RNA-Sequencing

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K. Reinert and S. Andreotti

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Outline

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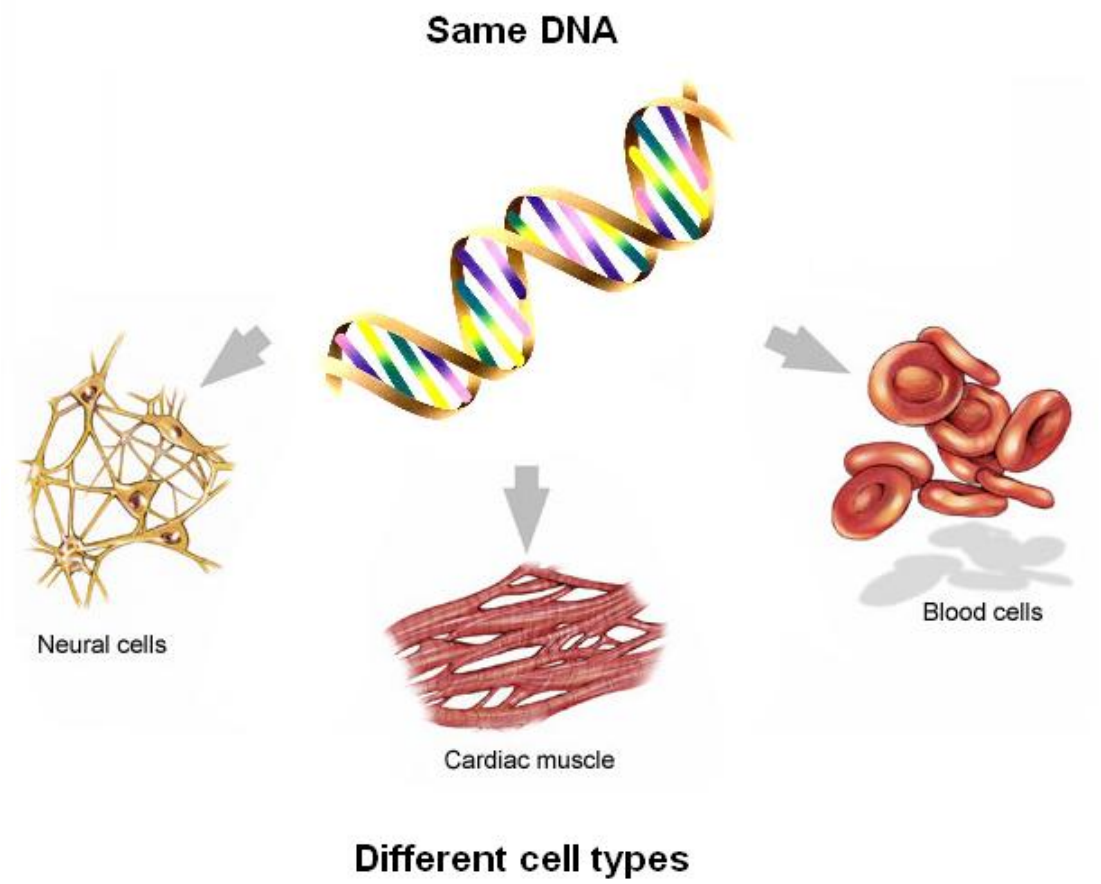
Motivation

- mRNAs specify cells' identity

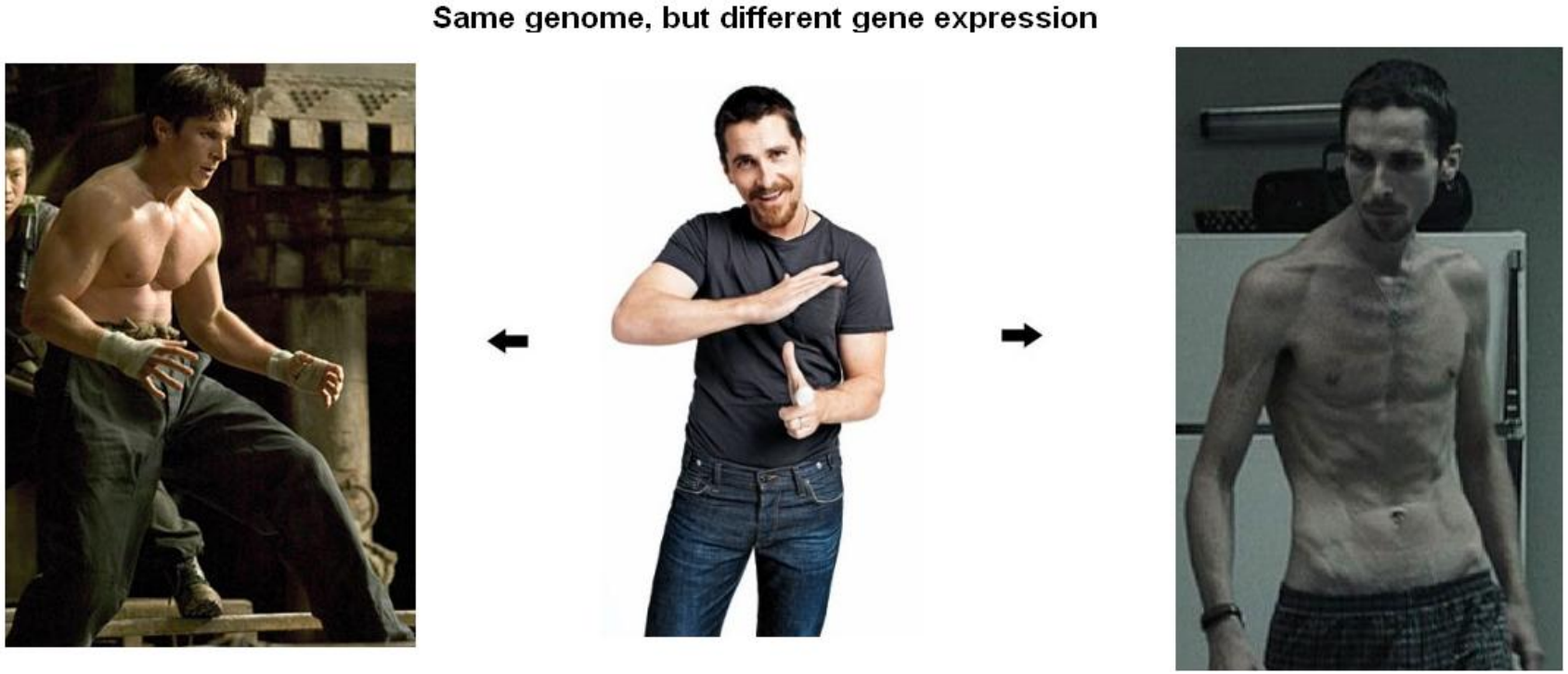


Motivation

- Crucial to understand the processes inside a cell
- DNA cannot give all that information, but transcriptome

Motivation

- mRNAs govern cells' activity due to environmental conditions



Motivation

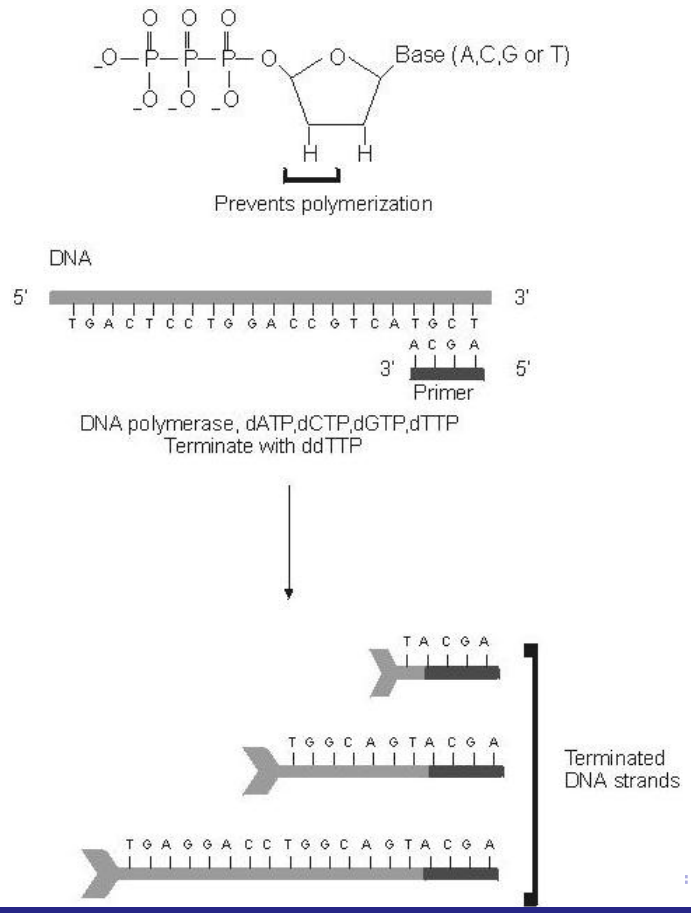
- Crucial to understand the processes inside a cell
- DNA alone doesn't give us all that information, but transcriptome does:
  - mRNAs specify cells' identity
  - mRNAs govern cells' activity due to environmental conditions

For transcriptome analyses RNA sequencing is needed!

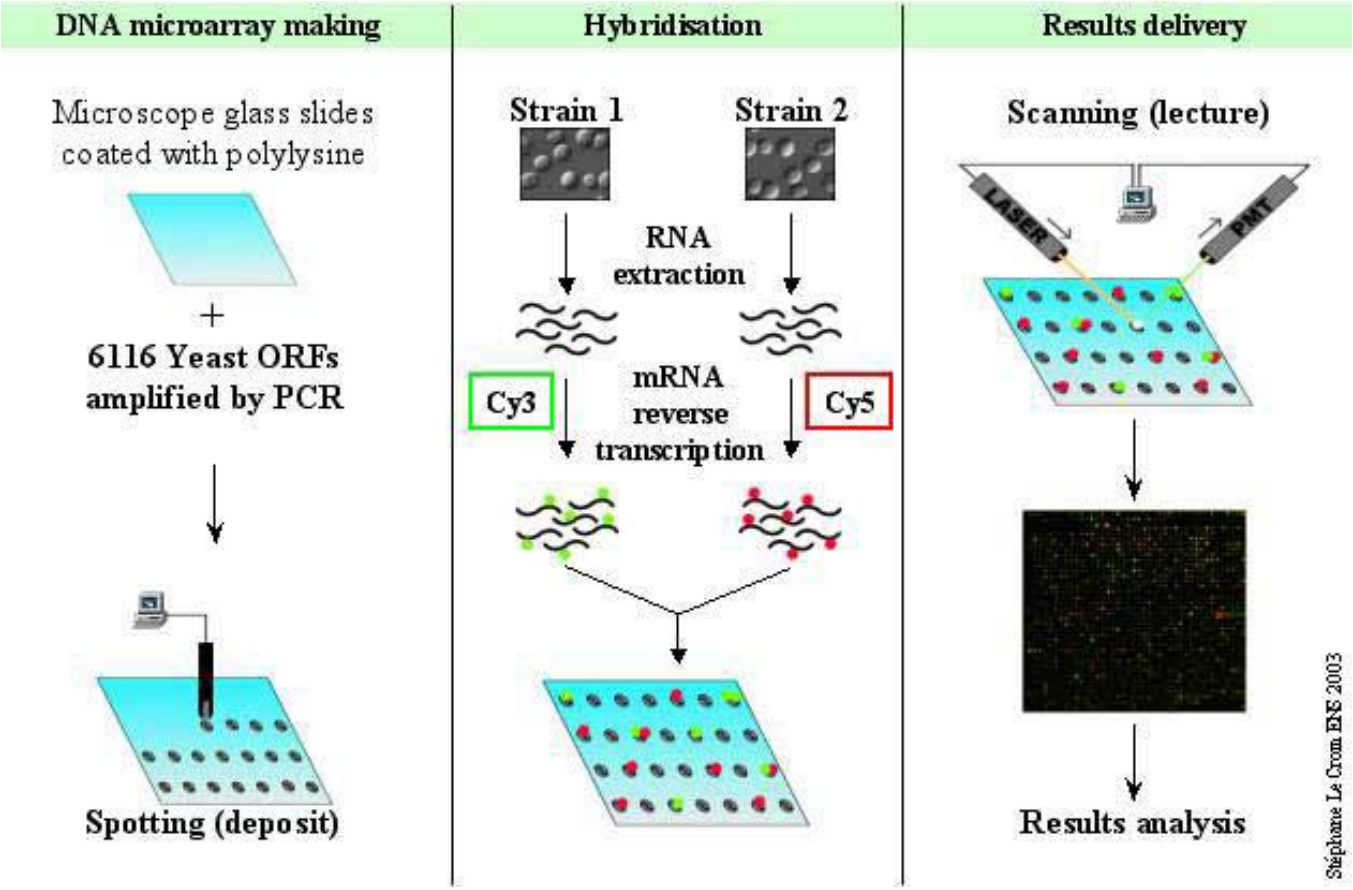
Outline

Sanger sequencing

- Create DNA strands of different lengths by tagged ddNTPs
- Not accurate for long strands
- Cost and time consuming



Microarrays



Microarrays

- (Cross-)hybridzation artifacts
- Dye-based detection issues
- Limited RNA splice detection
- No novel exon or novel gene detection

SAGE

Serial analysis of gene expression

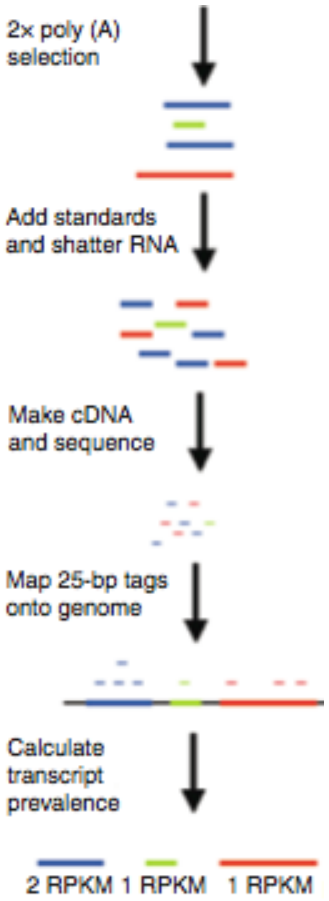
- Uses DNA sequencing of cloned tags 17-25bp from terminal ends
- Maps tags to mRNA database or source genome
- New or low abundant transcripts cannot be found

RNA sequencing

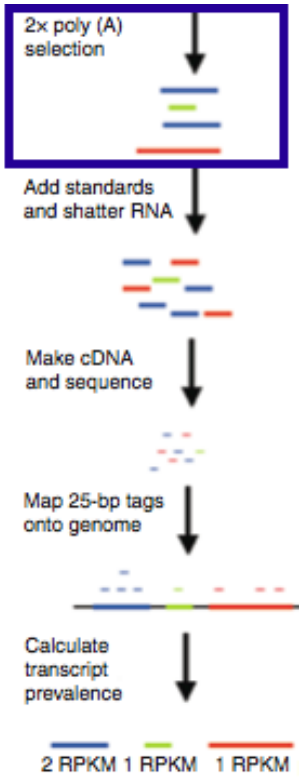
- Ultra-high-throughput sequencing
- No bacterial cloning of cDNA needed (no bacterial cloning constraints)
- Accurate due to short reads
- Can discover new exons, genes
- Can handle RNA splicing

Outline

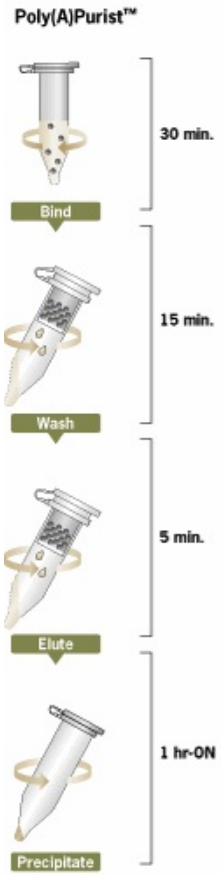
Pipeline - Outline



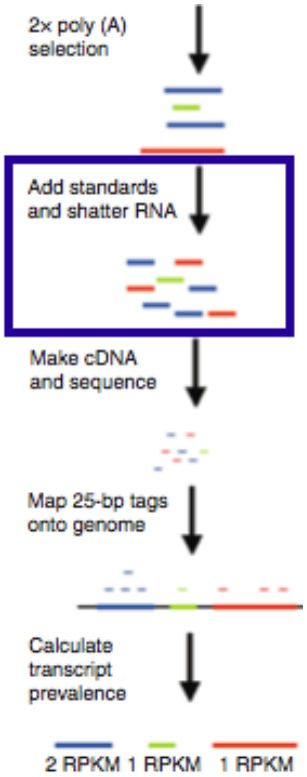
Poly(A)-Selection



- Input: Cell lysate
- Output: RNA

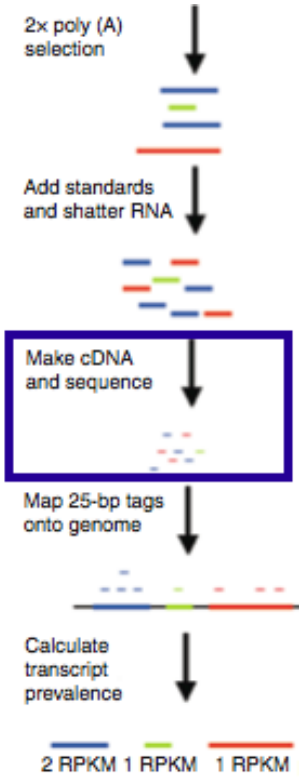


Fragmentation of RNA

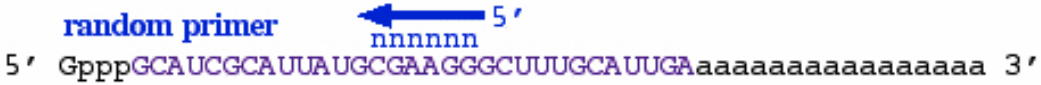


- Important for the uniform sequence coverage
- Why do we shatter before cDNA conversion?
  - Over-representation of 5'-ends of transcripts
  - Occurrence of strongly favored sites

Conversion to cDNA



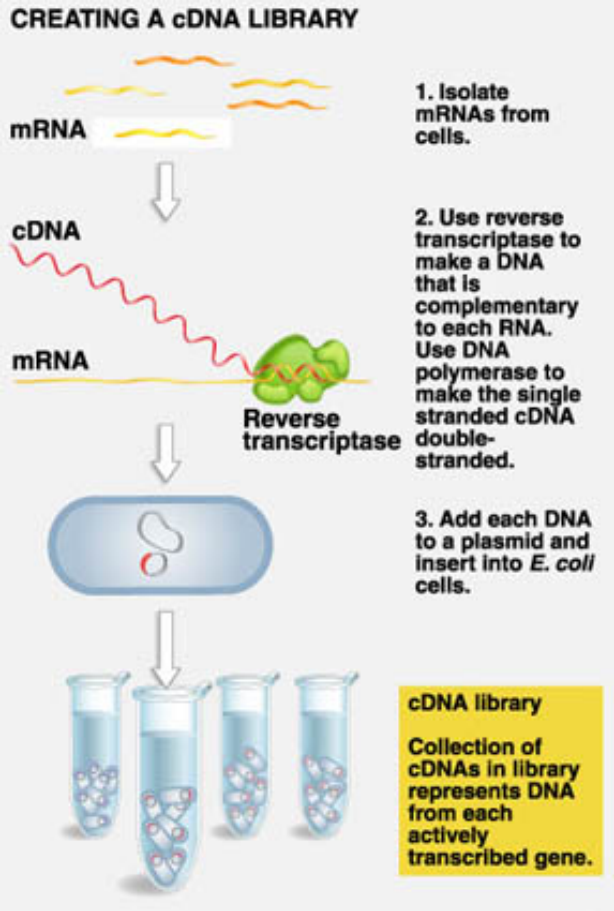
- Conversion by random priming

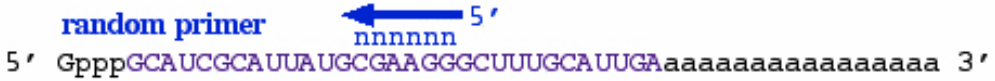


- Why do we use cDNA?
  - RNA: unstable, fast degradation
  - RNA forms secondary and tertiary structures
  - RNA: uridine instead of thymidine

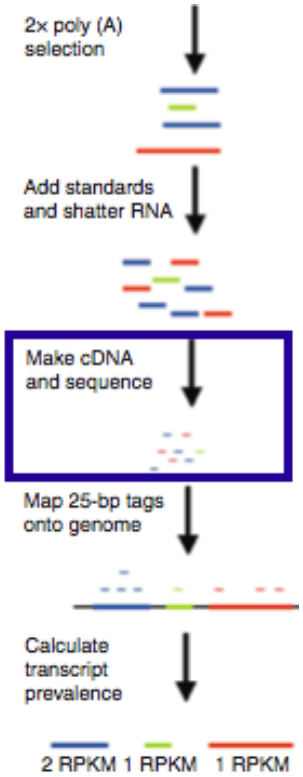
  $\longrightarrow$ More difficult to map

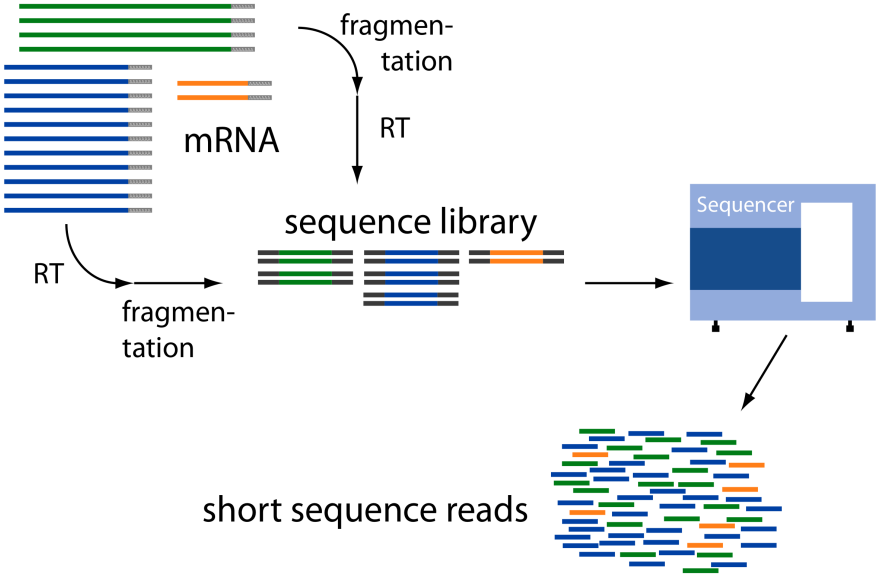
Conversion to cDNA



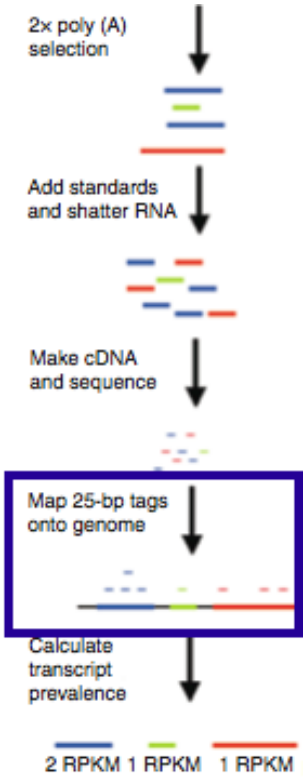


Sequencing

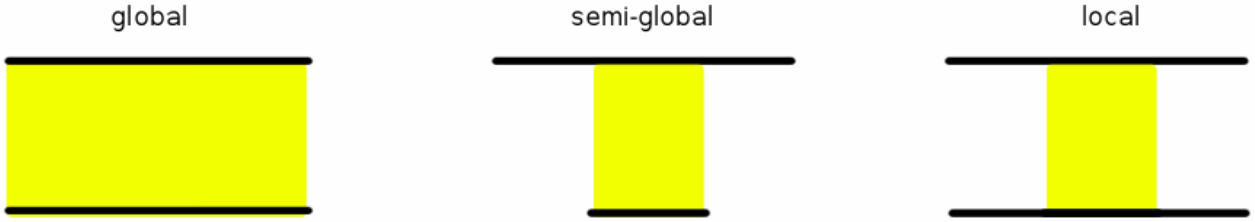

2x poly (A) selection
Add standards and shatter RNA
Make cDNA and sequence
Map 25-bp tags onto genome
Calculate transcript prevalence
2 RPKM
1 RPKM
1 RPKM


fragmen-
tation
RT
mRNA
sequence library
RT
fragmen-
tation
Sequencer
short sequence reads

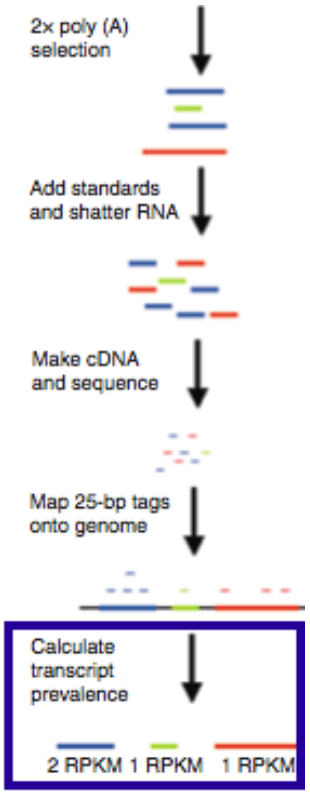
Mapping



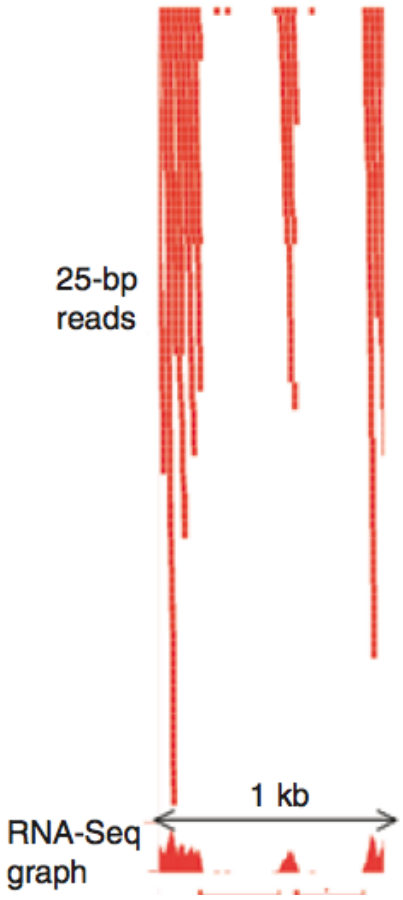
- *Read-mapping* is the semi-global alignment of many (very) short sequences (reads) to a long sequence (the genome)
- Alignment: approximate string matching



Quantification



- Gene coverage
- RPKM
  Reads per kilobase of exon model per million mapped reads



Outline

Overview

Two main phases

1. Filtration
2. Verification

$\Rightarrow$ different (combinations of) algorithms available

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Filtration

For every read:

- identify candidate regions on the genome by an heuristic(!) approach
- discard the rest of genome

Trade-off: Sensitivity vs Speed

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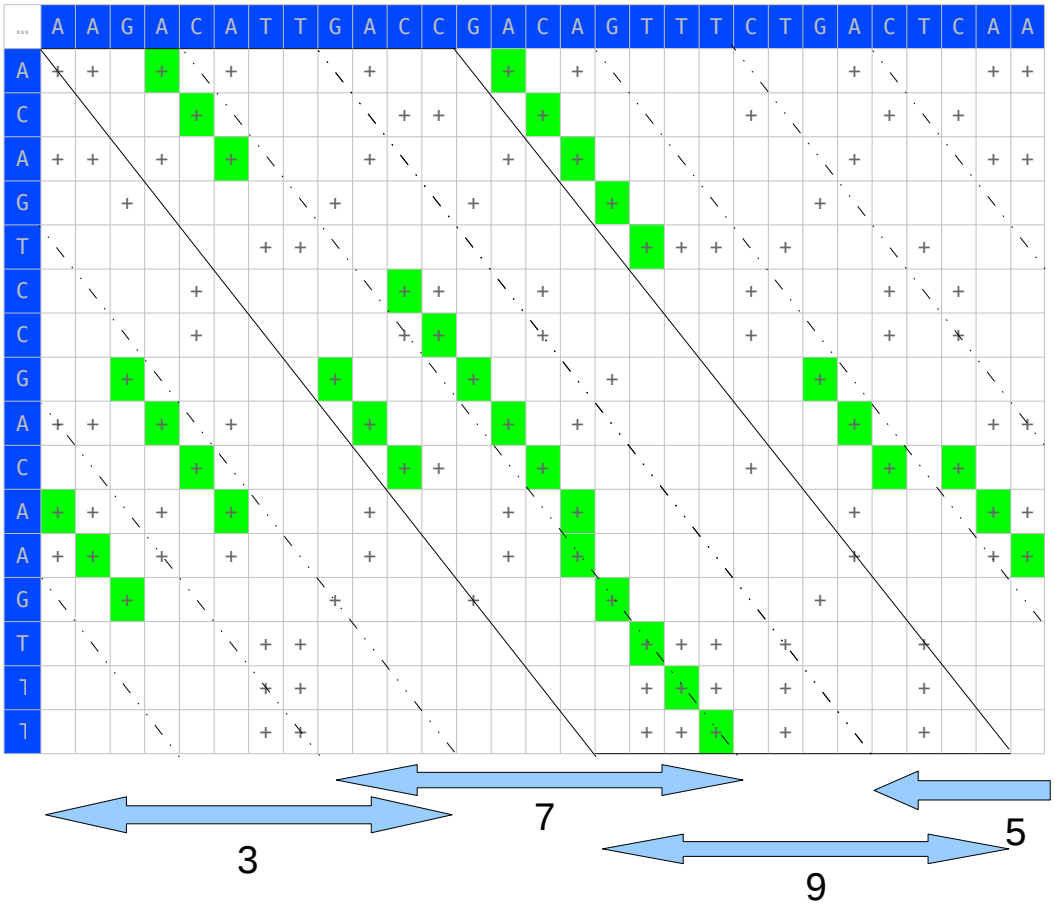
Trade-off: Sensitivity vs Speed

Filtration, Q-Gram-Counting



Verification

For every candidate region identified by the filter:

1. calculate alignment with read
2. depending on score, accept or reject

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Verification, Alignment Algorithms

- “typical” algorithms can be used
- e.g. just scoring the diagonal with hamming distance (no gaps) [Maq]
- or exact DP-Algorithms like Gotoh (with gaps)
- or heuristic searches like local BLAST [QUASAR] or Banded alignments
- bit-parallel DP-algorithms exist also (Myer’s Bitvector algorithm) [RazerS]

Questions regarding choice of algorithm and implementation:

input how long are the reads? how many are there?
how big is/are the genome(s)?
what technology was used?
known error-profile or quality values?

output gapped or ungapped alignments?
what kind of scoring / distance measurement?

Outline

“Not-so-short” reads

Read lengths in NGS

- used to be 20-40bp
- now industry standard is 70-100bp
- read length increases (already up to few hundreds)

Problems

- not possible to use hamming distance
- chance of spanning an intron increases

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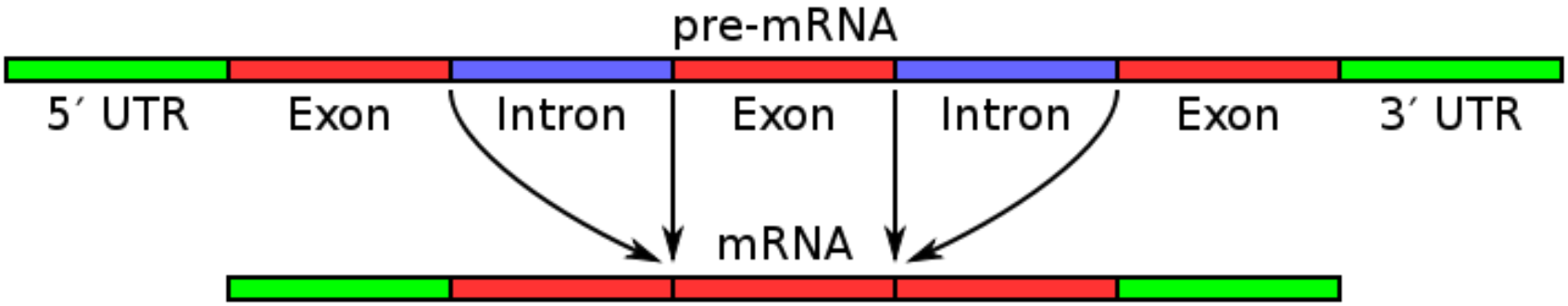
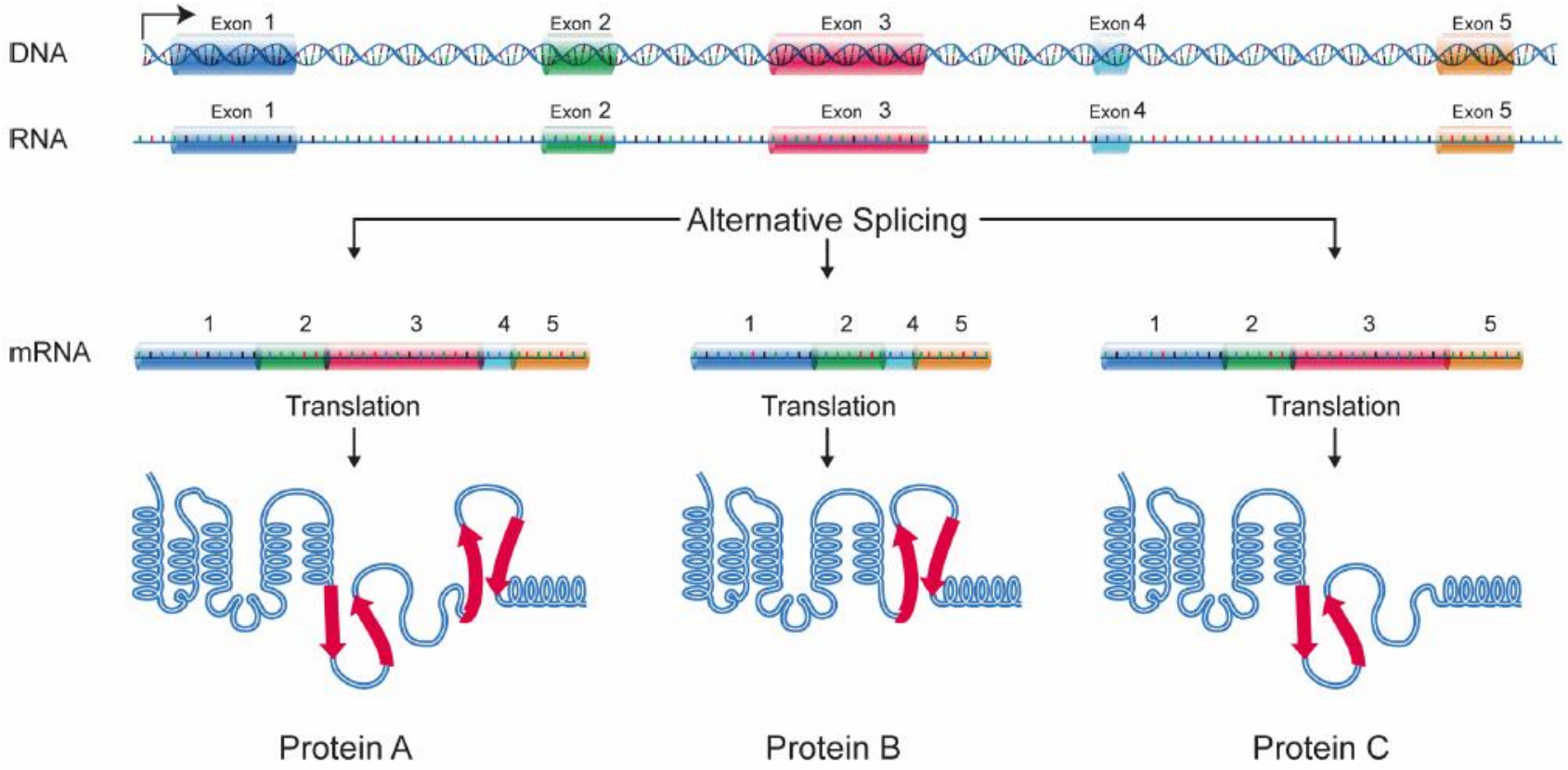
Outline

Brief repetition of splicing



Problem: How to find splicing junctions?

Alternative splicing



Problem: Which isoforms exist?

Spliced mapping

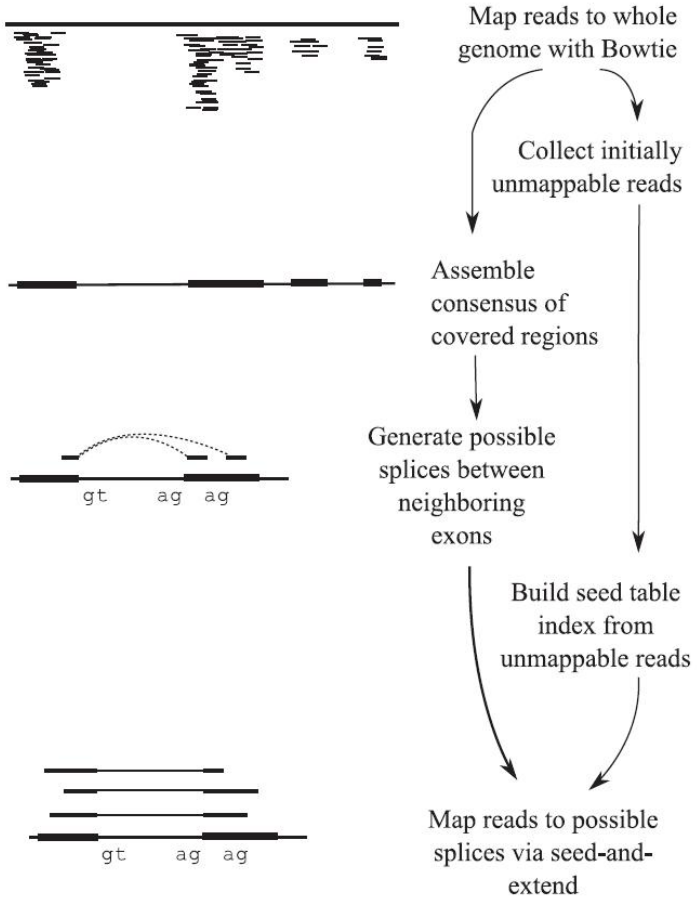
Solutions 1:

- Map reads to known splicing patterns
- Good results for (moderately) abundant transcripts, but not appropiate for rare or unknown transcripts!

Spliced Mapping

Solution 2: TopHat

- Finds novel splice junctions fast
- Synthesizes possible splicing from mapped reads and aligns them with unmapped ones
- Junctions missed when sequencing coverage too low
- Introns could contain non-canonical splicing

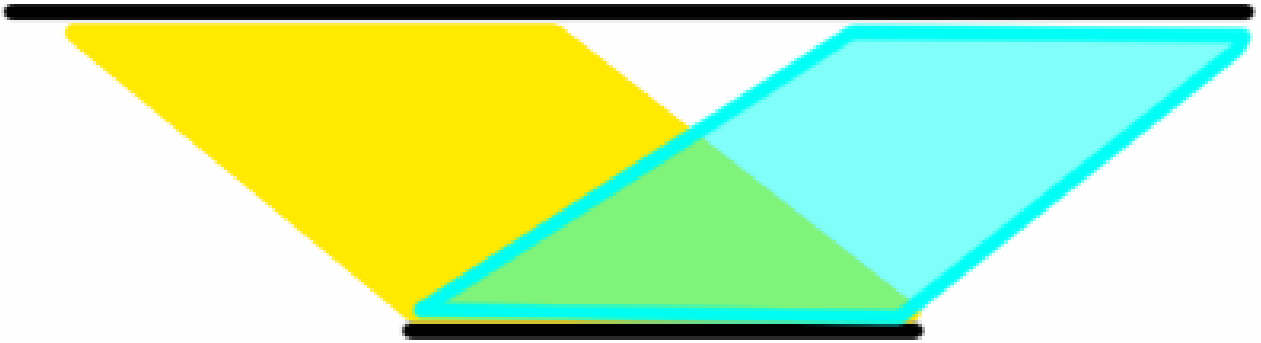


Outline

Multireads

Definition: Reads that have high-scoring alignments to multiple positions in a reference genome

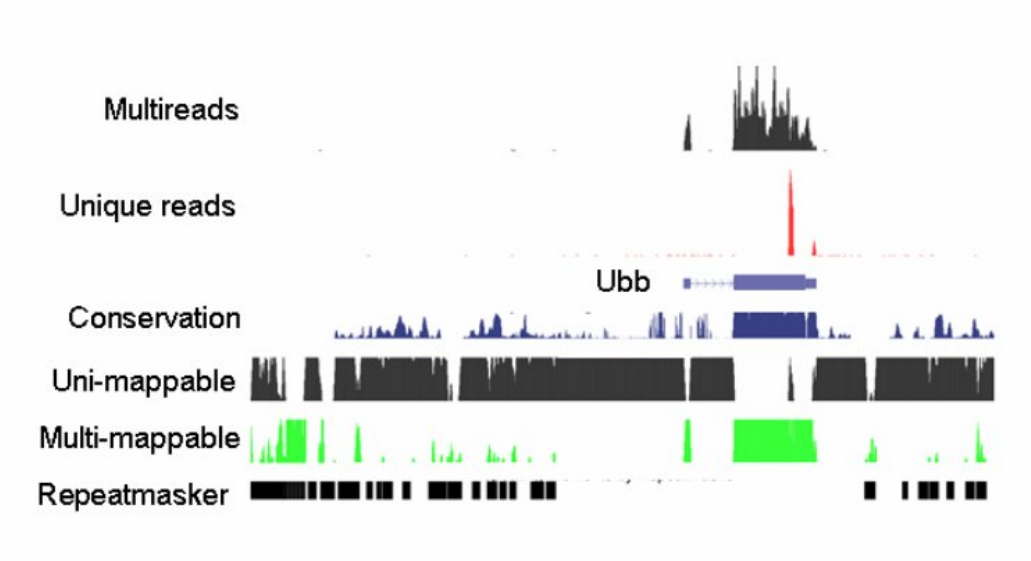
- Gene multiread: map to multiple genes
- Isoform multiread: map to isoforms of one gene



Multireads

Strategies of handling multireads:

- Discard of the multireads
- Allocation of multiread in proportion to the number of unique and splice reads recorded at similiar loci



Multialignment

What happend there?

- Sequence erros (Multiple genes)
- Mapping of reads form other isoforms of that gene
- Repetitive Sequences
  - Identification of columns where differences occur
    Algorithm of Tammi
  - Separation and Identification of the different instances
    Algorithm of Kececioglu

```
...AGCCGTCAGA...
...AGCCGTCAGA...
...AGCCCTCTGA...
...TGTCGTCTGA...
...AGTCGTCTCA...
...AGTCGTCTGA...
```

Sources

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- Bo Li, Victor Ruotti, Ron M. Stewart, James A. Thomson & Colin N. Dewey.
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- Ben Langmead, Cole Trapnell, Mihai Pop & Steven L Salzberg.
  Ultrafast and memory-efficient alignment of short DNA sequences to the human genome.
  Genome Biology 2009, 10:R25.

Thank you

Questions?

Google | What is Read Mapping | Search | Advanced Search

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