Working with CYCLeR

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Contents

CYCLeR is a pipeline for reconstruction of circRNA transcripts from RNA-seq data and their subsequent quantification. The algorithm relies on comparison between control total RNA-seq samples and circRNA enriched samples to identify circRNA specific features. Then the selected circRNA features are used to infer the transcripts through a graph-based algorithm. Once the predicted transcript set is assembled, the transcript abundances are estimated through an EM algorithm with **kallisto** [1]. **CYCLeR** takes as an input BAM files and back-splice junction (BSJ) files and outputs transcript infomation in different formats and a transcript abundance file.

Installation of CYCLeR

Command line tools needed

```
**STAR** - https://github.com/alexdobin/STAR
**samtools** - https://sourceforge.net/projects/samtools/files/samtools/
**kallisto** - http://pachterlab.github.io/kallisto/download
#We also suggest RStudio to supplement the interactive experience
**RStudio** - https://rstudio.com/products/rstudio/download/
```

R packages installation

```
library(devtools)
install_github("stiv1n/CYCLeR")
```

Pre-processing the data

Mapping with STAR

The STAR [2] mapping parameters are up to a personal preference. It is imperative to include the **intronMotif** tag. My suggestion would be 2-pass mapping with these parameters:

```
#first pass
STAR --alignSJoverhangMin 5 --outSAMstrandField intronMotif --outFilterMismatchNmax 3
--outFilterMismatchNoverLmax 0.1
--chimSegmentMin 15 --chimScoreMin 1 --chimJunctionOverhangMin 15
--outFilterMultimapNmax 50 --alignIntronMax 100000 --alignIntronMin 15
#second pass
STAR --outSAMstrandField intronMotif --outFilterMismatchNmax 7
--outFilterMismatchNoverLmax 0.3
--alignSJoverhangMin 15 --alignSJDBoverhangMin 3
--chimSegmentMin 15 --chimScoreMin 1 --chimJunctionOverhangMin 15
--outFilterMultimapNmax 50 --alignIntronMax 100000 --alignIntronMin 15
#converting to sorted BAM
samtools view -u -h Aligned.out.sam | samtools sort <name>_sorted.bam
```

Processing the BAM info in R

We need the information for read length, fragment length and library sizes from the BAM files.

```
#load BAM files
bam_file_prefix<-system.file("extdata", package = "CYCLeR")
filenames<-c("sample1_75","sample2_75","sample3_75","sample4_75")
BSJ_files_ciri<-paste0(bam_file_prefix,"/",filenames)
bam_files<-paste0(bam_file_prefix,"/",filenames,".bam")
#mark the samples control and enriched or bare the consequences
sample_table<-data.frame(filenames,c("control","control","enriched","enriched")
                         ,bam_files,stringsAsFactors = F)
colnames(sample_table)<-c("sample_name","treatment","file_bam")
si<- DataFrame(sample_table[,c("sample_name","file_bam")])
si$file_bam <-BamFileList(si$file_bam, asMates = F)
#this holds all the needed info of the bam files for downstream processing
sc <- getBamInfo(si)
sample_table$lib_size<-sc@listData$lib_size
sample_table$read_len<-sc@listData$read_length
```

Use the provided sample table template.

```
##   sample_name treatment
## 1  sample1_75   control
## 2  sample2_75   control
## 3  sample3_75  enriched
## 4  sample4_75  enriched
##                                                                        file_bam
## 1 /home/stefan/miniconda3/envs/cycler/lib/R/library/CYCLeR/extdata/sample1_75.bam
## 2 /home/stefan/miniconda3/envs/cycler/lib/R/library/CYCLeR/extdata/sample2_75.bam
## 3 /home/stefan/miniconda3/envs/cycler/lib/R/library/CYCLeR/extdata/sample3_75.bam
## 4 /home/stefan/miniconda3/envs/cycler/lib/R/library/CYCLeR/extdata/sample4_75.bam
##   lib_size read_len
## 1    13884       75
## 2    13959       75
## 3     8494       75
```

```
## 4     8637       75
```

Selecting a BSJ set

Selecting a BSJ set is very important, because the algorithm assumes that the provided set of BSJ is *correct*. I suggest BSJ identification with **CIRI2** [3] and **CIRCexplorer2** [4], but the choice is up to a personal preference. I have provided some useful functions for parsing the output from BSJ identification software.

```
#load the BSJ files
BSJ_files_prefix<-paste0(system.file("extdata", package = "CYCLeR"),"/ciri_")
ciri_table<-parse_files(sample_table$sample_name,BSJ_files_prefix,"CIRI")
colnames(ciri_table)<-c("circ_id", "sample1_75","sample2_75","sample3_75","sample4_75")
ciri_bsjs<-process_BSJs(ciri_table,sample_table)
# i would suggest combine the output of pipelines using different mapping tools
BSJ_files_prefix_CE<-paste0(system.file("extdata", package = "CYCLeR"),"/CE_")
ce_table<-parse_files(sample_table$sample_name,BSJ_files_prefix_CE,"CE")
colnames(ce_table)<-c("circ_id", "sample1_75","sample2_75","sample3_75","sample4_75")
ce_bsjs<-process_BSJs(ce_table,sample_table)
#we need to unify the results from the BSJ identification and counting
table_circ<-combine_two_BSJ_tables(ce_bsjs,ciri_bsjs)
#further downstream we need just the mean values for enriched samples
table_circ<-table_circ[,c("chr","start","end","meanRR")]
colnames(table_circ)<-c("chr","start","end","count")
#combine
BSJ_set<-union(ciri_bsjs$circ_id,ce_bsjs$circ_id)
BSJ_set<-BSJ_set[!grepl("caffold",BSJ_set)]
#just in case
BSJ_set<-BSJ_set[!grepl("mitochondrion",BSJ_set)]
#####################################################################
#converting the BSJ set into a GRanges object
BSJ_gr<-make_BSJ_gr(BSJ_set)
```

The parse.files can work with **CIRI2**, **CIRCexplorer2** or **TSV** file. Naturally a person may have different criterion for *correct* BSJs based on different criteria. It is not an issue as long as the data is presented in the following template:

```
head(table_circ)
```

```
## # A tibble: 6 x 4
##   chr   start    end        count
##   <chr> <chr>    <chr>      <dbl>
## 1 3L    24725824 24726292 435115.
## 2 3L    24725824 24728508   9150.
## 3 3L    24728297 24734187 171930.
## 4 3L    24728297 24741000   7992.
## 5 3R    4622509  4628349   64579.
## 6 3R    4626973  4628349  160551.
```

Transcript assembly

BSJ loci extraction

Prior to the feature detection the files need to be trimmed to speed up the process. Afterwards the transcript features (e.g. exons, junctions) are identified with **SGSeq** [5]. The files are convereted with **samtools** [6].

```
#################################################
samtools_prefix<-""
```

```
trimmed_bams<-filter_bam(BSJ_gr,sample_table,samtools_prefix)
sc@listData[["file_bam"]]<-trimmed_bams
```

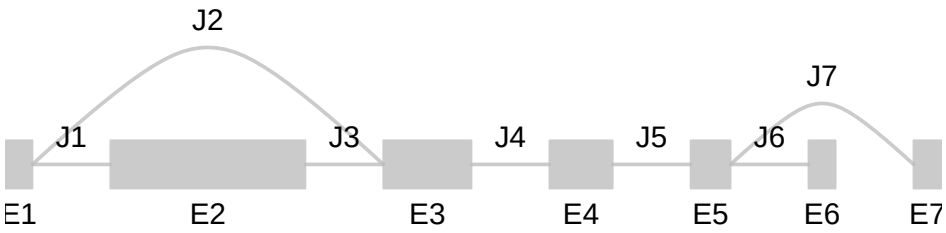
Feature identification with SGSeq

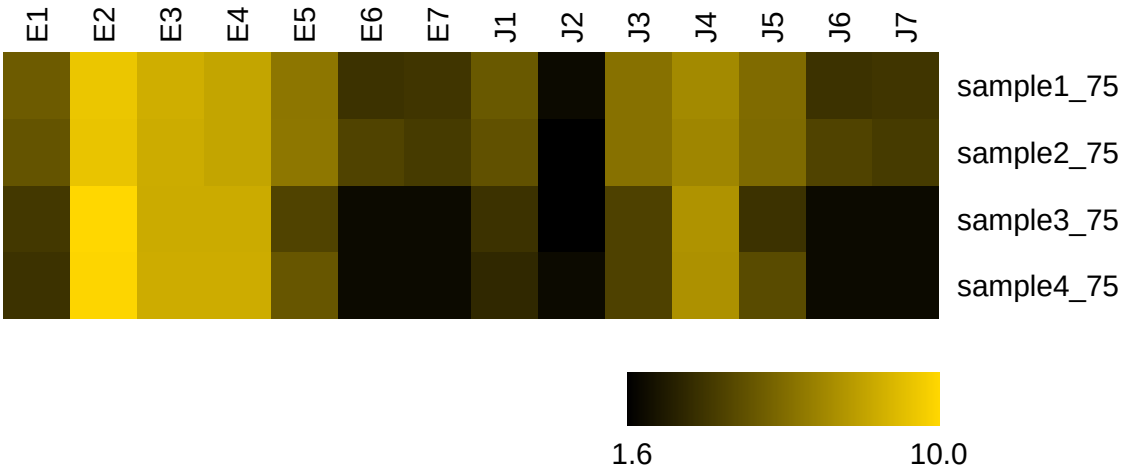
The use of the TxDb package is to annotate the identified features. The annotation step is not mandatory, but it does provide useful information. The feature detection is based on the SGSeq package.

```
#######################################################
#get the gene/transcript info
library("TxDb.Dmelanogaster.UCSC.dm6.ensGene")
#restoreSeqlevels(txdb)
txdb <- TxDb.Dmelanogaster.UCSC.dm6.ensGene
txdb <- keepSeqlevels(txdb, c("chr2L","chr2R","chr3R","chr3L","chr4","chrX","chrY"))
seqlevelsStyle(txdb) <- "Ensembl"
gene_ranges <- genes(txdb)
txf <- convertToTxFeatures(txdb)
#asnnotation as sg-object
sgf <- convertToSGFeatures(txf)
sgfc_pred <- analyzeFeatures(sc, min_junction_count=2, beta =0.1 ,
                             min_n_sample=1,cores=1,verbose=F)
sgfc_pred <- SGSeq::annotate(sgfc_pred, txf)
```

SGSeq feature plotting function can be used for visual representation of the control VS enriched difference

```
plotFeatures(sgfc_pred,  geneID = "1",assay = "counts",color_novel = "red",
             include = "both",tx_view=F,Rowv=NA, square=T)
```


J2
J7
J1
J3
J4
J5
J6
E1
E2
E3
E4
E5
E6
E7


E1
E2
E3
E4
E5
E6
E7
J1
J2
J3
J4
J5
J6
J7
sample1_75
sample2_75
sample3_75
sample4_75
1.6
10.0

Re-couting and Processing the features

I prefer the **RSubread** [7] counting method, thus I re-count the identified exon features. I use the **SGseq** counted junctions. The features that are depleted in circRNA enriched samples need to be removed. **CYCLeR** provides 2 approaches for identifying depleted features: DEU strategy and simple comparison of normalized coverage values.

```
#extract BSJ-corrected splice graphs (sg)
full_sg<-overlap_SG_BSJ(sgfc_pred,BSJ_gr,sgf) #includes linear and circular features
# we have made new feature set so we need to recount the exons
full_fc<-recount_features(full_sg,sample_table)#fc==feature counts
# time to prepare the circular splice graph
```

```
#get the correct genome for sequence info
#requires the appropriate BSgenome library
library(BSgenome.Dmelanogaster.UCSC.dm6)
bs_genome=Dmelanogaster
circ_sgfc<-prep_circular_sg(full_sg, full_fc,sgfc_pred, bs_genome, BSJ_gr, th=15)
```

The circRNA exon features are stored in SummarizedExperiment format

Transcript prediction

Transcript prediction is processed one samples at a time. The transcript sets from different samples are then merged.

```
qics_out1<-transcripts_per_sample(sgfc=circ_sgfc,BSJ_gr = BSJ_gr,"sample3_75")
qics_out2<-transcripts_per_sample(sgfc=circ_sgfc,BSJ_gr = BSJ_gr,"sample4_75")
qics_out_final<-merge_qics(qics_out1,qics_out2,sgfc_pred)
```

Output and Quantification

CYCLeR transcript output

CYCLeR provides 3 forms of output of the annotated transcript: a comprehensive flat file, a GTF-like file, and FASTA file.

```
gtf.table<-prep_output_gtf(qics_out_final,circ_sgfc)
write.table(qics_out_final[,-9],file = "dm_circles.txt", sep = "\t",
            row.names = F, col.names = T,quote=F)
qics_out_fa<-DNAStringSet(qics_out_final$seq)
names(qics_out_fa)<-qics_out_final$circID

#prepping the circRNA sequences for quantification
extended_seq<-paste0(qics_out_final$seq,substr(qics_out_final$seq,1,30),
      strrep("N",mean(sc@listData$frag_length[sample_table$treatment=="enriched"])))
qics_out_fa_extended<-DNAStringSet(extended_seq)
names(qics_out_fa_extended)<-qics_out_final$circID
writeXStringSet(qics_out_fa_extended,'circles_seq_extended_padded.fa')
```

If you have a known set of circRNA in FASTA format the CYCLeR output can be combined with it.

```
fasta_circ<-readDNAStringSet("...")
final_ref_fa<-merge_fasta(qics_out_fa,fasta_circ)
writeXStringSet(final_ref_fa,'...')
```

The same function can be used for merging with known linear annotation for the quantification step.

```
fasta_lin<-readDNAStringSet("...")
final_ref_fa<-merge_fasta(qics_out_fa_extended,fasta_lin)
writeXStringSet(final_ref_fa,'...')
```

CYCLeR quantification

The final transcript abundance estimation is performed with **kallisto**. An extended and padded circRNA reference sequences are build and combined with linear RNA sequences *kallisto index* is created to be used for any desired sample quantification.

```
#alternative way of merging linear and circular sequences
cat linear_transcripts.fa circles_seq_extended_padded.fa > for_kallisto.fa
#Kallisto comands
kallisto index -i kallisto_index -k 31 for_kallisto.fa
kallisto quant -i kallisto_index -o ./ sample_1.fastq sample_2.fastq
```

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