Counting with `summarizeOverlaps`

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1 Introduction

This vignette illustrates how reads mapped to a genome can be counted with `summarizeOverlaps`. Different "modes" of counting are provided to resolve reads that overlap multiple features. The built-in count modes are fashioned after the "Union", "IntersectionStrict", and "IntersectionNotEmpty" methods found in the HTSeq package by Simon Anders (see references).

2 A First Example

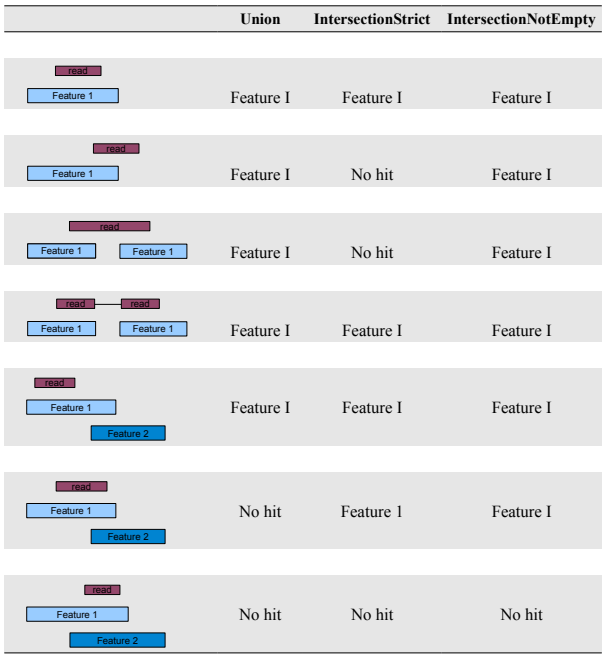
In this example reads are counted from a list of BAM files and returned in a `matrix` for use in further analysis such as those offered in DESeq and edgeR.

```
> library(Rsamtools)
> library(DESeq)
> library(edgeR)
> fls = list.files(system.file("extdata",package="GenomicRanges"),
```

```
+     recursive=TRUE, pattern="*bam$", full=TRUE)
> bfl <- BamFileList(fls)
> features <- GRanges(
+     seqnames = Rle(c("chr2L", "chr2R", "chr2L", "chr2R", "chr2L", "chr2R",
+         "chr2L", "chr2R", "chr2R", "chr3L", "chr3L")),
+     strand = strand(rep("+", 11)),
+     ranges = IRanges(start=c(1000, 2000, 3000, 3600, 7000, 7500, 4000, 4000,
+         3000, 5000, 5400), width=c(500, 900, 500, 300, 600, 300, 500, 900, 500,
+         500, 500))
+ )
> olap <- summarizeOverlaps(features, bfl)
> deseq <- newCountDataSet(countData=assays(olap)$counts,
+                          conditions=rownames(colData(olap)))
> edger <- DGEList(counts=assays(olap)$counts, group=rownames(colData(olap)))
```

3 Counting Modes

The modes of "Union", "IntersectionStrict" and "IntersectionNotEmpty" provide different approaches to resolving reads that overlap multiple features. Figure 1 illustrates how both simple and gapped reads are handled by the modes. Note that a read is counted a maximum of once; there is no double counting. These methods do not currently handle paired-end reads. For additional detail on the counting modes see the `summarizeOverlaps` man page.



* Picture reproduced from HTSeq web site :
http://www-huber.embl.de/users/anders/HTSeq/doc/count.html

Figure 1: Counting Modes

4 Counting Features

Features can be exons, transcripts, genes or any region of interest. The number of ranges that define a single feature is specified in the `features` argument.

When annotation regions of interest are defined by a single range a *GRanges* should be used as the `features` argument. With a *GRanges* it is assumed that each row (i.e., each range) represents a distinct feature. If `features` was a *GRanges* of exons, the result would be counts per exon.

When the region of interest is defined by one or more ranges the `features` argument should be a *GRangesList*. In practice this could be a list of exons by gene or transcripts by gene or other similar relationships. The count result will

be the same length as the *GRangesList*. For a list of exons by genes, the result would be counts per gene.

The combination of defining the features as either *GRanges* or *GRangesList* and choosing a counting mode controls how `summarizeOverlaps` assigns hits. Reguardless of the mode chosen, each read is assigned to at most a single feature. These options are intended to provide flexibility in defining different biological problems.

This next example demonstrates how the same read can be counted differently depending on how the `features` argument is specified. We use a single read that overlaps two ranges, gr1 and gr2.

```
> rd <- GappedAlignments("a", seqnames = Rle("chr1"), pos = as.integer(100),
+     cigar = "300M", strand = strand("+"))
> gr1 <- GRanges("chr1", IRanges(start=50, width=150), strand="+")
> gr2 <- GRanges("chr1", IRanges(start=350, width=150), strand="+")
```

When provided as a *GRanges* both gr1 and gr2 are considered distinct features. In this case none of the modes count the read as a hit. Mode `Union` discards the read becasue more than 1 feature is overlapped. `IntersectionStrict` requires the read to fall completely within a feature which is not the case for either gr1 or gr2. `IntersetctionNotEmpty` requires the read to overlap a single unique disjoint region of the `features`. In this case gr1 and gr2 do not overlap so each range is considered a unique disjoint region. However, the read overlaps both gr1 and gr2 so a decision cannot be made and the read is discarded.

```
> gr <- c(gr1, gr2)
> data.frame(union = assays(summarizeOverlaps(gr, rd))$counts,
+            intStrict = assays(summarizeOverlaps(gr, rd,
+                mode="IntersectionStrict"))$counts,
+            intNotEmpty = assays(summarizeOverlaps(gr, rd,
+                mode="IntersectionNotEmpty"))$counts)
```

```
  union intStrict intNotEmpty
1     0         0           0
2     0         0           0
```

Next we count with `features` as a *GRangesList*; this is list of length 1 with 2 elements. Modes `Union` and `IntersectionNotEmpty` both count the read for the single feature.

```
> grl <- GRangesList(c(gr1, gr2))
> data.frame(union = assays(summarizeOverlaps(grl, rd))$counts,
+            intStrict = assays(summarizeOverlaps(grl, rd,
+                mode="IntersectionStrict"))$counts,
+            intNotEmpty = assays(summarizeOverlaps(grl, rd,
+                mode="IntersectionNotEmpty"))$counts)
```

```
  union intStrict intNotEmpty
1     1         0           1
```

In this more complicated example we have 7 reads, 5 are simple and 2 have gaps in the CIGAR. There are 12 ranges that will serve as the `features`.

```
> group_id <- c("A", "B", "C", "C", "D", "D", "E", "F", "G", "G", "H", "H")
> features <- GRanges(
+     seqnames = Rle(c("chr1", "chr2", "chr1", "chr1", "chr2", "chr2",
+         "chr1", "chr1", "chr2", "chr2", "chr1", "chr1")),
+     strand = strand(rep("+", length(group_id))),
+     ranges = IRanges(
+         start=c(1000, 2000, 3000, 3600, 7000, 7500, 4000, 4000, 3000, 3350, 5000, 5400),
+         width=c(500, 900, 500, 300, 600, 300, 500, 900, 150, 200, 500, 500)),
+    DataFrame(group_id)
+ )
> reads <- GappedAlignments(
+     names = c("a","b","c","d","e","f","g"),
+     seqnames = Rle(c(rep(c("chr1", "chr2"), 3), "chr1")),
+     pos = as.integer(c(1400, 2700, 3400, 7100, 4000, 3100, 5200)),
+     cigar = c("500M", "100M", "300M", "500M", "300M", "50M200N50M", "50M150N50M"),
+     strand = strand(rep.int("+", 7L)))
>
```

Using a *GRanges* as the `features` all 12 ranges are considered to be different features and counts are produced for each row,

```
> data.frame(union = assays(summarizeOverlaps(features, reads))$counts,
+            intStrict = assays(summarizeOverlaps(features, reads,
+                mode="IntersectionStrict"))$counts,
+            intNotEmpty = assays(summarizeOverlaps(features, reads,
+                mode="IntersectionNotEmpty"))$counts)

   union intStrict intNotEmpty
1      1         0           1
2      1         1           1
3      0         0           0
4      0         0           0
5      0         1           1
6      0         0           0
7      0         0           0
8      0         0           0
9      0         0           0
10     0         0           0
11     0         1           1
12     0         0           0
```

When the data are split by group to create a *GRangesList* the highest list-levels are treated as different features and the multiple list elements are considered part of the same features. Counts are returned for each group.

```
> lst <- split(features, values(features)[["group_id"]])
> length(lst)

[1] 8

> data.frame(union = assays(summarizeOverlaps(lst, reads))$counts,
+            intStrict = assays(summarizeOverlaps(lst, reads,
+                mode="IntersectionStrict"))$counts,
+            intNotEmpty = assays(summarizeOverlaps(lst, reads,
+                mode="IntersectionNotEmpty"))$counts)

  union intStrict intNotEmpty
A     1         0           1
B     1         1           1
C     1         0           1
D     1         1           1
E     0         0           0
F     0         0           0
G     1         1           1
H     1         1           1
```

If desired, users can supply their own counting function as the `mode` argument and take advantage of the infrastructure for counting over multiple BAM files and parsing the results into a *SummarizedExperiment*. See `?'BamViews-class'` or `?'BamFile-class'` in Rsamtools.

5 pasilla Data

In this excercise we use the pasilla data to create an *ExonCountSet* and *CountDataSet* similar to those available in the pasilla data package. These objects can be used in differential expression methods offered in the DESeq or DEXSeq packages. Details of read alignment and the creation of the annotaion file are available in the pasilla package vignette.

5.1 source files

BAM files were downloaded from `http://www.embl.de/~reyes/Graveley/bam`. Of the seven files available, 3 are single-reads and 4 are paired-end. `summarizeOverlaps` does not currently handle paired-end reads so in this example we use the following 3 single-read files,

- treated1.bam
- untreated1.bam
- untreated2.bam

We use the Dmel.BDGP5.25.62.DEXSeq.chr.gff annotation file created by the pasilla authors and stored in the `/extdata` directory of the package. This file contains non-overlapping exon regions identified as "exonic_part" and a collective range for the exons identified as "aggregate_gene". The "exonic_part" ranges will be used to create the *ExonCountSet* and the "aggregate_gene" ranges to create the *CountDataSet*.

```
> library(pasilla)
> library(rtracklayer)
> library(Rsamtools)
> gff <- import(system.file("extdata", "Dmel.BDGP5.25.62.DEXSeq.chr.gff",
+     package = "pasilla"), "gff1")
> features <- as(gff, "GRanges")
> head(features[,1])

GRanges with 6 ranges and 1 elementMetadata col:
      seqnames        ranges strand |
         <Rle>     <IRanges>  <Rle> |
  [1]    chr2L [7529,  9484]      + |
  [2]    chr2L [7529,  8116]      + |
  [3]    chr2L [8193,  8589]      + |
  [4]    chr2L [8590,  8667]      + |
  [5]    chr2L [8668,  9484]      + |
  [6]    chr2L [9836, 21372]      - |
                                          source
                                        <factor>
  [1] Drosophila_melanogaster.BDGP5.25.62.gtf.gz
  [2] Drosophila_melanogaster.BDGP5.25.62.gtf.gz
  [3] Drosophila_melanogaster.BDGP5.25.62.gtf.gz
  [4] Drosophila_melanogaster.BDGP5.25.62.gtf.gz
  [5] Drosophila_melanogaster.BDGP5.25.62.gtf.gz
  [6] Drosophila_melanogaster.BDGP5.25.62.gtf.gz
  ---
  seqlengths:
                           chr2L ... chrdmel_mitochondrion_genome
                              NA ...                             NA
```

5.2 Exon counts

For counting exons we retain the ranges marked as "exonic_part". The exon and gene id's are extracted from the 'group' metadata column for later use.

```
> exons <- features[values(features)[["type"]] == "exonic_part"]
> st <- strsplit(gsub("\"", "", values(exons)[["group"]]), ";")
> exonID <- do.call(c,
+                lapply(st, function(x) {
+                   gsub("[^0-9]", "", x[2])}))
```

```
> geneID <- do.call(c,
+               lapply(st, function(x) {
+                  gsub(" gene_id ", "", x[3])}))
```

The `params` argument can be used to subset the reads in the bam file on characteristics such as position, unmapped or paired-end reads. Quality scores or the "NH" tag, which identifies reads with multiple mappings, can be included as metadata columns for further subsetting. See `?ScanBamParam` for details about specifying the `param` argument.

```
> param <- ScanBamParam(
+              what='qual',
+              which=GRanges("chr2L", IRanges(1, 1e+6)),
+              flag=scanBamFlag(isUnmappedQuery=FALSE, isPaired=NA))
> bamTag(param) <- "NH"
```

We use `summarizeOverlaps` to count with the default mode of "Union". If a `param` argument is not included all reads from the BAM file are counted.

```
> fls <- c("treated1.bam", "untreated1.bam", "untreated2.bam")
> path <- "pathToBAMFiles"
> bamFiles <- BamFileList(file.path(paste(path, fls, sep="")))
> se_exons <- summarizeOverlaps(exons, bamFiles, mode="Union")
```

An `ExonCountSet` is constructed from the counts and experiment data in *pasilla*.

```
> library(DEXSeq)
> expdata = new("MIAME",
+               name="pasilla knockdown",
+               lab="Genetics and Developmental Biology, University of
+                   Connecticut Health Center",
+               contact="Dr. Brenton Graveley",
+               title="modENCODE Drosophila pasilla RNA Binding Protein RNAi
+                   knockdown RNA-Seq Studies",
+               url="http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE18508",
+               abstract="RNA-seq of 3 biological replicates of from the Drosophila
+                   melanogaster S2-DRSC cells that have been RNAi depleted of mRNAs
+                   encoding pasilla, a mRNA binding protein and 4 biological replicates
+                   of the the untreated cell line.")
>              pubMedIds(expdata) <- "20921232"
> design <- data.frame(
+               condition=c("treated", "untreated", "untreated"),
+               replicate=c(1,1,2),
+               type=rep("single-read", 3),
+               countfiles=colData(se_exons)[,1], stringsAsFactors=TRUE)
> pasillaECS <- newExonCountSet(
+                   countData=assays(se_exons)$counts,
+                   design=design,
```

```
+                   exonIDs=factor(exonID),
+                   geneIDs=factor(geneID))
> experimentData(pasillaECS) <- expdata
> sampleNames(pasillaECS) = colnames(se_exons)
```

5.3 Gene counts

The *CountDataSet* will hold counts for the aggregate gene regions. The counts can be obtained by summing the exon hits by gene id using `geneCountTable` in DEXSeq,

```
> genetable = geneCountTable(pasillaECS)
> pasillaCDS = newCountDataSet(countData=genetable, conditions=design)
> experimentData(pasillaCDS) = expdata
```

If the primary interest was in counts per gene and counts per exon were not needed an alternative approach could be taken. The annotation file could be subset on "aggregate_gene" ranges and then counted.

```
> genes <- features[values(features)[["type"]] == "aggregate_gene"]
> se_genes <- summarizeOverlaps(genes, bamFiles, mode="Union")
> pasillaCDS_alt <- newCountDataSet(countData=assays(se_genes)$counts,
+     conditions=design)
> experimentData(pasillaCDS_alt) = expdata
```

6 Refererences

`http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html` `http://www-huber.embl.de/users/anders/HTSeq/doc/count.html`