Cell Systems

Transparent Peer Review Record

A framework for ultra-low input spatial tissue proteomics

Anuar Makhmut, Di Qin, Sonja Fritzsche, Jose Nimo, Janett König, and Fabian Coscia

Summary

Initial Submission: Received June 1, 2023
Preprint: doi.org/10.1101/2023.05.13.540426
Scientific editor: Bernadett Gaal, DPhil

First round of review: Number of reviewers: 3
3 confidential, 0 signed
Revision invited July 13, 2023
Minor changes anticipated
Revision received Aug 3, 2023

Second round of review: Number of reviewers: 2
2 original, 0 new
2 confidential, 0 signed
Accepted Oct 6, 2023

Cell Systems Transparent Peer Review Record

Editorial decision letter with reviewers' comments, first round of review

Dear Fabian,

I hope this email finds you well. The reviews are back on your manuscript and I've appended them below. You'll see that the reviewers find the manuscript compelling and their comments are intended to strengthen an already strong piece of work. We're happy to invite a revision.

If you have any questions or concerns about the revision, I'd be happy to talk about them, either over email or by Zoom. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

I look forward to seeing your revised manuscript.

All the best,

Bernadett

Bernadett Gaal, DPhil
Editor-in-Chief, *Cell Systems*

Reviewers' comments:

Reviewer #1: Coscia and his team present an interesting and valuable methods paper, which provides a detailed protocol for proteomic analysis of very small FFPE samples after laser microdissection. This work is a direct continuation of the DVP work that Coscia was a part of, and here he provides many more details and calibrations that further optimize this approach. As the spatial proteomics field is a only now emerging, every protocol and optimization has a lot of value to the community. Overall, the manuscript is very nicely written and presented, takes the reader step by step through the entire sample preparation. In addition, they also show the value of the work through examination of specific biological examples.
My comments are primarily related to the specific topics that were emphasized more in the text vs. the supplementary material.
1. In the first section of the results the authors discuss the impact of antigen retrieval and type of slides. The selection of of Hier vs. Pier is typically dictated by the specific antibody, and their own result of higher background with Pier is not relevant to many other antibodies. Also the discussion of slide distortion, doesn't seem like a general issue. Given that the end result is that there is no difference between these methods, I advise to present the results but shorten the text to a couple of sentences. Generally, I think the paper should emphasize only the generalizable information, and anything potentially specific to an antibody, batch of slides should be shortened.
2. In continuation of the previous comments, there are topics that I think require more emphasis. For example the comparison of lysis buffers is much more important and should be presented in the main figures. This is an example of a generalizable result, which actually has impact on the analytical depth.

Reviewer #2: Makhmut and co-authors present a study on the feasibility and advancement of spatial proteome analysis at the resolution of single cells or small phenotypically defined tissue regions using a combination of multiplex IHC imaging, laser capture microdissection and low input proteomics sample process and LC-MS/MS acquisition.
The study addresses a very timely need and describes considerable methodological and conceptual advances. I particularly commend the authors for their attention to detail and in-depth SOPs that will make this work a go-to resource!

The data is overall of excellent quality and the manuscript is well written. I did not identify any major concerns but have a few suggestions for minor edits and additional analyses that would provide considerable additional insight and increase the value of the work further.

The effect of sample overloading described on pages 6, 9 and following should be investigated and characterized more closely. Is there any indication of in TIMS cell fragmentation? Does the observed loss in IDs and increase in CV occur evenly across proteins or is it correlated with peptide/protein intensity? If so, a consideration for possible implications for biological interpretation should be added to the discussion.

The manuscript uses tissue area/volume as and cell number estimates as measures of sample input throughout. This makes absolute sense in the context of the study but makes it difficult to compare to other studies that use isolated/suspension cells. It would be very helpful if the authors could conduct a calibration experiment where they measure/estimate the protein amount extracted from the tissue regions of varying area/volume. This could be done by sensitive protein or peptide quantification or by calibrating their MS signal intensity against a peptide dilution series.

The manuscript lacks any mention of other spatial profiling methods. I would be important to compare and contrast their method with e.g. spatial transcriptomic approaches. While a direct quantitative comparison of RNA to protein levels is probably beyond the scope of this paper it would be important to compare metrics like coverage (quantified transcripts or proteins), CV, dynamic range, cost, time.

An analysis of the data completeness between single cell contours should be added (acknowledging that some of the missing data may be true heterogeneity). This should be demonstrated with and without match between runs.

Minor comments:
Figure 1D: I would recommend to also provide CV information

Figure 1F: This would be better visualized by an upset plot

Figure 1G: I would recommend to change the sort order. Here the key comparison is for each compartment term between methods rather than different terms within methods (as facilitated by the current order). Grouping by compartment would facilitate direct comparison. Also, the gradient fading of the color is irritating and should be removed.

Figure 2 C-E: remove color. It is not required and distracts

Figure 2F: it is not clear which of the hepatocyte markers got identified by single cell contours. It is

also not clear if this represents identification by a single cell contour or the union of all single cell contours.

Figure 4: it would be helpful to add a figure showing the distribution of missing values (e.g. heatmap with missing values clearly colored)

Page 6 line 8: Please specify the nature of replica measurements (different regions or injections?) that the CV is based on. This becomes clear later in the text but would be very helpful to already specify at this point.

The effect of varying extracellular matrix composition and cellularization of tissue regions should be mentioned when optimal tissue amounts are discussed.

The use and type of imputation should already be mentioned in the results section and not only in the methods. This would improve understanding of the presented data.

In the SOP supplement it would be interesting if the authors to elaborate on their choice of evotips for peptide cleanup (without use on an evosep-one LC system) rather than more commonly used custom made C18 or SDB-RPS tips.

The authors should consider organizing their files in the pride repository in a way that complies with a full submission to facilitate re-analysis (even though I recognize that this is a lot of work and unnecessarily complex to do)

Reviewer #3: This manuscript tried to address the challenges of single-cell proteomics of FFPE samples using the laser microdissection. Their work integrates high-content imaging (QuPath/BIAS), laser microdissection (LMD7), optimized sample preparation methods, and ultra-high sensitivity mass spectrometer (timsTOF SCP). This method, leveraging the enhanced sensitivity of timsTOF SCP, has identified around 2,000 proteins in single murine hepatocytes and approximately 5,000 proteins in 50-cell contained tissues. The methodology was further applied in human tonsils, successfully identifying an average of 1,952 proteins in 146 microregions, resolving their spatial heterogeneity. This work provides a tissue-level sample processing and analysis solution with single-cell resolution. The manuscript is well-organized and written.
The reviewer requests that the authors address the following issues to further improve the manuscript.

Major issues:
1. The manuscript should provide a more comprehensive and direct comparison between the proposed workflow and the methodology employed by the DVP workflow published by Mann's group (Mund, A., et al. Deep Visual Proteomics defines single-cell identity and heterogeneity. Nat Biotechnol 40, 1231-1240 (2022). https://doi.org/10.1038/s41587-022-01302-5).
2. Considering that PEN glass slides cannot tolerate high temperatures (over 70°C) and may become distorted during processing, a study (Nordmann, T.M., et al., A standardized and reproducible workflow for membrane glass slides in routine histology and spatial proteomics. bioRxiv, 2023: p. 2023.02.20.529255.) addresses the glass slide issues. It would be appreciated to discuss how the study addresses this limitation compared to the other study.
3. In comparison to a similar study conducted by Mann's group (Rosenberger, F.A., et al., Spatial single-cell mass spectrometry defines zonation of the hepatocyte proteome. bioRxiv, 2022: p. 2022.12.03.518957.), which appears to have a more comprehensive workflow and achieves better performance in terms of results with murine hepatocytes, the authors may clearly state the strengths

and advantages of the current work over that study.

Minor issues:
1. "Importantly, irrespective of the choice of antigen retrieval (HIER or PIER), staining technique (H&E or IF), or LMD slide type (PEN or PPS), proteomics results from three different liver tissue amounts were highly consistent."-- What are the data supporting this claim in the Results?
2. In Fig 1H, can you also test slides from other companies, such as MMI's frame slide?
3. On page 21: "Afterwards, the specimens were cut into 5-μm-thick slices, and two representative slices were embedded in paraffin for further microscopic diagnosis." This procedure seems strange. Can you please double-check this is the correct protocol?
4. In Figure S1B, the max values in the y axis are all 6. This is incorrect. Please double check.
5. Error bars should be added to Figures 2C and 2D.
6. The authors should clarify the comparison in Figure S2G, specifically indicating which protocol is organic solvent-based and why it is preferred over the DDM/ACN combination for higher tissue amounts.
7. The manuscript should elucidate the methodology used to determine the number of cells in the 50-cell samples (50,000 μm^2, 5 μm thick). The authors use area or volume in different places to show the cell numbers, which is confusing; please make them consistent. A BNID accession number may be referenced for the calculations if applicable.
8. The number of replicates used in Figure 3D should be specified, particularly given the observed lack of linear relationships in human tonsil data.
9. The authors should clarify the meaning of regions labeled in black in Figure 4C, particularly the light, grey, and dark zones. It is also essential to rectify the discrepancy in the sum of squamous cell epithelium (5), follicles (10+106), and interfollicular T-cell zone (34), which currently does not add up to 146.
10. Statistical analysis should be added to Figures 4E and 4F to provide information regarding significance.
11. On page 22, it is suggested to include the exact concentrations of antibodies used in conjunction with the dilutions for enhanced reproducibility.

Authors' response to the reviewers' first round comments

Attached.

Editorial decision letter with reviewers' comments, second round of review

Dear Fabian,

I'm very pleased to let you know that the reviews of your revised manuscript are back, the peer-review process is complete, and only a few minor, editorially-guided changes are needed to move forward towards publication.

In addition to the final comments from the reviewers, I've made some suggestions about your manuscript within the "Editorial Notes" section, below. Please consider my editorial suggestions

carefully, ask any questions of me that you need, make all warranted changes, and then upload your final files into Editorial Manager.

I'm looking forward to going through these last steps with you. Although we ask that our editorially-guided changes be your primary focus for the moment, you may wish to consult our FAQ (final formatting checks tab) to make the final steps to publication go more smoothly. More technical information can be found below my signature, and please let me know if you have any questions.

All the best,

Bernadett

Bernadett Gaal, DPhil
Editor-in-Chief, Cell Systems

Editorial Notes

Transparent Peer Review: Thank you for electing to make your manuscript's peer review process transparent. As part of our approach to Transparent Peer Review, we ask that you add the following sentence to the end of your abstract: "A record of this paper's Transparent Peer Review process is included in the Supplemental Information." Note that this ***doesn't*** count towards your 150 word total!

Also, if you've deposited your work on a preprint server, that's great! Please drop me a quick email with your preprint's DOI and I'll make sure it's properly credited within your Transparent Peer Review record.

Article type: The manuscript is currently submitted as a Research Article. We feel that it would be better suited to be published a Methods paper or possibly a Report if you prefer. Please let me know if you have any questions about this!

Manuscript Text:

- House style disallows editorializing within the text (e.g. strikingly/striking, surprisingly, importantly, etc.), especially the Results section. These terms are a distraction and they aren't needed—your excellent observations are certainly impactful enough to stand on their own. Please remove these words and others like them. "Notably" is suitably neutral to use once or twice if absolutely necessary.
- Please double check that you use the word "significantly" in the statistical sense only.

Figures and Legends:

*P*lease look over your figures keeping the following in mind:

- When color scales are used, please define them, noting units or indicating "arbitrary units," and specify whether the scale is linear or log.
- Bar graphs are not acceptable because they obscure important information about the distributions of the underlying data. Please display individual points within your graphs unless their large number obscures the graph's interpretation. In that case, box-and-whisker plots are a good alternative.
- Please ensure that every time you have used a graph, you have defined "n's" specifically and listed statistical tests within your figure legend.
- When figures include micrographs, please ensure that scale bars are included and defined within the legend, montages are made obvious, and any digital adjustments (e.g. brightness) have been applied equally across the entire image in a manner that does not obscure characteristics of the original image (e.g. no "blown out" contrast). ***Note that all accepted papers are screened for image irregularities, and if this advice is not followed, your paper will be flagged.***
- Please ensure that if you include representative images within your figures, a "representative of XXX individual cells"-type statement is made in the legend.
- Please ensure that all figures included in your point-by-point response to the reviewers' comments are present within the final version of the paper, either within the main text or within the Supplemental Information.

Supplemental Information: Please name the "Detailed sample preparation protocol" section Method S1 and refer to it as such in the main text if needed.

STAR Methods: Note that Cell Press has recently changed the way it approaches "availability" statements for the sake of ease and clarity. Please revise the first section of your STAR Methods as follows, noting that the particular examples used might not pertain to your study. Please consult the STAR Methods guidelines for additional information.

RESOURCE AVAILABILITY

Lead Contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jane Doe (janedoe@qwerty.com).

Materials Availability: This study did not generate new materials. *-OR-* Plasmids generated in this study have been deposited at [Addgene, name and catalog number]. *-OR-* etc.

Data and Code Availability:

- **Source data statement** (described below)
- **Code statement** (described below)
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Data and Code Availability statements **have three parts and each part must be present. Each part should be listed as a bullet point, as indicated above.**

Instructions for section 1: Data. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. Please ensure that all datatypes reported in your paper are represented in section 1. For more information, please consult this list of standardized datatypes and repositories recommended by Cell Press.

- [Standardized datatype] data have been deposited at [datatype-specific repository] and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

- [Adjective] data have been deposited at [general-purpose repository] and are publicly available as of the date of publication. DOIs are listed in the key resources table.

- [De-identified human/patient standardized datatype] data have been deposited at [datatype-specific repository]. They are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

- [De-identified human/patient standardized datatype] data have been deposited at [datatype-specific repository], and accession numbers are listed in the key resources table. They are available upon request until [date or delete "until"] if access is granted. To request access, contact [insert name of governing body and instructions for requesting access]. [Insert the following when applicable] In addition, [summary statistics describing these data/processed datasets derived from these data] have been deposited at [datatype-specific repository] and are publicly available as of the date of publication. These accession numbers are also listed in the key resources table.

- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.

- [Adjective or all] data reported in this paper will be shared by the lead contact upon request.

Instructions for section 2: Code. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. ***If you are using GitHub, please follow the instructions here to archive a "version of record" of your GitHub repo at Zenodo, then report the resulting DOI. Additionally, please note that the Cell Systems strongly recommends that you also include an explicit reference to any scripts you may have used throughout your analysis or to generate your figures within section 2.***

- All original code has been deposited at [repository] and is publicly available as of the date of publication. DOIs are listed in the key resources table.

- All original code is available in this paper's supplemental information.

- This paper does not report original code.

Instructions for section 3. Section 3 consists of the following statement: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

In addition,

STAR Methods follows a standardized structure. Please reorganize your experimental procedures to include these specific headings in the following order: LEAD CONTACT AND MATERIALS AVAILABILITY (including the three statements detailed above); EXPERIMENTAL MODEL AND SUBJECT DETAILS (when appropriate); METHOD DETAILS (required); QUANTIFICATION AND STATISTICAL ANALYSIS (when appropriate); ADDITIONAL RESOURCES (when appropriate). We're happy to be flexible about how each section is organized and encourage useful subheadings, but the required sections need to be there, with their headings. They should also be in the order listed. Please see the STAR Methods guide for more information or contact me for help.

Please ensure that the standardized datasets generated in this paper has been archived in at least one datatype-specific repository recommended by Cell Press (e.g. GEO, PRIDE, etc.). If your data are not standardized, we recommend that you deposit them in a general purpose repository recommended by Cell Press. Please provide your datasets' accession numbers/DOIs in Deposited Data section of the Key Resources Table. Thank you!

Thank you!

Reviewer comments:

Reviewer #2: We thank the reviewers for a careful and thorough revision of their manuscript. The authors addressed all major concerns and improved the manuscript.

My only recommendation for further improvement would be to include Response to Reviewers Figure 2 (Estimated sample yield) as a supplementary figure so that all reader can benefit from this information.

Reviewer #3: Thanks for the revisions. I have no more comments. Congratulations for the good work.

Point-by-point response to the reviewers' comments

We thank all reviewers for their thorough, constructive and very positive evaluation of our manuscript entitled "*A framework for ultra-low input spatial tissue proteomics*". We believe that in the revised version, all points are now addressed, further strengthening our manuscript. In a nutshell, we included some additional analyses and edited figures and text passages according to the suggestions. In the pages below, each of the reviewers' comments are addressed in more detail. We provide data directly in those cases where it wasn't possible to incorporate it into the revised manuscript.

Reviewer #1: Coscia and his team present an interesting and valuable methods paper, which provides a detailed protocol for proteomic analysis of very small FFPE samples after laser microdissection. This work is a direct continuation of the DVP work that Coscia was a part of, and here he provides many more details and calibrations that further optimize this approach. As the spatial proteomics field is a only now emerging, every protocol and optimization has a lot of value to the community. Overall, the manuscript is very nicely written and presented, takes the reader step by step through the entire sample preparation. In addition, they also show the value of the work through examination of specific biological examples.

We are very pleased to read the reviewer's very positive remarks and thank the reviewer for these additional comments to further strengthen our manuscript. We fully agree that these suggestions have further improved our manuscript.

My comments are primarily related to the specific topics that were emphasized more in the text vs. the supplementary material.
1. In the first section of the results the authors discuss the impact of antigen retrieval and type of slides. The selection of of Hier vs. Pier is typically dictated by the specific antibody, and their own result of higher background with Pier is not relevant to many other antibodies. Also the discussion of slide distortion, doesn't seem like a general issue. Given that the end result is that there is no difference between these methods, I advise to present the results but shorten the text to a couple of sentences. Generally, I think the paper should emphasize only the generalizable information, and anything potentially specific to an antibody, batch of slides should be shortened.

We thank the reviewer for this comment. We have revised the main text and Fig. 1 accordingly. We agree that different antibodies could result in different staining backgrounds when comparing Hier vs. Pier, which we have made clearer in the text. We therefore also moved the images from former panel 1C to the supplement to focus more on generalizable information as requested. Additionally, we now better emphasize that despite the general concordance of protein identifications and quantitative reproducibility obtained from the different conditions (comparing membrane slides and antigen retrieval methods), the choice of the right membrane slide and staining procedure is still important for reasons such as slide distortion and laser microdissection collection efficiency dependent on the desired application.

2. In continuation of the previous comments, there are topics that I think require more emphasis. For example the comparison of lysis buffers is much more important and should be presented in the main figures. This is an example of a generalizable result, which actually has impact on the analytical depth.

We fully agree and now show the lysis buffer comparison in Fig. 2F-G.

Reviewer #2: Makhmut and co-authors present a study on the feasibility and advancement of spatial proteome analysis at the resolution of single cells or small phenotypically defined tissue regions using a combination of multiplex IHC imaging, laser capture microdissection and low input proteomics sample process and LC-MS/MS acquisition. The study addresses a very timely need and describes considerable methodological and conceptual advances. I particularly commend the authors for their attention to detail and in-depth SOPs that will make this work a go-to resource!

The data is overall of excellent quality and the manuscript is well written. I did not identify any major concerns but have a few suggestions for minor edits and additional analyses that would provide considerable additional insight and increase the value of the work further.

We are delighted to hear this very positive feedback from this reviewer and are grateful for the minor point suggestions. We have now incorporated these additional suggestions into our revised manuscript.

The effect of sample overloading described on pages 6, 9 and following should be investigated and characterized more closely. Is there any indication of in TIMS cell fragmentation? Does the observed loss in IDs and increase in CV occur evenly across proteins or is it correlated with peptide/protein intensity? If so, a consideration for possible implications for biological interpretation should be added to the discussion.

We thank the reviewer for raising this important point. We used our tonsil tissue titration dataset from Fig. 3C to address this point. As seen in the figure below (left, upper panel), there is no evidence that the lower precursor ids from an 'overloaded' sample is associated with precursor intensity, which is a characteristic for in TIMS fragmentation. Similarly, the mobilogram of an 'overloaded' sample does not show typical signs of TIMS overloading (lower panel), which would otherwise show vertical 'stripes' of high abundant precursors with large 1/K0 ranges. However, we noticed a stepwise increase in missed cleavages, which suggests that for the highest tissue amounts tested, trypsin amounts could be further optimized. As we believe this is an important point to interpret these data, we edited the corresponding main text and included this analysis in the revised manuscript as new Fig. S3E. Additionally, we added a separate comment in the detailed sample preparation protocol.

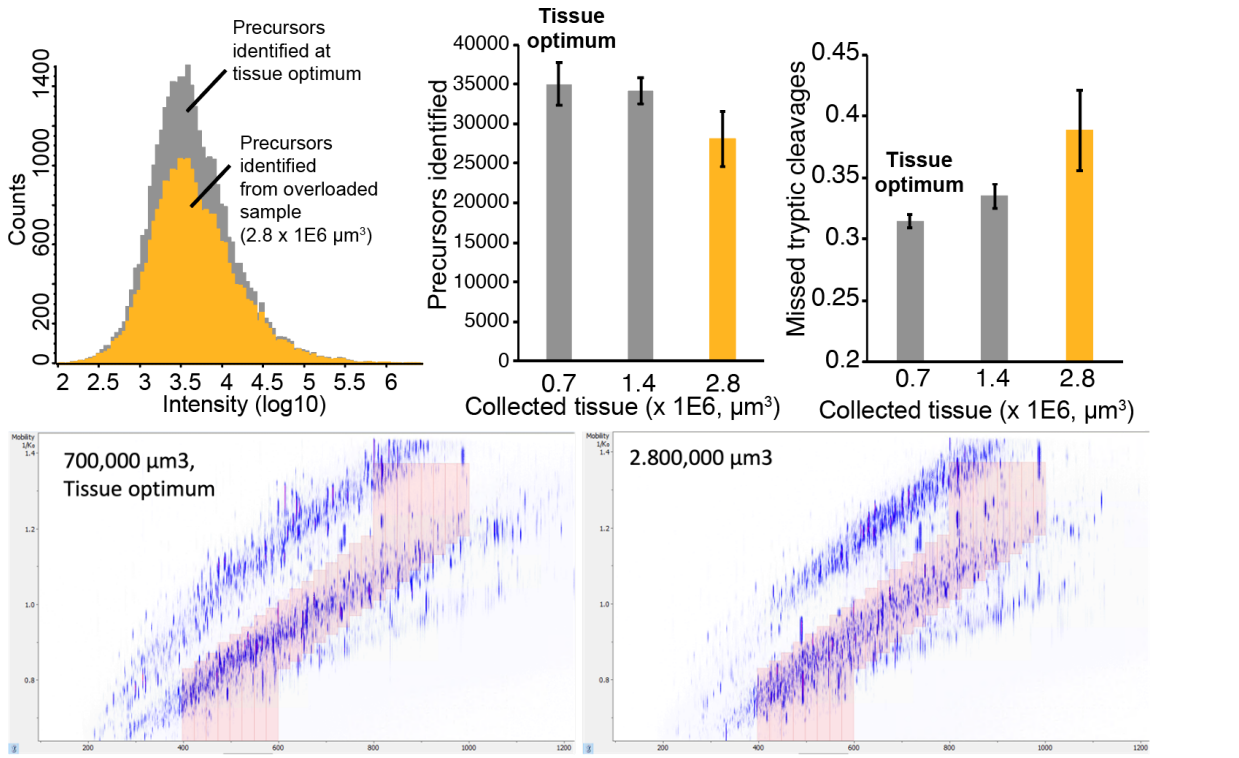
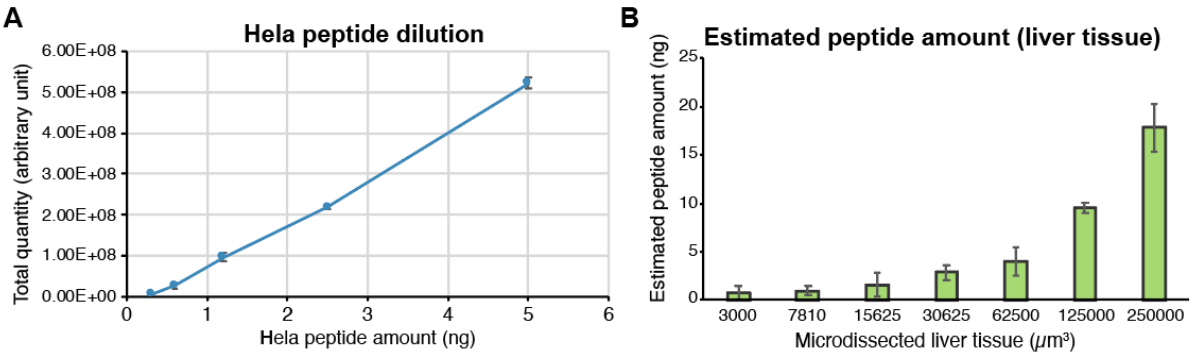


***Figure 1**: Characterizing low identification rates from high tissue amounts. **Upper left)** Distribution of precursor Intensities (log10) from tonsil tissue samples. Grey: 700,000 µm3 sample, yellow: 2.800,000 µm3. **Upper middle)** Number of identified precursors from the two tissue amounts. The tissue optimum was around 700,000 µm3, beyond which identification rates dropped significantly. **Upper right)** Tryptic miscleavage rates for both tissue amounts. **Lower panel)** Ion mobilogram of two representative raw files from a 700,000 µm3 and 2.800,000 µm3 sample.*

The manuscript uses tissue area/volume as and cell number estimates as measures of sample input throughout. This makes absolute sense in the context of the study but makes it difficult to compare to other studies that use isolated/suspension cells. It would be very helpful if the authors could conduct a calibration experiment where they measure/estimate the protein amount extracted from the tissue regions of varying area/volume. This could be done by sensitive protein or peptide quantification or by calibrating their MS signal intensity against a peptide dilution series.

We fully agree that the comparison between laser microdissected FFPE tissue and isolated/suspension cells is not straightforward. To estimate the peptide amounts from our low-input tissue samples, we measured Pierce Hela dilutions with the exact same LC gradient and diaPASEF MS setup (Thermo Easy-nLC1200 and Bruker timsTOF SCP). We diluted Hela peptide standard from 5 ng down to 300 pg (single-cell equivalents) and measured each amount in triplicates. Similar to our tissue normalization strategy (Fig. 3), we then used the total quantity (sum of MS2 quantities of identified precursors) reported by DIA-NN to compare Hela and tissue injections for peptide amount estimations. Panel A of the figure below shows a linear increase of the total MS2 quantity from Hela dilutions, which then allowed us to estimate peptide amounts from the liver tissue dataset (panels B-C). We estimated that the 50-cell liver FFPE tissue samples (250,000 μm3) were equivalent to 17.85 ng +/- 2.47 ng and single contour samples (3,000 μm3) 0.74 ng +/- 0.66 ng, which is in very good agreement with reported literature numbers (700 pg per hepatocyte, Bionumbers, PMID: 19854939).



C

Microdissected liver tissue (μm3)	Average peptide amount (ng)	Standard deviation
3000 (single contour)	0.74	0.66
7810	0.98	0.50
15625	1.56	1.24
30625	2.85	0.73
62500	3.97	1.43
125000	9.60	0.57
250000	17.85	2.47

***Figure 2**: Estimation sample input from low-input tissue samples. **A**) Hela (Pierce) peptide dilution experiment showing a linear increase of total quantity (sum of M2 quantities of identified precursors) with increasing peptide amounts. Peptide standard was diluted from 5 ng down to 0.3 ng. **B**) Estimation of sample input from low-input mouse liver FFPE tissue. The Hela calibration experiment was used to estimated peptide amounts via the total quantity measure reported by DIA-NN. **C**) Summary table of sample input estimations from Hela dilutions.*

The manuscript lacks any mention of other spatial profiling methods. I would be important to compare and contrast their method with e.g. spatial transcriptomic approaches. While a direct quantitative comparison of RNA to protein levels is probably beyond the scope of this paper it would be important to compare metrics like coverage (quantified transcripts or proteins), CV, dynamic range, cost, time.

We apologize if this has not been clear. We tried to address this both in the introduction and discussion. In the introduction we wrote the following paragraph:

"*To analyze cell dynamics in space and time, powerful spatial genomics [2], epigenomics [3], transcriptomics [4–6] and imaging-based proteomics [7,8] methods have been developed to better understand cellular and molecular drivers of health and disease states. As proteins are the biomolecules closest to the cellular phenotype determining cell identity and function [9,10], spatial proteomics (SP) methods are particularly promising for the study of human (patho)physiology. SP methods with the single-cell resolution are dominated by targeted antibody-based methods such as imaging mass cytometry [11] (IMC) or multiplex immunofluorescence (mIF) imaging [8,12], where several dozen proteins can be analyzed at (sub)cellular resolution".*

Later in the discussion we then compare the spatial resolution to state-of-the-art transcriptomics:

"The data from single excised hepatocytes also revealed that a ~25-µm spatial resolution is principally achievable for tissue types such as liver, on par with the spatial resolution of state-of-the-art spatial transcriptomics [41,42]."

Based in the reviewer's suggestion, we have further extended this paragraph on page 11:

"Instead, the integration of whole-slide IF imaging for detailed cell and cellular neighborhood phenotyping allows to prioritize cells and ROIs subjected to global proteome analysis, thereby offering a powerful, cost-effective and accessible spatial profiling strategy."

An analysis of the data completeness between single cell contours should be added (acknowledging that some of the missing data may be true heterogeneity). This should be demonstrated with and without match between runs.

We thank the reviewer for this great suggestion. We have added new supplemental Figs. S2G to the manuscript, in which we show the missing data from the single-cell contours (10 µm thick tissue sections). It is important to mention that we used a project-specific spectral library, which, based on a global predicted library (uniprot FASTA), was further refined in DIA-NN. Injections from higher load samples (more than twenty 50-cell liver tissue regions) were used to generate this library, resulting in 68,006 precursors, 61,554 elution groups and 8,225 protein groups. The final search of the single-cell contour raw files was then without the match-between-runs (MBR) function. We here followed the recommended DIA-NN software settings (https://github.com/vdemichev/DiaNN): As it can be expected that this library provides complete coverage for these ultra-low input amounts, we did not use MBR.

However, following the suggestion of the reviewer, we have now re-analyzed our single-cell contours with and without MBR. As expected, this showed no difference in proteome coverage (panels A-C of the figure below). However, we noticed higher data completeness when MBR was enabled (panel D). From 10 µm thick contours, data completeness was 89% for the 2,000 most abundant proteins in the dataset, compared to 83% without MBR. We included the data completeness comparison in the revised manuscript as new Fig. S2G.

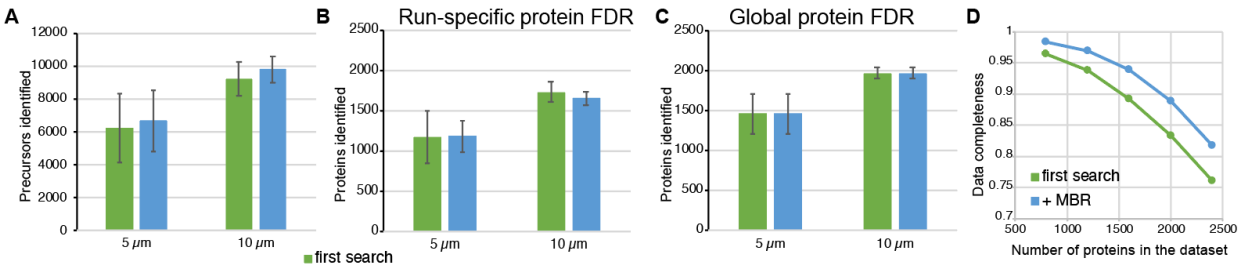


***Figure 3**: Analysis of data completeness in single-cell contours (10 µm thick section of mouse liver FFPE tissue). **A)** Number of identified precursors from single-cell contours with (MBR) and without (first search) match-between-runs in DIA-NN. **B)** Number of identified proteins (run-specific protein FDR) from single-cell contours with (MBR) and without (first search) match-between-runs in DIA-NN. **C)** Number of identified proteins (global protein FDR) from single-cell contours with (MBR) and without (first search) match-between-runs in DIA-NN. **D)** Comparison of data completeness with (MBR) and without (first search) match-between-runs. Note, data completeness improved when MBR was enabled.*

Minor comments:
Figure 1D: I would recommend to also provide CV information

This is now provided in revised Fig. 1E.

Figure 1F: This would be better visualized by an upset plot

We have revised this figure accordingly, which is now Fig. 1F.

Figure 1G: I would recommend to change the sort order. Here the key comparison is for each compartment term between methods rather than different terms within methods (as facilitated by the current order). Grouping by compartment would facilitate direct comparison. Also, the gradient fading of the color is irritating and should be removed.

We have revised this figure accordingly, thanks for this suggestion.

Figure 2 C-E: remove color. It is not required and distracts

We have removed the colors for most groups, but believe that the color coding for single-cell and 50-cell contours is useful to relate them to the other panels of Fig. 2. This is because figures 2H-J make use of the same colors so that the readers would understand that the single-cell or 50-cell contours relate to the samples as shown in the dilution experiments (panels C-E).

Figure 2F: it is not clear which of the hepatocyte markers got identified by single cell contours. It is also not clear if this represents identification by a single cell contour or the union of all single cell contours.

We have revised this figure (now Fig. 2H) and the corresponding figure legend to make this clearer. Using the same color code, we now indicate which hepatocyte markers were identified in the single-cell contours after stringent data filtering. We required a minimum of three quantified values per quadruplicate measurements from the single-contour samples, which resulted in 1,123 unique protein groups after removing potential contaminants. Eight marker proteins were quantified this way (Aspg, Acat3, Ttpa, Ido2, Akr1d1, Ftcd, Ugp2, Aspdh).

Figure 4: it would be helpful to add a figure showing the distribution of missing values (e.g. heatmap with missing values clearly colored).

We thank the reviewer for this suggestion. In contrast to the data completeness comparison for single hepatocyte contours (described above), which we now include as new Fig. S2G, we believe that a separate figure showing the distribution of missing values for the tonsil dataset could be misleading for the following reason. We on purpose chose regions comprised of different cell types and states (as opposed to the liver example, where we chose relatively homogenous areas mainly comprised of hepatocytes). It is therefore expected that these proteomics data will show a high percentage of missing values as many of the identified proteins are exclusive markers for the different cell types, functional states and spatially-defined niches. As requested, we added a heatmap below, which shows the missing values in light grey after different data filtering strategies. Panel C shows bona-fide cell type markers of epithelium, T-cell and B-cell zones, quantified in the respective samples, but which (as expected) are almost absent in the other groups. In such cases, low data completeness (or a high percentage of missing values) is expected, reflecting a biological cause of missingness, as this reviewer also acknowledged in a comment above.

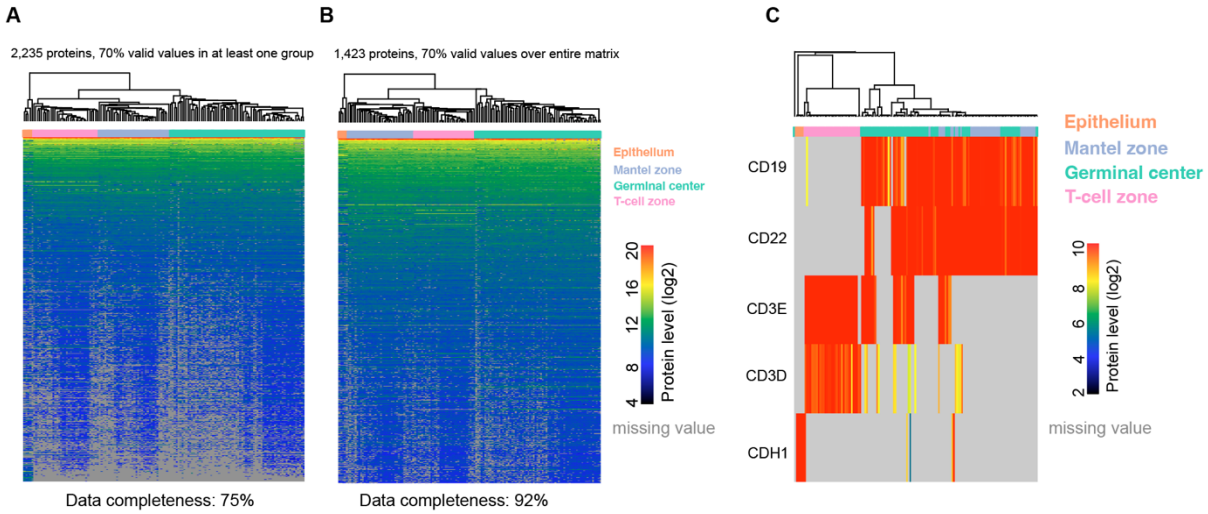


***Figure 4**: Data completeness of the cell-type resolved tonsil proteomics dataset. **A)** Data matrix after filtering for 70% valid values in one of the four cell type groups. **B)** Data matrix after filtering for 70% over the entire dataset. **C)** Heatmap of known cell type markers for the four cell type groups. Grey values show missing values in all three panels.*

Page 6 line 8: Please specify the nature of replica measurements (different regions or injections?) that the CV is based on. This becomes clear later in the text but would be very helpful to already specify at this point.

We have added this information. These replicates are from adjacent tissue regions.

The effect of varying extracellular matrix composition and cellularization of tissue regions should be mentioned when optimal tissue amounts are discussed. The use and type of imputation should already be mentioned in the results section and not only in the methods. This would improve understanding of the presented data.

We have revised the text accordingly. On page 7, we now included the following statement:

*"To minimize variability from different extracellular matrix compositions, we focused on homogenous tissue regions of high cellularity (**Fig. 3B**), similar to the liver titration experiment (**Fig. S1G**)."*

On page 8, we also included the imputation strategy:

"*After data filtering and imputation by normal distribution (Methods), proteomes clearly separated by microanatomical region dominated by distinct cell types (**Fig. 4D**)."*

In the SOP supplement it would be interesting if the authors to elaborate on their choice of evotips for peptide cleanup (without use on an evosep-one LC system) rather than more commonly used custom made C18 or SDB-RPS tips.

We have revised the document accordingly and now include a separate note on the tip choice.

The authors should consider organizing their files in the pride repository in a way that complies with a full submission to facilitate re-analysis (even though I recognize that this is a lot of work and unnecessarily complex to do)

Thanks for this advice. We will certainly consider it prior publication of the manuscript.

Reviewer #3: This manuscript tried to address the challenges of single-cell proteomics of FFPE samples using the laser microdissection. Their work integrates high-content imaging (QuPath/BIAS), laser microdissection (LMD7), optimized sample preparation methods, and ultra-high sensitivity mass spectrometer (timsTOF SCP). This method, leveraging the enhanced sensitivity of timsTOF SCP, has identified around 2,000 proteins in single murine hepatocytes and approximately 5,000 proteins in 50-cell contained tissues. The methodology was further applied in human tonsils, successfully identifying an average of 1,952 proteins in 146 microregions, resolving their spatial heterogeneity. This work provides a tissue-level sample processing and analysis solution with single-cell resolution. The manuscript is well-organized and written. The reviewer requests that the authors address the following issues to further improve the manuscript.

Major issues:

1. The manuscript should provide a more comprehensive and direct comparison between the proposed workflow and the methodology employed by the DVP workflow published by Mann's group (Mund, A., et al. Deep Visual Proteomics defines single-cell identity and heterogeneity. Nat Biotechnol 40, 1231-1240 (2022). https://doi.org/10.1038/s41587-022-01302-5).

We thank the reviewer for bringing up this important point. We have now revised the main text to better explain the differences between our original DVP workflow and the optimized pipeline as presented here. Please also see the answer to point 3 below.

In a nutshell, our original organic solvent-based sample preparation protocol included relatively high lysis buffer volumes per 384-well (around 20 μl) to reduce tissue loss from contours that were not collected at the bottom of the well. Our new protocol reduces tissue loss substantially by adding an additional organic solvent-based washing step, which brings most microdissected contours to the well bottom. This now also allows to work in small 1-2 μl volumes to further minimize sample loss from surface adsorption. We also show the importance of combining our organic solvent-based protocol with the mass spec compatible detergent DDM (revised Fig. 2F-G) for improved proteome coverage of single hepatocyte contours. Lastly, in our original article we used relatively long LC gradients in combination with diaPASEF (70 min total). We here show that using a short and optimized 15-min active gradient combined with diaPASEF not only increases sample throughput, but also proteome coverage for low-input samples (Fig. S1C-E). This can be attributed to narrower peaks with better signal-to-noise.

In the revised manuscript we now write on page 4:

*Compared to our previous DVP protocol, we optimized our workflow for lower microliter volumes (1-2 µl) to minimize peptide loss from surface adsorption, while still being pipette-able with standard laboratory equipment. At the same time, this also allowed the integration of robotic sample preparation workflows (Methods). In addition, we used an optimized 15-min active nano-LC gradient (**Fig. S1C-E**) in combination with an optimal window design dia-PASEF [26] method on a trapped-ion mobility spectrometry (TIMS) mass spectrometer (Bruker timsTOF SCP) for improved sensitivity and sample throughput.*

2. Considering that PEN glass slides cannot tolerate high temperatures (over 70°C) and may become distorted during processing, a study (Nordmann, T.M., et al., A standardized and reproducible workflow for membrane glass slides in routine histology and spatial proteomics. bioRxiv, 2023: p. 2023.02.20.529255.) addresses the glass slide issues. It would be appreciated to discuss how the study addresses this limitation compared to the other study.

The addition of glycerol as suggested by Nordmann et al. is a good and simple way to reduce membrane distortion from heat-induced antigen retrieval. In our experience, the only way to fully avoid loss of tissue in the area of membrane distortion (near the label end), is to mount the tissue on the other side of the glass membrane slide. We have revised the main text to make this point clearer. Moreover, for the majority of experiments we used frame slides, which do not require any specific precautions for sample preparation.

3. In comparison to a similar study conducted by Mann's group (Rosenberger, F.A., et al., Spatial single-cell mass spectrometry defines zonation of the hepatocyte proteome. bioRxiv, 2022: p. 2022.12.03.518957.), which appears to have a more comprehensive workflow and achieves better performance in terms of results with murine hepatocytes, the authors may clearly state the strengths and advantages of the current work over that study.

The study by Rosenberger et al. is different in several ways as it focuses on one single tissue and cell type to profile single-cell proteome differences along the liver zonation axis of fresh frozen tissue. Our main intention was rather to develop and provide a scalable framework and SOP like protocols for various ultra-low input spatial proteomics applications, not only covering single-cell applications. We show the usability of our method for a wide range of spatial proteomics experiments beyond single cells, such as illustrated in the case of human tonsil (Fig. 4). Secondly, instead of frozen tissue our protocol was optimized for FFPE tissue (which includes long heating steps for formalin de-crosslinking and antigen retrieval) and the performance we achieved from single hepatocyte contours is on par with the depth presented by Rosenberger et al. (~2,000 proteins from single hepatocyte contours). It is noteworthy though that FFPE tissue generally results in lower proteome coverage compared to frozen tissue, further emphasizing the high quality of our data.

To put our study in the context of the recent study by Rosenberger et al. and our previous Deep Visual Proteomics pipeline (Mund & Coscia et al., 2022), as also mentioned in point 1 above, we referenced both of these studies in the introduction and also later in the discussion to emphasize our rationale and unique findings. Please see pages 4 (introduction) and 9/10 (discussion).

In the introduction, we state our rationale compared to the previous two studies:

"To realize DVP, we developed an automated laser microdissection (LMD) workflow for the streamlined collection of nuclei, cells or larger regions of interest (ROI) directly into 96 or 384-well plates, thereby connecting whole-slide imaging and deep-learning-based image analysis [16] with ultra-sensitive MS-based proteomics [17]. This allowed the profiling of as little as 100 phenotype-matched cells from archival tissue material, while also preserving detailed cell type and spatial information. Further advances in sample preparation and MS acquisition recently pioneered the profiling of single-cell proteome heterogeneity in cryosections of murine liver tissue [18], emphasizing the strong spatial influence on the hepatocyte-specific proteome. Despite these promising proof-of-concept studies, a systematic evaluation and optimization of all experimental steps of immunofluorescence microscopy-guided spatial tissue proteomics is still missing. In particular, the analysis of few or even single cells of FFPE tissue collected by laser microdissection has remained elusive and relies on optimized and robust 'end-to-end' protocols."

In the discussion, we further state:

"Our group recently co-developed Deep Visual Proteomics, an approach that combines high-parametric imaging and machine-learning-based single-cell phenotyping to guide precise tissue sampling for ultra-sensitive LC-MS analysis. This enabled the profiling of as little as 100 tissue cells per sample to a depth of 3,000 - 5,000 proteins, dependent on the tissue and cell type of interest. However, the flexible and highly modular design of the DVP pipeline, enabling the profiling of single or few cells on one hand, or hundreds of phenotype-matched cells for deeper proteome interrogation (i.e., 5,000 proteins or more) on the other hand, also necessitates carefully designed tissue benchmarking experiments and detailed guidelines to extract most information for diverse biomedical applications."

Minor issues:
1. "Importantly, irrespective of the choice of antigen retrieval (HIER or PIER), staining technique (H&E or IF), or LMD slide type (PEN or PPS), proteomics results from three different liver tissue amounts were highly consistent."-- What are the data supporting this claim in the Results?

We now include the corresponding figure references to clarify this. This relates to revised Fig. 1D-G.

2. In Fig 1H, can you also test slides from other companies, such as MMI's frame slide?

A common provider for laser microdissection slides is the company MicroDissect GmbH (Herborn, Germany). The slides used by MMI are essentially the same membrane slides. Our goal was to compare the two most common slides for laser microdissection based on PEN or PPS membranes, irrespective of the LMD system.

3. On page 21: "Afterwards, the specimens were cut into 5-μm-thick slices, and two representative slices were embedded in paraffin for further microscopic diagnosis." This procedure seems strange. Can you please double-check this is the correct protocol?

We apologize for this confusion and thank the reviewer for spotting this mistake. We have corrected it in the revised version of the manuscript.

4. In Figure S1B, the max values in the y axis are all 6. This is incorrect. Please double check.

We have also corrected this typo. The new y-axis labeling is now 'Intensity (10^6)' instead of just '6'.

5. Error bars should be added to Figures 2C and 2D.

We now consistently show standard deviations as error bars.

6. The authors should clarify the comparison in Figure S2G, specifically indicating which protocol is organic solvent-based and why it is preferred over the DDM/ACN combination for higher tissue amounts.

In response to reviewer 1, we have moved this figure now to main Fig. 2F-G. We have revised the labeling of this figure so that it is clear that the acetonitrile (ACN) based protocol is the organic solvent one and DDM refers to the detergent based protocol. Our conclusion is that for very low sample amounts (single contours or 1562 μm2 regions), the combined lysis buffer is advantageous over ACN or DDM alone, likely due to reduced surface adsorption in the 384-well plate. This effect is not apparent for higher input amounts (50,000 μm2), where proteome coverage is nearly identical between protocols. As DDM is not removed during peptide clean-up steps, it accumulates on the analytical column over time. This can be monitored in the ion mobilogram and necessitates additional wash steps to clean the LC column. Therefore, our recommendation is to use the combined protocol for any experiment near single-cell amounts and the cleaner, organic solved based protocol (ACN) for higher sample amounts around the sampling optimum (e.g. 10 - 20 ng amounts).

7. The manuscript should elucidate the methodology used to determine the number of cells in the 50-cell samples (50,000 μm², 5 μm thick). The authors use area or volume in different places to show the cell numbers, which is confusing; please make them consistent. A BNID accession number may be referenced for the calculations if applicable.

We have revised all figures and for the majority of plots now report volume (in μm^3), instead of area. There are few cases where we show area, for example to illustrate contour sizes of the laser microdissection dilution experiment (Fig. 2A-B). For the cell number estimations, we used 4,000 – 6,000 μm^3 as typical size for murine hepatocytes, as recently described (PMID: 24748404). This number is similar to BNID104616 (PMID: 19854939), which reports ~7,000 μm^3 for the average hepatocyte volume in rats. We reference these numbers either the figure legends of Figs. S1F-G or in the main text on page 5:

"For the lowest tissue amounts measured (7,500 μm^3 samples, 1-2 hepatocytes [33,34]), close to 1,000 proteins could still be quantified […]".

8. The number of replicates used in Figure 3D should be specified, particularly given the observed lack of linear relationships in human tonsil data.

We have now added the number of replicates to the figure legend. For tonsil tissue, we included six replicates per group and a minimum of five replicates for liver tissue.

9. The authors should clarify the meaning of regions labeled in black in Figure 4C, particularly the light, grey, and dark zones. It is also essential to rectify the discrepancy in the sum of squamous cell epithelium (5), follicles (10+106), and interfollicular T-cell zone (34), which currently does not add up to 146.

We thank the reviewer for spotting this discrepancy. We have now revised this figure so that the numbers are correct and that the sample groups are easier to understand.

10. Statistical analysis should be added to Figures 4E and 4F to provide information regarding significance.

We now provide ANOVA p-values comparing the four sample groups after data filtering and imputation. *** indicate p-values of < 0.001.

11. On page 22, it is suggested to include the exact concentrations of antibodies used in conjunction with the dilutions for enhanced reproducibility.

We now provide this information in the methods section.