nature portfolio

Peer Review File

Spatial profiling of chromatin accessibility in formalin-fixed paraffin-embedded tissues

Corresponding Author: Dr Yanxiang Deng

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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 manuscript “Spatial profiling of chromatin accessibility in formalin-fixed paraffin-embedded tissues” introduces ATAC-seq chromatin profiling of FFPE-preserved sections. This is a modification of a previously published method (PMID: 35978191). Since FFPE samples are heavily crosslinked and embedded, the current manuscript explores crosslink reversal and epitope retrieval steps to enable tagmentation.

The limited data here is not adequate to judge the performance of these modifications for spatial-ATAC-seq. The analysis here relies on bioinformatic packages designed for single cell ATAC analysis, but does not provide quantification of the quality of results. The main problem with FFPE material is that DNA is degraded, and the integrity of DNA is a standard measure for genomic analysis of FFPE samples, and should be assessed, because there are a number of features in the data that suggest the quality is low. Extended Data Figure 2e, 4a, and 4c show ATAC fragment size profiles which the authors described as “optimized” (line 80), although these profiles do not meet the standards for ATAC-seq pipelines, which expect a significant nucleosomal band. The authors should assess and discuss whether these standards are feasible for samples with heavily damaged DNA. Only one figure shows genomic tracks of the data (Figure 4f); qualitatively these tracks look sparse with modest peaks. Comparison to high-quality ATAC-seq data should be included.

Figure 2f raises fundamental concerns that must be addressed. The panel for Bcl11b shows no similarity between inferred ATAC signal and ISH. Car10, maybe: it’s hard to tell since these slides are unrelated and these images cannot be aligned. Prox1 is clearly a mixture of some overlap and some non-overlap, Tbr1 shows no similarity. Further, the dynamic range of inferred marker signal is very small. These lack of correspondence raise questions on what is the quality of the data and whether it is being analyzed appropriately.

Quantitative performance of data quality should be presented, and the manuscript makes a number of statements on data quality that appear unsupported:
Line 75: “extensive optimization of the target retrieval conditions to achieve a high signal-to-noise ratio, as measured by the transcription start site (TSS) enrichment score, and a high yield, indicated by the number of unique fragments”. The presented values are substantially lower than controls, and should also be compared to high-quality ATAC-seq for quality assessment.
Line 87 “Unsupervised clustering identified eight distinct clusters, with spatial projections closely aligning with tissue morphology observed in adjacent H&E-stained sections (Fig. 2b,e). For example, chromatin accessibility highlighted the cortex and cerebellum regions, with Bcl11b and Car10 serving as respective marker genes, which is consistent with gene expression data from the Allen Brain Atlas (Fig. 2f and Extended Data Fig. 3).” The spatial pattern of UMAP clusters in Figure 2b does not look similar to H&E staining in Figure 2e; what do the authors mean by “closely aligning”?

Line 92: “The consistency between the reference and our data confirms our data quality.”
Line 95: “The successful optimization of spatial FFPE-ATAC-seq enabled high-quality chromatin accessibility profiling from FFPE mouse tissues, matching fresh frozen data, while accurately identifying distinct spatial patterns and neuronal subtypes.”
These statements imply quantitation, but no quantitation is presented.

Line 103: “By integrating the spatial-FFPE-ATAC-seq data with scRNA-seq data from human cerebellar cells, we identified

the major cell types—including oligodendrocytes, granule cells, and Purkinje cells." It is not apparent how these conclusions are being made. It doesn't seem that spatial-FFPE-ATAC-seq has cellular resolution, so how can the authors be identifying cell types?

The previous paper that describes the design and performance of the spatial-ATAC-seq method is not adequately cited.

The description of the method is lacking in details for a reader; this paper gives the pixel size of the grid, but doesn't state the area that it covers or its effective cellular resolution. In the previous paper it is apparent that the spatial grid only covers a small fraction of the tissue sample, but these details should be made clear here.

It is not made clear in the paper how many samples are analyzed, and the GEO entry lists 8 with no apparent replicates. The authors should justify their choice to not perform replicates for reproducibility. I would like to see more comprehensive descriptions of sequencing results in the manuscript; how many reads, mapping rates, duplication rates. All of this inform the reader on data quality for this method.

Reviewer #2

(Remarks to the Author)
The authors reported on the adaptation of their previously developed spatial-level chromatin accessibility analysis method, spatial ATAC-seq (Deng et al., 2022), to FFPE samples, resulting in spatial FFPE-ATAC-seq. By comparing the data quality with that obtained using spatial ATAC-seq on fresh frozen (FF) sections, they demonstrate the feasibility of obtaining data of a certain quality using FFPE sections. As the authors also assert, enabling spatial chromatin analysis using FFPE sections holds significant potential for clinical applications and for leveraging the wealth of past archive of tissue blocks. However, the results presented in this paper primarily focus on optimizing experimental conditions for applying their spatial ATAC-seq to FFPE samples and the quality assesments of the obtained data. Without a novel experimental ideas, sufficient investigation of the impact of FFPE-induced cellular damage on analysis results, or demonstration on the potential applicability for clinical samples, that this reports may be perceived as merely a minor update to their original paper.

Below are the specific comments.

1. Throughout the paper, the data analysis results are largely similar to those presented in their previous study (Deng et al., 2022). As mentioned above, providing a demonstration of insights that become possible by this spatial FFPE-ATAC-seq, such as cancer tissue analysis, or a detailed investigation of how cellular damage affects the quality of the data and the analysis results, is essential.

2. Fig. 1b-c: How does the performance compare to other spatial ATAC-seq methods (e.g., Thornton, Nat. Commun., 2021, Llorens-Bobadilla, Nat. Biotech., 2023)? Since this is FFPE, it does not need to surpass others, but it would be informative for readers to understand the extent of quality degradation compared to FF, or how well the authors' optimization of the FFPE processing mitigates this degradation.

3. Fig. 2: Including data from different tissues, where the correlation coefficients are expected to be significantly lower, would help assess the relative highs and lows of the correlation coefficients. Since correlation coefficients can vary greatly depending on the subtle changes to the data preprocessing, they are difficult to use as absolute indicators of reproducibility.

4. Ex Fig. 2e-f: In ATAC-seq using FFPE, as shown in the Figure that lacks mono- or di-nucleosomal size fragments, subnucleosomal fragments are mainly obtained from nucleosome depleted regions (NDRs). How does this characteristic affect the results compared to FF tissues? While the correlation coefficients roughly correspond to those of FF tissues, there is insufficient examination of how FFPE-induced cellular damage creates differences and how spatial ATAC-seq might yield different results compared to FF.

5. Fig. 3-4: The cell type annotation relies mainly on label transfer, but to what extent is the chromatin accessibility information, which would have been preserved in FF, lost in FFPE, and how does this affect the label transfer results? There is concern that the granularity of the annotation obtained may decrease due to the insufficient read count or by the bias, such as the chromatin accessibility information towards regions like NDR. Additionally, especially in the case of clinical samples, wouldn't it be challenging to prepare corresponding single-cell RNA-seq?

Reviewer #3

(Remarks to the Author)
In this manuscript, Guo et al. developed a spatial-ATAC-seq technique that can profile FFPE samples. The authors optimised a few steps prior to in situ tagmentation, which seems to be the key to work with FFPE samples. Using spatial FFPE-ATAC-seq, they profiled diverse sample types, including different brain regions and thymus from mice and humans. They showed that the data is of good quality and is able to uncover known cell types in the complex tissues.

The flow of the manuscript is easy to follow, and the writing is clear. I don't have any problems with the data presentation. The manuscript is about the method, so my comments are mainly on the description and the principle of the methodology.

In its current form, there are still many technical details missing from the manuscript, especially about the reasons why the current method works and the modifications over previous methods.

Things to improve:

(1) Spatial FFPE-ATAC-seq seem to be based on Spatial-ATAC-seq (doi: 10.1038/s41586-022-05094-1) developed by the last author of the current manuscript. What makes the method in the current manuscript work for FFPE? What are the improvements? Did the authors simply include some target recovery steps prior to the regular spatial ATAC-seq procedures? Did the authors also make some improvement on the spatial ATAC-seq method as well? In addition, there are other methods, though not spatial, that work with FFPE, such as:

doi: 10.1038/s41467-023-41666-z
doi: 10.1101/gr.275269.121

I didn't list all of them. Those methods also have some sort of target recovery steps. What are the differences between the target recovery steps described in this manuscript and in other publications? How about using a table for a comparison?

Since spatial FFPE-ATAC-seq is presented as a new method in the current manuscript, I think it is important to include the aforementioned technical details to let the readers know what exactly are invented, changed and improved over previous methods. Without that, the manuscript misses the mark of being a new method.

(2) In Figure 1b, different temperatures were tested during target recovery, and 65C turned out to be the best in terms of TSS enrichment. What about the alignment rate to the reference genome? Henikoff et al. (doi: 10.1038/s41467-023-41666-z) seemed to get lower alignment rates with lower temperature like 65C in FFPE-CUTAC. I know it is a different assay, but it is still interesting and useful to show the alignment rates to the reference genome in different conditions. Maybe there are some trade-offs between TSS enrichment and alignment rates.

(3) Figure 1c contained important optimisation information. How many times have the experiments in Figure 1c repeated? How reproducible are the results? Currently, it does not seem to have a clear pattern. If we compare S7 with S4, the conclusion is 250 nM has a huge impact on median fragments. However, when compare S8 to S5, it does not seem so. Maybe it is a combination of things. Nevertheless, it is worth mentioning how reproducible the results are. On a related, what is the rationale of adding NaCl and why only at 250 nM which seems to be a very low concentration compared to normal usage which is at the scale of mM.

(4) The integration with scRNA-seq reference is a bit strange. For example, in Figure 2c, red dots are from spatial cluster 0, indicating they have similar ATAC profiles. Then why they are scattered into different cell types in the scRNA-seq reference UMAP? Any discussions? In addition, is there a particular reason to integrate with scRNA-seq reference? Intuitively, integration with scATAC-seq reference would make more sense since the data is spatial ATAC-seq. There are well-annotated scATAC-seq data sets in the brain as well.

(5) Figure 3c is a bit confusing. At the left-hand side, it seems there are very few spatial FFPE-ATAC-seq points at 3 o'clock position of the UMAP. However, at the right-hand side, there are way more points at 3 o'clock position of the UMAP. Is this a mistake or is it due to colour limitations? Maybe change to a different colour palette?

(6) In Figure 1c, what is "AR" in the table stand for? Is it supposed to be "TR"?

(7) Line 66: "liker" should be "linker".

(8) Gene names should be in italic in figures.

(9) If possible, please use a different colour palette that is colourblind safe for Figures 1b, 2b, 2f, 3a, 3d-f, 4a, 4e.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript "Spatial profiling of chromatin accessibility in formalin-fixed paraffin-embedded tissues" has added new experiments and analysis that more thoroughly document the performance of spatial-FFPE-ATAC-seq. The additional text and comparison of FFPE and flash-frozen samples have addressed my comments.

Reviewer #2

(Remarks to the Author)
This manuscript has included sufficient validation data that were previously missing, such as the comparison of sample quality and analysis results between FF and FFPE, and the demonstration for cancer tissue analysis. These additions will be valuable information for readers considering clinical applications using spatial FFPE ATAC-seq.

In my previous comments, I highlighted concerns about the impact of sample quality degradation on analysis results.

However, at this stage, the authors' evaluation focuses only on general aspects such as overall correlation and TSS enrichment, which does not fully address my concerns. If the following points are improved, I believe this paper will be worthy of publication.

Specific points:

* New Figure 2b: The current evaluation is based on a qualitative approach. It would be more convincing to include quantitative evaluation metrics. For example, what percentage of ATAC-seq signals around marker gene loci are concentrated in cortex or cerebelium regions? In addition, calculating metrics such as Moran's I to determine the extent of non-random spatial patterning would provide a more reliable assessment.
* New Fig. 2c: Since the subnucleosomal fraction represents the majority of reads in standard ATAC-seq, reducing the nucleosomal fraction is expected to have a minimal impact on the overall correlation coefficient. Therefore, it would be beneficial to focus on areas where differences occur (e.g., localization of peaks that do not overlap with standard ATAC-seq peaks as shown in Supplementary Figure 4) and discuss the differences between them.
* New Figure 4c-e: There is some arbitrariness in determining peak calling thresholds. For example, in Supp. Fig. 4c, I suspect that spatial FFPE-ATAC-seq has low S/N, and using the same peak calling threshold may result in excessive sensitivity and large overlap (e.g., 68%) with bulk ATAC-seq. I suggest setting the gold standard peaks from bulk ATAC-seq and generating ROC or precision-recall curves by varying the read count threshold of spatial FFPE-ATAC in 1K or 10K genomic bins. This would allow a more detailed assessment of the optimal threshold setting and the differences in signal distribution between the two methods.

Reviewer #3

(Remarks to the Author)
The authors have successfully addressed all my concerns. It seems the main improvement is on the target retrieval steps, which is clear now. I also understand the rationale of making "minimal modifications, specifically refining the target retrieval (TR) process" to facilitate adaptation of the previous Spatial-ATAC technology on FFPE samples. Therefore, I have no further questions.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I thank the authors for their thoughtful and comprehensive revisions. The additional analyses—including GO enrichment based on FF/FFPE-specific peaks, spatial topic modeling, and ROC curve evaluation—clearly and convincingly address my prior concerns regarding the biological relevance and the maintained integrity of the spatial FFPE-ATAC-seq data. These additions meaningfully strengthen both the scientific rigor of the manuscript and the reliability of this method.

Responses to Reviewers' comments

Overall summary of the revision

We thank all our reviewers for their careful review of our manuscript. We were pleased to read their positive remarks about the work, and greatly appreciated all the constructive feedback and suggestions. On the basis of the collective comments, we have now revised the manuscript accordingly with the addition of a considerable amount of new data.

Key to the rebuttal

- Reviewers' comments: black italic text

- Our responses: blue text

*- New manuscript figure citations: **red text***

- Previous manuscript figure citations: black text

*- Reviewer only figures ("**Figure RX**"): **purple text***

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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The limited data here is not adequate to judge the performance of these modifications for spatial-ATAC-seq. The analysis here relies on bioinformatic packages designed for single cell ATAC analysis, but does not provide quantification of the quality of results. The main problem with FFPE material is that DNA is degraded, and the integrity of DNA is a standard measure for genomic analysis of FFPE samples, and should be assessed, because there are a number of features in the data that suggest the quality is low. Supplementary Figure 2e, 4a, and 4c show ATAC fragment size profiles which the authors described as "optimized" (line 80), although these profiles do not meet the standards for ATAC-seq pipelines, which expect a significant nucleosomal band. The authors should assess and discuss whether these standards are feasible for samples with heavily damaged DNA. Only one figure shows genomic tracks of the data (Figure 4f); qualitatively these tracks look sparse with modest peaks. Comparison to high-quality ATAC-seq data should be included.

We thank the reviewer for the thoughtful feedback. We agree that the quality of FFPE samples presents a unique challenge for chromatin accessibility profiling, particularly due to DNA degradation. To address the reviewer's concern regarding the quality of the FFPE data, we performed a comprehensive analysis by conducting three replicates using the spatial-ATAC-seq method and six replicates using the spatial-FFPE-ATAC-seq method. The inclusion of multiple

replicates in both fresh frozen and FFPE conditions is crucial for ensuring the robustness and reproducibility of our findings. This rigorous approach strengthens the validity of our conclusions and helps mitigate potential variability introduced by sample preservation techniques. By performing these replicates, we are confident that our results reflect reliable and meaningful patterns in chromatin accessibility, offering a more accurate comparison between fresh frozen and FFPE samples. Below are the details of our analysis:

1. **DNA integrity assessment:** We extracted genomic DNA from both fresh frozen and FFPE mouse brain tissues and confirmed the presence of DNA breaks in the FFPE samples, consistent with the degradation typically observed in FFPE material (Supplementary Fig. 2a-b). This supports the notion that DNA quality in FFPE samples is compromised compared to fresh frozen samples.

2. **Fragment size profiles:** While the PCR amplicon size distribution in both fresh frozen and FFPE samples showed the same main peak (Supplementary Fig. 2c-d), we observed a loss of the nucleosomal band in the FFPE samples. This is expected due to DNA fragmentation, which impacts the formation of nucleosomal patterns. Other FFPE related papers also reported the loss of nucleosomal band[1,2], indicating a common feature of DNA fragmentation of high fixation by formalin.

3. **Data quality verification:** To further assess the quality of the FFPE data, we compared the peak correlation between FFPE and FF samples. The correlations were all greater than 0.61, suggesting reasonable consistency between the datasets. Furthermore, we focused on comparing open chromatin regions in the white matter of the mouse cerebellum, a known cell type with well-defined chromatin characteristics. Open chromatin regions are typically defined as nucleosome-depleted regions (NDRs), which are enriched in enhancers and promoters. The proportion of promoters identified in FFPE samples (ranging from 60.8% to 83.1%) showed no significant difference compared to that of fresh frozen samples (75.6% to 81.9%) (Fig. 2e), indicating that the FFPE samples retained relevant chromatin features.

4. **Genomic tracks for marker genes:** To verify the consistency of the data, we added tracks for selected marker genes. The distribution of peaks in the FFPE samples was highly consistent with the corresponding FF samples (Fig. 2d and Supplementary Fig. 3b), further supporting the reliability of the FFPE data for spatial ATAC-seq analysis. For human samples, more genome tracks have been added (Fig. 3f,3g,4h,4i,5f and Supplementary Fig. 4f).

In conclusion, while FFPE samples exhibit expected DNA degradation, our analysis demonstrates that key chromatin features, such as NDRs including promoters and intergenic regions, are still detectable and comparable to fresh frozen tissue. We believe that these results support the feasibility of using FFPE samples for spatial ATAC-seq.

Figure 2f raises fundamental concerns that must be addressed. The panel for Bcl11b shows no

similarity between inferred ATAC signal and ISH. Car10, maybe: it's hard to tell since these slides are unrelated and these images cannot be aligned. Prox1 is clearly a mixture of some overlap and some non-overlap, Tbr1 shows no similarity. Further, the dynamic range of inferred marker signal is very small. These lack of correspondence raise questions on what is the quality of the data and whether it is being analyzed appropriately.

We acknowledge the reviewer's concerns regarding the comparison between inferred ATAC signals and ISH data. To address this, we performed a new comparison of the spatial distribution of marker gene chromatin accessibility from FF and FFPE samples. As shown in Fig. 2b, marker genes such as *Bcl11b, Car10*, and *Prox1* exhibit similar spatial distributions between FF and FFPE samples.

The lack of similarity between inferred ATAC signals and ISH data is expected, as ATAC-seq measures chromatin accessibility rather than direct gene expression levels. Unlike scRNA-seq or ISH, which provide transcript abundance, scATAC-seq primarily detects accessible regulatory elements. While some peaks correspond to gene promoters, many represent enhancers or other distal regulatory regions, leading to discrepancies between ATAC signals and ISH patterns. To prevent potential misinterpretation, we have removed the ISH data from the revised manuscript.

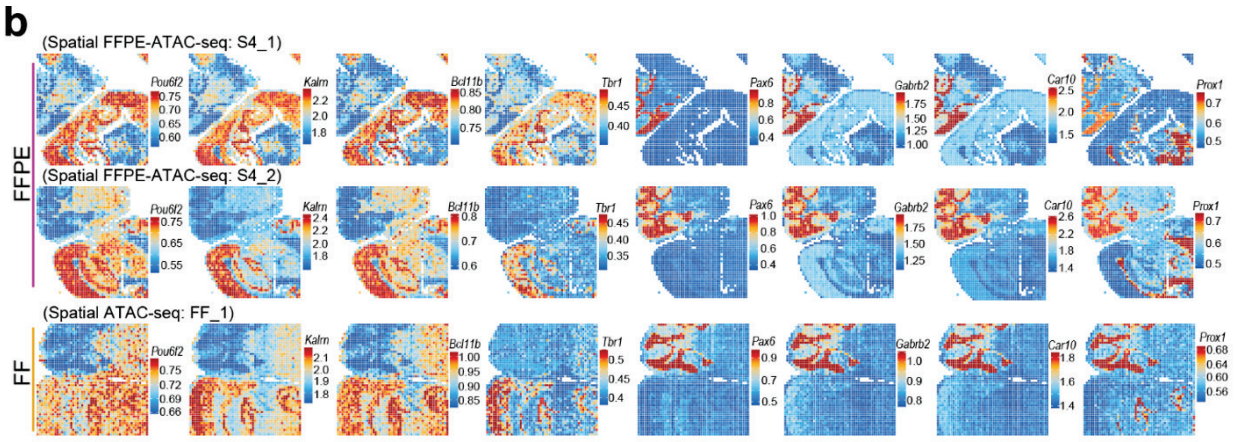


Fig. 2b

Quantitative performance of data quality should be presented, and the manuscript makes a number of statements on data quality that appear unsupported:
Line 75: "extensive optimization of the target retrieval conditions to achieve a high signal-to-noise ratio, as measured by the transcription start site (TSS) enrichment score, and a high yield, indicated by the number of unique fragments". The presented values are substantially lower than controls, and should also be compared to high-quality ATAC-seq for quality assessment.

We thank the reviewer for these comments. To address these concerns, we employed a rigorous experimental design to ensure the reliability of our results:

1. **Biological Replicates**: We included biological replicates from both FF (n = 3) and FFPE (n = 6) samples to account for variability and enhance reproducibility.
2. **Data Normalization**: For the comparison of TSS score and unique fragments, all datasets were downsampled to the same sequencing depth (50M reads) to ensure a fair

comparison during downstream analysis. We found the similar unique fragments (FF:7,761 and FFPE: 7,695) and the consistent TSS score (~4).

3. **Chromatin accessibility across various genomic elements**, including promoters, introns, exons, and intergenic regions, was compared between FF and FFPE samples. We found no significant differences in the proportions of these genomic elements between FF and FFPEs (Fig. 2c).

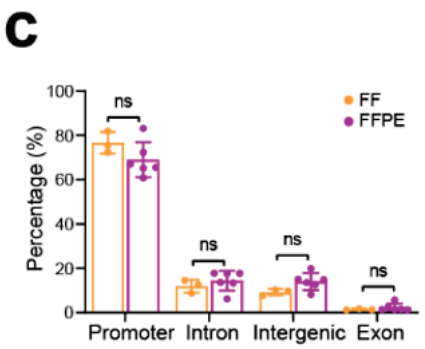


Fig. 2c

Line 87 "Unsupervised clustering identified eight distinct clusters, with spatial projections closely aligning with tissue morphology observed in adjacent H&E-stained sections (Fig. 2b,e). For example, chromatin accessibility highlighted the cortex and cerebellum regions, with Bcl11b and Car10 serving as respective marker genes, which is consistent with gene expression data from the Allen Brain Atlas (Fig. 2f and Supplementary Fig. 3)." The spatial pattern of UMAP clusters in Figure 2b does not look similar to H&E staining in Figure 2e; what do the authors mean by "closely aligning"?

We thank the reviewer for their comments. The H&E-stained sections were included to illustrate the overall structure of the adjacent tissue sections. However, in certain well-defined tissue types, such as the mouse brain, the morphology is already distinguishable. As shown in the revised Fig. 2a, the spatial pattern of UMAP clusters closely resembles the underlying tissue architecture, supporting our conclusion that spatial UMAPs align well with tissue morphology. Additionally, slight distortions may occur during tissue sectioning, causing adjacent sections to appear misaligned. To address this concern, we have removed the adjacent H&E-stained sections of the mouse brain in the revised manuscript.

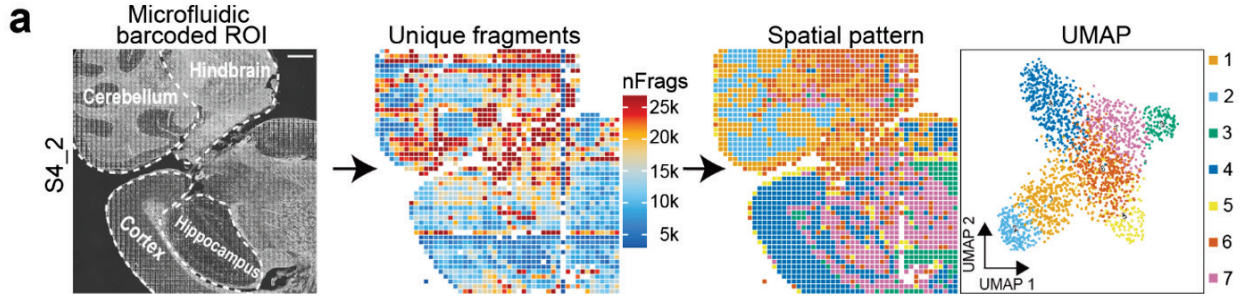


Fig. 2a

Line 92: "The consistency between the reference and our data confirms our data quality."

We have deleted "The consistency between the reference and our data confirms our data quality." In replacement, more detailed information has been added to the revised manuscript, such as "Next, we mapped spatial FFPE-ATAC-seq data to specific cell types by integrating it with scRNA-seq data[15] (Fig. 2e). For example, cerebellar inhibitory neurons (CBINH1) and granule cells (CBGRC) were enriched in the gray and white matter of the cerebellum, respectively, while telencephalon excitatory neurons 7 (TEGLU7) were localized in the cortex region (Fig. 2f). We observed high reproducibility between replicates via correlation analysis ($r > 0.9$) (Supplementary Fig. 3d). Notably, the high concordance between spatial FFPE-ATAC-seq data with single-cell ATAC-seq[16] data provided additional evidence of the high data quality obtained with our FFPE-based protocol (Supplementary Fig. 3c). Taken together, our results demonstrate that spatial FFPE-ATAC-seq faithfully captures key features of chromatin accessibility in FFPE tissues, providing a robust approach for epigenomic profiling in archived samples.".

Line 95: "The successful optimization of spatial FFPE-ATAC-seq enabled high-quality chromatin accessibility profiling from FFPE mouse tissues, matching fresh frozen data, while accurately identifying distinct spatial patterns and neuronal subtypes." These statements imply quantitation, but no quantitation is presented.

We thank the reviewer for these comments. A comparison of ATAC-seq between FF and FFPE samples was performed, including analysis of spatial distribution and genome tracks of marker genes, quantification of genomic features, and integration of spatial FFPE-ATAC-seq with scATAC-seq data (Fig. 2b-d and Supplementary Fig. 3b-d). All analyses demonstrated similar data quality between FF and FFPE samples.

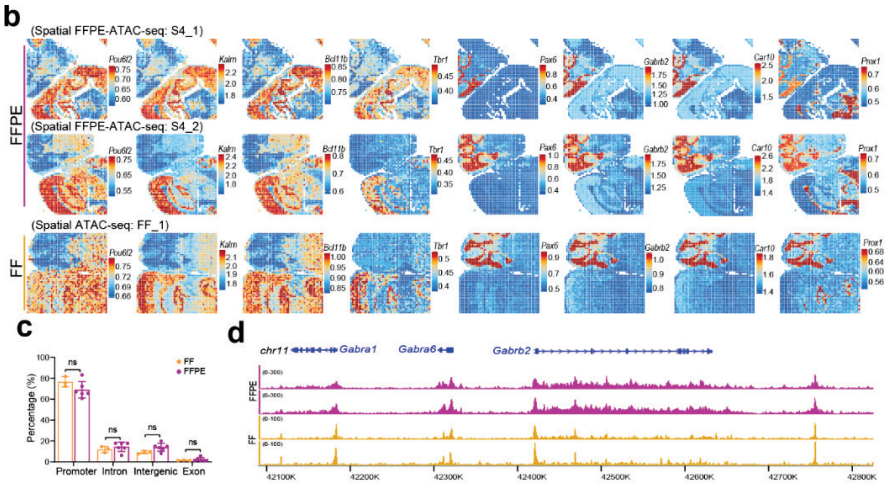
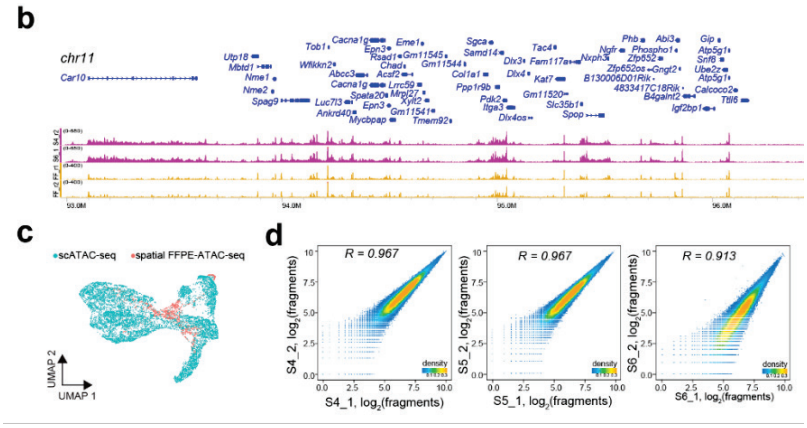


Fig. 2b-d



Supplementary Fig. 3b-d

In the revised manuscript, we have added the following conclusion:

"Taken together, our results demonstrate that spatial FFPE-ATAC-seq faithfully captures key features of chromatin accessibility in FFPE tissues, providing a robust approach for epigenomic profiling in archived samples."

Line 103: "By integrating the spatial-FFPE-ATAC-seq data with scRNA-seq data from human cerebellar cells, we identified the major cell types—including oligodendrocytes, granule cells, and Purkinje cells." It is not apparent how these conclusions are being made. It doesn't seem that spatial-FFPE-ATAC-seq has cellular resolution, so how can the authors be identifying cell types?

We thank the reviewer for these comments. While our 50-μm resolution means each pixel captures chromatin accessibility from a region approximately 50 μm across, this resolution still provides valuable insights into tissue architecture. The 50-μm resolution can identify cell types for several reasons:

1. Spatial chromatin accessibility signatures are detectable at the regional level.
2. Cluster-based analyses group pixels with similar accessibility patterns, which can correspond to specific cell types or tissue regions.
3. Cell-type marker regions in the chromatin accessibility data help infer the presence and proportions of different cell types.
4. The tissue architecture and regional chromatin accessibility patterns offer additional context to aid cell-type identification.

To validate these points, we compared 50-μm and 10-μm resolutions using human thymus samples. By performing label transfer with scRNA-seq data, we found that both 50-μm and 10-μm resolutions identified similar cell types, including T cells, TECs, and mesenchymal cells. Given that 10-μm resolution is single-cell resolution, we conclude that the 50-μm resolution is sufficient to identify cell types.

[Figure Redacted]

Fig. 4a-g

The previous paper that describes the design and performance of the spatial-ATAC-seq method is not adequately cited.

We thank the reviewer for this comment. In the revised manuscript, we have added the following details to clarify the purpose behind the design of spatial FFPE-ATAC-seq:

To maximize the utility and compatibility of spatial ATAC-seq for FFPE samples, we made minimal modifications, focusing primarily on refining the target retrieval (TR) process to accurately capture open chromatin regions.

In the workflow, only the first step is specifically optimized for FFPE samples, while the remaining steps are consistent with standard protocols.

To compare the performance of spatial FFPE-ATAC-seq with spatial ATAC-seq, we performed additional analyses that demonstrate their similar effectiveness in terms of spatial results (Fig. 2b-d and Supplementary Fig. 3b).

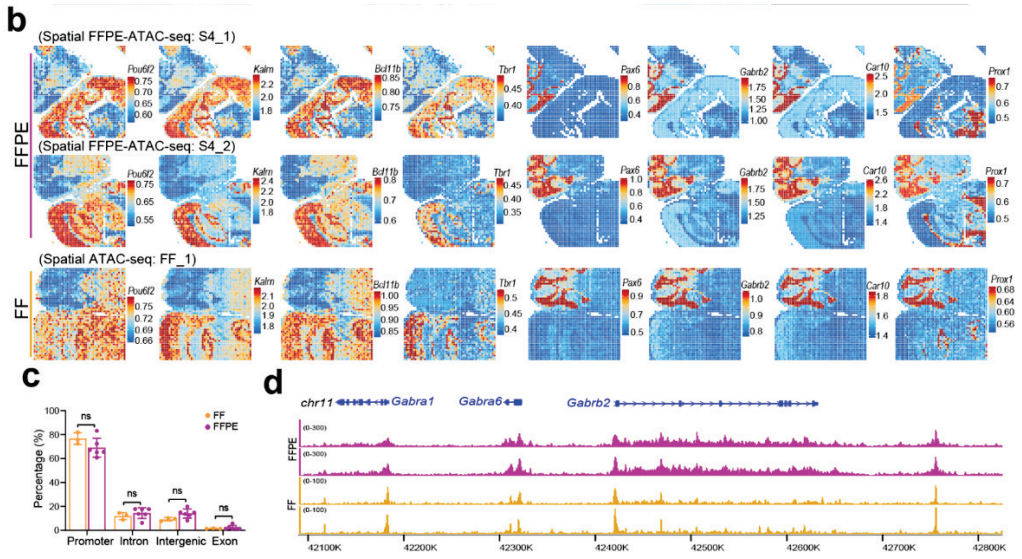
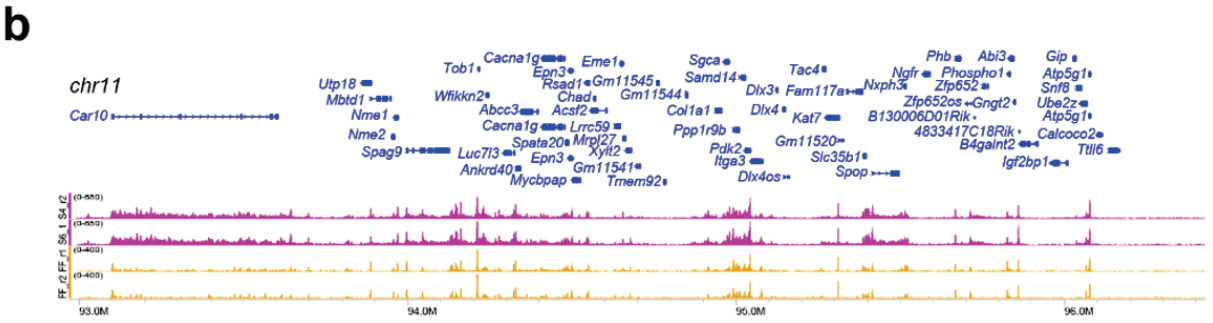


Fig. 2b-d



Supplementary Fig. 3b

The description of the method is lacking in details for a reader; this paper gives the pixel size of the grid, but doesn't state the area that it covers or its effective cellular resolution. In the previous paper it is apparent that the spatial grid only covers a small fraction of the tissue sample, but these details should be made clear here.

We would like to thank the reviewer for these comments. In the revised manuscript, we have provided additional details about the method, including information on the covered region, spatial resolution, and the number of pixels.

It is not made clear in the paper how many samples are analyzed, and the GEO entry lists 8 with no apparent replicates. The authors should justify their choice to not perform replicates for reproducibility. I would like to see more comprehensive descriptions of sequencing results in the manuscript; how many reads, mapping rates, duplication rates. All of this inform the reader on data quality for this method.

We thank the reviewer for these suggestions. A summarized table has been added (Supplementary Table 2).

Reviewer #2 (Remarks to the Author):

The authors reported on the adaptation of their previously developed spatial-level chromatin accessibility analysis method, spatial ATAC-seq (Deng et al., 2022), to FFPE samples, resulting in spatial FFPE-ATAC-seq. By comparing the data quality with that obtained using spatial ATAC-seq on fresh frozen (FF) sections, they demonstrate the feasibility of obtaining data of a certain quality using FFPE sections. As the authors also assert, enabling spatial chromatin analysis using FFPE sections holds significant potential for clinical applications and for leveraging the wealth of past archive of tissue blocks. However, the results presented in this paper primarily focus on optimizing experimental conditions for applying their spatial ATAC-seq to FFPE samples and the quality assesments of the obtained data. Without a novel experimental ideas, sufficient investigation of the impact of FFPE-induced cellular damage on analysis results, or demonstration on the potential applicability for clinical samples, that this reports may be perceived as merely a minor update to their original paper.

We sincerely appreciate the reviewer's comments. The key point we would like to emphasize is that our primary motivation is to develop a user-friendly and easily implementable method for researchers. Given that FFPE samples are heavily crosslinked, selecting the correct target retrieval (TR) conditions is crucial for exposing the original chromatin states. Notably, we tested over 50 TR conditions to identify the optimal approach. Because the modifications we introduced are minimal, they can be widely applied across various platforms, enhancing the method's versatility and allowing more researchers to seamlessly integrate it into their existing protocols.

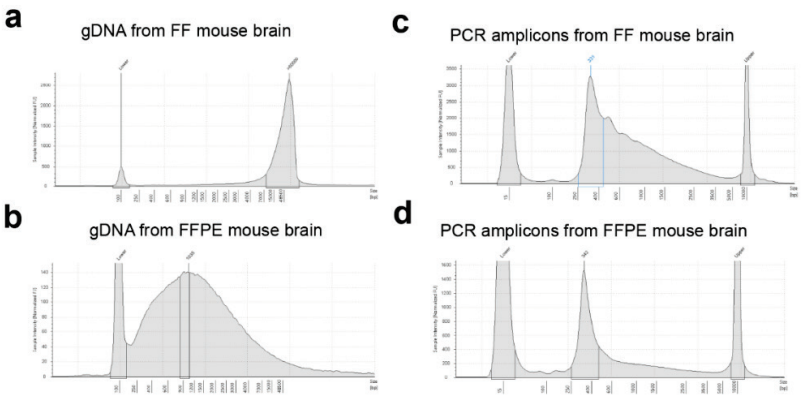
For us, our primary goal is to maximize the utility and compatibility of spatial ATAC-seq for FFPE samples after TR treatments. We conducted a thorough comparison of ATAC-seq data from FF and FFPE samples, demonstrating consistent results across both sample types. In human samples, we further assessed the method's performance by evaluating various resolution levels, which uncovered clear spatial patterns at a single-cell resolution. Finally, we applied this technique to human melanoma samples, where it successfully distinguished tumor from non-tumor regions. Moreover, this approach revealed subclusters within each region that were not detectable by traditional staining methods, such as H&E.

Below are the specific comments.

1. Throughout the paper, the data analysis results are largely similar to those presented in their previous study (Deng et al., 2022). As mentioned above, providing a demonstration of insights that become possible by this spatial FFPE-ATAC-seq, such as cancer tissue analysis, or a detailed investigation of how cellular damage affects the quality of the data and the analysis results, is essential.

We appreciate the reviewer's suggestion to address the quality of data in more detail.

1) Formalin fixation resulted in the fragmentation of gDNA, which is observed as small fragments in the ATAC-seq library, overlapping with nucleosome-free fragments (Supplementary Fig. 2a-d).



Supplementary Fig. 2a-d

2) A comparison of ATAC-seq between FF and FFPE samples was performed, including analysis of spatial distribution and genome tracks of marker genes, quantification of genomic features, and integration of spatial FFPE-ATAC-seq with scATAC-seq data (Fig. 2b-d and Supplementary Fig. 3b-d). All analyses demonstrated similar data quality between FF and FFPE samples.

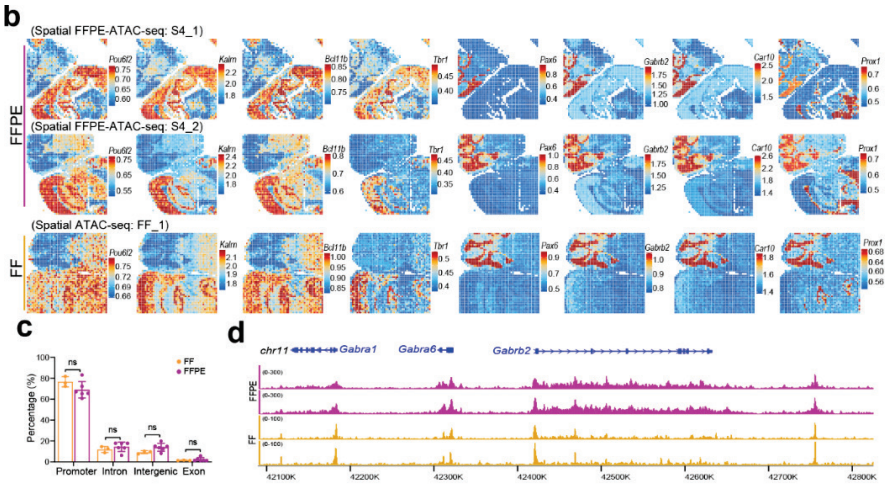
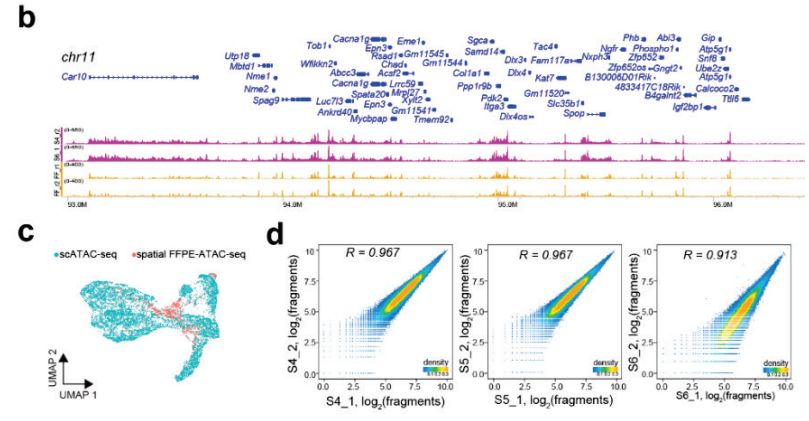
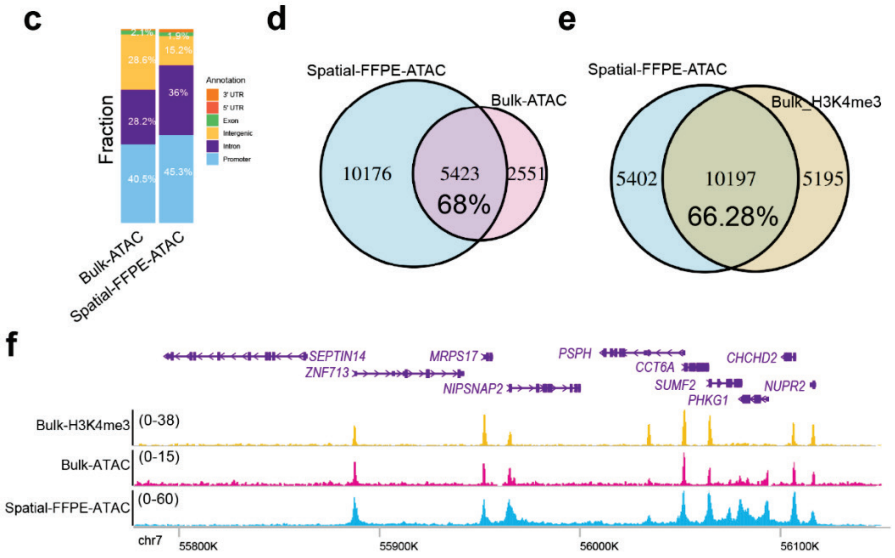


Fig. 2b-d



Supplementary Fig. 3b-d

3) For human samples, we compared bulk-ATAC, H3K4me3 ChIP-seq, and spatial FFPE-ATAC-seq data from the same tissue type (cerebellum; Supplementary Fig. 4c-f), confirming that spatial FFPE-ATAC-seq also yielded reliable results in human samples.



Supplementary Fig. 4c-f

3) For FFPE tumors, we applied spatial FFPE-ATAC-seq with human melanoma. Spatial results showed a clear pattern of tumor and non-tumor regions. And each region can be subdivided into different clusters which are undistinguished by regular H&E staining.

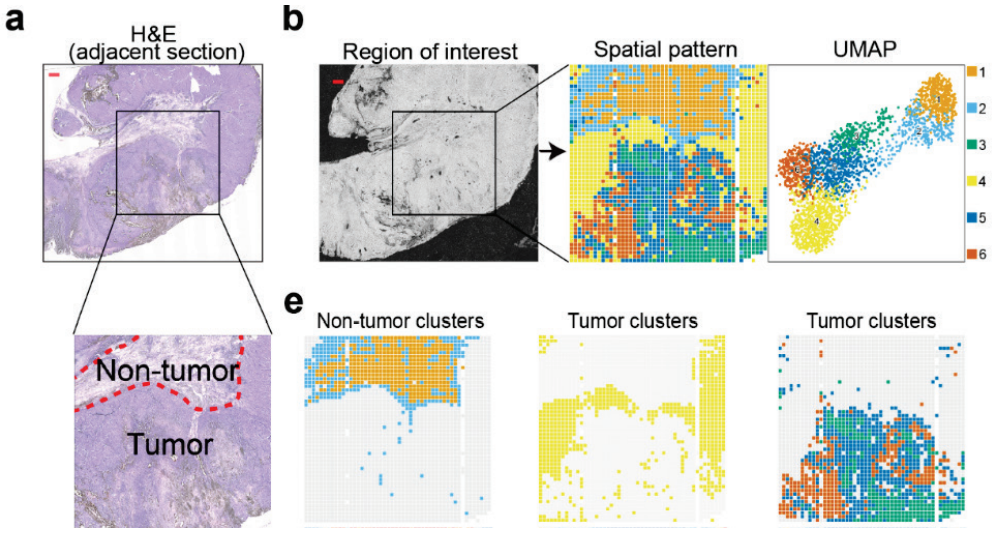
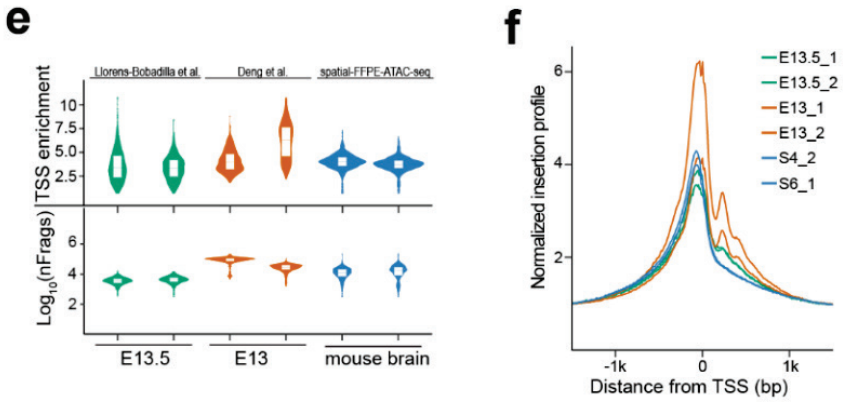


Fig. 5a,5b,5e

2. Fig. 1b-c: How does the performance compare to other spatial ATAC-seq methods (e.g., Thornton, Nat. Commun., 2021, Llorens-Bobadilla, Nat. Biotech., 2023)? Since this is FFPE, it does not need to surpass others, but it would be informative for readers to understand the extent of quality degradation compared to FF, or how well the authors' optimization of the FFPE processing mitigates this degradation.

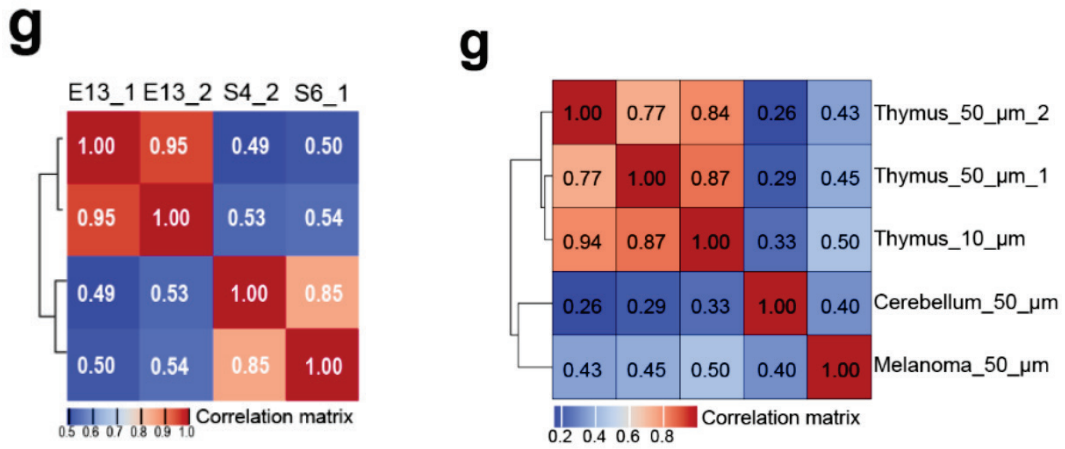
We thank the reviewer for these comments. We have added new comparisons between spatial FFPE-ATAC-seq and other spatial ATAC-seq techniques, such as those from Llorens-Bobadilla et al.[3] and Deng et al.[4]. We found that the TSS enrichment scores and median numbers of unique fragments in spatial FFPE-ATAC-seq are comparable to those in previously published spatial ATAC-seq datasets, as shown in Supplementary Fig. 2e-f.



Supplementary Fig. 2e-f

3. Fig. 2: Including data from different tissues, where the correlation coefficients are expected to be significantly lower, would help assess the relative highs and lows of the correlation coefficients. Since correlation coefficients can vary greatly depending on the subtle changes to the data preprocessing, they are difficult to use as absolute indicators of reproducibility.

That's a great suggestion! To comprehensively assess correlation coefficients across different tissues, we compared both mouse and human samples included in the manuscript. As shown in Supplementary Fig. 2g and Supplementary Fig. 5g, we observed strong correlations between replicates of the same tissue, whereas different tissues exhibited weaker correlations. This highlights the distinct chromatin landscapes across tissues, further supporting the biological relevance of our approach.



Supplementary Fig. 2g and Supplementary Fig. 5g

4. Ex Fig. 2e-f: In ATAC-seq using FFPE, as shown in the Figure that lacks mono- or di-nucleosomal size fragments, subnucleosomal fragments are mainly obtained from nucleosome depleted regions (NDRs). How does this characteristic affect the results compared to FF tissues? While the correlation coefficients roughly correspond to those of FF tissues, there is insufficient examination of how FFPE-induced cellular damage creates differences and how spatial ATAC-seq might yield different results compared to FF.

We appreciate the reviewer's concern regarding the absence of mono- or di-nucleosomal size fragments. To address this, we conducted the following analyses:

1. Assessment of FFPE gDNA integrity – We confirmed that FFPE processing leads to DNA fragmentation (Supplementary Fig. 2a-d).
2. Comparison of genomic accessibility features – We analyzed the distribution of accessibility across genomic features in FFPE and fresh-frozen (FF) samples, finding no significant differences (Fig. 2c).
3. Spatial analysis of marker genes – We examined the spatial distribution of marker genes and their genome tracks from the same tissue regions (Fig. 2b,d and Supplementary Fig. 3b).
4. Validation against external datasets – We compared ATAC-seq peak overlap and genome tracks of selected genes with ENCODE bulk ATAC-seq and H3K4me3 ChIP-seq datasets (Supplementary Fig. 4c-f).

Together, these analyses demonstrate that spatial FFPE-ATAC-seq produces results comparable to FF samples.

5. Fig. 3-4: The cell type annotation relies mainly on label transfer, but to what extent is the chromatin accessibility information, which would have been preserved in FF, lost in FFPE, and how does this affect the label transfer results? There is concern that the granularity of the annotation obtained may decrease due to the insufficient read count or by the bias, such as the chromatin accessibility information towards regions like NDR. Additionally, especially in the case of clinical samples, wouldn't it be challenging to prepare corresponding single-cell RNA-seq?

We thank the reviewer for these comments. The reason why we chose scRNA-seq for label transfer is based on the following reasons:

1. scATAC-seq captures open chromatin regions, not direct gene expression levels – Unlike scRNA-seq, which provides direct transcript abundance, scATAC-seq primarily detects accessible regulatory elements. While some peaks can be linked to gene promoters, many correspond to enhancers or other distal regulatory elements, making direct cell type assignment more challenging.

2. scRNA-seq provides a more established reference – Since scRNA-seq directly measures gene expression, well-annotated cell type references exist, making it easier to transfer these labels to scATAC-seq data using computational methods.

3. Independent scATAC-seq annotation is challenging – De novo clustering of scATAC-seq data without scRNA-seq references is difficult due to sparse data, technical noise, and variability in chromatin accessibility landscapes between cell states.

Since our results have verified no significant difference of NDR distributions across the genomes, label transfer results of FFPEs are not affected (Fig. 2e, 3c, 4e).

For the question "*Additionally, especially in the case of clinical samples, wouldn't it be challenging to prepare corresponding single-cell RNA-seq?*", we have not tested this yet. However, we are currently exploring spatial RNA-seq and spatial co-profiling of ATAC and RNA-seq in human clinical samples. Our preliminary results are very promising.

Reviewer #3 (Remarks to the Author):

In this manuscript, Guo et al. developed a spatial-ATAC-seq technique that can profile FFPE samples. The authors optimised a few steps prior to in situ tagmentation, which seems to be the key to work with FFPE samples. Using spatial FFPE-ATAC-seq, they profiled diverse sample types, including different brain regions and thymus from mice and humans. They showed that the data is of good quality and is able to uncover known cell types in the complex tissues.

The flow of the manuscript is easy to follow, and the writing is clear. I don't have any problems with the data presentation. The manuscript is about the method, so my comments are mainly on the description and the principle of the methodology.

In its current form, there are still many technical details missing from the manuscript, especially about the reasons why the current method works and the modifications over previous methods.

We appreciate the reviewer's positive feedback on our manuscript. We are glad to hear that the manuscript is well-structured, clearly written, and that the data presentation is effective. We also appreciate the reviewer's recognition of the methodological advancements in spatial FFPE-ATAC-seq and its application to diverse sample types.

In response to the reviewer's concerns, we have conducted additional experiments, including replicates, analysis using human tumor samples, and single-cell resolution samples. Additionally, we have reorganized the manuscript to provide more detailed explanations. Below, we present a point-by-point response to address the specific issues raised.

Things to improve:

(1) Spatial FFPE-ATAC-seq seem to be based on Spatial-ATAC-seq (doi: 10.1038/s41586-022-05094-1) developed by the last author of the current manuscript. What makes the method in the current manuscript work for FFPE? What are the improvements? Did the authors simply include some target recovery steps prior to the regular spatial ATAC-seq procedures? Did the authors also make some improvement on the spatial ATAC-seq method as well? In addition, there are other methods, though not spatial, that work with FFPE, such as:

doi: 10.1038/s41467-023-41666-z
doi: 10.1101/gr.275269.121

I didn't list all of them. Those methods also have some sort of target recovery steps. What are the differences between the target recovery steps described in this manuscript and in other publications? How about using a table for a comparison?

We thank the reviewer for these comments. These questions are replied one by one.

What makes the method in the current manuscript work for FFPE?

Performing chromatin accessibility profiling on FFPE tissues presents significant challenges due to the extensive crosslinking caused by formalin fixation, which can obscure chromatin structure and hinder the Tn5 transposase from efficiently accessing the genomic DNA. While normal chromatin consists of euchromatin and heterochromatin, formalin fixation introduces additional crosslinking that does not alter the original chromatin states. To access these regions, different

energy levels may be required. By carefully controlling the energy supplied during antigen retrieval, it is possible to reverse the crosslinking and restore the chromatin to its normal states.

What are the improvements?

Spatial profiling, including spatial ATAC-seq, has been hindered by the challenges of FFPE samples. Our novel target retrieval modification enables effective spatial analysis of these abundant clinical resources, providing researchers with a straightforward and efficient FFPE-compatible ATAC-seq method.

Did the authors also make some improvement on the spatial ATAC-seq method as well?

We sincerely appreciate the reviewer's comments. The key point we would like to emphasize is that our primary motivation is to develop a user-friendly and easily implementable method for researchers. Given that FFPE samples are heavily crosslinked, selecting the correct target retrieval (TR) conditions is crucial for exposing the original chromatin. Given the promising spatial results obtained from FFPE samples, it offers valuable insights that could guide further optimization of spatial ATAC-seq, such as adjustments to fixation concentration and duration.

Did the authors simply include some target recovery steps prior to the regular spatial ATAC-seq procedures?

We want to maximize the utility and compatibility of spatial ATAC-seq for FFPE samples, so that we made minimal modifications, specifically refining the target retrieval (TR) process to accurately capture open chromatin regions. Of note, we tested over 50 target retrieval (TR) conditions to identify the optimal one.

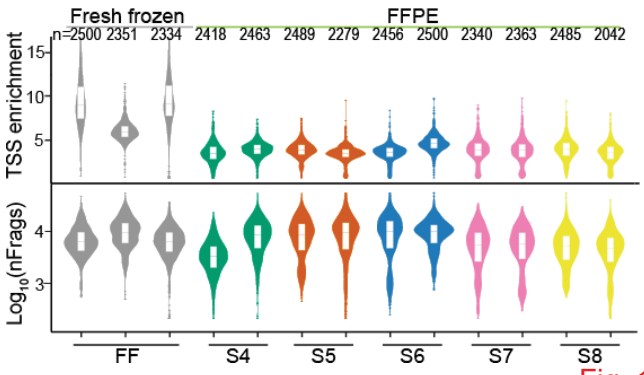
In addition, there are other methods, though not spatial, that work with FFPE, such as: doi: 10.1038/s41467-023-41666-z. doi: 10.1101/gr.275269.121

I didn't list all of them. Those methods also have some sort of target recovery steps. What are the differences between the target recovery steps described in this manuscript and in other publications? How about using a table for a comparison?

In brief, FFPE-CUTAC targets histone modifications and FFPE-ATAC extracts nuclei from FFPEs, and both cannot maintain spatial context.

1) Compared with FFPE-CUTAC:

   Since high temperatures can lead to a lower TSS (Fig. 1b), we performed spatial experiments (sample S8) by replacing the target retrieval buffer with FFPE-CUTAC buffer (0.8 M Tris-HCl, pH 8.0 at 65°C for 15 minutes, followed by 10 ng/μL proteinase K treatment at 37°C for 45 minutes). Compared to the Tris-EDTA buffer (pH 9.0), this condition resulted in a similar TSS score but a much lower number of unique fragments (7,695 *vs.* 4,340; Fig. 1b).



Sample ID	TR Condition	Median TSS	Median Fragments
FF_1	N/A	9.505	5860
FF_2		6.139	9661
FF_3		9.737	6004
S4_1	1x Tris-EDTA, pH 9.0	3.606	2908
S4_2		4.042	8336
S5_1	1x Tris-EDTA, pH 9.0+250nM NaCl	3.96	8052
S5_2		3.573	9393
S6_1	1x Tris-EDTA, pH 9.0+250mM NaCl	3.75	9699
S6_2		4.73	9472
S7_1	1x Rodent, pH 6.0	4.015	5280
S7_2	1x Rodent, pH 6.0+250nM NaCl	3.944	5330
S8_1	0.8M Tris-HCl, pH 8.0	4.048	4322
S8_2	0.8M Tris-HCl,pH 8.0 +250nM NaCl	3.651	4358

Fig. 1b

2) Compared with FFPE-ATAC (arrow indicated figure):

FFPE-ATAC relies on enzymatic digestion to extract nuclei from FFPE samples, a process that is time-consuming (taking up to 16 hours) and may result in nuclear fragmentation. We compared the marker gene *Gabrb2* from mouse cerebellum using both methods. While both methods indicate high chromatin accessibility at this locus (Fig. 2d), our approach provides more detailed information. Specifically, it reveals that high chromatin accessibility is primarily localized in the white matter region, offering a more accurate spatial pattern (Fig. 2b).

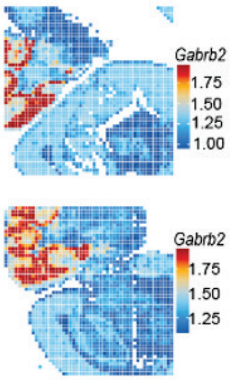


Fig. 2b

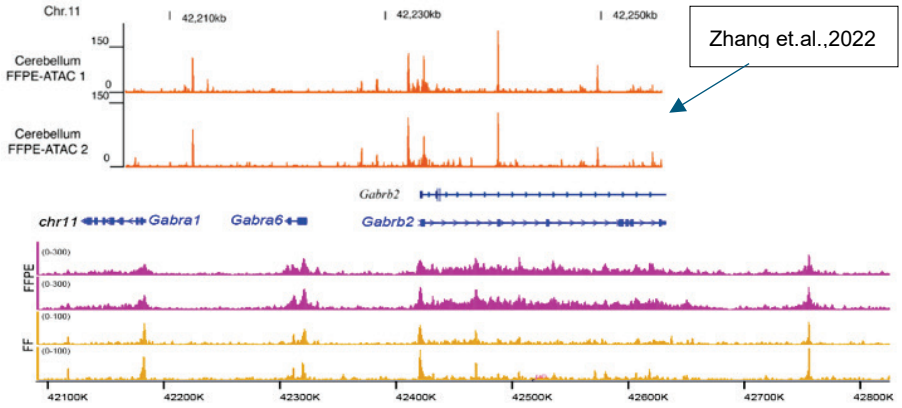


Fig. 2d

A direct comparison of different methods is provided in Supplementary Table 1.

Since spatial FFPE-ATAC-seq is presented as a new method in the current manuscript, I think it is important to include the aforementioned technical details to let the readers know what exactly are invented, changed and improved over previous methods. Without that, the manuscript misses the mark of being a new method.

We thank the reviewer for these comments. These comments have been addressed by adding the following in the revised manuscript.

1) We refined our research aim: To maximize the utility and compatibility of spatial ATAC-seq for FFPE samples, we made minimal modifications, specifically refining the target retrieval (TR) process to accurately capture open chromatin regions.

2) We added the details about target retrieval process: To enable chromatin accessibility profiling in FFPE tissues, we implemented several key modifications to the target retrieval (TR) process: (i) optimizing heat-induced retrieval temperature, (ii) incorporating additional proteinase K (PK) treatment to break protein-DNA crosslinks, and (iii) evaluating different TR solutions, including Tris-EDTA buffer (pH 9.0), 0.8 M Tris-HCl buffer (pH 8.0) (as used in FFPE-CUTAC2), and citrate buffer (pH 6.0). To assess the effectiveness of these modifications, we generated both bulk and spatial FFPE-ATAC-seq libraries from three-year-old archived mouse brain FFPE tissues. We used the transcription start site (TSS) enrichment score and the number of unique fragments as key quality metrics, leading to several important findings. First, TSS scores improved as the retrieval temperature decreased from 99°C to 65°C. The highest TSS enrichment score (~4) was achieved using a combination of 1× target retrieval at 65°C and PK digestion at 10 ng/μL for 45 minutes (Fig. 1b). Second, while different TR reagents produced comparable TSS scores (~4), the average number of unique fragments per condition varied: 7,695 (Tris-EDTA buffer), 5,305 (0.8 M Tris-HCl buffer), and 4,340 (citrate buffer) per 50-μm pixel resolution (Fig. 1c). Notably, only Tris-EDTA treatment yielded the unique fragments comparable to those from spatial ATAC-seq in fresh-frozen mouse brain (average: 7,761). We further examined the impact of varying sodium chloride concentrations (0 nM, 250 nM, and 250 mM) across different AR reagents and found no obvious differences in the median number of fragments (Fig. 1c).

(2) In Figure 1b, different temperatures were tested during target recovery, and 65C turned out to be the best in terms of TSS enrichment. What about the alignment rate to the reference genome? Henikoff et al. (doi: 10.1038/s41467-023-41666-z) seemed to get lower alignment rates with lower temperature like 65C in FFPE-CUTAC. I know it is a different assay, but it is still interesting and useful to show the alignment rates to the reference genome in different conditions. Maybe there are some trade-offs between TSS enrichment and alignment rates.

We thank the reviewer for these insightful comments. We performed genome alignment for all samples included in the manuscript and found that alignment rates remained consistently high (>85%) across all conditions. Since our target retrieval process spans a temperature range from 65°C to 99°C, temperature variations do not appear to affect alignment rates.

A key distinction between FFPE-CUTAC and our spatial FFPE-ATAC-seq is that, in our method, Tn5 transposase directly binds to chromatin, whereas CUTAC relies on histone tail binding before cutting the adjacent chromatin region. As a result, we do not observe any trade-offs between TSS enrichment and alignment rates in our experiments.

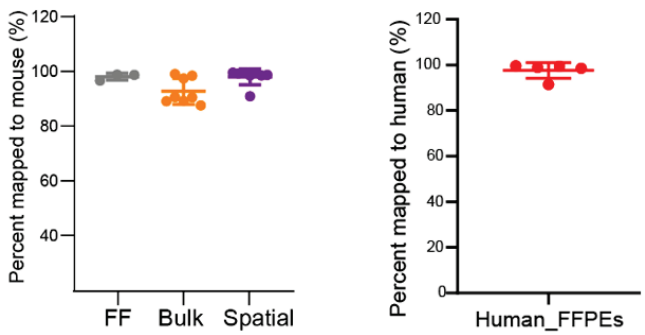
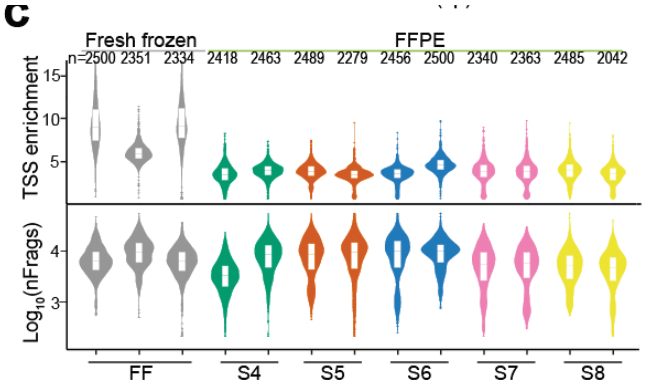


Fig. 1f and Supplementary Fig. 5f

(3) Figure 1c contained important optimisation information. How many times have the experiments in Figure 1c repeated? How reproducible are the results? Currently, it does not seem to have a clear pattern. If we compare S7 with S4, the conclusion is 250 nM has a huge impact on median fragments. However, when compare S8 to S5, it does not seem so. Maybe it is a combination of things. Nevertheless, it is worth mentioning how reproducible the results are. On a related, what is the rationale of adding NaCl and why only at 250 nM which seems to be a very low concentration compared to norm al usage which is at the scale of mM.

We thank the reviewer for their valuable comments. To address the concerns, we conducted new experiments for each condition and additionally included a 250 mM sodium chloride condition (samples: S6_1 and S6_2). Our results showed an average of 7,695 unique fragments, comparable to the fresh frozen samples (7,761). Based on these findings, we concluded that varying sodium chloride concentrations (0 nM, 250 nM, and 250 mM) across different AR reagents did not result in obvious differences in the median number of fragments (Fig. 1c).



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S6_2		4.73	9472
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S7_2	1x Rodent, pH 6.0+250nM NaCl	3.944	5330
S8_1	0.8M Tris-HCl, pH 8.0	4.048	4322
S8_2	0.8M Tris-HCl,pH 8.0 +250nM NaCl	3.651	4358

Fig. 1c

(4) The integration with scRNA-seq reference is a bit strange. For example, in Figure 2c, red dots are from spatial cluster 0, indicating they have similar ATAC profiles. Then why they are scattered into different cell types in the scRNA-seq reference UMAP? Any discussions? In addition, is there a particular reason to integrate with scRNA-seq reference? Intuitively, integration with scATAC-seq reference would make more sense since the data is spatial ATAC-seq. There are well-annotated scATAC-seq data sets in the brain as well.

We appreciate these insightful comments. We use ArchR and snapATAC to analyze spatial chromatin accessibility data, which do not directly assign cell types. Instead, these tools focus on clustering cells based on chromatin accessibility patterns and help infer the regulatory landscapes

of cells. In contrast, scRNA-seq clusters are based on the actual transcriptional profile of the cell, meaning they are derived from gene expression patterns. These clusters reflect the functional identity of cells, such as whether a cell is actively transcribing certain genes or engaged in specific biological processes.

To explain the difference in clustering between ATAC and RNA-seq data, we refer to the new Fig. 2e-f. For example, Cluster 4 in the ATAC data is labeled as mouse cortex (Fig. 2a). By adjusting the parameters, we can obtain more clusters (**Figure R1**) and integrate with scRNA-seq data (**Figure R2**). In this case, the cortex can be subdivided into three additional clusters, which can then be mapped to different cell types (indicated by arrows). However, this spatial pattern appears quite complex, particularly when considering different tissue regions.

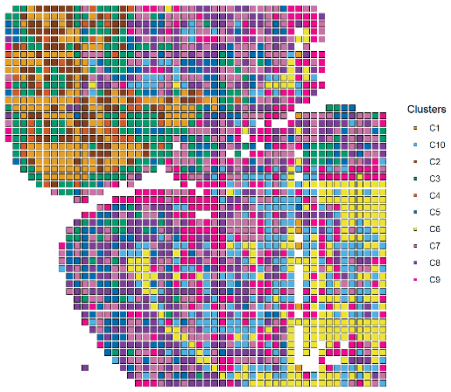


Figure R1

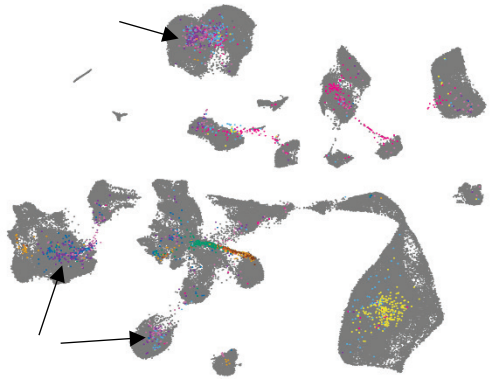
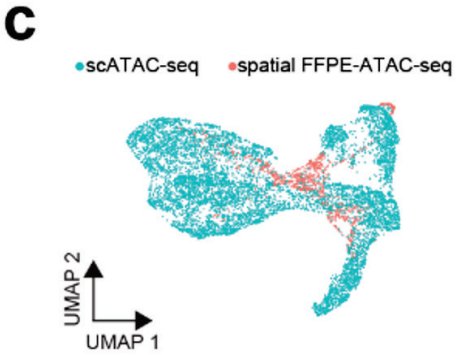


Figure R2

For the integration with scATAC-seq, we did observed the high overlap between spatial FFPE-ATAC-seq and scATAC-seq data (Supplementary Fig. 3c).



Supplementary Fig. 3c

(5) Figure 3c is a bit confusing. At the left-hand side, it seems there are very few spatial FFPE-ATAC-seq points at 3 o'clock position of the UMAP. However, at the right-hand side, there are way more points at 3 o'clock position of the UMAP. Is this a mistake or is it due to colour limitations? Maybe change to a different colour palette?

We thank the reviewer for pointing this out. In the original figure, reference UMAP and the spatial FFPE-ATAC-seq data points at the 3 o'clock position were both colored blacks, making it indistinguishable from each other. We have now changed the color for better clarity.

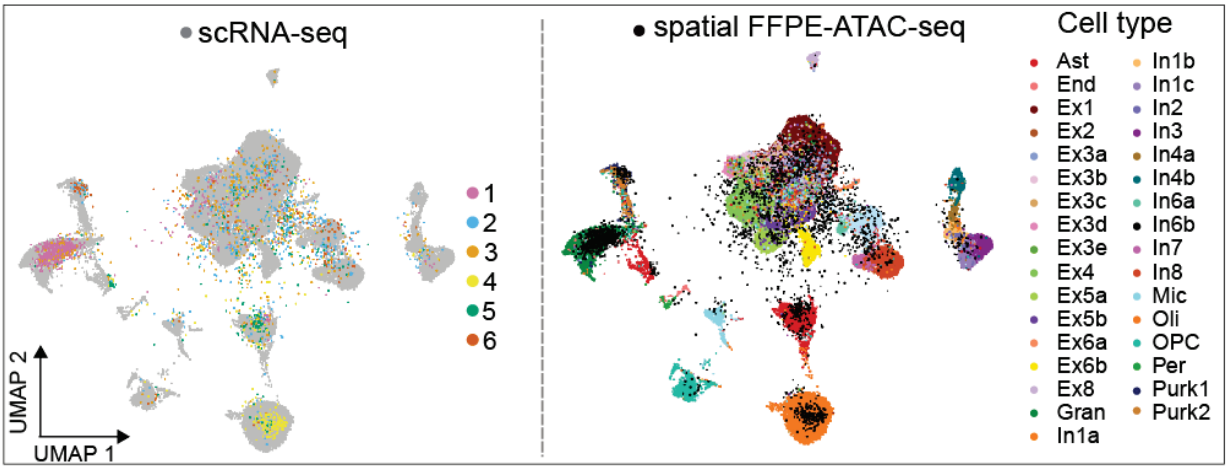


Fig. 3c

(6) In Figure 1c, what is "AR" in the table stand for? Is it supposed to be "TR"?
This has been corrected with "TR" in the table.

(7) Line 66: "liker" should be "linker".
This has been corrected.

(8) Gene names should be in italic in figures.
This has been corrected with italic gene names in figures.

(9) If possible, please use a different colour palette that is colourblind safe for Figures 1b, 2b, 2f, 3a, 3d-f, 4a, 4e.
This has been corrected with new colour palette.

Reference

1 Henikoff, S. *et al.* Epigenomic analysis of formalin-fixed paraffin-embedded samples by CUT&Tag. *Nature Communications* **14**, 5930 (2023). https://doi.org:10.1038/s41467-023-41666-z

2 Zhang, H. *et al.* Profiling chromatin accessibility in formalin-fixed paraffin-embedded samples. *Genome Res* **32**, 150-161 (2022). https://doi.org:10.1101/gr.275269.121

3 Llorens-Bobadilla, E. *et al.* Solid-phase capture and profiling of open chromatin by spatial ATAC. *Nature Biotechnology* **41**, 1085-1088 (2023). https://doi.org:10.1038/s41587-022-01603-9

4 Deng, Y. *et al.* Spatial profiling of chromatin accessibility in mouse and human tissues. *Nature* **609**, 375-383 (2022). https://doi.org:10.1038/s41586-022-05094-1

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript "Spatial profiling of chromatin accessibility in formalin-fixed paraffin-embedded tissues" has added new experiments and analysis that more thoroughly document the performance of spatial-FFPE-ATAC-seq. The additional text and comparison of FFPE and flash-frozen samples have addressed my comments.

We sincerely thank the reviewer for their positive feedback. We are glad that the additional experiments and analyses — particularly the comparison between FFPE and flash-frozen samples — have addressed your concerns and helped clarify the performance of spatial-FFPE-ATAC-seq. We appreciate your thoughtful evaluation and support for the revised manuscript.

Reviewer #2 (Remarks to the Author):

This manuscript has included sufficient validation data that were previously missing, such as the comparison of sample quality and analysis results between FF and FFPE, and the demonstration for cancer tissue analysis. These additions will be valuable information for readers considering clinical applications using spatial FFPE ATAC-seq.

In my previous comments, I highlighted concerns about the impact of sample quality degradation on analysis results. However, at this stage, the authors' evaluation focuses only on general aspects such as overall correlation and TSS enrichment, which does not fully address my concerns. If the following points are improved, I believe this paper will be worthy of publication.

Specific points:

** New Figure 2b: The current evaluation is based on a qualitative approach. It would be more convincing to include quantitative evaluation metrics. For example, what percentage of ATAC-seq signals around marker gene loci are concentrated in cortex or cerebelium regions? In addition, calculating metrics such as Moran's I to determine the extent of non-random spatial patterning would provide a more reliable assessment.*

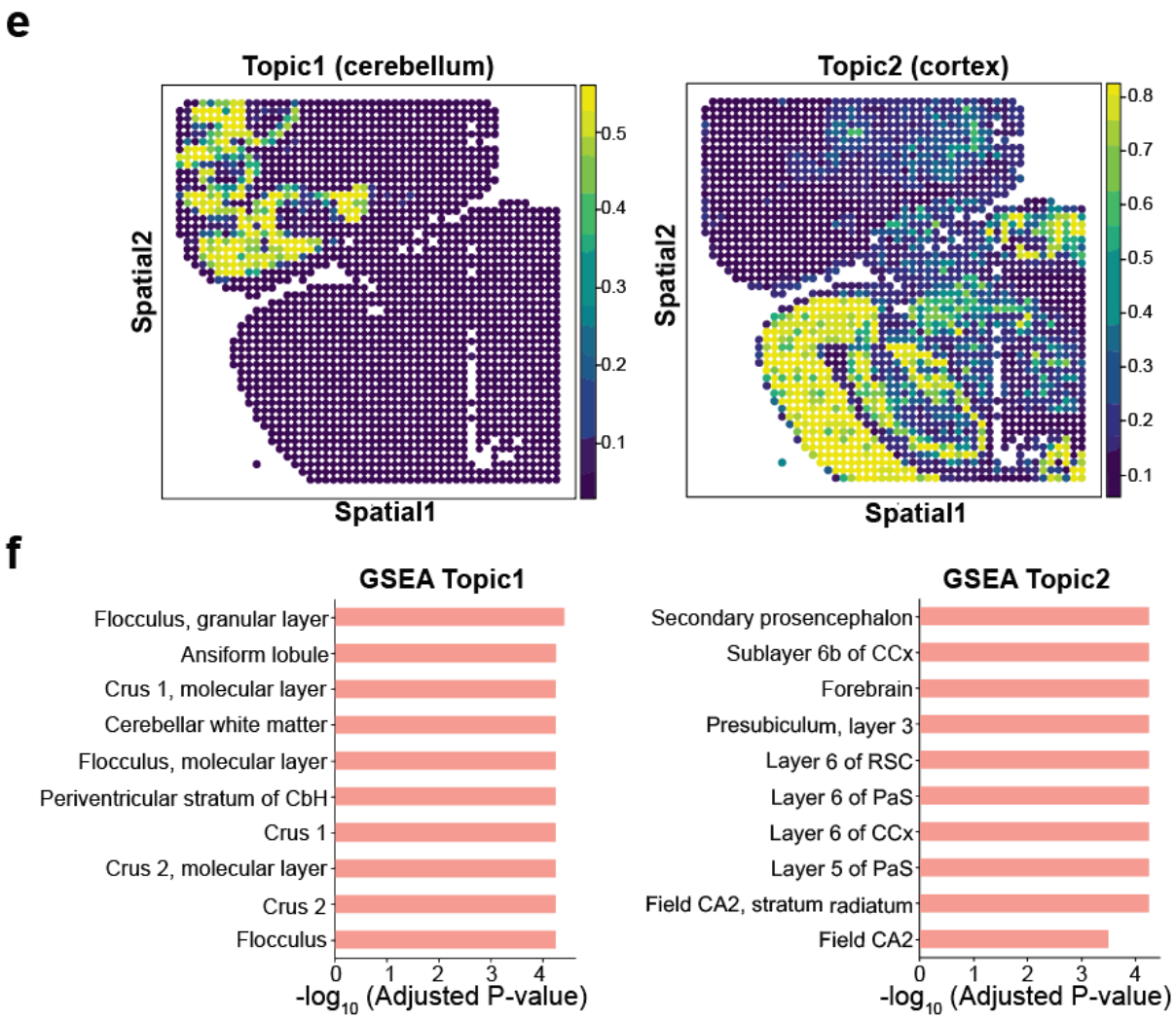
Thank you for your valuable comments. We completely agree that it is essential to add more comprehensive quantitative evaluations to demonstrate the capability of our proposed FFPE-based protocol in capturing biologically meaningful epigenomic signals at spatial resolution. Following your suggestions, we conducted quality assessments of the FFPE-based ATAC-seq data based on two key analyses:

1. Applying an advanced deep learning-based spatial factor decomposition to the FFPE spatial gene scores to demonstrate that the captured gene scores exhibit meaningful spatial distribution patterns corresponding to tissue architecture, such as brain regions (e.g., cortex and cerebellum), thereby providing strong evidence of the model's potential to uncover biologically relevant spatial insights.
2. Comparing spatial autocorrelation scores—specifically Moran's I and Geary's C—of gene activity scores between FFPE and FF samples to highlight the spatial consistency of gene activity captured by the FFPE protocol relative to FF samples.

For the spatial factor decomposition, we employed STAMP[1], a recently proposed state-of-the-art method for spatial topic modeling based on interpretable deep learning. STAMP decomposes spatial omics data into distinct spatial topics, analogous to meta-genes in NMF (Non-negative Matrix Factorization), but with spatial context considered. Each topic is represented as a linear combination of gene values, with each gene contributing to varying degrees across topics. Prior to applying STAMP, we discretized the gene activity

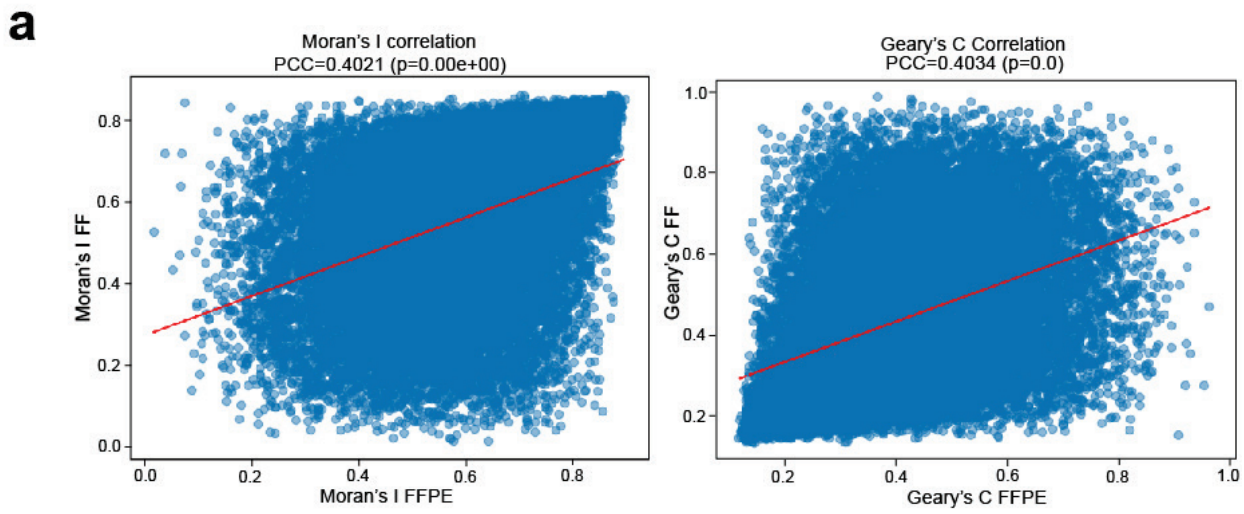
scores (rounding them to the nearest integer) to meet input format requirements and specified two topics for decomposition. Visualization of the resulting spatial topic distributions (Supplementary Fig. 4e) revealed highly variable spatial patterns aligned with specific brain regions (e.g., cortex and cerebellum).

To further validate that the spatial gene activity scores captured by our method reflect brain region-specific distributions, we conducted Gene Set Enrichment Analysis (GSEA) on the top 50 contributing genes for each topic. The reference gene sets were drawn from the Allen Brain Atlas, which defines markers for various brain regions. We reported all significant enrichment results (Supplementary Fig. 4f) (with at least one enriched term at FDR-adjusted p-value < 0.1) for each topic, strongly validating that the spatial gene activity profiles derived from FFPE data capture complex tissue structures and spatial biological patterns. These results underscore the potential of our FFPE-based spatial epigenomics protocol in capturing biologically meaningful spatial information, offering valuable insights into complex biological applications involving spatial organization.



Supplementary Fig. 4e-f

For the spatial autocorrelation assessment, we utilized squidpy.gr.autocorr to calculate Moran's I and Geary's C scores for each gene in both FFPE and FF samples, based on their gene activity scores. We then computed the Pearson correlation between the Moran's I scores of FFPE and FF samples, as well as between the Geary's C scores of FFPE and FF samples (Supplementary Fig. 5a). The results indicate a statistically significant correlation (p-value < 0.05) for both metrics between FFPE and FF, demonstrating that the FFPE protocol effectively preserves spatial biological signals.



Supplementary Fig. 5a

** New Fig. 2c: Since the subnucleosomal fraction represents the majority of reads in standard ATAC-seq, reducing the nucleosomal fraction is expected to have a minimal impact on the overall correlation coefficient. Therefore, it would be beneficial to focus on areas where differences occur (e.g., localization of peaks that do not overlap with standard ATAC-seq peaks as shown in Supplementary Figure 4) and discuss the differences between them.*

To address the reviewer's concern regarding the minimal impact of nucleosomal read reduction on overall correlation due to the dominance of subnucleosomal fragments in standard ATAC-seq, we focused on a more biologically informative comparison: the differences in **peak localization and gene-level functional relevance**.

As shown in the updated Supplementary Fig. 4a-d, we annotated the peaks to nearby genes and performed GO enrichment analysis separately for:

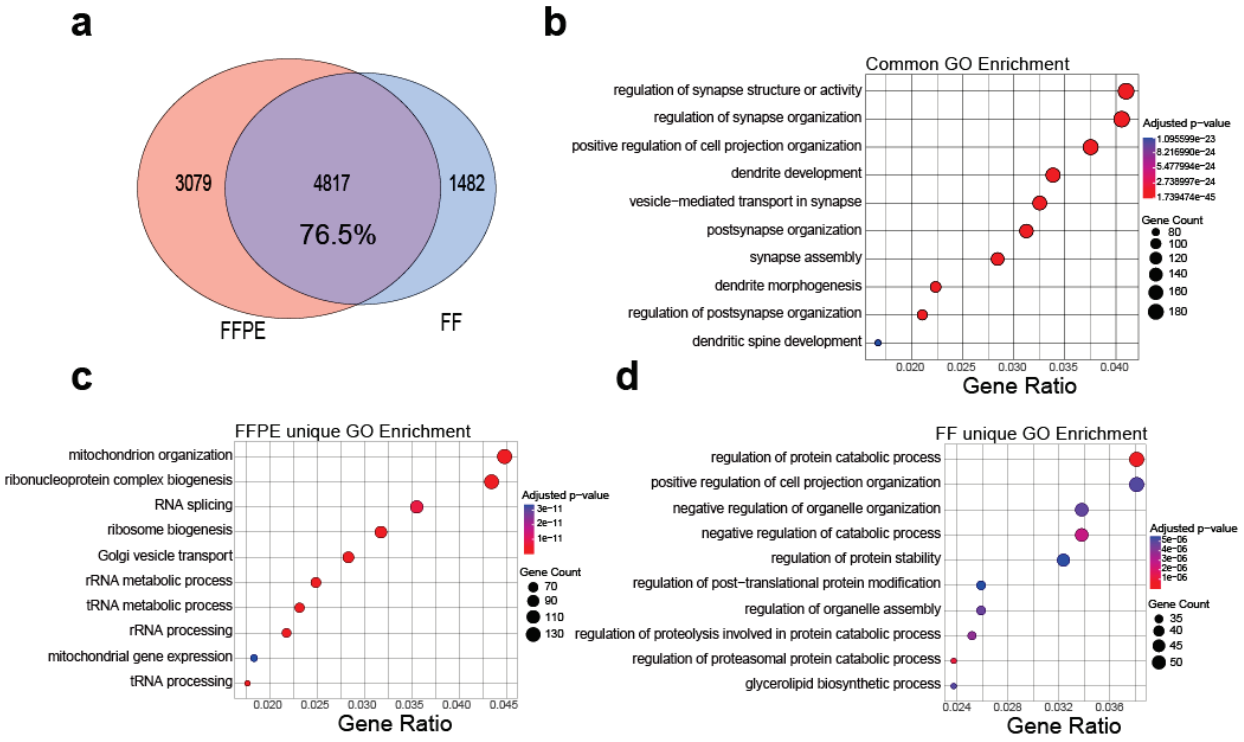
- **Common genes** identified from peaks shared between FF and FFPE samples,
- **Unique genes** identified from FF-only or FFPE-only peaks.

This gene-centric approach revealed biologically meaningful differences that are not apparent from global correlation metrics:

- **Common peaks** were enriched in synapse-related processes, indicating shared functional elements across both sample types.
- **FF-unique peaks** were enriched for protein catabolic processes and organelle regulation.

- **FFPE-unique peaks** showed strong enrichment for RNA processing and mitochondrial functions.

These results demonstrate that peak annotation and functional analysis can more effectively capture biologically relevant differences between FF and FFPE ATAC-seq data, beyond what is captured by overall read correlation. Therefore, **annotating peaks before comparison provides deeper insight** into how sample preservation methods may impact biological signal interpretation.



Supplementary Fig. 4a-d

** New Figure 4c-e: There is some arbitrariness in determining peak calling thresholds. For example, in Supp. Fig. 4c, I suspect that spatial FFPE-ATAC-seq has low S/N, and using the same peak calling threshold may result in excessive sensitivity and large overlap (e.g., 68%) with bulk ATAC-seq. I suggest setting the gold standard peaks from bulk ATAC-seq and generating ROC or precision-recall curves by varying the read count threshold of spatial FFPE-ATAC in 1K or 10K genomic bins. This would allow a more detailed assessment of the optimal threshold setting and the differences in signal distribution between the two methods.*

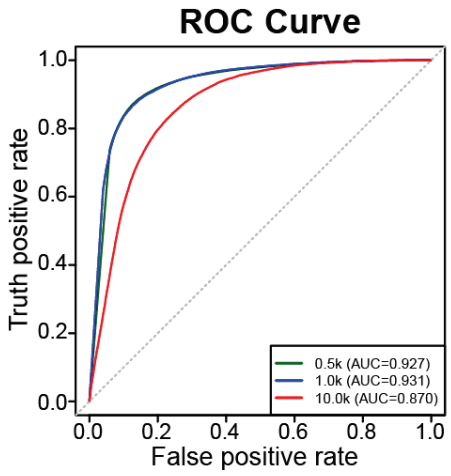
We thank the reviewer for the insightful comment. To address the concern regarding the arbitrariness of peak calling thresholds, we performed a quantitative benchmarking analysis using a gold standard set of peaks. Specifically, we utilized the ENCODE

narrowPeak file from bulk ATAC-seq (ENCFF858CCP.bed), which represents high-confidence peaks identified using standardized peak calling parameters.

To systematically assess the performance of spatial FFPE-ATAC-seq, we divided the genome into non-overlapping bins of 0.5 kb, 1 kb, and 10 kb. For each bin size, we calculated the read counts from the spatial FFPE-ATAC-seq data and generated receiver operating characteristic (ROC) curves by varying the read count threshold across bins. Bins overlapping bulk ATAC-seq peaks were treated as positives, and those not overlapping were treated as negatives.

Our analysis revealed area under the ROC curve (AUC) values ranging from 0.87 to 0.931 across the different bin sizes, demonstrating that spatial FFPE-ATAC-seq signals show strong concordance with bulk ATAC-seq peaks despite the inherently lower signal-to-noise ratio. This approach provides a more nuanced and threshold-independent evaluation of signal quality and supports the reliability of our spatial profiling.

c



Supplementary Fig. 6c

Reviewer #3 (Remarks to the Author):

The authors have successfully addressed all my concerns. It seems the main improvement is on the target retrieval steps, which is clear now. I also understand the rationale of making "minimal modifications, specifically refining the target retrieval (TR) process" to facilitate adaptation of the previous Spatial-ATAC technology on FFPE samples. Therefore, I have no further questions.

We thank the reviewer for their supportive comments and are pleased that the revisions clarified the improvements made, particularly in the target retrieval steps. We appreciate your understanding of our rationale to implement minimal yet effective modifications to enable the application of Spatial-ATAC to FFPE samples. Thank you again for your thoughtful review and encouraging feedback.