nature portfolio

Peer Review File

Seq-Scope-eXpanded: Spatial Omics Beyond Optical Resolution

Corresponding Author: Dr Jun Hee Lee

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)
The authors presented Seq-Scope-X, a new technology to surpass the limition of resolution. By introducing physically tissue expansion, Seq-Scope-X not only achieves a remarkable improvement in spatial resolution, surpassing the optical limits faced by previous sST methods, but also offers an effective solution to controlling RNA diffusion and enhancing transcriptome signal retention. The study provides useful insights for the field. I have a few major and minor suggestions.

Major Comments:
1.Tissue expansion microscopy is the key innovation that enables the resolution breakthrough in Seq-Scope-X. Thus, the uniformity of tissue expansion and the potential introduction of artifacts are critical concerns. Although the authors demonstrate overall consistency with DAPI staining (Fig. S1A, S1B), anisotropic expansion remains a major challenge for such techniques. I recommend providing more quantitative analyses to assess potential structural distortions and discussing their potential impact, especially at tissue edges and around internal voids. Furthermore, a discussion on the method's performance across tissue types with different physical properties would add significant value to the claims of broad applicability

2.The manuscript presents a benchmark comparison suggesting high per-area capture efficiency (Fig. 1L, Fig. S1D-S1K). However, a key question is whether the multi-step process of tissue expansion and transfer leads to a net loss of RNA molecules compared to the direct-capture approach of the original Seq-Scope. A more direct and compelling validation would be a head-to-head comparison of the total UMI capture efficiency (e.g., UMI/μm^2 or UMI/cell) between conventional Seq-Scope and Seq-Scope-X performed on adjacent tissue sections. This would provide a definitive assessment of the trade-offs between resolution gain and potential molecule loss

Minor Comments:
1.The Introduction section is missing a sub-title.
2.The order of figure citations in the text should be revised to improve readability. For instance, Figures 2I, 2J, and 2K are first mentioned after Figures 3A and 3B, which disrupts the logical flow.
3.The scale and appearance of the x- and y-axes in Fig. S1D–S1K should be further improved for clarity and aesthetics.
4.For Fig. S2B, is the Spearman correlation between each pair of datasets calculated using the ranking of gene expression based on all shared genes across all datasets, or only the genes shared between each pair? If the latter, please clarify the number of shared genes for each comparison and whether differences in this number could affect the correlation evaluation.

Reviewer #2

(Remarks to the Author)
In this paper, authors incorporate tissue expansion into Sequencing-based spatial transcriptomics with Seq-Scope to increase resolution. Seq-Scope constitutes one example from a group of methods using NGS for spatial transcriptomics. In these methods, a spatial barcode is used to localize the subsequently sequenced transcripts within the tissue space. The authors incorporated expansion into this procedure for the first time, and this is important to the field. Nevertheless, expansion has been previously incorporated into imaging based spatial transcriptomic methods (which the authors cite – refs 21 and 22) as well as into an NGS based approach (cited ref 23). Therefore, the comparison between the proposed method and these previously established methods; as well as the demonstration of novel abilities and applications for which

the previously developed methods are inapplicable is very important for establishing the significance of the contribution to the field.

Indeed, evidence demonstrating the validity and resolution enhancement obtained via the proposed method (especially as shown in figures 1 and 3-4) was convincing. In particular, the paper demonstrates the proposed method on liver tissue, and shows utility for cell-type classification as well as identifies within cell differences between nuclear and cytoplasmic transcript distributions (confirmed with a comparison to analysis of data acquired with MERFISH rather than Seq-Scope-X). Moreover, application of the method to brain and colon tissues is described, again demonstrating cell-type identification and spatial distributions of cell types identified. In addition, they used their method for spatial proteome analysis at high resolution using the novel Seq-Scope-X method (however, see our notes regarding validation of this specific application). Finally, they demonstrated the possibility of obtaining even higher resolution spatial transcriptomics information by replacing the expansion protocol of Seq-Scope-X with the 10X expansion protocol to allow obtaining higher expansion factors. While the sample was only expanded by a factor of 3.3, the demonstration that different gel chemistries can be incorporated into the proposed protocol is useful. Finally, the discussion nicely captures the proposed method advantages, limitations, and potential future extensions.

I have one key question / comment regarding the methodology:
An oligo-dT sequence is used to hybridize the poly-A tail of mRNAs to the gel used for expansion, and then to the Seq-scope chip. It seems like this should mean partial capture of mRNAs by the chip, as some would rebind to the gel during the step of hybridization to the chip. The quantification of RNA capture efficiency (Fig. 1L) as well as comparison to bulk RNA-seq accuracy address this issue in a convincing manner – except there does seem to be high variability in efficiency with Seq-Scope-X. Is there any explanation for how the proposed method works in spite of this potential issue, or any important design considerations in the oligo-dT sequences or the hybridization procedure used that allow this to work correctly? It would be useful to expand on this.

A few more technical questions and comments regarding the method described:

1. The expansion protocol requires some clarifications: Any justification for protocol details that deviate from standard proExM or other implementations of ExM? Was the protocol applied based on a specific recipe from a specific previous paper (none is cited in the relevant “Tissue Embedding” section)? This should be indicated or the logic behind any optimization performed should be specified. For example, the specific concentrations of expansion gel monomers detailed under “Tissue Embedding” seem to slightly deviate from previous protocols. Similarly, tissue expansion typically uses 4-HT to delay polymerization and allow monomer diffusion throughout the tissue prior to gelation, and this is not done here, and APS and TEMED concentrations are typically similar but in this paper 2% APS was used with 0.2% TEMED, which is a very high concentration of APS. Moreover, gelling was performed at room temperature, and this is also an irregular choice as it is typically performed at 37°C. Can all of the above points be clarified?

2. I find the description of tissue expansion with DMMA gels (lines 598-599) and its justification (599-602) a bit confusing. DMMA fraction is very high, and it is not indicated whether it is 80% w/v or v/v or … ? Was oxygen purging conducted as in the original protocol? If not – any ideas why it was not necessary in this case? It is also unclear to what extent was expansion “speed” optimized, as the expansion step is generally a rapid process taking less than 1 hour, and its speed depends in part on the rate of medium (water / 0.1X SSC) exchange. The mentioned dependence of the expansion factor on the volume of liquid used for expansion is also unclear, as concentration (of SSC or PBS applied in the expansion step) effects final expansion factors and it may be the case that expansion was limited at an intermediate stage where it is not yet uniform. Can such potential non-uniformity be reuled out? Otherwise, can a change of SSC concentration be used instead of volume to limit expansion factors and obtain comparable / better results?

I also have multiple technical questions / comments regarding the manuscript text and figures (including a few suggested additional validation experiments and analyses):

1. In comparing the sequencing-based approach to imaging based approaches for spatial transcriptomics, the authors claim (line 49) that: “iST methods are generally constrained to analyzing a predefined gene set…”. Nevertheless, this is explicitly NOT the case for the ExSeq method cited just before this sentence (ref 21). Thus, this sentence should be modified.

2. The validation of expansion uniformity in an example slice (Fig. S1) is convincing. However, it could be useful to add some quantification on a few examples to further support this.

3. In panel G of Figure 1 there seems to be nothing labeled in green, or no “unspliced” transcripts (or image contrast for the green channel is poor). Is this a visualization error and should the green signal overlap with the red? This needs clarification.

4. In panel H of figure 1, in addition to red and green dots (spliced and unspliced transcripts signal) there are blue colored dots – this seems like an error, as this should be a 2 color image.

5. The reported resolution and pixel density improvements for seq-scope-X versus seq-scope are mostly based on the computed expansion factor. The comparison of Seq-Scope and Seq-Scope application to the same tissue type (liver) is important for demonstrating this resolution enhancement and its utility – but technical issues with the figures (no green signal in Fig. 1G, apparent low resolution in Fig. 1H compared to Fig. 1I) are limiting. It would be useful to generate a Seq-Scope data based image similar to that shown in panel I, as well as a zoomed in area, as in panel K, applying the same enhancement tools, if necessary, to also increase the Seq-Scope image quality, so as to more clearly demonstrate the

resolution enhancement. Also, if additional quantitative comparisons could be made to demonstrate improved spatial resolving of transcripts that would be useful. We note that the comparisons of Seq-Scope-X to Seq-Scope in figure 3 clearly demonstrates the enhanced spatial resolution and its utility, but improvements to figure 1 panels would still be beneficial.

6. The abbreviation “UMI” used in panel L of figure 1 should be defined in the figure legend.

7. In line 144-145 it is claimed that Seq-Scope-X surpasses the RNA capture efficiency of other spatial transcriptomic methods. However, the data presented in Figure 1L does not seem to show any statistically significant difference in capture between the different methods, and therefor it seems more appropriate to describe the capture efficiency (which also seems more variable than in some of the other methods) as “comparable to” and not “surpassing” other methods.

8. In figure 2, panel A, the rectangle that indicates the region zoomed-in on on the right should be white so that it would be more conspicuous.

9. In figure S2, panel C, it is unclear what the gray dots describe and this is not mentioned on the figure nor in its caption.

10. In figure S2, panel E: there is an orange boxed region in the top left panel that is supposed to be then expanded with an orange boundary in the top right panel, but the boundary is gray instead of orange. The caption of this panel should also be extended to describe not just the segment labeling but also what is shown, more broadly.

11. Figure 3 panels clearly show enhanced resolution with Seq-Scope-X compared to Seq-Scope. However, the global spatial distributions of cell types identified seem very different, even though two samples of the same tissue type. E.g., in panel F, the PM cell type seems very dominant, and clusters of CL clels are surrounding by SM cells. Are these just inter-sample differences? If so – is it possible to find two examples that show more similar global organization, or demonstrate that across samples there is at least similarity in the abundance of different cell types – identified with the two distinct methods?

12. The contrast in figure S3A and S3B, left panels, can be improved, particularly for the green channel that is barely visible. It is again unclear how in panels associated with Seq-Scope-X (middle and right panels in S3A) there are not just green and red but also blue dots.

13. In figure 4, The global tissue organization seems better preserved for the brain sample (panel B) than for the colon sample (panel C). In fact, the structure in the panel C Seq-Scope-X images seems quite obscure (particularly in comparison with the structure of the standard Seq-Scope image, but also broadly), I am not convinced that the method worked correctly on these specific samples. It would be useful to show other examples / somehow indicate the correctness of the information contained in these images. Panels in figure S4H-I from the same sample type seem better, but there is no comparison to standard Seq-Scope data in this case, making the validity / global structure preservation more challenging to assess.

14. Figure 5 demonstrates the application of Seq-Scope-X for spatial proteome capture. While the results seem promising in terms of identifying distinct spatial distributions for many different proteins tagged in two types of tissues (mouse spleen and human tonsil); additional validation of the correctness of the results seems to be required, in particular in light of the discrepancy shown, described and explained, between ExM and Seq-Scope-X image of the sample analyzed, still suggesting potential loss of some spatial information. In particular, it would be useful to compare some of the spatial distributions of proteins shown (Fig. 5D) to their distribution captured via a distinct method (possibly proExM applied with standard antibodies). Moreover, applying spatial transcriptomics to the same tissue samples as the ones to which spatial protemics were captured, and conducting similar cell-type clustering and spatial distribution capture with FICTURE, could be used to show that the spatial proteome reliably captures the spatial distribution of cell types in the tissue, preserving spatial information.

15. In figure 6 the use of a DMAA gel (corresponding to the 10x expansion protocol) rather than a standard ExM gel is demonstrated on mouse spleen tissue. While the images look very convincing, it is a shame that a distinct tissue type was chosen for this demonstration (mouse spleen), compared to other imaging with Seq-Scope-X which was conducted on liver, brain and colon images. It would be useful to show images of the same type of tissue obtained with the two gel chemistries for mutual validation that both chemistries similarly preserve spatial transcriptome information while allowing its capture at high resolution.

Finally, a few minor questions / comments / suggested corrections:
1. In line 85 the gel is referred to as a “polyacrylamide gel”. However, what makes expansion microscopy gels unique is their ability to expand that is a consequence of the absorptive properties of sodium acrylate, the main gel monomer. Thus, describing the gel as a “polyacrylamide gel” is not the right terminology to use. Similarly, in lines 779 and 811 the gel is referred to as “acrylite hydrogel” which is also not the correct nomenclature.
2. Line 510: “After disassembling humidified chamber” ==> “After disassembling the humidified chamber”
3. Citation 24 redundantly includes the words “Optical imaging” that are not part of the manuscript title. This should be removed.
4. Line 778: “Tissue” ==> “The tissue” and line 779 (same sentence): “probe” ==> “probes”
5. Line 349: “significant advancement” ==> “significant advance”

Reviewer #3

(Remarks to the Author)
In the present manuscript, the authors introduce a sequencing-based spatial transcriptomics method that achieves submicrometer-scale resolution through tissue expansion and incorporates antibody-derived barcode tags for proteome profiling. Overall, the new technique seems to be an interesting contribution to the field and the performance is demonstrated reasonably. However, several aspects of the study could still be improved.
Major comments:
- Line 97 mentions that the expansion factor ranged between 2- and 3-fold without causing substantial distortion of the overall tissue structure. Was there any testing to determine at what expansion level distortion would begin to affect the results of transcriptome analysis—for example, under the theoretically expected 10-fold expansion using DMAA chemistry (and 10-fold resolution increase that is mentioned in the abstract). The comparison in Figures S1A and S1B is very clear and intuitive; why does Figure 6B lack a similar direct overlap comparison? Could the 3.3-fold DMAA expansion have introduced tissue distortion?
- L135-147: For the benchmarking of sensitivity (Figure 1L), is UMI per μm² "back-calculated" to original tissue size before expansion? Also, besides UMIs per μm², including additional metrics would provide a more comprehensive benchmarking—for example, UMI and gene detection across different bin sizes and per segmented cell.
- L163-167: Advise caution with this type of analysis. The UMAP space is NOT interpretable due to non-linear compression. Are the 5 major cell types known archetypes or ad hoc definitions of the authors?
- L176-182: This statement lacks experimental data or supporting references and appears to be overinterpreted. Solid support would require, for example, the observation of known markers of the state transition in the nuclear/cytoplasmic analyses.
- One of the strengths of Seq-Scope-X is its improved ability to segment cells within tissues, which is a major challenge in spatial analysis (whereas relatively few researchers may have biological questions that actually require subcellular-level analysis). It would be helpful to explicitly demonstrate this improvement.
- L327: In the section titled "Seq-Scope-X with DMAA Chemistry Enables Greater Expansion"), only proteomic results are presented, with no transcriptomic results comparable to that shown for the standard expansion gels.
- L369: This is an important caveat of the technique (inherent limitation of Seq-Scope-X is that tissue expansion inherently requires a larger capture area). It would be important for the reader to understand how much cost difference the increased resolution comes with due to larger chip areas being occupied.
- L420–427: Having only IgM is insufficient to demonstrate the ability to detect intracellular proteins. It would be preferable to include multiple intracellular proteins or additional ER-localizing markers as supporting evidence. Furthermore, it is important to clearly indicate the resolution of the nucleotide-tagged antibodies to avoid misunderstanding—does it, like the transcriptomic data, reach the subcellular level?
Minor comments:
- General: Seq-Scope-X offers significant improvements in resolution and multi-omics capabilities. However, there are many overstated claims that seem unnecessary, such as those in lines 308–313, 414–419, 422–423, 457–460, and 467–472.
- L107: "Supra-Optical Resolution" do they mean superresolution? supra = greek for above?
- L270: The abbreviation 'bST' appears to be a misspelling? Or if not, provide its full form.
- L462-464: Why did the DMAA expansion not reach the theoretically expected 10-fold expansion, but instead only achieved approximately 3.3-fold.

Reviewer #4

(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 #5

(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.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The additional quantitative analyses address our concerns.To further strengthen the manuscript, we recommend refining the Methods section regarding the distortion calculations. While we can infer the authors' intent, the description in Lines 907-911 remains ambiguous. We suggest rephrasing this section to ensure the methodology is described clearly and without ambiguity.It would be helpful to define exactly what constitutes 'displacement error' and how 'RMS' is calculated in this context.

We are also confused by the phrasing 'generated all possible pairs of the down sampled displacement vectors.' It is unclear

how 'all possible pairs' were generated if the data was first 'down-sampled'. Please clarify if this refers to all pairwise combinations within the down-sampled subset, or if a different sampling strategy was used. A clear calculation flow is needed.

Reviewer #2

(Remarks to the Author)
We find that the authors fully addressed the concerns raised by us and the other reviewers, significantly improving the manuscript.

In particular, the concern of non-isotropic expansion was addressed by additional experiments and analyses comparing isotropy in multiple tissue types. The presented error rates are consistent with the literature and the reported tissue differences would be valuable for future application of the novel Seq-Scope-X method.

The authors also very convincingly addressed the concern I raised regarding a potential limitation of RNA capture due to the method's design - I found the added explanation regarding the use of a temperature gradient to ensure directional mRNA transfer from the gel to the array probe useful. The concern was further indirectly addressed by additional improvement of the quantitative validation of RNA capture performance, including the reporting of UMI/μm2 and UMI/cell and showing a UMI/cell decrease with respect to Seq-Scope without expansion that is lower than expected according to the expansion factor.

Concerns regarding protocol details were also fully addressed by correcting an error in reported APS concentration and explaining protocol optimizations / modifications made for this specific application of expansion, prioritizing the preservation of RNA molecules. Similarly, we found the re-wording of the tissue expansion methods section useful.

All detailed text and figure corrections requested were thoroughly and rigorously performed.

Reviewer #3

(Remarks to the Author)
The authors have addressed all of the raised concerns with new experiments and analyses.

Reviewer #4

(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 #5

(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 #1 (Remarks to the Author):

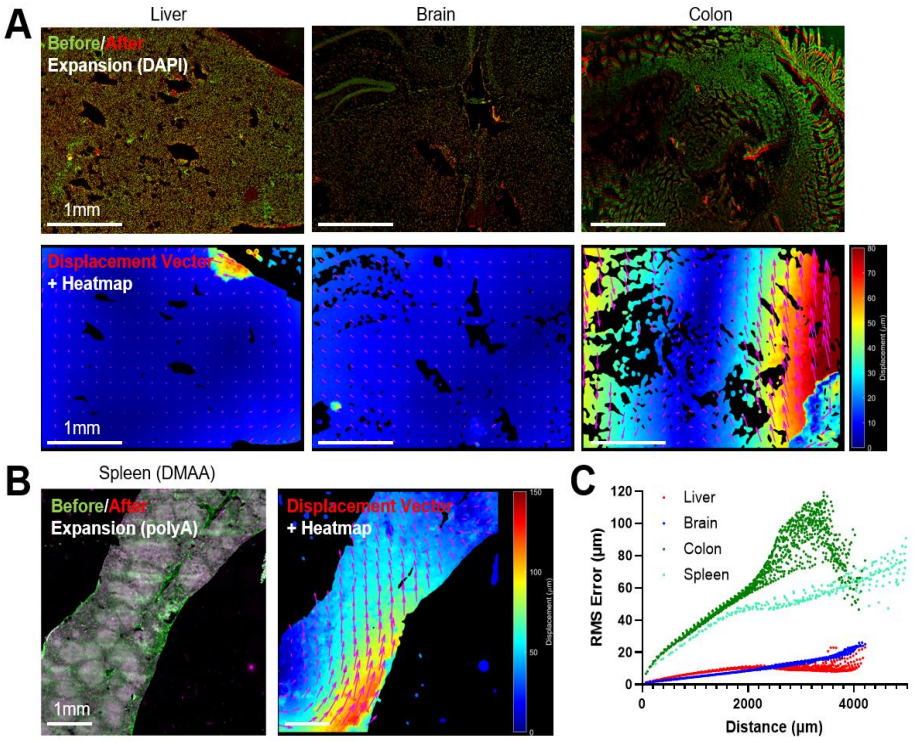
The authors presented Seq-Scope-X, a new technology to surpass the limitation of resolution. By introducing physically tissue expansion, Seq-Scope-X not only achieves a remarkable improvement in spatial resolution, surpassing the optical limits faced by previous sST methods, but also offers an effective solution to controlling RNA diffusion and enhancing transcriptome signal retention. The study provides useful insights for the field. I have a few major and minor suggestions.

Major Comments:

1.Tissue expansion microscopy is the key innovation that enables the resolution breakthrough in Seq-Scope-X. Thus, the uniformity of tissue expansion and the potential introduction of artifacts are critical concerns. Although the authors demonstrate overall consistency with DAPI staining (Fig. S1A, S1B), anisotropic expansion remains a major challenge for such techniques. I recommend providing more quantitative analyses to assess potential structural distortions and discussing their potential impact, especially at tissue edges and around internal voids. Furthermore, a discussion on the method's performance across tissue types with different physical properties would add significant value to the claims of broad applicability.

The risk of anisotropic gel expansion was raised as a concern by all three reviewers, and we take this seriously. To address it, we performed additional quantitative analyses comparing tissue architecture across multiple organs before and after expansion, including displacement vector mapping, anisotropy heatmaps, and RMS distortion statistics across tissues and regions. Detailed methods for these calculations are now provided in the Methods section of the revised manuscript.

We would like to clarify that our tissue expansion was performed on a very thin layer (~10 µm) of tissue sections; so, unlike thick tissue expansion where polymer penetration and tissue clearing is more challenging, our approach allows more isotropic expansion. The distortion analyses demonstrate that, for standard (~2-3X) expansion of liver, colon, and brain tissues, expansion was largely isotropic. In the liver and brain, the distortion (as RMS error) was <1% of distance (>99% fidelity); also in the colon, it was well below 5% of distance (>95% fidelity), even though the level of distortion was somewhat greater than the liver or brain (**Response Letter Fig. 1A, 1C**). Most distortions were concentrated at the outermost expansion edges; therefore, the distortions in the central area were even smaller. Notably, distortions at tissue boundaries and around internal voids (e.g., blood vessels) were relatively minimal. Larger distortion for the colon tissue could be because it has dense layers of tissues with varying density of biological materials including the circular and longitudinal muscle layers, which may impose uneven in-plane resistance during expansion and may also affect polymer penetration or tissue clearing; more optimization might be able to bring the distortion level further down. But it is clear that even the current level of distortion does not grossly alter biological interpretations of the general geometry that the original tissues and cells have. For instance, for ~20 µm size cells, <1% or error means <0.2 µm distortion, which is smaller than the optical resolution, and such values would be even smaller in the focus area at the center. Similarly, spleen tissue expanded by the DMAA protocol (~3.3X) exhibited minimal distortion with less than 5% of RMS error, confirming that expansion was largely isotropic across all experiments described in the current paper (**Response Letter Fig. 1B, 1C**).



Response Letter Figure 1. Distortion analysis of expanded tissues. (A and B) DAPI staining was performed on liver (A, left), brain (A, center) and colon (A, right), and polyA detection was performed on spleen (B), before (green) and after (red) tissue expansion through standard (A) and DMAA (B) protocol. Corresponding displacement vectors (red) and distortion heat maps are shown in a separate panel. (C) Distance–RMS error scatterplot demonstrating that the average distortion is <5% of distance across all samples and <1% for liver and brain tissues.

We have incorporated these new analyses and the associated discussion - including edge effects, behavior around internal voids, tissue type variability, and the potential trade-off between resolution and spatial fidelity - into the revised manuscript.

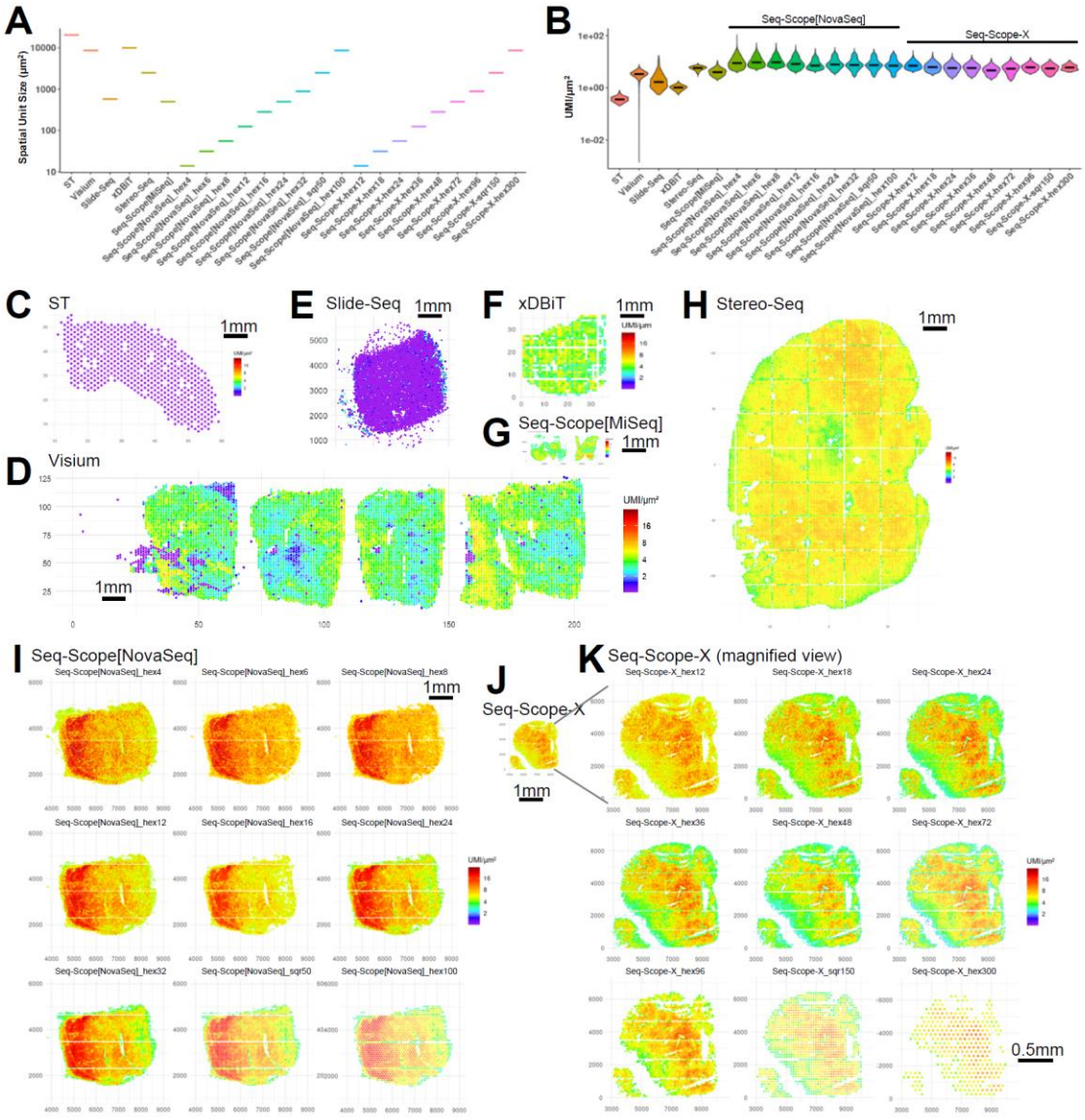
Relevant sections: Fig. S1C, S1D, S1E. Line 110-117, 474-485, 894-911.

2.The manuscript presents a benchmark comparison suggesting high per-area capture efficiency (Fig. 1L, Fig. S1D-S1K). However, a key question is whether the multi-step process of tissue expansion and transfer leads to a net loss of RNA molecules compared to the direct-capture approach of the original Seq-Scope. A more direct and compelling validation would be a head-to-head comparison of the total UMI capture efficiency (e.g., UMI/µm² or UMI/cell) between conventional Seq-Scope and Seq-Scope-X performed on adjacent tissue sections. This would provide a definitive assessment of the trade-offs between resolution gain and potential molecule loss

The Seq-Scope and Seq-Scope-X data were generated from the same mouse strain, age, housing condition, and liver sample, using identical freezing protocols, sectioning equipment, and NovaSeq6000-based arrays produced from the same procedure. Thus, the datasets are directly comparable. In the revised manuscript, we show that the datasets generated from conventional Seq-

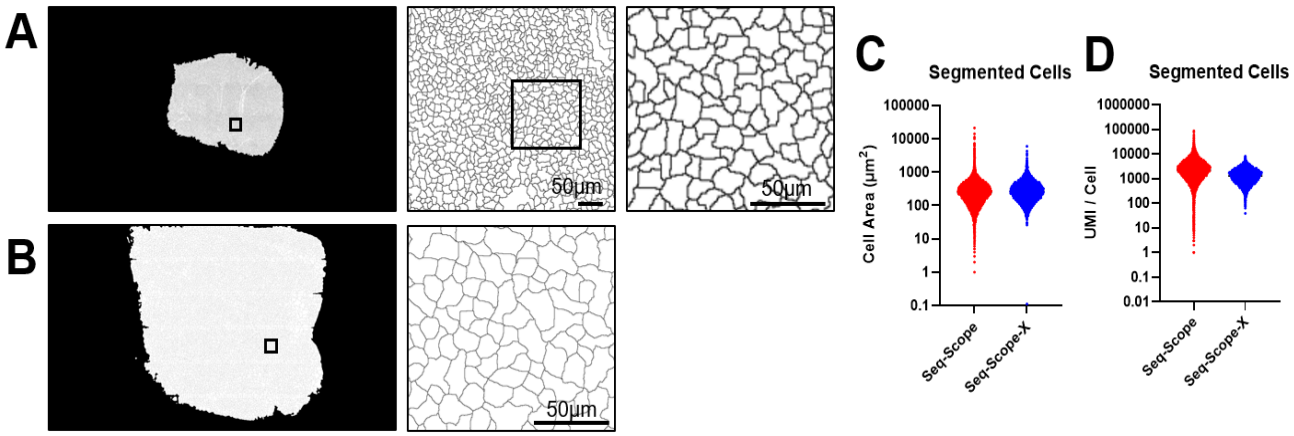
Scope and Seq-Scope-X could be successfully integrated (see below in responses to Reviewer #2 and #3; especially, **Response Letter Figure 9 and 10**), showing comparable performance in RNA capture and cell type identification. The integration analysis was used to strengthen the other aspects of the paper; however, it also provides additional support for the robustness of Seq-Scope-X comparable to original Seq-Scope, albeit with increased resolution performance.

We agree, however, with the reviewer in that our original benchmarking was not sufficiently rigorous, as we used different grid sizes for estimating UMI/μm² of different technologies, and also did not report UMI/cell. In response, we have now performed a more comprehensive benchmarking analysis: datasets were re-binned into comparable units, UMI/μm² was compared at each level (**Response Letter Fig. 2**), and cell areas and UMI/cell were quantified and compared between Seq-Scope and Seq-Scope-X (**Response Letter Fig. 3**).



Response Letter Figure 2. UMI/μm² analysis of Seq-Scope and Seq-Scope-X using different bin sizes. (A) Spatial unit sizes used to calculate UMI/μm² for each of the technologies. Seq-Scope[NovaSeq]_hex100 and Seq-Scope-X_hex300 have spatial

unit sizes comparable to Visium or xDBiT. Seq-Scope[NovaSeq]_sqr50 and Seq-Scope-X_sqr150 have spatial unit sizes comparable to Stereo-Seq, which was also binned with 50 µm-sided squares. Seq-Scope[MiSeq] (binned with 24 µm flat-to-flat height hexagons), Seq-Scope[NovaSeq]_hex24, and Seq-Scope-X_hex72 have spatial unit sizes comparable to Slide-Seq. (B) UMI/µm² values calculated for each bin size, demonstrating relative transcript capture performance across technologies. (C-K) Comparison of transcriptome capture coverage in liver tissue across various spatial transcriptomics (ST) technologies. Six publicly available ST datasets (C-I) and the current Seq-Scope-X dataset (J, K) are shown to illustrate the spatial distribution of transcriptome capture coverage for the datasets shown in (A, B), with the comparable magnification scale. The datasets include: Original ST (C), 10x Visium (D), Slide-Seq (v1; E), xDBiT-Seq (F), Seq-Scope[MiSeq] (G; binned by 10 µm-sided squares), Stereo-Seq (H; binned by 50 µm-sided squares), Seq-Scope[NovaSeq] (I; binned as indicated), and the Seq-Scope-X dataset (J, K; binned as indicated).



Response Letter Figure 3. UMI/cell analysis of Seq-Scope and Seq-Scope-X using cell segmentation methods. (A) Conventional Seq-Scope data segmentation was according to Kim et al. 2025., where H&E histology was used to estimate the cell boundary. (B) Seq-Scope-X data was segmented using RNA density images. (C) The cell area is comparable between the two technologies, while UMI/cell is slightly less in Seq-Scope-X.

These analyses confirm that Seq-Scope-X achieves capture throughput comparable to state-of-the-art spatial transcriptomics platforms. As expected and consistent with prior observations, capture efficiency (1489 ± 953.6 UMIs per cell) was lower than in the original Seq-Scope (3110 ± 3281 UMIs per cell). It is important to emphasize that tissue expansion increases not only the XY dimensions but also the Z dimension. If the molecular diffusion radius remains constant, the capture efficiency per unit area would be expected to scale inversely with the expansion factor, even after XY scale was adjusted for expansion; for example, a 3-fold expansion should reduce UMI concentration to one third. Our results indicate that this dilution effect is present but not too severe, since the capture efficiency decreased by approximately one half rather than the one third predicted from a 3-fold expansion. We speculate that complete tissue digestion by proteinase K, together with loosening of the hydrogel network during expansion, may have facilitated more efficient RNA release, enabling Seq-Scope-X to maintain high-quality data despite the additional processing steps.

In the revised manuscript, we have presented these new benchmarking results. The added analyses directly compare UMI/µm² and UMI/cell between matched Seq-Scope and Seq-Scope-X sections and provide a clearer evaluation of transcriptome capture performance.

Relevant sections: Fig. S2. Line 169-184.

Minor Comments:

1.The Introduction section is missing a sub-title.

Accordingly, “Introduction” subtitle was added to the manuscript.

Relevant section: Line 33.

2.The order of figure citations in the text should be revised to improve readability. For instance, Figures 2I, 2J, and 2K are first mentioned after Figures 3A and 3B, which disrupts the logical flow.

Thank you for pointing this out. We revised the text so that Figures 3A and 3B are mentioned only after the description of Figures 2I-2K is completed, which restores a more natural logical flow in the manuscript.

Relevant section: Line 257-264.

3.The scale and appearance of the x- and y-axes in Fig. S1D–S1K should be further improved for clarity and aesthetics.

We appreciate the suggestion. We have replaced the former Fig. S1D-S1K with a new figure that shows the results in a similar scale, as presented above in **Response Letter Fig. 2C-2K**. The newer figure provides improved clarity and aesthetics.

Relevant sections: Fig. S2C-S2K.

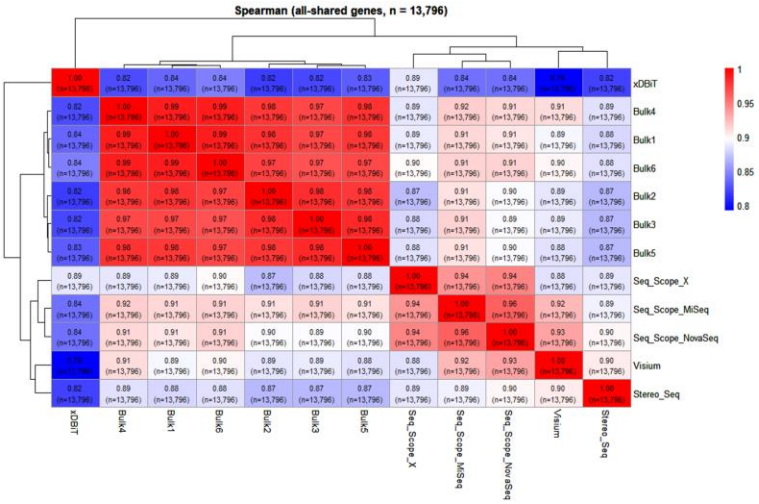
4. For Fig. S2B, is the Spearman correlation between each pair of datasets calculated using the ranking of gene expression based on all shared genes across all datasets, or only the genes shared between each pair? If the latter, please clarify the number of shared genes for each comparison and whether differences in this number could affect the correlation evaluation.

Spearman correlations were computed on a pairwise basis using only the genes shared between the two datasets being compared, after harmonizing gene identifiers. For the heat map, each cell’s correlation was likewise calculated from the genes present in both datasets for that pair.

All datasets here are whole-transcriptome, untargeted sequencing assays (no fixed gene panels or targeted capture). Thus, differences in the number of shared genes primarily reflect detection depth and coverage—factors such as library complexity, read depth, and capture efficiency—rather than selective inclusion or exclusion of genes by platform. This means variation in *n* does not imply biological or platform bias; it affects precision, not direction.

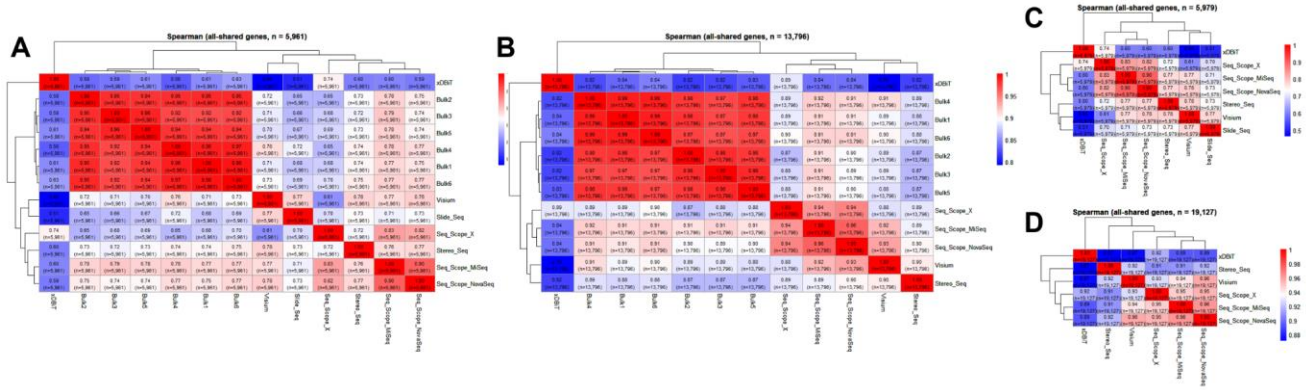
Among spatial transcriptomics (ST) technologies excluding Slide-seq, the shared-gene numbers are high (≥19,966 up to 33,951). Slide-seq itself contains only 6,878 detected genes in our data, which limits its overlap; accordingly, it has markedly fewer shared genes with other datasets (≈6,075–6,186 vs

ST and 6,357 vs bulk). Among bulk RNA-seq datasets, n is 16,838 for every pair, and between bulk RNA-seq and ST datasets, n is 14,449–14,799. We now display n in each heat-map cell alongside ρ (**Response Letter Fig. 4**).



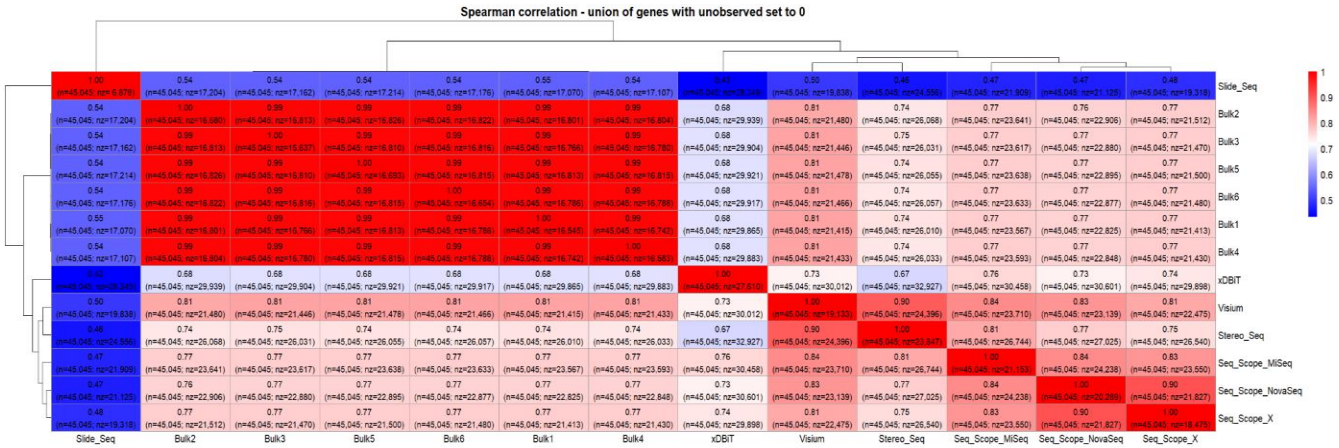
Response Letter Figure 4. Updated Spearman correlation heatmap table (former Fig. S2B) with the number of genes (*n* in parentheses) used for comparison in every pair.

To directly test whether differing gene-set sizes drive the lower Slide-seq correlations, we repeated the analysis after restricting all datasets to the same, globally shared gene set. The resulting intersections were: all datasets including Slide-seq, n = 5,961; all datasets excluding Slide-seq, n=13,796; ST-only including Slide-seq, n = 5,979; ST-only excluding Slide-seq, n = 19,127. Under these matched-gene-set analyses (**Response Letter Fig. 5**), the qualitative clustering of Seq-Scope-X with other ST technologies is preserved, and correlations involving Slide-seq fall within the range observed for other ST–ST pairs using the same gene set. This indicates that the initially lower Slide-seq correlations were primarily due to its smaller overlap in detected genes, not a systematic platform effect.



Response Letter Figure 5. Additional Spearman correlation heatmap tables using the globally shared gene set. (A) All datasets. (B) All datasets except Slide-Seq. (C) All ST datasets. (D) ST datasets except Slide-Seq. All data are consistent with our previous conclusion indicating that Seq-Scope-X produces transcriptome data similar to Bulk RNA-Seq and other ST technologies.

Finally, we have constructed the Spearman correlation heatmap calculated after treating all unobserved genes as having expression value 0, thereby including the full union of genes across datasets (**Response Letter Fig. 6**). Even in this case, the correlation values did not substantially decrease from when we used only the intersection of the genes (compared to **Response Letter Fig. 4**). This result further confirms our initial observations.



Response Letter Figure 6. Spearman correlation heatmap computed using the full union of genes, with all unobserved values treated as zero. The correlation pattern remains consistent with results obtained using pairwise shared or globally shared gene sets, confirming the robustness of dataset relationships. n, total number of genes considered in each comparison (union size); nz, number of genes with non-zero expression in at least one dataset of the pair.

We have updated Fig. S2 accordingly: each cell now reports ρ with n in parentheses, the caption and Methods clarify the pairwise shared-gene calculation and the untargeted nature of the assays, and we include an additional panel showing the “all-shared genes” heat map as a robustness check. Overall conclusions are unchanged.

Relevant sections: Fig. S3B-S3G. Line 189-193.

Reviewer #2 (Remarks to the Author):

In this paper, authors incorporate tissue expansion into Sequencing-based spatial transcriptomics with Seq-Scope to increase resolution. ... Finally, the discussion nicely captures the proposed method advantages, limitations, and potential future extensions.

I have one key question / comment regarding the methodology:
An oligo-dT sequence is used to hybridize the poly-A tail of mRNAs to the gel used for expansion, and then to the Seq-scope chip. It seems like this should mean partial capture of mRNAs by the chip, as some would rebind to the gel during the step of hybridization to the chip. The quantification of RNA capture efficiency (Fig. 1L) as well as comparison to bulk RNA-seq accuracy address this issue in a convincing manner – except there does seem to be high variability in efficiency with Seq-Scope-X. Is there any explanation for how the proposed method works in spite of this potential issue, or any important design considerations in the oligo-dT sequences or the hybridization procedure used that allow this to work correctly? It would be useful to expand on this.

We appreciate the reviewer's thoughtful question regarding the hybridization design and its impact on capture efficiency. The oligo-dT sequence used to hybridize poly-A tails in the expansion gel was specifically designed to bind strongly enough at room temperature and 37°C, yet dissociate during the transfer step at 45°C. It is a 15-mer dT probe containing several LNA linkages, with an empirically reported melting temperature of approximately 39°C. In contrast, the Seq-Scope array contains 30-mer dT probes with melting temperatures exceeding 50°C. Thus, during the 45°C incubation, most mRNAs are released from the gel and are efficiently captured by the array probes. This design explains how Seq-Scope-X maintains robust capture efficiency despite the possibility of mRNAs rebinding to the gel. We apologize that this design principle was not clearly described in the original manuscript, and we have now added this explanation in the revised version.

Relevant sections: Line 92-97.

A few more technical questions and comments regarding the method described:

1. The expansion protocol requires some clarifications: Any justification for protocol details that deviate from standard proExM or other implementations of ExM? Was the protocol applied based on a specific recipe from a specific previous paper (none is cited in the relevant "Tissue Embedding" section)? This should be indicated or the logic behind any optimization performed should be specified. For example, the specific concentrations of expansion gel monomers detailed under "Tissue Embedding" seem to slightly deviate from previous protocols. Similarly, tissue expansion typically uses 4-HT to delay polymerization and allow monomer diffusion throughout the tissue prior to gelation, and this is not done here, and APS and TEMED concentrations are typically similar but in this paper 2% APS was used with 0.2% TEMED, which is a very high concentration of APS. Moreover, gelling was performed at room temperature, and this is also an irregular choice as it is typically performed at 37°C. Can all of the above points be clarified?

Thank you for pointing this out. We did make several modifications to the original ExM and ExST protocols by adapting components that we previously used for expansion MERFISH, an ExM-based spatial transcriptomics method described earlier (Wang et al. 2018, Sci Rep). For example, the specific monomer concentrations were adjusted to balance gel swelling, tissue integrity, and RNA retention. Unlike traditional ExM protocols, we did not use 4-HT to delay polymerization. In our hands, direct initiation with APS/TEMED was sufficient to produce consistent gels while still allowing adequate monomer diffusion into the relatively thin tissue sections (10 μm) used in our applications. Preserving RNA integrity was a central consideration, since RNA is inherently unstable and even limited fragmentation would compromise sequencing performance. For this reason, we selected room-temperature polymerization rather than 37 °C incubation, as this approach reduces thermal stress on RNA and helps maintain stable oligo-dT probe hybridization without affecting gel integrity. In the revised manuscript, we have cited these source protocols and clarified the specific modifications made and the rationale behind them.

We apologize for the error in describing APS concentrations, which was 0.2% as in the standard, which is consistent with conventional ExM - the error was corrected (2% to 0.2%).

To confirm that these protocol modifications did not compromise expansion quality, we performed new quantitative analyses comparing tissue architecture before and after expansion across multiple organs (see above for **Response Letter Fig. 1**). Displacement vector mapping, anisotropy heatmaps, and RMS distortion statistics consistently showed that ~3X expansion of liver, colon, brain and spleen tissues was largely isotropic with less than 5% of errors (<1% for liver and colon). Only moderate distortions were detected at the outermost expansion edge, while distortions at tissue boundaries or around internal voids were minimal. These results demonstrate that, despite minor protocol deviations from canonical ExM, our optimized procedure achieves uniform expansion and preserves tissue architecture at the level required for subcellular spatial analysis.

We have clarified these points in the revised Methods and incorporated the new isotropy analyses and supporting references into the updated manuscript.

Relevant sections: Fig. S1C, S1D, S1E. Line 110-117, 474-485, 592-607, 894-911.

2. I find the description of tissue expansion with DMMA gels (lines 598-599) and its justification (599-602) a bit confusing. DMMA fraction is very high, and it is not indicated whether it is 80% w/v or v/v or … ? Was oxygen purging conducted as in the original protocol? If not – any ideas why it was not necessary in this case? It is also unclear to what extent was expansion "speed" optimized, as the expansion step is generally a rapid process taking less than 1 hour, and its speed depends in part on the rate of medium (water / 0.1X SSC) exchange. The mentioned dependence of the expansion factor on the volume of liquid used for expansion is also unclear, as concentration (of SSC or PBS applied in the expansion step) effects final expansion factors and it may be the case that expansion was limited at an intermediate stage where it is not yet uniform. Can such potential non-uniformity be ruled out? Otherwise, can a change of SSC

concentration be used instead of volume to limit expansion factors and obtain comparable / better results?

We apologize for the confusing description and appreciate the opportunity to clarify. Our DMAA-based expansion followed the X10 protocol (Truckenbrodt et al. *Nature Protocols* 14: 832–863) in which the hydrogel comprises DMAA and sodium acrylate, polymerized by a persulfate/TEMED redox pair (X10 specifies KPS/TEMED, which we followed). Consistent with the X10 protocol, our monomer solution contained 1.335 g DMAA and 0.32 g sodium acrylate. In place of the 2.85 mL (2.85 g) of water specified in the original X10 formulation, we used 2.85 mL of buffer containing 1.75 M NaCl and 100 mM Tris-Cl (pH 8.0). This maintained the 4:1 molar ratio of DMAA to sodium acrylate (approximately 80:20 within the monomer fraction). We have revised the Methods to report this unambiguously.

As in the X10 protocol, we performed oxygen removal by nitrogen bubbling, which is critical to prevent inhibition of free-radical polymerization. Through this, we achieved robust and reproducible gelation of thin (~10 µm) tissue sections.

Finally, to avoid ambiguity, we have revised our wording and removed references to “expansion speed.” The extent of expansion is governed primarily by the ionic strength of the medium (e.g., ddH2O vs. SSC/PBS). Bath volume and exchange frequency influence how rapidly the gel reaches its equilibrium state by accelerating the effective reduction in salt concentration. In our workflow, we cannot use very low–salt conditions because reduced ionic strength can destabilize the hybridization between mRNA and the anchor probe, resulting in molecular loss. For this reason, we carried out expansion in 0.1X SSC with two buffer exchanges. During the final incubation, we monitored the gel until the desired scale factor was reached in plateau; if additional expansion was required, we added more 0.1X SSC to allow the gel to reach the target expansion factor.

Also we performed additional experiments to show that both standard and DMAA expansion conditions we used for liver, brain, colon and spleen tissues can produce largely isotropic tissue expansion with >95% accuracy, analyzed through quantitative methods including displacement vector mapping, anisotropy heatmaps, and RMS distortion statistics across tissues and regions (see **Response Letter Fig. 1**).

Altogether, these clarifications bring our description into closer alignment with prior X10 protocols, and the new isotropy analyses demonstrate that our implementation preserved tissue architecture sufficiently for high-resolution Seq-Scope-X analysis.

Relevant section: Fig. S1D, S1E. Line 397-399, 616-628, 737-749, 894-911.

I also have multiple technical questions / comments regarding the manuscript text and figures (including a few suggested additional validation experiments and analyses):
1. In comparing the sequencing-based approach to imaging based approaches for spatial transcriptomics, the authors claim (line 49) that: “iST methods are generally constrained to analyzing a predefined gene set…”. Nevertheless, this is explicitly NOT the case for the ExSeq method cited just before this sentence (ref 21). Thus, this sentence should be modified.

Thank you for pointing this out. Indeed, ExSeq was designed to perform unbiased spatial transcriptomics, even though it was also used for targeted analyses. We have modified the sentence accordingly.

Relevant sections: Line 51-52.

2. The validation of expansion uniformity in an example slice (Fig. S1) is convincing. However, it could be useful to add some quantification on a few examples to further support this.

We thank the reviewer for this constructive suggestion. In addition to the qualitative validation shown in previous Fig. S1, we have now performed quantitative analyses of expansion uniformity across multiple tissue types (see above in **Response Letter Fig. 1**). Specifically, we carried out displacement vector mapping, anisotropy heatmaps, and RMS distortion statistics in liver, colon, and brain sections of standard expansion and a spleen section of DMAA expansion. These analyses consistently demonstrated that our expansion conditions were largely isotropic with less than 5% of error (<1% for liver and brain). We observed only moderate distortions at the outermost gel boundaries, while distortions at tissue edges and around internal voids (e.g., blood vessels) were minimal.

Together, these results reinforce and extend the conclusion drawn from former Fig. S1: our optimized expansion protocols preserve tissue architecture with sufficient fidelity to enable subcellular spatial omics, while also highlighting the trade-off between resolution gain and structural distortion at higher expansion factors. We have incorporated these quantitative results and discussion into the revised manuscript.

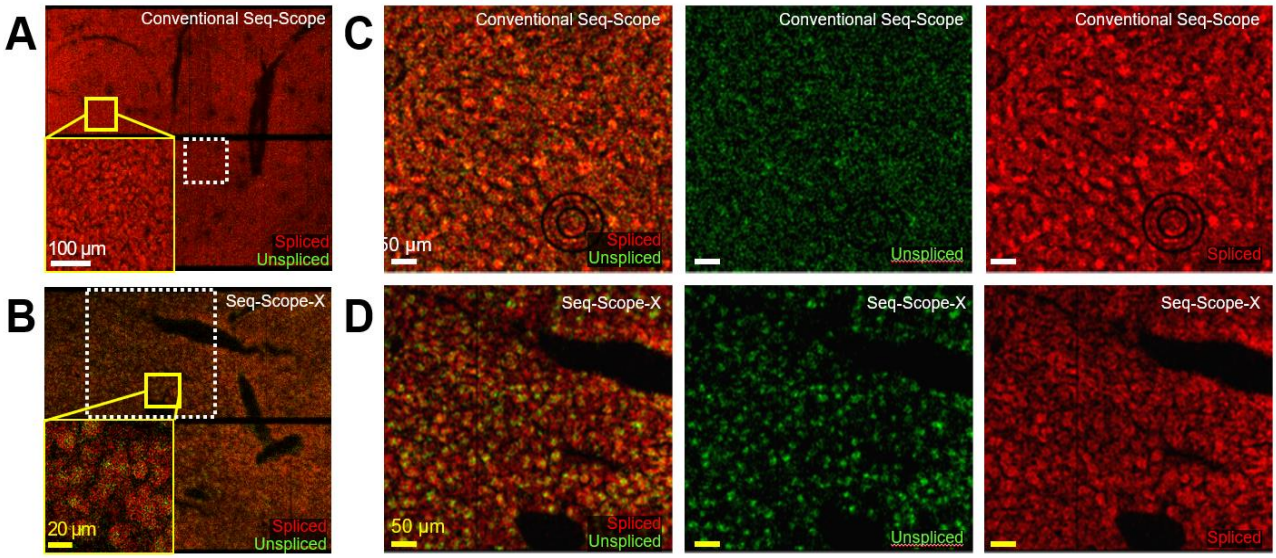
Relevant sections: Fig. S1C, S1D, S1E. Line 110-117, 474-485.

3. In panel G of Figure 1 there seems to be nothing labeled in green, or no "unspliced" transcripts (or image contrast for the green channel is poor). Is this a visualization error and should the green signal overlap with the red? This needs clarification.

We thank the reviewer for raising this point. The green channel in Fig. 1G (shown below in **Response Letter Fig. 7A**) represents unspliced transcripts. These signals are present; however, because spliced transcripts are far more abundant, the red channel dominates the merged image and visually masks the weaker green signal. In addition, Fig. 1G was displayed without compensating for the expansion factor. As a result, the transcript density in the conventional Seq-Scope data appears higher, which further intensifies the red signal and obscures the green channel. In contrast, Seq-Scope-X data (Fig. 1H; **Response Letter Fig. 7B**) show clear intranuclear enrichment of unspliced transcripts because tissue expansion reduces effective diffusion and concentrates unspliced RNAs within nuclei. Thus, the apparent absence of green signal in Fig. 1G reflects biological and technical differences in transcript abundance, diffusion, and scaling, rather than a visualization error.

In the revised manuscript, we now provide additional images showing magnified views of the same regions, scaled to compensate for tissue expansion and displayed separately for spliced (red) and

unspliced (green) transcripts. These images were further enhanced using identical linear contrast adjustments for both datasets (**Response Letter Fig. 7C, 7D**; current Fig. 1I-1J). In these views, unspliced transcripts are clearly detectable in both Seq-Scope and Seq-Scope-X. However, the characteristic nuclear enrichment remains evident only in Seq-Scope-X, whereas conventional Seq-Scope continues to show diffuse unspliced signals with only occasional nuclear-like enrichment. We believe these clarifications and added data address the reviewer's concern and explain the visual differences between the panels.



Response Letter Figure 7. Comparison of Seq-Scope-X (A, C) and conventional Seq-Scope (B, D) data visualized at the same scale according to the final (A, B) or original (C, D) tissues. Red and green dots indicate spliced and unspliced transcripts, respectively. Spatial plots of spliced (red) and unspliced (green) transcripts, adjusted to represent identical physical scales in the original tissue. Yellow boxes in (A, B) were magnified in insets. White dotted boxes in (A, B) were magnified in the left panels of (C, D).

Relevant sections: Fig. 1G-1J. Line 133-145.

4. In panel H of figure 1, in addition to red and green dots (spliced and unspliced transcripts signal) there are blue colored dots – this seems like an error, as this should be a 2 color image.

Thank you for pointing this out. In those previous panels, unrelated transcript data were inadvertently plotted in blue. In the revised figures, we have removed the blue channel so that it will be the 2-color image.

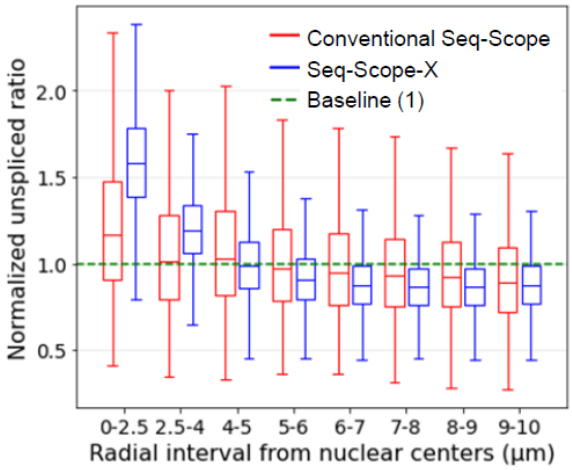
Relevant sections: Fig. 1G, 1H.

5. The reported resolution and pixel density improvements for seq-scope-X versus seq-scope are mostly based on the computed expansion factor. The comparison of Seq-Scope and Seq-Scope application to the same tissue type (liver) is important for demonstrating this resolution enhancement and its utility – but technical issues with the figures (no green signal in Fig. 1G, apparent low resolution in Fig. 1H compared to Fig. 1I) are limiting. It would be useful to generate a Seq-Scope data based image similar to that shown in panel I, as well as a zoomed in

area, as in panel K, applying the same enhancement tools, if necessary, to also increase the Seq-Scope image quality, so as to more clearly demonstrate the resolution enhancement. Also, if additional quantitative comparisons could be made to demonstrate improved spatial resolving of transcripts that would be useful. We note that the comparisons of Seq-Scope-X to Seq-Scope in figure 3 clearly demonstrates the enhanced spatial resolution and its utility, but improvements to figure 1 panels would still be beneficial.

We thank the reviewer for this valuable suggestion. As the reviewer noted, we previously provided multiple lines of evidence showing that Seq-Scope-X not only achieves higher technical resolution but also yields cleaner, biologically interpretable data compared to conventional Seq-Scope and other prior technologies. To improve the visual clarity of this comparison, we have revised Fig. 1 so that Seq-Scope and Seq-Scope-X images are presented at identical magnification (relative to original tissue) and processed with the same visualization pipeline (**Response Letter Fig. 7**; included as Fig. 1G-1J in the revised manuscript). The updated Seq-Scope images now incorporate identical Gaussian filtering and contrast adjustments used for Seq-Scope-X, allowing a fair side-by-side comparison. These enhanced images reveal that Seq-Scope-X clearly resolves nuclear localization of unspliced transcripts, whereas such subcellular features remain indistinct in conventional Seq-Scope data.

We have also added a quantitative analysis to further demonstrate Seq-Scope-X's improved spatial resolving capability. Specifically, we measured the subcellular distribution of unspliced transcripts, which are expected to concentrate near the nuclear center. In conventional Seq-Scope data, only modest enrichment of unspliced reads was observed within a 2.5 µm radius from the nuclear center (red box plots in **Response Letter Fig. 8**) with a high degree of variation. In contrast, Seq-Scope-X data showed a markedly higher and more consistent nuclear enrichment pattern (blue box plots), confirming its superior spatial precision.



Response Letter Figure 8. Seq-Scope-X more clearly captures unspliced RNA enrichment in nuclear area. For each segmented cell (n>1,000), cellular area was stratified into 8 bins with different radial intervals from nuclear centers. Relative enrichment of unspliced RNA, over the whole-cell baseline (expressed as 1), was expressed in a box plot. Conventional Seq-Scope data (red) and Seq-Scope-X data (blue) were compared side-by-side. A two-way ANOVA showed a significant interaction between method and radial interval ($p < 1 \times 10^{-16}$), supporting the observation that Seq-Scope-X exhibits stronger enrichment within the central nuclear region (within 2.5 µm).

Finally, as further detailed below in our response to Reviewer #3 and in **Response Letter Fig. 12**, we also emphasized the resolution advantage of Seq-Scope-X by demonstrating its improved ability to segment individual cells within tissues, a long-standing challenge in spatial analysis. In particular, centrilobular hepatocytes expressing *Glul* and *Slc1a2* could be delineated with clear cell boundaries in Seq-Scope-X (**Response Letter Fig. 12**), whereas the same regions appeared substantially more diffuse in conventional Seq-Scope data. This provides a direct example of how the increased resolution of Seq-Scope-X enhances cell segmentation performance, enabling more accurate interpretation of cellular architecture compared to conventional Seq-Scope, where boundaries remain blurred.

Together, these updates make the comparison between Seq-Scope and Seq-Scope-X more balanced, quantitative and biologically relevant, reinforcing the conclusion—across both main and supplemental figures—that Seq-Scope-X provides markedly improved subcellular transcriptomic resolution compared to conventional Seq-Scope.

Relevant sections: Fig. 1G-1P. Line 133-158.

6. The abbreviation "UMI" used in panel L of figure 1 should be defined in the figure legend.

We apologize for the omission and thank the reviewer for pointing it out. We have now defined "UMI" (unique molecular identifier) in the legend of Fig. 1L (Fig. 1Q in the revised manuscript).

Relevant section: Line 976.

7. In line 144-145 it is claimed that Seq-Scope-X surpasses the RNA capture efficiency of other spatial transcriptomic methods. However, the data presented in Figure 1L does not seem to show any statistically significant difference in capture between the different methods, and therefor it seems more appropriate to describe the capture efficiency (which also seems more variable than in some of the other methods) as "comparable to" and not "surpassing" other methods.

We appreciate the reviewer's point. We have revised the text to state that Seq-Scope-X capture efficiency is comparable to other state-of-the-art methods, rather than "surpassing" them.

Relevant sections: Line 169-175.

8. In figure 2, panel A, the rectangle that indicates the region zoomed-in on on the right should be white so that it would be more conspicuous.

We thank the reviewer for the suggestion. The rectangle in Fig. 2A has been changed to white to improve visibility.

Relevant section: Fig. 2A

9. In figure S2, panel C, it is unclear what the gray dots describe and this is not mentioned on the figure nor in its caption.

We thank the reviewer for noticing this omission. In the former Fig. S2C (Fig. S4A in the revised manuscript), the gray dots represent transcripts below the classification threshold and therefore not assigned as either nuclear or cytoplasmic markers. We have clarified this in the revised figure caption.

Relevant section: Line 1089-1090.

10. In figure S2, panel E: there is an orange boxed region in the top left panel that is supposed to be then expanded with an orange boundary in the top right panel, but the boundary is gray instead of orange. The caption of this panel should also be extended to describe not just the segment labeling but also what is shown, more broadly.

Thank you for catching this. We corrected the top-right zoom boundary to orange to match the inset box and improve consistency. We also broadened the caption to describe what the panels show beyond the segment labels. Please note that the figure number was changed during revision due to new data and organization.

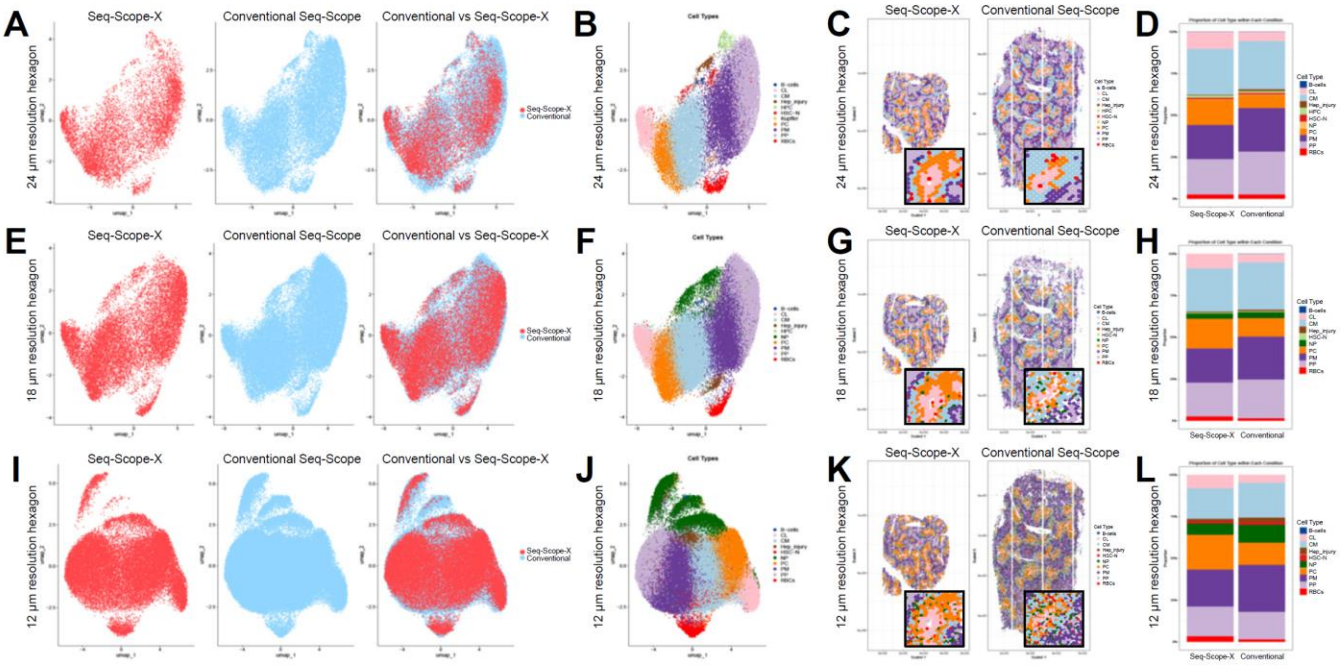
Relevant sections: Fig. S4C. Line 1095-1100.

11. Figure 3 panels clearly show enhanced resolution with Seq-Scope-X compared to Seq-Scope. However, the global spatial distributions of cell types identified seem very different, even though two samples of the same tissue type. E.g., in panel F, the PM cell type seems very dominant, and clusters of CL cells are surrounding by SM cells. Are these just inter-sample differences? If so – is it possible to find two examples that show more similar global organization, or demonstrate that across samples there is at least similarity in the abundance of different cell types – identified with the two distinct methods?

We appreciate the reviewer's careful observation. Both Seq-Scope and Seq-Scope-X data were generated from the same mouse strain, age, housing condition, and tissue type (liver), processed with identical freezing, sectioning, and library preparation and sequencing protocols using NovaSeq 6000-based arrays prepared in parallel. Therefore, while minor variations may arise from various technical sources, we do not expect substantial inter-sample differences.

Fig. 3C and 3F show FICTURE projections of hepatocellular cell types inferred from the multidimensional clustering results in Fig. 3A and 3D. The overall clustering structures are highly consistent between the two methods, indicating no substantial differences in cell type composition (Fig. 3A and 3D). The spatial organization is also broadly similar; however, projections generated from conventional Seq-Scope appear noticeably more diffuse (Fig. 3E and 3F), whereas Seq-Scope-X reveals sharper and more coherent structures (Fig. 3B and 3C). The diffuse patterns in the conventional data likely reflect lower spatial precision and greater transcript diffusion, which degrade the spatial assignment of closely related hepatocyte subtypes. These deviations represent technical limitations of conventional Seq-Scope rather than true biological differences.

To directly address the reviewer's concern, we performed an integrated analysis combining Seq-Scope and Seq-Scope-X datasets, ensuring both were processed jointly within the same analytical framework. Using three different hexagonal grid sizes (flat-to-flat heights of 24 µm, 18 µm, and 12 µm, corresponding to the original tissue size; **Response Letter Fig. 9A-9L**), we found that the datasets integrated well, forming reproducible clusters corresponding to the same hepatocellular and non-parenchymal cell types (**Response Letter Fig. 9B, 9F, and 9J**). Importantly, spatial plots from these integrated datasets revealed the same expected portal-central zonation pattern (**Response Letter Fig. 9C, 9G, and 9K**), and the relative proportions of each cell type were highly similar between Seq-Scope and Seq-Scope-X (**Response Letter Fig. 9D, 9H, and 9L**). As expected, Seq-Scope-X still produced clearer and less noisy boundaries between zonation layers, consistent with its enhanced spatial resolution (insets in **Response Letter Fig. 9C, 9G, and 9K**).



Response Letter Figure 9. Integrated analysis of liver spatial transcriptomes profiled using conventional Seq-Scope and Seq-Scope-X. The datasets were analyzed using hexagonal grids with flat-to-flat heights of 24 µm (A–D), 18 µm (E–H), and 12 µm (I–L), corresponding to the original tissue scale (adjusted for the expansion factor). (A, E, I) UMAP plots showing effective data integration between conventional Seq-Scope (blue) and Seq-Scope-X (red) datasets. (B, F, J) UMAP plots illustrating distinct hepatocellular and non-parenchymal cell types identified by multidimensional clustering. (C, G, K) Spatial maps of hexagonal grids color-coded by cell type. Boxed areas were magnified in insets. (D, H, L) Proportions of different cell types derived from each analysis, demonstrating consistent cell type composition between conventional Seq-Scope and Seq-Scope-X.

Together, these results confirm that the apparent differences in Fig. 3 arise mainly from technical limitations of conventional Seq-Scope, not from biological or sampling differences. The new integrated analysis demonstrates that the two datasets show consistent cell type composition and organization, while confirming the superior spatial precision achieved with Seq-Scope-X.

Finally, we identified labeling errors in Fig. 3B and 3E, which have been corrected in the revised manuscript. We apologize for any confusion this error may have caused.

Relevant sections: Fig. 3B, 3E, S6. Line 274-285.

12. The contrast in figure S3A and S3B, left panels, can be improved, particularly for the green channel that is barely visible. It is again unclear how in panels associated with Seq-Scope-X (middle and right panels in S3A) there are not just green and red but also blue dots.

We thank the reviewer for these helpful comments. We agree that the green signal (representing unspliced RNA) in the left panels of the former Fig. S3A and S3B were difficult to distinguish. This reflects the intrinsic difference in abundance between spliced and unspliced transcripts - spliced RNAs are substantially more abundant and therefore visually dominate the raw images.

In the revised version, we have adjusted the image contrast to make the green signal more visible without altering the underlying data (**Response Letter Fig. 7**). These new renderings more clearly illustrate that Seq-Scope-X produces concentrated unspliced signals around nuclear regions, while conventional Seq-Scope displays a more diffuse and scattered pattern. The revised panels are now included in the main Figure 1, which provides a more direct and balanced comparison.

We also acknowledge that the previous figure inadvertently included a blue channel representing an unrelated analysis. This has been removed in the updated version. Together, these revisions improve image clarity and remove the source of confusion in the former Fig. S3A–B.

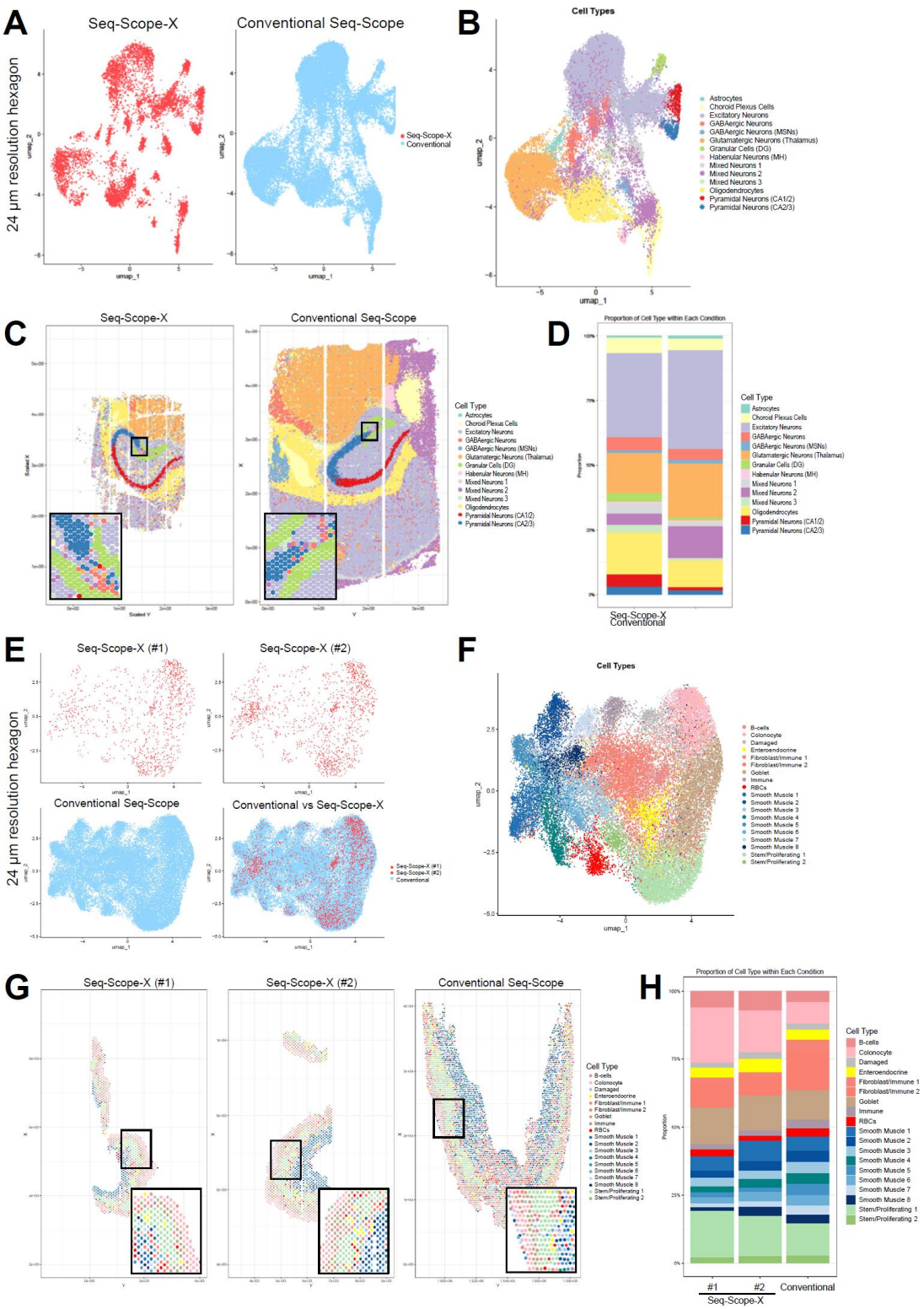
Relevant sections: Fig. 1G-1M. Line 133-145

13. In figure 4, The global tissue organization seems better preserved for the brain sample (panel B) than for the colon sample (panel C). In fact, the structure in the panel C Seq-Scope-X images seems quite obscure (particularly in comparison with the structure of the standard Seq-Scope image, but also broadly), I am not convinced that the method worked correctly on these specific samples. It would be useful to show other examples / somehow indicate the correctness of the information contained in these images. Panels in figure S4H-I from the same sample type seem better, but there is no comparison to standard Seq-Scope data in this case, making the validity / global structure preservation more challenging to assess.

We appreciate the reviewer's careful evaluation. To directly address whether Seq-Scope-X preserves global tissue organization in both brain and colon samples, we performed an integrated analysis using conventional Seq-Scope and Seq-Scope-X datasets processed side-by-side. Both datasets were binned using hexagonal grids with a 24 μm flat-to-flat height, corresponding to the same physical tissue scale after adjusting for the expansion factor.

The integrated analyses (**Response Letter Fig. 10**) show that conventional Seq-Scope and Seq-Scope-X data align closely in a shared UMAP space (**Response Letter Fig. 10A, 10E**), indicating strong cross-dataset concordance. Multidimensional clustering identified the same major cell types across both methods (**Response Letter Fig. 10B, 10F**). Importantly, spatial projection of these clusters reproduced the same tissue structures in brain and colon (**Response Letter Fig. 10C, 10G**), and the

overall cell type compositions were highly similar between the two platforms (**Response Letter Fig. 10D, 10H**).



Response Letter Figure 10. Integrated analysis of brain (A-D) and colon (E-H) spatial transcriptomes profiled using conventional Seq-Scope and Seq-Scope-X. The datasets were analyzed using hexagonal grids with flat-to-flat heights of 24

μm, corresponding to the original tissue scale (adjusted for the expansion factor). (A, E) UMAP plots showing effective data integration between conventional Seq-Scope (blue) and Seq-Scope-X (red) datasets. Two batches of tissues were analyzed in Seq-Scope-X colon datasets (#1 and #2). (B, F) UMAP plots illustrating distinct cell types identified by multidimensional clustering. (C, G) Spatial maps of hexagonal grids color-coded by cell type. Boxed areas were magnified in insets. (D, H, L) Proportions of different cell types derived from each analysis, demonstrating consistent cell type composition between conventional Seq-Scope and Seq-Scope-X.

These new results confirm that Seq-Scope-X preserves global tissue architecture and validate the structural information in the Seq-Scope-X brain and colon datasets.

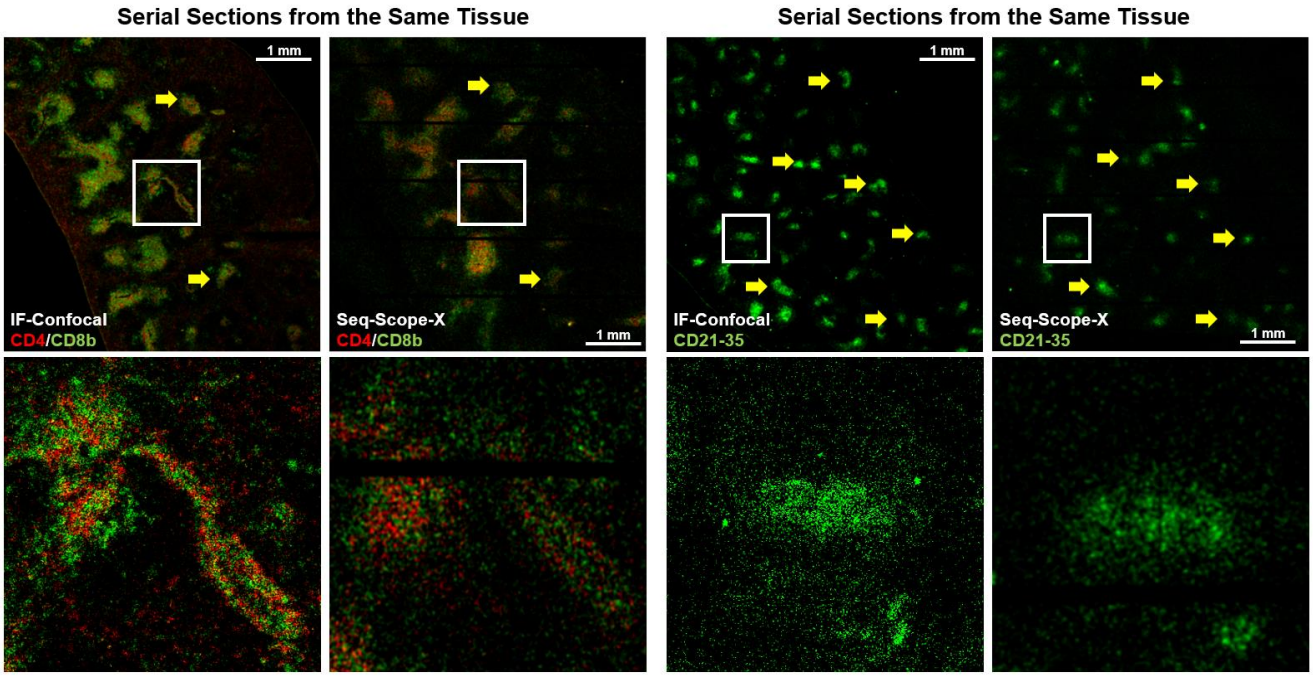
Relevant sections: Fig. S8. Line 307-312.

14. Figure 5 demonstrates the application of Seq-Scope-X for spatial proteome capture. While the results seem promising in terms of identifying distinct spatial distributions for many different proteins tagged in two types of tissues (mouse spleen and human tonsil); additional validation of the correctness of the results seems to be required, in particular in light of the discrepancy shown, described and explained, between ExM and Seq-Scope-X image of the sample analyzed, still suggesting potential loss of some spatial information. In particular, it would be useful to compare some of the spatial distributions of proteins shown (Fig. 5D) to their distribution captured via a distinct method (possibly proExM applied with standard antibodies). Moreover, applying spatial transcriptomics to the same tissue samples as the ones to which spatial proteomics were captured, and conducting similar cell-type clustering and spatial distribution capture with FICTURE, could be used to show that the spatial proteome reliably captures the spatial distribution of cell types in the tissue, preserving spatial information.

We thank the reviewer for raising this important point. To directly validate the spatial proteomic patterns observed with Seq-Scope-X, we performed immunofluorescence confocal microscopy on nearby tissue sections from the same samples using well-established antibody clones targeting CD4, CD8b, and CD21-CD35. These markers represent major lymphocyte and follicular populations and are routinely used for high-confidence spatial immunophenotyping. We compared the immunofluorescence staining patterns with the corresponding Seq-Scope-X antibody-derived tag (AbTag) signals side-by-side using serial sections, and the results are presented in **Response Letter Fig. 11**.

This analysis showed that Seq-Scope-X faithfully recapitulates the expected spatial distributions of these proteins, with patterns that closely match those observed by confocal imaging. The agreement was consistent across cell-rich and boundary regions, supporting that Seq-Scope-X preserves spatial protein localization with high fidelity. Importantly, the precision of protein mapping achieved by Seq-Scope-X is comparable to that of confocal microscopy, despite being generated through a sequencing-based capture process.

These validation data demonstrate that Seq-Scope-X proteome mapping preserves spatial integrity and reliably captures biologically meaningful protein distributions. Together with our other analyses (including the expansion-associated distortion quantification shown in **Response Letter Fig. 1**), these results also confirm that the method does not exhibit systematic spatial distortion.



Response Letter Figure 11. Side-by-side comparison of CD4, CD8b, and CD21-35 immunodetection using immunofluorescence confocal microscopy (IF-Confocal) and Seq-Scope-X. Serial sections from the same mouse spleen were processed by either IF-Confocal or Seq-Scope-X to visualize CD4 and CD8b (left panels) and CD21-35 (right panels). The spatial patterns show strong overall concordance between the two methods, with minor differences expected from the use of neighboring, rather than identical, sections. Yellow arrows mark salient structures present in both images. Boxed regions are shown at higher magnification in the lower panels.

Relevant sections: Fig. S9D, S9E. Line 360-371.

15. In figure 6 the use of a DMAA gel (corresponding to the 10x expansion protocol) rather than a standard ExM gel is demonstrated on mouse spleen tissue. While the images look very convincing, it is a shame that a distinct tissue type was chosen for this demonstration (mouse spleen), compared to other imaging with Seq-Scope-X which was conducted on liver, brain and colon images. It would be useful to show images of the same type of tissue obtained with the two gel chemistries for mutual validation that both chemistries similarly preserve spatial transcriptome information while allowing its capture at high resolution.

We appreciate the reviewer's suggestion. At present, establishing robust transcriptome capture with DMAA-based gels has been more challenging, likely because very high expansion factors reduce the effective mRNA concentration and alter ionic conditions required for stable RNA anchoring. This remains an active area of optimization and development.

By contrast, proteomic applications have been more successful with both standard and DMAA gels. Several factors likely contribute to this difference. First, protein targets are far more abundant per cell than mRNA. Second, antibody-bound DNA oligonucleotides are substantially more chemically stable than RNA, which improves their retention during tissue digestion and expansion. Third, DNA barcodes enable straightforward library preparation without random-primed extension, reducing vulnerability to sequence loss.

For proteomic applications, we selected spleen and tonsil because commercially available antibody panels with poly-A–tailed oligonucleotide tags are primarily optimized for hematopoietic cell surface proteins. These cocktails include more than 100 validated antibodies and were originally developed for single-cell proteogenomic profiling of mouse and human PBMCs, which made them natural starting points for establishing high-plex spatial proteomics with Seq-Scope-X.

Importantly, we performed DMAA-based expansion experiments using mouse spleen tissue and generated matched datasets from both standard and DMAA gels (shown in Fig. 5 and Fig. 6). These datasets exhibit concordant antibody staining patterns and consistent cell type assignments, for example identifying distinct T cell, B cell, and myeloid-lineage populations. Together with our quantified distortion analysis showing that DMAA gels maintain near-isotropic expansion with less than 5 percent error (**Response Letter Fig. 1**), these data support that both gel chemistries preserve spatial proteome information.

Nevertheless, we agree that demonstrating transcriptome capture using both gel chemistries in the same tissue type would be an informative future enhancement. As improvements in molecular anchoring strategies are developed, we anticipate that transcriptome applications will also be able to take full advantage of DMAA-based expansion. We have incorporated this discussion into the revised manuscript.

Relevant sections: Line 437-446, 457-465.

Finally, a few minor questions / comments / suggested corrections:
1. In line 85 the gel is referred to as a "polyacrylamide gel". However, what makes expansion microscopy gels unique is their ability to expand that is a consequence of the absorptive properties of sodium acrylate, the main gel monomer. Thus, describing the gel as a "polyacrylamide gel" is not the right terminology to use. Similarly, in lines 779 and 811 the gel is referred to as "acrylite hydrogel" which is also not the correct nomenclature.

We thank the reviewer for pointing this out. We have corrected the terminology throughout the manuscript. The gel is now described as a polyacrylate-containing expandable hydrogel, consistent with standard expansion microscopy terminology.

Relevant sections: Line 88, 945, 997.

2. Line 510: "After disassembling humidified chamber" ==> "After disassembling the humidified chamber"

We thank the reviewer for these suggestions. The text has been corrected accordingly.

Relevant sections: Line 597

3. Citation 24 redundantly includes the words "Optical imaging" that are not part of the manuscript title. This should be removed.

We thank the reviewer for these suggestions. The text has been corrected accordingly.

Relevant sections: Reference 24

4. Line 778: “Tissue” ==> “The tissue” and line 779 (same sentence): “probe” ==> “probes”

We thank the reviewer for these suggestions. The text has been corrected accordingly.

Relevant section: Line 943-944.

5. Line 349: “significant advancement” ==> “significant advance”

We thank the reviewer for these suggestions. The text has been corrected accordingly.

Relevant sections: Line 429

Reviewer #3 (Remarks to the Author):

In the present manuscript, the authors introduce a sequencing-based spatial transcriptomics method that achieves submicrometer-scale resolution through tissue expansion and incorporates antibody-derived barcode tags for proteome profiling. Overall, the new technique seems to be an interesting contribution to the field and the performance is demonstrated reasonably. However, several aspects of the study could still be improved.

Major comments:
- Line 97 mentions that the expansion factor ranged between 2- and 3-fold without causing substantial distortion of the overall tissue structure. Was there any testing to determine at what expansion level distortion would begin to affect the results of transcriptome analysis—for example, under the theoretically expected 10-fold expansion using DMAA chemistry (and 10-fold resolution increase that is mentioned in the abstract). The comparison in Figures S1A and S1B is very clear and intuitive; why does Figure 6B lack a similar direct overlap comparison? Could the 3.3-fold DMAA expansion have introduced tissue distortion?

We thank the reviewer for raising this important point. As noted, acrylamide-acrylate gels used for ~2-3X expansion showed largely isotropic behavior across multiple tissues, and we further substantiated these findings by performing displacement vector mapping, anisotropy heatmaps, and RMS distortion statistics (also see above in **Response Letter Fig. 1A, 1C**).

Regarding the lack of a direct overlap comparison in Fig. 6B: while pre/post overlap images were feasible using samples dedicated for imaging, we found that repeated high-resolution fluorescence imaging significantly damaged or depleted nucleic acids, which reduced library quality and resulted in unusable sequencing data. For this reason, the specific samples used for Fig. 6B did not yield pre/post images of sufficient quality to enable a robust isotropy assessment.

Instead, we examined additional parallel samples dedicated to isotropy imaging analysis, using the identical expansion condition (3.3X DMAA expansion). These results were described above in **Response Letter Fig. 1B and Fig. 1C**, and confirm that DMAA expansion is isotropic with less than 5 percent spatial distortion overall, which is comparable to conventional acrylamide gel expansion (**Response Letter Fig. 1A and Fig. 1C**). Therefore, the 3.3-fold DMAA expansion used in Fig. 6B is unlikely to introduce distortion that would affect the spatial transcriptome or proteome analysis.

Relevant sections: Fig. S1C, S1D, S1E. Line 110-117, 397-399, 474-485, 894-911.

- L135-147: For the benchmarking of sensitivity (Figure 1L), is UMI per μm² "back-calculated" to original tissue size before expansion? Also, besides UMIs per μm², including additional metrics would provide a more comprehensive benchmarking—for example, UMI and gene detection across different bin sizes and per segmented cell.

Yes, UMI per μm² was recalculated to the pre-expansion (original) tissue area by dividing the measured area by the square of the sample's expansion factor, so the Seq-Scope and Seq-Scope-X values are

directly comparable on the same physical scale. In addition, to provide a more robust benchmarking, we re-binned all datasets to matched spatial bin sizes referenced to the original tissue scale and reported sensitivity across multiple bin sizes. The results were described above in **Response Letter Fig. 2 and 3**. Specifically, to address the reviewer's request for broader metrics, we now include: (i) UMI and genes per bin across several bin sizes (which matches with resolutions of the other previous ST technologies; **Response Letter Fig. 2A, 2B**), and (ii) UMI and genes per segmented cell (including cell area distributions), enabling a like-for-like comparison of capture efficiency and library complexity between conventional Seq-Scope and Seq-Scope-X (**Response Letter Fig. 3**). These analyses corroborate our conclusion that Seq-Scope-X achieves comparable capture efficiency to state-of-the-art methods while providing higher spatial resolution.

Relevant sections: Fig. S2. Line 169-184.

- L163-167: Advise caution with this type of analysis. The UMAP space is NOT interpretable due to non-linear compression. Are the 5 major cell types known archetypes or ad hoc definitions of the authors?

We agree that UMAP is a non-linear dimensionality reduction and should be interpreted cautiously. Accordingly, we did not draw conclusions solely from the UMAP visualization but instead used it as a convenient display of transcriptomic similarity. In the revised manuscript, we have updated the corresponding text to clarify that cell type identities are not defined by the UMAP embedding.

The five hepatocellular subtypes we report are not ad hoc definitions; they correspond to well-established liver zonation archetypes along the portal–central axis. Specifically, we identified periportal (PP), portal-side midzone (PM), central-side midzone (CM), pericentral (PC), and centrilobular (CL) hepatocytes, together with non-parenchymal cells. These zonal hepatocyte populations are consistently observed in both bulk and single-cell transcriptomic studies through various methodologies and are supported by well-known marker gradients.

To further support our analysis, we additionally performed integrated data analysis combining both conventional Seq-Scope and Seq-Scope-X results (**Response Letter Fig. 9**), which produced results consistent with our previous analyses, identifying all hepatocellular cell types. Thus, while UMAP provides a convenient visualization of the data, the biological identities of the hepatocyte groups are grounded in well-established liver biology rather than defined by the manifold structure.

Relevant sections: Fig. S6. Line 202-208, 274-285.

- L176-182: This statement lacks experimental data or supporting references and appears to be overinterpreted. Solid support would require, for example, the observation of known markers of the state transition in the nuclear/cytoplasmic analyses.

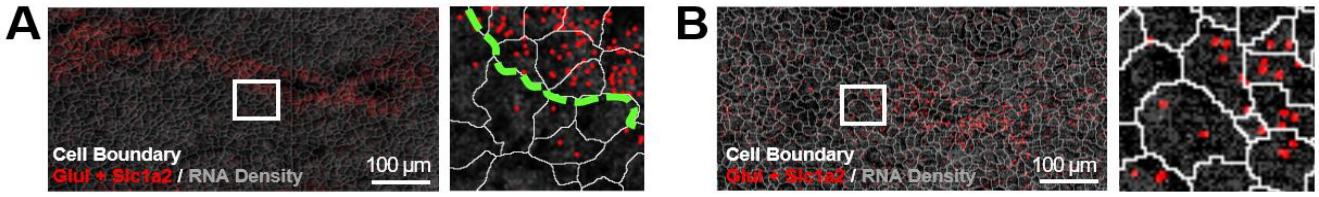
We appreciate the reviewer's caution. We agree that strong claims about dynamic state transitions require direct validation with established markers. In the revised manuscript, we have clarified that our current data primarily demonstrate widespread nuclear–cytoplasmic transcriptome differences, which

are consistent with—but do not in themselves prove—transcriptional state switching. We note that several well-characterized zonation markers showed distinct nuclear-cytoplasmic distributions in our analyses, an evidence from orthogonal technologies such as MERFISH (Fig. 2J) and smFISH (Fig. 2K, 2L), supporting the biological plausibility of this observation. Nonetheless, we have toned down our interpretation, now presenting these findings as an intriguing observation rather than definitive evidence of hepatocyte state transitions, and highlighting the need for future studies to more rigorously link nuclear/cytoplasmic disparities with dynamic functional states.

Relevant sections: Line 216-222.

- One of the strengths of Seq-Scope-X is its improved ability to segment cells within tissues, which is a major challenge in spatial analysis (whereas relatively few researchers may have biological questions that actually require subcellular-level analysis). It would be helpful to explicitly demonstrate this improvement.

We agree with the reviewer that improved cell segmentation is one of the key advantages of Seq-Scope-X, as accurate cell boundaries remain a central challenge in spatial analysis. In the revised manuscript, we now more directly demonstrate this improvement. Specifically, we highlight the clear single-cell–level expression of *Glul* and *Slc1a2*, which shows well-delineated, cell-confined expression domains in Seq-Scope-X data (**Response Letter Fig. 12**). This provides a strong example of how the increased resolution directly improves cell segmentation compared to conventional technologies, where such boundaries are either undetected or blurred.



Response Letter Figure 12. Side-by-side comparison of Seq-Scope-X (A) and conventional Seq-Scope (B) data in estimating cell boundary and *Glul* + *Slc1a2* expression. Seq-Scope-X shows superior performance in clearly separating centrilobular (CL) hepatocytes from other cell types with lower *Glul* + *Slc1a2* expression.

Relevant sections: Fig. 1N, 1O. Line 146-153.

- L327: In the section titled "Seq-Scope-X with DMAA Chemistry Enables Greater Expansion"), only proteomic results are presented, with no transcriptomic results comparable to that shown for the standard expansion gels.

We thank the reviewer for pointing out this discrepancy. At present, robust transcriptome capture with DMAA chemistry has been more difficult to establish, likely due to reduced transcript concentration and weaker RNA anchoring at very high expansion factors. This remains an area of active optimization. In contrast, our proteomic workflow has been successfully combined with DMAA gels, yielding consistent and interpretable results. To avoid overstating the scope of our current data, we have revised the

section title to clarify that “DMAA Chemistry Enables Greater Expansion for Seq-Scope-X Proteomic Workflow.” This wording more accurately reflects the present stage of development.

Relevant section: Line 385.

- L369: This is an important caveat of the technique (inherent limitation of Seq-Scope-X is that tissue expansion inherently requires a larger capture area). It would be important for the reader to understand how much cost difference the increased resolution comes with due to larger chip areas being occupied.

The reviewer has correctly identified the tradeoff between the improved resolution and cost. We have expanded our discussion to highlight this limitation more clearly. Because Seq-Scope-X relies on tissue expansion, the same biological region occupies a proportionally larger area on the capture array, increasing chip usage and sequencing cost when broad coverage is needed. We now provide an estimate of this cost scaling in the revised text so readers can appreciate the trade-off between resolution and throughput.

Relevant section: Line 466-473.

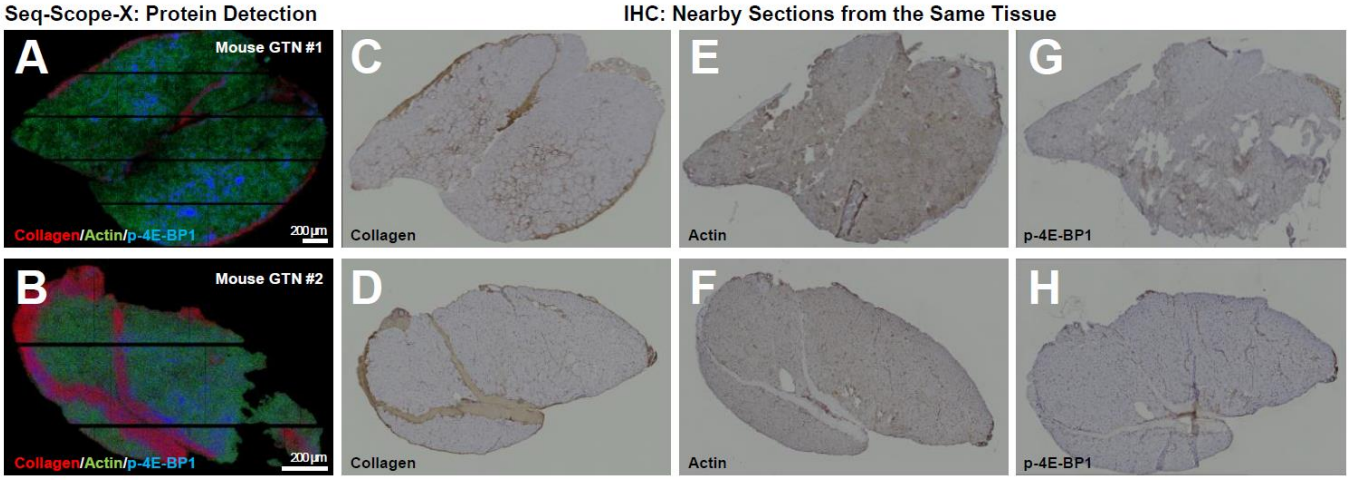
- L420–427: Having only IgM is insufficient to demonstrate the ability to detect intracellular proteins. It would be preferable to include multiple intracellular proteins or additional ER-localizing markers as supporting evidence. Furthermore, it is important to clearly indicate the resolution of the nucleotide-tagged antibodies to avoid misunderstanding—does it, like the transcriptomic data, reach the subcellular level?

We appreciate the reviewer’s request for additional evidence supporting intracellular protein detection. To address this point, we tested whether Seq-Scope-X can detect extracellular and intracellular proteins, as well as post-translational modifications, using a mouse skeletal muscle tissue characterized by strong mammalian target of rapamycin complex 1 (mTORC1) signaling (Cho et al. Autophagy 2022, 18:2303-2322). This tissue displays marked extracellular collagen deposition, intracellular actin filament bundles, and high mTORC1 activity that produces robust phosphorylation of downstream targets such as 4E-BP1 within spatially restricted cytosolic regions, as described in our previous study (Cho et al. Autophagy 2022, 18:2303-2322).

For this experiment, we conjugated barcoded oligonucleotides to antibodies targeting an intracellular structural protein (ACTA1), an intracellular signaling protein (phospho-4E-BP1; p-4E-BP1), and an extracellular matrix protein (COL1A1). These nucleotide-tagged antibodies were generated in-house using protein G-mediated oligonucleotide conjugation (oYo-Link technology). Using these reagents, we performed Seq-Scope-X multiplexed digital protein detection (**Response Letter Fig. 13A, 13B**) and classical immunohistochemistry on serial sections from the same muscle tissue (**Response Letter Fig. 13C-13H**).

Both analyses revealed consistent spatial patterns. Collagen antibodies marked extracellular matrix structures (**Response Letter Fig. 13A-13D**), ACTA1 antibodies labeled intracellular actin fibers

(Response Letter Fig. 13A, 13B, 13E, 13F), and phospho-4E-BP1 antibodies highlighted localized cytosolic activation domains consistent with mTORC1 activity (**Response Letter Fig. 13A, 13B, 13G, 13H**). These results indicate that Seq-Scope-X proteomic detection extends to extracellular and intracellular proteins, including post-translationally modified targets, and that the spatial patterns obtained with Seq-Scope-X are in close agreement with those observed using conventional immunohistochemistry.



Response Letter Figure 13. Seq-Scope-X detection of extracellular, intracellular and post-translational modification (PTM) targets. (A, B) COL1A1 (red), ACTA1 (green) and p-4E-BP1 (blue) antibodies were labeled with oligonucleotide tags, and used for Seq-Scope-X analysis of gastrocnemius (GTN) muscle from *CKM-Cre/Tsc1*$^{F/F}$*/Depdc5*$^{F/F}$ (mTORC1-activated) mice. Spatially detected antibody tags were visualized through spatial plotting. (C-H) Expression patterns of each of these proteins were examined through traditional immunohistochemistry using the sections from the same tissue, showing similar patterns of expression.

With respect to resolution, the nominal spatial resolution of Seq-Scope-X proteomics extends beyond the subcellular scale. In practice, however, detection efficiency and pre-capture molecular diffusion limit confident visualization of fine subcellular features. Still, our comparisons show that even individual antibody measurements in Seq-Scope-X can yield spatial patterns that are similar to immunofluorescence confocal microscopy (**Response Letter Fig. 11**) or traditional immunohistochemical detection (**Response Letter Fig. 13**). Such a level of spatial precision, together with the ability to detect intracellular, extracellular, and post-translationally modified targets, has not been achieved by earlier sequencing-based protein detection methods.

We have revised the manuscript to clarify these point and to frame these effects as current technical limitations that motivate future methodological improvements.

Relevant section: Fig. S12. Line 408-426, 654-662, 702-722.

Minor comments:

- General: Seq-Scope-X offers significant improvements in resolution and multi-omics capabilities. However, there are many overstated claims that seem unnecessary, such as those in lines 308–313, 414–419, 422–423, 457–460, and 467–472.

We appreciate the reviewer's evaluation recognizing the significant improvements in resolution and multi-omics capabilities. We agree that the indicated overstatements are not necessary and can be removed or rephrased without weakening the overall impact of the paper. These modifications improve clarity and precision. We have made the suggested revisions accordingly as listed below.

Former 308-313: Deleted.
Former 414-419: Rewritten in Line 514-519.
Former 422-423: Rewritten in Line 523-528.
Former 457-460: Deleted.
Former 467-472: Rewritten in Line 553-558.

- L107: "Supra-Optical Resolution" do they mean superresolution? supra = greek for above?

We thank the reviewer for pointing this out. Our intended meaning was "superresolution", referring to resolution beyond the diffraction limit of conventional optical microscopy. The term "supra-optical resolution" could be interpreted ambiguously, so we have revised it to "super-resolution" for clarity.

Relevant section: Line 121.

- L270: The abbreviation 'bST' appears to be a misspelling? Or if not, provide its full form.

We thank the reviewer for pointing this out. This was a typographical error. It should be written as sST, which refers to sequencing-based ST (defined earlier in the text). We apologize for the mistake and have made the correction.

Relevant section: Line 319.

- L462-464: Why did the DMAA expansion not reach the theoretically expected 10-fold expansion, but instead only achieved approximately 3.3-fold.

The theoretically reported approximately 10-fold linear expansion of DMAA hydrogels assumes maximal osmotic swelling in very low ionic strength media such as pure water. In our transcriptomics workflow, however, robust capture and chip transfer depend on stable poly(T)–poly(A) hybridization for both mRNAs and antibody tags. These interactions are destabilized under low salt conditions, which prevents full equilibration to the theoretical 10-fold expansion.

Expansion also occurs in three dimensions. A 3.3-fold linear expansion corresponds to roughly 36-fold volumetric dilution, whereas a full 10-fold linear expansion would create nearly 1,000-fold dilution. Such extreme dilution markedly reduces the effective concentrations of targets and probes and increases the

probability of molecular dissociation from the gel matrix during processing. In addition, hydrogel sections become increasingly fragile as swelling increases. Near 10-fold expansion, the gel becomes mechanically unstable, making distortion or tearing during handling more likely.

These biochemical and physical constraints together impose a practical upper limit in our current implementation. We therefore selected approximately 3.3-fold as the optimal balance between achievable expansion, capture efficiency, molecular retention, and library quality. We also moderated the swelling by adding salt to the monomer solution and expansion buffer to maintain poly(T)–poly(A) duplex stability.

Looking ahead, we consider larger expansion both desirable and feasible. Achieving it will likely require additional optimization, including alternatives to hybridization-based capture. For example, RNA could in principle be covalently immobilized through chemical capture strategies. However, such approaches would require dedicated capture-release chemistries compatible with the Seq-Scope array and must account for the inherently lower chemical stability of RNA compared to protein, which still necessitates relatively mild processing conditions. Conditional-release chemistries that sustain molecular retention under low ionic strength conditions, as well as in situ molecular amplification to compensate for increased dilution, may further enhance feasibility. Practical handling challenges at high swelling may also be addressed by iterative expansion, which provides additional hydrogel scaffold support. Although these developments are beyond the scope of the present study, we now note them as promising directions for enabling larger, transcriptomically robust expansion in future work.

Relevant section: Line 437-446, 457-465.

Reviewer #1 (Remarks to the Author):

The additional quantitative analyses address our concerns. To further strengthen the manuscript, we recommend refining the Methods section regarding the distortion calculations. While we can infer the authors' intent, the description in Lines 907-911 remains ambiguous. We suggest rephrasing this section to ensure the methodology is described clearly and without ambiguity.It would be helpful to define exactly what constitutes 'displacement error' and how 'RMS' is calculated in this context.
We are also confused by the phrasing 'generated all possible pairs of the down sampled displacement vectors.' It is unclear how 'all possible pairs' were generated if the data was first 'down-sampled'. Please clarify if this refers to all pairwise combinations within the down-sampled subset, or if a different sampling strategy was used. A clear calculation flow is needed.

We thank the reviewer for this constructive comment. We have revised the Methods section (previously Lines 907–911, now Lines 943–952 in the revised manuscript) to provide a clear and unambiguous description of the distortion analysis workflow. In the revised text, we explicitly define each displacement vector as consisting of a measurement location and its associated displacement derived from image alignment. We clarify that the displacement error is computed as the Euclidean distance between two displacement vectors, while the measurement distance is defined as the Euclidean distance between their corresponding spatial locations. We further specify that, after down-sampling the displacement field, all pairwise combinations were generated within the down-sampled subset, and that the RMSE for each measurement distance was calculated as the square root of the mean squared displacement error. These revisions clarify both the sampling strategy and the calculation flow, addressing the ambiguity noted by the reviewer.

Reviewer #2 (Remarks to the Author):

We find that the authors fully addressed the concerns raised by us and the other reviewers, significantly improving the manuscript.
In particular, the concern of non-isotropic expansion was addressed by additional experiments and analyses comparing isotropy in multiple tissue types. The presented error rates are consistent with the literature and the reported tissue differences would be valuable for future application of the novel Seq-Scope-X method.
The authors also very convincingly addressed the concern I raised regarding a potential limitation of RNA capture due to the method's design - I found the added explanation regarding the use of a temperature gradient to ensure directional mRNA transfer from the gel to the array probe useful. The concern was further indirectly addressed by additional improvement of the quantitative validation of RNA capture performance, including the reporting of UMI/μm2 and UMI/cell and showing a UMI/cell decrease with respect to Seq-Scope without expansion that is lower than expected according to the expansion factor.
Concerns regarding protocol details were also fully addressed by correcting an error in reported APS concentration and explaining protocol optimizations / modifications made for this

specific application of expansion, prioritizing the preservation of RNA molecules. Similarly, we found the re-wording of the tissue expansion methods section useful.
All detailed text and figure corrections requested were thoroughly and rigorously performed.

We thank the reviewer for their positive evaluation and thoughtful feedback. We are pleased that the additional experiments, analyses, and clarifications adequately addressed the concerns raised and improved the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have addressed all of the raised concerns with new experiments and analyses.

We thank the reviewer for their positive evaluation.

Reviewer #4 (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.

We thank the reviewer for their time and contribution to the peer-review process.

Reviewer #5 (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.

We thank the reviewer for their time and contribution to the peer-review process.