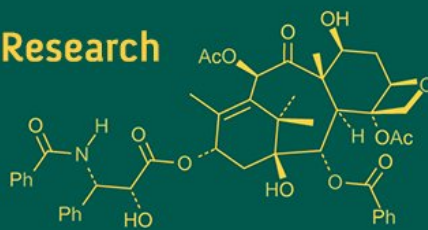


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Spatial transcriptomics reveals region-specific gene expression patterns in the mouse brain using 10x visium

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Abstract

Spatial transcriptomics enables the characterization of gene expression while preserving its spatial relationship within tissue architecture, providing insights into regional transcriptional heterogeneity. In the present study, a publicly available fresh-frozen mouse (*Mus musculus*) brain spatial transcriptomic dataset generated using the 10x Genomics Visium platform was analyzed using the Seurat package in R. The raw feature-barcode matrix comprised 32,589 genes across 14,273 spatial spots. Following quality control filtering based on transcript counts and detected genes per spot ($nCount_Spatial > 500$ and $nFeature_Spatial > 200$), 6,631 high-quality spots were retained for downstream analysis. Data were normalized and variance-stabilized using SCTransform, followed by principal component analysis, with the first 20 principal components selected for subsequent analyses. Graph-based clustering and Uniform Manifold Approximation and Projection identified distinct transcriptional clusters representing spatially heterogeneous regions of the mouse brain. Cluster-specific marker genes were identified using the Wilcoxon rank-sum test and visualized using spatial feature and dimensional plots. Functional enrichment analysis of representative marker genes revealed significant enrichment of extracellular signaling, hormone and receptor activity, and neurochemical communication. KEGG pathway analysis highlighted neuroactive ligand-receptor interaction as an enriched pathway, indicating the representation of genes involved in neuronal communication and receptor-mediated signaling. Protein-protein interaction network analysis further revealed functional relationships among selected marker genes. Overall, this study demonstrates the utility of spatial transcriptomics and the Seurat analytical framework for characterizing spatial gene-expression heterogeneity in the mouse brain and provides a computational basis for comparative neurobiology and veterinary biomedical research.

Keywords: Spatial transcriptomics, 10x Genomics Visium, mouse brain, Seurat, spatial gene expression, neuroanatomy

Introduction

Spatial transcriptomics has emerged as a powerful approach for investigating gene expression while retaining the spatial context of cells within intact tissues. Unlike conventional bulk RNA sequencing, which provides an average transcriptional profile across heterogeneous cell populations, spatial transcriptomics enables the localization of gene expression patterns within histological tissue sections. This capability is particularly valuable for complex organs such as the brain, where cellular composition, anatomical organization, and local cell-cell interactions play fundamental roles in biological function and disease processes. Advances in spatially resolved transcriptomic technologies have therefore enabled the characterization of tissue architecture and molecular heterogeneity at increasingly high resolution (Giacomello *et al.*, 2017; Liao *et al.*, 2021) [2, 6]. The mouse brain represents an important model for evaluating spatial transcriptomic technologies because of its highly organized anatomical structure and extensive transcriptional heterogeneity. Different brain regions contain distinct neuronal and non-neuronal populations, each characterized by specific gene expression profiles and functional roles. Preservation of both RNA quality and tissue architecture is therefore essential for obtaining biologically meaningful spatial transcriptomic data. Tissue handling and preservation procedures before sequencing can substantially influence RNA integrity, transcript detection, molecular sensitivity, and the

quality of spatial gene expression patterns obtained from tissue sections (Srinivasan *et al.*, 2002; Thavarajah *et al.*, 2012) ^[8, 9].

Fresh frozen tissue has traditionally been widely used for transcriptomic analysis because rapid freezing can minimize RNA degradation and preserve native molecular profiles. The absence of chemical fixation also reduces the possibility of fixation-induced modification of nucleic acids and proteins, potentially allowing more direct detection of endogenous gene expression patterns. Consequently, fresh frozen tissue is frequently considered suitable for studies investigating dynamic biological processes, including metabolism, cellular signaling, neurotransmission, and tissue-specific gene expression (Ferreira *et al.*, 2019) ^[1]. In contrast, fixed frozen tissue involves chemical stabilization of tissue components before freezing. Chemical fixation can improve the preservation of tissue morphology and cellular architecture by stabilizing cellular and extracellular structures. Such preservation may be advantageous for spatial transcriptomic studies, in which accurate anatomical localization of transcriptional profiles is important. However, fixation may also alter nucleic acid accessibility and influence transcript detection because chemical crosslinking can affect molecular extraction and downstream sequencing procedures (Srinivasan *et al.*, 2002; Thavarajah *et al.*, 2012) ^[8, 9]. Therefore, the relative performance of fresh frozen and fixed frozen tissues may depend on the balance between molecular preservation, RNA accessibility, and maintenance of tissue architecture.

The development of commercial spatial transcriptomic platforms has made it possible to systematically investigate these effects. The 10x Genomics Visium Spatial Gene Expression platform combines gene expression profiling with spatial coordinates and histological imaging, enabling the identification of transcriptionally distinct regions within tissue sections (10x Genomics). Computational approaches implemented in the Seurat framework facilitate quality control, normalization, dimensionality reduction, clustering, identification of marker genes, and visualization of spatial gene expression patterns (Hao *et al.*, 2021) ^[4]. SCTransform normalization provides an additional approach for variance stabilization and reduction of technical variation in sequencing datasets, thereby improving downstream comparative analyses (Hafemeister & Satija, 2019) ^[3]. Dimensionality reduction methods such as Uniform Manifold Approximation and Projection (UMAP) further enable visualization of transcriptional relationships among spatial spots and identification of distinct molecular domains (McInnes *et al.*, 2018) ^[7]. Functional interpretation of spatially expressed genes is equally important for understanding the biological information retained under different preservation conditions. Cluster-specific marker genes can be subjected to functional enrichment analysis using Gene Ontology (GO) and pathway databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG). GO analysis enables the classification of genes according to biological processes, cellular components, and molecular functions, whereas KEGG analysis provides information on metabolic and signaling pathways represented within gene sets. Resources such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) facilitate the systematic identification and interpretation of enriched functional categories from large gene lists (Huang *et al.*, 2009) ^[5].

Despite the increasing application of spatial transcriptomics, the influence of tissue preservation on downstream clustering patterns, transcript detection, marker gene identification, and functional enrichment remains an important methodological consideration. Differences observed between preservation conditions may reflect true effects of tissue handling; however, they may also be influenced by technical factors, sample-specific biological variation, sequencing characteristics, and analytical parameters. Consequently, comparative studies should interpret differences in pathway enrichment and cluster number cautiously, particularly when individual datasets are analyzed without biological replication. The present study therefore aimed to comparatively evaluate fresh frozen and fixed frozen mouse brain spatial transcriptomic datasets generated using the 10x Genomics Visium platform. The datasets were independently processed using the Seurat analytical framework, including quality control, SCTransform normalization, dimensionality reduction, clustering, and identification of cluster-specific marker genes. Functional enrichment analysis of the top marker genes from each cluster was subsequently performed using GO and KEGG databases to characterize the biological processes and pathways represented under each preservation condition. The study specifically compared spot retention, gene detection, clustering patterns, and functional enrichment profiles between fresh frozen and fixed frozen tissues. By examining these features, this analysis provides an exploratory assessment of how tissue preservation conditions may influence the molecular and spatial interpretation of publicly available mouse brain spatial transcriptomic data.

Materials and Methods

Study Design and Data Acquisition

The present study was conducted as a secondary analysis of a publicly available spatial transcriptomic dataset derived from fresh-frozen mouse brain tissue (*Mus musculus*). The dataset was imported from 10x Genomics Visium Spatial Gene Expression platform and included the raw feature-barcode expression matrix, spatial barcode coordinates, and corresponding histological tissue images. The dataset was analyzed to characterize spatial patterns of gene expression and transcriptional heterogeneity across the mouse brain tissue section. The experimental procedures used to generate the original spatial transcriptomic dataset were performed according to the 10x Genomics Visium Spatial Gene Expression workflow. The accession number, source database, sample metadata, mouse strain, sex, age, brain region, and original sequencing information should be reported in the final manuscript to ensure complete reproducibility.

Data Import and Preprocessing

Spatial transcriptomic data were analyzed in R using the Seurat package. The raw feature-barcode matrix and associated spatial information were imported using the Load10X_Spatial() function. The spatial assay was designated as "Spatial", and the tissue section was assigned the slice identifier "slice1". The initial dataset consisted of 32,589 genes measured across 14,273 spatial spots. Spatial coordinates and histological images were retained during data import to enable subsequent integration of

transcriptomic profiles with their corresponding locations within the tissue section.

Quality Control and Spot Filtering

Quality control was performed to identify and exclude spatial spots with low transcript capture or limited gene detection. The following quality metrics were evaluated for each spatial spot:

- **nCount_Spatial:** Total number of unique molecular identifiers (UMIs) detected per spatial spot.
- **nFeature_Spatial:** Number of genes detected per spatial spot.
- **percent.mt:** Percentage of transcripts derived from mitochondrial genes.

The mitochondrial gene percentage was calculated using the `PercentageFeatureSet()` function, with mitochondrial genes identified using the pattern `"^mt-"`. Spatial spots were retained for downstream analysis if they satisfied the following criteria:

- `nCount_Spatial > 500`
- `nFeature_Spatial > 200`

Spots failing to meet either criterion were excluded. Following quality filtering, 6,631 high-quality spatial spots were retained for subsequent analyses. The distributions of transcript counts, detected genes, and mitochondrial transcript percentages were examined to assess overall data quality and to identify potential low-quality or damaged spatial regions.

Data Normalization and Variance Stabilization

Normalization and variance stabilization were performed using the `SCTransform` method implemented in the Seurat `SCTransform()` function. This approach models technical variation associated with sequencing depth and applies regularized negative binomial regression to generate normalized and variance-stabilized gene-expression values. The spatial assay was used as the input for normalization. The argument `return.only.var.genes = FALSE` was specified to retain normalized expression information for all genes available in the processed assay. The `future.globals.maxSize` parameter was increased to accommodate the memory requirements associated with `SCTransform` normalization. Following normalization, highly variable genes were identified for downstream dimensionality reduction. The processed dataset contained expression information for 17,020 genes in the normalized analysis object. This value is reported as the number of genes available following preprocessing and normalization and should not be interpreted solely as the result of an independent feature-filtering step.

Feature Selection and Dimensionality Reduction

Highly variable genes were identified using the variance-stabilizing transformation approach implemented in Seurat. A total of 3,000 highly variable genes were selected for dimensionality reduction and downstream clustering. Principal component analysis (PCA) was subsequently performed using the `RunPCA()` function on the selected features. The relative contribution of principal components was evaluated using the `ElbowPlot()` function. Based on the observed inflection point in the `ElbowPlot`, the first 20

principal components were selected for downstream graph construction, clustering, and low-dimensional visualization.

Graph-Based Clustering Analysis

Transcriptionally similar spatial spots were grouped using a graph-based clustering approach implemented in Seurat. A k-nearest-neighbor graph was constructed using the `FindNeighbors()` function based on the first 20 principal components. Community detection was subsequently performed using the `FindClusters()` function to identify transcriptionally distinct spatial domains. The clustering resolution parameter and community detection algorithm used in the final analysis should be explicitly reported in the final manuscript. The same analytical parameters should be retained throughout the analysis to ensure reproducibility. The identified clusters were subsequently evaluated in both reduced-dimensional and spatial contexts to determine whether transcriptionally distinct groups exhibited anatomically structured spatial distributions.

UMAP Analysis and Data Visualization

Uniform Manifold Approximation and Projection (UMAP) was performed using the `RunUMAP()` function based on the first 20 principal components. UMAP was used to visualize relationships among spatial spots according to their transcriptional similarity. Cluster assignments were visualized using `DimPlot()`, whereas their corresponding spatial distributions within the tissue section were examined using `SpatialDimPlot()`. The integration of cluster identities with histological images enabled the visualization of transcriptionally distinct spatial domains within the mouse brain tissue section. Data visualization was performed using Seurat and the `ggplot2` and `patchwork` packages.

Identification of Cluster-Specific Marker Genes

Cluster-specific marker genes were identified using differential expression analysis implemented in Seurat. The `FindAllMarkers()` function was used to identify genes preferentially expressed in each cluster relative to all other clusters. For individual comparisons, the `FindMarkers()` function was used where appropriate. Differential expression testing was performed using the Wilcoxon rank-sum test. Genes were considered as candidate cluster markers based on statistical significance and effect-size thresholds, including adjusted *P*-values and log-fold-change criteria. The exact values of the minimum log-fold-change threshold, minimum percentage of expressing spots, and multiple-testing correction procedure should be reported in the final manuscript. Representative marker genes were selected based on statistical significance and relative expression patterns and were subsequently used for spatial visualization and functional interpretation.

Spatial Visualization of Marker Gene Expression

The spatial distribution of selected marker genes was examined using the `SpatialFeaturePlot()` function. This approach enabled visualization of transcript abundance across spatial spots while retaining the anatomical context of the tissue section. Cluster identities and spatial domains were visualized using `SpatialDimPlot()`, whereas `VlnPlot()` was used to examine the distribution of marker gene expression across transcriptionally defined clusters. Together, these visualization approaches enabled the

integration of gene-expression patterns, transcriptional clusters, and tissue spatial organization.

Functional Enrichment Analysis

Functional enrichment analysis was performed using representative cluster-specific marker genes to investigate the biological functions and molecular pathways associated with the identified transcriptional domains. The top-ranked marker genes from each cluster were selected for downstream enrichment analysis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

The following Gene Ontology categories were examined:

- **Biological Process (BP):** Biological processes in which the identified genes are involved.
- **Molecular Function (MF):** Molecular activities associated with the gene products.
- **Cellular Component (CC):** Cellular structures or subcellular locations associated with the identified genes.

KEGG pathway analysis was performed to identify significantly overrepresented signaling and metabolic pathways. Multiple-testing correction was applied using the Benjamini-Hochberg procedure, and terms with an adjusted P -value < 0.05 were considered statistically significant. Enrichment results were interpreted in conjunction with the known biological context of mouse brain tissue and the spatial expression patterns of genes contributing to the enriched pathways.

Software and Statistical Analysis

All computational analyses were performed using R (version 4.2.0) and relevant R packages. The principal packages used included:

- **Seurat:** Spatial transcriptomic data processing, normalization, dimensionality reduction, clustering, and visualization.
- **ggplot2:** Graphical visualization.
- **Patchwork:** Combination and organization of multiple graphical panels.
- **dplyr:** Data manipulation and processing.

The exact versions of all packages used in the final analysis should be reported to ensure computational reproducibility. Unless otherwise specified, statistical analyses were two-sided, and statistical significance was determined using an adjusted P -value < 0.05 following correction for multiple comparisons. All analyses were conducted using a standardized computational workflow to facilitate reproducibility and to ensure consistency across quality control, normalization, dimensionality reduction, clustering, marker identification, spatial visualization, and functional enrichment analyses.

Results and Discussion

Quality Control and Data Processing

Spatial transcriptomic datasets generated from fresh-frozen and fixed-frozen mouse brain samples were processed using the 10x Genomics Visium platform and the Seurat workflow. Quality-control assessment examined the relationship between sequencing depth (nCount_Spatial) and the number of detected genes (nFeature_Spatial) for spatial spots in both datasets (Figure 1). A positive relationship was observed between the total number of UMIs and the number of detected genes, indicating that spots with greater sequencing depth generally exhibited higher transcript detection sensitivity. This relationship is expected in spatial transcriptomics datasets and indicates that sequencing depth contributed substantially to variation in gene detection across spatial spots.

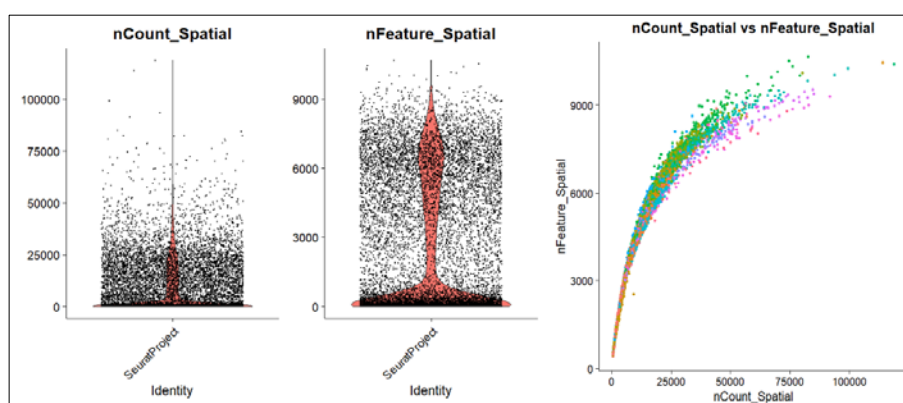


Fig 1: Quality-control assessment of spatial transcriptomics datasets from fresh-frozen and fixed-frozen mouse brain samples

Scatter plots illustrating the relationship between total UMI counts (nCount_Spatial) and the number of detected genes (nFeature_Spatial) across spatial spots. Each point represents an individual spatial spot.

The fresh-frozen dataset initially contained 14,273 spatial spots and 32,589 genes, whereas the fixed-frozen dataset contained 14,328 spots and the same number of genes. After quality filtering using $nCount_Spatial > 500$ and $nFeature_Spatial > 200$, 6,631 spots were retained in the fresh-frozen dataset and 7,239 spots were retained in the

fixed-frozen dataset, corresponding to approximately 46.5% and 50.5% retention, respectively (Table 1).

The difference in spot retention indicates variation in the proportion of spots passing the selected quality thresholds. However, because only one dataset was analysed for each preservation condition, this difference should not be interpreted as definitive evidence that one preservation method consistently provides better data quality. Spot retention may also be influenced by tissue composition, section quality, sequencing depth, permeabilization, capture efficiency, and dataset-specific technical variation.

Table 1: Summary of quality-control and clustering results for fresh-frozen and fixed-frozen mouse brain samples

Parameter	Fresh Frozen	Fixed Frozen
Initial spots	14,273	14,328
Post-filtering spots	6,631	7,239
Spots retained (%)	46.5	50.5
Initial genes	32,589	32,589
Features retained after processing	17,020	18,578
Clusters identified	23	28

Following SCTransform normalization, 17,020 and 18,578 features were retained in the fresh-frozen and fixed-frozen datasets, respectively. The larger number of detected features in the fixed-frozen dataset suggests broader

transcript detection under the analytical conditions used. Nevertheless, differences in detected gene numbers should be interpreted cautiously because they may reflect both biological and technical factors, including sequencing depth, tissue composition, RNA capture efficiency, library preparation, and preservation procedures.

Dimensionality Reduction and Cluster Identification
Principal component analysis was performed following SCTransform normalization, and the selected principal components were subsequently used for graph-based clustering and UMAP visualization. The resulting UMAP projections revealed distinct transcriptional structures in both fresh-frozen and fixed-frozen brain datasets (Figure 2).

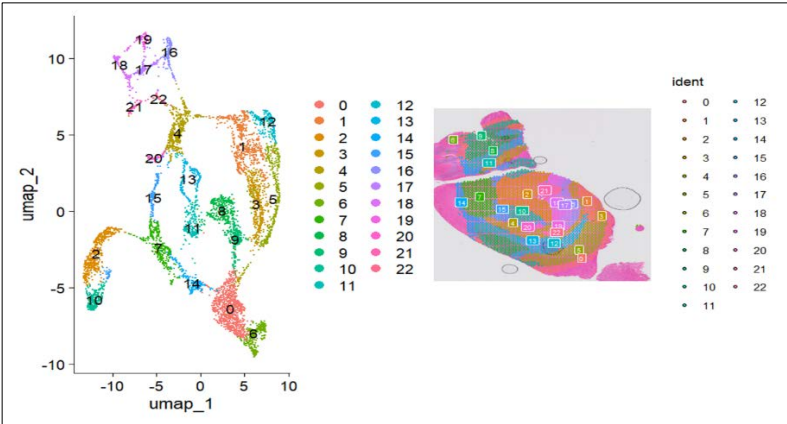


Fig 2: UMAP and spatial visualization of fresh-frozen and fixed-frozen mouse brain samples

UMAP representation of the fresh-frozen sample showing 23 clusters and its spatial distribution of clusters mapped onto the fresh-frozen tissue section.

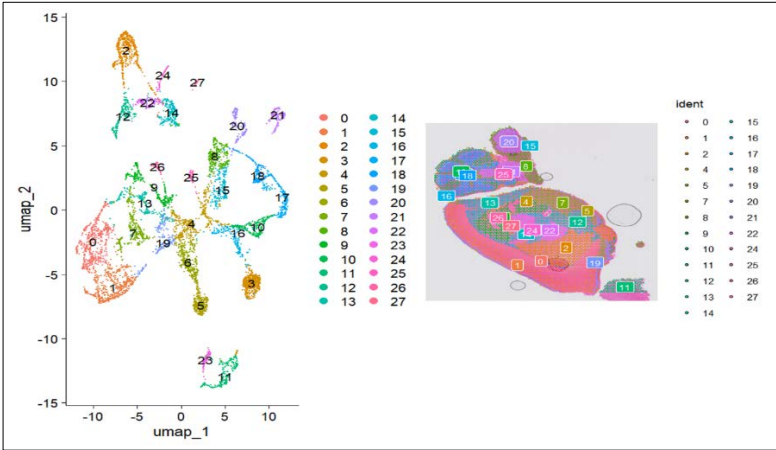


Fig 3: UMAP and spatial visualization of fresh-frozen and fixed-frozen mouse brain samples

UMAP representation of the fixed-frozen sample showing 28 transcriptionally distinct clusters and its spatial distribution of clusters mapped onto the fixed-frozen tissue section
The fresh-frozen sample contained 23 clusters, whereas 28 clusters were identified in the fixed-frozen sample. The larger number of clusters observed in the fixed-frozen dataset indicates greater subdivision of transcriptional profiles under the selected clustering parameters. However, cluster number alone should not be interpreted as a direct measure of biological complexity or preservation quality. Cluster resolution is influenced by several factors, including the number of retained spots, feature detection, sequencing

depth, dimensionality reduction parameters, graph construction, and clustering resolution.
The UMAP structure of the fresh-frozen sample exhibited several elongated and continuous distributions, suggesting gradual transcriptional transitions among subsets of spatial regions. In contrast, the fixed-frozen dataset showed a more compact arrangement of several clusters, with some transcriptionally distinct groups appearing relatively isolated. These differences indicate that the two datasets captured different patterns of transcriptional organization. Importantly, UMAP geometry should not be interpreted as a direct developmental trajectory unless trajectory-specific analyses are performed. Therefore, elongated or branching UMAP structures are more appropriately described as

representing continuous transcriptional relationships rather than definitive developmental transitions.

Spatial Distribution of Transcriptional Clusters

Spatial mapping of clusters onto tissue sections demonstrated that transcriptionally distinct clusters occupied different anatomical locations within both mouse brain samples. The spatial distribution of clusters reflected the heterogeneous organization of brain tissue and indicated that transcriptomic signatures corresponded to region-specific molecular environments. The fresh-frozen dataset displayed several spatially organized cluster domains corresponding to anatomically distinct regions and layers. Similarly, the fixed-frozen dataset demonstrated multiple transcriptionally distinct spatial domains distributed throughout the tissue section.

Differences in the spatial arrangement and number of clusters between the datasets may reflect differences in tissue preservation, but may also arise from variation in tissue section location, anatomical composition, capture efficiency, and analytical resolution. Consequently, the observed differences should be considered dataset-specific unless validated using biological replicates processed under standardized experimental conditions.

Overview of Functional Enrichment Between Preservation Conditions

Gene Ontology (GO), KEGG, and Reactome enrichment analyses were performed using the combined top 10 marker genes identified from each spatial cluster to compare the functional profiles observed in fresh-frozen and fixed-frozen mouse brain samples. The fresh-frozen dataset contained 23 spatial clusters, whereas the fixed-frozen dataset contained 28 clusters. Marker genes were identified independently for each cluster, and the top 10 marker genes from each cluster were used as input for downstream functional enrichment analysis.

The enrichment analyses revealed partially overlapping but distinct functional profiles between the two preservation conditions. These differences should be interpreted primarily as differences in the transcriptional signatures captured in the two datasets rather than definitive evidence of preservation-specific biological superiority, because the present analysis was based on one sample representing each preservation condition. The functional enrichment profile of the fixed and fresh frozen mouse brain sample is presented in Figures 4-7.

1. Biological Process Enrichment Fresh-Frozen Sample

The fresh-frozen sample showed enrichment of biological processes associated predominantly with neuroendocrine signaling, behavioural regulation, immune responses, metabolism, and neuronal function. Prominent terms included neuropeptide signaling pathway, feeding behavior, eating behavior, drinking behavior, response to glucose, synaptic transmission, cholinergic signaling, neuron projection development, and negative regulation of neuron apoptosis. Together, these enriched terms are consistent with the functional complexity of brain tissue and indicate that genes associated with neuronal communication, neuropeptide signaling, behavioural regulation, and metabolic sensing were represented among the cluster marker genes.

The enrichment of immune system process, innate immune response, T-cell-mediated immunity, antibacterial humoral response, and defense response to Gram-positive bacterium indicates the representation of immune-related transcriptional programs. In the brain, such enrichment may

reflect genes associated with resident immune cells, vascular-associated cells, or shared inflammatory and defense-response pathways. The presence of response to glucose and other metabolically associated terms suggests representation of genes involved in cellular energy sensing and metabolic regulation.

Fixed-Frozen Sample

The fixed-frozen sample showed broader enrichment of biological processes associated with developmental patterning, transcriptional regulation, morphogenesis, signal transduction, and structural differentiation. Prominent enriched terms included anterior/posterior pattern specification, embryonic skeletal system morphogenesis, anatomical structure morphogenesis, epithelial cell differentiation, regulation of DNA-templated transcription, and regulation of transcription by RNA polymerase II.

The presence of developmental and embryonic GO annotations in adult brain tissue should not be interpreted as evidence that embryonic developmental processes are actively occurring. Rather, these results indicate that the identified marker genes are annotated to developmental and morphogenetic pathways and may participate in related functions such as cellular differentiation, tissue maintenance, structural organization, and cell-type specification. The fixed-frozen dataset also showed enrichment of signal transduction and adenylate cyclase-activating GPCR signaling, indicating the representation of receptor-mediated signaling pathways.

Interpretation

Overall, the fresh-frozen dataset showed relatively greater representation of neuropeptide, behavioural, neuronal, metabolic, and immune-associated GO annotations, whereas the fixed-frozen dataset showed greater representation of developmental, transcriptional, morphogenetic, and signaling-related annotations. These differences may reflect variation in the preservation conditions, spatial regions represented in each sample, tissue composition, clustering resolution, and the specific marker genes identified in each dataset.

2. Cellular Component Enrichment

The fresh-frozen sample demonstrated enrichment of cellular component terms associated primarily with membranes, neuronal structures, and secretory compartments. These included axon terminus, secretory granule, early endosome, plasma membrane, apical plasma membrane, basolateral plasma membrane, cell surface, and vesicle. The enrichment of these terms is consistent with the importance of membrane-associated signaling, vesicular transport, secretion, and neuronal communication in brain tissue. In contrast, the fixed-frozen dataset showed enrichment of terms associated with structural and transcription-related cellular components, including intermediate filament, intermediate filament cytoskeleton, keratin filament, transcription regulator complex, and cornified envelope. The enrichment of keratinization-related and cornified envelope terms should be interpreted cautiously in brain tissue. Such enrichment does not necessarily indicate the presence of skin-like tissue. Instead, it may result from the expression of structural genes shared with keratinization-related annotation categories or from the enrichment of particular structural gene families among the identified marker genes. Both datasets showed enrichment of extracellular and membrane-associated terms, including extracellular region and extracellular space, suggesting that genes encoding extracellular proteins, signaling molecules,

receptors, and cell-surface components were represented under both preservation conditions.

3. Molecular Function Enrichment

Fresh-Frozen Sample

The fresh-frozen dataset showed enrichment of molecular functions related to signaling molecules, neuropeptides, growth factors, enzymes, and metabolic activities. Important enriched functions included hormone activity, neuropeptide hormone activity, growth factor activity, structural constituent of myelin sheath, signaling receptor binding, and several enzyme-associated functions. The enrichment of hormone activity, neuropeptide hormone activity, and growth factor activity is consistent with the representation of neuroendocrine and extracellular signaling genes among the marker genes. The identification of structural constituent of myelin sheath suggests the representation of genes associated with myelinated neuronal structures and glial cells.

Fixed-Frozen Sample

The fixed-frozen dataset showed substantial enrichment of molecular functions associated with transcriptional regulation and DNA binding. Prominent functions included sequence-specific double-stranded DNA binding, DNA-binding transcription factor activity, RNA polymerase II-specific transcription factor activity, chromatin binding, and cis-regulatory region binding. This pattern indicates that a substantial proportion of the marker genes identified in the fixed-frozen dataset encode transcription factors or proteins involved in gene regulatory processes. The dataset also contained enrichment of structural and receptor-related functions, including structural molecule activity, signaling receptor activity, G protein-coupled peptide receptor activity, and neuropeptide receptor activity.

Interpretation

The molecular function analysis therefore suggests that the fresh-frozen dataset was characterized by greater representation of signaling molecules, neuropeptides, growth factors, metabolic enzymes, and myelin-associated functions, whereas the fixed-frozen dataset showed prominent enrichment of transcription factor and DNA-binding activities.

4. KEGG Pathway Enrichment

Fresh-Frozen Sample

The top KEGG pathways identified in the fresh-frozen dataset were:

1. Neuroactive ligand-receptor interaction
2. Vascular smooth muscle contraction
3. Fructose and mannose metabolism
4. Tyrosine metabolism
5. Complement and coagulation cascades
6. Cytoskeleton in muscle cells
7. TGF-beta signaling pathway
8. Tryptophan metabolism
9. Calcium signaling pathway
10. Regulation of lipolysis in adipocytes

The enrichment of Neuroactive ligand-receptor interaction is biologically consistent with brain tissue because this pathway contains genes encoding neurotransmitters, neuropeptides, hormones, and their receptors. The presence of Tyrosine metabolism and Tryptophan metabolism reflects genes involved in metabolic pathways associated with precursor molecules for several important neurotransmitter-related compounds. Calcium signaling and vascular smooth muscle contraction represent pathways involved in

intracellular signaling, cellular excitability, and vascular regulation. The enrichment of Fructose and mannose metabolism and regulation of lipolysis in adipocytes indicates representation of carbohydrate and lipid metabolism-related genes. The Complement and coagulation cascades pathway reflects immune, inflammatory, and vascular-associated gene functions.

Fixed-Frozen Sample

The fixed-frozen dataset showed fewer significantly enriched KEGG pathways than the fresh-frozen dataset. The identified pathways included:

1. Neuroactive ligand-receptor interaction
2. Staphylococcus aureus infection
3. Estrogen signaling pathway
4. Complement and coagulation cascades
5. Pancreatic secretion
6. Thyroid hormone synthesis
7. cAMP signaling pathway

However, the number of pathways listed in an enrichment output does not necessarily correspond to the number of statistically significant pathways after multiple-testing correction. Therefore, only pathways meeting the selected adjusted significance threshold, preferably adjusted P-value or false discovery rate (FDR) < 0.05, should be interpreted as statistically significant. The presence of only two or three strongly significant KEGG pathways in the fixed-frozen dataset is therefore not unexpected. It may indicate that the marker gene set had strong enrichment for a limited number of pathways rather than broad pathway-level enrichment.

Important Interpretation of the KEGG Results

Although the fresh-frozen sample showed a larger number of enriched KEGG pathways, this should not automatically be interpreted as evidence that fresh-frozen tissue is biologically superior.

The number of enriched pathways can be influenced by several factors, including:

- Number of clusters identified
- Number and diversity of marker genes
- Gene overlap between clusters
- Database annotation coverage
- Statistical correction for multiple testing
- Tissue composition
- Spatial region represented in the sample
- Differences in sequencing depth and data quality

Because the fresh-frozen dataset contained 23 clusters and the fixed-frozen dataset contained 28 clusters, both datasets contributed different numbers and compositions of marker genes to the enrichment analysis. Consequently, differences in pathway enrichment may reflect differences in cellular composition and marker gene profiles in addition to preservation-related effects.

5. Reactome Pathway Enrichment

Both datasets showed enrichment of pathways associated with GPCR-mediated signaling, including:

- GPCR ligand binding
- Signaling by GPCR
- Class A/1 rhodopsin-like receptors
- Peptide ligand-binding receptors

The presence of these pathways in both datasets is consistent with the importance of G-protein-coupled receptors in neurotransmission, neuropeptide signaling, sensory processes, and neuroendocrine regulation.

Fresh-Frozen-Specific Patterns

The fresh-frozen dataset additionally showed enrichment of:

- FGFR2 ligand binding and activation
- Immunoregulatory interactions between lymphoid and non-lymphoid cells
- Degradation of the extracellular matrix

These pathways suggest representation of genes associated with growth factor signaling, immune communication, and extracellular matrix remodeling.

Fixed-Frozen-Specific Patterns

The fixed-frozen dataset showed a broader range of Reactome annotations, including:

- Formation of the cornified envelope
- Keratinization
- Developmental Biology
- GPCR downstream signaling
- G alpha signaling events
- Class B/2 secretin family receptors
- Sensory perception-related pathways
- Peptide receptor signaling pathways

The enrichment of developmental and keratinization-related pathways should again be interpreted as gene annotation enrichment rather than direct evidence that these biological processes are actively occurring in adult brain tissue.

6. Comparative Summary

Functional Category	Fresh-Frozen Dataset	Fixed-Frozen Dataset
Biological Processes	Neuroendocrine signaling, behavioural functions, neuronal processes, metabolic and immune responses	Developmental annotations, transcriptional regulation, morphogenesis, signal transduction
Cellular Components	Membrane structures, axon-associated components, secretory compartments and vesicles	Intermediate filaments, structural components and transcription-related complexes
Molecular Functions	Hormones, neuropeptides, growth factors, enzymes and myelin-associated functions	DNA-binding proteins, transcription factors and gene regulatory functions
KEGG Pathways	Neuroactive signaling, metabolism, vascular and calcium signaling	Neuroactive signaling, immune-associated and endocrine-related pathways
Reactome Pathways	GPCR signaling, growth factor signaling, immune regulation and extracellular matrix remodeling	Broad GPCR signaling, structural differentiation and developmental annotations

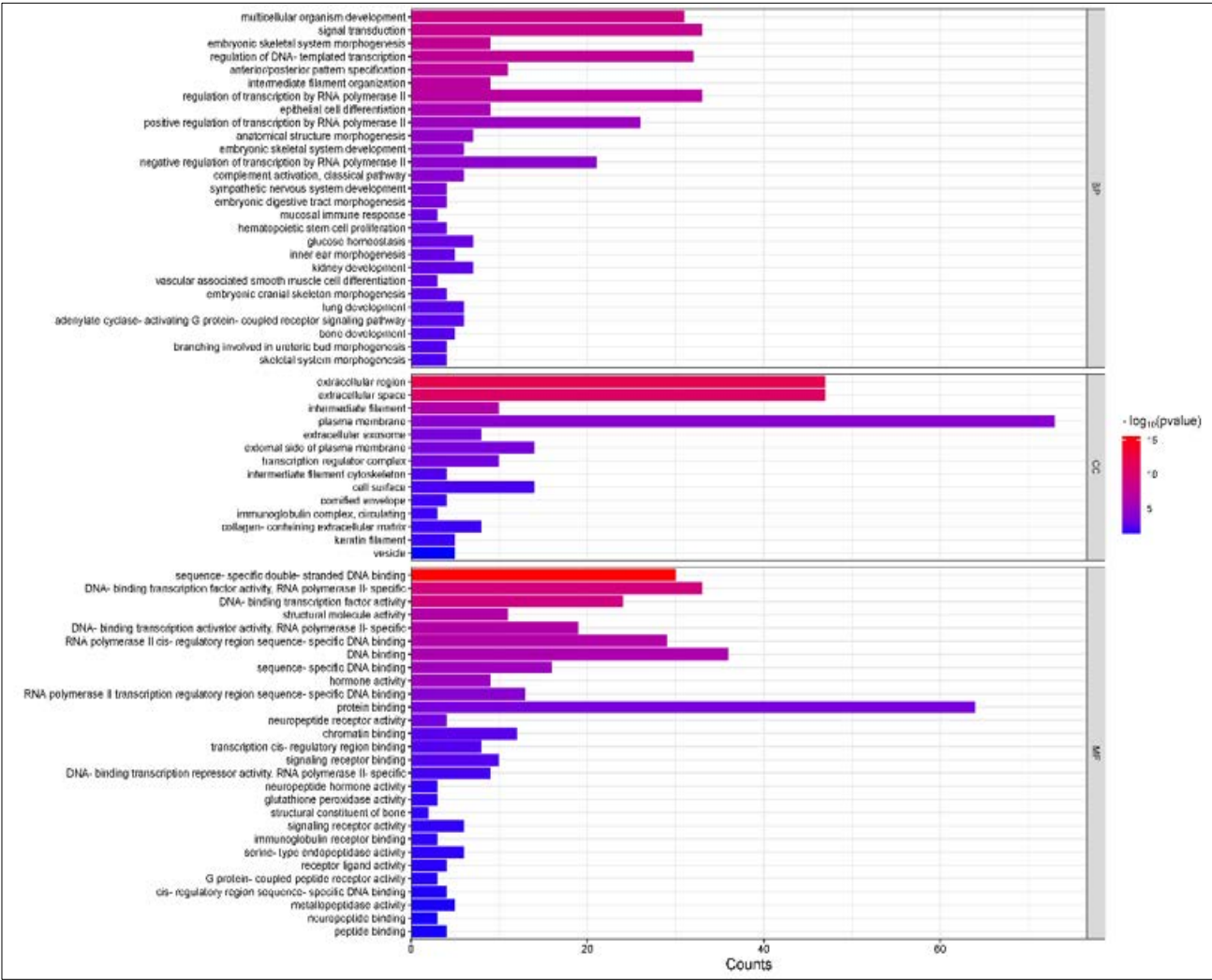


Fig 3: GO enrichment analysis of the fixed-frozen mouse brain sample

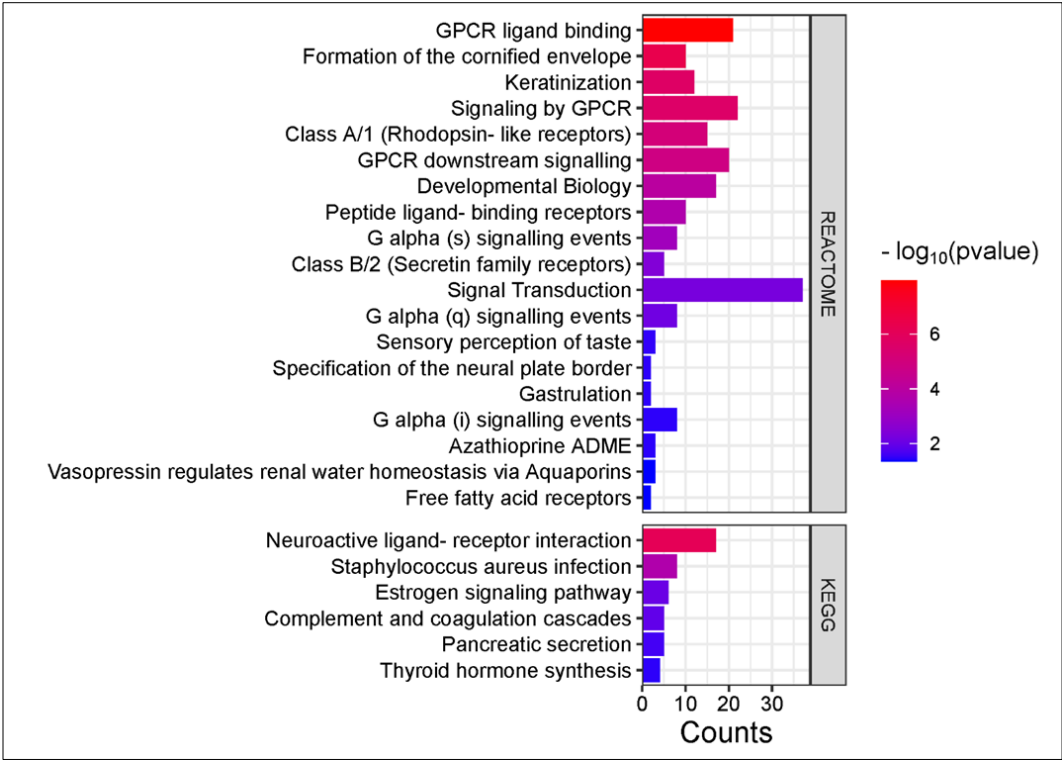


Fig 4: KEGG and Reactome pathway enrichment analysis of the fixed-frozen mouse brain sample

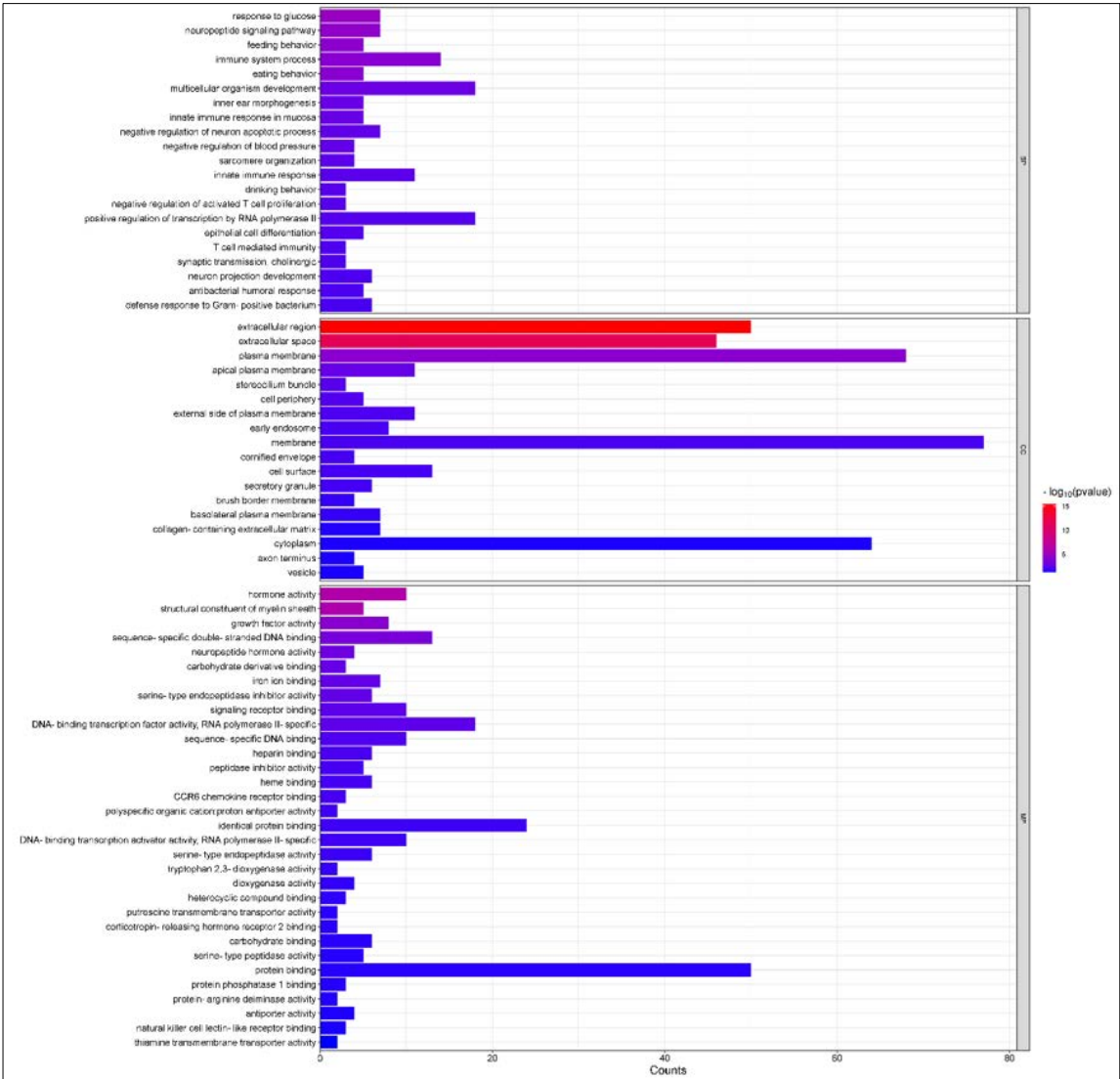


Fig 5: GO enrichment analysis of the fresh-frozen mouse brain sample

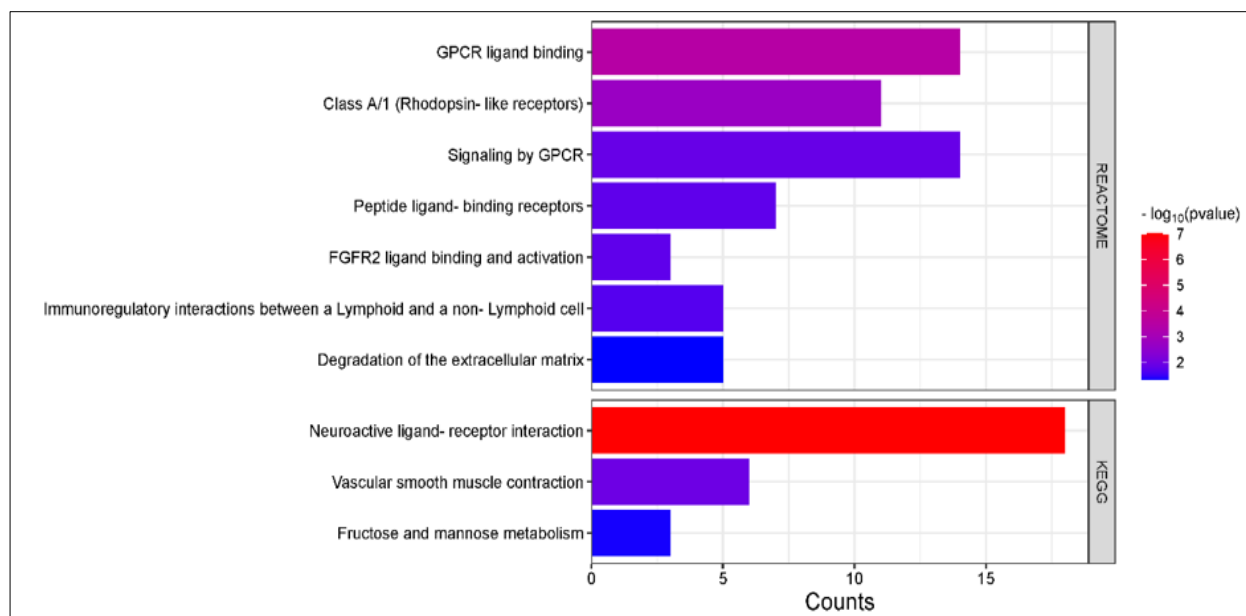


Fig 6: KEGG and Reactome pathway enrichment analysis of the fresh-frozen mouse brain sample

7. Overall Interpretation and Conclusion

The comparative enrichment analysis demonstrated that both fresh-frozen and fixed-frozen mouse brain datasets retained biologically meaningful transcriptional signatures associated with neural signaling and extracellular communication. The fresh-frozen dataset showed relatively greater representation of pathways associated with neuropeptide and neuroactive signaling, behavioural regulation, amino acid and carbohydrate metabolism, calcium signaling, vascular-associated processes, and growth factor signaling. In contrast, the fixed-frozen dataset showed relatively greater representation of transcription factor activity, developmental and morphogenetic gene annotations, intermediate filament organization, structural processes, GPCR signaling subclasses, and endocrine-associated pathways.

Importantly, these findings should be interpreted as comparative transcriptional signatures observed in the two analysed datasets rather than definitive evidence that one preservation method universally performs better than the other. The study included one publicly available sample for each preservation condition, and therefore differences between the datasets may also reflect biological variation, spatial sampling differences, cellular composition, sequencing characteristics, and analytical factors. Nevertheless, the analysis demonstrates that both preservation conditions can capture important brain-associated molecular signatures. Fresh-frozen tissue showed a broader representation of metabolic, neuropeptide, vascular, and calcium-signaling pathways in this dataset, whereas the fixed-frozen sample showed broader representation of transcriptional, structural, developmental annotation, and GPCR-related signatures. Therefore, the results provide an exploratory comparison of how preservation conditions may influence the transcriptional and functional profiles captured by spatial transcriptomic analysis. Further studies using multiple biological replicates from matched brain regions and standardized analytical procedures will be necessary to determine whether the observed differences represent reproducible preservation-related effects.

Limitations

Several limitations should be considered when interpreting the findings. First, only one dataset was analysed for each preservation condition. Consequently, biological and technical variation cannot be separated from preservation-associated effects. Second, the number of identified clusters is influenced by analytical parameters and should not be interpreted independently as evidence of superior tissue preservation. Third, pathway enrichment analysis was based on marker genes and therefore depends on clustering results, marker-selection thresholds, database annotation, and the gene universe used for enrichment testing. Fourth, pathway names should be interpreted carefully. For example, enrichment of infection-related pathways does not necessarily indicate infection, and enrichment of keratinization-related pathways does not necessarily indicate epithelial differentiation within brain tissue.

Data Availability

The publicly available spatial transcriptomics datasets analysed in this study were obtained from the 10x Genomics Visium Spatial Gene Expression dataset repository. Two healthy mouse brain datasets were analysed: (i) the FFPE mouse brain, sagittal, 11-mm capture area dataset and (ii) the Fixed Frozen mouse brain, sagittal, 11-mm capture area dataset. The original datasets are publicly available through the 10x Genomics dataset repository under the Creative Commons Attribution 4.0 International (CC BY 4.0) licence

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