Stranded Mapping from Long Reads Version 2

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Abstract

This protocol demonstrates how to convert raw long reads produced using a strand-specific sequencing protocol (e.g. ONT's strand-switching protocol) into strand-specific mapped reads.

The general ides is to use LAST to identify the adapter orientation relative to the genome, and then use that information to split BAM files up and recombine them to create two strand-specific files that are displayable in a genome browser.

Citation: David Eccles Stranded Mapping from Long Reads. **protocols.io**
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Before start

I have written my own script to process LAST results into a CSV format, which makes it easier to do line-by-line data filtering. I have also created a fastq filtering script that helps for filtering reads into different files.

You will also need access to the following free and open-source software programs:

- LAST
- minimap2
- samtools

And the following additional data files:

- a FASTA file containing strand-specific primer / adapter sequences.

Protocol

Read Correction

Step 1.

Download canu v1.6 or later

SOFTWARE PACKAGE (Linux / Darwin)

canu, 1.6

Maryland Bioinformatics Labs
https://github.com/marbl/canu

Read Correction

Step 2.

I prefer starting off my data analysis with a read correction with Canu (ideally v1.7 now that it's out, because that attempts correction of *all* reads, but here I use canu v1.6). I use `minimap` as the mapper to speed this up. The `genomeSize` parameter should be approximately a tenth to a fortieth of the number of bases in your dataset to make sure that no sequences are excluded (bigger is better, as long as Canu doesn't freak out about memory consumption):

(This creates a file `4T1_BC06/4T1_BC06.correctedReads.fasta.gz`.)

COMMAND

```
~/install/canu/canu-1.6/Linux-
amd64/bin/canu overlapper=minimap genomeSize=100M minReadLength=100 minOverlapLength=30 -
correct -p 4T1_BC06 -d 4T1_BC06 -nanopore-raw  workspace/pass/barcode06/fastq_runid_*.fastq
```

Read correction using Canu v1.6

Chimeric read filtering

Step 3.

The next step I carry out is a basic read-level QC to exclude chimeric reads. Porechop can be used for this, although that removes adapters by default, which is not particularly useful in this case.

I use LAST to search for adapter sequences within the corrected reads, pass it through my conversion script, and extract out duplicated mappings (i.e. where the same read/adapter pair appears more than once in the mapping results):

COMMAND

```
lastal -
P 10 ONT_barcodes_adapters.fa <(zcat 4T1_BC06/4T1_BC06.correctedReads.fasta.gz) | ~/scripts
/maf_bcsplit.pl | awk -F',' '{print $1,$2}' | sort | uniq -
d | awk '{print $1}' | uniq > reads_with_duplicated_adapters.txt
```

Mapping adapter sequences with LAST

Chimeric read filtering

Step 4.

I use this file to filter out chimeric reads from the corrected dataset using another fastq filtering script I've created:

cmd COMMAND

```
~/scripts/fastx-fetch.pl -v -
i reads_with_duplicated_adapters.txt 4T1_BC06/4T1_BC06.correctedReads.fasta.gz |  gzip > 4T
1_BC06.correctedReads.uniqueOnly.fasta.gz
```

Removing reads with duplicated adapters

Adapter filtering

Step 5.

Along roughly similar lines to the chimeric read filtering, I then look for the strand switch and VNT adapters in the sequences. For a forward-orientated query, I expect the strand switch primer to be in the forward direction, and the VNP primer to be in the reverse direction.

Adapter filtering

Step 6.

Transcript-forward reads are identified. In this case a sequence is transcript-forward if the `ONT_SSP`primer is in the forward orientation, or the `ONT_VNP` primer is in the reverse orientation.

cmd COMMAND

```
lastal -
P 10 ONT_barcodes_adapters.fa <(zcat 4T1_BC06.correctedReads.uniqueOnly.fasta.gz) | ~/scrip
ts/maf_bcsplit.pl | grep -e 'ONT_SSP,+' -e 'ONT_VNP,-' |   awk -
F',' '{print $1}' | sort | uniq > fwdQry_seqs_BC06.txt
```

Identifying transcript-forward reads with LAST

Adapter filtering

Step 7.

Transcript-reverse reads are identified. In this case a sequence is transcript-reverse if the `ONT_SSP`primer is in the reverse orientation, or the `ONT_VNP` primer is in the forward orientation.

cmd COMMAND

```
lastal -
P 10 barcodes_primerSeqs.fa <(zcat 4T1_BC06.correctedReads.uniqueOnly.fasta.gz) | ~/scripts
/maf_bcsplit.pl | grep -e 'ONT_SSP,-' -e 'ONT_VNP,+' |  awk -
F',' '{print $1}' | sort | uniq > revQry_seqs_BC06.txt
```

Identifying transcript-reverse reads with LAST

Adapter filtering

Step 8.

The read name lists are used by my read filtering script to split the original reads up into transcript-forward and transcript-reverse subsets:

cmd COMMAND

```
~/scripts/fastx-fetch.pl -
i fwdQry_seqs_BC06.txt 4T1_BC06.correctedReads.uniqueOnly.fasta.gz |  gzip > fwd_4T1_BC06.c
orrectedReads.uniqueOnly.fasta.gz
```

```
~/scripts/fastx-fetch.pl -
i revQry_seqs_BC06.txt 4T1_BC06.correctedReads.uniqueOnly.fasta.gz | gzip > rev_4T1_BC06.co
rrectedReads.uniqueOnly.fasta.gz
```
Filtering reads into transcript-forward and transcript-reverse subsets

Read Mapping

Step 9.

Download minimap2 v2.10 or later

SOFTWARE PACKAGE (Linux)

minimap2, 2.10

Heng Li

https://github.com/lh3/minimap2

Read Mapping

Step 10.

Now that the reads have been oriented, the mapping can be done. I use `minimap2` for mapping long reads to a transcriptome.

COMMAND
```
~/install/minimap2/minimap2 -t 10 -a mmus_ucsc_all_cdna.idx -
x splice fwd_4T1_BC06.correctedReads.uniqueOnly.fasta.gz  > fwd_4T1_BC06.CU_vs_mmus.bam

~/install/minimap2/minimap2 -t 10 -a mmus_ucsc_all_cdna.idx -
x splice rev_4T1_BC06.correctedReads.uniqueOnly.fasta.gz  > rev_4T1_BC06.CU_vs_mmus.bam
```
Mapping reads to a transcriptome using minimap2

Splitting and recombining strands

Step 11.

Download samtools v1.8 or later

SOFTWARE PACKAGE (Linux)

SAMtools, 1.8

Wellcome Trust Sanger Institute

https://github.com/samtools/samtools

Splitting and recombining strands

Step 12.

Mapped BAM files are split based on how they are oriented to the *genome* using SAMtools. This uses the `-F` or `-f` options of `samtools view` to exclude or include reads (respectively) that are mapped to the genome in the forward or reverse orientation (respectively).

COMMAND
```
samtools view -F 0x10 -b fwd_4T1_BC06.CU_vs_mmus.bam > fwd_fwd_4T1_BC06.CU_vs_mmus.bam

samtools view -f 0x10 -b fwd_4T1_BC06.CU_vs_mmus.bam > rev_fwd_4T1_BC06.CU_vs_mmus.bam

samtools view -F 0x10 -b rev_4T1_BC06.CU_vs_mmus.bam > fwd_rev_4T1_BC06.CU_vs_mmus.bam

samtools view -f 0x10 -b rev_4T1_BC06.CU_vs_mmus.bam > rev_rev_4T1_BC06.CU_vs_mmus.bam
```
Splitting BAM files with samtools based on genome-relative orientation

Splitting and recombining strands

Step 13.

And finally the files are recombined to identify forward and reverse-encoded transcripts via `samtools merge`. I'm calling the "pos" strand the one that is encoded in the same direction as the genome, and the "neg" strand the reverse-complement direction:

cmd COMMAND

```
samtools merge pos_4T1_BC06.CU_vs_mmus.bam  rev_fwd_4T1_BC06.CU_vs_mmus.bam fwd_rev_4T1_BC06.CU_vs_mmus.bam

samtools merge neg_4T1_BC06.CU_vs_mmus.bam  fwd_fwd_4T1_BC06.CU_vs_mmus.bam rev_rev_4T1_BC06.CU_vs_mmus.bam
```

Merging forward and reverse-oriented reads with `samtools merge`

Splitting and recombining strands

Step 14.

NOTES

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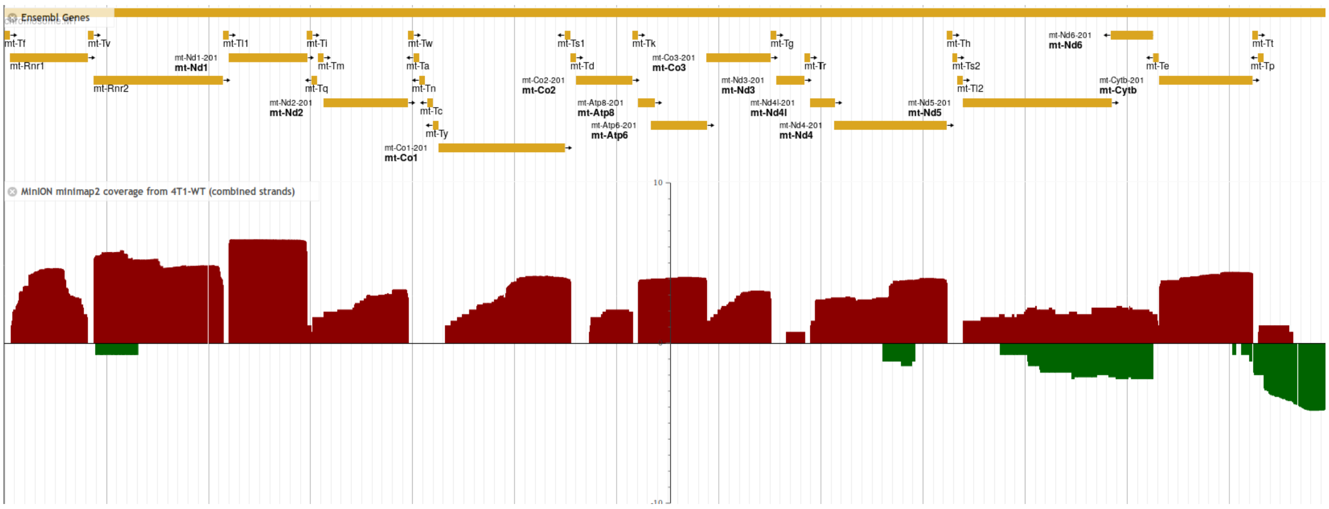
Alternatively, it's possible to set the BAM flags for read 1 and read 2 for the reverse and forward-encoded adapters respectively, which allows everything to then be combined into a single BAM file (and treated in the same way as a strand-specific Illumina BAM file).

Sanity Check

Step 15.

EXPECTED RESULTS

If this has worked properly, then mapping human or mouse to the mitochondrial genome should show most expression appearing on the positive strand, with a small scattering of negative-strand expression, a bit like this:



Warnings

These scripts have been slightly modified from scripts that I have run. Consider them demonstrative: pay attention to the words rather than the script.