Advances in high-throughput sequencing technologies now yield unprecedented volumes of OMICs data with opportunities to conduct systematic data analyses and derive novel biological insights. Here, we provide protocols to perform differential-expressed gene analysis of TCGA and GTEx RNA-Seq data from human cancers, complete integrative GO and network analyses with focus on clinical and survival data, and identify differential correlation of trait-associated biomarkers.
Protocol

Network analysis of TCGA and GTEx gene expression datasets for identification of trait-associated biomarkers in human cancer

Huey-Miin Chen1,2,3,* and Justin A. MacDonald1,*

1Department of Biochemistry & Molecular Biology, Cumming School of Medicine, University of Calgary, Calgary, AB T2N 4Z6, Canada
2Technical contact
3Lead contact
*Correspondence: thmchen@ucalgary.ca (H.-M.C.), jmacdo@ucalgary.ca (J.A.M.)
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SUMMARY

Advances in high-throughput sequencing technologies now yield unprecedented volumes of OMICS data with opportunities to conduct systematic data analyses and derive novel biological insights. Here, we provide protocols to perform differential-expressed gene analysis of TCGA and GTEx RNA-Seq data from human cancers, complete integrative GO and network analyses with focus on clinical and survival data, and identify differential correlation of trait-associated biomarkers. For complete details on the use and execution of this protocol, please refer to Chen and MacDonald (2021).

BEFORE YOU BEGIN

@ Timing: 0.5–1 h

1. The hardware specifications for the computing platform used to estimate the timing for each step are provided in the key resources table.
2. The R software environment for statistical computing and graphics is required for this protocol. The latest R version (4.1.2), downloaded from https://CRAN.R-project.org/bin/windows/base/, was used to perform the protocol below. This protocol describes the specific steps for network analysis of TCGA colon cancer gene expression. Tissue-specific parameters for the extension of the protocol application to 18 additional human cancers are also contained within this document.
3. The R Studio integrated development environment (IDE), that provides a graphical interface to R, can be downloaded from https://www.rstudio.com/products/rstudio/.
4. R packages utilized in this protocol are listed under the Software and algorithms heading of the key resources table. To install the listed R packages, first install BiocManager with commands:

```R
> chooseCRANmirror();
> install.packages("BiocManager")
```

5. Then, run the following command to install the R packages listed under the Software and algorithms heading of the key resources table.
6. R is always pointed at a designated directory. Specify a working directory at the start of each R session with the following command:

```r
> setwd('Path_to_the_Desired_Folder')
```

7. In addition to R, the Cytoscape (3.9.0) software platform and the stringApp add-on (1.7.0) are required to retrieve molecular networks from the STRING database. Cytoscape 3.9.0 can be downloaded from https://cytoscape.org/download.html. Then, start Cytoscape and go to App > App Manager to search for and install stringApp (1.7.0).

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Deposited data**  |        |            |
| TcgaTargetGtex_gene_expected_count | Xena Toil data hub | https://toil-xena-hub.s3.us-east-1.amazonaws.com/download/TcgaTargetGtex_gene_expected_count.gz |
| TcgaTargetGTEX_phenotype | Xena Toil data hub | https://toil-xena-hub.s3.us-east-1.amazonaws.com/download/TcgaTargetGTEX_phenotype.txt.gz |
| COAD_clinicalMatrix | Xena TCGA data hub | https://tcga-xena-hub.s3.us-east-1.amazonaws.com/download/TCGA.COAD.sampleMap%2FCOAD_clinicalMatrix |
| TCGA_survival_data | Xena Toil data hub | https://toil-xena-hub.s3.us-east-1.amazonaws.com/download/TCGA_survival_data |
| **Software and algorithms** |        |            |
| Windows OS 10 Home, 64-bit | Microsoft | https://www.microsoft.com/ |
| R (4.1.2) | The R Project | https://CRAN.R-project.org/bin/windows/base/ |
| RStudio (2021.09.1 Build 372) | RStudio Team | https://www.rstudio.com/products/rstudio/ |
| BiocManager (1.30.16) | Morgan (2021) | https://CRAN.R-project.org/package=BiocManager |
| UCSCXenaTools (1.4.7) | Wang and Liu (2019) | https://CRAN.R-project.org/package=UCSCXenaTools |
| data.table (1.14.2) | Dowle and Srivinasan (2021) | https://CRAN.R-project.org/package=data.table |
| R.utils (2.11.0) | Bengtsson (2021) | https://CRAN.R-project.org/package=R.utils |
| dplyr (1.0.7) | Wickham et al. (2021) | https://CRAN.R-project.org/package=dplyr |
| limma (3.48.3) | Ritchie et al. (2015) | https://bioinf.wehi.edu.au/limma/ |
| edgeR (3.34.1) | McCarthy et al. (2012) and Robinson et al. (2010) | https://bioinf.wehi.edu.au/edgeR/ |
| topGO (2.44.0) | Alexa and Rahnenfuhrer (2021) | https://bioconductor.org/packages/topGO/ |
| grex (1.9) | Xiao et al. (2019) | https://CRAN.R-project.org/package=grex |
| biomaRt (2.48.3) | Durinck et al. (2005, 2009) | https://bioconductor.org/packages/biomaRt/ |
| ggplot2 (3.3.5) | Wickham et al. (2016) | https://ggplot2.tidyverse.org/ |
| RegParallel (1.10.0) | Bligh and Lasky-Su (2021) | https://github.com/kevinbligh/RegParallel |
| survminer (0.4.9) | Kasambara et al. (2021) | https://CRAN.R-project.org/package=survminer |
| Cytoscape (3.9.0) | Shannon et al. (2003) | https://cytoscape.org/ |
| stringApp (1.7.0) | Doncheva et al. (2019) | https://apps.cytoscape.org/apps/stringapp |
| DGCA (1.0.2) | McKenzie et al. (2016) | https://CRAN.R-project.org/package=DGCA |
| org.Hs.eg.db (3.13.0) | Carlson (2021) | https://bioconductor.org/packages/org.Hs.eg.db/ |
| GOSTats (2.58.0) | Falcon and Gentleman (2007) | https://bioconductor.org/packages/GOSTats/ |
| HGNChelper (0.8.1) | Oh et al. (2020) | https://CRAN.R-project.org/package=HGNChelper |
| plotrix (3.8-2) | Lemon (2006) | https://CRAN.R-project.org/package=plotrix |

(Continued on next page)
MATERIALS AND EQUIPMENT

The analyses outlined in this protocol utilize publicly available RNA-Seq, clinical, and survival datasets and several well-established CRAN and BioConductor R packages. All human subjects have been de-identified. The ethical principles set forth by the International Ethical Guidelines for Health-related Research Involving Humans (CIOMS, 2016) should preside over any activities that fall under health-related research with humans, such as biobanking and epidemiological studies. Researchers who study these data should also comply with TCGA and GTEx policies such as maintaining participants’ privacy, accessing data securely, and following TCGA and GTEx publication guidelines. To cite R packages, run the following command in R.

> citation("Package_Name")

STEP-BY-STEP METHOD DETAILS

Data import, cleaning, and preprocessing

© Timing: 1–4 h

Note: The Methods S1 file “R Markdown Code Script for UCSCXenaTool” contains the exact script used to generate the expected outcomes for this section.

1. Download Genes.xlsx (Piovesan et al., 2019) from https://osf.io/edjzv/ and save the downloaded file as zz_gene.protein.coding.csv to your working directory.
2. Download the ID/gene mapping for the TcgaTargetGtex_gene_expected_count dataset of the TCGA TARGET GTEx cohort (see key resources table for link) and save the downloaded file as zz_genencode.v23.annotation.csv to your working directory.
3. Load the following R packages: 1) UCSCXenaTools, 2) data.table, 3) R.utils, and 4) dplyr.
4. Generate a record of datasets hosted on UCSC Xena Data Hubs via the UCSCXenaTools R package (Wang and Liu, 2019).

Note: Datasets from UCSC Xena Data Hubs can be filtered by their XenaHostNames, XenaCohorts, and/or XenaDatasets. The object XenaData provides information on the contents of each dataset and can be saved to the working directory to assist in the selection of target datasets.

5. Retrieve gene expression, clinical, survival, and phenotype data from the UCSC Xena platform via the UCSCXenaTools R package (Wang and Liu, 2019). For network analysis of TCGA colon cancer gene expression, we selected and downloaded:
a. The TcgaTargetGtex_gene_expected_count dataset for the TCGA TARGET GTEx cohort from host toilHub.
b. The COAD_clinicalMatrix dataset for the TCGA Colon Cancer cohort from host tcgaHub by setting the filters paraCohort = "TCGA Colon Cancer" and paraDatasets = "TCGA.COAD.sampleMap/COAD_clinicalMatrix".
c. The TCGA_survival_data dataset for the TCGA TARGET GTEx cohort from host toilHub.
d. The TcgaTargetGTEX_phenotype dataset for the TCGA TARGET GTEx cohort from host toilHub.

Note: Within the TcgaTargetGtex_gene_expected_count dataset, GTEx normal tissue data complimentary to TCGA tumor data are available for 16 primary sites (see Table 1). This protocol can be used (with minor modifications) to conduct paired comparison on these 16 primary sites. Table 2 can be used to set values for the arguments paraCohort and paraDatasets (defined during step 5.b) to retrieve dataset(s) that contain clinical information of the desired cancer type.

6. Subset the gene expression matrix to include only observations of desired tissue type(s).
a. The Genotype-Tissue Expression (GTEx) project provides gene expression data from healthy, cancer-free individuals. For differential gene expression analysis, we selected GTEx normal colon tissue samples by setting the filters paraStudy = "GTEX", paraPrimarySiteGTEx = "Colon" and paraPrimaryTissueGTEx = "Colon" to the TcgaTargetGTEX_phenotype dataset. Table 3 can be used to set values for the arguments paraPrimarySiteGTEx and paraPrimaryTissueGTEx.

b. The Cancer Genome Atlas (TCGA) program provides gene expression data from primary tumors. For differential gene expression analysis, we selected TCGA colon cancer primary tumor samples by setting the filters paraSampleType = "Primary Tumor", paraPrimarySiteTCGA = "Colon", and paraHistologicalType = "Colon Adenocarcinoma". Table 4 can be used to set values for the arguments paraPrimarySiteTCGA and paraHistologicalType.

c. The TcgaTargetGtex_gene_expected_count dataset from the toilHub data hub combines RNA-Seq data from TCGA and GTEx by uniformly realigning reads to the hg38 genome and re-calling expressions using RSEM and Kallisto methods (Vivian et al., 2017). To compare gene expression between GTEx normal and TCGA tumor for network analyses, subset the

| Primary site    | GTEx normal tissue | TCGA primary tumor |
|-----------------|--------------------|--------------------|
| Adrenal gland   | 126                | 77                 |
| Bladder         | 9                  | 404                |
| Brain           | 1148               | 660                |
| Breast          | 178                | 1090               |
| Colon           | 307                | 282                |
| Esophagus       | 652                | 181                |
| Kidney          | 28                 | 884                |
| Liver           | 110                | 369                |
| Lung            | 288                | 1011               |
| Ovary           | 88                 | 419                |
| Pancreas        | 167                | 177                |
| Prostate        | 100                | 494                |
| Skin            | 555                | 102                |
| Stomach         | 174                | 410                |
| Testis          | 165                | 132                |
| Uterus          | 78                 | 57                 |
TcgaTargetGtex_gene_expected_count dataset via lists generated during step 6.a and step 6.b.

Note: When generating the GTEx and TCGA sample lists to subset the gene expression matrix TcgaTargetGtex_gene_expected_count, the subset function may not compute, or may return zero observations. Please refer to troubleshooting, problem 1 (step 6) for potential solution.

7. Subset the gene expression matrix to include only protein-coding genes. This can be achieved by utilizing the zz_gene.protein.coding.csv saved during step 1.

8. The gene expression matrix can now be saved for downstream analyses.

9. The COAD_clinicalMatrix dataset contains 133 administrative and phenotypic annotations (see the Methods S1 file “R Markdown Code Script for UCSCXenaTool” for a full list), ranging from sample IDs to pathologic stage to KRAS mutation codon. Keep only the variable(s) of interest. For example, to identify potential biomarkers for lymphatic invasion during network analysis of TCGA colon cancer gene expression, we retained the following variables:

Note: Phenotype variables included in clinical matrices differ among the 19 cancer types laid out in Table 2. For examples of how one can view, select, and re-code other phenotype variable(s) into an annotation matrix that is appropriate for downstream analyses, please refer to troubleshooting, problem 2 (step 9).

10. The phenotype annotation matrix can now be saved for downstream analyses.

△ CRITICAL: Tissue samples from TCGA are classified by cancer types, as well as by sample types (e.g., primary tumor, solid tissue normal). Solid tissue normal samples (referred to as NAT, normal adjacent to tumor) are collected from histologically normal tissues adjacent
to tumor margins and are often utilized as healthy controls for comparison with tumor samples. However, analyses of expression profiles from GTEx healthy, TCGA NAT, and TCGA tumor tissues indicate that NAT constitutes an intermediate state between healthy and tumor (Aran et al., 2017). That is, the expression profiles obtained from healthy, NAT, and tumor tissues segregate into distinct clusters, with closer resemblance of NAT to tumor in some tissues (e.g., prostate and colon) and greater similarity of NAT to healthy in other tissues (e.g., uterus and breast). Over half of the differentially expressed genes (DEGs) in the healthy to tumor comparison were not identified in the NAT to tumor comparison, while ~40% of DEGs in the NAT to tumor comparison were insignificant in the healthy to tumor comparison (Aran et al., 2017). Given the middling nature of solid tissue normal (i.e., NAT), these samples should be removed as they may distort subsequent analyses. Furthermore, solid tissue normal samples often have matched primary tumor samples. As such, inclusion of these solid tissue normal samples will introduce duplicate patient IDs.

△ CRITICAL: Tissue samples from TCGA are also classified by histological type. Histological heterogeneity can confound gene module-trait correlation if specific clinical traits are over-represented in certain histological type. For example, colon mucinous adenocarcinoma has
| paraPrimarySiteTCGA | paraDatasets | paraHistologicalType | Sample size |
|---------------------|--------------|----------------------|-------------|
| Adrenal gland       | TCGA.ACC.sampleMap/ACC_clinicalMatrix | Adrenocortical Carcinoma- Myxoid Type | 1 |
|                     |              | Adrenocortical Carcinoma- Oncocytic Type | 3 |
|                     |              | Adrenocortical carcinoma- Usual Type | 73 |
| Bladder             | TCGA.BLCA.sampleMap/BLCA_clinicalMatrix | Muscle invasive urothelial carcinoma | 404 |
| Brain               | TCGA.GBM.sampleMap/GBM_clinicalMatrix | Glioblastoma Multiforme | 1 |
|                     |              | Treated primary GBM | 1 |
|                     |              | Untreated primary \(de novo\) GBM | 150 |
|                     | TCGA.LGG.sampleMap/LGG_clinicalMatrix | Astrocytoma | 193 |
|                     |              | Oligoastrocytoma | 126 |
|                     |              | Oligodendroglioma | 189 |
| Breast              | TCGA.BRCA.sampleMap/BRCA_clinicalMatrix | Infiltrating Carcinoma NOS | 1 |
|                     |              | Infiltrating Ductal Carcinoma | 780 |
|                     |              | Infiltrating Lobular Carcinoma | 203 |
|                     |              | Medullary Carcinoma | 6 |
|                     |              | Metaplastic Carcinoma | 9 |
|                     |              | Mixed Histology | 29 |
|                     |              | Mucinous Carcinoma | 17 |
|                     |              | Other | 45 |
| Colon               | TCGA.COAD.sampleMap/COAD_clinicalMatrix | Colon Adenocarcinoma | 244 |
|                     |              | Colon Mucinous Adenocarcinoma | 38 |
| Esophagus           | TCGA.ESCA.sampleMap/ESCA_clinicalMatrix | Esophage Adenocarcinoma, NOS | 89 |
|                     |              | Esophage Squamous Cell Carcinoma | 92 |
| Kidney              | TCGA.KICH.sampleMap/KICH_clinicalMatrix | Kidney Chromophobe | 66 |
|                     | TCGA.KIRC.sampleMap/KIRC_clinicalMatrix | Kidney Clear Cell Renal Carcinoma | 530 |
|                     | TCGA.KIRP.sampleMap/KIRP_clinicalMatrix | Kidney Papillary Renal Cell Carcinoma | 288 |
| Liver               | TCGA.LIHC.sampleMap/LIHC_clinicalMatrix | Fibrolamellar Carcinoma | 3 |
|                     |              | Hepatocellular Carcinoma | 359 |
|                     |              | Hepatobiliary Carcinoma \(Mixed\) | 7 |
| Lung                | TCGA.LUNG.sampleMap/LUNG_clinicalMatrix | Lung Acinar Adenocarcinoma | 18 |
|                     |              | Lung Adenocarcinoma Mixed Subtype | 105 |
|                     |              | Lung Adenocarcinoma- Not Otherwise Specified | 320 |
|                     |              | Lung Basaloid Squamous Cell Carcinoma | 14 |
|                     |              | Lung Bronchioloalveolar Carcinoma Mucinous | 5 |
|                     |              | Lung Bronchioloalveolar Carcinoma Nonmucinous | 19 |
|                     |              | Lung Clear Cell Adenocarcinoma | 2 |
|                     |              | Lung Micropapillary Adenocarcinoma | 3 |
|                     |              | Lung Mucinous Adenocarcinoma | 2 |
|                     |              | Lung Papillary Adenocarcinoma | 23 |
|                     |              | Lung Papillary Squamous Cell Carcinoma | 6 |
|                     |              | Lung Signet Ring Adenocarcinoma | 1 |
|                     |              | Lung Small Cell Squamous Cell Carcinoma | 1 |
|                     |              | Lung Solid Pattern Predominant Adenocarcinoma | 5 |
|                     |              | Lung Squamous Cell Carcinoma- Not Otherwise Specified | 477 |
|                     |              | Mucinous \(Colloid\) Carcinoma | 10 |
| Ovary               | TCGA.OV.sampleMap/OV_clinicalMatrix | Serous Cystadenocarcinoma | 419 |
| Pancreas            | TCGA.PAAD.sampleMap/PAAD_clinicalMatrix | Pancreas-Adenocarcinoma Ductal Type | 147 |
|                     |              | Pancreas-Adenocarcinoma-Other Subtype | 25 |
|                     |              | Pancreas-\(colloid mucinous non-cystic\) Carcinoma | 4 |
|                     |              | Pancreas-Undifferentiated Carcinoma | 1 |
| Prostate            | TCGA.PRAD.sampleMap/PRAD_clinicalMatrix | Prostate Adenocarcinoma Acinar Type | 479 |
|                     |              | Prostate Adenocarcinoma, Other Subtype | 15 |

(Continued on next page)
distinct molecular aberrations (e.g., overexpression of the MUC2 protein) and higher ratio of lymphatic invasion as compared to its non-mucinous counterpart (Luo et al., 2019). Out of the 282 primary tumor samples from the TCGA Colon Cancer cohort, 38 samples (13%) were annotated with histological type colon mucinous adenocarcinoma. Pursuing weighted gene co-expression network analysis (WGCNA) without first resolving histological type may lead to detection of gene-module trait correlations that are associated with histological type in addition to the clinical trait of interest. Table 4 can be used to set values for paraHistologicalType, defined during step 6.b.

Note: For complete details on the use and execution of the UCSCXenaTools R package, please refer to (Wang and Liu, 2019).

Differential gene expression analysis with limma-voom

*Timing: 0.5–2 h*

Differential gene expression analysis is routinely used to investigate the biological differences between healthy and diseased states (McDermaid et al., 2019). Identification of DEGs can be valuable for uncovering potential biomarkers, therapeutic targets, and gene signatures for diagnostics. In this section, we utilize the limma workflow (Law et al., 2016) to detect DEGs across TCGA primary tumor and GTEx normal colon tissue samples.

Note: The Methods S2 file “R Markdown Code Script for LIMMA_ColonCancer” contains the exact script used to generate the expected outcomes for this section.

11. Load the following R packages: 1) dplyr, 2) limma, and 3) edgeR.
12. The TcgaTargetGtex_gene_expected_count dataset for the TCGA TARGET GTEx cohort from host toilHub was previously log2(x+1) transformed. As such, it is necessary to back-transform the gene expression matrix that was saved to the working directory during step 8 into RSEM gene-level expected count before passing the dataset to limma.
13. Convert the back-transformed gene expression matrix into a DGEList-object using the DGEList function.
**Note:** In `limma-voom`, all samples are assumed to have a similar range and distribution of log-CPM values (Law et al., 2016). Samples that have significantly different range and/or distribution of log-CPM values should be removed prior to the generation of the DGEList-object. To resolve the potential issue of sample outliers, please refer to troubleshooting, problem 3 (step 13). It should also be noted that if there are any sample outliers, and these outliers were not removed prior to the generation of the DGEList-object, then warning messages will start appearing when undertaking step 17, up until step 22, when the `eBayes` function will fail on execution.

14. Group samples by condition (i.e., TCGA tumor or GTEx normal).
15. Convert expected counts to counts per million (CPM) and log2-counts per million using the function `cpm`.
16. Remove genes that are lowly expressed using the function `filterByExpr`.
17. Perform normalization on gene expression using the function `calcNormFactors`.
18. Generate a design matrix using the function `model.matrix`.
19. Set up contrast for comparison using the function `makeContrasts`.
20. Transform gene expression data for linear modeling using the function `voom`.
21. Perform linear modeling using the function `lmFit`, then the function `contrasts.fit`.
22. Compute empirical Bayes statistics for differential expression using the function `eBayes`.
23. Inspect the number of significantly up- and down-regulated genes.
24. Save the list of DEGs for subsequent analyses.
25. Save the `voom` transformed gene expression matrix for subsequent analyses.

**Note:** For complete details on the use and execution of the limma R package, please refer to Ritchie et al. (2015) and Law et al. (2016).

### Identification of gene set(s) highly correlated to specific traits of human cancer with WGCNA

© Timing: 1–3 h

In addition to the identification of DEGs, the analysis of correlated gene expression (i.e., co-expression) can provide a framework for describing changes in expression of gene sets within the confine of human cancer traits. WGCNA is a bioinformatic algorithm, developed by Langfelder and Horvath (2008), that can be used to find clusters (modules) of correlated genes and to associate the identified modules with specific sample traits. Importantly, the gene expression data input for WGCNA is not pre-filtered by differential expression. As such, gene sets that may be highly correlated to specific traits of human cancer, but do not pass the differential expression threshold, may still be revealed via WGCNA. In this section, the `voom` normalized expression data, generated from the `limma-voom` workflow presented in the previous section, is subjected to WGCNA to identify gene set(s) highly correlated to lymphatic invasion in human colon cancer.

**Note:** The Methods S3 file “R Markdown Code Script for WGCNA” contains the exact script used to generate the expected outcomes for this section.

26. Load the following R packages: 1) `WGCNA`, and 2) `dplyr`.
27. Subset the `voom` transformed gene expression matrix, saved during step 25, to include only TCGA gene expression data.
28. Remove genes that are lowly-expressed and/or genes with low variation between samples.

**Note:** Genes that are lowly-expressed and/or display low variation between samples have a tendency to generate noise in WGCNA (Langfelder and Horvath, 2008). Lowly-expressed genes can be removed by defining a mean expression cutoff value. Filtering for low variance can be achieved by setting a variance cutoff value. The method and threshold-value chosen
for gene-filtering may vary according to study-specific characteristics. Users should examine expression values across the entire dataset for features that would distinguish uninformative variables. For WGCNA on TCGA gene expression data, we removed genes that had normalized expression value < 0 in any of the 244 colon tissue samples.

29. Identify outlier samples by clustering samples using the function `hclust`. Then, remove the outlier samples by setting a value for the `cutHeight` argument in the function `cutreeStatic`.

\textbf{Note}: Users should refer to the sample clustering dendrogram generated via the function `plot` to discern the most reasonable cut height for their dataset. In general, each dataset will require a different cut height. A histogram of height (the distance between samples and/or clusters) can also assist in the setting of a value for the `cutHeight` argument.

30. Convert the phenotype annotation matrix, saved during step 10, into a trait data frame that is analogous to the gene expression data frame.

\textbf{Note}: Sample traits data can be plotted alongside the sample clustering dendrogram, generated during step 29, to investigate whether samples with distinct trait values have globally distinct gene expression patterns. If so, there is likely a high correlation among large groups of genes, which invalidates the scale-free topology assumption and can interfere with the appropriate selection of the soft-thresholding power $b$ (step 31). If the lack of scale-free topology is caused by the sample trait of interest, then the recommended power $b$ is six for unsigned or signed hybrid networks, and 12 for signed networks (for sample size listed in Table 1). If the lack of scale-free topology is caused by traits that are uninteresting for the study, then the WGCNA consensus network analysis (not described in this protocol) should be employed. WGCNA consensus network analysis allows for detection of common co-expression patterns across multiple conditions (e.g., the driver of sample differences that is not being studied). Tutorials for the identification of consensus modules that cluster genes with dense connectivity in multiple conditions can be found at https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/, under the heading II. Consensus analysis of female and male liver expression data. (Langfelder and Horvath, 2008)

31. Use the function `pickSoftThreshold` to analyze scale-free topology for a set of candidate $b$-values.

32. Plot, and then inspect the results returned by the function `pickSoftThreshold`. Select the smallest $b$-value that satisfies the scale-free topology assumption (i.e., $R^2 > 0.8$).

\textbf{Note}: A reasonable $b$-value is < 15 for unsigned or signed hybrid networks, and < 30 for signed networks. If the scale-free topology assumption cannot be satisfied with a reasonable $b$-value, then the input data should be investigated for heterogeneity drivers that instigated the globally distinct expression pattern (see Note under step 30).

33. Calculate adjacencies with the selected $b$-value via function `adjacency`. The adjacency matrix is a square, symmetrical matrix with values ranging between 0 and 1 that correspond to the connection strengths between each pair of genes.

34. Calculate the topological overlap matrix (TOM) from the adjacency matrix using the function `TOMsimilarity`. Then calculate the dissimilarity matrix via command `dissTOM = 1 – TOM`.

\textbf{Note}: When executing functions `pickSoftThreshold`, `adjacency` and `TOMsimilarity`, the network type needs to be defined via arguments `networkType`, `type`, and `networkType`, respectively. In “signed” networks, a pair of genes with positive correlation is considered connected whereas a pair of genes with negative correlation is considered unconnected. In
“unsigned” networks, positive and negative correlations are both regarded as connected (i.e., mixed). Although the default network type in WGCNA is “unsigned”, the use of “signed” networks is recommended for analysis of gene expression data (see https://peterlangfelder.com/2018/11/25/signed-or-unsigned-which-network-type-is-preferable/).

35. Detect modules within the network by clustering genes based on dissTOM using the function hclust. Module assignments can be plotted under the gene dendrogram using the function plotDendroAndColors.

Note: WGCNA may return modules that capture a large fraction of the input data (i.e., containing 10, 20, or 50% of the total input data), which may not provide adequate resolution for the identification of core mechanisms associated with the sample trait of interest. To resolve the potential issue of large modules, please refer to troubleshooting, problem 4 (step 35).

36. Calculate the weighted average value (eigengene) for each module using the function moduleEigengenes.

37. Calculate the correlation between trait and module eigengenes (MEs) using the function cor and corPvalueStudent to uncover module(s) with significant association to the sample trait of interest. A graphical view of module-trait relationship can be generated with the function labeledHeatmap.

38. Calculate the correlation between trait and gene expression levels with the function cor and corPvalueStudent to define gene significance (GS).

39. Calculate the correlation between MEs and gene expression levels using the function cor and corPvalueStudent to define module membership (MM).

40. Plot a scatterplot of variables GS vs. MM to examine if genes that are highly associated with the trait of interest are also highly associated with their assigned module.

41. Annotate results from WGCNA with Ensembl IDs. This can be done by utilizing the zz_gencode.v23.annotation.csv downloaded and saved during step 2 of this protocol.

42. Save the annotated WGCNA result for subsequent analyses.

Note: For complete details on the use and execution of the WGCNA R package, please refer to Langfelder and Horvath (2008).

Gene set enrichment analysis with topGO

© Timing: 0.5–1 h

Having identified clusters (modules) of genes that are highly correlated to specific traits of human cancer, the next step is to infer underlying molecular mechanisms based on the biological attributes of these genes. Gene ontology (GO) is a set of structured and controlled vocabulary, which describes gene characteristics in terms of their function and localization. In the hierarchical tree structure of GO, each child node is a more specific term than its parents. At the highest level, GO terms are classified into three major categories: cellular components (where the gene product is localized), molecular functions (function of the gene product), and biological processes (the activity with which the gene product is involved). In this section, GO enrichment analysis is completed with the topGO R package (Alexa and Rahnenfu¨hrer, 2021) for the category “biological processes”. topGO was chosen to owe to its ‘elim’ method, that considers GO hierarchy when calculating enrichment. In brief, the algorithm accounts for the ‘inheritance problem’ (i.e., where root terms inherit annotations from descendent terms; a situation which can generate false positives) in GO enrichment analysis by disregarding genes that had already been annotated with significantly enriched descendent GO terms (Alexa et al., 2006).
Note: The Methods S4 file “R Markdown Code Script for topGO” contains the exact script used to generate the expected outcomes for this section.

43. Load the following R packages: 1) data.table, 2) grex, 3) biomaRt, 4) topGO, 5) dplyr, and 6) ggplot2.

44. Annotate the WGCNA result, saved during step 42, with Entrez IDs, via function grex.

45. Connect to the ENSEMBL_MART_ENSEMBL BioMart database to query GO IDs for the list of Entrez IDs returned by the function grex. This constitutes the ‘gene universe’ that we will compare our list of genes of interest to.

46. Define the WGCNA module of interest then set up named factors for genes located within and outside of the module of interest.

47. Build a topGO data object with the base function new, then run GO analysis.

48. Test significance of GO terms using the function runTest.

49. Generate a summary table of results obtained from topGO enrichment analysis with the function GenTable.

Note: If users decide to re-run GO term enrichment analysis after some time (e.g., six months), the outcome of this later analysis may not always agree with those obtained from analysis conducted at an earlier time. For explanation and potential solution, please refer to troubleshooting, problem 5 (step 49, step 61, and step 71).

50. Calculate odds ratios via the command:

   ```r
   > all_res$OR = log2((all_res$Significant/tot_candidate)/(all_res$Annotated/tot_background))
   ```

51. Generate a summary figure of topGO results via the ggplot2 R package (Wickham et al., 2016).

   Note: For complete details on the use and execution of the topGO R package, please refer to Alexa and Rahnenfu¨hrer (2021) and Alexa et al. (2006).

**Survival analysis with RegParallel**

© Timing: 0.5–1 h

Potential biomarkers for specific human cancer traits can be detected by cross-referencing genes that reside within the WGCNA module(s) of interest with the list of DEGs derived with limma-voom. To further restrict this list of potential biomarkers, the prognostic ability of gene expression on survival may be evaluated. In this section, the RegParallel R package (Blighe and Lasky-Su, 2021) was utilized to examine how the expression of genes within the WGCNA module of interest influenced the rate of overall survival.

Note: The Methods S5 file “R Markdown Code Script for Survival” contains the exact script used to generate the expected outcomes for this section.

52. Load the following R packages: 1) dplyr, 2) data.table, 3) RegParallel, and 4) survminer.

53. Subset the voom transformed gene expression matrix, saved during step 25, to include only TCGA sample IDs and only genes within the module of interest.

54. Transform the gene expression data to Z-score with the function scale.
55. Merge the transformed gene expression matrix with the file TCGA_survival_data that was downloaded and saved during step 5.c.

*Note:* For analysis of how gene expression within the WGCNA module of interest influences the rate of overall survival for members of the TCGA colon cancer cohort, we subset the TCGA_survival_data to include only data ≤ 3,650 days (i.e., 10 years). The subset criterion of 10-year was set based on data that were available within the TCGA_survival_data dataset for the TCGA colon cancer cohort. A histogram summarizing the range and distribution of available survival data can be generated to assist in the setting of the subset criterion. Five-year survival is commonly used, but long-term survival outcomes (e.g., 10, 15, or 20 years) could also be interrogated when data are available (Miller et al., 2019; Myers and Ries, 1989).

56. Run the function `RegParallel` to fit Cox proportional hazards regression model to gene expression to independently test the association between survival time and each gene within the WGCNA module of interest.

*Note:* For network analysis of TCGA colon cancer gene expression, we examined how gene expression influences the rate of overall survival. However, other events of interest can be found within the file TCGA_survival_data (e.g., disease-specific survival, disease-free interval, and progression-free interval).

57. Load the summary of limma-voom differential expression analysis, saved during step 24.

58. Merge the limma-voom differential expression data with `RegParallel` output, then save the composite data table for Cytoscape network visualization.

59. Generate a short-list of genes that satisfy the conditions:
   a. Is associated with the trait of interest (see step 53).
   b. Is differentially expressed (adj.P.Val < 0.05).
   c. Statistically significant prognostic separation between high and low gene expression (LogRank < 0.05).

60. Generate Kaplan-Meier (KM) plots for shortlisted genes using the function `ggsurvplot`.

*Note:* For complete details on the use and execution of the RegParallel and survminer R package, please refer to Blighe and Lasky-Su (2021) and Kassambara et al. (2021), respectively.

**Molecular network visualization with Cytoscape**

© Timing: 1–2 h

The effective visual display of data architecture is critical for information dissemination and broader contextual understanding. This section describes the use of stringApp for Cytoscape (Doncheva et al., 2019; Shannon et al., 2003) to retrieve molecular networks from the search tool for the retrieval of interacting genes/proteins (STRING) database (Szklarczyk et al., 2021) given a list of proteins of interest. Moreover, the means to import external data (e.g., results from differential expression and survival analysis) for mapping additional information onto the STRING network is provided.

61. Construct the protein-protein interaction (PPI) network with the STRING database.
   a. Start Cytoscape and go to File > Import > Network from Public Database.
   b. Select STRING: protein query as Data Source.
   c. Copy/paste the variable column of data table saved during step 58 into the box titled: Enter protein names or identifiers.
   d. Press Import.
Note: The default confidence (score) cutoff value is 0.40. Lowering the cutoff will increase the sensitivity but will also increase the likelihood of false positives. The setting of the cutoff value is arbitrary and is often based on the number of interactions required for the analysis. For example, to visualize PPIs for genes within our WGCNA module of interest, the cutoff was set to 0.15. At this cutoff-value, a single molecular network incorporating 145 out of 151 genes in our module of interest was constructed via the STRING database.

Note: If users decide to re-construct their PPI network after some time (e.g., six months), the newly constructed network may not be the same as what was returned via earlier import of network from the STRING database. For explanation and potential solution, please refer to troubleshooting, problem 5 (step 49, step 61, and step 71).

62. Import the composite data table, saved during step 58, to the constructed STRING network to visualize differential expression and survival analysis overlap.
   a. Go to File > Import > Table from File.
   b. Select the composite data table, saved during step 58. Then press Open.
   c. Select To a Network Collection for field Where to Import Table Data:
   d. Select Node Table Columns for field Import Data as:
   e. Select shared name for field Key Column for Network.
   f. Under the Preview window, click on the Variable column then change the Meaning: to Key. Then press OK.

63. Compute a set of topological parameters for the STRING network by selecting Tool > Analyze Network. Then press OK.

64. Adjust Layout and Styling as needed. For our network analysis of TCGA colon cancer gene expression, we emphasized the degree of interaction by mapping node size to the network parameter degree. We also highlighted genes that were differentially expressed via colored nodes, and genes that were associated with overall survival with colored node border.

65. Export topology data from Cytoscape by selecting File > Export > Table to File.... Then change the Select a table to export: to String Network default node. Then press OK.

Note: The exported table can be used to prioritize hub gene(s) by cross-referencing DEGs that significantly impact overall survival probability with their node degree. Node degree represents the number of interactions linked to a given gene.

Note: For complete details on the use and execution of the Cytoscape (3.9.0) software platform, please refer to Cytoscape User Manual (http://manual.cytoscape.org/en/stable/index.html) and Shannon et al. (2003).

Differential (co-expression) correlation analysis with DGCA

© Timing: 1–2 h

Over the past decade, increased efficiencies in global gene expression profiling have made common the study of differential gene expression. However, this approach overlooks the fact that biological processes are defined by complex interactions among molecules. Thus, methods such as WGCNA (Langfelder and Horvath, 2008) emerged to facilitate the exploration of relationships between gene sets and sample traits. Complementary to differential expression and co-expression studies, differential co-expression (correlation) studies analyze the rewiring of molecular interactions associated with perturbations such as disease or oncogene activation (Savino et al., 2020). In this section, we perform differential correlation analysis with the R package DGCA (McKenzie et al., 2016) on our hub gene (identified during step 65) within the gene universe, and between normal and primary tumors. The identification of differentially-correlated genes (DCGs) and the comparison
of enrichment of GO terms derived from DCGs can provide insight into the functional relevance of a hub gene in different cellular contexts (e.g., normal vs. tumor). These linkages can further promote the development of an effective hypothesis for integration with experimental validation.

Note: The Methods S6 file “R Markdown Code Script for DCGA” contains the exact script used to generate the expected outcomes for this section.

66. Load the following R packages: 1) dplyr, and 2) DGCA, 3) org.Hs.eg.db, 4) GOstats, 5) HGNChelp, and 6) plotrix.
67. Import the voom transformed gene expression matrix, saved during step 25.
68. Extract column names from the gene expression matrix to construct a design matrix for DGCA.
69. Filter the gene expression matrix using the function filterGenes:
   a. First filter genes by central tendency (e.g., filterCentralPercentile = 0.25; this removes genes in the bottom 25th percentile of median expression).
   b. Then filter genes by dispersion (e.g., filterDispersionPercentile = 0.25; this removes genes in the bottom 25th percentile of the dispersion index of expression).
70. Perform differential correlation analysis on the hub gene between normal and primary tumor using the function ddcorAll.

Optional: Visualize the correlation in each condition (i.e., normal, and primary tumor) between the hub gene and its top DCGs via the following command:

```r
> plotCors(inputMat = dataExpr, design = dataMatrix, compare = c("dataGTEx", "dataTCGA"), geneA = "hub_gene", geneB = "top_DCG")
```

71. Perform GO enrichment on genes with significant gain of correlation to the hub gene in primary tumor and on genes with significant loss of correlation to the hub gene in primary tumor via the DGCA wrapper function ddcorGO.

Note: If users decide to re-run GO term enrichment analysis after some time (e.g., 6 months), the outcome of this later analysis may not always agree with those obtained from analysis conducted at an earlier time. For explanation and potential solution, please refer to troubleshooting, problem 5 (step 49, step 61, and step 71).

72. Visualize the odd-ratios from enrichment of GO terms derived from DCGs via the wrapper function plotGOTwoGroups.

Note: For complete details on the use and execution of the DGCA R package, please refer to McKenzie et al. (2016).

EXPECTED OUTCOMES
The filterByExpr function removes genes that are either unexpressed or lowly expressed while keeping as many genes as possible with worthwhile counts (i.e., keeping genes that have CPM above k in n samples, where n is determined by the minimum group sample size and k is determined by minimum count (default: 10) in a minimum proportion (default: 70% of the smallest group size) of samples). The density plot of log-CPM values for raw vs. filtered expected count should show a sizable proportion of lowly-expressed genes (Figure 1A) that is removed after application of filterByExpr (Figure 1B). For network analysis of TCGA colon cancer gene expression, 15,164/18,205 genes were retained for differential expression analysis after application of the filterByExpr function. The definition of “lowly-expressed” is subjective. In addition to modifying the values of
arguments for the `filterByExpr` function, the filtering of lowly expressed genes can also be achieved by functions external to `edgeR::filterByExpr` (e.g., the genefilter R package (Gentleman et al., 2021)) to maximize the number of DEGs. However, it should be noted that inadequate removal of lowly expressed genes will negatively impact linear modeling in `limma-voom`, which is carried out on log-CPM values which are assumed to be normally distributed. If the filtering of lowly-expressed genes is insufficient for linear modeling in `limma-voom`, then the mean-variance trend plot, generated as part of the `voom` function, will show a drop in variance levels at the low end of the expression scale. Figure 2 shows the expected mean-variance trend for network analysis of TCGA colon cancer gene expression. Please refer to materials (i.e., Figure 1) in Law et al. (2014) for acceptable trends of mean-variance relationship that can be applied to linear modeling with `limma-voom`.

Figure 1. Impact of removing lowly-expressed genes on the distribution of expression values
(A and B) Density plot of log-CPM values before (A) and after (B) removal of genes that are lowly-expressed in TCGA primary tumor and GTEx normal colon tissue samples.

Figure 2. The mean-variance relationship of the input gene expression data
Mean-variance relationship of log-CPM values for the input dataset (TCGA primary tumor and GTEx normal colon tissue gene expression data) is appropriate for subsequent linear modeling with `limma-voom` since a drop in variance levels at the low end of the expression scale was not observed.
The number of DEGs detected across TCGA primary tumor and GTEx normal colon tissue samples accounts for 92% of total gene input (see the Methods S2 file “R Markdown Code Script for LIMMA_ColonCancer”). To focus on genes that have significant differential expressions relative to a non-zero-fold change threshold (and thus are more likely to be biologically meaningful), the \texttt{treat} function can be used to trim large DEG lists (McCarthy and Smyth, 2009). Upon application of an arbitrary threshold of log$_{2}$FC = 0.58, the number of DEGs were reduced to account for 56% of total gene input (see the Methods S2 file “R Markdown Code Script for LIMMA_ColonCancer”). The higher the chosen threshold, the stronger the evidence needed for any particular gene to be defined as a DEG. As such, the decision to apply the \texttt{treat} function and the selection of threshold value are dependent on the magnitude of contrast between conditions and the size of the gene input. Another illustration of the \texttt{treat} function is presented in the Methods S7 file “R Markdown Code Script for LIMMA_OvarianCancer”.

The results of differential expression analyses can be visualized as volcano plots, a standard in DEG visualization. A volcano plot is a type of scatter plot that illustrates the statistical significance (−log$_{10}$(adjusted p value)) and magnitude of change (log$_{2}$(fold-change)) associated with the conditions under comparison. The volcano plot seen in Figure 5B of Chen and MacDonald (2021) is the expected outcome of differential gene expression analysis on TCGA tumor vs. GTEx normal tissue. The Methods S8 file “R Markdown Code Script for Enhanced Volcano” contains the exact script used to construct a volcano plot with the refined list (i.e., have undergone \texttt{treat}) of DEGs saved during step 24 of step-by-step method details using the EnhancedVolcano R package (Blighe and Lasky-Su, 2021). However, any software that can create scatter plots may be used to create volcano plots.

Quantification and statistical analyses can be performed as optional functions within specific R packages. For differential gene expression analysis with \texttt{limma-voom}, the \texttt{treat} method with a non-parametric empirical Bayes approach for the analysis of factorial data provided a paired t-test for every gene within the limma R environment. To calculate the correlation between clinical trait and WGCNA module eigengenes, Pearson coefficients (r) and the corresponding p-values were used to determine statistically significant linear relationships. For gene set enrichment analysis with \texttt{topGO}, the \texttt{elim} method was applied alongside Fisher’s exact test to assess significance of GO term enrichment. For survival analyses with \texttt{RegParallel}, a univariate Cox survival model was used to compute hazard ratios, and outputs from log-rank testing was used to describe overall significance of the model. For DGCA, the pipeline provided Pearson coefficients (r) and the corresponding p values for each pair of genes across samples.

**LIMITATIONS**

The presented protocol utilizes TCGA and GTEx RNA-Seq datasets. These datasets excel at having sufficient observations for statistically sound correlation studies. However, should additional data-sets that offer a juxtaposition of tumor vs. normal samples become available, these resources should be utilized for the validation of results obtained from the application of the presented protocol. The presented protocol employs gene expression profiles of tumor bulk tissues. Given that genes may demonstrate diverse functions across different cell types, gene sets identified from averaged data-sets need to be reexamined in a cell type-specific manner for the identification of susceptible cell types and converged pathways among different cells. Deconvolution methods may be utilized to reconstruct cell type-specific gene expression from tumor bulk tissues (Aran et al., 2015). Finally, the presented protocol examines gene-to-gene correlations, which do not indicate causal relationships.

**TROUBLESHOOTING**

**Problem 1**
The \texttt{subset} function does not compute, or the \texttt{subset} function returns zero observations (step 6).
Potential solution

In R, unique names (i.e., identifiers) given to variables must not start with an underscore (_). However, many variables found in the TcgaTargetGTEX_phenotype and clinicalMatrix datasets contain a starting underscore (e.g., _primary_site).

One can utilize the `gsub` function to remove or replace underscores in identifiers. For example:

```r
> gsub("\_","",names(YourDataset))
```

The R language is generally case sensitive. However, TCGA and GTEx data stored with the UCSC-Xena platform carry capitalization inconsistencies, which can interfere with subsetting in R. For example, in TcgaTargetGTEX_phenotype, the _primary_site for _sample_type “Normal Tissue” under _study “GTEX” is “Adrenal Gland”, whereas the _primary_site for _sample_type “Primary Tumor” under _study “TCGA” is “Adrenal gland”.

One can use `subset` with `grepl` to ignore case. For example:

```r
> subset(YourDataset, grepl("adrenal gland", primarysite, ignore.case = TRUE) & study == "GTEX")
```

Samples stored with the skin cancer clinical matrix do not have assigned histological type. When retrieving IDs for skin cancer primary tumor samples. One can use the following script:

```r
> filterTCGA02 = subset(filterTCGA01, sampletype == paraSampleType & primarysite == paraPrimarySiteTCGA)
```

In R, certain characters hold special meaning to certain functions (i.e., metacharacters). For example, when subsetting with `grepl`, the first argument is a pattern, a regular expression. If we set the value for this first argument as "Colon", then `grepl` gives back the rows (observations) that begins with the pattern "Colon"; If we set the value for this first argument as "Colon'", then `grepl` gives back the rows (observations) that contains the pattern "Colon". When utilizing `grepl`, parentheses are also metacharacters. To match literal parentheses, we need to escape the metacharacters by placing backslashes (\) in front of parentheses to suppress their special meaning. When setting the values for paraPrimaryTissueGTEX (step 6.a) or paraHistologicalType (step 6.b), please refer to Tables 3 or 4, respectively, to obtain non-zero returns.

Problem 2

In general, phenotypic information stored in TCGA clinical matrices can be classified into three categories: discrete numeric (e.g., age), binomial (e.g., additional therapy?), or ordinal (e.g., clinical stage) variables. While discrete numeric variables can be used directly (i.e., without modification) to estimate the relationship of gene expression to phenotype, binomial and ordinal variables need to be re-coded for downstream analyses. Given that 1) the variables included in clinical matrices differ among the 19 cancer types listed in Table 2, and 2) the importance of research question in the context of a study design, we provide here a more generalized solution to the preparation of binomial and ordinal variables for downstream analyses (step 9).

Potential solution

The clinical matrix TCGA.OV.sampleMap/OV_clinicalMatrix for ovarian cancer is used to exemplify the generalized solution for re-coding nominal and ordinal variable for downstream
analyses. Please refer to the Methods S9 file “R Markdown Code Script for troubleshooting problem 2” for the exact script used to generate the expected outcome.

To view a list of variables included in the clinical matrix (downloaded during step 5.b), one can use the following script:

```r
> names(filterTCGA02)
```

To keep variable(s) of interest (e.g., “Additional Radiation Therapy” and “Clinical Stage”), one can use the following script:

```r
> varClinKeep = c("sampleID", "additionalradiationtherapy", "clinicalstage")
```

To re-code the binomial variable “Additional Radiation Therapy” from YES/NO to 1/0, one can use the following script:

```r
> clinFinal$additionalradiationtherapy = if_else(clinFinal$additionalradiationtherapy == "YES", 1, 0, missing = NULL)
```

To re-code the ordinal variable “Clinical Stage” from Stages I/II/III/IV to 1/2/3/4, one can use the following script:

```r
> clinFinal$clinicalstage[grepl("Stage IV", clinFinal$clinicalstage)] <- 4;
> clinFinal$clinicalstage[grepl("Stage III", clinFinal$clinicalstage)] <- 3;
> clinFinal$clinicalstage[grepl("Stage II", clinFinal$clinicalstage)] <- 2;
> clinFinal$clinicalstage[grepl("Stage I", clinFinal$clinicalstage)] <- 1
```

### Problem 3

In limma-voom, all samples are assumed to have a similar range and distribution of log-CPM values (Law et al., 2016). If a subset of samples appears as outliers on the density plot for filtered expected count (Figure 3), these sample outliers should be removed prior to running limma-voom on the DGEList-object (step 13).

#### Potential solution

The script used as a solution to this problem is provided in the Methods S10 file “R Markdown Code Script for troubleshooting problem 3 – Colon Cancer”. In which, a lcpm.cutoff of log2(10/M + 2/L), where M is the median library size in millions and L is the mean library size in millions, was applied to the lcpm data matrix generated during step 15 of step-by-step method details to identify lowly-expressed genes. Next, the proportion of genes below lcpm.cutoff by sample was calculated then summarized with a histogram to identify sample outlier(s). For example, in determination of DEGs across TCGA primary tumor and GTex normal colon tissue samples, we filtered out four GTEx samples that had >80% of genes below the defined lcpm.cutoff (Figure 3C). Another illustration of this solution is presented in the code script included in the Methods S11 file “R Markdown Code Script for troubleshooting problem 3 – Ovarian Cancer”. In which, one TCGA sample was identified as outlier and was removed prior to the generation of the DGEList-object in the code script provided in the Methods S7 file “R Markdown Code Script for LIMMA_OvarianCancer” to facilitate clean execution of functions called during step 17–22.
Problem 4

WGCNA returns gene clusters (modules) that are labeled with colors. The module labeled turquoise contains the highest number of genes, the module labeled blue contains the second highest number of genes, then brown, green, etc. The module labeled gray contains non-module genes. Depending on the nature of the input data, and/or the argument settings in functions `adjacency`, `TOMsimilarity`, and `cutreeDynamic`, WGCNA may return modules that capture a large fraction of the input data (i.e., containing 10, 20, or 50% of the total input data). From the perspective of WGCNA, there is no “correct” upper-limit on the size of any module or the number of non-module genes. However, given that the typical objective of performing WGCNA is to infer underlying molecular mechanisms based on biological attributes of a given gene set (module), large modules may not provide adequate resolution for the identification of core mechanisms associated with the sample trait of interest (step 35).

Potential solution

A solution to this problem is to conduct multiple runs of WGCNA, and adjust the argument `minClusterSize` (i.e., `minModuleSize`) and/or the argument `deepSplit` under the function `cutreeDynamic` between runs. The `deepSplit` argument controls the sensitivity of module splitting. Users can select an integer value between 0 and 4 for `deepSplit`, with 4 being the most sensitive. The `minModuleSize` argument controls the lower limit of module size. For WGCNA on TCGA colon primary tumors, the `deepSplit` was set at 4. By decreasing the `minModuleSize` value, we observed 1) an increased in number of modules detected, 2) a decrease in the number of genes per module, 3) the elimination of the gray module, and 4) the detection of a gene set (module) significantly correlated with the trait of interest lymphatic invasion (Figure 4).

Problem 5

Discordant results from GO term enrichment analysis and/or molecular network analysis when re-running analyses later (step 49, step 61, and step 71).

Potential solution

In this protocol, GO term enrichment was completed by retrieving GO annotations from the Ensembl database (Durinck et al., 2005, 2009) or the org.Hs.e.g.,db database (Carlson, 2021). And
a PPI network was constructed utilizing the STRING database (Szklarczyk et al., 2021). Databases implement different approaches to manage annotation. Furthermore, databases are updated over time. As such, the outcome of enrichment or network analysis can change depending on which database was employed for annotation and/or when the analysis was conducted (Tomczak et al., 2018). One way to improve the reproducibility of gene set analysis is to increase the input sample size (Maleki et al., 2019). Comparing annotations from multiple databases may also improve reproducibility. Finally, one should keep track of data provenance as the outcome of enrichment or network analysis will differ when R packages are updated (e.g., org.Hs.e.g.,.db for gene annotation by Entrez ID, and biomaRt for annotation by Ensemble ID).

**RESOURCE AVAILABILITY**

**Lead contact**

Requests for further information should be directed to and will be fulfilled by the lead contact, Huey-Min Chen (thmchen@ucalgary.ca).

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**Figure 4. Comparison of module-trait relationship matrices**

For the Module-Trait Relationships heat maps, the Pearson correlation value and p value (in brackets) are provided; p values below 0.05 were considered significant. Gene significance (GS) vs. module membership (MM) scatterplots were generated with varied minModuleSize values. Red boxes mark modules that were significantly correlated with the clinical trait of interest *lymphatic invasion*. 

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**Materials availability**
This protocol did not generate any new materials.

**Data and code availability**

1. Data

This paper analyzes existing, publicly available RNA-Seq data. The sources for the datasets are listed in the key resources table.

2. Code

This paper does not report original code. All codes were used in this study in alignment with recommendations made by authors of R packages in their respective user’s guides.

3. Additional information requests

Any additional information required to reanalyze the data used in this paper is available from the lead contact upon request.

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101168.

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**AUTHOR CONTRIBUTIONS**
H.C. completed the protocol outline, validated the materials, and wrote the manuscript. J.A.M. coordinated the study, secured grant funding support, edited the manuscript, and provided intellectual contributions to the project. All authors reviewed and approved the final version of the manuscript.

**DECLARATION OF INTERESTS**
Justin A. MacDonald is cofounder and a shareholder of Arch Biopartners Inc. Huey-Miin Chen has no competing interests.

**INCLUSION AND DIVERSITY**
One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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