nature portfolio

Peer Review File

Spatial Proteomics of Single Cells and Organelles on Tissue Slides Using Filter-Aided Expansion Proteomics

Corresponding Author: Professor Tiannan Guo

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study entitled “Filter-aided expansion proteomics” by Dong & Jiang et al. describes an advanced LC-MS based spatial proteomics workflow as a follow-up from the recently published ProteomEx approach. The authors combined their previous ProteomEx workflow with an in-tip digestion method that leads to higher sample throughput, reproducibility and depth of analysis. Further protocol optimizations were performed to most efficiently perform the tissue expansion procedure (protein anchoring, hydrogel embedding, homogenization etc.). The authors finally show two applications for 1) the profiling of single nuclei when combined with LCM and 2) FFPE colorectal cancer tissues.

Overall, this is a well written and concise manuscript that is easy to follow and which adequately cites previous literature in the spatial proteomics field. Figures are clear and easy to understand.
To a large part, the improvements of the workflow are convincingly shown by the provided data. However, an exception is the single nucleus application. Additional experiments are needed to really convince the reader that FAXP is indeed a reliable approach to analyse individual cells or sub-cellular structures such as nuclei. A closer look at the provided DIANN output tables (pg.matrix for relative protein levels) of the nucleus experiment brought up several questions:

1) I find it concerning that no histone and only few nuclear proteins were found in the provided dataset, which are typically among the most abundant proteins in the cell/nucleus. Instead, it is mostly keratins that show up in these proteome measurements, indicating a high contamination level. Please comment and revise.
2) Also, proteome correlations are very poor (Pearson r = 0.6-0.8). These data are missing and should be added to better judge the current limitations when analysing single cells or nuclei.
3) To convince the reader that the FAXP approach is useful to reliably analyse single nucleus proteomes when coupled to LCM, the authors should compare single nuclei to single whole cells. A quantitative comparison should then reveal major differences in proteins related to e.g. cytosol, mitochondria and nucleoplasm and chromatin related proteins. An example of such analysis can be found in the cited Deep Visual Proteomics publication (see Fig. 3C). I suggest to also show this for tissue derived hepatocytes as the authors first introduce murine liver in Fig. 4, but then don’t provide any proteomics data and instead focus on Hela cells.
4) The reported depth of analysis of 1400 protein groups per Hela nucleus seems very overstated due to the presence of many non-unique rows where the same protein id shows up several times. It is therefore recommended to report only unique proteins groups, which can be done using the --relaxed-prot-inf command under Additional Options. Please see the DIANN manual for more info: https://github.com/vdemichev/DiaNN. How any proteins would then be identified? Please revise accordingly.
5) An interesting application of the FAXP approach is together with immunofluorescence staining. To show that this is possible, an interesting approach would be to use the DAPI stained hepatocyte nuclei together with a plasma membrane marker and then distinguish diploid cells from polyploid hepatocytes. Can FAXP be used to quantitatively compare these proteomes of these different phenotypes?

Reviewer #2

(Remarks to the Author)
The manuscript by Dong et al. describes FAXP, a novel proteomics approach via filter-aided hydrogel-based tissue

expansion. The major advantage of this protocol is that hydrogel-based tissue expansion increases the sample volume by up to 15 times (compared to the previously published ProteomeEx method), allowing for manual or laser-based dissection of very small, locally confined tissue areas. The authors demonstrate that with this protocol, they can obtain an average of 1420 proteins from a single HeLa nucleus. This is a significant achievement for the field. They further applied the protocol to different malignant areas of colorectal cancer tissues from three patients and measured 6751 proteins, revealing various molecular and biological properties in these areas.
The authors have previously developed the ProteomeEx method and show how FAXP is superior to ProteomeEx in terms of sensitivity, speed, throughput, and volume expansion. In summary, this manuscript describes highly innovative and relevant work contributing to the timely topic of spatial omics and offering major advantages in clinical proteomics and beyond. The manuscript is well-written, and the figures are of high quality and underline the statements.

Major Comments:
1) PRIDE Repository: The authors have used PRIDE to publish RAW data. However, the CRC raw data are all labeled as ProteomeEx, and no FAXP-named files can be found. It seems 131 raw files should exist for CRC, which is not reflected in the current PRIDE repository. Please upload all files to PRIDE and name them carefully to allow for data re-use and reproducibility.
2) CRC Application:
a)Replicates: Only CRC tissues from three patients were analyzed. However, as not all tissue types were present in all samples, several tissue types were only present in one or two patients. PC and CC occur only in patient 1. Is this why three slides were used to generate enough biopsies? If so, this means the same tissue areas might be present for PC and CC, unlike other tissue areas where this should not occur.
b) T-test: How was the t-test performed? It seems the technical replicates (from the same biological patient with different tissue regions) were treated as independent samples, which invalidates the t-test. I suggest including more patients to make this study more robust and allow for proper statistical analysis.
c) Clinical Applications: From a clinical proteomics perspective, it would have been more exciting to dissect single CRC tumor cells to show their heterogeneity or showcase an entity with particular intra-tumoral heterogeneity, rather than examining larger areas of different tissue types, which could evenly be done with existing proteomics methods. The authors should also comment on sensitivity and whether important clinical biomarkers and drug targets, such as ADC targets (e.g., HER2/ERBB2), can still be found with FAXP.

Minor Comments:
1) Title: The title seems more like a working title. I suggest adding more details about what can be achieved and the advantages of FAXP.
2) Comparison with ProteomeEx: Throughout the manuscript, there is a strong focus on comparing FAXP with ProteomeEx. I suggest reducing this focus and describing FAXP more generally and in comparison to other proteomics methods, such as DVP or methods focusing on cellular organelles.
3) Abbreviations: Please introduce abbreviations when used for the first time, e.g., MS, ROI.
4) Introduction: The last part of the introduction gives an idea about the study, however is too long and detailed for this section of the manuscript. I suggest shortening it and keeping the results at a very high level (lines 71-91).
5) GitHub Repository: It would be beneficial to provide the input data in the GitHub repository to enable anyone to reproduce the figures.
6) Results: Optimization of Tissue Expansion Procedures: Lines 94 to 129 could be improved for clarity. How were the methods optimized? For example, for reduction/alkylation, there is only a link to Figure 2H-I. However, the different conditions tested should also be described in the text. I can't find a description of how the tissue staining time could be improved by 86%. Please ensure each step is described in enough detail.
7) Throughout the results section, it is unclear on which tissue and how many replicates the results are presented. Please include this information in the description of each result in the manuscript text and not only in the figure legends.
8) Line 192: This sentence is unclear: "Moreover, it is usually very challenging to safely the thus isolated nucleus for downstream proteomic analysis."
9) Figure 1:
a) Clarify if LEF is always compared to the original sample size or the previous step in the protocol
b) In 1A, avoid comparison in red to ProteomeEx; make 1A specific to FAXP and use 1B for the comparison
c) In 1A, clarify what "newly prepared sample" means. Are these cell culture experiments in contrast to FFPE blocks?
d) In 1A, change "Proteomic analysis" to "Proteomic measurement" to avoid confusion with data analysis
e) Number Figure 1A and 1B with the same steps for direct comparison, e.g., Step 2 in A corresponds to 2-4 in B. In 1B, reduction and alkylation are mentioned twice, which is not reflected in 1A
f) Double-check the numbers in 1B, e.g., why does step 9 microdissection take no time for FAXP?
g) Consider moving Figure 1B to the supplementary section as these details may not be necessary for the main manuscript
10) Figure 2: Zoom into the y-axis to make differences more visible.
11) Figure 3: Zoom into the y-axis in panels B and C to make differences more visible.
12) Figure 6: Ensure the color scheme is accessible for color-blind readers. Add carcinoma (C) to plot F.
13) Discussion:
a) Maximum Tissue Size: Is there a maximum tissue size compatible with FAXP? Comment on this in the discussion/limitations. Cryopreserved Tissues: Is this method compatible with cryopreserved tissues?
b) Heterogeneity: The statement about FAXP's ability to discern heterogeneity in disease progression based on tissue regions of only three patients is an overstatement.
c) Single-Cell Nucleus Study: Is this the first study on a single-cell nucleus? If so, emphasize this throughout the manuscript. If not, compare it to similar methods.
d) Subcellular Organelles: Discuss the feasibility of dissecting other subcellular organelles for MS analysis in more detail,

considering the nucleus is the largest organelle and thus the easiest to analyze.
e) Spatial Transcriptomics: The statement about spatial transcriptomics is incorrect. While compatible with FFPE, RNA degrades, leading to lower quality. Comparison with spatial transcriptomics is unnecessary here. Discuss other proteomics methods instead.
14) Methods:Lines 376-378: These lines read like results. Is this sentence in the wrong place?
15) Limitations Section: This section is a bit unstructured and contradicts parts of the results (e.g., regarding the extracellular matrix). Reorganize for clarity.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
Dong and colleagues introduce a method termed Filter-aided expansion proteomics (FAXP), which combines hydrogel-based cell or tissue expansion with mass spectrometry (MS) for high spatial resolution proteomics analysis. As stated by the authors, the idea of combining expansion with MS has been demonstrated before by Drelich et al., 2021 (PMID: 34449217) and by the authors themselves, Li et. al., 2022 (PMID: 36450705). However, the authors have significantly enhanced their protocols and introduced novel steps (namely, in-gel in-tip digestion-combined with the physical expansion of specimens), resulting in a substantial 50% reduction in sample preparation time. Additionally, they achieved around two-fold increase in lateral resolution, and increased the protein identification.

The manuscript is written clearly. The figures are nicely executed and clear. The experimental data is convincing. FAXP strongly improves the existing expansion-proteomics analysis tools introduced by the authors in Nature Communications in 2022. The current results are better than the ones published in 2022, albeit the authors do not show a fundamental improvement in biological analyses.

Major comments:

1. The authors show that shortening the anchoring time to 1-hour has yielded a 4.4 expansion factor, while a 2-hour anchoring has decreased the expansion factor to 3.1 which what would be expected for X4 expansion protocols in tissue sections. A concern arises from the fact that shorter anchoring time leads to less amine-reactive entities getting the anchor, hence less proteins being incorporated into the gel. This could explain the higher expansion factor. Did the authors apply their FAXP analysis onto 2 hours anchored 10 microns thick tissues? Would the peptide yield be comparable to that of 1-hour anchoring? Figure S1. does not provide enough information to rule this out.

2. It is not clear how the volumetric enhanced resolution was calculated to reach a claimed 14.5-fold increase. The authors state that the lateral resolution is enhanced by 2.2-fold. One would assume that the enhancement in volumetric resolution would be 2.2(x) x 2.2(y) x 2.2(z) = 10.6. The authors should clarify how they calculated the 14.5 value. Ultimately, the volume resolution is rather meaningless, given that the authors excise a piece of a defined area from a flat sample. In other words, the authors' technique presents a 2D picture of the specimen, in which the volume is almost meaningless.

3. The manuscript adequately details the technical methodology but lacks a critical discussion on the sensitive steps of the expansion and MS procedures, experiment critical steps, failures and troubleshooting. To enhance reproducibility, the authors must include a Supplementary Discussion section addressing the pivotal bottleneck steps of FAXP and tips to overcome them.

4. The authors should add a section in which they compare their technology with other visual proteomics protocols (for example from M. Mann's laboratory), explaining the advantages and disadvantages of different tools.

Minor comments:

1. All statistical tests and their respective types should be mentioned in the figure legend for each panel in each figure.

2. Although the robot-automated steps constituted a minor part of the FAXP method, it would be beneficial for the authors to discuss the time savings achieved through automation. This is particularly important as not all laboratories have access to such advanced tools.

3. The authors should consider the fact that their technology would be most attractive if laboratories could reproduce their robotic applications. Otherwise, interest in this technology may be limited. I suggest that the authors make greater efforts to present this technology (the robotics part) in an easily reproducible fashion.

4. I am missing accurate values in some critical steps of the expansion procedure, and in some instances, there are contradictions. The authors are responsible to provide exact information about the used protocols. Careful revision of the Methods section is required. I provide couple of examples below:

a. Line 423-424: “For gel-making, 50-100 µL of protein anchoring solution was added to the fixed cells and incubated at room temperature for 1.5 hours.” Is it 1.5 hours or 1 and 2 hours as stated in Figure 2E?
b. Line 430: “…nitrogen gas atmosphere for 2-3 hours”, is it 2 or 3 hours? One of the hallmarks of this paper is speed. For reproducibility, exact values are required.

5. In Table S4, section for the text “Mix 25 mL 0.2 M MES stock solution with 25 mL ddH2O to make the final volume at 50 mL and test if the pH is at 6.0.”, cite Tillberg et al., 2016: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5068827/, as this anchoring buffer was first described here.

6. Line 103: “adjustment significantly enhanced the sturdiness of the hydrogel, in consistent with a..:”, remove “in” from “in consistent”.

7. Line 223: “…based on median values, respectively (Figure 5D), in consistent with previous reports…”, remove “in” from “in consistent”.

8. While most of the figures are nicely executed, Figure 2, gave me trouble understanding it. For instance, it took me a while to understand what does a, b, c, d, and e, mean in panel (I). Naturally, the reader would think that panels (A,B,C, and D) where involved, then I was wondering what refers to (e), as panel E was a graph; only later to find out that the annotations are present in panel (H).

9. Panel (H) in Figure 2, could get a little adjustment by either removing the four contour boxes from conditions b-e, or by adding a contour box around condition a for consistency.

10. One possible suggestion (and not a must) is to slightly edit the panel positions in Figure 2. For instance, moving panel G and placing it next to panel F, while moving panel H to occupy a complete row under panels F and H. This would of course require reordering the content of panel H in a landscape fashion.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did a great job in addressing my concerns. I have no further comments and congratulate them on their work.

Reviewer #2

(Remarks to the Author)
The authors have addressed all reviewer comments appropriately and the revised manuscript is well-written and provides valuable insights for spatial proteomic investigations upon publication.
As a minor improvement and to clarify the biomarker aspects in Figure 6, the authors could think about adding a table of the DEPs / biomarkers and the aspects (Prognosis, Response to therapy) that they are associated with.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
Dong and colleagues have thoroughly addressed all the points I raised to my satisfaction. It’s evident that they put in extra effort and conducted additional experiments to resolve all concerns. The manuscript is now clearer, and the added points in the discussion and supplementary notes are excellent. All the suggested corrections and edits have been implemented. With good faith, I unequivocally recommend this study for publication in Nature Communications as fast as possible.

Point-by-point response to the reviewers' comments:

We sincerely thank the reviewers for their thorough and constructive feedback on our manuscript, "Filter-aided expansion proteomics." In the revised version, we have addressed all the points raised, further strengthening our work. We included additional analyses and revised figures and text as suggested. Below, we detail our responses to each comment, providing data where incorporation into the manuscript was not possible.

As you will see from the reports copied below, the reviewers raise some concerns that limit the strength of the study, and therefore we ask you to address them with additional work. In particular, we ask you to alleviate reviewer #1's concerns about whether FAXP is a reliable method for analysing single cells or subcellular structures.

Reply: We appreciate the editor's concerns and have conducted additional work to address them. Specifically, we conducted a comparison between a single mouse liver nucleus and cell to demonstrate that FAXP is a reliable method for analyzing single cells and subcellular structures. The details of these comparisons can be found in the corresponding replies below.

Reviewer #1 (Remarks to the Author):

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Overall, this is a well written and concise manuscript that is easy to follow and which adequately cites previous literature in the spatial proteomics field. Figures are clear and easy to understand.

Reply: We thank Reviewer #1 for the thorough and positive evaluation of our manuscript.

To a large part, the improvements of the workflow are convincingly shown by the provided data. However, an exception is the single nucleus application. Additional experiments are needed to really convince the reader that FAXP is indeed a reliable approach to analyse individual cells or sub-cellular structures such as nuclei. A closer look at the provided DIANN output tables (pg.matrix for relative protein levels) of the nucleus experiment brought up several questions:

1) I find it concerning that no histone and only few nuclear proteins were found in the provided dataset, which are typically among the most abundant proteins in the cell/nucleus. Instead, it is mostly keratins that show up in these proteome measurements, indicating a high contamination level. Please comment and revise.

Reply: We appreciate the reviewer's observation regarding the presence of keratins and the limited detection of histones and other nuclear proteins in our dataset. To address this concern, we have implemented several measures to reduce contamination, including wearing protective gear, conducting key procedures in a clean bench, and switching to MS-grade buffers. These steps have significantly decreased the abundance and number of keratins detected in our study. However, some keratin contamination persists due to the complexity of the FAXP workflow, which presents challenges in achieving the same level of cleanliness as conventional methods. This residual contamination might be attributed to the minimal amount of nuclear proteins present. Nevertheless, we are committed to further minimizing contamination and will continue refining our procedures in future studies.

In the current dataset, we have successfully identified histones and other nuclear proteins in relatively high abundance in the single-nucleus samples, exceeding levels observed in single-cell samples. This can be seen in Figure 4 and Supplementary Figure 8B. We hope this addresses the concern and provides reassurance regarding the quality of our data.

2) Also, proteome correlations are very poor (Pearson r = 0.6-0.8). These data are missing and should be added to better judge the current limitations when analysing single cells or nuclei.

Reply: As suggested by the reviewer, we have updated the single HeLa nucleus data with data from mouse liver FFPE tissue. In the new dataset, after QC filtering, the single-cell proteome correlation exceeds 0.92, and the single-nucleus correlation exceeds 0.86. Please refer to Figure 4G and Supplementary Figure 7 for details.

3) To convince the reader that the FAXP approach is useful to reliably analyse single nucleus proteomes when coupled to LCM, the authors should compare single nuclei to single whole cells. A quantitative comparison should then reveal major differences in proteins related to e.g. cytosol, mitochondria and nucleoplasm and chromatin related proteins. An example of such analysis can be found in the cited Deep Visual Proteomics publication (see Fig. 3C). I suggest to also show this for tissue derived hepatocytes as the authors first introduce murine liver in Fig. 4, but then don't provide any proteomics data and instead focus on Hela cells.

Reply: We thank Reviewer #1 for the valuable suggestion. As suggested by the reviewer, we have replaced the HeLa data with mouse liver FFPE tissue data for better consistency. We have conducted a quantitative comparison between the single nucleus and the single hepatocyte derived from mouse liver. This analysis reveals higher expression of nucleoplasm and chromatin-related proteins in the single nucleus, and higher expression of mitochondrial, endoplasmic reticulum, Golgi apparatus and peroxisome-related proteins in the single hepatocyte. This is consistent with the findings in the Deep Visual Proteomics (DVP) publication (Fig. 3C in DVP)[1]. The details of this analysis are presented in Figure 4H of the revised manuscript.

4) The reported depth of analysis of 1400 protein groups per Hela nucleus seems very overstated due to the presence of many non-unique rows where the same protein id shows up several times. It is therefore recommended to report only unique proteins groups, which can be done using the --relaxed-prot-inf command under Additional Options. Please see the DIANN manual for more info: https://github.com/vdemichev/DiaNN. How any proteins would then be identified? Please revise accordingly.

Reply: Thank you for the suggestion. We have acquired new MS data using further optimized protocols which led to much better proteomic depth. We have also analyzed our data using the "--relaxed-prot-inf" command in DIA-NN to ensure we report only unique protein groups. After applying this command, from the new data, we identified an average of 2368 unique protein groups per hepatocyte nucleus (n = 5) and 3312 unique protein groups per whole hepatocyte (n = 6). We have updated the manuscript to reflect these revised numbers, ensuring accurate reporting of our proteomics data.

Additionally, to improve sample preparation for these minute samples from FFPE tissue, we included the surfactant n-dodecyl-β-d-maltoside (DDM) to enhance enzymatic digestion efficiency and maximize peptide recovery, as recommended by Tsai, et al.[2]

5) An interesting application of the FAXP approach is together with immunofluorescence staining. To show that this is possible, an interesting approach would be to use the DAPI stained hepatocyte nuclei together with a plasma membrane marker and then distinguish diploid cells from polyploid hepatocytes. Can FAXP be used to quantitatively compare these proteomes of these different phenotypes?

Reply: Thank you for the insightful suggestion. We used asialoglycoprotein receptor 1 (ASGR1), a hepatocytic plasma membrane marker[3], in combination with nuclear staining to distinguish mononuclear hepatocytes from binuclear hepatocytes.

Our analysis revealed 26 differentially expressed proteins (DEPs) with a p-value < 0.05 (Figure R1), though no significant differences were observed with adjusted p-values, likely due to the relatively small sample size. The correlation between these cells was high (Supplementary Figure 8A). The histone expression levels (Supplementary Figure 8B) among these different phenotypes displayed similar patterns as previously reported[4]. Additionally, we identified proteins exclusively detected in binucleated cells (129 proteins) or mononucleated cells (37 proteins; Supplementary Figure 8C).

We believe that these results might have been influenced by the sample selection from different areas, as we used only a general hepatocyte marker and randomized cell selection. This underscores the

importance of precise sample selection and suggests that additional markers may be needed for more accurate classification.

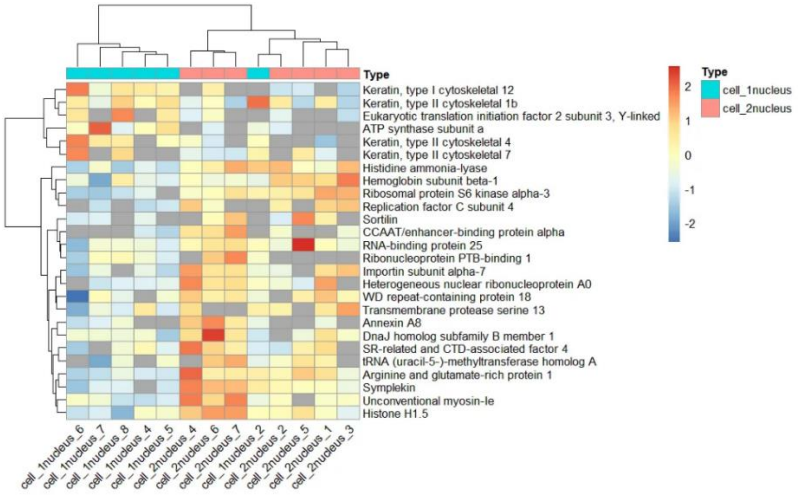

Figure R1 – DEPs between mononuclear and binuclear cells. P-values are estimated by Welch's t-test (two-sided).

Reviewer #2 (Remarks to the Author):

The manuscript by Dong et al. describes FAXP, a novel proteomics approach via filter-aided hydrogel-based tissue expansion. The major advantage of this protocol is that hydrogel-based tissue expansion increases the sample volume by up to 15 times (compared to the previously published ProteomeEx method), allowing for manual or laser-based dissection of very small, locally confined tissue areas. The authors demonstrate that with this protocol, they can obtain an average of 1420 proteins from a single HeLa nucleus. This is a significant achievement for the field. They further applied the protocol to different malignant areas of colorectal cancer tissues from three patients and measured 6751 proteins, revealing various molecular and biological properties in these areas.

The authors have previously developed the ProteomeEx method and show how FAXP is superior to ProteomeEx in terms of sensitivity, speed, throughput, and volume expansion. In summary, this manuscript describes highly innovative and relevant work contributing to the timely topic of spatial omics and offering major advantages in clinical proteomics and beyond. The manuscript is well-written, and the figures are of high quality and underline the statements.

Reply: We thank Reviewer #2 for the thorough review and positive comment of our manuscript.

Major Comments:

1) PRIDE Repository: The authors have used PRIDE to publish RAW data. However, the CRC raw data are all labeled as ProteomeEx, and no FAXP-named files can be found. It seems 131 raw files should exist for CRC, which is not reflected in the current PRIDE repository. Please upload all files to PRIDE and name them carefully to allow for data re-use and reproducibility.

Reply: Thank you for highlighting this issue. We have carefully reviewed and corrected the labeling discrepancies. All 131 CRC raw files have been re-uploaded to the PRIDE repository in six batches. Detailed annotations for each CRC raw data file are now provided in the "20230404_FAXP_CRC_Batch_design.tsv" file. Additionally, we have revised the other data files that were previously labeled as ProteomEx.

2) CRC Application:

a)Replicates: Only CRC tissues from three patients were analyzed. However, as not all tissue types were present in all samples, several tissue types were only present in one or two patients. PC and CC occur only in patient 1. Is this why three slides were used to generate enough biopsies? If so, this means the same tissue areas might be present for PC and CC, unlike other tissue areas where this should not occur.

Reply: We appreciate the reviewer's observation. The tissue areas initially categorized as PC (peripheral carcinoma) and CC (central carcinoma) were both defined as C (carcinoma). We manually distinguished them to explore potential differences within these subcategories. To enhance clarity and comparability, we

have combined the PC (peripheral carcinoma) and CC (central carcinoma) categories into the original "C" (carcinoma) category for our analyses. The use of three consecutive slides from patient 1 ensured sufficient biopsies and diverse tissue representation. This consolidation addresses concerns about potential overlap and improves consistency across samples.

b) T-test: How was the t-test performed? It seems the technical replicates (from the same biological patient with different tissue regions) were treated as independent samples, which invalidates the t-test. I suggest including more patients to make this study more robust and allow for proper statistical analysis.
Reply: We appreciate the reviewer's valuable feedback. We recognize that using a t-test for the technical replicates, which come from the same biological patient but different tissue regions as well as different patients, may not be appropriate due to the dependency of samples. Our study involves three patients, with the first patient providing three consecutive slides, and the remaining two patients providing one slide each. Each slide contains different tissue regions, making a t-test unsuitable for this sample structure. To address this, we employed the limma package, which allows for the application of linear models and fits a mixed-effects model incorporating one random effect term. By adopting this mixed linear model approach, our statistical analysis is more robust and appropriate for the current dataset.

We agree that including more patients would contribute to the robustness of the study. However, due to current constraints, this is beyond the scope of our present work. We plan to address this in future studies to enhance the robustness and generalizability of our findings. We have revised our manuscript, including Figures 5-6 and Supplementary Figures 9-10, to reflect these changes.

c) Clinical Applications: From a clinical proteomics perspective, it would have been more exciting to dissect single CRC tumor cells to show their heterogeneity or showcase an entity with particular intra-tumoral heterogeneity, rather than examining larger areas of different tissue types, which could evenly be done with existing proteomics methods. The authors should also comment on sensitivity and whether important clinical biomarkers and drug targets, such as ADC targets (e.g., HER2/ERBB2), can still be found with FAXP.
Reply: We appreciate the reviewer's valuable feedback regarding the clinical applications of our study. We agree that dissecting single CRC tumor cells to showcase their heterogeneity would be an exciting direction, and it is indeed our next step. In this study, the sample sizes we analyzed were relatively small compared to bulk analysis. Based on our calculations, the manually dissected sample sizes were equivalent to approximately 258 mammalian cells in volume (BNID 100434). This scale allows us to capture nuanced differences within tissue regions and between patients without the need for high-end micro-dissection instruments.

Regarding sensitivity and the detection of important clinical biomarkers and drug targets, we utilized Ingenuity Pathway Analysis (IPA) to explore the landscape of DEPs across different stages of malignant changes compared to normal regions, finding potential clinical biomarkers (Figure 6D). Additionally, we used the ADCdb[5] to identify potential ADC targets among different stages of malignant changes (Figure 6E).

Minor Comments:
1) Title: The title seems more like a working title. I suggest adding more details about what can be achieved and the advantages of FAXP.
Reply: Thank you for the suggestion. We propose the title "Spatial proteomics analysis of single cells and subcellular organelles on tissue slides using filter-aided expansion proteomics" to better reflect the achievements and advantages of FAXP in the current study.

2) Comparison with ProteomeEx: Throughout the manuscript, there is a strong focus on comparing FAXP with ProteomeEx. I suggest reducing this focus and describing FAXP more generally and in comparison to other proteomics methods, such as DVP or methods focusing on cellular organelles.
Reply: Thank you for the suggestion. FAXP is indeed an advancement of ProteomEx, making it appropriate to compare the two to highlight FAXP's improvements. We also acknowledge the relationship between FAXP and DVP. In our view, FAXP is not competing with DVP, which uses LCM to dissect cell shapes. Instead, FAXP is directly compatible with DVP. Cells or tissues could be expanded with FAXP

and then followed by the DVP workflow. We have incorporated this comparison in the discussion of the revised manuscript. Specifically, we have emphasized FAXP's advantages in spatial resolution, particularly when combined with LCM, compared to other LCM-based methods, which was crucial for our subcellular level analysis.

3) Abbreviations: Please introduce abbreviations when used for the first time, e.g., MS, ROI.
Reply: Thank you for the reminder. We have introduced all abbreviations, such as MS and ROI, upon their first use in the revised manuscript.

4) Introduction: The last part of the introduction gives an idea about the study, however is too long and detailed for this section of the manuscript. I suggest shortening it and keeping the results at a very high level (lines 71-91).
Reply: Thank you for the suggestion. We have shortened the last part of the introduction.

5) GitHub Repository: It would be beneficial to provide the input data in the GitHub repository to enable anyone to reproduce the figures.
Reply: We have added the input data to our GitHub repository to facilitate the reproduction of the figures and ensure transparency and reproducibility of our study.

6) Results: Optimization of Tissue Expansion Procedures: Lines 94 to 129 could be improved for clarity. How were the methods optimized? For example, for reduction/alkylation, there is only a link to Figure 2H-I. However, the different conditions tested should also be described in the text. I can't find a description of how the tissue staining time could be improved by 86%. Please ensure each step is described in enough detail.
Reply: We appreciate the feedback regarding the optimization of tissue expansion procedures. To enhance clarity, we have added more details to the "Optimization of the Tissue Expansion Procedures" section by incorporating previous Supplementary Note 1 and Figure 2H legend.

Regarding the improvement in staining time, we acknowledge the reviewer's point. We have revised Figure 1B to focus on the comparison of time consumed for each step in the process to provide a clearer understanding of the improvements made. Specifically, the 86% improvement refers to the combined time for staining, expansion, and imaging, which decreased from 10.5 hours (41-30.5 hours) in ProteomEx to 1.5 hours (11.7-10.2 hours) in FAXP.

7) Throughout the results section, it is unclear on which tissue and how many replicates the results are presented. Please include this information in the description of each result in the manuscript text and not only in the figure legends.
Reply: Thank you for pointing this out. We have updated the results section to specify the tissue type and number of replicates for each result directly in the text, as well as in the figure legends.

8) Line 192: This sentence is unclear: "Moreover, it is usually very challenging to safely the thus isolated nucleus for downstream proteomic analysis."
Reply: We have revised the sentence for clarity.

9) Figure 1:
a) Clarify if LEF is always compared to the original sample size or the previous step in the protocol
Reply: We thank the reviewer for highlighting this point. In our experiments, the Linear Expansion Factor (LEF) is calculated by comparing the expanded sample size to the original, unexpanded sample size, rather than to any intermediate steps in the protocol. This method ensures that the LEF consistently reflects the degree of expansion relative to the initial sample, providing a clear and accurate measure of the expansion achieved. Please refer to legend of Figure 1 for further details.

b) In 1A, avoid comparison in red to ProteomeEx; make 1A specific to FAXP and use 1B for the comparison
Reply: We appreciate the reviewer's feedback. To address this concern, we have revised Figure 1A to focus solely on the FAXP workflow without comparisons to ProteomEx. This separation ensures that

Figure 1A is dedicated exclusively to outlining the FAXP process, while Figure 1B highlights the differences and improvements offered by FAXP in comparison to ProteomEx.

c) In 1A, clarify what "newly prepared sample" means. Are these cell culture experiments in contrast to FFPE blocks?

Reply: We appreciate the reviewer's request for clarification. In our manuscript, the term "newly prepared sample" specifically refers to FFPE blocks that were freshly cut into sections before the experiment. These samples are not from cell culture experiments, although we are capable of processing those as well. Rather, they are freshly sectioned FFPE tissue blocks, selected to ensure optimal sample quality for our analyses. This distinction emphasizes that the samples were not archived or stored for extended periods before use. We have updated this term in Figure 1A accordingly.

d) In 1A, change "Proteomic analysis" to "Proteomic measurement" to avoid confusion with data analysis

Reply: We appreciate the reviewer's suggestion. To avoid confusion between the experimental process and the subsequent data analysis, we have revised the term "Proteomic analysis" to "Proteomic measurement" in Figure 1A. This change clarifies that the reference is specifically to the measurement stage within the FAXP workflow.

e) Number Figure 1A and 1B with the same steps for direct comparison, e.g., Step 2 in A corresponds to 2-4 in B. In 1B, reduction and alkylation are mentioned twice, which is not reflected in 1A

Reply: We appreciate the reviewer's insightful suggestions. We have made the following changes to the figures, as reflected in the updated Figure 1 shown below:

1. In Figure 1A, we have changed the numbering from Arabic to Roman numerals to clearly distinguish the steps from those in Figure 1B.
2. In Figure 1B, we have listed which procedures correspond to the steps in Figure 1A.
3. Additionally, we have labeled the steps used in FAXP (indicated by a short red vertical line) or ProteomEx (indicated by a short blue vertical line) in front of each procedure listed in Figure 1B. This adjustment also clarifies why reduction and alkylation are mentioned twice: once for FAXP (Procedure No. 6 in Figure 1B) and once for ProteomEx (Procedure No. 10 in Figure 1B), which is not shown in Figure 1A.

f) Double-check the numbers in 1B, e.g., why does step 9 microdissection take no time for FAXP?

Reply: We appreciate the reviewer's comments. To clarify, the discrepancy in Figure 1B is due to a misunderstanding related to the steps. Specifically, it is not step 9 but rather step 10 that shows no time allocated for FAXP. This is because step 10 is exclusive to the ProteomEx workflow, as indicated by the short blue vertical line. Conversely, step 11 is unique to the FAXP workflow, and thus, there is no time allocated to this step for ProteomEx, which explains the time difference from 56.5 hours at step 10 to 56.5 hours at step 11.

g) Consider moving Figure 1B to the supplementary section as these details may not be necessary for the main manuscript

Reply: Thank you for the suggestion. We believe that Figure 1B is crucial for illustrating the advancements of FAXP compared to ProteomEx, especially regarding time efficiency. Given the significant improvements and detailed comparisons it provides, we feel that retaining it in the main manuscript enhances the clarity and impact of our work. Thus, we think it may be better to keep Figure 1B in the main text rather than moving it to the supplementary section.

10) Figure 2: Zoom into the y-axis to make differences more visible.

Reply: Thank you for the suggestion. We have zoomed into the y-axis in Figure 2 to make the differences more visible.

11) Figure 3: Zoom into the y-axis in panels B and C to make differences more visible.

Reply: Thank you for the suggestion. We have zoomed into the y-axis in Figure 3 to make the differences more visible.

12) Figure 6: Ensure the color scheme is accessible for color-blind readers. Add carcinoma (C) to plot F.

Reply: We have adjusted the color scheme regarding CRC application to ensure it is accessible for color-blind readers.

13) Discussion:
a) Maximum Tissue Size: Is there a maximum tissue size compatible with FAXP? Comment on this in the discussion/limitations. Cryopreserved Tissues: Is this method compatible with cryopreserved tissues?
Reply: We appreciate the comments. FAXP does not impose a limitation on tissue size as long as the tissue can be attached to a standard glass slide (25 mm × 75 mm). It is compatible with thin FFPE tissue sections below 10 microns and can handle thicker sections up to 120 microns when processed by vibratome sectioning. Additionally, FAXP is compatible with cryopreserved tissues, which is easier since it eliminates the need for dewaxing and rehydration steps. We have included these details in the discussion.

b) Heterogeneity: The statement about FAXP's ability to discern heterogeneity in disease progression based on tissue regions of only three patients is an overstatement.
Reply: Thank you for your feedback. We acknowledge that the original statement was overstated given the limited number of patients. We have revised the wording to "variability".

c) Single-Cell Nucleus Study: Is this the first study on a single-cell nucleus? If so, emphasize this throughout the manuscript. If not, compare it to similar methods.
Reply: Yes, this is the first spatial proteomics study focusing on a microdissected single-cell nucleus. We have highlighted this throughout the manuscript.

d) Subcellular Organelles: Discuss the feasibility of dissecting other subcellular organelles for MS analysis in more detail, considering the nucleus is the largest organelle and thus the easiest to analyze.
Reply: This study primarily targets the nucleus due to its size and the challenges of expanding FFPE tissues, especially in clinical specimens like CRC. However, FAXP combined with LCM shows promise for analyzing smaller organelles, such as vacuoles (2.1 μm; BNID: 107609), peroxisomes (0.1-1 μm; BNID: 106729), and mitochondria (0.5 μm; BNID: 110892). Future research could focus on optimizing FAXP for these smaller structures, but current advancements are centered on larger organelles. These considerations are discussed in the limitations section.

e) Spatial Transcriptomics: The statement about spatial transcriptomics is incorrect. While compatible with FFPE, RNA degrades, leading to lower quality. Comparison with spatial transcriptomics is unnecessary here. Discuss other proteomics methods instead.
Reply: We appreciate the feedback. We have revised the manuscript in the discussion to focus on comparisons with other spatial proteomics methods rather than spatial transcriptomics.

14) Methods:Lines 376-378: These lines read like results. Is this sentence in the wrong place?
Reply: Thank you for pointing this out. The lines in question describe FAXP's compatibility with DAPI staining, which relates to earlier optimization results. Since we have achieved subcellular proteomics identification using immunofluorescence and nuclear staining for mouse liver tissue, we have removed these lines and the associated supplementary figure to avoid redundancy.

15) Limitations Section: This section is a bit unstructured and contradicts parts of the results (e.g., regarding the extracellular matrix). Reorganize for clarity.
Reply: Thank you for your feedback. We have reorganized the limitations section for clarity.

Reviewer #3 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
Reply: Thank you for your effort in providing valuable reviews for us.

Reviewer #4 (Remarks to the Author):
Dong and colleagues introduce a method termed Filter-aided expansion proteomics (FAXP), which

combines hydrogel-based cell or tissue expansion with mass spectrometry (MS) for high spatial resolution proteomics analysis. As stated by the authors, the idea of combining expansion with MS has been demonstrated before by Drelich et al., 2021 (PMID: 34449217) and by the authors themselves, Li et. al., 2022 (PMID: 36450705). However, the authors have significantly enhanced their protocols and introduced novel steps (namely, in-gel in-tip digestion-combined with the physical expansion of specimens), resulting in a substantial 50% reduction in sample preparation time. Additionally, they achieved around two-fold increase in lateral resolution, and increased the protein identification.
The manuscript is written clearly. The figures are nicely executed and clear. The experimental data is convincing. FAXP strongly improves the existing expansion-proteomics analysis tools introduced by the authors in Nature Communications in 2022. The current results are better than the ones published in 2022, albeit the authors do not show a fundamental improvement in biological analyses.
Reply: Thank you for your positive feedback on the manuscript.

Major comments:
1. The authors show that shortening the anchoring time to 1-hour has yielded a 4.4 expansion factor, while a 2-hour anchoring has decreased the expansion factor to 3.1 which what would be expected for X4 expansion protocols in tissue sections. A concern arises from the fact that shorter anchoring time leads to less amine-reactive entities getting the anchor, hence less proteins being incorporated into the gel. This could explain the higher expansion factor. Did the authors apply their FAXP analysis onto 2 hours anchored 10 microns thick tissues? Would the peptide yield be comparable to that of 1-hour anchoring? Figure S1. does not provide enough information to rule this out.
Reply: To address this concern, we conducted an additional experiment comparing peptide yields from mouse liver tissues anchored for 1 hour versus 2 hours (Supplementary Figure 1C). We analyzed six consecutive 10-micron thick sections with three biological replicates for each condition. The results showed an average peptide yield of 21.68 µg for 1-hour anchoring and 20.64 µg for 2-hour anchoring. Statistical analysis indicated no significant difference between the two conditions. This data confirms that a shorter anchoring time does not adversely affect peptide yield and addresses the reviewer's concern effectively.

2. It is not clear how the volumetric enhanced resolution was calculated to reach a claimed 14.5-fold increase. The authors state that the lateral resolution is enhanced by 2.2-fold. One would assume that the enhancement in volumetric resolution would be 2.2(x) x 2.2(y) x 2.2(z) = 10.6. The authors should clarify how they calculated the 14.5 value. Ultimately, the volume resolution is rather meaningless, given that the authors excise a piece of a defined area from a flat sample. In other words, the authors' technique presents a 2D picture of the specimen, in which the volume is almost meaningless.
Reply: Thank you for raising this point. The lateral and volumetric resolution were calculated based on data from Figure 4B. In the ProteomEx study[6], it was documented that "ProteomEx enables quantitative profiling of the spatial variability of the proteome in mammalian tissues at ~160 µm lateral resolution, equivalent to a tissue volume of 0.61 nL." Based on similar calculations, FAXP achieves a lateral resolution of ~73 µm, which corresponds to a 2.2-fold improvement (160/73). For volumetric resolution, ProteomEx recorded 0.61 nL, whereas FAXP achieves 0.042 nL, resulting in a 14.5-fold improvement (0.61/0.042).

The volumetric resolution is not simply the cubic of the lateral resolution increase (10.6-fold) because the thickness of the current study's section is 10 µm, whereas ProteomEx processes 30-µm sections. Additionally, the 14.5-fold increase in volumetric resolution indicates that we can achieve this resolution manually, without the need for high-end instruments like LCM, making it more accessible for laboratories. We have included these calculations and further clarifications in the discussion.

Regarding the relevance of volume in a 2D sample, it's important to note that while we excise a defined area from a flat sample, the thickness of the tissue section remains critical for constructing a 3D picture in proteomics analysis. Additionally, ProteomEx did not report its capability for processing thin FFPE sections, so FAXP's ability to handle reduced thickness in FFPE sections represents an advancement. Therefore, the introduction of volumetric resolution in our study is primarily intended for benchmarking purposes.

3. The manuscript adequately details the technical methodology but lacks a critical discussion on the sensitive steps of the expansion and MS procedures, experiment critical steps, failures and troubleshooting. To enhance reproducibility, the authors must include a Supplementary Discussion section addressing the pivotal bottleneck steps of FAXP and tips to overcome them.
Reply: Thank you for your suggestion. We have included a Supplementary Discussion section as "Supplementary Note 1. Critical steps and troubleshooting in FAXP and MS procedures".

4. The authors should add a section in which they compare their technology with other visual proteomics protocols (for example from M. Mann's laboratory), explaining the advantages and disadvantages of different tools.
Reply: Thank you for the suggestion. We have added a comparison section in the discussion, where we discussed the FAXP and LCM-based spatial proteomics, like DVP.

Minor comments:
1. All statistical tests and their respective types should be mentioned in the figure legend for each panel in each figure.
Reply: Thank you for pointing this out. We have updated the figure legends to include the details.

2. Although the robot-automated steps constituted a minor part of the FAXP method, it would be beneficial for the authors to discuss the time savings achieved through automation. This is particularly important as not all laboratories have access to such advanced tools.
Reply: Thank you for your comment. The development of FAXP tips indeed facilitates automation within the FAXP workflow. However, even without these advanced tools, high throughput can still be achieved using an 8-channel pipette and a centrifuge that accommodates 96-well plates. In practice, the time savings offered by automation may not be significantly greater than with these more accessible tools. The primary purpose of automation is to ensure the accuracy and consistency of the experiments, as well as minimizing contamination at the moment. In the future, a fully automated protocol is required to reach higher throughput, and for handling samples with higher spatial resolution.

3. The authors should consider the fact that their technology would be most attractive if laboratories could reproduce their robotic applications. Otherwise, interest in this technology may be limited. I suggest that the authors make greater efforts to present this technology (the robotics part) in an easily reproducible fashion.
Reply: Thank you for your suggestion. As mentioned in our previous response, FAXP tips can be effectively used with more accessible tools such as an 8-channel pipette and a centrifuge that accommodates 96-well plates. This setup allows laboratories to achieve high throughput without requiring specialized robotics. Further efforts are needed to standardize robotics for broader applications.

4. I am missing accurate values in some critical steps of the expansion procedure, and in some instances, there are contradictions. The authors are responsible to provide exact information about the used protocols. Careful revision of the Methods section is required. I provide couple of examples below:
a. Line 423-424: "For gel-making, 50-100 µL of protein anchoring solution was added to the fixed cells and incubated at room temperature for 1.5 hours." Is it 1.5 hours or 1 and 2 hours as stated in Figure 2E?
Reply: Thank you for pointing out the inconsistency. We apologize for the confusion. The 1.5-hour anchoring time mentioned was specific to an earlier version of our study using HeLa cell expansion, which has since been replaced by mouse liver hepatocyte data (now shown in Figure 4), as suggested by reviewer #1. In the revised manuscript, we have standardized the anchoring time for all FFPE tissue samples to 1 hour, as indicated in Figure 2E.

b. Line 430: "…nitrogen gas atmosphere for 2-3 hours", is it 2 or 3 hours? One of the hallmarks of this paper is speed. For reproducibility, exact values are required.
Reply: Thank you for your observation. While both 2 and 3 hours are acceptable, a 3-hour incubation under a nitrogen gas atmosphere yields better outcomes. To ensure consistency and reproducibility, we have standardized this step to 3 hours across the manuscript.

5. In Table S4, section for the text “Mix 25 mL 0.2 M MES stock solution with 25 mL ddH2O to make the final volume at 50 mL and test if the pH is at 6.0.”, cite Tillberg et al., 2016: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5068827/, as this anchoring buffer was first described here.
Reply: Thank you for bringing this to our attention. We have now cited the Tillberg et al., 2016 paper in Supplementary Table 4 to acknowledge the original description of the anchoring buffer.

6. Line 103: “adjustment significantly enhanced the sturdiness of the hydrogel, in consistent with a..:”, remove “in” from “in consistent”.
Reply: We have removed the word "in" from "in consistent" as suggested.

7. Line 223: “…based on median values, respectively (Figure 5D), in consistent with previous reports…”, remove “in” from “in consistent”.
Reply: We have removed the word "in" from "in consistent" as suggested.

8. While most of the figures are nicely executed, Figure 2, gave me trouble understanding it. For instance, it took me a while to understand what does a, b, c, d, and e, mean in panel (I). Naturally, the reader would think that panels (A,B,C, and D) where involved, then I was wondering what refers to (e), as panel E was a graph; only later to find out that the annotations are present in panel (H).
Reply: We appreciate the reviewer's feedback on Figure 2. To address the confusion, we have updated the condition labels in panel (I) from letters (a-e) to numbers (①-⑤) to clarify their correspondence with the annotations in panel (H). We believe this change will make the figure more intuitive and easier to interpret.

9. Panel (H) in Figure 2, could get a little adjustment by either removing the four contour boxes from conditions b-e, or by adding a contour box around condition a for consistency.
Reply: Thank you for the suggestion. We have added a contour box around condition a in panel (H) to maintain consistency and improve the overall clarity of the figure. We believe this adjustment enhances the visual coherence of the panel.

10. One possible suggestion (and not a must) is to slightly edit the panel positions in Figure 2. For instance, moving panel G and placing it next to panel F, while moving panel H to occupy a complete row under panels F and H. This would of course require reordering the content of panel H in a landscape fashion.
Reply: Thank you for the suggestion. We have repositioned the panels in Figure 2 as advised. Specifically, we moved panel G next to panel F and rearranged panel H to occupy a full row beneath panels F and G, reordering the content of panel H in a landscape orientation. This reorganization enhances the figure’s layout and improves readability.

References

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2 Tsai, C.-F. *et al.* Surfactant-assisted one-pot sample preparation for label-free single-cell proteomics. *Communications Biology* **4** (2021). https://doi.org:10.1038/s42003-021-01797-9
3 Zhang, Z. *et al.* Deficiency of ASGR1 promotes liver injury by increasing GP73-mediated hepatic endoplasmic reticulum stress. *Nature Communications* **15**, 1908 (2024). https://doi.org:10.1038/s41467-024-46135-9
4 Rosenberger, F. A. *et al.* Spatial single-cell mass spectrometry defines zonation of the hepatocyte proteome. *Nature Methods* **20**, 1530-1536 (2023). https://doi.org:10.1038/s41592-023-02007-6
5 Shen, L. *et al.* ADCdb: the database of antibody–drug conjugates. *Nucleic Acids Research* **52**, D1097-D1109 (2023). https://doi.org:10.1093/nar/gkad831
6 Li, L. *et al.* Spatially resolved proteomics via tissue expansion. *Nature Communications* **13**, 7242 (2022). https://doi.org:10.1038/s41467-022-34824-2

Point-by-point response to the reviewers' comments:

We sincerely thank the reviewers for their positive feedback on our manuscript. We have addressed all the comments and made the suggested revisions.

Reviewer #1 (Remarks to the Author):
The authors did a great job in addressing my concerns. I have no further comments and congratulate them on their work.
Reply: Thank you for the kind feedback and support.

Reviewer #2 (Remarks to the Author):
The authors have addressed all reviewer comments appropriately and the revised manuscript is well-written and provides valuable insights for spatial proteomic investigations upon publication.
As a minor improvement and to clarify the biomarker aspects in Figure 6, the authors could think about adding a table of the DEPs / biomarkers and the aspects (Prognosis, Response to therapy) that they are associated with.
Reply: Thank you for the suggestion. The table has been included in the corresponding source data as requested.

Reviewer #3 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
Reply: Thank you for your contribution.

Reviewer #4 (Remarks to the Author):
Dong and colleagues have thoroughly addressed all the points I raised to my satisfaction. It's evident that they put in extra effort and conducted additional experiments to resolve all concerns. The manuscript is now clearer, and the added points in the discussion and supplementary notes are excellent. All the suggested corrections and edits have been implemented. With good faith, I unequivocally recommend this study for publication in Nature Communications as fast as possible.
Reply: Thank you for your kind and encouraging feedback.