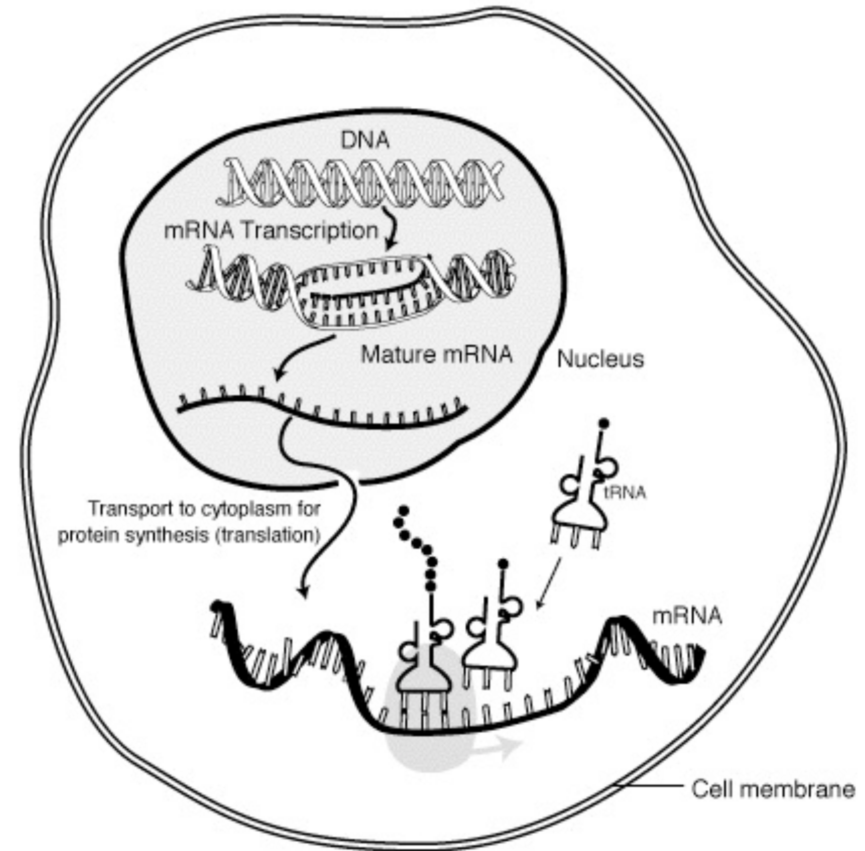
RNA-seq

What is RNA-seq and how does it work?

What is RNA-seq?

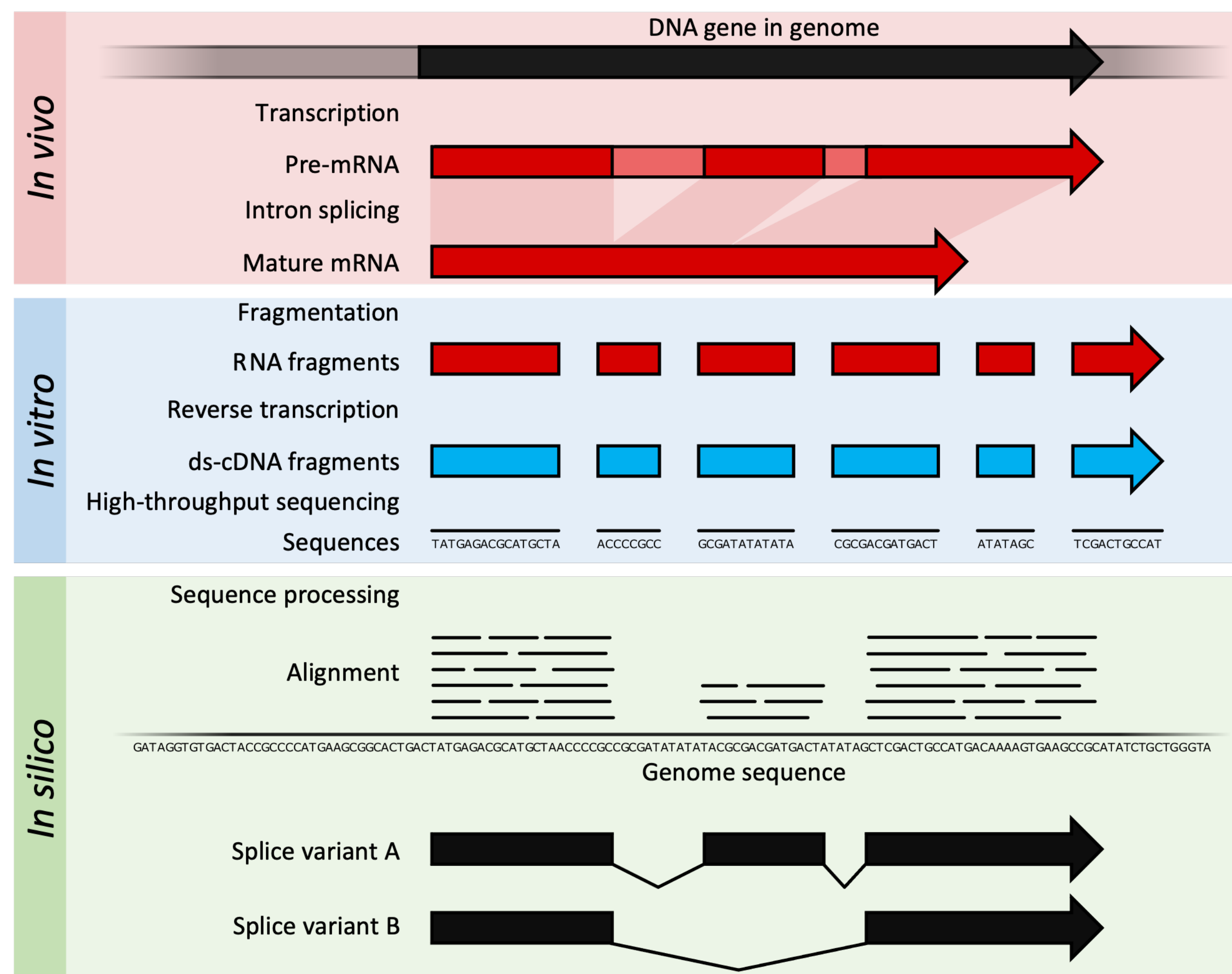
- RNA-seq = RNA sequencing
- Use next generation sequencing to quantify RNA in a cell
- Mainly when people do RNA-seq they’re concerned with mature messenger RNA, because they want to know what genes are expressed



Stark 2019, Image: Wikipedia

How are RNA-seq libraries prepared?

1. Capture RNA
   - Poly-A Capture
   - Ribo-Zero
2. Fragment
3. Reverse transcribe to make cDNA
4. PCR amplification
   - Sequencing adapters
   - PCR bias
5. Illumina sequencing
6. Align to a reference genome
7. Count number of reads that correspond to each gene



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Designing an RNA-seq Experiment

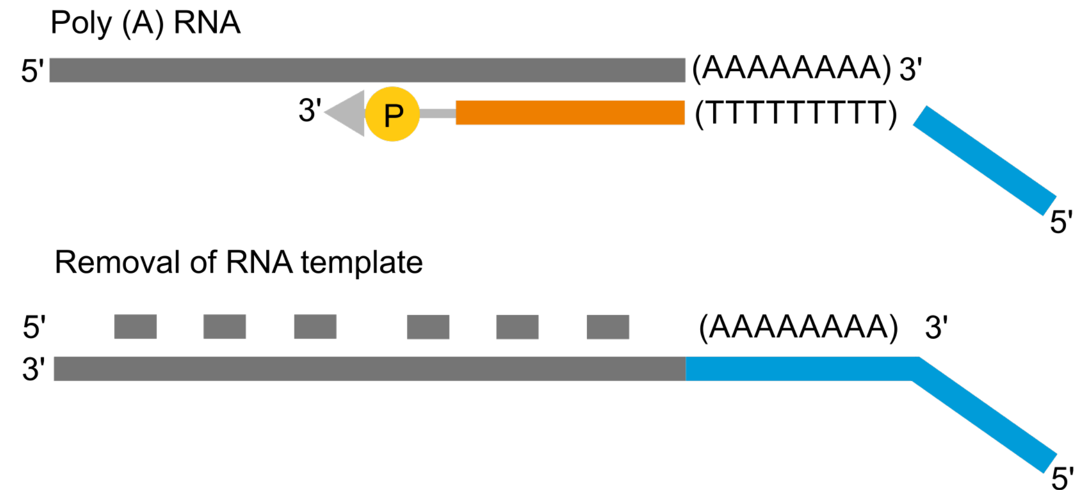
- Minimum experiment is 3 control samples vs 3 experimental samples
  - Examples:
    1. 3 control samples vs 3 samples treated with a drug
    2. 3 samples in young mouse intestine vs 3 samples in old mouse intestine
    3. 3 samples from germ free (no microbiome) mice vs 3 samples from normal mice
- You need 3 replicates per condition MINIMUM
  - Statistically need at least replicates in order to calculate variance
- Can have multiple conditions
  - Limited by budget
  - Increases the complexity of analysis

Designing an RNA-seq Experiment: Isolating RNA

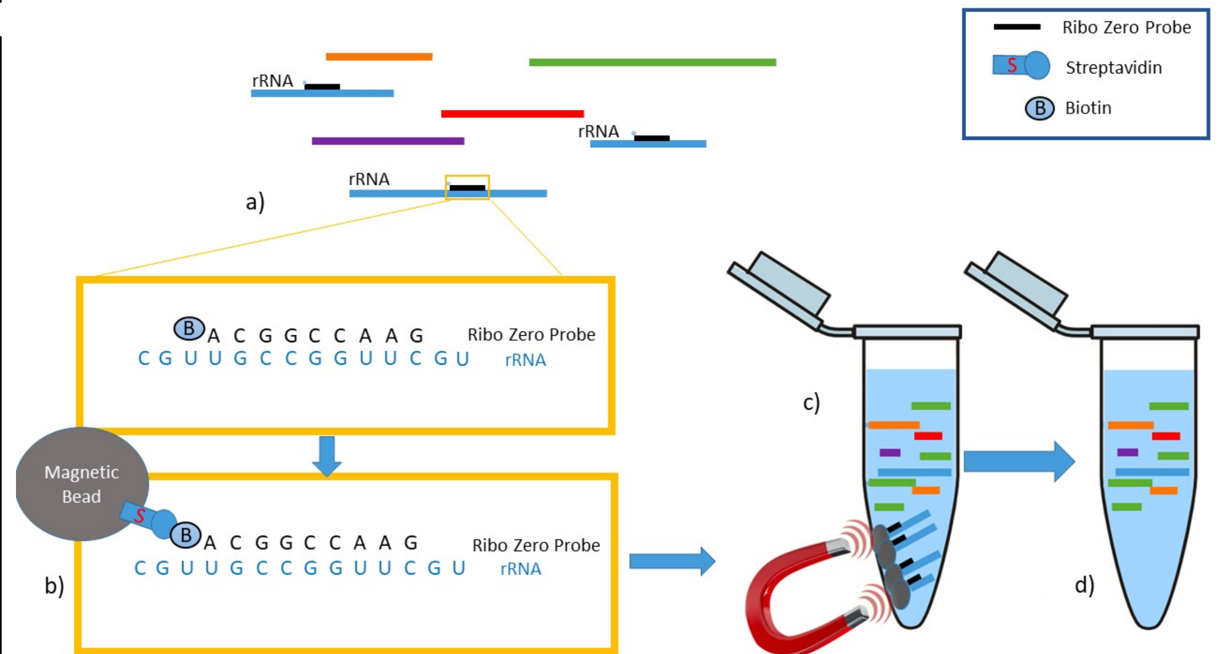
ISSUE: Most RNA (80-90%) is ribosomal RNA that we're not interested in

- Poly-A Capture
  - Use an oligo-dT primer complimentary to the poly-A tail of mature mRNA
  - Better at capturing only RNAs that correspond to expressed genes
- Ribosomal Depletion
  - Have sequences complimentary to ribosomal RNA on magnetic beads.
  - Sequence more non-coding RNAs, but some experiments want that
- The strategy you choose DOES effect your results

Poly-A Capture



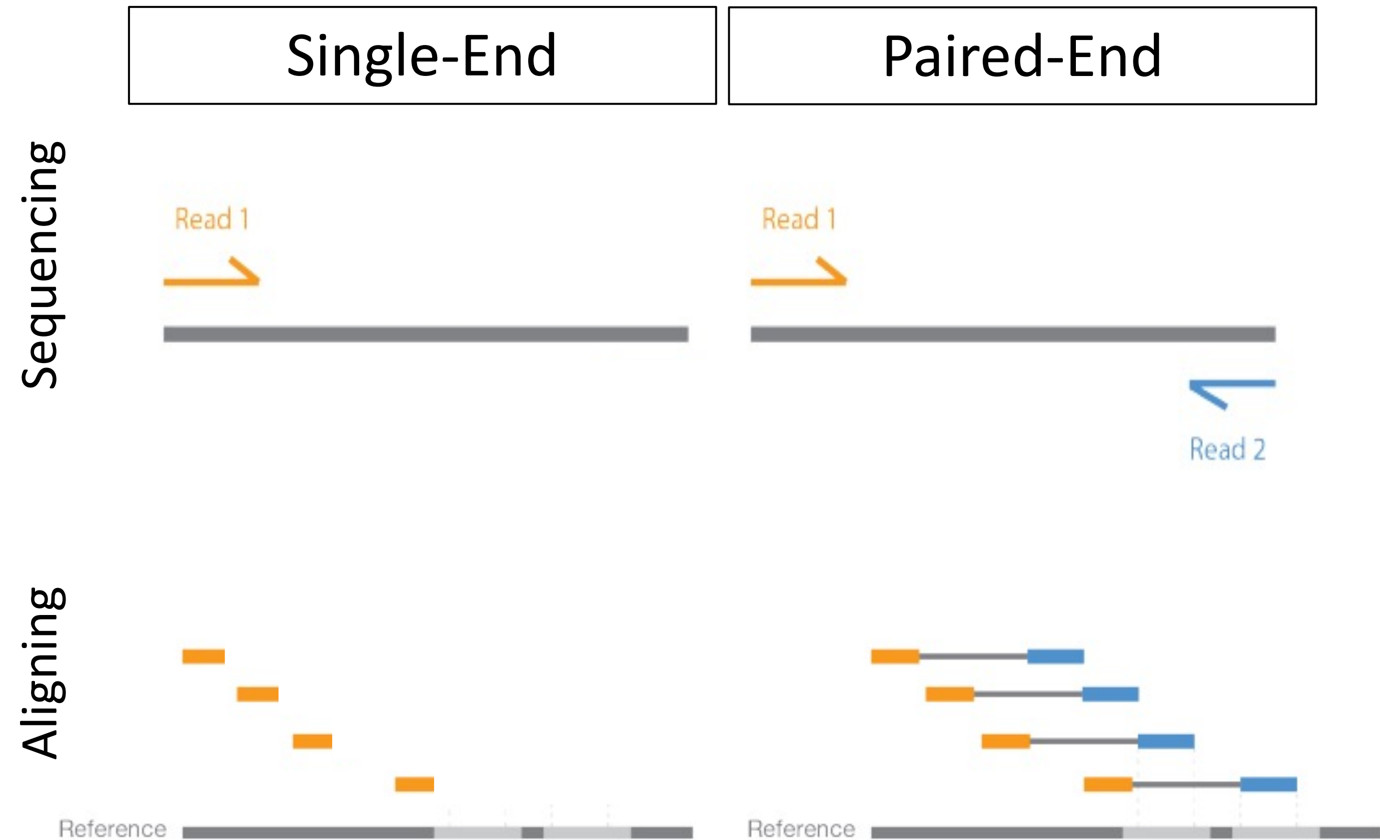
Ribosomal Depletion



Zhao 2018, top image: Thermo Fisher, bottom image: Illumina

Designing an RNA-seq Experiment: Picking the Sequencing Type

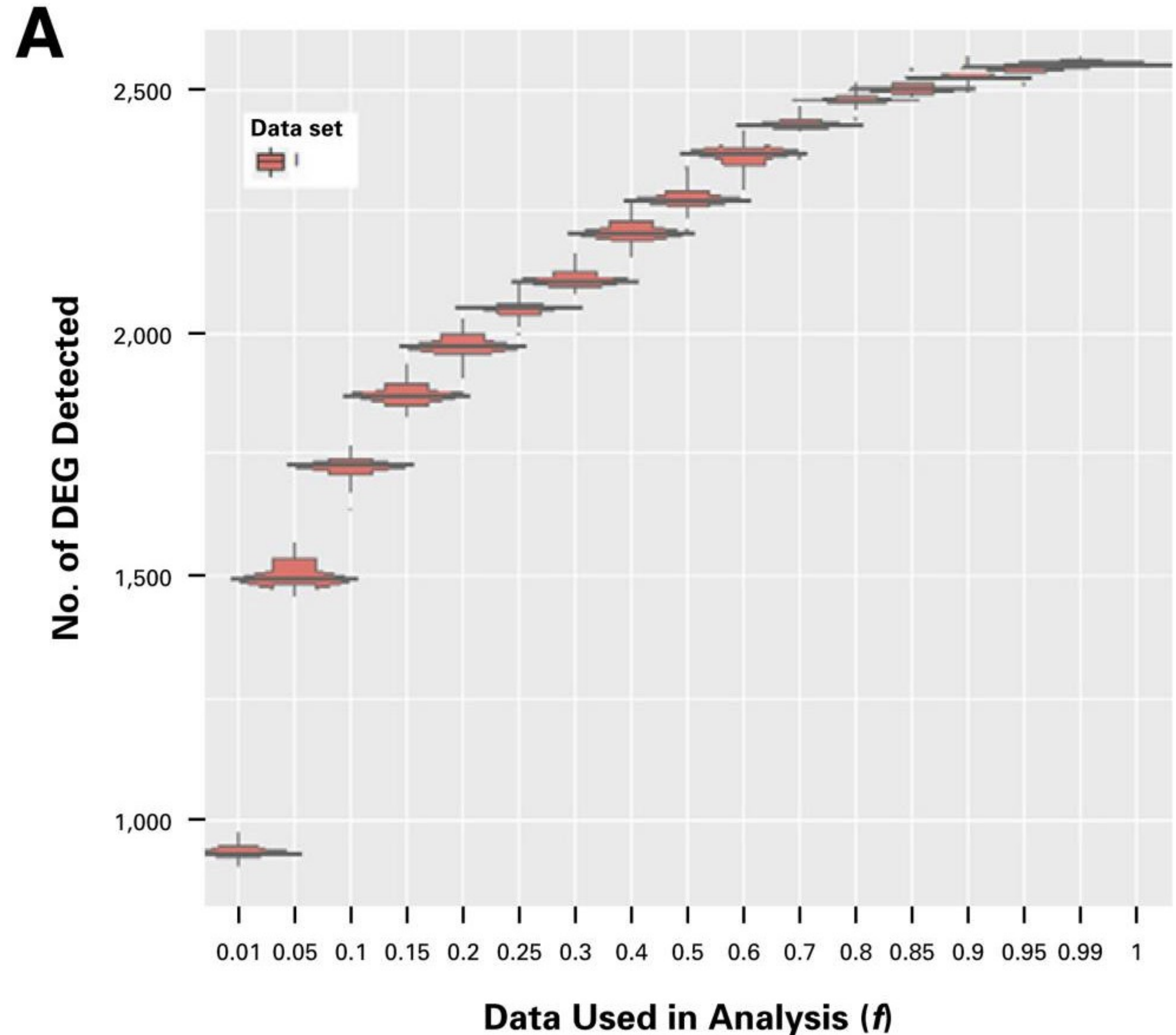
- Read Length
  - Longer the read length the more sequence overlap / coverage
  - More expensive as read length increases
    - Sequencing company charges more
    - Also more like to read into adapters which is a waste of money
- Single vs Paired-End Reads
  - Single-end is cheap and simple
  - Paired-end
    - Get 2 or more times the information from the same DNA
    - Good for repetitive RNA
    - Necessary for splicing



Illumina

Designing an RNA-seq Experiment: Sequencing Depth

- Sequencing depth
  - Standard depth is ~30 million reads per sample
  - If you're looking for very lowly expressed genes, may need to sequence more, but this should be fine for most experiments
- For plot - 50 million reads total. 1 = all 50 millions reads used



Stupnikov 2018

Analyzing RNA-seq

Processing Sequencing Data

1. Remove unwanted sequences
2. Align to reference genome
3. Count feature of interest
4. Filter and normalize.
    - Remove low counts
    - Remove features with low read depth
    - Compensate for differences in library size

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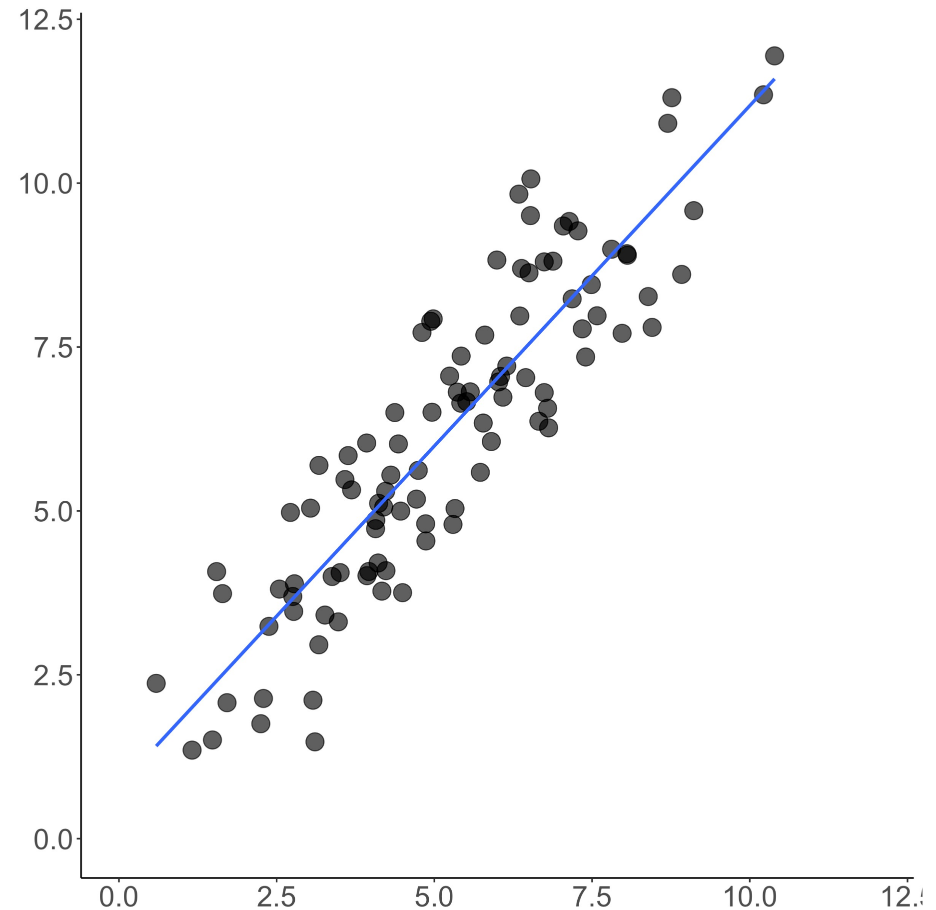
Differential Gene Expression

Is there is difference in expression of this gene between my conditions?

- Can use a variety of statistical tests, but most common practice is a linear model
- Test association of each gene with phenotype or condition
- Correct for multiple testing



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What else can you look at with RNA-seq?

- Transcript level quantification
- Alternative splicing
- Small RNAs like miRNA, etc…
- Transposable elements
- Ribosomal RNA
- Mutations like single nucleotide variants (SNVs), small insertions or deletions (indels), or copy number variations (CNVs)

Single Cell RNA-seq

What is single cell RNA-seq and why do we need it?

- Issues with bulk RNA-seq that single-cell RNA-seq overcomes
  - Average of multiple cell types
  - Diversity in expression
  - Where in the tissue was it?
- Challenges that still need to be addressed
  - What cell type is this? (cell atlases are working on it)
  - Data sparsity and measurement uncertainty
  - Cost, ~10x more expensive than bulk RNA-seq
- When should I use single cell sequencing? When you want to test something in **multiple cell types**

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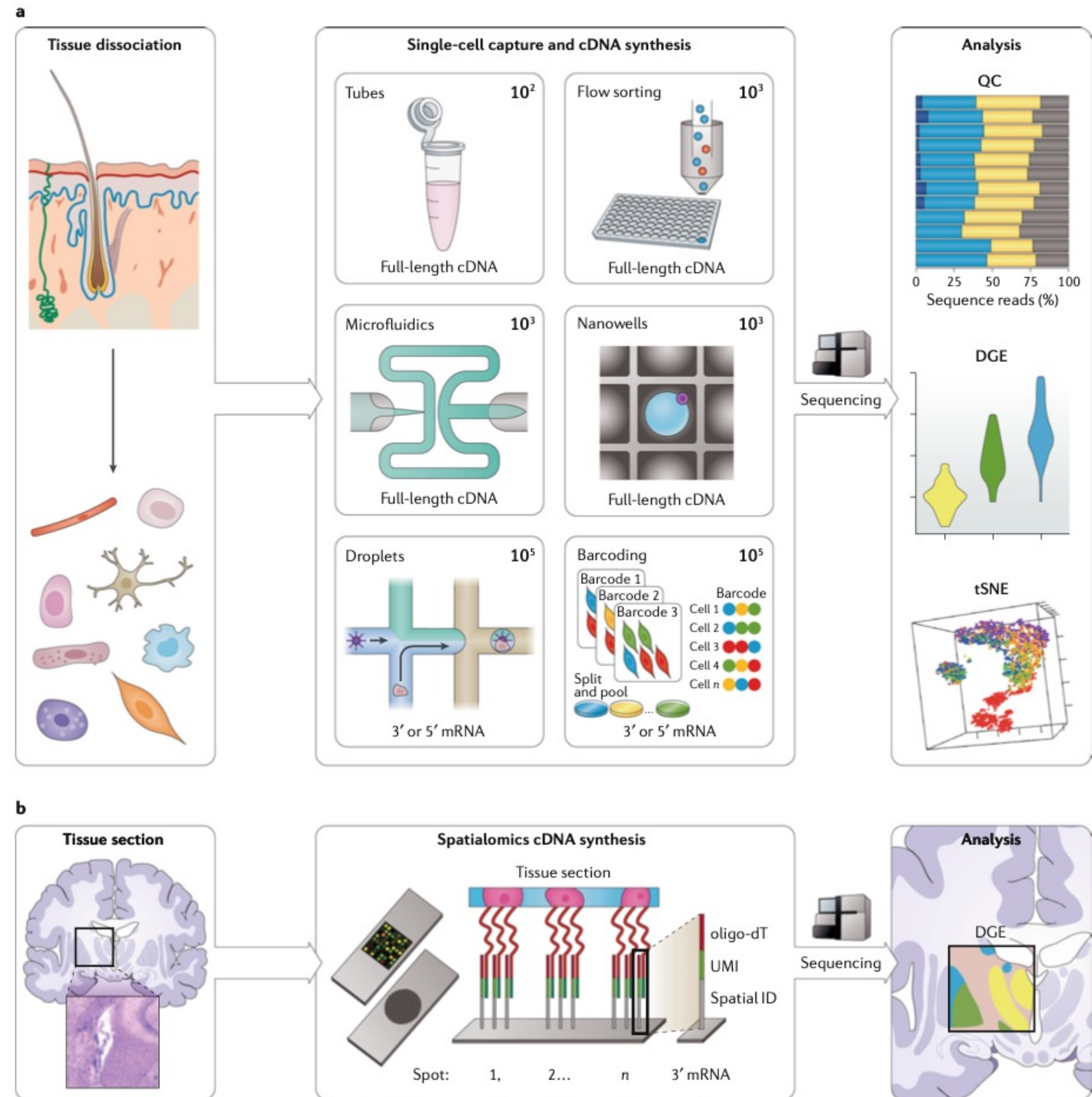
Single Cell Analysis

Overall strategy: Isolate single cells and attach a barcode to them.

1. Isolation
   - Flow sorting
   - Microfluidics / droplet encapsulation
2. Barcoding
   - Capture oligos either on a slide or a bead
   - Indexed PCR primers
3. Sequence everything together
4. Computationally sort cells apart when processing data



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