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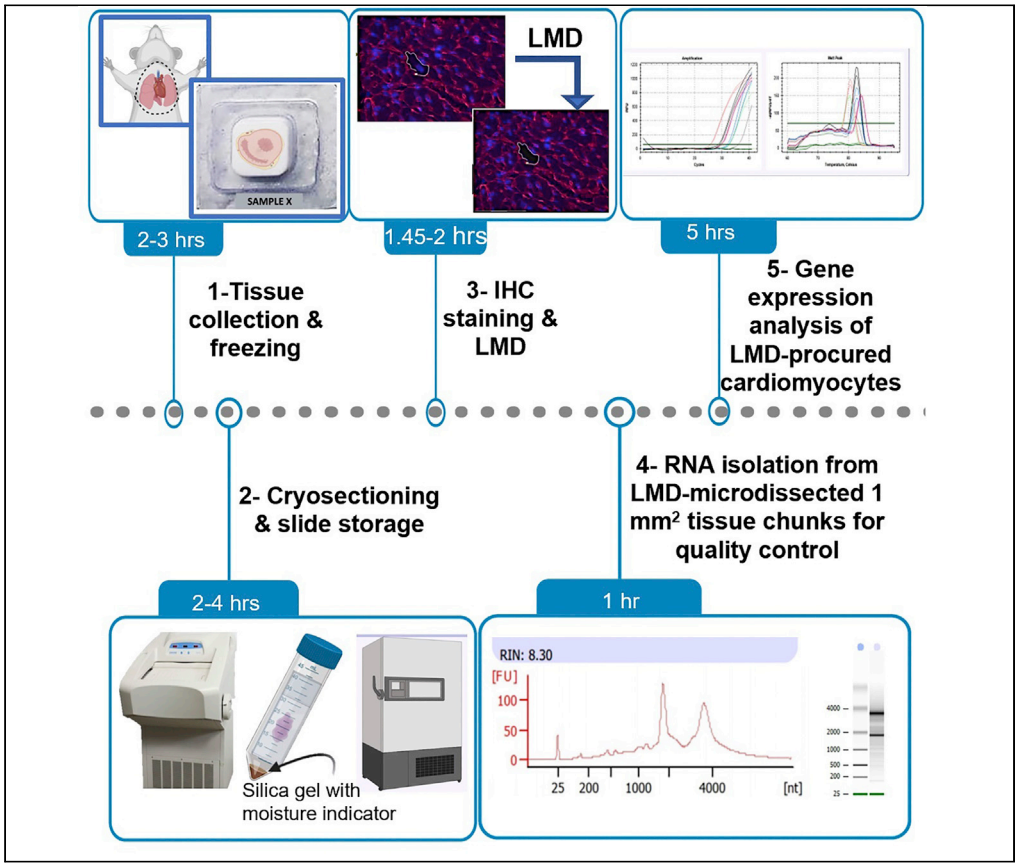
Protocol

A protocol for extracting immunolabeled murine cardiomyocytes of high-quality RNA by laser capture microdissection



We developed a highly efficient, ultrashort immunohistochemistry-laser capture microdissection (IHC-LMD) protocol, which allows microdissection of up to 250 single cardiomyocytes. Before LMD, murine hearts are excised, snap-frozen, and cryosectioned. RNA isolated from LMD material is of high RNA quality, making it usable for gene expression analysis and RNA sequencing. Challenges and limitations of this protocol include visualization of the immunostaining and nuclei DAPI dye on the PEN slides, and timing and speed to limit RNA degradation as much as possible.

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Highlights

LMD-procurement of immunolabeled cardiomyocytes from murine frozen sections

High quality RNA suitable for next-generation sequencing analysis

Gene expression analysis using SYBR™ Green Fast Advanced Cells-to-CT™ technology

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A protocol for extracting immunolabeled murine cardiomyocytes of high-quality RNA by laser capture microdissection

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SUMMARY

We developed a highly efficient, ultrashort immunohistochemistry-laser capture microdissection (IHC-LMD) protocol, which allows microdissection of up to 250 single cardiomyocytes. Before LMD, murine hearts are excised, snap-frozen, and cryosectioned. RNA isolated from LMD material is of high RNA quality, making it usable for gene expression analysis and RNA sequencing. Challenges and limitations of this protocol include visualization of the immunostaining and nuclei DAPI dye on the PEN slides, and timing and speed to limit RNA degradation as much as possible.

BEFORE YOU BEGIN

LMD enables isolation of subpopulations of cells from tissue, while maintaining the *in vivo* molecular state of the cells and spatial distribution (Espina et al., 2006). Preservation of spatial and temporal information is not achievable if cells are isolated using other methods, for example enzymatic tissue dissociation. Moreover, disruption of aggregated living cells using enzymes from tissue samples to isolate cells followed by FACS can profoundly influence downstream gene and protein expression patterns, confounding interpretation of the results (Geho et al., 2005). Indeed, the transcriptome of quiescent muscle stem cells can change with disruption of their niche due to the enzymatic digestion isolation procedure, which leads to their early activation resulting in a bias in their transcriptional profile (van Velthoven et al., 2017). Also, LMD can precisely extract cells down to the single cell level, allowing characterization of single-cell heterogeneity.

LMD combined with quantitative RT-PCR represents a powerful method to analyze the gene expression in defined cell types at the transcriptional level. However, when analyzing few cells (i.e., 250 LMD-procured cells) any loss during RNA isolation caused by washing, performing DNase treatment, or membrane binding during elution can introduce serious uncertainty and even total loss of some transcripts (Bengtsson et al., 2008). Hence, a protocol based on lysis of LMD elements that disrupts the membranes of the procured cells, makes RNA accessible for reverse transcription without inhibiting the downstream enzymatic reactions offers great advantages in quantitative gene expression profiling of few/rare cells. In the present protocol, we adapted the Cells-to-C_T technology to prepare LMD-procured cells for RT-qPCR analysis without the need for traditional sample heating, column-based RNA isolation and purification. The Cells-to-C_T technology has been shown to provide equivalent or better performance across a wide variety of cell lines and amplification targets (Abruzzese et al., 2010).

We started by testing an immunolabeling protocol based on using high salt buffer during washes and incubation steps (Brown and Smith, 2009), but morphology, immunostaining or RNA integrity were

compromised. Therefore, to avoid the abovementioned problems and minimize RNA loss and degradation here we describe an immunolabeling-LMD protocol whereby: (i) an immunostaining enhancer was used as a diluent for antibodies which boosted the antibody signal in 5 min, (ii) washing steps were dramatically reduced in time, (iii) RNase inhibitors were incorporated in all washing and staining solutions, and (iv) direct lysis of LMD-procured cells followed by cells-to-CT and qPCR.

The comprehensive immunolabeling LMD protocol described herein is comprised of five main steps: (1) harvesting and processing of fresh murine heart tissues; (2) cryo-sectioning and slide storage; (3) IHC staining protocol and LMD; (4) RNA isolation of leftover tissues and quality control and (5) gene expression analysis of LMD-procured cells. Breakpoints are introduced at some stages throughout the protocol, rendering it simpler to follow and carry out, without compromising RNA quality. This IHC-LMD methodology potentially provides unprecedented power in performing combined spatial and quantitative analyses on individual cell populations from specific regions of the heart. Moreover, the methodology can be adapted to other solid tissues and organs.

⚠ CRITICAL: Prior to starting the protocol, extensive cryo-sectioning training, and sufficient knowledge of microscopy and immunohistopathology are strongly required. The distinct, stepwise description of equipment, reagents and materials allows the user to be highly organized, especially if each phase in the procedure must be performed within different research facilities.

Institutional permissions

Mouse hearts were collected from C57BL/6 animals housed in the Biological Services Unit at King's College London. Mice were culled via schedule 1 culling methods, as approved by the Home Office and King's College London. Animal experiments conform to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Prepare PBS-RNAsecure™ wash buffer

Timing: ~15 min

1. Prepare 30 mL 1× PBS:
   a. Add 3 mL 10× PBS to 27 mL UltraPure™ DNase/RNase-Free Distilled Water.
   b. Aliquot 1.5 mL 1× PBS into 20 RNase-free 1.5 mL tubes.
2. Dilute RNAsecure™ Reagent to 1× final concentration:
   a. For each 1.5 mL 1× PBS, add 60 μL RNAsecure™ Reagent.
   b. Incubate the mixture in a heating block at 60°C for 10 min, then cool to room temperature.
   c. The buffer/solution is then ready to use or can be stored at 4°C until use.

Refer to "materials and equipment" to prepare other solutions, materials, and tools prior to the procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Wheat Germ Agglutinin, Texas Red™-X Conjugate Working dilution: 1:50	Invitrogen	Cat# W21405 RRID: AB_2334867 https://www.thermofisher.com/order/catalog/product/W21405
6. 4′,6-diamidino-2-phenylindole (DAPI) (1 mg/mL)	Sigma-Aldrich	Cat# D9542 https://www.sigmaaldrich.com/US/en/product/sigma/d9542

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Optimum Cutting Temperature (OCT) Embedding Compound	VWR	Cat# 00411243 https://uk.vwr.com/store/product/11852042/o-c-t-compound-q-path-mounting-medium-for-cryotomy-vwr
Acetone	Sigma-Aldrich	Cat# 179124 https://www.sigmaaldrich.com/US/en/product/sigald/179124
10× PBS buffer, PH 7.4 500 ML	Thermo Fisher Scientific (Life Technologies)	Cat# AM9624 https://www.thermofisher.com/order/catalog/product/AM9624
RNaseZap™	Sigma-Aldrich	Cat# R2020-250ML https://www.sigmaaldrich.com/US/en/product/sigma/r2020
UltraPure™ DNase/RNase-Free Distilled Water	Invitrogen	Cat# 10-977-015 https://www.thermofisher.com/order/catalog/product/10977015
DEPC-treated water	Ambion™	Cat# AM9922 https://www.thermofisher.com/order/catalog/product/AM9922
BioUltra Ethanol (ETOH), for molecular biology	Sigma-Aldrich	Cat# 51976-500ML-F https://www.sigmaaldrich.com/DE/en/search/51976-500ml-f?focus=products&page=1&perpage=30&sort=relevance&term=51976-500ml-f&type=product
RNAsecure™ RNase Inhibitor	Ambion™	Cat# AM7005 https://www.thermofisher.com/order/catalog/product/AM7005
RNaseOut™	Invitrogen	Cat# 10777019 https://www.thermofisher.com/order/catalog/product/10777019
Pierce™ Immunostain Enhancer	Thermo Fisher Scientific (Life Technologies)	Cat# 46644 https://www.thermofisher.com/order/catalog/product/46644
2-Mercaptoethanol (β-mercaptoethanol)	Sigma-Aldrich	Cat# M3148-25ML https://www.sigmaaldrich.com/US/en/product/sigma/m3148
Critical commercial assays		
Qiagen RNeasy® Plus Micro Kit	QIAGEN	Cat# 74034 https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-plus-kits/
Agilent RNA 6000 Pico Kit	Agilent	Cat# 5067-1513 https://www.agilent.com/store/en_US/Prod-5067-1513/5067-1513
High Sensitivity RNA ScreenTape Analysis kit		Cat# 5067-5579, 5067-5581 and 5067-5580 https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-rna-screentape-reagents/high-sensitivity-rna-screentape-analysis-228267
SYBR™ Green Fast Advanced Cells-to-CT™ Kit	Invitrogen	Cat# A35379 https://www.thermofisher.com/order/catalog/product/A35379
Experimental models: Organisms/strains		
Mouse: female and male, C57BL/6, 24–25 months old.	Charles River	N/A
Oligonucleotides		
Primers for qPCR, see Table 1	MilliporeSigma	PROBES https://www.sigmaaldrich.com/GB/en/technical-documents/technical-article/genomics/pcr/standard-dna-synthesis

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
PALM Robo Software	version: 4.8.	Carl Zeiss Microscopy, GmbH https://www.zeiss.com/microscopy/int/products/microscope-software/palm-robosoftware.html
Agilent 2100 Expert Software	version: B.02.08.SI648(SR2)	Agilent Technologies https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-software/2100-expert-software-228259
TapeStation Software	Revision 4.1.1	Agilent Technologies https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-software/tapestation-software-379381
CFX Manager Software	N/A	BioRad https://www.bio-rad.com/en-us/sku/1845000-cfx-manager-software?ID=1845000
Other		
Cryo-block embedding mold	Wolf Laboratories Limited	Cat# 50894-2 https://www.emsdiasum.com/microscopy/products/histology/tissue-tek.aspx
Sterile, individually wrapped, 3.0 mL transfer pipettes	VWR	Cat# 612-2851P https://ie.vwr.com/store/product/565734/transfer-pipettes-high-performance
Kimwipes	SLS	Cat# CLE7600 https://www.scientificlabs.co.uk/product/CLE7600
Personna® plus, low profile microtome blades	VWR	Cat# 76169-160 https://us.vwr.com/store/product/24039069/personna-plus-microtome-blades
Membrane Slides, nuclease and human nucleic acid-free polyethylene naphthalate (PEN) Membrane	Zeiss	Cat# 415190-9041-000 https://www.fishersci.fi/shop/products/membrane-slides/15350731
Sterile 50 mL Polypropylene conical tube	Falcon	Cat# 352070 https://www.scientificlabs.co.uk/product/352070
Silica beads desiccant for slide storage	Supelco	Cat# 1019691000 https://www.sigmaaldrich.com/US/en/product/mm/101969
UV-lamp (UV2 PCR CABINET)	VWR	Cat# 132-0435 https://uk.vwr.com/store/product/2188939/uv-pcr-cabinets-workstations
Cryostat	Bright Instruments	Cat# OTF5000 https://brightinstruments.co.uk/product/otf5000-cryostat/
PAP Pen	Vector	Cat# H-4000 https://vectorlabs.com/immedge-hydrophobic-barrier-pen.html
AdhesiveCap 500 opaque collection tube	Zeiss	Cat# 415190-9201-000 https://www.fishersci.dk/shop/products/adhesive-caps/16360024
RNase-free 1.5 mL tubes	SLS	Cat# E0030123328 https://www.scientificlabs.co.uk/product/E0030123328
Agilent 2100 Bioanalyzer system	Agilent	Cat# G2939BA https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250
Agilent 4150 TapeStation System	Agilent	N/A
Real-time PCR multiwell plates	Starlab	Cat# E1403-1200-C https://www.starlabgroup.com/GB-en/product/96-well-pcr-plate-non-skirted-natural-case-size-e1403-1200-c.html

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
BioRad Thermal Cycler	Bio-Rad	Cat# 1855201 https://www.bio-rad.com/en-us/sku/1855201-cfx-connect-real-time-pcr-detection-system?ID=1855201
Vortex	N/A	N/A
Microcentrifuge	N/A	N/A
Paint brushes	N/A	N/A
ZEISS PALM MicroBeam Laser Microdissection system	Carl Zeiss Microscopy, GmbH	N/A

MATERIALS AND EQUIPMENT

Alternatives: This protocol uses a Zeiss PALM MicroBeam system which utilizes laser light to catapult dissected tissues. Other popular types of laser microdissection equipment are Arcturus XT system and Leica LMD systems which also use membrane slides for sampling tissue sections. Our tissue preparation steps, IHC protocol and Cells-to-C_T workflow can be applied, if required, to these systems without further adaptations.

Diluted Ethanol (ETOH) Series Solution		
Solution	Reagents	Final volume (mL)
50% ETOH	Mix 25 mL of BioUltra Ethanol and 25 mL of DEPC-treated water	50
75% ETOH	Mix 37.5 mL of BioUltra Ethanol and 12.5 mL of DEPC-treated water	50
95% ETOH	Mix 47.5 mL of BioUltra Ethanol and 2.5 mL of DEPC-treated water	50
Store the prepared ethanol series at 4°C and keep on ice during the IHC staining process.		

Primary antibody cocktail		
Reagent	Final concentration	Volume (µL)
Pierce™ Immunostain Enhancer	N/A	100
RNaseOut™	2 U/µL	5
Wheat Germ Agglutinin antibody	50 µg/mL	5
DAPI	10 µg/mL	1
This cocktail is prepared fresh prior to IHC staining of tissue sections and kept on ice at all times.		

STEP-BY-STEP METHOD DETAILS

Dissection and snap-freezing of mouse heart

Timing: 2–3 h, depending on number of mice sacrificed

In this step, murine hearts were excised, washed, and embedded in OCT-filled cryomolds (Figure 1). Hearts were collected from C57BL/6 mice (n=6) housed in the Biological Services Unit at King's College London. Mice were culled via schedule 1 method, in accordance with the regulations for animal testing, directed by the Home Office and stipulated under the Animals (Scientific Procedures) Act 1986.

1. Dissecting out the murine heart:
   a. Use sterile forceps and small, sharp scissors to cut the chest skin and expose the heart.
   b. Cut the thoracic cage using bigger scissors and dissect the heart out after severing the arteries and veins connected to it.

Table 1. Sequences of all primers used in this study

Oligonucleotide	Sequence (5′->3′)	GenBank accession N°
Actin, beta (Actb)	F: TCAGCAAGCAGGAGTACGATG R: AACGCAGCTCAGTAACAGTCC	NM_007393.5
Troponin C, cardiac/slow skeletal (TNNC1)	F: CGGATCTCTTCCGCATGTTTG R: TAATGGTCTCACCTGTGGCCT	NM_009393.3
Vimentin (Vim)	F: AGAGAGGAAGCCGAAAGCAC R: GGTCAAGACGTGCCAGAGAA	NM_011701.4
platelet/endothelial cell adhesion molecule 1 (Pecam1)	F: GCCAGCAGTATGAGGACCAG R: ACCGCAATGAGCCCTTTCTT	NM_008816.3
CD68	F: TGCCTGACAAGGGACACTTC R: CAGGCCAATGATGAGAGGCA	NM_001291058.1

c. Transfer the heart to a Petri dish containing 1× PBS.
d. Wash the heart several times with fresh PBS using individually wrapped transfer pipettes.
e. Dispose of the carcass using labeled biohazard bags.

2. Embedding the excised heart:
   a. Fill half of an embedding cryomold with OCT slowly and carefully.
   b. Use the disposable scalpels to cut the heart into 2 transverse sections.
   c. Orient each individual section on the floor of one embedding cryomold pre-filled with OCT.
   d. Fill the rest of the cryomold slowly with OCT till the whole tissue is covered, avoiding creation of bubbles.
   e. Using forceps, quickly and carefully lower the tissue in the cryomold into the liquid nitrogen so it is partially submerged and sitting just on top of the liquid nitrogen.

⚠ CRITICAL: Do not submerge the whole of the cryomold into liquid nitrogen. Make sure the top of the cryomold is visible. Total immersion of OCT embedded tissues into liquid nitrogen results in cracked OCT, formation of bubbles within the specimen and resulting in very difficult cryosectioning.

Pause point: After the OCT becomes solid white (~10–15 s), place the frozen tissue into −80°C freezer for storage.

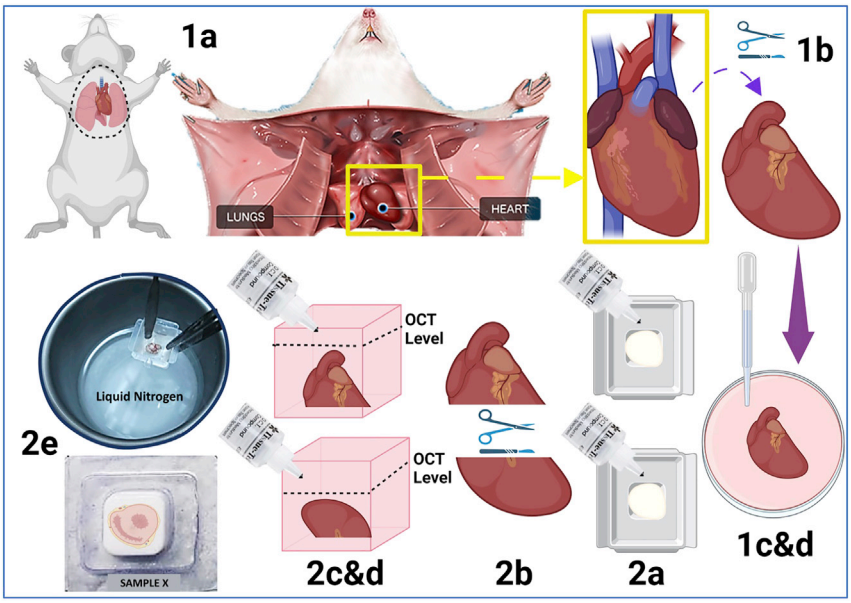


Figure 1. Stepwise illustration of step 1—Murine heart dissection and embedding in OCT
Timing: [2–3 h, depending on number of mice]. 1 mouse heart takes 15–20 min to dissect.

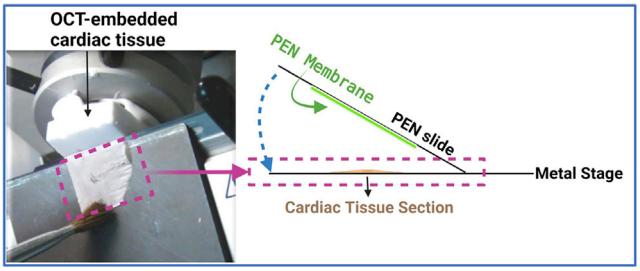


Figure 2. Cartoon depicting mounting of OCT-embedded cardiac cryosection on PEN slides

Note: Prior to dissection, label the cryomolds and sterilize the dissection instruments. Spray the dissection tools with RNaseZap™, then RNase-free, DNase-free water and dry with clean Kimwipes. Spray the dissection area with RNaseZAP™ solution and let dry.

Cryosectioning and slide storage

Timing: 2–4 h depending on the number of blocks cut and number of sections required

In this step, murine hearts are cryosectioned on PEN slides and stored in sterile Falcon tubes containing silica desiccant beads.

3. Prepare PEN membrane slides as follows:
   a. Spray RNaseZap™ on the PEN slide and leave for 5 min.
   b. Wash with DEPC water.
   c. UV light for 30 min (distance between the PEN slide and the UV lamp should not be more than 20 cm).
   d. When the slides are UV-treated, label and keep them face up at room temperature.
4. Prepare the cryostat and tissue block:
   a. Set temperature of the cryochamber at −22°C and the specimen temperature at −20°C.
   b. Clean the cryostat, microtome blade and tools with 70% ETOH. Wipe dry.
   c. Set the section thickness on the cryostat to 8 μm. If cutting a new tissue block, set the cryostat to trim 20 μm thick sections until the whole tissue is exposed. Then adjust the thickness to 8 μm.
   d. Remove the tissue block from the cryomold, mount it on the chuck using OCT and place the tissue/chuck assembly in the cryostat chamber and allow to equilibrate for 15 min.
5. Cryosectioning:
   a. Secure the chuck in the chuck stage and carefully bring the specimen very close to the blade.
   b. To mount sections on the PEN slides, brush away tissue trimmings, then slowly cut and tease the tissue section away from the blade using paintbrushes.
   c. Gently unroll the cut section using the paint brushes and pivot the PEN slide (membrane side facing the tissue section) slowly on top of the section (Figure 2).
   d. Carefully remove the mounted tissue from the chuck using a razor blade, transfer the tissue back to the cryomold, wrap the cryomold with aluminum foil and keep on dry ice until storage in −80°C freezer.

Pause point: Collected sections on PEN slides can be stored individually in 50 ml Falcon tubes containing ~5 ml silica desiccant beads and stored in −80°C freezer.

⚠ CRITICAL: humidity condensation on PEN slides will activate RNases in tissue sections. After getting the stored Falcon tubes out of the −80°C freezer, allow them to equilibrate to 25°C completely before opening the lid (~15 min).

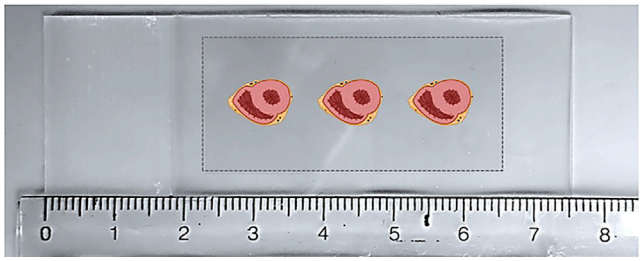


Figure 3. Murine cardiac sections mounted on PEN slides

Care must be taken to mount the sections within the PEN membrane area (dotted outline). Ruler is in centimeters.

⚠ CRITICAL: avoid mounting sections near the edges or the end of the slide (Figure 3). Sections mounted near the edges will not stain properly and will not be seen in the field of view during LMD.

Note: in this protocol, we found that 8 μm cardiac section thickness allowed good tissue adhesion to the PEN membranes, adequate microscopic resolution and reduced the laser energy sufficient for cutting through the tissue section, without compromising the RNA yield or quality. Further optimization is warranted on thicker sections.

Note: murine hearts are small and up to 4 sections can fit within the membrane area. If working with larger organs, care must be taken to cut fewer section per PEN slide.

IHC staining and cell procurement using LMD

Timing: For one slide, IHC: 15 min and LMD < 90 min

To visualize cells and regions of interest on tissue sections prior to LMD, histochemical stains like toluidine blue, Cresyl violet and H&E can be used. However, beside the compromised histomorphology due to extensive dryness of the tissue sections, histochemical stains fail to detail the identity and molecular characteristics of the cells of interest, which can be crucial in the downstream analyses and prior to an LMD study. This makes immunolabeling a key prerequisite to identify cell types that cannot be marked by histomorphology alone during LMD. Nevertheless, conventional IHC protocols comprise several rinsing steps and overnight incubations that can dramatically deteriorate the nucleic acids. This, in addition to the intrinsic degrading activity of RNases which come back to life as soon as the tissue is rehydrated and not frozen anymore. In this step, cardiac sections are immunolabeled with WGA and DAPI. Thereafter, cardiomyocytes will be selected, microdissected and catapulted into AdhesiveCap 500 opaque collection tube. All solutions used in this step must be kept on ice at all times and all incubations must be performed on ice.

6. Immunolabeling murine cardiac sections (Figure 4):
   a. Fix sections in 100% acetone on ice for 5 min.
   b. Use a PAP Pen to outline membrane area and prevent spread of solutions.
   c. Pipette 500 μL, PBS-RNAsecure™ on the section and decant after 30 s, repeat twice.
   d. Incubate sections with primary antibody cocktail for 5 min.
   e. Decant the antibody, pipette 500 μL PBS-RNAsecure™ on the section and incubate for 30 s, repeat twice.
   f. Dehydrate sections by sequentially dipping the slide into 50 mL of 50%, 75%, 95%, 100% ETOH for 30 s, each.
   g. Proceed directly to LMD.

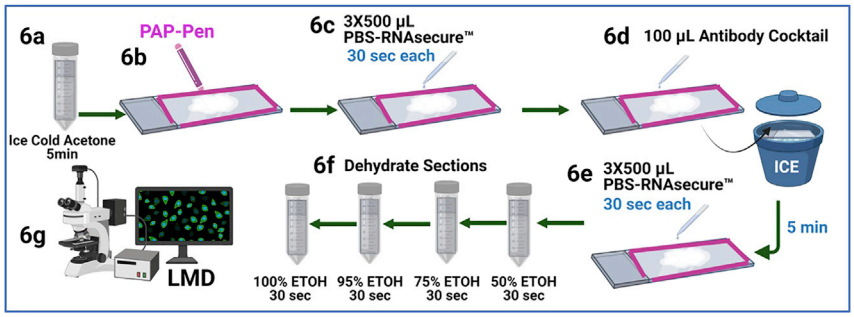


Figure 4. IHC workflow for staining of frozen heart sections with WGA antibody and DAPI prior to LMD

Optional: In this protocol, we used Texas Red™-X conjugate of WGA which exhibits the bright, red fluorescence of the Texas Red™ dye (excitation/emission maxima ~595/615 nm). Molecular Probes® offers a broad selection of fluorescent wheat germ agglutinin conjugates that can be used depending on the filter sets available in the Laser Microdissection system used.

Optional: Two types of RNase inhibitors were used in the current protocol. The first one, RNASecure™ was used in the treatment of all buffers. Then for extra protection of the precious RNA prior to antibody staining, another RNase inhibitor RNaseOUT™ was added at a concentration 2 U/µL to the antibody incubation buffer. RNASecure™ requires activation by heating at 60°, therefore it was not possible to use it with the antibody incubation buffer used. A recent study demonstrated that the inclusion of a moderate amount of a small ribonuclease inhibitor, Ribonucleoside Vanadyl Complex (RVC), in staining and washing steps effectively protected RNA and yielded high quality RNA profiles from as little as a few tens of dissected cells (Zhang et al., 2021). The excellent RNA preservation quality of RVC is mostly owing to its small size and fast diffusion in dense tissue sections. One potential problem with the use of RVC is its effect on downstream procedures, including reverse transcription and/or PCR that are required for the construction of cDNA sequencing libraries, owing to its adverse effect on polymerases (Lau et al., 1993). Therefore, RVC must be removed from the dissected materials before the downstream procedures can be performed properly.

Note: As it is often done in diagnostic histopathology, cells and regions of interest are identified in tissue sections prior to LMD according to morphological or histochemical peculiarities. To start with, the accuracy of visualizing individual cardiomyocytes in cardiac sections was assessed either by histochemical staining using Cresyl violet (CV) or hematoxylin and

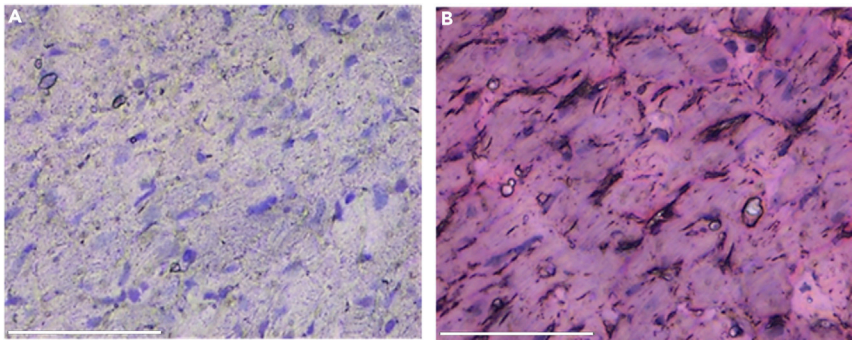
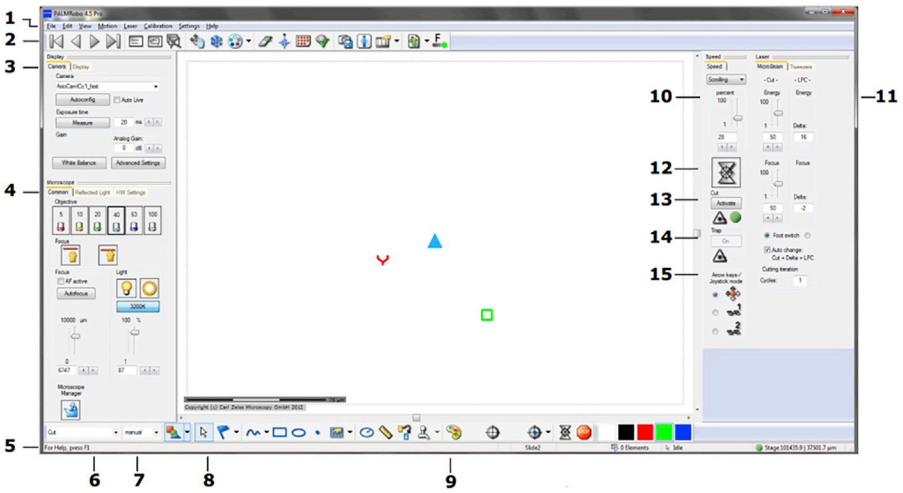


Figure 5. Histochemical staining of cardiac sections

(A and B) Raw images captured using ZEISS PALM MicroBeam Laser Micro-dissection system. Frozen cardiac sections stained with (A) CV or (B) H&E. (A) Staining with CV revealed dark violet nuclei and light violet cardiac tissue without accurate identification of cardiomyocytes. (B) Staining with H&E revealed dark blue nuclei and pink cardiac tissue labeled by eosin. Scale bar=75 µm.



1. **Menus**
2. **Toolbar**
3. **Camera & Display Settings**
4. **Settings for the microscope, for fluorescence and hardware settings.**
5. Status Bar
6. **Cut tools**
7. To select a well for catapulting with PALM RoboMover.
8. **Graphic Tools**
9. Color Palette
10. **Speed Tools**
11. **Laser Tools**
12. **Start Cutting Laser**
13. **Cutting Laser status**
14. Switch on/off Trapping Laser, Trapping Laser status
15. Arrow keys/Joystick mode.

Figure 6. PALM RoboSoftware layout

The software controls all parameters of the LMD, camera, slide and tube holders, laser and imaging. Parameters used in the current study are marked in Bold in the list of tools on the right.

eosin (H&E). The two staining procedures were performed in 5 min and sections were inspected under brightfield light microscopy. The histochemical stains did not make it possible to recognize the tissue architecture or identify the different cell types in the cardiac section (Figure 5). We were unable to confidently identify cardiomyocytes for microdissection using histochemical staining.

7. Laser Capture Microdissection (LMD):
   a. Gloves must be worn throughout the whole LMD procedure which, ideally, should not exceed 90 min.
   b. Initiating PALM RoboSoftware:
      i. Switch on the computer, microscope and the laser 5 min before performing LMD.
      ii. Launch PALM RoboSoftware and wait few minutes until the stage calibrates.

   Note: This is accompanied by loud noise that will stop once the stage returns to home position.

      iii. PALM RoboSoftware controls all operations in the system such as platform movement, objective selection, light intensity, exposure time, condenser control, white balance and contrast, autofocusing, slide and region of interest selection, camera specifications, and laser parameters like speed, thickness, and energy (Figure 6).
   c. Loading PEN slides and tubes:
      i. For loading the slide: push the metal bar to the left and slot the slide into one of the 3 slide holders (Figure 7A). The slide is locked in place by releasing the metal bar slowly. Unless the slide is firmly held in the slide holder and the metal bar is stable in place, the outlines drawn around selected cells will be changed during laser cutting. This will result in inclusion of irrelevant cells/areas in the procured elements.
      ii. For loading the tube: the tube cap was slotted into the horseshoe and the tube was pinned back (Figure 7B).
      iii. Click "Capture Device" which opens the PALM RoboMover then PALM CapMover II window.
      iv. Use the PALM RoboMover to select Collector type: TUBE 2 × 500 CMII.
      v. Select position (1) or (2) according to which tube position was chosen (Figures 7C and 7D).
   d. Camera and microscope adjustments:

i. On the screen, from the "Display" menu, choose "Camera" and select the installed camera from the drop-down menu.
ii. Use the Joystick to move the PEN slide to a section-free area and select "White Balance". Move back to the tissue section area.
iii. From the "Microscope" menu, choose "Reflected Light" and select 5× objective lens to identify the area or cell of interest prior to dissection. Use these options later to change the microscope magnification.
iv. Select "Focus" then select channels for inspection of fluorescently immunolabeled cells. For our experiments, the FS45adv filter set was chosen for inspection of Texas Red™-conjugated WGA and the FS96adv filter set for inspection of DAPI-positive nuclei.
v. To activate or deactivate the fluorescence beam, open or close the fluorescence shutter, respectively.
vi. Imaging: at any time during LMD, the image displayed on the screen (either showing the slide or the cap) can be captured and saved in one step by clicking "Save Current Image".

e. Calibration:
i. Click "Calibration" from the main menu, then select "Cut Laser Adjustment Wizard" from the drop-down menu.
ii. Proceed with rough laser adjustments, then set the laser marker by positioning the cursor precisely over the center of the marker hole created.
iii. Calibration is an indispensable step that should be repeated each time a new experiment is conducted, or a new magnification is selected. The alignment and position of the laser marker must be calibrated with respect to stage movement; this is very important as an inappropriate alignment will not allow for isolation of selected cell populations.

f. Setting-up a multi-dimensional acquisition experiment to select cells:
i. This setup is key to visualize overlayed images of WGA+ DAPI+ cardiomyocytes.
ii. To acquire, select the "Multi-Dimensional Acquisition" icon from the toolbar, then create/modify multi-dimensional acquisition experiments from the drop-down menu.
iii. Enter and save parameters of the dyes (Texas Red™ and DAPI) and exposure times for each fluorescence channel used.
iv. Save the experiment and click on the icon again to start an experiment.
v. Acquired images can be saved and viewed individually or in an overlay to further select the cells of interest.

g. Laser cutting:
i. This process should be started in a non-precious area of the slide until the laser cut is optimized, before moving to the area(s) of interest.
ii. Select "MicroBeam" from the "Laser" menu to adjust laser settings.
iii. For the cardiac tissue sections, laser settings were typically for the CUT Energy: 38, Focus 60, and for the LPC Energy: 23 and Focus 5.
iv. Adjust the laser speed to 8–10 μm/s to minimize the cutting energy used.
v. Draw a line around cardiomyocytes or cells of interest using freehand drawing tool.
vi. Select "RoboLPC" cutting function from the cut tools to simultaneously cut and catapult the cell or area of interest and click "Start Cutting Laser".
vii. Select "Oneclick-LPC" if the cell/area was partially catapulted and click inside the cell/area outline to fully catapult it.
viii. All selected cardiomyocytes (DAPI nuclei in the center surrounded by WGA membrane stain; see Figure 7) were laser cut and catapulted into the adhesive cap.
ix. Laser cutting of all selected elements should be monitored carefully during the whole LMD procedure because cutting parameters may require readjustments for some areas in the tissue section.

h. Checking the cap and elements:
i. Checking the cap needs to be done regularly by clicking "Go to Cap Check". Cap check enables the visualization of catapulted cells by moving the underlying stage and leaving only the cap in the microscopy light path.

ii. In our case, we change to the lowest magnification (5×) to visualize the largest area possible of the cap.
iii. If elements start to accumulate in an area of the cap, move the stage to reach an empty area in the cap and click "Capture Device", choose "Adjust" option, click "Save Position as delta position for the collector", click "Close".
iv. To move the stage from "Cap Check" back to the point of origin (cutting position), click "Point of Origin".
v. To check if target number/area of elements are collected: Click "Element List", choose the "Summary" sheet then click "Print" to save summary of total elements collected.
vi. Once enough material has been collected, click "Capture Device", click on "Home" to return the tube holder to start position.
vii. In our experiments, 50–250 cardiomyocytes were captured for downstream gene expression analysis and the tube was displaced from its position.
viii. For quality control, a new microfuge tube was quickly mounted on the tube holder and a total of 1 mm^2 tissue chunks were microdissected from the same section.
ix. Proceed directly with cell lysis for microdissected tissue chunks and captured cardiomyocytes according to the workflow in steps 4 and 5, respectively.

⚠ CRITICAL: If cryosectioning and LMD are done on separate days, avoid thawing of PEN slide/tissue section by using dry ice during transportation.

Optional: In our experiments, cardiomyocytes were collected into AdhesiveCaps of 500 opaque collection tube (Figure 7C). Alternatively, commercially available RNase-free tubes can be used and a small drop of RNA stabilizers e.g., TriZol can be dispensed in the cap before collection of elements to minimize RNA degradation. Microfuge tubes with AdhesiveCaps were chosen for these experiments because they allow LMD without applying any capturing liquid into the caps which minimizes the risk of evaporation and crystal formation during extended specimen harvesting.

Note: We recommend having one section only on each PEN slide and collecting < 250 cells in each LMD session to avoid taking too much time, which will lead to extensive degradation of RNA of the LMD-procured cells. Later, all stored lysates from cells procured for the same experiment can be pooled before proceeding with RNA isolation.

Note: More details for the LMD and software operation are available in the instrument user guide provided by the manufacturer.

RNA isolation from LMD-microdissected 1 mm^2 tissue chunks for quality control

Timing: For 1 sample, 1 h

In this step, we describe RNA isolation from LMD-microdissected chunks of cardiac tissue (approximately 1 mm^2). Isolated RNA will be used for quality control to assess the impact of the protocol on RNA integrity.

8. Extracting RNA using Qiagen RNeasy® Plus Micro Kit:
   a. Add 350 µL Buffer RLT containing 2-Mercaptoethanol to the bottom of the tube into which 1 mm^2 tissue chunks were catapulted in the AdhesiveCaps, close the cap and incubate in an upside-down position at 25°C for 30 min.
   b. Spin down the lysate in a microcentrifuge for 5 min at no more than 8,500×*g* at 25°C to avoid detachment of the adhesive cap.
   c. Store the tube on ice until RNA isolation which we recommend performing on the same day, avoiding any freeze-thawing cycles.

Figure 7. Loading PEN slides and tubes

(A and B) The slides were loaded on the slide holder (A) on the movable stage of the LMD (B).

(C and D) Tubes are inserted into the tube holder (C) and on the PC screen, position (1) was chosen because the tube and adhesive cap were loaded at position (1) on the tube holder (D).

d. Perform the rest of RNA isolation steps according to the manufacturer's instructions.

e. Elution of RNA in the final step was performed using 14 μL RNase-free water supplied with the kit, with the following modification:

   i. Dispense 7 μL on the column's filter and centrifuge at 16,000 × *g* for 30 s.

   ii. Dispense another 7 μL of RNase-free water and centrifuge at 16,000 × *g* for 30 s.

   iii. Finally, pipette the eluted 14 μL on the column's filter and centrifuge at 16,000 × *g* for 1 min.

f. Assess the quality and concentration of 1 μL of the isolated RNA using Bioanalyzer and Agilent RNA 6000 Pico kit and store the remaining RNA at −80°C.

Note: The concentration and integrity of RNA isolated from < 0.5–1 mm^2 microdissected tissues were below the detection limit of the Bioanalyzer. Therefore, it was not possible to perform quality control on as few as 250 cardiomyocytes.

Note: This is a brief outline of RNA isolation of LMD-procured tissue chunks. For detailed procedure and troubleshooting, follow the manufacturer's instructions.

Gene expression analysis of LMD-procured cardiomyocytes

Timing: For 1 sample, ~4 h

In this step, we adapt the Cells-to-C_T workflow to prepare LMD-procured cardiomyocytes for RT-qPCR analysis without the need for traditional sample heating, column-based RNA isolation and purification (Figure 8). Cell lysis must start directly in the AdhesiveCaps containing the microdissected elements once the microfuge tube is removed from the tube holder of the LMD.

9. Prepare the Cells-to-C_T lysate:

   a. Prepare fresh Lysis reaction after all cardiomyocytes are captured by LMD.

Lysis reaction	
Reagent	Amount
Lysis Solution	49.5 μL
DNase I	0.5 μL
Total	50 μL

b. Dispense 50 μL of lysis reaction on top of the microdissected cardiomyocytes in the Adhesive-Cap, pipette up and down several times for 1 min.
c. Incubate the lysis reaction mix upside down for 5 min at room temperature.
d. Spin down the lysate and add 5 μL Stop Solution to the lysis reaction, pipette up and down five times and incubate for 2 min at room temperature.
e. Store the lysate on ice for ≤2 h, or at −20°C to −80°C for ≤5 months.

10. Perform reverse transcription (RT):
    a. In a nuclease-free microcentrifuge tube on ice, prepare an RT Master Mix.

RT Master Mix	
Reagent	Amount
2× Fast Advanced RT Buffer	25 μL
20× Fast Advanced RT Enzyme Mix	2.5 μL
Lysate	22.5
Total	50 μL

    b. Mix the RT Master Mix gently but thoroughly, then spin down and place on ice.
    c. Incubate the RT mix at 37°C for 30 min then 95°C for 5 min.
    d. Assembled RT reactions can be stored at 4°C for up to 4 h.

11. Perform qPCR:
    a. In a nuclease-free microcentrifuge tube at room temperature, prepare the PCR Cocktail

PCR reaction master mix	
Reagent	Amount
PowerUp™SYBR™ Green Master Mix	10 μL
PCR primers Forward and Reverse primers[a]	Variable
Nuclease-free Water Variable	Variable
Total	16 μL

[a] Recommended final concentration of each primer is 200–400 nM.

    b. Primers used in the current study are listed in Table 1.
    c. Distribute the PCR Cocktail into individual PCR tubes or wells of a real-time PCR plate at room temperature.
    d. Pipette cDNA up and down slowly 4–5 times and add 6 μL cDNA to 14 μL PCR Cocktail.
    e. Close the tubes or cover the plate, then mix gently.
    f. Spin down the tube contents to remove bubbles and collect the contents at the bottom of the wells.
    g. Set up the real-time PCR instrument as indicated in the following table, then load and run the reactions.

PCR cycling conditions			
Steps	Cycles	Temperature	Time
UDG activation	1	50°C	2 min
Enzyme activation (hold)	1	95°C	10 min
PCR	40	95°C	3 s
		60°C	30 s
Dissociation curve		Use default setting	

Note: for more details about SYBR™ Green Fast Advanced Cells-to-C_T™ procedure and troubleshooting, refer to the manufacturer's guidelines.

Optional: In this study, the Cells-to-C_T kit was used to process and measure gene expression in 10, 50 and 250 LMD-procured cardiomyocytes. To detect less abundant transcripts in smaller numbers of cells, a workflow using TaqMan Pre-Amp Cells-to-C_T kit and TaqMan Gene Expression Cells-to-C_T, specifically designed to extend material from valuable or small amounts of sample, has been previously described (Abruzzese et al., 2010).

EXPECTED OUTCOMES

Because the last dehydration step in the IHC protocol leaves the tissue sections significantly dry (Micke et al., 2004), visualization of WGA in the Tx-Red channel and nuclei/DAPI in the UV channel becomes extremely difficult (Figure 9A). To overcome this, a drop (50 µL) of 100% ETOH was dispensed onto the section, making it possible to clearly visualize and select the cells of interest in the cardiac sections (Figure 9B). The impact of this step with 100% ETOH, which was done intermittently throughout the LMD procedure (up to 2 h), had no effect on the sample RNA integrity (RIN 7.0) as assessed using the High Sensitivity RNA ScreenTape assay and the TapeStation (Figure 9C).

Immunolabeling cardiac sections with WGA, which stains the cell membrane, together with DAPI to identify the cell nuclei allowed us to identify cardiomyocytes and to laser cut along the membrane and dissect out single cardiomyocytes (Figure 10). A multi-dimensional acquisition experiment using

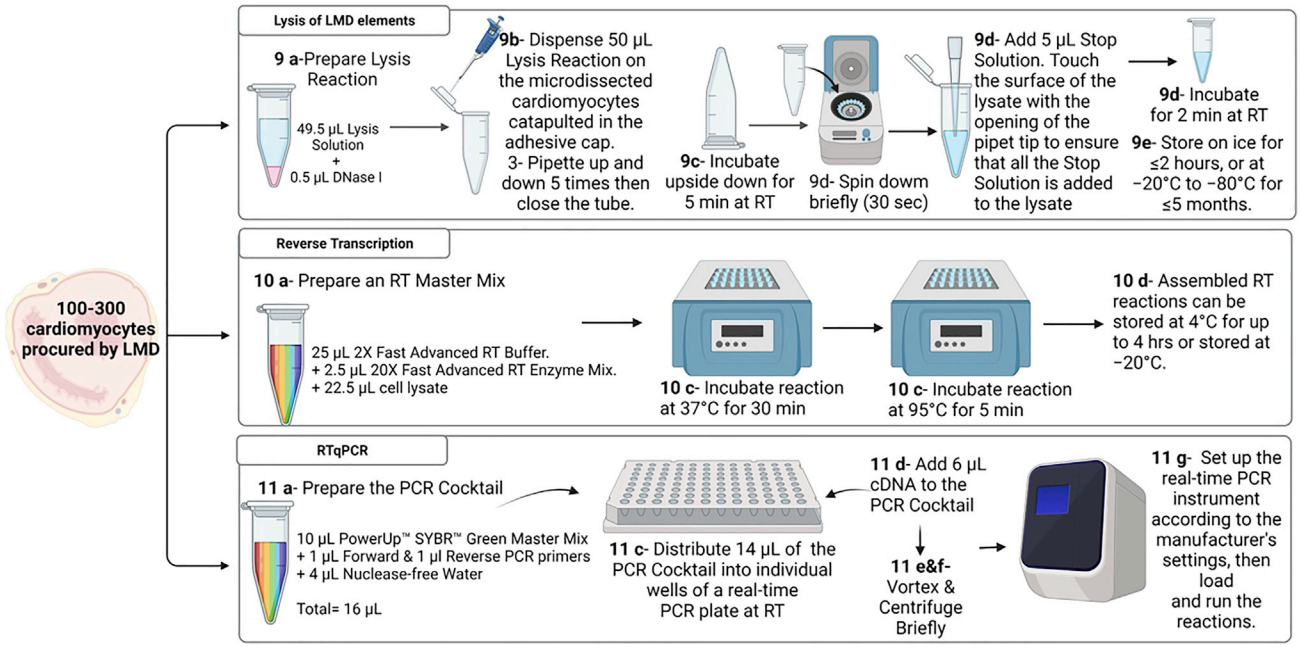


Figure 8. Schematic representation the RT-qPCR workflow using SYBR™ Green Fast Advanced Cells-to-CT™ Kit

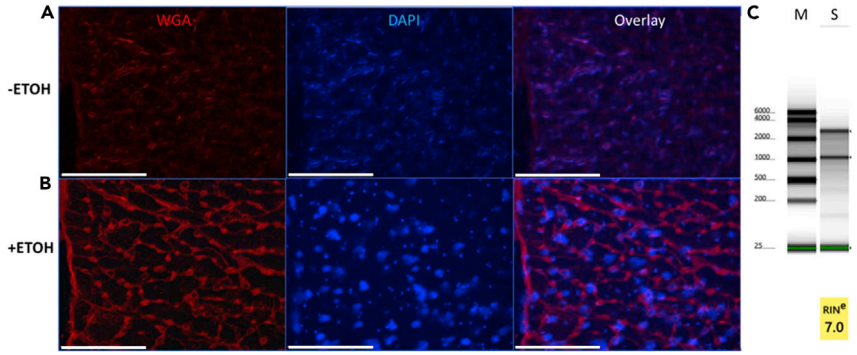


Figure 9. Adding a drop of ETOH onto the cardiac section improves visibility of cardiomyocyte membrane and nuclei for LMD dissection

(A and B) Representative images showing WGA membrane immunolabelling and DAPI nuclei staining of cardiac tissues without ETOH (A) and with ETOH (B). Note the dramatic enhancement of cell visualization when a 50 μL drop of 100% ETOH was applied to the section. Scale bar=75 μm.

(C) Electropherogram from the Agilent 2200 TapeStation showing 28S rRNA upper band and 18S rRNA lower band and RIN value=7.0. A cardiac tissue section was immunolabeled with WGA and DAPI and drops of 100% ETOH were intermittently applied throughout the 90 min LMD session to improve visualization of cardiomyocytes. Following LMD, RNA was isolated from leftover tissue (step 4) and analyzed using the TapeStation. M: marker and S: sample.

PALM Robo software allowed us to distinguish between cardiomyocytes and other cells making up the cardiac tissues (Figure 10).

One of the primary goals of this study was to microdissect out single cardiomyocytes by LMD that possessed intact, high-quality RNA, which would be able to proceed to downstream gene

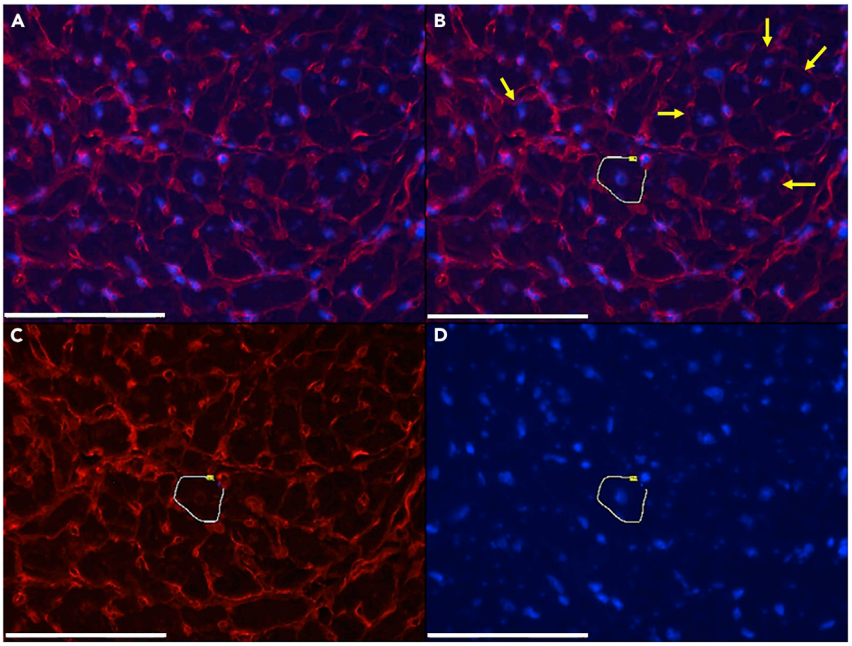


Figure 10. Identification of cardiomyocytes using WGA and DAPI immunolabeling

(A–D) Representative raw images of WGA and DAPI immunolabeled cardiac tissue sections. The cardiomyocyte is outlined and the element is given a number by the PALM Robo software. (A and B) Overlayed images created after merging the Tx-Red (C) and DAPI (D) channels using the multi-dimensional acquisition experiment setup in the PALM Robo software. Representative cardiomyocytes (membranous WGA staining and centrally located nuclei) are shown in B by arrows. An outlined micro-dissected cardiomyocyte visualized in the merged (B), Tx-Red (Red; C) and DAPI (Blue; D) channels. Scale bar=75 μm.

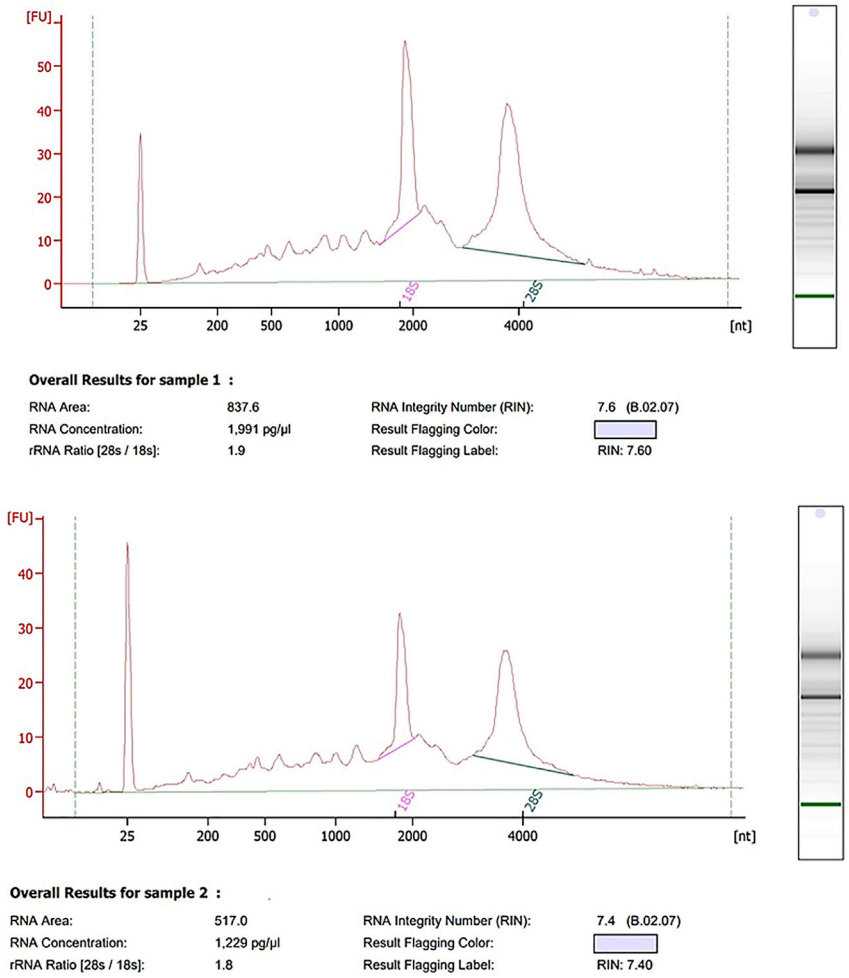


Figure 11. RNA isolated from immunolabeled, LMD-procured cardiac tissue is of good quality

Representative Bioanalyzer electropherograms (fluorescence intensity versus migration time) are shown for two representative samples from murine cardiac sections. One mm2 was microdissected from each immunolabeled section using LMD and RNA was isolated from each sample using Qiagen RNeasy® Plus Micro Kit and analyzed by the Agilent 2100 Bioanalyzer system and software. Positions of 18S and 28S ribosomal RNA are indicated. RNA yield and integrity measured by RIN value are shown below each plot.

expression analyses, including RNA-sequencing. Since 100–300 cells did not provide sufficient RNA that was detectable by the Bioanalyzer, quality control checks of RNA from a 1 mm^2 micro-dissected area (Garrido-Gil et al., 2017) was isolated and evaluated. The microdissected 1 mm^2 area was from the same tissue section that the cardiomyocytes were microdissected from. Following RNA isolation, the quantity and quality of RNA was evaluated on a 2100 Bioanalyzer. Figure 11 demonstrates that the quality of RNA was good (RIN >7.0) and consistent following our IHC protocol and following 1–1.30 h of the LMD experimentation process for 2 separate samples.

Binding and release of nucleic acids in techniques based on membrane RNA isolation techniques can lead to a considerable loss in the RNA that is recovered (Abruzzese et al., 2010). Therefore, we adapted a protocol based on Cells-to-C_T technology to process low numbers of single cardiomyocytes effectively for real-time analysis without the need for traditional RNA isolation techniques. We applied the Cells-to-C_T technology to 10, 50 and 250 microdissected cardiomyocytes (elements) and raw C_T values were obtained by RT-qPCR for cardiomyocyte transcript, TNNC1, and housekeeping gene β-Actin (Figure 12). The C_T values decreased with the increased number of

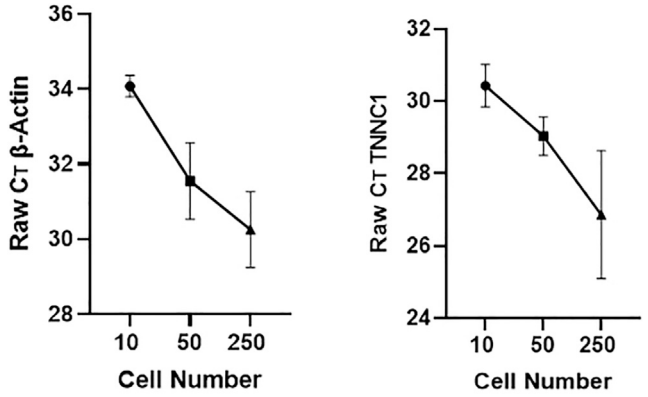


Figure 12. Validation of Cells-to-CT technology for gene expression of LMD-procured cardiomyocytes

CT values of the housekeeping gene (β-ACTIN) and a cardiomyocyte gene (TNNC1) in 10, 50 and 250 LMD-procured cardiomyocytes. Cardiomyocytes were dissected from 3 different animals/tissue sections and RTqPCR was run in technical duplicates. Error bars represent standard deviation of six qPCR reactions per tested cell number (2 technical duplicates × 3 animals).

cardiomyocytes per group, showing that the amount of gene expression increased with the increase in the number of cardiomyocytes analyzed (Figure 12).

To verify the purity of LMD-procured cardiomyocytes, expression of Troponin C (cardiomyocyte marker), Vimentin (fibroblast marker), CD68 (macrophage marker) and CD31 (endothelial cell marker) were assessed in 250 cardiomyocytes using RTqPCR. From the analyzed genes, Troponin C was detected at significantly higher levels compared to other non-cardiomyocyte genes (Figure 13). These results indicate that the LMD-procured material was enriched with cardiomyocytes with rare contaminations of fibroblasts, macrophages and endothelial cells.

LIMITATIONS

One of the challenges in performing IHC-LMD is clear visualization of immunolabeled tissues cut and mounted on PEN slides, which limits the repertoire of cell types that can be isolated without

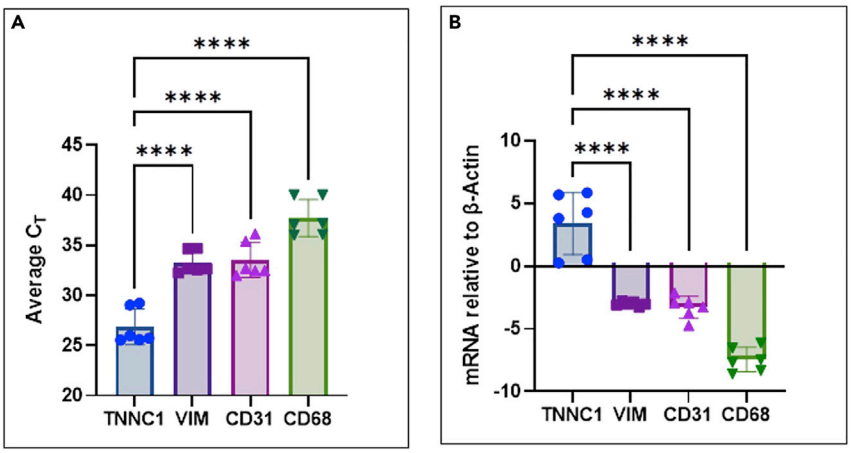


Figure 13. Transcript levels are reflective of LMD-procured cells being cardiomyocytes

(A) Average CT of 250 cardiomyocytes processed by Cells-to-CT technology followed by RT-qPCR for Troponin C (TNNC1), Vimentin (VIM), CD31 and CD68.

(B) Transcript expression relative to the housekeeping gene (β-ACTIN). Error bars represent standard deviation of six qPCR reactions per tested gene (2 technical duplicates × 3 animals). Statistical analysis was performed in Graph Pad Prism 9 using ANOVA and Dunnett's multiple comparison test, ****p<0.0001.

contamination. This problem did not exist when IHC was performed on cardiac sections cut and mounted on glass slides. This is due to the mounting medium and glass coverslips used to cover the sections and prevent their dryness. Because LMD requires physical access to the tissue surface, mounting and applying a glass coverslip over the section is inapplicable. This visualization problem is further aggravated by the fact that the membrane of the LMD PEN slides creates autofluorescence especially in the UV (DAPI) channel. To overcome this limitation, a simple method was adopted and involved applying a drop of 100% ethanol to the section, which enhanced tissue morphology temporarily while outlining the target cells on the computer screen (Figure 9). It is worth noting that subsequent cutting and catapulting steps are only possible after the fluid has evaporated and sections have become completely air-dry. Other available, but expensive, options can be used like covering the stained sections on PEN slides with a fluid cover medium that has been documented to enable better morphological analysis without compromising the RNA integrity (Micke et al., 2004).

TROUBLESHOOTING

Problem 1

Intact tissue sections are difficult to obtain section in cryosectioning and slide storage).

Potential solution

- Avoid air bubbles that can occur during embedding the tissues in OCT.
- Never immerse OCT embedded tissues totally into liquid nitrogen since this will crack OCT and the tissues embedded in it.

Problem 2

Cryosectioned tissues detach from the PEN slides during immunostaining procedure (IHC staining and cell procurement using LMD).

Potential solution

To overcome the hydrophobic nature of the membrane in PEN slides, the manufacturer strongly recommends irradiating with UV light at 254 nm for 30 min (e.g., in a cell culture hood). This renders the membrane more hydrophilic, therefore the cryosections adhere better. According to the manufacturer, UV pre-treatment of PEN slides have additional positive side effects including sterilization and destruction of potentially contaminating nucleic acids.

Problem 3

Areas adjacent to outlined cells are lifted and catapulted by laser together with the cells/area of interest (IHC staining and cell procurement using LMD).

Potential solution

- Always use PEN slides before their expiry date.
- Avoid storing the tissue sections on PEN slides for > 14 days in the −80°C freezer.
- Avoid extensively long LMD procedure and limit the session to < 90 min.

Problem 4

Quality control experiments show RNA degradation from 1 mm^2 leftover tissues (RNA isolation from LMD-microdissected 1 mm^2 tissue chunks for quality control).

Potential solution

Time is an important factor to consider when adapting this protocol to other studies since endogenous RNase activity varies between different tissues (Marek et al., 2019). Several pilot trials should be conducted by the researcher prior to embarking on performing LMD of precious samples. This is

because the time required to search and identify cells of interest in a section, and to perform LMD is often relatively long, even after the cells in question have been specifically immunolabeled.

Problem 5

Duplicate samples show variable C_T values after qPCR (gene expression analysis of LMD-procured cardiomyocytes).

Potential solution

Always pipette the cDNA 4–5 times before aliquoting into the PCR tubes. This is more critical when the cDNA tubes are left for a long time on ice or in the freezer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources, reagents and data should be directed to and will be fulfilled by the lead contact, [Prof. Georgina Ellison-Hughes] (georgina.ellison@kcl.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all **figures and data** generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conception and design, A.S. and G.M.E.-H.; collection and/or assembly of data, A.S.; data analysis and interpretation, A.S.; manuscript writing, A.S. and G.M.E.-H.; final approval of the manuscript, G.M.E.-H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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