Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60939R1
Full Title:	LASER CAPTURE MICRODISSECTION OF GLIOMA SUBREGIONS FOR SPATIAL AND MOLECULAR CHARACTERIZATION OF INTRATUMORAL HETEROGENEITY, ONCOSTREAMS AND INVASION
Section/Category:	JoVE Cancer Research
Keywords:	glioma, laser microdissection, transcriptomic analysis, RNA integrity, tumor heterogeneity, invasion, molecular targets
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ann Arbor, Michigan, United States

TITLE:

Laser Capture Microdissection of Glioma Subregions for Spatial and Molecular Characterization of Intratumoral Heterogeneity, Oncostreams, and Invasion

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KEYWORDS:

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SUMMARY:

Laser microdissection (LMD) is a sensitive and highly reproducible technique that can be used to uncover pathways that mediate glioma heterogeneity and invasion. Here, we describe an optimized protocol to isolate discrete areas from glioma tissue using laser LMD followed by transcriptomic analysis.

ABSTRACT:

Gliomas are primary brain tumors characterized by their invasiveness and heterogeneity. Specific histological patterns such as pseudopalisades, microvascular proliferation, mesenchymal transformation and necrosis characterize the histological heterogeneity of high-grade gliomas. Our laboratory has demonstrated that the presence of high densities of mesenchymal cells, named oncostreams, correlate with tumor malignancy. We have developed a unique approach to understand the mechanisms that underlie glioma's growth and invasion. Here, we describe a comprehensive protocol that utilizes laser capture microdissection (LMD) and RNA sequencing to analyze differential mRNA expression of intra-tumoral heterogeneous multicellular structures

(i.e., mesenchymal areas or areas of tumor invasion). This method maintains good tissue histology and RNA integrity. Perfusion, freezing, embedding, sectioning, and staining were optimized to preserve morphology and obtain high-quality laser microdissection samples. The results indicate that perfusion of glioma bearing mice using 30% sucrose provides good morphology and RNA quality. In addition, staining tumor sections with 4% Cresyl violet and 0.5% eosin results in good nuclear and cellular staining, while preserving RNA integrity. The method described is sensitive and highly reproducible and it can be utilized to study tumor morphology in various tumor models. In summary, we describe a complete method to perform LMD that preserves morphology and RNA quality for sequencing to study the molecular features of heterogeneous multicellular structures within solid tumors.

INTRODUCTION:

Gliomas are the most aggressive primary tumors of the central nervous system. They are highly invasive and heterogeneous[1]. Analysis of cellular and molecular components of the tumor will reveal novel therapeutic targets.

Among different methods currently available, laser capture microdissection (LMD) of frozen brain tumor tissue is a cost-effective, reliable technique that allows the isolation of discrete anatomical areas or specific cell populations from tumor tissues to study their molecular profile[2,3]. LMD allows the analysis of mRNA gene expression profiles of selected single cells or multicellular structures[4,5]. LMD can be utilized to gain in-depth mechanistic knowledge about the molecular events that take place during tumor progression. Improvement in processing of tumor tissues is necessary to obtain optimal optical resolution of tissue morphology and RNA-quality[6]. Although paraformaldehyde fixation is the best option for morphological analysis, RNA quality is affected and degraded under these conditions, resulting in poor RNA quality for RNA-seq analysis. The use of frozen tissue sections avoids ice crystal formation, which could break cell membranes and produce holes within cells, and remains the best option for RNA-Seq analysis[7].

Here, we describe an optimized section fixation and staining method to process frozen mouse brain tumor tissues for LMD. To prevent ice crystals from forming in the tissue, we perfused mice with a solution of 30% sucrose. This solution disrupts interactions between polar water molecules and prevents the formation of ice crystals, preserving the tissue morphology. Tissue staining is necessary to differentiate and obtain specific population of cells or anatomically distinct areas within the tumor. It is essential to fix and stain the tissue with innocuous dyes to maintain RNA integrity. It has been previously shown that staining tissue with hematoxylin/eosin (H&E) deteriorates RNA integrity[8]. We fixed and stained the tissue of interest with ethanol, Cresyl violet 4% and eosin Y 0.5% solutions. Cresyl violet is an acidophilic dye that stains the cell nucleus with a dark blue color. Eosin Y is a basophilic dye that stains basic components of the cells, providing a distinction between cytoplasm and other cellular structures[8]. Both dyes are soluble in ethanol and do not deteriorate RNA quality. To avoid tissue damage and maintain high optical resolution of the cellular structures, we mounted the tissue sections prior to LMD[9].

PROTOCOL:

All methods described here that use experimental animals have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan.

NOTE: Glioma neurospheres generated from a GEMM or stable lines can be used for intracranial tumor engraftment in mice[10] and processed for LMD and RNA sequencing. These cells constitutively express firefly luciferase and GFP proteins, which will be further utilized for tumor growth analysis and localization.

1. Generation of intracranial mouse glioma model from neurospheres derived from genetically engineered glioma models

1.1. To generate a primary culture of neurosphere cells from a genetically engineered mouse (GEMM) model, use the protocol described previously[10,11].

1.2. Prepare neurosphere culture medium as described: 500 mL of Dulbecco's Modified Eagle Medium F-12 (DMEM/F12) supplemented with 10 mL of 50x B-27 and 5 mL of 100x N-2 neuronal culture supplements, 5 mL of 100x Antibiotic-Antimycotic, and 1 mL of Normocin. Before plating the neurospheres, add 20 ng/mL human recombinant EGF and FGF to the culture medium.

1.3. Culture glioma neurospheres in a tissue culture incubator at 37 °C and 5% CO_2 for 2-3 days prior to intracranial tumor engraftment.

1.4. On the day of the surgery, collect the glioma neurospheres and spin them at 550 *x g* for 5 min at room temperature. Carefully remove the supernatant without disturbing the cell pellet.

1.5. To dissociate the neurospheres, resuspend the cell pellet in 1 mL of cell detachment solution and incubate at 37 °C and 5% CO_2 for 2-4 min. Following the incubation period, pipette the neurospheres up and down with a 1 mL micropipette to ensure a single cell suspension.

1.6. Inactivate the cell detachment solution by diluting the neurosphere suspension with 10 mL of un-supplemented DMEM/F12 media. Spin the neurospheres at 550 x *g* for 5 min at room temperature. Carefully, remove the supernatant.

1.7. Resuspend the cell pellet in 100 µL of DMEM/F12 media with no supplements. Make a 1:50 dilution of the cell suspension, and then add 50 µL of Trypan Blue to count viable cells. Use the following formula to determine the concentration of cells:

cells/mL = [((∑cells counted per square) / # of squares counted) x 50 x 10,000 cells/mL].

1.8. After determining the cell count, to achieve a target concentration of 30,000 cells/µL in 100 µL volume, spin the neurospheres down and resuspend them in the appropriate volume of DMEM/F12 containing no supplements.

1.9. Place neurospheres in a pre-labeled 0.6 mL tube on ice.

1.10. Prepare working solutions of anesthesia, analgesic, and anesthesia reversal prior to implantation: For ketamine/dexmedetomidine anesthesia, add 0.6 mL of 100 mg/mL ketamine hydrochloride and 0.8 mL of 0.5 mg/mL dexmedetomidine hydrochloride to sterile 8.6 mL of 0.9% NaCl containing vial. For buprenorphine analgesic, add 1 mL of 0.3 mg/mL buprenorphine to 9 mL of 0.9% NaCl containing vial. For atipamezole anesthesia reversal, add 1 mL of 5 mg/mL atipamezole to 9 mL of 0.9% NaCl containing vial.

1.11. Use 6-8-week-old C57BL/6J female mice for intracranial tumor engraftment.

1.12. In a room approved for rodent survival surgery, set up sterile supplies for intracranial tumor engraftment. Perform surgeries on a rodent stereotaxic frame equipped with a sterilized 10 µL Hamilton syringe with a removable 33 G needle. Utilize a bead sterilizer to sterilize tools between surgeries.

1.13. Anesthetize mice with a single intraperitoneal (i.p.) injection of the anesthetic solution prepared in step 1.11 (ketamine:75.0 mg/kg and dexmedetomidine:0.5 mg/kg). Approximately, a 250 µL volume of the anesthetic solution will be delivered to a mouse weighing 20 g. Ensure that the mouse is unresponsive to pedal reflex prior to proceeding.

1.14. Once the mouse is in a deeply anesthetized state, apply sterile petrolatum ophthalmic lubricant to the eyes to prevent drying. Shave the fur on the mouse cranium. Apply 10% povidone-iodine topical solution to the shaved area in order to disinfect.

1.15. Secure the mouse's skull in a stereotactic frame. First, carefully open the mouth with forceps and gently pull the tongue and move it to one side of the mouth to prevent choking. Keep the mouth open with the forceps and place the top incisors into the keyhole of the tooth bar on the stereotactic frame.

1.16. Holding the mouse head by the ears, place the ear bars against the postorbital bones and secure them. Ensure that the mouse's cranium is level with the surgical tabletop. Secure the ear bars carefully not applying pressure against the skull. Next, carefully secure the nose bar.

1.17. Using a size 15 scalpel blade, make an incision along the mouse head, exposing the cranium. Retract the skin at the incision site using Colibri retractors. Use a sterile applicator to remove all pericranial tissue.

1.18. Identify the bregma and lower the needle of the Hamilton syringe directly over it. Using the frame, position the needle 1 mm anterior of the bregma and 1.5 mm lateral. Using a 26G needle, mark this spot by scoring the cranium.

1.19. Use a cordless power drill equipped with a 0.45 mm drill bit to create a burr hole at the target site. Drill until reaching the underlying dura mater. Carefully extract the remaining bone in the burr hole with a 26 G needle.

1.20. Using a pipette, homogenize the cells thoroughly and draw up 7 µL of the neurosphere suspension into the syringe. To ensure that that syringe is working properly, dispense 1 µL of the suspension onto a 70% alcohol-soaked pad.

1.21. Lower the needle to the surface of the dura mater. Lower the syringe 3.5 mm ventral and retract 0.5 mm. This will create a 0.5 mm space in the brain for the neurospheres to deposit when injected.

1.22. Leave the needle in place for 2 min for pressure equilibration. Slowly and smoothly deliver 1 µL of the cells over the course of 1 min. Allow the cells to settle in for 6 min. Remove the syringe slowly and smoothly from the brain over the course of 2 min.

1.23. Using sterile saline solution, wash the surface of the cranium three times. Dispense the excess cells in the syringe onto a 70% alcohol pad, and wipe the needle clean with another 70% alcohol pad. Rinse the syringe with sterile PBS to avoid clogging of the needle.

1.24. Remove the retractors and carefully take the mouse out of the stereotactic frame. Close the incision using 3-0 nylon sutures, forceps, and a needle driver.

1.25. Administer atipamezole (1.0 mg/kg), approximately 100 µL for a 20 g mouse. Administer buprenorphine (0.1 mg/kg subcutaneous) subcutaneously, approximately 70 µL for a 20 g mouse. Place post-surgical mice into a clean recovery cage and monitor them until alert and active.

2. Animal perfusion and brain preservation

2.1. To monitor tumor progression, determine in vivo bioluminescence using an in vivo imaging system until animals show signs of tumor burden. Euthanize mice when they reach a signal between 10^6 to 10^7 photon/s.

2.2. Anesthetize mice displaying signs of tumor burden with intraperitoneal (i.p.) injection of ketamine (75.0 mg/kg) and dexmedetomidine (0.5 mg/kg) solution. Deliver approximately, 250 µL to a mouse weighing 20 g. Then, ensure that the mouse in unresponsive to pedal reflex.

2.3. Using forceps, hold the skin above the peritoneal cavity, and using a large pair of dissection scissors make a “Y” incision by penetrating the peritoneal wall, puncture the diaphragm, and then cut the rib cage.

2.4. Insert a blunt 20 G cannula into the left ventricle of the mouse’s heart. Then snip the right atrium of the heart to allow for exsanguination.

2.5. Allow oxygenated Tyrode’s solution (0.8% NaCl, 0.0264% $CaCl_2$, 0.005% NaH_2PO_4, 0.1% glucose, 0.1% $NaHCO_3$, 0.02% KCl) to flow through the mouse circulatory system until the liver and lungs have completely cleared due to the removal of blood (~ 5 min).

2.6. Continue perfusing the animal with 30% sucrose solution dissolved in Tyrode's solution for an additional 15 min. To evaluate the success of the perfusion, confirm that the neck, tail and legs are rigid post 30% sucrose circulation.

2.7. Using a small pair of dissection scissors, cut the scalp at the midline. Starting at the occipital bone and working forwards towards the snout. This will expose the cranium.

2.8. Using a pair of rongeurs, break through the cranium beginning at the occipital bone and continue forward to totally expose the surfaces of the brain. Then turn the head side up and dissect the nerves at the base of the brain to release it from the cranium.

2.9. Prepare 30% sucrose solution with RNase free water and filter it through a 40 µm nylon mesh filter to reduce RNA degradation.

2.10. To maximize sucrose solution infiltration, place the dissected brains into 30% sucrose solution and store them at 4 ˚C overnight. Before further processing ensure that the brain reaches the bottom of the tubes containing the sucrose solution.

3. Cryopreservation of brains harboring glioma tumors

3.1. Prior to cryopreserving the brains, prepare a jar filled with cold isopentane/2-methylbutane and place the jar into a container filled with liquid nitrogen. Let the solvent cool down.

3.2. Remove the brain from the 30% sucrose solution and blot it dry on a filter paper.

3.3. Label the cryomold with a permanent marker. Carefully, add approximately 5 mL of OCT (optimal cutting temperature compound) into the center of the cryomold avoiding air bubbles.

3.4. Place the brain into cryomold containing OCT in the desired orientation. Fill the mold with OCT until the brain is fully submerged. Using clean forceps, quickly place the cryomold with OCT and the brain into the cold isopentane/2-methylbutane.

3.5. Once the OCT solidifies (~30-40 s), remove the cryomold with the brain and place it in dry ice. Do not leave the mold containing the brain in 2-methylbutane past 2 min as this may cause cracks in solid OCT. Wrap the cryomold with the brain in aluminum foil and store it at -80 °C.

4. Sectioning frozen brain tumor tissues

4.1. Label 2 µm polyethylene naphthalate (PEN) slides with the sample information. Tissue sections will be placed directly on these slides following sectioning.

4.2. Set the temperature of the cryostat chamber between -20 to -24 °C. Before sectioning, place the sample block in the cryostat chamber and let it equilibrate to the temperature in the chamber for 30-60 min.

4.3. Clean the cryostat chamber and the knife holder with 100% ethanol and spray the brushes to be used with RNase cleaning solution. Working inside the cryostat chamber, remove the mold and attach the OCT block containing the brain to the cryostat specimen disk with OCT. Place the block in the disk holder and align the block with the knife blade.

4.4. Install a disposable blade into the sectioning holder.

4.5. Section the brain at 10 µm thickness. Make sure there are no streaks or scratch lines in the tissue. Using a paintbrush, cautiously flatten and uncurl the tissue onto the cutting surface.

4.6. Carefully mount the tissue containing the brain sections onto RNase free PEN glass slides. Flip the positive charged glass slides with the fingers in direction of the tissue and smoothly press the glass slide down towards the tissue section.

NOTE: Temperature of hands will help the tissue attach to the glass.

4.7. After mounting the brain sections onto the slides, keep slides in a box inside the cryostat chamber and then store them at -80 °C. Never keep the slides at room temperature.

NOTE: Folding of the tissue, and tearing are common. For accurate posterior analysis, it is important to minimize these artefacts.

5. Fixation and staining of cryopreserved brain tissue sections

5.1. To preserve RNA integrity, clean all instruments to be used with RNase cleaning solution. Proceed with the fixation and staining protocol inside a fume hood.

5.2. Prepare the described fixative solutions in clean RNase free 50 mL tubes. Make all solutions with RNase free water on the day of the staining.

5.3. Same day the laser microdissection will be performed, prepare 100%, 95%, 70% and 50% ethanol solutions. Keep the solutions in tightly closed tubes at room temperature.

5.4. Prepare 4% Cresyl violet and 0.5% eosin Y in 75% ethanol solution. Vortex the solution vigorously for 1 min and filter them through a 0.45 µm nylon filter to eliminate traces of undissolved powder.

5.5. Place the tissue slides into a container with 95% ethanol for 30 s. Transfer slides to the tube containing 75% ethanol; leave slides there for 30 s.

5.6. Transfer slides to 50% ethanol and leave them there for 25 s. At this point, the OCT will be dissolved. Transfer the slide to 4% Cresyl violet solution for 20 s, and then transfer to 0.5% eosin Y solution for 5 s.

5.7. Take the slide out of the dye solution and blot the slide dry with a filter paper. Then, place the slides in 50% ethanol for 25 s. Transfer the slides to 75% ethanol for 25 s. Transfer the slides to 95% ethanol for 30 s. Transfer the slides to 100% ethanol for 60 s.

5.8. Rinse the slide with xylene. Transfer them to a container with xylene and wait 3 min.

5.9. Prepare mounting medium (e.g., Pinpoint gum) in RNase-free water. To mount mouse brain sections, dilute the mounting medium in RNase-free water at a ratio of 1:10.

5.10. Dry the slides on a RNase-free surface at room temperature for 10 s. Before the xylene dries, proceed to mounting the slides with the tissue sections.

5.11. Gently disperse mounting solution on top of the tissue on the slide with a sterile and RNase-free thin paintbrush. Wait 10-20 s, and then immediately transfer the tissue slides to the microscope microdissection platform.

NOTE: The ratio of mounting medium to RNase-free water used for mounting varies depending on the tissue of interest. The mounting medium/water ratio preserves glioma tissue morphology for laser microdissection without affecting the RNA integrity.

6. Laser capture microdissection

NOTE: A laser capture microdissection microscope needs to be utilized to laser microdissect specific areas of interest within the tumor tissue. To minimize the time for tissue laser microdissection, have the LMD microscope prepared before fixation and staining.

6.1. To start the system, first turn on the power strip, followed by turning on the laser. Then, turn on the microscope controller and the computer. Start the LMD software.

6.2. Under **Microscope control**, select **10x** magnification. Under **Laser control**, set the laser parameters for tissue dissection. Set a laser frequency of 120 Hz for best cutting results. Always set the laser current to 100%.

6.3. For accurate laser microdissection, set the speed at 10 and an aperture setting at 2.0-10.0 µm. Set the power to 53. Having the laser power at a higher setting may cause glass etching.

6.4. Load the tissue collector that will capture the tissue following dissection. Click the second unload button. Remove the empty collector and place the DNase/RNase free 0.5 mL PCR flat head tubes containing 30 µL of lysis buffer into the collector. Place the collector back into the machine and click **Continue** on the software to proceed.

6.5. Load the processed (fixed and stained) specimen onto the microscope. First, click **unload** on the LMD software. Next, mount the sample on the slide holder and place the slide holder onto the stage. Click **Continue** on the software to proceed.

6.6. Under **Cut Shapes** windows, select **Draw + Cut**. Use the microscope controls to find the area of interest. Draw the region of interest (ROI) and select a destination collector tube. It is possible to draw multiple ROIs to micro dissect different areas from a single slide at the same time.

6.7. Click **Start Cut** to proceed to tissue micro dissection. After sectioning the areas of interest remove the collector tubes from the holder and place the tubes on dry ice. Transfer the RNA tissue samples collected to -80 ˚C for long term storage.

7. RNA isolation of micro-dissected glioma tissue

7.1. For RNA extraction from LMD use an RNA isolation kit optimized for small samples and low RNA yield (see **Table of Materials**). Follow the manufacture instructions. Carry out all the isolation steps at room temperature (25 °C). To maintain RNA quality, work rapidly. Prepare all the solutions as indicated by the manufacturer.

7.2. Adjust the sample volume to 350 µL with lysis buffer with 1% β-mercaptoethanol. Vortex the sample for 40 s in order to reduce sample viscosity and increase RNA elution spin column efficiency.

7.3. Transfer the entirety of the sample to a gDNA eliminator spin column placed in a 2 mL collection tube. Centrifuge the tube for 30 s at 8,000 *x g*. Save the flow-through and ensure that no liquid is left on the column following centrifugation.

7.4. Add 350 µL of 70% ethanol to the flow-through and mix well by pipetting up and down. Transfer the sample, to an RNA elution spin column placed in a 2 mL collection tube. Close the lid gently, and centrifuge for 15 s at 8,000 *x g*. Discard the flow-through, saving the column.

7.5. Add 700 µL of RNA washing buffer 1 (20% ethanol, 900 mM GITC, 10 mM Tris-HCl pH 7.5) to the RNA elution spin column. Close the lid gently, and centrifuge for 15 s at 8,000 *x g* to wash the spin column membrane. Discard the flow-through.

7.6. After centrifugation, carefully remove the RNA elution spin column from the collection tube so that the column does not contact the flow-through.

7.7. Add 500 µL of the second RNA washing buffer (ethanol 80%, NaCl 100 mM, Tris-HCl 10 mM pH 7.5) to the spin column. Close the lid of the column and spin 8,000 *x g* for 20 s to wash the column membrane. Dispose the flow-through.

7.8. To wash the RNA elution column, add 500 µL of 80% ethanol, close the lid of the column and centrifuge at 8000 *x g* for 2 min. Throw out the tubes with the elution solution.

7.9. Use a new collection tube, open the lid of the spin column and centrifuge at 8000 *x g* for 5 min to dry the column. Discard the elution tube.

7.10. Place the RNA elution spin column in a new 1.5 mL collection tube. Add 12 µL of RNase-free water warmed at 37 ˚C directly to the center of the spin column membrane. Wait for 4 min and centrifuge for 1 min at full speed to elute RNA.

8. RNA quality control, library preparation and RNA-Seq analysis

8.1. Following RNA extraction and purification, amplify RNA and create a cDNA library using a special kit suitable for RNA isolation at pico-molar concentrations following manufacturer instructions (see **Table of Materials**). Follow manufacturer's instructions. Clean workstation in order to avoid contamination with other PCR products and nuclease degradation of samples.

8.2. If RNA has a RIN value greater than 4, meaning the RNA is of good quality or only partially degraded, proceed with the fragmentation step. Prepare all items on ice.

8.3. Create a master mix as indicated (**Table 1**). Create reaction mixture in a nuclease-free thin-wall 0.2 mL PCR tube. Incubate the tubes at 94 °C in a hot-lid thermal cycler. Fragmentation incubation time depends on the quality of the RNA. RIN ≥ 7: 4 min, RIN 5-6: 3 min, RIN 4-5: 2 min.

8.4. Following incubation, place the tubes on a PCR chiller rack that was previously cooled at -20 °C and let it sit for 2 min.

8.5. For each tube of RNA sample, prepare first strand synthesis reaction master mix (**Table 2**). Incubate the tubes in a hot-lid thermal cycler under conditions described in **Table 3**. cDNA products can be frozen at -20 °C until for two weeks before proceeding to the next step.

8.6. Create the PCR master mix as indicated in **Table 4**. Place the tubes in a hot lid thermal cycler and run the PCR reaction under the settings indicated in **Table 5**.

8.7. Allow the beads, which will be used to purify the DNA, to warm to room temperature. Once warmed, add 40 µL of the beads to each sample. Vortex the tubes to mix and spin down briefly to collect liquid at bottom of the tube. Let the tubes incubate at room temperature for 8 min to allow the DNA to bind to the beads.

8.8. Place the tubes on a magnetic separation device and let sit for about 5 min, or until the solution becomes completely clear. Keeping the tubes on the separation device, use a pipette to remove the supernatant carefully without disturbing the beads.

8.9. Keeping the tubes on the separation device, add 200 µL of freshly made 80% ethanol to the beads to wash without disturbing the beads. Wait for 30 s before removing the 80% ethanol. Repeat this step.

8.10. Briefly spin down the tubes and place the tubes back on the separation device. Remove any residual 80% ethanol without disturbing the beads.

8.11. Let the tubes air dry with the caps open for 5 min. Do not let it sit longer as the beads will over dry.

8.12. Keeping the tubes on the separation device, add 52 µL of nuclease-free water to cover the beads. Remove the tubes from the separation device and pipette up and down until all the beads are resuspended. Incubate at 5 min at room temperature.

8.13. Place the tubes back on the separation device until solution becomes clear, about 1 min. Transfer 50 µL of the resulting supernatant to the wells of an 8-well strip. Add 40 µL of new beads to each sample. Vortex thoroughly to mix. Allow beads to bind to DNA by incubating at room temperature for 8 min.

8.14. During this incubation period, begin thawing the components to be used for any rRNA present in the samples. Once thawed, place them on ice. Also, pre-heat a thermal cycler to 72 °C.

8.15. Place the samples on the magnetic separation device until the solution clears (about 5 min). Aliquot 1.5 µL of probes per sample to a chilled PCR tube. Place the tube in the preheated thermal cycler under the settings of 72 °C for 2 min and 4 °C.

8.16. Keeping the sample tubes in the magnetic separation device, remove the supernatant with a pipet without disturbing the beads. Then add 200 µL of freshly made 80% ethanol to the beads to wash without disturbing the beads. Wait 30 s before removing the 80% ethanol. Repeat this step.

8.17. Briefly spin down the tubes and place the tubes back on the separation device. Remove any residual 80% ethanol without disturbing the beads. Let the tubes air dry with the caps open for 2 min. Do not let sit longer as the beads will over dry.

8.18. Prepare master mix for all samples by combining the following components in the order presented (**Table 6**).

8.19. Mix the master mix by vortexing and add 22 µL of the mix to the dried beads of each sample and mix thoroughly to resuspend. Let it incubate at room temperature for 5 min.

8.20. Spin down the tubes and place them on the magnetic separation device for 1 minute or until the samples become clear. Transfer 20 µL of the supernatant, without disturbing the beads to new PCR tubes. Place the tubes in a pre-heated thermal cycler under settings in **Table 7**.

8.21. Prepare a PCR master mix for enough for reactions for each sample. Add the following components to the master mix in the indicated **Table 8**. Add 80 µL of the PCR master mix to each of the sample tubes from step 8.20 and place in the thermal cycler at the following settings (**Table 9**).

8.22. Allow beads, which will be used to purify the DNA, to warm to room temperature. Once warmed, add 100 µL of the beads to each sample. Let the tubes incubate at room temperature for 8 min to allow the DNA to bind to the beads.

8.23. Place the tubes on a magnetic separation device and let sit for about 5 min, or until the solution becomes completely clear.

8.24. Keeping the tubes on the separation device, use a pipette to remove the supernatant carefully without disturbing the beads.

8.25. Keeping the tubes on the separation device, add 200 µL of freshly made 80% ethanol to the beads to wash without disturbing the beads. Wait for 30 s before removing the 80% ethanol. Repeat this step.

8.26. Briefly, spin down the tubes and place the tubes back on the separation device. Remove any residual 80% ethanol without disturbing the beads.

8.27. Let the tubes air dry with the caps open for 5 min. Do not let sit longer as the beads will overdry. Pipette 20 µL of Tris Buffer to the dried pellet. Remove the tube from the separation device and mix thoroughly with a pipet to resuspend the beads. Let it incubate at room temperature for 5 min.

8.28. Place the tubes on the separation device for 2 min, or until solution becomes clear. Acquire the supernatant and transfer it to new tubes. Then store the collected solution at -20 °C.

8.29. Check the final libraries need for quality and quantity control. Pool the samples, clustered on and sequence, as paired-end 50 nt reads, according to manufacturer's recommended protocols.

REPRESENTATIVE RESULTS:

Our laboratory has generated a genetically engineered mouse models (GEMMs) using the sleeping beauty transposase system (**Figure 1A**). This system incorporates specific genetic alterations into the genome of neural progenitor cells in neonatal mice. These altered progenitor cells form endogenous glioma tumors. Plasmid sequences used to generate the tumors were: (1) pT2C-LucPGK-SB100X for Sleeping Beauty transposon & luciferase expression, (2) pT2-NRASSV12 for NRAS expression, (3) pT2-shp53-GFP4 for p53 knock-down and GFP protein expression, and (4) pT2-shATRx-GFP4 for ATRX knock-down. Plasmids were injected into the lateral ventricle of 1-day old neonatal pups as described previously[11]. Plasmid uptake and tumor formation was

monitored via in vivo bioluminescence imaging system. Once tumor-bearing mice displayed signs of tumor burden, they were sacrificed. Tumors were either used to generate neurosphere cultures or directly cryo-preserved for LMD processing (**Figure 1A**).

Cell cultures started from the GEMM were used to generate a translatable glioma model (**Figure 1B**). Glioma neurospheres derived from GEMM tumors were cultured and implanted intracranially into the striatum of immune-competent mice. Single cell suspensions obtained from neurospheres culture were used to generate glioma tumors by intracranial implantation as described before by our laboratory[10-12].This methodology allows the careful quantification of the number of cells to be implanted per mouse (30,000 cells/1 µL/mouse). This protocol permits the reproducibility of the results between different experimental implantations. However, implantation of neurospheres could be an alternative option to generate mouse glioma tumors and subsequent LMD and RNA-Seq analysis. Nevertheless, this method is considered to be more accurate. Tumor progression was monitored by in vivo bioluminescence spectrum imaging system. Mice displaying signs of tumor burden were transcardially perfused and the brain was cryo-preserved for LMD processing (**Figure 1C**).

Tissue sections were laser microdissected to characterize the transcriptome of multicellular structures within gliomas. The perfusion, freezing and embedding procedures described were optimized to preserve tissue morphology and obtain good quality RNA after laser microdissection. Different perfusion approaches were evaluated in order to acquire tissues with superior morphology and RNA integrity (**Table 10**). To dissect the areas of interest, it was necessary to stain the tissue with innocuous dyes for RNA (**Figure 2**). We observed that perfusing tumor bearing mouse with Tyrode's solution for 5 min, and then 30% sucrose for 15 min, followed by overnight storage of the dissected brain in 30% sucrose preserves morphology and RNA integrity of the tumor tissue (**Figure 3D**). Perfusion with 30% sucrose solution prevented ice-crystal formation within the tissue. Although, paraformaldehyde tissue fixation resulted in high quality tissue morphology, RNA integrity was negatively affected (**Figure 3B**). Other approaches such as Tyrode's solution for 5 min or Tyrode's solution for 5 min + 30% sucrose solution for 15 min (**Figure 3A and 3C**) did not affect the RNA quality. Under these conditions, brains were not preserved in 30% sucrose overnight; we observed reduced resolution in tissue morphology.

We performed various staining techniques followed by RNA integrity quality control analysis. We observed that 4% Cresyl violet and 0.5% eosin Y staining was sufficient to identify glioma multicellular structures and maintain RNA integrity. Cresyl violet is an acidophilic dye that stains the nucleus of cells with a dark blue color. Eosin Y is a basophilic dye that stains basic components of the cells.

We observed that if the tissue was not mounted with mounting medium, the sections became dehydrated and the morphology deteriorated (**Figure 4A**). To maintain high quality tissue morphology, we mounted the tissue with mounting medium. We observed that 15% mounting medium dissolved in water (30 µL in 200 µL of water) maintained high quality tissue morphology (**Figure 4B**).

In **Figure 5A**, areas of glioma heterogeneity are shown. Images were acquired using a laser capture microdissection microscope preceding dissection. Red lines depict areas with abundant mesenchymal cells (elongated cells). Blue lines depict areas with no mesenchymal cells (rounded cells) (**Figure 5A, middle image**). **Figure 5B** shows images of laser micro-dissected tumor areas (red lines) and normal brain tissue areas (blue lines). ROI selection is made to dissect areas of interest (**Figure 5A and 5B**, **lower images**). We can observe in these images the success in the dissection of the selected areas.

RNA extraction was performed using a commercial kit. It was determined that a total area of dissected tissue between 2.5 x 10^6 – 7 x 10^6 μm^2 was required for mRNA extraction and cDNA library preparation for subsequent RNA sequencing. RNA quality control determined a RIN between 6 to 7 in glioma tissue after laser microdissection. A RIN of 6 was determined to be appropriate for cDNA library preparation. Following RNA extraction, a kit for RNA isolation at picomolar concentrations was utilized to generate a cDNA library suitable for next generation sequencing.

FIGURE AND TABLE LEGENDS:

Table 1: RNA quality control and library preparation: Master Mix preparation for step 8.3

Table 2: RNA quality control and library preparation: Master Mix preparation for step 8.5

Table 3: RNA quality control and library preparation: Thermocycler conditions for step 8.5

Table 4: RNA quality control and library preparation: Master Mix preparation for step 8.6

Table 5: RNA quality control and library preparation: Thermocycler conditions for step 8.6

Table 6: RNA quality control and library preparation: Master Mix preparation for step 8.18

Table 7: RNA quality control and library preparation: Thermocycler conditions for step 8.20

Table 8: RNA quality control and library preparation: Master Mix preparation for step 8.21

Table 9: RNA quality control and library preparation: Thermocycler conditions for step 8.21

Table 10: Methods for different perfusion approaches.

Figure 1: Mouse glioma models used for laser microdissection. (**A**) Sleeping beauty GEMM used to develop de novo glioma. Images display tumor progression: (i) plasmid injection into the lateral ventricle of neonates, (ii) tumor burden. (**B**) Generation of transplantable glioma model using glioma cell cultures. Neurosphere cells cultured from GEMM are implanted intracranially into the striatum. (**C**) Depiction of cryo-sectioning of the brain embedded in coronal orientation following perfusion.

Figure 2: Schematic representation of the method used to stain cryo-preserved tissue sections with Cresyl violet and Eosin. Slides used for staining were handled with tools cleaned with RNase-free water. All solutions were prepared with RNase/DNase free water on day of staining. Cresyl violet stains nuclei violet and eosin Y stains cytoplasm pink. Dyes were dissolved in 70% ethanol.

Figure 3: Comparison of various perfusion approaches to preserve tissue morphology and RNA integrity. H&E images represent comparative morphology of brain tissue that was subjected to perfusion under different methods: (**A**) Tyrode's solution for 15 min, (**B**) Tyrode's solution for 5 min, 4% PFA for 10 min, (**C**) Tyrode's solution for 5 min, 30% sucrose for 15 min, and (**D**) Tyrode's solution for 5 min, 30% sucrose for 15 min with overnight immersion in 30% sucrose. RNA quality assessment is displayed for each perfusion method. Plots depict 18s and 28s rRNA peaks and the initial marker peak. The ratio of 18s and 28s rRNA is used to determine RNA quality. A gel image of the RNA fragments is displayed to the right of the plots. RNA quality was assessed using the RIN values. RNA quality was high in methods A, C, and D. Tissue morphology was superior in methods C and D.

Figure 4: Representative images of mounted and non-mounted glioma tissue. (**A**) Representative images of tissue stained with H&E. This tissue was left unmounted and became dehydrated displaying poor morphology with breaks in the tissue. (**B**) Representative images of tissue stained with H&E and mounted with mounting medium.

Figure 5: Representative images of glioma tissue used for laser microdissection. (**A-B**) Representative images of tissue stained with H&E and areas of multicellular structures selected for LMD. (**A**) On the left, areas of elongated cells (red) and control areas (blue) were selected for LMD. (**B**) On the right, areas of collective invasion (red) and control areas (blue) were selected for LMD. (**C**) Representative RNA quality control for laser microdissected areas. A total area of 6.5×10^6 μm^2 was microdissected. The analysis shows RNA concentration of 2,346 pg/µL and RNA Integrity Number (RIN): 6.8.

DISCUSSION:

Understanding the molecular mechanisms underlying glioma heterogeneity and invasion are of critical importance to uncover novel therapeutic targets[13]. In this manuscript, we describe a detailed and optimized method to analyze the molecular landscape of glioma heterogeneity and invasion using laser capture microdissection (LMD) followed by transcriptomic analysis.

Laser capture microdissection (LMD) can be used to identify different areas or single cells within the tumor, providing a specific sample to further analyze the molecular pattern maintaining the spatial context of the tumor[14]. This technique is reliable and low-priced compared with other methods used to analyze the spatial transcriptome in solid tumors[15]. We analyzed glioma heterogeneity and invasion in genetically engineered mouse tumor models or intracranial implantable models using LMD. These models recapitulate the salient characteristics of human gliomas, allowing the study of glioma heterogeneity and invasion[10,12].

Maintaining high-quality tumor tissue morphology and RNA integrity is one of the limitations for LMD. To improve tissue morphology, we perfused mice with Tyrode's solution for 5 minutes, followed by 30% sucrose dissolved in the same solution. Once the brain was dissected, we preserved it in 30% sucrose dissolved in RNase/DNase-free water overnight or until the brain reached the bottom of the storage container. These steps significantly improve the morphology of the tissue and reduced the formation of ice-crystals in the tissue during cryo-preservation. Although human glioma tissue was not used for this protocol, incubation of human samples in 30% sucrose solution overnight could be a feasible methodology to improve cryosections morphology. Another possible limitation for LMD is the preservation of RNA integrity post-staining[8]. Although other research teams have performed laser microdissection on glioma tissue followed by RNA-seq analysis they do not illustrate any RNA and/or morphology nor do they comment on morphological quality, or particular controls for glioma frozen sections[16]. In this protocol accurate morphological identification was essential in order to laser microdissect precise macrocellular structures within gliomas. We observed that fixing glioma tissue with ethanol solution and staining it with 4% Cresyl violet and 0.5% eosin Y dissolved in ethanol-maintained RNA quality and enabled the microscopic identification of single cells and multicellular structures. We demonstrated that mounting glioma sections with mounting solution is a critical step which prevents cracking and fissure formation in the tissue. Please note that the mounting medium needs to be prepared in water, as using the mounting medium dissolved in ethanol will result in poor tissue morphology. Laser microdissection has to be performed as fast as possible in an RNase-free environment. We also recommend to section up to 2.5 x 10^6 μm^2 total tumor/tissue area in order to obtain appropriate amounts of RNA, both for RNA quality control and transcriptomic analysis.

LMD enables the analysis of the molecular signaling pathways that regulate glioma heterogeneity and invasion. This analysis could reveal novel potential targets for diagnosis, prognosis and future translational development in preclinical glioma models.

ACKNOWLEDGMENTS:

Work was supported by National Institutes of Health, NIH/NINDS Grants: R37-NS094804, R21-NS107894 and R01-NS105556 to M.G.C.; NIH/NINDS Grants R01-NS076991, and R01-NS096756 to P.R.L.; NIH/NIBIB: R01-EB022563; NIH/NCI U01CA224160; the Department of Neurosurgery, Rogel Cancer Center at The University of Michigan, ChadTough Foundation, The Pediatric Brain Tumor Foundation, the Smiles for Sophie Forever Foundation and Leah's Happy Hearts Foundation to M.G.C. and P.R.L. University of Michigan, MICHR Postdoctoral Translational Scholars Program, TL1 TR002240-02, Project F049768 to A.C. University of Michigan Forbes Cancer Research Institute, a Physician-Scientist Award from Research to Prevent Blindness, Inc. (RPB), grant R01 EY022633 from the NEI of the NIH (AK), and an unrestricted grant from RPB to the Department of Ophthalmology and Visual Sciences. This research utilized the Vision Research Core (P30 EY007003), and the Cancer Center Research Core (P30 CA046592). AK is supported by the Mrs. William Davidson Emerging Scholar Award from the A. Alfred Taubman Medical Research Institute.

DISCLOSURES:

All authors of this paper declare no potential conflicts of interest.

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Figure 1

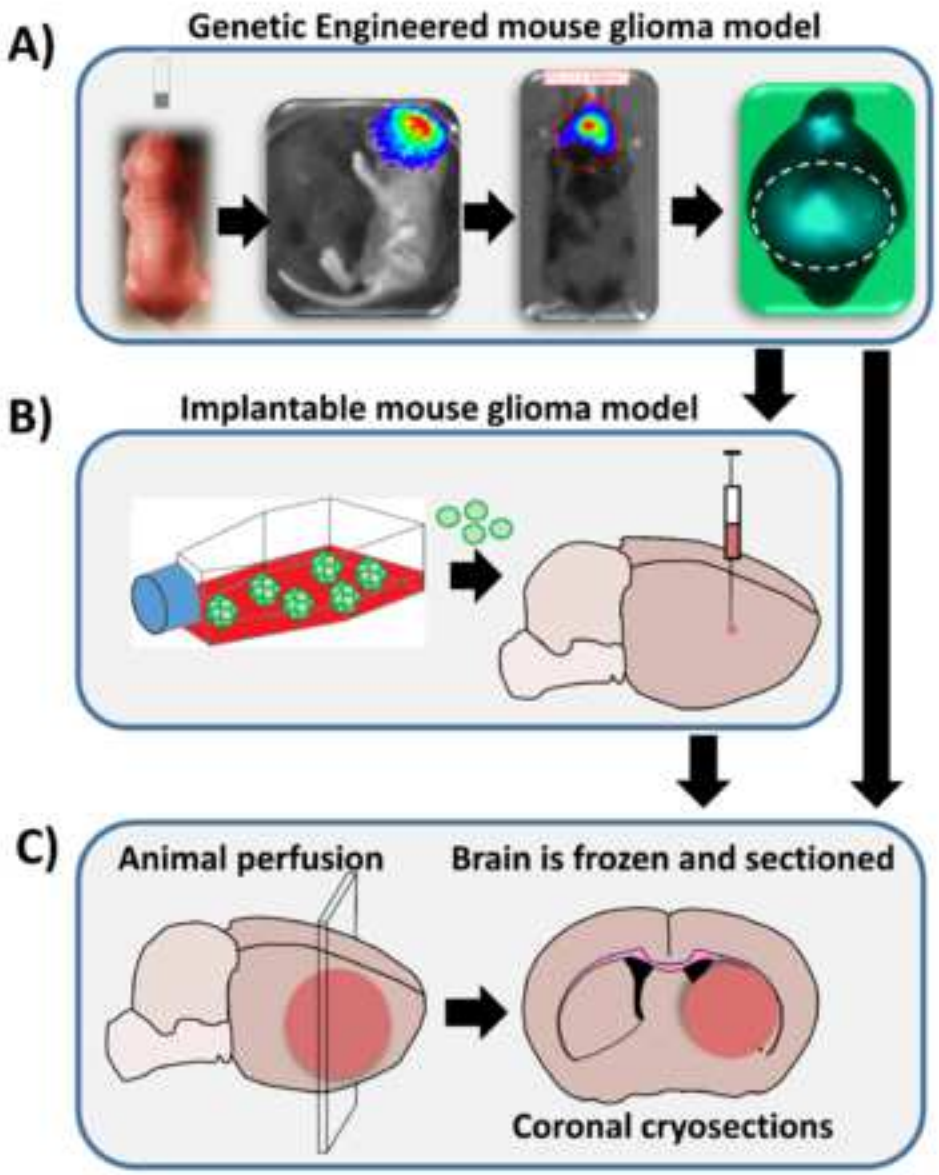

Figure 2

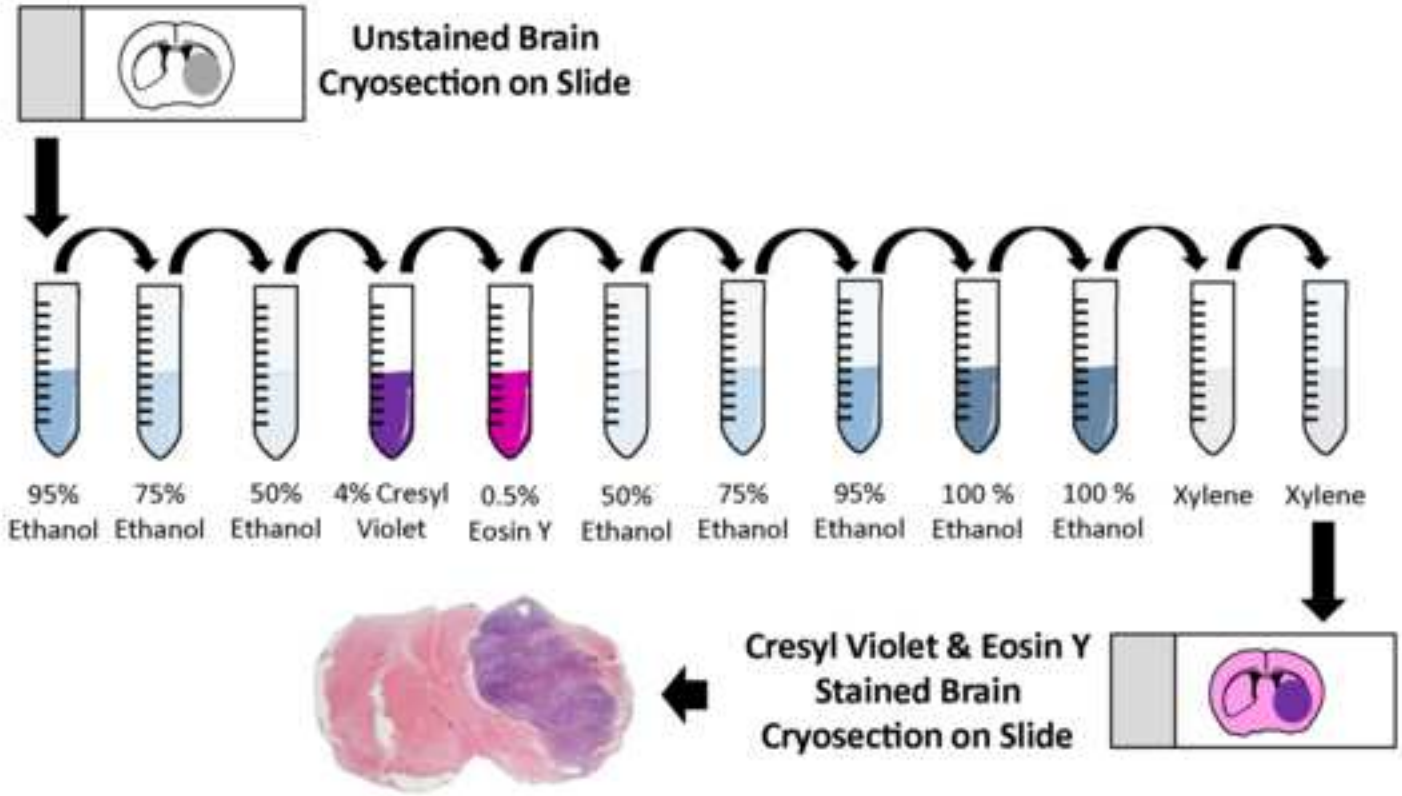

Figure 3

Figure 3

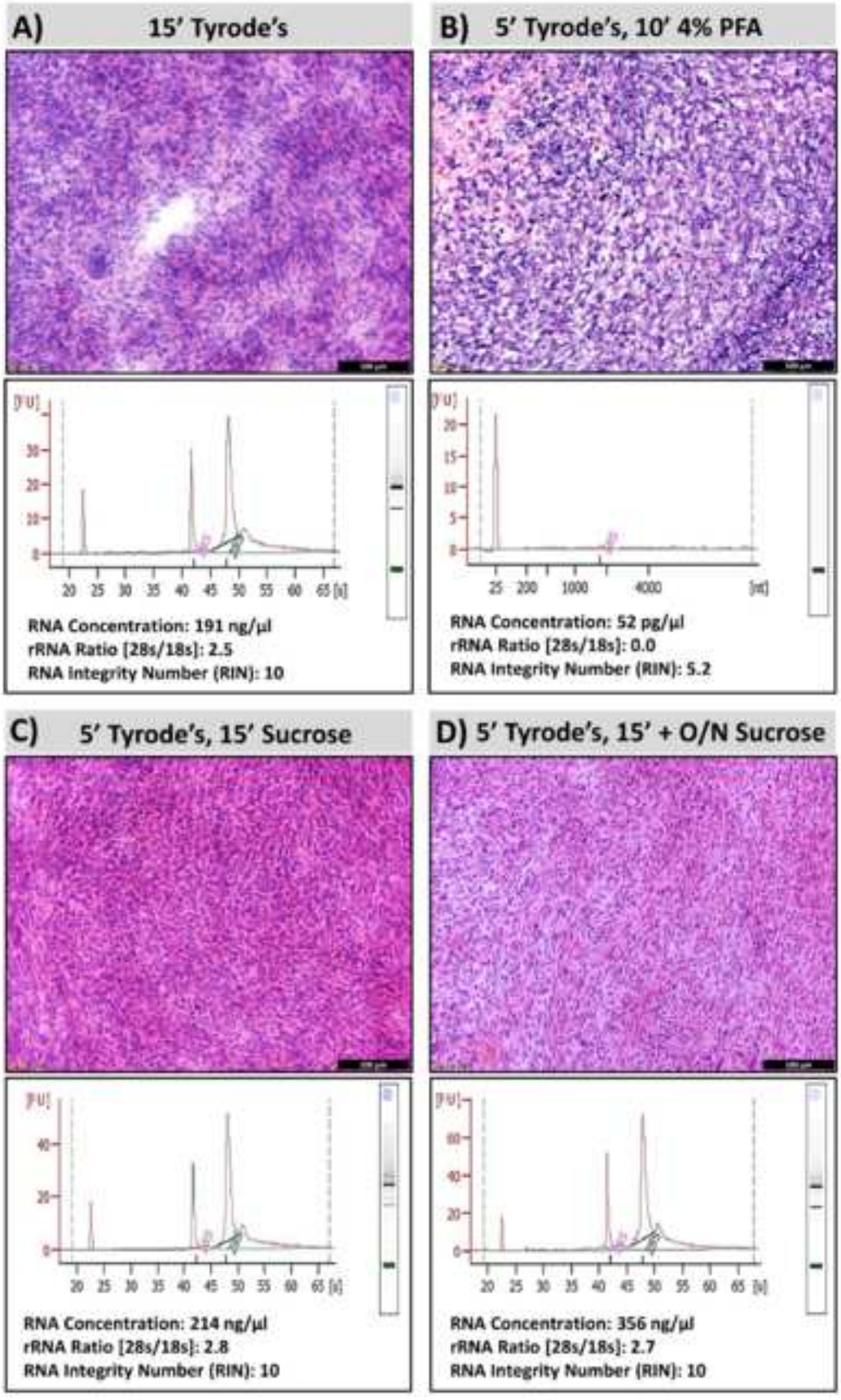

Figure 4

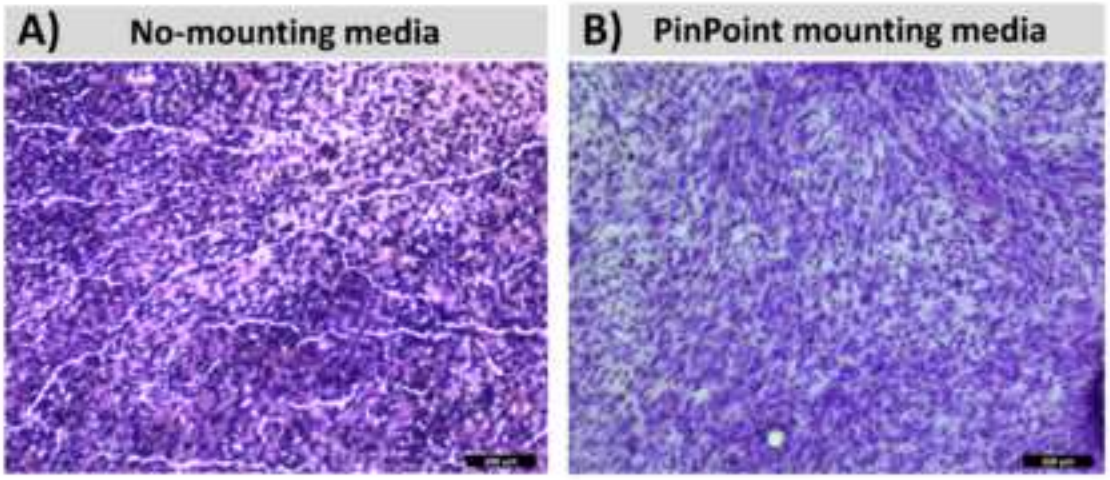

Figure 5

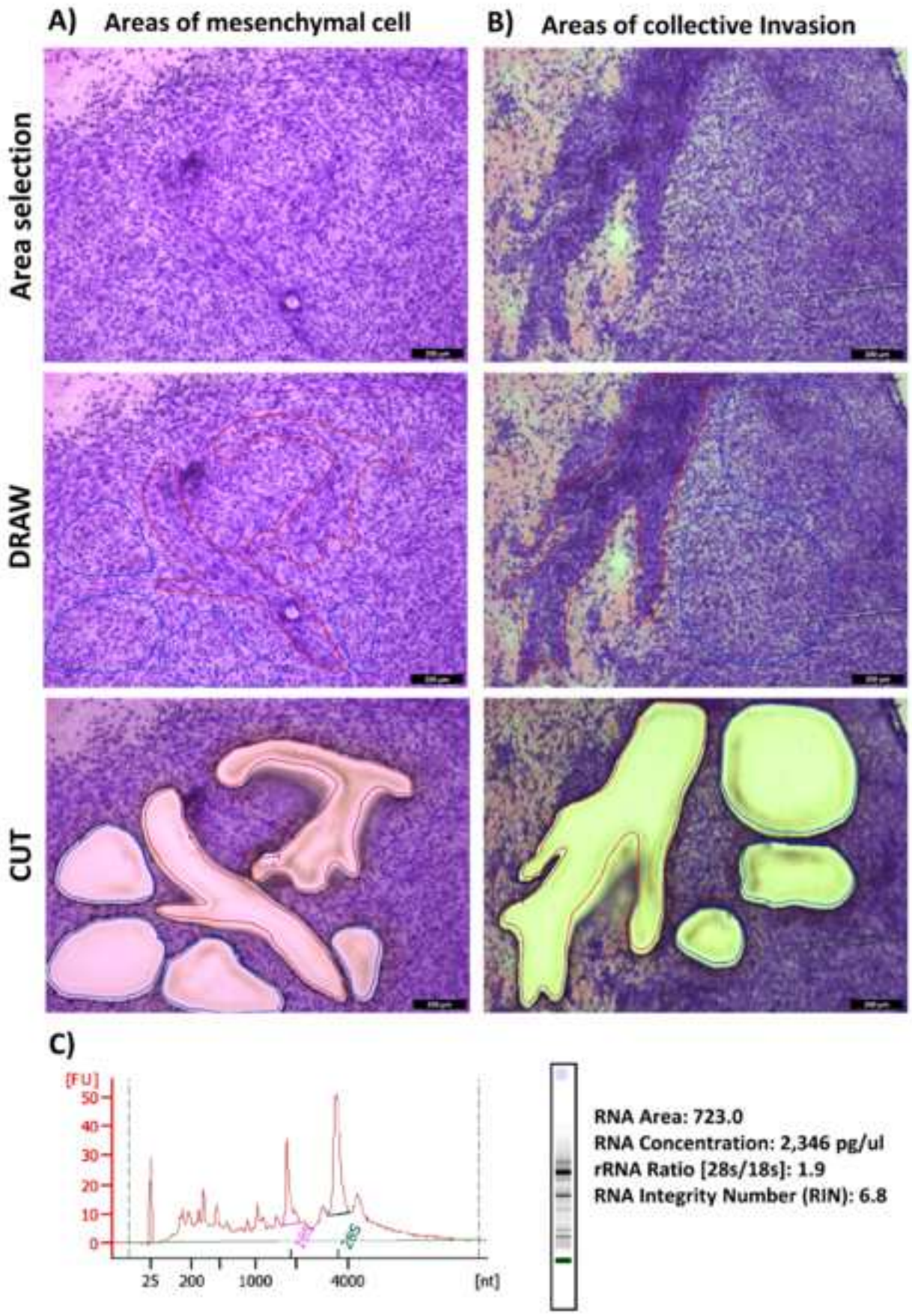

Table 1
0.25 ng - 10 ng (1-8 µL) of RNA
4 µL of the 5x First-Strand Buffer
1 µL of the Oligos Mix V2
Nuclease-free water to final volume of 13 µL

Table 2
4.5 µL Mix V2
0.5 µL RNase inhibitor
2 µL Reverse Transcriptase
Final volume of 7 µL
Add the 7 µL to the RNA sample tube

Table 3
42 °C for 90 min
70 °C for 10 min
4 °C hold

Table 4
2 µL nuclease-free water
25 µL 2x PCR Buffer,
1 µL DNA Polymerase for each reaction
Mix gently and spin down briefly
Add 28 µL of the master mix to each of the sample tubes
Add 1 µL of each 5’ and 3’ primer to each tube.
Mix the reactions gently and spin down briefly

Table 5
1 cycle
94 °C for 1 min
5 cycles
98 °C for 15 s
55 °C for 15 s
68 °C for 30 s
1 cycle
68 °C for 2 min, 4 °C hold

Table 6
16.8 µL nuclease-free water
2.2 µL 10x R Buffer
1.5 µL R v2
1.5 µL R-Probes v2

Table 7
37 °C 60 min
72 °C 10 min
4 °C hold

Table 8
26 µL nuclease-free water
50 µL CB PCR Buffer
2 µL PCR2 Primers v2
2 µL DNA Polymerase

Table 9
1 cycle
94 °C for 1 min
X cycles
98 °C for 15 s
55 °C for 15 s
68 °C for 30 s
1 cycle
68 °C for 2 min, 4 °C hold

Table 10

	Step 1	Step 2	Step 3
Method 1	Tyrode's 15'	--	--
Method 2	Tyrode's 5'	Sucrose 30% 15'	--
Method 3	Tyrode's 5'	4% PFA 10'	--
Method 4	Tyrode's 5'	30% Sucrose 15'	Brain in 30% Sucrose Overnight

Name of Material/ Equipment	Company	Catalog Number
Accutase Cell Detachment Solution	Biolegend	423201
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15
Antibiotic-Antimycotic (100X)	Gibco	15240062
B-27 Supplement (50X), serum free	Gibco	17504044
Buffer RLT	Qiagen	79216
Corning PCR Tubes	Sigma Aldrich	CLS6530
Cresyl Violet Acetate	Sigma Aldrich	C5042
DMEM/F12 - Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12	Gibco	11330057
Eosin Y	Sigma Aldrich	E4009
HiSeq 4000	Illumina	N/A
Laser Microdissection (LMD) System	Leica	LMD7000
N-2 Supplement (100X)	Gibco	17502048
Normocin - Antimicrobial Reagent	Invivogen	ant-nr-1
Peel Away Disposable Embedding Molds	Electron Microscopy Sciences	70182
PEN Membrane Glass Slide (2 µm)	Lieca	1150518
Pinpoint Solution	Zymo Research	D3001-1
Recombinant Human FGF-basic	Peprotech	100-18B-1MG
Research Cryostat	Leica	CM3050s
RNaseZap RNase Decontamination Solution	Fisher Scientific	AM9780
RNeasy Plus Micro Kit	Qiagen	74034
SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian	Takara Bio	634411
Tissue-Plus O.C.T. Compound	Fisher Scientific	23-730-571

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November 28, 2019

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Vineeta,

Thank you for your thorough review of our manuscript, entitled: **"**LASER CAPTURE MICRODISSECTION OF GLIOMA SUBREGIONS FOR SPATIAL AND MOLECULAR CHARACTERIZATION OF INTRATUMORAL HETEROGENEITY, ONCOSTREAMS AND INVASION**",** by Drs. A. Comba, P. Dunn, P.E. Kish, P. Kadiyala, A. Kahana, M.G. Castro, and P.R. Lowenstein.

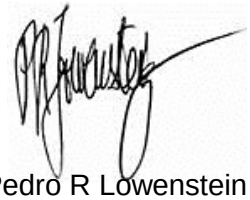
We appreciated the insightful suggestions from the Editor and each of the reviewers, and our revised manuscript takes into account these comments. We include below an itemized list of detailed responses to each of the reviewers' and Editor's comments. **We would like to request that the revised version of our manuscript be sent to the same initial reviewers who reviewed our paper.**

We look forward to future correspondence in regards to our manuscript for publication in JoVE.

Thank you for your consideration.

Looking forward to hearing from you at your eraliest convenience.

Yours sincerely,



Pedro R Lowenstein

We appreciated the comments from editor and each reviewer and have included an itemized response to each comment below (reviewer's comments italicized).

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Answer: We checked the manuscript and corrected *spelling and grammar issues.*

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.
Answer: *We formatted the revised manuscript as requested by the editor.*

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Accutase, IVIS® 174 Spectrum In Vivo Imaging System (Perkin Elmer, USA), RNaseZap®, Leica LMD7000 microscope, RLT lysis buffer (Qiagen), RNeasy Plus Micro Kit (QIAGEN), RNeasy MinElute, RW1, RPE, (Clontech/Takara), SMART Pico Oligos Mix V2, SMARTScribe Reverse Transcriptase, MART TSO Mix V2, SeqAmp CB, AMPure beads, R-Probes V2 and ZapR V2 Buffer, Illumina Sequencing platforms (Kapa Biosystems, 485 Wilmington MA), TapeStation, Kapa's library, etc
Answer: *As specified by the editor we removed in the revised manuscript any commercial language.*

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Answer: *We edited the text in protocol section to ensure that the imperative tense is used in all sentences*.

5. The Protocol should contain only action items that direct the reader to do something.
Answer: *The revised protocol section of the manuscript now contains only action items.*

6. Please ensure that individual steps of the protocol contain only 2-3 actions per step.
Answer: *We edited the text to ensure that each individual step in the protocol section contain no more than 3 actions.*

7. Please ensure that all steps in the protocol are numbered action steps.
Answer: *We confirm that all steps in the protocol section are numbered.*

8. Please make tables for all the master mixes and thermocycler conditions and refer to the tables wherever applicable. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
Answer: All master mixes and thermocycler conditions have been included as tables in the revised manuscript.

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential

steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Answer: We highlighted in yellow until 2.75 of the protocol to identify the critical steps for the video.

10. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
Answer: In the revised manuscript we include all figure legends at the end of the Representative results section.

11. Please do not abbreviate the journal titles in the references section.
Answer: We incorporated the full names of journal titles in the references section of the revised manuscript.

12. Please sort the materials table in alphabetical order. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Answer: We addressed all these changes in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this manuscript, the authors describe the method to generate a high quality of RNA dissected from glioma tissue using laser capture microdissection for subsequent RNAseq. The manuscript is timely and of high importance, as the method will allow for the spatial and molecular analysis of glioma tissue known to be highly heterogeneous. The manuscript is well written and with great attention to details, which would allow the readers to follow and reproduce the protocol closely. I have only a few minor comments described below.

Major Concerns:
none

Minor Concerns:

1.2. Please mention that GEMM-derived cell lines express firefly luciferase or GFP as shortly after that you describe monitoring tumor growth by BLI.
Answer: As requested by this reviewer, we describe now in Protocol Section 1 that GEMM-derived cell lines express firefly luciferase and GFP proteins. These features enable further tumor growth analysis using an *in vivo* imaging system and/or tumor localization and dissection using a fluorescence stereo-zoom microscope.

1.3. Since you giving volumes for B27 and N2 instead of concentration, please mention stock of B27(50x) and N2(100x).
Answer: As per the reviewer's suggestion, we have added this information in the revised manuscript (please see section 1.2).

6.6. *This line seems out of place as tissue is already fixed and you describe it earlier.*
Answer: As per the reviewer's suggestion, we have removed this line (previous section 6.6) in the revised manuscript.

7.4.-7.12. I am wondering if it is necessary to write here the manufacturer's protocol for RNA isolation unless you introduce modifications to this protocol.
Answer: The manufacturer's protocol for RNA isolation contains various options, such as samples lysis, homogenization, use of carrier RNA etc. We have selected and adapted manufacturer's protocol to

achieve optimum results in RNA yield and purity for our experimental model. Therefore, we would prefer to keep the described steps for RNA isolation from laser microdissected glioma tissue.

Line 548: please correct um2 to µm2
Answer: We have changed *um^2 to μm^2* in the revised manuscript.

8.23. What is step 18 you mention here? Is it 8.18?
Answer: Number 18 mentioned in section 8.23 has been now changed to 8.21.

It might be interesting to readers to hear authors' opinion about adopting this protocol to human GBM (by incubating samples overnight in 30% sucrose solution?) knowing that it is not possible to perfuse human GBM resection tissue.
Answer: We agree with the reviewer that this protocol could be adapted for human GBM samples incubating tumor tissue overnight in 30% sucrose. This is an interesting proposal that will be addressed in future experimental procedures using human samples. This consideration was incorporated into the discussion section of the revised manuscript.

Reviewer #2:

Manuscript Summary:

In this article, Comba A. and collaborators describe a method for extracting and characterizing sub-regions of glioblastoma without damaging RNA quality, for further RNAseq. This is an interesting and very well described article. Future readers/experimentators will find easily all necessary details for reproducing these experiments.

I recommend accepting this article after few changes.

Major Concerns:
No major concerns.

Minor Concerns:
The authors could discuss several steps of their protocol.

1/ Cells are cultures as neurospheres, some teams are directly implanting neuropheres into mouse brains for generating tumors. Can the authors explain why they are not implanting neuropheres with adapted syringe?
Answer: Herein we describe the methods utilized in our laboratory. In our laboratory we generate glioma tumors by intracranial implantation of dissociated single cells obtained from neurospheres culture as was also described before by our laboratory in several publications (A. Calinescu et al, FJ. Nunez et al, C. Koshman et al). This methodology allows the careful quantification of the number of cells to be implanted per mouse (30.000 cells/1 ul/ mouse). This protocol permits the reproducibility of the results between different experimental implantations. However, implantation of neurospheres could be an alternative option to generate mouse glioma tumors and subsequent LMD and RNA-Seq analysis. Nevertheless, we believe our method to be more accurate.

2/ Other teams working on glioblastoma described LCM followed by RNAseq without all preservative steps, without major decrease of RNA quality. Can it be discussed?
Answer: In this manuscript we describe a protocol for LMD of frozen glioma tissue using an integrated approach, which allows to obtain reliably good tissue morphology and a high RNA yield and quality. Although other research teams have performed laser microdissection on glioma tissue followed by RNA-seq analysis they do not illustrate any RNA and/or morphology, nor do they comment on morphological quality, or particular controls for glioma frozen sections. Please note that in our case accurate morphological identification was essential in order to laser microdissect precise macrocellular structures

within gliomas. In this manuscript, we also discuss the difficulties observed by us and other groups regarding tissue fixation, staining and mounting. These considerations have now been included into the discussion section in the revised manuscript.

3/ The microdissection of tumor tissues induces the extraction of both tumoral and stromal compartments. Can the authors discuss about this limitation?
Answer: We agree with the reviewer suggestion. This limitation has now been included into the revised manuscript in the discussion section. As is the case in other tumors, i.e., pancreatic cancer, in glioma tumors it remains impossible to separate glioma tumors, from the stromal microenvironment.

Reviewer #3:

Manuscript Summary:
Authors provide a very interesting and detailed methodology to allow for a genetic understanding of intratumoral heterogeneity, which is particularly relevant for GBM.

Major Concerns:
None

Minor edits to consider below:

Abstract: the meaning of "oncostreams" in abstract is not clear. It would appear these methods can be used for a variety of other applications beyond this stated hypothesis involving oncostreams.
Answer: Oncostreams represent areas of mesenchymal transformation within glioma tissue. They are an example of a multicellular structure and glioma heterogeneity. We believe oncostreams have particular functions within gliomas, as our data suggest they participate in glioma growth and invasion. In particular, the technique described herein, has allowed us to demonstrate that oncostreams are molecularly distinct. This methodology could be used to analyze glioma heterogeneity by dissecting different particular neuropathological areas, such as pseudopalisades, areas of microvascular proliferation, or necrosis. An expanded explanation was included in the revised manuscript.

Page 2, line 67: ice crystals should be and 'the' cell membrane
Answer: We have changed *ice crystals to ice crystal* and we incorporated ‘the’ cell membrane in line of the revised manuscript.

Line 71: ice crystals
Answer: We have changed *ice crystal to ice crystals* of the revised manuscript.

Line 207: grammar
Answer:

Line 208: cool
Answer: We have changed *cold to cool* in the revised manuscript.

Relevance of yellow highlighting in text is unclear.
Answer: Highlighted text is a requirement of the Journal to identifies the essential steps of the protocol for the video.