TRAINING COURSE IN
Computational Methods for Epitranscriptomics

Bari, 11th-13th September 2024

From raw data to RNA modifications: how the analysis works

Introduction to the bioinformatics concepts, pipelines and software tools to analyse Nanopore data in order to detect RNA modifications.

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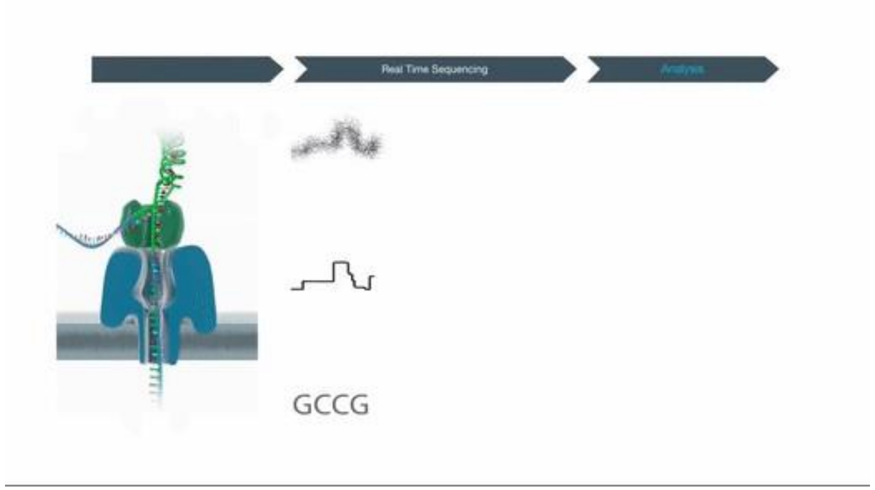
Nanopore data

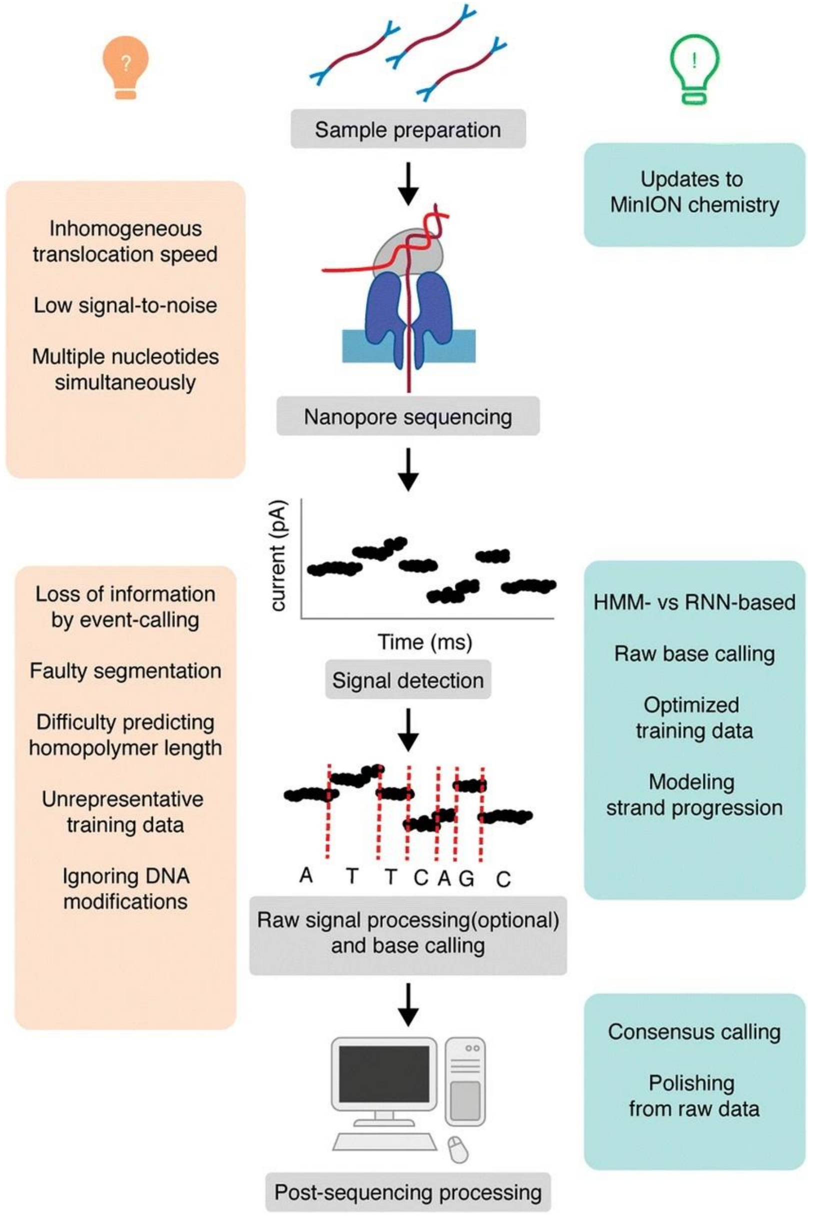
- Raw data is current (pA) over time
- RNA moves through the pore at 70bps
- The current is recorded at 4kHz
- ~57 data points per kmer
- Data stored in Fast5 format (HDF5)



From the sample to the data

Multiple factors influence the properties and quality of the data:

- Library chemistry
- Electronics
- Pore
- Basecalling


?
Sample preparation
!
Updates to MinION chemistry
Inhomogeneous translocation speed
Low signal-to-noise
Multiple nucleotides simultaneously
Nanopore sequencing
current (pA)
Time (ms)
Signal detection
Loss of information by event-calling
Faulty segmentation
Difficulty predicting homopolymer length
Unrepresentative training data
Ignoring DNA modifications
HMM- vs RNN-based
Raw base calling
Optimized training data
Modeling strand progression
A T T C A G C
Raw signal processing(optional) and base calling
Consensus calling
Polishing from raw data
Post-sequencing processing

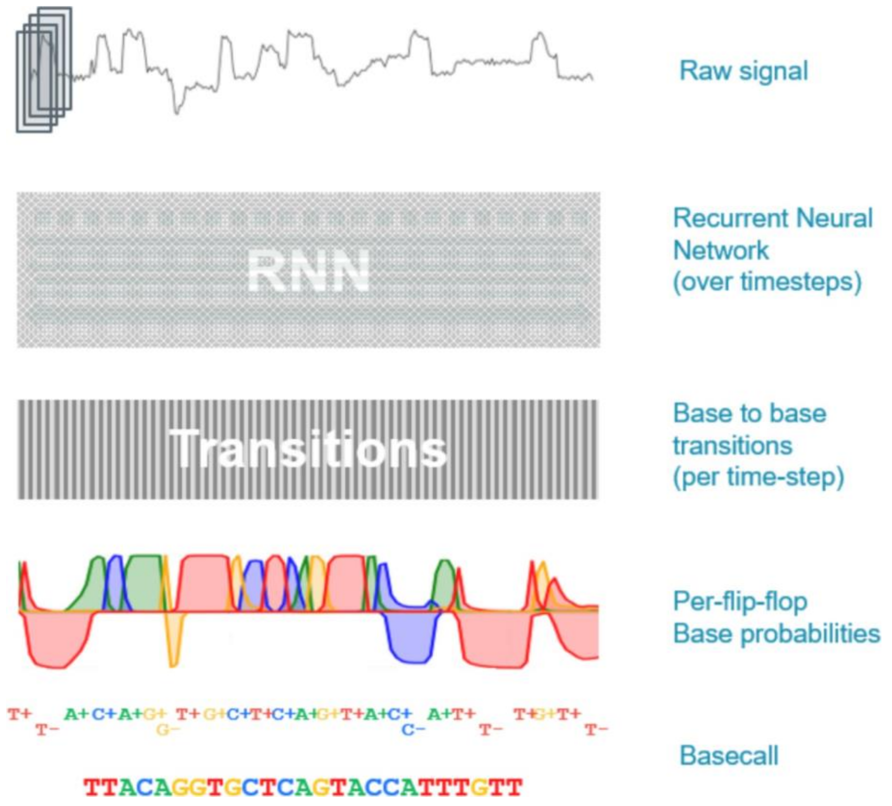
Rang et al., Genome Biology 2018

Analysis steps

☑ Sequencing	Starting and monitoring a run
☐ Basecalling	From signal to sequence
☐ QC	Assess quality of the data
☐ Mapping	Map the reads to the genome/transcriptome
☐ Quantification	Estimate gene/transcript expression
☐ Modification detection	Detect RNA modifications

Current basecalling algorithm

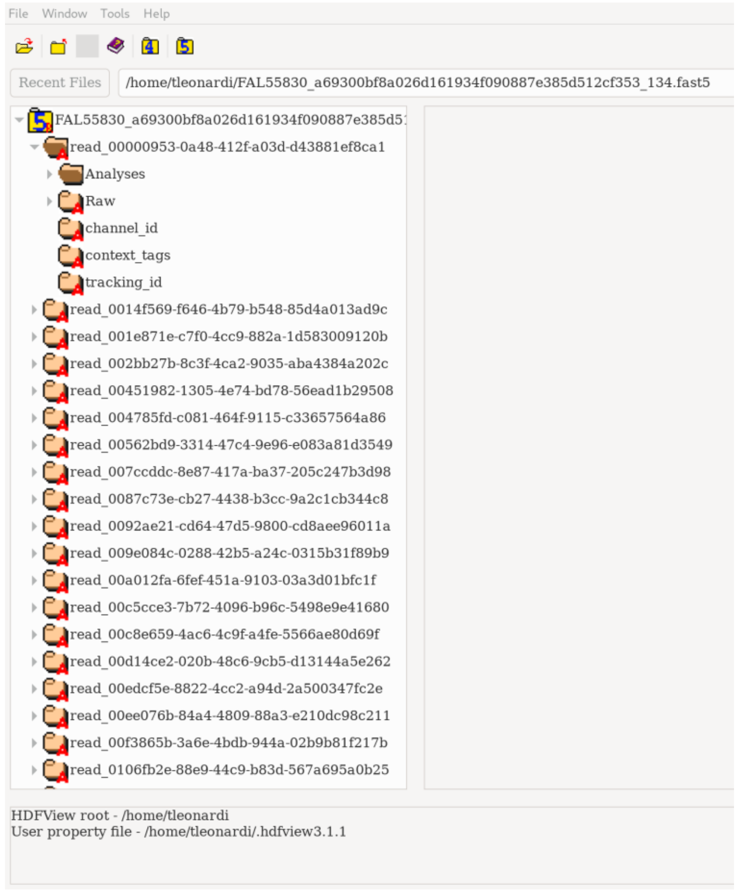
- Dorado is the current production basecaller (RNA004)
- Guppy is the previous production basecaller (RNA002), included in MinKnow
- Based on RNN
- Trained on a collection of human, yeast, *E. coli* and *C. elegans* samples
- Implements a fast model and a Higher Accuracy model (HAC)
- Supports GPU accelaration


Raw signal
RNN
Recurrent Neural Network (over timesteps)
Transitions
Base to base transitions (per time-step)
Per-flip-flop Base probabilities
Basecall
TTACAGGTGCTCAGTACCATTTGTT

nanoporetech.com

Fast5 files (now pod5)

- Binary format based on HDF5
- Slots contain various types of data/metadata
- Always contain raw signal
- Can also contain basecalled sequence
- One file can contain data for multiple reads (multi fast5)
- Default: 1 fast5 -> 4000 reads



```
/home/tleonardi/FAL55830_a69300bf8a026d161934f090887e385d512cf353_134.fast5

FAL55830_a69300bf8a026d161934f090887e385d51
  read_00000953-0a48-412f-a03d-d43881ef8ca1
    Analyses
    Raw
    channel_id
    context_tags
    tracking_id
  read_0014f569-f646-4b79-b548-85d4a013ad9c
  read_001e871e-c7f0-4cc9-882a-1d583009120b
  read_002bb27b-8c3f-4ca2-9035-aba4384a202c
  read_00451982-1305-4e74-bd78-56ead1b29508
  read_004785fd-c081-464f-9115-c33657564a86
  read_00562bd9-3314-47c4-9e96-e083a81d3549
  read_007ccddc-8e87-417a-ba37-205c247b3d98
  read_0087c73e-cb27-4438-b3cc-9a2c1cb344c8
  read_0092ae21-cd64-47d5-9800-cd8aee96011a
  read_009e084c-0288-42b5-a24c-0315b31f89b9
  read_00a012fa-6fef-451a-9103-03a3d01bfc1f
  read_00c5cce3-7b72-4096-b96c-5498e9e41680
  read_00c8e659-4ac6-4c9f-a4fe-5566ae80d69f
  read_00d14ce2-020b-48c6-9cb5-d13144a5e262
  read_00edcf5e-8822-4cc2-a94d-2a500347fc2e
  read_00ee076b-84a4-4809-88a3-e210dc98c211
  read_00f3865b-3a6e-4bdb-944a-02b9b81f217b
  read_0106fb2e-88e9-44c9-b83d-567a695a0b25

HDFView root - /home/tleonardi
User property file - /home/tleonardi/.hdfview3.1.1
```

Basecalling with Guppy

Guppy is free to use but it is not open source. It can be downloaded from the ONT Community website after registering.

Important Options

Flag	Function
-i input path	Path to the root folder containing fast5 files to basecall
-s output path	Path to the output folder
--flowcell name	Flowcell model used (e.g. FLO-MIN106)
--kit name	Kit used for library prep (e.g. SQK-RNA002)

Guppy: other important options

Important Options

Flag	**Function**
--qscore_filtering	Flag that enables pass/fail filtering
--min_qscore int	Min q-score to consider a read as pass
--calib_detect	Filter calibration strand reads in a dedicated folder
--reverse_sequence	Reverse the called sequence (used for RNA sequencing due to 3’ to 5’ direction)
--u_substitution	Substitute 'U' for 'T' in the called sequence
--recursive, -r	Look for fast5 files recursively inside the input path

Guppy: less important options

Important Options

Flag	Function
--trim_strategy	Stretegy to trim adapters: dna, rna or none
--disable_pings	Do not send telemtry info to ONT
--compress_fastq	Compress fastq files with gzip
--fast5_out	Save basecalled fast5 files
--resume	Resume a stopped run

Guppy input/output

Input: directory containing fast5 files to basecall

Output:

Guppy output location (MinKnow)

Guppy stores its output at:

Var	Meaning
output_dir	base output directory
experiment_id	experiment id specified when starting the run
sample_id	sample_id specified when starting the run
start_time	time when the run started (YYYYMMDD_HHMM)
device_id	serial ID of the MinION or device position for GridION/PromethION (e.g. X1-5)
flow_cell_id	flow cell ID

Guppy summary file

A tsv file containing useful information on the run

Guppy: basecalling on CPU

Important Options

Flag	Function
--num_callers	Number of parallel basecaller processes
--num_cpu_threads_per_caller	Number of CPU worker threads per basecaller

- The total number of threads used is num_callers * cpu_threads_per_caller The total
- number of threads should not exceed the number of cores at your disposal Guppy
- uses 4GB of RAM plus 1 extra GB per thread

Guppy: basecalling on GPU

GPU basecalling gives a significant speed up. Use it whenever possible.

Important Options

Flag	**Function**
--device cuda:0	Instructs Guppy to use the first GPU
--chunks_per_caller	Number of signal chunks to collect before sending to basecaller

Supported GPUs:

- NVIDIA Tesla V100
- NVIDIA Quadro GV100
- NVIDIA Jetson TX2
- NVIDIA Jetson Xavier
- Other GPUs w/ CUDA Compute Capability >6.1 (limited support)

Other tools and resources

ONT provides various open source tools for analyses and manipulation of sequencing data and they are available on github.com/nanoporetech/. The following is a non exhaustive list of some interesting ones:

- Dorado: latest beta production basecaller (nanoporetech/**dorado**)
- Rerio: experimental basecalling models for Guppy (nanoporetech/**rerio**)
- Pychopper: orient and trim cDNA reads (epi2me-labs/**pychopper**)
- Remora: modified base detection with dRNA support (nanoporetech/**remora**)
- Modkit: converting modBAM to bedMethyl files (nanoporetech/**modkit**)

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Data QC

Quality controls give an immediate feedback on the performance of the run through a number of metrics:

- Experiment throughput
- Read length
- Read quality
- Error rate
- Flowcell stats (yield over time, n. pores, etc)

cartoonstock.com

Where is the QC info stored?

Metrics for assesing QC can be retrieved from multiple locations:

- Sequencing summary file
- Fast5 files
- BAM files (after mapping)
- Telemetry files

QC tools

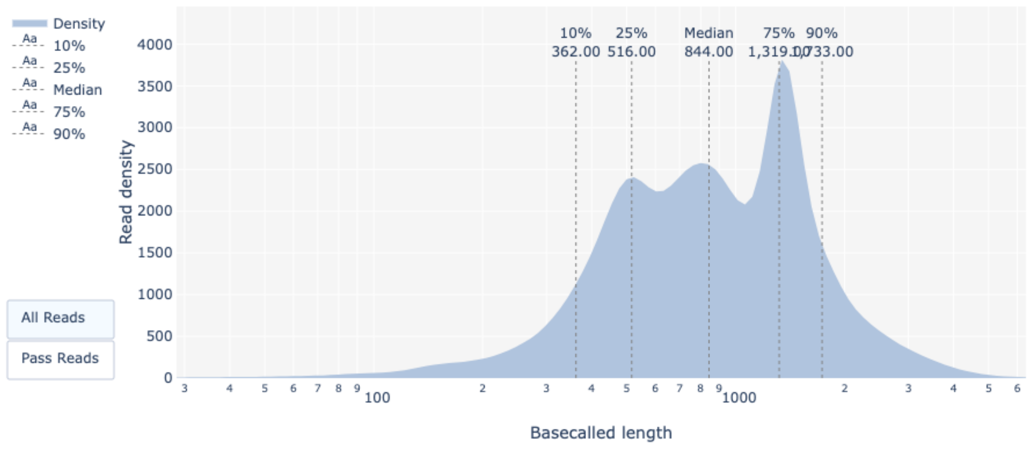
Several tools take care of collecting and plotting QC data:

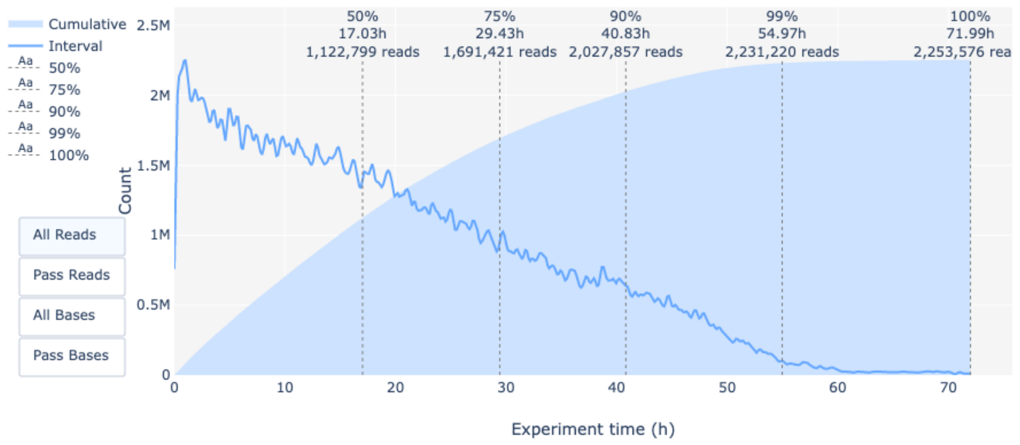
Pipeline	**Github Repo**
★ pycoQC	a-slide/pycoQC
★ nanoQC	wdecoster/nanoQC
☆ LongQC	yfukasawa/LongQC
☆ minion_qc	roblanf/minion_qc

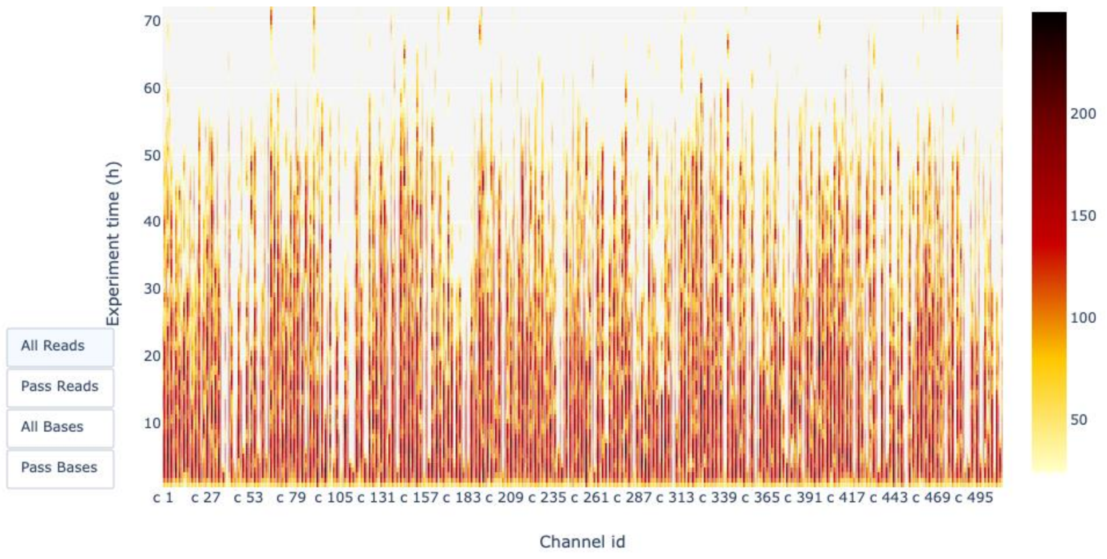
pycoQC

Features:

- Reads sequencing_summary or fast5s
- Very fast
- html report with interactive plots
- Optionally supports reading BAM file for alignment/identity stats







Running pycoQC

Main Options

Flag	**Function**
--summary_file sequencing_summary.txt	Path to summary file generated by Guppy
--html_outfile output path	Path to the output html file
--filter_calibration	Remove reads marked as calibration by Guppy

Analysis steps

☑ Sequencing	Starting and monitoring a run
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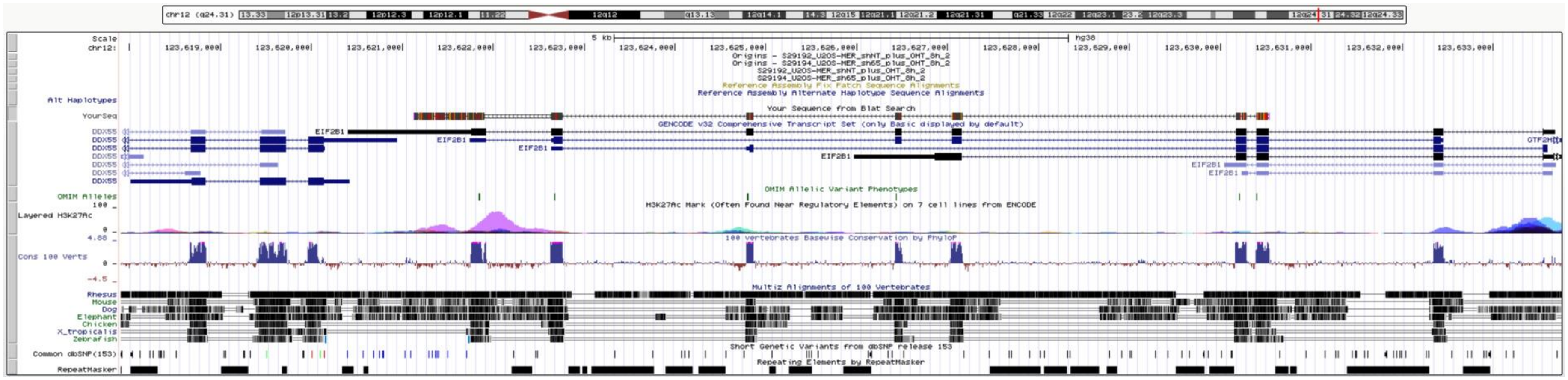
Mapping the reads



- Nanopore error rate is higher than Illumina
- Reads are very long (up to Mb)
- Need optimised mappers
- Most common mapper: Minimap2
- Reads can be mapped to genome and/or transcriptome
- Output: BAM file

Genome vs transcriptome

Genome

Advantages

- Detect new genes/transcripts
- Visualise data on a genome browser

Disadvantages

- Relies on good annotation
- Requires splice-aware alignment
- Assigning reads to individual TXs is complex

Transcriptome

Advantages

- Direct assignment of read to transcripts
- Trivial quantification of isoforms
- No need to consider splicing

Disadvantages

- Limited to annotated genes/transcripts
- Can't be loaded on a genome browser

Assemble the Transcriptome

Long reads are mostly full-length: assembly step is *in theory* not required
But they suffer from higher error rates, that makes building a consensus more difficult

Scenario:

- A *genome* is not available or it is not desirable to take it into account
- A *transcriptome* is not available or it is not desirable to take it into account

Final goals:

- To cluster long read RNAseq reads into groups corresponding to *gene families*
- To obtain a collection of *consensus transcripts*

Assemble the Transcriptome

Tool	**Github Repo**
★ IsoQuant	ablab/IsoQuant
★ Bambu	GoekeLab/bambu
★ Flair	BrooksLabUCSC/flair
★ TALON	mortazavilab/TALON
★ stringtie2	skovaka/stringtie2

Evaluate Transcriptome Quality

Gffcompare

Estimate accuracy of one or more GFF files (the *"query"* files) when compared with a reference annotation (also provided as GFF)

(Pertea et al., 2020; doi:10.12688/f1000research.23297.1)

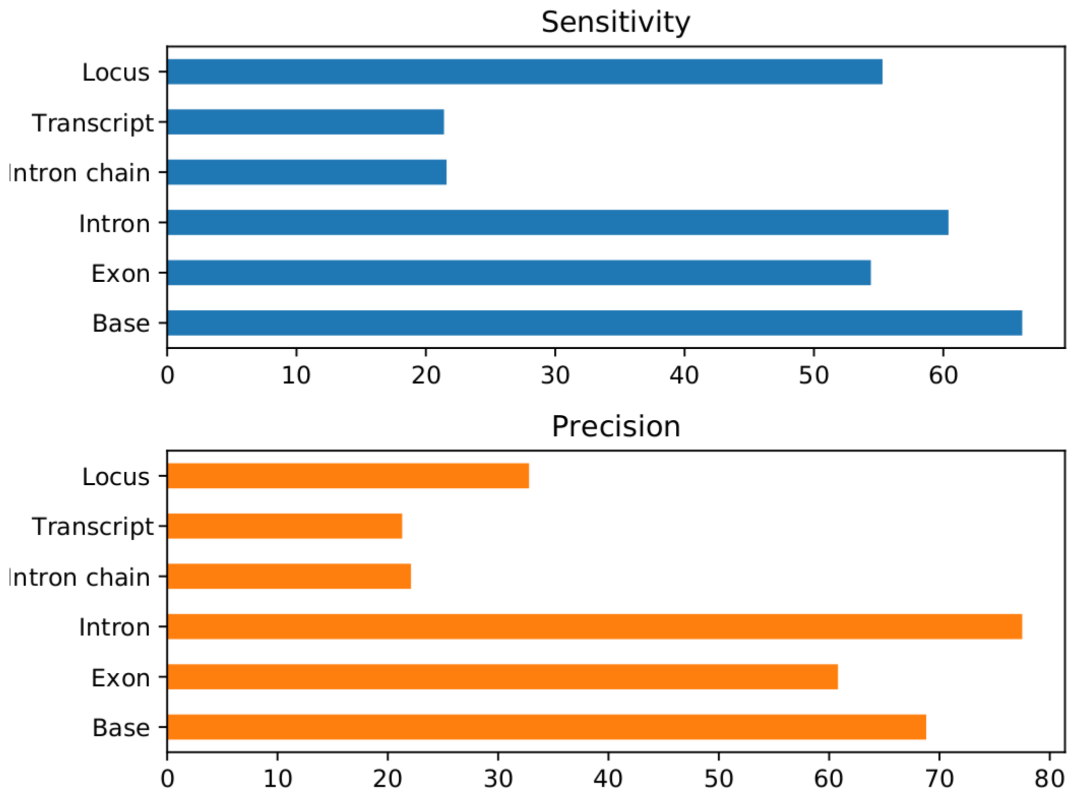
SQANTI3

Multilevel comparison between the *"query"* file and a reference annotation (provided as GFF)

(Pardo-Palacios, F.J., Arzalluz-Luque, A. et al. doi:10.1038/s41592-024-02229-2)

Evaluate Transcriptome Quality

Sensitivity and Precision of the Assembly



Per-item statistics:

Sensitivity = TP / (TP+FN) Precision = TP / (TP+FP)

- **TP (true positives)**: total bases that are reported on both the query and *any* reference item
- **FN (false negatives)**: total bases in reference by any of the predicted items
- **FP (false positives)**: total bases covered by an but not covered by any reference item

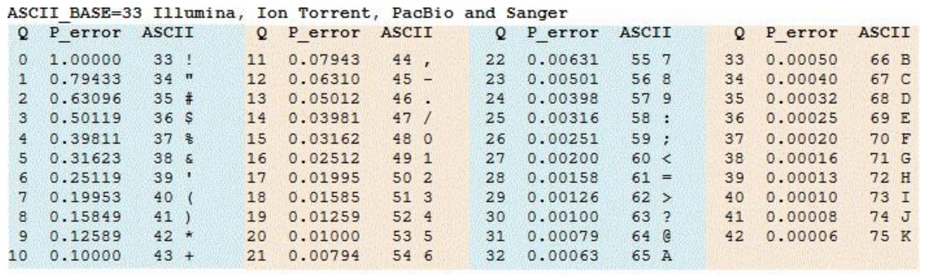
Input data: the fastq format

Read name Sequence Quality

```
@32e69ca0-0744-4020-8956-17ab085a841e runid=2749b read=100 ch=207 start_time=2017-10-10T06:56:00Z
GGAGAGGGAGGAAAGCACATTCGGGAACGAGTTCTCAAACTAATGCTCAGAACCGGTGTCCAGTGACGTAGAAACCACTTTCCCAAATGGCGGGGCTTTTC
+   97;;9:999988;:;:;8=;86;89;97::978799;;778:9:9688:9:9;4:579784785671967264322538626226/.2350412310100;
```

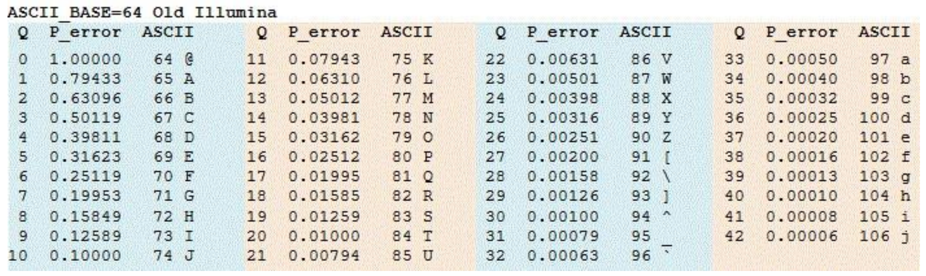
Phred scores

```
@32e69ca0-0744-4020-8956-17ab085a841e runid=2749b read=100 ch=207 start_time=2017-10-10T06:56:00Z
GGAGAGGGAGGAAAGCACATTCGGGAACGAGTTCTCAAACTAATGCTCAGAACCGGTGTCCAGTGACGTAGAAACCACTTTCCCAAATGGCGGGGCTTTTC
+   97;;9:999988;:;:;8=;86;89;97::978799;;778:9:9688:9:9;4:579784785671967264322538626226/.2350412310100;
```



ASCII_BASE=33 Illumina, Ion Torrent, PacBio and Sanger

Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	33 !	11	0.07943	44 ,	22	0.00631	55 7	33	0.00050	66 B
1	0.79433	34 "	12	0.06310	45 -	23	0.00501	56 8	34	0.00040	67 C
2	0.63096	35 #	13	0.05012	46 .	24	0.00398	57 9	35	0.00032	68 D
3	0.50119	36 $	14	0.03981	47 /	25	0.00316	58 :	36	0.00025	69 E
4	0.39811	37 %	15	0.03162	48 0	26	0.00251	59 ;	37	0.00020	70 F
5	0.31623	38 &	16	0.02512	49 1	27	0.00200	60 <	38	0.00016	71 G
6	0.25119	39 '	17	0.01995	50 2	28	0.00158	61 =	39	0.00013	72 H
7	0.19953	40 (	18	0.01585	51 3	29	0.00126	62 >	40	0.00010	73 I
8	0.15849	41)	19	0.01259	52 4	30	0.00100	63 ?	41	0.00008	74 J
9	0.12589	42 *	20	0.01000	53 5	31	0.00079	64 @	42	0.00006	75 K
10	0.10000	43 +	21	0.00794	54 6	32	0.00063	65 A			

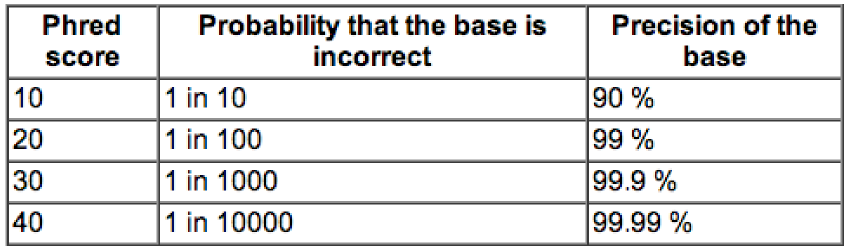


ASCII_BASE=64 Old Illumina

Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	64 @	11	0.07943	75 K	22	0.00631	86 V	33	0.00050	97 a
1	0.79433	65 A	12	0.06310	76 L	23	0.00501	87 W	34	0.00040	98 b
2	0.63096	66 B	13	0.05012	77 M	24	0.00398	88 X	35	0.00032	99 c
3	0.50119	67 C	14	0.03981	78 N	25	0.00316	89 Y	36	0.00025	100 d
4	0.39811	68 D	15	0.03162	79 O	26	0.00251	90 Z	37	0.00020	101 e
5	0.31623	69 E	16	0.02512	80 P	27	0.00200	91 [	38	0.00016	102 f
6	0.25119	70 F	17	0.01995	81 Q	28	0.00158	92 \	39	0.00013	103 g
7	0.19953	71 G	18	0.01585	82 R	29	0.00126	93]	40	0.00010	104 h
8	0.15849	72 H	19	0.01259	83 S	30	0.00100	94 ^	41	0.00008	105 i
9	0.12589	73 I	20	0.01000	84 T	31	0.00079	95 _	42	0.00006	106 j
10	0.10000	74 J	21	0.00794	85 U	32	0.00063	96 `			

$$Q = -10 \log_{10} P$$

$$P = 10^{\frac{-Q}{10}}$$



Phred score	Probability that the base is incorrect	Precision of the base
10	1 in 10	90 %
20	1 in 100	99 %
30	1 in 1000	99.9 %
40	1 in 10000	99.99 %

Real reads

```
@32e69ca0-0744-4020-8956-17ab085a841e          runid=2749b          read=100          ch=207          start_time=2017-10-10T06:56:00Z
UGGCAAGCACCUGGGCUCCGCAGCAGGAAGCCUCCGGCCCAGUAAUGGAAAUACUUUUGAAGUUCAGAUGAAAGUGGAUUCCUGAUGCCACGGCCCU
GCAAGUGGCCGCCACUCCGGACGUGCUGGAGUACUGAAGAAUAAAAGGGGAGUUGCACAGGGUCUGGGCGAAUCUUACCAGUGCCGCAGAAACCUGU
GUGGUGGUGUGACUCCUUCUGUGAAGUGUCCUCUGGCGGGGGAGCUUUCCUCCGCUUCAUCAGUUUUGCCUCCCUGGAAAUACUCCGAUUACUCCAA
AUGUAAAAAGAACAUGAUUGAGCGGGGGGGGGAGAACUUUUUCAGGAUAUCACUGUCUAGAAACAAAAUUAAGAUCUGUGCCAUACUUUCAUCAAAG
AUGGAGCGACAAAUAUUGACUUUCACGCCUACUCCAGAGUGACUGAGAGUCUGGAAGCAGCCGUGGCGGCCAAGAAGCGAUUUAGUGUAUACGUUAC
AGAGUCACAGCCUGAUUUUGUCAGGUAAGAAAAUGGCCAAAGCCCUUUGCCACCUUCAACGUCCUGUCACUGUGGUGCUAGAUGCUGCUGUCGGCUA
CAUCAUGGAGAAAGCAGAUUUUGUUUAGUUGGUGCUGAAAGGAGUUGUUGAAACGGAGGAAUUAUUAACAAGAUUGGAACCAACCAGAUGGCUGUGU
GUGCCAAAGCACAGAACAAACCUUCUAUGUGGUUGCAGAAAGUUCUGGUUUUGUCCGGCUCUUCCACUAAACCAGCAAGACGUCCCAGAUAAGUUAA
GUAUACGCUGAUAGACCAUUUUCCAAGGUCGCGCAGACUGGACAAGACCUUCAAAGAGGAGCAUCCGUGGGUCGACUAUGUCCCUUCCUUAUCACUC
UCUGCUGUUAUAGACCUGGGUGCUGACACCACAGCAGUCAGCGAUGAGCUCAUAUCAAGCUCUAUCUGUAACCUGUGAGCCUUUCCUGCCAAGGUGC
AGCUUUACGUAGUUAGGCGGGUGAGUAGCUGCUUGACACCCCAGUGAAUAGGCCAAACUGAGAUGUGUUUUAAUGAAGAUCUAUGGAGUAAGGCUUA
AAAUCAUCAUUUGGAGAUUCUUACUCAUUCAGUCCCAUCUAAAAUGUGUUCAGCUAUUUUAAAUCCCAACUUAAAUUACGGUUUCUAGAAAACUUUC
CUUUUUCAGUUUCACCAGAGCUACAAGUUAGAUAAUUGUUACUUAUUGAUGAAAGAUGAGCCCUAGUCCACCUGUUUCAUCCUCCCUGCACUCCGGA
CUGAUCUGCCUAAAGCACGCAAGAUGCAGGCGAGCAGCCAUACCCCUCUGCCACAAACGACCAGCUGGUCAGGACGUUACACGCGGUGCCAUUGUAA
GAGGCAAGAAACACUUGCCGAAUCUGCGUCUGGCUUCAGUGGUAAGCACAUUCCAGCAGGAUCAGCCAAACAGUAAAACUACCAAGAGAACGAGGAA
AGCAGAAAAAACGAUGUUUAGCAACAACAGUAUUCUGCAUGGUUCUUGUUUAAGAAAAUGCCUUCUGAAUAUUUGUAACUGAAAUUCUGUAUGUGUG
UAAAC
+
#$($'-+*(,6+.,3#$(1,0///0-%**.(4#%*)*03,"'%%#.0./:1-,(/@..(+0*,-.((,..1.0(%$213().**%#"%($%%('5/%
%$.%)%)2(6+-0)%(54:4,+(+,/83=)*."$))17*+/(015-'.2*')$%#$#/%)??++1,41+,.:(%'*.+-,.22;/"#)*/?4.40+*
.....
```

Genome mapping: minimap2

- Genome fasta can be downloaded from Ensembl
- You can find it in ~/data

Important Options

Flag	Function
-a	Output alignments in SAM format
-x splice	Use a preset of options that allow spliced alignments
-k14	Use a smaller k-mer size, reccomended for noisy dRNA-Seq reads
-uf	Consider forward strand only when looking for canonical splice-sites (GT-AG)

Genome mapping: inspect results

Results are stored in SAM formatSamtools can be used to view/filter the file

Important Options

Field	**Definition**
1	QUERY name
2	FLAG
3	REFERENCE position
4	Mapping position
5	Mapping quality
6	CIGAR string
..	Additional mandatory fields up to 11

Transcriptome mapping step 1: generate the transcriptome

- Genome fasta and transcriptome GTF can be downloaded from Ensembl
- You can find them in `~/data`

Transcriptome mapping step 2: minimap2

Important Options

Flag	Function
-a	Output alignments in SAM format
-x map-ont	Use a preset combination of options optimised for ONT data
-k14	Use a smaller k-mer size, reccomended for noisy dRNA-Seq reads
--for-only	Only map to the forward strand of the reference

SAM/BAM filtering

The SAM files generated by minimap2 usually need to be filtered for:

- Unmapped reads
- Secondary alignments
- Supplementary alignments

Filtering can be done using the SAM FLAG

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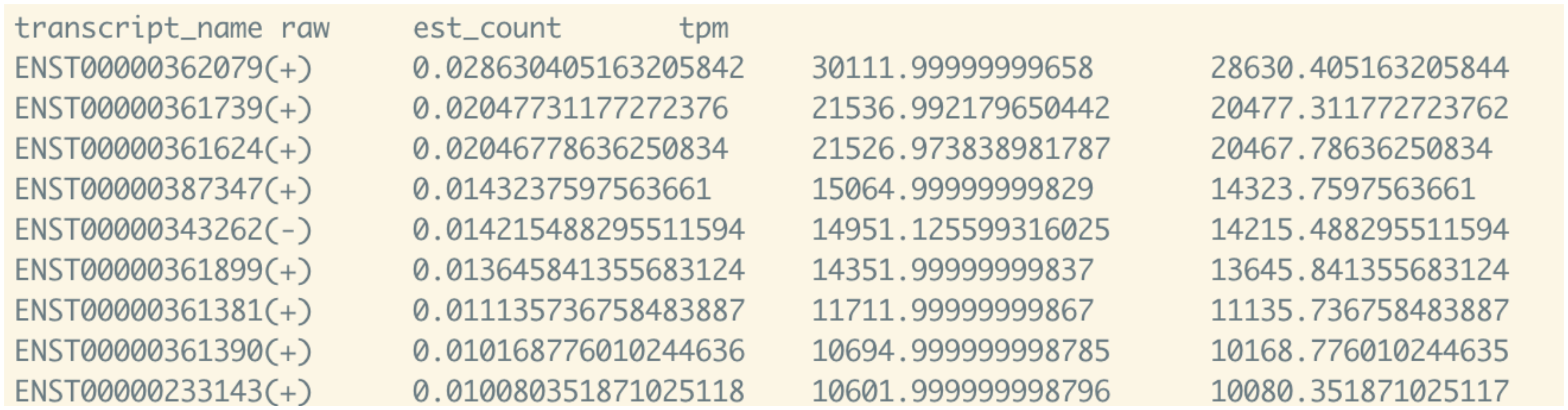
Expression quantification: a naïve approach

You can just count how many reads map to each gene!

Expression quantification: Nanocount

Our naïve above completely ignores the problem of multimapping reads (we filtered out all secondary alignments). Dedicated tools such as **Nanocount** take secondary alignments into account using Expectation Maximization algorithms, providing more robust estimates of transcript abundance.



```
transcript_name raw     est_count       tpm
ENST00000362079(+)      0.028630405163205842    30111.99999999658       28630.405163205844
ENST00000361739(+)      0.02047731177272376     21536.992179650442      20477.311772723762
ENST00000361624(+)      0.02046778636250834     21526.973838981787      20467.78636250834
ENST00000387347(+)      0.0143237597563661      15064.99999999829       14323.7597563661
ENST00000343262(-)      0.014215488295511594    14951.125599316025      14215.488295511594
ENST00000361899(+)      0.013645841355683124    14351.99999999837       13645.841355683124
ENST00000361381(+)      0.011135736758483887    11711.99999999867       11135.736758483887
ENST00000361390(+)      0.010168776010244636    10694.999999998785      10168.776010244635
ENST00000233143(+)      0.010080351871025118    10601.999999998796      10080.351871025117
```

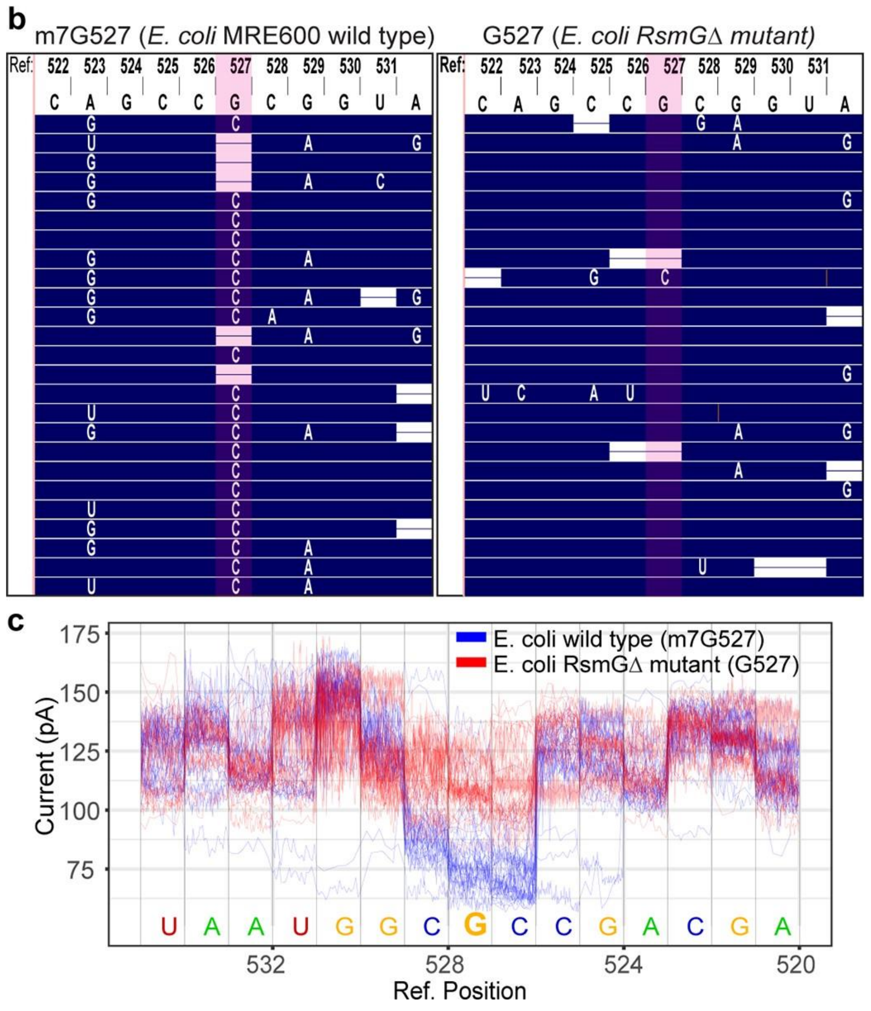
Analysis steps

☑ Sequencing	Starting and monitoring a run
☑ Basecalling	From signal to sequence
☑ QC	Assess quality of the data
☑ Mapping	Map the reads to the genome/transcriptome
☑ Quantification	Estimate gene/transcript expression
☐ Modification detection	Detect RNA modifications

Connecting the signal to the sequence

Why bother?

- Visualisation
- Identification of modifications
- Detection of basecalling inaccuracies
- Comparison of samples

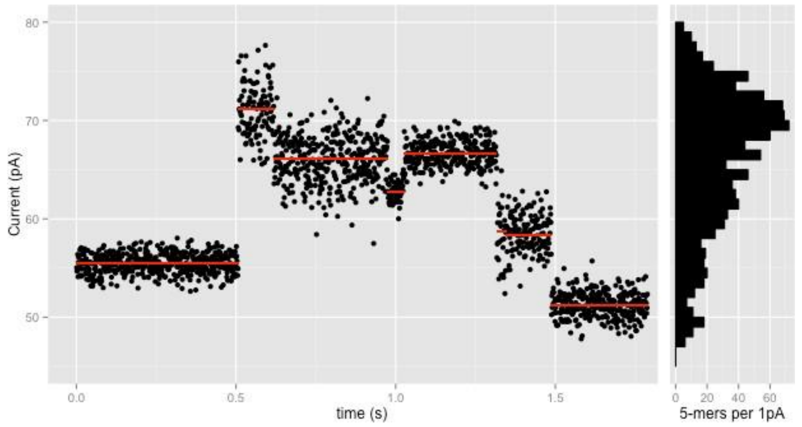


Smith et al., Plos One 2019

Connecting the signal to the sequence

Why is it hard?

- Would be (was) easier with older basecalling algorithms
- Current NN don't provide a direct "association" between signal and seq
- Resquiggling needs to be done a posteriori using HMM
- Tool of choice: **Nanopolish**



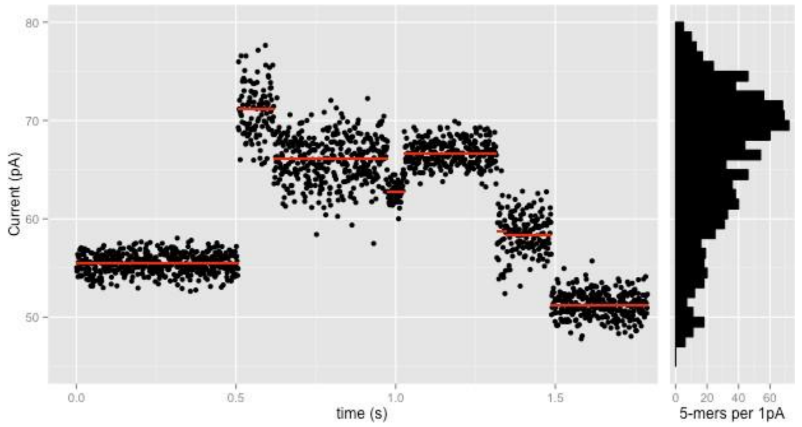
simpsonlab.github.io

Nanopolish eventalign

Align signal-level events to k-mers of a reference genome

Input

- Basecalled sequences
- Reference sequence (i.e. mapped reads)
- Raw data (fast5 files)



simpsonlab.github.io

Nanopolish eventalign

```
> nanopolish eventalign
  Usage: nanopolish eventalign [OPTIONS] --reads reads.fa --bam alignments.bam --genome genome.fa
  Align nanopore events to reference k-mers
    -v, --verbose                       display verbose output
        --version                       display version
        --help                          display this help and exit
        --sam                           write output in SAM format
    -r, --reads=FILE                    the ONT reads are in fasta FILE
    -b, --bam=FILE                      the reads aligned to the genome assembly are in bam FILE
    -g, --genome=FILE                   the genome we are computing a consensus for is in FILE
    -t, --threads=NUM                   use NUM threads (default: 1)
        --scale-events                  scale events to the model, rather than vice-versa
    -n, --print-read-names              print read names instead of indexes
        --samples                       write the raw samples for the event to the tsv output
        --signal-index                  write the raw signal start and end index values for the

 event to the tsv output

  [ Some options not relevant to this course were omitted to save space on the slide ]
```

Main Options

Flag	Function
--scale-events	scale events to the model, rather than vice-versa
--print-read-names	print read names instead of indexes
--samples	write the raw samples for the event to the tsv output
--signal-index	write the raw signal start and end coordinates

Hands on: Building an index for Nanopolish

Nanopolish requires an index to associate reads to the corresponding fast5 files

1. Combine all the basecalled fastq files (`~/data/consortium_basecalled/pass/`) into a single one.
2. Run `nanopolish index`

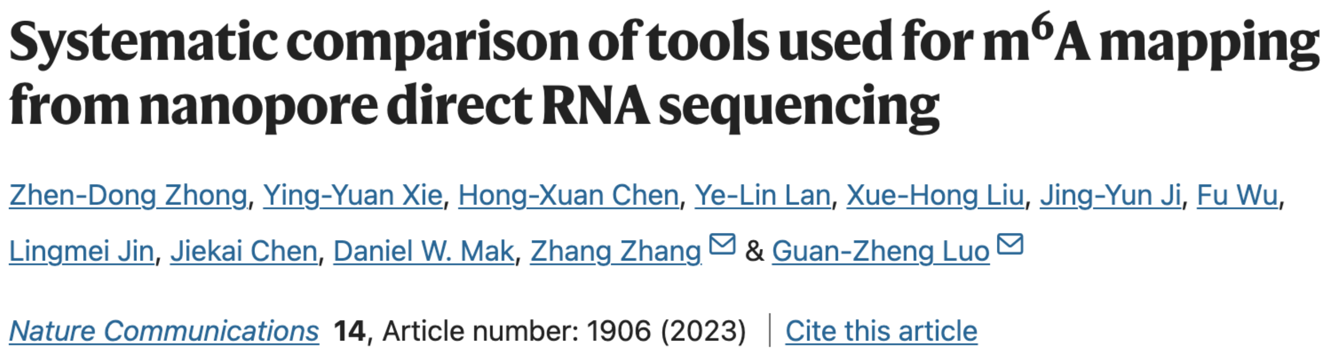
```
# Combine all basecalled fastq files
> cat ~/data/consortium_basecalled/pass/*fastq > basecalled.fastq

> nanopolish index \
  -d ~/data/consortium_basecalled/workspace/ \
  -s ~/data/consortium_basecalled/sequencing_summary.txt \
  basecalled.fastq

  [readdb] indexing /home/ubuntu/data/consortium_basecalled/workspace/
  [readdb] indexing /home/ubuntu/data/consortium_basecalled/workspace//1
  [readdb] indexing /home/ubuntu/data/consortium_basecalled/workspace//2
  [readdb] indexing /home/ubuntu/data/consortium_basecalled/workspace//4
  [readdb] indexing /home/ubuntu/data/consortium_basecalled/workspace//3
  [readdb] indexing /home/ubuntu/data/consortium_basecalled/workspace//0
  [readdb] num reads: 11699, num reads with path to fast5: 11699

> ls
  basecalled.fastq   basecalled.fastq.index   basecalled.fastq.index.fai
  basecalled.fastq.index.gzi   basecalled.fastq.index.readdb
```

Hands on: Running eventalign

1. Convert the SAM file to BAM and sort it
2. Generate and index for the BAM file
3. Run `eventalign` with `--scale-events --print-read-names --samples --signal-index`

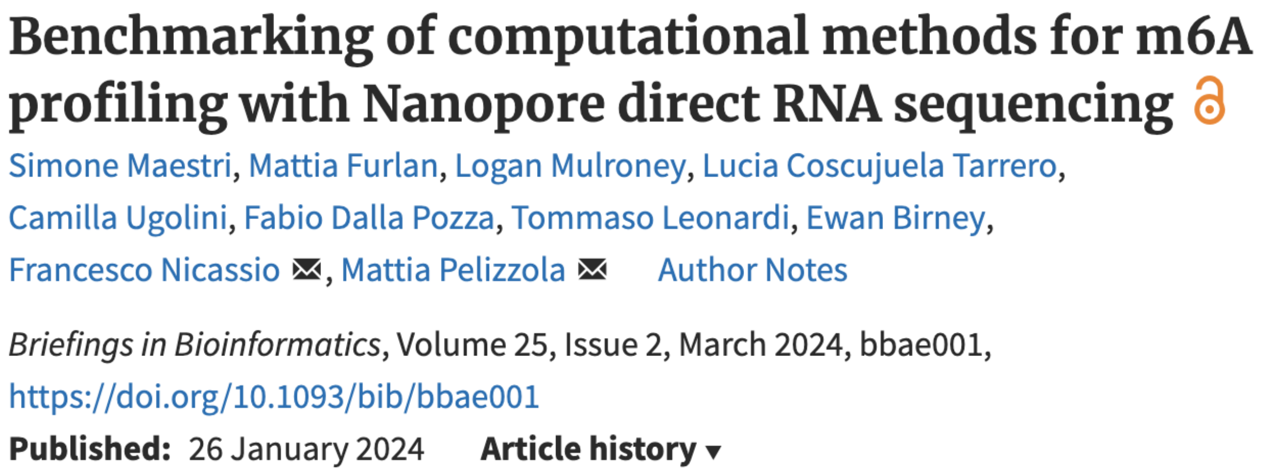
```
# Convert the SAM file to BAM and sort it
> samtools view \
  -b ~/data/consortium_minimap_transcriptome_filtered_oneread.sam | samtools sort > mapped_reads.bam

# Index the BAM file
> samtools index mapped_reads.bam

# Run nanopolish
> nanopolish eventalign \
  --scale-events --print-read-names --samples --signal-index \
  --reads basecalled.fastq \
  --bam mapped_reads.bam \
  --genome ~/data/Homo_sapiens.GRCh38.98_transcriptome.fa > eventalign.txt
```

The evolution of RNA methylation detection tools


Systematic comparison of tools used for m^{6}A mapping from nanopore direct RNA sequencing

Zhen-Dong Zhong, Ying-Yuan Xie, Hong-Xuan Chen, Ye-Lin Lan, Xue-Hong Liu, Jing-Yun Ji, Fu Wu, Lingmei Jin, Jiekai Chen, Daniel W. Mak, Zhang Zhang & Guan-Zheng Luo

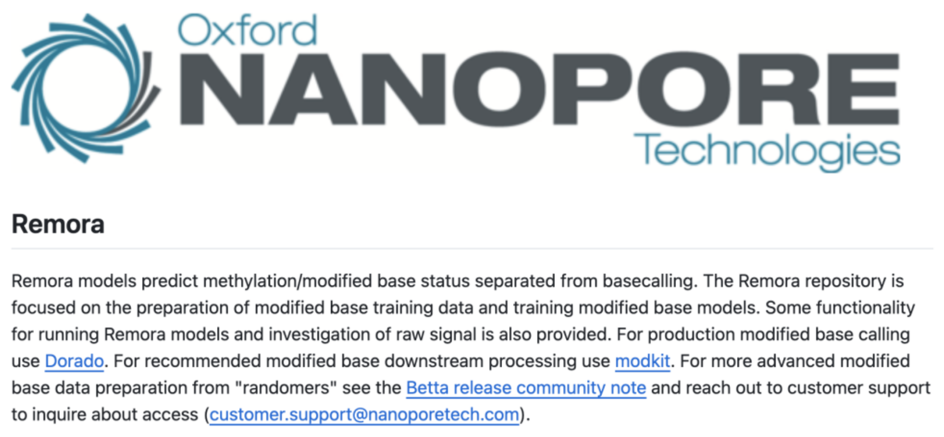
Nature Communications **14**, Article number: 1906 (2023) | Cite this article


Benchmarking of computational methods for m6A profiling with Nanopore direct RNA sequencing

Simone Maestri, Mattia Furlan, Logan Mulroney, Lucia Coscujuela Tarrero, Camilla Ugolini, Fabio Dalla Pozza, Tommaso Leonardi, Ewan Birney, Francesco Nicassio, Mattia Pelizzola Author Notes

Briefings in Bioinformatics, Volume 25, Issue 2, March 2024, bbae001, https://doi.org/10.1093/bib/bbae001

Published: 26 January 2024 **Article history ▾**


Remora

Remora models predict methylation/modified base status separated from basecalling. The Remora repository is focused on the preparation of modified base training data and training modified base models. Some functionality for running Remora models and investigation of raw signal is also provided. For production modified base calling use Dorado. For recommended modified base downstream processing use modkit. For more advanced modified base data preparation from "randomers" see the Betta release community note and reach out to customer support to inquire about access (customer.support@nanoporetech.com).

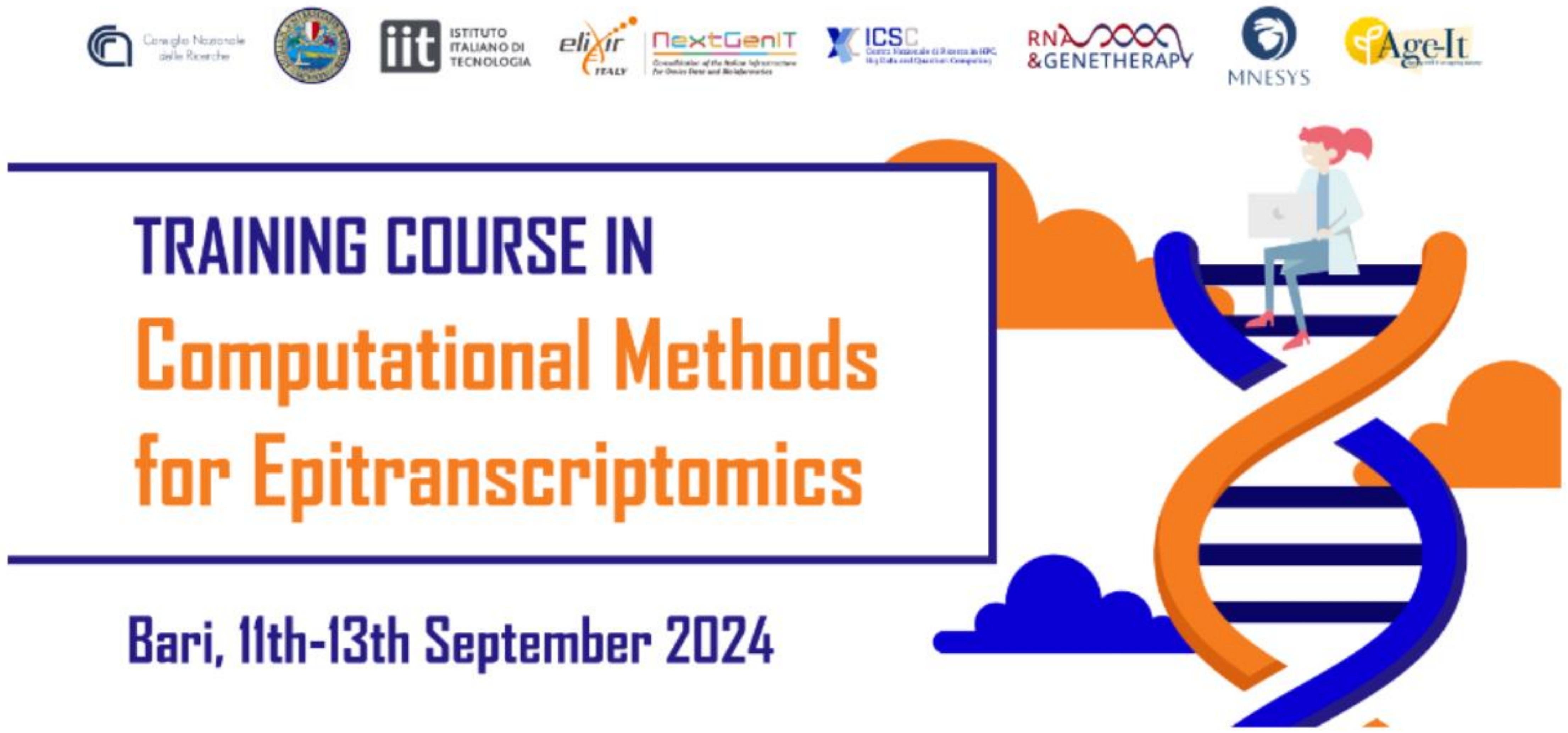
m6Anet and Remora

m6Anet (Goeke Lab)

- Machine learning-based tools for m6A detection across nanopore reads
- Runs on single samples
- Best performing on human datasets according to Maestri e al.
- Available for RNA002 and RNA004 (development version)

Remora (Oxford Nanopore Technologies)

- Coupled with Dorado basecalling
- Runs on single samples
- New signal resquiggling
- Available for RNA004
- PseudoU calling also available!


TRAINING COURSE IN
Computational Methods for Epitranscriptomics
Bari, 11th-13th September 2024

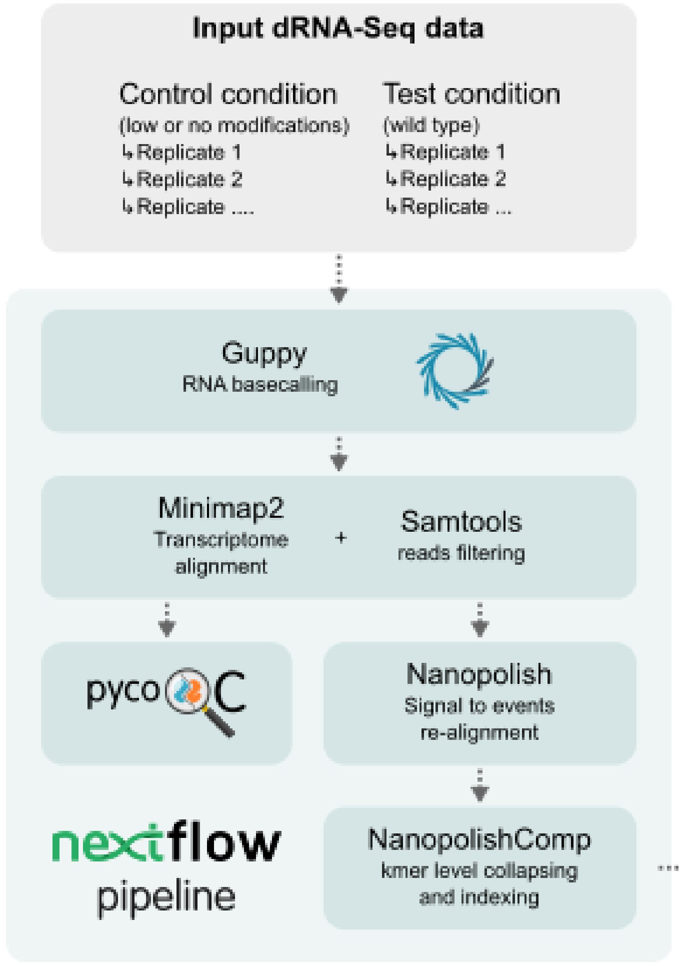
From raw data to RNA modifications: Practical session

Camilla Ugolini

Credits: Tommaso Leonardi & Luca Pandolfini

How to go from the raw data to modification calls

- Basecall the fast5 files
- Map to the transcriptome
- Filter alignments
- Resquiggle with Nanopolish
- Collapse and index results with eventalign_collapse
- Analyse with Nanocompore



Leger et al., bioRxiv 2019

Step 1 (recap): basecalling with Guppy

```
> guppy_basecaller -i input_folder \
                   -s out_folder \
                   --flowcell FLO-MIN106 \
                   --kit SQK-RNA002 \
                   --fast5_out \
                   --recursive \
                   --num_callers 2 \
                   --disable_pings \
                   --qscore_filtering
```

Step 1 (recap): basecalling with Guppy

```
> guppy_basecaller -i input_folder \
                   -s out_folder \
                   --flowcell FLO-MIN106 \
                   --kit SQK-RNA002 \
                   --fast5_out \
                   --recursive \
                   --num_callers 2 \
                   --disable_pings \
                   --qscore_filtering
```

Expected output:

Step 1 (recap): basecalling with Guppy

```
> guppy_basecaller -i input_folder \
                   -s out_folder \
                   --flowcell FLO-MIN106 \
                   --kit SQK-RNA002 \
                   --fast5_out \
                   --recursive \
                   --num_callers 2 \
                   --disable_pings \
                   --qscore_filtering
```

Expected output:

1. fast5 files (basecalled)

Step 1 (recap): basecalling with Guppy

```
> guppy_basecaller -i input_folder \
                   -s out_folder \
                   --flowcell FLO-MIN106 \
                   --kit SQK-RNA002 \
                   --fast5_out \
                   --recursive \
                   --num_callers 2 \
                   --disable_pings \
                   --qscore_filtering
```

Expected output:

1. fast5 files (basecalled)
2. fastq files

Step 1 (recap): basecalling with Guppy

```
> guppy_basecaller -i input_folder \
                   -s out_folder \
                   --flowcell FLO-MIN106 \
                   --kit SQK-RNA002 \
                   --fast5_out \
                   --recursive \
                   --num_callers 2 \
                   --disable_pings \
                   --qscore_filtering
```

Expected output:

1. fast5 files (basecalled)
2. fastq files
3. sequencing_summary.txt

Step 2 (recap): Map to the transcriptome

Required Input: (1) fastq files, (2) transcriptome in fasta format

Step 2 (recap): Map to the transcriptome

Required Input: (1) fastq files, (2) transcriptome in fasta format

```
# Convert the Ensembl GTF to BED
bedparse gtf2bed \
  ~/data/Homo_sapiens.GRCh38.102.chr.gtf \
  > reference_transcriptome.bed

# Generate a Fasta from the GTF
bedtools getfasta \
  -fi ~/data/Homo_sapiens.GRCh38.dna.primary_assembly.fa \
  -s -split -name \
  -bed reference_transcriptome.bed \
  > Homo_sapiens.GRCh38.98_transcriptome.fa

# Generate a fasta index
samtools faidx Homo_sapiens.GRCh38.98_transcriptome.fa

# Map
minimap2 -a -x map-ont -k14 --for-only \
  Homo_sapiens.GRCh38.98_transcriptome.fa   basecalled.fastq > minimap_transcriptome.sam
```

Step 2 (recap): Map to the transcriptome

Required Input: (1) fastq files, (2) transcriptome in fasta format

```
# Convert the Ensembl GTF to BED
bedparse gtf2bed \
  ~/data/Homo_sapiens.GRCh38.102.chr.gtf \
  > reference_transcriptome.bed

# Generate a Fasta from the GTF
bedtools getfasta \
  -fi ~/data/Homo_sapiens.GRCh38.dna.primary_assembly.fa \
  -s -split -name \
  -bed reference_transcriptome.bed \
  > Homo_sapiens.GRCh38.98_transcriptome.fa

# Generate a fasta index
samtools faidx Homo_sapiens.GRCh38.98_transcriptome.fa

# Map
minimap2 -a -x map-ont -k14 --for-only \
  Homo_sapiens.GRCh38.98_transcriptome.fa  basecalled.fastq > minimap_transcriptome.sam
```

Expected output:

- Alignments in SAM format

Step 3 (recap): Filter alignments

Required Input: Alignments in SAM format

Step 3 (recap): Filter alignments

Required Input: Alignments in SAM format

```
# Filter, convert to BAM and sort
samtools view -b -F 2324 minimap_transcriptome.sam | samtools sort \
  > minimap_transcriptome_filtered_sorted.bam

# Index
samtools index minimap_transcriptome_filtered_sorted.bam
```

Step 3 (recap): Filter alignments

Required Input: Alignments in SAM format

```
# Filter, convert to BAM and sort
samtools view -b -F 2324 minimap_transcriptome.sam | samtools sort \
  > minimap_transcriptome_filtered_sorted.bam

# Index
samtools index minimap_transcriptome_filtered_sorted.bam
```

Expected output:

- Filtered and sorted alignments in BAM format

Step 3 (recap): Filter alignments

Required Input: Alignments in SAM format

```
# Filter, convert to BAM and sort
samtools view -b -F 2324 minimap_transcriptome.sam | samtools sort \
  > minimap_transcriptome_filtered_sorted.bam

# Index
samtools index minimap_transcriptome_filtered_sorted.bam
```

Expected output:

- Filtered and sorted alignments in BAM format

Questions:

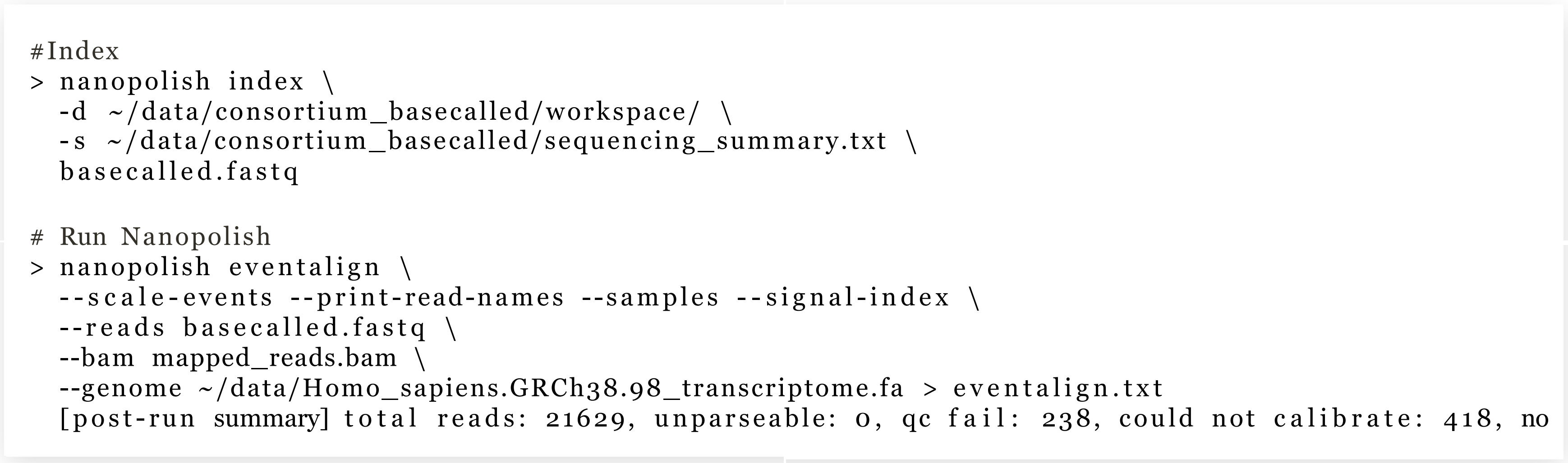
- What does 2324 mean?
- Would it be ok to use 2308?

Step 4 (recap): Resquiggle with Nanopolish

Required Input: (1) Fast5 files (2) Fastq (3) Alignments in BAM format (4) sequencing_summary.txt

Step 4 (recap): Resquiggle with Nanopolish

Required Input: (1) Fast5 files (2) Fastq (3) Alignments in BAM format (4) sequencing_summary.txt



```
#Index
> nanopolish index \
  -d ~/data/consortium_basecalled/workspace/ \
  -s ~/data/consortium_basecalled/sequencing_summary.txt \
  basecalled.fastq

# Run Nanopolish
> nanopolish eventalign \
  --scale-events --print-read-names --samples --signal-index \
  --reads basecalled.fastq \
  --bam mapped_reads.bam \
  --genome ~/data/Homo_sapiens.GRCh38.98_transcriptome.fa > eventalign.txt
  [post-run summary] total reads: 21629, unparseable: 0, qc fail: 238, could not calibrate: 418, no
```

Step 5: Collapse eventalign files

Required Input: eventalign file (from Nanopolish)

Output: Collapsed file where all the events of each kmer are summarised (mean, median)

```
> nanocompore eventalign_collapse -h
  usage: nanocompore eventalign_collapse [-h] [--eventalign EVENTALIGN] [--n_lines N_LINES]
                                         [--nthreads NTHREADS] [--outpath OUTPATH]
                                         [--outprefix OUTPREFIX] [--overwrite]
                                         [--log_level {warning,info,debug}] [--progress]

  Collapse the nanopolish eventalign output at kmers level and compute kmer level statistics
  * Minimal example
      nanocompore eventalign_collapse -i nanopolish_eventalign.tsv -outprefix out

  Input options:
    --eventalign EVENTALIGN, -i EVENTALIGN
                            Path to a nanopolish eventalign tsv output file, or a list of file,
                            or a regex (can be gzipped). It can be ommited if piped to standard
                            input (default: piped to stdin)

  Output options:
    --outpath OUTPATH, -o OUTPATH
                            Path to the output folder (default: ./)
    --outprefix OUTPREFIX, -p OUTPREFIX
                            text outprefix for all the files generated (default: out)
    --overwrite, -w         Use --outpath even if it exists already (default: False)
```

Step 5: Collapse eventalign files

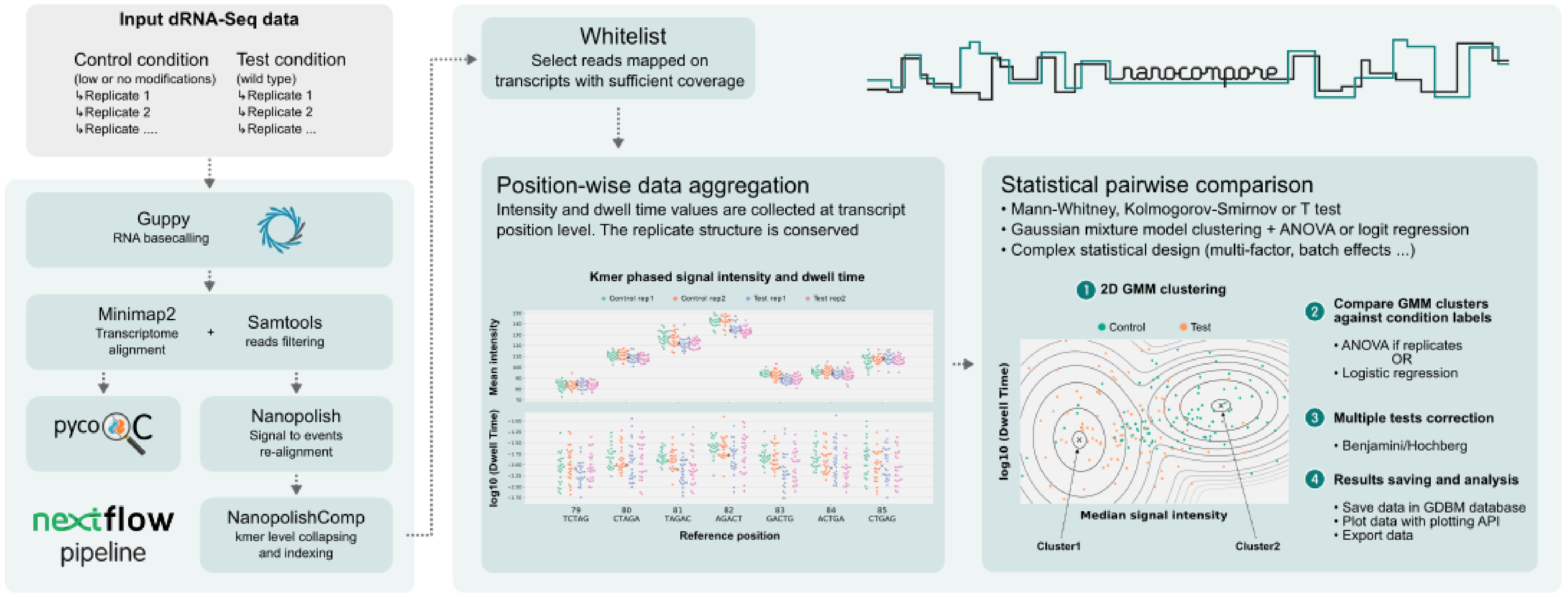
Input

```
> head eventalign.txt
contig              position   reference_kmer   read_name  strand   event_index   event_level_mean   event
ENST00000451311(+)  22         AGACC            b69985a2   t        31            127.42             2.202
ENST00000451311(+)  22         AGACC            b69985a2   t        32            121.97             3.668
ENST00000451311(+)  22         AGACC            b69985a2   t        33            123.74             1.832
ENST00000451311(+)  22         AGACC            b69985a2   t        34            121.53             1.789
ENST00000451311(+)  22         AGACC            b69985a2   t        35            122.73             2.728
ENST00000451311(+)  22         AGACC            b69985a2   t        36            123.63             9.997
ENST00000451311(+)  23         GACCA            b69985a2   t        37            78.56              2.769
ENST00000451311(+)  23         GACCA            b69985a2   t        38            76.02              2.127
ENST00000451311(+)  24         ACCAG            b69985a2   t        39            71.39              2.717
```

Output

```
> head out_eventalign_collapse.tsv
b69985a2-7e7a-4f08-bd6d-ea7598d65b5c   ENST00000451311(+)
ref_pos   ref_kmer           num_events   num_signals   dwell_time             NNNNN_dwell_time   misma
22        AGACC              6            167           0.055449999403208494   0.0                0.0
23        GACCA              2            28            0.00929000019095838    0.0                0.0
24        ACCAG              1            24            0.007969999685883522   0.0                0.0
25        CCAGA              1            16            0.005309999920427799   0.0                0.0
26        CAGAC              5            128           0.042489999905228615   0.0                0.0
27        AGACT              5            139           0.04614999983459711    0.0                0.0
28        GACTT              2            26            0.008630000287666917   0.0                0.0
29        ACTTC              1            7             0.002319999970495701   0.0                0.0
```

Step 6: Run Nanocompore


Input dRNA-Seq data
Control condition
(low or no modifications)
↳Replicate 1
↳Replicate 2
↳Replicate ....
Test condition
(wild type)
↳Replicate 1
↳Replicate 2
↳Replicate ...
Guppy
RNA basecalling
Minimap2
Transcriptome alignment
+
Samtools
reads filtering
pycoQC
Nanopolish
Signal to events re-alignment
nextflow pipeline
NanopolishComp
kmer level collapsing and indexing
Whitelist
Select reads mapped on transcripts with sufficient coverage
nanocompore
Position-wise data aggregation
Intensity and dwell time values are collected at transcript position level. The replicate structure is conserved
Kmer phased signal intensity and dwell time
Control rep1
Control rep2
Test rep1
Test rep2
Mean intensity
log10 (Dwell Time)
Reference position
Statistical pairwise comparison
• Mann-Whitney, Kolmogorov-Smirnov or T test
• Gaussian mixture model clustering + ANOVA or logit regression
• Complex statistical design (multi-factor, batch effects ...)
1 2D GMM clustering
Control
Test
log10 (Dwell Time)
Median signal intensity
Cluster1
Cluster2
2 Compare GMM clusters against condition labels
• ANOVA if replicates
OR
• Logistic regression
3 Multiple tests correction
• Benjamini/Hochberg
4 Results saving and analysis
• Save data in GDBM database
• Plot data with plotting API
• Export data

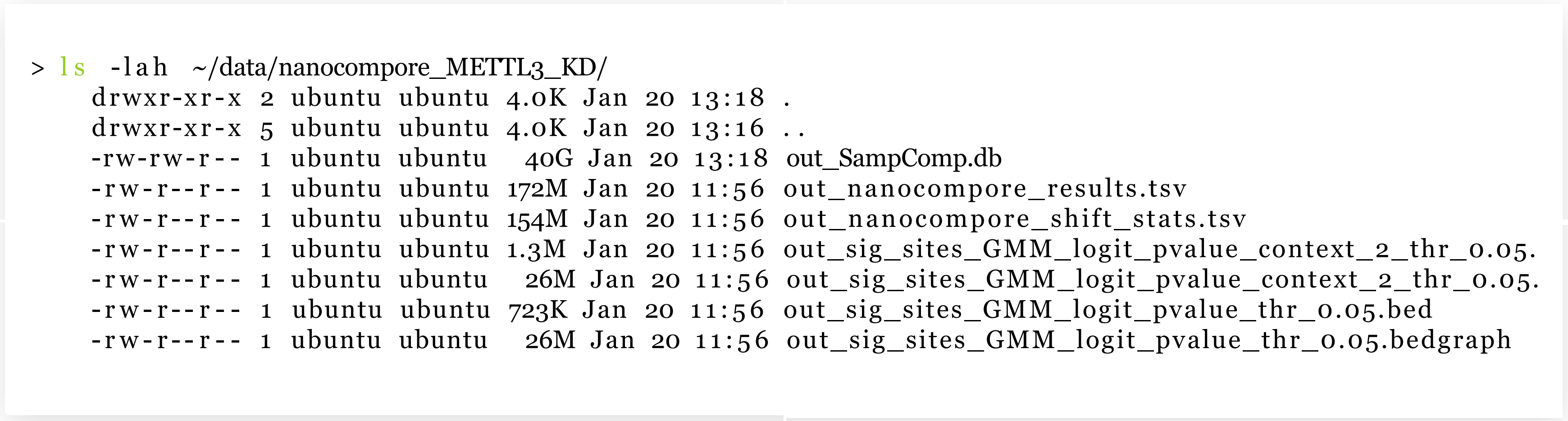
Step 6: Run Nanocompore

Required Input:

- eventalign_collapse files (one per sample)
- Transcriptome Fasta
- Transcriptome BED (optional)

```
> nanocompore sampcomp \
    --file_list1 WT_rep1_ealign_collapsed.tsv,WT_rep2_ealign_collapsed.tsv
    --file_list2 KD_rep1_ealign_collapsed.tsv,KD_rep2_ealign_collapsed.tsv
    --label1 WT \
    --label2 KD \
    --fasta reference_transcriptome.fa \
    --bed reference_transcriptome.bed \
    --outpath ~/data/nanocompore_METTL3_KD/ \
    --sequence_context 2 \
    --allow_warnings \
    --pvalue_thr 0.01 \
    --min_coverage 30 \
    --logit \
    --nthreads 3
```

Nanocompore output



```
> ls -lah ~/data/nanocompore_METTL3_KD/
    drwxr-xr-x 2 ubuntu ubuntu 4.0K Jan 20 13:18 .
    drwxr-xr-x 5 ubuntu ubuntu 4.0K Jan 20 13:16 ..
    -rw-rw-r-- 1 ubuntu ubuntu  40G Jan 20 13:18 out_SampComp.db
    -rw-r--r-- 1 ubuntu ubuntu 172M Jan 20 11:56 out_nanocompore_results.tsv
    -rw-r--r-- 1 ubuntu ubuntu 154M Jan 20 11:56 out_nanocompore_shift_stats.tsv
    -rw-r--r-- 1 ubuntu ubuntu 1.3M Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_context_2_thr_0.05.
    -rw-r--r-- 1 ubuntu ubuntu  26M Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_context_2_thr_0.05.
    -rw-r--r-- 1 ubuntu ubuntu 723K Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_thr_0.05.bed
    -rw-r--r-- 1 ubuntu ubuntu  26M Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_thr_0.05.bedgraph
```

Nanocompore output

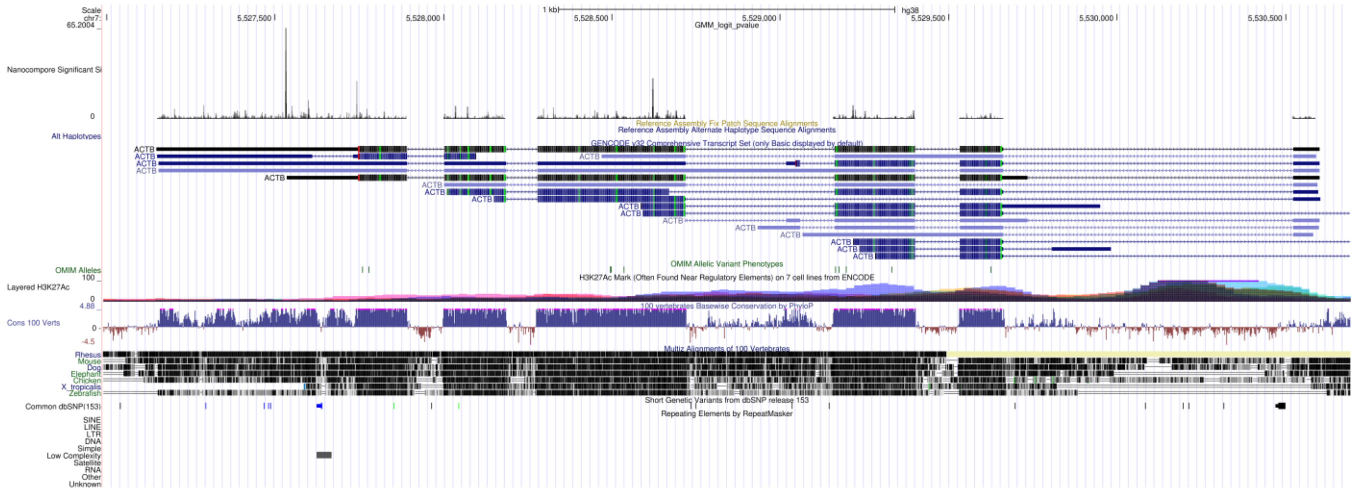
```
> ls  -lah  ~/data/nanocompore_METTL3_KD/
    drwxr-xr-x 2 ubuntu ubuntu 4.0K Jan 20 13:18 .
    drwxr-xr-x 5 ubuntu ubuntu 4.0K Jan 20 13:16 ..
    -rw-rw-r-- 1 ubuntu ubuntu  40G Jan 20 13:18 out_SampComp.db
    -rw-r--r-- 1 ubuntu ubuntu 172M Jan 20 11:56 out_nanocompore_results.tsv
    -rw-r--r-- 1 ubuntu ubuntu 154M Jan 20 11:56 out_nanocompore_shift_stats.tsv
    -rw-r--r-- 1 ubuntu ubuntu 1.3M Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_context_2_thr_0.05.
    -rw-r--r-- 1 ubuntu ubuntu  26M Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_context_2_thr_0.05.
    -rw-r--r-- 1 ubuntu ubuntu 723K Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_thr_0.05.bed
    -rw-r--r-- 1 ubuntu ubuntu  26M Jan 20 11:56 out_sig_sites_GMM_logit_pvalue_thr_0.05.bedgraph
```

Main results table

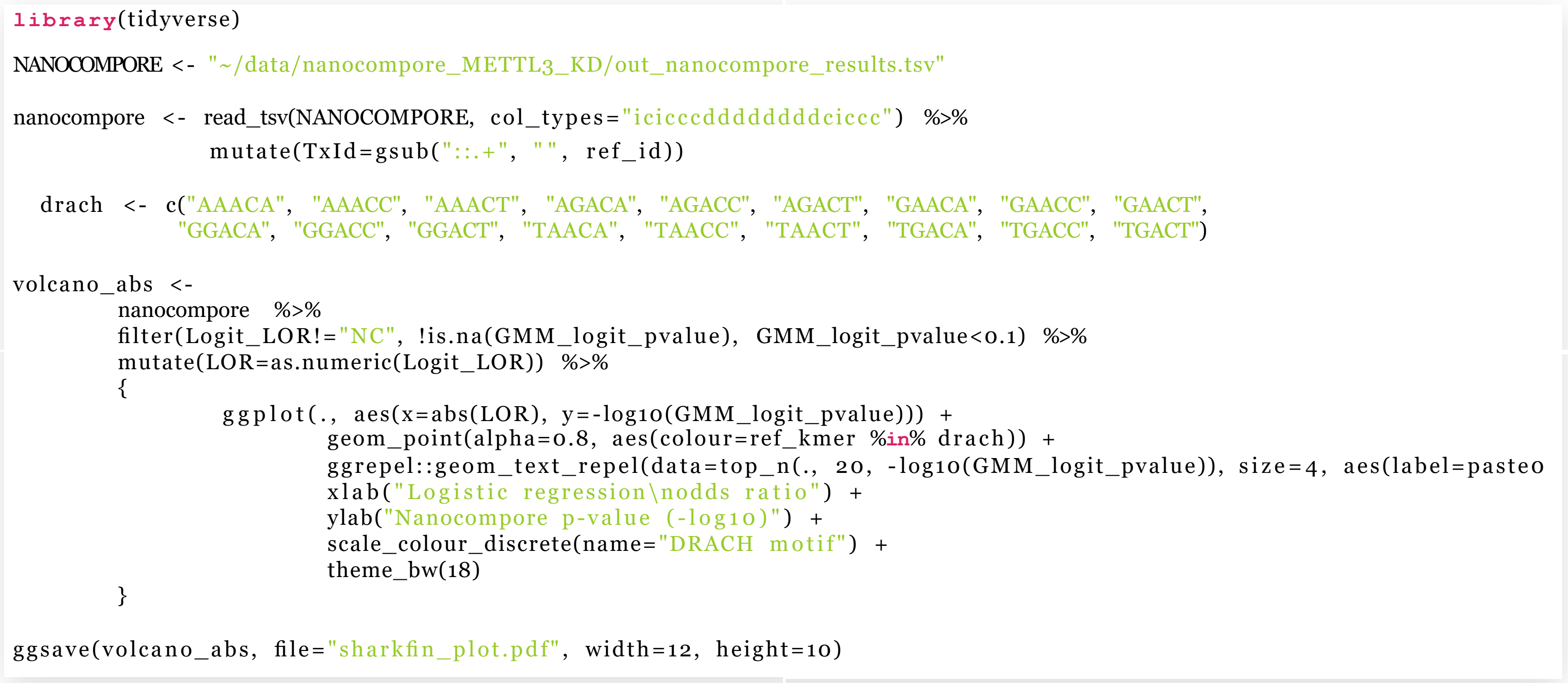
```
 > head  ~/data/nanocompore_METTL3_KD/out_nanocompore_results.tsv
pos     chr     genomicPos      ref_id          strand  ref_kmer  GMM_anova_pvalue  GMM_anova_pvalue_context
1417    1       8861365         ENST00000647408 -       CAAGT     1.0               0.9999999999998306
1422    1       8861360         ENST00000647408 -       AAGCT     1.0               0.9999999999998306
1424    1       8861358         ENST00000647408 -       GCTGT     1.0               0.9999999999998306
1425    1       8861357         ENST00000647408 -       CTGTG     nan               nan
1426    1       8861356         ENST00000647408 -       TGTGG     1.0               0.9999999999998306
1427    1       8861355         ENST00000647408 -       GTGGG     1.0               0.9999999999998306
1429    1       8861353         ENST00000647408 -       GGGCA     1.0               0.9999999999998306
1430    1       8861352         ENST00000647408 -       GGCAG     1.0               0.9999999999998306
1440    1       8861342         ENST00000647408 -       CCCTT     1.0               0.9999999999998306
```

Nanocompore BED tracks

Nanocompore also creates BED and BEDGRAPH tracks of significant hits.
Download the file:
`out_sig_sites_GMM_logit_pvalue_thr_0.05.bedgraph`
and load it into the UCSC genome browser.

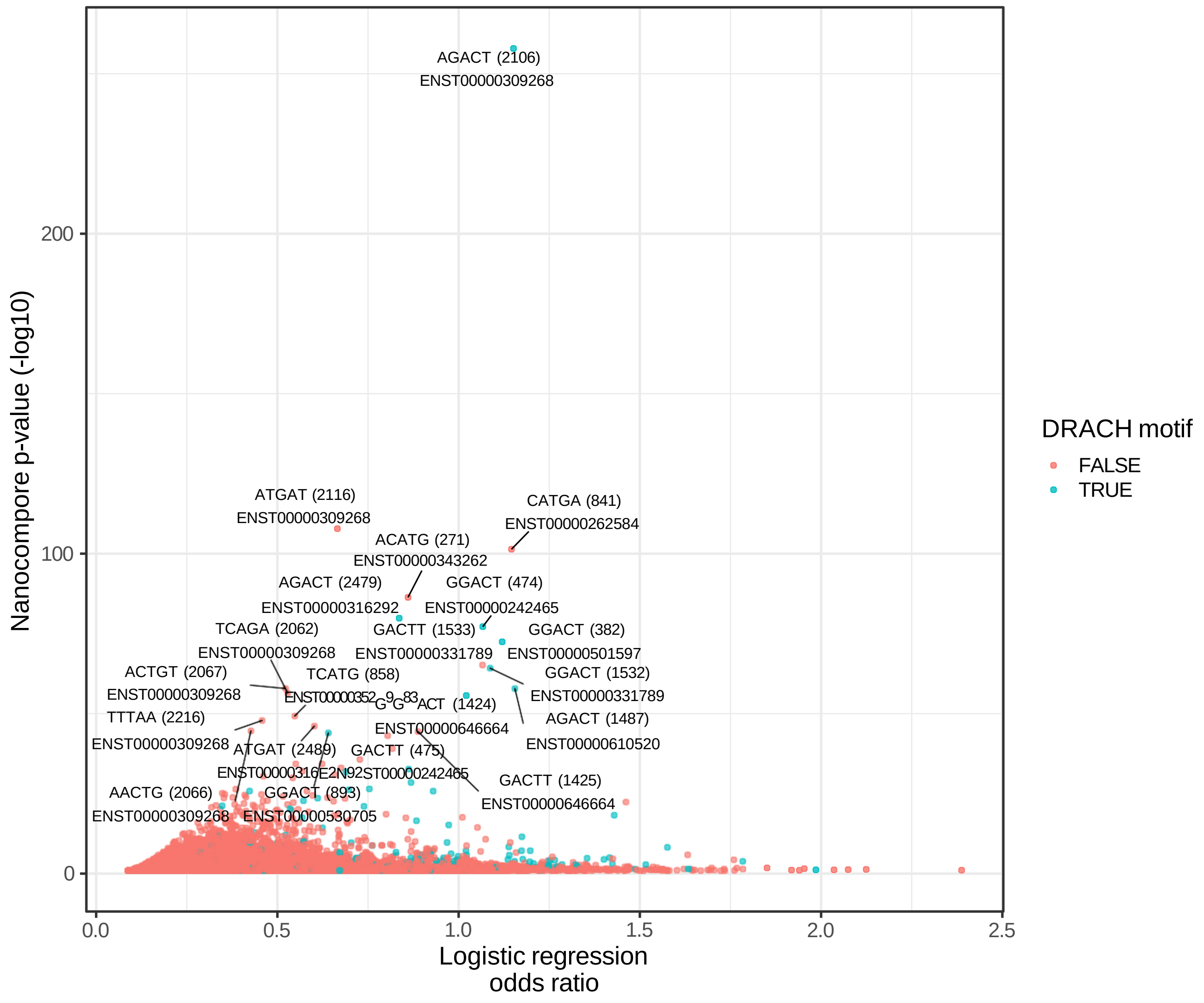

Scale
chr7:
65.2004
1 kb
hg38
5,527,500
5,528,000
5,528,500
5,529,000
5,529,500
5,530,000
5,530,500
GMM_logit_pvalue
Nanocompore Significant Si
0
Reference Assembly Fix Patch Sequence Alignments
Reference Assembly Alternate Haplotype Sequence Alignments
Alt Haplotypes
GENCODE v32 Comprehensive Transcript Set (only Basic displayed by default)
ACTB
OMIM Allelic Variant Phenotypes
OMIM Alleles
H3K27Ac Mark (Often Found Near Regulatory Elements) on 7 cell lines from ENCODE
100
Layered H3K27Ac
100 vertebrates Basewise Conservation by PhyloP
4.88
Cons 100 Verts
-4.5
Multiz Alignments of 100 Vertebrates
Rhesus
Mouse
Dog
Elephant
Chicken
X_tropicalis
Zebrafish
Short Genetic Variants from dbSNP release 153
Common dbSNP(153)
Repeating Elements by RepeatMasker
SINE
LINE
LTR
DNA
Simple
Low Complexity
Satellite
RNA
Other
Unknown

Visualise Nanocompore results



```r
library(tidyverse)

NANOCOMPORE <- "~/data/nanocompore_METTL3_KD/out_nanocompore_results.tsv"

nanocompore <- read_tsv(NANOCOMPORE, col_types="icicccdddddddciccc") %>%
                mutate(TxId=gsub("::.+", "", ref_id))

  drach <- c("AAACA", "AAACC", "AAACT", "AGACA", "AGACC", "AGACT", "GAACA", "GAACC", "GAACT",
             "GGACA", "GGACC", "GGACT", "TAACA", "TAACC", "TAACT", "TGACA", "TGACC", "TGACT")

volcano_abs <-
        nanocompore  %>%
        filter(Logit_LOR!="NC", !is.na(GMM_logit_pvalue), GMM_logit_pvalue<0.1) %>%
        mutate(LOR=as.numeric(Logit_LOR)) %>%
        {
                ggplot(., aes(x=abs(LOR), y=-log10(GMM_logit_pvalue))) +
                        geom_point(alpha=0.8, aes(colour=ref_kmer %in% drach)) +
                        ggrepel::geom_text_repel(data=top_n(., 20, -log10(GMM_logit_pvalue)), size=4, aes(label=paste0
                        xlab("Logistic regression\nodds ratio") +
                        ylab("Nanocompore p-value (-log10)") +
                        scale_colour_discrete(name="DRACH motif") +
                        theme_bw(18)
        }

ggsave(volcano_abs, file="sharkfin_plot.pdf", width=12, height=10)
```

The sharkfin plot


AGACT (2106)
ENST00000309268
ATGAT (2116)
ENST00000309268
CATGA (841)
ENST00000262584
ACATG (271)
ENST00000343262
AGACT (2479)
ENST00000316292
GGACT (474)
ENST00000242465
TCAGA (2062)
ENST00000309268
GACTT (1533)
ENST00000331789
GGACT (382)
ENST00000501597
ACTGT (2067)
ENST00000309268
TCATG (858)
GGACT (1532)
ENST00000331789
TTTAA (2216)
ENST00000309268
GGACT (1424)
ENST00000646664
AGACT (1487)
ENST00000610520
ATGAT (2489)
ENST00000316292
GACTT (475)
ENST00000242465
GACTT (1425)
ENST00000646664
AACTG (2066)
ENST00000309268
GGACT (893)
ENST00000530705
Nanocompore p-value (-log10)
Logistic regression
odds ratio
DRACH motif
FALSE
TRUE