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.