

geneXtendeR

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Introduction

This vignette describes geneXtendeR (Khomtchouk et al. 2016), an R/Bioconductor package for optimized annotation of genomic features (primarily peaks called from a ChIP-seq experiment, but any coverage island regions would work) with the nearest gene. “Extending” refers to performing gene-feature overlaps after adding to the gene-span a user-specified region upstream of the start of the gene model and a fixed (500 bp) region downstream of the gene, resulting in assigning to a gene the features that do not physically overlap with it but are sufficiently close. Extending is an automated iterative procedure in geneXtendeR, allowing the user to repeatedly align peaks to multiple gene transfer format (GTF) files to assess what global gene-spans optimize the genomewide alignment of peaks with their closest genes. This facilitates the process of deciphering which differentially enriched peaks are dysregulating which specific genes. This, in turn, aids experimental follow-up and validation in designing primers for a set of prospective genes during qPCR (Barbier et al. 2016).

Rationale

With an abundance of Bioconductor software currently available for peak annotation to nearby features (e.g., ChIPpeakAnno (Zhu et al. 2010)) as well as the existence of various command line tools (e.g., BEDTools *closest* function (Quinlan and Hall, 2010), HOMER (Heinz et al. 2010)), what makes geneXtendeR different? The simple answer is: geneXtendeR is designed for assessing the variability of peak overlap with cis-regulatory elements and proximal-promoter regions. It is well-known that peak coordinates (peak start position, peak end position) exhibit a considerable degree of variance depending on the peak caller used (e.g., SICER (Zang et al. 2009), MACS2 (Zhang et al. 2008), etc.), both in terms of length distribution of peaks as well as the total number of peaks called, even when run at identical default parameter values (Koohy et al. 2014; Thomas et al. 2017). Tuning algorithm-specific

parameters produces even greater variance amongst peak callers, thereby complicating the issue further. This variance becomes a factor when annotating peak lists genome-wide with their nearest genes as, depending on the peak caller, peaks can be either shifted in genomic position (towards 5' or 3' end) or be of different lengths. As such, geneXtender represents a first step towards tailoring (or customizing) the functional annotation of a ChIP-seq peak dataset according to the details of the peak coordinates (chromosome number, peak start position, peak end position).

The primary focus of geneXtender is to optimize the process of functional annotation of a ChIP-seq peak list whereby instead of just annotating peaks with their nearest genomic features (as statically defined by a given genome build's coordinates), geneXtender investigates how peaks dynamically align to various user-specified gene extensions (e.g., 500 bp upstream extensions, 2000 bp upstream extensions, etc. for all genes in the genome). This shows where peaks localize across the genome with respect to their nearest gene, as well as what gene ontologies (BP, CC, and MF) are impacted at these various extension levels. This, in turn, informs the user what gene extensions ideally capture the GO terms involved in the biology of their experiment. For example, if a user's study is investigating the role of epigenetic enzymes in alcohol addiction and dependence, then functionally annotating a peak list using gene extensions that maximize the number of brain-related ontologies (for both BP, CC, and MF categories) makes sense.

With regards to histone modification ChIP-seq analysis, geneXtender computes optimal gene extensions tailored to the broadness of the specific epigenetic mark (e.g., H3K9me1, H3K27me3), as determined by a user-supplied ChIP-seq peak input file. To accomplish this level of custom-tailored data analysis, geneXtender first optimally extends the boundaries of every gene in a genome by some genomic distance (in DNA base pairs) for the purpose of flexibly incorporating cis-regulatory elements, such as promoter regions, as well as downstream elements that are important to the function of the gene relative to an epigenetic histone modification ChIP-seq dataset. This action effectively transforms genes into "gene-spheres", a new term that we coin to emphasize the 3D-nature of heterochromatin. A gene-sphere is composed of cis-regulatory elements (e.g., proximal promoters $\pm \approx 3$ kb from TSS), distal regulatory elements (e.g., enhancers), transcription start/end sites (TSS/TES), exons, introns, and downstream elements of a gene. As such, geneXtender maximizes the signal-to-noise ratio of locating genes closest to and directly under peaks. By performing a computational expansion of this nature, ChIP-seq reads that would initially not map strictly to a specific gene can now be optimally mapped to the regulatory regions of the gene, thereby implicating the gene as a potential candidate, and thereby making the ChIP-seq analysis more successful. Such an approach becomes particularly important when working with epigenetic histone modifications that have inherently broad peaks with a diffuse range of signal enrichment (e.g., H3K9me1, H3K27me3).

Quick start

First, install the geneXtender package via:

```
> ## try http:// if https:// URLs are not supported
> source("https://bioconductor.org/biocLite.R")
> biocLite("geneXtender")
```

```
> library(geneXtender)
```

This automatically loads the `rtracklayer` R package, which contains the `readGFF()` command used to retrieve GTF files of any model organism. As such, load in a GTF file into your R environment, e.g.:

```
> rat <- readGFF("ftp://ftp.ensembl.org/pub/release-84/gtf/  
+               rattus_norvegicus/Rattus_norvegicus.Rnor_6.0.84.chr.gtf.gz")
```

URLs may be obtained as direct links from: <http://useast.ensembl.org/info/data/ftp/index.html>. Click on the “GTF” link under the “Gene sets” column for a particular species and then right-click (or command-click on Mac OS X) the name of the file containing the species name/version number and file extension `chr.gtf.gz` (e.g., `Homo_sapiens.GRCh38.84.chr.gtf.gz`, `Mus_musculus.GRCm38.84.chr.gtf.gz`, etc.), and copy the link address. Then, paste the link address into the `readGFF()` as shown above. This will create an R dataframe object containing the respective GTF file.

Next, the user must input their peak data from a peak caller (e.g., SICER, MACS2, etc.). The peak data must contain only three tab-delimited columns (chromosome number, peak start, and peak end) and a header containing: “chr”, “start”, and “end”. See `?samplepeaksinput` for an example. Once the peak input data (e.g., “somepeaksfile.txt”) has been assembled properly (i.e., to contain only the three tab-delimited columns and header above), it must be properly formatted prior to the execution of downstream analyses.

First, the user must set their working directory to point to the location of their peak data file. Then type the following command:

```
> peaksInput("somepeaksfile.txt")
```

This command properly formats the user’s peak file in preparation for subsequent analyses, producing a resultant “peaks.txt” file in the user’s working directory.

To see how the above command works using a built-in example, the `geneXtender` package provides a peak input dataset¹ called “somepeaksfile.txt”, which can be loaded into memory like this:

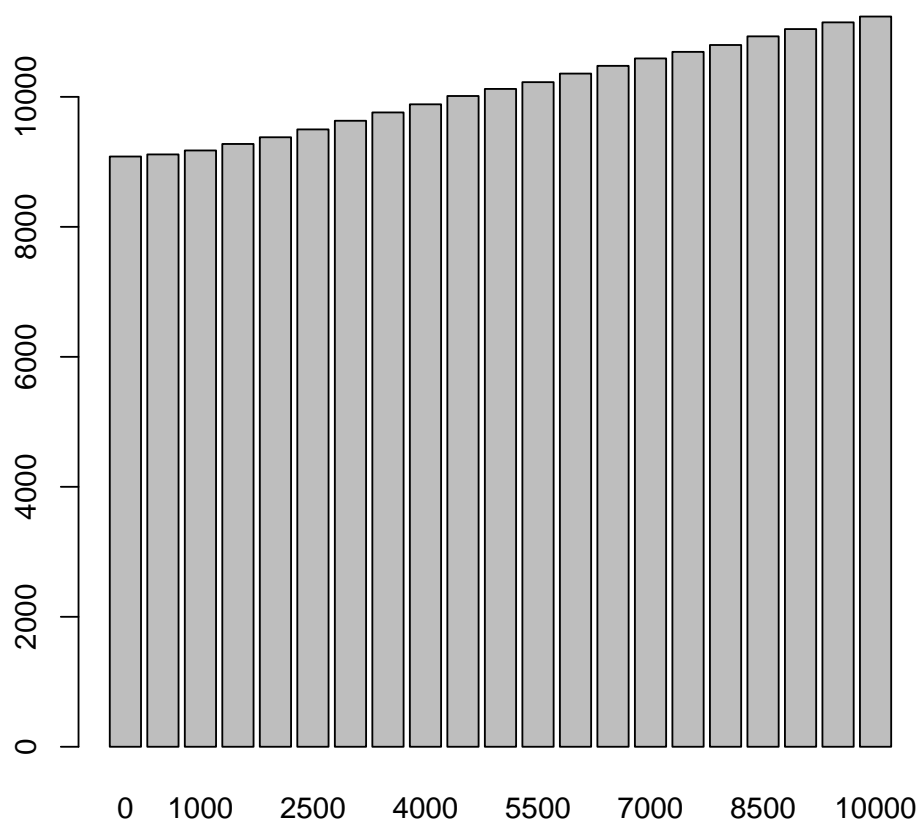
```
> fpath <- system.file("extdata", "somepeaksfile.txt", package="geneXtender")  
> peaksInput(fpath)
```

This creates a properly formatted (i.e., properly sorted) “peaks.txt” file in the user’s working directory.

Now, we may use the R object that we created with `readGFF()` earlier to create a bar chart visualization showing the number of peaks that are sitting directly on top of genes across a series of upstream extensions (of each gene in a genome):

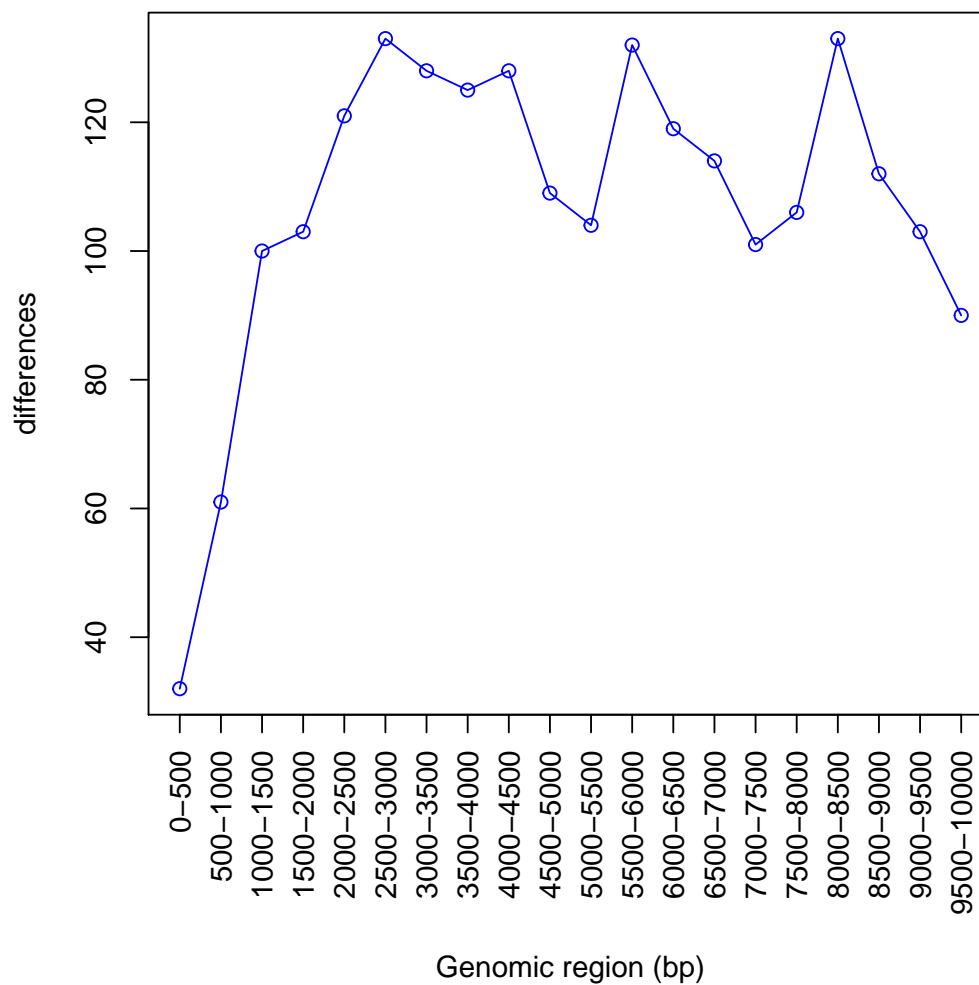
¹This peaks dataset comes from a ChIP-seq investigation of brain tissue (prefrontal cortex) in alcohol addiction and dependence (Barbier et al. 2016), see References section for details.

```
> barChart(rat, 0, 10000, 500)
```



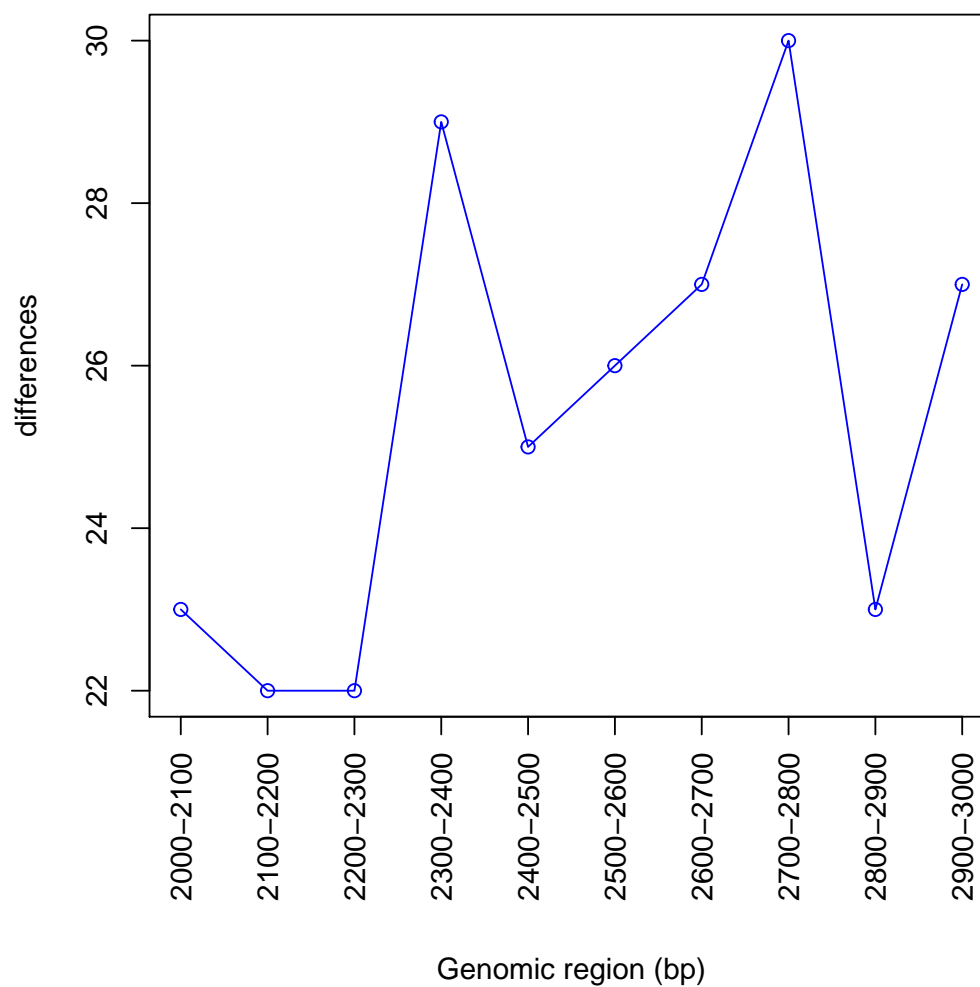
This command first generates 21 individual whole-genome files: 0, 500, 1000, ..., and 10000 bp upstream extension files for the rat (*Rattus norvegicus*) genome, each having an automatic 500 bp downstream extension. In other words, each gene in the rat genome is extended upstream and downstream by some user-specified distance, thereby creating a “gene-sphere.” As such, this bar chart command visualizes the raw count of the number of peaks that are sitting on top of genes at each individual upstream cutoff. Clearly, the wider the gene-sphere, the more peaks-on-top-of-genes are found throughout the genome. However, the law of diminishing returns begins to kick in at increasing upstream extension levels (see `linePlot()` for a visual representation):

```
> linePlot(rat, 0, 10000, 500)
```



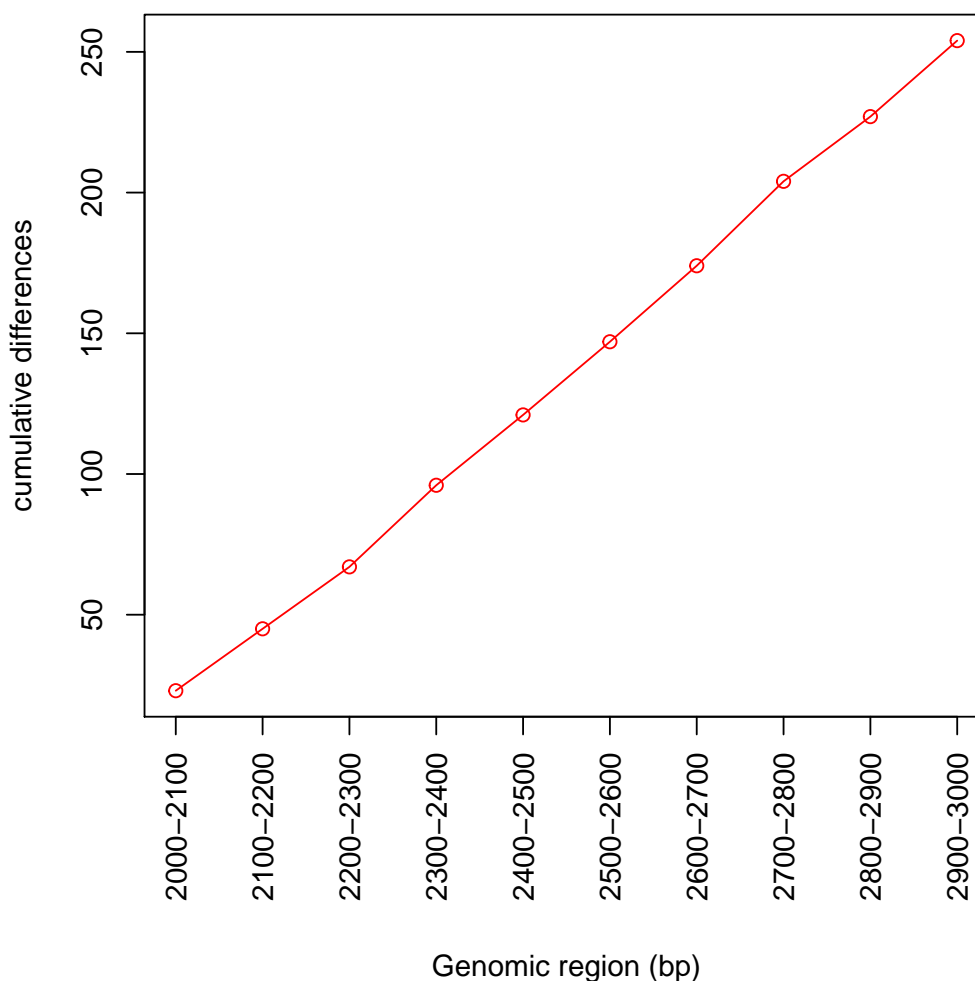
In this line plot, there is a sharp rise in the number of peaks-on-top-of-genes from a 0 bp upstream extension to a 1500 bp upstream extension, and from a 2000 bp upstream extension to a 3000 bp upstream extension. This steady rise up until 3000 bp is followed by a steady decline at subsequent extension levels followed by some noisy fluctuations. It may be interesting to investigate what is going on in the interval from 2000 bp to 3000 bp:

```
> linePlot(rat, 2000, 3000, 100)
```



Visually, there is a relative spike in the number of peaks-on-top-of-genes at the 2400 bp upstream extension (as compared to the 2300 bp extension). This spike then drops back down at subsequent extension levels and fluctuates in a noisy manner. However, a cumulative line plot shows that this “spike” is more of a visual effect than anything else, since the graph is almost perfectly linear:

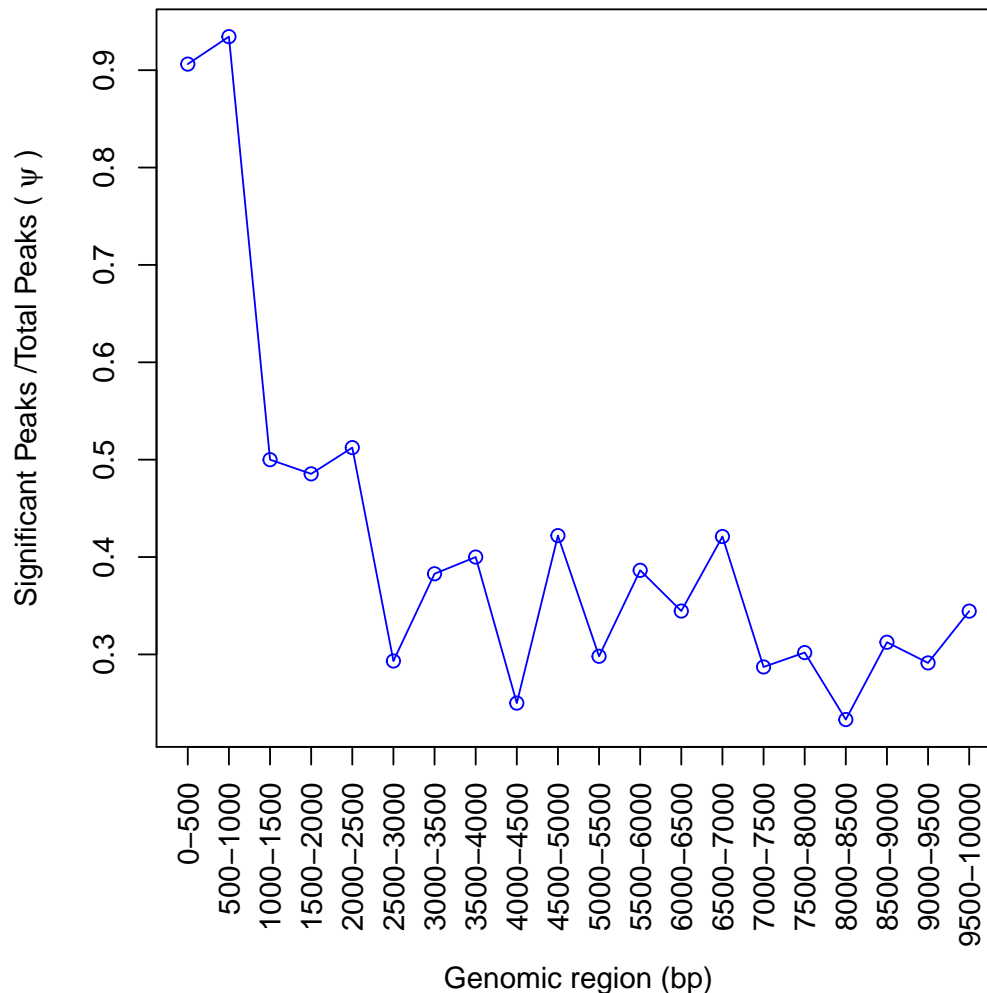
```
> cumlinePlot(rat, 2000, 3000, 100)
```



Hence, one very useful function in `geneXtenderR` is called `hotspotPlot()`, which allows users to examine the ratio of statistically significant peaks² to the total number of peaks at each genomic interval (e.g., 0-500 bp upstream of every gene in the genome, 500-1000 bp upstream of every gene in the genome, etc.).

²Note that statistical significance is set apriori by the user at the peak calling stage (prior to `geneXtenderR`) to give the user the freedom to choose how to filter out peak coordinates that only pass specific p-value and FDR cutoffs from a peak caller. Peak caller output (e.g., from SICER) gives both p-value and FDR measures for each peak, thereby making it easy to extract only the peak coordinates that pass a specific set of statistical cutoff criteria.

```
> allpeaks <- system.file("extdata", "totalpeaksfile.txt", package="geneXtender")
> sigpeaks <- system.file("extdata", "significantpeaksfile.txt", package="geneXtender")
> hotspotPlot(allpeaks, sigpeaks, rat, 0, 10000, 500)
```



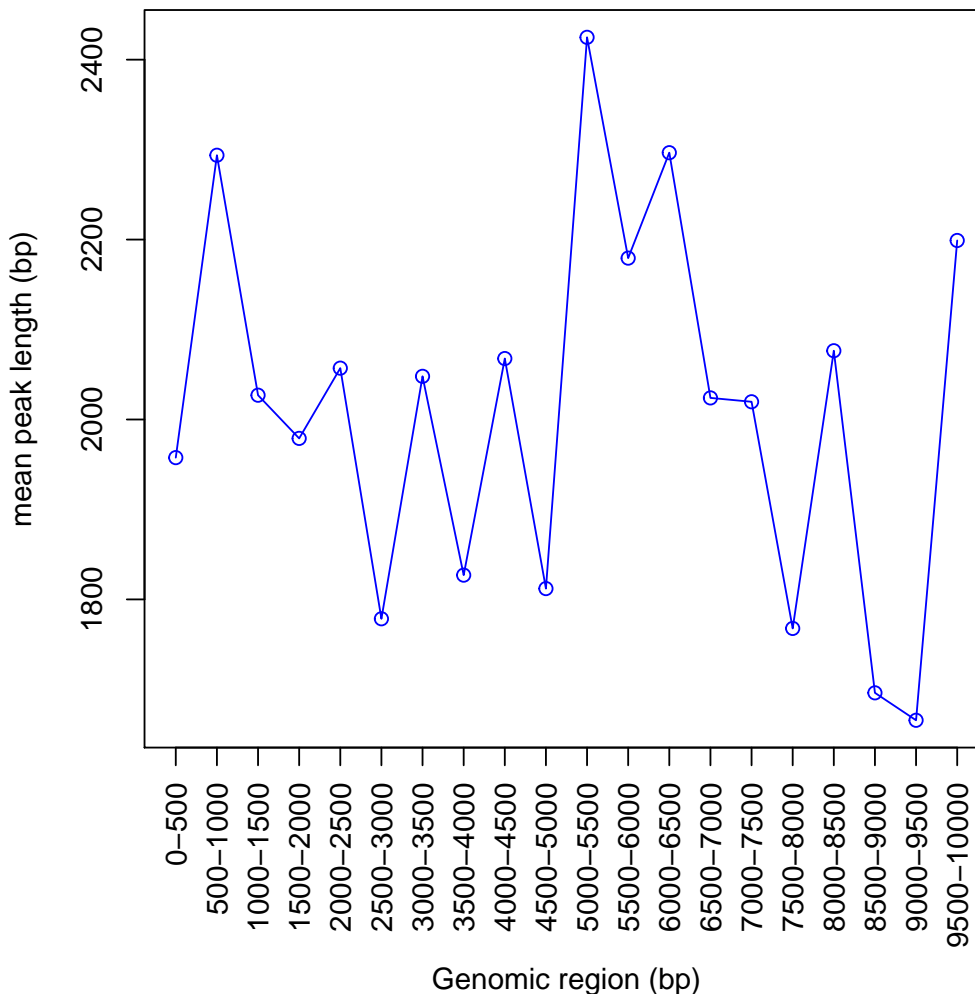
This line plot shows that the concentration of significant peaks in this dataset (Barbier et al. 2016) is highest between 0 and 1000 bp upstream of a gene, with over 90% of peaks in these regions being statistically significant. In contrast, between 1000 bp and 2500 bp, only about half of the total peaks contained in these intervals are significant. Statistical significance then fluctuates noisily at further upstream genomic intervals, but with at least a quarter (25%) of the total peaks in these further upstream regions being statistically significant. As such, the take-home message is that genomic regions within the first 1000 bp upstream of their respective genes are most likely to contain significant peaks (relative to the total peak count in these regions) and are therefore hotspots, but regions beyond this also contain a fair share of statistically significant peaks.

One interesting area to investigate is the variance in the broadness of significant (or total) peaks across different genomic intervals³. In other words, asking questions like “are statistically significant peaks that

³One can either observe the global distribution of peak lengths within specific genomic intervals (see ?peakLength-

are located very close to their nearest gene (e.g., 0-500 bp away) wider or narrower than peaks located 500-1000 bp away from their nearest gene?”. To answer this question we can do:

```
> sigpeaks <- system.file("extdata", "significantpeaksfile.txt", package="geneXtenderR")
> peaksInput(sigpeaks)
> meanPeakLengthPlot(rat, 0, 10000, 500)
```



This line plot displays the mean (average) length of all significant peaks found within each genomic interval. Clearly, the “average peak” is slightly narrower in 0-500 bp intervals than in 500-1000 bp intervals yet, overall, peak lengths tend to fluctuate more or less stochastically at various intervals. To get the exact peak length, we can do:

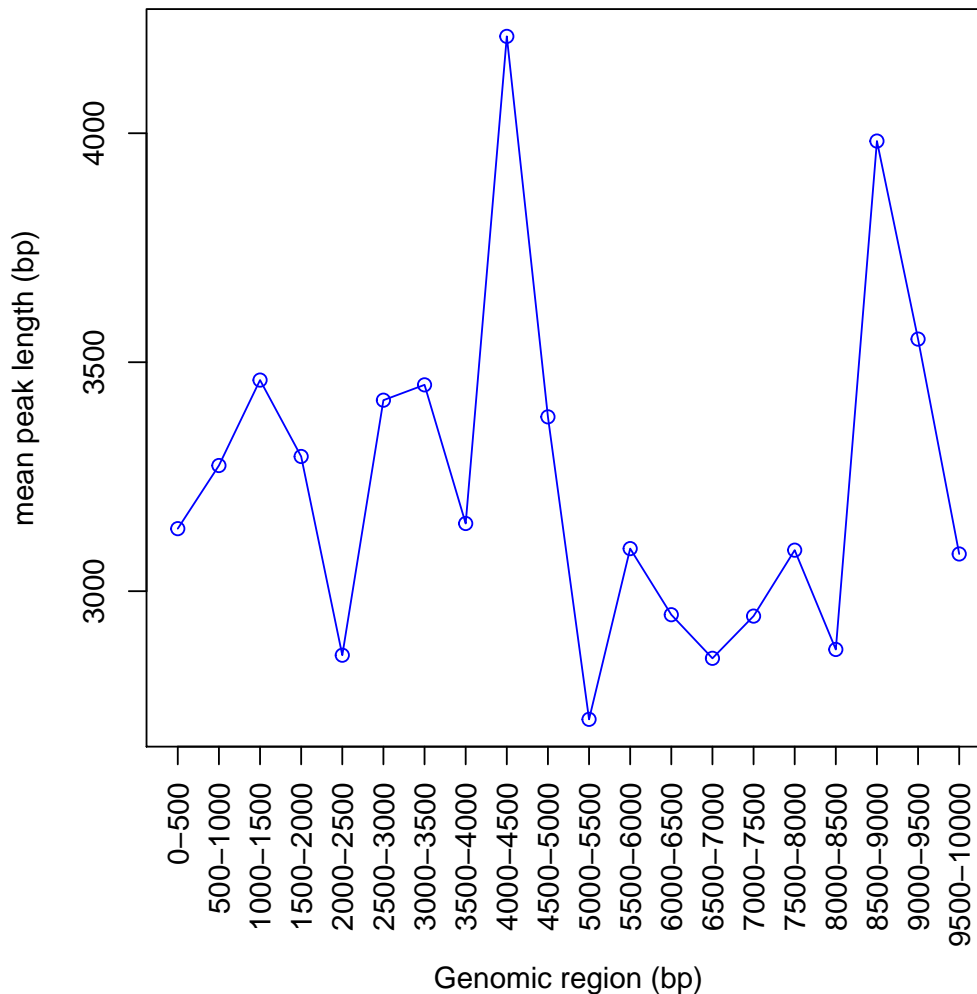
```
> sigpeaks <- system.file("extdata", "significantpeaksfile.txt", package="geneXtenderR")
> peaksInput(sigpeaks)
> meanPeakLength(rat, 0, 500)
```

```
[1] 1957.621
```

Boxplot()), or observe the global distribution of peak lengths across all intervals (see ?allPeakLengths()).

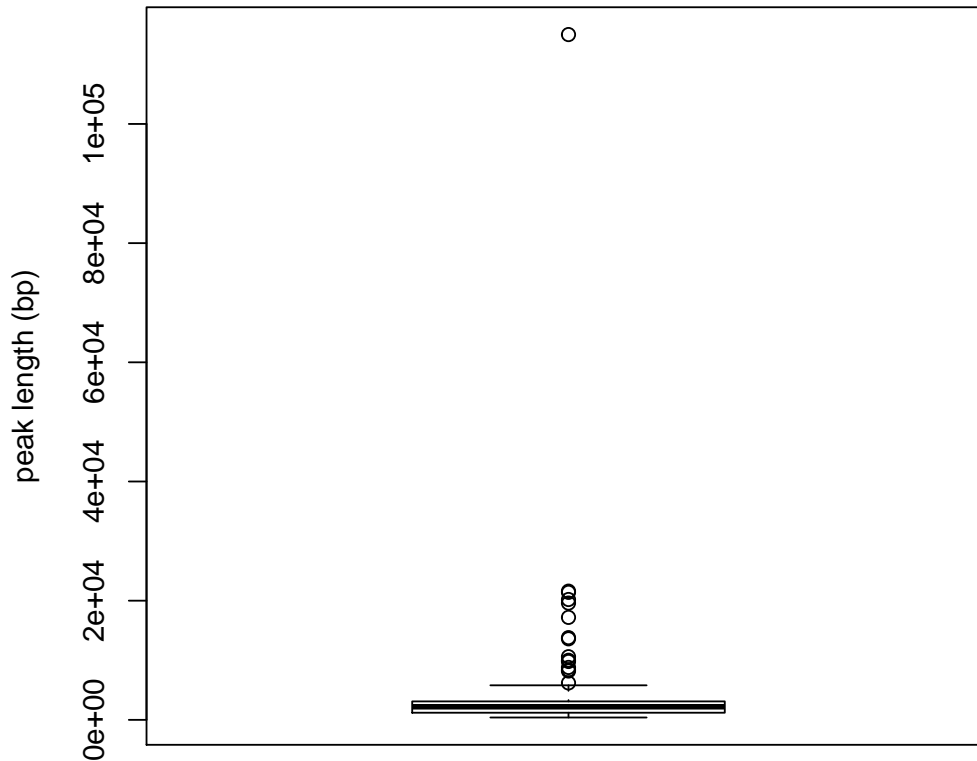
So the mean peak length in the interval 0-500 bp is approximately 1958 bp. Although we see that there is no specific interval with peaks of extraordinary average lengths, it is still possible to see peak length outliers in certain cases (especially when looking at total peak sets):

```
> allpeaks <- system.file("extdata", "totalpeaksfile.txt", package="geneXtenderR")
> peaksInput(allpeaks)
> meanPeakLengthPlot(rat, 0, 10000, 500)
```



We see that the 4000-4500 bp and 8500-9000 bp intervals both look quite different in terms of their mean peak lengths relative to the other intervals. To see if the mean might be influenced by a strong outlier(s), we can do:

```
> allpeaks <- system.file("extdata", "totalpeaksfile.txt", package="geneXtenderR")
> peaksInput(allpeaks)
> peak_lengths <- peakLengthBoxplot(rat, 4000, 4500)
```



This box-and-whisker plot shows a clear outlier, which is an example of a very broad peak. We can find the exact length of this outlier peak using:

```
> peak_lengths <- peakLengthBoxplot(rat, 4000, 4500)
> max(peak_lengths)
```

```
[1] 114999
```

So this outlier peak measures 114999 bp in total length, therefore making it an extremely broad peak. To see what nearest gene it resides to, we can first extract the peak's index by:

```
> peak_lengths <- peakLengthBoxplot(rat, 4000, 4500)
> match(114999, peak_lengths)
```

```
[1] 126
```

which returns the index of where this peak length is found. Then the following command finds all unique peaks that reside between 4000 and 4500 bp upstream of their nearest gene:

```
> distinct(rat, 4000, 4500)
```

	V1	V2	V3	V4	V5	V6	V7	V8	V9
1:	1	19526200	19526799	1	19520708	19526671	ENSRNOG00000030796	AABR07000595.1	0
2:	1	61630800	61631999	1	61624941	61630954	ENSRNOG00000025949	Vom1r22	0
3:	1	71346800	71347999	1	71334629	71347133	ENSRNOG00000049014	LOC100912263	0
4:	1	98385400	98394199	1	98394160	98403468	ENSRNOG00000037331	Cd33	0
5:	1	101099600	101101399	1	101086377	101100094	ENSRNOG00000020583	Fcgrt	0

124:	18	60006800	60007199	18	59985860	60007069	ENSRNOG00000017852	Nars	0
125:	19	45499400	45499799	19	45499420	45507827	ENSRNOG00000053551	AABR07043877.1	0
126:	19	54877400	54992399	19	54871853	54877469	ENSRNOG00000028578	AABR07044065.1	0
127:	20	30610800	30620799	20	30606026	30611101	ENSRNOG00000049167	AABR07044988.1	0
128:	100	73017400	73018799	100	73018667	73024598	ENSRNOG00000027980	AABR07039245.1	0

where we see that index 126 belongs to gene AABR07044065.1⁴. Checking the arithmetic difference between column 3 and column 2 for this specific row verifies 114999, as these two columns represent the peak start position and peak end positions. Now let's identify what the other columns represent by running the `distinct()` function again (but this time on a smaller interval to have less output printed to the screen):

```
> fpath <- system.file("extdata", "somepeaksfile.txt", package="geneXtender")
> peaksInput(fpath)
> distinct(rat, 2300, 2400)
```

	V1	V2	V3	V4	V5	V6	V7	V8	V9
1:	1	79718600	79725199	1	79725197	79728613	ENSRNOG00000026891	AC093995.1	0
2:	1	188715600	188716999	1	188688243	188715680	ENSRNOG00000016013	Gprc5b	0
3:	1	214368800	214373199	1	214373115	214386385	ENSRNOG00000018367	Taldo1	0
4:	1	221669800	221671199	1	221671190	221694018	ENSRNOG00000027456	Cdc42bpg	0
5:	1	236532800	236534799	1	236529431	236532885	ENSRNOG00000022308	LOC103691298	0
6:	3	82239000	82242199	3	82096568	82239064	ENSRNOG00000008758	Tspan18	0
7:	3	82780200	82784599	3	82762362	82780214	ENSRNOG00000042533	Accs1	0
8:	3	146409600	146412399	3	146376328	146409652	ENSRNOG00000006795	Apmmap	0
9:	3	165702800	165706799	3	165678807	165702889	ENSRNOG00000042101	Zfp93	0
10:	4	84850400	84851999	4	84851986	84872257	ENSRNOG00000010205	Mturn	0
11:	4	118157000	118157799	4	118157747	118166562	ENSRNOG00000016273	Fam136a	0
12:	4	171955800	171956999	4	171956961	171961084	ENSRNOG00000057540	AABR07062363.1	0
13:	4	180237200	180239199	4	180231882	180237204	ENSRNOG00000048961	Bhlhe41	0
14:	5	36437600	36438199	5	36433358	36437694	ENSRNOG00000055329	AABR07047528.1	0
15:	5	69038200	69039399	5	69035218	69038218	ENSRNOG00000060997	U6	0

⁴This peak may not be statistically significant, but how could it be if it's so huge? In situations like this, it may be a good idea to check what is known about the gene already: <http://panthertest2.usc.edu/genes/gene.do?acc=RAT%7CEnsembl=ENSRNOG00000028578%7CUniProtKB=A0A0G2K0W2>. Clearly, not much is known yet.

16:	5	121456000	121457199	5	121451803	121456072	ENSRNOG000000045614	LOC102552337	0
17:	5	153628200	153630199	5	153568245	153628269	ENSRNOG000000018109	Clic4	0
18:	7	14586000	14587199	7	14587120	14615369	ENSRNOG000000048450	Cyp4f37	0
19:	7	75225000	75225799	7	75225775	75249569	ENSRNOG000000061463	AABR07057510.3	0
20:	8	133130600	133133199	8	133126720	133130690	ENSRNOG000000006730	Ccr111	0
21:	10	1830200	1832199	10	1832118	1841132	ENSRNOG000000040121	RGD1565158	0
22:	11	80315400	80316799	11	80316777	80332099	ENSRNOG000000022160	Rtp2	0
23:	14	76654000	76654999	14	76654911	76833661	ENSRNOG000000051169	Clnk	0
24:	14	103716400	103719199	14	103711769	103716440	ENSRNOG000000054704	AABR07016558.1	0
25:	16	631200	642399	16	517332	631224	ENSRNOG000000061982	AABR07024473.2	0
26:	16	9020200	9020999	16	9020987	9055164	ENSRNOG000000042628	RGD1561145	0
27:	16	75363800	75364599	16	75364529	75368406	ENSRNOG000000029462	Defa11	0
28:	20	1747000	1747399	20	1747316	1751142	ENSRNOG000000050043	Olr1735	0
29:	20	22423400	22426199	20	22420251	22423425	ENSRNOG000000057124	AABR07044824.1	0
	V1	V2	V3	V4	V5	V6	V7	V8	V9

This data table shows 29 separate entries sorted by chromosome and start position. V1-V3 denote the chromosome/start/end positions of the peaks, V4-V6 denote the respective values for the genes, V7 is the gene ID (e.g., Ensembl ID), V8 is the gene name, and V9 is the distance of each respective peak to its nearest gene. It should be noted that the X chromosome is designated by the integer 100, the Y chromosome by the integer 200, and the mitochondrial chromosome by the integer 300. This is done for sorting purposes (see ?peaksInput for details). In short, the distinct() command finds what peaks-on-top-of-genes would be missed if a 2300 bp upstream extension is used instead of a 2400 bp extension. In other words, these 29 genes all reside between 2300-2400 bp upstream of their nearest gene.

It may be of interest to note the differential gene ontologies between these two upstream extensions:

```
> library(org.Rn.eg.db)
> library(GO.db)
> x <- diffGO(rat, 2300, 2400, BP, org.Rn.eg.db)
> head(x, 20)
```

	gene\$SYMBOL	GOID	TERM
1	Gprc5b	GO:0001934	positive regulation of protein phosphorylation
2	Gprc5b	GO:0007186	G-protein coupled receptor signaling pathway
3	Gprc5b	GO:0007626	locomotory behavior
5	Gprc5b	GO:0010976	positive regulation of neuron projection development
7	Gprc5b	GO:0032147	activation of protein kinase activity
9	Gprc5b	GO:0042593	glucose homeostasis
11	Gprc5b	GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB signaling
13	Gprc5b	GO:0045666	positive regulation of neuron differentiation
14	Gprc5b	GO:0045860	positive regulation of protein kinase activity
15	Gprc5b	GO:0050729	positive regulation of inflammatory response
17	Gprc5b	GO:0060907	positive regulation of macrophage cytokine production
19	Gprc5b	GO:0061098	positive regulation of protein tyrosine kinase activity
21	Gprc5b	GO:0090263	positive regulation of canonical Wnt signaling pathway

23	Taldo1	GO:0005975	carbohydrate metabolic process
24	Taldo1	GO:0006002	fructose 6-phosphate metabolic process
25	Taldo1	GO:0006098	pentose-phosphate shunt
26	Taldo1	GO:0009052	pentose-phosphate shunt, non-oxidative branch
27	Taldo1	GO:0019682	glyceraldehyde-3-phosphate metabolic process
28	Cdc42bpg	GO:0006468	protein phosphorylation
30	Cdc42bpg	GO:0035556	intracellular signal transduction

This dataframe shows the first 20 unique gene ontology terms, their IDs, and respective gene symbols. Clearly, gene name *Gprc5b* has several BP ontologies related explicitly to the brain, while *Taldo1* does not. Considering that the ChIP-seq peaks dataset used as input into geneXtender comes from a ChIP-seq study investigating the prefrontal cortex, this suggests that a 2400 bp extension may be more suitable for this brain dataset. However, such decisions are left entirely to the discretion and judgment of the user in deciding the relative importance of specific genes and their respective GO terms (BP, CC, or MF) to the goals of the computational analysis (as well as plans for experimental follow-up and validation). See Discussion section for details.

It is also critical to note that the `diffGO()` function returns ALL known gene ontologies, NOT a gene ontology enrichment analysis (more about this in Discussion section). The goal is to provide users with knowledge regarding all possible known roles of any given gene. For example, by knowing that a potential gene candidate has previously been linked with known brain-related ontologies, a user may be prompted to look more closely into the relevant literature behind this gene and its implications to the biological question under study (before embarking on making a decision about its potential impact and suitability as a good candidate for experimental validation).

Furthermore, a user may plot the differential gene ontology results as an interactive network:

```
> library(networkD3)
> library(org.Rn.eg.db)
> library(dplyr)
> makeNetwork(rat, 2300, 2400, BP, org.Rn.eg.db)
```

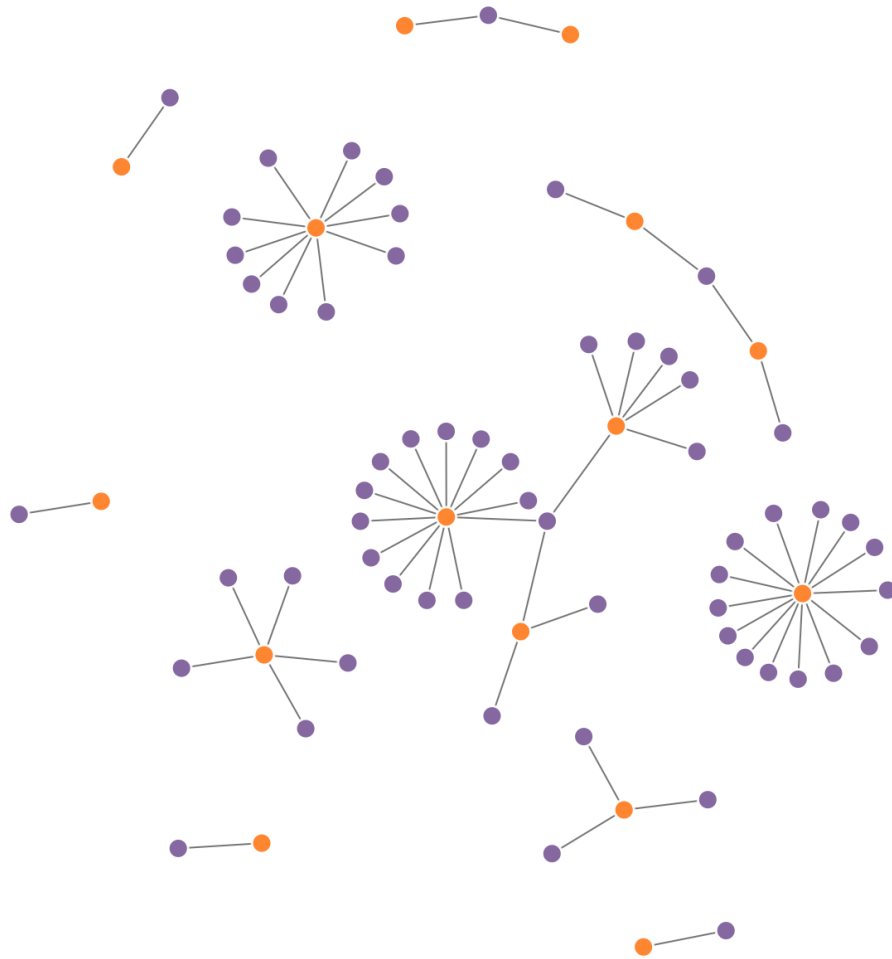


Figure 1: Orange color denotes gene names, purple color denotes GO terms. A user can hover the mouse cursor over any given node to display its respective label directly within R Studio. Likewise, users can dynamically drag and reorganize the spatial orientation of nodes, as well as zoom in and out of them for visual effect.

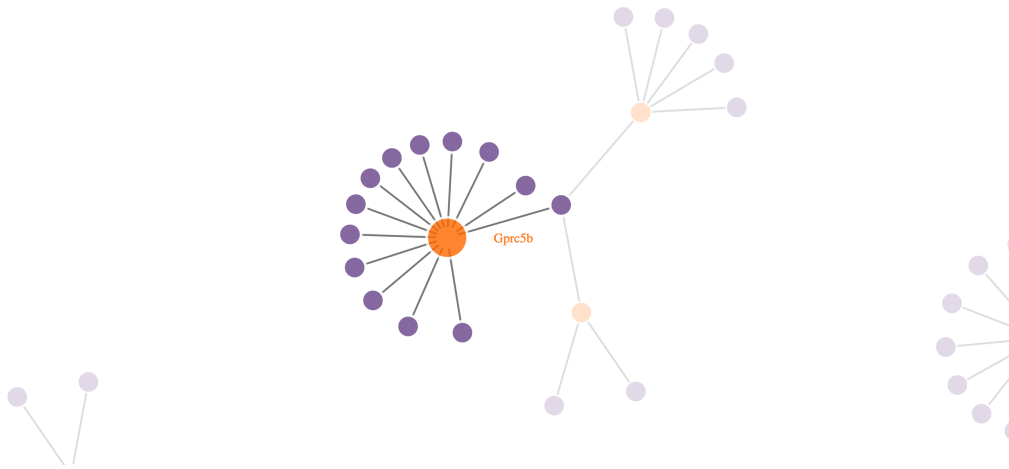


Figure 2: Orange color denotes gene names, purple color denotes GO terms. A user can hover the mouse cursor over any given node to display its respective label directly within R Studio. Likewise, users can dynamically drag and reorganize the spatial orientation of nodes, as well as zoom in and out of them for visual effect.

In addition, users can generate word clouds comprised from words present in their GO terms:

```
> library(tm)
> library(SnowballC)
> library(wordcloud)
> library(RColorBrewer)
> makeWordCloud(rat, 2300, 2400, BP, org.Rn.eg.db)
```




Figure 3: Word cloud generated from words comprising gene ontology terms of category BP. This word cloud shows the words that are used within BP gene ontology terms of peaks found to be present between 2300 and 2400 bp upstream of their nearest genes.

It may also be of interest to visually examine the most frequently used words found within GO terms:

```
> library(tm)
> library(SnowballC)
> library(wordcloud)
> library(RColorBrewer)
> plotWordFreq(rat, 2300, 2400, BP, org.Rn.eg.db, 10)
```

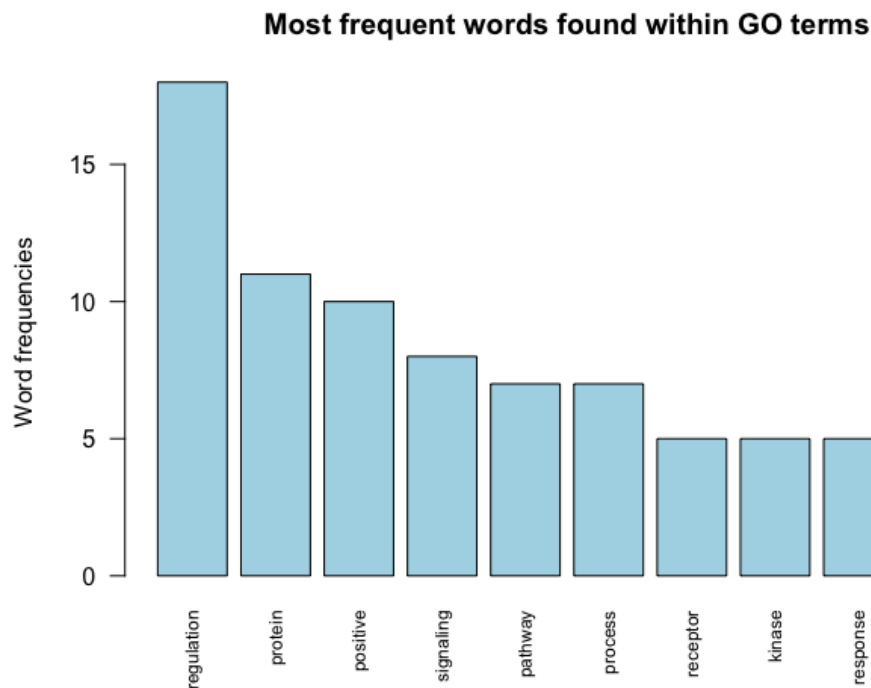


Figure 4: This barplot shows the top 10 words used within gene ontology terms (specific to BP) of peaks found to be present between 2300 and 2400 bp upstream of their nearest genes.

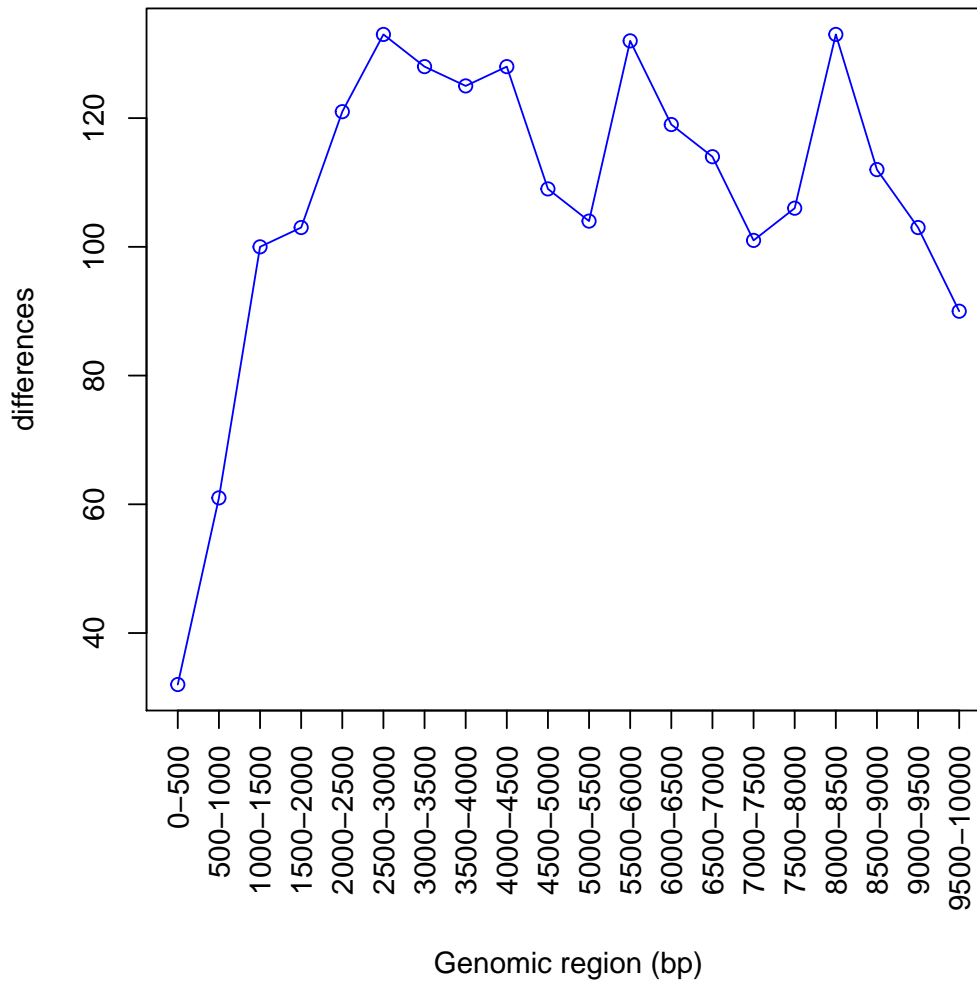
Once the user has chosen the specific upstream extension to be used, the peak file is ready to be fully annotated:

```
> annotate(rat, 2400)
```

which generates a fully annotated peaks outfile (in the user's working directory) containing various genomic features and labeled headers.

Discussion

Even though geneXtenderR is designed to compute (and analyze/display) optimal gene extensions tailored to the characteristics of a specific peak input file, geneXtenderR will not explicitly impose on the user the optimal extension to select, since this information is highly study-dependent and, as such, is ultimately reserved to the user's discretion. For example, a user may choose a conservatively lower upstream extension (e.g., for studies investigating narrow peaks such as H3K4me3 or H3K9ac that exhibit a compact and localized enrichment pattern, where high upstream extensions may begin to lose biological relevance). An example of such a user-driven decision would be the selection of a 1500 bp upstream extension instead of a 3500 bp extension in situations like this:



This line plot is derived from the input peak dataset used from the H3K9me1 study examined earlier (Barbier et al. 2016). If the study had examined a narrower chromatin mark (e.g., H3K4me3) then the decision process for choosing an optimal extension may have been different.

In certain cases, additional extensions are unlikely to add significant value to the annotation of the peak file. Taking the example of the 0-10000 bp line plot, an upstream extension beyond 3500 bp globally across every gene in a genome would most likely not accurately reflect the biology of the peak input file (since such large global upstream extensions are likely to reach considerably beyond known proximal promoter elements, especially for relatively narrow histone marks or transcription factors). Such assumptions may be validated directly by the user by investigating the p-value and FDR of specific peaks using a combination of HT-seq (to count the reads) and edgeR/DESeq2 (to assess statistical significance). As such, geneXtender is designed to be used as part of a biological workflow involving subsequent statistical analysis:

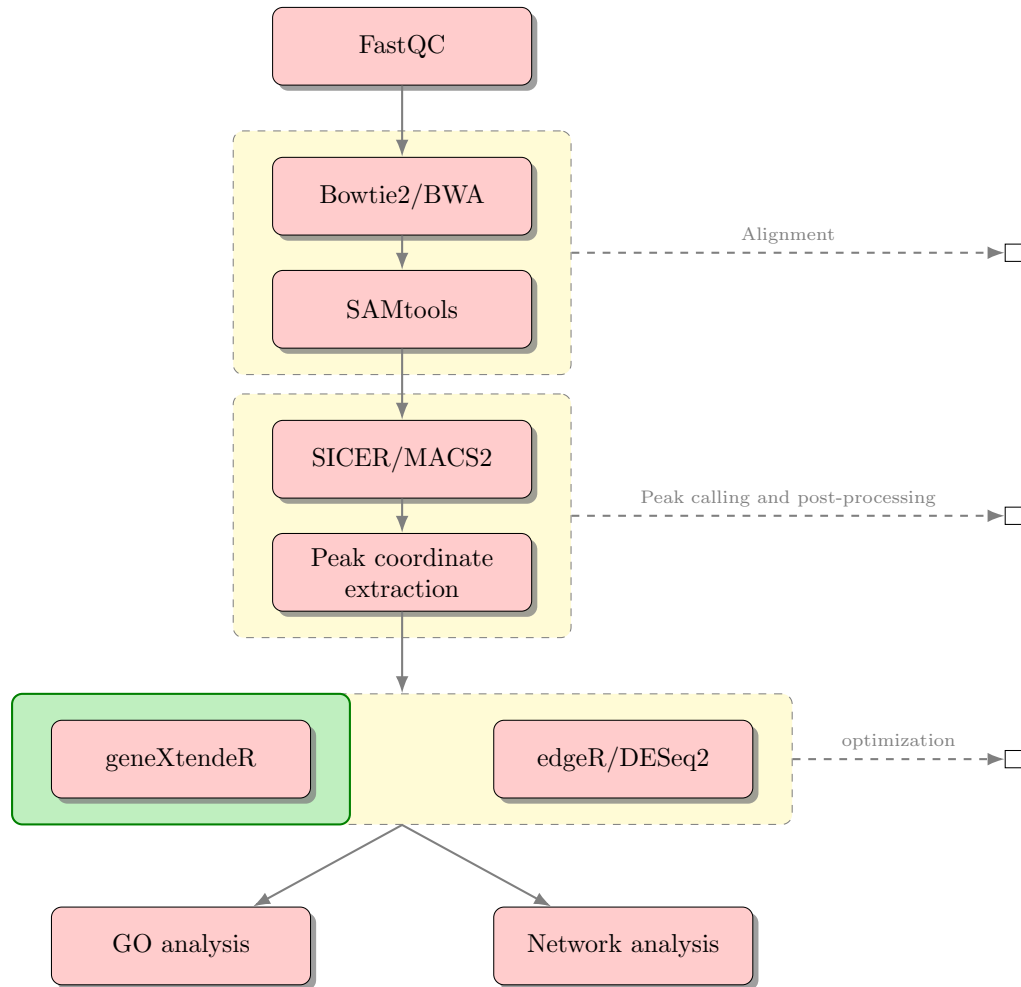
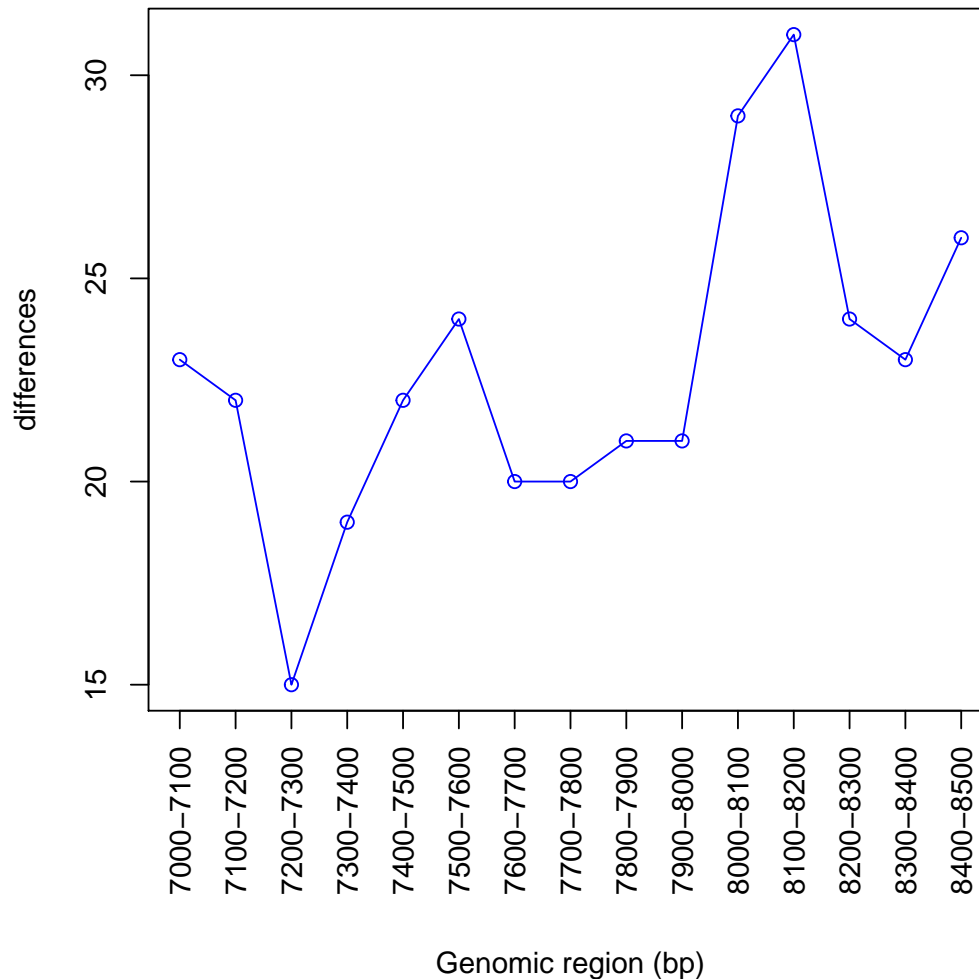


Figure 5: Sample biological workflow using geneXtenderR in combination with existing statistical software to analyze peak significance. Subsequent gene ontology enrichment or network analysis may be conducted on genes associated with statistically significant peaks.

It is entirely possible (and probable) for significant peaks to be present at relatively high upstream extension levels (i.e., large gene-spheres), albeit these significant peaks may be associated with biology not directly relevant to the study at-hand, due mainly to the sheer magnitude of the distance of the peak from traditional gene boundaries (where traditional gene boundaries may be loosely defined as ± 3 kb from TSS and ± 0.5 kb from TES). Consequently, it is likely for peaks-on-top-of-genes to exhibit higher levels of noise at higher upstream extension levels. Nevertheless, this does not mean that potential enhancer activity should be discounted. For instance, it is not uncommon to see a steady rise or even a surge in the number of peaks-on-top-of-genes at higher upstream extension levels:

```
> linePlot(rat, 7000, 8500, 100)
```



This line plot shows that there are over 30 peaks in this dataset (across the rat genome) that reside between 8100 and 8200 bp upstream of their nearest gene. In far-out cases like this, it is particularly recommended to examine the statistical significance of peaks to get a sense for the possibility of potential enhancer activity/regulation. Of course, such computational findings would require experimental follow-up and/or database mining for known motifs. Assessment of such statistical significance values is beyond the scope of *geneXtenderR*, in order to allow the user freedom to choose the most appropriate statistical package/technique for their analysis. As before, first use the `distinct()` function to create a table of unique genes located under peaks between the two upstream extension levels:

```
> distinct(rat, 8100, 8200)
```

Then, assess the statistical significance of these peaks using a combination of HT-seq (Anders et al. 2015) and edgeR (Robinson et al. 2010), or HT-seq and DESeq2 (Love et al. 2014), or some other appropriate combination of existing software tools. Genes associated with the resultant statistically significant peaks may then be further assessed with gene ontology enrichment analysis to help answer a variety of interesting research questions. It should once again be noted that the `diffG0()` function

does NOT perform gene ontology enrichment analysis. Instead, it returns all known gene ontologies for each gene. The purpose and utility of this is described in the previous section.

Moreover, DNA sequences under peaks may be checked for the presence of known regulatory motifs (e.g., using TRANSFAC (Matys et al. 2006) or MEME/JASPAR (Sandelin et al. 2004, Bailey et al. 2009)), or for the presence of biological repeats (e.g., using RepeatMasker (Smit et al. 2015)). Pending a prospective GO enrichment and network analysis, functional validation may be followed up in the lab to test any potential regulatory sites or prospective enhancer elements, thereby bringing the computational analysis pipeline back to the bench.

In addition to the computational workflows discussed above, geneXtender's wide array of functions makes it possible to conduct some rather interesting and creative combinations of genomic analysis. Let's say, for example, that a user wants to explore all known ontological differences across specific disparate sectors of the genome (e.g., 0-500 bp vs. 2000-3000 bp, but removing 501-1999 bp from consideration). In other words, look at all peaks (across the entire genome) that reside between 0-500 bp upstream of their nearest gene (and 2000-3000 bp upstream of their nearest gene), and extract unique gene ontologies that differ between these two variable-length sectors (where one is 500 bp long and the other is 1000 bp in length). This can be accomplished rather conveniently using dplyr:

```
> library(dplyr)
> library(org.Rn.eg.db)
> library(GO.db)
> a <- diffGO(rat, 0, 500, BP, org.Rn.eg.db)
> b <- diffGO(rat, 2000, 3000, BP, org.Rn.eg.db)
> dplyr::filter(b, TERM %in% a$TERM)
```

	gene\$SYMBOL	GOID	TERM
1	Sod2	GO:0001889	liver development
2	Sod2	GO:0007507	heart development
3	Sod2	GO:0008285	negative regulation of cell proliferation
4	Sod2	GO:0042311	vasodilation
5	Sod2	GO:0042493	response to drug
6	Sod2	GO:0043066	negative regulation of apoptotic process
7	D111	GO:0001757	somite specification
8	D111	GO:0008284	positive regulation of cell proliferation
9	D111	GO:0008285	negative regulation of cell proliferation
10	D111	GO:0045596	negative regulation of cell differentiation
11	Olr40	GO:0007186	G-protein coupled receptor signaling pathway
12	Hbb	GO:0070527	platelet aggregation
13	Olr139	GO:0007186	G-protein coupled receptor signaling pathway
14	Olr282	GO:0007186	G-protein coupled receptor signaling pathway
15	Gprc5b	GO:0007186	G-protein coupled receptor signaling pathway
16	Aqp8	GO:0055085	transmembrane transport
17	Aqp8	GO:0071320	cellular response to cAMP
18	Ano9	GO:1902476	chloride transmembrane transport
19	Osbp15	GO:0006869	lipid transport
20	Cdc42bpg	GO:0006468	protein phosphorylation

21	Dusp5	GO:0045892	negative regulation of transcription, DNA-templated
22	Adgrl2	GO:0007166	cell surface receptor signaling pathway
23	Adgrl2	GO:0007186	G-protein coupled receptor signaling pathway
24	Nfe2l2	GO:0016567	protein ubiquitination
25	Nfe2l2	GO:0071456	cellular response to hypoxia
26	Olr559	GO:0007186	G-protein coupled receptor signaling pathway
27	Tspan18	GO:0007166	cell surface receptor signaling pathway
28	Kcnq2	GO:0060081	membrane hyperpolarization
29	RGD1565415	GO:0006412	translation
30	Reg3b	GO:0008284	positive regulation of cell proliferation
31	Reg3b	GO:0043066	negative regulation of apoptotic process
32	Olr828	GO:0007186	G-protein coupled receptor signaling pathway
33	Tspan9	GO:0007166	cell surface receptor signaling pathway
34	Bhlhe41	GO:0045892	negative regulation of transcription, DNA-templated
35	Aptx	GO:0006974	cellular response to DNA damage stimulus
36	Ccl21	GO:0007186	G-protein coupled receptor signaling pathway
37	Aldob	GO:0001889	liver development
38	Aldob	GO:0042493	response to drug
39	Clic4	GO:1902476	chloride transmembrane transport
40	Htr1d	GO:0042310	vasoconstriction
41	Slc2a5	GO:0055085	transmembrane transport
42	Nlrc4	GO:0016567	protein ubiquitination
43	Esyt1	GO:0006869	lipid transport
44	Sbno2	GO:0045892	negative regulation of transcription, DNA-templated
45	Olr1085	GO:0007186	G-protein coupled receptor signaling pathway
46	Fbxo7	GO:0016567	protein ubiquitination
47	Dnmt1	GO:0042493	response to drug
48	Dnmt1	GO:0045892	negative regulation of transcription, DNA-templated
49	Xcr1	GO:0007186	G-protein coupled receptor signaling pathway
50	Ccr11l	GO:0007186	G-protein coupled receptor signaling pathway
51	Il17a	GO:0007166	cell surface receptor signaling pathway
52	RGD1559955	GO:0006412	translation
53	LOC684471	GO:0007186	G-protein coupled receptor signaling pathway
54	Il3	GO:0008284	positive regulation of cell proliferation
55	Il3	GO:0043066	negative regulation of apoptotic process
56	Olr1501	GO:0007186	G-protein coupled receptor signaling pathway
57	Socs3	GO:0016567	protein ubiquitination
58	Socs3	GO:0042493	response to drug
59	Socs3	GO:0043066	negative regulation of apoptotic process
60	Fbxw8	GO:0016567	protein ubiquitination
61	Fcgr2b	GO:0007166	cell surface receptor signaling pathway
62	Eef1e1	GO:0008285	negative regulation of cell proliferation
63	F13a1	GO:0007596	blood coagulation
64	Vom2r66	GO:0007186	G-protein coupled receptor signaling pathway
65	Csnk2a2	GO:0006468	protein phosphorylation

```

66      Csnk2a2 G0:0051726                      regulation of cell cycle
67      01r1735 G0:0007186          G-protein coupled receptor signaling pathway
>

```

This displays all biological process (BP) ontologies present in b that are not present in a. Similarly, one can look at all BP, CC, or MF ontologies present in a that are not present in b.

Concluding remarks

geneXtender is continually evolving, so any suggestions or new feature requests are always appreciated. Likewise, any bug reports may be posted to <https://github.com/Bohdan-Khomtchouk/geneXtender/issues> or emailed to the package maintainer directly.

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