ASpli: An integrative R package for analysing alternative splicing using RNA-Seq

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Contents

1 Introduction

Alternative splicing (AS) is a common mechanism of post-transcriptional gene regulation in eukaryotic organisms that expands the functional and regulatory diversity of a single gene by generating multiple mRNA isoforms that encode structurally and functionally distinct proteins. The development of novel high-throughput sequencing methods for RNA (**RNA-Seq**) has provided a powerful means of studying AS under multiple conditions and in a genome-wide manner [1]. However, using **RNA-Seq**to study changes in AS under different experimental conditions is not trivial. In this vignette, we describe how to use ASpli, an integrative and user-friendly R package that facilitates the analysis of changes in both annotated and novel AS events. This package combines statistical information from exon, intron, and splice junction differential usage (p-value, FDR), with information from splice junction reads to calculate differences in the percentage of exon inclusion (ΔPSI) and intron retention (ΔPIR). The proposed methodology reliably reflect the magnitude of changes in the relative abundance of different annotated and novel AS events. This method can be used to analyze both simple and complex experimental designs involving multiple experimental conditions.

2 Getting started

2.1 Installation

ASpli is available at Bioconductor site and can be downloaded using `biocLite()`:

```
> source("https://www.bioconductor.org/biocLite.R")
> biocLite("ASpli")
```

```
> library(ASpli)
```

`biocLite()` will take care of installing all the packages that ASpli depends on e.g. edgeR, GenomicFeatures, GenomicRanges, GenomicAlignments, Gviz, and other R package required. Some packages depend on operating system packages, like `curl`, that are not installed automatically, and should be installed by the user.

2.2 Required data

ASpli requires a single `BAM` file for each sample in the experiment. Also, a gene annotation file is mandatory (usually a `GTF/GFF` file). Make sure all files use the same coordinate system.

2.3 Building a `TxDb` of your genome

Gene annotation is handled using `TxDb` objects, which are provided by `GenomicFeatures` package. The building of a `TxDb` object might be time consuming, depending on the size of your genome. If a `TxDb` has been already created, we strongly recommend saving it as a `sqlite` file and reloading it any time the analysis is run. The `GenomicFeatures` package provides functions to create TxDb objects based on data downloaded from UCSC Genome Bioinformatics, BioMart or gff/gtf files. See "Creating New TxDb Objects or Packages" in the `GenomicFeatures` vignette for more details. In this example, a TxDb is built from a gtf file:

```
> library(GenomicFeatures)
> TxDb <- makeTxDbFromGFF(
   file="genes.gtf",
   format="gtf")
```

3 Running ASpli

The workflow intended to be used with ASpli is divided in five steps (Figure 1). For each of these steps ASpli provides a single or few functions to use:

1. Genomic alignments reading: `loadBAM()`
2. Feature extraction: `binGenome()`
3. Read counting: `readCounts()`
4. Alternative splicing discovery using junctions: `AsDiscover()`
5. DE and DU estimation `DUreport(), junctionDUReport() and DUreportBinSplice() )`

At each step it is possible to export results in a friendly manner. See Section 3.6:Output and results for more details.

ASpli defines four major objects classes used to store data and perform operations ralated to a single task. Throughout this vignette each of these will be discussed in detail, however they are presented here:

ASpliFeatures Contains data extracted from annotation, such a genomic coordindates of genes.
ASpliCounts Contains counts of aligned reads to each genomic feature.
ASpliDU Contains results of Differential expression and usage analysis.
ASpliAS Contains data useful to search novel splicing events.

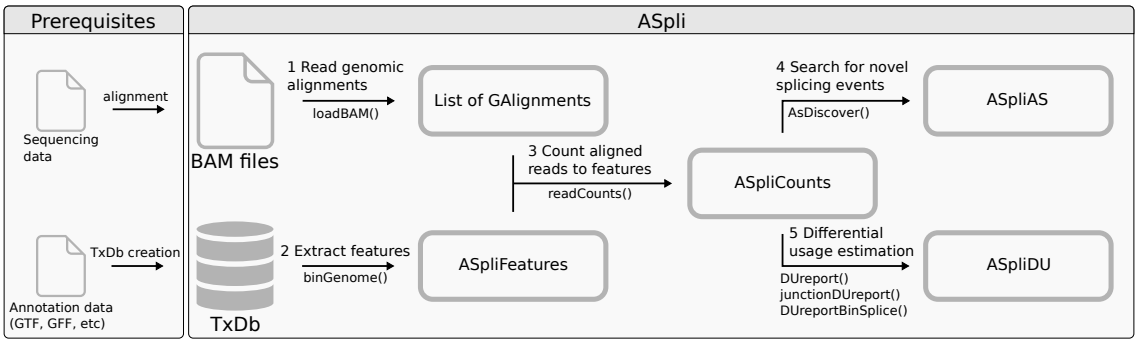


Figure 1: Workflow of ASpli pipeline and prerequisites. Each arrow represents one of the main steps of a typical analysis. Rounded boxes are objects created by ASpli functions. Names under arrows are functions included in ASpli used to generate each kind of object.

3.1 To BIN or not to BIN

Sub-genic features such as exons and introns are analyzed using existing annotations by splitting the genome into non-overlapping features called bins, as described previously for DEXSeq [2]. Exon and intron coordinates are then extracted from the annotation, but only those from multi-exonic genes are saved for further evaluation. When more than one isoform exists, some exons and introns will overlap. Exons and introns are then subdivided into new features called exon and intron bins, and are then classified into exclusively exonic bins, exclusively intronic bins, or alternative splicing (AS) bins (See Figure 2). In addition to these non overlapping bins, an additional type of bin is also defined. The latter corresponds to full introns extracted from annotation, before they are splitted by the presence of overlapping exons in another isoform. These bins as referred as `Intron original` bins.

3.1.1 Splicing event assignation

Each AS bin is assigned to a kind of splicing event. The assignation is made using the minimum gene model, extracted from the annotation, corresponding to the bin being evaluated and the two neighbor bins (one to the left, one to the right). The minimum gene model is constructed by extracting from the corresponding isoforms the regions of the selected bins and removing those that are identical.

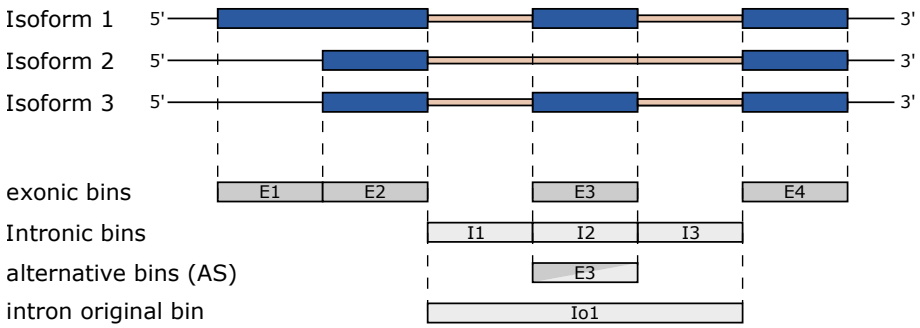


Figure 2: Schema of resulting bins from a gene with three hypothetical transcripts. Those bins that are exonic and intronic in different isoforms are named *AS bins*.

The criteria for the assignation is that the assigned splicing event must explain a differential usage found for this bin, while the rest of the bins in the gene are not altered.

When the minium gene model contains two isoforms the bin is labelled as follow:

- **ES** Exon skipping
- **IR** Intron retention
- **ALt5'SS** Alternative 5' splicing site
- **ALt3'SS** Alternative 3' splicing site

When the minimum gene model contains three, four or five isoforms (note that no more than five non redundant isoforms can exist in the minimum gene model), labels of bins are similar to the ones defined previously but have an '*' character added to end (**ES***, **IR***, **Alt5'SS***, **Alt3'SS***) in order to show that more than two isoforms exists and, therefore, the results should be used with caution. In some cases, is not possible to make an assignation for a bin with assumptions made here, those bins are labelled as **as**. The figure 3 shows a summary of the assignation. Finally, those bins that overlap with the beginning or ending of any transcript are labelled as **external**. Please note that an external bin of a transcript may overlap to a non external bin of another transcript, in these cases the bin is still labelled as **external**.

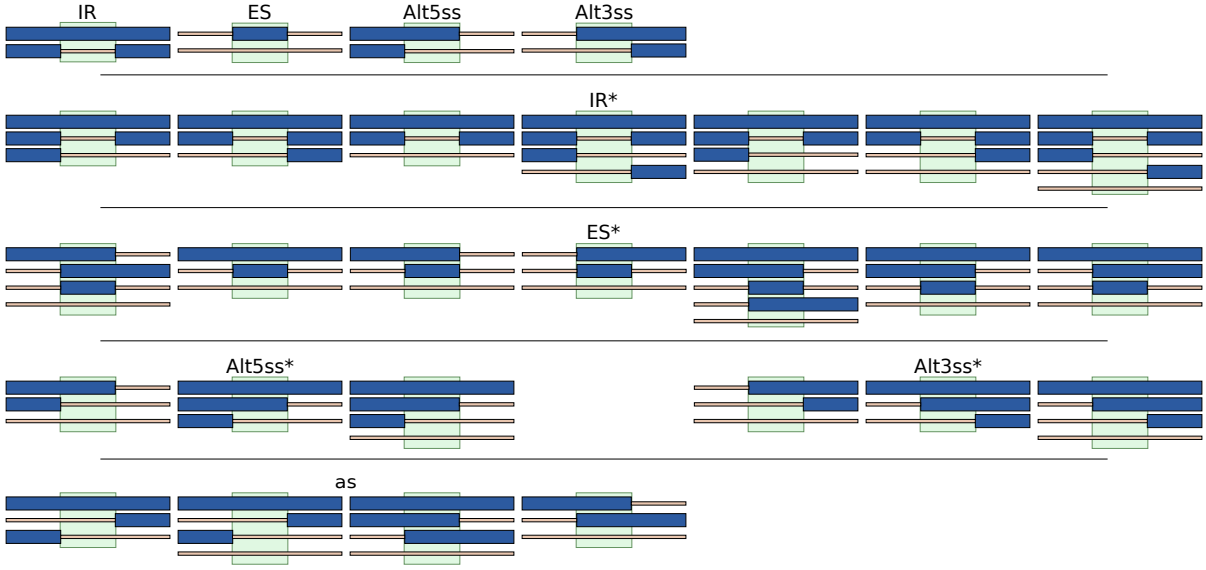


Figure 3: Summary of assignation of splicing events to bins from minimum gene model. The bin being evaluated has a green background highlight. The blue boxes represents exons, while the little light orange boxes represent introns. Gene models shown are plus sense strand.

3.1.2 Naming conventions for bins and junctions

Subgenic features are named as follow for a hypothetical gene named GeneAAA:

- **GeneAAA:E001**: defines first exonic bin
- **GeneAAA:I001**: defines first intronic bin
- **GeneAAA:Io001**: defines first `Intron original` before being divided
- **GeneAAA:J001**: defines first annotated junction of GeneAAA
- **chr.start.end**: defines an experimental junction, `chr` is the chromosome name, `start` is the leftmost position of the junction and `end` is the rightmost position of the junction.

Bins and junctions are consecutively named from 5' to 3' sense of the plus strand of the reference sequence. This implies that bins and junctions with lower numbering are always closer to the 5' of that strand. Alternative splicing bins are named as exons.

All tasks presented in this section: subgenic splitting of genes into bins, coordinates extraction of junctions, genes and bins, naming of genes and bins and splicing event assignation to bins are performed by single method, `binGenome`. The result is an object of class `ASpliFeatures`. Method `binGenome` accepts an additional optional argument `geneSymbols` used to assign symbols to genes (i.e. common name or a short description). Then, features coordinates could be obtained using their accesors methods. Method `featuresg` returns coordinates for genes as a GRangesList object, and methods `featuresb` and `featuresj` returns coordinates for bins and bins respectively as a GRanges object. GRangesList and GRanges are objects defined in GenomicRanges package. These objects contains aditional information stored as metadata and can be accessed with `mcols` function.

```
> library(GenomicFeatures)
> annFile       <- aspliExampleGTF()
> aTxDb         <- makeTxDbFromGFF(annFile)
> # extract features from annotation
> features      <- binGenome( aTxDb )
> # Accesors of ASpliFeatures object
> geneCoord     <- featuresg( features )
> binCoord      <- featuresb( features )
> junctionCoord <- featuresj( features )
> # Extract metadata annotation, such as bins names, event and feature type
> binMetadata   <- mcols( binCoord )

> # extract features and map symbols to genes
> symbols       <- data.frame( row.names = genes( aTxDb ),
                               symbol = paste( 'This is symbol of gene:',
                                               genes( aTxDb ) ) )
> features      <- binGenome( aTxDb, geneSymbols = symbols )
```

3.2 Defining the targets

Before to proceed, ASpli is requires to define the samples, experimental factors and to associate them with the corresponding genomic alignment files. This must be specified in a `data.frame` that has as many rows as samples. Each row name is the name of a sample. The first column must be named `bam` and contain the path to a single bam file. The remaining columns are used to define the experimental factors for each sample and must have a proper name. This `data.frame` is referred to as the **targets** `data.frame`.

For example, for a one factor experiment with two conditions (Control and Mutant) and two replicates for each condition, the **targets** `data.frame` can be defined as follow:

```
> bamFiles <- c( "Ct1.bam", "Ct2.bam", "Mut1.bam","Mut2.bam" )
> targets <- data.frame( row.names =  c("CT_rep1","CT_rep2", "Mut_rep1", "Mut_rep2"),
                         bam = bamFiles,
```

```
                        genotype = c("CT","CT", "Mut", "Mut") ,
                        stringsAsFactors = FALSE )
```

More sofisticated designs should be specified as follow. For example: for a two factor experiment (factors genotype and time), with two values for each factor adn two replicates for each condition, the **targets** `data.frame` can be defined as follow:

```
> bamFiles <- c("Ct_time1_rep1.bam", "Ct_time1_rep2.bam",
                "Ct_time2_rep1.bam", "Ct_time2_rep2.bam",
                "Mut_time1_rep1.bam","Mut_time1_rep2.bam",
                "Mut_time2_rep1.bam","Mut_time2_rep2.bam")
> targets_2 <- data.frame( row.names = c( 'CT_t1_r1',  'CT_t1_r2',
                                          'CT_t2_r1',  'CT_t2_r2',
                                          'Mut_t1_r1', 'Mut_t1_r2',
                                          'Mut_t2_r1', 'Mut_t2_r2' ),
                         bam = bamFiles,
                         genotype = c( 'CT',  'CT',  'CT',  'CT',
                                       'MUT', 'MUT', 'MUT', 'MUT' ),
                         time     = c( 't1', 't1', 't2', 't2',
                                       't1', 't1', 't2', 't2' ),
                         stringsAsFactors = FALSE )
```

The name of the each experimental condition is determined by the experimental factors defined in the **targets** `data.frame`. Those can be consulted using the `getConditions` function.

```
> # Show the name of each unique condition in the console
> getConditions( targets_2 )
```

3.3 Read Counting

3.3.1 Genomic alignment files loading

Once the **targets** `data.frame` is defined the alignment data from bam files can be loaded easily with `loadBAM` function. BAM files are loaded and stored as a named list of `GAlignments` objects. `GAlignments` objects are defined in `GenomicAlignments` package.

```
> targets <- aspliTargetsExample()
> bam <- loadBAM(targets)
```

3.3.2 Counting alignment reads overlapping features

The method `readCounts` allows to count the number of reads from genomic alignments that overlaps each feature of interest. These features are genes, bins, junctions, and intron/exon flanking regions. For genes and bins, a read densitie value is calculated as shown below:

$$f_d = \frac{N_r}{l}$$

Where f_d is the feature read density, N_r is the number of reads overlaping the feature and l is the length of the feature.

Results generated by `readCounts` are stored into an `ASpliCounts` object. The usage of this method is shown below:

```
> counts <- readCounts (
     features,
     bam,
     targets,
     cores = 1,
     readLength = 100L,
```

```
    maxISize = 50000,
    minAnchor = NULL )
```

Where the accepted parameters are:

- features: An ASpliFeatures object.
- bam: A list of GAlignments generated with loadBAM function.
- targets: A **targets** data.frame as defined in section 3.2 : Defining the targets.
- cores: Number of proccessor cores used to speed up the computation.
- readLength: Read length of sequenced library
- maxISize: maximum intron expected size. Junctions longer than this size will be dicarded [3].
- minAnchor: minimum anchoring overlap for intron flanking regions (see 3.3.3 : Counting intron flanking regions).

The result of readCounts method is an object of class ASpliCounts. Count and read density data could be extracted from it as data.frame objects using accesors methods. Also, it is possible to export count and read densities tables to text files in a tidy manner.

```
> # Accessing count and read density data
> GeneCounts <- countsg(counts)
> GeneRd <- rdsg(counts)
> BinCounts <- countsb(counts)
> BinRd <- rdsb(counts)
> JunctionCounts <- countsj(counts)
> e1iCounts <- countse1i(counts)
> ie2Counts <- countsie2(counts)
> # Export count data to text files
> writeCounts(counts=counts, output.dir = "example")
> writeRds(counts=counts, output.dir = "example")
```

3.3.3 Counting intron flanking regions

Every intron is considered as a potential retained intron. Analysis of putative intron retention events consider adjacent 5' and 3' exons (E1 and E2, following the order in the genomic coordinates, regardless of the sense of the strand) and the intron itself (I). Following [4], two retention regions E1I (connecting exon E1 and I) and IE2 (connecting the intron and exon 2) and one constitutive (i.e., no retention) junction, E1E2 (connecting exons 1 and 2) are defined. The read length of sequenced library is used for the definition of those new artifitial ranges:

- E1I: intron start - (readLength - minAnchor) — intron start + (readLength - minAnchor)
- IE2: intron end - (readLength - minAnchor) — intron end + (readLength - minAnchor)
- minAnchor is 10% of read length by default (parameter minAnchor)

Only those reads which minimum overlap *readLength* and without gap in this interval are considered. Accordingly, only sequences aligned within those two exons/introns are counted. If the reads of your genomic alignment are trimmed by quality rendering reads with a length inferior to the nominal read length, then E1I and IE2 values will be underestimated.

3.3.4 Additional details of readCounts method

Number of reads by gene are computed as the number of reads overlapping any exon included in the annotation for a given gene. Note that if a single read overlaps more than one exon is counted only once. A read can overlaps two different genes, in this case is counted for both of them.

In order to estimate read densities of a gene, an effective length is considered. The effective length is calculated as the sum of the length of exonic bins, alternative bins (all bins except intronic bins).

Junctions are extracted from BAM files. They are defined as those reads aligned skipping region from the reference (N operator of CIGAR notation for aligned reads [5]). They are essential for alternative splicing event quantification and

discovery. Junction alignment quality/confidence is extremely important and it should be controlled at the moment of the alignment step.

3.3.5 Contents of ASpliCounts object

Accessor methods of `ASpliCounts` object return `data.frame` objects for genes counts, genes read density, bin counts, bin read density, E1I flanking region counts and IE2 flanking region counts. All of them share the same structure, however the specific content is different for each kind of feature. The first columns contains feature information extracted from the annotation, and then there several columns with count or read density data for each experimental sample. A more detailed overview is shown below:

- Gene counts and gene read densities `data.frames` contains the following columns:
  row.names Gene name as is found in annotation.
  symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1 : To BIN or not to BIN).
  locus_overlap Show the names of all other overlapping *loci*.
  gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
  start Left-most position of the gene.
  end Right-most position of the gene.
  length Number of bases covering the gene.
  effective_length Number of bases that overlaps with annotated exons.
  sample data One column by sample containing gene counts or gene read densities.

- Bin counts and bin read densities `data.frames` contains the following columns:
  row.names Bin name as given in section 3.1.2 : Naming conventions for bins and junctions.
  feature Is the kind of bin: **E** for exonic bins, **I** for intronic bins and **Io** for introns before splitting.
  event Splicing event asigned to the bin (see section 3.1:Splicing event assignation)
  locus The name of the locus that contains the bin.
  locus_overlap Show the names of all other overlapping *loci*.
  symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1 : To BIN or not to BIN).
  gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
  start Left-most position of the bin.
  end Right-most position of the bin.
  length Number of bases covering the bin.
  sample data One column by sample containing bin counts or bin read densities.

- Junction count `data.frame` contains the following columns:
  row.names Junction name in format `chromosome.start.end` (see 3.1.2 : Naming conventions for bins and junctions).
  junction If junction coincides with a junction inferred from the annotation, the name is shown as is given in section 3.1.2:Naming conventions for bins and junctions, otherwise contains `noHit`.
  gene The name of the locus that contains the junction.
  strand Sense strand of the gene.
  multipleHit Contains `yes` if junction spans multiple genes.
  symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1 : To BIN or not to BIN).
  gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
  bin_spanned The names of the bins spanned by the junction.
  j_within_bin If junction fall within a single bin, the name of that bin is shown.
  sample data One column by sample containing junction counts.

- E1I and IE2 flanking region count `data.frame` contains the following columns:
  row.names Junction name in format `chromosome.start.end` (see **??** : **??**).

junction If junction coincides with a junction inferred from the annotation, the name is shown as is given in section 3.1:To BIN or not to BIN, otherwise contains `noHit`.
gene The name of the locus that contains the junction.
strand Sense strand of the gene.
multipleHit Contains `yes` if junction spans multiple genes.
symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1:To BIN or not to BIN).
gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
bin_spanned The names of the bins spanned by the junction.
j_within_bin If junction fall within a single bin, the name of that bin is shown.
sample data One column by sample containing junction counts.

3.4 Alternative splicing analysis and discovery using junction

Count data of bins and junctions, generated with `readCounts` method, can be used to get an integrative view of the alternative splicing events. The method `AsDiscover` will produce an `ASpliAS` object containing comprehensive results of evidence of alternative splicing usage of bins and junctions.

The analysis will consider junctions that are completely included within a unique gene and have more than a minimum number of reads supporting them, by default this number is five.

For each experimental junction identified, it is also reported if it is novel and which bins are spanned. In addition, it is stated if the junction is completely included in an annotated bin, which would indicate that the AS event is a possible exintron [6].

```
> as <- AsDiscover( counts, targets, features, bam, readLength=100L,
                    threshold = 5)
```

Where the accepted parameters are:

- `counts`: An ASpliCount object.
- `targets`: A **targets** `data.frame` as defined in section 3.2 : Defining the targets.
- `features`: An ASpliFeatures object.
- `bam`: A list of `GAlignments` generated with loadBAM `function`.
- `readLength`: Read length of sequenced library
- `threshold`: minimum number of reads supporting a junction.
- `cores`: Number of proccessor cores used to speed up the computation.

The contents of an `ASpliAS` object can be retrieved using accessor methods: `irPIR`, `altPSI`, `esPSI`, `joint` `junctionPIR`, `junctionPSI` (see section 3.4.3 : Contents of ASpliAS object for a detailed description of each method and retrieved data). In addition, method `writeAS`, allows to easily export all the data into text files tables.

```
> # Access result from an ASpliAS object
> irPIR  <- irPIR( as )
> altPSI <- altPSI( as )
> esPSI  <- esPSI( as )
> junctionsPIR <- junctionsPIR( as )
> junctionsPSI <- junctionsPSI( as )
> allBins      <- joint( as )
> # Export results to files
> writeAS(as=as, output.dir="example")
```

3.4.1 PIR and PSI metrics

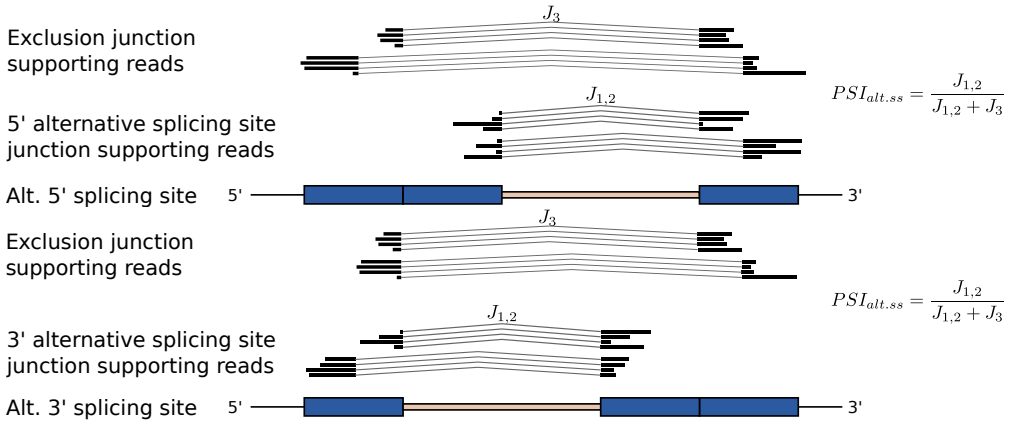
To provide an integrative view of the AS events being analyzed, splice junction information is used to analyze differential bin usage. PSI (percent of inclusion) and PIR (percent of intron retention) metrics, which are extensively used to quantify AS [7], are calculated (see figure 4) for bins junctions. Only junctions that pass an abundance filter are considered for PSI/PIR calculation.

$$J_{ij}^{min} = min(J_{ij})$$

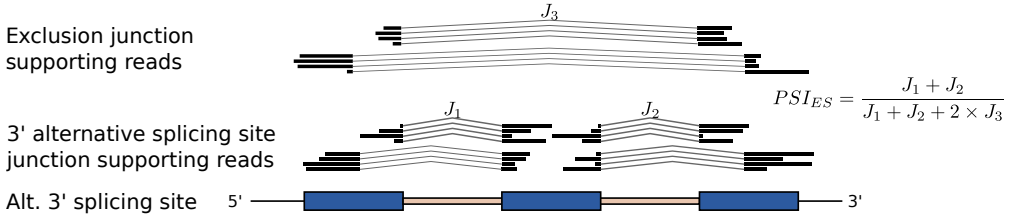
$$acceptByCondition(i,j) = \begin{cases} 1 & \text{if } J_{ij}^{min} > \text{threshold} \\ 0 & otherwise \end{cases}$$

$$accepted(i) = \sum_{j} acceptByCondition(i,j) > 0$$

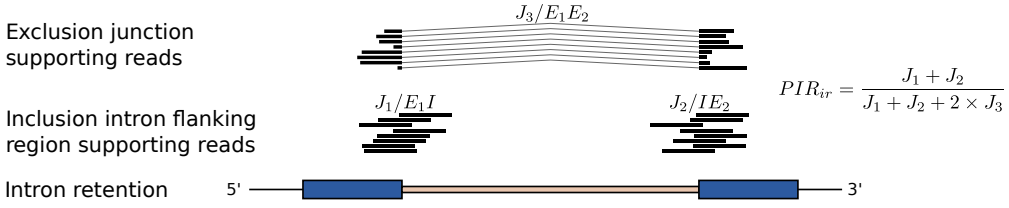
Lets define J_{ij} as the set of junction supporting reads count values for junction i in condition j. The minimum value of counts for a given junction i for all samples of a condition j is calculated (J_{ij}^{min}), then a junction i is accepted if J_{ij}^{min} is greater or equal than a threshold value for at least one condition j.



(a) PSI metric for alternative 5' and 3' splicing sites estimation and its relationship with junctions



(b) PSI metric for exon skipping estimation and its relationship with junctions



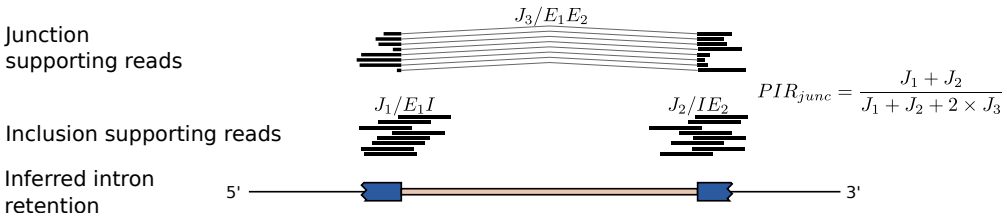
(c) PIR metric for intron retention estimation and its relationship with junctions

Figure 4: PSI and PIR metrics estimation and their relationship with junctions

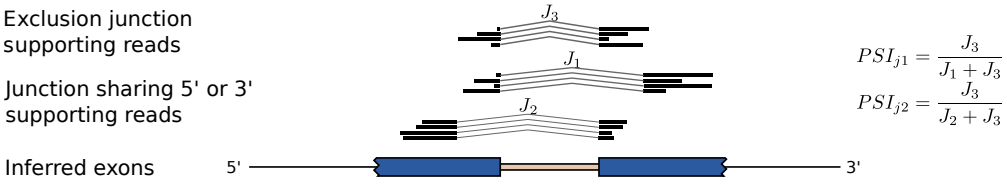
For each bin, one of PIR or PSI variants metrics is calculated, the decision of which one is selected depends on the splice event assigned to that bin (see section 3.1.1 : Splicing event assignation). If no splice event is assigned, meaning that the bin is not alternative, an exon are considered to be involved in a putative exon skiping splicing event, and an intron are considered to be involved in a putative intron retention splicing event.

Every junction implies the existence of an intron that could be potentially retained, therefore a PIR value is computed for every junction. Also, every junction defines a 5' and a 3' splicing site. Each one of these can be found exclusively in this junction or found also in other junctions. The latter is evidence of an exon skipping event or and alternative 5' or 3' splicing site event. Althought is not possible to infer the kind of event, the computation of a PSI value is helpful to detect this cases. The details of the formulas used to calculted these values are shown in 5

This information allows the user to obtain reliable information on the relative abundance of the AS event being evaluated. Both metrics strongly enrich the count-centric analysis and provide independent experimental support for the existence of novel AS events, based on the identification of corresponding changes in nearby splice junctions.



(a) PIR metric to estimate intron retention for junctions



(b) PSI metric to compute inclusion of exons bearing a junction.

Figure 5: PSI and PIR metrics for junctions

3.4.2 New splicing events discovery

ASpli allows novel AS events to be identified based on the splice junction repertoire. A novel AS event can be is identified whenever a novel splice junction that shares its beginning or end with another splice junction is discovered. When a novel AS event is identified using the splice junction repertoire, the PSI metric is calculated and reported. This ability to detect novel AS events and to estimate the magnitude of the changes in the usage of these AS events under different experimental conditions are original functions of the package.

3.4.3 Contents of ASpliAS object

Accessor methods of `ASpliAS` object return `data.frame` objects containing detailed information for bins involved in intron retention, 5' or 3' alternative splicing site and exon skipping events, and for experimental junctions.

- Results for bins involved in intron retention, alternative 5' or 3' splicing site, and exon skipping events can be accessed with `irPIR`, `altPSI` and `esPSI` respectively, or using `joint` method for all of them altogether. These methods return a `data.frame` object which columns are specified below. The description given uses **J1**, **J2**, **J3** count values, which have different meaning for different metrics, see figures 4 and 5 for more detail.
  row.names Bin name as is defined in 3.1.2 : Naming conventions for bins and junctions.
  event Splicing event asigned to the bin (see section 3.1:Splicing event assignation)
  J1 The name of J1 junction or E/I flanking region.
  sample data for J1 J1 counts for each sample.
  J2 The name of J2 junction or E/I flanking region.
  sample data for J2 J2 counts for each sample.
  J3 The name of J3 junction or E/I flanking region.
  sample data for J3 J3 counts for each sample.

PSI or PIR data A column by condition with the corresponding PSI variant metric or PIR metric.

- Results for experimental junctions assuming that they correspond to introns involved in intron retention events can be accessed with `junctionPIR` method. The returning value is a `data.frame` with columns:
  row.names Junction name in format `chromosome.5'site-3'site`.
  hitIntron The name of a bin that match exactly with positions of the junction, if it exists.
  hitIntronEvent If `hitIntron` is not empty, contains the splicing event assigned to that bin.
  event Splicing event asigned to the bin (see section 3.1:Splicing event assignation)
  sample data for J1 J1 counts for each sample.
  sample data for J2 J2 counts for each sample.
  sample data for J3 J3 counts for each sample.
  PSI data A column by condition with the corresponding PIR metric.

- Results for experimental junctions assuming that the bearing exons are involved in alternative splice events can be accessed with `junctionPSI` method. The returning value is a `data.frame` with columns:
  row.names Junction name in format `chromosome.5'site-3'site`.
  junction If junction coincides with a junction inferred from the annotation, the name is shown as is given in section 3.1:To BIN or not to BIN, otherwise contains `noHit`.
  gene The name of the locus that contains the junction.
  strand Sense strand of the gene.
  multipleHit Contains `yes` if junction spans multiple genes.
  symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1 : To BIN or not to BIN).
  gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
  bin_spanned The names of the bins spanned by the junction.
  j_within_bin If junction fall within a single bin, the name of that bin is shown.
  sample data for J3 J3 counts for each sample.
  StartHit The name of J1 junction.
  sample data for J1 J1 counts for each sample.
  PSI data A column by condition with the PSI metric for J1. The name of the column is the name of the condition with '.start' suffix.
  EndHit The name of J2 junction.
  sample data for J2 J2 counts for each sample.
  PSI data A column by condition with the PSI metric for J2. The name of the column is the name of the condition with '.end' suffix.

3.5 Estimating differential gene expression and bin/junction usage

Using the generated counting data, it is possible to estimate the differential usage (**DU**) of bins and junctions, and the differential expression at gene level (**DE**) using any analysis methodology of choice. For the sake of user convinience, ASpli provides methods to perform statistical analysis using the functionality of `edgeR` package.

The methods provided are `DUreport`, `DUreportBinSplice`, `junctionDUreport`. `DUreport` and `DUreportBinSplice` have the same purposes, perform DE statistical tests for genes and DU statistical tests for bins using a count centric approach. The differende is the methodology used for bin DU. `junctionDUreport` do a similar analysis for junctions. The usage of these methods will be discussed in detail in the following sections. Results are stored into an `ASpliDU` object and can be accessed individual for genes, bins and junctions using the accesors methods `genesDE`, `binsDU` and `junctionDU` respectively. Also, can be exported as text files tables using `writeDU` function.

3.5.1 Differential gene expression (DE)

The first step of the gene DE analysis pipeline is the selection of those annotated genes that are considered to be expressed, or at least that can be confidently detected. For this, the average number of reads for each gene and condition and the average read density for each gene and condition are calculated as shown below:

$$\hat{G}^{c}_{ij} = \sum_{k \in K_j} \frac{S^{c}_{ik}}{|K_j|}$$

$$\hat{G}^{rd}_{ij} = \sum_{k \in K_j} \frac{S^{rd}_{ik}}{|K_j|}$$

Where $\hat{G}^{c}_{ij}$ is avg. gene count for gene i in condition j, $\hat{G}^{rd}_{ij}$ is avg. read density for gene i in condition j, S^{c}_{ik} is gene count for gene i in sample k, S^{rd}_{ik} is read density for gene i in sample k. K_j is the set of samples corresponding to condition j.

Gene i is considered expressed if any of the corresponding $\hat{G}^{c}_{ij}$ and $\hat{G}^{rd}_{ij}$ values are equal or greater than a minimum cutoff (by default, these numbers are 10 for gene count and 0.05 for read density) and for any condition j.

Differential gene expression is estimated with edgeR [8] package using an exact test (for paired comparison) or generalized linear model (GLM) for more complex designs. For each gene **logFC**, **pvalue**, and **adjusted pvalue** by false discovery rate [9] are reported.

`DUreport` and `DUreportBinSplice` methods perform genes DE analysis with the same methodology and gives the same results.

Each condition in the analysis is weighted by contrast vector that ultimately defines the comparison that will be done. For a simple pair comparison between `Control` and `Trat` conditions, represeted as $Trat - Control$ can be expressed as:

```
> contrast <- c( -1, 1 )
```

Assuming that the order of the conditions given by `getConditions` methods is `[ 'Control', 'Trat' ]`.

3.5.2 Differential bin and junction usage (DU)

Bins and junctions are treated similarly for DU analysis. In order to select informative events, will be taken into account bins and junctions from expressed genes (see section 3.5.1 : Differential gene expression (DE)) if they have more reads than a threshold (five reads by default) supporting them in at least one of the conditions.

External bins do not participate in alternative splicing, therefore are usually excluded from the analysis. However, a external bin in one isoform can overlap to a non external bin in other isoform that can participate in alternative splicing regulation. ASpli, allows to optionally include the external bins in the analysis. Also, original intron bins can be optionally excluded. The inclusion of those bins can affect the estimation of corrected p-values (fdr). The information provided by intron original bins are highly correlated with the one of their sub bins, and increase largely the number of events to be analyzed. Therefore the fdr correction is more strict. Also, there is a violation on the fdr correction assupmtion that all individual tests are independent from each other. If a intron original bin show a significant change, there is a very high chance that at least one of their sub bins also shows a significant change.

Differential bin/junction usage are estimated using the statistical methodology proposed in the `edgeR` package. Read counts from bins/junctions can not be used naively for differential usage analysis. Counts needs to be adjusted to remove the signal from changes in gene expression. To perform this, Aspli provides three alternatives:

1. Adjust bin/junction counts to gene counts
2. Use an offset value given by gene expression
3. Delegate differential bin usage estimation to `edgeR`'s `diffSpliceDGE` . This is available only for bins.

The adjustment of bin counts to gene counts is performed by transforming the raw bin counts. The transformation is done by:

$$B^A_{ijk} = \frac{B_{ijk} \times \bar{G}_j}{G_{jk}} \quad (1)$$

Where B^A_{ijk} is the adjusted bin count for bin i of gene j in sample k, B_{ijk} is the raw bin count for bin i of gene j in sample k, $\bar{G}_j$ is the mean raw count for gene j for all samples and G_{jk} is the raw gene count for gene j in sample k.

Then the adjusted bin counts are used as input for an statical analysis identical to the one performed on genes using the methods provided by edgeR package.

The offset methodology consist in define a vector that reflects the change of gene expression levels and use it to correct the observed change in bin usage in the statistical analysis. The are several ways to estimate the offset vector:

- Use gene counts (gene mode):
  1. Use only raw counts.
  2. Fit a generalized linear model from gen counts.
- Use the sum of exonic bins from each gene (bin mode). Is important to note that in this mode some reads are counted more than one time, because the overlap more than one bin. This mode is not available for junctions.

EdgeR package also provides a separate framework to detect splicing events, this rely on their `diffSpliceDGE` method. This method uses an strategy similar to the offset methodology. ASpli allow its use with the method `DUreportBinSplice`.

3.5.3 Usage of `DUreport`, `DUreportBinSplice` and `junctionDUreport` methods

`DUreport` method performs DE estimation for genes and DU estimation for bins in the same run. The call of this method is done by:

```
> du <- DUreport( counts,
                  targets,
                  minGenReads = 10,
                  minBinReads = 5,
                  minRds = 0.05,
                  offset = FALSE,
                  offsetAggregateMode =  c( "geneMode", "binMode" )[2],
                  offsetUseFitGeneX = TRUE,
                  contrast = NULL,
                  forceGLM = FALSE,
                  ignoreExternal = TRUE,
                  ignoreIo = FALSE,
                  ignoreI = FALSE,
                  filterWithConstrasted = FALSE,
                  verbose = FALSE )
```

Where the accepted parameters are:

counts an ASpliCounts object.
targets a targets data frame built as explained in 3.2 : Defining the targets.
minGenReads minimum value for reads in any condition required for a gene to be considered as expressed (see 3.5.1 : Differential gene expression (DE)). The default value is 10.
minBinReads minimum value for reads in any condition required for a bin to be included in the analysis (see 3.5.2 : Differential bin and junction usage (DU)). The default value is 5.
minRds minimum value for read density for genes and bins required to be considered in the analysis. The default value is 0.05.

offset a logical value that indicates, if TRUE, that an offset vector should be estimated and used in the analysis. If FALSE, bin counts by gene counts adjustment is used instead. The default value is FALSE (see 3.5.2 : Differential bin and junction usage (DU))

offsetAggregateMode a character value that specifies if gene mode or bin mode of offset estimation should be used. It is only relevant when

offset argument is TRUE (see 3.5.2 : Differential bin and junction usage (DU)). The default value is geneMode.

offsetUseFitGeneX a logical value that indicates if the offset vector should be computed using a GLM. This is only relevant when offset argument is TRUE and offsetAggregateMode argument is geneMode (see 3.5.2 : Differential bin and junction usage (DU)). The default value is TRUE.

contrast is a vector representing the coefficients for each unique condition in the analysis. They are assigned in the order given by getConditions method (see 3.2 : Defining the targets). The default value is NULL which correspond to a vector with value -1 in the first position, 1 in the second, and zero for the remaining positions. This is for the paired comparison of the second condition versus the first condition.

forceGLM a logical value that indicates that fitting a GLM should be used in the analysis for paired comparison, instead of an extact test (used by default for this type of comparison). The default value if FALSE.

ignoreExternal a logical value indicating that external bins should be ignored in the analysis. The default value is TRUE.

ignoreIo a logical value indicating that original intron bins should be ignored in the analysis. The default value is TRUE.

ignoreI a logical value indicating that only exons should be included in the analysis. The default value is FALSE.

filterWithConstrasted a logical value that specifies that gene and bin filtering when more than two conditions are present, should be done only with the data of those conditions and ignore the others. The default value id FALSE, it is strongly recommended to do not change this value.

verbose a logical value that indicates that detailed information about each step in the analysis will be presented to the user.

DUreportBinSplice method performs DE estimation for genes and DU estimation for bins in the same run. The call of this method is done by:

```
> du <- DUreportBinSplice( counts,
                           targets,
                           minGenReads = 10,
                           minBinReads = 5,
                           minRds = 0.05,
                           contrast = NULL,
                           forceGLM = FALSE,
                           ignoreExternal = TRUE,
                           ignoreIo = TRUE,
                           ignoreI = FALSE,
                           filterWithContrasted = FALSE )
```

Where the accepted parameters are:

counts an ASpliCounts object.

targets a targets data frame built as explained in 3.2 : Defining the targets.

minGenReads minimum value for reads in any condition required for a gene to be considered as expressed (see 3.5.1 : Differential gene expression (DE)). The default value is 10.

minBinReads minimum value for reads in any condition required for a bin to be included in the analysis (see 3.5.2 : Differential bin and junction usage (DU)). The default value is 5.

minRds minimum value for read density for genes and bins required to be considered in the analysis. The default value is 0.05.

contrast is a vector representing the coefficients for each unique condition in the analysis. They are assigned in the order given by getConditions method (see 3.2 : Defining the targets). The default value is NULL which correspond to a vector with value -1 in the first position, 1 in the second, and zero for the remaining positions. This is for the paired comparison of the second condition versus the first condition.

forceGLM a logical value that indicates that fitting a GLM should be used in the analysis for paired comparison, instead of an extact test (used by default for this type of comparison). The default value if FALSE.

ignoreExternal a logical value indicating that external bins should be ignored in the analysis. The default value is TRUE.

ignoreIo a logical value indicating that original intron bins should be ignored in the analysis. The default value is TRUE.
ignoreI a logical value indicating that only exons should be included in the analysis. The default value is FALSE.
filterWithContrasted a logical value that specifies that gene and bin filtering when more than two conditions are present, should be done only with the data of those conditions and ignore the others. The default value id FALSE, it is strongly recommended to do not change this value.

junctionDUreport method performs DU estimation for junctions. This is independent of the genes DE and bins DU analysis. However, the results can be merged with existing genes and bins results. The call of this method is done by:

```
> du <- junctionDUReport(  counts,
                           targets,
                           appendTo = NULL,
                           minGenReads = 10,
                           minRds = 0.05,
                           threshold = 5,
                           offset   = FALSE,
                           offsetUseFitGeneX = TRUE,
                           contrast = NULL,
                           forceGLM = FALSE )
```

Where the accepted parameters are:

counts an ASpliCounts object.
targets a targets data frame built as explained in 3.2 : Defining the targets.
appendTo an ASpliDU object to which the results will be attached.
minGenReads minimum value for reads in any condition required for a gene to be considered as expressed (see 3.5.1 : Differential gene expression (DE)). The default value is 10.
minRds minimum value for read density for genes required to be considered as expressed. The default value is 0.05.
threshold minimum number of junction supporting reads required to be present in at least one sample of every conditions in order to include the junction in the analysis.
offset a logical value that indicates, if TRUE, that an offset vector should be estimated and used in the analysis. If FALSE, junction counts by gene counts adjustment is used instead. The default value is FALSE (see 3.5.2 : Differential bin and junction usage (DU))
offsetUseFitGeneX a logical value that indicates if the offset vector should be computed using a GLM. This is only relevant when offset argument is TRUE (see 3.5.2 : Differential bin and junction usage (DU)). The default value is TRUE.
contrast is a vector representing the coefficients for each unique condition in the analysis. They are assigned in the order given by getConditions method (see 3.2 : Defining the targets). The default value is NULL which correspond to a vector with value -1 in the first position, 1 in the second, and zero for the remaining positions. This is for the paired comparison of the second condition versus the first condition.
forceGLM a logical value that indicates that fitting a GLM should be used in the analysis for paired comparison, instead of an extact test (used by default for this type of comparison). The default value if FALSE.

The three methods described above return an ASpliDU object. This object contains the results for genes DE, bins DU and junction DU, which can be consulted through their respective accessor methods: genesDE, binsDU and junctionsDU. See next section (3.5.4 : Contents of ASpliDU object) for details about the specific contents of the data obtained with these methods.

3.5.4 Contents of ASpliDU object

ASpliDU accessor methods genesDE, binsDU and junctionsDU returns a data.frame object with the corresponding results.

- Results for genes differential expression analysis can be accessed with genesDE. This method returns a data.frame object which columns are specified below.
  row.names Gene name as is found in annotation.

symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1 : To BIN or not to BIN).
locus_overlap Show the names of all other overlapping *loci*.
gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
start Left-most position of the gene.
end Right-most position of the gene.
length Number of bases covering the gene.
effective_length Number of bases that overlaps with annotated exons.
logFC Show the observed change of expression level in a log_2 scale.
pvalue The pvalue for the DE test.
gen.fdr The pvalue adjusted by **Benjamini-Hochberg** method.

- Results for experimental junctions assuming that they correspond to introns involved in intron retention events can be accessed with `junctionPIR` method. The returning value is a `data.frame` with columns:
row.names Bin name as given in section 3.1.2:Naming conventions for bins and junctions.
feature Is the kind of bin: **E** for exonic bins, **I** for intronic bins and **Io** for introns before splitting.
event Splicing event asigned to the bin (see section 3.1:Splicing event assignation)
locus The name of the locus that contains the bin.
locus_overlap Show the names of all other overlapping *loci*.
symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1:To BIN or not to BIN).
gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
start Left-most position of the bin.
end Right-most position of the bin.
length Number of bases covering the bin.
logFC Show the observed change of usage in a log_2 scale.
pvalue The pvalue for the DU test.
gen.fdr The pvalue adjusted by **Benjamini-Hochberg** method.

- Results for experimental junctions assuming that the bearing exons are involved in alternative splice events can be accessed with `junctionPSI` method. The returning value is a `data.frame` with columns:
row.names Junction name in format `chromosome.5'site-3'site`.
junction If junction coincides with a junction inferred from the annotation, the name is shown as is given in section 3.1.2:Naming conventions for bins and junctions, otherwise contains `noHit`.
gene The name of the locus that contains the junction.
strand Sense strand of the gene.
multipleHit Contains `yes` if junction spans multiple genes.
symbol An optional common name for the gene, that must be provided at the moment of feature extraction (see section 3.1:To BIN or not to BIN).
gene_coordinates Show the genomic coordinates of the gene with format `chromosome:start-end`.
bin_spanned The names of the bins spanned by the junction.
j_within_bin If junction fall within a single bin, the name of that bin is shown.
junction count data One column per sample containing raw junction counts if `offset` argument is `TRUE`, or junction counts adjusted by gene counts otherwise.
logFC Show the observed change of usage in a log_2 scale.
pvalue The pvalue for the DU test.
gen.fdr The pvalue adjusted by **Benjamini-Hochberg** method.
bin_start_hit List of junction names that shares the start position with current junction, in format `chromosome:start-end`.
junction sharing start data One column per samples containing the counts of junctions sharing the start with the current junction. Junction counts can be raw or adjusted following the same criteria described for `junction count data` column.
junction sharing start ratio One column by condition containing the ratio of current junction among all junctions sharing their start position (see 3.4.1 : PIR and PSI metrics). The ratio is calculated using raw or adjusted

counts.

end_start_hit List of junction names that shares the start position with current junction, in format `chromosome:start-end`.

junction sharing end data One column per samples containing the counts of junctions sharing the end with the current junction. Junction counts can be raw or adjusted following the same criteria described for `junction count data` column.

junction sharing end ratio One column by condition containing the ratio of current junction among all junctions sharing their end position (see 3.4.1 : PIR and PSI metrics). The ratio is calculated using raw or adjusted counts.

3.6 Output and results

At each module, results are stored in `ASpliObjects`. Self-explanatory tables can be exported at each step of the analysis. Using `write` functions, it is possible to export tab delimited tables in a features-level output folder:

```
> writeCounts(counts, "example_counts")
> writeDU(du, output.dir="example_du");
> writeAS(as=as, output.dir="example_as");
> writeAll(counts=counts, du=du, as=as, output.dir="example_all")
```

Column of text tables has the same name and meaning that those described in 3.3.5 : Contents of ASpliCounts object, 3.4.3 : Contents of ASpliAS object and 3.5.4 : Contents of ASpliDU object. Exported text tables files are arranged in subfolders "exons", "genes", "introns" and "junctions". `writeAS` creates an additional text table:

as_discovery.tab Contains junction counts and PSI/PIR metrics for all bins.

`writeAll` methods writes some additional tables:

summary.tab Contains bin DU usage for all bins together.
bins_du_psi_pir.tab Contains bin DU usage for all bins merged with PSI/PIR metrics.

3.7 Plots

Inspection of coverage plots is a good practice for the interpretation of analytical results. After selection of AS candidates it is possible to plot the results in a genome browser manner highlighting alternatively spliced regions using the function `plotGenomicRegions`. Coverage data is taken from **bam** files. `plotGenomicRegions` function will draw a coverage plot for each condition, not for each sample. therefore coverage data from samples needs to be merged. `plotGenomicRegions` can extract and merge the reads of the corresponding *locus* of interest on-the-fly from sample's **bam** files or can use preiviously merged **bam** generated by the user. When the number of *loci* to be plotted is small (ten or less) the first approach is usually faster, otherwise is better to merge and index **bam** files beforehand. **Bam** files can be merged using `samtools` utility or `RSamtools` package.

```
> library(GenomicFeatures)
> bamfiles <- system.file( 'extdata',
     c('A_C_0.bam', 'A_C_1.bam', 'A_C_2.bam',
       'A_D_0.bam', 'A_D_1.bam', 'A_D_2.bam',
       'B_C_0.bam', 'B_C_1.bam', 'B_C_2.bam',
       'B_D_0.bam', 'B_D_1.bam', 'B_D_2.bam' ),
     package = "ASpli")
> targets <- data.frame(
   row.names = c( 'A_C_0', 'A_C_1', 'A_C_2',
                  'A_D_0', 'A_D_1', 'A_D_2',
                  'B_C_0', 'B_C_1', 'B_C_2',
                  'B_D_0', 'B_D_1', 'B_D_2' ),
```

```
   bam = bamfiles,
   f1  = rep( c('A','B'), each=6 ),
   f2  = rep( c('C','D'), 2, each=3 ),
   stringsAsFactors = FALSE )
> genomeTxDb <- makeTxDbFromGFF( system.file( 'extdata', 'genes.mini.gtf',
 package='ASpli'))
> features <- binGenome( genomeTxDb )
> # Draw a single plot on a window
> # xIsBin arguments is used to specify if names given are bins or genes.
> # When multiple names are given, all must correspond to bins or correspond to
> # genes. Mixed names are not allowed.
>
> plotGenomicRegions( features, 'GENE02:E002' , genomeTxDb, targets,
     xIsBin = TRUE, verbose = TRUE)
> # Draw a single plot on a window, with a custom layout
> plotGenomicRegions( features, 'GENE02:E002' , genomeTxDb, targets, xIsBin =
    TRUE, layout = matrix( c('A_C','A_D','B_C','B_D') ,nrow = 1 ),
    verbose = TRUE, color = '#AA8866')
> # Draw many plots on files.
> # Argument outfileType define the file format of the generated files.
> # Argument useTransparency specified that transparency can be used to draw the
> # plot. No all graphic devices support transparency.
> # Argument annotationHeight specifies the proportional height of the plot used
> # to draw the annotation.
> # Argument deviceOpt gives additional arguments (width and height in this
> # example) to the underlying graphic device (pdf in this example)
> # One of the plots generated here is shown in a figure below.
> plotGenomicRegions( features, c( 'GENE02:E002', 'GENE01:E002', 'GENE02:E003' ),
     genomeTxDb, targets, xIsBin = TRUE, outfolder = 'grplots', verbose = TRUE,
     color = '#334466', outfileType='pdf', useTransparency = TRUE,
     annotationHeight = 0.12, deviceOpt = c( width = 8, height = 6) )
> # Draw a single plot on a window, with premerged bams
> mergedBams <- data.frame(
   row.names = c( 'A_C','A_D','B_C','B_D' ),
   bam = c( 'A_C.bam',  # Warning, these files do not exists.
            'A_D.bam',  # Bam files should be indexed.
            'B_C.bam',
            'B_D.bam' ))
> plotGenomicRegions( features, 'GENE02:E002' , genomeTxDb, targets,
    xIsBin = TRUE, verbose = TRUE, preMergedBAMs = mergedBams )
```

Figure 6 shows a plot from one of the previuos examples.

In addition to coverage plots, ASpli is able to draw plots containing raw gene counts, raw bin counts, PSI/PIR value, inclusion and exclusion junction counts for a single bin in all, or a subset, conditions of the analysis. This plot is useful to integrate evidences of different sources in order to explain an experimental result. The function to make these plots is plotBins, below there are some examples of use.

Example 1:

```
> # Defines an experiment with one experimental factor (genotype, with two
> # values: wild-type (WT) and mutant (MT) ), and three replicate samples for each
> # condition.
> targets <- data.frame(
   row.names = c( 'WT1', 'WT2', 'WT3',
                  'MT1', 'MT2', 'MT3' ),
```

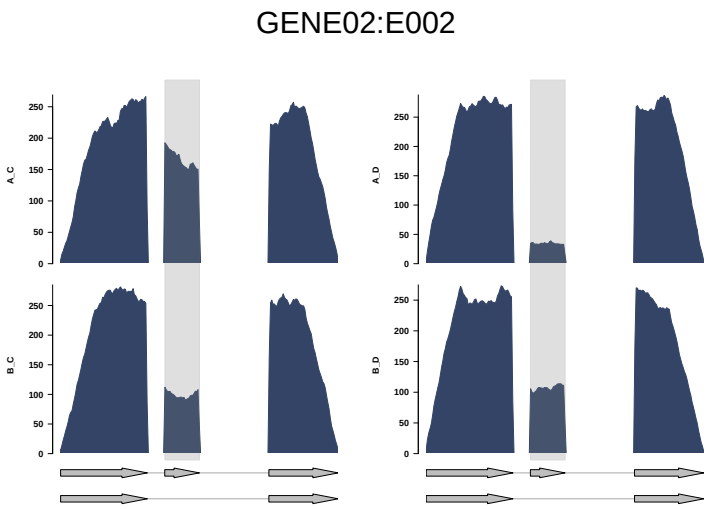


Figure 6: Genomic plot of a gene containing a exon skipping splicing event highlighted for four different conditions.

```
   bam = c( 'WT1.bam', 'WT2.bam', 'WT3.bam',
            'MT1.bam', 'MT2.bam', 'MT3.bam' ),
   genotype = c( 'WT','WT','WT','MT','MT','MT' ),
   stringsAsFactors = FALSE )
> # Specifies what factors and values will be plotted, in this example all of
> # them.
> fv = list( genotype = c( 'WT', 'MT' ) )
> plotbins(
          counts,
          as,
          'GENE02:E002',
          factorsAndValues = fv,
          targets )
```

Example 2:

```
> # Defines an experiment with two experimental factor (treat, with four
> # values: A, B, C and D; and time, with twelve values from T1 to T12.), and
> # two replicate samples for each condition. In the definition, many values are
> # not shown to reduce the space of the example, and are replaced by '...' .
> targets <- data.frame(
   row.names = c( 'A_T1.r1', 'A_T1.r2',
                  'A_T2.r1', 'A_T2.r2',
                  ... ,
                  'D_T12.r1', 'D_T12.r2' ),
   bam = c( 'A_T1.r1.bam', 'A_T1.r2.bam',
            'A_T2.r1.bam', 'A_T2.r2.bam',
            'D_T12.r1.bam', 'D_T12.r2.bam' ),
   treat = c( 'A', 'A',
              'A', 'A',
              ... ,
              'D', 'D' ),
   time = c( 'T1', 'T1',
             'T2', 'T2',
```

```
            ... ,
            'T12', 'T12' ),
   stringsAsFactors = FALSE )
> # Draw the plots.
> plotbins(
         counts,
         as,
         'GENE02:E002',
         factorsAndValues = fv,
         targets )
> # Specifies what factors and values will be plotted. In this example there are
> # two factor, the first one in 'fv' list is the main factor, values will
> # be grouped by this factor.
> fv = list( time = c( 'T1', 'T2', ... , 'T12' ) ,
           treat = c( 'A', 'B', 'C', 'D' ) )
> # Draw the plots
> plotbins(
         counts,
         as,
         'GENE02:E002',
         factorsAndValues = fv,
         targets )
```

Figures 7 and 8 shows the images that can be obtained in setups like shown in the examples above. Note that one image contains barplots, and the other contains lines, they are selected automatically according to the number of conditions and values per condition. However, it is possible to force to one of them.

3.8 Utility functions

ASpli includes many functions that help the use to manipulate and interrogate the different ASpli objects.

3.8.1 Consulting data in `ASpliDU` objects

ASpliDU objects can contain data for gene, bins and/or junctions depending on what function was used to create it. `DUreport` and `DUreportBinSplice` methods returns `ASpliDU` objects with data for gene and bins. `junctionDUreport` function returns an object with junction data only or with gene, bins and junction depending on the input. There are two function that are useful to know what kind of data an `ASpliDU` contains: `containsJunctions` and `containsGenesAndBins`.

```
> du <- aspliDUexample1()
> containsJunctions( du )
> containsGenesAndBins( du )
```

3.8.2 Filtering `ASpliCount` objects

Genes, bins and junctions present in an `ASpliCount` object can be filtered by abundance according to several statments:

- number of counts or read density.
- mean value or minimum value.
- grouping by condition or on the whole set.
- all conditions must meet the selection criteria or at least one condition must do it.

`filterReadCounts` method allow this filtering. Here there are some examples:

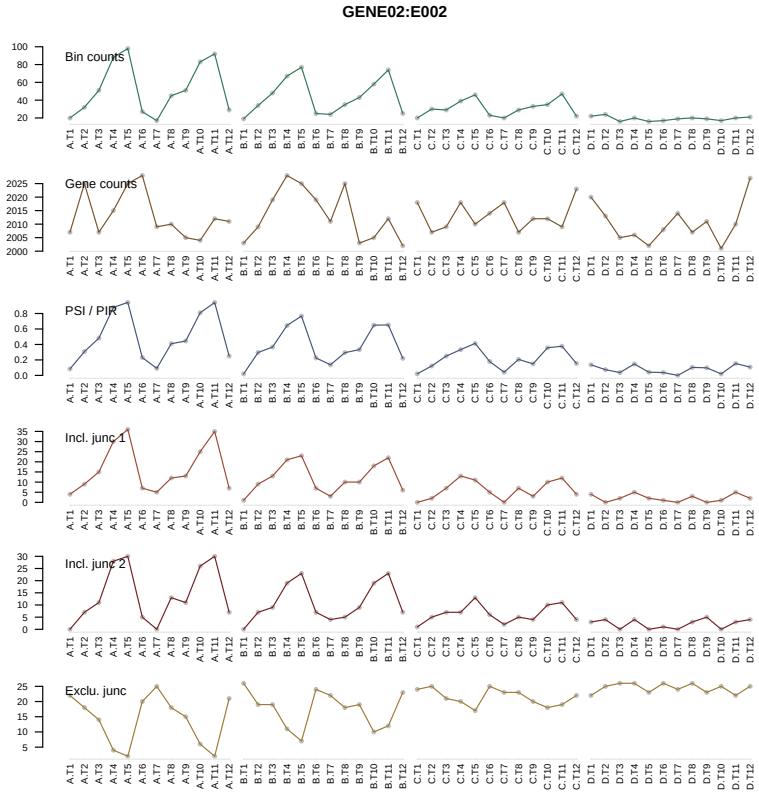


Figure 7: Plots of bin gene, and junction counts, and PSI/PIR metric for a single bin of an experiment containing a time course of 12 posints in four conditions. Gene expression seem to be constant across all conditions, however, the usage of the bin oscilates with time. Inclusion and exclusion junctions show a concordant behaviour.

3.8.3 Filtering ASpliDU objects

Results present in ASpliDU can be filtered by **logFC** and **FDR** with filterDU function. Elements with and **FDR** value lower or equal to a given threshold, and with an absolute **logFC** greater or equal to a given value are kept. Default values for **logFC** and **FDR** threshold values are 0 and 1 respectively, in other words, is not filtered by that value.

```
> # Filter genes that changes at least a 50 percent their expression with a
> # FDR value of 0.05 or less, and bins changes at least a 50 percent their expression with a
> # FDR value of 0.05
> du <- aspliDUexample1()
> duFilt <- filterDU(du, 'genes', '0.05', log(1.5,2))
> duFilt <- filterDU(duFilt, 'bins', '0.1', log(1.5,2))
```

It is possible to filter only those elements that has a positive (or negative) **logFC** using the arguments absLogFC and logFCgreater. absLogFC forces to take the absolute value of **logFC**, this is TRUE by default. logFCgreater specifies, if TRUE, that the **logFC** value must be greater than the threshold.

```
> # Filter genes that reduces their expression to the half
> du <- aspliDUexample1()
> duFilt <- filterDU( du, 'genes', '0.05', -1, absLogFC = FALSE,
                      logFCgreater = FALSE  )
```

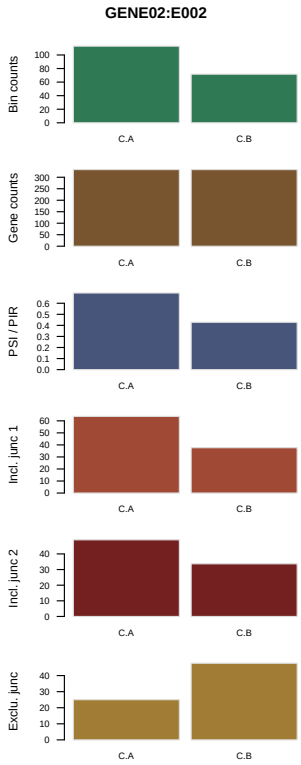


Figure 8: Plots of bin gene, and junction counts, and PSI/PIR metric for a single bin of an experiment containing two combinatorial factors. Of those, only two conditions are shown.

3.8.4 Subsetting ASpli objects

ASpliCounts, ASpliAS, targets data.frame and list of GAlignments (i.e. bam files data) can be subsetted by conditions or samples.

```
> # Subset counts
> counts <- aspliCountsExample()
> countsSmall <- subset(counts, targets, select = c("A_C", "A_D") )
> # Subset AS results. Note that PIR/PSI metrics are not recalculated.
> as <- aspliASexample()
> asSmall <- subset(as, targets, select = c("A_C_0", "A_C_1") )
> # Subset targets
> targets      <- aspliTargetsExample()
> targetsSmall <- subsetTargets( targets, c("A_C", "A_C") )
> # Subset BAMs
> bams     <- aspliBamsExample()
> bamSmall <- subsetBams( bams, targets, c("A_C", "A_C") )
```

3.8.5 Merging DU and junction data for bins

mergeBinDUAS function merge bin data from DU analysis contained in an ASpliDU object and bin data from junction repertoire usage and PSI/PIR metrics contained in an ASpliAS object. Also a delta PSI/PIR value is calculated from a contrast vector.

```
> du <- aspliDUexample1()
> as <- aspliASexample()
> mergeBinDUAS(du,as, targets)
```

4 Example data

4.0.1 Synthetis data set

ASpli contains a small synthetic data set to test de complete working pipeline. It consist of a genome with ten genes with multiple isoforms, a set of **BAM** files from an experiment with two×two factorial design, and a **GTF** file with the corresponding annotation. The two factors in this examples are called **f1** and **f2**. **f1** can have value **A** or **B** and **f2** can have value **C** or **D**, defining four conditions: **A.C**, **A.D**, **B.C** and **B.D**. There are three replicates for each condititon (see table 1) .

Sample	f1	f2	replicate	condition
1	A	C	0	A.C
2	A	C	1	A.C
3	A	C	2	A.C
4	A	D	0	A.D
5	A	D	1	A.D
6	A	D	2	A.D
7	B	C	0	B.C
8	B	C	1	B.C
9	B	C	2	B.C
10	B	D	0	B.D
11	B	D	1	B.D
12	B	D	2	B.D

Table 1: Experimental design of the example data set.

The firts step of the workflow is to load the annotation from the reference sequence and extract their features.

```
> library( GenomicFeatures )
> # gtfFileName contains the full path to the example gtf file in your system.
> gtfFileName <- aspliExampleGTF()
> # Create a TxDb object using makeTxDbFromGFF function from GenomicFeatures
> # package
> genomeTxDb <- makeTxDbFromGFF( gtfFileName )
> # Extract the genomic features
> features <- binGenome( genomeTxDb )
>
```

Also, the exprimental sequencing data must be loaded, and the experimental samples, and factors should be defined.

```
> # bamFiles contains the full path of the bam files in your system
> bamFiles <- aspliExampleBamList( )
> # Define the targets
> targets <- data.frame(
   row.names = paste0('Sample',c(1:12)),
   bam = bamFiles,
   f1 = c( 'A','A','A','A','A','A',
           'B','B','B','B','B','B'),
   f2 = c( 'C','C','C','D','D','D',
```

```
          'C','C','C','D','D','D'),
   stringsAsFactors = FALSE
 )
> # Examinate the condition names
> getConditions(targets)
> # Load the bam files
> bams = loadBAM(targets)
```

After the targets are defined and the sequence data is read, you can continue to assign the number of counts to each feature.

```
> counts <- readCounts( features, bams, targets, readLength = 100,
                        maxISize = 5000 )
```

Now you can run the DE/DU analysis, here will be avaualted a test to hypothesis that the expression of some genes respond to the interaction of **f1** and **f2**. In other words, that the observed effect of applying the two factors can not be explained by the independent contribution of each factor. The interaction can be expressed as:

$$I = (B.D - B.C) - (A.D - A.C) \tag{2}$$

The coefficients of the terms in this expression can be represented in the contrast vector $[1, -1, -1, 1]$, assuming that they correspond to the order given by `getConditions` function.

```
> # DU/DE analysis for genes and bins.
> du <- DUreport( counts, targets, contrast = c( 1, -1, -1, 1 ) )
> # DU analysis for junctions
> du <- junctionDUReport( counts, targets, appendTo = du,
                          contrast = c( 1, -1, -1, 1 ) )
```

The discovery of AS events can be done with:

```
> # AS analysis
> as <- AsDiscover( counts, targets, features, bams, readLength = 100)
```

Finally, you can select your top tags integrating differential usage data and junction data.

```
> # Select top tags from DU
> topTagsBins <- filterDU( du, what = 'bins', fdr = 0.05, logFC = log(1.5,2) )
> # DU results are merged with AS results
> topTagsBins <- mergeBinDUAS( du, as, targets, contrast = c( 1, -1, -1, 1 ) )
> # We filter those top tags that have also junction evidence that supports the
> # differential usage, a change of at least 0.1 between observed and expected
> # in the PSI or PIR metric is required.
> topTagsBins <- topTagsBins[ abs(topTagsBins[,'delta' ]) > 0.10,]
```

Bin **GENE10:E002** is the one with the lowest **FDR** value, now we can examine it in a plot. The results in figure 9 show that a change in **f1** from **A** to **B** has not the same outcome for **C** or **D** in **f2**.

```
> plotGenomicRegions( features,'GENE10:E002', genomeTxDb, targets )
```

4.0.2 Real data set

It is possible to run a demo of ASpli using public RNA-Seqdata available via Bioconductor. It requieres installing of RNAseqData.HNRNPC.bam.chr14 package and loading an small TranscriptDb of Human Genome only for chromosome 14. This subset of reads is part of an experiment intended to analyze splicing factors and their relationship with exonization of Alu elements [10]. We load the package and the genome:

```
> library(RNAseqData.HNRNPC.bam.chr14)
```

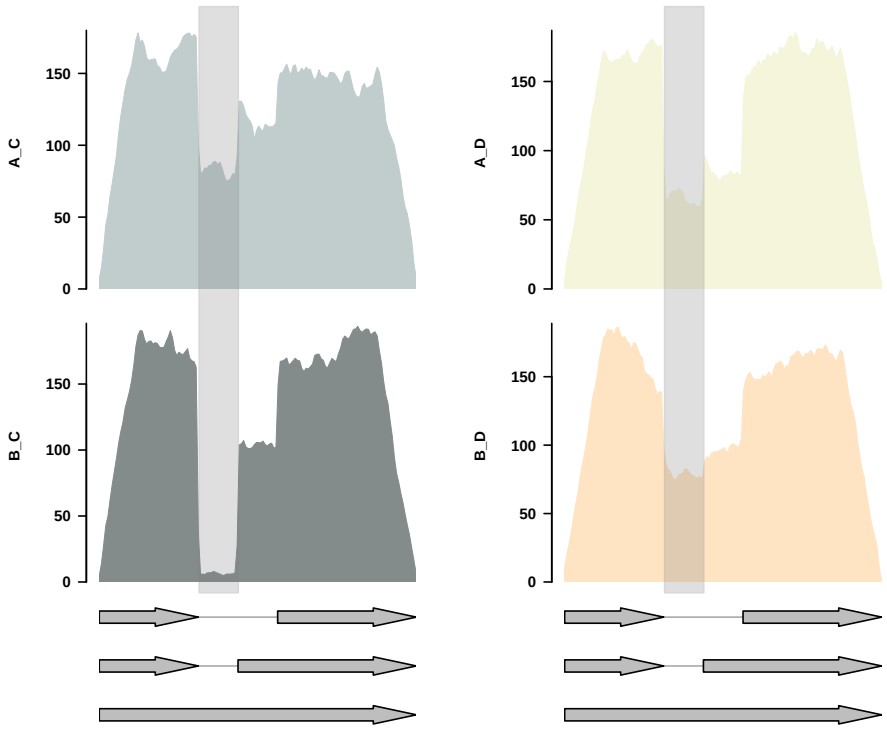


Figure 9: Genomic region of example bin **GENE10:E002**.

In this case we are loading a genome `TranscriptDb` already created and available in the `ASpli` package for the *Homo sapiens* chromosome 14:

```
> chr14 <- system.file("extdata","chr14.sqlite", package="ASpli")
> genome <- loadDb(chr14)
```

And now we are ready to start the complete analysis.

```
> features <- binGenome(genome)
```

This data set contains data for of two conditions **KD** and **CT** with four replicates each one. Create targets object using the information of the available files:

```
> targets <- data.frame(
              bam = RNAseqData.HNRNPC.bam.chr14_BAMFILES,
              treat = c( "CT", "CT", "CT", "CT",
                         "KD", "KD", "KD", "KD" ),
              stringsAsFactors = FALSE )
```

Then, load the bam files:

```
> bam <- loadBAM(targets)
```

Overlap alignments against features:

```
> counts <- readCounts( features, bam, targets, readLength=100L, maxISize=50000 )
```

Perform DE/DU analysis

```
> du <- DUreport(counts, targets)
```

Analyze AS using junctions:

```
> as <- AsDiscover (counts, targets, features, bam, readLength=100L, threshold = 5, cores = 1 )
```

Select top tags, using DU and AS information:

```
> duFiltered <- filterDU( du, 'bins', fdr = 0.05, logFC = log(1.5,2) )
> merged     <- mergeBinDUAS( duFiltered, as, targets )
```

Bin ZNF410:E013, from top tags, has a usage change that is statistically significant and the change of PSI/PIR metric is greater than 0.3. We can inspect it visually, see figure 10.

```
> # Show the complete gene, with the bin hightlighted
> plotGenomicRegions( features, 'ZNF410:E013', genome, targets, verbose=TRUE,
                      colors = matrix(c('#223344','#223344'), ncol=2 ), outfileType='pdf', outfolder='grplo
> # Show a zoom in in the bin
> plotGenomicRegions( features, 'ZNF410:E013', genome, targets, verbose=TRUE,
                      colors = matrix(c('#223344','#223344'), ncol=2 ),
                      zoomOnBins = 0.05)
```

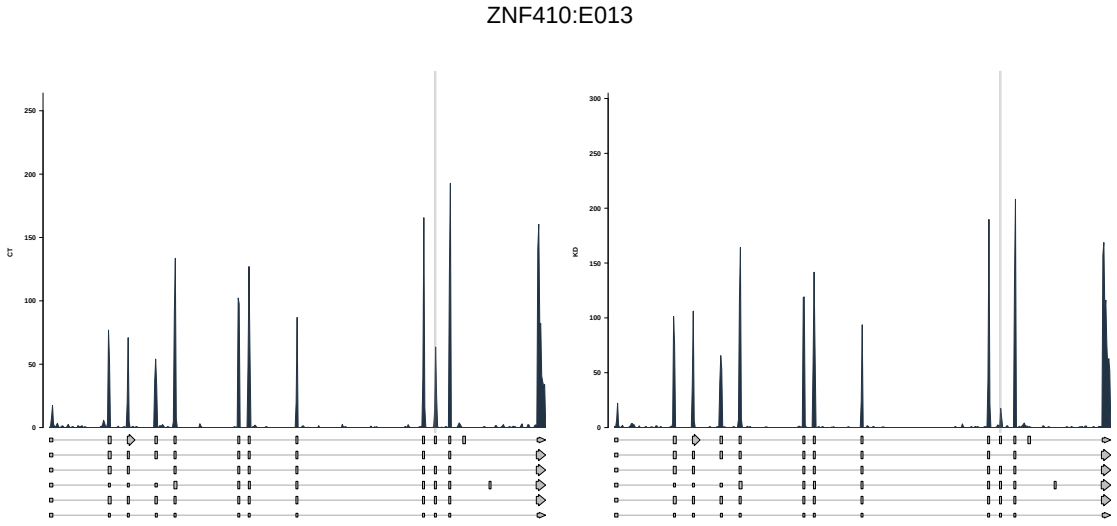


Figure 10: Plot of ZNF410:E013 exon skipping

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