

scisorseqr-StandardWorkflow

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

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 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 |
|------------------|-----------|
| TGGACGCAGCACCGTC | PFC_Astro |
| GGGACCTCATGTTGAC | PFC_Astro |

| V1 | V2 |
|------------------|------------------|
| GTCACGGTCTGTCTCG | Hipp_NIPCs |
| GGAAAGCGTGCACCAC | PFC_InhibNeuron |
| TATCTCACATGTCTCC | Hipp_Oligo |
| GCATGTACAAGCTGTT | PFC_InhibNeuron |
| GTAGTCAGTTCTGAAC | PFC_ExciteNeuron |
| TGCACCTAGTGGAGTC | Hipp_Astro |

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 |

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_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*

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_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*

Step 4: 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 |
|----------|------|----------|---------|-----|-------------|--------|-----------|
|----------|------|----------|---------|-----|-------------|--------|-----------|

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-ModalityID.csv*
- *NumModalityPerCluster*

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

| Comparison | Celltypes | Comparison | 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 *TreeTraversal_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 |
|------------------------|-------------------|--------------------|----------------|----------------|-----|
| ENSMUSG000000000001.4 | 0.563702861650773 | 0 | 0 | NA | 1 |
| ENSMUSG000000000078.7 | 1 | 0 | 0 | NA | 1 |
| ENSMUSG000000000088.7 | 1 | 0.0714285714285714 | 1 | NA | 1 |
| ENSMUSG000000000326.13 | 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

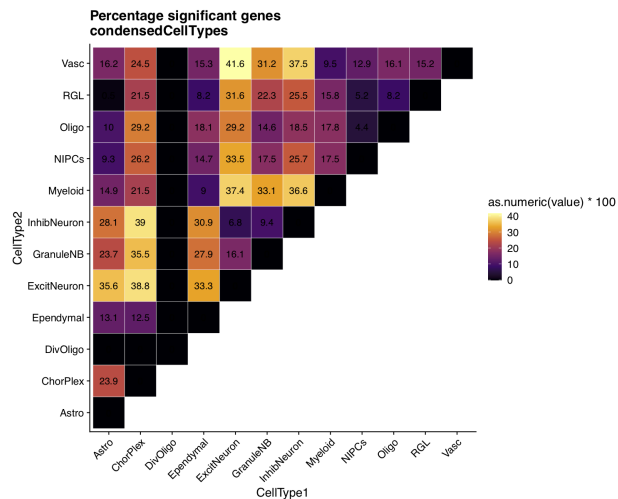
This function plots a triangular heatmap of percentage DIE genes of pairwise comparisons in a directory It also takes in an untitled file list of cell types to be plotted as follows:

```
Hipp_Astro
Hipp_ChorPlex
Hipp_DivOligo
Hipp_Ependymal
.
.
```

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

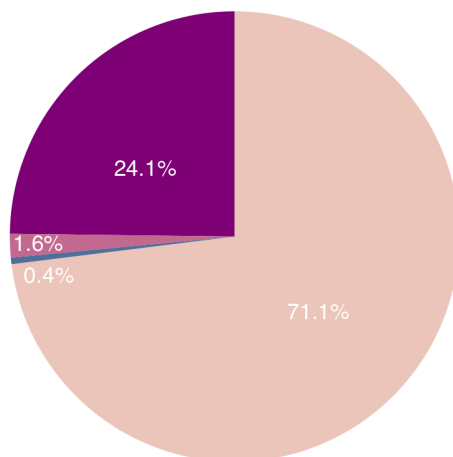
triHeatmap(treeDir = 'TreeTraversal_Iso/',
            comparisonList = condensedCellTypes,
            outName = condensedCellTypes)
```

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) **NOTE** 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 $\Delta\text{PI} = 0.1$ threshold

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

NOTE 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!