scisorseqr: Standard Workflow

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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.

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

Step1: 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 analysis 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
GCCTCTACAACAACCT	Hipp_Astro
GAGTCCGAGTTATCGC	Hipp_InhibNeuron
TAGTTGGGTAAGAGAG	PFC_Astro
TGACAACAGAAGGTTT	PFC_ExciteNeuron
TACTCATCAGCCTTGG	PFC_Oligo
CACAGTACACGAAAGC	Hipp_Astro
ATTGGTGAGGTGCTAG	Hipp_ExciteNeuron
CACCTTGTCAGCATGT	Hipp_NIPCs

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_position	Barcodes
ReadX	poly_T_found	rev	47	GTCGGGTTCCAAAGTC

Barcode_position	Cluster	UMI	TSO_Status	TSO_position	Length
22	Hipp_GranuleNB	ATGGCGGGAT	TSO_found	26	583

Step2: 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_sequins/',
          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 into *STARoutput*

Step3: Map and filter for full-length, spliced reads

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

NOTE 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

This will result in multiple files being stored in yet another output directory: *LRProcessingOutput*

Step4: Concatenate all the information collected so far

```
InfoPerLongRead(barcodeOutputFile =
                  'OutputFiltered/FilteredDeconvBC_AllFiles.csv',
                mapAndFilterOut = 'LRProcessingOutput/',
                minTimesIsoObserve = 3)
```

This will yield a directory *LongReadInfo* with file **AllInfo** which has the following structure

ReadName	Gene	Celltype	Barcode	UMI	IntronChain	Status	# Introns

Step5a: 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

Step5b: [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*

Step6: 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 5b)

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

Step7: Visualizations

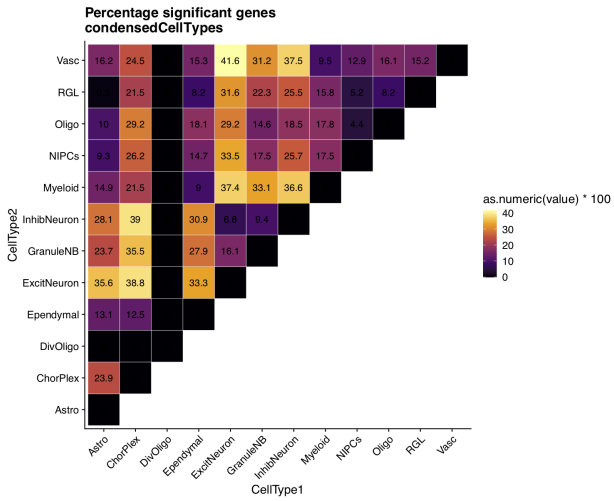
Triangular Heatmap

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
.
.

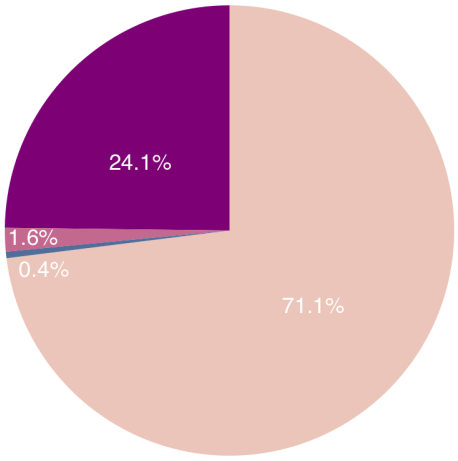
```
condensedCellTypes = system.file("extdata/", "userInput/condensedCellTypes",
                      package = "scisorseqr")

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

NOTE Cell-type labels should correspond to the barcode-celltype list. 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)



Pie Chart breaking down the significant genes

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



NOTE Percentage labels do not always arrange themselves neatly in the figure (requires editing by hand)

Done!