rCube - RNA-Rates in R

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Abstract

rCube provides a framework for the estimation of RNA metabolism Rates in R (R^3). The rCube package complements the recently published transient transcriptome sequencing (TT-seq) protocol. It has been shown, that 4sU-labeling and subsequent purification of RNA allows to monitor local RNA synthesis. Therefore, the information from TT-seq/4sU-seq and total RNA-seq samples is used to model RNA synthesis, splicing, and degradation rates based on first-order kinetics. The rCube package works on count data and provides a series of functionalities to extract them from the desired features. It allows to extract junctions and constitutive exons from feature annotations, count reads from BAM-files, and normalize different samples against each other using a variety of different methods.

rCube version: 1.1.0

If you use rCube in published research, please cite:

B. Schwalb, M. Michel, B. Zacher, K. Frühauf, C. Demel, A. Tresch, J. Gageur, and P. Cramer:
TT-seq maps the human transient transcriptome.
Science (2016). doi:10.1126/science.aad9841 [1]

Contents

1 Background

As described in .... 4sU-seq allows to monitor changes in the RNA metabolism. If cells are exposed to 4sU, they rapidly take up this Uridine analog and incorporate it into newly-synthesized RNAs. This way, newly-synthesized RNAs are labeled and can be extracted from the total RNA in the sample. The longer the labeling time, e.i. the time from 4sU addition to harvesting the cells, the bigger is the proportion of labeled RNAs among all RNAs.

explain the why we have spike ins (diagram?) Artificial RNA spike-in sequences can be used to adjust for global sequencing variations between samples. One source of variation is the sequencing depth. Even replicates from the same experimental condition may exhibit different read counts based on how deep the samples were sequenced. These variations (up to biological variations) can be overcome by normalization to sequencing depth. In a typical RNA-seq experiment, one wants to compare different samples. After extracting the RNA from the cells, the same starting material is used for the library prepartion, therefore the information is lost, if cells from different samples were expressing different amounts of RNA. Adding the same volumes of spike-ins to a defined number of cells can help to resolve this problem. In our case, we additionally want to rescale 4sU-labeled and total RNA-seq samples, so that the ratio of labeled RNA to total RNA read counts reflects the ratio of labeled RNA to total RNA amounts in the cell.

explain time series vs only label total

ref to TTseq [1]

ref to MSB In another study, we [2]

define synthesis, splicing, decay rate

define the read classifications: junction reads E-E, E-I, I-E junction

2 Getting started

This vignette provides a pipeline how to... starting from BAM files... You will learn how to estimate sample specific sequencing depths and cross-contamination rates from spike-in counts. These values can be used to normalize gene expression values obtained by RNA-Seq and thus estimate gene-specific synthesis and degradation rates. By extracting reads spanning junctions, splicing times can be estimated. For more robust estimation, multiple samples with different labeling times are taken into account. Before starting, the package must be loaded by:

```
library("rCube")
```

2.1 Example Data

The `inst/extdata` of the *rCube* package provides two example data sets that should illustrate the two different functionalities of rCube:

The first example data set, "Jurkat", contains bam files from resting and activated Jurkat T-cells for TT-seq and RNA-seq samples. The bamfiles are restricted to the FOS gene (chr14:75278000-75283000) and the artifical spike-ins, subsampled to reduce file size. The full data sets are published in [2]. This example data is used to demonstrate the spike-ins normalization method, and the estimation of synthesis and degradation rates for individual 4sU-labeled (TT-seq) and total RNA-seq pairs.

The second data set , ...

3 Conditional synthesis and degradation rates for Jurkat data

Example data sets from a T-cell activation experiment are stored in the `inst/extdata` of the *rCube* package. In this part of the vignette, we will demonstrate

- how reads can be counted for (constitutive) exons and spike-ins,
- how the samples are normalized against each other based on the spike-in read counts,
- how synthesis and degradation rates are obtained for (constitutive) exons
- how gene-specific rates are obtained from exon-specific rates

3.1 Gene model

working on exons/introns/genes... The estimation of synthesis and degradation rates with the *rCube* package relies on read counts. Dependent on the features, for which read counts are provided, the rates can reflect synthesis rates of exons, introns, or full genes. Especially degradation rates may differ between exons and introns. Therefore, the features, which should be used to estimate synthesis and degradation rates, and for which read counts are provided or should be obtained, need to be provided as a *GRanges* object.

Due to numerous transcript isoforms per gene, and the arising problem that for some bases their exonic or intronic nature cannot be unambigously identified, we propose to use the model of constitutive exons/introns from [3]. Hereby, all bases, that belong to an exon/intron in all (annotated) transcript isoforms of the same gene, are thought to be part of "constitutive" exons/introns. In the following, we have an example annotation from the FOS gene (not comprehensive) to illustrate how constitutive exons can be extracted from an exon annotation.

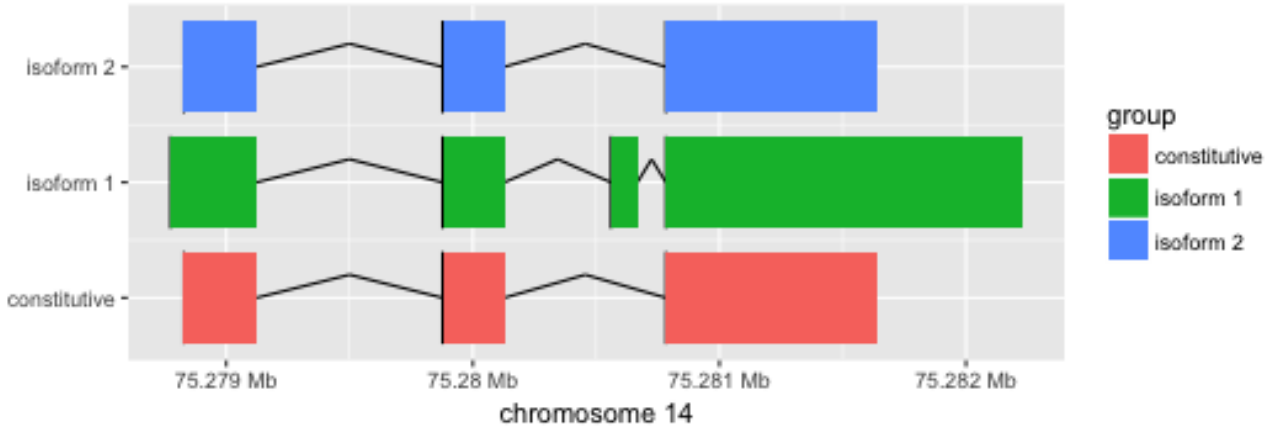
```
data("exampleExons")
exampleExons
```

```
## GRanges object with 7 ranges and 3 metadata columns:
##       seqnames              ranges strand |     type           gene_id
##          <Rle>           <IRanges>  <Rle> | <factor>       <character>
```

```
##   [1]    chr14 [75278774, 75279123]      + |     exon ENSG00000170345.9
##   [2]    chr14 [75278828, 75279123]      + |     exon ENSG00000170345.9
##   [3]    chr14 [75279877, 75280128]      + |     exon ENSG00000170345.9
##   [4]    chr14 [75279877, 75280128]      + |     exon ENSG00000170345.9
##   [5]    chr14 [75280560, 75280667]      + |     exon ENSG00000170345.9
##   [6]    chr14 [75280783, 75282230]      + |     exon ENSG00000170345.9
##   [7]    chr14 [75280783, 75281636]      + |     exon ENSG00000170345.9
##           transcript_id
##             <character>
##   [1] ENST00000303562.8
##   [2] ENST00000535987.5
##   [3] ENST00000303562.8
##   [4] ENST00000535987.5
##   [5] ENST00000303562.8
##   [6] ENST00000303562.8
##   [7] ENST00000535987.5
##   -------
##   seqinfo: 1 sequence from an unspecified genome; no seqlengths
```

```
constitutiveExons <- createConstitutiveFeaturesGRangesFromGRanges(exampleExons,
                                                                  BPPARAM=NULL,
                                                                  ncores=1)
constitutiveExons
```

```
## GRanges object with 3 ranges and 2 metadata columns:
##            seqnames              ranges strand |                 type
##               <Rle>           <IRanges>  <Rle> |             <factor>
##   CF00001    chr14 [75278828, 75279123]      + | constitutive feature
##   CF00002    chr14 [75279877, 75280128]      + | constitutive feature
##   CF00003    chr14 [75280783, 75281636]      + | constitutive feature
##                    gene_id
##                <character>
##   CF00001 ENSG00000170345.9
##   CF00002 ENSG00000170345.9
##   CF00003 ENSG00000170345.9
##   -------
##   seqinfo: 1 sequence from an unspecified genome; no seqlengths
```



Please note, for the subsequent workflow it is not necessary to extract constitutive exons. Any kind of *GRanges* object can be used as feature annotation (e.g. full genes, introns, ...).

3.2 Experimental Design

The rCube package works on *rCubeExperiment* containers, that rely on the *SummerizedExperiment* class. Objects of this class are used as input for the whole workflow, starting from read counting, normalization, dispersion estimation, to rate estimation. Most of these steps return an updated and extended *rCubeExperiment* object.

The `rowRanges` of the *rCubeExperiment* is a *GRanges* annotation of features, for which RNA rates should be estimated. Experimental sample information can be either provided by a experimental design matrix or this information can be extracted from the BAM-file names (when they fulfil the required structure).

We first look at the experimental design file `experimentalDesign.txt`, that can be imported as a *data.frame*.

```
folder <- system.file("extdata/Jurkat", package='rCube')
folder
```

```
## [1] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat"
```

```
expDesign <- read.delim(file.path(folder, "experimentalDesign.txt"))
expDesign
```

```
##            sample condition LT labelingTime replicate            filename
## 1   Resting_L_5_1   Resting  L            5         1   Resting_L_5_1.bam
## 2   Resting_L_5_2   Resting  L            5         2   Resting_L_5_2.bam
## 3   Resting_T_5_1   Resting  T            5         1   Resting_T_5_1.bam
## 4   Resting_T_5_2   Resting  T            5         2   Resting_T_5_2.bam
## 5 Activated_L_5_1 Activated  L            5         1 Activated_L_5_1.bam
## 6 Activated_L_5_2 Activated  L            5         2 Activated_L_5_2.bam
## 7 Activated_T_5_1 Activated  T            5         1 Activated_T_5_1.bam
## 8 Activated_T_5_2 Activated  T            5         2 Activated_T_5_2.bam
```

Together with the feature annotation, for which we want to estimate synthesis and degradation rates, we can construct the *rCubeExperiment*:

```
exonCounts <- setupExperiment(constitutiveExons, designMatrix=expDesign, files=NULL)
class(exonCounts)
```

```
## [1] "rCubeExperiment"
## attr(,"package")
## [1] "rCube"
```

Alternatively, the experimental design matrix can be constructed from the bam file names internally, if they follow the following convention `{condition}_{L|T}_{labelingTime}_{replicate}.bam`

```
bamfiles <- list.files(folder, pattern="*.bam$", full.names=TRUE)
basename(bamfiles)
```

```
## [1] "ActivatedJurkat_L_5_1.bam" "ActivatedJurkat_L_5_2.bam"
## [3] "ActivatedJurkat_T_5_1.bam" "ActivatedJurkat_T_5_2.bam"
## [5] "RestingJurkat_L_5_1.bam"   "RestingJurkat_L_5_2.bam"
## [7] "RestingJurkat_T_5_1.bam"   "RestingJurkat_T_5_2.bam"
```

```
exonCounts <- setupExperiment(constitutiveExons, designMatrix=NULL, files=bamfiles)
```

The resulting *rCubeExperiment* object can now be used to count reads.

3.3 Counting

For read countings, we use the `readGAlignmentPairs` in a parallel fashion:

```
assay(exonCounts)
```

```
##         ActivatedJurkat_L_5_1 ActivatedJurkat_L_5_2 ActivatedJurkat_T_5_1
## CF00001                    NA                    NA                    NA
## CF00002                    NA                    NA                    NA
## CF00003                    NA                    NA                    NA
##         ActivatedJurkat_T_5_2 RestingJurkat_L_5_1 RestingJurkat_L_5_2
## CF00001                    NA                  NA                  NA
## CF00002                    NA                  NA                  NA
## CF00003                    NA                  NA                  NA
##         RestingJurkat_T_5_1 RestingJurkat_T_5_2
## CF00001                  NA                  NA
## CF00002                  NA                  NA
## CF00003                  NA                  NA
```

```
exonCounts <- countFeatures(exonCounts,
                            scanBamParam=ScanBamParam(flag=scanBamFlag(isSecondaryAlignment=FALSE)),
                            BPPARAM=NULL,
                            verbose=FALSE)
assay(exonCounts)
```

```
##         ActivatedJurkat_L_5_1 ActivatedJurkat_L_5_2 ActivatedJurkat_T_5_1
## CF00001                    74                   123                     6
## CF00002                   112                   195                     4
## CF00003                   481                   610                    17
##         ActivatedJurkat_T_5_2 RestingJurkat_L_5_1 RestingJurkat_L_5_2
## CF00001                     6                   0                   4
## CF00002                     9                   4                   6
## CF00003                    23                  15                  17
##         RestingJurkat_T_5_1 RestingJurkat_T_5_2
## CF00001                   4                   2
## CF00002                   2                   3
## CF00003                   1                   1
```

3.4 Spike-ins

The artifical spike-in annotations and labeling information can be loaded via:

```
data("spikeins")
data("spikeinLabeling")
spikeinLengths <- width(spikeins)
```

3.5 Spike-in design

An empty *rCubeExperiment* for the artificial spike-ins additionally requires information about the length and the labeling status of each spikein, and can be constructed as follows:

```
spikeinCounts <- setupExperimentSpikeins(rows=spikeins,
                                         designMatrix=expDesign,
                                         length=spikeinLengths,
                                         labelingState=spikeinLabeling)
```

The individual information from the *rCubeExperiment* can be assessed by:

```r
# feature information
rowRanges(spikeinCounts)
```

```
## GRanges object with 6 ranges and 9 metadata columns:
##           seqnames    ranges strand |       source       type     score
##              <Rle> <IRanges>  <Rle> |     <factor>   <factor> <numeric>
##    Spike2    chrS2 [1,  982]      + | Fruehauf2013 transcript      <NA>
##   Spike12   chrS12 [1,  947]      + | Fruehauf2013 transcript      <NA>
##    Spike4    chrS4 [1, 1011]      + | Fruehauf2013 transcript      <NA>
##    Spike5    chrS5 [1, 1012]      + | Fruehauf2013 transcript      <NA>
##    Spike8    chrS8 [1, 1076]      + | Fruehauf2013 transcript      <NA>
##    Spike9    chrS9 [1, 1034]      + | Fruehauf2013 transcript      <NA>
##                phase     gene_id transcript_id    length labelingState
##           <integer> <character>   <character> <integer>      <factor>
##    Spike2      <NA>      Spike2        Spike2      <NA>          TRUE
##   Spike12      <NA>     Spike12       Spike12      <NA>         FALSE
##    Spike4      <NA>      Spike4        Spike4      <NA>          TRUE
##    Spike5      <NA>      Spike5        Spike5      <NA>         FALSE
##    Spike8      <NA>      Spike8        Spike8      <NA>          TRUE
##    Spike9      <NA>      Spike9        Spike9      <NA>         FALSE
##           labeledSpikein
##                <logical>
##    Spike2          FALSE
##   Spike12          FALSE
##    Spike4          FALSE
##    Spike5          FALSE
##    Spike8          FALSE
##    Spike9          FALSE
##   -------
##   seqinfo: 6 sequences from an unspecified genome; no seqlengths
```

```r
# sample information
colData(spikeinCounts)
```

```
## DataFrame with 8 rows and 6 columns
##                          sample condition       LT labelingTime replicate
##                        <factor>  <factor> <factor>    <integer> <integer>
## Resting_L_5_1     Resting_L_5_1   Resting        L            5         1
## Resting_L_5_2     Resting_L_5_2   Resting        L            5         2
## Resting_T_5_1     Resting_T_5_1   Resting        T            5         1
## Resting_T_5_2     Resting_T_5_2   Resting        T            5         2
## Activated_L_5_1 Activated_L_5_1 Activated        L            5         1
## Activated_L_5_2 Activated_L_5_2 Activated        L            5         2
## Activated_T_5_1 Activated_T_5_1 Activated        T            5         1
## Activated_T_5_2 Activated_T_5_2 Activated        T            5         2
##                            filename
##                            <factor>
## Resting_L_5_1     Resting_L_5_1.bam
## Resting_L_5_2     Resting_L_5_2.bam
## Resting_T_5_1     Resting_T_5_1.bam
## Resting_T_5_2     Resting_T_5_2.bam
## Activated_L_5_1 Activated_L_5_1.bam
## Activated_L_5_2 Activated_L_5_2.bam
## Activated_T_5_1 Activated_T_5_1.bam
## Activated_T_5_2 Activated_T_5_2.bam
```

```
# read counts
assay(spikeinCounts)
```

```
##         Resting_L_5_1 Resting_L_5_2 Resting_T_5_1 Resting_T_5_2 Activated_L_5_1
## Spike2             NA            NA            NA            NA             NA
## Spike12            NA            NA            NA            NA             NA
## Spike4             NA            NA            NA            NA             NA
## Spike5             NA            NA            NA            NA             NA
## Spike8             NA            NA            NA            NA             NA
## Spike9             NA            NA            NA            NA             NA
##         Activated_L_5_2 Activated_T_5_1 Activated_T_5_2
## Spike2               NA              NA              NA
## Spike12              NA              NA              NA
## Spike4               NA              NA              NA
## Spike5               NA              NA              NA
## Spike8               NA              NA              NA
## Spike9               NA              NA              NA
```

3.6 Spike-in counting

```
colData(spikeinCounts)$filename
```

```
## [1] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/ActivatedJurka
## [2] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/ActivatedJurka
## [3] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/ActivatedJurka
## [4] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/ActivatedJurka
## [5] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/RestingJurkat_
## [6] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/RestingJurkat_
## [7] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/RestingJurkat_
## [8] "/Library/Frameworks/R.framework/Versions/3.4/Resources/library/rCube/extdata/Jurkat/RestingJurkat_
```

```
spikeinCounts <- countSpikeins(spikeinCounts)#,
                           # scanBamParam=ScanBamParam(flag=scanBamFlag(isSecondaryAlignment=FALSE)),
                           # BPPARAM=NULL,
                           # verbose=FALSE)
assay(spikeinCounts)
```

```
##         Resting_L_5_1 Resting_L_5_2 Resting_T_5_1 Resting_T_5_2 Activated_L_5_1
## Spike2           3648          4416           492           541            4416
## Spike12            75           109           369           408             129
## Spike4           4060          4399           414           456            4031
## Spike5             43            33           472           401             105
## Spike8           4455          5205           397           432            4927
## Spike9             18            13           249           283              66
##         Activated_L_5_2 Activated_T_5_1 Activated_T_5_2
## Spike2             3791             695             498
## Spike12              93             496             349
## Spike4             3800             548             425
## Spike5               30             573             363
## Spike8             4272             582             412
## Spike9               14             333             187
```

+ diagnostic plot?

3.7 Size factor based on spike-in

3.8 Estimate dispersion

3.9 Fit the rates

3.10 describe the different fitting functions

3.11 describe the class of the returned object (rCubeRates)

4 Labeling time series

4.1 Experimental Design

4.2 Read classification

4.3 Gene model

(exon, into and junctions) by gff de novo + gff

4.4 Counting

4.5 Spike-ins

4.6 Spike-in design

4.7 Spike-in counting

4.8 Size factor based on spike-in

4.9 Estimate dispersion

4.10 Fit the rates

4.11 describe the different fitting functions

4.12 describe the class of the returned object (rCubeRates)

this part is old from before talking to julien:

4.13 Input Data

The rCube package works on *rCubeExperiment* containers, that rely on the *SummerizedExperiment* class. The `rowRanges` of the *rCubeExperiment* is a *GRanges* object of features, for which RNA rates should be estimated. Experimental sample information can be either provided by a design matrix or this information can be extracted from the BAM-file names (when they fulfil the required structure). The file name should be a string containing condition, labelingTime (as integer), L/T sample information, and replicate (integer/string), separated by a "_". Then, an empty *rCubeExperiment*, e.g. for the artificial spike-ins, can be constructed as follows:

```
data("designMatrix")

spikeinCounts <- setupExperimentSpikeins(rows=spikeins,
                                         designMatrix=designMatrix,
                                         length=spikeinLengths,
                                         labelingState=spikeinLabeling)
```

The `setupExperiment` can be used analogically for genes/exons/introns/junctions. Here, only the `rows` and either `designMatrix` or `files` has to be set. See also section 4.14.

The individual information from the *rCubeExperiment* can be assessed by:

```
# feature information
rowRanges(spikeinCounts)
```

```
## GRanges object with 6 ranges and 9 metadata columns:
##           seqnames    ranges strand |       source       type     score
##              <Rle> <IRanges>  <Rle> |     <factor>   <factor> <numeric>
##    Spike2    chrS2 [1,  982]      + | Fruehauf2013 transcript      <NA>
##   Spike12   chrS12 [1,  947]      + | Fruehauf2013 transcript      <NA>
##    Spike4    chrS4 [1, 1011]      + | Fruehauf2013 transcript      <NA>
##    Spike5    chrS5 [1, 1012]      + | Fruehauf2013 transcript      <NA>
##    Spike8    chrS8 [1, 1076]      + | Fruehauf2013 transcript      <NA>
##    Spike9    chrS9 [1, 1034]      + | Fruehauf2013 transcript      <NA>
##               phase     gene_id transcript_id    length labelingState
##           <integer> <character>   <character> <integer>      <factor>
##    Spike2      <NA>      Spike2        Spike2      <NA>          TRUE
##   Spike12      <NA>     Spike12       Spike12      <NA>         FALSE
##    Spike4      <NA>      Spike4        Spike4      <NA>          TRUE
##    Spike5      <NA>      Spike5        Spike5      <NA>         FALSE
##    Spike8      <NA>      Spike8        Spike8      <NA>          TRUE
##    Spike9      <NA>      Spike9        Spike9      <NA>         FALSE
##           labeledSpikein
##                <logical>
##    Spike2          FALSE
##   Spike12          FALSE
##    Spike4          FALSE
##    Spike5          FALSE
##    Spike8          FALSE
##    Spike9          FALSE
##   -------
##   seqinfo: 6 sequences from an unspecified genome; no seqlengths
```

```
# sample information
colData(spikeinCounts)
```

```
## DataFrame with 8 rows and 5 columns
##           sample condition       LT labelingTime replicate
##         <factor>  <factor> <factor>    <numeric>  <factor>
## A_L_5_1  A_L_5_1         A        L            5         1
## A_L_5_2  A_L_5_2         A        L            5         2
## B_L_5_1  B_L_5_1         B        L            5         1
## B_L_5_2  B_L_5_2         B        L            5         2
## A_T_5_1  A_T_5_1         A        T            5         1
## A_T_5_2  A_T_5_2         A        T            5         2
## B_T_5_1  B_T_5_1         B        T            5         1
```

```
## B_T_5_2  B_T_5_2         B        T            5         2
```

```
# read counts
assay(spikeinCounts)
```

```
##         A_L_5_1 A_L_5_2 B_L_5_1 B_L_5_2 A_T_5_1 A_T_5_2 B_T_5_1 B_T_5_2
## Spike2       NA      NA      NA      NA      NA      NA      NA      NA
## Spike12      NA      NA      NA      NA      NA      NA      NA      NA
## Spike4       NA      NA      NA      NA      NA      NA      NA      NA
## Spike5       NA      NA      NA      NA      NA      NA      NA      NA
## Spike8       NA      NA      NA      NA      NA      NA      NA      NA
## Spike9       NA      NA      NA      NA      NA      NA      NA      NA
```

4.14 Read counting

All RNA rate estimations of this package rely on read counts. These can be either provided as count matrices, or read counts can be obtained from BAM files using the rCube pipeline.

```
#TODO
```

Alternatively, count matrices can be assigned to the correctly formatted, empty *rCubeExperiment* object:

```
#TODO
```

5 Normalization of TT-seq/4sU-seq and RNA-Seq samples

The three possible normalization methods are described in detail below.

5.1 Normalization by fitting a GLM to artifical spike-in read counts

By normalization, we want to account for the sequencing depth of different samples and especially adjust the ratio between Labeled to Total RNA-Seq libraries. Additionally, the labeled RNA extraction is not perfect and some unlabeled RNA may contaminate the labeled RNA fraction. As the gene-expression also always varies a little bit for biological samples, read counts from real genes might lead to confusing estimations. Therefore, we apply our normalization approach only to artificial spike-ins from the External RNA Control Consortium (ERCC), for which the initial amount of each spike-in is known and the same across all samples. Some of the spike-ins were 4sU-labeled by in-vitro transcription, so the cross-contamination of unlabeled spike-ins in labeled samples can be monitored. The distribution of 4sU-labeled and unlabeld spike-ins among different samples can be monitored with `plotSpikeinCountsVsSample`.

```
data(spikeinCounts)
plotSpikeinCountsVsSample(spikeinCounts)
```

The goal of this package is to reliably estimate sequencing depths and cross-contamination rates per sample, given 4sU- and total RNA-Seq data.

The sample specific parameters like sequencing depth and cross-contamination rate are estimated from spike-in counts only. Therefore, we fit a generalized linear model (GLM) of the Negative Binomial family with a log link function. The response of the GLM are the observed spike-in counts, and the terms that specify the linear predictor of the response are comprised of:

- a sample specific factor (that reflects the sample specific sequencing depth),
- a labeled sample specific factor (that reflects the control for cross contamination (only estimated for unlabeled spike-ins in labeled samples)), and
- a spike-in specific factor to allow for some spike-in specific variation e.g. due to sequence biases.

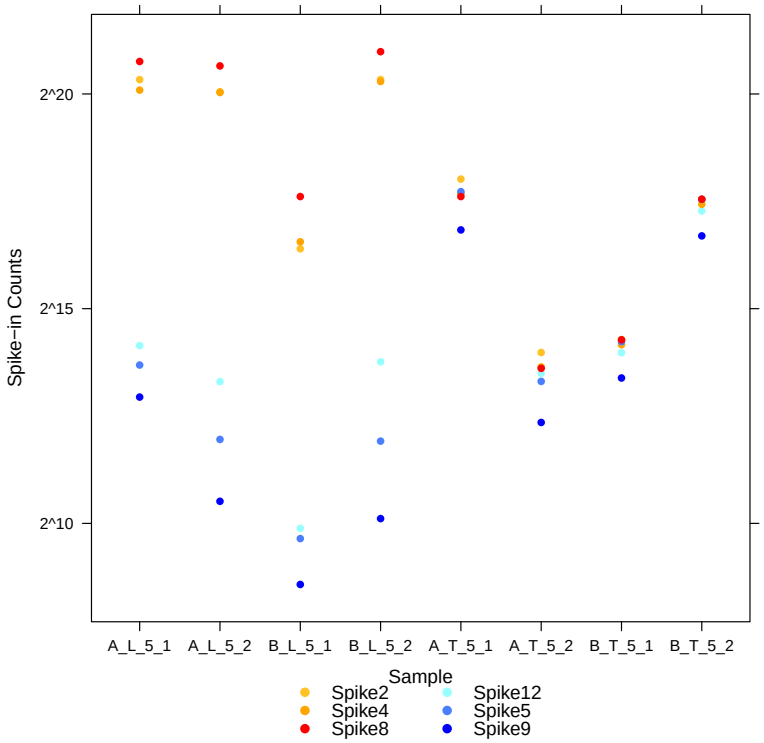


Figure 1: **Spikein-vs-Sample-plot.**

Additionally, the length of each spike-in is used as an offset, i.e. a known slope for the covariate.

```
data(geneCounts)
data(spikeinCounts)
geneCounts <- estimateSizeFactors(geneCounts, spikeinCounts, method="spikeinGLM")
colnames(colData(geneCounts))
```

```
## [1] "sample"              "condition"           "LT"
## [4] "labelingTime"        "replicate"           "sequencing.depth"
## [7] "cross.contamination"
```

```
geneCounts$sequencing.depth
```

```
## A_L_5_1 A_L_5_2 B_L_5_1 B_L_5_2 A_T_5_1 A_T_5_2 B_T_5_1 B_T_5_2
## 1.00000 0.90453 0.08914 1.10879 0.16871 0.00919 0.01453 0.14209
```

```
geneCounts$cross.contamination
```

```
## A_L_5_1 A_L_5_2 B_L_5_1 B_L_5_2 A_T_5_1 A_T_5_2 B_T_5_1 B_T_5_2
## 0.01200 0.00445 0.00721 0.00411 1.00000 1.00000 1.00000 1.00000
```

Note, the cross-contamination value for all total RNA-seq samples is 1, as 100% of the unlabeled RNAs are supposed to be in the sample. Additional fitting results are stored in the `metadata` of the resulting *rCubeExperiment* object.

```
metadata(geneCounts)
```

5.2 Normalization using mean counts of artifical spike-ins

5.3 Normalization using joint model

6 Estimating gene-specific synthesis and degradation rates

Using the sample-specific values for sequencing depth and cross-contamination as estimated in the previous section, we can now normalize all the samples. It is especially important to bring labeled (4sU-seq/TT-seq) and total RNA-seq samples to comparable scales. Labeled and total RNA-seq samples can be sequenced at the same depth, and the same amount of RNA is used for library preparation, but the resulting read counts do not reflect the true ratio of labeled vs all RNAs in the cells, where the amount of newly-synthesized, labeled RNA should be much less than the total RNA amount. Therefore it is necessary to upscale the read counts from total RNA-seq samples compared to the labeled RNA read counts.

6.1 Providing gene-wise dispersion estimates

Usually, read counts in different RNA-seq samples underly fluctuations due to biological or technical variances. To take these fluctuations into account, we estimate each gene's dispersion. For each gene, a single dispersion estimate for all 4sU-Seq samples and for all Total RNA-Seq samples is needed. Here, we can use the method provided in the DESeq2 package [4] The wrapper function `estimateSizeDispersions` applies the DESeq algorithm to all genes, while separating the count table according to the RNA-Seq protocol (labeled or total RNA). It is possible to choose between all provided DESeq dispersion estimates, namely the genewise maximum likelihood dispersion estimate ("dispGeneEst"), the smooth curve fitted through the gene-wise disperion estimates ("dispFit") and the genewise dispersion estimates shrunken towards the fitted curve ("dispMAP", default). The input is an *rCubeExperiment* object with read counts for the features of interest. The function returns an updated *rCubeExperiment* object with two additional columns in the `rowRanges`, namely `dispersion_L` and `dispersion_T`.

```
geneCounts <- estimateSizeDispersions(geneCounts, method='DESeqDispMAP')
rowRanges(geneCounts)
```

```
## GRanges object with 12 ranges and 2 metadata columns:
##           seqnames       ranges strand |      dispersion_L        dispersion_T
##              <Rle>    <IRanges>  <Rle> |         <numeric>           <numeric>
##    gene 1  chrTest [ 724, 2389]      * | 0.267593980890705 0.00285282168427403
##    gene 2  chrTest [1331, 2444]      * | 0.210169732934922 0.00246148010370263
##    gene 3  chrTest [1209, 2066]      * | 0.217187602571734 0.00209222050855509
##    gene 4  chrTest [1222, 3157]      * | 0.249077753495831 0.00207165673496985
##    gene 5  chrTest [ 828, 1607]      * | 0.203015823141568  0.0021568661882415
##       ...      ...          ...    ... .               ...                 ...
##    gene 8  chrTest [1055, 2066]      * | 0.217187602571734 0.00246148010370263
##    gene 9  chrTest [1218, 1881]      * | 0.885921521910211    0.10447013633359
##   gene 10  chrTest [1712, 2766]      * | 0.217187602571734 0.00246148010370263
##   gene 11  chrTest [ 646, 1502]      * | 0.202381362920392 0.00289479811680433
##   gene 12  chrTest [1381, 2451]      * |                10                  10
##   -------
##   seqinfo: 1 sequence from an unspecified genome; no seqlengths
```

6.2 Feature-specific synthesis and degratation rate estimates

After estimating sequencing depth and cross-contamination rates per sample (see Section 5) and extracting feature-specific dispersion estimates (see Section 6.1), we can now estimate RNA synthesis and degradation rate for each feature and condition individually. Multiple replicates for the same condition can be used for a joint estimation. The user has to specify for which replicate or combination of replicates the results should be estimated. Therefore, the `replicate` parameter is a vector of all combinations that should be evaluated. For the joint estimation for multiple replicates, these have to be given as a string separeted by a ":". In the following example, we will obtain individual results for replicate 1 and 2 and also results for a joint estimation.

```
rates <- estimateRateByFirstOrderKinetics(geneCounts,
                                          replicate=c(1, 2, "1:2"),
                                          method='single',
                                          BPPARAM=BiocParallel::MulticoreParam(1))
```

7 Estimating splicing times from junction read counts

8 Session Information

This vignette was generated using the following package versions:

```
sessionInfo()
```

```
## R version 3.4.1 (2017-06-30)
## Platform: x86_64-apple-darwin15.6.0 (64-bit)
## Running under: OS X El Capitan 10.11.6
##
## Matrix products: default
## BLAS: /Library/Frameworks/R.framework/Versions/3.4/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/3.4/Resources/lib/libRlapack.dylib
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## attached base packages:
## [1] parallel  stats4    stats     graphics  grDevices utils     datasets
## [8] methods   base
##
## other attached packages:
##  [1] ggbio_1.25.3               ggplot2_2.2.1
##  [3] rCube_1.1.0                SummarizedExperiment_1.7.5
##  [5] DelayedArray_0.3.19        matrixStats_0.52.2
##  [7] Biobase_2.37.2             GenomicRanges_1.29.12
##  [9] GenomeInfoDb_1.13.4        IRanges_2.11.12
## [11] S4Vectors_0.15.5           BiocGenerics_0.23.0
## [13] knitr_1.16
##
## loaded via a namespace (and not attached):
##  [1] ProtGenerics_1.9.0           bitops_1.0-6
##  [3] bit64_0.9-7                  RColorBrewer_1.1-2
##  [5] progress_1.1.2               httr_1.2.1
##  [7] rprojroot_1.2                tools_3.4.1
##  [9] backports_1.1.0              R6_2.2.2
## [11] rpart_4.1-11                 Hmisc_4.0-3
## [13] DBI_0.7                      lazyeval_0.2.0
## [15] colorspace_1.3-2             nnet_7.3-12
## [17] gridExtra_2.2.1              prettyunits_1.0.2
## [19] GGally_1.3.2                 DESeq2_1.17.12
## [21] curl_2.8.1                   bit_1.1-12
## [23] compiler_3.4.1               graph_1.55.0
## [25] htmlTable_1.9                rtracklayer_1.37.3
## [27] scales_0.4.1                 checkmate_1.8.3
```

```
## [29] genefilter_1.59.0            RBGL_1.53.0
## [31] stringr_1.2.0                digest_0.6.12
## [33] Rsamtools_1.29.0             foreign_0.8-69
## [35] rmarkdown_1.6                XVector_0.17.0
## [37] base64enc_0.1-3              dichromat_2.0-0
## [39] htmltools_0.3.6              ensembldb_2.1.10
## [41] BSgenome_1.45.1              highr_0.6
## [43] htmlwidgets_0.9              rlang_0.1.1
## [45] RSQLite_2.0                  BiocInstaller_1.27.2
## [47] shiny_1.0.3                  BiocParallel_1.11.4
## [49] acepack_1.4.1                VariantAnnotation_1.23.6
## [51] RCurl_1.95-4.8               magrittr_1.5
## [53] GenomeInfoDbData_0.99.1      Formula_1.2-2
## [55] Matrix_1.2-10                Rcpp_0.12.12
## [57] munsell_0.4.3                stringi_1.1.5
## [59] yaml_2.1.14                  MASS_7.3-47
## [61] zlibbioc_1.23.0              plyr_1.8.4
## [63] AnnotationHub_2.9.5          grid_3.4.1
## [65] blob_1.1.0                   lattice_0.20-35
## [67] Biostrings_2.45.3            splines_3.4.1
## [69] GenomicFeatures_1.29.8       annotate_1.55.0
## [71] locfit_1.5-9.1               geneplotter_1.55.0
## [73] reshape2_1.4.2               biomaRt_2.33.3
## [75] XML_3.98-1.9                 evaluate_0.10.1
## [77] biovizBase_1.25.1            latticeExtra_0.6-28
## [79] data.table_1.10.4            httpuv_1.3.5
## [81] gtable_0.2.0                 reshape_0.8.6
## [83] assertthat_0.2.0             mime_0.5
## [85] xtable_1.8-2                 AnnotationFilter_1.1.3
## [87] survival_2.41-3              OrganismDbi_1.19.0
## [89] tibble_1.3.3                 GenomicAlignments_1.13.4
## [91] AnnotationDbi_1.39.2         memoise_1.1.0
## [93] cluster_2.0.6                interactiveDisplayBase_1.15.0
## [95] BiocStyle_2.5.8
```

9 References

[1] Björn Schwalb, Margaux Michel, Benedikt Zacher, Carina Frühauf, Katja Demel, Achim Tresch, Julien Gagneur, and Patrick Cramer. TT-seq maps the human transient transcriptome. *Science*, 352(6290):1225–1228, 2016.

[2] Margaux Michel, Carina Demel, Benedikt Zacher, Björn Schwalb, Stefan Krebs, Julien Gagneur, and Patrick Cramer. TT-seq captures enhancer landscapes immediately after T-cell stimulation. *Molecular Systems Biology*, 13(3):920, 2017. doi:10.15252/msb.20167507.

[3] James H Bullard, Elizabeth Purdom, Kasper D Hansen, and Sandrine Dudoit. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC Bioinformatics*, 11:94, 2010. doi:10.1186/1471-2105-11-94.

[4] Michael I Love, Wolfgang Huber, and Simon Anders. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. *Genome Biology*, 15(12):550, 2014.