

# Counting reads with `summarizeOverlaps`

*Valerie Obenchain*

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### 1 Introduction

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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

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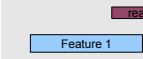
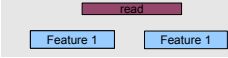
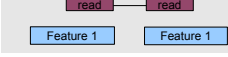
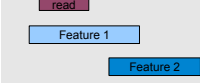


In this example reads are counted from a list of BAM files and returned in a `matrix` for use in further analysis such as those offered in `DESeq2` and `edgeR`.

```
> library(GenomicAlignments)
> library(DESeq2)
> library(edgeR)
> fls <- list.files(system.file("extdata", package="GenomicAlignments"),
+   recursive=TRUE, pattern="*bam$", full=TRUE)
> features <- GRanges(
+   seqnames = c(rep("chr2L", 4), rep("chr2R", 5), rep("chr3L", 2)),
+   ranges = IRanges(c(1000, 3000, 4000, 7000, 2000, 3000, 3600, 4000,
+     7500, 5000, 5400), width=c(rep(500, 3), 600, 900, 500, 300, 900,
+     300, 500, 500)), "-"),
+   group_id=c(rep("A", 4), rep("B", 5), rep("C", 2)))
> olap <- summarizeOverlaps(features, fls)
> deseq <- DESeqDataSet(olap, design= ~ 1)
> edger <- DGEList(assay(olap), group=rownames(colData(olap)))
```

By default, the `summarizeOverlaps` function iterates through files in 'chunks' and with files processed in parallel. For finer-grain control over memory consumption, use the `BamFileList` function and specify the `yieldSize` argument (e.g., `yieldSize=1000000`) to determine the size of each 'chunk' (smaller chunks consume less memory, but are a little less efficient to process). For controlling the number of processors in use, use `BiocParallel::register` to use an appropriate back-end, e.g., in linux or Mac to process on 6 cores of a single machine use `register(MulticoreParam(workers=6))`; see the [BiocParallel](#) vignette for further details.

### 3 Counting Modes

The modes of "Union", "IntersectionStrict" and "IntersectionNotEmpty" provide different approaches to resolving reads that overlap multiple features. Figure ?? 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. For additional detail on the counting modes see the `summarizeOverlaps` man page.

	Union	IntersectionStrict	IntersectionNotEmpty
	Feature 1	Feature 1	Feature 1
	Feature 1	No hit	Feature 1
	Feature 1	No hit	Feature 1
	Feature 1	Feature 1	Feature 1
	Feature 1	Feature 1	Feature 1
	No hit	Feature 1	Feature 1
	No hit	No hit	No hit

\* 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. Regardless 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 <- GAlignments("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 because 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`. `IntersectionNotEmpty` 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 = assay(summarizeOverlaps(gr, rd)),
+   intStrict = assay(summarizeOverlaps(gr, rd,
+   mode="IntersectionStrict")),
+   intNotEmpty = assay(summarizeOverlaps(gr, rd,
+   mode="IntersectionNotEmpty")))
  reads reads.1 reads.2
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 = assay(summarizeOverlaps(grl, rd)),
+   intStrict = assay(summarizeOverlaps(grl, rd,
+   mode="IntersectionStrict")),
```

## Counting reads with `summarizeOverlaps`

```
+      intNotEmpty = assay(summarizeOverlaps(grl, rd,  
+      mode="IntersectionNotEmpty"))  
  
reads reads.1 reads.2  
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 <- GAlignments(  
+   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 = assay(summarizeOverlaps(features, reads)),  
+   intStrict = assay(summarizeOverlaps(features, reads,  
+   mode="IntersectionStrict")),  
+   intNotEmpty = assay(summarizeOverlaps(features, reads,  
+   mode="IntersectionNotEmpty"))  
  
reads reads.1 reads.2  
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.

## Counting reads with `summarizeOverlaps`

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

[1] 8
```

```
> data.frame(union = assay(summarizeOverlaps(lst, reads)),
+           intStrict = assay(summarizeOverlaps(lst, reads,
+           mode="IntersectionStrict")),
+           intNotEmpty = assay(summarizeOverlaps(lst, reads,
+           mode="IntersectionNotEmpty")))

  reads reads.1 reads.2
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 *RangedSummarizedExperiment* object. See `?BamViews-class` or `?BamFile-class` in the *Rsamtools* package.

## 5 pasilla Data

In this exercise we count the *pasilla* data by gene and by transcript then create a *DESeq-DataSet*. This object can be used in differential expression methods offered in the *DESeq2* package.

### 5.1 source files

Files are available through NCBI Gene Expression Omnibus (GEO), accession number GSE18508. <http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE18508>. SAM files can be converted to BAM with the `asBam` function in the *Rsamtools* package. Of the seven files available, 3 are single-reads and 4 are paired-end. Smaller versions of untreated1 (single-end) and untreated2 (paired-end) have been made available in the *pasillaBamSubset* package. This subset includes chromosome 4 only.

`summarizeOverlaps` is capable of counting paired-end reads in both a `BamFile`-method (set argument `singleEnd=TRUE`) or a `GAlignmentPairs`-method. For this example, we use the 3 single-end read files,

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

Annotations are retrieved as a GTF file from the ENSEMBL web site. We download the file our local disk, then use *Rtracklayer*'s `import` function to parse the file to a *GRanges* instance.

## Counting reads with `summarizeOverlaps`

```
> library(rtracklayer)
> fl <- paste0("ftp://ftp.ensembl.org/pub/release-62/",
+             "gtf/drosophila_melanogaster/",
+             "Drosophila_melanogaster.BDGP5.25.62.gtf.gz")
> gffFile <- file.path(tempdir(), basename(fl))
> download.file(fl, gffFile)
> gff0 <- import(gffFile)
```

Subset on the protein-coding, exon regions of chromosome 4 and split by gene id.

```
> idx <- mcols(gff0)$source == "protein_coding" &
+       mcols(gff0)$type == "exon" &
+       seqnames(gff0) == "4"
> gff <- gff0[idx]
> ## adjust seqnames to match Bam files
> seqlevels(gff) <- paste("chr", seqlevels(gff), sep="")
> chr4genes <- split(gff, mcols(gff)$gene_id)
```

## 5.2 counting

The `param` 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("chr4", IRanges(1, 1e6)),
+   flag=scanBamFlag(isUnmappedQuery=FALSE, isPaired=NA),
+   tag="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"
> bamlst <- BamFileList(fls)
> genehits <- summarizeOverlaps(chr4genes, bamlst, mode="Union")
```

A `CountDataSet` is constructed from the counts and experiment data in *pasilla*.

```
> expdata <- 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",
+   pubMedIds="20921232",
+   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
```

## Counting reads with `summarizeOverlaps`

```
+           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.")
> design <- data.frame(
+           condition=c("treated", "untreated", "untreated"),
+           replicate=c(1,1,2),
+           type=rep("single-read", 3),
+           countfiles=path(colData(genehits)[,1]), stringsAsFactors=TRUE)
> geneCDS <- DESeqDataSet(genehits, design=design, metadata=list(expdata=expdata))
```

If the primary interest is to count by transcript instead of by gene, the annotation file can be split on transcript id.

```
> chr4tx <- split(gff, mcols(gff)$transcript_id)
> txhits <- summarizeOverlaps(chr4tx, bamlst)
> txCDS <- DESeqDataSet(txhits, design=design, metadata=list(expdata=expdata))
```

## 6 References

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