

Overlap encodings

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1 Introduction

In the context of an RNA-seq experiment, encoding the overlaps between the aligned reads and the transcripts can be used for detecting those overlaps that are “splice compatible”, that is, compatible with the splicing of the transcript.

Various tools are provided in the *GenomicAlignments* package for working with *overlap encodings*. In this vignette, we illustrate the use of these tools on the single-end and paired-end reads of an RNA-seq experiment.

2 Load reads from a BAM file

2.1 Load single-end reads from a BAM file

BAM file `untreated1_chr4.bam` (located in the *pasillaBamSubset* data package) contains single-end reads from the “Pasilla” experiment and aligned against the dm3 genome (see `?untreated1_chr4` in the *pasillaBamSubset* package for more information about those reads):

```
> library(pasillaBamSubset)
> untreated1_chr4()

[1] "/home/biocbuild/bbs-3.22-bioc/R/site-library/pasillaBamSubset/extdata/untreated1_chr4.bam"
```

We use the `readGAlignments` function defined in the *GenomicAlignments* package to load the reads into a *GAlignments* object. It’s probably a good idea to get rid of the PCR or optical duplicates (flag bit 0x400 in the SAM format, see the SAM Spec ¹ for the details),

¹<http://samtools.sourceforge.net/>

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as well as reads not passing quality controls (flag bit 0x200 in the SAM format). We do this by creating a `ScanBamParam` object that we pass to `readGAlignments` (see `?ScanBamParam` in the `Rsamtools` package for the details). Note that we also use `use.names=TRUE` in order to load the *query names* (aka *query template names*, see QNAME field in the SAM Spec) from the BAM file (`readGAlignments` will use them to set the names of the returned object):

```
> library(GenomicAlignments)
> flag0 <- scanBamFlag(isDuplicate=FALSE, isNotPassingQualityControls=FALSE)
> param0 <- ScanBamParam(flag=flag0)
> U1.GAL <- readGAlignments(untreated1_chr4(), use.names=TRUE, param=param0)
> head(U1.GAL)
```

GAlignments object with 6 alignments and 0 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	njunc
	<Rle>	<Rle>	<character>	<integer>	<integer>	<integer>	<integer>	<integer>
SRR031729.3941844	chr4	-	75M	75	892	966	75	0
SRR031728.3674563	chr4	-	75M	75	919	993	75	0
SRR031729.8532600	chr4	+	75M	75	924	998	75	0
SRR031729.2779333	chr4	+	75M	75	936	1010	75	0
SRR031728.2826481	chr4	+	75M	75	949	1023	75	0
SRR031728.2919098	chr4	-	75M	75	967	1041	75	0

seqinfo: 8 sequences from an unspecified genome

Because the aligner used to align those reads can report more than 1 alignment per *original query* (i.e. per read stored in the input file, typically a FASTQ file), we shouldn't expect the names of `U1.GAL` to be unique:

```
> U1.GAL_names_is_dup <- duplicated(names(U1.GAL))
> table(U1.GAL_names_is_dup)
```

U1.GAL_names_is_dup
FALSE TRUE
190770 13585

Storing the *query names* in a factor will be useful as we will see later in this document:

```
> U1.uqnames <- unique(names(U1.GAL))
> U1.GAL_qnames <- factor(names(U1.GAL), levels=U1.uqnames)
```

Note that we explicitly provide the levels of the factor to enforce their order. Otherwise `factor()` would put them in lexicographic order which is not advisable because it depends on the locale in use.

Another object that will be useful to keep near at hand is the mapping between each *query name* and its first occurrence in `U1.GAL_qnames`:

```
> U1.GAL_dup2uniq <- match(U1.GAL_qnames, U1.GAL_qnames)
```

Our reads can have up to 2 *skipped regions* (a *skipped region* corresponds to an N operation in the CIGAR):

```
> head(unique(cigar(U1.GAL)))
```

[1] "75M" "35M6727N40M" "22M6727N53M" "13M6727N62M" "26M292N49M" "62M21227N13M"

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```
> table(njunc(U1.GAL))
      0      1      2
184039 20169  147
```

Also, the following table indicates that indels were not allowed/supported during the alignment process (no I or D CIGAR operations):

```
> colSums(cigarOpTable(cigar(U1.GAL)))
      M      I      D      N      S      H      P      =      X
15326625      0      0 21682582      0      0      0      0      0
```

2.2 Load paired-end reads from a BAM file

BAM file `untreated3_chr4.bam` (located in the `pasillaBamSubset` data package) contains paired-end reads from the “Pasilla” experiment and aligned against the dm3 genome (see `?untreated3_chr4` in the `pasillaBamSubset` package for more information about those reads). We use the `readGAlignmentPairs` function to load them into a `GAlignmentPairs` object:

```
> U3.galp <- readGAlignmentPairs(untreated3_chr4(), use.names=TRUE, param=param0)
> head(U3.galp)
```

GAlignmentPairs object with 6 pairs, strandMode=1, and 0 metadata columns:

```
      seqnames strand :      ranges --      ranges
      <Rle>  <Rle> : <IRanges> -- <IRanges>
SRR031715.1138209 chr4   + : 169-205 -- 326-362
SRR031714.756385  chr4   + : 943-979 -- 1086-1122
SRR031714.2355189 chr4   + : 944-980 -- 1119-1155
SRR031714.5054563 chr4   + : 946-982 -- 986-1022
SRR031715.1722593 chr4   + : 966-1002 -- 1108-1144
SRR031715.2202469 chr4   + : 966-1002 -- 1114-1150
```

```
-----
seqinfo: 8 sequences from an unspecified genome
```

The `show` method for `GAlignmentPairs` objects displays two ranges columns, one for the *first* alignment in the pair (the left column), and one for the *last* alignment in the pair (the right column). The strand column corresponds to the strand of the *first* alignment.

```
> head(first(U3.galp))
```

GAlignments object with 6 alignments and 0 metadata columns:

```
      seqnames strand      cigar      qwidth      start      end      width      njunc
      <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer>
SRR031715.1138209 chr4   +      37M      37      169      205      37      0
SRR031714.756385  chr4   +      37M      37      943      979      37      0
SRR031714.2355189 chr4   +      37M      37      944      980      37      0
SRR031714.5054563 chr4   +      37M      37      946      982      37      0
SRR031715.1722593 chr4   +      37M      37      966     1002      37      0
SRR031715.2202469 chr4   +      37M      37      966     1002      37      0
```

```
-----
seqinfo: 8 sequences from an unspecified genome
```

```
> head(last(U3.galp))
```

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```
GAlignments object with 6 alignments and 0 metadata columns:
      seqnames strand      cigar  qwidth  start  end  width  njunc
      <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer>
SRR031715.1138209 chr4 -      37M      37      326   362    37      0
SRR031714.756385  chr4 -      37M      37      1086  1122   37      0
SRR031714.2355189 chr4 -      37M      37      1119  1155   37      0
SRR031714.5054563 chr4 -      37M      37      986   1022   37      0
SRR031715.1722593 chr4 -      37M      37      1108  1144   37      0
SRR031715.2202469 chr4 -      37M      37      1114  1150   37      0
-----
seqinfo: 8 sequences from an unspecified genome
```

According to the SAM format specifications, the aligner is expected to mark each alignment pair as *proper* or not (flag bit 0x2 in the SAM format). The SAM Spec only says that a pair is *proper* if the *first* and *last* alignments in the pair are “properly aligned according to the aligner”. So the exact criteria used for setting this flag is left to the aligner.

We use `isProperPair` to extract this flag from the `GAlignmentPairs` object:

```
> table(isProperPair(U3.galp))
FALSE TRUE
29581 45828
```

Even though we could do *overlap encodings* with the full object, we keep only the *proper* pairs for our downstream analysis:

```
> U3.GALP <- U3.galp[isProperPair(U3.galp)]
```

Because the aligner used to align those reads can report more than 1 alignment per *original query template* (i.e. per pair of sequences stored in the input files, typically 1 FASTQ file for the *first* ends and 1 FASTQ file for the *last* ends), we shouldn't expect the names of `U3.GALP` to be unique:

```
> U3.GALP_names_is_dup <- duplicated(names(U3.GALP))
> table(U3.GALP_names_is_dup)
U3.GALP_names_is_dup
FALSE TRUE
43659 2169
```

Storing the *query template names* in a factor will be useful:

```
> U3.uqnames <- unique(names(U3.GALP))
> U3.GALP_qnames <- factor(names(U3.GALP), levels=U3.uqnames)
```

as well as having the mapping between each *query template name* and its first occurrence in `U3.GALP_qnames`:

```
> U3.GALP_dup2unq <- match(U3.GALP_qnames, U3.GALP_qnames)
```

Our reads can have up to 1 *skipped region* per end:

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```
> head(unique(cigar(first(U3.GALP))))
[1] "37M"      "6M58N31M" "25M56N12M" "19M62N18M" "29M222N8M" "9M222N28M"
> head(unique(cigar(last(U3.GALP))))
[1] "37M"      "19M58N18M" "12M58N25M" "27M2339N10M" "29M2339N8M" "9M222N28M"
> table(njunc(first(U3.GALP)), njunc(last(U3.GALP)))
      0      1
0 44510  596
1   637   85
```

Like for our single-end reads, the following tables indicate that indels were not allowed/supported during the alignment process:

```
> colSums(cigarOpTable(cigar(first(U3.GALP))))
      M      I      D      N      S      H      P      =      X
1695636  0      0 673919  0      0      0      0      0
> colSums(cigarOpTable(cigar(last(U3.GALP))))
      M      I      D      N      S      H      P      =      X
1695636  0      0 630395  0      0      0      0      0
```

3 Find all the overlaps between the reads and transcripts

3.1 Load the transcripts from a *TxDB* object

In order to compute overlaps between reads and transcripts, we need access to the genomic positions of a set of known transcripts and their exons. It is essential that the reference genome of this set of transcripts and exons be **exactly** the same as the reference genome used to align the reads.

We could use the `makeTxDbFromUCSC` function defined in the *GenomicFeatures* package to make a *TxDB* object containing the dm3 transcripts and their exons retrieved from the UCSC Genome Browser². The Bioconductor project however provides a few annotation packages containing *TxDB* objects for the most commonly studied organisms (those data packages are sometimes called the *TxDB* packages). One of them is the *TxDB.Dmelanogaster.UCSC.-dm3.ensGene* package. It contains a *TxDB* object that was made by pointing the `makeTxDbFromUCSC` function to the dm3 genome and *Ensembl Genes* track³. We can use it here:

```
> library(TxDB.Dmelanogaster.UCSC.dm3.ensGene)
> TxDb.Dmelanogaster.UCSC.dm3.ensGene

TxDb object:
# Db type: TxDb
# Supporting package: GenomicFeatures
```

²<http://genome.ucsc.edu/cgi-bin/hgGateway>

³See <http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgside=276880911&g=ensGene> for a description of this track.

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```
# Data source: UCSC
# Genome: dm3
# Organism: Drosophila melanogaster
# Taxonomy ID: 7227
# UCSC Table: ensGene
# Resource URL: http://genome.ucsc.edu/
# Type of Gene ID: Ensembl gene ID
# Full dataset: yes
# miRBase build ID: NA
# transcript_nrow: 29173
# exon_nrow: 76920
# cds_nrow: 62135
# Db created by: GenomicFeatures package from Bioconductor
# Creation time: 2015-10-07 18:15:53 +0000 (Wed, 07 Oct 2015)
# GenomicFeatures version at creation time: 1.21.30
# RSQLite version at creation time: 1.0.0
# DBSCHEMAVERSION: 1.1

> txdb <- TxDb.Dmelanogaster.UCSC.dm3.ensGene
```

We extract the exons grouped by transcript in a *GRangesList* object:

```
> exbytx <- exonsBy(txdb, by="tx", use.names=TRUE)
> length(exbytx) # nb of transcripts

[1] 29173
```

We check that all the exons in any given transcript belong to the same chromosome and strand. Knowing that our set of transcripts is free of this sort of trans-splicing events typically allows some significant simplifications during the downstream analysis ⁴. A quick and easy way to check this is to take advantage of the fact that `seqnames` and `strand` return *RleList* objects. So we can extract the number of *Rle* runs for each transcript and make sure it's always 1:

```
> table(elementNROWS(runLength(seqnames(exbytx))))
 1
29173
> table(elementNROWS(runLength(strand(exbytx))))
 1
29173
```

Therefore the strand of any given transcript is unambiguously defined and can be extracted with:

```
> exbytx_strand <- unlist(runValue(strand(exbytx)), use.names=FALSE)
```

We will also need the mapping between the transcripts and their gene. We start by using `transcripts` to extract this information from our *TxDb* object `txdb`, and then we construct a named factor that represents the mapping:

⁴Dealing with trans-splicing events is not covered in this document.

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```
> tx <- transcripts(txdb, columns=c("tx_name", "gene_id"))
> head(tx)

GRanges object with 6 ranges and 2 metadata columns:
      seqnames      ranges strand |      tx_name      gene_id
      <Rle>      <IRanges> <Rle> | <character> <CharacterList>
 [1] chr2L      7529-9484      + | FBtr0300689    FBgn0031208
 [2] chr2L      7529-9484      + | FBtr0300690    FBgn0031208
 [3] chr2L      7529-9484      + | FBtr0330654    FBgn0031208
 [4] chr2L 21952-24237      + | FBtr0309810    FBgn0263584
 [5] chr2L 66584-71390      + | FBtr0306539    FBgn0067779
 [6] chr2L 67043-71081      + | FBtr0306536    FBgn0067779
-----
seqinfo: 15 sequences (1 circular) from dm3 genome

> df <- mcols(tx)
> exbytx2gene <- as.character(df$gene_id)
> exbytx2gene <- factor(exbytx2gene, levels=unique(exbytx2gene))
> names(exbytx2gene) <- df$tx_name
> exbytx2gene <- exbytx2gene[names(exbytx)]
> head(exbytx2gene)

FBtr0300689 FBtr0300690 FBtr0330654 FBtr0309810 FBtr0306539 FBtr0306536
FBgn0031208 FBgn0031208 FBgn0031208 FBgn0263584 FBgn0067779 FBgn0067779
15682 Levels: FBgn0031208 FBgn0263584 FBgn0067779 FBgn0031213 FBgn0031214 FBgn0031216 ... FBgn0264003

> nlevels(exbytx2gene) # nb of genes

[1] 15682
```

3.2 Single-end overlaps

3.2.1 Find the single-end overlaps

We are ready to compute the overlaps with the `findOverlaps` function. Note that the strand of the queries produced by the RNA-seq experiment is typically unknown so we use `ignore.strand=TRUE`:

```
> U1.OV00 <- findOverlaps(U1.GAL, exbytx, ignore.strand=TRUE)
```

`U1.OV00` is a `Hits` object that contains 1 element per overlap. Its length gives the number of overlaps:

```
> length(U1.OV00)
```

```
[1] 563552
```

3.2.2 Tabulate the single-end overlaps

We will repeatedly use the 2 following little helper functions to “tabulate” the overlaps in a given `Hits` object (e.g. `U1.OV00`), i.e. to count the number of overlaps for each element in the query or for each element in the subject:

Number of transcripts for each alignment in `U1.GAL`:

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```
> U1.GAL_ntx <- countQueryHits(U1.OV00)
> mcols(U1.GAL)$ntx <- U1.GAL_ntx
> head(U1.GAL)
```

GAlignments object with 6 alignments and 1 metadata column:

	seqnames	strand	cigar	qwidth	start	end	width	njunc
	<Rle>	<Rle>	<character>	<integer>	<integer>	<integer>	<integer>	<integer>
SRR031729.3941844	chr4	-	75M	75	892	966	75	0
SRR031728.3674563	chr4	-	75M	75	919	993	75	0
SRR031729.8532600	chr4	+	75M	75	924	998	75	0
SRR031729.2779333	chr4	+	75M	75	936	1010	75	0
SRR031728.2826481	chr4	+	75M	75	949	1023	75	0
SRR031728.2919098	chr4	-	75M	75	967	1041	75	0

```
      ntx
      <integer>
SRR031729.3941844      0
SRR031728.3674563      0
SRR031729.8532600      0
SRR031729.2779333      0
SRR031728.2826481      0
SRR031728.2919098      0
```

```
-----
seqinfo: 8 sequences from an unspecified genome
```

```
> table(U1.GAL_ntx)
```

```
U1.GAL_ntx
  0    1    2    3    4    5    6    7    8    9   10   11   12
47076 9493 26146 82427 5291 14530 8158 610 1952 2099 492 4945 1136
```

```
> mean(U1.GAL_ntx >= 1)
```

```
[1] 0.7696362
```

76% of the alignments in `U1.GAL` have an overlap with at least 1 transcript in `exbytx`.

Note that `countOverlaps` can be used directly on `U1.GAL` and `exbytx` for computing `U1.GAL_ntx`:

```
> U1.GAL_ntx_again <- countOverlaps(U1.GAL, exbytx, ignore.strand=TRUE)
> stopifnot(identical(unname(U1.GAL_ntx_again), U1.GAL_ntx))
```

Because `U1.GAL` can (and actually does) contain more than 1 alignment per *original query* (aka read), we also count the number of transcripts for each read:

```
> U1.OV10 <- remapHits(U1.OV00, Lnodes.remapping=U1.GAL_qnames)
> U1.uqnames_ntx <- countQueryHits(U1.OV10)
> names(U1.uqnames_ntx) <- U1.uqnames
> table(U1.uqnames_ntx)
```

```
U1.uqnames_ntx
  0    1    2    3    4    5    6    7    8    9   10   11   12
39503 9298 18394 82346 5278 14536 9208 610 2930 2099 488 4944 1136
```

```
> mean(U1.uqnames_ntx >= 1)
```

```
[1] 0.7929287
```

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78.4% of the reads have an overlap with at least 1 transcript in `exbytx`.

Number of reads for each transcript:

```
> U1.exbytx_nOV10 <- countSubjectHits(U1.OV10)
> names(U1.exbytx_nOV10) <- names(exbytx)
> mean(U1.exbytx_nOV10 >= 50)

[1] 0.009015185
```

Only 0.869% of the transcripts in `exbytx` have an overlap with at least 50 reads.

Top 10 transcripts:

```
> head(sort(U1.exbytx_nOV10, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0112904 FBtr0289951 FBtr0089243 FBtr0333672 FBtr0089186
  40654      40529      40529      11735      11661      11656      10087      10084
FBtr0089187 FBtr0089172
 10084      6749
```

3.3 Paired-end overlaps

3.3.1 Find the paired-end overlaps

Like with our single-end overlaps, we call `findOverlaps` with `ignore.strand=TRUE`:

```
> U3.OV00 <- findOverlaps(U3.GALP, exbytx, ignore.strand=TRUE)
```

Like `U1.OV00`, `U3.OV00` is a *Hits* object. Its length gives the number of paired-end overlaps:

```
> length(U3.OV00)

[1] 113827
```

3.3.2 Tabulate the paired-end overlaps

Number of transcripts for each alignment pair in `U3.GALP`:

```
> U3.GALP_ntx <- countQueryHits(U3.OV00)
> mcols(U3.GALP)$ntx <- U3.GALP_ntx
> head(U3.GALP)

GAlignmentPairs object with 6 pairs, strandMode=1, and 1 metadata column:
      seqnames strand :   ranges --   ranges |      ntx
      <Rle>  <Rle> : <IRanges> -- <IRanges> | <integer>
SRR031715.1138209 chr4   + : 169-205 -- 326-362 |      0
  SRR031714.756385 chr4   + : 943-979 -- 1086-1122 |      0
SRR031714.5054563 chr4   + : 946-982 -- 986-1022 |      0
SRR031715.1722593 chr4   + : 966-1002 -- 1108-1144 |      0
SRR031715.2202469 chr4   + : 966-1002 -- 1114-1150 |      0
SRR031714.3544437 chr4   - : 1087-1123 -- 963-999 |      0
-----
seqinfo: 8 sequences from an unspecified genome

> table(U3.GALP_ntx)
```

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```
U3.GALP_ntx
  0    1    2    3    4    5    6    7    8    9   10   11   12
12950 2080 5854 17025 1078 3083 2021  70  338  370  59  803  97

> mean(U3.GALP_ntx >= 1)

[1] 0.7174217
```

71% of the alignment pairs in U3.GALP have an overlap with at least 1 transcript in `exbytx`.

Note that `countOverlaps` can be used directly on U3.GALP and `exbytx` for computing U3.GALP_ntx:

```
> U3.GALP_ntx_again <- countOverlaps(U3.GALP, exbytx, ignore.strand=TRUE)
> stopifnot(identical(unname(U3.GALP_ntx_again), U3.GALP_ntx))
```

Because U3.GALP can (and actually does) contain more than 1 alignment pair per *original query template*, we also count the number of transcripts for each template:

```
> U3.OV10 <- remapHits(U3.OV00, Lnodes.remapping=U3.GALP_qnames)
> U3.uqnames_ntx <- countQueryHits(U3.OV10)
> names(U3.uqnames_ntx) <- U3.uqnames
> table(U3.uqnames_ntx)

U3.uqnames_ntx
  0    1    2    3    4    5    6    7    8    9   10   11   12
11851 2061 4289 17025 1193 3084 2271  70  486  370  59  803  97

> mean(U3.uqnames_ntx >= 1)

[1] 0.7285554
```

72.3% of the templates have an overlap with at least 1 transcript in `exbytx`.

Number of templates for each transcript:

```
> U3.exbytx_nOV10 <- countSubjectHits(U3.OV10)
> names(U3.exbytx_nOV10) <- names(exbytx)
> mean(U3.exbytx_nOV10 >= 50)

[1] 0.00712988
```

Only 0.756% of the transcripts in `exbytx` have an overlap with at least 50 templates.

Top 10 transcripts:

```
> head(sort(U3.exbytx_nOV10, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0112904 FBtr0089243 FBtr0289951 FBtr0333672 FBtr0089186
  7574      7573      7572      2750      2732      2732      2260      2260
FBtr0089187 FBtr0310542
  2260      1698
```

4 Encode the overlaps between the reads and transcripts

4.1 Single-end encodings

The *overlap encodings* are strand sensitive so we will compute them twice, once for the “original alignments” (i.e. the alignments of the *original queries*), and once again for the “flipped alignments” (i.e. the alignments of the “flipped *original queries*”). We extract the ranges of the “original” and “flipped” alignments in 2 *GRangesList* objects with:

```
> U1.grl <- grglist(U1.GAL, order.as.in.query=TRUE)
> U1.grlf <- flipQuery(U1.grl) # flipped
```

and encode their overlaps with the transcripts:

```
> U1.ovencA <- encodeOverlaps(U1.grl, exbytx, hits=U1.OV00)
> U1.ovencB <- encodeOverlaps(U1.grlf, exbytx, hits=U1.OV00)
```

`U1.ovencA` and `U1.ovencB` are 2 *OverlapsEncodings* objects of the same length as *Hits* object `U1.OV00`. For each hit in `U1.OV00`, we have 2 corresponding encodings, one in `U1.ovencA` and one in `U1.ovencB`, but only one of them encodes a hit between alignment ranges and exon ranges that are on the same strand. We use the `selectEncodingWithCompatibleStrand` function to merge them into a single *OverlapsEncodings* of the same length. For each hit in `U1.OV00`, this selects the encoding corresponding to alignment ranges and exon ranges with compatible strand:

```
> U1.grl_strand <- unlist(runValue(strand(U1.grl)), use.names=FALSE)
> U1.ovenc <- selectEncodingWithCompatibleStrand(U1.ovencA, U1.ovencB,
+                                               U1.grl_strand, exbytx_strand,
+                                               hits=U1.OV00)
> U1.ovenc
```

OverlapEncodings object of length 563552 with 0 metadata columns:

	Loffset	Roffset	encoding	flippedQuery
	<integer>	<integer>	<factor>	<logical>
[1]	0	3	1:i:	TRUE
[2]	4	0	1:k:	FALSE
[3]	4	0	1:k:	TRUE
[4]	4	0	1:k:	TRUE
[5]	4	0	1:k:	TRUE
...
[563548]	22	0	1:i:	TRUE
[563549]	23	0	1:i:	TRUE
[563550]	24	0	1:i:	TRUE
[563551]	24	0	1:i:	TRUE
[563552]	23	0	1:i:	TRUE

As a convenience, the 2 above calls to `encodeOverlaps` + merging step can be replaced by a single call to `encodeOverlaps` on `U1.grl` (or `U1.grlf`) with `flip.query.if.wrong.strand=TRUE`:

```
> U1.ovenc_again <- encodeOverlaps(U1.grl, exbytx, hits=U1.OV00, flip.query.if.wrong.strand=TRUE)
> stopifnot(identical(U1.ovenc_again, U1.ovenc))
```

Overlap encodings

Unique encodings in U1.ovenc:

```
> U1.unique_encodings <- levels(U1.ovenc)
> length(U1.unique_encodings)

[1] 120

> head(U1.unique_encodings)

[1] "1:c:" "1:e:" "1:f:" "1:h:" "1:i:" "1:j:"

> U1.ovenc_table <- table(encoding(U1.ovenc))
> tail(sort(U1.ovenc_table))

      1:f:   1:k:c:   1:k:   1:c: 2:jm:af:   1:i:
      1555   1889   8800   9523   72929  455176
```

Encodings are sort of cryptic but utilities are provided to extract specific meaning from them. Use of these utilities is covered later in this document.

4.2 Paired-end encodings

Let's encode the overlaps in U3.OV00:

```
> U3.grl <- grglist(U3.GALP)
> U3.ovenc <- encodeOverlaps(U3.grl, exbytx, hits=U3.OV00, flip.query.if.wrong.strand=TRUE)
> U3.ovenc
```

OverlapEncodings object of length 113827 with 0 metadata columns:

	Loffset	Roffset	encoding	flippedQuery
	<integer>	<integer>	<factor>	<logical>
[1]	4	0	1--1:i--k:	TRUE
[2]	4	0	1--1:i--i:	TRUE
[3]	4	0	1--1:i--k:	FALSE
[4]	4	0	1--1:i--k:	FALSE
[5]	4	0	1--1:a--c:	TRUE
...
[113823]	22	0	1--1:i--i:	TRUE
[113824]	23	0	1--1:i--i:	TRUE
[113825]	24	0	1--1:i--i:	TRUE
[113826]	24	0	1--1:i--i:	TRUE
[113827]	23	0	1--1:i--i:	TRUE

Unique encodings in U3.ovenc:

```
> U3.unique_encodings <- levels(U3.ovenc)
> length(U3.unique_encodings)

[1] 123

> head(U3.unique_encodings)

[1] "1--1:a--c:" "1--1:a--i:" "1--1:a--j:" "1--1:a--k:" "1--1:b--i:" "1--1:b--k:"

> U3.ovenc_table <- table(encoding(U3.ovenc))
> tail(sort(U3.ovenc_table))
```

Overlap encodings

```
1--1:i--m:      1--1:i--k:      1--1:c--i: 1--2:i--jm:a--af: 2--1:jm--m:af--i:
      852              1485              1714              2480              2700
1--1:i--i:
      100084
```

5 Detect “splice compatible” overlaps

We are interested in a particular type of overlap where the read overlaps the transcript in a “splice compatible” way, that is, in a way that is compatible with the splicing of the transcript. The `isCompatibleWithSplicing` function can be used on an `OverlapEncodings` object to detect this type of overlap. Note that `isCompatibleWithSplicing` can also be used on a character vector or factor.

5.1 Detect “splice compatible” single-end overlaps

5.1.1 “Splice compatible” single-end encodings

`U1.ovenc` contains 7 unique encodings compatible with the splicing of the transcript:

```
> sort(U1.ovenc_table[isCompatibleWithSplicing(U1.unique_encodings)])
      2:jm:ag:      2:gm:af: 3:jmm:agm:aaf:      1:j:      1:f:      2:jm:af:
      32              79              488              1538              1555              72929
      1:i:
      455176
```

Encodings `"1:i:"` (455176 occurrences in `U1.ovenc`), `"2:jm:af:"` (72929 occurrences in `U1.ovenc`), and `"3:jmm:agm:aaf:"` (488 occurrences in `U1.ovenc`), correspond to the following overlaps:

- `"1:i:"`
 - read (no skipped region): 00000000
 - transcript: ... >>>>>>>>>>>>>>>>>>>>>> ...
- `"2:jm:af:"`
 - read (1 skipped region): 00000---000
 - transcript: ... >>>>>>>>>>>>>>>>>>>>>> ...
- `"3:jmm:agm:aaf:"`
 - read (2 skipped regions): 00---00000---0
 - transcript: ... >>>>>>>>>>>>>>>>>>>>>> ...

For clarity, only the exons involved in the overlap are represented. The transcript can of course have more upstream and downstream exons, which is denoted by the ... on the left side (5' end) and right side (3' end) of each drawing. Note that the exons represented in the 2nd and 3rd drawings are consecutive and adjacent in the processed transcript.

Encodings `"1:f:"` and `"1:j:"` are variations of the situation described by encoding `"1:i:"`. For `"1:f:"`, the first aligned base of the read (or “flipped” read) is aligned with the first base of the exon. For `"1:j:"`, the last aligned base of the read (or “flipped” read) is aligned with the last base of the exon:

- `"1:f:"`

Overlap encodings

```
- read (no skipped region):      00000000
- transcript:                    ... >>>>>>>>>>>> ...
```

- "1:j:"

```
- read (no skipped region):      00000000
- transcript:                    ... >>>>>>>>>>>> ...
```

```
> U1.OV00_is_comp <- isCompatibleWithSplicing(U1.ovenc)
> table(U1.OV00_is_comp) # 531797 "splice compatible" overlaps

U1.OV00_is_comp
FALSE  TRUE
31755 531797
```

Finally, let's extract the "splice compatible" overlaps from U1.OV00:

```
> U1.compOV00 <- U1.OV00[U1.OV00_is_comp]
```

Note that high-level convenience wrapper `findCompatibleOverlaps` can be used for computing the "splice compatible" overlaps directly between a `GAlignments` object (containing reads) and a `GRangesList` object (containing transcripts):

```
> U1.compOV00_again <- findCompatibleOverlaps(U1.GAL, exbytx)
> stopifnot(identical(U1.compOV00_again, U1.compOV00))
```

5.1.2 Tabulate the "splice compatible" single-end overlaps

Number of "splice compatible" transcripts for each alignment in U1.GAL:

```
> U1.GAL_ncomptx <- countQueryHits(U1.compOV00)
> mcols(U1.GAL)$ncomptx <- U1.GAL_ncomptx
> head(U1.GAL)
```

GAlignments object with 6 alignments and 2 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	njunc
	<Rle>	<Rle>	<character>	<integer>	<integer>	<integer>	<integer>	<integer>
SRR031729.3941844	chr4	-	75M	75	892	966	75	0
SRR031728.3674563	chr4	-	75M	75	919	993	75	0
SRR031729.8532600	chr4	+	75M	75	924	998	75	0
SRR031729.2779333	chr4	+	75M	75	936	1010	75	0
SRR031728.2826481	chr4	+	75M	75	949	1023	75	0
SRR031728.2919098	chr4	-	75M	75	967	1041	75	0

	ntx	ncomptx
	<integer>	<integer>
SRR031729.3941844	0	0
SRR031728.3674563	0	0
SRR031729.8532600	0	0
SRR031729.2779333	0	0
SRR031728.2826481	0	0
SRR031728.2919098	0	0

seqinfo: 8 sequences from an unspecified genome

```
> table(U1.GAL_ncomptx)
```

Overlap encodings

```
U1.GAL_ncomptx
  0    1    2    3    4    5    6    7    8    9   10   11   12
51101 9848 33697 72987 5034 14021 7516  581 1789 2015  530 4389  847

> mean(U1.GAL_ncomptx >= 1)

[1] 0.7499401
```

75% of the alignments in U1.GAL are “splice compatible” with at least 1 transcript in exbytx.

Note that high-level convenience wrapper `countCompatibleOverlaps` can be used directly on U1.GAL and exbytx for computing U1.GAL_ncomptx:

```
> U1.GAL_ncomptx_again <- countCompatibleOverlaps(U1.GAL, exbytx)
> stopifnot(identical(U1.GAL_ncomptx_again, U1.GAL_ncomptx))
```

Number of “splice compatible” transcripts for each read:

```
> U1.comp0V10 <- remapHits(U1.comp0V00, Lnodes.remapping=U1.GAL_qnames)
> U1.uqnames_ncomptx <- countQueryHits(U1.comp0V10)
> names(U1.uqnames_ncomptx) <- U1.uqnames
> table(U1.uqnames_ncomptx)

U1.uqnames_ncomptx
  0    1    2    3    4    5    6    7    8    9   10   11   12
42886 9711 26075 72989 5413 14044 8584  581 2706 2015  530 4389  847

> mean(U1.uqnames_ncomptx >= 1)

[1] 0.7751953
```

77.5% of the reads are “splice compatible” with at least 1 transcript in exbytx.

Number of “splice compatible” reads for each transcript:

```
> U1.exbytx_ncomp0V10 <- countSubjectHits(U1.comp0V10)
> names(U1.exbytx_ncomp0V10) <- names(exbytx)
> mean(U1.exbytx_ncomp0V10 >= 50)

[1] 0.008706681
```

Only 0.87% of the transcripts in exbytx are “splice compatible” with at least 50 reads.

Top 10 transcripts:

```
> head(sort(U1.exbytx_ncomp0V10, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0089243 FBtr0289951 FBtr0112904 FBtr0089186 FBtr0089187
  40309      40158      33490      11365      11332      11284      10018      9627
FBtr0333672 FBtr0089172
  9568      6599
```

Note that this “top 10” is slightly different from the “top 10” we obtained earlier when we counted **all** the overlaps.

Overlap encodings

```
- paired-end read (1 skipped region on the first end, 1 skipped region
  on the last end):          000---0  00---00
- transcript:                ... >>>>> >>>>>> >>>>> ...
```

Note: switch use of “first” and “last” above if the read was “flipped”.

```
> U3.OV00_is_comp <- isCompatibleWithSplicing(U3.ovenc)
> table(U3.OV00_is_comp) # 106835 "splice compatible" paired-end overlaps

U3.OV00_is_comp
FALSE  TRUE
 6928 106899
```

Finally, let’s extract the “splice compatible” paired-end overlaps from U3.OV00:

```
> U3.compOV00 <- U3.OV00[U3.OV00_is_comp]
```

Note that, like with our single-end reads, high-level convenience wrapper `findCompatibleOverlaps` can be used for computing the “splice compatible” paired-end overlaps directly between a `GAlignmentPairs` object (containing paired-end reads) and a `GRangesList` object (containing transcripts):

```
> U3.compOV00_again <- findCompatibleOverlaps(U3.GALP, exbytx)
> stopifnot(identical(U3.compOV00_again, U3.compOV00))
```

5.2.2 Tabulate the “splice compatible” paired-end overlaps

Number of “splice compatible” transcripts for each alignment pair in U3.GALP:

```
> U3.GALP_ncomptx <- countQueryHits(U3.compOV00)
> mcols(U3.GALP)$ncomptx <- U3.GALP_ncomptx
> head(U3.GALP)

GAlignmentPairs object with 6 pairs, strandMode=1, and 2 metadata columns:
      seqnames strand :   ranges --   ranges |      ntx  ncomptx
      <Rle>  <Rle> : <IRanges> -- <IRanges> | <integer> <integer>
SRR031715.1138209 chr4    + : 169-205 -- 326-362 |         0         0
SRR031714.756385  chr4    + : 943-979 -- 1086-1122 |         0         0
SRR031714.5054563 chr4    + : 946-982 -- 986-1022 |         0         0
SRR031715.1722593 chr4    + : 966-1002 -- 1108-1144 |         0         0
SRR031715.2202469 chr4    + : 966-1002 -- 1114-1150 |         0         0
SRR031714.3544437 chr4    - : 1087-1123 -- 963-999 |         0         0
-----
seqinfo: 8 sequences from an unspecified genome

> table(U3.GALP_ncomptx)

U3.GALP_ncomptx
  0    1    2    3    4    5    6    7    8    9   10   11   12
13884 2029 8094 14337 1099 2954 1865  84  296  332  89  699  66

> mean(U3.GALP_ncomptx >= 1)

[1] 0.6970411
```

Overlap encodings

69.7% of the alignment pairs in `U3.GALP` are “splice compatible” with at least 1 transcript in `exbytx`.

Note that high-level convenience wrapper `countCompatibleOverlaps` can be used directly on `U3.GALP` and `exbytx` for computing `U3.GALP_ncomptx`:

```
> U3.GALP_ncomptx_again <- countCompatibleOverlaps(U3.GALP, exbytx)
> stopifnot(identical(U3.GALP_ncomptx_again, U3.GALP_ncomptx))
```

Number of “splice compatible” transcripts for each template:

```
> U3.comp0V10 <- remapHits(U3.comp0V00, Lnodes.remapping=U3.GALP_qnames)
> U3.uqnames_ncomptx <- countQueryHits(U3.comp0V10)
> names(U3.uqnames_ncomptx) <- U3.uqnames
> table(U3.uqnames_ncomptx)
```

```
U3.uqnames_ncomptx
  0    1    2    3    4    5    6    7    8    9   10   11   12
12769 2027 6534 14337 1210 2954 2114   84  444  332   89  699   66
```

```
> mean(U3.uqnames_ncomptx >= 1)
```

```
[1] 0.7075288
```

70.7% of the templates are “splice compatible” with at least 1 transcript in `exbytx`.

Number of “splice compatible” templates for each transcript:

```
> U3.exbytx_ncomp0V10 <- countSubjectHits(U3.comp0V10)
> names(U3.exbytx_ncomp0V10) <- names(exbytx)
> mean(U3.exbytx_ncomp0V10 >= 50)
```

```
[1] 0.007061324
```

Only 0.7% of the transcripts in `exbytx` are “splice compatible” with at least 50 templates.

Top 10 transcripts:

```
> head(sort(U3.exbytx_ncomp0V10, decreasing=TRUE), n=10)
```

```
FBtr0308296 FBtr0089175 FBtr0089176 FBtr0289951 FBtr0089243 FBtr0112904 FBtr0089187 FBtr0089186
      7425      7419      5227      2686      2684      2640      2257      2250
FBtr0333672 FBtr0310542
      2206      1650
```

Note that this “top 10” is slightly different from the “top 10” we obtained earlier when we counted **all** the paired-end overlaps.

6 Compute the *reference query sequences* and project them on the transcriptome

6.1 Compute the *reference query sequences*

The *reference query sequences* are the query sequences **after** alignment, by opposition to the *original query sequences* (aka “true” or “real” query sequences) which are the query sequences **before** alignment.

The *reference query sequences* can easily be computed by extracting the nucleotides mapped to each read from the reference genome. This of course requires that we have access to the reference genome used by the aligner. In Bioconductor, the full genome sequence for the dm3 assembly is stored in the *BSgenome.Drosophila.melanogaster.UCSC.dm3* data package⁵:

```
> library(BSgenome.Drosophila.melanogaster.UCSC.dm3)
> Dmelanogaster

| BSgenome object for Fly
| - organism: Drosophila melanogaster
| - provider: UCSC
| - genome: dm3
| - release date: Apr. 2006
| - 15 sequence(s):
|   chr2L   chr2R   chr3L   chr3R   chr4   chrX   chrU   chrM   chr2LHet
|   chr2RHet chr3LHet chr3RHet chrXHet chrYHet chrUextra
|
| Tips: call 'seqnames()' on the object to get all the sequence names, call 'seqinfo()' to get the
| full sequence info, use the '$' or '[' operator to access a given sequence, see '?BSgenome' for
| more information.
```

To extract the portions of the reference genome corresponding to the ranges in *U1.grl*, we can use the `extractTranscriptSeqs` function defined in the *GenomicFeatures* package:

```
> library(GenomicFeatures)
> U1.GAL_rqseq <- extractTranscriptSeqs(Dmelanogaster, U1.grl)
> head(U1.GAL_rqseq)

DNAStringSet object of length 6:
      width seq
[1] 75 GGACAACCTAGCCAGGAAAGGGGCAGAGAACCC...GCCGAACCATCTGTGGTGTGGTCCACCACAG SRR031729.3941844
[2] 75 CAACAACATCCCGGAAATGAGCTAGCGGACAA...GAAAGGGCAGAGAACCCTCTAATTGGGCCCGA SRR031728.3674563
[3] 75 CCCAATTAGAGGGTTCTCTGCCCCCTTCTGCTGGC...CGTAGCTCATTTCCGGGATGTTGTTGTGTCC SRR031729.8532600
[4] 75 GTTCTCTGCCCTTTCCTGGCTAGGTTGTCCGC...TCCGGGATGTTGTTGTGTCCCGGACCCACCT SRR031729.2779333
[5] 75 TTCCTGGCTAGGTTGTCCGCTAGCTATTTCCC...TTGTGTCCCGGACCCACCTTATTGTGAGTTTG SRR031728.2826481
[6] 75 CAAACTTGGAGCTGTCAACAAACTACAATAAG...GGGACACAACAACATCCCGGAAATGAGCTAGC SRR031728.2919098
```

When reads are paired-end, we need to extract separately the ranges corresponding to their *first ends* (aka *first segments* in BAM jargon) and those corresponding to their *last ends* (aka *last segments* in BAM jargon):

⁵See <http://bioconductor.org/packages/release/data/annotation/> for the full list of annotation packages available in the current release of Bioconductor.

Overlap encodings

```
> U3.grl_first <- grglist(first(U3.GALP, real.strand=TRUE), order.as.in.query=TRUE)
> U3.grl_last <- grglist(last(U3.GALP, real.strand=TRUE), order.as.in.query=TRUE)
```

Then we extract the portions of the reference genome corresponding to the ranges in *GRangesList* objects `U3.grl_first` and `U3.grl_last`:

```
> U3.GALP_rqseq1 <- extractTranscriptSeqs(Dmelanogaster, U3.grl_first)
> U3.GALP_rqseq2 <- extractTranscriptSeqs(Dmelanogaster, U3.grl_last)
```

6.2 Project the single-end alignments on the transcriptome

The `extractQueryStartInTranscript` function computes for each overlap the position of the *query start* in the transcript:

```
> U1.OV00_qstart <- extractQueryStartInTranscript(U1.grl, exbytx,
+                                               hits=U1.OV00, ovenc=U1.ovenc)
> head(subset(U1.OV00_qstart, U1.OV00_is_comp))
```

	startInTranscript	firstSpannedExonRank	startInFirstSpannedExon
1	100	1	100
8	4229	5	137
9	4229	5	137
10	4207	5	115
11	4207	5	115
12	4187	5	95

`U1.OV00_qstart` is a data frame with 1 row per overlap and 3 columns:

1. `startInTranscript`: the 1-based start position of the read with respect to the transcript. Position 1 always corresponds to the first base on the 5' end of the transcript sequence.
2. `firstSpannedExonRank`: the rank of the first exon spanned by the read, that is, the rank of the exon found at position `startInTranscript` in the transcript.
3. `startInFirstSpannedExon`: the 1-based start position of the read with respect to the first exon spanned by the read.

Having this information allows us for example to compare the read and transcript nucleotide sequences for each “splice compatible” overlap. If we use the *reference query sequence* instead of the *original query sequence* for this comparison, then it should match **exactly** the sequence found at the *query start* in the transcript.

Let's start by using `extractTranscriptSeqs` again to extract the transcript sequences (aka transcriptome) from the dm3 reference genome:

```
> txseq <- extractTranscriptSeqs(Dmelanogaster, exbytx)
```

For each “splice compatible” overlap, the read sequence in `U1.GAL_rqseq` must be an *exact* substring of the transcript sequence in `exbytx_seq`:

```
> U1.OV00_rqseq <- U1.GAL_rqseq[queryHits(U1.OV00)]
> U1.OV00_rqseq[flippedQuery(U1.ovenc)] <- reverseComplement(U1.OV00_rqseq[flippedQuery(U1.ovenc)])
> U1.OV00_txseq <- txseq[subjectHits(U1.OV00)]
```

Overlap encodings

```
> stopifnot(all(
+   U1.OV00_rqseq[U1.OV00_is_comp] ==
+     narrow(U1.OV00_txseq[U1.OV00_is_comp],
+           start=U1.OV00_qstart$startInTranscript[U1.OV00_is_comp],
+           width=width(U1.OV00_rqseq)[U1.OV00_is_comp])
+ ))
```

Because of this relationship between the *reference query sequence* and the transcript sequence of a “splice compatible” overlap, and because of the relationship between the *original query sequences* and the *reference query sequences*, then the edit distance reported in the NM tag is actually the edit distance between the *original query* and the transcript of a “splice compatible” overlap.

6.3 Project the paired-end alignments on the transcriptome

For a paired-end read, the *query start* is the start of its “left end”.

```
> U3.OV00_Lqstart <- extractQueryStartInTranscript(U3.grl, exbytx,
+                                               hits=U3.OV00, ovenc=U3.ovenc)
> head(subset(U3.OV00_Lqstart, U3.OV00_is_comp))
```

	startInTranscript	firstSpannedExonRank	startInFirstSpannedExon
2	4118	5	26
7	3940	4	31
8	3940	4	31
9	3692	3	320
10	3692	3	320
11	3690	3	318

Note that `extractQueryStartInTranscript` can be called with `for.query.right.end=TRUE` if we want this information for the “right ends” of the reads:

```
> U3.OV00_Rqstart <- extractQueryStartInTranscript(U3.grl, exbytx,
+                                               hits=U3.OV00, ovenc=U3.ovenc,
+                                               for.query.right.end=TRUE)
> head(subset(U3.OV00_Rqstart, U3.OV00_is_comp))
```

	startInTranscript	firstSpannedExonRank	startInFirstSpannedExon
2	4267	5	175
7	3948	4	39
8	3948	4	39
9	3849	3	477
10	3849	3	477
11	3831	3	459

Like with single-end reads, having this information allows us for example to compare the read and transcript nucleotide sequences for each “splice compatible” overlap. If we use the *reference query sequence* instead of the *original query sequence* for this comparison, then it should match **exactly** the sequences of the “left” and “right” ends of the read in the transcript.

Let’s assign the “left and right reference query sequences” to each overlap:

Overlap encodings

```
> U3.OV00_Lrqseq <- U3.GALP_rqseq1[queryHits(U3.OV00)]
> U3.OV00_Rrqseq <- U3.GALP_rqseq2[queryHits(U3.OV00)]
```

For the single-end reads, the sequence associated with a “flipped query” just needed to be “reverse complemented”. For paired-end reads, we also need to swap the 2 sequences in the pair:

```
> flip_idx <- which(flippedQuery(U3.ovenc))
> tmp <- U3.OV00_Lrqseq[flip_idx]
> U3.OV00_Lrqseq[flip_idx] <- reverseComplement(U3.OV00_Rrqseq[flip_idx])
> U3.OV00_Rrqseq[flip_idx] <- reverseComplement(tmp)
```

Let’s assign the transcript sequence to each overlap:

```
> U3.OV00_txseq <- txseq[subjectHits(U3.OV00)]
```

For each “splice compatible” overlap, we expect the “left and right reference query sequences” of the read to be *exact* substrings of the transcript sequence. Let’s check the “left reference query sequences”:

```
> stopifnot(all(
+   U3.OV00_Lrqseq[U3.OV00_is_comp] ==
+     narrow(U3.OV00_txseq[U3.OV00_is_comp],
+           start=U3.OV00_Lqstart$startInTranscript[U3.OV00_is_comp],
+           width=width(U3.OV00_Lrqseq)[U3.OV00_is_comp])
+ ))
```

and the “right reference query sequences”:

```
> stopifnot(all(
+   U3.OV00_Rrqseq[U3.OV00_is_comp] ==
+     narrow(U3.OV00_txseq[U3.OV00_is_comp],
+           start=U3.OV00_Rqstart$startInTranscript[U3.OV00_is_comp],
+           width=width(U3.OV00_Rrqseq)[U3.OV00_is_comp])
+ ))
```

7 Align the reads to the transcriptome

Aligning the reads to the reference genome is not the most efficient nor accurate way to count the number of “splice compatible” overlaps per *original query*. Supporting junction reads (i.e. reads that align with at least 1 skipped region in their CIGAR) introduces a significant computational cost during the alignment process. Then, as we’ve seen in the previous sections, each alignment produced by the aligner needs to be broken into a set of ranges (based on its CIGAR) and those ranges compared to the ranges of the exons grouped by transcript.

A more straightforward and accurate approach is to align the reads directly to the transcriptome, and without allowing the typical skipped region that the aligner needs to introduce when aligning a junction read to the reference genome. With this approach, a “hit” between a read and a transcript is necessarily compatible with the splicing of the transcript.

Overlap encodings

In case of a “hit”, we’ll say that the read and the transcript are “string-based compatible” (to differentiate from our previous notion of “splice compatible” overlaps that we will call “encoding-based compatible” in this section).

7.1 Align the single-end reads to the transcriptome

7.1.1 Find the “hits”

The single-end reads are in `U1.oqseq`, the transcriptome is in `exbytx_seq`.

Since indels were not allowed/supported during the alignment of the reads to the reference genome, we don’t need to allow/support them either for aligning the reads to the transcriptome. Also since our goal is to find (and count) “splice compatible” overlaps between reads and transcripts, we don’t need to keep track of the details of the alignments between the reads and the transcripts. Finally, since BAM file `untreated1_chr4.bam` is not the full output of the aligner but the subset obtained by keeping only the alignments located on `chr4`, we don’t need to align `U1.oqseq` to the full transcriptome, but only to the subset of `exbytx_seq` made of the transcripts located on `chr4`.

With those simplifications in mind, we write the following function that we will use to find the “hits” between the reads and the transcriptome:

```
> ### A wrapper to vwhichPDict() that supports IUPAC ambiguity codes in 'qseq'
> ### and 'txseq', and treats them as such.
> findSequenceHits <- function(qseq, txseq, which.txseq=NULL, max.mismatch=0)
+ {
+   .asHits <- function(x, pattern_length)
+   {
+     query_hits <- unlist(x)
+     if (is.null(query_hits))
+       query_hits <- integer(0)
+     subject_hits <- rep.int(seq_len(length(x)), elementNROWS(x))
+     Hits(query_hits, subject_hits, pattern_length, length(x),
+          sort.by.query=TRUE)
+   }
+
+   .isHitInTranscriptBounds <- function(hits, qseq, txseq)
+   {
+     sapply(seq_len(length(hits)),
+            function(i) {
+              pattern <- qseq[[queryHits(hits)[i]]]
+              subject <- txseq[[subjectHits(hits)[i]]]
+              v <- matchPattern(pattern, subject,
+                                max.mismatch=max.mismatch, fixed=FALSE)
+              any(1L <= start(v) & end(v) <= length(subject))
+            })
+   }
+
+   if (!is.null(which.txseq)) {
+     txseq0 <- txseq
+     txseq <- txseq[which.txseq]
+   }
+ }
```


Overlap encodings

```
+ names(qseq) <- NULL
+ other <- alphabetFrequency(qseq, baseOnly=TRUE)[ , "other"]
+ is_clean <- other == 0L # "clean" means "no IUPAC ambiguity code"
+
+ ## Find hits for "clean" original queries.
+ qseq0 <- qseq[is_clean]
+ pdict0 <- PDict(qseq0, max.mismatch=max.mismatch)
+ m0 <- vwhichPDict(pdict0, txseq,
+                 max.mismatch=max.mismatch, fixed="pattern")
+ hits0 <- .asHits(m0, length(qseq0))
+ hits0@nLnode <- length(qseq)
+ hits0@from <- which(is_clean)[hits0@from]
+
+ ## Find hits for non "clean" original queries.
+ qseq1 <- qseq[!is_clean]
+ m1 <- vwhichPDict(qseq1, txseq,
+                 max.mismatch=max.mismatch, fixed=FALSE)
+ hits1 <- .asHits(m1, length(qseq1))
+ hits1@nLnode <- length(qseq)
+ hits1@from <- which(!is_clean)[hits1@from]
+
+ ## Combine the hits.
+ query_hits <- c(queryHits(hits0), queryHits(hits1))
+ subject_hits <- c(subjectHits(hits0), subjectHits(hits1))
+
+ if (!is.null(which.txseq)) {
+   ## Remap the hits.
+   txseq <- txseq0
+   subject_hits <- which.txseq[subject_hits]
+   hits0@nRnode <- length(txseq)
+ }
+
+ ## Order the hits.
+ oo <- orderIntegerPairs(query_hits, subject_hits)
+ hits0@from <- query_hits[oo]
+ hits0@to <- subject_hits[oo]
+
+ if (max.mismatch != 0L) {
+   ## Keep only "in bounds" hits.
+   is_in_bounds <- .isHitInTranscriptBounds(hits0, qseq, txseq)
+   hits0 <- hits0[is_in_bounds]
+ }
+ hits0
+ }
```

Let's compute the index of the transcripts in `exbytx_seq` located on `chr4` (`findSequenceHits` will restrict the search to those transcripts):

```
> chr4tx <- transcripts(txdb, vals=list(tx_chrom="chr4"))
> chr4txnames <- mcols(chr4tx)$tx_name
> which.txseq <- match(chr4txnames, names(txseq))
```

Overlap encodings

We know that the aligner tolerated up to 6 mismatches per read. The 3 following commands find the “hits” for each *original query*, then find the “hits” for each “flipped *original query*”, and finally merge all the “hits” (note that the 3 commands take about 1 hour to complete on a modern laptop):

```
> U1.sbcompHITSa <- findSequenceHits(U1.oqseq, txseq,
+                                 which.txseq=which.txseq, max.mismatch=6)
> U1.sbcompHITSb <- findSequenceHits(reverseComplement(U1.oqseq), txseq,
+                                 which.txseq=which.txseq, max.mismatch=6)
> U1.sbcompHITS <- union(U1.sbcompHITSa, U1.sbcompHITSb)
```

7.1.2 Tabulate the “hits”

Number of “string-based compatible” transcripts for each read:

```
> U1.uqnames_nsbcomptx <- countQueryHits(U1.sbcompHITS)
> names(U1.uqnames_nsbcomptx) <- U1.uqnames
> table(U1.uqnames_nsbcomptx)

U1.uqnames_nsbcomptx
 0    1    2    3    4    5    6    7    8    9   10   11   12
40555 10080 25299 74609 5207 14265 8643 610 3410 2056 534 4588 914

> mean(U1.uqnames_nsbcomptx >= 1)

[1] 0.7874142
```

77.7% of the reads are “string-based compatible” with at least 1 transcript in `exbytx`.

Number of “string-based compatible” reads for each transcript:

```
> U1.exbytx_nsbcompHITS <- countSubjectHits(U1.sbcompHITS)
> names(U1.exbytx_nsbcompHITS) <- names(exbytx)
> mean(U1.exbytx_nsbcompHITS >= 50)

[1] 0.008809516
```

Only 0.865% of the transcripts in `exbytx` are “string-based compatible” with at least 50 reads.

Top 10 transcripts:

```
> head(sort(U1.exbytx_nsbcompHITS, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0089243 FBtr0289951 FBtr0112904 FBtr0089186 FBtr0333672
 40548      40389      34275      11605      11579      11548      10059      9742
FBtr0089187 FBtr0089172
 9666      6704
```

7.1.3 A closer look at the “hits”

[WORK IN PROGRESS, might be removed or replaced soon...]

Any “encoding-based compatible” overlap is of course “string-based compatible”:

```
> stopifnot(length(setdiff(U1.compOV10, U1.sbcompHITS)) == 0)
```

Overlap encodings

but the reverse is not true:

```
> length(setdiff(U1.sbcompHITS, U1.compOV10))
[1] 13549
```

7.2 Align the paired-end reads to the transcriptome

[COMING SOON...]

8 Detect “almost splice compatible” overlaps

In many aspects, “splice compatible” overlaps can be seen as perfect. We are now interested in a less perfect type of overlap where the read overlaps the transcript in a way that *would* be “splice compatible” if 1 or more exons were removed from the transcript. In that case we say that the overlap is “almost splice compatible” with the transcript. The `isCompatibleWithSkippedExons` function can be used on an `OverlapEncodings` object to detect this type of overlap. Note that `isCompatibleWithSkippedExons` can also be used on a character vector of factor.

8.1 Detect “almost splice compatible” single-end overlaps

8.1.1 “Almost splice compatible” single-end encodings

`U1.ovenc` contains 7 unique encodings “almost splice compatible” with the splicing of the transcript:

```
> sort(U1.ovenc_table[isCompatibleWithSkippedExons(U1.unique_encodings)])
      2:jm:am:am:am:am:af: 2:jm:am:am:am:am:am:af:      2:gm:am:af:      2:jm:am:am:am:af:
                1                1                4                7
      3:jmm:agm:aam:aam:aaf: 3:jmm:agm:aam:aaf:      2:jm:am:am:af:      2:jm:am:af:
                9                21               144            1015
```

Encodings "2:jm:am:af:" (1015 occurrences in `U1.ovenc`), "2:jm:am:am:af:" (144 occurrences in `U1.ovenc`), and "3:jmm:agm:aam:aaf:" (21 occurrences in `U1.ovenc`), correspond to the following overlaps:

- "2:jm:am:af:"
 - read (1 skipped region): 00000-----000
 - transcript: ... >>>>>> >>>> >>>>>>> ...
- "2:jm:am:am:af:"
 - read (1 skipped region): 00000-----000
 - transcript: ... >>>>>> >>>> >>>> >>>>>>> ...
- "3:jmm:agm:aam:aaf:"
 - read (2 skipped regions): 00---0000-----00
 - transcript: ... >>>>>> >>>> >>>> >>>>>>> ...

```
> U1.OV00_is_acomp <- isCompatibleWithSkippedExons(U1.ovenc)
> table(U1.OV00_is_acomp) # 1202 "almost splice compatible" overlaps
```

Overlap encodings

```
U1.OV00_is_acomp
FALSE TRUE
562350 1202
```

Finally, let's extract the "almost splice compatible" overlaps from U1.OV00:

```
> U1.acompOV00 <- U1.OV00[U1.OV00_is_acomp]
```

8.1.2 Tabulate the "almost splice compatible" single-end overlaps

Number of "almost splice compatible" transcripts for each alignment in U1.GAL:

```
> U1.GAL_nacomptx <- countQueryHits(U1.acompOV00)
> mcols(U1.GAL)$nacomptx <- U1.GAL_nacomptx
> head(U1.GAL)
```

GAlignments object with 6 alignments and 3 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	njunc
	<Rle>	<Rle>	<character>	<integer>	<integer>	<integer>	<integer>	<integer>
SRR031729.3941844	chr4	-	75M	75	892	966	75	0
SRR031728.3674563	chr4	-	75M	75	919	993	75	0
SRR031729.8532600	chr4	+	75M	75	924	998	75	0
SRR031729.2779333	chr4	+	75M	75	936	1010	75	0
SRR031728.2826481	chr4	+	75M	75	949	1023	75	0
SRR031728.2919098	chr4	-	75M	75	967	1041	75	0

	ntx	ncomptx	nacomptx
	<integer>	<integer>	<integer>
SRR031729.3941844	0	0	0
SRR031728.3674563	0	0	0
SRR031729.8532600	0	0	0
SRR031729.2779333	0	0	0
SRR031728.2826481	0	0	0
SRR031728.2919098	0	0	0

seqinfo: 8 sequences from an unspecified genome

```
> table(U1.GAL_nacomptx)
```

U1.GAL_nacomptx	0	1	2	3	4	5	6	7	8	9	10	11	12
	203800	283	101	107	19	24	2	3	1	3	4	4	4

```
> mean(U1.GAL_nacomptx >= 1)
```

```
[1] 0.002715862
```

Only 0.27% of the alignments in U1.GAL are "almost splice compatible" with at least 1 transcript in exbytx.

Number of "almost splice compatible" alignments for each transcript:

```
> U1.exbytx_nacompOV00 <- countSubjectHits(U1.acompOV00)
> names(U1.exbytx_nacompOV00) <- names(exbytx)
> table(U1.exbytx_nacompOV00)
```

Overlap encodings

```
U1.exbytx_nacomp0V00
  0   1   2   3   4   5   6   7   8   9  10  12  13  14  17  18
29039 50   8  15  12   2   3   7   5   7   3   2   1   1   1   2
    20  21  32  34  44  55  59  77  170
    1   3   2   1   3   2   1   1   1

> mean(U1.exbytx_nacomp0V00 >= 50)

[1] 0.0001713914
```

Only 0.017% of the transcripts in `exbytx` are “almost splice compatible” with at least 50 alignments in `U1.GAL`.

Finally note that the “query start in transcript” values returned by `extractQueryStartInTranscript` are also defined for “almost splice compatible” overlaps:

```
> head(subset(U1.OV00_qstart, U1.OV00_is_acomp))

      startInTranscript firstSpannedExonRank startInFirstSpannedExon
144226                133                 1                 133
144227                133                 1                 133
144240                151                 1                 151
144241                151                 1                 151
146615                757                 7                  39
146616                689                 8                  39
```

8.2 Detect “almost splice compatible” paired-end overlaps

8.2.1 “Almost splice compatible” paired-end encodings

`U3.ovenc` contains 5 unique paired-end encodings “almost splice compatible” with the splicing of the transcript:

```
> sort(U3.ovenc_table[isCompatibleWithSkippedExons(U3.unique_encodings)])

  2--1:jm--m:am--m:af--i:      1--2:i--jm:a--am:a--am:a--af:
                                1                                5
2--2:jm--mm:am--mm:af--jm:aa--af:      1--2:i--jm:a--am:a--af:
                                9                                53
  2--1:jm--m:am--m:af--i:
                                73
```

Paired-end encodings “2--1:jm--m:am--m (73 occurrences in `U3.ovenc`), “1--2:i--jm:a--am (53 occurrences in `U3.ovenc`), and “2--2:jm--mm:am--mm:af--j (9 occurrences in `U3.ovenc`), correspond to the following paired-end overlaps:

- “2--1:jm--m:am--m
 - paired-end read (1 skipped region on the first end, no skipped region on the last end):
ooo-----o oooo
 - transcript: ... >>>> >>>> >>>>>>> ...
- “1--2:i--jm:a--am
 - paired-end read (no skipped region on the first end, 1 skipped region on the last end):
oooo oo-----oo

Overlap encodings

```

- transcript:      ... >>>>>>>>> >>> >>>>> ...
- paired-end read (1 skipped region on the first end, 1 skipped region
  on the last end):      0-----000 00---00
- transcript:      ... >>>>> >>>> >>>>>>>> >>>>>> ...

```

Note: switch use of “first” and “last” above if the read was “flipped”.

```

> U3.OV00_is_acomp <- isCompatibleWithSkippedExons(U3.ovenc)
> table(U3.OV00_is_acomp) # 141 "almost splice compatible" paired-end overlaps
U3.OV00_is_acomp
FALSE  TRUE
113686  141

```

Finally, let's extract the “almost splice compatible” paired-end overlaps from U3.OV00:

```

> U3.acompOV00 <- U3.OV00[U3.OV00_is_acomp]

```

8.2.2 Tabulate the “almost splice compatible” paired-end overlaps

Number of “almost splice compatible” transcripts for each alignment pair in U3.GALP:

```

> U3.GALP_nacomptx <- countQueryHits(U3.acompOV00)
> mcols(U3.GALP)$nacomptx <- U3.GALP_nacomptx
> head(U3.GALP)
GAlignmentPairs object with 6 pairs, strandMode=1, and 3 metadata columns:
      seqnames strand :   ranges --   ranges |      ntx  ncomptx  nacomptx
      <Rle>  <Rle> : <IRanges> -- <IRanges> | <integer> <integer> <integer>
SRR031715.1138209 chr4   + : 169-205 -- 326-362 |         0         0         0
SRR031714.756385  chr4   + : 943-979 -- 1086-1122 |         0         0         0
SRR031714.5054563 chr4   + : 946-982 -- 986-1022 |         0         0         0
SRR031715.1722593 chr4   + : 966-1002 -- 1108-1144 |         0         0         0
SRR031715.2202469 chr4   + : 966-1002 -- 1114-1150 |         0         0         0
SRR031714.3544437 chr4   - : 1087-1123 -- 963-999 |         0         0         0
-----
seqinfo: 8 sequences from an unspecified genome

> table(U3.GALP_nacomptx)
U3.GALP_nacomptx
  0    1    2    3    4    5   11
45734  74   4  13   1   1   1

> mean(U3.GALP_nacomptx >= 1)
[1] 0.002051148

```

Only 0.2% of the alignment pairs in U3.GALP are “almost splice compatible” with at least 1 transcript in `exbytx`.

Number of “almost splice compatible” alignment pairs for each transcript:

Overlap encodings

```
> U3.exbytx_nacomp0V00 <- countSubjectHits(U3.acomp0V00)
> names(U3.exbytx_nacomp0V00) <- names(exbytx)
> table(U3.exbytx_nacomp0V00)

U3.exbytx_nacomp0V00
  0    1    5    8   12   13   66
29143  22   4   1   1   1   1

> mean(U3.exbytx_nacomp0V00 >= 50)

[1] 3.427827e-05
```

Only 0.0034% of the transcripts in `exbytx` are “almost splice compatible” with at least 50 alignment pairs in `U3.GALP`.

Finally note that the “query start in transcript” values returned by `extractQueryStartInTranscript` are also defined for “almost splice compatible” paired-end overlaps:

```
> head(subset(U3.OV00_Lqstart, U3.OV00_is_acomp))

      startInTranscript firstSpannedExonRank startInFirstSpannedExon
27617                1549                 12                    45
27629                1562                 12                    58
27641                1562                 12                    58
27690                1567                 12                    63
27812                1549                 12                    45
42870                 659                  4                   101

> head(subset(U3.OV00_Rqstart, U3.OV00_is_acomp))

      startInTranscript firstSpannedExonRank startInFirstSpannedExon
27617                2135                 14                   115
27629                2135                 14                   115
27641                2141                 14                   121
27690                2048                 14                    28
27812                2136                 14                   116
42870                 866                  6                    19
```

9 Detect novel splice junctions

9.1 By looking at single-end overlaps

An alignment in `U1.GAL` with “almost splice compatible” overlaps but no “splice compatible” overlaps suggests the presence of one or more transcripts that are not in our annotations.

First we extract the index of those alignments (*nsj* here stands for “novel splice junction”):

```
> U1.GAL_is_nsj <- U1.GAL_nacompTx != 0L & U1.GAL_ncompTx == 0L
> head(which(U1.GAL_is_nsj))

[1] 57972 57974 58321 67251 67266 67267
```

We make this an index into `U1.OV00`:

Overlap encodings

```
> U1.OV00_is_nsj <- queryHits(U1.OV00) %in% which(U1.GAL_is_nsj)
```

We intersect with `U1.OV00_is_acomp` and then subset `U1.OV00` to keep only the overlaps that suggest novel splicing:

```
> U1.OV00_is_nsj <- U1.OV00_is_nsj & U1.OV00_is_acomp
> U1.nsj0V00 <- U1.OV00[U1.OV00_is_nsj]
```

For each overlap in `U1.nsj0V00`, we extract the ranks of the skipped exons (we use a list for this as there might be more than 1 skipped exon per overlap):

```
> U1.nsj0V00_skippedex <- extractSkippedExonRanks(U1.ovenc)[U1.OV00_is_nsj]
> names(U1.nsj0V00_skippedex) <- queryHits(U1.nsj0V00)
> table(elementNROWS(U1.nsj0V00_skippedex))

  1  2  3  4  5
234 116  7  1  1
```

Finally, we split `U1.nsj0V00_skippedex` by transcript names:

```
> f <- factor(names(exbytx)[subjectHits(U1.nsj0V00)], levels=names(exbytx))
> U1.exbytx_skippedex <- split(U1.nsj0V00_skippedex, f)
```

`U1.exbytx_skippedex` is a named list of named lists of integer vectors. The first level of names (outer names) are transcript names and the second level of names (inner names) are alignment indices into `U1.GAL`:

```
> head(names(U1.exbytx_skippedex)) # transcript names
[1] "FBtr0300689" "FBtr0300690" "FBtr0330654" "FBtr0309810" "FBtr0306539" "FBtr0306536"
```

Transcript `FBtr0089124` receives 7 hits. All of them skip exons 9 and 10:

```
> U1.exbytx_skippedex$FBtr0089124

$`104549`
[1] 9 10

$`104550`
[1] 9 10

$`104553`
[1] 9 10

$`104557`
[1] 9 10

$`104560`
[1] 9 10

$`104572`
[1] 9 10

$`104577`
```


Overlap encodings

```
[1] 9 10
```

Transcript FBtr0089147 receives 4 hits. Two of them skip exon 2, one of them skips exons 2 to 6, and one of them skips exon 10:

```
> U1.exbytx_skippedex$FBtr0089147
```

```
$`72828`
```

```
[1] 10
```

```
$`74018`
```

```
[1] 2 3 4 5 6
```

```
$`74664`
```

```
[1] 2
```

```
$`74670`
```

```
[1] 2
```

A few words about the interpretation of `U1.exbytx_skippedex`: Because of how we've conducted this analysis, the alignments reported in `U1.exbytx_skippedex` are guaranteed to not have any "splice compatible" overlaps with other known transcripts. All we can say, for example in the case of transcript FBtr0089124, is that the 7 reported hits that skip exons 9 and 10 show evidence of one or more unknown transcripts with a splice junction that corresponds to the gap between exons 8 and 11. But without further analysis, we can't make any assumption about the exons structure of those unknown transcripts. In particular, we cannot assume the existence of an unknown transcript made of the same exons as transcript FBtr0089124 minus exons 9 and 10!

9.2 By looking at paired-end overlaps

[COMING SOON...]

10 sessionInfo()

```
> sessionInfo()
```

```
R version 4.5.0 beta (2025-04-02 r88102)
```

```
Platform: x86_64-pc-linux-gnu
```

```
Running under: Ubuntu 24.04.2 LTS
```

```
Matrix products: default
```

```
BLAS: /home/biocbuild/bbs-3.22-bioc/R/lib/libRblas.so
```

```
LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.12.0 LAPACK version 3.12.0
```

```
locale:
```

```
[1] LC_CTYPE=en_US.UTF-8
```

```
LC_NUMERIC=C
```

```
LC_TIME=en_GB
```

```
[4] LC_COLLATE=C
```

```
LC_MONETARY=en_US.UTF-8
```

```
LC_MESSAGES=en_US.UTF-8
```

```
[7] LC_PAPER=en_US.UTF-8
```

```
LC_NAME=C
```

```
LC_ADDRESS=C
```

```
[10] LC_TELEPHONE=C
```

```
LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
```

Overlap encodings

```
time zone: America/New_York
tzcode source: system (glibc)
```

```
attached base packages:
```

```
[1] stats4      stats      graphics  grDevices  utils      datasets  methods   base
```

```
other attached packages:
```

```
[1] BSgenome.Dmelanogaster.UCSC.dm3_1.4.0    BSgenome_1.77.0
[3] rtracklayer_1.69.0                       BiocIO_1.19.0
[5] TxDb.Dmelanogaster.UCSC.dm3.ensGene_3.2.2 GenomicFeatures_1.61.0
[7] AnnotationDbi_1.71.0                     pasillaBamSubset_0.45.0
[9] GenomicAlignments_1.45.0                 SummarizedExperiment_1.39.0
[11] Biobase_2.69.0                            MatrixGenerics_1.21.0
[13] matrixStats_1.5.0                        Rsamtools_2.25.0
[15] Biostrings_2.77.0                         XVector_0.49.0
[17] GenomicRanges_1.61.0                    GenomeInfoDb_1.45.0
[19] IRanges_2.43.0                           S4Vectors_0.47.0
[21] BiocGenerics_0.55.0                      generics_0.1.3
[23] RNAseqData.HNRNPC.bam.chr14_0.45.0       BiocStyle_2.37.0
```

```
loaded via a namespace (and not attached):
```

```
[1] KEGGREST_1.49.0          rjson_0.2.23             xfun_0.52                bslib_0.9.0
[5] lattice_0.22-7          vctrs_0.6.5             tools_4.5.0             bitops_1.0-9
[9] curl_6.2.2              parallel_4.5.0          RSQLite_2.3.9           blob_1.2.4
[13] pkgconfig_2.0.3         Matrix_1.7-3            lifecycle_1.0.4         GenomeInfoDbData_1.2.14
[17] compiler_4.5.0          codetools_0.2-20        htmltools_0.5.8.1       sass_0.4.10
[21] RCurl_1.98-1.17        yaml_2.3.10             crayon_1.5.3            jquerylib_0.1.4
[25] BiocParallel_1.43.0     DelayedArray_0.35.0     cachem_1.1.0            abind_1.4-8
[29] digest_0.6.37          restfulr_0.0.15         bookdown_0.43           fastmap_1.2.0
[33] grid_4.5.0             cli_3.6.4               SparseArray_1.9.0       S4Arrays_1.9.0
[37] XML_3.99-0.18          UCSC.utils_1.5.0        bit64_4.6.0-1           rmarkdown_2.29
[41] httr_1.4.7             bit_4.6.0               png_0.1-8               memoise_2.0.1
[45] evaluate_1.0.3         knitr_1.50              rlang_1.1.6            DBI_1.2.3
[49] BiocManager_1.30.25    jsonlite_2.0.0          R6_2.6.1
```