Network Methods for Pathway Analysis of Genomic Data

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Abstract

Rapid advances in high–throughput technologies have led to considerable interest in analyzing genome–scale data in the context of biological pathways, with the goal of identifying functional systems that are involved in a given phenotype. In the most common approaches, biological pathways are modeled as simple sets of genes, neglecting the network of interactions comprising the pathway and treating all genes as equally important to the pathway’s function. Recently, a number of new methods have been proposed to integrate pathway topology in the analyses, harnessing existing knowledge and enabling more nuanced models of complex biological systems. However, there is little guidance available to researchers choosing between these methods. In this review, we discuss eight topology-based methods, comparing their methodological approaches and appropriate use cases. In addition, we present the results of the application of these methods to a curated set of ten gene expression profiling studies using a common set of pathway annotations. We report the computational efficiency of the methods and the consistency of the results across methods and studies to help guide users in choosing a method. We also discuss the challenges and future outlook for improved network analysis methodologies.

Introduction

Modern high–throughput (HT) technologies enable researchers to make comprehensive measurements of the molecular state of biological samples and have yielded a wealth of information regarding the association of genes with specific phenotypes. However, the complex and adaptive nature of living systems presents a significant challenge to deriving accurate and predictive mechanistic models from genomic data. Because cellular processes are governed by networks of molecular interactions, critical alterations to these systems may arise at different points yet result in similar phenotypes. At the same time, the adaptability and robustness of living systems enables variations to be tolerated. Typical gene–level analyses of HT data, such as tests of differential expression, are unable capture these effects. As a result, there has been growing interest in systems–level analyses of genomic data.

Pathway analysis techniques, which aim to examine HT data in the context of mechanistically related gene sets, have been enabled by the growth of databases describing functional networks of interactions. These include KEGG [1], BioCarta [2], Reactome [3], the NCI Pathway Interaction Database (NCI-PID) [4], and InnateDB [5], amongst others. To address the challenge of querying these databases using a common framework, markup languages such as KGML (used by KEGG) and BioPAX have been developed to describe pathways using a consistent format. In particular, the Biological Pathway Exchange (BioPAX) project now provides a unified view of the data from many of the above sources [6], including NCI-PID, Reactome, BioCarta, and WikiPathways.

Over the past decade, a number of pathway analysis methods have been developed to integrate this information with data derived from genomic studies [7]. These methods can be broadly grouped into two categories. The first category comprises analyses designed to identify pathways in which significant genes are overrepresented. A comprehensive review of these methods was recently published Khatri & al [7]; examples

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include hypergeometric tests, Gene Set Enrichment Analysis (GSEA) [8], and Ingenuity Pathway Analysis [9]. The second category of approaches use dimension reduction algorithms to summarize the variation of the genes across the pathway and test for pathway–level differences without relying on single–gene association statistics. Examples include GPC-Score [10], Pathifier [11], and PDM [12]. In contrast to enrichment analyses like GSEA, these methods are capable of identifying differences at the systems–level that would be indetectable by methods that rely upon single–gene association statistics, such as differences in the coordination of the expression of two genes.

Despite these advances, the majority of these methods treat pathways as simple lists of genes, neglecting the network of interactions codified in pathway databases (cf. Fig 1) despite the fact that the importance of pathway network structure to biological function has long been appreciated. In [13], the authors presented systematic mathematical analysis of the topology of metabolic networks of 43 organisms representing all three domains of life, and found that despite significant variation in the pathway components, these networks share common mathematical properties which enhance error-tolerance. In [14], the authors compared the lethality of mutations in yeast with the positions of the affected protein in known pathways, and found that the biological necessity of the protein was well modeled by its connectivity in the network.

To incorporate known interaction network topology with traditional pathway analyses, a multitude of approaches have been proposed for overlaying gene–specific data (either the raw data itself or p-values derived from gene–level statistical tests) onto pathway networks [15–34]. However, to date few comparisons between them have been made. One recent review [35] attempted to compare three network–based analyses (SPIA, PARADIGM, and PathOlogist); unfortunately, the comparison was stymied by the methods’ disparate implementations and reference databases, and yielded inconclusive results. To address this gap, we review eight topology–based pathway analysis methods [8,15–26,36]. While this is not a comprehensive review of all such algorithms, the methods we consider have the common feature of being implemented in R and permitting the user to provide the pathway models (such as those obtained from KEGG), thus allowing them to be compared directly without the issues encountered in [35]. Their free availability from CRAN and BioConductor [37] also makes them the most popular network analysis methods in the bioinformatics and computational biology research community.

Below, we briefly describe each method and discuss its features and limitations. In addition, we also provide a comparison of these methods applied to a curated set of gene expression data from 10 ovarian cancer studies [38], using a common set of 247 KEGG pathways. Using this data, we were able to evaluate both the computational efficiency of the methods and the consistency of the results between the methods and across the studies. The suite of data and scripts used in this comparison are available from our website, enabling researchers to compare updated versions and new network analysis packages using this common framework.

Overview of Network Pathway Analysis Methods

We consider 8 popular network-based pathway analysis methods, described below. In the following discussion, we consider a pathway to be a network of genes (nodes), where edges represent a biochemical interaction. These edges may be directed (e.g., gene \(i\) induces gene \(j\), but not vice-versa) or signed (e.g., \(+1\) for activation or induction, \(-1\) for repression or inhibition). Also, while the following methods were designed with differential expression analyses in mind, it should be noted that most are flexible enough to accommodate other statistics or data types (e.g., allele frequencies and \(\chi^2\) statistics from GWAS). A summary of the key features of the methods is given in Table 1. For comparison, we also consider GSEA [8], a commonly–used pathway analysis algorithm that does not incorporate network topology.

GSEA  Gene Set Enrichment Analysis (GSEA) [8] is an extremely popular method for detecting pathways that are enriched for differentially expressed genes. In contrast to simple hypergeometric overrepresentation analysis, GSEA does not require a threshold for calling a gene significant, but rather considers the magnitude of the expression changes for all the genes in the pathway. Briefly, the algorithm ranks all assayed genes according to the significance of the gene–level associations, and computes a running sum statistic to test whether the genes on the pathway of interest tend to lie at the top of the ranked list (vs. a null hypothesis of being randomly distributed). Significant pathways are those whose genes occur higher in the ranked list
than expected by chance, resulting in a sharply peaked running sum statistic. The size of this peak, called the enrichment score (ES), is tested for significance by permuting the sample labels and recalculating the test statistic to generate a null distribution. This procedure identifies pathways with an accumulation of differentially expressed genes that are associated with the phenotypes of interest. As its name suggests, however, GSEA treats pathways as sets of genes, without incorporating network topology.

SPIA  Signaling Pathway Impact Analysis (SPIA) [15], and the related ROnToTools/Pathway-Express method described below, are methods designed to quantify the impact that differentially expressed (DE) genes have on the downstream elements of the pathway by taking into account the number of DE genes, the magnitude of the expression differences, and the direction and type of edges in the pathway. In SPIA, a “perturbation factor” is computed for each gene $g$, defined as the weighted sum of the expression differences of all the DE genes which are direct upstream parents of $g$ in the network. The weighting coefficients are a function of both the number of outgoing edges of the parent node, as well as the type of edge (positive for induction/activation, negative for repression/inhibition) connecting the parent to the child node $g$. The total accumulated perturbations of all genes in the pathway is compared against a null distribution obtained by repeatedly randomizing the observed expression differences to different genes in the network. SPIA combines the resulting perturbation $p$ value, $p\text{.PERT}$, with the $p$ value from the simple hypergeometric overrepresentation test for the pathway, $p\text{.ORA}$, using Stouffer’s weighted-$z$ method [39], yielding a pathway graph $p$ value ($p\text{.Comb}$). Thus, a pathway with DE genes at its entry point could be deemed more significant than one with DE genes further downstream, even if the fold changes are smaller.

Although SPIA is described in the context of differential gene expression, the method can in principle be used with any type of gene-level association statistic (e.g., hazard ratios for survival, minor allele frequencies of SNP variants, or multivariate $F$ or $T^2$ statistics testing association across more than two phenotypes). However, SPIA requires that the user set a significance threshold to select the genes that are considered in the analysis.

ROntoTools/Pathway Express  ROnToTools/Pathway Express (ROT/pe) [16–18] is a refinement upon SPIA. In SPIA, a gene significance threshold is used to select DE genes, and the DE genes are treated equally, while those that are not DE are not considered in the analysis. To enable a more nuanced analysis, ROT/pe weights the change in expression of each gene by the significance of the gene level statistics. As a result, marginally significant changes in expression are given less weight than a highly significant changes of the same magnitude. Furthermore, this method permits a “cut-off free” analysis that assigns non-significant genes a low weight rather than aggressively discarding them as non-DE. When no cutoff is used, ROT/pe does not calculate the overrepresentation significance $p\text{.ORA}$, but simply reports the significance of the perturbation (obtained, as in SPIA, by randomly permuting the gene expression differences across the network).

PathNet  PathNet [19] incorporates pathway topology by computing both a “direct” and “indirect” association statistic for each gene. The “direct” statistic is the result of a single–gene association test (e.g., a classical $t$ test of differential expression). Once these are obtained, PathNet then computes “indirect” statistic for each gene, defined as the sum of the $-\log_{10} p$ values of the direct associations for all the gene’s neighbors in the pathway. The direct and indirect scores for each gene are summed and tested for significance by permuting the direct evidence statistic across all genes in a “global” network formed by merging pathways with common genes. The resulting gene–level $p$ values are then thresholded for significance, and the pathway is tested for enrichment using a hypergeometric test.

Like SPIA and ROT/pe, PathNet can accommodate a variety of gene–level metrics and is not restricted to simple differential expression. In contrast to SPIA and ROT/pe, PathNet considers all possible edges in the global network, not simply those confined to a single pathway, enabling it to find pathways that are strongly affected through indirect links. Also in contrast to both SPIA and ROT/pe, PathNet does not distinguish between inhibitory and activating edges. PathNet’s use of thresholding for testing pathway significance is also a drawback, but could easily be overcome by using a GSEA-like procedure instead of the hypergeometric test.
**NEA**  As in PathNet, Network Enrichment Analysis (NEA) [20,21] also attempts to incorporate information from the “global” network of pathways formed by merging the common genes in the individual functional pathways represented in the database, but uses a different approach to quantify enrichment. Rather than counting significant nodes (i.e. genes) in each pathway of interest, NEA counts the number of edges in the pathway connecting to a significance gene. This is done by summing the degree (i.e., number of edges) for each DE gene on the pathway and amongst its neighbors, subtracting out links from genes that do not connect to a gene in the pathway. Genes outside the pathway will thus contribute to the pathway’s score; connections between two DE genes on the pathway are counted twice (once per each gene). NEA assess the significance of this statistic by randomly rewiring the topology of the “global” network, conditioned on preserving the degree of each node.

Like all the foregoing methods, any gene-level statistic of interest may be used as an input to NEA. However, NEA as implemented is dependent upon setting cutoffs for gene significance in order to count the number of DE links, making the results susceptible to noise induced by thresholding. It may be of interest to investigate possible refinements of NEA using a weighting scheme rather than a strict binary cutoff. Also, NEA as currently implemented does not consider the direction of the edges in the graph, but rather treats all edges as bi-directional. Direction-based refinements to NEA may thus also be of interest.

**CePa**  All of the above methods improve upon GSEA by not only considering each gene’s differential expression, but also examining the differential expression of its nearest–neighbors (“perturbations” from upstream genes in SPIA and ROT/pe, “indirect evidence” in PathNet, number of DE links in NEA). The resulting pathway scores thus represent an accumulation of localized effects within the pathway. In contrast to these approaches, CePa [22, 23] attempts to take a broader view of the network by incorporating graph centrality measures into the statistics.

Briefly, the “centrality” of a node in a graph is a measurement of its relative importance to the rest of the network [40]. One simple centrality measure is the node’s degree: the number of edges connecting to that node. On a directed graph, the in–/out–degrees, which are the counts of a node’s incoming/outgoing edges respectively, are also useful for quantifying how susceptible/influential a node is. “Betweenness,” another centrality measure, quantifies how frequently the shortest path between any two nodes goes through the node of interest.

CePa uses the centrality measures of the genes in the network to perform either an “over representation analysis” (CePa-ORA) or a “gene set analysis” (CePa-GSA). In CePa-ORA, the user specifies a set of significant genes (based on some pre-determined significance threshold); CePa-ORA then sums the centrality measures of the significant genes and calculates the significance of the sum by randomly selecting a new set of “significant” genes based on the proportion of truly observed significant genes in the network. That is, CePa-ORA tests whether significant differential expression is more likely to be high–centrality nodes in the pathway than would be expected by chance.

The CePa–GSA variant incorporates the gene–level statistics (such as the $t$ statistic from a test of differential expression) rather than using only the list of significant genes. CePa-GSA multiplies the gene statistics by the gene centrality measures. In this way, CePa directly weights each gene’s statistic by its importance in the network, reflecting the observation made by Jeong and others [14] that alterations to more central genes have a more profound impact on an organism. CePa–GSA then aggregates the weighted gene–level statistics into a single pathway–level statistic by taking, for example, their maximum or median. As implemented in the CePa package, the default gene–level statistic is the absolute value of the $t$ statistic from a two–sample test of differential expression, and the default pathway–level statistic is the mean of the centrality–weighted gene–level statistics. The significance of this pathway–level score is calculated by permuting the sample labels of the gene expression matrix, recomputing the gene–level statistics with the permuted samples, and then recomputing the pathway statistic. The resulting $p$ value quantifies the differences in centrality–weighted gene expression that are associated with the phenotypic differences of interest.

While in principle both CePa-ORA and CePa-GSA can use any gene–level statistics, at present the implementation of CePa-GSA is such that the user is confined only to two–sample $t$–tests of differential expression. If, for instance, the outcome of interest is survival (yielding hazard ratios for each gene), or if
differences across multiple conditions or phenotypes are being assessed with a multivariate linear model or ANOVA, or if the input data is categorical rather than continuous (such as allele frequencies from SNP GWAS studies), the user has no choice other than to precompute the gene–level $p$ values and use the thresholded CePa-ORA analysis rather than the more nuanced CePa-GSA approach. It would be of considerable interest to address this limitation by providing a more flexible interface to CePa-GSA that permits user–defined models. In addition, while CePa provides a very rich view of the centrality–weighted pathway analysis, the variety of statistics it obtains (one per each of 6 centrality metrics considered) and the diverse options for pathway aggregation (max, mean, etc., as well as the option for user–defined function) can make the results difficult to interpret. Finally, it may also be argued that linear centrality–weighting (as opposed to, e.g., weighing by a power function of the centrality) is an arbitrary choice that may influence the results.

DEGraph
An alternative topology–weighting approach is implemented in DEGraph [24], which uses a multivariate Hotelling $T^2$ test to identify pathways in which a significant subset of genes are differentially expressed. In DEGraph, both the standard $T^2$ and a network–smoothed graph $T^2$ are computed. Based on the intuition that two genes connected in the network should behave in a correlated fashion, DEGraph’s network–smoothing filters the gene expression differences by keeping only the first few components of its projection onto a basis defined by the graph Laplacian [41]. Spectral decomposition of the pathway’s graph Laplacian encapsulates the geometry of the network topology at progressively finer scales, and the projection of the gene expression shifts onto the coarsest components may be analogized to keeping only the lowest frequency Fourier components of a function. These network–smoothed shifts are then tested using the Hotelling $T^2$ test.

Unlike CePa, DEGraph provides a means to obtain pathway–wide topology–weighted scores without making arbitrary choices about the weighting. In particular, the mathematical and statistical properties of graph Laplacian spectra are well–characterized [41,42] and can be precisely related to the dynamical properties of the network [43,44], making this approach more justifiable than the weighting scheme proposed in CePa. However, this spectral approach is necessarily confined to operate only on connected components, posing problems for pathways comprising several disjoint subgraphs (e.g., two sub-processes connected by an exogenous stimulus that is not represented in the gene network). In such cases, the pathway will have several $T^2$ statistics—one for each connected component—leaving a choice as to whether the pathway $p$ value should be defined by that of its largest subnetwork or some combination of all its subnetworks. In addition, the use of the Hotelling $T^2$ test requires that the gene–level statistics follow a multivariate normal distribution, which limits the types of data and analyses to which DEGraph could be applied. In consequence, the DEGraph implementation is only capable of testing pathway–wide multi–gene differential expression between two sample classes. Recently, a more flexible spectral decomposition method has been proposed [31,45], but which relies upon computationally intensive permutation tests. It would be highly interesting to extend DEGraph to accommodate other data types and statistical tests.

Topology GSA
Although analyzing the data at the pathway level can identify important systems–level changes that would be missed by single–gene analyses, the pathway–level findings can be difficult to interpret and validate for large networks. While identifying functionally significant pathways is the goal of systems biology, it is often necessary to identify specific features within those networks that can be targeted experimentally. To facilitate the discovery of targetable sets of genes and interactions, a number of techniques have been proposed to search for significant submodules within pathways. One such method is TopologyGSA [25,26], which uses a Gaussian network model to identify significant subnetworks in the graph. TopologyGSA begins by transforming the directed pathway network graph into its so–called “moral” graph by connecting all “parent” nodes of a vertex and removing the edge directionality. The moral graph is then decomposed into cliques: subsets of nodes in the graph for which every pair is connected by an edge (triangles are the simplest cliques; a clique of four nodes has 6 edges; a clique of five has 10; etc). Each clique is then tested for differential expression by modeling the expression of the genes in the network as Gaussian random variables, subject to the class–conditional covariance between the clique’s genes for each phenotype. If no significant cliques are found, edges are iteratively added and the statistics recomputed. The result is the identification not only of pathways with significant gene expression differences, but of specific connected
subgraphs within the pathways that can then be investigated in greater detail.

An appealing feature of TopologyGSA is the use of gene covariance in the analysis. Despite the fact that genes often exhibit considerable correlations in expression, most pathway analyses consider the genes as independent variables. However, TopologyGSA’s approach of conditioning the clique statistics on phenotype-specific covariances also introduces a limitation: like DEGraph, it can only be used if the outcome of interest can be dichotomized.

Moreover, while testing cliques of a triangulated graph to detect significant subnetworks is an interesting idea, it has a very serious drawback: finding all maximal cliques in a graph is an \( NP \)-hard problem, meaning that a solution is not guaranteed in polynomial time. In particular, a brute-force search for a clique of size \( k \) in a graph with \( n \) nodes has a computational complexity of \( O(n^k k^2) \). While approximations can be made, a recent proof by Chen [46] demonstrated that the clique problem cannot be solved in less than \( f(k)n^{o(k)} \) time for any function \( f \) and linear function \( o(k) \). As the size of the pathway \( n \) grows, the size of the submodule cliques \( k \) also increases, and time needed to search for those submodules increases exponentially. This means that for larger pathways—often the very ones for which submodule detection is desirable—the problem TopologyGSA attempts to solve may be intractable. Because TopologyGSA iteratively adds edges as part of the significance computation, the exponential cost is incurred on each iteration. A far more efficient approach would be to use spectral methods to identify communities of nodes [41, 42, 47, 48] rather than articulating all maximal cliques. Such an approach, which would be closely related to yet still distinct from the spectral used in DEGraph [24] and Pathway/PDM [31, 45], would permit the identification of connected subnets of significant genes far more efficiently than solving the clique problem.

Evaluating the Performance of Network Analysis Methods

As described above and shown in Table 1, these network analysis methods have different features that make them better suited to some use cases than others. Nevertheless, for many common analyses, most of these approaches could be applied, and the user is faced with a choice between several promising methods. Unfortunately, benchmark tests to systematically evaluate the performance of network analysis methods remain lacking, limiting the community’s ability to compare methods.

The development of a systematic evaluation framework faces a number of challenges. First, the methods themselves have highly disparate implementations, often using different databases and pathway semantics, making them difficult to compare in a consistent way. For example, PathOlogist [32, 33] treats pathways as bipartite graphs of genes and interactions in contrast to the gene–mode networks considered by the methods described above, and is restricted in its implementation to pathways from the NCI/PID [4] database. Secondly, it is not clear what the “gold standard” for these methods should be. Unlike machine learning and network inference problems which are readily tested against simulated benchmark data with known solutions (such as the in-silico data suites used in the DREAM Challenges [49]), there is no agreement on what the “correct” results of these analyses would be.

To provide intuition regarding the performance of the methods reviewed here, we systematically applied them to a curated suite of gene expression data from 10 ovarian cancer studies [38] using a common set of pathway definitions obtained from the KEGG database. The goal of these tests was not only to supply provisional guidance about the relative performance of the methods, but also to suggest a strategy for a testing framework for pathway analysis methods.

Methods

Our approach is motivated by the observation that systems–level analyses improve the concordance of results between different studies of the same phenotype [45, 50]. Although multiple studies of the same phenotype may yield very different lists of significant genes, pathway analyses tend to to show much greater agreement. This effect is not unexpected, considering the complexity of biological systems [51] and the noisiness of HT data; individual disease–associated genes may be detected in some studies but miss the significance threshold in others. However, if a pathway is functionally related to the disease, we may reasonably expect to detect its association across multiple studies, even if the specific genes contributing to its significance vary from one study to the next.
This observation leads to the following conjectures: If a specific pathway is functionally related to a particular phenotype, we expect that some manifestation of its involvement will be present in the data for all studies of that disease, and that an accurate and sensitive network analysis approach will detect those signals consistently across the studies. A poor network analysis method, on the other hand, will yield results that are strongly influenced by noise in the data, and hence will detect pathways that are particular to each study rather than the common biological signal. On the basis of this conjecture, we use the cross–study concordance of each method’s results to measure its ability to detect a common (and presumably “true”) signal in each of the studies.

**Ovarian Cancer Data**

For the purposes of our analysis, we used gene expression and clinical information from curatedOvarian-Data [38], an expert–curated collection of uniformly prepared microarray data and documented clinical metadata from 23 ovarian cancer studies totalling 2970 patients. The curatedOvarianData project was designed to facilitate gene expression meta-analysis as well as software development. By providing a consistent representation of data that has been processed to ensure comparability between studies, the package enables users to immediately analyze the data without needing to reconcile different microarray technologies, study designs, expression preprocessing methods, or clinical data formats.

Because several of the methods under consideration were limited to two–sample comparisons, we selected data sets with sample classes that could be meaningfully dichotomized. Since the vast majority of the samples came from patients with stage III cancers, tumor/normal and stage–based comparisons were not feasible; instead, we chose to compare low– and high–grade ovarian serous carcinomas. These grades have distinct histological features, molecular characteristics, and clinical outcomes [52, 53]. Low–grade serous carcinomas typically evolve slowly from adenofibromas, acquiring over time frequent mutations to KRAS, BRAF, or ERBB2 genes, but not TP53 mutations. In contrast, high–grade serous carcinomas are characterized by TP53 mutations, often without mutations to KRAS, BRAF, or ERBB2. They arise from unknown precursor lesions, progress rapidly, and have worse clinical outcomes. For this analysis, we selected studies with a minimum of 15 high– and low–grade serous carcinomas and 1000 genes assayed, keeping only the patients who fell into those categories and who had survival data. 10 of the 23 available studies met these criteria. The study accession numbers and sample counts are given in Table 2.

The microarray data was filtered to keep only the genes common to all 10 studies; no other filtering was done. This resulted in 7680 genes common to all 10 studies.

To obtain the gene–level statistics required by several of the analyses, the R limma package [54] was used. \( \log_2 \) fold changes were used for the magnitude of differential expression when required; the significance of the association was quantified using the \( p \) value for the empirical Bayes estimated \( t \) statistic [54]. Where thresholds for significance were needed, the 0.05 most significant genes were selected. (NB, this corresponds to the 0.05 quantile of significance, \( not p = 0.05 \). Because the studies varied considerably in their sample sizes, and hence power, we chose to use a quantile–based threshold rather than a \( p \) value threshold to render them comparable. While the \( p \) value for the 0.05 quantile varied from study to study, in all cases this corresponded to \( p \ll 0.05 \).)

**Network Models**

In order to ensure that each of the eight methods tested used a common, comparable set of pathway definitions, we created the pathway annotation objects required for each method by hand from a fresh download of the KEGG pathway database [1]. The KEGGgraph R package [55] was used to obtain the pathway KGML files for 247 human pathways. The KGML files were first processed into R graphNEL objects for use by ROn-toTools/PathwayExpress (ROT/pe), TopologyGSA, and DEGraph. The graphNEL objects were then used to generate the lists of genes, edges, and adjacency matrices variously required by GSEA, PathNet, NEA, and CePa. The path.info data used by SPIA was also generated from the KEGG graphNEL objects and written to disk as required by SPIA. In this way, we ensured that the set of pathways considered by each method would be directly comparable to each other. The complete set of pathway annotation objects for all the methods, along with an R script to generate a complete set of updated mappings from a fresh KEGG...
download, is available from http://braun.tx0.org/netRev.

Application

Each of the methods shown in Table 1 was applied to all 10 data sets described in Table 2 for all 247 KEGG pathways in our database. Where permutation tests were required, 1000 permutations were used. Several of the methods have options that permit different styles of analysis, which we also explored. The details of our application is given below:

GSEA

As a point of reference, the non–networked GSEA was applied as described in [56], using the gene \( p \)-values obtained from limma as described above. Significance was tested using 1000 permutations of the sample classes.

SPIA

SPIA was applied using an 0.05–quantile threshold for significance as described above. A number of the pathways had no edges considered by SPIA (which only considers directed edges, cf. [15,36]), and so were preemptively excluded from the analysis by the package. The overrepresentation and perturbation \( p \)-values were combined using Stouffer’s normal–inverse method.

ROT/pe, cutoff

ROT/pe permits analysis with and without a \( p \) value threshold [16–18]; we applied both. Here, we used the same threshold as in SPIA, meaning that the results should be roughly comparable to SPIA for the \( p \).ORA overrepresentation analysis. In contrast to SPIA, however, pPert is now weighted by the gene’s significance, rather than treating all significant genes equally. Genes not meeting the significance threshold are excluded from \( p \).Pert with 0 weight, similar to the exclusion in SPIA. \( p \).Pert and \( p \).ORA were combined as in SPIA.

ROT/pe, no cut

We performed ROT/pe without a significance threshold. Because the hypergeometric test cannot be performed without setting a cut-off, only pPert is reported. In contrast to the thresholded analysis, pPert now involves data from all the genes, although those with low significance will have low weighing.

PathNet

PathNet was carried out as described above and in [19]. PathNet returns both the PathNet \( p \) value combining the “direct” and “indirect” evidence, along with the simple hypergeometric \( p \) value. The quantity of interest is \( p \text{PathNet} \).

NEA

NEA was carried out as described above and in [20,21], using the same gene thresholds and number of permutations as in the other studies.

CePa-ORA

Like ROT/pe, CePa also has options to perform the analyses with or without setting a gene–significance threshold [22,23]. We performed both; here, we use the same thresholds used in the other analyses to carry out CePa-ORA. As described above, CePa will report the significance using a variety of network centrality measures. Because there is no clear choice of which one is correct, we chose to combine all six measures into a single \( p \) value for CePa-ORA using Stouffer’s method.

CePa-GSA

We also performed the non-thresholded CePa-GSA. The analysis differs from CePa-ORA not only in the number of genes considered, but also in the type of hypothesis test performed. While CePa-ORA tests a “competitive” hypothesis [7] comparing pathways to random subsets of genes while holding fixed the sample labels (and hence the gene–level statistics), CePa-GSA tests the “self–contained” null hypothesis by permuting the sample labels while holding the pathway definition fixed (see Table 1).
The two tests are orthogonal to each other, and we do not anticipate that the results of CePa-GSA will necessarily be the same as those for CePa-ORA. As in CePa-ORA, we chose to combine the six $p$'s into one $p$ value for CePa-GSA using Stouffer’s method.

**DEGraph**

DEGraph also presents several alternative analysis approaches, specifically, whether or not the network should be signed (corresponding to inversely–related nodes) or unsigned for the purposes of computing the smoothing vector [24]. We performed both the signed and unsigned analyses. For each of these, we also had to make a decision regarding how to handle pathways with more than one connected component, and hence more than one $p$-value. We tried both simply taking the $p$ value for the largest connected component as the $p$ value for the pathway, as well as combining the $p$ values for all the components using Stouffer’s method. DEGraph will report both the non-networked $p(T^2)$ as well as the graph–smoothed $p(T^2)$ graph; the latter is the primary quantity of interest.

**TopologyGSA**

Finally, we attempted to apply TopologyGSA [25,26] as implemented to our data. In principle, TopologyGSA reports $p$ values for both differential variance and differential mean expression across the pathway submodules, both of which are of interest.

**Results**

**Computational efficiency**

The computational time to complete each each of the analyses on a desktop machine (3.4 GHz quad-core Intel Core i7 iMac with 16GB RAM) is given in Table 3. Each of the methods shown in Table 1 was applied to all 10 data sets described in Table 2 for 247 pathways. Where permutation tests were required, 1000 permutations were used. All jobs completed the calculation in under an hour per study with the exception of NEA, which required $\sim$2.5 hrs/study, and TopologyGSA, which failed to complete even the first analysis when it was finally halted after 100hrs (>4 days). Interestingly, ROT/pe, which is a weighted modification of the same computation carried out in SPIA, required less time than SPIA (and, additionally, was able to treat more pathways), which we attribute to code improvements by the authors of both methods [15–18,36] and made ROT/pe amongst the fastest of the methods we tested. Nevertheless, with most methods taking only a few minutes per study, the differences in computational cost between them are minor.

With the exception of TopologyGSA, which we will return to below, and DEGraph, the major computational cost is due to permutation testing. (In DEGraph, the computation of the smoothing vector scales as $O(n^3)$ where $n$ is the number of genes in the pathway graph, and so can be cumbersome for very large pathways; however, DEGraph does not require permutation tests.) Permutation testing is trivially parallelizable, and the development of parallel R libraries such as snow [57] facilitates development of packages that can be run on clusters. Yet, of the packages considered here, only CePa provides a parallelized implementation.

On inspection, it became clear that TopologyGSA had been working for over 90 CPU hours to obtain the maximal cliques for a single pathway. As expected, the clique problem had become intractable; an example of the computational cost of the clique algorithm used in TopologyGSA for a moderately sized KEGG network is given in Supplementary Table S1. Unfortunately, TopologyGSA has no mechanism built in to monitor and bail out of the computation, nor does it check the size of the transformed pathway before invoking the max clique algorithm so that potentially problematic pathways can be skipped. Running TopologyGSA thus requires that the user confine the analyses to smaller pathways, but because the computation depends on the density of the graph after moralization and triangularization, doing so is not as simple as merely selecting smaller pathways. While the user could transform the graphs by hand to make a selection of tractable pathways, these checks should really be built into the package. The issue of exponential cost should also be well–documented, ideally with a small benchmark that the user can run to estimate the computational time required on their machine (e.g., extrapolating from a table such as Supplementary Table S1), so that the user can make informed decisions about the application of the method to particular pathways.
Analyses

As discussed above, we posit that if a pathway is functionally related to a particular phenotype (here, high vs. low grade ovarian cancer), we expect that a manifestation of its involvement will be present in the data for all studies of that disease, and that an accurate and sensitive network analysis approach will detect those signals consistently across the studies while a poor network analysis method will yield results that are strongly influenced by noise in the data and will vary strongly from one study to another. Based on this intuition, we consider the cross–study concordance of each method’s results to measure its ability to detect a common (and presumably “true”) signal in each of the studies.

Cross–study concordance

For each method tested, we examined the correlation of \( p \)-values obtained for the 247 pathways between all 45 possible pairs of the 10 studies listed in Table 2. For reference, we also began by examining the correlation in gene–level statistics between the studies. Because each method has a different range/resolution of possible \( p \) values owing to the different uses of parametric and nonparametric tests, we use the nonparametric Spearman’s rank correlation as a measure of concordance.

Figure 2 depicts the correlation of results for all 45 study pairs. For completeness, we show the cross–study correlations for each method’s subanalyses, in addition to the “final” combined result. This includes the \( p \)ORA and \( p \)Pert values for SPIA and ROT/pe (both with and without a cutoff) that are combined to form the final \( p \)Comb value for the pathway; the standard \( p \)Hyper hypergeometric test along with the network edge–based hypergeometric test from PathNet; the results for all six centrality measures considered by CePa ORA and GSA analyses, which are then combined into \( p \)Comb; and the DEGraph results for the standard Hotelling \( T^2 \) and the network–smoothed \( pT^2\)graph for the largest connected component (lcc) and the full pathway (\( p \)'s for all connected components combined) for both the signed and unsigned analyses. The “final” results for each analysis are denoted by bolding. Supplementary Figure File S1 provides a more detailed view of the these correlations. To aid in the interpretation of Fig. 2, Fig. 3 presents a summary of the cross–study correlations for each of the “final” results. Here, the distribution of correlations for each of the 10 studies with respect to all other studies is shown for each of the methods.

As can be seen in the top row of Fig. 2 and first panel of Fig. 3, the correlation of gene–level statistics is often poor; we obtained a maximum rank correlation \( \rho = 0.20 \) for the gene \( p \)-values, with a median of \( \sim 0.02 \). This lack of correspondence even amongst studies of the same phenotype has been observed in other investigations of microarray data [50]. Also as previously observed in other studies [45,50], we found that the correlations were generally improved in pathway analyses, both in terms of the number of positively–correlated study pairs (fewer blue cells in Fig. 2) and in the magnitude of the positive correlations (cf Figs. 2,3). Considering that each study is interrogating gene expression in the same phenotypes, we ideally desire that the correlations between all study pairs should be positive, and indeed many were, with the CePa GSA analyses being the strongest and most consistent (cf Fig. 3).

In addition, we also observe that the concordance for the network–based analyses was always stronger than that exhibited by non–network analyses. In Fig. 2, this can be seen in the darker blue cells of GSEA and both SPIA and ROT/pe \( p \)ORA sub-analysis values, all of which measure enrichment without considering the network topology. In Fig. 3, this difference is manifest by the lower medians and tails for the GSEA boxplot versus the others.

It is also instructive to consider the cross–study correlations for each method in the context of the gene–level concordance and the sample size of the studies. The study pairs depicted in Fig. 2 are sorted in order of gene–level concordance. Generally, we expect that studies which have greater similarity at the gene level will also exhibit greater similarity in the pathway statistics, and in most cases this pattern holds, with lower correlations at the left end of the plot. Qualitative departures from this trend can be seen in the SPIA, NEA, and CePa–ORA betweenness results. Moreover, we expect that two large studies will exhibit greater correlation than two smaller studies, on the basis of the intuition that two large studies are better powered to distinguish subtle but biologically meaningful, and hence consistent, effects from noise. In Fig. 2, the sum of the sample sizes for each study pair is given in the bottom row, and the pattern of study sizes can be seen to be most strongly manifest in the various CePa analyses.
**Consistency between methods**  A similar intuition regarding the concordance of findings may be had regarding the results of the various methods within a given study. That is, despite the differences between the methods, we might reasonably expect that if a pathway is strongly affected in a particular study, it will be detected by more than one of these methods, i.e., that for a given study the results from SPIA, ROT/pe, NEA, &c. will be correlated. Figure 4 shows, for each study, the distribution of pathway \( p \)-value correlations that each method had with all other methods. A more detailed view is provided in Supplementary Figure File S2. As above, we use Spearman’s rank correlation as a measure of concordance to account for methodological differences influencing the dynamic range of \( p \) values. NEA analyses tended to give results that disagree with methods obtained from the other analyses (cf Fig. 4), for reasons we ill discuss further below, while the other methods are fairly comparable.

While the above comparisons consider correlations for the whole range of \( p \) values returned by each method, we now consider how consistent the selection of “significant” pathways is for the various methods. The heatmap in Figure 5 depicts the number of times that a pathway was ranked in the top 20\% for the 10 studies listed in Table 2. that a pathway was ranked in the top 20\%. Results from all subanalyses of each method are given, including the \( p \)ORA and \( p \)Pert values for SPIA and ROT/pe (with and without a cutoff) that are combined to form the final \( p \)Comb value for the pathway; the standard \( p \)Hyper hypergeometric test and the network edge–based hypergeometric test from PathNet; the results for all six centrality measures considered by CePa ORA and GSA analyses that are then combined into \( p \)Comb; and DEGraph results for the standard Hotelling \( T^2 \) test \( pT2 \) and the network–smoothed \( pT2graph \) for the largest connected component (lcc) and the full pathway (\( p \)’s for all connected components combined) for both the signed and unsigned analyses. The “final” results for each analysis are denoted by bolding. The pathways are sorted by the average across the bolded analyses, such that (eg) CePa–GSA contributes to that average once as opposed to seven times. The probabilities for the counts under the null hypothesis are also shown; our expectation is that we will see, by chance, counts of 0–4 with 95\% probability, while counts > 5 should occur only once by chance amongst the 247 pathways. On the other hand, pathways for which the null hypothesis is indeed false should exhibit high counts more frequently, corresponding to their detection in multiple studies. Generally, this pattern appears to qualitatively hold, and the pathways which are deemed significant in > 5 studies tend to detected consistently by most of the methods (with the exception of NEA). This suggests that the results are being driven by commonalities across studies, and that those common patterns are detectable by many of the methods considered.

**Distribution of results**  A more detailed understanding of these patterns emerges from looking at the concordance between methods for all pathways in all studies, shown in Fig. 6. In the upper triangle of plots, the joint distributions of \( -\log_{10} \) \( p \) values are reported; the corresponding correlation coefficients are shown in the lower triangle. Note that, as above, rank correlation coefficients are reported (and so may differ from “by eye” estimates). On the diagonal, histograms of \( -\log_{10} p \) are plotted in red; the black lines show the expected distribution of \( -\log_{10} p \) corresponding to the uniform distribution of \( p \) values expected under the null. Strikingly, it can be seen from the histograms in Fig. 6 that the \( p \) values obtained from NEA are strongly biased toward highly significant \( p \) values; indeed, over half of all NEA \( pZ \) values fall \( \leq 10^{-3} \), whereas we expect that the proportion would be \( \sim 1/1000 \). This causes an extremely large fraction of pathways to be deemed statistically significant even after adjusting for multiple hypotheses in NEA, making it difficult to discriminate truly significant pathways using the current implementation. Such severely skewed \( p \)-value distributions are generally attributable to an incorrect null model.\(^1\) The other methods follow the theoretical \( p \) value distributions relatively well, with a slight deviation observed in CePa–GSA.

**Discussion**  
Our review indicates a number of benefits and drawbacks associated with each method, some of which are inherent to the underlying methodology, and others of which are consequences of the implementations.

\(^1\)In the case of NEA, we believe there may be a simple remedy for the incorrect null model. Specifically, we note that the NEA package does not distinguish genes that are assayed and deemed non-significant from genes those that are simply not assayed. By treating non-assayed genes as insignificant, the proportion of significant genes is significantly reduced, lowering the probability of significant edges in the resampled graphs.
Methodological considerations

In Table 1, the design features of each method are listed. A major distinction between the methods is the need for gene $p$-value thresholding. Threshold–free analyses are generally considered preferable to those that require gene–level significance thresholds, since the threshold introduces an arbitrary choice and excludes the full spectrum of data from the analysis; for this reason, GSEA is preferred to hypergeometric enrichment tests [7,8] for non-network pathway analyses. Of the network–based methods, SPIA, PathNet, and NEA have the common drawback of requiring thresholding on gene–level significance; in contrast, CePa and ROT/pe provide threshold–free options, and the DEGraph and TopologyGSA analyses are threshold–free by design. However, it should be noted that the cutoff–free ROT/pe analysis differs substantially from the cutoff based ROT/pe analysis. Specifically, ROT/pe with a cutoff computes both the downstream perturbation analysis ($p_{\text{Pert}}$) and a hypergeometric over-representation analysis ($p_{\text{ORA}}$) which are then combined to measure the impact of differential expression on a pathway, while in the cutoff–free analysis, only the downstream perturbations $p_{\text{Pert}}$ are considered. This limitation could be overcome simply by using a GSEA-like analysis in place of the hypergeometric test, enabling both $p_{\text{ORA}}$ and $p_{\text{Pert}}$ to be computed with or without a threshold.

The second major methodological distinction is in the type of hypothesis being tested by the methods. “Competitive” null hypotheses compare the pathway of interest to a random pathway while holding the sample classes (or gene–level associations) fixed, whereas “self-contained” null hypotheses test if the pathway is more strongly associated with a particular phenotypic attribute than expected by chance given the genes and topology of the pathway [7]. The two tests represent two different conditional probabilities (“competitive” being conditioned on the sample labels, allowing the definition of the pathway to vary; the “self–contained” being conditioned on the pathway definition, but allowing the sample classes to vary), and may thus give different results. The “self–contained” null is considered superior since it is both better justified biologically (“competitive” permutations tests will create physiologically unrealistic pathways) and directly answers the question of whether a particular pathway is associated with the phenotype of interest. Unfortunately, though, methods testing the “self–contained” null tend also to be limited in the type of data that can be used: DEGraph, TopologyGSA, and CePa-GSA are limited to two–sample comparisons of continuous data, making them unsuitable for survival analysis or application to GWAS SNP data. However, while this limitation is inherent to the distributional assumptions made in DEGraph (which uses Hotelling’s $T^2$ test) and TopologyGSA (which uses a Gaussian network model), it is only an implementation limitation in CePa-GSA rather than a methodological constraint. A revision of the CePa package with a more flexible interface would provide a threshold–free, “self–contained” network analysis tool that could be applied to a broad variety of studies.

Ease of use

All network methods tested are provided as R or BioConductor packages, making them easily adoptable. In addition, SPIA, ROT/pe, TopologyGSA, and DEGraph accept R graphNEL [58] objects describing pathways, making them easy to use with pathway annotation packages such as KEGGgraph [55], GRAPHITE [59], NCIgraph [60], &c., without additional preprocessing.

A more serious consideration regarding the ease of use is the computation time, shown in Table 3. As discussed above, most methods are comparably efficient with the exception of NEA and TopologyGSA. We could identify no methodological factor that contributes to NEA’s lengthy run-time, and believe that it is likely due to inefficiencies in the R implementation. In contrast, TopologyGSA’s inefficiency is an unavoidable consequence of the method’s reliance on solving the NP-hard maximal clique problem. The exponential complexity can easily become intractable for even moderately sized pathways, illustrated in Supplementary Table S1. At minimum, checks should be built into the TopologyGSA package to conservatively skip pathways that may fail to be solved in a reasonable amount of time (e.g., by rejecting pathways with more than a certain number of nodes or edges after moralization and triangulation). However, it is not clear that the maximal clique problem necessarily needs to be solved. Arguably, simply detecting community structure within the network (i.e., finding dense subgraphs without requiring that all pairs of nodes are completely connected as in a clique) is sufficient to define pathway “modules,” which can then be analyzed in the same
way as the cliques currently are. Detecting community structure/clusters within a graph is readily achieved by spectral methods \([41,42,45,47,48]\), that solve a relaxation of the problem in \(O(n^3)\) time. Computational efficiency of many of these methods could be further improved by parallelized implementations, a feature that only CePa has to date.

**Reliability of results**

In absence of “gold–standard” data against which to benchmark these analyses, we attempted to characterize their reliability based on the concordance of their results in a suite of comparable ovarian cancer studies (Table 2).

Based on the intuition that studies of similar phenotypes should yield similar results, we examined the correlation of pathway statistics obtained by each method amongst the 10 studies. In general, we found greater concordance in the pathway network analysis results than in the simple gene–level analysis (cf. Figs. 2,3), which was the expected and desirable result from studies of the same phenotype \([50]\). We also observed that the network–based analyses generally gave more concordant results than the non-network GSEA analysis (Fig. 3). The greatest increases in cross–study concordance were obtained with ROT/pe and CePa (Figs. 2,3). In addition, we expected that meaningful cross–study concordance of pathway results would be positively influenced by both gene–level concordance and the power of the studies under consideration.

With the exceptions of NEA and the SPIA analyses, the correlation of pathway–level concordance with gene–level concordance is visible for all methods in Fig. 2, meeting our expectations. The correlation of concordance with sample size is most clearly manifest only in the CePa-GSA analyses, and, weakly, in DEGraph. We are thus inclined to believe that the improved cross–study concordance amongst the CePa and DEGraph are attributable to their detection of common biological signals across the 10 studies.

Based on the conjecture that biological “truths” should be detectable despite slight methodological differences between the analyses, we also examined the concordance of findings between various methods. Fig. 4 shows the correlations in pathway \(p\) values amongst the methods for each study. Most methods yielded comparable results, with the exception of NEA. Correlations in the pathways consistently identified as being in the top 20% significant can also be seen amongst SPIA, ROT/pe, PathNet, CePa, and DEGraph in Fig. 5. By contrast, NEA tends to detect consistently significant pathways that are infrequently found amongst the top 20% in any study using the other methods.

In addition to investigating the cross–study and cross–method concordance, we also examined the distribution of \(p\) values obtained for the methods, shown in Fig. 6. It is expected that most of the 247 pathways tested are not significantly associated with ovarian cancer, and thus the distribution of \(p\) values should be, by definition, uniformly distributed on \([0,1]\) with the exception of a handful of significant pathways. However, as seen in Fig. 6 and discussed above, the network enrichment \(pZ\) computed in NEA are exceedingly small a majority of the time, indicating a likely problem with the null model used in NEA.\(^1\)

More generally, this observation raises questions about what constitutes an appropriate null model for network analyses. In the context of the “self–contained” hypothesis tests, the answer is straight–forward: one permutes the sample labels, leaving the network itself intact. For the “competitive” hypothesis, however, the answer is far less clear. The difficulties in constructing null models that accurately preserve the statistical and graph theoretic properties of networks have been considered by ourselves and others \([45,61]\). Most notably, simple randomization of node properties or graph rewiring will produce null models that lack the assortativity found in biological interaction networks. That is, because of the underlying biology, groups of genes that are connected in pathway databases will likely exhibit correlated expression (and hence correlated gene–level significance) in experimental data. By randomizing the data across the network, that property is destroyed, resulting in network models that are not as structured as those found in nature. Methods using such null models will yield inflated significance, since the data is being compared against naïve random networks rather than biologically plausible random networks. The test of the “competitive” null hypothesis thus demands more sophisticated null models than those currently employed. These issues also underscore the benefit of using a “self–contained” test, which preserves the associations between gene expression and network structure while randomizing their association with the phenotype of interest.

In general, we find that CePa-GSA exhibits the best cross–study concordance (Fig. 3), does so in a
way that meets reasonable expectations of being correlated with gene–level concordance and sample sizes (Fig. 2), and has the benefit of testing the preferred “self-contained” null. The drawbacks of the method, however, include limitations regarding the input data as discussed above, as well as the fact that CePa returns several sub-analyses that must be selected or combined by the user, as we did here. CePa-GSA also required nearly an hour per study when using 1000 permutations, though this may be possible to reduce with CePa’s parallel implementation. Other methods provide speedier computations and more flexible inputs, albeit at the expense of improved concordance or other methodological limitations (cf. Table 1).

Conclusions

New network-based methods have garnered increasing interest as tools to analyze complex genomic datasets at the systems level. Despite the development of a number of promising tools, however, there is little guidance available to researchers for choosing between the methods. In this review, we sought to compare all the network analysis methods available in R/BioConductor at the time of this writing [8,15–26,36]. In addition to discussing their methodological and implementation features, we also proposed and applied a novel means to compare their performance using a suite of curated microarray data-sets [38] and a set of updated KEGG mappings developed to enable consistent pathway models for each method. The data we used for the analysis, the prepared KEGG pathways, and the scripts to carry out the computations (including functions to refresh the curated data and KEGG mappings from their source repositories) have been made available on the author’s website (http://braun.tx0.org/netRev) to enable other researchers to apply these comparisons to new methods as they are developed. In addition, we plan to make available updated versions of our findings as these packages are updated. The results of our tests clearly indicated the benefits and limitations of each approach. The tests also revealed idiosyncracies that would have been unnoticed except in comparison; for example, our comparisons revealed a bug in the previous version of the ROT/pe computation, which led us to suggest a fix that has now been implemented in the current version (reviewed here).

In addition to providing guidance about the features of the methods (Table 1), the efficiency of the computations (Table 3), and the consistency of the results (Figs. 2–6), our review also suggests a number of directions for future methodological development. Most notably, there is a need for benchmark and testing standards against which network analysis methods should be tested. We used the consistency of the results across a set of comparable studies, but this approach is plagued by a serious limitation: namely, we have no way to assess whether the “consistent” results are consistent owing to biological commonalities amongst ovarian cancers or due to a fluke of the microarray data, since the set is homogenous with respect to the disease type. A more insightful analysis could be obtained by the development of a database of diverse studies that are all curated to the same standards, just as was done for the curated ovarian data [38]. While diverse datasets are readily obtained, the work required to ensure that they are all comparable is non-trivial (and was beyond the scope of this paper); however, such data would be immensely useful to the research community. Relatedly, agreement on a common pathway representation format such as BioPAX [6] and developers’ adoption of a consistent API accepting these pathway files would aid comparison between these methods without requiring that the pathways be prepared by the user in different ways.

Secondly, we note that the most significant methodological distinctions between the packages involve a choice between using the preferred “self-contained” null hypothesis versus having the flexibility to apply the method in contexts other than two-sample differential expression studies. We recommend using methods that test the self-contained null (both for statistical and biological reasons as discussed above and in [7]), but at present none of these packages are able to test, for example, a self-contained hypothesis that a pathway is significantly associated with survival. This compromise could be easily resolved by further development of CePa–GSA allowing the user indicates to the function the statistical test (or model to be fit) rather than assuming that a two-sample t-test is desired. In the case of DEGraph, which uses Hotelling’s two-sample $T^2$ statistic to compare the graph “smoothed” gene expressions in two phenotypes, such an extension is less obvious but would be a valuable addition to DEGraph’s functionality.

Relatedly, we note that care must be exercised when constructing null models for the pathways for the “competitive” tests. An easy check of whether or not the null model is correct is to examine the distribution
of $p$ values across a large set of pathways; strong deviation from the expected uniform distribution of $p$ values is indicative of an incorrect null model. However, this rough assessment will only reveal egregious flaws. In the methods discussed above, and indeed many network biology methods generally, null graphs are generated by simply resampling node or edge properties. This destroys the correlation structure in the data (as [8] discussed) as well as the expected assortativity of gene expression in the pathway, yielding excessively conservative null models. There is thus a need to develop methods that can produce null graphs that are more biologically plausible.

Finally, we observe that a common drawback to all of these methods is their reliance upon single–gene statistical tests. As a result, while all of these methods are able to articulate differences in gene expression that have an impact at the pathway level, they cannot detect differentially regulated pathways when there are no detectable marginal effects at the gene level. An alternative approach would be to overlay the gene expression data itself onto the network (instead of using statistics corresponding to the gene’s differential expression), obtain a summary statistic for the network as a whole, and compare those. This approach has proved powerful in a non–network context [10,45], where it was able to detect pathways in which non-linear patterns of gene expression were associated with phenotype. While network extensions have been proposed [31], R implementations remain lacking.

Network analysis is rapidly becoming a valuable tool for harnessing existing biological information to yield mechanistic, systems–level insights from HT data. A number of promising methods have been developed, and we have found that most yield more consistent results (as measured by cross–study concordance) than both gene–level analyses and non–network pathway analyses (GSEA [8]). Nevertheless, challenges remain, and further work in this area has the potential to significantly improve the systems–based analysis of HT data, facilitating better understanding of the structure and function of the complex networks that coordinate living processes.
| Method   | Gene p-value thresholding | Expression\(^1\) | 2-sample data only restriction | Directed | Signed\(^2\) | Null type\(^3\) | Citation |
|----------|--------------------------|------------------|-------------------------------|----------|-------------|----------------|----------|
| GSEA     | no                       | no               | no                            | N/A      | N/A         | self-contained | [8]      |
| SPIA     | yes                      | no               | no                            | yes      | yes         | competitive    | [15, 16, 36] |
| ROT/pe   | optional                 | no               | no                            | yes      | yes         | competitive    | [16–18] |
| PathNet  | yes                      | no               | no                            | yes      | no          | competitive    | [19]     |
| NEA      | yes                      | no               | no                            | no       | no          | competitive    | [20, 21] |
| CePa-ORA | yes                      | no               | no                            | no       | no          | competitive    | [22, 23] |
| CePa-GSA | no                       | as written\(^4\) | as written\(^4\)             | yes      | no          | self-contained | [22, 23] |
| DEGraph  | no                       | yes              | yes                           | no       | optional    | self-contained | [24]     |
| TopologyGSA | no                    | yes              | yes                           | no       | no          | self-contained | [25, 26] |

Table 1: Properties of the various methods, including whether genes are thresholded on p-values, restrictions on the type of data and comparisons that can be made, the type of edges used by the model, and the type of null hypothesis tested. Notes:

1 “Expression data” is used here to denote any data that meets the assumptions used in gene expression testing, i.e., that the data is continuous and normally distributed. Other data meeting these assumptions can also be used, but methods which have this restriction cannot accept SNP data, etc.

2 “Signed” edges have signs assigned based on the interaction type in the pathway reference graph, distinguishing activating/inducing edges (+1) from repressing/inhibiting edges (-1).

3 “Null type” refers to the type of null hypothesis tested (cf. [7]. “Competitive” null hypotheses compare the pathway of interest to randomly generated pathways, without permuting sample labels, i.e., testing that the pathway statistic is more significant than a random pathway given the sample labels. “Self-contained” null hypotheses compare the statistic for the pathway to that obtained by randomly permuting sample labels, i.e., testing that the pathway is more strongly associated with a particular phenotypic attribute than expected by chance given the genes and topology of the pathway. The self-contained null is considered to be a stronger test [7].

4 CePa-GSA is not inherently restricted to 2-sample tests of differential expression methodologically, however the present implementation will only carry out the non-thresholded “GSA” type analysis for 2-sample tests of differential expression. As currently implemented, other types of analyses (e.g., using SNP data or modeling survival) must be carried out in CePa using the “ORA” analysis, which requires gene p-value thresholding and can only test the competitive null hypothesis\(^3\).
## Table 2: Studies and samples sizes of the data used in this investigation. The data are publicly accessible from GEO [62] and available as part of the curatedOvarianData package.

| Study Accession No. | grade | N(low) | N(high) |
|---------------------|-------|--------|---------|
| GSE13876 eset       |       | 59     | 85      |
| GSE14764 eset       |       | 24     | 44      |
| GSE17260 eset       |       | 67     | 43      |
| GSE30161 eset       |       | 19     | 27      |
| GSE32062.GPL6480 eset |   | 131   | 129     |
| GSE32063 eset       |       | 23     | 17      |
| GSE9891 eset        |       | 103    | 154     |
| PMID17290060 eset   |       | 57     | 57      |
| PMID19318476 eset   |       | 17     | 24      |
| TCGA eset           |       | 75     | 470     |

## Table 3: Package versions and run times (in seconds) for 247 pathways in all ten studies. Average times (in h/m/s) for a single study (i.e., 1/10th wallclock) are also noted. Times for computation (“user”) and kernel system calls (“system”) are given; the total CPU time consumed is the sum of these. The computations were carried out on a lightly–loaded 3.4 GHz quad-core Intel Core i7 iMac with 16GB RAM running R version 3.0.3 under OS X 10.8.5 (Darwin 12.5.0). Note that the times for DEGraph include both the signed and unsigned graph analyses. 1000 random permutations were used for the methods that perform resampling (all except DEGraph).

| Method       | Package Version | time (sec), 247 pathways, all 10 studies | avg runtime per study |
|--------------|-----------------|------------------------------------------|-----------------------|
|              |                 | user | system | CPU (usr+sys) | wallclock |                             |
| SPIA         | SPIA_2.14.0     | 1229.803 | 123.825 | 1353.628 | 1356.908 | 2m 15s                     |
| ROT/pe, cutoff | ROntoTools_1.4.0 | 948.702 | 9.497 | 958.199 | 959.367 | 1m 35s                     |
| ROT/pe, no cut | ROntoTools_1.4.0 | 1054.795 | 16.861 | 1071.656 | 1076.913 | 1m 47s                     |
| PathNet      | PathNet_1.2.0   | 747.983 | 9.572 | 757.555 | 796.516 | 1m 20s                     |
| NEA          | neaGUI_1.0.0    | 78099.148 | 5280.723 | 83379.871 | 83389.635 | 2h 18m 58s                |
| CePa-ORA     | CePa_0.5        | 3217.099 | 21.367 | 3238.466 | 3238.537 | 5m 24s                     |
| CePa-GSA     | CePa_0.5        | 29127.875 | 512.618 | 29640.493 | 29650.268 | 49m 25s                    |
| DEGraph (s,u) | DEGraph_1.14.0  | 2467.075 | 43.743 | 2510.818 | 2514.990 | 4m 11s                     |
| TopologyGSA  | topologyGSA_1.4.3 | [N/A: job halted after 100hrs]          | ≫360000.000          |
Classical pathway analyses such as GSEA do not take the network topology into account, but rather treat the pathway as a simple list of genes. As a result, changes to a gene such as p53 (red), which has a high degree and a direct influence on a number of other high-degree genes, a large downstream network, and an outgoing connection to a whole other network (the apoptosis pathway) are treated in the same way as changes to a gene such as Bub1 (blue), which has far fewer connections. In contrast, topology-based analyses attempt to incorporate the structure of the network and the relative importance of each gene to the pathway.
Figure 2: Cross–study concordance for each sub-analysis. For each subcomputation of each method, we show the correlation between pathway p values for all possible study pairs (45 total). Study pairs are ordered along the x axis according to their correlation in gene–level p values, shown in the top row. Methods are labeled in alternating colors, with the final/combined p values denoted in bold. The bottom row of the plot shows the sum of the sample sizes for each pair of studies, with dark green being high.
Figure 3: Cross-study correlations. Each plot displays the cross-study correlation of the results for each major analysis method. Boxplots within each frame indicate, for a given method, the distribution of correlations each study had with the nine other studies. EG, consider the “GSEA.pES” plot; the blue (leftmost) box indicates the distribution of correlations of GSEA pathway enrichment score p values (pES) that study ‘GSE13876’ had with each of the other data sets. The red box indicates the correlations between ‘GSE14764’ GSEA results and those of other nine studies, etc. Cross-study correlations of the gene–level statistics are also shown. Note that the scale on each of the plots is the same.
Figure 4: Cross–method correlations. Each plot displays the correlation in p values amongst different methods applied to each of the data sets. Here, the boxplots within each frame indicate, for a given study, the distribution of correlations the results from each method had with the others. EG, in the top left frame, the blue (left most) box plot indicates the distribution of correlation between the pathway enrichment score p values (pES) vs. the pathway p values obtained from the other nine analyses when applied to the GSE13876 data. Note that the scale on each of the plots is the same.
**Figure 5: Number of studies in which a pathway ranks in the top 20% for each analysis.** For each subcomputation of each method, we show for each pathway the number of times the pathway was amongst the top 20% most significant in each of the 10 studies. Pathways were only considered significant if they met the 20% cutoff unambiguously; if there were more than 20% of pathways tied for the top spot, none were considered to be meaningfully in the top 20%. Methods are labeled in alternating colors, with the final/combined $p$ values denoted in bold. The number of studies (out of 10 possible) in which the pathway was in the top 20% for that analysis is given by color; black indicates that the method could not give an answer for that pathway (typically a result of gene thresholding leaving no meaningful edges). The $p$ values in the color scale correspond to the probability of that specific overlap assuming 10 Bernoulli trials with $p = 0.2$ success. The 247 pathways are ordered along the $x$ axis by the mean overlap from the final (bolded) analyses, while the bottom row shows the average across all sub-analyses.
Figure 6: p-value distributions by method (all pathways, all studies). Depicted are joint and marginal distributions of $-\log_{10}(p)$ values for all pathways in all studies. (Note that higher values are more significant.) In the upper triangle, smoothed scatter plots depict the joint distribution of $-\log_{10}(p)$ for each pair of methods; darker red corresponds to higher density of points. In the lower triangle, Spearman’s rank correlations $\rho$ between the $p$ values obtained from each pair of methods is given, with positive correlations shown in increasing blue intensity and negative correlations shown in increasing red intensity (there are no negative values). Note that because rank correlations provide a measure of concordance that is independent of the dynamic range of the quantities being correlated and hence less influenced by outliers, the $\rho$ reported in the lower triangle may differ from a “by eye” estimate of the correlation based on the plots in the upper triangle. On the diagonal, the marginal distributions of $-\log_{10}(p)$ are shown as red histograms, with the theoretically expected distributions (uniform $p$ under the null) shown as a black line.
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Table S1:  R output showing exponential cost of the maximal clique problem for a the oxidative phosphorylation KEGG pathway.  hsa00190 is a modestly sized 133 nodes and 132 edges (below the median across all KEGG graphs).  After moralization, however, it becomes considerably more dense, jumping to 1078 edges.  Finding cliques in a graph of this size is a challenging task.  TopologyGSA does this with `qpGetCliques()`, which is an R interface to the GNU GPL Cliquer library implementing Östergård’s algorithm [63], the fastest maximal clique algorithm to date.  The algorithm uses a branch-and-bound procedure, in which first small subgraphs of $S$ nodes are searched for maximal cliques.  Once found, the size of the subgraph is incremented and searched again, until either all nodes are in the subgraph $S = 133$ or when increasing the subgraph would not permit the maximal clique found to that point.  Because maximal clique is an NP-hard problem, the cost increases exponentially with each increase in subgraph size, as can be seen here with the dense moralized hsa00190 graph.  The first column of the timing output shows the progress of the calculation as the subgraph size is increased.  In the second column, the accumulated runtime is recorded, while the last column gives the amount of time required since the previous search round finished clique was found.  The exponential increase in hardness as is clearly visible.  Timing was stopped after approximately 8h, before the algorithm could complete.  Extrapolating from this data, running to completion could take as much as $\sim 57042500$ sec, nearly two years.
**Figures S1: supp.xStudyPairs.pdf**
Plots of $p$-value correlations between different studies for each of the 10 methods, along with the gene-wise $p$-value correlations. $-\log_{10} p$ values are plotted against each other above the diagonal; below, the correlation coefficients are given. Note that in NEA (pg 9), a huge number of pathways “saturated” the test, with none of 1000 permutations yielding statistics comparable to those observed, suggesting possible issues with the null model.

**Figures S2: supp.xMethodPairs.pdf**
Plots of $p$-value correlations between different methods for each of the 10 studies. $-\log_{10} p$ values are plotted against each other above the diagonal; below, the correlation coefficients are given.