Application of NGS to Transcriptomics

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International Course on MASSIVE Data Analysis
Valencia, March, 2011

Acknolowegdments

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Outline

1. Introduction

2. RNA-Seq Data Mapping

3. RNA-Seq Data Analysis

Outline

1. Introduction
   - Basic Biology
   - From Microarrays to RNA-Seq
   - RNA-Seq
2. RNA-Seq Data Mapping
3. RNA-Seq Data Analysis

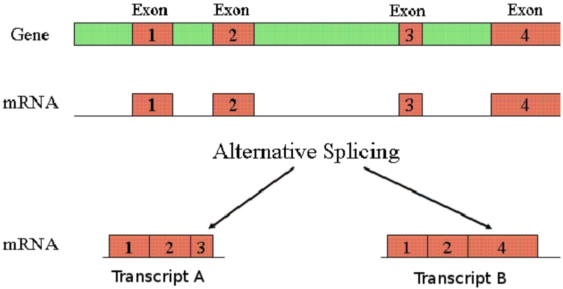
Inicial Concepts

Definitions

- Gene: a hereditary DNA sequence that determines a particular characteristic in an organism.
- Exon: a region of a gene that codes information for protein synthesis that is transcribed to mRNA.
- Intron: a region of a gene which is not translated into protein and is removed before translation of mRNA.
- Splicing: a process in which the introns are removed and exons are joined to be translated into a single transcript.

Alternative Splicing

Alternative splicing: process in which exons can be spliced out in different combinations named transcripts to generate the mature RNA molecule.



Microarrays

Features

- Allow measuring the abundance of thousands of DNA and RNA sequences simultaneously in different cell samples.
- Make use of the hibridatory properties of the nucleic acids to observe their abundance.
- Probes: Short (known) DNA sequences fixed in the array.
- Targets: DNA sample that one wants to monitorize.
- The abundance of each sequence is a function of the fluorescence level recovered after the hybridization process.

Hight-throughput sequencing

Brief Summary

- Improvements in the efficiency, quality and cost of genemo-wide sequencing have made biologist to abandon microarrays in favor of so-called next-generation sequencing (NGS)
- Plataforms: SOLiD, Illumina, Roche's 454, HeliScope
- Allow to obtain *digital* measures for the secuence abundances (read counts)

Pros / Cons

Microarrays

Pros

- Price
- Well-established protocols
- Wide computational analysis tools accessible.

Cons

- Limited to known genomes/transcriptomes.
- Limited sensitivity
- Problems in the hybridization (e.g. cross-hybridization, affinity effects, ...)
- Specific designs for each particular problem

Sequencing

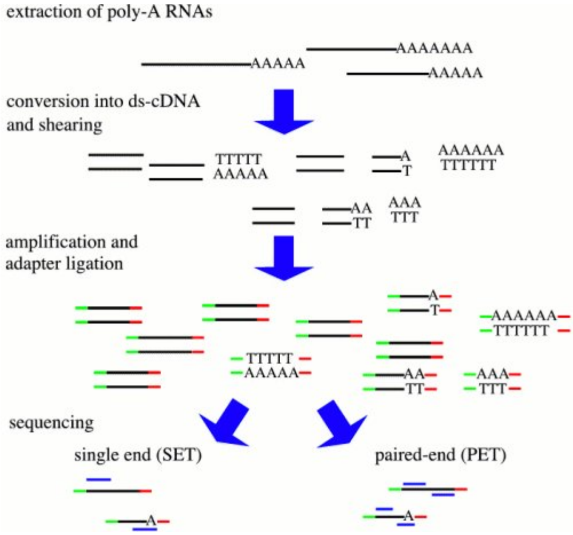
Pros

- Potential for the discovery of novel / not annotated regions
- Discrete measure of abundance (read counts)
- Improved quality and versatility of the data

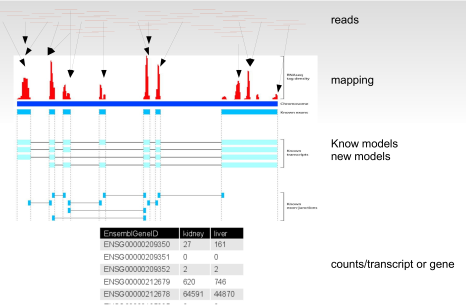
Cons

- Dependence in the sequencing depth
- Price
- Complex data processing and analysis
- Lack of a well-defined benchmark

RNA-Seq. General Protocol



RNA-Seq. Schema



reads

mapping

Know models
new models

EnsemblGeneID	kidney	liver
ENSG00000209350	27	161
ENSG00000209351	0	0
ENSG00000209352	2	2
ENSG00000212679	620	746
ENSG00000212678	64591	44870

counts/transcript or gene

General Objectives

- Quantify transcript abundances
- Identify gene transcriptional structure: splicing, 5’ and 3’ sites, etc
- Quantify expression level changes in each transcript

RNA-Seq. Data

Raw Data

Reads from the sequencer (sequences + qualities)

Formats

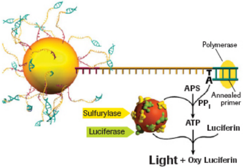
- FASTQ $\Longrightarrow$ nucleotides
- Colorspace $\Longrightarrow$ colors for each change

Basic Features

- Single-end / Parired-end
- Length: 35bp, 50bp, 75bp, 400bp,....
- Strand specificity
- Quality
- Depth $\Longrightarrow$ Tipically 10 millions per *lane* (growing)

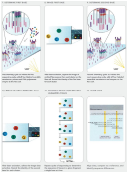
Platforms

Roche



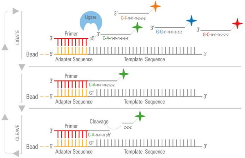
- “Long reads” (400nts)
- Good for *de novo*
- Errors: Poly-n’s

Illumina



- Reads 35-150nts
- Paired-end
- Errores: hexámeros

SOLiD



- Reads 50-100nts
- Strand specific
- Colorspace

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1 Introduction

2 RNA-Seq Data Mapping

- Before we start
- Background
- TopHat

3 RNA-Seq Data Analysis

What do we have?

Sequencer Output

- Obtained sequence (read) → Different techniques and protocols
- Estimated quality → Sequencer calibration

Main Problem

VERY big files → How can we have an idea of what is in them?

Related problems

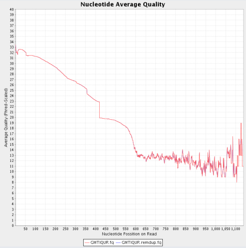
- Detect wrong reads
- What to do with the wrong ones (trimming, removing, ...)
- Take into account specific problems of each platform

Read Quality

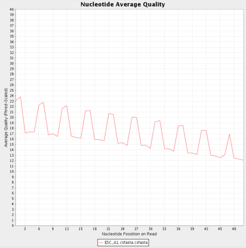
Theory: Same scale ⟹ Comparable results

Reality: Different platforms ⟹ Different behviours

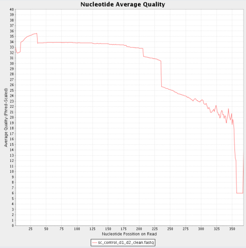
Roche



SOLiD



Illumina



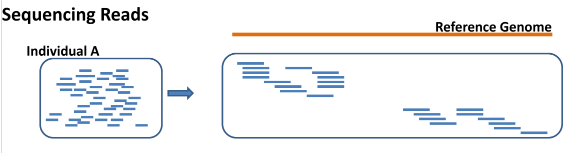
FastQC

- Covered in the previous class
- Software for the sequencing quality control
- Very useful to get an quick idea of the quality of the data and where problems can be expected

Ejemplos

- Datos OK:
  http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/good_sequence_short_fastqc/fastqc_report.html
- Datos with problems:
  http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/bad_sequence_fastqc/fastqc_report.html

RNA-Seq. Mapping



Main Issues

- Number of allowed mismatches
- Number of multi-hits
- Distance between pairs
- Consider exon junctions

Mapping Algorithms

BWA

- Short reads up to 200bp with error $< 5\,\%$
- Do not account for read quality
- Gapped alignment

Bowtie

- Very fast for short reads
- Does not align with gaps
- Use the read quality to evaluate the alignment

Tophat

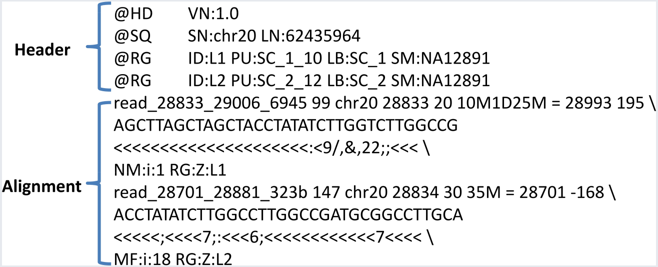
- Improved Bowtie with gap alignment

Other

- ELAND (Illumina software), SOAP, MAQ, etc.

SAM format

SAM file example



Header

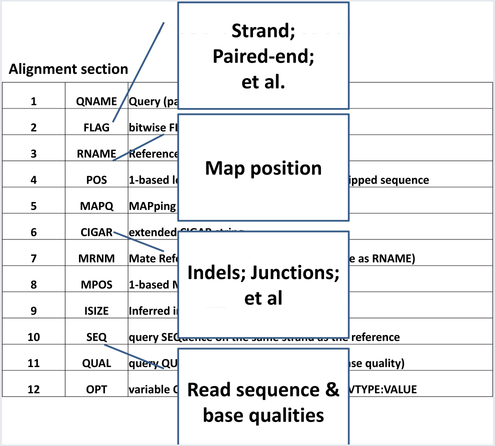
```
@HD     VN:1.0
@SQ     SN:chr20 LN:62435964
@RG     ID:L1 PU:SC_1_10 LB:SC_1 SM:NA12891
@RG     ID:L2 PU:SC_2_12 LB:SC_2 SM:NA12891
```

Alignment

```
read_28833_29006_6945 99 chr20 28833 20 10M1D25M = 28993 195 \
AGCTTAGCTAGCTACCTATATCTTGGTCTTGGCCG
<<<<<<<<<<<<<<<<<<<<<:<9/,&,22;;<<< \
NM:i:1 RG:Z:L1
read_28701_28881_323b 147 chr20 28834 30 35M = 28701 -168 \
ACCTATATCTTGGCCTTGGCCGATGCGGCCTTGCA
<<<<<;<<<<7;:<<<6;<<<<<<<<<<<<<7<<<< \
MF:i:18 RG:Z:L2
```

SAM Format

Information about the alignment



Alignment section

1	QNAME	Query (pa
2	FLAG	bitwise Fl
3	RNAME	Reference
4	POS	1-based l ipped sequence
5	MAPQ	MAPping
6	CIGAR	extended CIGAR string
7	MRNM	Mate Ref e as RNAME)
8	MPOS	1-based M
9	ISIZE	Inferred i
10	SEQ	query SEQuence on the same strand as the reference
11	QUAL	query QU se quality)
12	OPT	variable O VTYPE:VALUE

Strand; Paired-end; et al.

Map position

Indels; Junctions; et al

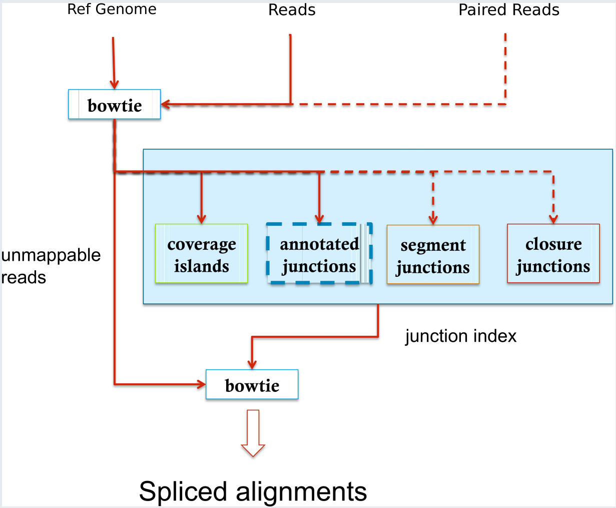
Read sequence & base qualities

Tophat (http://tophat.cbcb.umd.edu/)

Features

- Align the sequences against the genome AND the exon unions (with/without reference)
- Uses Bowtie, an ultrafasr aligner with low memory consumption
- Align segments (25bp by default) of each read, allowing up to 2 mismatches (by default)
- Recent support for colorspace
- It does not consider indels
- Highly configurable
- Continous improvement of the software → good but... Caution! New bugs sometimes

Tophat. Schema



Tophat. Example

Input data → `rawReads1.fastq`, `rawReads2.fastq`

`rawReads1.fastq`:

1000 reads

50 % gene `ARHGAP5` (two exons) and 50 % gene `CMA1` (two exons)

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500 reads

20 % gene `ARHGAP5` (two exons) and 80 % gene `APEX1` (three exons)

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Reference → `HS_chr14.*`

Homo sapiens, chromosome 14 (pre-indexed)

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rawReads1.fastq:
1000 reads
50 % gene ARHGAP5 (two exons) and 50 % gene CMA1 (two exons)

rawReads2.fastq:
500 reads
20 % gene ARHGAP5 (two exons) and 80 % gene APEX1 (three exons)

Reference → HS_chr14.*

Homo sapiens, chromosome 14 (pre-indexed)

Command

1. `cd /home/biouser/rnaseq`
2. `mkdir results`
3.
```
tophat -o /home/biouser/rnaseq/results/exp1/ -p 1 /home/biouser/rnaseq/data/HS.chr14
/home/biouser/rnaseq/data/rawReads1.fastq
```
4.
```
tophat -o /home/biouser/rnaseq/results/exp2/ -p 1 /home/biouser/rnaseq/data/HS.chr14
/home/biouser/rnaseq/data/rawReads2.fastq
```

Tophat. Exercices

Alignments

- Examinate and understand the generated SAM files (`accepted_hits.sam`)
- Load the SAM files with IGV and observe the alignment

  Hint: Look for the regions of interest

Junctions

- Observe and understand the generated BED files (`junctions.bed`)

  Hint: `http://genome.ucsc.edu/FAQ/FAQformat.html#format1`

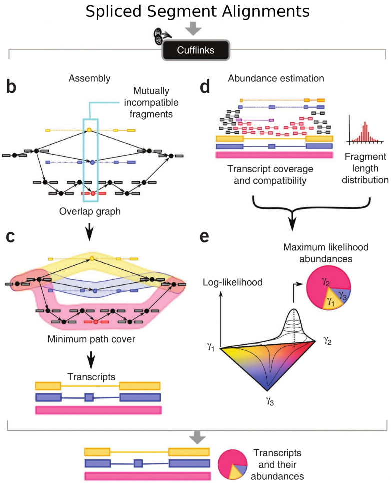
BONUS

- Which are the genomic coordinates of the junctions?
- Can you explain the situation for the CMA1 gene?

Outline

1. Introduction
2. RNA-Seq Data Mapping
3. RNA-Seq Data Analysis
   - Transcript Reconstruction
   - Counting Regions of Interest
   - Final Exercise

Cufflinks → http://cufflinks.cbcb.umd.edu/


Spliced Segment Alignments
Cufflinks
b
Assembly
Mutually incompatible fragments
Overlap graph
c
Minimum path cover
Transcripts
d
Abundance estimation
Transcript coverage and compatibility
Fragment length distribution
e
Maximum likelihood abundances
Log-likelihood
γ_1
γ_2
γ_3
Transcripts and their abundances

- Not restricted to a previous annotation
- Accounts for alternative splicing
- Receives a set of mapped reads (SAM/BAM)
- Detects compatible fragments and search for a parsimonous explanation

Cufflinks. Example

Exercise

Assembly the transcripts found in the previous alignment

Cufflinks. Example

Exercise

Assembly the transcripts found in the previous alignment

Commands

From /home/biouser/rnaseq/results/exp1/ and /home/biouser/rnaseq/results/exp2/:

```
cufflinks -p 1 accepted_hits.sam
```

Cufflinks. Example

Exercise

Assembly the transcripts found in the previous alignment

Commands

From /home/biouser/rnaseq/results/exp1/ and /home/biouser/rnaseq/results/exp2/:

```
cufflinks -p 1 accepted_hits.sam
```

Question

Were the transcripts reconstructed as you expected?

Hints:

Observe the transcripts.gtf files
GTF definition: http://genome.ucsc.edu/FAQ/FAQformat.html#format3
Finding the actual transcripts in *ensembl* might help

Comparing Experiments → cuffcompare

Exercise

cuffcompare cufflinks application for comparing results from different experiments

Compare the outputs obtained in the previous experiment

Comparing Experiments → cuffcompare

Exercise

cuffcompare cufflinks application for comparing results from different experiments

Compare the outputs obtained in the previous experiment

Commands

Create /home/biouser/rnaseq/results/compare/ and from there:

```
cuffcompare -r /home/biouser/rnaseq/data/HS.chr14.gtf
   ../exp1/transcripts.gtf ../exp2/transcripts.gtf
```

Comparing Experiments → cuffcompare

Exercise

cuffcompare cufflinks application for comparing results from different experiments

Compare the outputs obtained in the previous experiment

Commands

Create /home/biouser/rnaseq/results/compare/ and from there:

```
cuffcompare -r /home/biouser/rnaseq/data/HS.chr14.gtf
    ../exp1/transcripts.gtf ../exp2/transcripts.gtf
```

Questions

- How many transcripts in total? Why?
  Hint: Observe the file `stdout.combined.gtf`
- Can you identify the specific transcripts of each experiment?
  Hint: Observe the file `stdout.tracking`

Counting Regions of Interest → htseq-count

htseq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html)

htseq-count: Receives an alignment file (`SAM/BAM`) and a list of genomics features (p.ej. `GTF`)
Returns the number of reads that fall within the selected feature

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Exercise

Compute the counts at the gene level for the previous experiments

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Exercise

Compute the counts at the gene level for the previous experiments

Commands

From `/home/biouser/rnaseq/results/exp1/` and `/home/biouser/rnaseq/results/exp2/`:

```
htseq-count -s no -i gene_name accepted_hits.sam
/home/biouser/rnaseq/data/HS.chr14.gtf > counts.txt
```

Counting Regions of Interest → htseq-count

htseq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html)

htseq-count: Receives an alignment file (`SAM/BAM`) and a list of genomics features (p.ej. `GTF`)
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```
htseq-count -s no -i gene_name accepted_hits.sam
/home/biouser/rnaseq/data/HS.chr14.gtf > counts.txt
```

Questions

- How many reads has each gene in each experiment
- Does it matches with what you expected? Can you explain it? Hint: Think of the effects of the transcript length and the sequencing depth

BONUS

RPKM

Compute the RPKM (Reads per kilobase per million reads) value for a gene in a given experiment and compare your results with those obtained by `cufflinks`

$$RPKM = \frac{\text{number of reads of the region}}{\frac{\text{total reads}}{1000000} \times \frac{\text{region length}}{1000}}$$

Real problem

Objective

Use what you have learnt in a real dataset

Data

Two real sequencing experiments (reduced due to memory issues):

- `brain.fastq`
- `uhr.fastq`

Hints

- Reference genome: hg19.*
- Annotation: hg19.gtf
- Examine the problem and follow the previously used pipeline
- Ask if you find yourself lost