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Canadian Bioinformatics Workshops

Module 2c: Ranked Gene Lists

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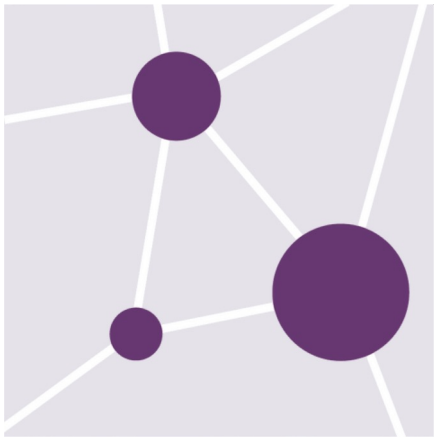
Pathway and Network Analysis

May 12, 2026



UNIVERSITY OF CALGARY

Arnie Charbonneau Cancer Institute

Learning Objectives

1. List differences between ORA and FCS
2. Learn how GSEA calculates enrichment scores
3. Understand pathway databases and GMT files
4. Interpret GSEA results
5. Identify when to use GSEA vs other methods

DE-derived gene lists

Pathway analysis tools require different inputs depending on the method

Defined Gene List

For Over-Representation Analysis

- A simple list of significant gene names
- Filtered by thresholds (FDR, FC)
- Unordered
- Often separated: up vs down
- **Tool: g:Profiler**
- **Method: Hypergeometric test (Fisher's Exact Test)**

```
TP53
BRCA1
MYC
...
```

Ranked Gene List

For Functional Class Scoring

- ALL genes with a numeric score
- No arbitrary threshold cutoff
- Ranked from most up to most down
- **Tool: GSEA**
- **Method: Running sum statistic (modified Kolmogorov-Smirnov)**

```
TP53    14.2
BRCA1   -9.8
MYC      0.5
...
```

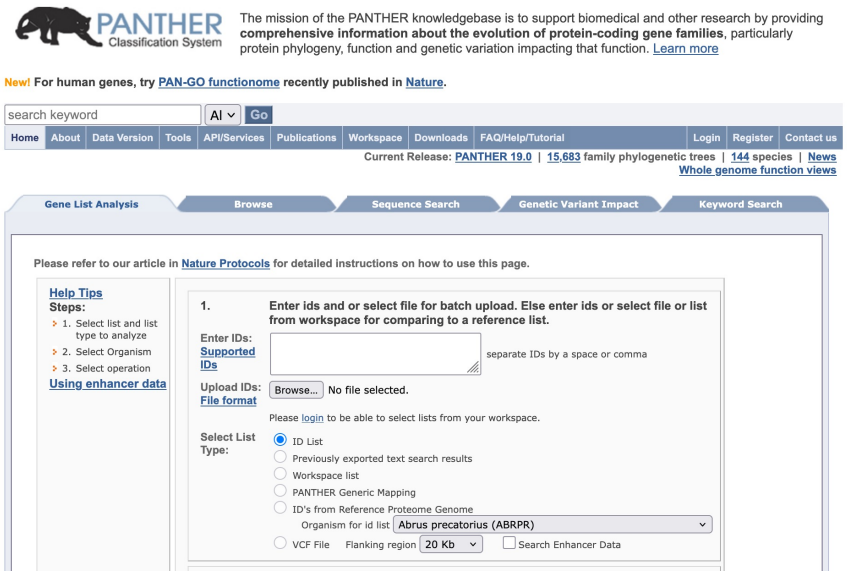
Functional Class Scoring methods test whether genes in a gene set are enriched at the extremes of a ranked gene list rather than randomly distributed

Why test enrichment in ranked gene list?

- Possible limitations of a defined gene list approach
  - No “natural” value to set thresholds
  - Different thresholds give different results
  - Possible loss of statistical power due to thresholding
    - All significant signals are weighted the same
    - Weak signals are neglected
- Key insight: If many pathway genes show modest, co-ordinated changes, that pathway may be dysregulated
  - *ie*: A pathway can be dysregulated even when no individual gene passes the significance threshold

Some tools for ranked lists

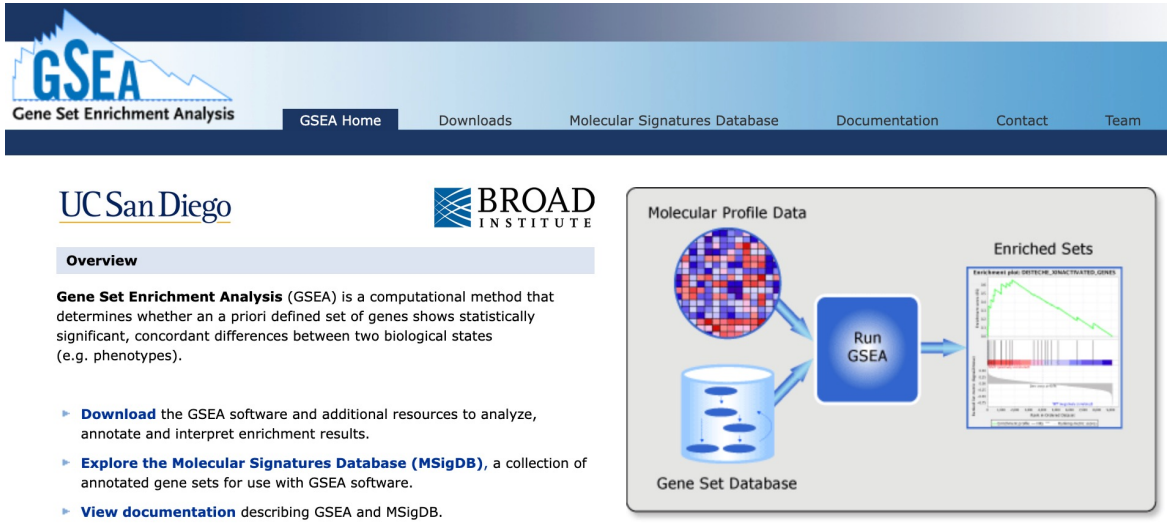
GSEA is the most commonly used tool for FCS



PANTHER is primarily ORA, but can accept a ranked list

We'll focus on GSEA

- Gene Set Enrichment Analysis



https://www.gsea-msigdb.org/gsea/index.jsp

GSEA: The core idea

Upregulated → → → Ranked Gene List → → → **Downregulated**

We have genes ranked from most upregulated to most downregulated

- GSEA asks: Do the genes in a given pathway cluster at the top or bottom of this list more than expected by chance?
- Three scenarios:

Enriched at TOP	**Random distribution**	**Enriched at BOTTOM**
Pathway upregulated	Pathway not enriched	Pathway downregulated

GSEA has three key steps

1. Calculate an enrichment score (ES)

2. Estimate significance level of ES

3. Adjust for multiple testing

Step 1: The GSEA enrichment score

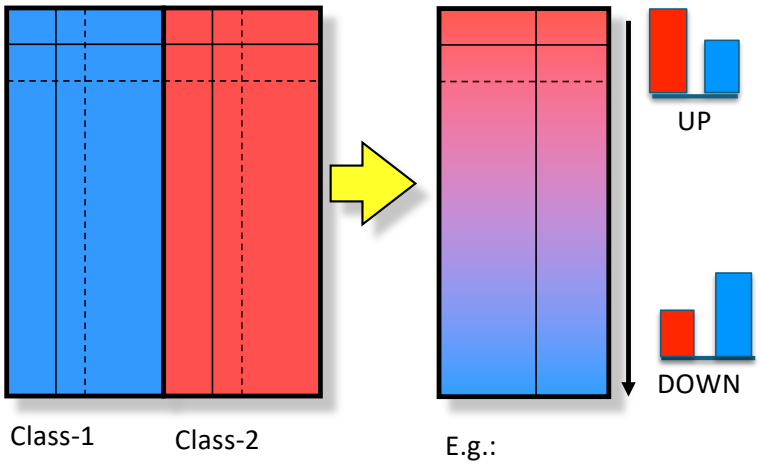
For a given gene set *S* and a ranked gene list *L*, the ES reflects how much *S* is overrepresented at the extremes of *L*

I. Rank all genes in *L* by their score
   - *eg.* $\mathbf{ranking\ value} = -\log_{10}(\mathrm{p{-}value}) * \mathbf{sign}(\mathrm{log2FC})$

II. Walk down *L*
   - When we encounter a gene in *S*, increase a running sum statistic
   - When we encounter a gene not in *S*, decrease the running sum statistic

III. The ES is the maximum deviation from zero

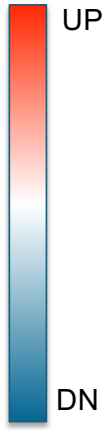
Recall the ranked gene list



Ranking value/score =

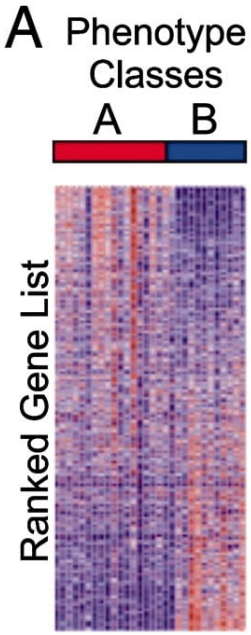
$$-\log_{10}(\text{p-value}) * \text{sign}(\log2\text{FC})$$

	LogFC	Pvalue
BGN	+1	1.73E-33
ANTXR1	+1	4.39E-31
FZD1	+1	4.41E-30
COL16A1	+1	1.33E-29
KLF3	+1	8.32E-02
RASEF	+1	9.01E-01
ISOC1	+1	9.01E-01
ANO1	+1	9.01E-01
CBWD3	-1	8.18E-02
GBP4	-1	2.45E-16
TAP1	-1	1.04E-19
PSMB9	-1	1.84E-20



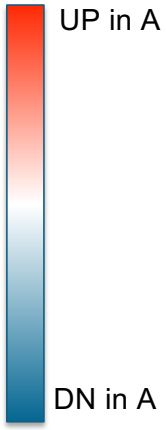
Calculating the GSEA ES

⚠ For a negative ES, the leading edge subset is the subset of members of S that appear after the peak score



Ranking score = sign(logFC)*-log10(pvalue)

	LogFC	Pvalue	score
BGN	+1	1.73E-33	32.76
ANTXR1	+1	4.39E-31	30.36
FZD1	+1	4.41E-30	29.36
COL16A1	+1	1.33E-29	28.88
KLF3	+1	8.32E-02	1.08
RASEF	+1	9.01E-01	0.05
ISOC1	+1	9.01E-01	0.05
ANO1	+1	9.01E-01	0.04
CBWD3	-1	8.18E-02	-1.09
GBP4	-1	2.45E-16	-15.61
TAP1	-1	1.04E-19	-18.98
PSMB9	-1	1.84E-20	-19.73



Modified from https://www.pnas.org/doi/10.1073/pnas.0506580102

GSEA ES walkthrough example

Ranked gene list *L*

UP

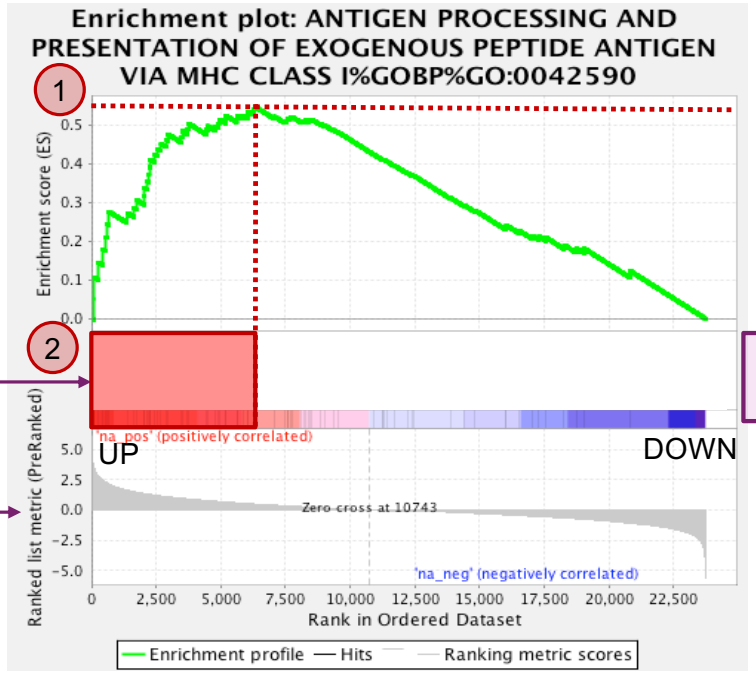
BGN	32.76
ANTXR1	30.36
FZD1	29.36
COL16A1	28.88
KLF3	1.08
RASEF	0.05
...	...
...	...
ISOC1	0.05
ANO1	0.04
CBWD3	-1.09
GBP4	-15.6
TAP1	-19
PSMB9	-19.7

DOWN

Ranked genes

Ranking value

Gene set of interest *S*



Enrichment Score

Mark the genes in *L* that occur in *S*

1. Maximum (or minimum) ES score is the final **ES score** for the gene set
2. Can define "leading edge subset" as all those genes ranked as least as high as the enriched set.

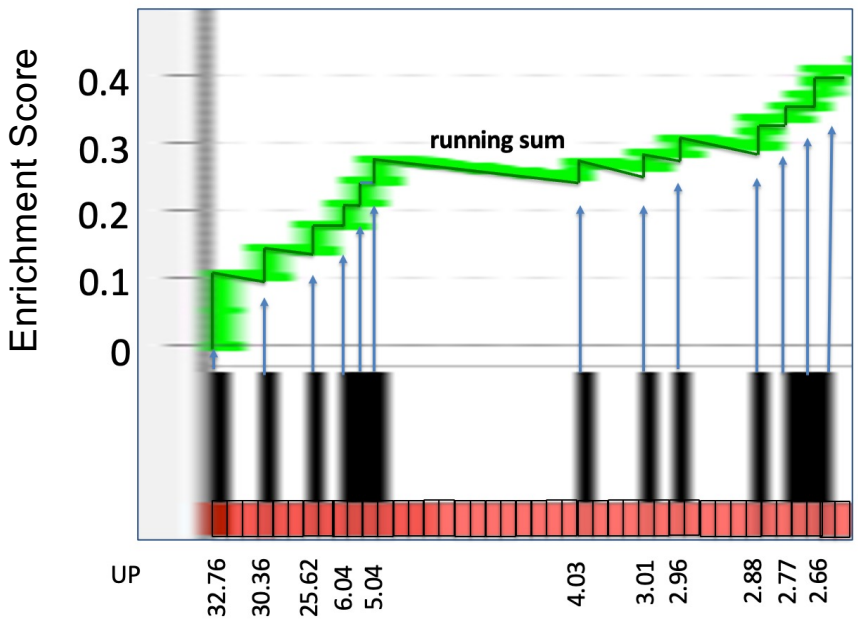
The GSEA running sum

Ranked gene list *L*

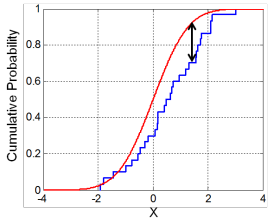
UP

BGN	32.76
ANTXR1	30.36
FZD1	29.36
COL16A1	28.88
KLF3	1.08
RASEF	0.05
...	...
...	...
ISOC1	0.05
ANO1	0.04
CBWD3	-1.09
GBP4	-15.6
TAP1	-19
PSMB9	-19.7

DOWN



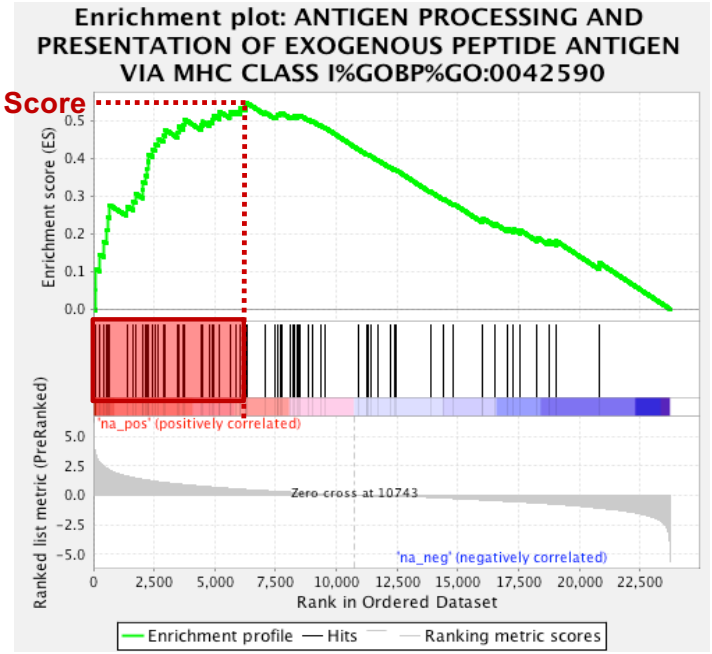
Conceptually a modified Kolmogorov–Smirnov test



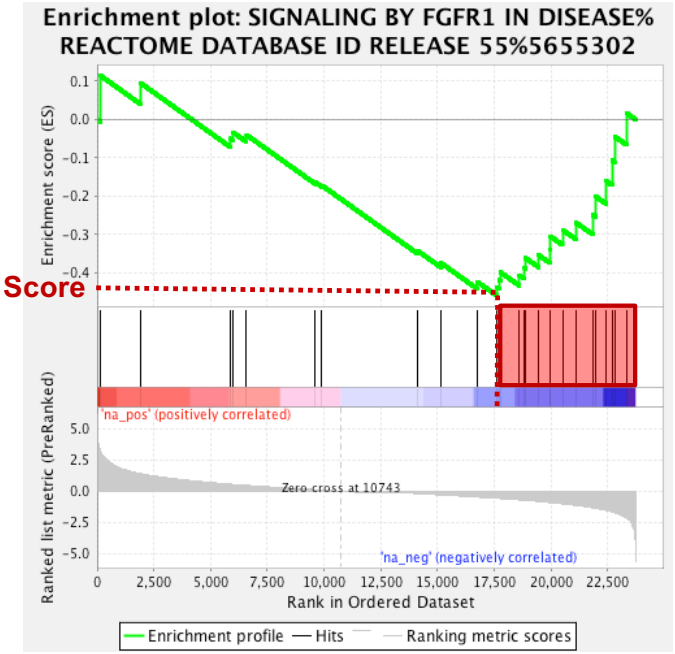
- The KS test compares distributions by finding the maximum distance between two cumulative distribution functions
- The GSEA score incorporates weights into the running sum
- Running sum step sizes are unequal

Positive and negative enrichment

Enrichment Score



Enrichment Score



Step 2: Estimate significance level of ES

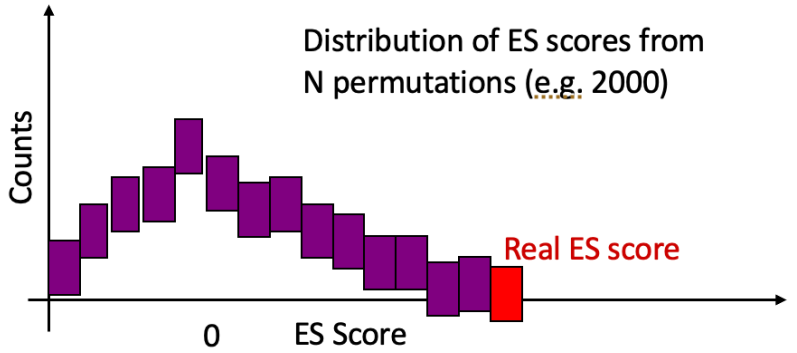
GSEA determines significance by comparing observed ES to a null distribution generated by permutation

I. Calculate the real ES

II. Randomly permute phenotype labels (or gene set membership)
- Recalculate ES for each permutation
- Repeat 1,000+ times to build the null distribution

III. Compare real ES to null distribution to derive p-value

For positive ES:
p-value = (# of null ES ≥ observed ES) / (# of permutations)
= 4 / 2,000
= 0.002



For negative ES:
p-value = (# of null ES ≤ observed ES) / (# of permutations)
= 4 / 2,000
= 0.002

Step 3: Adjust for multiple testing

When evaluating many gene sets, remember we must account for multiple hypothesis testing!

Interpreting GSEA output

- Sample gene set enrichment: *TP53*-mut vs *TP53*-WT
  - Enrichment in *TP53*-WT

Normalized Enrichment Score

Family-wise Error Rate correction

NAME	GS	GS DESC	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
hsp27Pathway	hsp27Pathway	Details ...	15	0.7782923	2.1820793	0	0.003337581	0.004
p53hypoxiaPathway	p53hypoxiaPathway	Details ...	20	0.67939055	2.0815823	0	0.008710083	0.013
p53Pathway	p53Pathway	Details ...	16	0.7459421	2.0595584	0	0.009290409	0.018
P53_UP	P53_UP	Details ...	40	0.5995161	1.8757944	0	0.065383784	0.195
radiation_sensitivity	radiation_sensitivity	Details ...	26	0.5673681	1.8265389	0.001689189	0.088138245	0.296
ck1Pathway	ck1Pathway	Details ...	15	0.53940195	1.6332773	0.015180266	0.4493492	0.835
inflamPathway	inflamPathway	Details ...	28	0.54343283	1.5540237	0.049069375	0.7013238	0.955
no2il12Pathway	no2il12Pathway	Details ...	17	0.62922	1.5182602	0.048824593	0.7759864	0.975
badPathway	badPathway	Details ...	21	0.47360346	1.514384	0.055658627	0.7095338	0.977
lairPathway	lairPathway	Details ...	15	0.5720317	1.497634	0.05719921	0.70921886	0.986
chemicalPathway	chemicalPathway	Details ...	21	0.45646906	1.48676	0.045289855	0.69150734	0.989
GPCRs_Class_A_Rhodopsin-like	GPCRs_Class_A_Rhodopsin-like	Details ...	111	0.42651632	1.4715267	0.062305298	0.6924941	0.991
cytokinePathway	cytokinePathway	Details ...	21	0.5414412	1.4707	0.09540636	0.64282876	0.991
p53_signalling	p53_signalling	Details ...	87	0.34070346	1.4658169	0.028037382	0.6143147	0.994
MAP00561_Glycerolipid_metabolism	MAP00561_Glycerolipid_metabolism	Details ...	43	0.41517496	1.4188069	0.072607264	0.752188	0.998
ST_Interleukin_4_Pathway	ST_Interleukin_4_Pathway	Details ...	24	0.4032152	1.3417566	0.09965035	1	1
HTERT_DOWN	HTERT_DOWN	Details ...	64	0.3427992	1.3177729	0.110526316	1	1

NES normalizes ES by the mean of the null distribution

- Allows comparison between gene sets of different sizes

$$\text{NES} = \frac{\text{observed ES}}{\text{mean}(|\text{null ES values}|)}$$

The leading edge

- The leading edge subset contains the genes that "drive" the enrichment signal.
- Leading Edge Statistics:
  - Tags: % of pathway genes appearing before (or after) the ES peak
  - List: % of ranked list genes appearing before (or after) the ES peak
  - Signal: Enrichment signal strength combining Tags and List

Positive ES (Upregulated)

Leading edge = genes BEFORE the peak (at the top of the ranked list, contributing to the upward climb)

Negative ES (Downregulated)

Leading edge = genes AFTER the trough (at the bottom of the ranked list, contributing to the downward dive)

Gene set databases

- Gene sets are stored in GMT (Gene Matrix Transposed) format:

```
PATHWAY_NAME    Description    GENE1    GENE2    GENE3    ...
```

- Some resources:

Resource	Description	Website
MSigDB	Broad Institute collection (Hallmarks, C2 curated, C5 GO, etc.)	https://www.gsea-msigdb.org/gsea/msigdb
Bader Lab	Combined GO + pathway databases, updated monthly	https://baderlab.org/GeneSets
Reactome	Curated human pathway database	https://reactome.org/
KEGG	Manually drawn pathway maps	https://www.genome.jp/kegg/pathway.html
WikiPathways	Open-source, community-curated biological pathways	https://www.wikipathways.org/

Gene set size considerations

- The size of gene sets affects statistical power and interpretability:

Too Small (<15)

- Low statistical power
- High variance in ES
- Sensitive to noise

Recommended
(15-500 genes)

- Good statistical power
- Interpretable pathways
- Robust ES estimates

Too Large (>500)

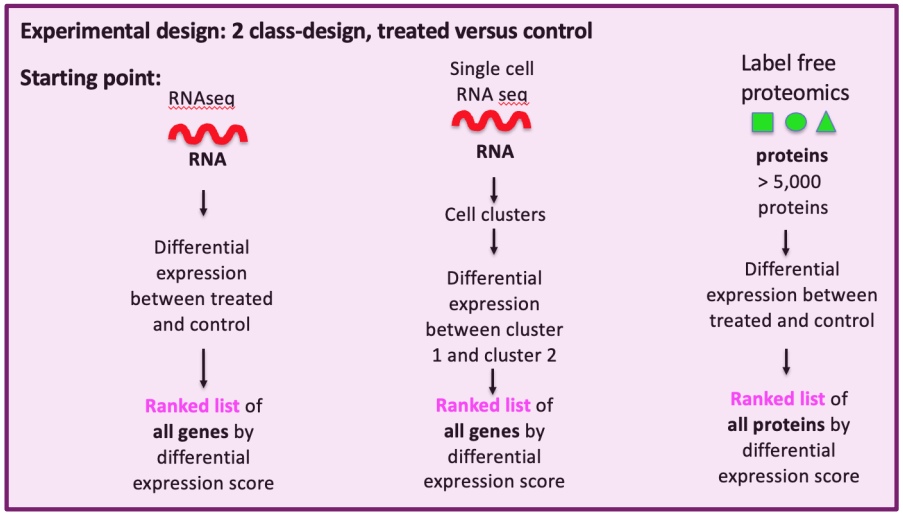
- Overly broad terms
- Less specific biology
- Harder to interpret

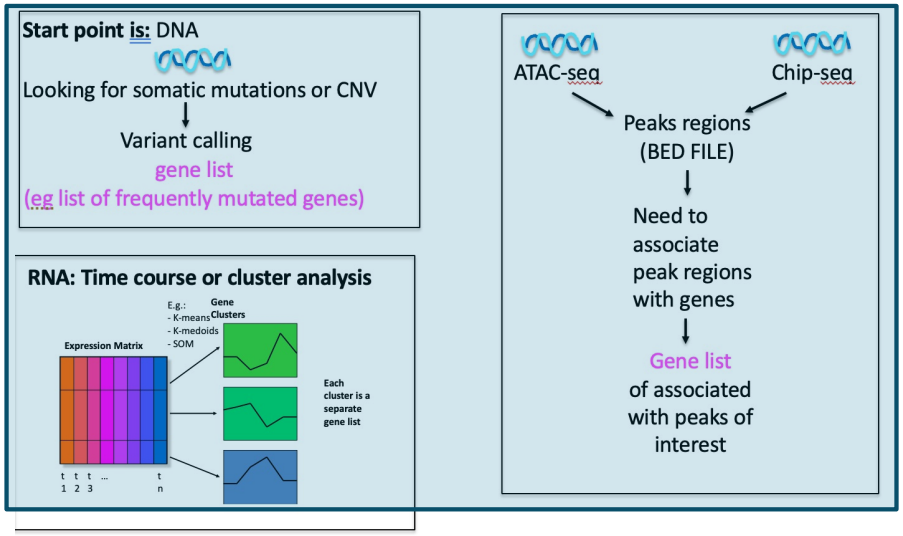
GSEA Settings

Min size: Default 15, can lower to 10 for exploratory analysis
Max size: Default 500, consider 200-250 for more specific results

ORA or FCS?

- First, consider your gene set: Is it ranked or not ranked?





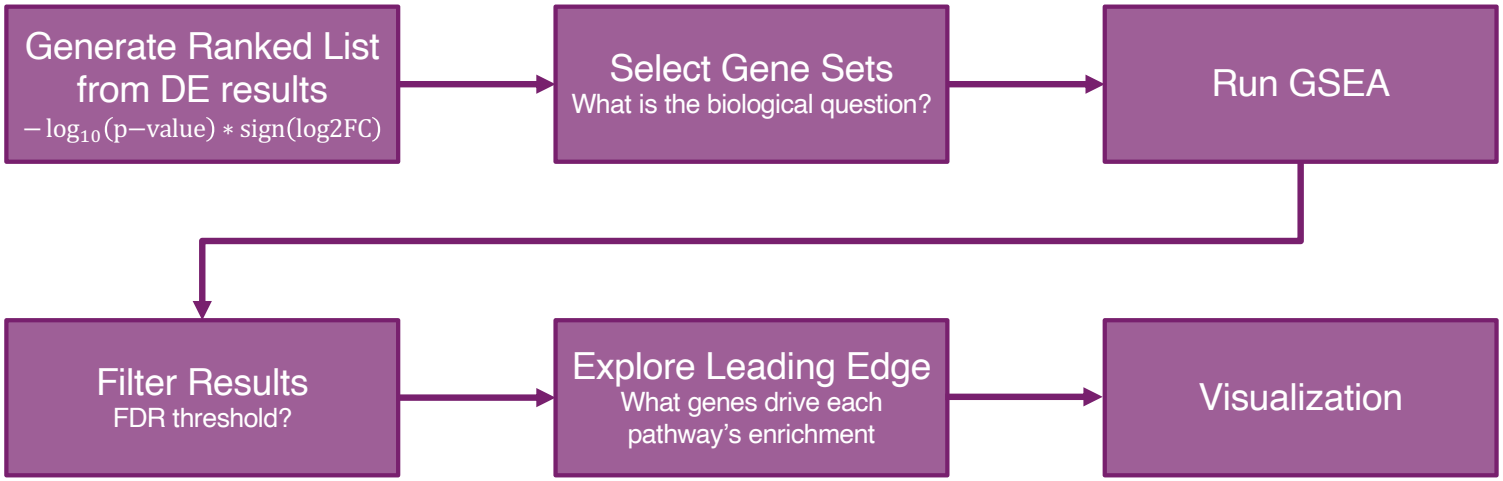
ORA or FCS (specifically GSEA)?

- Also consider characteristics of the method

	ORA	**GSEA**
Input	Defined gene list (threshold-based)	Ranked gene list (all genes)
Information used	Binary (in list or not)	Continuous (ranking scores)
Threshold dependence	High - results change with cutoff	None - uses full ranking
Subtle effects	May miss coordinated changes	Detects subtle, consistent changes
Speed	Very fast	Slower (permutations)
Interpretation	Straightforward	Richer (leading edge, NES)

- Suggestion: Try both! ORA for quick exploration, GSEA for deeper investigation

GSEA workflow summary



Summary

- Functional Class Scoring methods are appropriate for ranked gene lists
  - Uses ALL genes, not just significant ones (no arbitrary threshold)
  - GSEA is the most commonly used tool
- GSEA detects coordinated changes in gene sets
- Enrichment Score reflects clustering at list extremes
  - NES allows comparison across gene sets of different sizes
- Leading edge genes drive the enrichment signal

Some other ranked list methods

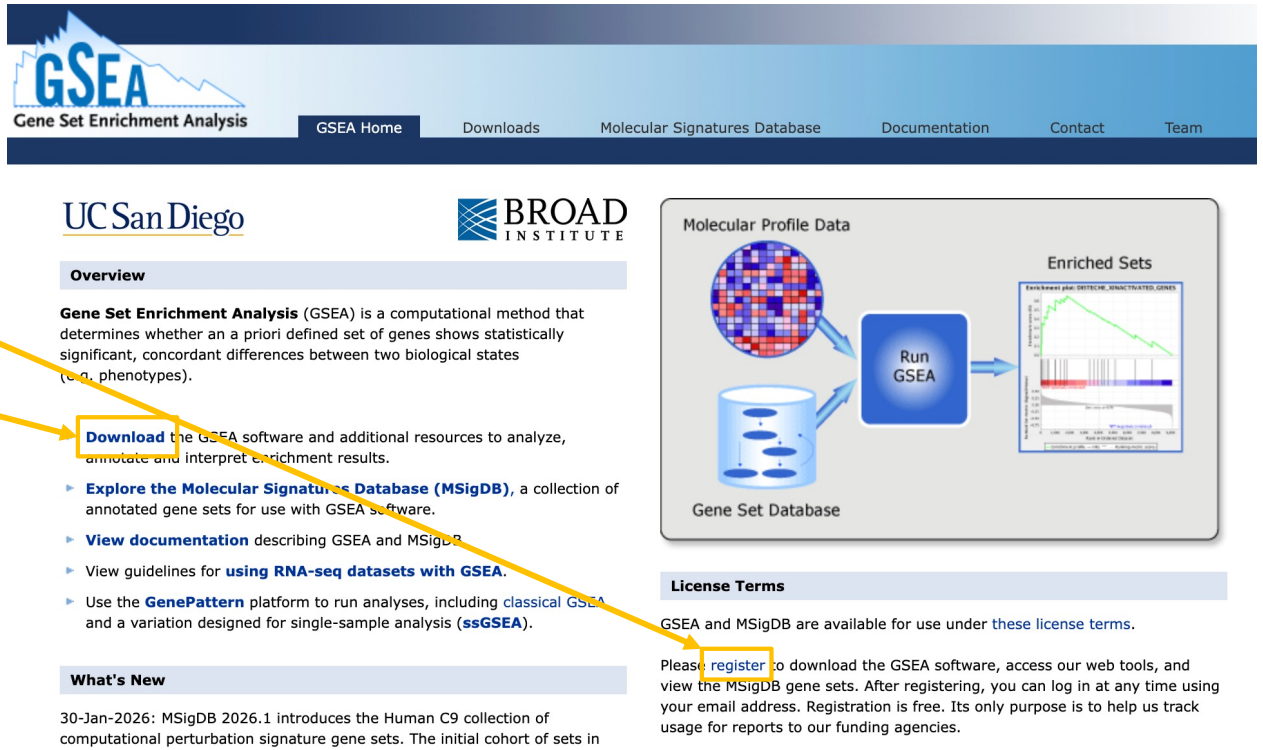
Method	Algorithm	Key features
GSAV	Gene set variation analysis	Per-sample enrichment scores; good for clustering
ssGSEA	Single-sample GSEA	Variant of GSEA that scores each sample individually
CAMERA	Correlation-adjusted mean rank	Accounts for inter-gene correlation; parametric
GAGE	Generally Applicable Gene-set Enrichment	Two-sample t-test on gene sets; directional

Time to try it!

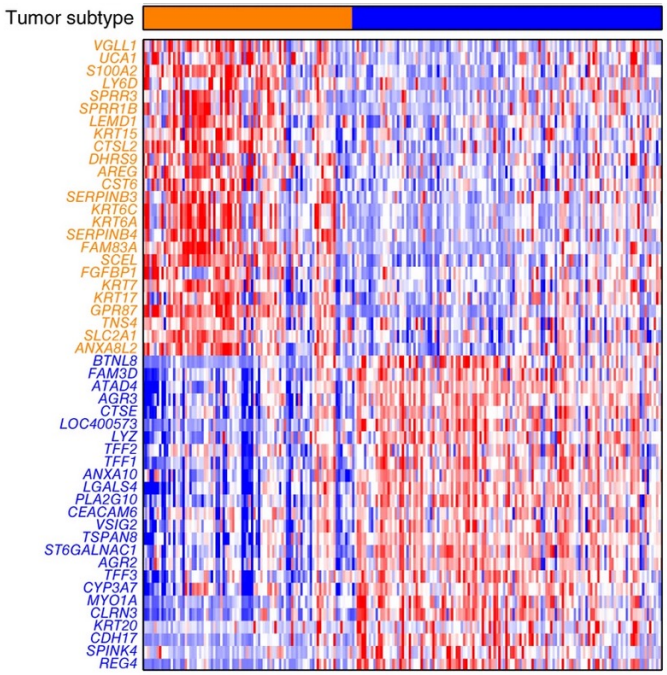
- Download the lab materials:
  - https://bioinformaticsdotca.github.io/PNA_CalSask-2605/module-2.html
  - Module 2c: Lab protocol pdf
    - You can use our input .rnk or use the one you generated in Lab 2a
- You'll need to register and download GSEA

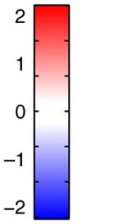
Prep for Lab

- Go to https://www.gsea-msigdb.org
  - Register to download
  - Then Download
- Go to https://download.baderlab.org/EM_Genesets/current_release/Human/symbol/
  - Download the geneset Human_GOBP_AllPathways_noPFOCR_no_GO_iea_May_01_2026_symbol.gmt



GSEA Lab: Continue our PAAD exploration





Module 2a Lab

DESeq2

Defined Gene List → Module 2b Lab

Ranked Gene List → Module 2c Lab