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Computational Frameworks for Pathway Analysis: Evaluating Over-representation, Topology-aware Scoring, and Crosstalk Modeling

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ABSTRACT

Pathway analysis translates high-throughput molecular measurements into interpretable hypotheses about coordinated biological processes, but its conclusions depend strongly on the statistical and biological assumptions embedded in each method. This narrative review provides a practical, tool-focused update rather than a proposal of a new formal taxonomy. It revisits threshold-dependent over-representation analysis, functional class scoring, topology-aware scoring, and emerging crosstalk-aware approaches, with emphasis on when each is defensible for experimental interpretation. Threshold-dependent enrichment is fast and transparent, but relies on an arbitrary significance cutoff and treats pathway members as exchangeable counts. Functional class scoring retains ranked or continuous measurements and can partially accommodate coordinated intra-pathway behavior, yet most implementations still evaluate pathways independently and do not model inter-pathway dependence. Topology-aware methods add mechanistic structure by incorporating directionality, edge sign or weight, node centrality, or perturbation propagation; their interpretability is constrained by incomplete, static, and context-agnostic pathway maps. Because signaling pathways share components and influence one another through regulatory edges, crosstalk can produce both false pathway prioritization and biologically meaningful inter-pathway signals. We therefore compare four representative crosstalk or pathway-deregulation tools Pathifier, PathTracer, PAGI, and PathwAX/PathwAX II across input requirements, outputs, crosstalk definitions, accessibility, computational burden, strengths, and limitations. Recent multi-omics and single-cell methods are also considered because pathway inference increasingly requires direction-aware, sample-level, and reproducible integration across molecular layers. No method is universally optimal: selection should be driven by data type, biological question, availability of curated topology, tolerance for model assumptions, and intended interpretation. Transparent reporting of input gene universes, pathway databases, software versions, parameter settings, and multiple-testing procedures remains essential for reproducible pathway-level inference.

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Introduction

High-throughput expression, sequencing, proteomic, and metabolomic platforms now make it routine to measure thousands of molecular features in a single experiment. Biological interpretation of these data, however, rarely emerges directly from isolated lists of differentially expressed genes or proteins. Pathways provide curated models of molecular relationships, reactions, and regulatory events, and major resources such as Gene Ontology, KEGG, PANTHER, Reactome, WikiPathways, BioCarta, and the NCI Pathway Interaction Database have

therefore become central to downstream interpretation of omics data (Ashburner *et al.,* 2000; Mi *et al.,* 2013).

Pathway analysis is best understood not as a single statistical test but as a family of assumptions for asking whether a biological process is disproportionately represented, coordinately perturbed, topologically influenced, or connected to other processes. Earlier reviews organized the field into over-representation analysis, functional class scoring, and topology-based approaches (Khatri *et al.,* 2012; Nguyen *et al.,* 2019). The present review retains that taxonomy as a historical and pedagogical scaffold, but its contribution is primarily practical: it clarifies where the scaffold remains useful, where it becomes insufficiently granular, and how crosstalk-aware and multi-omics tools should be selected by researchers who need defensible biological interpretation rather than an exhaustive catalogue of software. This emphasis requires supplementing secondary summaries with primary methodological literature. Foundational functional class scoring methods, most notably Gene Set Enrichment Analysis (GSEA), established a rank-based framework for detecting coordinated pathway-level changes without imposing a hard differential-expression threshold (Subramanian *et al.,* 2005). Signaling pathway impact analysis and related perturbation methods introduced direction and position within curated pathway graphs (Draghici *et al.,* 2007; Tarca *et al.,* 2009). Later tools, including Pathifier, PathTracer, PAGI, BinoX, and PathwAX, moved the discussion toward sample-wise deregulation, network crosstalk, and practical web-based annotation (Drier *et al.,* 2013; Han *et al.,* 2015; Ogris *et al.,* 2016; Ogris *et al.,* 2017; Ogris *et al.,* 2022; Nygard *et al.,* 2019). A recurring trade-off across these methods is that increased biological realism typically requires greater dependence on curated databases, statistical modeling choices, and data quality. For this reason, the central question is not which method is universally best, but which assumptions are acceptable for a given experimental design, molecular modality, and research question. This review therefore places methodological limitations, crosstalk definitions, software accessibility, and reproducibility requirements in the foreground.

Materials and Methods

This review adopts a narrative methodological approach. It synthesizes established and recent pathway-analysis literature with priority given to primary tool papers, benchmark studies, and methodological reviews that explicitly discuss assumptions, input requirements, software implementation, or reproducibility. The review focuses on over-representation analysis, functional class scoring, topology-aware scoring, crosstalk-aware annotation, and multi-omics pathway integration. Comparative performance rankings are deliberately omitted, as benchmark outcomes are highly contingent on data modality, pathway database version, gene universe definition, network coverage, and evaluation endpoint.

To maintain traceability, we distinguish historical frameworks from the present interpretive contribution in this review. Khatri *et al.* and Nguyen *et al.* are cited as influential secondary syntheses. Primary methodological sources anchor claims regarding GSEA, SPIA, Pathifier, PAGI, BinoX, PathwAX, PathTracer, CTpathway, SCPA, ActivePathways, MOPA, PathIntegrate, DMPA, alongside recent direction-aware multi-omics frameworks.

Classification of pathway analysis methods and their assumptions

Over-representation analysis (ORA) tests whether a predefined set of selected genes is represented in a pathway more often than expected under a specified background universe. Standard implementations rely on Fisher's exact test, the hypergeometric test, or chi-square approximations, which are computationally efficient and readily interpretable for experimental collaborators (Huang *et al.,* 2009). This simplicity introduces well-documented constraints: outcomes are sensitive to the differential-expression threshold, to the definition of the measured gene universe, and to the assumption that genes behave as exchangeable counts rather than functionally interdependent molecular actors.

Functional class scoring (FCS) was developed partly to avoid the information loss produced by thresholding. Methods such as GSEA, GlobalTest, SAFE, PAGE, sigPathway, SAM-

GS, GSVA, and related approaches utilize ranked or continuous molecular measurements to detect coordinated shifts among members of a gene set (Subramanian *et al.,* 2005; Tian *et al.,* 2005; Kim *et al.,* 2005; Jiang *et al.,* 2007; Hanzelmann *et al.,* 2013). The frequently noted ambiguity in the literature can be clarified: while FCS incorporate coordinated intra-pathway behavior, and some implementations use sample or phenotype permutations that partially preserve inter-gene correlation, the vast majority of methods still evaluate predefined pathways independently and do not explicitly model inter-pathway dependence or crosstalk (Efron *et al.,* 2007; Gatti *et al.,* 2010; Khatri *et al.,* 2012).

Topology-aware methods integrate pathway architecture directly into the statistical model. Implementations vary in their use of topological features: some incorporate directionality, others leverage edge sign or weight, node centrality, perturbation propagation, or graph-based diffusion. This additional structure can sharpen mechanistic interpretation, but it also inherits the limitations of pathway databases. Curated maps are incomplete, often static, and rarely specific to the cell type, tissue state, or stimulation context under study. Dynamic phenomena such as feedback loops, transient activation states, and condition-specific network rewiring remain poorly captured by static topological priors (Draghici *et al.,* 2007; Tarca *et al.,* 2009; Shojaie *et al.,* 2010; Khatri *et al.,* 2012). These constraints are further amplified when pathways are analyzed as interconnected systems rather than isolated modules. Biological networks inherently share components, reactions, and regulatory edges, meaning that pathway boundaries are often artificial constructs of curation rather than discrete biological units. Crosstalk-aware and network-based approaches therefore function as a cross-cutting analytical layer that spans the preceding categories, rather than constituting a rigid fourth generation. Their primary value lies in discriminating whether an apparent pathway signal is pathway-specific, overlap-driven, edge-mediated, or propagated through a wider network. Table 1 consolidates these assumptions and recommended reporting safeguards to reduce repetition in later sections.

Table 1. Comparison of pathway-analysis method families and recommended reporting safeguards.

Method family	Typical input	Core question	Information retained or modeled	Key limitations & recommended reporting safeguards
Over-representation analysis	List of selected genes and a background universe	Are selected genes over- or under-represented in a pathway?	Gene counts and pathway membership	Threshold dependence; gene independence; pathway independence; no topology or crosstalk. Report the background universe, database version, threshold, and multiple-testing procedure.
Functional class scoring	Ranked or continuous molecular measurements for most genes	Is the pathway's measurement distribution shifted relative to the genome or phenotype labels?	Continuous signal; coordinated modest changes; some preservation of within-pathway correlation	Often analyzes pathways separately; still vulnerable to gene-set overlap and database bias. Clarify whether permutations preserve sample correlation and whether inter-gene dependence is modeled.
Topology-aware methods	Molecular measurements plus curated pathway topology	Do perturbed genes influence pathway behavior according to network architecture?	Directionality, edge sign or weight, node centrality, perturbation propagation	Incomplete, static, and context-agnostic maps; limited ability to model feedback loops and dynamic states. Interpret topology as curated prior knowledge, not direct causal proof.
Crosstalk-aware or network-based methods	Gene lists or molecular measurements plus pathway overlap, interaction networks, or regulatory edges	Is a pathway signal explained by shared genes, network connectivity, or cross-pathway propagation?	Gene overlap, regulatory links, physical interactions, random walks, or crosstalk enrichment/depletion	Sensitive to network coverage, hub bias, and tissue specificity. Distinguish statistical association from causal cross-activation and inspect leading genes or edges.

Understanding pathway crosstalk

Pathway crosstalk refers to functional communication between biological pathways. In computational practice, crosstalk is frequently operationalized as gene set overlap between two annotated pathways. This operational definition is

computationally tractable and addresses a recognized source of statistical bias: overlapping gene sets systematically inflate enrichment p-values.

Donato (Donato *et al., 2013*) demonstrated that uncorrected gene sharing can yield false-positive pathway assignments and quantified this effect using the Jaccard similarity coefficient (Jaccard, 1908).

For mechanistic and signaling interpretation, a broader biological definition is required. Crosstalk can also arise when a signaling component (e.g., receptor, kinase, or transcription factor) in one pathway modulates the activity or output of another, even in the absence of shared annotated genes. This edge-mediated perspective aligns with experimental characterizations of signaling specificity, robustness, and adaptive rewiring (Tegge *et al.,* 2016; Rowland *et al.,* 2017). In disease studies, altered crosstalk may therefore represent either a statistical artifact requiring methodological correction or a genuine biological signal of pathway rewiring (De Anda-Jauregui *et al.,* 2019; Wang *et al.,* 2019).

Databases and crosstalk representation

No single resource comprehensively captures all forms of pathway crosstalk. Interaction databases such as STRING, BioGRID, FunCoup, and GeneMANIA catalog pairwise physical or functional associations, whereas pathway repositories like Reactome, WikiPathways, KEGG, and PANTHER encode curated biological processes and reaction topologies (Szklarczyk *et al.,* 2023; Persson *et al.,* 2021; Mi *et al.,* 2013). These resources address distinct analytical questions: interaction networks capture molecular associations, whereas pathway maps contextualize those molecules within structured biological processes.

XTALKDB catalogs signaling pathway crosstalk under stringent biological criteria (Sam *et al.,* 2017). While KEGG provides manual cross-map linkages, these connections do not establish context-specific regulatory crosstalk. Consequently, pathway crosstalk should be reported with its operational definition: shared genes, shared reactions, physical interactions, regulatory edges, or statistical network enrichment.

Computational implications

The distinction between gene-overlap and regulatory crosstalk is not merely semantic; it dictates fundamentally different statistical and computational challenges. When crosstalk is defined strictly by gene-set overlap, the primary statistical challenge is redundancy among gene sets and consequent p-value inflation. If crosstalk is defined by network edges, the problem shifts toward network completeness, degree-bias correction (hub genes), and the biological plausibility of inferred interactions. This difference underlies the need for conceptual separation between conventional enrichment, pathway deregulation scoring, and network crosstalk annotation (Fig. 1).

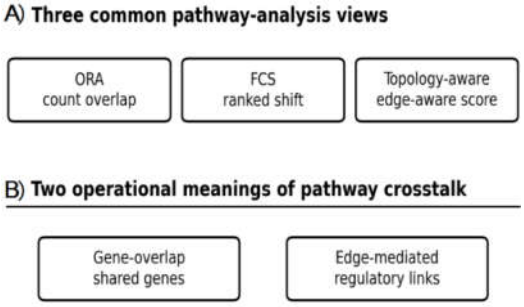

Fig. 1. Conceptual framework of pathway analysis methods and crosstalk definitions: A) Three common analytical views: over-representation analysis (ORA) based on count overlap, functional class scoring (FCS) based on ranked distribution shifts, and topology-aware scoring based on edge-aware network propagation; B) Two operational meanings of pathway crosstalk: gene-overlap crosstalk driven by shared pathway membership versus edge-mediated crosstalk driven by regulatory or physical interactions between distinct pathway modules.

Recent approaches increasingly combine pathway membership with network topology or prior crosstalk maps. CTpathway, for example, uses a global pathway crosstalk map to enrich pathway scoring with prior knowledge and was evaluated in both bulk and single-cell contexts (Liu *et al.,* 2022). Such approaches are promising, but they should not be interpreted as eliminating uncertainty. Rather, they shift uncertainty from arbitrary gene-list thresholds toward database provenance, edge-confidence scores, and the biological relevance of the crosstalk prior to the experimental system.

Common limitations of expression-based, overlap-based, and network-based approaches

Several constraints are inherent to the underlying data structures and statistical frameworks, rather than specific to any single tool. Expression-dependent methods are inherently sensitive to preprocessing choices-including normalization strategies, batch correction algorithms, and sample composition-and rely on the assumption that transcript abundance reliably proxies functional pathway activity. Overlap-dependent approaches are constrained by pathway database coverage and are susceptible to bias from oversized gene sets, redundant pathway annotations, and pleiotropic or highly connected genes. Network-based methods require careful handling of incomplete interactomes, degree-bias (hub effects), edge-confidence calibration, and the context-inappropriate transfer of interactions across tissues or species without empirical validation. Furthermore, multiple-testing correction is complicated by the inherent dependence among overlapping pathway tests. While false-discovery rate control remains standard, its underlying independence or positive-dependence assumptions should be explicitly acknowledged rather than treated as an automatic or assumption-free step (Benjamini *et al.,* 1995; Efron *et al.,* 2007). Consequently, computational reproducibility functions as a methodological prerequisite, not an administrative formality. Standardized reporting should explicitly document: pathway database and version; defined gene universe; identifier mapping and synonym-resolution rules; missing-value handling protocols; software or package versions; accessible code or workflow scripts; random seeds for stochastic algorithms; and the precise multiple-testing correction applied. This reporting standard aligns with established computational reproducibility frameworks, including the FAIR principles, the ten simple rules for reproducible computational research, and the five-pillar framework proposed by Ziemann *et al.* (Ziemann *et al.,* 2023).

Representative crosstalk-aware and deregulation-focused tools

Pathifier estimates a pathway deregulation score for each sample by comparing disease samples against a normal reference set within a multidimensional gene-expression space constrained to pathway membership. It uses principal curves to represent a data-driven trajectory, then quantifies how far a sample lies along that trajectory from the normal baseline (Drier *et al.,* 2013). In this context, describing Pathifier as computationally scalable reflects its ability to perform sample-wise inference across large transcriptomic datasets. However, this scalability does not imply methodological independence from expression data quality, pathway annotation accuracy, or gene-set overlap artifacts.

The key strength of Pathifier is that it produces continuous, sample-specific pathway scores without requiring complete knowledge of the causal wiring inside a pathway. This property is particularly valuable in oncology and complex disease cohorts, where pathway activity often exhibits continuous inter-individual heterogeneity rather than binary case-control dichotomization (Drier *et al.,* 2013; Fa *et al.,* 2019). Its primary methodological constraint concerns principal-curve geometry: when the fitted curve self-intersects or poorly approximates the underlying manifold, resulting deregulation scores may become biologically ambiguous (Nygard *et al.,* 2019).

PathTracer was developed as a methodological successor to Pathifier that enhances differential pathway activity detection in tumor cohorts through improved interpretability and computational efficiency (Nygard *et al.,* 2019). It retains the principal-curve concept but computes deregulation using Euclidean projection onto the fitted curve, thereby mitigating scoring artifacts associated with curve self-intersection. It also provides diagnostic visualizations (heatmaps, hierarchical clustering, and principal-curve projections) that facilitate biological validation of pathway-level signals.

The reported ~10-fold reduction in processing time must be interpreted within its original benchmark parameters rather than as a universal performance guarantee. In validation on the METABRIC breast cancer cohort (n= 2,115 samples, 1,288 Reactome pathways), Nygard *et al,* attributed this improvement primarily to dimensionality stabilization (fixed principal components) and parallelized computation.

Absolute processing times remain contingent on computational infrastructure, pathway database scale, and cohort size; therefore, hardware-specific benchmarks are preferred over generalized runtime claims.

PathTracer is retained as a representative method because it directly addresses principal-curve instability while preserving the sample-wise deregulation paradigm. However, alternative dimensionality-reduction and pathway-activity frameworks may be preferable depending on data structure and sparsity. For example, ZIFA addresses zero-inflation in sparse single-cell profiles, whereas GSVA, single-sample GSEA, decoupleR, and SCPA offer complementary strategies for pathway or regulator activity estimation across bulk and single-cell modalities (Hanzelmann *et al.,* 2013; Bibby *et al.,* 2022).

PAGI (Pathway Analysis based on Global Influence) integrates within-pathway perturbation and inter-pathway crosstalk within a topology-informed statistical framework (Han *et al.,* 2015). It constructs a gene-gene interaction network from KEGG-derived relationships, maps differential-expression signals onto the network, and uses a random-walk-with-restart algorithm to propagate differential signals across the network topology. The resulting global dysregulation score is intended to capture both localized pathway perturbations and secondary effects propagated through interconnected network modules.

PAGI is particularly applicable when the analytical objective prioritizes network-mediated signal propagation over simple gene-set membership. Its limitations follow from that strength: curated topology may omit context-specific interactions, and static graph structures inherently cannot capture dynamic or tissue-conditioned signaling rewiring. PAGI should therefore be interpreted as a hypothesis-generating prioritization framework rather than a mechanistic or causal model of cross-pathway regulation.

PathwAX was designed to make network-crosstalk pathway annotation accessible to researchers who would otherwise rely solely on gene-overlap enrichment. It uses the BinoX framework to compare observed network links between a query gene set and pathway genes with a randomized background, allowing both crosstalk enrichment and crosstalk depletion to be detected (Ogris *et al.,* 2016; Ogris *et al.,* 2017). This network-based strategy can identify pathway associations even when the query list and pathway share minimal or zero direct gene overlap.

PathwAX II represents the current, actively maintained implementation and should be prioritized for contemporary analyses. It extends the original web server with an interactive network viewer and Reactome pathways, allowing users to visually trace the specific gene-to-pathway edges underlying crosstalk statistics (Ogris *et al.,* 2022). The original PathwAX deployment relied on Python 2.7 CGI scripts; this should be documented as a legacy technical note rather than a current limitation. For contemporary analyses, users should consult PathwAX II or the BinoX package and application programming interface when larger gene lists or local workflows are required.

The strongest advantage of PathwAX is edge-level interpretability, enabling users to distinguish signals distributed across multiple interactions from those concentrated in high-degree network hubs. Its main limitation is that the result is inherently bounded by the coverage and confidence scores of the underlying functional association network and pathway annotations. Negative crosstalk signals (depletion) require cautious interpretation, as they may indicate genuine biological isolation, incomplete network curation, or context-specific interactions absent from the background reference. A comprehensive side-by-side comparison of these four representative tools, detailing their input requirements, analytical outputs, crosstalk definitions, computational demands, and key limitations, is provided in Table 2**.**

Multi-omics, single-cell, and reproducibility considerations

Pathway analysis is increasingly extended to multi-omics and single-cell modalities. Multi-omics integration can reveal regulatory relationships undetectable in transcriptomic profiling alone, but it introduces methodological complexities including scale heterogeneity, structured missingness, discordant sample matching, and layer-specific technical noise (De Maturana et al., 2019). Tools such as ActivePathways, multiGSEA, MOPA,

PathIntegrate, DMPA, and direction-aware multi-omics frameworks illustrate different strategies for combining evidence across molecular layers (Paczkowska *et al.,* 2020; Slobodyanyuk *et al.,* 2024).

Direction-aware methods are particularly valuable when the biological hypothesis requires coherent, concordant shifts across molecular layers rather than isolated layer-specific significance.

Single-cell pathway inference introduces distinct analytical constraints. Technical dropout, continuous cell-state heterogeneity, compositional bias, and donor-level pseudo-replication can render uncorrected gene-set scoring statistically unreliable. SCPA was developed to identify pathway activity changes across single-cell populations and to characterize regulatory mechanisms during T-cell activation (Bibby *et al.,* 2022). Robust single-cell pathway inference typically requires cell-type stratification, pseudobulk aggregation or mixed-effects modeling to preserve biological replication, and systematic sensitivity testing across pathway databases and gene-set definitions.

Computational reproducibility demands increase proportionally with analytical complexity, as workflows transition from single-gene lists to integrated multi-omics matrices and network models. Ziemann *et al.*, formalize these requirements as five foundational pillars: literate programming workflows, rigorous version control, containerized or explicitly documented computational environments, persistent data archiving, and comprehensive methodological documentation. These practices are especially important for pathway analysis because seemingly minor technical variations-such as gene identifier mapping protocols, pathway database versions, or background universe definitions-can substantially alter pathway rankings without reflecting underlying biological differences (Wadi et al., 2016).

Practical decision framework for method selection

Method selection should be driven by data modality and biological question rather than software familiarity. Table 3 provides a structured decision framework and corresponding reporting safeguards for researchers designing publication-ready pathway analyses.

Conclusion

Pathway analysis translates high-dimensional molecular profiles into testable biological hypotheses, but this dimensionality reduction inherently amplifies the impact of underlying methodological assumptions. While over-representation analysis provides rapid, interpretable enrichment screening, functional class scoring preserves continuous signal intensity, topology-aware methods incorporate mechanistic network priors, and crosstalk-aware frameworks distinguish localized pathway activity from network-propagated or overlap-driven artifacts. No computational framework substitutes for biological context, systematic sensitivity testing, or transparent reporting of analytical boundaries.

In practice, robust pathway analysis requires aligning methodological choice with data architecture and biological hypothesis rather than software convention. Critical decision points include the availability of ranked versus thresholding inputs, the contextual validity of curated topologies, the distinction between statistical overlap and regulatory crosstalk, and the necessity for direction-aware or sample-resolved modeling in multi-omics or single-cell contexts. Ultimately, defensible pathway inference transcends statistical significance: it requires explicit documentation of assumptions, version-controlled inputs, and biological coherence that withstands independent scrutiny.

Table 2. Side-by-side comparison of four representative crosstalk-aware or pathway-deregulation tools.

Feature	Pathifier	PathTracer	PAGI	PathwAX / PathwAX II
Input data type	Normalized expression matrix, phenotype labels, normal reference cohort, pathway gene sets	Normalized expression matrix, reference category, user-defined pathways, optional comparison groups	Differential-expression values mapped to KEGG-derived gene-gene interaction network	Query gene/protein list with supported identifiers; precomputed functional association networks & pathway collections
Primary output	Continuous sample-wise pathway deregulation score (PDS)	Sample-wise deregulation scores with diagnostic visualizations, clustering, and category testing	Global dysregulation score integrating local perturbation and network propagation	Pathway crosstalk enrichment/depletion statistics with interactive edge-level network maps
Key strength	Quantifies continuous pathway activity per sample without requiring complete causal wiring	Mitigates principal-curve instability; improves runtime scalability and diagnostic transparency	Captures secondary pathway effects via network-mediated signal propagation	Detects enrichment/depletion without requiring gene overlap; enables edge-level signal inspection
Main limitation	Principal-curve geometry may become unstable or biologically ambiguous in high-noise datasets	Runtime gains are cohort- and hardware-dependent; small pathways may be excluded during dimensionality reduction	Relies on completeness and tissue-relevance of static KEGG-derived topology	Signal reliability bounded by network coverage, hub bias, identifier mapping accuracy, and web-visualization constraints
Software accessibility	Local execution via Bioconductor R package	Local execution via Bioconductor R package; optional web deployment	Originally web-deployed; current implementations may require local script execution	Web server (PathwAX II) for standard queries; REST API & BinoX package for local/large-scale workflows
Programming language	R	R	R / Python (legacy web); Python recommended for local use	Python (PathwAX II backend); API accessible via standard HTTP requests
Crosstalk modeling approach	Indirect; overlap may confound deregulation scores if not explicitly corrected	Indirect; focuses on improved sample-wise deregulation rather than explicit crosstalk testing	Explicit; integrates within-pathway topology and inter-pathway propagation via random walk	Explicit; compares observed vs. randomized network links to quantify enrichment/depletion
Ideal use case	Identifying continuous pathway deregulation trajectories across heterogeneous patient cohorts	Robust detection and visualization of differential pathway activity in large tumor datasets	Prioritizing pathways affected by both direct perturbation and network-mediated cross-talk	Mapping functional associations between query gene sets and pathways independent of direct gene overlap

Table 3. Practical checklist for choosing a pathway-analysis strategy.

Decision point	Recommended methodological approach	Essential reporting & validation step
Only a reliable list of selected genes is available	Over-representation analysis	Define the measured background universe; avoid interpreting p-values as pathway activity.
Ranked or continuous expression/proteomic data are available	Functional class scoring, GSEA-like methods, GSVA-like sample scoring	Report ranking metric, permutation strategy, and whether inter-gene dependence is preserved.
The question concerns signaling direction or network position	Topology-aware scoring (e.g., SPIA, perturbation methods)	Verify that the pathway database contains relevant directed edges for the target tissue or condition.
Pathway overlap is likely to inflate results	Overlap-aware scoring (e.g., PADOG), crosstalk correction, or network-based interpretation	Inspect leading-edge or shared genes; separate pathway-specific and shared components.
The question concerns regulatory or edge-mediated crosstalk	Network-based methods (e.g., PathwAX, PAGI, CTpathway)	Explicitly state whether crosstalk is defined by shared genes, physical interactions, regulatory edges, or propagated signals.
Patient- or sample-level pathway activity is required	Pathifier, PathTracer, GSVA, ssGSEA, or MOPA	Use a biologically appropriate reference group and assess robustness across pathway databases.
Multi-omics layers are available	ActivePathways, multiGSEA, MOPA, PathIntegrate, DMPA, or direction-aware models	Integrate only biologically relevant layers; report normalization, missingness handling, and directionality assumptions.
Single-cell data are analyzed	SCPA, single-cell pathway scoring, or pseudobulk enrichment	Stratify by cell type or state; account for dropout, compositional effects, and sample-level replication.
The analysis must support publication-grade reproducibility	Any method, with complete methodological reporting	Share code, software versions, pathway database versions, gene universe, random seeds, and parameters.

Conflict of interests

The authors declare that there are no conflicts of interest.

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