20 mM CaCl₂, 50% Glycerol at 20 mg/ml. Proteinase K from AmericanBio comes with 100 mg powder per bottle, so add 5 ml buffer to the bottle, mix to dissolve, and store at −20 °C. Proteinase K from this supplier has 30 units of activity/mg; to use proteinase K from other suppliers it may be necessary to adjust the mg/ml to achieve 600 units/ml. Preparation of MLB: Thaw 40 µl Signosis Cell Lysis buffer per cap (plus enough for 1 additional cap) immediately before use, and keep on ice. Add 1/50 volume proteinase K, for a final concentration of 0.4 mg/ml.

6. RNaseAway, Life Technologies.
7. TURBO DNA-free DNase treatment kit, Life Technologies.
8. RNAClean XP magnetic beads, Beckman Coulter.
9. 100% ethanol, highly purified (any reputable supplier).

10. RNase-free water (any reputable supplier).
11. Magnet unit for use with 0.2, 0.5, or 1.5 ml microfuge tube. We use a MiniMacs apparatus, Miltenyi Biotec, though the Agencourt SPRIPlate 96R Ring Magnet Plate, Beckman Coulter, may have some advantages.

2.6 cDNA Synthesis

1. dNTPs, any reputable supplier, such as Roche, Promega, NEB, Life Technologies.
2. Superscript III Reverse Transcriptase, Life Technologies.
3. Random hexamers, any reputable supplier.
4. NuGEN Ovation RNA-Seq FFPE System.

3 Methods

3.1 Preparation of Tissue/Slides for LCM

1. Mice are cardiac perfused with ~10 ml of PBS, pH 7.4, followed by 50 ml of 3.2% PFA in PBS, using a Masterflex L/S peristaltic pump at speed setting 5.
2. Calvaria containing implants are dissected out and fixed for 2–4 h in 3.2% PFA in PBS at 4 °C.
3. The calvaria are removed from the PFA, briefly dried, and embedded in Shandon Cryomatrix in a base mold, snap frozen for at least 20 min in methyl-butane that had been prechilled on dry ice, and stored at –80 °C until use. The samples should be oriented in the mold so the sections will be cut in the desired plane of the specimen. In our case the calvaria with the human cell implant is placed to give coronal sections through both the mouse calvaria and the human implant.
4. Before sectioning, the tissue blocks are removed from the mold and frozen onto the specimen disc in Cryomatrix and placed on the specimen head, all in the Cryostat chamber.
5. Thick (40–60 µm) sections are cut until the appropriate level of the specimen is reached. Check using a microscope to make sure the correct level is reached.
6. The cryostat is adjusted to give thinner sections; we usually use 7 µm sections.
7. A precut piece of CryoJane Adhesive Tape is chilled in the Cryostat chamber, placed on the specimen, a chilled roller is rolled over the tape to make sure it has good contact with the sample.
8. The section is cut, the tape is placed with the section down on a CryoJane slide that had been placed on the CryoJane slide station within the Cryostat chamber, the roller is rolled over the tape to ensure good contact. A gloved finger is used to press briefly onto the tape to warm it briefly (a few sec). Alternatively, the roller is not used, and only the gloved finger is used to gently press down on the tape. If more than one section is put on one slide they are all done in succession.

9. The slide is put in the CryoJane UV station to fix the section onto the slide. The on switch is activated twice, with 20 s between activations.
10. The tape and section are again briefly warmed with a gloved finger, and the tape is gently peeled off, leaving the tissue section on the slide. The brief warming is necessary to prevent peeling the section off with the tape, and it is probably necessary to experiment to determine the minimum time to warm the slide before peeling the tape off and still get good transfer to the slide.
11. The slide is then dipped in a graded series of ethanol in RNase-free water, 75% for 30 s, 95% for 30 s, 100% 2× for 1 min, and 100% xylene 2× for 1 min each, store in xylene until right before LCM.

3.2 LCM

1. Immediately before LCM, dry the slide in the chemical hood until the xylene has evaporated.
2. Put the slide on the LCM microscope stage. It is important to carry out the LCM procedure as quickly as possible after letting the xylene evaporate, to avoid letting the section rehydrate by absorbing water from the air. It is also valuable to run a dehumidifier in the room containing the LCM instrument and isolate that room as much as possible, to keep the air as dry as possible.
3. Identify the regions containing specific tissue types to be captured. In our case, since our sections are not decalcified, we identify mineralized tissues as dark areas, because the mineral blocks the light. Mineralized cartilage has a distinct appearance because the large hypertrophic chondrocyte cell bodies form distinctive large round holes in the mineral layer, while bone is generally more densely mineralized, and does not have the round holes (Fig. 2).
4. Use the computer mouse to outline the area containing the tissue to be captured. Then activate the UV laser to cut around the outline, and observe whether the tissue is completely cut, which can usually be detected as a visible line through the matrix. Mineralized tissue is particularly resistant to the UV laser. If cutting is not complete, it may be helpful to repeat the UV laser cutting multiple times. It is important to not try to outline an area that is too small, because the UV laser can damage RNA that is too close to the laser cut line.
5. When satisfactory cutting is achieved, activate the IR laser to lift the target tissue and attach it to the LCM cap. You should adjust the size of the IR spot depending on the size and shape of the area you want to capture, to avoid producing IR spots that cross the UV cut line.
6. Activate the cap removal function of the LCM instrument, then follow the procedures to inspect the material removed on the cap and what is left behind, to make sure the appropriate area is taken.

7. If it is desired to obtain more of the same type of tissue, and there is more of the desired tissue type on the slide as well as room on the cap, you can reposition the cap and repeat the process.
8. Once enough sample has been obtained, remove the LCM cap from the instrument, and either place the cap in an appropriate sized RNase-free microfuge tube, freeze it on dry ice and store at -80°C for later use, or proceed to the RNA extraction protocol.

3.3 RNA Extraction (see Note 1)

1. Preheat oven with tape-covered block at 55°C and TempBlock or water bath at 75°C .
2. Prepare sufficient MLB for the number of caps you are going to process, plus one sample extra to allow for pipetting inaccuracy. Immediately before use, pre-warm the MLB at 55°C .
3. Remove the LCM cap from the microfuge tube (if the tube is frozen, it may help to use a cap removal device, which is available from companies such as NEB), add $40\text{ }\mu\text{l}$ pre-warmed MLB to the tube, snap the LCM cap back on tightly, and use a quick snap of the wrist to bring the MLB to the top of the tube, so it is in contact with the LCM cap. Place the tube in the oven upside down on the tape, and incubate for 30 min.
4. Spin the tube briefly in a microfuge at maximum speed to bring the MLB to the bottom of the tube, then incubate at 75°C for 20 min to inactivate the proteinase K. Proceed to DNase step, or quick freeze and store at -80°C until further processing.

3.4 DNase Treatment (see Note 2)

1. Thaw the $10\times$ DNase buffer and DNase Inactivation Reagent from the TURBO DNA-free kit. Transfer $15\text{ }\mu\text{l}$ of extracted RNA to a fresh tube.
2. Add $1.7\text{ }\mu\text{l}$ $10\times$ DNase buffer and $0.5\text{ }\mu\text{l}$ DNase enzyme to the tube and mix. If processing multiple samples, make a master mix containing these volumes of buffer and enzyme times sample number +1, mix, and add $2.2\text{ }\mu\text{l}$ of the mix to each sample.
3. Incubate at 37°C for 30 min.
4. Add $2\text{ }\mu\text{l}$ of DNase Inactivation Reagent, which contains beads that bind DNase. Mix by flicking carefully, and incubate at room temperature for 2 min (periodically mixing to keep the beads suspended).
5. Spin at $10,000\times g$ for 1.5 min. Transfer the supernatant to a fresh tube (you should be able to remove $11\text{--}15\text{ }\mu\text{l}$ without disturbing the beads). Proceed directly to Magnetic Bead Purification or freeze.

3.5 Magnetic Bead Purification

1. Warm RNAClean XP magnetic beads to room temperature; this is necessary to ensure maximum efficiency of binding of RNA to the beads. Shake bottle of beads gently to resuspend them.
2. Add 1.6–1.8 volumes of the beads to the RNA sample. For 15 μ l of RNA, this is 27 μ l, but this procedure can be scaled up substantially to purify RNA from larger volumes, as long as the proportion of beads to RNA is kept the same. The microfuge tube size can be adjusted to accommodate larger volumes, as long as the magnetic apparatus you have is compatible. After the beads are added, flick the tube to mix the beads with the RNA and let it sit for ~10 min to allow the RNA to bind the beads, with occasional flick-mixing to keep the beads suspended.
3. Place the tube in the magnet, and wait ~5 min, or until the beads have moved to the side of the tube next to the magnet.
4. Remove most of the supernatant. It is best to leave a few microliters in the tube to avoid removing some of the beads, which would decrease RNA yield.
5. Wash 3 $\times$ with 200 μ l of 70% ethanol. It is important to make the 70% ethanol immediately before using, making sure to derive from a recently opened or tightly closed bottle of ultra-pure 100% ethanol. Long exposure of ethanol to air allows uptake of water from the air, and ethanol concentrations lower than 70% may lead to partial elution of RNA from the magnetic beads. The washes are done by gently adding the ethanol to the tube, ensuring that the beads have not been washed away from the magnet. The ethanol is then removed with the tube still on the magnet, putting the pipette down to the bottom of the tube, avoiding the magnetic beads. After the third wash, the last few microliters should be carefully removed to speed up drying. If a large volume of RNA is being purified in a large microfuge tube, and depending on the magnetic apparatus being used, it may be necessary to increase the volume of 70% ethanol to cover all of the magnetic beads.
6. Allow the ethanol to dry thoroughly for up to 20–30 min.
7. Elute by taking the tube off the magnet and adding water or dilute buffer such as TE (10 mM Tris-HCl pH 7.5, 1, or 0.1 mM EDTA). Resuspend the beads by pipetting up and down or by gently flicking the tube. Let the RNA elute for ~3 min, putting the tube back on the magnet for ~2 min or until all of the beads are drawn to the magnet. Remove the supernatant. The elution volume can be adjusted depending on the downstream application for the RNA. If maximum yield is of priority, you can elute in a larger volume such as 30–40 μ l. Multiple elutions can be done to increase yield. For maximum concentration, elution in a smaller volume can be done, with some decrease in yield. Elution in as little as 10 μ l can yield sufficient of RNA for some applications.

4 Notes

1. This protocol obviates the need for protracted RNA purification steps from fixed tissue acquired by LCM.
2. DNase treatment of extracted tissue effectively eliminates detectable genomic DNA contamination (Fig. 3).
3. RNA from different tissues are enriched in mRNA that is specifically expressed in those tissues (Fig. 4).
4. RNA amount may be estimated from LCM-acquired tissue, as shown using quantitative Real-Time PCR with 18 ribosomal and rPL19 primers and standard curves (Fig. 5).

Acknowledgments

This work was supported by grants R01-MH061525 from the National Institutes of Health, RG-4503A4/1 from the National Multiple Sclerosis Society, and 2010-0913 from the Connecticut Department of Public Health to J.S.P. and grant R01-AR064381 from the National Institutes of Health to D.R. Xiaonan Xin and Xi Jiang contributed equally to this work.

References

1. Esposito G (2007) Complementary techniques:: laser capture microdissection--increasing specificity of gene expression profiling of cancer specimens. *Adv Exp Med Biol* 593:54–65 161:1961–1971 [PubMed: 17265716]
2. Datta S, Malhotra L, Dickerson R, Chaffee S, Sen CK, Roy S (2015) Laser capture microdissection: big data from small samples. *Histol Histopathol* 30:1255–1269. 10.14670/HH-11-622. [PubMed: 25892148]
3. Swennenhuis JF, Terstappen L (2015) Sample preparation methods following cellsearch approach compatible of single-cell whole-genome amplification: an overview. *Methods Mol Biol* 1347:57–67 [PubMed: 26374309]
4. Salmon CR, Silverio KG, Giorgetti AP, Sallum EA, Casati MZ, Nociti FH Jr (2012) Gene expression analysis in microdissected samples from decalcified tissues. *Diagn Mol Pathol* 21:120–126 [PubMed: 22555095]
5. Belluoccio D, Rowley L, Little CB, Bateman JF (2013) Maintaining mRNA integrity during decalcification of mineralized tissues. *PLoS One* 8:e58154 [PubMed: 23505463]
6. Srinivasan M, Sedmak D, Jewell S (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol* 161:1961–1971 [PubMed: 12466110]
7. Bussolati G, Annaratone L, Medico E, D'Armento G, Sapino A (2011) Formalin fixation at low temperature better preserves nucleic acid integrity. *PLoS One* 6:e21043 [PubMed: 21698245]
8. Gage GJ, Kipke DR, Shain W (2012) Whole animal perfusion fixation for rodents. *J Vis Exp* 65:3564
9. Belder N, Coskun O, Doganay Erdogan B et al. (2016) From RNA isolation to microarray analysis: comparison of methods in FFPE tissues. *Pathol Res Pract* 212:678–685 [PubMed: 27161306]
10. Xin X, Jiang X, Wang L, Stover ML et al. (2014) A site-specific integrated col2.3gfp reporter identifies osteoblasts within mineralized tissue formed in vivo by human embryonic stem cells. *Stem Cells Transl Med* 3:1125–1137 [PubMed: 25122686]

Fig. 1. Extraction of LCM-acquired material. Once sufficient material is acquired by LCM, Modified Lysis Buffer, preheated to 55 °C, is added to a sterile Eppendorf tube. The LCM cap, containing the tissue, is snapped into the top of the Eppendorf tube. The tube is then inverted and placed in the pre-warmed oven at 55 °C for 30 min

Fig. 2.

LCM retrieval of specific cell types from human iPSC-derived skeletal tissues produced in our mouse calvarial defect model. The upper panel shows the outlines drawn with the mouse around four different tissue types. The identities of the tissues in this image were determined based primarily on the mineral density observed under transmitted light optics, but we have also used multiple staining modalities, including H&E, toluidine blue, safranin O, alkaline phosphatase, human-specific bone sialoprotein antibody, and darkfield imaging on similar sections to identify these tissue types (data not shown). Low mineral cartilage (LMC) refers to unmineralized tissue containing cells with chondrocyte morphology. High mineral cartilage (HMC) refers to mineralized areas with large round cell with hypertrophic cartilage morphologies. High mineral bone (HMB) refers to more densely mineralized tissue with

cells that lack the distinctive hypertrophic chondrocyte morphology, and dense fibrous tissue (DFb) refers to areas with a fibrous appearance that are distinct from looser fibrous areas. The bottom panels show, as an example, the LMC sample on the cap (lower right panel), and the remaining section (lower left panel). Note that there is some tissue removed outside of the UV laser cut even after multiple passages of the laser, because of the toughness of the matrix

Fig. 3.

Confirmation of DNase elimination of DNA contamination of samples. cDNA was generated from RNA purified using our procedures. PGK1 PCR primers produce a 255 bp band from cDNA or processed pseudogenes, and a 375 bp band from the functional gene with and intron. The top panel shows the first round of RT PCR of RNA from the same tissues shown in Fig. 2; in some cases, the bands are very faint. To improve sensitivity, a second round of PCR was done, which produced a robust band of the size produced by the cDNA, but no band was produced of the size produced by the intron-containing genomic DNA

Laser-Capture Microdissection and RNA Extraction from Perfusion-Fixed...

Fig. 4.

PCR analysis of tissue-specific RNAs from material obtained by LCM from calvarial defect model. Following extraction of calvarial tissues acquired by LCM (Fig. 2), cDNA was generated from RNA purified using our procedures using SuperScript III (Life Technologies), following the manufacturer's instructions. We designed human specific primers for COL1A1, COL10 and osteocalcin (OCN) genes. As expected, all tissues express the COL1A1 gene, which was confirmed using COL1a1 antibody staining (data not shown). COL1A1 is expressed in chondrocytes because the cartilage made in our model is fibrocartilage, which is known to express the COL1A1 gene. COL10 collagen is a marker for hypertrophic chondrocytes, so it is expected that it would be most highly expressed in HMC tissue. Osteocalcin is a marker for osteoblasts, so it is expected that it would be most highly expressed in HMB tissue (*see Note 3*)

Fig. 5.

Quantitative Real-Time PCR allows estimation of the amount of RNA extracted from LCM samples. BioRad SsoFast EvaGreen Supermix real-time PCR mastermix was used in a BioRad CFX96 machine. Standard curves for 18S ribosomal and rPL19 ribosomal protein were produced using cDNA produced from cultured human cells using Trizol. Comparing the CT values of the LCM samples to the standard curve, and multiplying by the dilution factor we used for the different primer sets, indicated that we obtained between 1 and 3 pg of RNA from our samples (*see Note 4*)