scisorseqr: Standard Workflow

The *scisorseqr* package allows you to go from short-read, single cell assignments and a set of fastq files to a full-blown cell-type specific analysis of alternative splicing patterns.

Setup

Software required

- STARlong (and corresponding genome index file)
- Minimap2 if not using STARlong
- samtools
- bedtools
- python version >= 3.7 with the following libraries
  - pandas
  - multiprocessing

Quick cheat for viewing documentation

All the possible arguments, usage, and description for each function can be viewed interactively by putting a "?" in front of the function name in the R console like so:

```
?GetBarcodes
```

Step 1: Barcode deconvolution

The first step is to deconvolve barcodes from either the PacBio or ONT fastq output For that the user needs to specify

- Tab separated Barcode-Celltype file
- Directory containing fastq.gz files

Create a barcode - celltype file

Assuming that short read single-cell analysis has been performed using Seurat:

```
library(Seurat)
library(tidyr)
library(dplyr)

bc <- data.frame("Barcode"=rownames(my_object@meta.data),
                 "Celltype"=my_object@meta.data$celltype) %>%
  separate(Barcode,into=c("BC","suffix"),sep="-") %>%
```

```
  select(-suffix) %>% as.data.frame()

write.table(bc, file = "PATH_TO_FILE", sep="\t", row.names = FALSE,
            col.names = FALSE, quote = FALSE)
```

The table should look something like this

V1	V2
AAAGTAGGTCAGCTAT	PFC_Vasc
CGGACGTGTCCGCTGA	Hipp_Oligo
GCGACCACAGCGATCC	Hipp_ExciteNeuron
GTCTCGTTCGGACAAG	PFC_Oligo
TTGCCGTGTACAGCAG	Hipp_NIPCs
GTGGGTCAGTCAATAG	Hipp_InhibNeuron
AGGTCCGGTATTCGTG	Hipp_Oligo
GCCTCTAAGGAGTTGC	Hipp_ChorPlex

Use that file to locate barcodes from the fastq files

Example files included in the package and loaded as follows:

```
bc_clust <- system.file("extdata/", "userInput/bc_celltype_assignments",
                        package = "scisorseqr")
fastqFolder <- system.file("extdata/", "userInput/FastqFiles/",
                           package = "scisorseqr")

## run command
GetBarcodes(fqFolder = fastqFolder, BCClustAssignFile = bc_clust,
            chemistry = "v2", concatenate = TRUE, filterReads = FALSE)
```

You will see two output directories

- OutputRaw
  - file.CSV (One line per read)
  - file_summary (Stats for run)
- OutputFiltered
  - file.CSV (One line per barcoded read with the following structure:)

ReadName	T9_Status	Strand	T9_pos	Barcodes	BC_pos	Cluster	UMI	TSO_Status	TSO_pos	Length
ReadX	poly_T_found	rev	17	GTCGGGTTCCAAAAGT	22	Hipp_Granule	ATGGCGGGTA	TSO_found	26	913
ReadY	poly_T_found	fwd	18	AGTGCCGATAGTTATG	22	Hipp_Astro	GGGATATGTC	TSO_found	26	1312

NOTE 1 If you want to limit the downstream analysis to barcoded reads only, set the 'filterReads' parameter to TRUE. This will output a fastq.gz file in the OutputFiltered directory.

NOTE 2 Setting 'concatenate' to FALSE ensures that your input files are treated as separate samples.

Step 2: Align your fastq files to the reference

We have made two aligners available and compatible with our package

- STARlong
- Minimap2

```
## Load example data
fastqFolder <- system.file("extdata/", "userInput/FastqFiles/",
                           package = "scisorseqr")

STARalign(fqFolder = fastqFolder, starProgPath = '~/bin/Linux_x86_64/STARlong',
          refGenomeIndex = '~/starIndex_gencode10/',
          numThreads = 24)

MMalign(fqFolder = fastqFolder, mmProgPath = '~/minimap2/minimap2',
        refGenome='~/genomes/mm10.fa',
        numThreads = 16)
```

This process will convert all the aligned sam files to bam files, and dump the output along with run reports into *STARoutput* or *MMoutput*

Step 3: Map and filter for full-length, spliced reads

We used annotated CAGE peaks and PolyA sites for our study

```
MapAndFilter(annoGZ='[PATH_TO_annotation.gtf.gz]',numThreads=24,
             seqDir = '[PATH_TO_GENOME_DIR/chr*.fa.gz]',
             filterFullLength=TRUE,
             cageBed = '[PATH_TO_Cage.bed.gz]',
             polyABed = '[PATH_TO_PolyA.bed.gz]',
             cp_distance=50, genomeVersion="mm10")
```

NOTE 1: the parameter seqDir takes in a reference genome broken down by chromosome so as to parallelize the process. This function has not yet been made generalizable to loading in the full reference

NOTE 2: The genome and version is not auto-detected and hence defaults to 'mm10'. It does not affect any processing but might lead to confusion if processing a different species.

This will result in multiple files being stored in yet another output directory: *LRProcessingOutput*. A lot of these files are not super relevant and there is an option to remove them during Step 4.

Step 4: Concatenate all the information collected so far

```
InfoPerLongRead(barcodeOutputFile =
                  'OutputFiltered/FilteredDeconvBC_AllFiles.csv',
                mapAndFilterOut = 'LRProcessingOutput/',
                minTimesIsoObserve = 5, rmTmpFolder = TRUE)
```

This will yield a directory *LongReadInfo*. Along with some files containing basic statistics on a per-barcode basis, this folder contains two main files:

- *AllInfo.gz*: Concatenated information for all poly-exonic, spliced, barcoded, mapped, and 5' **and** 3' complete reads
- *AllInfo_Incomplete.gz*: Same as above, except with a relaxed criterion for the 5' and 3' completeness

If annotated CAGE and PolyA peaks are not provided, then only an AllInfo file is generated, and the reads obviously do not contain information on assigned peaks

The *AllInfo.gz* file has the following structure, where Intron Chain contains the assigned TSS and PolyA site:

ReadName	Gene	Celltype	Barcode	UMI	IntronChain	ExonChain	Status	# Introns

The *AllInfo_Incomplete.gz* file has the following structure, where the intron chain purely contains a string of introns. If a CAGE peak or PolyA site are not assigned, then the status is indicated as "NoTSS" or "NoPolyA" in the 7th and 8th column respectively

ReadName	Gene	Celltype	Barcode	UMI	IntronChain	TSS	PolyA	ExonChain	Status	# Introns

NOTE: In case you want to retain all the temporary files and / or re-run this step, set the argument 'rmTmpFolder' to FALSE.

Step 5a: Quantify the various isoforms observed in your data

If you provided CAGE and PolyA peaks at the MapAndFilter() stage, you can choose to quantify unique cage and polyA sites per gene. Otherwise the default option is False

```
IsoQuant(AllInfoFile = 'LongReadInfo/AllInfo',
         Iso = TRUE, TSS = TRUE, PolyA = TRUE)
```

Depending on which modality you chose as *TRUE*, you will see the following files in the *IsoQuantOutput* folder:

- *Modality*-*Modality*ID.csv
- Num*Modality*PerCluster

These will be used for the differential splicing analysis step

Step 5b: [OPTIONAL] Quantify the various exons observed in your data

For ONT reads we don't have too much confidence in this method and recommend using our sister package IsoQuant.

This function however, works for annotated as well as novel exons

```
ExonQuant(AllInfoFile = 'LongReadInfo/AllInfo',
         groupingFactor = "Celltype", threshold = 10)
```

This will output the quantifications to a separate folder *ExonQuantOutput*

Step 6: Differential isoform analysis

This step can be done using various settings for various modalities. Options include

- Full-length isoform (Iso)
- TSS
- PolyA
- Exon (Refer to step 6b)

Depending on the modality chosen, this function automatically detects the input

It also requires a tab-separated config file in the following format to be provided by the user

NOTE: Header here just for demonstration

Comparison1	Celltypes	Comparison2	Celltypes
HippNeuron	Hipp_ExciteNeuron,Hipp_InhibNeuron	PFCNeuron	PFC_ExciteNeuron,PFC_InhibNeuron
HippInhib	Hipp_InhibNeuron	PFCInhib	PFC_InhibNeuron
HippExcite	Hipp_ExciteNeuron	HippInhib	Hipp_InhibNeuron

```
config <- system.file("extdata/", "userInput/config",
                      package = "scisorseqr")

DiffSplicingAnalysis(configFile = config,
                     typeOfTest = 'Iso',
                     minNumReads = 25, is.hier = FALSE)

## or for exons
DiffSplicingAnalysis(configFile = config,
                     typeOfTest = 'Exon',
                     minNumReads = 25,
                     is.hier = FALSE)
```

Assuming you run this command, each comparison (line in the config file) yields a separate sub-directory in the folder *TreeTraveral_Iso*

The most important file in that output directory is the results file, which has the following structure:

Gene	pvals	dPI	maxDeltaPI_ix1	maxDeltaPI_ix2	FDR
ENSMUSG00000000001.4	0.563702861650773	0	0	NA	1
ENSMUSG00000000078.7	1	0	0	NA	1
ENSMUSG00000000088.7	1	0.0714285714285714	1	NA	1
ENSMUSG00000000326.13	0.343030146138244	0.666666666666667	1	3	1

NOTE Deeper data will yield significant values. These ^ are just a product of the example data subset we have provided with the package

You can use the above output to plot your significant genes

Step 7: Visualizations

Triangular Heatmap

This function plots a triangular heatmap of percentage DIE genes of pairwise comparisons in a directory It also takes in an untitled file list of cell types to be plotted as follows:

Hipp_Astro
Hipp_ChorPlex
Hipp_DivOligo
Hipp_Ependymal
.
.

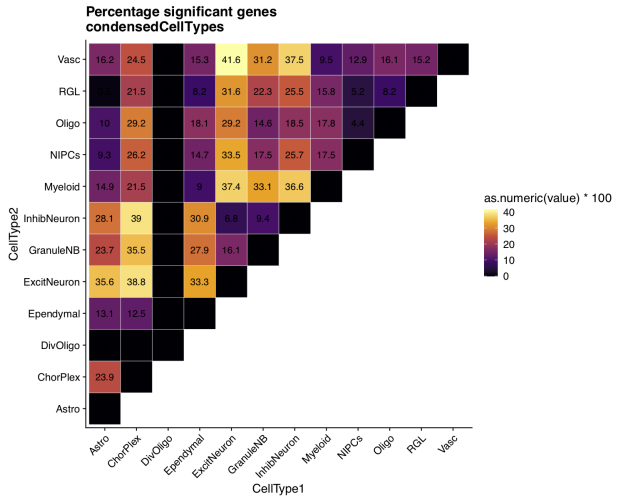
```
condensedCellTypes = system.file("extdata/", "userInput/condensedCellTypes",
                      package = "scisorseqr")

triHeatmap(treeDir = 'TreeTraversal_Iso/',
           comparisonList = condensedCellTypes,
           typeOfTest = "Iso",
           outName = condensedCellTypes)
```

This was not quite designed for visualizing differential exon analysis output and thus may give some errors. Please do not hesitate to reach out or open an issue if there is difficulty with this stuff.

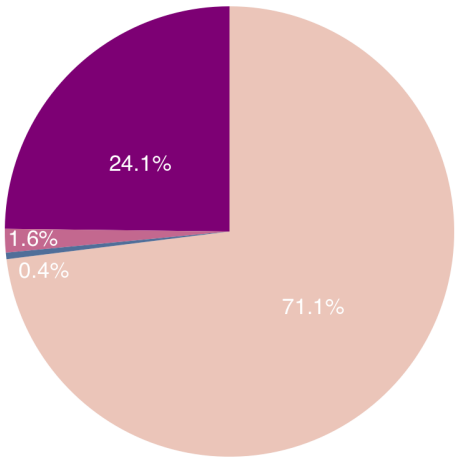
NOTE 1: Cell-type labels should correspond to the barcode-celltype list i.e. the way they appear in the AllInfo files. If you input a cell-type for which pairwise DIE has not been calculated, it will output all zeros (e.g. DivOligo in this case)

NOTE 2: The example data included in this package does not yield the plot below. This is just an example plot generated from the full dataset.



Pie Chart breaking down the significant genes

```
sigSplitPie(compDir = 'Uniq_TreeTraversal_Iso/Astro_ExcitNeuron_10/')
```



Each number in the legend corresponds to the number of isoforms needed to cross the deltaPI = 0.1 threshold
NOTE 1: Percentage labels do not always arrange themselves neatly in the figure (requires editing by hand)

NOTE 2: The example data included in this package does not yield the plot above. This is just an example plot generated from the full dataset.

Done!