nature portfolio

Peer Review File

A spatially resolved human glioblastoma atlas reveals distinct cellular and molecular patterns of anatomical niches

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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)
Park et al. analyzed different anatomic regions of GBM through ST and paired scRNA-seq, adding more data to the fast-growing GBM ST datasets. However, the results are mostly descriptive offering limited biological insights, lack comparison with existing datasets, and many of the claims are not substantiated by data. Also, the writing and figure presentation needs to be substantially revised to improve clarity.

1. The introduction is poorly written. The first paragraph has no reference. The logic flow from the second to third paragraph is mixed up. Many recent publications using spatial transcriptomics to investigate GBM have not been properly discussed and compared with this study to highlight the knowledge gap.
2. The sampling and sequencing strategy appears confusing. The authors should cite and describe their Table S1 in the first paragraph of Results, and summarize the mutational profiles of GBM samples analyzed, especially the IDH mutation status.
3. Fig.2, it is unclear how the authors distinguish neoplastic vs TME cells.
4.“We were able to detect a granulocyte population from the freshly processed samples, a cell type missing, from most human GBM scRNA-seq public datasets” Which granulocyte population? Neutrophils? The authors need to back up their claim with specific markers and compare with other scRNA-seq datasets.
5. What is the rationale for dividing Oligo into 4 subtypes? Have these subtypes been previously shown? Is there any disease relevance? Also, I don’t understand why the authors put the comparison in Fig. S7A. They should immediately show it in the main figure.
6. Fig.3D, I am not sure what the authors are showing here.
7. The web link provided does not appear to allow users to explore the spatial gene expression.
8. Fig.6A, OPALIN and MOBP are not classical markers for myelinating OCs.
9. What is the cellular frequency of Oligo_2_3_2 in the tumor core and periphery? How does it impact on the tumor cells? I am not fully convinced this is an important cell subpopulation.

Reviewer #2

(Remarks to the Author)
Park et al. present a comprehensive single-cell and spatial transcriptomic dataset of glioblastoma samples, complementing previous studies by Ravi (2022) and Greenwald (2024). While this dataset, which employs CITE-seq, sc/snRNA-seq, Visium, and Xenium technologies, is a valuable resource, it offers limited biological insights.
The authors identified a potential novel cell type, Oligo_2_3_2, interacting with another new cell type, gbmEndoPeri, but further characterization is needed to understand their phenotypes and clinical implications. The proposed link between neuroinflammation and the transformation of oligodendrocytes into pathogenic states associated with impaired remyelination and neurodegenerative processes remains unsupported by current analysis.

Major Points:

The authors provide a rich dataset of spatial transcriptomics. However, there have been previously published spatial transcriptomic datasets:
- Ravi, V. M. et al. Spatially resolved multi-omics deciphers bidirectional tumor-host interdependence in glioblastoma.

Cancer Cell 40, 639-655.e13 (2022).
- Greenwald, A. C. et al. Integrative spatial analysis reveals a multi-layered organization of glioblastoma. Cell (2024).

The analysis of spatial organization leads to similar conclusions made in the studies above. The conclusion and the knowledge contribution to the field did not come across strongly. Can the authors clarify specifically how this dataset can enable novel discovery for the community compared to the other datasets?

To increase value to the community and further validate findings, increasing the Xenium dataset would be beneficial.

More attention is required in the figures and text. Some legends and labels are unclear (see section below).

The AUC analysis for spatial enrichment of cell types in AFs is missing in the manuscript. More evidence and analysis are required to support the claims about spatial co-localization.

The pseudotime analysis in Figure 6C is not supported. These are non-malignant cells that are terminally differentiated and thus, the pseudotime framework to model trajectories should not apply.

The main finding of this manuscript is the novel oligodendrocyte subtype. It would be good to validate the existence of this subtype with protein marker staining. Although the authors have identified Oligo_2_3_2, it is not clear from the analysis how the novel subtype contributes to the pathology of GBM. Is there an association between the proportion of Oligo_2_3_2 and overall survival?

Minor Points:

Figure 1B: It is unclear where the cell count per cell type is coming from. Why is the microglia count much higher than tumour cells?
Figure 2A/B: Why does the UMAP look different between different levels of labels?
Figure 2C: Need to briefly describe what the states stand for and how they align with previously reported cell states.
Figure 2C-D: Add a statistical test to support the claim that core samples have a higher proportion of tumor cells compared to peri samples.
Figure 3D: This plot is unclear. Please make the message clearer, either through text or an alternative visualization.
Figure 5B: Can be moved to supplementary.
Figure 6A: “We scrutinized the differentially expressed genes (DEGs) between oligodendrocytes from the tumor core and periphery”: Oligo_2_1 is not enriched towards the periphery. What is the rationale for specifically comparing Oligo_2_3_2 to Oligo_2_1?
Figure 6 Caption: Ensure captions are consistent with the figure.
Figure S1: Legend missing for dot size and colour.
Figure S3: Legend missing for Visium spot annotations.
Figure S6B: Needs a legend. If the enrichment of Oligo_2_3_2 towards the core is significant.
Figure S7A: The Y-axis is missing or too small.
Figure S7B: Change the colour of the colour bar to be more easily interpretable. Report FDR instead of p-value.

In the text, it should be Neftel and not Verhaak.
"TC_mesnh, resembling Mes1-like cells in Verhaak classification, demonstrate an association with immune infiltration, whereas TC_mesh (Mes2-like cells in Verhaak classification) are linked with chronic hypoxia."

“Spots from patients with astrocytoma (Ast), IDH mutant (IDHm) (SNU19, SNU22) were classified into LE_GM, LE_WM, IT, and CT”: Does it make sense to label IDHmut cases with GBM anatomical features?

“This suggests that despite sharing mesenchymal gene signatures, these two tumor cell states may have arisen under different environmental pressures.”: How does this connect with Sten Linnarsson’s GW-like and FB-like states?

Reviewer #3

(Remarks to the Author)
In this manuscript (NCOMMS-24-33567-T, “A spatially resolved human glioblastoma atlas reveals distinct cellular and molecular patterns of anatomical niches”) Park*, Sonpatki* et al., perform spatial transcriptomics with matched single cell RNA sequencing and cellular indexing of transcriptomes and epitopes (CITE-Seq) on FFPE embedded glioma samples. They present a spatial map of 56 cellular compartment based on 115,914 spatial transcriptomes from 32 tissues sections that could be of use to the research community. The authors suggest that their study provides novel insight into the cellular architecture of GBM compared to similar studies that have been published in Nature Communications and other journals (PMCID: PMC9974099, PMCID: PMC9950149, PMID: 35700707, PMCID: PMC10336135, PMCID: PMC9767870). Although the authors show some unique data, including some previously unreported diversity in oligodendrocytes, there are some issues ranging from major to minor that reduce my enthusiasm for the manuscript in its present form.

1. In general, in its current form I fail to understand the overall questions or hypothesis that is trying to be answered through this analysis. I understand the authors are claiming they have a unique dataset, which I do not want to diminish as it could be

useful to the research community. However, multiple recent papers, some listed above, have extensively conducted spatial transcriptomics with matched RNA sequencing.

2. The authors highlight their unique oligodendrocyte cell type that they have identified throughout their analysis, but that is not discussed in the abstract or the title.

3. It is current form, it is not clear how the Oligo2_3_2 population marked by expression of HLA-A is novel and/or clinically relevant. What is the function of this population? Many manuscripts have extensively categorized and identified the OPC lineage. If the authors want to focus on this, I suggest making the paper about this and providing more evidence as to how this population is unique and why it is important (linked to any patient outcome, genetic mutation, or any other characteristic (treatment response, biological sex, etc.)).

4. The authors suggest that this shared resource will help develop new and effective combinatorial therapies but do not give an example as to how. What makes this analysis and dataset different than what is already out there? How can this dataset provide information that no other dataset can when looking for combination therapies. Not to belabor the point, this gets back to the authors not answering a clearly delineated question or hypothesis.

5. The patient classification should be based on sex (not gender) as this is the biological variable.

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.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have properly addressed my concerns. I have no further comments.

Reviewer #2

(Remarks to the Author)
This revised manuscript presents a multi-modal glioblastoma atlas integrating sc/snRNA-seq, CITE-seq, Visium profiling of both frozen and FFPE samples, and subcellular-resolution Xenium data. The dataset is undeniably large, encompassing over 115,000 Visium spots, more than 220,000 single cells, and nearly three-quarters of a million Xenium profiles generated with a custom 348-gene panel. The authors have also genotyped all tumors, incorporated high-resolution imaging, and provided a public browser. In terms of scale, technical breadth, and potential clinical applicability through FFPE inclusion, the resource is both large and diverse.

The revision addresses most of the concerns raised in the previous round. The authors now more clearly articulate how their dataset differs from and extends prior atlases such as Ravi (2022) and Greenwald (2024). They highlight the deeper sequencing, broader histological sampling across canonical niches, the advantages of FFPE for translational research, and the more balanced Xenium panel design that better captures stromal and vascular components. The Xenium dataset has been extended to four GBM patients, providing some subcellular validation of cell-type assignments and niche architecture. The presentation is cleaner, and the discussion has been moderated; speculative links have been either removed or clearly marked as limitations.

The central biological claim remains the identification of a distinct oligodendrocyte subtype, Oligo_2_3_2, enriched in the tumor core and perivascular regions, characterized by immune-associated transcripts and loss of myelin genes. In this revision, the authors show that the Oligo_2_3_2 signature stratifies survival in TCGA and CGGA, is enriched at recurrence in GLASS, and associates with IDH-wildtype and MGMT-unmethylated tumors. They supplement these findings with Xenium validation and orthogonal evidence from human IHC and mouse IF. These additions reduce the likelihood that Oligo_2_3_2 is a computational artifact.

That said, the advance remains primarily descriptive. The dataset is broad and technically impressive, but the biological insights are incremental rather than transformative, and several of the supporting analyses remain statistically underpowered or methodologically weak. The work's value lies chiefly in the scale and accessibility of the resource, not in mechanistic discovery. The following issues must be addressed for the manuscript to meet Nature Communications standards and for the atlas to serve as a credible and durable community resource.

1. The differential expression analysis strategy is unclear and methodologically weak. The manuscript describes per-cell t-tests with random subsampling to equalize group sizes and, in some cases, "paired = TRUE wherever applicable". It is not

evident what “pairing” means at the single-cell level, and treating individual cells as independent replicates inflates degrees of freedom and increases the risk of false positives. This approach also assumes normally distributed data, which is inappropriate for sparse, zero-inflated single-cell counts. Subsampling further introduces unnecessary stochasticity. For a flagship resource, key claims (particularly the definition of the Oligo_2_3_2 population) should be supported by a patient-aware framework. Best practice would involve pseudobulk aggregation per patient per state analyzed with edgeR or DESeq2, or mixed-effects models with patient identity included as a random effect.

2. Although the prognostic analyses suggest that high expression of the Oligo_2_3_2_up gene set and low expression of the Oligo_2_3_2_down gene set correlate with poorer outcomes across glioma subgroups, it is difficult to reconcile these results with the single-cell finding that Oligo_2_3_2 is absent from IDH-mutant high-grade cases. If this population does not exist in IDH-mutant tumors, it is unclear how its signatures could have prognostic impact in these cases. This raises the concern that the observed associations may be driven by confounding factors, such as general stress-related transcriptional programs, rather than by Oligo_2_3_2 as a unique cell type. Moreover, the current survival analyses are descriptive rather than inferential: Kaplan–Meier curves are shown, but without hazard ratios, confidence intervals, or multivariable Cox regression, it is impossible to determine whether the Oligo_2_3_2 signal is independent of established clinical covariates. With TCGA and CGGA data available, the authors should adjust, at minimum, for age, IDH status, 1p/19q codeletion, and MGMT methylation, and should state explicitly how cut-points were defined (median, quartiles, or optimized). Reporting hazard ratios with 95% confidence intervals, global p-values, and cohort or batch terms will be essential to clarify whether the observed associations reflect a truly GBM-specific oligodendrocyte population or instead broader gene expression patterns that correlate with tumor aggressiveness.

3. The new AUC and neighbourhood analyses are useful additions but currently lack transparency. The manuscript does not state the number of permutations performed, the criteria used to define significance, or whether multiple-testing correction was applied across the many AF×cell-type comparisons. In the neighbourhood analysis, a fixed 50-µm radius is used without justification or sensitivity analysis. The authors should specify permutation counts (at least 1,000), clearly define how p-values were derived, apply appropriate FDR correction, and demonstrate that conclusions are robust across a range of neighbourhood radii.

4. Data availability is inadequate and falls below Nature Communications standards. The manuscript states only that raw data will be made “available upon request” and provides links to multiple ad hoc portals for processed data. This is unacceptable for a resource paper. Accession numbers must be provided for all RAW datasets together with complete metadata. Raw data should encompass FASTQs, Visium brightfield and IF images, Xenium raw outputs and segmentation masks, probe design files, and per-sample annotations. In addition, all code should be placed in a versioned public repository with a DOI (e.g., via Zenodo) and must include executable scripts or notebooks that can reproduce every main figure. Without these changes, the resource will not be reproducible or durable.

5. Several clarifications are needed to strengthen the central conclusion around the Oligo_2_3_2 claim. First, the per-patient distribution of Oligo_2_3_2 frequencies in core and periphery should be shown, not just pooled fractions, and the defining DEGs confirmed with patient-aware analysis. Second, given that only four GBM patients were profiled with Xenium, an independent FFPE validation (either with the same panel or via orthogonal ISH/IF) would substantially improve confidence in the generalizability of this signature. Third, because Oligo_2_3_2 is proposed as a malignant oligodendrocyte-like state, CNV score distributions should be provided comparing this cluster to bona fide neoplastic and non-neoplastic populations to rule out misclassification. More broadly, several of the reported cell type annotations are not fully convincing. For example, in Figure 4B, the BV population does not show strong enrichment for the vascular signature described by Greenwald et al., whereas neuronal and NPC populations unexpectedly display higher enrichment. Similarly, the PNZ and PAN populations appear depleted of tumor cells, which is difficult to reconcile with expectations. These observations raise the possibility of discrepancies in cell-type labelling that could contribute to downstream inconsistencies in interpretation. To strengthen confidence in the annotations, the authors should provide additional validation, for instance, a heatmap of CNV profiles with AF and cell type labels overlaid to assess biological coherence, as well as a dot plot or heatmap showing expression of key marker genes for each annotated cell type (similar to Figure S1 for single-cell data).

With the revisions outlined above, this study will provide a credible and durable resource for the GBM research community.

Reviewer #3

(Remarks to the Author)
In general, the authors have been responsive to the previous review and have made satisfactory modifications to their manuscript to increase clarity. I have no additional issues.

I would like to point out that part of the response to point 3.1 is not logical, the claims that work was started before other papers were published has no bearing on the clarity of the message, which was the original issue.

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.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Overall, the authors have been highly responsive, and the manuscript is substantially improved. I view the manuscript as acceptable pending a few minor additions/clarifications.

- The pseudobulk DESeq2 analysis is appreciated. For transparency, it would be helpful if the authors could explicitly state the design formula used (e.g., whether patient identity was included as a covariate).
- The multivariable Cox models strengthen the prognostic analysis. I suggest tempering the interpretation to acknowledge that some IDH-related confounding is difficult to eliminate fully in bulk RNA datasets.
- While raw data deposition now meets Nature Communications standards, reproducibility would be further enhanced if Visium annotations (spot-level metadata or cell-type assignments, when available) were documented more explicitly in the repository.
- While the single-cell CNV profiles are clearly presented in Fig. 2B, the approach used to assign CNV status in the spatial datasets is less explicitly documented. If feasible, providing a brief visualization or description analogous to Fig. 2B for the spatial data would strengthen confidence in the malignant vs. non-malignant spatial assignments.
- A few small presentation items (gene name capitalization, missing scale bars in select supplemental figures) can be addressed during final revision.

I support publication once these minor points are addressed.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Park et al. analyzed different anatomic regions of GBM through ST and paired scRNA-seq, adding more data to the fast-growing GBM ST datasets. However, the results are mostly descriptive, offering limited biological insights, lack comparison with existing datasets, and many of the claims are not substantiated by data. Also, the writing and figure presentation needs to be substantially revised to improve clarity.

- We thank the reviewer for the constructive feedback. As suggested, we have revised the manuscript accordingly and addressed each of the points raised. Specifically, we have expanded the biological insights through additional analyses, incorporated systematic comparisons with existing GBM ST datasets, carefully revised or removed claims to ensure they are fully supported by data, and substantially improved the writing and figure presentation to enhance clarity and readability. A detailed point-by-point response is provided below.

Comment #1.1. The introduction is poorly written. The first paragraph has no reference. The logic flow from the second to third paragraph is mixed up. Many recent publications using spatial transcriptomics to investigate GBM have not been properly discussed and compared with this study to highlight the knowledge gap.

Response #1.1. We thank the reviewer for this valuable comment. In the revised manuscript, we added appropriate references to the opening paragraph and have thoroughly rewritten the Introduction to improve clarity and logical flow. In the updated manuscript, we included detailed comparisons with recent spatial transcriptomics studies, mapping our AFs to modules and layers defined by Ravi et al. and Greenwald et al. and clarified how our study builds upon and differs from them (Figure 4C, Supplementary File 1, Table S6). Specifically, our work uniquely integrates both fresh frozen and FFPE samples with matched single-cell multi-omics and subcellular-resolution Xenium profiling, enabling us to identify novel vascular and glial niches, including the clinically relevant Oligo_2_3_2 subtype. These revisions highlight the knowledge gaps left by prior studies and frame the distinct contributions of our atlas.

Comment #1.2. The sampling and sequencing strategy appears confusing. The authors should cite and describe their Table S1 in the first paragraph of Results, and summarize the mutational profiles of GBM samples analyzed, especially the IDH mutation status.

Response#1.2. We agree that the description in the submitted version may not have adequately conveyed the deliberate, stepwise nature of our experimental design.

Our sampling and sequencing strategy followed a discovery-to-validation progression:

1. **Single-cell RNA-seq and CITE-seq** were performed first, and integrated with public domain datasets to establish a high-resolution cellular atlas of the glioma microenvironment and to define reference cell states.

2. **Visium spatial transcriptomics** was then applied to FFPE and frozen tissue sections from the same cohort and some additional samples to map those cellular programs back into their tissue architecture and spatial niches.

Xenium in situ transcriptomics was conducted lastly, as a cross validation platform, enabling higher-resolution single-molecule detection of key marker sets within the tissue context.

This tiered design of single-cell discovery, spatial mapping, and targeted high-resolution validation was intentional. While not every assay was possible for every patient due to tissue availability, the overall sequencing order was consistent and conceptually aligned. Each modality was chosen for its complementary strengths, with Xenium providing crucial independent validation of our single-cell–derived findings in situ.

To further clarify the approach, we have expanded the description of the cohort in both the Introduction and Results sections. In addition, we now provide the comprehensive mutational profiles of all patients in Figure 1F and Supplementary Table S1.

Comment #1.3. Fig.2, it is unclear how the authors distinguish neoplastic vs TME cells.

Response #1.3. We appreciate the reviewer raising this important point.
To distinguish neoplastic from tumor microenvironment (TME) populations, we combined recursive clustering, marker-based annotation, and CNV analysis.

1. **Level-1 clustering at low resolution:**
   We applied a modified Recursive Consensus Clustering (RCC) with a **resolution of 0.1** at Level-1 to generate only broad, stable partitions and avoid over-fragmentation. This yielded **seven macro-lineages**.
2. **Marker-based annotation of Level-1 clusters:**
   Cluster identities were assigned using differential gene expression and canonical lineage markers:
   - **Cluster 1 – Microglia:** *P2RY12, CSF1R, TREM2, CX3CR1*
   - **Cluster 2 – Oligodendrocytes:** *MOG, MAG, PLP1, MBP*
   - **Cluster 3 – Endothelial/stromal + neoplastic admixture:** vascular/stromal (*CLU, TNR, MARCKS*) together with neoplastic (*SOX2, PTPRZ1*)
   - **Cluster 4 – Microglia + granulocytes:** *CD74, CCL3, FCGR2B, TYROBP*
   - **Cluster 5 – Myeloid lineage:** *S100A8, S100A9, S100A11, SPI1, CD68*
   - **Cluster 6 – Lymphocytes:** *HLA-B, CD3E, IL7R, CXCR4, CD52*

- **Cluster 7 – Immune + neoplastic admixture:** proliferative/neoplastic (*STMN1, TOP2A, MKI67, UBE2C*) with immune (*HLA-A, HLA-C*)
  The full gene lists per cluster are provided in Table S2.

Recursive refinement through three levels:
Each of the seven macro-lineages was recursively subdivided up to **three levels**, progressively resolving finer cell states. The final level of clusters was used for cell type assignments.

3. **CNV validation after full cell-type assignment:**
   InferCNV was then applied **after the final, finer assignments**. This allowed CNV analysis at the level of minor cell types, as shown in Figure 2B. Neoplastic subclusters displayed consistently elevated CNV scores, while immune and stromal populations remained low, thus providing validation of the malignant lineages.

Thus, neoplastic vs. TME assignment was based on transcriptomic clustering and canonical markers, with inferCNV providing a validation across the entire dataset. The graphical representation of this validation is included in Figure 2B, and the Methods and figure legend have been revised accordingly.

Comment #1.4. "We were able to detect a granulocyte population from the freshly processed samples, a cell type missing, from most human GBM scRNA-seq public datasets" Which granulocyte population? Neutrophils? The authors need to back up their claim with specific markers and compare with other scRNA-seq datasets.

Response #1.4. We appreciate the reviewer's suggestion to clarify the identity of the granulocyte population. As detailed in the CITE-seq section, the granulocytes we detected show high CD16 and low CD14 expression, consistent with neutrophils, in line with Maas et al., Cell 2023 (Highlighted in green, Figure 2C). We also compared our findings with the GBmap dataset (Ruiz-Moreno et al., Neuro Oncol 2025), where granulocytes are rare (~0.45%), underscoring that our freshly processed samples enabled detection of this population often missed in other GBM scRNA-seq studies (Figure S2A). The corresponding description can be found in the revised manuscript (page 7, lines 161–175).

Comment #1.5. What is the rationale for dividing Oligo into 4 subtypes? Have these subtypes been previously shown? Is there any disease relevance? Also, I don't understand why the authors put the comparison in Fig. S7A. They should immediately show it in the main figure.

Response # 1.5. We thank the reviewer for this comment. Recursive clustering of oligodendrocytes in our dataset resulted in four distinct clusters. To the best of our knowledge, these subtypes have not been described previously. Their disease relevance is demonstrated in Figure 9, where the Oligo_2_3_2 gene signatures stratify patient survival in TCGA/CGGA cohorts and increased in recurrent gliomas, particularly in IDH-wildtype and MGMT-unmethylated tumors. In addition, the comparison previously shown in Figure S7A has been moved to the main text as Figure 8A for clarity.

Comment #1.6. Fig.3D, I am not sure what the authors are showing here.

Response #1.6. We thank the reviewer for pointing this out. Figure 3D has been removed and is no longer part of the revised manuscript.

Comment #1.7. The web link provided does not appear to allow users to explore the spatial gene expression.

Response #1.7. Thank you for bringing this to our attention. We have resolved the issue with the provided web link, which now allows users to fully explore the spatial gene expression data. The users can now access the data at https://www.amaranth-insights.com/ with the following credentials:
Email: amaranthinsights@gmail.com
Password: password@123
They can view all the spatial datasets under the Spatial Glioblastoma Atlas tenant and Glioma manuscript project.

Comment #1.8. Fig.6A, OPALIN and MOBP are not classical markers for myelinating OCs.

Response #1.8. Thank you for your comment regarding the use of OPALIN and MOBP as markers in our study. While we acknowledge that OPALIN and MOBP are not traditionally considered classical markers for myelinating oligodendrocytes (OCs), both are well-documented in the literature as being highly expressed during oligodendrocyte maturation and myelination. We appreciate the reviewer's suggestion and have now expanded our discussion of these markers in the revised text.

Functional studies indicate that Opalin (also known as TMEM10) plays a potentially redundant, non-essential role in oligodendrocyte differentiation and maintenance of oligodendrocyte states. TMEM10/Opalin was first identified as a novel type I transmembrane protein and the most enriched transcript upon OPC differentiation into oligodendrocytes, as shown by microarray profiling and genetic ablation approaches (PMCID: PMC2396630; PMC2830273). TMEM10/Opalin expression increases during early OPC differentiation and continues to rise as oligodendrocytes myelinate axons in vivo. Although conventional TMEM10/Opalin knockout mice show normal oligodendrocyte maturation and myelination, they display learning deficits (PMCID: PMC5113975). Overexpression of TMEM10/Opalin in Oli-neu cells promotes myelin gene expression (MAG, CNP, CGT), while siRNA knock-down impairs differentiation and branching (PMCID: PMC6400977). Furthermore, TMEM10/Opalin functions as a receptor for neuronal LGI1, orchestrating oligodendrocyte differentiation and myelination (PMCID: PMC11317624). TMEM10/Opalin-positive oligodendrocytes have also been detected in remyelinating MS plaques and EAE models (PMCID: PMC6400977), implicating them in disease contexts. Human OPALIN is listed in the Human Protein Atlas as an oligodendrocytic myelin paranodal protein, selectively expressed in CNS oligodendrocytes and highly expressed in glioblastoma, consistent with our findings.

Similarly, MOBP is a known myelin gene enriched in newly-formed oligodendrocytes, expressed at higher levels than CNP during OPC differentiation (PMCID: PMC5221728), and

has been identified alongside MOG and other myelin-associated genes in single-nucleus sequencing studies of the human cortex and substantia nigra (PMCID: PMC7442652).

Taken together, these data support the use of OPALIN/TMEM10 and MOBP as relevant oligodendrocyte markers. We have revised the manuscript to include this discussion and the corresponding references. In addition, for comparison and validation, we also analyzed classical markers such as PLP1, MBP, and MAG in our dataset.

Comment #1.9. What is the cellular frequency of Oligo_2_3_2 in the tumor core and periphery? How does it impact the tumor cells? I am not fully convinced this is an important cell subpopulation.

Response #1.9. We thank the reviewer for this insightful comment and for prompting us to elaborate on the significance of the Oligo_2_3_2 population.

Spatial distribution

In our analysis, Oligo_2_3_2 was found almost exclusively in the tumor core, accounting for ~1.9% of total cells and 24.6% within oligodendrocytes, whereas in the periphery its frequency was negligible (~0.2% of total cells and 1.7% within oligodendrocytes) (Figure 2F, Figure 8A). Within the core, this population was predominantly localized to perivascular regions, where it showed a close spatial association with immune cells, particularly macrophages.

Clinical implications

We further demonstrate that enrichment of Oligo_2_3_2 is significantly associated with adverse clinical outcomes in two independent cohorts (TCGA and CGGA). Patients with high enrichment of the Oligo_2_3_2 up signature and low enrichment of the Oligo_2_3_2 down signature had the poorest prognosis, whereas the reverse pattern predicted the most favorable survival (Figure 9A). In addition, analysis of the GLASS [PMCID: PMC6280138] longitudinal dataset revealed that Oligo_2_3_2 up signatures were further increased at recurrence, particularly in IDH-wildtype and MGMT-unmethylated tumors (Figure 9B), suggesting a role in therapy resistance and tumor relapse. Consistently, this population was primarily observed in glioblastoma, IDH-wildtype, but not in IDH-mutant astrocytoma or oligodendroglioma, further underscoring its disease specificity and potential clinical relevance.

We have revised the manuscript to better contextualize these findings and emphasize their implications. In the revised manuscript, we added the following passages: “The analysis revealed that patients with high scores for the Oligo_2_3_2 up gene set and low scores for the Oligo_2_3_2 down gene set exhibited significantly poorer survival outcomes… recurrent samples with unmethylated MGMT promoters showed elevated expression of the Oligo_2_3_2 gene set, suggesting a potential association with treatment resistance and tumor recurrence.” on page 16 corresponding to lines 420-434.

Reviewer #2 (Remarks to the Author):

Park et al. present a comprehensive single-cell and spatial transcriptomic dataset of glioblastoma samples, complementing previous studies by Ravi (2022) and Greenwald (2024). While this dataset, which employs CITE-seq, sc/snRNA-seq, Visium, and Xenium technologies, is a

valuable resource, it offers limited biological insights. The authors identified a potential novel cell type, Oligo_2_3_2, interacting with another new cell type, gbmEndoPeri, but further characterization is needed to understand their phenotypes and clinical implications. The proposed link between neuroinflammation and the transformation of oligodendrocytes into pathogenic states associated with impaired remyelination and neurodegenerative processes remains unsupported by current analysis.

-We thank the reviewer for this thoughtful comment. We agree that our current data do not fully establish a mechanistic link between neuroinflammation and the transformation of oligodendrocytes into pathogenic states. We have revised the Discussion to explicitly acknowledge this as a limitation and to note that further functional validation will be required to confirm these findings.

Despite these limitations, our study provides several important advances. We identify and spatially localize a previously unrecognized oligodendrocyte subtype (Oligo_2_3_2) enriched in perivascular and immune-glial niches of the GBM core; we show that this population displays an immune-activated profile with reduced myelin gene expression; and we demonstrate its potential clinical relevance through associations with patient survival and recurrence (Figure 9). By integrating multiple modalities (CITE-seq, sc/snRNA-seq, Visium, and Xenium) across both FF and FFPE tissues, our study provides a resource that can guide future functional investigations into this population and its role in GBM.

Major Points:

Comment #2.1. The authors provide a rich dataset of spatial transcriptomics. However, there have been previously published spatial transcriptomic datasets:
–Ravi, V. M. et al. Spatially resolved multi-omics deciphers bidirectional tumor-host interdependence in glioblastoma. Cancer Cell 40, 639-655.e13 (2022).
–Greenwald, A. C. et al. Integrative spatial analysis reveals a multi-layered organization of glioblastoma. Cell (2024).
The analysis of spatial organization leads to similar conclusions made in the studies above. The conclusion and the knowledge contribution to the field did not come across strongly. Can the authors clarify specifically how this dataset can enable novel discovery for the community compared to the other datasets?

Response #2.1. We appreciate the reviewer's acknowledgment of the value of our dataset and share the concern regarding explaining how our work builds upon existing studies. In the revised manuscript, we now provide a clearer description of what distinguishes our dataset, for example, sequencing depth, the inclusion of both FF and FFPE samples, and the use of complementary platforms such as Visium and Xenium, and how these features together create a resource that can facilitate future discovery for the GBM research community.

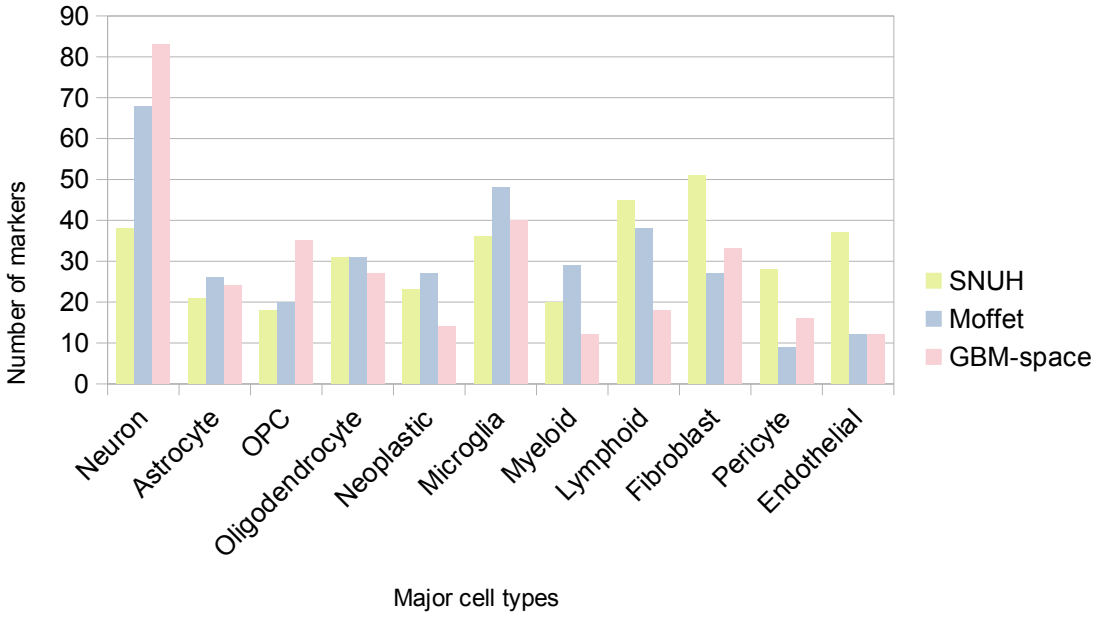
Dataset Strengths and Novel Contributions

While we acknowledge the value of prior studies in the field, including those mentioned in your comments, our dataset provides several critical advancements:

1) **Scale and Resolution:**

- Our dataset comprises **115,914 spatial transcriptomes from 32 glioma sections**, representing one of the largest GBM spatial resources to date.
- Deeper sequencing enabled detection of more genes and finer cell type resolution, allowing us to map **56 distinct cellular components**, including novel oligodendrocyte and vascular subtypes. By contrast, Greenwald et al. analyzed 70,618 spots from 19 cases and identified 14 metaprograms broadly overlapping with single-cell states. This difference is largely attributable to lower transcript and feature counts in earlier studies, which may be explained by technical differences such as the exclusive use of frozen sections (as in Greenwald, Ravi, and Linnarson), thereby limiting the ability to reliably assign cell types. **A detailed comparison of these datasets is provided in Supplementary File 1.**

2) **Application of Xenium technology:**
   - We use **custom gene panel** with more even marker distribution to include more **markers for endothelial cells, fibroblasts, and pericytes** compared to other studies (Data provided in Supplementary File 1, sheet 8). The number of markers identified for each major cell type was compared across three Xenium panels; our cohort (SNUH), the Moffet dataset [PMC10699851], and the GBM-space dataset [https://doi.org/10.1101/2025.05.13.653495]. While all datasets captured canonical neoplastic, oligodendrocyte, and immune cell markers, our cohort (SNUH) showed a more balanced distribution across lineages, including robust representation of stromal (fibroblast, pericyte, endothelial) and immune (myeloid, lymphoid) markers. In contrast, GBM-space and Moffet displayed strong enrichment for neuronal and astrocytic markers but under-represented vascular and myeloid populations. Thus our design choice ensured broader coverage of the tumor microenvironment in our analyses.



- We leverage **Xenium in situ technology** for subcellular spatial resolution. By utilizing **Xenium**, we demonstrate a level of granularity and cell-type resolution confirming the accuracy of cell-type assignments, particularly for complex tumor niches. We also show that our findings, such as **hypoxic regions devoid of vasculature** and the **exclusion of immune cells and oligodendrocytes** in these regions, align with findings from **Greenwald et al.**'s CODEX data, further validating our conclusions.

3) **Advantages of FFPE Data:**
   - **Preservation of Tissue Architecture**: FFPE tissue fixation preserves the tissue's histopathological structure, enabling us to directly correlate **molecular data** with **tissue morphology** in a way that frozen sections cannot always achieve.
   - **Broad Applicability**: We utilized **FFPE** tissue samples, the gold standard in clinical pathology. FFPE samples offer a **much wider applicability** for retrospective clinical studies and offer the potential to re-analyze rare, historically archived samples for further discovery. This makes our dataset especially valuable for expanding spatial transcriptomics into clinical research.
   - We did high-resolution imaging so that our data is **compatible with AI-driven image analysis** based on H&E staining. This integration bridges molecular data with histopathological context, offering significant utility for future computational analyses.

4) **Integration with Genotypic Data**:
   - Although our cohort size is not yet sufficient for a full genotype-stratified analysis, all tumor samples were comprehensively genotyped, providing a framework for linking spatial and molecular states to mutational backgrounds. This annotation enhances the long-term value of the dataset, as it can be integrated with future studies to investigate genotype-phenotype-spatial relationships in GBM. We also include outcome data for future analysis.

5) **Niche-Specific Insights:**
   - Unlike previous spatial transcriptomic studies that largely focus on the tumor core and hypoxic regions, we carefully selected tumor regions to include all known anatomical and histological niches. This broader sampling approach provides a more complete understanding of the tumor microenvironment.
   - By systematically sampling across diverse histological regions, we were able to characterize all canonical GBM niches and introduce two additional compartments, the immune-glial niche (IGN) and the immune-fibroblast niche (IFN), which were not captured in earlier atlases. These refinements provide a more complete view of how stromal, immune, and glial elements organize around the tumor.

These advancements collectively distinguish our study and dataset, providing a unique resource that integrates high-resolution spatial and molecular data. This level of granularity and compatibility with computational tools makes the dataset well-suited for advanced analyses,

including AI-based models for predicting tumor behavior and patient outcomes. We have updated the manuscript to more explicitly highlight these technical strengths and their implications for advancing GBM research.

Comment #2.2. To increase value to the community and further validate findings, increasing the Xenium dataset would be beneficial.

Response #2.2. Thank you for your suggestion. We expanded the Xenium dataset by profiling four GBM patients (SNU21, SNU33, SNU18, and SNU25) using our custom 348-gene panel, resulting in the annotation of 729,307 cells across a total area of 155 mm². The tissue cores sampled from these tumors encompassed cellular tumor regions, infiltrative tumor margins, and peripheral tissue, thereby enabling us to capture the full spectrum of tumor-associated microenvironments. This expansion provided subcellular-resolution validation of major and minor cell types and allowed us to refine niche-specific organization within the tumor. We have updated the manuscript accordingly, with revisions in the Methods (Xenium data analysis), Results (GBM landscape visualized at subcellular resolution), and Discussion sections. The cellular and spatial landscape of the samples can be viewed in Figures 5 and 7.

Comment #2.3. More attention is required in the figures and text. Some legends and labels are unclear (see section below).

Response #2.3. Thank you for highlighting the need for clearer legends and labels in the figures and text. We have carefully reviewed all figures and their corresponding legends to ensure clarity and accuracy.

Comment #2.4. The AUC analysis for spatial enrichment of cell types in AFs is missing in the manuscript. More evidence and analysis are required to support the claims about spatial co-localization.

Response #2.4. We appreciate the reviewer's comment.

In the revised manuscript, we have now:

- Included a description of the **AUC analysis for cell-type enrichment in anatomical features** in the Methods section (under *Cell-type enrichment in anatomical features*).

- Performed an additional **neighborhood analysis** in both the Visium and Xenium datasets to more rigorously evaluate spatial co-localization. The methodology for this has been detailed in the Methods section (under *Neighborhood analysis*).

- Presented the results of these analyses in the revised **Figures 4** and **7**, which provide further evidence supporting the spatial enrichment and co-localization of cell types within specific anatomical features.

Comment #2.5. The pseudotime analysis in Figure 6C is not supported. These are non-malignant

cells that are terminally differentiated and thus, the pseudotime framework to model trajectories should not apply.

Response #2.5. We thank the reviewer for this important comment. In response, we have removed the pseudotime analysis previously shown in Figure 6C from the revised manuscript.

Comment #2.6. The main finding of this manuscript is the novel oligodendrocyte subtype. It would be good to validate the existence of this subtype with protein marker staining.

Response #2.6. Thank you for highlighting the importance of validating our main finding. We appreciate the suggestion to confirm the novel oligodendrocyte subtype Oligo_2_3_2 with protein marker staining. In the revised manuscript, we now include immunohistochemical staining of human GBM samples, which confirmed the presence of Oligo_2_3_2 in the tumor core (Figures S8 and S9). Additionally, we validated its existence using a murine glioblastoma model, where immunofluorescence demonstrated $Sox10^{+}/Cre^{-}$ oligodendrocytes co-expressing Oligo_2_3_2 markers (S100a1, Serpina3n, B2M) within the tumor core but not in normal brain. These validation experiments are presented in Figure S9 (human GBM IHC) and Supplemental Figure S10 (mouse model immunofluorescence), with both images and quantitative analyses supporting the robustness of this subtype identification.

Comment #2.7. Although the authors have identified Oligo_2_3_2, it is not clear from the analysis how the novel subtype contributes to the pathology of GBM. Is there an association between the proportion of Oligo_2_3_2 and overall survival?

Response #2.7. We thank the reviewer for this question. Our analysis demonstrate that enrichment of Oligo_2_3_2 in the tumor core is significantly correlated with worse overall survival in two independent datasets (TCGA and CGGA), highlighting its potential contribution to GBM pathology (revised manuscript Figure 9A). Importantly, Oligo_2_3_2 is predominantly observed in glioblastoma, IDH-wildtype, which is associated with a more aggressive clinical course, further underscoring its relevance to disease severity. Moreover, this population is more abundant in recurrent glioblastomas compared to primary tumors (revised manuscript Figure 9B), suggesting a role in tumor progression and recurrence. We acknowledge that a limitation of our manuscript is that we do not functionally validate these potential roles of Oligo_2_3_2. Nevertheless, its unique spatial distribution and strong association with adverse outcomes provide a compelling rationale for additional functional studies in the future. The manuscript has been revised to better articulate these findings and their clinical implications.

<u>Minor Points:</u>

Comment #2.8. Figure 1B: It is unclear where the cell count per cell type is coming from. Why is the microglia count much higher than tumour cells?

Response #2.8. The cell type counts shown in Figure 1B represent the major cell types included in our Glioma reference atlas. The earlier values were incorrect and have now been corrected in the updated figure.

Comment #2.9. Figure 2A/B: Why does the UMAP look different between different levels of labels?

Response #2.9. We applied recursive consensus clustering up to three levels. From the second level onward, each level-1 cluster was re-clustered independently, generating a new UMAP for the corresponding subpopulation. At every level, UMAP coordinates were recalculated to represent the full cellular population, which explains why UMAPs at different levels appear different. In the revised figure 2A/B, we now present only the level-3 UMAPs to avoid potential confusion.

Comment #2.10. Figure 2C: Need to briefly describe what the states stand for and how they align with previously reported cell states.

Response #2.10. Some of these cellular states are novel and have not been described in any previous publications (e.g., Oligo_2_3_2); the rest are described in the results section now. We have also included a detailed description of each cellular state and correspondence to cell states described in other studies in Table S3.

Comment #2.11. Figure 2C-D: Add a statistical test to support the claim that core samples have a higher proportion of tumor cells compared to peri samples.

Response #2.11. We now mark cell types with significant core–peri differences using asterisks. Significance was assessed with a two-sided paired t-test across patients on per-sample proportions (tumor-cell fraction per type), with Benjamini–Hochberg correction for multiple testing ($q < 0.05$). This is stated in the figure and detailed in Methods.

Comment #2.12. Figure 3D: This plot is unclear. Please make the message clearer, either through text or an alternative visualization.

Response #2.12. We have removed the figure from the revised manuscript.

Comment #2.13. Figure 5B: Can be moved to supplementary.

Response #2.13. This panel has been retained in the main figures (now Fig. 5E). It provides the primary cross-modality validation, demonstrating concordance between cell-type assignments from single-cell RNA-seq and Xenium spatial profiling. This evidence is central to our claim that the single-cell atlas transfers to spatial data and underpins downstream analyses

Comment #2.14. Figure 6A: "We scrutinized the differentially expressed genes (DEGs) between oligodendrocytes from the tumor core and periphery": Oligo_2_1 is not enriched towards the periphery. What is the rationale for specifically comparing Oligo_2_3_2 to Oligo_2_1?

Response #2.14. In the revised manuscript, we have addressed the concern by performing a differential gene expression analysis between Oligo_2_3_2 and all other oligodendrocyte subclusters (Oligo_2_1, Oligo_2_2, and Oligo_2_3_1), rather than limiting the comparison to

Oligo_2_1. This broader analysis provides a more comprehensive assessment of differences between the tumor core–enriched subcluster and the remaining oligodendrocyte populations.

Comment #2.15. Figure 6 Caption: Ensure captions are consistent with the figure.

Response #2.15. We have corrected the legends to align with the figures to avoid inconsistencies.

Comment #2.16. Figure S1: Legend missing for dot size and colour.

Response #2.16. The legends have been added to the respective figure.

Comment #2.17. Figure S3: Legend missing for Visium spot annotations.

Response #2.17. The legend has been added to the respective figure.

Comment #2.18. Figure S6B: Needs a legend. If the enrichment of Oligo_2_3_2 towards the core is significant.

Response #2.18. We have removed the figure from the revised manuscript.

Comment # 2.19. Figure S7A: The Y-axis is missing or too small.

Response # 2.19. The figure is now moved to Figure 8 panel A and the axis labels are adjusted.

Comment #2.20. Figure S7B: Change the colour of the colour bar to be more easily interpretable. Report FDR instead of p-value.

Response #2.20. We have redone the figure and are now reporting -log10 FDR values instead of p-values. The color scheme is adjusted to show better distinction in the significance values.

Comment #2.21. In the text, it should be Neftel and not Verhaak.
"TC_mesnh, resembling Mes1-like cells in Verhaak classification, demonstrate an association with immune infiltration, whereas TC_mesh (Mes2-like cells in Verhaak classification) are linked with chronic hypoxia."

Response #2.21. We thank the reviewer for catching this error. The text in the Methods section, “Cell state assignment for reference single-cell data” (page 30, line 755 in the revised manuscript) has been corrected to read “Neftel” instead of “Verhaak.”

Comment #2.22. “Spots from patients with astrocytoma (Ast), IDH mutant (IDHm) (SNU19, SNU22) were classified into LE_GM, LE_WM, IT, and CT”: Does it make sense to label IDHmut cases with GBM anatomical features?

Response #2.22. Thank you for raising this important point. We recognize that anatomical features (AFs) were introduced in GBM. We apply them to IDH-mutant gliomas as a neutral

spatial vocabulary to enable cross-dataset comparison, while restricting labels to BV, LE_GM, LE_WM, IT, and CT and explicitly excluding GBM-defining features such as necrosis and microvascular proliferation. Two observations support this choice: (i) Greenwald et al. report recurrent cell-state programs in IDH-mutant gliomas (vascular, neuronal, oligodendroglial, astrocytic/OPC-like, myeloid) that align with AF-linked niches (vascular ↔ perivascular regions, neuronal ↔ LE_GM, oligodendroglial ↔ LE_WM/IT, astrocytic/OPC-like ↔ CT, myeloid ↔ immune-enriched zones); and (ii) the foundational anatomic transcriptional atlas of human glioblastoma (doi:10.1126/science.aaf2666) included a small subset of IDH-mutant cases, indicating that AF-oriented analyses have been considered alongside IDH status. We have clarified this rationale and the exclusion of GBM-specific labels in the revised text.

Comment #2.23. "This suggests that despite sharing mesenchymal gene signatures, these two tumor cell states may have arisen under different environmental pressures.": How does this connect with Sten Linnarsson's GW-like and FB-like states?

Response # 2.23. Thank you for this insightful comment. Upon assigning our cell types to the Linnarsson data we found that FB-like 1 and FB-like 2 align with the mesenchymal, non-hypoxic malignant state, whereas FB-like 3 corresponds to GBM-associated fibroblasts. Most GW-like programs likewise map to our mesenchymal, non-hypoxic malignant state; the exceptions are GW-like 4 and GW-like 6, which correspond to our mesenchymal, hypoxia-associated malignant state. This cross-mapping separates stromal CAFs from tumor programs and clarifies how similar mesenchymal signatures can arise under distinct microenvironmental pressures (ECM remodeling versus hypoxia/vascular stress). We have not included this analysis in the manuscript as the paper is still not peer-reviewed.

Reviewer #3 (Remarks to the Author):

In this manuscript (NCOMMS-24-33567-T, "A spatially resolved human glioblastoma atlas reveals distinct cellular and molecular patterns of anatomical niches") Park*, Sonpatki* et al., perform spatial transcriptomics with matched single cell RNA sequencing and cellular indexing of transcriptomes and epitopes (CITE-Seq) on FFPE embedded glioma samples. They present a spatial map of 56 cellular compartments based on 115,914 spatial transcriptomes from 32 tissue sections that could be of use to the research community. The authors suggest that their study provides novel insight into the cellular architecture of GBM compared to similar studies that have been published in Nature Communications and other journals (PMCID: PMC9974099, PMCID: PMC9950149, PMID: 35700707, PMCID: PMC10336135, PMCID: PMC9767870). Although the authors show some unique data, including some previously unreported diversity in oligodendrocytes, there are some issues ranging from major to minor that reduce my enthusiasm for the manuscript in its present form.

Thank you for your insightful feedback. Below, we provide a point-by-point response to your comments.

Comment # 3.1. In general, in its current form I fail to understand the overall questions or hypothesis that is trying to be answered through this analysis. I understand the authors are claiming they have a unique dataset, which I do not want to diminish as it could be useful to the

research community. However, multiple recent papers, some listed above, have extensively conducted spatial transcriptomics with matched RNA sequencing.

Response # 3.1. We thank the reviewer for raising this important point and for allowing us to clarify the overall motivation of our study.

While we acknowledge that several recent publications have successfully applied spatial transcriptomics in glioblastoma, our single-cell sequencing and Visium profiling was initiated before the other studies were published. The central aim of our study was to understand how the remarkable cellular heterogeneity of GBM is organized across distinct anatomical niches and how this organization contributes to cellular states. Given the other datasets and analysis focused on tumor and immune cell populations and hypoxic niches, we set out to determine whether non-malignant cell populations, in particular oligodendrocytes, endothelial cells, fibroblasts, and pericytes, harbor novel disease-associated subtypes within the GBM microenvironment. Through recursive clustering of single-cell data, we identified 21 cellular subtypes/states for these major cell types. We identified Oligo_2_3_2, a previously unrecognized oligodendrocyte state enriched in the tumor core. This population displayed an immune-activated transcriptomic profile and loss of canonical myelin genes, features that distinguish it from oligodendrocytes in normal brain or IDH-mutant gliomas. Importantly, enrichment of the Oligo_2_3_2 signature correlated with poor survival in TCGA and CGGA cohorts and was further increased at recurrence in the GLASS dataset, suggesting a potential role in GBM aggressiveness and therapy resistance. We further validated our findings with subcellular-resolution Xenium profiling using a fully custom gene panel and were able to elucidate two additional niches; Immune-glial and Immune-fibroblast.

A further goal of our work was to extend the applicability of spatial transcriptomics to clinically relevant settings. By generating data from both fresh frozen and FFPE tissues and integrating these with matched single-cell multi-omic data, we demonstrate that high-resolution spatial mapping can be performed on routinely archived clinical material. This provides a framework for future studies to interrogate rare or historically collected specimens that were not accessible to earlier frozen-tissue–based atlases.

Taken together, our study was not only intended to introduce a large and technically diverse dataset, but also to address fundamental questions regarding the spatial organization of malignant and non-malignant states in GBM and to highlight the clinical significance of previously unrecognized cell populations. We have revised the Introduction and Discussion to state these overarching questions more explicitly.

Comment # 3.2. The authors highlight their unique oligodendrocyte cell type that they have identified throughout their analysis, but that is not discussed in the abstract or the title.

Response # 3.2. We thank the reviewer for this helpful suggestion. In response, we have revised the abstract to explicitly include our main finding. The revised abstract now states: *"Notably, we identified a distinct oligodendrocyte subset confined to core and perivascular regions, characterized by transcriptional signatures predictive of recurrence and adverse clinical outcome."* (see Abstract, line 46-48)

Comment # 3.3. It is current form, it is not clear how the Oligo2_3_2 population marked by expression of HLA-A is novel and/or clinically relevant. What is the function of this population? Many manuscripts have extensively categorized and identified the OPC lineage. If the authors want to focus on this, I suggest making the paper about this and providing more evidence as to how this population is unique and why it is important (linked to any patient outcome, genetic mutation, or any other characteristic (treatment response, biological sex, etc.)).

Response # 3.3. Thank you for raising this important point regarding the Oligo_2_3_2 population and its clinical relevance. This point is similar to comment #2.7, so we would like to refer the reviewer to our response #2.7. Briefly, we have included additional analyses to explore potential links between the Oligo_2_3_2 population and clinical characteristics such as disease status, genetic mutations, and patient outcomes. As mentioned above, we demonstrate that the Oligo_2_3_2 population is significantly associated with worse clinical outcomes in two independent datasets (TCGA and CGGA). Furthermore, this subpopulation is more abundant in recurrent glioblastomas compared to primary tumors and is predominantly observed in IDH wild-type glioblastomas, which are associated with a more aggressive clinical course. These findings underscore its potential role in tumor progression and recurrence. These data are shown in the revised manuscript in Figures 9A and 9B.

The reviewer also asks about the potential function of the Oligo_2_3_2 subpopulation. It is characterized by high expression of immune-modulating genes including Serpina3n, S100a1, and GSN. While the precise function of Oligo_2_3_2 remains to be fully defined, its selective enrichment in the tumor core, particularly in perivascular regions, and its close association with macrophages suggest a potential role in modulating the immune landscape of the tumor microenvironment. We acknowledge that further experimental investigation will be required to delineate the specific mechanisms by which Oligo_2_3_2 contributes to GBM pathology, but its unique spatial distribution and strong association with adverse outcomes provide a compelling rationale for additional studies.

Again, we appreciate your feedback and hope these additional analyses clarify the significance of the Oligo_2_3_2 population in the tumor microenvironment. We have revised the manuscript to emphasize these points.

Comment # 3.4. The authors suggest that this shared resource will help develop new and effective combinatorial therapies but do not give an example as to how. What makes this analysis and dataset different than what is already out there? How can this dataset provide information that no other dataset can when looking for combination therapies. Not to belabor the point, this gets back to the authors not answering a clearly delineated question or hypothesis.

Response # 3.4. We thank the reviewer for this valuable comment. We agree that our initial manuscript did not provide sufficient context regarding how our resource may inform the development of combinatorial therapies. The primary aim of our study was to identify the specific combinations of cellular populations that define distinct anatomical niches in GBM. By resolving the cellular composition and interactions within each niche, we demonstrate that critical tumor-stroma-immune interactions vary in a context-dependent manner. While we do not

provide direct functional validation, our hypothesis is that targeting these key interacting cell populations simultaneously, rather than individually, may offer greater therapeutic benefit. In this way, our atlas highlights cellular combinations that could serve as a foundation for formulating rational combinatorial therapeutic strategies. We have replaced the text,

“This valuable and openly shared resource will help develop new and effective combinatorial therapies to target multiple niches simultaneously to improve patient outcomes.”

With
“*Our publicly accessible atlas provides a high-resolution framework for investigating GBM spatial biology and offers a resource for identifying spatially restricted therapeutic vulnerabilities*.” (see Abstract, line 48-49)

We added the following paragraphs to Discussion section including the limitations of our study and future work that can be done based on the findings from our atlas,

"*Translational implications....them within their spatial and cellular contexts.*" (page 19, line 466-483 in the revised manuscript)

Comment # 3.5. The patient classification should be based on sex (not gender) as this is the biological variable.

Response # 3.5. We thank the reviewer for pointing this out. We agree that patient classification should be based on sex as the appropriate biological variable. The manuscript has been revised accordingly to replace “gender” with “sex” throughout the text and figures where applicable.

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.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have properly addressed my concerns. I have no further comments.

Reviewer #2 (Remarks to the Author):

This revised manuscript presents a multi-modal glioblastoma atlas integrating sc/snRNA-seq, CITE-seq, Visium profiling of both frozen and FFPE samples, and subcellular-resolution Xenium data. The dataset is undeniably large, encompassing over 115,000 Visium spots, more than 220,000 single cells, and nearly three-quarters of a million Xenium profiles generated with a custom 348-gene panel. The authors have also genotyped all tumors, incorporated high-resolution imaging, and provided a public browser. In terms of scale, technical breadth, and potential clinical applicability through FFPE inclusion, the resource is both large and diverse.

The revision addresses most of the concerns raised in the previous round. The authors now more clearly articulate how their dataset differs from and extends prior atlases such as Ravi (2022) and Greenwald (2024). They highlight the deeper sequencing, broader histological sampling across canonical niches, the advantages of FFPE for translational research, and the more balanced Xenium panel design that better captures stromal and vascular components. The Xenium dataset has been extended to four GBM patients, providing some subcellular validation of cell-type assignments and niche architecture. The presentation is cleaner, and the discussion has been moderated; speculative links have been either removed or clearly marked as limitations.

The central biological claim remains the identification of a distinct oligodendrocyte subtype, Oligo_2_3_2, enriched in the tumor core and perivascular regions, characterized by immune-associated transcripts and loss of myelin genes. In this revision, the authors show that the Oligo_2_3_2 signature stratifies survival in TCGA and CGGA, is enriched at recurrence in GLASS, and associates with IDH-wildtype and MGMT-unmethylated tumors. They supplement these findings with Xenium validation and orthogonal evidence from human IHC and mouse IF. These additions reduce the likelihood that Oligo_2_3_2 is a computational artifact.

That said, the advance remains primarily descriptive. The dataset is broad and technically impressive, but the biological insights are incremental rather than transformative, and several of the supporting analyses remain statistically underpowered or methodologically weak. The work's value lies chiefly in the scale and accessibility of the resource, not in mechanistic discovery. The following issues must be addressed for the manuscript to meet Nature Communications standards and for the atlas to serve as a credible and durable community resource.

1. The differential expression analysis strategy is unclear and methodologically weak. The manuscript describes per-cell t-tests with random subsampling to equalize group sizes and, in

some cases, “paired = TRUE wherever applicable”. It is not evident what “pairing” means at the single-cell level, and treating individual cells as independent replicates inflates degrees of freedom and increases the risk of false positives. This approach also assumes normally distributed data, which is inappropriate for sparse, zero-inflated single-cell counts. Subsampling further introduces unnecessary stochasticity. For a flagship resource, key claims (particularly the definition of the Oligo_2_3_2 population) should be supported by a patient-aware framework. Best practice would involve pseudobulk aggregation per patient per state, analyzed with edgeR or DESeq2, or mixed-effects models with patient identity included as a random effect.

Response #1: We thank the reviewer for this valuable comment and agree that our initial differential expression strategy had methodological limitations. In the revised analysis, we have replaced the earlier per-cell *t*-test–based approach with **clusterDE**, a method specifically designed to address the *double-dipping* issue inherent to differential expression testing following clustering. **clusterDE** statistically controls for false discoveries and identifies robust, cell type specific markers even when cluster boundaries are uncertain. Using this updated framework, we re-evaluated the two oligodendrocyte-associated cell states and redefined their marker genes. As a result, the **Oligodendrocyte** gene set was slightly modified, with two genes (**MOBP** and **PLP1**) removed from the list of differentially expressed genes. The **Oligo_2_3_2** gene set remained unchanged. These revisions have been incorporated into the manuscript, and **Figure 8B** has been updated accordingly. Alternatively, differential gene expression analysis was also performed using pseudo-bulk data derived from the single-cell transcriptomic dataset. **DESeq2** was used to normalize and identify differentially expressed genes. Genes with p-value < 0.01 and $|\log_2 FC| > 0.8$ were considered significant, with $\log_2 FC > 0.8$ classified as up-regulated and $\log_2 FC < -0.8$ as down-regulated. The results for the same are included in Figure S7B.

2. Although the prognostic analyses suggest that high expression of the Oligo_2_3_2_up gene set and low expression of the Oligo_2_3_2_down gene set correlate with poorer outcomes across glioma subgroups, it is difficult to reconcile these results with the single-cell finding that Oligo_2_3_2 is absent from IDH-mutant high-grade cases. If this population does not exist in IDH-mutant tumors, it is unclear how its signatures could have prognostic impact in these cases. This raises the concern that the observed associations may be driven by confounding factors, such as general stress-related transcriptional programs, rather than by Oligo_2_3_2 as a unique cell type. Moreover, the current survival analyses are descriptive rather than inferential: Kaplan–Meier curves are shown, but without hazard ratios, confidence intervals, or multivariable Cox regression, it is impossible to determine whether the Oligo_2_3_2 signal is independent of established clinical covariates. With TCGA and CGGA data available, the authors should adjust, at minimum, for age, IDH status, 1p/19q codeletion, and MGMT methylation, and should state explicitly how cut-points were defined (median, quartiles, or optimized). Reporting hazard ratios with 95% confidence intervals, global p-values, and cohort or batch terms will be essential to clarify whether the observed associations reflect a truly GBM-specific oligodendrocyte population or instead broader gene expression patterns that correlate with tumor aggressiveness.

Response #2: We thank the reviewer for this thoughtful and important observation. Ideally, one would not expect to see Oligo_2_3_2_up signature to be high in IDH-mutant cases but given the limitation of bulk RNAseq data, we cannot distinguish between co-localized expression of the geneset vs. non-overlapping expression. Also, given the small IDHmutant single-cell data cohort and AF associated heterogeneity of this cell population it is possible that a small fraction of IDHmutant cases may have Oligo_2_3_2 population. Limitations of this analysis is mentioned in Discussion section. We stratified the high and low groups using 2 methods with similar results. We agree that the previous stratification method, which used the median ssGSEA score as the threshold, led to more *IDH*-mutant samples being classified as **Oligo_2_3_2-high**. To address this, we have updated our approach to define patient groups based on **Oligodendrocyte** and **Oligo_2_3_2** gene set ssGSEA scores using optimized thresholds. These thresholds were selected such that the **Oligodendrocyte-high** group was enriched for *IDH*-mutant tumors, while the **Oligo_2_3_2-high** group predominantly represented *IDH*-wildtype cases (81% in TCGA and 73% in CGGA).

Furthermore, we have strengthened our prognostic analysis by performing **multivariable Cox proportional hazards regression** using TCGA and CGGA datasets. The models included clinical covariates such as age, *IDH* mutation status, *1p/19q* codeletion, and *MGMT* promoter methylation, along with the **Oligodendrocyte** and **Oligo_2_3_2** ssGSEA scores as continuous variables. The corresponding forest plots with hazard ratios, 95% confidence intervals, and global *p*-values are presented in **Figure 9A**, while the updated Kaplan–Meier curves based on the revised stratification are shown in **Figure 9B**.

3. The new AUC and neighbourhood analyses are useful additions but currently lack transparency. The manuscript does not state the number of permutations performed, the criteria used to define significance, or whether multiple-testing correction was applied across the many AF×cell-type comparisons. In the neighbourhood analysis, a fixed 50-μm radius is used without justification or sensitivity analysis. The authors should specify permutation counts (at least 1,000), clearly define how p-values were derived, apply appropriate FDR correction, and demonstrate that conclusions are robust across a range of neighbourhood radii.

Response #3: We thank the reviewer for these valuable suggestions, which have been implemented in the revised manuscript. Both the AUC-based and neighbourhood analyses were repeated using **1,000 permutation iterations** to ensure statistical robustness. The neighborhood/enrichment scores were calculated by taking the ratios of the original values and the average across 1000 iterations of the shuffled values. For each test, **empirical p-values** were computed as the proportion of shuffled statistics greater than or equal to the observed statistic, following the standard permutation formula:

p = (count of permuted values ≥ observed) + $1/N_{perm}$ + 1

False discovery rate (**FDR**) correction across all comparisons was then applied using the **Benjamini–Hochberg** method.

In the neighborhood analysis of the **Xenium** dataset, grids with an area of **2,376 µm²** were generated to match the spatial coverage of a single spot in the **Visium** dataset, ensuring comparability between platforms [Page 35, Line 877-879]. The analysis pipeline is updated to refine the estimation of spatial proximity, now considering only **immediate neighboring grids** when quantifying local spatial interactions. All methodological details, permutation parameters, and significance criteria have been updated in the revised *Methods* section and corresponding figures 4E and 7D.

4. Data availability is inadequate and falls below Nature Communications standards. The manuscript states only that raw data will be made “available upon request” and provides links to multiple ad hoc portals for processed data. This is unacceptable for a resource paper. Accession numbers must be provided for all RAW datasets together with complete metadata. Raw data should encompass FASTQs, Visium brightfield and IF images, Xenium raw outputs and segmentation masks, probe design files, and per-sample annotations. In addition, all code should be placed in a versioned public repository with a DOI (e.g., via Zenodo) and must include executable scripts or notebooks that can reproduce every main figure. Without these changes, the resource will not be reproducible or durable.

Response #4: We thank the reviewer for this constructive suggestion and have now made all data publicly available in compliance with *Nature Communications* data-sharing standards. The **raw single-cell RNA-seq** data have been deposited in the **NCBI Sequence Read Archive (SRA)** under submission **SUB15673052**, and the **Visium spatial transcriptomics** data under submission **SUB15686159**. The **processed spatial transcriptomic data**, corresponding images, and associated metadata and codes are available on **Zenodo** (**10.5281/zenodo.17572905**). Both datasets would be accessible upon publication of the manuscript.
For convenient exploration and visualization, the datasets can also be accessed through the following interactive portals:

- **SNUH-GliomaAtlas (GBM Visium):** [https://gbmvisium.snu.ac.kr/]
  A spatially resolved glioblastoma atlas providing transcriptomic maps across tissue sections.
- **UCSC Multi-Omic GBM Browser:** [https://multiomic-gbm.cells.ucsc.edu/]
  An integrated portal for multi-omic glioblastoma datasets.
- **Amaranth Insights:** [https://www.amaranth-insights.com/]
  - *(Login: amaranthinsights@gmail.com | Password: password@123)*
  - **Tenant:** Spatial Glioblastoma Atlas **Project:** Glioma manuscript

5. Several clarifications are needed to strengthen the central conclusion around the Oligo_2_3_2 claim. First, the per-patient distribution of Oligo_2_3_2 frequencies in core and periphery should be shown, not just pooled fractions, and the defining DEGs confirmed with patient-aware analysis. Second, given that only four GBM patients were profiled with Xenium, an independent FFPE validation (either with the same panel or via orthogonal ISH/IF) would substantially improve confidence in the generalizability of this signature. Third, because Oligo_2_3_2 is proposed as a malignant oligodendrocyte-like state, CNV score distributions should be provided comparing this cluster to bona fide neoplastic and non-neoplastic populations to rule out misclassification. More broadly, several of the reported cell type annotations are not fully convincing. For example, in Figure 4B, the BV population does not show strong enrichment for the vascular signature described by Greenwald et al., whereas neuronal and NPC populations unexpectedly display higher enrichment. Similarly, the PNZ and PAN populations appear depleted of tumor cells, which is difficult to reconcile with expectations. These observations raise the possibility of discrepancies in cell-type labelling that could contribute to downstream inconsistencies in interpretation. To strengthen confidence in the annotations, the authors should provide additional validation, for instance, a heatmap of CNV profiles with AF and cell type labels overlaid to assess biological coherence, as well as a dot plot or heatmap showing expression of key marker genes for each annotated cell type (similar to Figure S1 for single-cell data).

With the revisions outlined above, this study will provide a credible and durable resource for the GBM research community.

Response #5: We thank the reviewer for this comment.

a. The per-patient distribution of Oligodendrocyte sub-populations in core and peri is provided in Figure 2F.
b. We have now added more IHC data of NGFR, MAG, and GSN expression in representative Oligodendroglioma, IDH-mutant and 1p/19q-codeleted (n = 3), Astrocytoma, IDH-mutant (n = 3), and Glioblastoma, IDH-wildtype (n = 5) samples. The same is represented in Figure S10, and the images are part of the data repository.
c. The CNV score for all the cell types and their sub-populations is provided in Figure 2C. Also, the gene expression profile of all the cellular states is provided in Supplementary Figure 5C.
d. The vascular signature reported by **Greenwald et al.** primarily captures **aberrant, glioblastoma-associated vasculature** rather than normal blood vessels (BV). This distinction is supported by the enrichment pattern of the **Endo_cap** signature, which more accurately reflects canonical endothelial expression (Figure 4C). The apparent enrichment of the Greenwald signature in neuronal and NPC clusters arises from the fact that these vascular structures are predominantly located within **gray matter regions**. To further clarify this point, we have now included **representative snapshots of these vascular features** in **Figure 4B** of the revised manuscript. The **PNZ** and **PAN** regions are depleted specifically of **non-mesenchymal tumor cells**, while **mesenchymal tumor**

cells remain present within these zones. This observation is consistent with prior reports describing mesenchymal enrichment in perinecrotic and perivascular niches. Supporting this, the **MES-HYP** metaprogram defined by **Greenwald et al.** also shows strong enrichment in these regions, further validating the mesenchymal identity of the tumor component within PNZ and PAN (Figure 4C). In the updated figure, the overlapping lines depicting enrichment from both the Greenwald and Ravi datasets have been made thinner to improve visual clarity and highlight these changes more effectively. To further illustrate the morphological distinctions among the different vascular subtypes, **representative H&E snapshots** of **BV**, **HBV**, **MVP**, and **IGN** have been included in **Figure 4A**.

e. As suggested by the reviewer, we have included the heatmap of aggregated gene set expression profiles of cellular states across the anatomical features in Figure S4H.

Reviewer #3 (Remarks to the Author):

In general, the authors have been responsive to the previous review and have made satisfactory modifications to their manuscript to increase clarity. I have no additional issues.

I would like to point out that part of the response to point 3.1 is not logical, the claims that work was started before other papers were published has no bearing on the clarity of the message, which was the original issue.

Response: We appreciate the reviewer's overall positive feedback and their acknowledgment of our efforts to improve the manuscript. We accept the point made. Our intention was to provide context, but we agree that this did not address the core issue raised.

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.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Overall, the authors have been highly responsive, and the manuscript is substantially improved. I view the manuscript as acceptable pending a few minor additions/clarifications.

- The pseudobulk DESeq2 analysis is appreciated. For transparency, it would be helpful if the authors could explicitly state the design formula used (e.g., whether patient identity was included as a covariate).

Response #1:
We thank the reviewer for their comment. We have now explicitly stated the design formula used for the pseudobulk differential expression analysis in the Methods section. Specifically, DESeq2 was run using a design formula that included both patient identity and cell type as covariates to account for inter-patient variability and differences in cellular composition. This clarification has been added to improve transparency and reproducibility (Lines 841-843).

- The multivariable Cox models strengthen the prognostic analysis. I suggest tempering the interpretation to acknowledge that some IDH-related confounding is difficult to eliminate fully in bulk RNA datasets.

Response #2:
We thank the reviewer for this thoughtful suggestion. We agree that, while the multivariable Cox models adjust for key clinical and molecular covariates, residual confounding related to IDH-associated transcriptional programs cannot be fully excluded in bulk RNA-seq datasets. We have therefore tempered the interpretation accordingly in the Results section (Lines 446-448).

- While raw data deposition now meets Nature Communications standards, reproducibility would be further enhanced if Visium annotations (spot-level metadata or cell-type assignments, when available) were documented more explicitly in the repository.

Response #3:
We thank the reviewer for this suggestion. To enhance reproducibility, we have now explicitly provided Visium spot-level annotations, including anatomical feature labels and cell-type assignments where available, in the Source Data files deposited alongside the manuscript (10.5281/zenodo.18093125). These files document spot-level metadata used for all downstream analyses and figure generation.

- While the single-cell CNV profiles are clearly presented in Fig. 2B, the approach used to assign CNV status in the spatial datasets is less explicitly documented. If feasible, providing

a brief visualization or description analogous to Fig. 2B for the spatial data would strengthen confidence in the malignant vs. non-malignant spatial assignments.
- A few small presentation items (gene name capitalization, missing scale bars in select supplemental figures) can be addressed during final revision.

Response #4:
We thank the reviewer for this helpful suggestion. We agree that explicit documentation of CNV assignment in spatial datasets is important for transparency. To address this, we have provided spatial CNV profiles in Supplementary File 3, showcasing inferred CNV scores for all spatial transcriptomics spots. In addition, spatial overlays of CNV scores mapped onto tissue sections are available through the UCSC Cell Browser (https://cells.ucsc.edu/?ds=multiomic-gbm+spatial&meta=inferCNV), enabling direct visualization of malignant versus non-malignant spatial regions, analogous to the single-cell CNV presentation in Fig. 2B.
We have also addressed the minor presentation issues noted by the reviewer, including standardizing gene name capitalization and adding missing scale bars to the relevant supplementary figures.