12-16-2022

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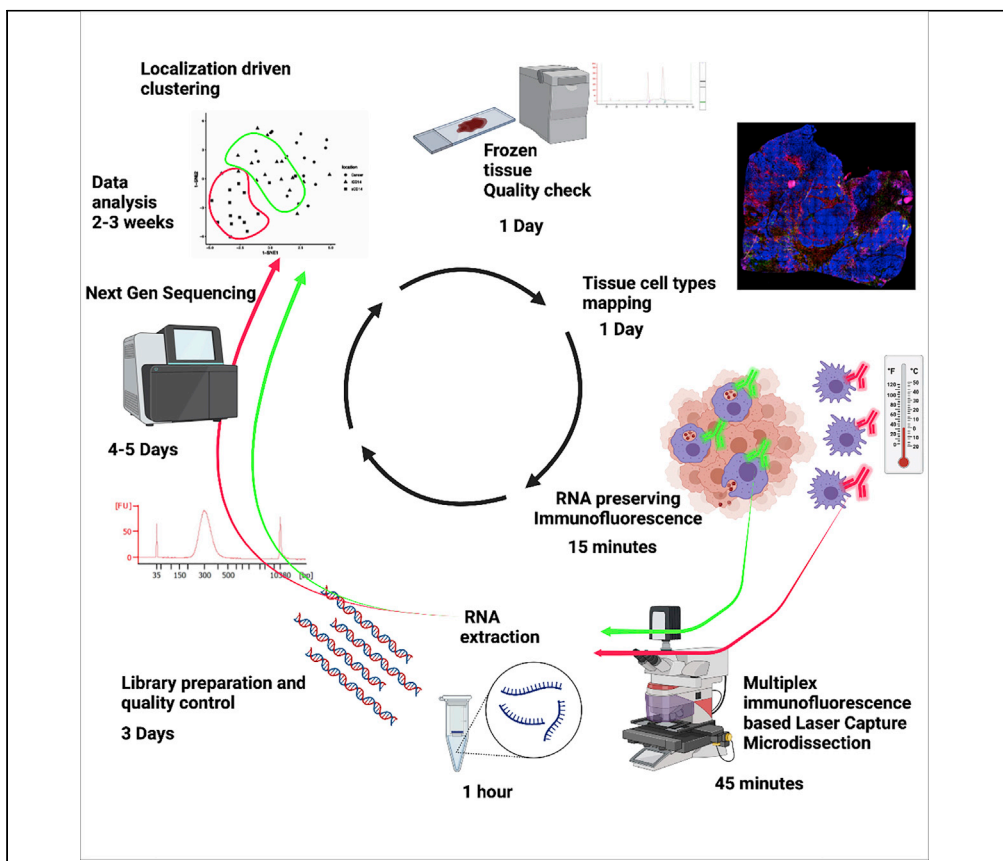Jianan Lin

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Protocol

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Highlights

Assessing the topology and transcriptome of individual cells in human melanoma tissue

Optimized immunofluorescence staining protocol for RNA preservation

Multiplexed immunofluorescence-based harvest of individual cells from tissues

RNA library preparation for next-generation sequencing

We describe a pipeline for optimized and streamlined multiplexed immunofluorescence-guided laser capture microdissection allowing the harvest of individual cells based on their phenotype and tissue localization for transcriptomic analysis with next-generation RNA sequencing. Here, we analyze transcriptomes of CD3+ T cells, CD14+ monocytes/macrophages, and melanoma cells in non-dissociated metastatic melanoma tissue. While this protocol is described for melanoma tissues, we successfully applied it to human tonsil, skin, and breast cancer tissues as well as mouse lung tissues.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Multiplex immunofluorescence-guided laser capture microdissection for spatial transcriptomics of metastatic melanoma tissues

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https://doi.org/10.1016/j.xpro.2022.101698

SUMMARY

We describe a pipeline for optimized and streamlined multiplexed immunofluorescence-guided laser capture microdissection allowing the harvest of individual cells based on their phenotype and tissue localization for transcriptomic analysis with next-generation RNA sequencing. Here, we analyze transcriptomes of CD3+ T cells, CD14+ monocytes/macrophages, and melanoma cells in non-dissociated metastatic melanoma tissue. While this protocol is described for melanoma tissues, we successfully applied it to human tonsil, skin, and breast cancer tissues as well as mouse lung tissues.
For complete details on the use and execution of this protocol, please refer to Martinek et al. (2022).

BEFORE YOU BEGIN

High dimensional analytic approaches such as single cell RNAseq and single cell mass cytometry have revolutionized the study of cells composing tissues and the functional status of immune infiltrates in human solid tumors including metastatic melanoma (Patel et al., 2014; Tirosh et al., 2016; Boddupalli et al., 2016; Lavin et al., 2017; Li et al., 2017; Elyada et al., 2019). However, an inherent need to dissociate the tissue for single cell suspension-based analyses destroys certain information such as tissue architecture, localization of particular cell types and region-specific cellular interactions. Further, in-situ sequencing based approaches such as slideSeq or 10× Visium still lack cellular resolution and are limited in their sequencing depth. To address this problem, here we developed an approach to investigate the transcriptome of CD14+ cells, and CD3+ T cells infiltrating metastatic melanoma samples, based on their tissue localization (intratumoral within melanoma nests vs tumor stroma). To do so, individual cells were harvested from pre-defined localizations in the tissue, based on their phenotype using a streamlined IF staining protocol under RNA preserving conditions and a customized Arcturus LCM microscope equipped with an infrared laser. Harvested cells' transcriptome was analyzed by Next gen RNA sequencing and revealed localization-specific signatures distinguishing macrophages but not T cells.

Our approach includes a tissue screening step, where samples are selected based on their RNA quality and overall structure.

Once the tissues are selected, the LCM step is performed on an Arcturus XT Laser Capture Microdissection system, the only system commercially available allowing harvest of individual cells using
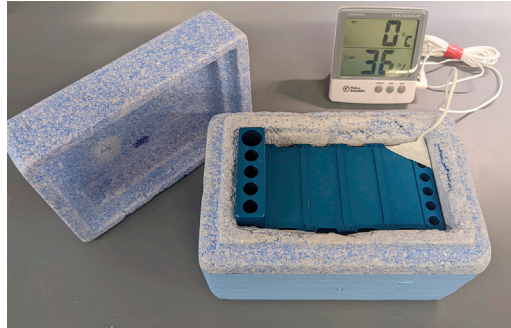
Figure 1. Cold Block setup for LCM IF staining with temperature monitoring

an infrared laser, to pick up the cells from the tissue rather than cutting them out using a high power UV laser which is associated with RNA damage within the vicinity of the cutting line (Bonner et al., 1997; Espina et al., 2006; Farris et al., 2017).

We customized our LCM system by fitting it with a SPECTRA X Light Engine (Lumencor) which has the ability to independently control the output power from multiple simultaneous light sources, allowing us to visualize simultaneously 3 fluorescently labeled markers during the harvest.

Here we describe in detail how to use LCM to harvest CD14+ and CD3+ cells from intra tumoral and stromal region in metastatic melanoma. In addition, we were successful in applying this protocol on different cancer types such as breast cancer, and head and neck cancer; using large tissue samples or needle biopsies; and could harvest different cell types by modifying our staining panel.

> *Note:* Prior starting this protocol, extensive cryo-sectioning experience is preferable as well as a basic multiplex Immunofluorescence (IF) staining and acquisition experience is needed.

Institutional permissions

Metastatic melanoma tissues obtained either from the Baylor University Medical Center (BUMC) or from the Cooperative Human Tissue Network (CHTN) were exempt under the Jackson Laboratory IRB review: IRB#2018-40 and IRB #2018-043.

Material preparation

> ⏱ Timing: 45 min

1. Cryostat RNAse decontamination.
   a. Remove microtome blade holder stage and microtome blade from cryostat.
      i. Allow them to get to 23C.
   b. Apply RNAse Away on both and wipe dry.
   c. Rinse both with 70% ethanol and wipe dry.
   d. Replace both inside the cryostat.
      i. Allow them to return to cryostat temperature.
2. LCM IF staining block (Figure 1).
   a. Fill a CoolSafe triple-density box with wet ice.
   b. Place Histogene™ Cold Block into the CoolSafe box.
   c. Apply RNAse Away on ClodBlock and inside of CoolSafe box lid.
   d. Let the cold block temperature drop to 0C.

> *Note:* Cold block temperature should be monitored during the LCM staining protocol and remain between 0–1C.

Reagents preparation

⏱ Timing: 20 min

3. Prepare fresh dehydration solutions in coplin jars.
   a. A clean jar with 75%, 95%, 100% Ethanol and 100% Xylene each.
   b. Prepare 3 Gene Amp tubes with 50 μL of lysis buffer from PicoPure RNA isolation kit.
4. Prepare staining reagents. For each tissue section prepare:
   a. 288 μL PBS + 12 μL Superase In.
   b. 78.4 μL PBS + 0.8 μL of anti-Melanoma Antigen AF488 + 0.8 μL anti-CD14 AF594 + 4 μL of Superase In.
   c. 76 μL of PBS with DAPI at 0.16 μg/mL + 4 μL Superase In.
   d. Keep all 3 solutions on ice.

⚠ CRITICAL: Step 1. Microtome blades are extremely sharp and should be handled with extreme vigilance, to reduce the risks of injury operator should wear cut resistant gloves. Step 3 Chemicals such as Xylene should be kept under a fume hood at all times.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti CD14 AF594 (5 μg/mL)	BioLegend	RRID: AB_2563225
Anti CD3 AF594 (2.5 μg/mL)	BioLegend	RRID: AB_2563236
Anti CD14 AF647 (5.6 μg/mL)	BioLegend	RRID: AB_830684
anti Melanoma Ag AF488 (1.25 μg/mL)	Novus Biologicals	cat#NBP2-34681AF488
Chemicals, peptides, and recombinant proteins		
Fc Receptor Blocker	Innovex	Cat#NB309
Background Buster	Innovex	Cat#NB306
Fluoromount G	SouthernBiotech	Cat#0100-01
Cover Glass	Thermo Scientific	Cat#152450
Super pap pen mini	Biotium	Cat#22005
Slides	Denville Scientific	Cat#M1021
Saponin	Sigma	Cat#S7900-100G
DAPI	Thermo Fisher Scientific	cat#D1306
BSA IgG free	Jackson ImmunoResearch	Cat#001-000-162
CapSure LCM Macro caps	Thermo Fisher Scientific	Cat#LCM0211
Superase in RNase inhibitor	Thermo Fisher Scientific	Cat#AM2696
Ethanol	Fisher	Cat#BP2818-4
Acetone	Fisher	Cat#A18-4
Xylene	Fisher	Cat#X3S-4
Eosin	Fisher	Cat#245-827
Hematoxylin	Fisher	Cat#245-653
Blueing reagent	VWR	Cat#95057-852
Cytoseal	Thermo Scientific	Cat#8310-16
Critical commercial assays		
RNase-free DNase	QIAGEN	Cat#79254
RNeasy Mini kit	QIAGEN	Cat#74104
Qubit RNA HS Assay Kit	Invitrogen	Cat#Q32852
Agilent RNA 6000 Pico kit	Agilent	Cat#50671513
CoolSafe Box	USA Scientific	Cat#9127-3260
Histogene Cold Block	Thermo Fisher Scientific	Cat#H1S0101
PicoPure™ RNA Isolation Kit	Thermo Fisher Scientific	Cat#KIT0204

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Gene Amp Thin-Walled Reactions Tubes	Thermo Fisher Scientific	Cat#N8010611
NEBNext® Ultra™ II DNA Library Prep Kit for Illumina	New England Biolabs	Cat#E76345S
96 microTUBE-50 AFA Fiber Plate	Covaris	Cat#520168
Bioanalyzer High Sensitivity DNA Analysis	Agilent	Cat#50674626
Qubit™ dsDNA HS Assay Kit	Invitrogen	Cat#Q32851
SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing	Takara	Cat#634889
Software and algorithms		
Imaris 9.0.2 and 9.4	Bitplane	https://imaris.oxinst.com/packages
Arcturuc XT	Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/gene-expression-analysis-genotyping-support-center/lcm-support.html#Tools_Software_and_Calculators
LAS X	Leica	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/
LLE 7ch controller	Lumencor	https://lumencor.com
Other		
Leica CM3050 S Cryostat	Leica	N/A
Leica SP8 Confocal microscope	Leica	N/A
Spectra X Light engine	Lumencor	N/A
Arcturus XT LCM system	Thermo Fisher Scientific	N/A
Bioanalyzer	Agilent	N/A

MATERIALS AND EQUIPMENT

As mentioned previously, we customized our LCM system by replacing the standard light source with a Lumencor SPECTRA X light Engine. This allows us to visualize simultaneously DAPI AF488 and AF594 live, during the harvest. This is an optional customization as the standard Arcturus XT LCM system is equipped with a mercury arc lamp and able to image individual fluorochromes. This configuration allows only for fixed power output across the spectrum, therefore risks overstimulating certain markers (DAPI for example) which will then overpower all other channels if attempting to visualize them simultaneously. Another option would be to acquire 3 separate images and use the overlay to select the cells for harvest for each area, but it will greatly increasing the harvest time along with the risk for RNA degradation. As our Arcturus system was customized from its initial setup, we cannot comment further on how to use the system with its original light source.

In the steps described below, we use the Arcturus system to scan the H&E staining and a Leica SP8 confocal to scan tissue whole section from our sample cohort. These steps can be performed on any slide scanning microscopes with equipped for H&E or fluorescence imaging.

Immunofluorescence staining buffer		
Reagent	Final concentration	Amount
Bovin Serum Albumin	5%	2.5 gr
Triton X-100	0.1%	0.05 gr
PBS 1×	N/A	50 mL
Total	N/A	50 mL

Note: Keep solution sterile at 4C up to 3 months.

Immunofluorescence antibody mix		
Reagent	Final concentration	Amount
Anti CD3 AF594	2.5 µg/mL	0.5 µL
Anti CD14 AF647	5.6 µg/mL	1 µL

(Continued on next page)

Continued

Reagent	Final concentration	Amount
Anti Melanoma AF488	1.25 µg/mL	0.2 µL
Immunofluorescence staining buffer	N/A	98.3 µL
Total	N/A	100 µL

Note: Prepare 100 µL per sample to stain, make fresh for each staining, keep in the dark.

LCM antibody mix

Reagent	Final concentration	Amount
Anti CD14 AF594	5 µg/mL	0.8 µL
Anti Melanoma AF488	6 µg/mL	0.8 µL
Superase In	5%	4 µL
PBS 1×	N/A	74.4 µL
Total	N/A	80 µL

Note: Make 80 µL per sample to stain, make fresh for each staining, keep on ice in the dark.

STEP-BY-STEP METHOD DETAILS
Samples quality control screening

⏱ Timing: 2 days

This step allows for the screening and selection of suitable tissues for LCM procedure based on: RNA quality, overall structure, absence of necrotic areas and presence of cells of interest.

1. Assess tissue RNA quality (Figure 2A).
   a. Prepare fresh RLT buffer.
   b. For each tissue, cut an 8 µm cryosection.
   c. Collect the section with a RNAse free 200 µL pipet tip.
      i. Maintain the tip at 23C. As the tip comes into contact with the tissue it will melt and aggregate on the tip.
   d. Immediately immerse the tip into 350 µL of RLT buffer and homogenize.
      i. Make sure all the tissue is dissolved. Keep on ice if you process multiple samples.
      ii. Purify RNA using Qiagen RNeasy mini kit following manufacturer protocol: https://www.qiagen.com/us/resources/download.aspx?id=5a5aebb5-1603-4a71-82f5-3eb56054417e&lang=en.
      iii. Assess RNA integrity using the Agilent bioanalyzer high sensitivity RNA 6000 pico kit following manufacturer protocol: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90046_RNA600Pico_KG_EN.pdf.
      iv. Select samples with RIN >8.
2. Assess tissue structure and integrity (Figure 2B).
   a. Stain 8 µm section following H&E staining protocol:
      i. 1 min in 70% ethanol.
      ii. 1 min DI water.
      iii. 2 min Hematoxylin.
      iv. 1 min DI water.
      v. 1 min Blueing agent.
      vi. 1 min 70% ethanol.
      vii. 1 min 95% ethanol.
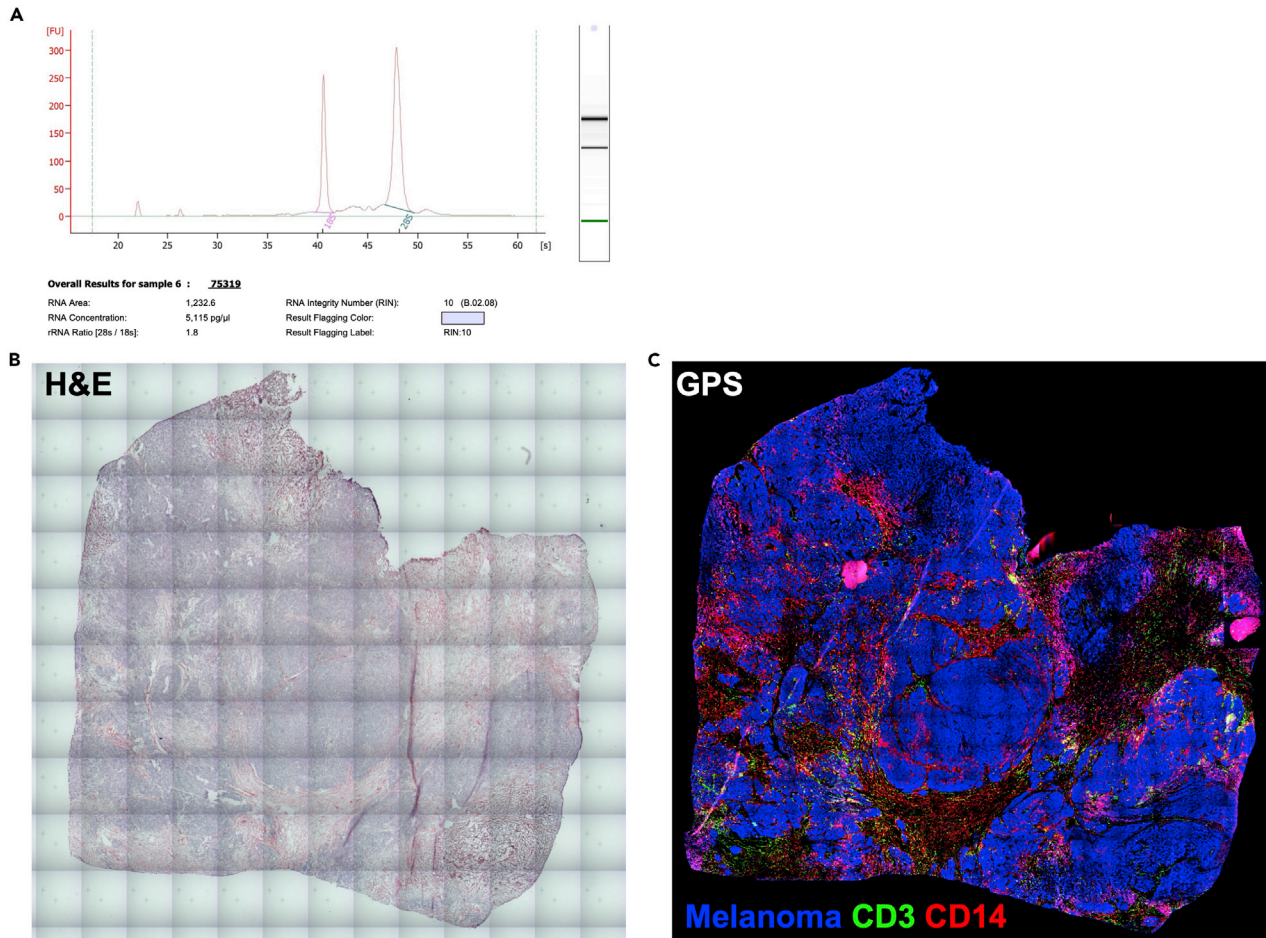
Figure 2. Tissue quality control process

(A) For each frozen melanoma sample, a section is used to establish the RNA quality by defining it's RIN number.

(B) Samples with satisfactory RNA quality are then screened based on their tissue integrity and structure with H&E staining and whole tissue imaging.

(C) For selected samples, we established a "GPS" slide. Mapping the CD14+ and CD3+ infiltrate in the tissue. The GPS slide is then used to guide the LCM harvest allowing to identify most suitable region of interest quickly and efficiently for the harvest. Figure adapted reprinted with permission from Martinek et al. (2022).

> viii. 4 min Eosin.
> ix. 1 min 70% ethanol.
> x. 1 min 95% ethanol.
> xi. 1 min 100% ethanol.
> xii. 5 min xylene.
> xiii. 15 min air dry.
> xiv. Mount in cytoseal.
> b. Using the tile image tool (Figure 3) on the Arcturus system, acquire whole section scan at 20× magnification.
> c. Select samples without necrotic areas.
3. Generate "GPS" slide (Figure 2C).
> a. Perform IF staining protocol on selected samples based on RIN and H&E.
> > i. Cut an 8 μm cryo-section for each tissue.
> > ii. Fix 5 min in cold acetone.
> > iii. Air dry for 20 min.

A

Present Stage (xviii)

Capture Image (xx)

Tiled Image

Exposure Time (xvii)

Fluorescent Mode On/off (xv and xxv)

B

(xi and xiii)

(ix)

(viii)

IR Capture Spots (xviii)

Ruler (xii)

C

Good Capture Spots

Insufficient IR Laser Power

Place Cap on Live Image Location (v)

IR Capture Only (xxvi)

Cap to QC Station (xxvi)

D

Adjust Each Laser Line Output (xvii)

Turn Violet Cyan and Green Laser On (xvi)

Figure 3. Arcturus LCM system setup and operation
(A) Main Arcturus software interface with critical commands indicated by arrows.
(B) Select option dialogue window used to adjust IR laser power.
(C) Snapshot of adequate and bad capture spots on cap, based on different levels of IR laser power.
(D) Lumencor interface.

> iv. Back circle samples with sharpie to delineate tissue on slide and avoid damaging it while pipetting and aspiration during staining procedure.
> v. Circle sample with pap pen.
> vi. Wash with PBS 2× 5 min.
> vii. Incubate 40 min with Fc Blocker.
> viii. Rinse with PBS.
> ix. Incubate 30 min with Background Buster.
> x. Rinse with PBS.
> xi. Incubate 1 h with anti-Melanoma Ag AF488, anti-CD14 AF647 and anti-CD3 AF594 in PBS 5% BSA 0.1% Saponin.
> xii. Wash with PBS 0.1% Saponin 2× 5 min.
> xiii. Counterstain 2 min with DAPI at 1 μg/mL in PBS.
> xiv. Rinse with PBS.
> xv. Mount in Fluoromount-G.
> b. Acquire whole section scan on Leica SP8 confocal microscope at 20× magnificiation.
> c. Stitch and max project each scan in LAS X.
> d. Evaluate each sample for the presence, abundance, and localization of each cell type to be harvested by LCM.

Note: The sample quality control is not mandatory to perform the LCM harvest per se but we found that screening samples and selecting only the ones with high RNA quality and no necrotic areas greatly increases the chances of getting high quality RNA sequencing from LCM harvested cells. The "GPS" slide, which consist of whole section scan after immunofluorescence staining plays several important roles. It helps selecting samples with sufficient numbers of specific cell types to be harvested (here intra tumoral CD14+ cells and stromal CD14+ cells). It also allowed to remove samples which were early melanoma metastasis to the lymph node and present only a minimal invasion by cancer cells. Finally, the "GPS" slide helps to guide the LCM operator during the harvest. The GPS slide can be displayed next to the LCM monitor and help the operator to quickly position the CapSure cap on best area of the tissue to harvest from, instead of browsing the entire slide, saving precious time, fluorescence signal and RNA quality in the process.

Laser capture microdissection

⊙ Timing: 65 min

Perform IF staining under RNA preserving conditions and LCM harvest of three different populations.

4. Power system on.
   a. Power Arcturus XT on.
      i. Open Arcuturs software.
   b. Power Lumencor SPECTRA on.
      i. Open LLE 7ch controller Lumencor software.
   c. Prepare stage for harvest.
      i. In Arcturus select "present stage".
      ii. Install the tray on LCM Cap sure.

      iii. Indicate number of Cap present in tray, in the software.

5. Perform IF staining for LCM.

  a. Place sample in cryostat.

    i. Let sample reach chamber temperature for 10 min.

  b. Cut a 5 μm section.

    i. Transfer immediately onto clean frosted supercharged glass slide.

  c. Fix 2 min in cold acetone.

    i. Air dry 2 min.

    ii. Back circle tissue with sharpie.

    iii. Place on cold block.

  d. Perform IF staining.

    i. Wash 2 min with PBS + Superase in.

    ii. Incubate 10 min with antibody mix + Superase in.

    iii. Wash 2× 2 min with PBS + Superase in.

    iv. Counterstain 1 min with PBS + DAPI + Superase in.

  e. Dehydrate sample.

    i. 30 s in 75% ethanol.

    ii. 30 s in 95% ethanol.

    iii. 1 min in 100% ethanol.

    iv. 5 min in xylene.

    v. 5 min air dry.

6. Proceed to LCM harvest. (Figure 3).

  a. Harvest cells.

    i. Install slide on stage.

    ii. Select the corresponding slide slot in the software.

    iii. Acquire slide overview.

    iv. Based on GPS slide, center on area of tissue where to perform the harvest.

    v. Place Cap on live image location.

    vi. Switch to 40× objective.

    vii. While staying in area covered by Cap, locate empty zone and center on it.

    viii. Open select option dialogue.

    ix. Test IR laser.

    x. Using right click on main screen, locate IR laser at the exact location where test IR shot appeared.

    xi. Adjust laser power/duration to generate adequate capture spot (meaning the polymer on cap made contact with glass).

    xii. Using ruler tool, determine capture spot's diameter.

    xiii. Adjust laser power to obtain a capture spot 8 μm in diameter.

    xiv. Click "OK".

    xv. Switch to fluorescence mode.

    xvi. In Lumencor software turn violet, cyan, and green lasers on.

    xvii. In Lumencor, adjust power for each laser line. In Arcturus adjust the exposure time so that all markers are visible, and their signals are balanced.

    xviii. Click on IR capture spots, Capture group "A" is selected by default.

    xix. Select intra tumoral CD14+ cells to be harvested in the field of view.

    xx. Capture image.

    xxi. Move to a different area.

    xxii. Select additional intra tumoral CD14+ cells to be harvest.

    xxiii. Capture image.

    xxiv. Repeat until around 50 cells are selected.

    xxv. Exit fluorescence mode.

    xxvi. Click on IR capture only.

    xxvii. Send Cap to QC station.
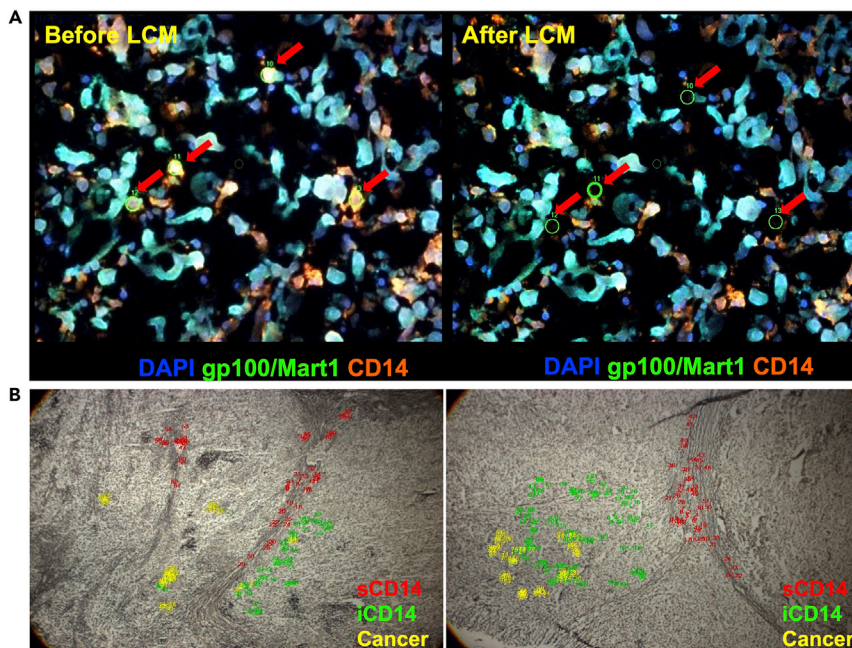
Figure 4. LCM harvest quality control

(A) Before/After LCM images used for LCM harvest QC. Left image shows iCD14+ cells marked for harvest (red arrows). Right image shows the same area after harvest where marked iCD14+ cells have been harvested (red arrows). This QC process is used to assess the precision and efficiency of each LCM run, n=8 in duplicates.

(B) LCM harvest overview. Representative images of 2 different melanoma samples after LCM harvest, low magnification image covers the entire area from cells were harvested. sCD14 cells are indicated in red, iCD14 in green and melanoma cells in yellow. Figure adapted reprinted with permission from Martinek et al. (2022).

> xxviii. Present stage.
> xxix. Carefully pick up cap and firmly place on Gene Amp tube containing lysis buffer.
> xxx. Invert tube so that all lysis buffer rest on cap's surface.
> xxxi. Incubate 30 min at 42C.
> xxxii. Select Capture group "B" Repeat steps iv to xxxi to harvest stromal CD14+ cells.
> xxxiii. Select Capture group "C" Repeat steps iv to xxxi to harvest cancer cells.
> xxxiv. Spin Gene Amp tubes 2 min at 800 g at 4C to collect all buffer, discard cap and freeze at -80C.

b. LCM Harvest quality control. (Figure 4).
> i. Do not remove slide from Arcturus System.
> ii. Find each field of view in which cells were harvested.
> iii. Acquire images after LCM harvest.
> iv. Compare before / after LCM images.
> v. Count the number of harvested cells for each population.
> vi. Evaluate the degree of contamination from co-harvested cells, if any.
> vii. Acquire an overview images of the total harvested area.

Note: Due to RNA instability at 23C, the LCM harvest should be performed immediately after the IF staining and should not last longer than 30 min. The total number of cells that can be harvested during this time greatly depends on their abundance in the tissue, but it is routinely feasible to harvest around 50 cells per population (or per cap), with 3 different populations per harvest. In some cases, where the cells of interest are very abundant (cancer cells in a tumor or immune cells in tonsil) it is possible to harvest up to 200 cells per cap. Additionally, as stained tissues undergo dehydration step, it is important to select fluorochromes able to withstand

incubations in ethanol and xylene. Here we used DAPI, AF488 and AF594 which have shown good stability. In addition, we found that DAPI can be replaced with an AF405 conjugated antibody, adding an extra marker to guide the LCM harvest instead of nuclear staining. In the absence of mounting media, photobleaching will occur much faster compared to conventional IF image acquisition.

▐▐ **Pause point:** Material harvested by LCM is stable at -80C, once lysed. We recommend harvesting all cell types of interest across multiple tissue and store the lysates in the freezer to extract RNA and prepare libraries in batches.

RNA purification and library preparation

7. RNA purification.
   a. RNA extraction was following the manufacturer's instructions of the PicoPure RNA isolation kit:

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2F1268200.pdf.

DNase I treatment at 23C was included to remove contamination of genomic DNA. RNA was eluted with 11.5 μL elution buffer and stored in -80C freezer.

8. library preparation.
   a. cDNA synthesis and amplification (SMART-Seq V4 ultra low input RNA kit).
      i. Prepare a stock solution of 10× Reaction Buffer by mixing the 10× Lysis Buffer with the RNase Inhibitor: 19 μL 10× lysis buffer+1 μL RNase inhibitor=20 μL total volume.
      ii. Transfer 10.5 μL total RNA to a 0.2 mL RNase-free PCR tube. Add 1 μL of 10× reaction buffer.
      iii. Place samples on ice, add 1 μL 3'SMART-Seq CDS Primer II A (12 μM). Mix well.
      iv. Incubate the tubes at 72C in a preheated, hot-lid thermal cycler 3 min.
      v. Prepare master mix per reaction: 4 μL 5× ultra low first-strand buffer, 1 μL SMART-Seq v4 oligonucleotide (48 μM), 0.5 μL RNase inhibitor (40 U/μL), 5.5 μL total volume added per reaction.
      vi. Immediately after the 3 min incubation at 72C, place the samples on ice for 2 min.
      vii. Preheat the thermal cycler to 42C.
      viii. Add 2 μL per reaction of the SMARTScribe Reverse Transcriptase to the master mix, add just prior to use.
      ix. Add 7.5 μL of the master mix to each reaction tube.
      x. Place the tube in a thermal cycler, run 42C 90 min, 70C 10 min, 4C forever [Stopping point: the tubes can be stored at 4C up to 15 h].
      xi. Thaw PCR reagent on ice, prepare PCR master mix: 25 μL 2× SepAmp PCR buffer, 1 μL PCR primer II A (12 μM), 1 μL SepAmp DNA polymerase, 3 μL nuclease-free water, 30 μL total volume per reaction.
      xii. Add 30 μL PCR master mix to each tube containing 20 μL of first strand cDNA product.
      xiii. Place the tubes in thermal cycler: 95C 1 min, cycle [98C 10 s, 65C 30 s, 68C 3 min], 72C 10 min, 4C forever. For <50 cells, proceed with 18 cycles. [Stopping point: the tubes can be stored at 4C up to 15 h].
   b. cDNA production purification.
      i. Add 1 μL 10× lysis buffer to each PCR product.
      ii. Add 50 μL AMPure XP beads to each sample.
      iii. Mix thoroughly, incubate at room temp for 8 min on vortexer 1,000 rpm to let cDNA bind to the beads.
      iv. Apply on magnetic separation device. Discard supernatant.
      v. Keep the samples on magnetic separation device. Add 200 μL of freshly made 80% ethanol to each sample. Waiting for 30 s and pipet out the supernatant.

    vi.   Repeat ethanol wash once.

    vii. Spin down 30 s and put on magnetic device again, remove all the remaining ethanol.

    viii. Place samples at 23C to dry, add 45 μL of elution buffer to cover the bead pellet.

    ix.   Incubate at 23C for 2 min to rehydrate.

    x.   Place back to magnetic device for 1 min.

    xi.   Collect supernatant containing purified cDNA. Determine amount of purified cDNA. Store at -20C.

[Stopping point: the tubes can be stored at -20C for future use].

c. DNA fragmentation.

    i.   Turn the power ON for the Covaris system and the main cooler. Add about 1.9 L of distilled or deionized water to the water bath. The water level in the cooler should be within +/-3 mm of the "FULL" waterline when the transducer is submerged. If needed, add distilled or deionized water to the water bath until the "FULL" line is reached. IMPORTANT: Never run a process without the water bath. This will permanently damage the transducer.

    ii.   Close the door and open the Sonolab software. Click "ON" for the degassed button and degas the water bath for 30 min.

    iii. (optional: Add 5 μL of Elution Buffer if the elution amount is low) to the cDNA from Section 8. b. Transfer the approximately 45 μL of the Elution Buffer + cDNA mixture into the E220 focused-ultrasonicators AFA 96 microTUBE Plate (Covaris). Put the plate into the appropriate location on the sample holder. *For shearing, we use the E220 Covaris Sonicator, the peak power is 450, duty factor is 30, cycles is 200 and time is 220 s.

d. Library preparation with NEBNext DNA library prep master mix set for Illumina.

    i.   starting materials: 500 pg-1 μg of fragmented DNA (If the DNA volume post shearing is less than 50 μL, add 1× TE to a final volume of 50 μL. Alternatively, samples can be diluted with 10 mM Tris-HCl, pH 8.0 or 0.1× TE.).

    ii.   NEBNext end prep: 3 μL NEBNext ultraII end prep enzyme mix, 7 μL NEXNext ultraII end prep reaction buffer, 50 μL fragmented DNA.

    iii. incubate in a thermal cycler for 30 min at 20C, 30 min 65C, hold at 4C.

    iv. Adaptor ligation: 60 μL end prep reaction mix (from step (iii)), 2.5 μL NEBNext adaptor for Illumina, 30 μL NEBNext ultra II ligation master mix, 1 μL NEBNext ligation enhancer. (* Adaptor Ligation of dA-Tailed DNA. Dilute the NEBNext Adapter to 1:10 (DNA 100 ng–5 ng) or 1:25 (DNA less than 5 ng) ratio with water and add.

    v.   incubate in a thermal cycler for 15 min at 20c with the heated lid off.

    vi. add 3 μL of (red) USER enzyme mix, incubate at 37C for 15 min with lid set to >47C.

    vii. clean up with AMPure XP beads: If the starting material is > 50 ng, follow the protocol for size selection. For input ≤ 50 ng, size selection is not recommended to maintain library complexity. Follow the protocol for cleanup without size selection.

    viii. PCR enrichment of adaptor ligated DNA: 23 μL adaptor ligated DNA fragments (from step (vii)), 25 μL NEBNext ultra II Q5 master mix, 1 μL index primer/i7 primer, 1 μL universal PCR primer/i5 primer.

    ix. place the tubes in thermal cycler: 98C 30 s, cycle [98C 10 s, 65C 75 s] 3–15 cycles depending on input DNA fragments, 65C 5 min, 4c forever.

    x.   clean up with AMPure XP beads.

e. Quality check: The library was gel qualified (bioanalyzer, high sensitivity DNA kit, Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp). (Figure 5).

Note: Newer cDNA synthesis kits for ultra low input RNA are available in addition to the kit we are using in our protocol. Although we have not tested them, it seems that NEBNext single Cell/Low input RNA Library prep Kit (New England Biolabs) would be a suitable alternative.
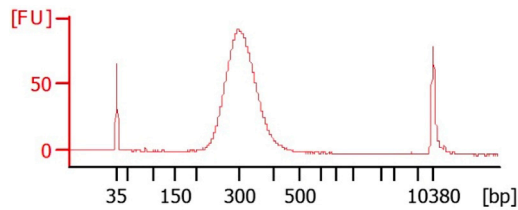
Figure 5. Library quality control
The library was qualified by running a gel on the bioanalyzer, high sensitivity DNA kit, Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

EXPECTED OUTCOMES

Successful LCM harvest should yield a highly enriched pool of individual cells of interest based on their tissue localization and with no or minimal contamination from adjacent cells (Figure 4). These harvested cells should yield high quality RNA, in sufficient amount to allow next generation RNA sequencing with high genome and transcriptome mapping rate and high number of unique genes detected for each cell types (Figure 6).

LIMITATIONS

The laser capture microdissection approach is possibly linked with "contamination" from co-harvested cells as well as from phagocytic cargo and captured exosomes, both containing RNA. Novel spatial transcriptomic technologies might reach subcellular resolution that might help overcome these limitations. Finally, we did not detect differentially expressed genes between T cells harvested from different locations. It is possible that the depth of sequencing was a limiting factor. Alternatively, TCR specificity rather than T cell transcriptome might represent a discriminating parameter.

TROUBLESHOOTING

Problem 1
Unable to generate good capture spot.

Cannot obtain harvest spot properly welded to in step 6.a.xi. even at high IR laser power.

Potential solution
In most cases this is due to residual humidity or xylene on the slide which will prevent melted polymer to adhere to the slide/tissue.

- Make sure slide is properly air dried for 5 min after final xylene incubation, under chemical fume hood with good air flow.
- Slightly move cap to a different area on tissue in case the cap was not properly set by the Arcturus system. This operation will also allow for residual xylene trapped between the cap and slide to quickly evaporate.

Problem 2
Inconsistent capture spots across cap.

Capture spot size during step 6. a. xiii. Or 6. a. xxvi. varies greatly depending on the location on the cap, leading to poor harvesting performance either in terms of efficiency or specificity.

Potential solution
Variable capture spot size is usually due to the cap not resting flat on the tissue.

- Make sure that polymer on cap is free of any artifact/dust/fold. Otherwise, send cap to QC station and use the next cap on tray.
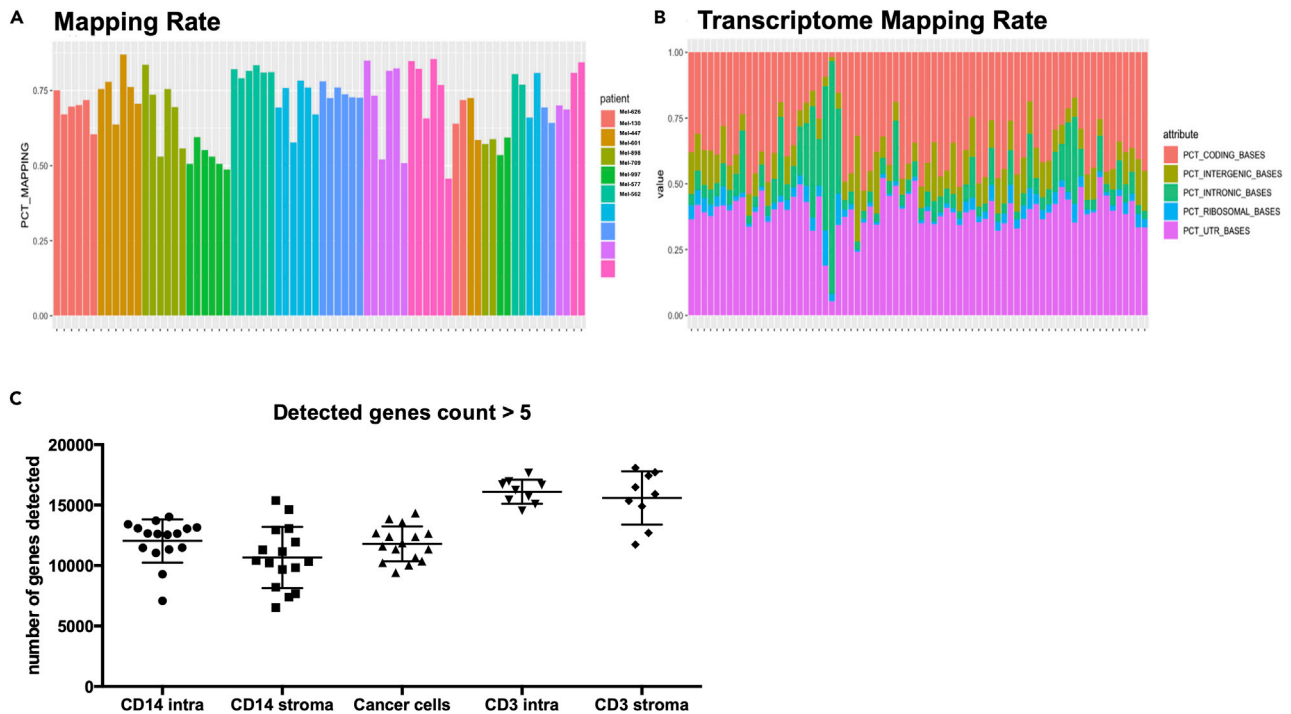
Figure 6. Expected RNA sequencing data quality

(A) Genome mapping rate for each LCM harvested sample after RNA sequencing. Each color indicates a different patient. Each column represents a different cell population. One sample had consistently lower mapping rate compared to all others and was therefore removed from all analysis.

(B) Transcriptome mapping rate for all LCM harvested sample. Same sample that showed low mapping rate also had low transcriptome mapping rate.

(C) Number of genes detected for each sample across different cell types harvested by LCM. Threshold for a gene to be considered detected: count >5, bar indicates median values with 95% CI. Figure adapted reprinted with permission from Martinek et al. (2022).

- Make sure cap is resting flat on tissue section. Avoid setting cap over tissue's edge in a way that cap can de tilted over the edge.
- Uneven spots can also occur if cap is set on top of folds in tissue section. Cryosections should be as even and flat as possible.

Problem 3

Cells are not harvested from tissue.

Despite good capture spots, no or very few cells are being harvested from the tissue at step 6. b. iv.

Potential solution

- Make sure caps are not expired and were stored in a dry space. Humidity affects cap's polymer and alter its ability to adhere to cells of interest.
- Use fresh 100% ethanol for each harvest. Complete sample dehydration is critical for good adhesion between tissue and cap's polymer.
- Certain cell types might require larger capture spots. For example, Breast cancer cells need a 10–12 µm capture spot.

Problem 4

Contamination from adjacent cells during harvest.

Harvest is being contaminated by cells in contact or surrounding the cells of interest when doing harvest QC at step 6. b. vi.

Potential solution

- Reduce the size of harvest spot by reducing the IR laser settings.
- Use CapSure HS caps (Thermo Fisher Scientific). HS caps have reduced working area but allow to generate spots under 8 μm in diameter, thus increasing harvesting precision.

Problem 5
RNA yield from harvest is too low.

If step 8. b. xi. Did not yield enough purified cDNA from extracted RNA.

Potential solution

- Make sure the entire staining protocol is done under RNAse free conditions and the sample and all solutions are kept at 0–1C until the dehydration step.
- If possible, harvest more cells per cap.
- If the cell type of interest is very rare, use the same cap to harvest from consecutive sections placed next to each other on the same slide.
- Pool product of cell lysis from 2 caps from 2 different harvest.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Karolina Palucka. (Karolina.palucka@jax.org).

Materials availability
This study did not generate new unique reagents.

Data and code availability
All raw sequencing data generated for this study have been deposited to dbGap: phs002564.v1.p1

All processed sequencing data generated for this study have been deposited to GEO and are publicly available. Accession # GSE180124.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

AUTHOR CONTRIBUTIONS
J.M.: experiment design and performance, data analysis, and manuscript writing. T.-C.W.: experiment performance, library preparations. J.L.: computational and statistical analysis. K.I.K.: computational and statistical analysis. L.S.: experiment performance, library preparations. F.M.: tissue samples processing. P.R.: experimental design, data analysis, manuscript writing. J.G.: computational analysis. K.P.: concept, study design, data analysis, and manuscript writing.

DECLARATION OF INTERESTS

K.P., MD, PhD serves as Advisory Board Member and a shareholder for Cue Biopharma, Inc. Cambridge, MA.

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