Journal of Visualized Experiments

Automated Dissection Protocol for Tumor Enrichment in Low Tumor Content Tissues

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62394R1
Full Title:	Automated Dissection Protocol for Tumor Enrichment in Low Tumor Content Tissues
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Additional Information:	
Question	**Response**
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TITLE:

Automated Dissection Protocol for Tumor Enrichment in Low Tumor Content Tissues

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KEYWORDS:

automated dissection, next-generation sequencing, tumor enrichment, low tumor content

SUMMARY:

Digital annotation with automated tissue dissection provides an innovative approach to enriching tumor in low tumor content cases and is adaptable to both paraffin and frozen tissue types. The described workflow improves accuracy, reproducibility and throughput and could be applied to both research and clinical settings.

ABSTRACT:

Tumor enrichment in low tumor content tissues, those below 20% tumor content depending on the method, is required to generate quality data reproducibly with many downstream assays such as next generation sequencing. Automated tissue dissection is a new methodology that automates and improves tumor enrichment in these common, low tumor content tissues by decreasing the user-dependent imprecision of traditional macro-dissection and time, cost, and expertise limitations of laser capture microdissection by using digital image annotation overlay onto unstained slides. Here, digital hematoxylin and eosin (H&E) annotations are used to target small tumor areas using a blade that is 250 μm^2 in diameter in unstained formalin fixed paraffin embedded (FFPE) or fresh frozen sections up to 20 µm in thickness for automated tumor enrichment prior to nucleic acid extraction and whole exome sequencing (WES). Automated dissection can harvest annotated regions in low tumor content tissues from single or multiple sections for nucleic acid extraction. It also allows for capture of extensive pre- and post-harvest collection metrics while improving accuracy, reproducibility, and increasing throughput with utilization of fewer slides. The described protocol enables digital annotation with automated dissection on animal and/or human FFPE or fresh frozen tissues with low tumor content and could also be used for any region of interest enrichment to boost adequacy for downstream sequencing applications in clinical or research workflows.

INTRODUCTION:

Next generation sequencing (NGS) is increasingly utilized for both patient care and in cancer research to help guide treatments and facilitate scientific discovery. Tissue is often limited and small specimens with variable tumor content are routinely used. Tumor adequacy and integrity, therefore, remain a barrier to obtaining meaningful data. Samples with lower tumor percentages may cause difficulty in distinguishing true variants from sequencing artifacts and are often ineligible for NGS[1]. Tumor enrichment of low tumor content cases, those below 20%, has been shown to help yield sufficient material in order to generate reproducible sequencing data and ensure low frequency variants are not missed[2,3]. However, limits will vary depending on the platforms utilized and planned use of the data generated.

Traditionally, enrichment of tumor regions for extraction is performed by manual macrodissection or laser capture microdissection (LCM) of formalin fixed paraffin embedded (FFPE) slides. Manual macrodissection, or scraping specified tissue areas from slides, allows tumor regions to be removed for use in downstream assays with relatively low cost, but with low accuracy and low precision[2,4]. Minimal technical accuracy can be very effective with higher tumor content cases where large swaths of tumor are present and/or minimal tissue loss does not significantly impact results, but low tumor content cases or cases with more dispersed tumor require increased precision. LCM was therefore invented in the 1990s and became a valuable way to precisely remove small, defined, microscopic regions of tissue from formalin fixed paraffin embedded (FFPE) slides[5–8]. LCM can be utilized to collect single cell populations when complex heterogeneity of the sample exists[9] allowing for collection of previously difficult to separate cell populations. However, LCM requires costly machinery that requires extensive technical expertise and hands-on time[10–14].

The instrument used for automated tissue dissection has precision in between that of LCM (~10 µm) and macrodissections (~1 mm)[15]. Additionally, it exhibits both cost and technical expertise requirements between that of macrodissection and LCM and is designed to perform rapid tissue enrichment from sequential FFPE slides to alleviate the disadvantages of previous methods[15]. Automated dissection in this fashion utilizes digital annotations or on-stage slide reference image overlays onto serially sectioned unstained tissue slides for dissecting and enriching regions of interest. The instrument uses plastic spinning blade milling tips, 1.5 mL collection tubes and can be used with a number of different fluids for dissection to collect regions of interest for downstream assays inclusive of nucleic extraction and sequencing. The spinning plastic milling tip utilizes inner and outer syringe barrel reservoirs and a plunger to collect buffer, then mills and collects tissue[16]. The variable milling tip size diameter (250 µm, 525 µm, 725 µm) can allow for dissection of separate tissue areas for comparison, multifocal regions that can be pooled or individual small areas from single or multiple FFPE slides. Section thicknesses used for harvest can be adjusted based on individual experiment needs and users can ensure regions of interest have not been depleted by performing an additional H&E on one serial section immediately after the last section used for harvest.

Automated dissection was identified as a way to enrich tumor content in low tumor content cases and tested and expanded the intended functionality of an automated tissue dissection instrument, which is currently marketed for use on FFPE clinical specimens up to 10 µm in thickness. The work shows that automated dissection can be applied to both FFPE and fresh frozen human or animal tissue sections up to 20 µm in thickness for research purposes. The protocol also demonstrates an approach to digitally annotate and automate dissection for tumor enrichment in tissues with low tumor content and/or cases with nested, dispersed tumor where meaningful macrodissection is challenging or not feasible and show both quality and yield of nucleic acid sufficient for NGS. Automated dissection can therefore provide mid-level precision and increased throughput for tumor enrichment and could also be applied to enrich other regions of interest or combined with other platforms to answer research or clinical questions.

PROTOCOL:

Prior to initiation, obtain appropriate tissue specimens according to Institutional Review Board (IRB) protocols. All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Genentech, Inc.

1. Tissue and slide preparation

1.1. Select FFPE or fresh frozen tissue blocks and utilize the corresponding processing method below.

1.2. Cut tissue block sections onto positively charged glass slides at the desired thickness. Serially section the FFPE tissue in ribbons with the first reference section cut at a thickness appropriate for H&E staining (i.e., 4 µm) followed by 1–4 sections at a thickness ranging from 4–

20 µm based on the need and tissue availability. Collect the tissue sections onto positively charged glass microscope slides.

NOTE: Fresh frozen reference tissue sections should be stained immediately with Hematoxylin and Eosin (H&E) using routine protocols for frozen sections and the unstained frozen sections held at -20 °C until they are needed for harvest.

1.3. Allow all the FFPE sections to dry at room temperature overnight.

1.4. Bake the FFPE reference slides at 60 °C for 30 min and then stain with H&E using routine protocols.

1.5. Scan the H&E stained slides on a whole slide imager at 20x magnification or greater.

1.6. Annotate the scanned slide images for tumor regions of interest using a vendor provided viewing platform or open-source viewer. Export these annotations as either a low-magnification screenshot or save them as a metadata file containing X-Y pixel coordinates corresponding to polygon vertices.

NOTE: The former is less technically challenging to work with, but the latter offers advantages in process automation.

1.7. Create digital masks of the annotated regions of interest in line with the approach used and export the manual annotations.

NOTE: If a screenshot/image of the annotations is used, simple image processing software can be used to select a region and to fill in the entire selection. Using X-Y coordinates for each ROI requires the use of a programming language to read both the image data and polygon coordinates to create a low-mag image with filled in regions of interest. The user should work with the automated dissection instrument vendor to establish a process based on their individual software availability and needs. If scanning, digital slide annotation and/or digital mask creation is unavailable, careful on-slide annotation using a marker can be performed and used in place of a digital mask as a reference image. Pseudocode for digital mask creation has been provided in **Supplementary File 1**.

2. Automated tissue dissection

2.1. Place the unstained sample tissue slides onto the stage in the first through fourth slide positions when using digital slide reference. When using on-slide annotation rather than a digital option, place the unstained sample tissue slides onto the stage in the second through fourth slide positions with a reference slide in the first position.

2.2. Create a milling job using the automated tissue dissection software: **Job Selection** > **Create New Job** > **Case ID** > **Name** the milling job; go to **Thickness** > **Section Thickness** using the

up or down arrow tab; then go to **Tissue Preparation** > **Paraffinized** or **Deparaffinized**, **Reference Image** > **From File** > **Import Image** > **File** to import from the dropdown as the digital reference, if applicable. Select **From Stage** for on stage slide reference. When fields are complete, scan the stage by selecting the **Scan Stage** button in the bottom-right corner to capture each sample tissue slide in the first through fourth position.

2.3. Select the tissue area for image capture.

2.3.1. If using an on-stage reference, drag the box from one corner to the opposite to create a rectangular area over tissue. Select the circular bubble under the rectangular area to capture the stage reference image. If using a digital reference image, overlay the image on the rectangular area selected. Resize and align the digital reference grossly in course zoom to best match the size and position over the sample tissue.

2.3.2. Copy this rectangle field onto remaining sample tissue slides in the second through fourth slide positions by selecting the copy option in the upper-right corner of the reference image. Align and resize grossly as necessary.

NOTE: When using an on-slide reference image rather than a digital mask, select which slide on the stage should be used as the reference.

2.4. Align the reference and sample slides

2.4.1. When the reference image is grossly aligned on tissue sample slides in all slide positions, select the **Scan Stage** button in the lower-right corner of the screen to move into the fine adjustment step. Select the first stage position and **Transform** tool icon (the third icon down in the right-hand toolbar) to make fine alignment and zoom adjustments of the reference to best match the sample slide overlay. Use the **Reference to Sample** sliding bar at the bottom of the screen toggling between reference image and sample image along with the **Zoom In** and **Zoom Out** feature to adjust and achieve alignment of each slide position. Replicate this process in the second through fourth sample slide positions.

2.5. Select the milling area of region of interest

2.5.1. Once optimal sample overlay of each of the four slide positions is achieved, draw milling path designations using the **Color Picker** tool icon (the tenth icon down in the right-hand toolbar) on the colored portion of masked reference image. Select the **Get Annotation(s)** button in lower right to draw milling paths onto sample slides.

2.5.2. Select the milling path in the first slide position.

NOTE: When the milling path is selected in the first slide position, it will be copied onto the remaining slide positions and milling tip usage will be calculated. The milling tip usage in the upper-left corner is calculated based on the area covered and the tip size selected. If more than

four tips are calculated, a larger tip size can be selected to capture the annotated ROI. Tip size can be selected or changed on the left side of the screen under the **Milling Tip** arrow and tip usage will be recalculated.

2.5.3. When the milling path is calculated, collect the annotated ROI with four or less milling tips. Select the **Setup Stage** button in the lower right of the screen to prompt loading of milling tips from placed collection tubes in their proper designation on stage.

2.6. Fill the reservoir with 3.0 mL of the dissection buffer most appropriate for the tissue type (FFPE or fresh frozen) and downstream nucleic acid extraction kit needs and select the **Dissect** button in the lower-right corner of the screen. Use molecular grade mineral oil or an appropriate buffer from commercially available nucleic acid extraction kits.

NOTE: Automated dissection of slides and selected regions of interest then begins and samples are collected by the instrument. The unit head will pick up milling tips from the back of the stage and fill with dissection fluid from the reservoir. Tips then spin along the milling path aspirating sample tissue from slides until complete or full. The collected sample with dissection fluid is then dispensed into collection tubes located at the back of the stage.

2.7. When automated dissection is complete, remove the collection tubes and the dissected sample slides from the stage and place them in a tube rack and slide rack, respectively.

NOTE: Fresh frozen harvests should be taken directly into nucleic acid extraction as per the manufacturer's instructions and post-dissection fresh frozen sections should be H&E stained immediately using routine protocols for frozen sections.

2.8. Bake the post dissected tissue slides at 60 °C for 30 min and then stain with H&E using routine protocols.

2.9. Scan the post dissected H&E stained slides on a whole slide imager at 20x magnification and/or archive for a reference of what tissue was not collected and remains on the slide.

NOTE: See step 1.5 above for alternative scanning options.

3. Nucleic acid extraction

3.1. Pool and pellet the tissue. Perform the nucleic acid extraction using a commercially available kit and following the manufacturer’s instructions.

REPRESENTATIVE RESULTS:

FFPE and FF mouse liver sections containing metastatic colorectal cancer in xenografts were selected. Sections were H&E stained (**Figure 1A,E,I**) and scanned on a whole slide imager at 20x magnification. A pathologist digitally annotated tumor regions of interest and a mask was generated using commercial software and formatted as a digital png reference image (**Figure 1B,**

F,J). Serial 10 µm and 20 µm thick unstained sample slides were placed on the stage and automated dissection was performed as described above. Fresh frozen tissues were collected in a lysis buffer from a commercially available kit and carried directly into nucleic acid extraction following the manufacturer's instructions. FFPE samples were collected using molecular grade mineral oil and dissected samples were pooled together and centrifuged at 25,000 x *g* for 20 min at 4 °C. The supernatant was removed and the minimal mineral oil required was used to resuspend, transfer, and collect the dissected tissue into a single collection tube for each sample appropriately. Samples were shipped at room temperature to a vendor for nucleic acid extraction, RNA and DNA sizing, quantity, integrity, and purity determination following the manufacturer's instructions. Sequencing libraries were created and used for hybridization and capture with commercial options following the manufacturer's instructions. Post dissected sample slides were H&E stained using routine staining protocols to confirm dissection areas in 10 µm (**Figure 1C,G,K**) and 20 µm (**Figure 1D,H,L**) slides and dissection metrics were captured (**Supplementary Table 1**). Exome sequencing generated approximately 75 million 100 bp paired-end reads, yielding an average depth of coverage (before removing duplicate reads) of 150x per sample, with 99.9% reads aligned and a 78% on-target rate. RNA sequencing metrics demonstrated just over 55 million bp paired-end reads, a 98% alignment rate, and a 19.4% duplication rate with 77% concordant reads.

FIGURE AND TABLE LEGENDS:

Figure 1; Successful dissection of tumor nests from fresh frozen and FFPE tissue. H&E stained mouse fresh frozen (**A–D**) and FFPE (**E–L**) liver tissue with colorectal cancer metastasis. 4 µm reference slides used for digital annotation (**A, E, I**) demonstrate examples with a low tumor percent for the total tissue area (**I**) and distributed tumor nests (**E**) that classically present challenges to tumor enrichment. Annotated and digitally masked H&E reference slides (**B, F, J**) were generated and post-dissection 10 µm (**C, G, K**) and 20 µm (**D, H, L**) H&E stained slides demonstrate successful harvest of selected areas.

Supplementary File 1: Pseudocode utilized to create digital masks from annotations to use in automated dissection.

Supplementary Table 1: Example captured metrics from automated dissection and nucleic acid extraction.

DISCUSSION:

Presented here is a protocol for the application of digital annotation and automated dissection to dissect tumor regions from low tumor content FFPE or fresh frozen tissues for tumor enrichment and use in WES. Combining digital annotation and mask creation with automated dissection significantly reduces the required hands-on time and expertise common to classical methods of tumor enrichment inclusive of manual macrodissection and LCM. The protocol demonstrates a potentially important mid-range tumor enrichment option that allows for not only low tumor content enrichment but also enrichment in cases where it is challenging to dissect distributed tumor nests away from the tumor adjacent normal tissue for meaningful tumor

enrichment with high throughput and a moderate level of precision. While the use of our workflow for low tumor content xenograft tissues is demonstrated here, it was also found that this protocol works across tissue types, including human, murine, and xenograft tissues for a variety of normal tissues and cancer indications.

Therefore, it could also apply to a broad range of applications where enriching for specific regions of interest without significant contamination of background tissue would be beneficial (i.e., to enrich for a specific brain region) or even for removal of areas of tissue prior to nucleic acid extraction using classical macrodissection.

Many platforms exist on the market for slide scanning and digital annotation. It is therefore important to remain aware that platform compatibility may present limitations and specified platforms within any protocol may not be broadly available in all laboratories. Therefore, significant efforts were made to provide alternative options within the described protocol that will guide users in making any necessary modifications based on their available resources. An option for removal of the digital annotation component have also been noted to allow for careful manual on-slide annotation. The options provided for modifications will maximize the ability of users to find an option that works with their current platform and software availability.

While digital annotation and automated dissection have been demonstrated to be broadly applied to both FFPE and fresh frozen tissue, it is important to note that the boundaries of the automated tissue dissection instrument have been pushed beyond its intended use with FFPE specimens and the protocol is meant for research use only. Here, successful tumor enrichment was demonstrated through automated tumor dissection of low tumor content FFPE as well as fresh frozen tissues for nucleic acid extraction, WES and RNA sequencing. The protocol shows that xenograft and human tissue regions of interest could be enriched prior to WES and RNA sequencing in basic and translational research settings and also note that other downstream molecular applications, including PCR, from both tissue types would be possible. The protocol expands FFPE automated dissection options and lays the groundwork for fresh frozen tissue automated dissection that could be developed and validated further for use in clinical settings.

ACKNOWLEDGMENTS:

The authors would like to thank Carmina Espiritu and Robin E. Taylor for their support in automated dissection development as well as the Genentech Pathology Core Laboratory staff that supported this work.

DISCLOSURES:

Charles A Havnar, Oliver Zill, Jeff Eastham, Jeffrey Hung, Jennifer Giltnane, Nicolas Lounsbury, Daniel Oreper, Sarajane Saturnio, and Amy A Lo are employees and stockholders of Genentech and Roche and Mana Javey and Emmanuel Naouri are employees and stockholders of Roche.

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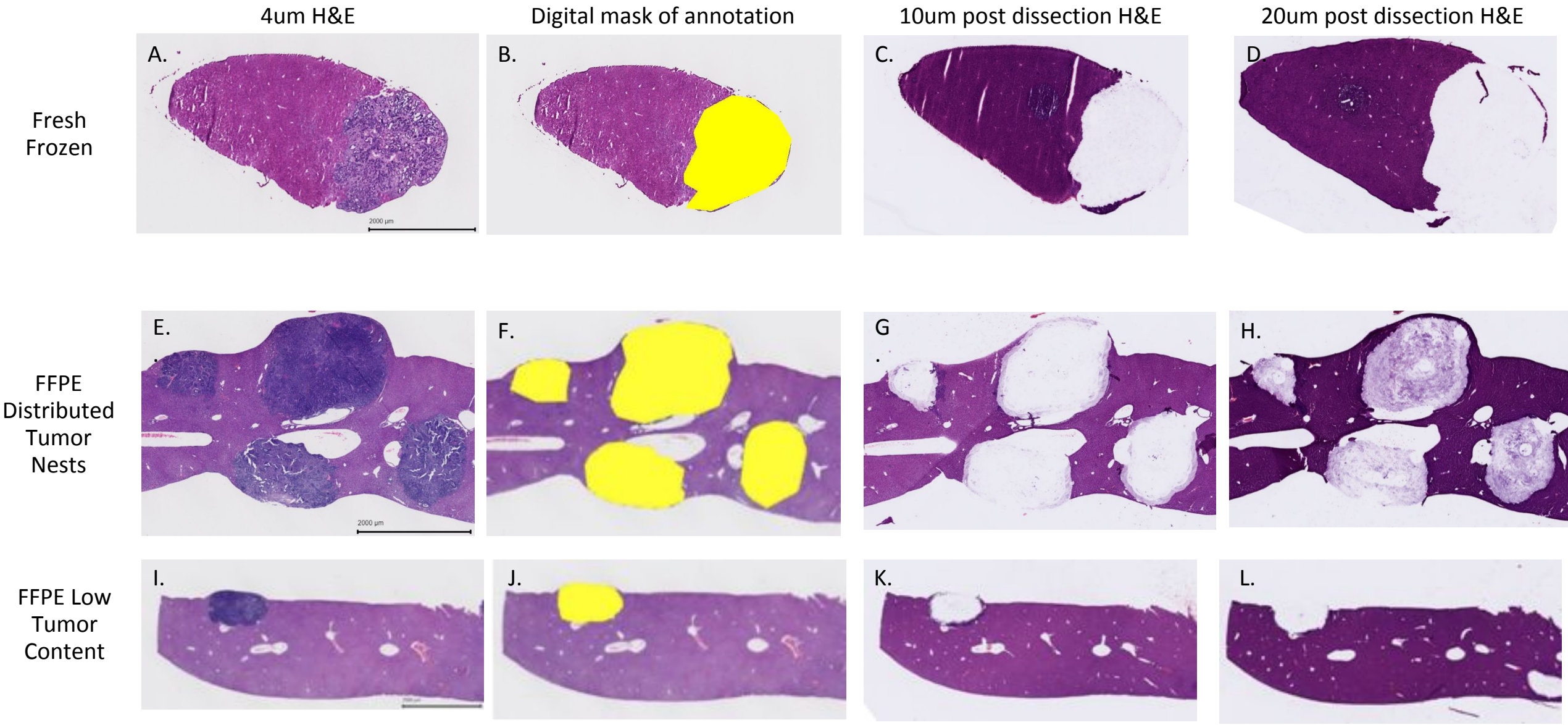
Figure 1



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agilent SureSelectXT	Agilent	G9611A	
AVENIO Millisect Fill Station	Roche	8106533001	
AVENIO Millisect Instrument, Base	Roche	8106568001	
AVENIO Millisect Instrument, Head	Roche	8106550001	
AVENIO Millisect Milling Tips Small	Roche	8106509001	
AVENIO Millisect PC	Roche	8106495001	
BioAnalyzer	Agilent	G2939BA	
Eppendorf 5427R	Eppendorf	22620700	Micro-centrifuge
Incubation Buffer	Promega	D920D	
Leica Autostainer XL	Leica	ST5010	Automated stainer
Molecular Grade Mineral Oil	Sigma	M5904-500ML	
Proteinase K	Promega	V302B	Digestion buffer
Qiagen AllPrep DNA/RNA Mini Kit	Qiagen	80284	
RLT Plus buffer	Qiagen	80204	
Superfrost Plus positively charged microscope slides	Thermo Scientific	6776214	

February 24, 2020

Lyndsay Troyer, Ph.D
Science Editor
JoVE

Dear Dr. Troyer,

Subject: submission of revised manuscript **JoVE62394 titled Automated Tissue Dissection Protocol for Tumor Enrichment in Low Tumor Content Tissues**

Response to Reviewers

Thank you for your email from February 8, 2021 enclosing reviewer comments and for giving us the opportunity to submit a revised draft of our manuscript "Automated Tissue Dissection Protocol for Tumor Enrichment in Low Tumor Content Tissues" for publication in the Journal of Visualized Experiments. We appreciate the time and effort the reviewers dedicated to providing feedback and are appreciative of the insightful comments and valuable suggestions for improvement to our manuscript. We have carefully reviewed the comments and revised the manuscript accordingly. Our responses are given in point by point manner below and also provided in tracked changes with all page numbers referring to the revised manuscript file containing the tracked changes.

Sincerely,

Amy A. Lo, MD, MS
Scientist-Pathologist
Research and Early Development
Department of Pathology
Genentech, Inc.

Point by point responses to reviewers:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author response: Thank you for the suggestion and the opportunity to to make these changes. We have carefully reviewed the manuscript to ensure spelling and grammar are correct with changes noted on lines 35, 37-38, 55, 64-64, 78, 109 and 155.

2. Please rephrase the following sentences to avoid previously published work: Lines 63-66

Author response: We have modified lines 65-70 stating "it is widely accepted that specimens with <10 % tumor are not eligible for NGS because sequencing of samples with lower percentages may cause difficulty in detecting copy number variation and distinguishing true variants from sequencing artifacts[1]" to "Samples with lower tumor percentages may cause difficulty in distinguishing true variants from sequencing artifacts and are often ineligible for NGS."

3. For in-text citations, cite consecutive references (e.g., 10,11,12,13) as (e.g., 10-13). See lines 78, 81, etc.

Author response: Thank you for noticing this citation need, which has been corrected on lines 78 and 83 using a citation manager.

4. Lines 105-107 should be included as a statement at the start of the protocol, and not as a step in the protocol.

Author response: "Prior to initiation, obtain appropriate tissue specimens according to Institutional Review Board (IRB) protocols. All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Genentech, Inc." has been moved to the start of the protocol on lines 121-123 rather than being included as step 1.1. Numbering thereafter has been adjusted accordingly to accommodate this alteration.

5. Line 141, 161: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? If button clicks/menu selections are identified (e.g., quotes or cursive text has been used), change them to bold text. Use either | or > between the clicks/selections, and do not use or other symbols. Example: "File Options Advanced" becomes File > Options > Advanced or File | Options | Advanced

Author response: Thank you for the suggestion. We have expanded details within the protocol and included button click/menu selections on lines 183-189 for step 2.2, line 210 for step 2.4.1, line 242 for step 2.6 and lines 250-255 for step 2.7.

6. Use appropriate SI units. E.g. "60 oC" instead of "60C" (line 120, 122), "4 oC" instead of "4 C" (line 240), etc. Use "mL" instead of "ml" (e.g. line 216). Also include a single space between the quantity and its unit. E.g. "4 mm" instead of "4mm".

Author response: We appreciate you pointing this out and have made changes to correct the degree designations on lines 138, 142 and 264 and the mL designation on line 240 and ensured other quantities and units have a space, appropriately.

7. Please avoid the use of personal pronouns in the protocol. E.g. “we”, our” etc. (e.g., line 248)

Author response: Personal pronouns “we” and “our” have been removed from the protocol in lines 147 and 281.

8. Line 220-221: Please clarify this sentence.

Author response: We appreciate you pointing out the need for improved clarity and we have modified lines 254-264 for step 2.7 to better clarify both our sentence and the process for this step to ensure we are pointing out what the instrument will do as well as what is required from the user. Step 2.6 now includes a note about what to expect when automated dissection begins allowing step 2.7 to focus on the actions required after automated dissection is complete. Step 2.7 has been clarified to now read: “When automated dissection is complete, remove collection tubes and dissected sample slides from the stage and place them in a tube rack and slide rack respectively. Note: Fresh frozen harvests should be taken directly into nucleic acid extraction per the manufacturer's instructions and post-dissection fresh frozen sections should be H&E stained immediately using routine protocols for frozen sections.”

9. 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. E.g. AVENIO Millisect, Superfrost Plus, Leica Autostainer, NanoZoomer, Eppendorf, Qiagen AllPrep etc.

Author response: Thank you for this suggestion. We have taken care to remove all of the commercial language and include commercial specifics in the Table of Materials. Specifically, we have changed “AVENIO Millisect” and “Millisect” to more generic phrasing (“automated tissue dissection instrument”) on lines 43, 87, 108, 183, 255, 342 and 398. We have also removed the commercial language for the slides on lines 132-133, mineral oil and lysis buffer on lines 243-244, slide scanner on lines 280-271, centrifuge on line 338, extraction and sequencing on lines 270-275 and 340-344, nucleic acid metrics on lines 325-326 and image analysis software on line 330.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate the journal name. Please include volume and issue numbers for all references.

Author response: We appreciate the opportunity to change our bibliography format and the references have been reformatted to that which is recommended using a citation manager (lines 424-462).

11. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Author response: The table has been updated, alphabetized and is submitted as an Excel file.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

1. I have been aware of the Millisect instrument since it came out and it's certainly a serious offering in the field and worthy of further published characterization. I have been keen to see results for tumour microdissection as it does appear to be efficient at extracting on the scale required for NGS library input. While it has considerably lower resolution than traditional LCM, extracting hundreds of nanograms of nucleic acids on conventional LCM is an exercise of remarkable tediousness. The description of the Millisect as falling in between LCM and manual dissection is therefore apt and frames the paper well. This manuscript does require a bit of reorganization though. As I understand it, as a JOVE publication should mostly be a method describing how to use an instrument or process in a general manner. This manuscript however also describes a particular experiment the authors performed, and this is mixed in with the general Millisect method in a manner that is confusing. For example there is discussion of FFPE extraction using Qiagen columns in section 3, but that's because that's what was done in the experiment. There are other methods for extraction of FFPE or OCT tissue that could be applied downstream of the Millisect so I would change step 3 to say "extract using Qiagen or Agencourt kits etc following manufacturers' directions".

Author response: We would like to sincerely thank the reviewer for this feedback and agree that separating the experiment we performed and the general automated tissue dissection protocol would help add clarity for the reader and we have taken steps to generalize the protocol whenever possible. We have now used step 3 to instruct the user to pool and pellet tissue and perform nucleic acid extraction using a commercially available kit following the manufacturer instructions (lines 274-275) and included details on how our samples were processed in the representative results section (lines 335-344).

2. Likewise the choice of scanner should be general so don't say to specifically use a NanoZoomer in step 2.9. In the discussion the authors actually state they are trying to make the method as general as possible, so decoupling from specific scanners and specific downstream chemistry is important.

Author response: Thank you for the suggestion. We have modified this to ensure generic scanner terminology is utilized (lines 270-271).

3. I suggest condensing the Millisect method to just the parts that are specific to preparing samples and running the instrument. Then go on to describe the specific experiment, describing the samples, and what was done for extraction, library construction, sequencing and analysis.

Author response: We agree with the reviewer that this would improve the clarity in our protocol and we have limited our protocol to sample preparation and running the instrument (lines 124-273). We have included details on how our samples were processed in the representative results section (lines 335-344).

4. There would desirably be more information about the samples, eg are these xenografts or animal models that developed CRC naturally due to hard living from attending too many genomics conference sponsor parties?

Author response: We have included additional detail highlighting that the tissue we used was xenografts (line 326) and also pointed out to the reader that we have found this protocol to work similarly across tissue types including human, xenograft for a variety of normal tissues and cancer indications (lines 379-381).

5. There should be a better discussion of sequencing analysis metrics in order to be able to declare that the process was indeed a success. For a recent example describing performance metrics in sequencing output from dissection, see PMID: 33135777.

Author response: Thank you for the suggestion. We have included additional summary metrics from our sequencing output in lines 348-352 stating that exome coverage was approximately 75 million 100bp paired-end reads, yielding an average depth (before removing duplicate reads) of 150X per sample with 99.9% reads aligned and a 78% on-target rate. We go on to state that RNA sequencing metrics demonstrated just over 55 million reads, a 98% alignment rate and a 19.4% duplication rate with 77% of successfully paired reads in order to demonstrate both the exome and RNA sequencing quality.

6. I note that the manuscript doesn't go into tumor enrichment, which one might like to see in a paper about a specific experiment, but as this is primarily a method paper for the Millisect that is OK. But as such a paper, it would be desirable to have results for the recovery of nucleic acids per unit volume (eg ng/mm3) of sample dissected and other performance metrics like how long it typically took for a dissection.

Author response: We thank the reviewer for this suggestion and have modified our table to include the nucleic acid metrics in a per unit volume of sample dissected format (Supplementary Table 2).

Minor Concerns:

7. Detailed Points with page and line numbers following taken from the document Reviewer PDF: Jove62394_reviwer.pdf

Title page Line 9: I think there's an "and" in there that shouldn't be. (Eg The same authors aren't both at Roche and Genetech!)

Author response: Thank you for noticing this. We have removed the “and” from line 10 and denoted “Genetech” on line 9 to clarify.

8. Line 64. The question of what is the limit of tumour content for NGS varies by assay. 10% would be for a targeted panel assay but is much to low for WGH or Exome. You have references for this so it's OK, but you might say that limits vary and then give examples. The MSKCC Impact Study as some published limits as does the PMID mentioned above. I don't want to get too picky here as everyone really does recognize that more tumor content is better!

Author response: We agree with the reviewer that more tumor content is better and have added a sentence to highlight variability across platforms on lines 70-71 which reads “however, limits will vary depending on the platforms utilized and planned use of the data generated.”

9. Line 80. It's true LCM is costly but we're talking about subsequent NGS on million dollar machines so that's not really a reason, especially since these processes still need an Aperio or similar to scan the slide.

Author response: We agree that the overall assay cost is still very significant whether the user chooses LCM or automated dissection, but automated dissection offers significant savings when the laboratory expertise and technical time required to utilize LCM is compared to that of automated dissection which is highlighted in lines 85-89.

10. Line 82: Take out "mid range" here, since the rest of the sentence describes nicely how and why it is actually mid range.

Author response: Thank you for this suggestion. “Mid-range” has been removed from line 87.

11. Line 84: "cons" is colloquial. Should be "disadvantages"

Author response: We would like to thank the reviewer for this suggestion and have changed “cons” to “disadvantages on line 92.

12. Line 86: "overlay" should be "overlays" unless this is meant to mean something different.

Author response: Thank you for noticing this typographical error. We have changed “overlay” to “overlays” on line 93.

13. Line 89: I would tend to use "regions of interest" rather than tumour as you're going to get a lot of normal, and you might not be targeting tumour.

Author response: We thank the reviewer for the suggestion and agree that this phrasing better conveys the options that automated dissection provides. We have therefore changed “tumor” to “regions of interest” on line 96.

14. Line 90-100: You could more explicitly state that conventional LCM has ~10um precision, so capable of single cell resolution, whereas manual macrodissection is more like 1mm so the millisect instrument is in between.

Author response: We agree that these details would provide further clarity and we have modified lines 87-89 to add these details.

15. Line 216: Section 2.6: The instructions for buffer choice is ill-worded. Say, "for FFPE use: XXX. For fresh frozen use YYYY" not in parentheses.

Author response: Thank you for the suggestion. We have reworded this to highlight that the user should choose a dissection buffer of their choice that is appropriate for their tissue type (FFPE or fresh frozen) and their downstream nucleic acid extraction kit of choice (lines 240-241).

16. Line: 227. I was confused initially why we are staining at this point and had to read through to see we are post-staining for QC in Step2. I would state this more explicitly.

Author response: We now see that this could be confusing and we have clarified more explicitly the rationale for staining at this point in lines 270-273.

17. Line 242 Section 3.3: "minimal mineral oil" should be more like "the minimum oil required to resuspend all the fragments or something.

Author response: Thank you for the suggestion. We have clarified this in lines 337-338 by rephrasing to "FFPE samples were collected using molecular grade mineral oil and dissected samples were pooled together and centrifuged at 25,000 x g for 20 min at 4 oC".

18. Line Section 3.4. See comments above about this not being specific to the millisect method.

Author response: We clarified that the user can utilize any commercial kits for extraction in lines 274-274 and separated out how our samples were processed in the representative results section (lines 335-344) as noted in response 1 above.

19. Line 268: The note about sequencing coverage should be in a Results section for this experiment and along with the sample yields in nanograms per mm^3 or similar.

Author response: Thank you for this suggestion. We have moved this to the results and included additional sequencing QC metrics in 348-342. This now reads: "Exome sequencing generated approximately 75 million 100bp paired-end reads, yielding an average depth of coverage (before removing duplicate reads) of 150X per sample, with 99.9% reads aligned and a 78% on-target rate. RNA sequencing metrics demonstrated just over 55 million X bp paired-end reads, a 98% alignment rate and a 19.4% duplication rate with 77% concordant reads." Additionally, we have included yields in ng/mm^3 in supplementary table 2.

20. Line 272 Representative results. Should be in materials and methods for this experiment. Need details or animal model and tumour (Xenograft vs naturally arising) From the pictures they look like Xenos.

Author response: We have added that the tissue used was from xenografts in line 326 and additionally have included comments that we have seen similar results with other tissue types including human tissues from a variety of indications in lines 379-381.

21. Line 284: How do we know that it's successful whole exome sequencing? What metrics are used here?

Author response: Thank you for pointing out that we should show our metrics rather than stating "data not shown". We have provided details of both exome and RNA sequencing metrics

in lines 348-342 as noted above in response 19 to demonstrate successful sequencing in our samples.

22. Line 328- "we have pushed the boundaries of the Millisect system beyond the intended use and our protocol is meant for research use only". This is an odd statement. What it its intended use other than dissection?

Author response: We apologize for the confusion here. The Millisect instrument has been released for use on clinical FFPE specimens up to 10 µm thick, but is not actively marketed for research purposes, for use on thicker sections or for use on fresh frozen tissue. However, we saw benefit for use of the instrument for research purposes on both FFPE and fresh frozen tissues from various sources including murine and human tissues. We have expanded our thoughts and clarified this in lines 107-110.

Reviewer #2:

1. To Authors,

This work describes an automation method based on the Avenio milisect commercial solution marketed by Roche. Further information and clarification are needed to facilitate understanding of this work. Indeed, more precision are necessary in the introduction to precise the context of automation for tissue dissection (state of the art)

Author response: We'd like to thank reviewer number 2 for this suggestion and agree that more information on the precision would provide further clarity on the abilities of the automated dissection instrument. We have therefore modified lines 87-89 to specify the levels of precision of LCM (10 µm) and macrodissection (1 mm) and also included a reference that directly compares macrodissection to automated dissection.

2. and the discussion need to be developed to bring information about the relevance of this approach with more precision about potential application in research.

Author response: Thank you to the reviewer for the suggestion to include more clear relevance of this approach in research. We now realize from reviewer responses that we needed to provide additional clarity on how this technique could be applied to various tissue types applicable to a research setting. We have expanded this information in lines 379-385 where we describe how the technique could be applicable to, and how we have had similar results on, many FFPE tissue types inclusive of xenografts and human tissues over a variety of indications applicable to the research setting.

3. Introduction:

The authors must specify and explain this tissue limitation, concrete examples of several cancers would be useful to better understand the scope of this work. Also, for the different alternative methods, additional information is necessary such as the type of tumours for which these methods are or are not used in clinical routine. What exactly is the clinical reality.

Author response: We appreciate the feedback and have incorporated information to highlight that various tissue types ranging from human to xenografts can be used (see response to question 2 above) in our protocol. We have also included additional information in the introduction on lines 107-110 to clarify that the instrument is marketed and intended for clinical

use on any human FFPE cancer tissues, but that we have developed a protocol for use in research settings applicable to a wider range of tissue types inclusive of FFPE and fresh frozen tissue and that this protocol can be expanded beyond human tumors to xenografts or normal tissue regions of interest.

4. For the LCM, this methodology is used in which type of cancers. Can this method also be automated? It is also important to describe the difference in tissue collection between these different methods.

Author response: All of the techniques mentioned can utilize FFPE material. We appreciate the reviewer pointing out that additional clarity was needed here and have added this to line 75-76. Specifics on tissue collection for macrodissection can also be found on line 76 and those for LCM can be found on 83-84 with references. Similar to our protocol, LCM can be applied to any tumor type, but is more technically challenging and requires extensive hands on time rather than being an automated process requiring less technical expertise (lines 85-86). We felt that a more extensive discussion of whether LCM could be automated to be beyond the scope of this more protocol focused manuscript.

5. They recommend this automated method for micro-tumours of less than 250 µm², but in the protocol they make 3 or 4 cuts at 20 µm thick and cut on non-stained sections: Is it not too thick in relation to the size of the tumour?

Author response: We apologize for the confusion here. We have modified our wording in lines 47 and 48 to clarify that the 250 µm² measurement represents the blade diameter on the automated-dissection instrument and the instrument can be used on tumor diameters both above and below 250 µm². The author makes an excellent point in that 20 µm² cuts are thick and when cutting through very small tumors and regions of interest depletion can be a concern. However, our goal was to show the full range of dissection options using the instrument and not to validate different tumor sizes or the impact of various section thicknesses on enrichment as this is something that would have to be adjusted based on the needs of individual experiments We have now included a sentence on lines 101-104 to clarify this point.

6. what is the size limit possible with this method? and what is the lateral resolution of this cutting method?

Author response: We have delineated the blade sizes on line 99 and note that investigation into the lateral resolution is beyond the scope of our protocol focused manuscript. Recently, since submission of this manuscript to JoVE, we have released a separate paper taking a deeper dive into the resolution of this automated dissection instrument and if it is of interest the reviewer can find it under PMID 33497835. We have also now referenced this recent publication in line 92.

7. What precautions must be taken to avoid contamination with healthy tissue?
Author response: We appreciate the concern for healthy tissue contamination and several approaches exist to limit the impact of healthy tissue in downstream assays including utilizing normal tissue or blood sequencing as a comparator. However, we felt that this was beyond the scope of our protocol focused manuscript which is targeted toward region of interest *enrichment*

rather than *isolation*. As we describe in lines 81-84 LCM would be a better choice if region of interest *isolation* down to a single cell level is required.

8. How do they make sure that the micro-tumour is present on all the sections since they only stain them after the microdissection?
Author response: See the response to the first major point of reviewer 2 above and the sentence we have now included on lines 101-104 to describe methods of confirmation.

9. if possible in addition to the data, indicate how to make an annotation on imageJ and transfer it to the device software.

Author response: We would like to thank the reviewer for his or her interest. Given the need to limit commercial language and feedback from other reviewers that we should focus our protocol on our process, we have removed the specific software recommendations and generalized the digital mask creation. We have provided details on our protocol surrounding specifics for our process, but transfers to the device from all platforms is something that is beyond the scope of our protocol and the would need to work with the vendor to establish a process based on individual software availability and needs. A sentence stating this has been added to line 171-172.

10. Is the NGS the only application possible with this methodology?

Author response: We appreciate the reviewer pointing out that this remained unclear from our text. NGS is not the only application possible and we have added clarity with PCR as a specific additional example application that could be possible in line 406.

11. In order to determine the relevance of this method, the results of the NSW analyses should be compared between an LCM macrodissection approach and the one described in this manuscript, in terms of quality and speed.

Author response: We thank the reviewer for his or her thoughtful comments. We have noted that for equivalent slides in our hands LCM takes twice as long as the automated dissection instrument we used in this protocol. However, we did not include these specifics because a head to head comparison of the metrics between LCM, macrodissection and automated dissection is beyond the scope of our protocol. We have therefore kept our language general in lines 87-89 noting that the automated dissection instrument we used falls in between that of LCM and macrodissection in terms of numerous metrics. We have also referenced our recently published manuscript that does a head to head comparison of macrodissection and automated dissection, but additional studies specifically designed to compare LCM and automated dissection would need to be done in a separate manuscript to address the comparative metrics.

12. Discussion :
According to the authors, what is the relevance and perspective of this work for a basic , translational research and clinical application?

Author response: Thank you for the opportunity to further clarify the relevance and applications of this work. We have modified lines 403-411 to state that we have expanded the

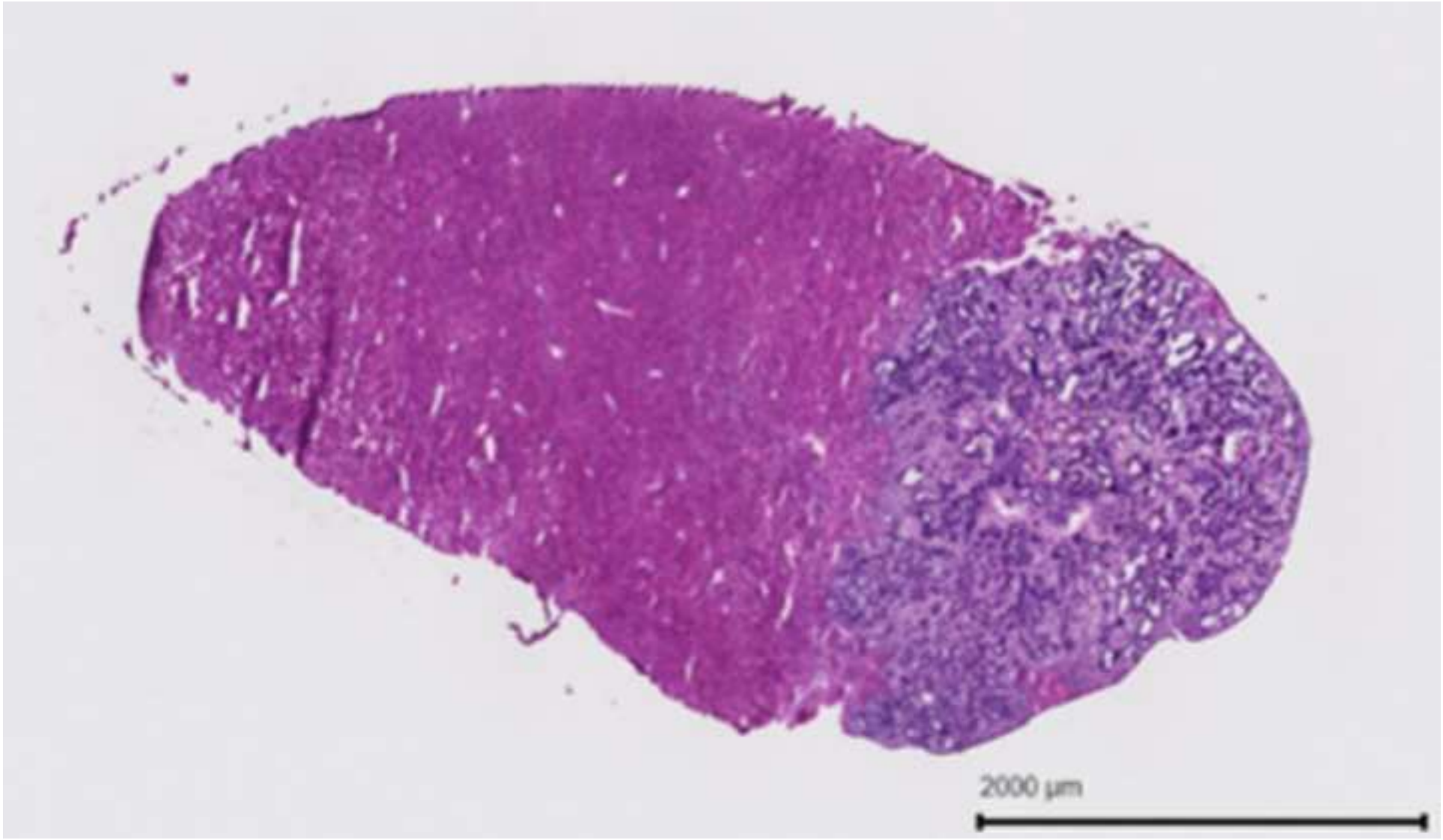
intended use of the automated dissection instrument from human FFPE to xenografts and human FFPE as well as fresh frozen tissues. We then go on to state that we have provided protocols for these tissue types that can be used for region of interest enrichment prior to WES or RNA sequencing in basic and translational research settings and that other molecular applications, including PCR, could also be possible. Finally we describe how these protocols could expand the region of interest enrichment options available and could be developed and validated further for use in clinical settings.

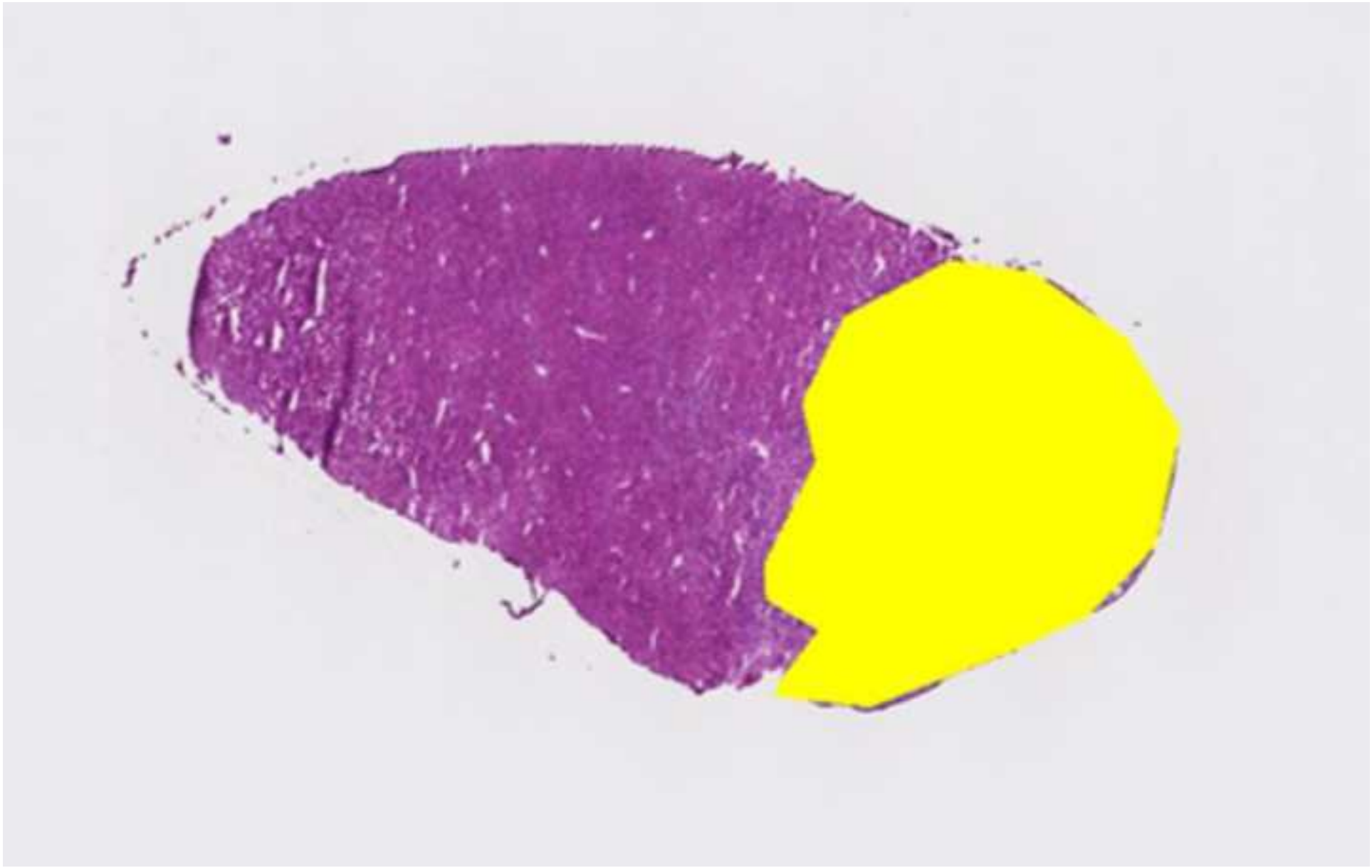
Supplementary File 1: Millisect Pseudocode

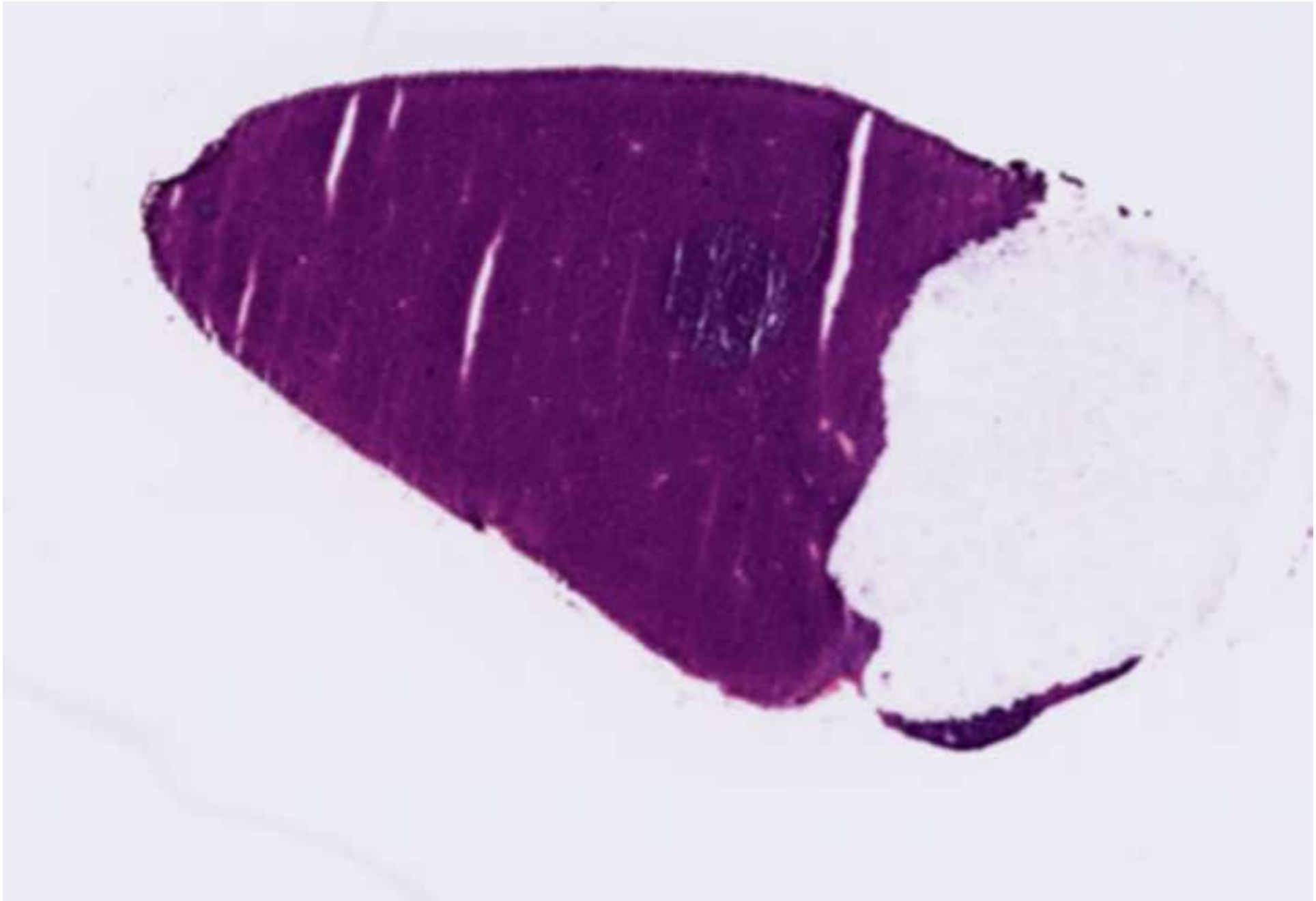
```
function Millisect_save_pseudo(slidepath)
%MILLISECT_SAVE_PSEUDO attempts to crop whitespace around tissue, creating
%one single PNG export with an mask of all ROIs present on a
%slide.  This function calls several custom APIs in order to access image
%data and ROI coordinates.  An end user will need to customize this access
%for their particular system.

%% create mask of ROIS and keep them

% Queries external viewer where ROIs are created.  IF there are no ROIs
% present on a slide, skip making an overlay image
AllRegions = VectorAPI(slidepath, 'GETALL'); %get all ROI ID's to be stamped

% if no ROIs, then skip the rest
if size(AllRegions, 2) > 0

    % get pixel data at a low mag power, high mag is overkill for image alignment
    imgin = CaptureAPI(slidepath,magpower);

    %% crop whitespace to focus on just regions with tissue
    % this step is optional, but can help the downstream alignment of the image
    % on the Millisect

    %create binary mask using global intensity threshold to roughly id tissue
    tmask = ~imbinarize(rgb2gray(imgin), intensity_thresh) ;

    % use alternating morphological operations of increasing size to remove
    % small isolated noise and non-tissue
    tmask = imclose(tmask, d3);
    tmask = imopen(tmask, d3);
    tmask = imclose(tmask, d5);

    tmask = ~(bwareaopen(~tmask, 1000)); % closes small holes in tissue
    tmask = imopen(imclose(tmask, d14), d9);
    tmask = bwareaopen(tmask, 3000); % removes small pieces of tissue
    tmask = imopen(tmask, d14);
    tmask = imclose(tmask, d30);
    tmask = bwareaopen(tmask, 10000); % removes small pieces of tissue
    tmask = ~bwareaopen(~tmask, 10000); % closes large holes

    %find convex hull to make one object, dilate to add a little around edges
```

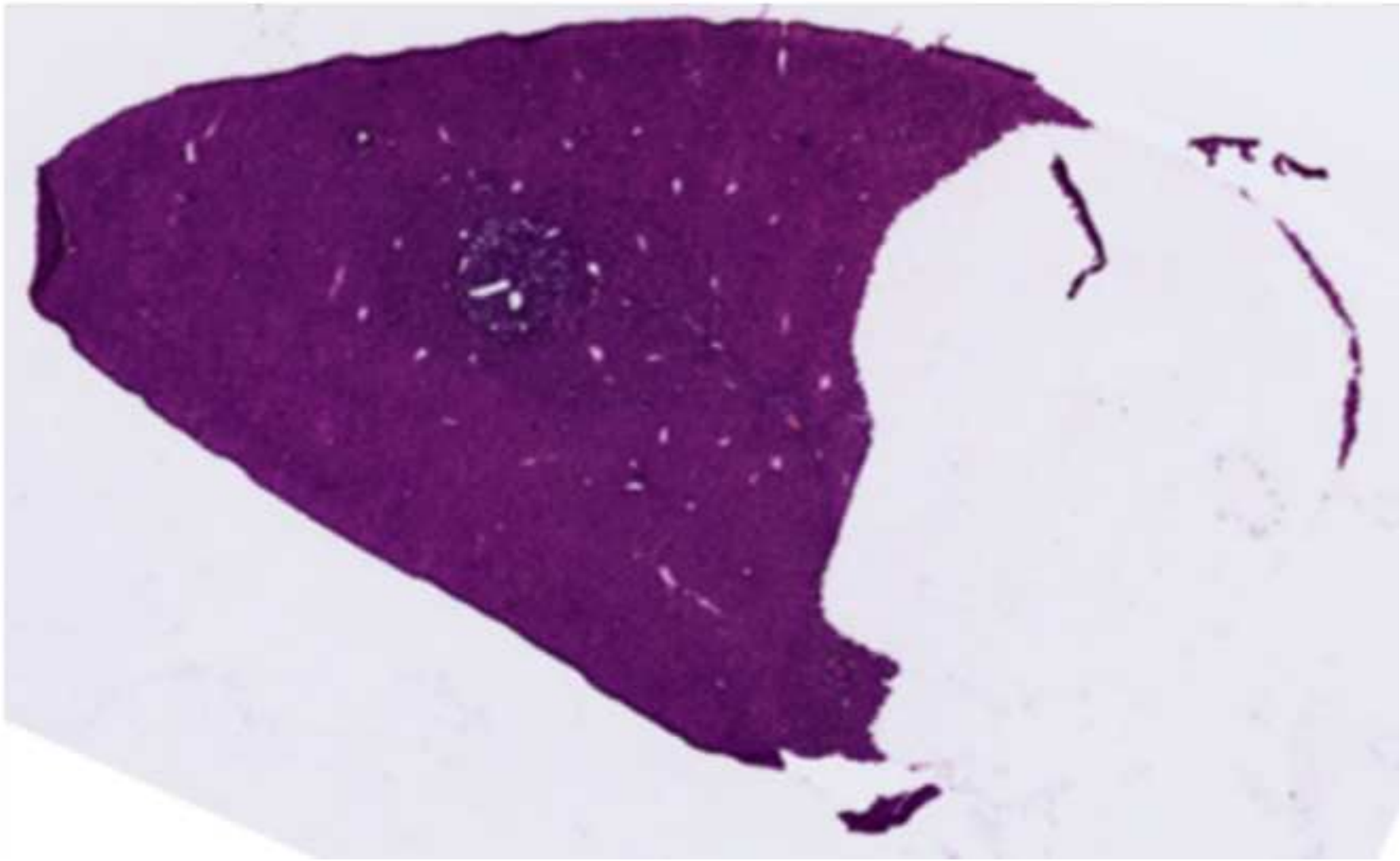
```
ss_mask = bwconvhull(tmask);
ss_mask = imdilate(ss_mask, d14);
% get x-y coordinates of bounding box to crop tissue
hstats = regionprops(ss_mask, 'BoundingBox') ;

%% get ROIs

% query an external viewing platform to return an array of vectors
% containing x-y coordinates for ROIs made on a slide, and return a binary
% mask of the union of them all at the same size as the raw image
add_rois = gSlide_API_ROIs_to_mask(slidepath ,lowmagpower );

%crop raw image and binary mask
imgin = imcrop(imgin , hstats.BoundingBox) ;
add_rois = imcrop(add_rois , hstats.BoundingBox);

%% process low-res image to make it a manageable size

dimy = size(imgin, 1);
dimx = size(imgin, 2);

%if longest dimension is greater than 1000 pixels, resize image so longest side is
%less than 1000
if ( dimy > 1000 || dimx > 1000 )
   %find longest side
   if dimy > dimx
      resize_factor = 1 / (dimy / 1000) ;
   else
      resize_factor = 1 / (dimx / 1000) ;
   end
   imgin = imresize(imgin, resize_factor);
   add_rois = imresize(add_rois, [size(imgin,1) size(imgin, 2)], 'nearest');
end


%% create overlays and save

% create filename with .jpg extension
[~, savename] = fileparts(slidepath);
savename = fullfile(savename, '.jpg');

%create image overlay of input image with solid green wherever add_rois
%binary mask is present
im2 = imoverlay(imgin, add_rois, [1 1 0 ]) ;
```

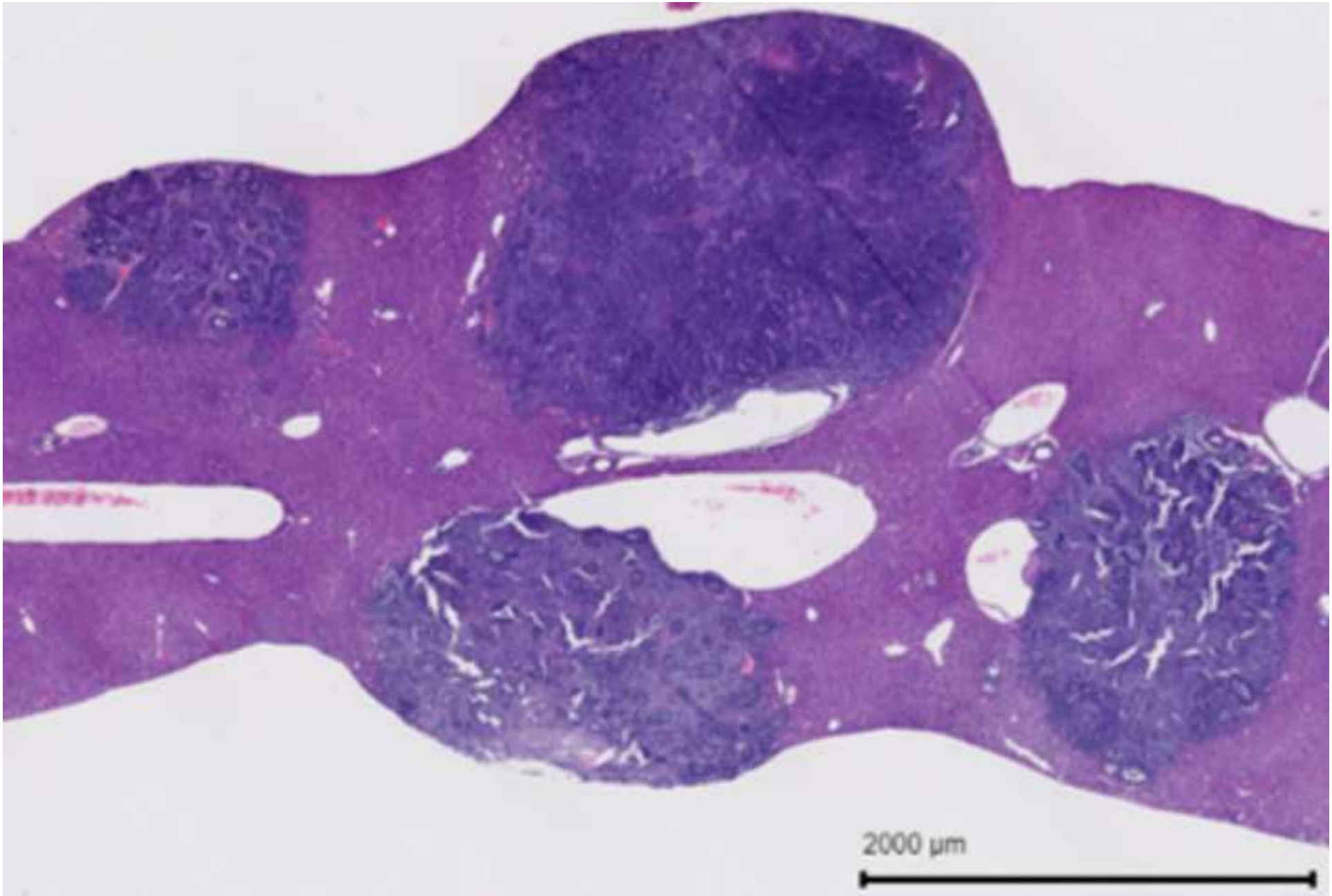
```
    imwrite(im2, savename, 'jpg');

end
end
```

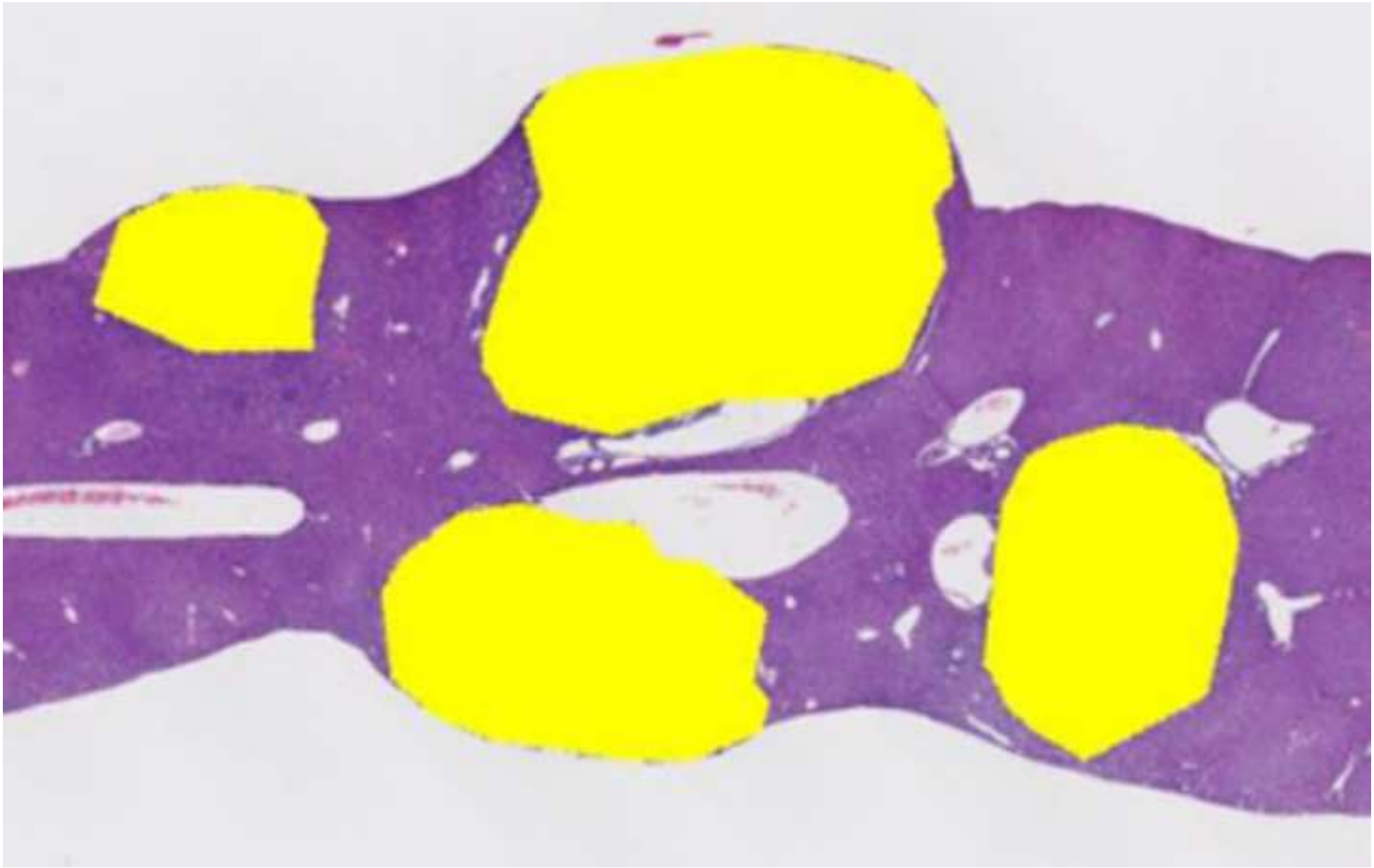


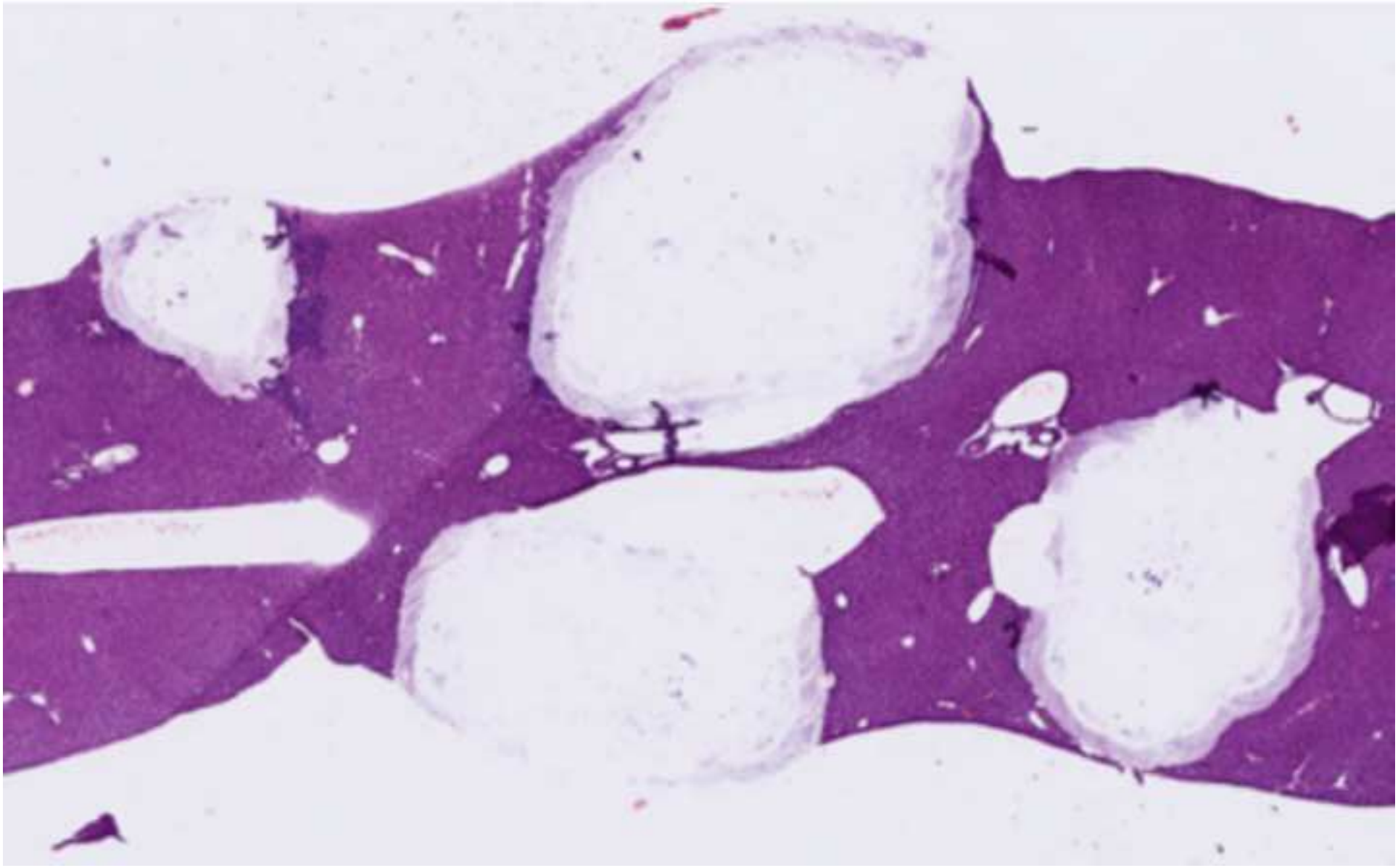


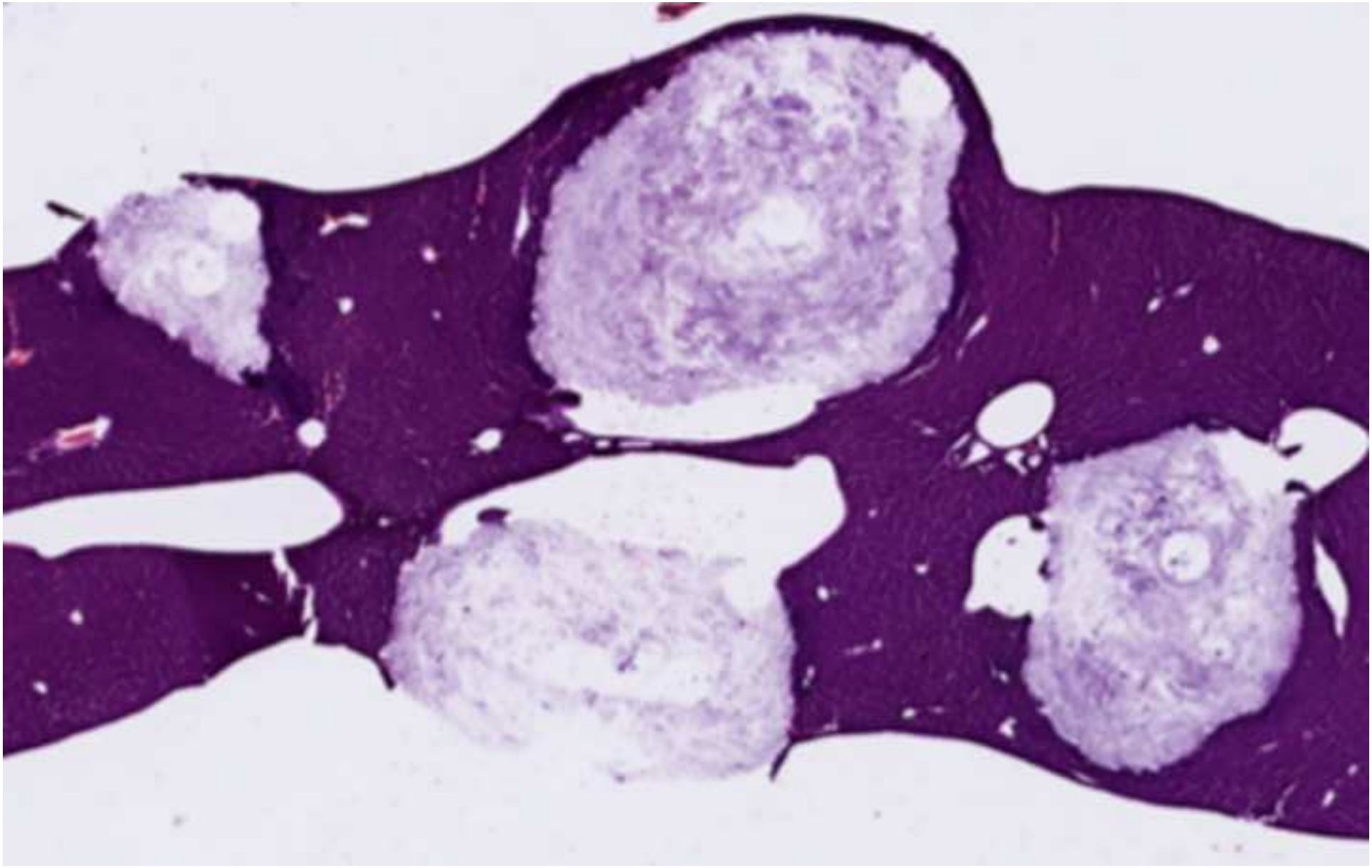


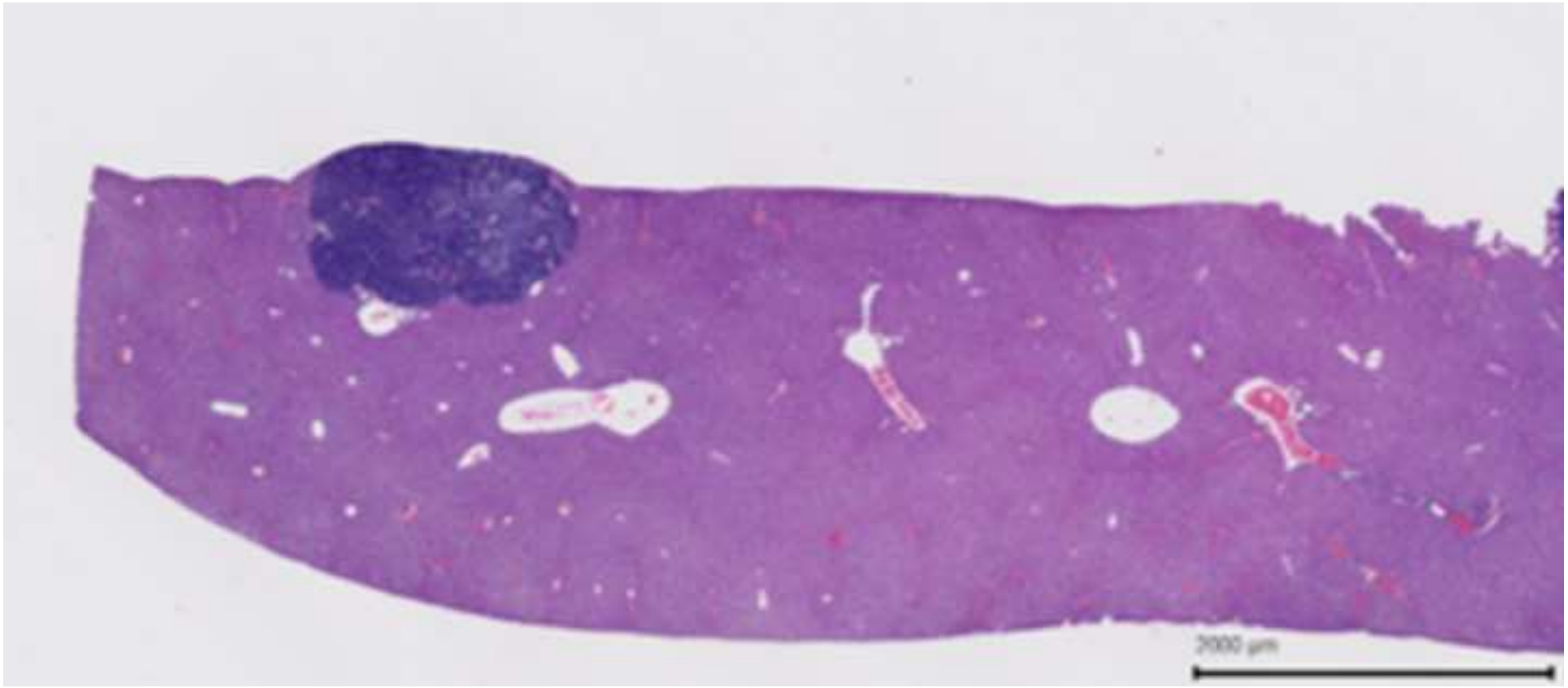


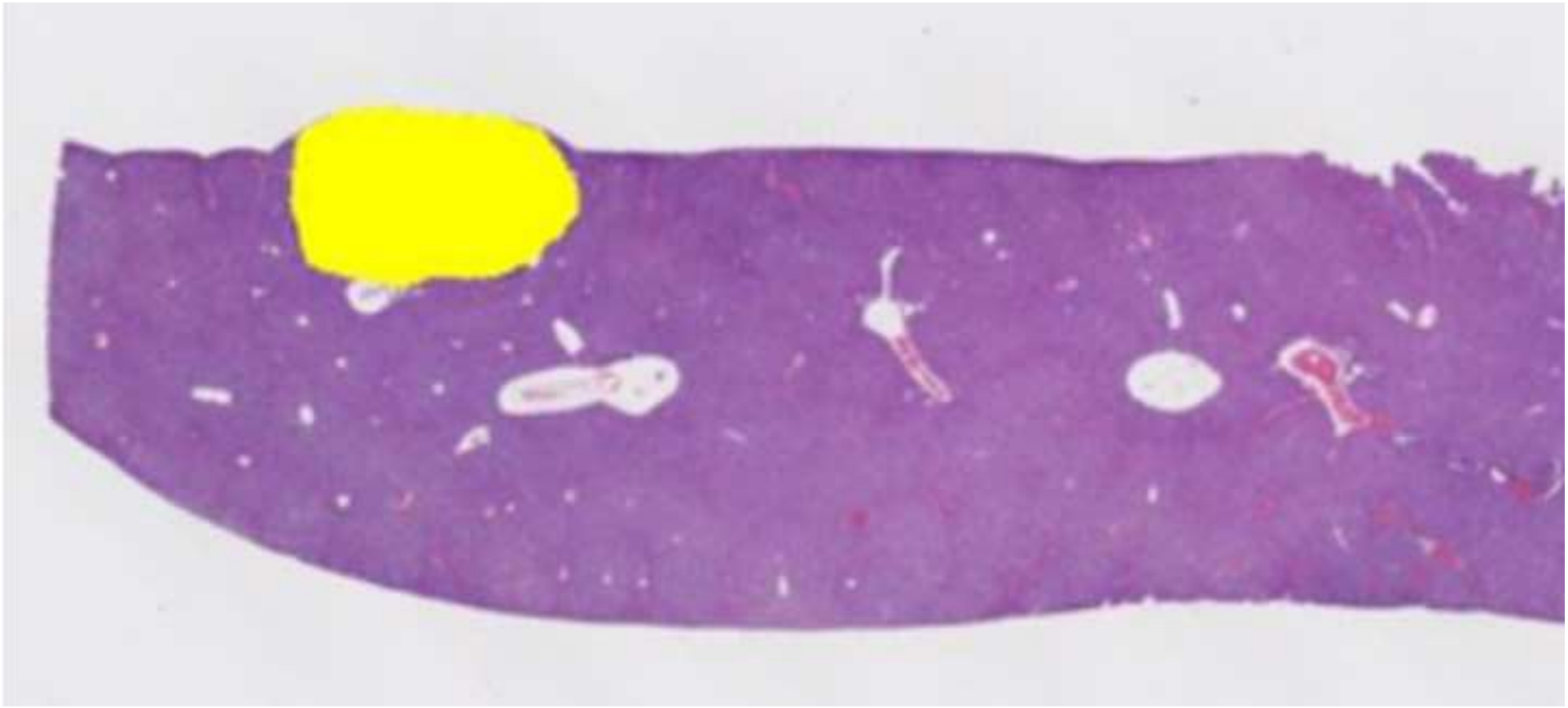


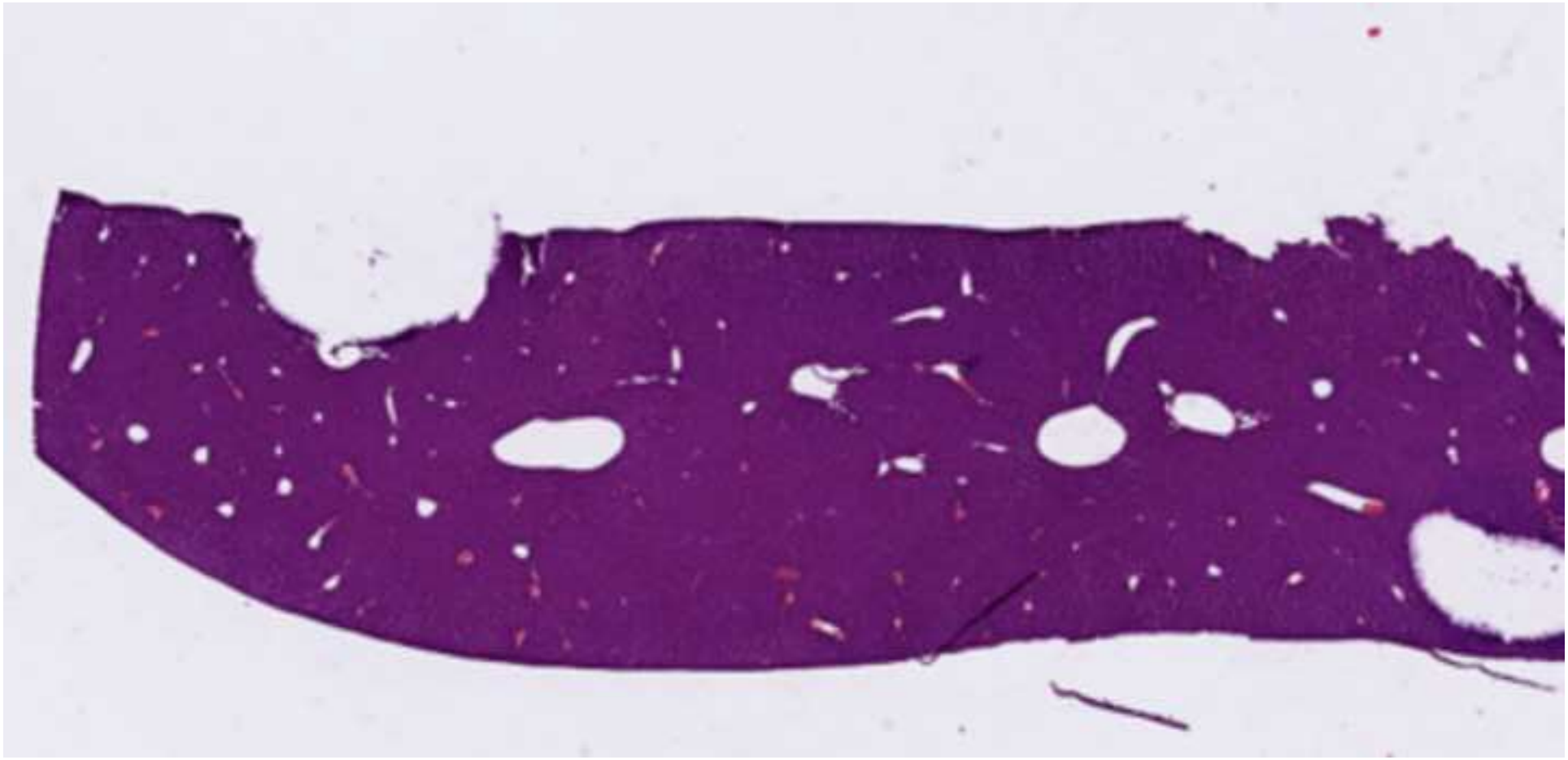












Supplementary Table 1.

Case Identifier	% Tumor Area per Total Tissue Area	Case Type	Harvested Area (mm2)	RNA (ng/mm^3)	DNA (ng/mm^3)
12547	20	FFPE	612	6.05	8.5
13072	15	FFPE	101	0.2	13.9
14124	5	FFPE	29	11.03	19.0
17321	15	FF	97	3.71	5.4
16327	10	FF	73	3.9	6.0