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REVIEW

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Spatial omics in high-grade glioma: study design, analytical pitfalls, and standards for reproducible neuro-oncology

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Abstract

High-grade gliomas (HGGs), particularly glioblastoma (GBM), remain highly lethal despite extensive molecular profiling and attempts at therapeutic innovation. Treatment failure is driven in part by spatially organized heterogeneity, including hypercellular cores, infiltrative margins, perivascular niches, and hypoxic/perinecrotic zones that concentrate stress adaptation, immune suppression, and therapy tolerance. Spatial omics can map these programs in situ, but reported findings often diverge because pre-analytical variation, sampling bias, platform artifacts, segmentation/spot mixing, and inappropriate spatial assumptions can dominate apparent biology, especially in necrotic, hemorrhagic, and myelin-rich brain tissue. Here, we propose a reproducibility framework for spatial omics in HGGs that links compartment-aware study design, platform selection, and robust analysis and validation. We outline study designs aligned to diagnostic, mechanistic, translational, and trial-embedded questions; compare major spatial transcriptomic, proteomic, and metabolomic platforms with glioma-specific failure modes; and propose practical pipelines for QC, segmentation, deconvolution, multimodal integration, and spatial interaction modeling. Finally, we provide a glioma-tailored reporting checklist to improve comparability across multicenter and clinical translation.

Keywords Glioblastoma, Spatial transcriptomics, Tumor microenvironment, Reproducibility, Multiplex imaging, Biomarkers

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Introduction

Scope and rationale for a methods-focused spatial omics paper

HGGs are defined not only by malignant cell-intrinsic alterations but by the spatial organization of tumor cells, vasculature, stromal interfaces, immune infiltrates, and metabolic gradients within the brain microenvironment. Although we use the term HGGs throughout, the methodological challenges and recommendations discussed here primarily apply to grade 4 diffuse astrocytic tumors, including GBM, IDH-wildtype, and astrocytoma, IDH-mutant, grade 4, which share the tissue-level features of necrosis, microvascular proliferation, hypercellularity, and diffuse infiltration that drive the analytical considerations presented in this review. Our recommendations are developed for adult-type diffuse gliomas as classified by the WHO 2021 classification. Pediatric-type diffuse HGGs, such as H3 K27-altered diffuse midline glioma, present distinct spatial biology and are not the primary focus of this review, although many analytical principles may generalize. Standard molecular profiling has elucidated recurrent pathway alterations and transcriptional programs in GBM, including PI3K/AKT/mTOR and MAPK signaling, dysregulatedcell-cycle pathways, and the coexistence of distinct transcriptional cell states within individual tumors [1, 2]. Nonetheless, outcomes remain poor, highlighting that treatment failure is often driven by heterogeneity, plasticity, and microenvironmental constraints rather than the mere absence of "targets" [3, 4]. Spatial omics preserves tissue context, allowing molecular programs to be measured in situ rather than averaged across bulk tissue or inferred from dissociated cells. In glioma, this added context is decisive: the infiltrative edge, perivascular niches, and perinecrotic hypoxic zones can harbor therapy-resistant reservoirs that are systematically underrepresented or mischaracterized by bulk profiling dominated by the resected enhancing core [2, 3]. However, spatial omics findings in HGGs frequently diverge across studies because technical and analytical factors can dominate the apparent biology. Pre-analytical variables, sampling bias, platform-specific artifacts, segmentation/spot mixing, and spatial statistical assumptions can introduce structured distortions that mimic biological phenomena, particularly in necrotic, hemorrhagic, and myelin-rich brain tissue [5, 6].

In glioma, observed spatial patterns are often confounded by measurement and analysis choices; without explicit control of these factors, plausible narratives may reflect artifact rather than reproducible biology. Spatial assays are increasingly proposed as diagnostics, stratifiers, or pharmacodynamic readouts. However, clinical deployment requires analytical validity, standardized pre-analytical handling, compatibility with turnaround time, and transparent reporting. Broader spatial omics guidelines emphasize that pre-analytical variables, platform-specific biases, and opaque computational pipelines remain significant barriers to adoption. In neuro-oncology, these challenges are compounded by limited tissue quantities, high necrosis fractions, and the need to integrate spatial outputs into tumor boards and neuropathology workflows [7, 8]. Accordingly, we synthesize study design, platform selection, and analysis choices into a reproducibility framework tailored to HGGs. We conclude with a glioma-specific reporting checklist that supports multicenter comparability and clinically credible translation. This review is written for neuro-oncologists and neuropathologists who increasingly encounter spatial outputs in diagnostic and trial-embedded settings, while remaining accessible to computational readers. While computational methods are discussed where necessary, the emphasis is on experimental design choices, failure modes, and interpretive principles that directly affect clinical inference. Our goal is to provide a practical framework that enables clinicians and pathologists to critically evaluate spatial omics studies and assess their readiness for clinical translation. We begin with study design because sampling and histologic anchoring determine what any downstream platform or pipeline can validly conclude.

Study design principles for spatial omics in HGGs

Spatial omics studies succeed or fail before sequencing or imaging: sampling strategy, tissue processing, and histopathologic anchoring determine interpretability and reproducibility (Fig. 1). In HGGs, the tumor is not a uniform mass but a spatially organized ecosystem with sharp transitions on the millimeter scale and complex interfaces with the reactive brain [9, 10]. The defining feature of diffuse gliomas is their infiltrative growth pattern, in which tumor cells migrate along white matter tracts and perivascular spaces, producing a continuous gradient of tumor cell density without a discrete margin. This growth pattern means that any spatial measurement at the tumor periphery captures a mixture of neoplastic cells, reactive astrocytes, microglia, oligodendrocytes, and neurons, making spatial omics measurements in these regions particularly susceptible to dilution effects, deconvolution errors, and misinterpretation of compositional changes as tumor-intrinsic biology. Method papers in spatial transcriptomics emphasize that inconsistencies across studies often arise from differences in tissue procurement and region selection rather than actual biological contradictions [7, 11]. Study design should begin with a spatially explicit clinical or mechanistic question and a sampling plan that captures the relevant compartments.

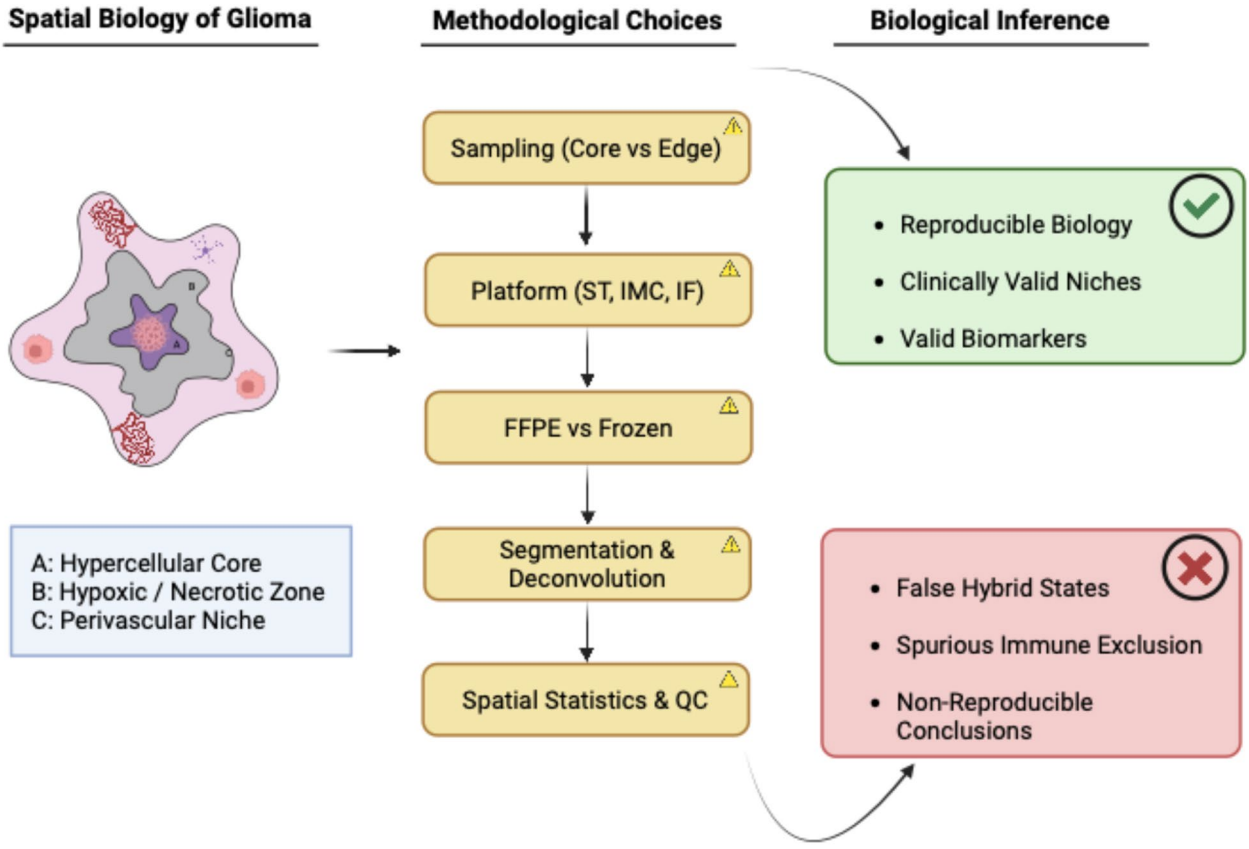

Fig. 1 Methodological determinants of biological inference in spatial omics studies of HGGs. HGGs are spatially organized into a hypercellular tumor core **A**, hypoxic or necrotic regions **B**, and perivascular niches enriched for vascular and immune interactions **C** (left). Spatial omics measurements interrogate these compartments through a series of methodological decisions, including tissue sampling, platform selection, tissue processing (FFPE vs. frozen), segmentation/deconvolution, and spatial statistical analysis with quality control (center). Compartment-aware and rigorously controlled workflows enable reproducible biology, clinically meaningful niche identification, and valid biomarker discovery. In contrast, methodological shortcomings can lead to false hybrid states, spurious immune exclusion, and non-reproducible conclusions (right)

Defining the question: diagnostic, mechanistic, translational, or trial-embedded

In glioma, spatial omics can serve several distinct purposes, each requiring different platforms and validation (Table 1). First, diagnostic refinement aims to improve classification, particularly when histology and conventional markers are ambiguous; multi-omic neuropathology has demonstrated improved diagnostic accuracy in neuro-oncology and provides a precedent for clinical integration of high-dimensional assays [12]. Second, mechanistic studies seek to map how tumor programs, immune states, and metabolic stress are arranged relative to vascular and necrotic structures. Third, translational biomarker studies aim to identify spatial signatures that predict response or recurrence, such as enrichment of therapy-tolerant niches or immune-exclusion patterns [8, 12]. Finally, trial-embedded applications treat spatial assays as stratifiers or pharmacodynamic readouts that must operate within clinical timelines, with limited tissue availability and standardized reporting. A common failure mode is conflating these goals: discovery-grade assays rarely meet clinical turnaround times, whereas targeted FFPE assays may be inadequate for unbiased discovery.

Multi-region sampling is strongly recommended in glioma

Single-section spatial studies are particularly misleading in GBM because of steep gradients across millimeters of tissue, section-plane variability, and the frequent coexistence of viable tumor, necrosis, microvascular proliferation, and reactive brain within the same specimen [1, 2]. At minimum, sample at least two anatomically distinct regions per patient (typically the enhancing core and the annotated invasive edge), each anchored to a histologic review. Clinically, this matters most at the infiltrative edge, the dominant reservoir for recurrence, which is protected by a relatively intact blood–brain barrier and represents a crucial region for translational studies of resistance and drug delivery [2]. Yet it is frequently underrepresented in routine molecular profiling, which is dominated by resected enhancing core tissue [13]. When feasible, at least three compartments should be explicitly

Table 1 Spatial study design recommendations tailored to glioma compartments and clinical questions

Study objective	Required compartments	Minimum spatial sampling	Preferred tissue type	Core validation strategy	References
Mechanistic mapping of niches (hypoxia/perivascular/edge)	Core + edge + hypoxic/perinecrotic when present	≥ 2 regions per tumor, ≥ 2 sections/region when feasible	Fresh frozen for unbiased RNA; FFPE for multiplex protein	Histology + orthogonal marker staining; replicate sections	[12, 15, 16, 18]
Immune architecture (myeloid vs. T cell spatial exclusion; TLS presence)	Core + perivascular interfaces; meningeal interfaces if available	Wide-area imaging + targeted high-resolution ROIs	FFPE often practical	Multiplex IF/IMC validation; segmentation QC; TLS confirmation by B/T cell zone markers (e.g., CD20/CD3)	[13, 52, 69, 70]
Biomarker development (risk stratification; response prediction)	Compartment defined by intended biomarker (e.g., edge or hypoxic core)	Multicenter harmonized protocol; predefined QC thresholds	FFPE favored for translation	Orthogonal assay validation + clinical correlation	[24, 27]
Imaging integration/radiomics	Sampling mapped to MRI features	Image-guided sampling with navigation, where possible	Either	Co-registration; reproducible annotation	[32–36]
Trial-embedded pharmacodynamics	Lesional region relevant to therapy action	Standardized SOP; turnaround compatible	FFPE favored	Locked pipeline; preregistered analysis plan	[24, 27, 33]

predefined and sampled: a hypercellular core region, an edge/margin region containing diffusely infiltrating cells, and a necrosis-adjacent or hypoxic region when present [14]. Anchor each compartment to histology and support it with marker staining (e.g., hypoxia markers such as CA9 or HIF-related signatures; vascular markers such as CD31). Importantly, "edge" is not purely anatomical: it is a mixture of low tumor fraction, reactive astrocytosis, microglia, and white matter tracts. Without careful histologic annotation, edge signals are easily misinterpreted as tumor-intrinsic downregulation rather than dilution by non-tumor tissue [14–16].

Accounting for treatment exposure and temporal remodeling

Recurrent GBMs exhibit therapy-induced spatial remodeling of vascular niches, immune organization, the extracellular matrix, and tumor cell states, underscoring the need to explicitly model post-treatment tumor dynamics [17–19]. Therefore, studies comparing primary and recurrent tumors should match key covariates and explicitly model treatment exposures such as radiotherapy/temozolomide (RT/TMZ), anti-angiogenic agents (e.g., anti-VEGF therapy), and corticosteroids, which can alter immune infiltration and vascular permeability [20, 21]. When possible, paired pre- and post-treatment samples from the same patient provide the strongest design for studying therapy-driven spatial evolution; however, these datasets are limited because sampled planes and surgical targets often differ.

Pairing spatial assays with imaging and histopathology

In parallel, integrating spatial molecular maps with neuropathology and radiology is particularly valuable for defining glioma target volumes and for relating resected tissue specimens to in vivo tumor regions. Accordingly, spatial omics study designs should prospectively incorporate preoperative imaging features and explicitly align sampled tissue regions with radiologic coordinates. In translational settings, radiomic and radiogenomic approaches are increasingly used to infer molecular states, stratify risk, and inform management decisions, with systematic evaluations suggesting that imaging-derived features can approximate tumor subtype, hypoxia, cellular density, and vascular activity. Spatial omics provides regionally resolved molecular reference maps that can serve as training and validation frameworks for such models, but only when tissue sampling is rigorously mapped to imaging coordinates and, when feasible, annotated using intraoperative navigation systems. Accurate co-registration and reproducible region annotation should therefore be treated as study design requirements rather than optional post hoc refinements. With a compartment-aware sampling framework established, the next critical determinant of validity becomes platform selection and careful accounting for glioma-specific artifact profiles [22–24].

Spatial transcriptomics platforms in HGG: strengths, limits, and glioma-specific failure modes

Spatial transcriptomics (ST) is often the entry point for mapping GBM cell states and immune architecture in situ [2, 10]. Spatial transcriptomic studies have also reported spatially restricted vulnerabilities and candidate therapeutic targets in diffuse HGGs [2, 19]. However, in HGGs, platform-specific artifacts, spot mixing, segmentation error, and spatially patterned quality loss near necrosis can produce convincing but incorrect "niche

biology" if they are not explicitly modeled and validated [25, 26]. At the same time, spatial transcriptomic studies have revealed bona fide microenvironment-specific niches in HGGs, including stem-like tumor compartments and hypoxia-associated immune states [27, 28]. Therefore, platform selection should align with the biological or clinical question, and interpretation should be constrained by each platform's dominant failure modes (Fig. 2; Table 2).

Capture-based spatial transcriptomics (ST)

Capture-based platforms (e.g., Visium-class array capture and newer high-definition barcoded arrays) typically profile thousands of genes (often transcriptome-wide) across millimeter-scale sections by capturing mRNA onto spatially barcoded features and then sequencing. In glioma, capture-based ST has enabled domain-level mapping and multilayer tumor organization, including spatial segregation of tumor cell states and immunosuppression concentrated within perinecrotic niches [29, 30]. The principal limitation is effective spatial resolution: barcoded features often capture transcripts from multiple cells, producing mixed spot profiles. In hypercellular GBM and myeloid-rich regions, spot mixing can spuriously inflate co-expression and generate false "hybrid" states unless deconvolution and orthogonal validation are performed [26, 31]. Higher-resolution variants reduce mixing but can introduce additional trade-offs, including increased sensitivity to RNA diffusion and a heavier computational burden. A glioma-specific failure mode is spatially structured quality degradation near necrosis and hemorrhage; low RNA regions are not randomly distributed, so naive QC filtering can erase biologically relevant zones or

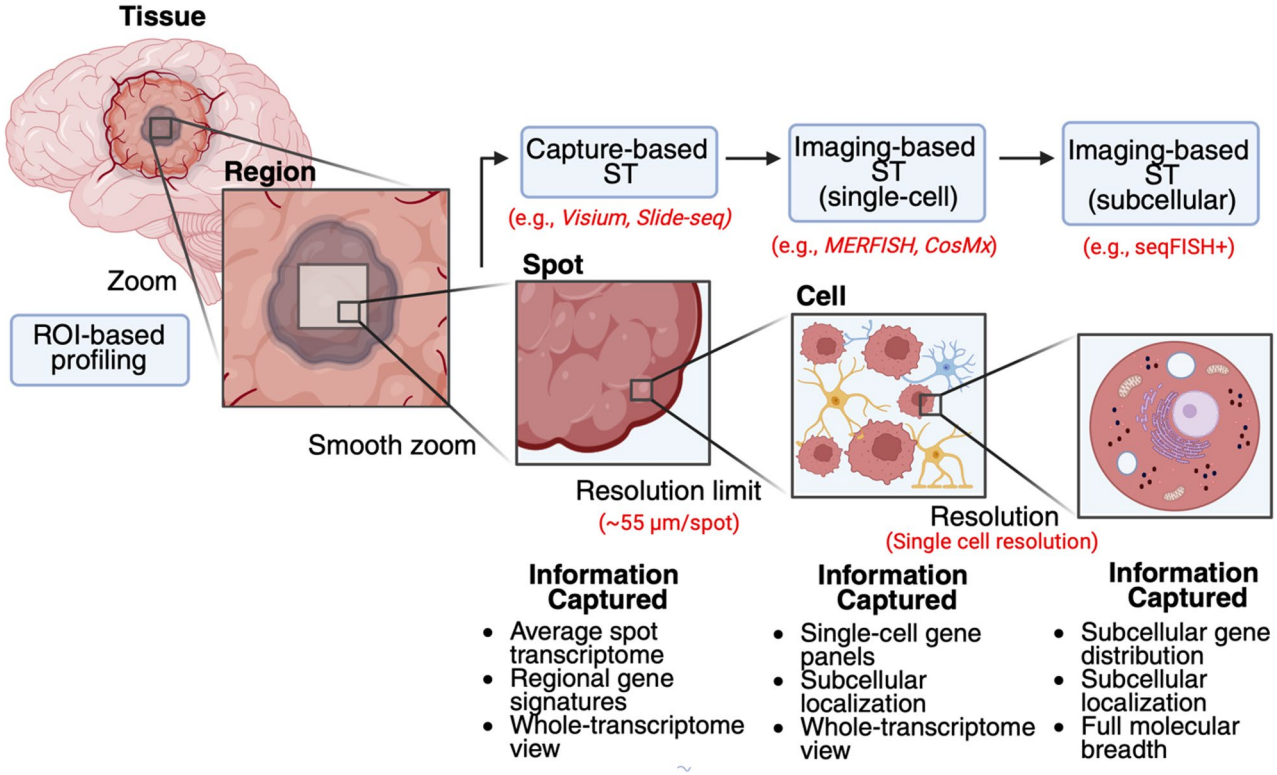

Fig. 2 Trade-offs between spatial resolution and molecular breadth across spatial transcriptomics platforms in glioma. Spatial transcriptomics platforms span a continuum from tissue-level profiling to subcellular resolution, each capturing different layers of biological information. **(Left)** At the tissue scale, region-of-interest (ROI)-based profiling enables compartment-specific interrogation of predefined microstructures. **(Center)** Capture-based spatial transcriptomics (e.g., Visium-class arrays) profiles transcriptome-wide gene expression at spot-level resolution, where each barcoded spot typically captures transcripts from multiple cells. A smooth zoom from tissue to spot illustrates the resolution limit inherent in these platforms; captured information includes average spot transcriptomes, regional gene signatures, and whole-transcriptome coverage. **(Right)** Imaging-based spatial transcriptomics methods (e.g., MERFISH, CosMx, seqFISH+) achieve single-cell to subcellular resolution using targeted gene panels, enabling measurement of single-cell gene expression, subcellular transcript localization, and, at the highest resolution, subcellular gene distribution with full molecular breadth. In high-grade gliomas, each platform tier introduces characteristic artifacts: capture-based methods are susceptible to spot mixing and RNA diffusion, particularly near necrotic zones; imaging-based approaches at the cellular level are vulnerable to segmentation error and panel-selection bias; and subcellular-resolution platforms, while offering the greatest spatial precision, require careful handling of autofluorescence and registration in myelin-rich and haemorrhagic brain tissue. Platform selection should therefore be guided by the biological question, required resolution, and the dominant failure modes relevant to the glioma compartments under study

Table 2 Integrated platform comparison for HGGs spatial omics

Modality	Typical strengths in HGGs	Key limitations in brain tumors	Tissue compatibility	Most common failure mode	Best-fit use case	References
Capture-based ST	Broad transcriptome; tissue-wide domains	Spot mixing; diffusion; necrosis-biased QC	Fresh-frozen; targeted FFPE	False hybrid states from spot mixing	Domain mapping; discovery of region-level programs	[12, 16, 21–24]
Imaging-based ST	Single-cell localization; small ROIs	Segmentation errors, autofluorescence, panel bias	FFPE feasible	Spurious neighborhoods from segmentation	Immune architecture; perivascular interfaces; edge microdomains	[13, 15, 21–24, 26, 31]
IMC/MIBI	High-plex immune mapping; low spectral overlap	Antibody validation; segmentation dependence	FFPE strong	False marker positivity/negativity	Immune niches; biomarker protein panels	[17, 18, 52]
Cyclic mIF/CODEX	Flexible high-plex; interpretable markers	Cycle drift, bleaching, and stripping failure	FFPE strong	Cycle-dependent artifacts	Immune phenotyping and validation	[24, 26]
MSI metabolomics (MALDI/DESI)	Oncometabolites/lipids; gradients	Ion suppression; registration; semi-quantitative	Frozen preferred	Matrix/ion suppression misread as depletion	Metabolic niche mapping; drug distribution hypotheses	[53–55]

exaggerate compartment boundaries [32, 33]. Permeabilization conditions are a key determinant of validity: over- or under-permeabilization can create structured artifacts and should be empirically optimized and reported [34]. Accordingly, capture-based ST in glioma should be framed as domain-level mapping unless cell-level claims are directly validated.

Imaging-based transcriptomics

Imaging-based transcriptomics (including in situ sequencing and multiplexed FISH-like approaches) measures transcripts directly in tissue with single-cell or subcellular localization. These platforms trade breadth for resolution: they are typically targeted but offer single-cell-to-subcellular localization [33, 35]. In glioma, imaging-based approaches are beneficial for resolving immune neighborhoods, perivascular interfaces, and infiltrative edge regions where tumor fraction is low and capture-based spot mixing is severe.

However, glioma tissue introduces modality-specific vulnerabilities that must be addressed explicitly. Hypercellularity and irregular morphology increase segmentation error and can distort neighborhood analyses if cell boundaries are misassigned. Autofluorescence and background signals in brain tissue can also bias detection thresholds, especially in FFPE sections, and panel choice can constrain inference toward predefined hypotheses. Segmentation quality should be explicitly quality-controlled, and key spatial claims should be validated with orthogonal markers or an independent modality, especially when conclusions imply clinical relevance [36–38].

ROI-based spatial profiling and targeted validation

Region-of-interest (ROI)-based profiling reduces spot mixing by restricting measurements to histologically defined microstructures, but at the cost of spatial continuity [17, 21]. In HGGs, ROI-based designs are well-suited to perivascular interfaces, necrosis-adjacent borders, or low-tumor-fraction edge regions when the study question is compartment-specific. However, ROI selection can amplify sampling bias and limit statistical replication if regions are chosen post hoc or without predefined criteria. This trade-off is acceptable for compartment-specific hypotheses, provided ROI selection is pre-specified and replicated. Therefore, ROI-based analyses should predefine ROI selection rules (ideally pathology-anchored), include replicate ROIs per compartment where feasible, and treat conclusions as hypothesis testing or validation rather than whole-tumor inference unless supported by broader spatial coverage [39].

Spatial proteomics platforms and analytical considerations

Because RNA abundance does not reliably reflect protein activity, spatial proteomics is necessary as the next layer for clinically actionable inference in HGGs. Multiplexed spatial proteomics, therefore, provides a direct bridge between discovery biology and clinically actionable measurements [40–42]. Yet proteomic imaging is highly sensitive to antibody performance, epitope retrieval, staining drift, and image-processing choices. In glioma, these issues are exacerbated by variable tissue fixation, necrosis, hemorrhage, and adjacent epitope loss, which can distort both marker-intensity and cell-segmentation outputs [43–45]. A key principle is that spatial proteomics couples biology with assay performance: it is a joint measurement of both. Consequently, high-impact glioma studies should treat antibody validation, batch controls, and segmentation robustness as validity-critical steps rather than optional technical details [46–48].

Metal-tagged mass spectrometry imaging (IMC/multiplexed ion beam imaging (MIBI)-type)

Imaging mass cytometry (IMC) and related metal-tagged imaging approaches enable simultaneous measurement of dozens of protein markers with minimal spectral overlap, making them attractive for immune architecture mapping and niche characterization in glioma, particularly on archived FFPE specimens. Spatial immune profiling using multiplex proteomics has identified perivascular inflammatory phenotypes associated with more prolonged survival, underscoring the clinical relevance of protein-defined spatial patterns [7, 39]. The dominant methodological determinant of validity in IMC-type assays is the antibody's performance in FFPE brain tumor tissue. Antibodies optimized for conventional IHC may perform poorly in IMC or multiplex contexts due to differences in antigen retrieval, conjugation chemistry, and detection sensitivity. In glioma, a myelin- and lipid-rich background can further influence apparent staining patterns. As a result, each antibody in a high-plex panel should be validated under thestaining and retrieval conditions use in the final assay, ideally including single-plex testing and known positive/negative controls [49, 50]. Batch effects across staining runs and slides can generate artifactual spatial gradients that resemble true niche biology; accordingly, rigorous standard operating procedures (SOPs), inclusion of common control tissues across batches, and explicit normalization frameworks are required to ensure analytical validity [43, 46, 50]. Cell-level quantification requires segmentation and inherits the same hypercellularity-driven failure modes described for other imaging-based assays. In hypercellular GBM, segmentation errors can systematically distort neighborhood analyses and interaction claims. Report segmentation methods and QC and demonstrate that key conclusions are robust to plausible segmentation parameter choices [51–53].

Cyclic immunofluorescence and CODEX-like methods

Cyclic multiplex immunofluorescence (mIF) methods, including CO-Detection by indEXing (CODEX)-type workflows, enable iterative staining and imaging of dozens of protein markers, offering flexibility and strong compatibility with FFPE tissue. These approaches are helpful for detailed immune phenotyping, vascular interface mapping, and orthogonal validation of transcriptomic hypotheses [44, 54, 55]. Cyclic workflows introduce modality-specific artifacts that must be actively monitored. Registration drift across cycles can generate artificial colocalization or apparent loss of co-expression; bleaching and signal decay can produce cycle-dependent intensity differences that mimic biological gradients; and incomplete antibody stripping can generate false positives in later cycles. Because these artifacts can be spatially structured, they can be misread as niche biology unless quantified and corrected. Accordingly, cyclic proteomics pipelines should report cycle-to-cycle registration metrics, include repeated markers across cycles as internal controls, and validate stripping efficiency [43, 56, 57].

Deep visual proteomics and microdissection-based mass spectrometry

Microdissection-coupled proteomics can provide deeper proteome coverage than imaging methods and enable compartment-resolved quantification of pathway activation or metabolic enzyme abundance, such as at necrotic borders or invasive margins. Although less spatially comprehensive than tissue-wide imaging, these approaches complement spatial transcriptomics by testing whether transcriptional programs translate into protein states, particularly in immune contexts where RNA–protein concordance may be limited. Throughput and morphological selection bias remain key constraints: microdissection tends to overrepresent visually distinct structures and may miss diffuse gradients characteristic of glioma infiltration. Nonetheless, incorporating a validation tier that quantifies proteins within spatially defined regions can substantially strengthen claims derived from RNA-based spatial maps, particularly when the conclusions have therapeutic implications [58–61].

Spatial metabolomics and lipidomics in HGG: method-driven considerations

Metabolic heterogeneity is a core feature of glioma biology and therapy resistance, including hypoxia-driven reprogramming, lipid metabolic shifts, and immunomodulatory pathways. Spatial metabolomics and lipidomics can localize metabolites and lipids to microanatomical structures, providing information that cannot be inferred reliably from RNA alone. However, metabolomic imaging is particularly vulnerable to technical artifacts in brain tissue, including matrix effects, lipid- and myelin-mediated ion suppression, and challenges in precise registration with histology. Interpretation should be conservative: most MSI maps are relative, and hotspots can reflect ionization and tissue composition [41, 62–64].

MALDI- and DESI-based mass spectrometry imaging (MSI) are the most widely used platforms for spatial metabolomics. In glioma, these approaches have been used to map lipid remodeling and metabolic gradients associated with invasion and stress adaptation. Translationally, these pathways are attractive because they may create metabolic vulnerabilities in therapy-tolerant niches. Yet MSI experiments in brain tumors must control tissue thickness, matrix deposition uniformity, and local tissue composition, because white matter-rich areas and necrosis can exhibit different ionization properties

that mimic metabolite depletion or enrichment. Therefore, MSI studies should use serial sections for H&E and marker staining, implement robust co-registration workflows, and validate key metabolites with orthogonal targeted assays when proposing biomarkers [65–67].

Immunometabolic pathways require additional methodological care. The tryptophan–kynurenine axis, mediated by indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TDO), is a well-established immunosuppressive mechanism in cancer and glioma, and spatial localization of these metabolites could, in principle, identify immune-evasive niches [68–70]. However, because metabolites diffuse and undergo rapid turnover, spatial interpretation requires strict control over ischemia duration and tissue handling. MSI is also sensitive to post-resection metabolic changes. Best practices, therefore, include rapid freezing, consistent documentation of time-to-processing, and, when feasible, the use of internal standards to reduce run-to-run variability. For a high-impact spatial metabolomics component, co-localizing metabolite patterns with RNA or protein markers of relevant enzymes (e.g., IDO1/TDO, lipid oxidation enzymes) strengthens interpretability by linking chemical maps to plausible biological mechanisms [66, 70].

FFPE constraints across spatial omics

Because FFPE is the dominant tissue format in clinical neuropathology, spatial assays compatible with FFPE material are essential for clinical translation. Yet FFPE introduces fundamental constraints that must be explicitly acknowledged and methodologically accommodated. Clinical translation reviews emphasize that pre-analytical variables often determine spatial data quality and that FFPE introduces fragmentation and cross-linking artifacts that are not random noise but rather systematic biases [6, 71]. In glioma, these biases can create apparent spatial patterns that reflect fixation gradients, differences in section thickness, or variability in antigen retrieval rather than true biology (Fig. 3).

In spatial transcriptomics, RNA fragmentation in FFPE reduces the effective length of targetable RNA and can increase dropout, particularly for low-abundance transcripts. Probe-based FFPE workflows can partially mitigate this but introduce panel selection bias and non-uniform capture efficiency across genes. Consequently, FFPE spatial transcriptomic results should not be treated as equivalent to fresh-frozen whole-transcriptome maps without bridging validation [71, 72]. Block age is a practical determinant of signal: older archival blocks often yield lower RNA and protein recovery, and degradation can be spatially heterogeneous because fixation and storage conditions vary within and across blocks. High-impact studies should report block age and, when possible, include

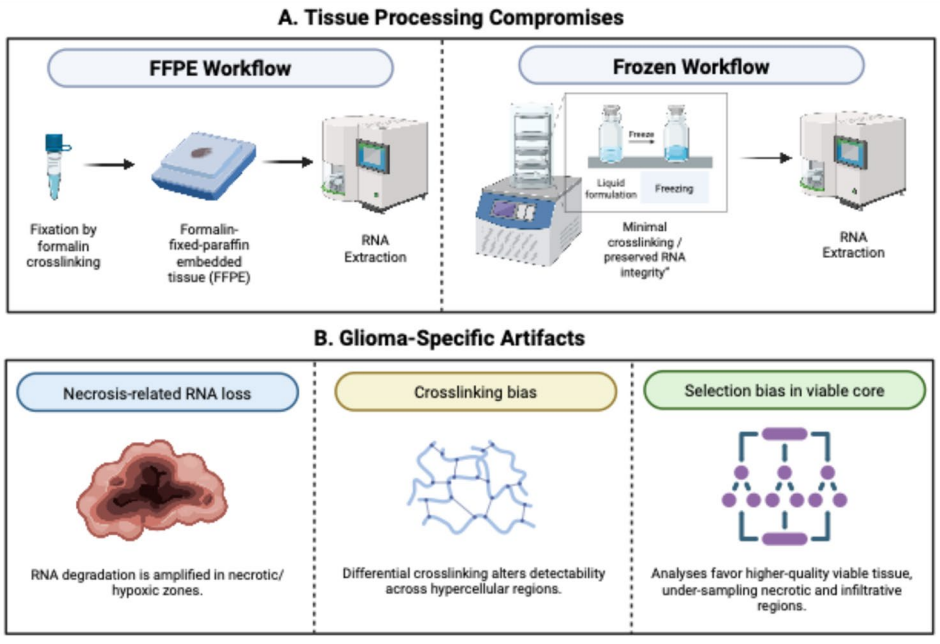

Fig. 3 Tissue processing–dependent biases and glioma-specific artifacts in spatial transcriptomic analyses. **A** Comparison of FFPE and frozen-tissue workflows highlights differences in RNA preservation before spatial transcriptomic profiling. FFPE processing involves formalin-induced crosslinking and paraffin embedding, whereas frozen workflows rely on rapid freezing with minimal crosslinking, resulting in improved RNA integrity, and **B** In HGGs, tissue heterogeneity exacerbates processing-related biases, including RNA loss in necrotic and hypoxic regions, differential crosslinking across hypercellular tumor areas, and preferential selection of higher-quality viable core tissue during analysis, resulting in spatially structured sampling bias

QC metrics that quantify transcript detection efficiency and background. For spatial proteomics, antigen retrieval is both necessary and a significant source of variability. Retrieval conditions that optimize one epitope may damage another, and retrieval can alter tissue morphology and increase background staining, affecting segmentation and spatial analysis. In cyclic IF workflows, repeated retrieval or stripping steps can progressively degrade tissue or increase autofluorescence, creating cycle-dependent spatial bias. For this reason, multiplex proteomics studies in glioma should explicitly report retrieval conditions, stripping validation, and image QC metrics [72, 73]. Section thickness effects span all modalities. As a result, thicker sections can increase signal but also increase diffusion, background, and segmentation errors. For MSI metabolomics, thickness influences ion yield and spatial resolution. Given these constraints, FFPE spatial omics should be framed as a clinically practical modality that requires its own QC thresholds and reporting standards, rather than as a direct surrogate for fresh-frozen "discovery-grade" spatial profiling [10, 74].

The feasibility of spatial omics on FFPE tissue varies considerably across modalities. Spatial proteomics platforms, including IMC and cyclic immunofluorescence, are well-established on FFPE and represent the most clinically practical spatial modalities for archived tissue. Probe-based spatial transcriptomics platforms, such as Visium FFPE and MERFISH/CosMx on FFPE, are compatible but introduce panel selection bias and reduced sensitivity for low-abundance transcripts compared to fresh-frozen whole-transcriptome profiling. Spatial metabolomics of FFPE tissue is technically feasible using MALDI-MSI after deparaffinization, but formalin fixation substantially alters the metabolome through crosslinking metabolites to proteins, extraction of small polar molecules during tissue processing, and chemical modification of lipid species. Consequently, FFPE-based metabolomic data provide a limited and biased representation of the in situ metabolome, and spatial metabolomic studies should prioritize fresh-frozen tissue when feasible or interpret FFPE-derived metabolomic findings with appropriate caution and orthogonal validation.

Computational analysis, integration, and spatial inference

In practice, most non-reproducibility enters through (i) spatially structured QC loss, (ii) segmentation/annotation choices, and (iii) invalid statistical assumptions. Spatial omics produces data that are simultaneously molecular, imaging-based, and spatially structured. A robust pipeline must therefore include preprocessing and QC, segmentation (when applicable), normalization and batch correction, feature extraction, and spatial statistics that respect tissue architecture. Reviews of spatial transcriptomics clinical translation emphasize that computational variability is a significant source of non-reproducibility, and that standardized pipelines and transparent reporting are prerequisites for clinical readiness. In glioma, these needs are intensified by necrosis, infiltrative margins, and immune cell mixtures that violate simplistic assumptions [75–77].

Preprocessing and quality control (QC)

Quality Control (QC) in spatial omics is not merely about removing low-quality observations; it is about avoiding systematic bias introduced by spatially structured tissue damage. In capture-based ST, standard QC metrics include total unique molecular identifier (UMI) counts per spot, detected genes per spot, and mitochondrial or ribosomal transcript proportions. Yet in GBM, necrotic and perinecrotic regions typically exhibit lower RNA content, and filtering using global thresholds can precisely exclude areas of greatest biological interest. Because damage and dropout patterns are often non-random and compartment-linked in glioma sections, QC thresholds can distort biology if applied blindly. Therefore, best practice is to perform QC in a spatially aware manner: visualize QC metrics as maps over the tissue, identify clusters of low-quality spots, and interpret whether these correspond to technical artifacts (folds, tears, staining failure) or biologically meaningful structures (necrosis). When necrosis is the object of study, using compartment-specific QC thresholds or explicitly modeling dropout may be preferable to aggressive filtering [34, 78]. In imaging-based assays, QC must include image-level checks: focus, illumination uniformity, autofluorescence, and staining artifacts. Segmentation QC is critical and should consist of manual review of representative fields, with quantification of segmentation error modes. For high-plex proteomics, QC should also address cycle drift, background, and marker-specific signal distributions [44].

Segmentation and annotation in hypercellular tumors

Segmentation is a major source of error in imaging-based spatial omics in glioma, where dense cellular packing and irregular morphology challenge general-purpose algorithms often trained on epithelial tissues. Nuclear-only segmentation can misassign cytoplasmic proteins or transcripts, whereas aggressive cytoplasmic expansion may merge adjacent cells, distorting neighborhood analyses. Because spatial interaction modeling depends directly on cell coordinates and identities, segmentation errors can generate spurious interaction networks and false immune exclusion patterns. Best practice is therefore to treat segmentation as a tunable modeling step rather than a fixed preprocessing operation, selecting and parameterizing algorithms according to tissue

architecture and testing downstream robustness across plausible parameter settings [79]. Annotation introduces additional uncertainty. Tumor cells in GBM adopt diverse and plastic transcriptional states [1], while microglia and macrophages share overlapping markers and exhibit functional plasticity, complicating deterministic labeling. Annotation strategies should therefore combine marker-based rules with probabilistic or reference-based mapping approaches and report uncertainty rather than imposing hard labels when evidence is ambiguous [80].

Deconvolution of mixed spots and its limits in glioma

Deconvolution is widely used to infer cell-type or state mixtures in capture-based spatial transcriptomics [26]. In glioma, this approach is attractive because spots frequently contain mixtures of tumor cells, microglia, macrophages, oligodendrocytes, neurons, endothelial cells, and other components. However, glioma deconvolution is inherently complex. First, single-cell or single-nucleus RNA-seq references are not ground truth; dissociation and nuclear isolation introduce transcriptional biases that can distort reference profiles [81]. Second, glioma cell states are continuous and plastic, and forcing them into discrete categories risks circular reasoning and obscures transitional programs [82]. Third, at the infiltrative edge where tumor fraction is low, inferred mixtures may largely reflect normal brain composition rather than tumor-intrinsic programs, leading to spurious interpretations of "state shifts." Best practice is therefore to treat deconvolution as a hypothesis-generating tool, validate key findings with orthogonal assays, and explicitly report uncertainty.

Multi-slice and multi-omic integration

A single tissue section cannot fully capture glioma biology, and computational frameworks increasingly integrate multiple slices and modalities. Graph-based methods that model spatial neighborhoods across sections enable identification of clinically relevant niches by incorporating tissue architecture into network analyses [76]. Multi-omics spatial integration is also expanding, with approaches that combine transcriptomics with mIF or proteomics on adjacent sections [83]. However, cross-modality integration introduces co-registration challenges: misalignment can generate spurious spatial correlations between RNA and protein signals. Registration error should therefore be quantified; shared anatomical landmarks, such as H&E features or vasculature, should guide alignment; and alignment metrics should be explicitly reported [84].

Integration with sc/snRNA-seq

Integrating spatial omics with single-cell or single-nucleus RNA-seq is essential for interpreting mixed spatial measurements, assigning cell states, and translating spatial domains into mechanistic hypotheses [84]. Together, these features complicate direct interpretation of spatial signals and necessitate joint modeling across modalities. sc/snRNA-seq reference atlases are shaped by tissue dissociation and nuclear isolation, which induce systematic transcriptional perturbations and can selectively underrepresent fragile or spatially anchored cell populations, thereby failing to accurately reflect true in situ composition [82, 85]. In glioblastoma, these biases intersect with intrinsic tumor plasticity: malignant cells occupy continuous transcriptional states rather than discrete categories [1], and immune populations such as microglia and macrophages exhibit overlapping markers and functional heterogeneity [86]. Consequently, reference selection should be treated as a modeling choice, with explicit documentation of reference origin, patient context, and preprocessing steps.

Spatial integration methods that transfer scRNA-defined labels onto spatial data inherently propagate the assumptions embedded in the reference atlas [84]. Decomposition frameworks similarly rely on reference-informed mixture models and require uncertainty-aware inference. In HGGs, label transfer and deconvolution can be dominated by normal tissue composition rather than tumor-intrinsic programs, generating apparent "state changes" that reflect dilution effects rather than reprogramming. Best practice is therefore to report probabilistic assignments or confidence scores, test robustness to alternative references and state granularities, and validate central spatial claims with orthogonal assays rather than presenting label transfer as independent validation of the scRNA taxonomy.

Robust spatial statistics and common failure modes

Spatial omics analyses in HGGs commonly aim to identify spatial domains, quantify cellular neighborhoods, and infer interactions between tumor, immune, and vascular compartments. However, spatial data violate the independence assumption because observations are autocorrelated and nested (e.g., spots/cells within fields, sections, and patients). Reviews of spatial omics translation emphasize that computational variability, opaque pipelines, and inappropriate statistical assumptions are significant sources of non-reproducibility, particularly when claims imply clinical relevance [76, 87]. In glioma, these risks are amplified by hypercellularity, necrosis-related loss of structured signal, infiltrative edges with low tumor fraction, and segmentation and spot-mixing artifacts [79]. Three principles substantially improve the likelihood that spatial claims survive review. First, the unit of inference must be defined a priori. Treating thousands of cells or spots as independent replicates constitutes pseudo-replication and inflates statistical

Table 3 Standard failure modes, how to detect them, and mitigation strategies

Failure mode	How it manifests	How to detect it	Mitigation/Best practice	Validation tier	References
Single-section overinterpretation	"New architecture" driven by the section plane	Compare adjacent sections; replicate regions	Multi-region + multi-section per tumor	Independent tumors; histology	[15, 16]
Edge dilution misread as biology	Apparent loss of tumor programs at the margin	Tumor fraction estimation; histology	Explicit edge definition; tumor content modeling	Orthogonal markers (IHC/IF)	[15, 16, 41]
Spot mixing (capture ST)	Spurious co-expression and interactions	Check spot cellularity; deconvolution uncertainty	Higher resolution or ROI validation; cautious claims	Imaging-based RNA/protein	[15, 16]
Segmentation error (imaging)	False neighborhoods and immune exclusion	Manual QC; sensitivity to parameters	Segmentation benchmarking; uncertainty reporting	Alternative segmentation + IF	[13, 16]
Necrosis-biased QC	Removing hypoxic/perinecrotic regions	QC maps overlaid on H&E	Compartment-aware QC; model dropout	Histology-anchored analyses	[12, 16]
Batch effects (slides/runs)	Clustering by batch rather than biology	Batch principal component analysis/UMAP (PCA/UMAP); controls	SOP harmonization; batch correction	Multicenter replication	[21, 31]

significance; robust inference should be anchored at the patient level or modeled hierarchically to account for nested spatial structure. Second, spatial autocorrelation must be explicitly modeled, and null models should preserve relevant tissue structure. Naïve random shuffling across an entire slide can generate spurious "significant" colocalization because it ignores architectural constraints; permutation schemes should instead respect compartment boundaries, sampling density, or local neighborhoods so that significance reflects biology rather than structure. Third, the scope of claims should be predefined: capture-based spatial transcriptomics is typically domain-level by default, whereas cell-level interaction claims require validated segmentation, robustness testing, and transparent reporting [88].

Neighborhood analyses in spatial omics (e.g., immune enrichment near vasculature or necrotic borders) should explicitly define the neighborhood model (radius vs. kNN; 2D vs. 3D assumptions), the null model used for permutation, the spatial scale tested, and the level of replication used for statistical inference (patients rather than cells) [76, 88]. Apparent "interactions" may reflect shared compartmentalization, batch effects, segmentation artifacts, or spot mixing rather than a biological mechanism. In hypercellular GBM, segmentation errors can generate spurious neighborhoods and false immune exclusion patterns [79]. In capture-based spatial transcriptomics, spot mixing can inflate tumor–immune co-expression, necessitating uncertainty-aware deconvolution and orthogonal validation [26]. Integration with sc/snRNA-seq improves interpretability but does not constitute ground truth; label transfer propagates reference assumptions and should be treated as hypothesis generation rather than validation [84]. Because glioma states are continuous and plastic [1], sensitivity analyses are essential, including varying tumor content thresholds at infiltrative edges, testing alternative references or coarser label granularity, and demonstrating replication across sections and patients rather than relying on a single representative region [88]. Robust manuscripts should explicitly test these vulnerabilities and support translational claims with orthogonal validation. (Table 3)

Table 4 Minimal reporting standards checklist (glioma-specific)

Category	Minimal required items for reproducibility
Clinical metadata	Primary vs. recurrent; treatment exposure (RT/TMZ, anti-VEGF, steroids); sampling context
Tissue handling	Fresh frozen vs. FFPE; fixation details if available; block age; ischemia time estimate if feasible; section thickness
Spatial annotation	Core/edge/perinecrotic definitions; H&E images; necrosis/hemorrhage/vascular proliferation annotation
Platform parameters	Platform + version; resolution; panel (if targeted); permeabilization/retrieval settings; controls
QC reporting	Per-spot/cell metrics, spatial QC maps, exclusion criteria, and handling of necrosis
Computation	Segmentation method + QC; deconvolution method + reference; batch correction strategy; spatial statistics choices
Validation	Orthogonal assays for key claims; independent cohort or replicate tumors when biomarker claims are made
Data/code	Public deposition of processed matrices + images where feasible; code or pipeline sufficient for reimplementation (versions + parameters).

Reporting Standards and ClinicalTranslation

Minimal reporting checklist (Table 4)

High-impact translational journals increasingly require scaffolding for reproducibility, including transparent reporting of tissue origin, platform parameters, QC metrics, computational workflows, and validation. Reviews of the clinical translation of spatial transcriptomics emphasize that inconsistent reporting and opaque analytical pipelines remain major barriers to comparability and clinical deployment [77, 88]. In neuro-oncology, these challenges are amplified by variability in surgical sampling, fixation, necrosis burden, and limited tissue

availability. At a minimum, glioma spatial omics studies should report anatomical region (core, edge, perinecrotic), tissue processing (fresh-frozen vs. FFPE; fixation parameters; block age), section thickness, and histologic annotations. Imaging-based datasets should document acquisition settings, segmentation methods, and error assessment; transcriptomic datasets should report sequencing depth, QC thresholds, and spatial QC maps. Computational workflows must specify software versions, key parameters, batch-correction strategy, and spatial-smoothing choices. For neighborhood and interaction analyses, authors should explicitly define the neighborhood model, null model, and unit of replication (patient rather than cell level) [76]. Together, these elements function as a practical reproducibility checklist for authors that mitigates common reviewer concerns regarding sampling bias, QC transparency, and statistical validity.

Translation pathway and clinical constraints

Clinical translation of spatial omics requires workflows aligned with specimen handling, turnaround constraints, and diagnostic laboratory standards. Reviews and translational guidelines emphasize that pre-analytical variability and opaque computational pipelines are major barriers to clinical trust and deployment [77, 88]. In neuro-oncology, additional constraints include limited tissue availability, necrosis and hemorrhage, and time-sensitive postoperative decisions. Fresh-frozen tissue enables unbiased transcriptome-wide discovery, whereas FFPE predominates in clinical archives and supports multiplex protein and targeted RNA assays [44, 89]. A pragmatic pathway uses fresh-frozen discovery followed by transfer of a reduced signature to an FFPE-compatible platform with predefined QC thresholds. Given the extreme heterogeneity of glioma, sampling strategies should incorporate multiple regions, particularly the invasive edge and perinecrotic compartments when biologically relevant [78]. To influence postoperative management, spatial results must be delivered within the short window between surgery and adjuvant therapy, typically 1–2 weeks, constraining platform complexity and necessitating automated, locked workflows. Reports should prioritize actionable outputs, compartment-level biomarker scores, or defined risk signatures over exploratory atlases.

Demonstrating clinical utility is essential: spatial biomarkers must sufficiently improve stratification or therapeutic decision-making to justify the cost and complexity [90]. The most credible path to adoption is prospective trials that embed spatial biomarkers as stratifiers or pharmacodynamic readouts under standardized, locked analysis plans to minimize overfitting and maximize reproducibility [88].

Conclusions

Box 1. Translational pathway for spatial omics in high-grade glioma

Step 1: Discovery (Exploratory).

High-breadth spatial omics performed on fresh-frozen tissue enables unbiased mapping of tumor, immune, vascular, and metabolic programs [34]. In high-grade glioma, such discovery-phase analyses have revealed transcriptional gradients, immune niches, and microenvironmental structure that support hypothesis generation and the identification of candidate biomarkers [78].

Step 2: Reduction (Signature refinement).

Discovery-phase spatial signals should be reduced to a minimal and reproducible marker set linked to clinically relevant phenotypes. Feature reduction must be supported by orthogonal validation and patient-level replication to avoid overfitting and ensure translational robustness [91]. Reviews of spatial omics clinical implementation emphasize that standardized pipelines, transparent reporting, and reproducibility metrics are prerequisites for clinical readiness [92].

Step 3: Deployment (Clinical grade)

Validated spatial signatures should then be deployed on FFPE-compatible platforms using standardized protocols and locked computational pipelines (fixed parameters, version-controlled code, and pre-specified outputs), ideally within prospective clinical trials where they function as stratification tools or pharmacodynamic readouts [90].

Although spatial omics provides powerful biological insights into HGGs, methodological rigor determines whether observed spatial patterns reflect biology or artifact. Necrosis, infiltrative margins, hypercellularity, and treatment-induced remodeling introduce structured biases that must be explicitly controlled [78]. This review outlines a reproducibility-centered framework spanning study design, analytical control, and reporting standards. When embedded within standardized workflows and prospectively validated, spatial omics can advance from exploratory mapping to robust, multicenter-ready biomarkers integrated into diagnostic, stratification, and therapeutic decision-making frameworks [88].

Abbreviations

BBB	Blood–brain barrier
CODEX	CO-Detection by indEXing
DESI	Desorption electrospray ionization
FFPE	Formalin-fixed paraffin-embedded
GBM	Glioblastoma
H&E	Hematoxylin and eosin
HGGs	High-grade glioma
IDO1	Indoleamine 2,3-dioxygenase 1
IF	Immunofluorescence
IHC	Immunohistochemistry
IMC	Imaging Mass Cytometry
kNN	K-nearest neighbors
MALDI	Matrix-assisted laser desorption/ionization
mIF	Multiplex immunofluorescence
MIBI	Multiplexed Ion Beam Imaging

MRI	Magnetic Resonance Imaging
MSI	Mass Spectrometry Imaging
PCA	Principal Component Analysis
QC	Quality Control
ROI	Region of Interest
RT/TMZ	Radiotherapy/Temozolomide
sc/snRNA-seq	Single-cell/Single-nucleus RNA Sequencing
SOP	Standard Operating Procedure
ST	Spatial Transcriptomics
TDO	Tryptophan 2,3-dioxygenase
TLS	Tertiary Lymphoid Structures
UMAP	Uniform Manifold Approximation and Projection
UMI	Unique Molecular Identifier
VEGF	Vascular Endothelial Growth Factor

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Ismail: Visualization; Methodology; Writing-Original Draft; Writing – Review & Editing. Itika Arora: Supervision; Writing – Original Draft, Writing – Review & Editing, Visualization. Saniyah Shaikh: Conceptualization; Visualization; Writing – Original Draft; Writing – Review & Editing. Thaabit Raziq: Visualization; Methodology; Writing – Original Draft; Writing – Review & Editing. Sulaf: Visualization; Methodology; Writing – Original Draft; Writing – Review & Editing. Touleen: Visualization; Methodology; Writing – Original Draft; Writing – Review & Editing. Mohammed Shariq Siddiqui & Arshiya Akbar: Visualization; Methodology; Writing – Original Draft; Writing – Review & Editing. Shamsa Hilal Saleh: Visualization; Methodology; Writing – Original Draft; Writing – Review & Editing. Mohammed Imran Khan: Supervision; Writing – Original Draft; Writing – Review & Editing, Validating, Visualization. Ahmed Yaqinuddin: Supervision; Writing – Original Draft; Writing – Review & Editing, Validating, Visualization.

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