

3D pathology-guided microdissection

Corresponding Author: Professor Jonathan Liu

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Decision Letter:

18th Dec 2025

Dear Jon,

Your Brief Communication, "3D pathology-guided microdissection", has now been seen by 3 reviewers. As you will see from their comments below, although the reviewers find your work of considerable potential interest, they have raised a number of concerns. We continue to be interested in potentially publishing your manuscript in Nature Methods, but would like to evaluate your response to these concerns before we reach a final decision on publication.

We therefore invite you to revise your manuscript to address these concerns.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your paper:

- * include a point-by-point response to the reviewers and to any editorial suggestions
- * please underline/highlight any additions to the text or areas with other significant changes to facilitate review of the revised manuscript
- * address the points listed described below to conform to our open science requirements
- * ensure it complies with our general format requirements as set out in our guide to authors at www.nature.com/naturemethods
- * resubmit all the necessary files by using the link below to access your home page

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We hope to receive your revised paper within two months. If you are substantially delayed, please let us know. In this event, we will still be happy to reconsider your paper at a later date so long as nothing similar has been accepted for publication at Nature Methods or published elsewhere.

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For any revision that includes light microscopy data, we ask our authors to please include a completed light microscopy reporting table [https://www.nature.com/documents/Light_microscopy_reporting_table.xlsx] to ensure the methods are described thoroughly. The table will be available to reviewers and ultimately published should the manuscript be accepted at the journal.

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We strongly encourage you to deposit all new data associated with the paper in a persistent repository where they can be freely and enduringly accessed. We recommend submitting the data to discipline-specific and community-recognized repositories; a list of repositories is provided here: <http://www.nature.com/sdata/policies/repositories>

All novel DNA and RNA sequencing data, protein sequences, genetic polymorphisms, linked genotype and phenotype data, gene expression data, macromolecular structures, and proteomics data must be deposited in a publicly accessible database, and accession codes and associated hyperlinks must be provided in the "Data Availability" section.

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Please include a "Data availability" subsection in the Online Methods. This section should inform readers about the availability of the data used to support the conclusions of your study, including accession codes to public repositories, references to source data that may be published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", describing which data is available

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Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing your revised manuscript and thank you for the opportunity to consider your work.

Happy Holidays!

Best regards,
Madhura

Madhura Mukhopadhyay, PhD
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Jonathan T. C. Liu and colleagues describes a 3D pathology-guided microdissection system that integrates open-top light-sheet microscopy with computer numerical controlled milling to enable volumetric microdissection of intact tissue blocks. The ability to isolate precise 3D regions based on pathologist-generated annotations is highly innovative and addresses a critical limitation in current spatial omics methodologies. Overall, the manuscript presents an exciting technical advance with substantial potential to impact spatial biology and diagnostic pathology. I commend the authors for developing this sophisticated workflow and for demonstrating its utility in resolving 3D tumor phylogeny. Overall, the manuscript is clearly written and well-organized, especially given the interdisciplinary nature of the work. The introduction nicely frames the problem and the need for 3D microdissection in the context of spatial omics. The description of the workflow (imaging, annotation, milling) is logical.

MAJOR COMMENTS

The validation of 3DPM across multiple tissue types and using several performance metrics is a major strength of the manuscript. The authors demonstrate successful microdissection in four tissue types of varying stiffness (prostate, head and neck, breast, and cartilage), supporting the generalizability of the approach. The reported average recall (tissue yield) and precision (sample purity) of ~65.1% and ~63.8%, respectively, are reasonable for a first-generation 3D microdissection method.

However, these values also indicate that a substantial fraction of the target tissue is not recovered and that roughly one-third of the extracted material may be collateral. The authors correctly note that even soft tissues such as breast can be processed (Extended Data Fig. 2g), albeit likely with lower precision. It would strengthen the validation to comment on how recall and precision vary across tissue types, for example, whether performance is lower in very soft tissues and higher in stiffer ones. Providing a breakdown or at least noting the range of observed values would add informative nuance.

The demonstration that DNA extracted from 3DPM-processed tissue is of high quality is also crucial. The manuscript shows that DNA integrity (DIN values) and PCR amplification from microdissected FFPE tissue are comparable to standard FFPE processing, alleviating concerns that clearing or milling might damage nucleic acids. This is an important validation step. If available, information on typical DNA yields from a VOI (e.g., nanograms of DNA or approximate cell numbers per ~1 mm³ region) would be helpful for readers planning downstream omics. Even a qualitative statement indicating that yields were sufficient for whole-genome sequencing (as evidenced by 40× WGS of each VOI) is reassuring.

Reproducibility is essential for a methods paper. Were replicate dissections performed to assess consistency? For example, if the same VOI were processed twice, would recall and precision remain similar? Any data or observations on reproducibility, quantitative or anecdotal, would increase confidence in the robustness of the method.

The description of the genetic algorithm–optimized toolpath would benefit from additional clarification. The manuscript mentions a two-step milling strategy (removing tissue above the VOI followed by angled cuts). It would be helpful to explicitly reference the schematic illustrating this process. If this is already depicted in Fig. 1b (“For each VOI”), please cite this figure in the text around line 93. In addition, the text and figures refer to VOIs 16 and 22, which is confusing given that only seven VOIs were sequenced. Clarifying the VOI numbering scheme and explaining how the seven sequenced VOIs were selected would prevent potential confusion.

Finally, beyond cancer phylogeny, the 3DPM method has the potential to be transformative in other fields, for example, mapping gene expression or mutational landscapes in developmental biology, neuroscience, or any context in which the 3D microenvironment is important. Expanding the Discussion to briefly highlight these broader applications would underscore the wide utility of the technique.

MINOR COMMENTS

- Line 116: Please define what “GU Pathology” stands for; I assume it refers to Genitourinary Pathology.
- Line 98: Change “To validate the 3D microdissection workflow,” to “To validate the 3DPM workflow,” for consistency.
- Figure 2a: The unit for the y-axis in the histogram is missing.
- Line 50: Replace “um” with “μm.”
- Figure 2b: Consider adding the x- and y-dimension lengths of the tissue block, not only the z-dimension.
- Extended Data Fig. 2d and 2g: Provide clearer indication of what the four colored bars represent. After reviewing the figure, I inferred that they correspond to the four tissue types, but this should be clarified.
- Extended Data Fig. 6c: The outline of the tissue block containing the 3D tumor skeleton should be made more prominent.

Reviewer #2 (Remarks to the Author):

The authors present a technically impressive workflow integrating OTLS (lightsheet microscope for high-throughput volumetric microscopy) with computer numerical controlled (CNC) milling for volumetric microdissection to realize 3D pathology where the holy grail is no need for physical cutting onto glass slides and with high-resolution histologic datasets from thick tissue specimens. The present method tried to take the specimen based on the 3D information from high-resolved lightsheet imaging for positioning the volume of interest (VOI) in 3D and use this 3D imaging landmark for further microdissection by CNC. First of all, the cleared tissue is required for 3D thick samples and the corresponding cleared tissue stiffness is demanded as well as shown in the Extended fig. 2d for Young’s module measurement. And the dissected specimens were subjected to the analysis such as whole genome sequencing in Extended fig. 7. Basically, they try to link the information at various scales ranging from mm size to molecular level, which the reviewer thinks a good direction for the medical research. Of course, AI triaging algorithms and explainable models to help pathologists in the interpretation of the obtained novel, but complex, large datasets is required, demonstrated herein the manuscript. However, there are some concerns raised by the reviewer as following for further discussions,

First, the entire pipeline depends on tissue clearing, which alters morphology and staining compatibility. Although the authors apply a false-color H&E algorithm to mimic conventional histology, cleared-tissue contrast is not directly comparable to true H&E—the gold standard for diagnostic interpretation, which has been published in the reference 9,29–33 in the manuscript. Since most tumor boundaries for grading criteria are defined in H&E staining, the reliance on cleared-tissue OTLS data raises concerns regarding annotation fidelity, especially for clinical translatability needed to be addressed more. And how the public acceptance for clearing process applicable for the human samples in the clinical usage? Also, how the clearing process affect the gene analysis such as phylogenetic tree?

Second, in terms of CNC milling precision, precision constraints that are fundamentally different from laser-capture

microdissection. The issue will be the findings in Extended Data Fig. 2e, highlights the limitations of mechanical milling in achieving the spatial purity, micron-level boundary control, and molecular integrity expected from LCM workflows. Deformation observed in soft tissues probably further underscores the difficulty of maintaining accurate 3D boundaries in compliant samples. There is one tissue clearing process in the manuscript, how about other clearing protocols applied to the soft tissues? A thorough samples among clearing process will be worthy of discussion, ethyl cinnamate used here. And another discussion will be how about Robotic micro-manipulators (e.g., da Vinci-style surgical systems) for another option? Last but not least, the reviewer tried to find the thickness of FFPE samples? This will be the key issue since the staining or many sample preparations such as reaction time or temperature related to the sample thickness. And there is be some swelling or shrinkage process during the tissue clearing, the VOI reported for the microdissection ($\sim 0.72 \text{ mm}^3$) in the manuscript, maybe a lot larger/smaller due to the shrinkage/swelling process.

Also, there are some questions below,

-The spatial resolution is $\sim 300 \text{ um}$ for lighthseet imaging/CNC maybe will be the issue for the further investigation, how to improve this ?

-There is no descriptions on the comparisons or differences on the 3D microdissection sequence data.

-While performing CNC mill, how to make sure there is no contamination from these debris by milling.

-How to decide the VOI (volume of interest), based on the reported mimic H&E staining ?

-Another concern for the targeted volume with $\sim 65 \%$ overlap region, maybe due to the sample clearing process for deforming, how to improve this ?

Overall, the method present is informative and good direction for the 3D pathology. The data is solid through the manuscript. The authors pave the road for bridging the gap between 3D volumetric annotation and microscale tissue isolation. To extend the possibility, does 3D model for tumor is needed for doctors? Maybe the brain neurodegeneration disease is needed.

Reviewer #2 (Remarks on code availability):

The code is running ok. Just it takes a lot of time $\sim 7\text{hr}$ for genetic-algorithm-based optimization over VOIs. maybe the authors could explain what is the rate determining step here?

Reviewer #2 (Remarks on figshare data availability):

the data is reasonable.

Reviewer #3 (Remarks to the Author):

Summary of the key results. In this study, Hsieh et al. developed a new approach demonstrating that 3D pathology-guided microdissection (3DPM) can precisely extract small, targeted 3D regions of tissue without causing damage, while preserving high-quality DNA suitable for sequencing. By using full 3D imaging instead of thin 2D slices, the method reveals tumor shapes and connections that are normally missed, such as branching or continuous tumor areas that look separate in 2D. The authors also show that 3DPM gives results similar to traditional laser microdissection but with far better spatial detail. Importantly, combining 3D structure with genetic data lets researchers map how different tumor subclones spread through the tissue. Overall, this work represents a timely extension of the OTLM approach previously developed by the Liu lab, and it will significantly advance studies of tumor evolution and drug response.

Originality and significance.

This study is original and represents a substantial advance beyond traditional laser-capture microdissection which have been used for decades. By enabling true volumetric sampling guided by intact 3D tissue architecture, the method overcomes key limitations of 2D section-based pathology and opens up new possibilities for spatially resolved molecular analysis. Its significance extends well beyond cancer research, this platform could have broad applicability across diverse fields, including neuroscience, developmental biology, and metabolic disease, where understanding 3D tissue organization and cellular interactions is essential.

Data & methodology. The methodology is well-justified, demonstrating a clear advance over existing 2D LCM-based methods. The authors provide validation that their 3D pathology-guided microdissection platform accurately captures predefined tissue volumes while preserving DNA quality suitable for downstream sequencing. Figures are clearly presented. Although it would have been valuable to include demonstrations beyond SNV analysis, such as examples of additional molecular or spatial information that can be extracted compared with standard 2D methods, it is understandable that this is a Methods in Brief article where the focus is appropriately placed on establishing the methodology rather than fully exploring every downstream application. Overall, the data and presentation are compelling and highlight the robustness and potential impact of the platform.

Conclusions: robustness, validity, reliability

The conclusions are well supported by the data. The method performs with reliable tissue extraction, good molecular quality, and strong agreement with existing approaches like 2D LCM. The results clearly show that the platform is robust and valid, even if the paper keeps the focus on methods rather than full downstream applications.

Appropriate use of statistics and treatment of uncertainties. Appropriate

Suggested improvements. It would be helpful to include at least one example demonstrating the platform's ability to extract additional molecular or spatial information beyond SNVs, such as known mutation or gene expression, methylation, or spatially resolved protein markers, to highlight the broader utility compared to standard 2D methods. Also, providing a bit more clarity on sources of technical variability, such as tissue consistency or imaging depth, and how these are controlled would further

improve confidence in the method. These are not essential for the core methodological contribution but would be valuable enhancements for a future studies.

Reviewer #3 (Remarks on figshare data availability):

The data presented in the manuscript are sufficient to assess the overall quality and robustness of the work

Version 1:

Decision Letter:

Our ref: NMETH-BC63095A

11th Mar 2026

Dear Jon,

Thank you for submitting your revised manuscript "3D pathology-guided microdissection" (NMETH-BC63095A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Methods, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements within two weeks or so. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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Thank you again for your interest in Nature Methods. Please do not hesitate to contact me if you have any questions. We will be in touch again soon.

Sincerely,
Madhura

Madhura Mukhopadhyay, PhD
Senior Editor
Nature Methods

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily responded to all my comments and revised the manuscript accordingly

Reviewer #2 (Remarks to the Author):

Thanks for the authors' reply. The reviewer is satisfied with the revision.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my suggestions by clearly highlighting the new analyses and discussion sections.

Version 2:

Decision Letter:

19th May 2026

Dear Jon,

I am pleased to inform you that your Brief Communication, "3D pathology-guided microdissection", has now been accepted for publication in Nature Methods. The received and accepted dates will be 14 Oct 2025 and 19 May 2026. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Methods style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Please feel free to contact me if you have questions about any of these points.

Best regards,
Madhura

Madhura Mukhopadhyay, PhD
Senior Editor
Nature Methods

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Reviewer #1 (Remarks to the Author):

The manuscript by Jonathan T. C. Liu and colleagues describes a 3D pathology-guided microdissection system that integrates open-top light-sheet microscopy with computer numerical controlled milling to enable volumetric microdissection of intact tissue blocks. The ability to isolate precise 3D regions based on pathologist-generated annotations is highly innovative and addresses a critical limitation in current spatial omics methodologies. Overall, the manuscript presents an exciting technical advance with substantial potential to impact spatial biology and diagnostic pathology. I commend the authors for developing this sophisticated workflow and for demonstrating its utility in resolving 3D tumor phylogeny. Overall, the manuscript is clearly written and well-organized, especially given the interdisciplinary nature of the work. The introduction nicely frames the problem and the need for 3D microdissection in the context of spatial omics. The description of the workflow (imaging, annotation, milling) is logical.

> Thank you for the positive comments.

MAJOR COMMENTS

The validation of 3DPM across multiple tissue types and using several performance metrics is a major strength of the manuscript. The authors demonstrate successful microdissection in four tissue types of varying stiffness (prostate, head and neck, breast, and cartilage), supporting the generalizability of the approach. The reported average recall (tissue yield) and precision (sample purity) of ~65.1% and ~63.8%, respectively, are reasonable for a first-generation 3D microdissection method. However, these values also indicate that a substantial fraction of the target tissue is not recovered and that roughly one-third of the extracted material may be collateral. The authors correctly note that even soft tissues such as breast can be processed (Extended Data Fig. 2g), albeit likely with lower precision. It would strengthen the validation to comment on how recall and precision vary across tissue types, for example, whether performance is lower in very soft tissues and higher in stiffer ones. Providing a breakdown or at least noting the range of observed values would add informative nuance.

> Thank you for the comment. We have added comments on performance across different tissue types, including how tissue stiffness affects recall and precision, as well as per-tissue-type average metrics and ranges, in Line 109-114. Briefly, recall is generally comparable across tissue types, with per-tissue-type average

recall exceeding 62% in all four tissues tested. In contrast, precision shows a stronger dependence on tissue stiffness: lower-stiffness normal breast tissue exhibits reduced average precision (~55%) and greater variability compared with the three stiffer tissue types (all >64%).

The demonstration that DNA extracted from 3DPM-processed tissue is of high quality is also crucial. The manuscript shows that DNA integrity (DIN values) and PCR amplification from microdissected FFPE tissue are comparable to standard FFPE processing, alleviating concerns that clearing or milling might damage nucleic acids. This is an important validation step. If available, information on typical DNA yields from a VOI (e.g., nanograms of DNA or approximate cell numbers per ~1 mm³ region) would be helpful for readers planning downstream omics. Even a qualitative statement indicating that yields were sufficient for whole-genome sequencing (as evidenced by 40× WGS of each VOI) is reassuring.

> Thank you for the comment. We have added a description of the volumetric DNA yield for the 7 VOIs selected for whole-genome sequencing (ng/mm³) in Line 124-126. DNA yields from these VOIs ranged from approximately 500 to 2000 ng/mm³, with an average yield of 994 ng/mm³. These yields were sufficient for downstream whole-genome sequencing.

Reproducibility is essential for a methods paper. Were replicate dissections performed to assess consistency? For example, if the same VOI were processed twice, would recall and precision remain similar? Any data or observations on reproducibility, quantitative or anecdotal, would increase confidence in the robustness of the method.

> Thank you for the comment. While the same VOI cannot be physically microdissected twice, as the tissue is irreversibly removed after microdissection, we agree that reproducibility is an essential aspect.

To address this, we have added an *in-silico* reproducibility analysis of the toolpath optimization in Extended Data Figure 3b, c and Line 372-384, in which the optimization was initialized with 10 different random states. We observed that the optimized toolpath parameters and performance metrics were comparable across runs.

Specifically, the mean standard deviation (SD) across 24 VOIs was 2.52 % for recall, 1.43 % for precision, and 0.37 % for damage to remaining VOIs. The SDs in toolpath parameters were also low (mean SD = 4.65° for θ_i and 26.05 μm for z_c).

Two slightly higher variations in recall (still less than 10%) were likely due to the presence of multiple local optima, where random initialization could lead to different convergence outcomes.

In addition, at the experimental level, we evaluated 3DPM performance across a wide range of VOI sizes and shapes in four different tissue types (Extended Data Fig. 2f). Recall and precision metrics were comparable for VOIs of similar size and geometry, particularly in cartilage, head-and-neck, and prostate specimens, supporting the robustness of the method across repeated applications.

The description of the genetic algorithm–optimized toolpath would benefit from additional clarification. The manuscript mentions a two-step milling strategy (removing tissue above the VOI followed by angled cuts). It would be helpful to explicitly reference the schematic illustrating this process. If this is already depicted in Fig. 1b (“For each VOI”), please cite this figure in the text around line 93.

> Thank you for the comment. We have cited Fig. 1b after the sentences “Here, we introduce ... to fully detach the VOI from the base” (Line 93) to improve clarity of the two-step milling strategy.

In addition, the text and figures refer to VOIs 16 and 22, which is confusing given that only seven VOIs were sequenced. Clarifying the VOI numbering scheme and explaining how the seven sequenced VOIs were selected would prevent potential confusion.

> Thank you for the comment. We have added clarification of the VOI numbering scheme in Line 121-124. Twenty-two candidate VOIs were initially annotated based on the 3D tumor architecture, from which 7 VOIs representing key spatial locations were selected for whole-genome sequencing following 3D microdissection.

Finally, beyond cancer phylogeny, the 3DPM method has the potential to be transformative in other fields, for example, mapping gene expression or mutational landscapes in developmental biology, neuroscience, or any context in which the 3D microenvironment is important. Expanding the Discussion to briefly highlight these broader applications would underscore the wide utility of the technique.

> Thank you for the comment. We have added a brief statement in the final part of

the Discussion (Line 208-211) to highlight the potential applicability of 3DPM beyond cancer phylogeny, including applications in developmental biology, neuroscience, and studies of tissue microenvironments.

MINOR COMMENTS

- Line 116: Please define what “GU Pathology” stands for; I assume it refers to Genitourinary Pathology.
- Line 98: Change “To validate the 3D microdissection workflow,” to “To validate the 3DPM workflow,” for consistency.
- Figure 2a: The unit for the y-axis in the histogram is missing.
- Line 50: Replace “um” with “ μm .”
- Figure 2b: Consider adding the x- and y-dimension lengths of the tissue block, not only the z-dimension.
- Extended Data Fig. 2d and 2g: Provide clearer indication of what the four colored bars represent. After reviewing the figure, I inferred that they correspond to the four tissue types, but this should be clarified.
- Extended Data Fig. 6c: The outline of the tissue block containing the 3D tumor skeleton should be made more prominent.

> Thank you for the comments. All minor comments have been addressed accordingly. Specifically, “GU Pathology” has been defined as Genitourinary Pathology (Line 119); “To validate the 3D microdissection workflow” has been revised to “To validate the 3DPM workflow” (Line 98); the unit (%) has been added to the y-axis of Fig. 2a; “um” has been replaced with “ μm ” (Line 49); and the x- and y-dimension lengths have been added to Fig. 2b in addition to the z-dimension. Legends have been added to Extended Data Figs. 2d and 2g to clarify the correspondence between bar colors and tissue types. In Extended Data Fig. 6c, the outline of the tissue block has been made more visually prominent.

Reviewer #2 (Remarks to the Author):

The authors present a technically impressive workflow integrating OTLS (lightsheet microscope for high-throughput volumetric microscopy) with computer numerical controlled (CNC) milling for volumetric microdissection to realize 3D pathology where the holy grail is no need for physical cutting onto glass slides and with high-resolution histologic datasets from thick tissue specimens. The present method tried to take the specimen based on the 3D information from high-resolved lightsheet imaging for positioning the volume of interest (VOI) in 3D and use this 3D imaging landmark for further microdissection by CNC. First of all, the cleared tissue is required for 3D thick samples and the corresponding cleared tissue stiffness is demanded as well as shown in the Extended fig. 2d for Young's module measurement. And the dissected specimens were subjected to the analysis such as whole genome sequencing in Extended fig. 7. Basically, they try to link the information at various scales ranging from mm size to molecular level, which the reviewer thinks a good direction for the medical research. Of course, AI triaging algorithms and explainable models to help pathologists in the interpretation of the obtained novel, but complex, large datasets is required, demonstrated herein the manuscript.

> Thank you for the positive comments.

However, there are some concerns raised by the reviewer as following for further discussions, First, the entire pipeline depends on tissue clearing, which alters morphology and staining compatibility. Although the authors apply a false-color H&E algorithm to mimic conventional histology, cleared-tissue contrast is not directly comparable to true H&E—the gold standard for diagnostic interpretation, which has been published in the reference 9,29–33 in the manuscript. Since most tumor boundaries for grading criteria are defined in H&E staining, the reliance on cleared-tissue OTLS data raises concerns regarding annotation fidelity, especially for clinical translatability needed to be addressed more. And how the public acceptance for clearing process applicable for the human samples in the clinical usage? Also, how the clearing process affect the gene analysis such as phylogenetic tree?

> Thank you for the comment. We would like to clarify that 3DPM is positioned as a research and discovery tool, for example, for applications in cancer evolution and spatial biology (similar to laser capture microdissection, which is

predominantly used for research purposes), rather than as a diagnostic tool or a workflow directly integrated into current clinical practice. To help clarify this point, we have added the phrase “...for research and discovery in spatial biology” in Line 52-53 of the Introduction.

In terms of annotation fidelity, two genitourinary (GU) pathologists contributed to the tumor annotation in the prostate cancer example presented in this study (one helped with initial tumor annotation, and the other annotated patterns shown in Fig. 2b). They did not express concerns regarding the use of false-color datasets for tumor segmentation or annotation.

As noted above, 3DPM is not positioned as a diagnostic tool for clinical settings; however, several prior publications from the Liu lab have focused on translational research and involved multiple pathologists from different subspecialties who successfully applied relevant grading and classification schema to OTLS-generated false-colored H&E images, demonstrating promising diagnostic performance^{1,2,3}. With respect to morphological changes induced by ethyl cinnamate-based tissue clearing, prior studies have evaluated tissue morphology interpretation and immunohistochemistry utilization before and after clearing and reported no observable alterations^{4,5} (note that Ref #4 is not from Liu lab). It is also worth noting that, in routine clinical practice, intraoperative frozen sections often exhibit substantial artifacts and morphological distortions, yet still retain clinical utility.

More broadly, regarding the use of tissue-clearing protocols in human tissues, multiple human tissue types have been processed in prior studies for research investigations⁶. In addition, rapid tissue-clearing methods have also been developed in recent years to improve imaging depth in more translational contexts⁷ – demonstrating the emerging translational potential of tissue-clearing approaches beyond fundamental research.

As a brief additional note, a variety of pseudo-H&E methods based on different imaging modalities, beyond OTLS-based approaches, have also been developed and increasingly investigated in both basic research and clinical contexts^{8, 9}, reflecting the growing interest in pseudo-H&E technologies.

Finally, the effects of the 3DPM workflow (including tissue clearing) on tumor phylogenetic analysis were evaluated thoroughly by comparison with conventional 2D LCM-based approaches (Extended Data Fig. 7). Clustering and subclonal reconstruction results were consistent across methods. DNA quality following the 3DPM workflow was also comparable, as shown in Extended Data

Fig. 5.

References:

1. Gao, Gan et al. "Deep-learning triage of 3D pathology datasets for comprehensive and efficient pathologist assessments." *bioRxiv : the preprint server for biology* 2025.07.20.665804. 22 Jul. 2025
2. Lindsey A, et al. "Artificial Intelligence–Triage 3-Dimensional Pathology to Improve Detection of Esophageal Neoplasia While Reducing Pathologist Workloads." *Modern Pathology*, vol. 36, no. 12, 1 Dec. 2023, pp. 100322–100322
3. Xie, Weisi et al. "Diagnosing 12 prostate needle cores within an hour of biopsy via open-top light-sheet microscopy." *Journal of biomedical optics* vol. 25,12 (2020): 126502.
4. Kontny, Andreas, et al. "On-Slide Clearing and Imaging of 100-Mm-Thick Histological Sections Using Ethyl Cinnamate and Epifluorescence." *Folia Medica*, vol. 66, no. 3, 30 June 2024, pp. 380–385
5. Reder, Nicholas P et al. "Open-Top Light-Sheet Microscopy Image Atlas of Prostate Core Needle Biopsies." *Archives of pathology & laboratory medicine* vol. 143,9 (2019): 1069-1075.
6. Mai, Hongcheng, and Dan Lu. "Tissue Clearing and Its Applications in Human Tissues: A Review." *View*, vol. 5, no. 2, 15 Jan. 2024
7. Huang, Chi Z et al. "Rapid clearing and imaging of Mohs and melanoma surgical margins using a low-cost tissue processor." *Biomedical optics express* vol. 15,2 700-714. 8 Jan. 2024
8. Yin, Zichen, et al. "Fast and Label-Free 3D Virtual H&E Histology via Active Phase Modulation-Assisted Dynamic Full-Field OCT." *Npj Imaging*, vol. 3, no. 1, 8 Apr. 2025
9. Abraham, Tanishq Mathew et al. "Label- and slide-free tissue histology using 3D epi-mode quantitative phase imaging and virtual hematoxylin and eosin staining." *Optica* vol. 10,12 (2023): 1605-1618.

Second, in terms of CNC milling precision, precision constraints that are fundamentally different from laser-capture microdissection. The issue will be the findings in Extended Data Fig. 2e, highlights the limitations of mechanical milling in achieving the spatial purity, micron-level boundary control, and molecular integrity expected from LCM workflows. Deformation observed in soft tissues probably further underscores the difficulty of maintaining accurate 3D boundaries

in compliant samples. There is one tissue clearing process in the manuscript, how about other clearing protocols applied to the soft tissues? A thorough samples among clearing process will be worthy of discussion, ethyl cinnamate used here. And another discussion will be how about Robotic micro-manipulators (e.g., da Vinci-style surgical systems) for another option?

> Thank you for the comment. We agree that CNC milling and laser-capture microdissection (LCM) have different precision characteristics, reflecting well-known trade-offs between mechanical milling and laser-based manufacturing techniques. While laser-based methods provide high lateral resolution, they are inherently two-dimensional due to the nature of laser cutting, which relies on localized heating and material ablation and offers limited control in the depth dimension. In contrast, CNC milling typically exhibits lower lateral precision (on the order of several tens of microns to $\sim 100\text{ }\mu\text{m}$), but enables precise and deterministic control in the depth dimension. Therefore, 3DPM is positioned as a complementary tool to existing methods, rather than a replacement for LCM.

In terms of different clearing methods, we agree that exploration of various clearing methods and/or additives to further increase tissue stiffness is an important future step for softer tissues, as added in Line 160-161 of the Discussion. Tissue clearing methods can be broadly classified into two categories: dehydration-based methods and aqueous methods. Due to the need for three-dimensional machining, dehydration-based methods are more suitable because they increase tissue stiffness during the process. Ethyl cinnamate is one of the commonly used dehydration-based clearing agents across a wide range of tissue types, and was therefore selected for this study (Masselink, Wouter et al. "Broad applicability of a streamlined ethyl cinnamate-based clearing procedure." *Development (Cambridge, England)* vol. 146,3 dev166884. 1 Feb. 2019).

Regarding robotic micro-manipulators, existing surgical robotic devices generally have lower precision than CNC milling. For example, the reported repeatability of da Vinci-style surgical systems is on the order of $\sim 1\text{ mm}$, which is not well suited for microdissection purposes. It is also worth noting that such high-end three-dimensional micro-manipulator systems typically have much higher costs (e.g., on the order of USD 1.5 M for da Vinci systems) compared with desktop CNC platforms (approximately USD 14,000 for the system used in this study), further supporting the generalizability of the proposed approach (This point has been added in Line 166-169.)

Last but not least, the reviewer tried to find the thickness of FFPE samples? This will be the key issue since the staining or many sample preparations such as reaction time or temperature related to the sample thickness. And there is some swelling or shrinkage process during the tissue clearing, the VOI reported for the microdissection ($\sim 0.72 \text{ mm}^3$) in the manuscript, maybe a lot larger/smaller due to the shrinkage/swelling process.

> Thank you for the comment. We have added the thickness of the tissue samples used in this study in Line 238-239, ranging from approximately 3–7 mm. Staining was performed following an established protocol published in *Nature Protocols* (Bishop, K.W., Erion Barner, L.A., Han, Q. *et al.* An end-to-end workflow for nondestructive 3D pathology, *Nat Protoc* **19**, 1122–1148, 2024), which has been tested on samples of varying thickness to ensure staining uniformity. In addition, the stains used in this work are small-molecule dyes, which allow better penetration and diffusion compared with antibody-based stains.

Regarding potential volume changes during tissue clearing, both imaging and 3D microdissection were performed on post-cleared tissue. As a result, any global dimensional changes induced by the clearing process are already accounted for in the definition of the VOIs and do not affect the calculation of microdissection performance. In practice, only minor shrinkage of the tissue was observed after clearing (< 10% compared to the original FFPE tissue).

Also, there are some questions below,

-The spatial resolution is $\sim 300 \text{ um}$ for light-sheet imaging/CNC maybe will be the issue for the further investigation, how to improve this ?

> Thank you for the question. To clarify, the spatial resolution of the light-sheet imaging is approximately $2 \text{ }\mu\text{m}$. This is achieved with the “low-resolution mode” of our OTLS microscope, and finer resolutions are possible if desired by the user. The current limitation on the minimum achievable microdissection size arises primarily from tissue deformation during the 3D microdissection process, rather than from the imaging resolution itself. As discussed in Line 160-161, future exploration of additives or methods to further increase tissue stiffness is expected to help reduce the minimum microdissection size.

-There is no descriptions on the comparisons or differences on the 3D microdissection sequence data.

> Thank you for the question. We have added a brief description and directed

readers to Extended Data Fig. 7, which presents a comparison of tumor phylogenetic reconstruction results between 2D LCM-based and 3DPM-based approaches in Line 148-151. The comparison shows a high level of agreement in clustering and subclonal reconstruction between the two methods.

-While performing CNC mill, how to make sure there is no contamination from these debris by milling.

> Thank you for the question. To avoid inter-VOI or inter-block contamination, the endmill is cleaned and/or replaced between samples, as described in Line 434-441. In addition, microdissection is performed on relatively small volumes with minimal debris generation, which limits the risk of carryover. This differs from typical industrial machining scenarios.

-How to decide the VOI (volume of interest), based on the reported mimic H&E staining?

> Thank you for the question. VOI selection is driven by the end-user's specific application. In the prostate cancer example presented here, tumor regions were first segmented, and VOIs were selected at key spatial nodes. In practice, annotations were performed in QuPath at 50- μ m intervals, followed by interpolation to generate a 3D VOI (Line 268-271). For other applications, end users may annotate different tissue structures depending on their specific research goals.

-Another concern for the targeted volume with ~65 % overlap region, maybe due to the sample clearing process for deforming, how to improve this ?

> Thank you for the question. To clarify, the current errors mainly arise from tissue deformation during the milling process, rather than from the relatively minor shrinkage associated with tissue clearing. Both imaging and microdissection were performed on post-cleared tissue. Recall and precision in 3DPM can potentially be improved through increased tissue stiffness to enhance machining accuracy (Line 155-161), as well as through future refinements of toolpath design for microdissection.

Overall, the method present is informative and good direction for the 3D pathology. The data is solid through the manuscript. The authors pave the road for bridging

the gap between 3D volumetric annotation and microscale tissue isolation. To extend the possibility, does 3D model for tumor is needed for doctors? Maybe the brain neurodegeneration disease is needed.

> Thank you for the comment. We have added a statement in the Discussion (Line 208-211) to highlight the potential of applying the 3DPM method to neuroscience and other biological contexts, in addition to cancer-related studies. Three-dimensional investigation of tumors using the proposed 3DPM framework could provide value in different spatial biology research areas, such as studies of cancer evolution and progression, and may ultimately contribute to a deeper understanding of cancer biology relevant to clinical contexts.

Reviewer #2 (Remarks on code availability):

The code is running ok. Just it takes a lot of time ~7hr for genetic-algorithm-based optimization over VOIs. maybe the authors could explain what is the rate determining step here?

> Thank you for the comment and for testing our code. The rate-determining step of the genetic-algorithm-based optimization is the simulation of the microdissection process for each parameter set, which involves repeated 3D array operations. The current implementation already avoids whole-volume computation by cropping regions surrounding each VOI, which improves computational efficiency. Future performance improvements could be achieved through GPU-based acceleration of 3D array operations (e.g., CuPy for parallel processing).

Reviewer #3 (Remarks to the Author):

Summary of the key results. In this study, Hsieh et al. developed a new approach demonstrating that 3D pathology-guided microdissection (3DPM) can precisely extract small, targeted 3D regions of tissue without causing damage, while preserving high-quality DNA suitable for sequencing. By using full 3D imaging instead of thin 2D slices, the method reveals tumor shapes and connections that are normally missed, such as branching or continuous tumor areas that look separate in 2D. The authors also show that 3DPM gives results similar to traditional laser microdissection but with far better spatial detail. Importantly, combining 3D structure with genetic data lets researchers map how different tumor subclones spread through the tissue. Overall, this work represents a timely extension of the OTLM approach previously developed by the Liu lab, and it will significantly advance studies of tumor evolution and drug response.

Originality and significance.

This study is original and represents a substantial advance beyond traditional laser-capture microdissection which have been used for decades. By enabling true volumetric sampling guided by intact 3D tissue architecture, the method overcomes key limitations of 2D section-based pathology and opens up new possibilities for spatially resolved molecular analysis. Its significance extends well beyond cancer research, this platform could have broad applicability across diverse fields, including neuroscience, developmental biology, and metabolic disease, where understanding 3D tissue organization and cellular interactions is essential.

Data & methodology. The methodology is well-justified, demonstrating a clear advance over existing 2D LCM-based methods. The authors provide validation that their 3D pathology-guided microdissection platform accurately captures predefined tissue volumes while preserving DNA quality suitable for downstream sequencing. Figures are clearly presented. Although it would have been valuable to include demonstrations beyond SNV analysis, such as examples of additional molecular or spatial information that can be extracted compared with standard 2D methods, it is understandable that this is a Methods in Brief article where the focus is appropriately placed on establishing the methodology rather than fully exploring every downstream application. Overall, the data and presentation are compelling and highlight the robustness and potential impact of the platform.

Conclusions: robustness, validity, reliability

The conclusions are well supported by the data. The method performs with reliable tissue extraction, good molecular quality, and strong agreement with existing approaches like 2D LCM. The results clearly show that the platform is robust and valid, even if the paper keeps the focus on methods rather than full downstream applications.

Appropriate use of statistics and treatment of uncertainties. Appropriate

> Thank you for the positive comments.

Suggested improvements. It would be helpful to include at least one example demonstrating the platform's ability to extract additional molecular or spatial information beyond SNVs, such as known mutation or gene expression, methylation, or spatially resolved protein markers, to highlight the broader utility compared to standard 2D methods.

> Thank you for the comment. Note that the seven VOIs used in the demonstrated prostate cancer case were fully consumed for DNA extraction, leaving no remaining material for RNA- or protein-based analyses.

In addition to SNV calling, we also performed copy-number analysis (primarily to infer local copy-number states for phylogenetic tree reconstruction, Line 126-127, 520-534), as well as annotation of non-synonymous variants among the SNVs used in clustering. In this case, all seven VOIs exhibited whole-genome duplication. Although we attempted more detailed copy-number analysis for descriptive purposes, the resulting copy-number alterations were highly complex and did not reveal clear conclusions or spatial correlations; therefore, they were not reported.

In terms of known mutations, we observed a non-synonymous SNV in KDM4B within cluster B (Extended Data Fig. 7e). We have added a brief description in the caption of Extended Data Fig. 7 as an illustrative example (Line 675-677).

Also, providing a bit more clarity on sources of technical variability, such as tissue consistency or imaging depth, and how these are controlled would further improve confidence in the method. These are not essential for the core methodological contribution but would be valuable enhancements for a future studies.

> Thank you for the comment. We have added a brief discussion on how tissue stiffness affects recall and precision (Line 109-114). Briefly, recall is generally comparable across tissue types, with per-tissue-type average recall exceeding 62% in all four tissues tested. In contrast, precision shows a stronger dependence on tissue stiffness: lower-stiffness normal breast tissue exhibits reduced average precision (~55%) and greater variability compared with the three stiffer tissue types (all >64%), which could potentially be improved through increased tissue stiffness to enhance machining accuracy (Line 160-161 in the Discussion).

The typical achievable imaging depth with the current system and tissue processing workflow is approximately 1.2 mm, and this information has been added in Line 262.

[Reviewer #3 \(Remarks on figshare data availability\):](#)

[The data presented in the manuscript are sufficient to assess the overall quality and robustness of the work](#)

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily responded to all my comments and revised the manuscript accordingly

Thank you.

Reviewer #2 (Remarks to the Author):

Thanks for the authors' reply. The reviewer is satisfied with the revision.

Thank you.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my suggestions by clearly highlighting the new analyses and discussion sections.

Thank you.