

Using R and Bioconductor for Proteomics Data Analysis

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July 8, 2013

This vignette shows and executes the code presented in the manuscript *Using R for proteomics data analysis*. It also aims at being a general overview useful for new users who wish to explore the R environment and programming language for the analysis of proteomics data.

Keywords: bioinformatics, proteomics, mass spectrometry, tutorial.

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1 Introduction

This document illustrates some existing R infrastructure for the analysis of proteomics data. It presents the code for the use cases taken from [8]. A pre-print of the manuscript is available on arXiv¹.

There are however numerous additional R resources distributed by the Bioconductor² and CRAN³ repositories, as well as packages hosted on personal websites. Section 7 on page 42 tries to provide a wider picture of available packages, without going into details.

1.1 General R resources

The reader is expected to have basic R knowledge to find the document helpful. There are numerous R introductions freely available, some of which are listed below.

From the R project web-page:

- **An Introduction to R** is based on the former *Notes on R*, gives an introduction to the language and how to use R for doing statistical analysis and graphics. [[browse HTML](#) — [download PDF](#)]
- Several introductory tutorials in the [contributed documentation](#) section.
- The `TeachingMaterial` repository⁴ contains several sets of slides and vignettes about R programming.

Relevant background on the R software and its application to computational biology in general and proteomics in particular can also be found in [8]. For details about the Bioconductor project, the reader is referred to [10].

1.2 Getting help

All R packages come with ample documentation. Every command (function, class or method) a user is susceptible to use is documented. The documentation can be accessed by preceding the command by a `?` in the R console. For example, to obtain help about the `library` function, that will be used in the next section, one would type `?library`. In addition, all Bioconductor packages come with at least one vignette (this document is

¹<http://arxiv.org/abs/1305.6559>

²<http://www.bioconductor.org>

³<http://cran.r-project.org/web/packages/>

⁴<https://github.com/lgatto/TeachingMaterial>

the vignette that comes with the **RforProteomics** package), a document that combines text and R code that is executed before the pdf is assembled. To look up all vignettes that come with a package, say **RforProteomics** and then open the vignette of interest, one uses the `vignette` function as illustrated below. More details can be found in `?vignette`.

```
> ## list all the vignettes in the RforProteomics
> ## package
> vignette(package = "RforProteomics")
> ## Open the vignette called RforProteomics
> vignette("RforProteomics", package = "RforProteomics")
> ## or just
> vignette("RforProteomics")
```

R has several mailing lists⁵. The most relevant here being the main R-help list, *for discussion about problem and solutions using R*. This one is for general R content and is not suitable for bioinformatics or proteomics questions. Bioconductor also offers several mailing lists⁶ dedicated to bioinformatics matters and Bioconductor packages. The main Bioconductor list is the most relevant one. It is possible to post⁷ questions without subscribing to the list. Finally, the dedicated **RforProteomics** google group⁸ welcomes questions/comments/announcements related to R and mass-spectrometry/proteomics.

It is important to read and comply to the posting guides ([here](#) and [here](#)) to maximise the chances to obtain good responses. It is important to specify the software versions using the `sessionInfo()` functions (see an example output at the end of this document, on page 45). If the question involves some code, make sure to isolate the relevant portion and report it with your question, trying to make your code/example reproducible⁹.

All lists have browsable archives.

1.3 Installation

The package should be installed using as described below:

⁵<http://www.r-project.org/mail.html>

⁶<http://bioconductor.org/help/mailing-list/>

⁷<http://bioconductor.org/help/mailing-list/mailform/>

⁸<https://groups.google.com/forum/#!forum/rbioc-sig-proteomics>

⁹<https://github.com/hadley/devtools/wiki/Reproducibility>

```
> ## only first time you install Bioconductor
> ## packages
> source("http://www.bioconductor.org/biocLite.R")
> ## else
> library("BiocInstaller")
> biocLite("RforProteomics")
```

To install all dependencies (78 packages) and reproduce the code in the vignette, replace the last line in the code chunk above with:)

```
> biocLite("RforProteomics", dependencies = TRUE)
```

Finally, the package can be loaded with

```
> library("RforProteomics")
```

*This is the 'RforProteomics' version 1.0.7.
Run 'RforProteomics()' in R or visit
'http://lgatto.github.com/RforProteomics/' to get started.*

See also the 'RforProteomics' web page¹⁰ for more information on installation.

1.4 External dependencies

Some packages used in the document depend on external libraries that need to be installed prior to the R packages:

mzR depends on the Common Data Format¹¹ (CDF) to CDF-based raw mass-spectrometry data. On linux, the `libcdf` library is required. On debian-based systems, for instance, one needs to install the `libnetcdf-dev` package.

IPPD (and others) depend on the **XML** package which requires the `libxml2` infrastructure on linux. On debian-based systems, one needs to install `libxml2-dev`.

biomaRt performs on-line requests using the `curl`¹² infrastructure. On debian-based systems, you one needs to install `libcurl-dev` or `libcurl4-openssl-dev`.

¹⁰<http://lgatto.github.io/RforProteomics/>

¹¹<http://cdf.gsfc.nasa.gov/>

¹²<http://curl.haxx.se/>

1.5 Obtaining the code

The code in this document describes all the examples presented in [8] and can be copy, pasted and executed. It is however more convenient to have it in a separate text file for better interaction with R (using ESS¹³ for emacs or RStudio¹⁴ for instance) to easily modify and explore it. This can be achieved with the `Stangle` function. One needs the Sweave source of this document (a document combining the narration and the R code) and the `Stangle` then specifically extracts the code chunks and produces a clean R source file. If the package is installed, the following code chunk will create a `RforProteomics.R` file in your working directory containing all the annotated source code contained in this document.

```
> ## gets the vignette source
> rnwfile <- dir(system.file(package = "RforProteomics",
+                             dir = "doc/vigsrc"),
+               full.name = TRUE,
+               pattern = "RforProteomics.Rnw")
> ## produces the R file in the working directory
> Stangle(rnwfile)
```

Writing to file RforProteomics.R

Alternatively, you can obtain the `Rnw` file on the github page <https://github.com/lgatto/RforProteomics/blob/master/inst/doc/vigsrc/RforProteomics.Rnw>.

1.6 Prepare the working environment

The packages that we will depend on to execute the examples will be loaded in the respective sections. Here, we pre-load packages that provide general functionality used throughout the document.

```
> library("RColorBrewer") ## Color palettes
> library("ggplot2")      ## Convenient and nice plotting
> library("reshape2")     ## Flexibly reshape data
```

¹³<http://ess.r-project.org/>

¹⁴<http://rstudio.org/>

2 Data standards and input/output

2.1 The mzR package

The **mzR** package [4] provides a unified interface to various mass spectrometry open formats. This code chunk, taken mainly from the **openMSfile** documentation illustrated how to open a connection to an raw data file. The example **mzML** data is taken from the **msdata** data package. The code below would also be applicable to an **mzXML**, **mzData** or **netCDF** file.

```
> ## load the required packages
> library("mzR") ## the software package
> library("msdata") ## the data package
> ## below, we extract the releavant example file
> ## from the local 'msdata' installation
> filepath <- system.file("microtofq", package = "msdata")
> file <- list.files(filepath, pattern = "MM14.mzML",
+   full.names = TRUE, recursive = TRUE)
> ## creates a connection to the mzML file
> mz <- openMSfile(file)
> ## demonstration of data access
> basename(fileName(mz))

[1] "MM14.mzML"

> isInitialized(mz)

[1] TRUE

> runInfo(mz)

$scanCount
[1] 112

$lowMz
[1] 0

$highMz
```

```

[1] 0

$dStartTime
[1] 270.3

$dEndTime
[1] 307.7

$msLevels
[1] 1

> instrumentInfo(mz)

$manufacturer
[1] "Unknown"

$model
[1] "instrument model"

$ionisation
[1] "electrospray ionization"

$analyzer
[1] "mass analyzer type"

$detector
[1] "detector type"

> ## once finished, it is good to explicitly close
> ## the connection
> close(mz)

```

mzR is used by other packages, like **MSnbase** [9], **TargetSearch** [6] and **xcms** [12, 1, 13], that provide a higher level abstraction to the data.

3 Raw data abstraction with MSnExp objects

MSnbase [9] provides base functions and classes for MS-based proteomics that allow facile data and meta-data processing, manipulation and plotting (see for instance figure 1 on page 11).

```
> library("MSnbase")
> ## uses a simple dummy test included in the
> ## package
> mzXML <- dir(system.file(package = "MSnbase", dir = "extdata"),
+   full.name = TRUE, pattern = "mzXML$")
> basename(mzXML)

[1] "dummyiTRAQ.mzXML"

> ## reads the raw data into and MSnExp instance
> raw <- readMSData(mzXML, verbose = FALSE)
> raw

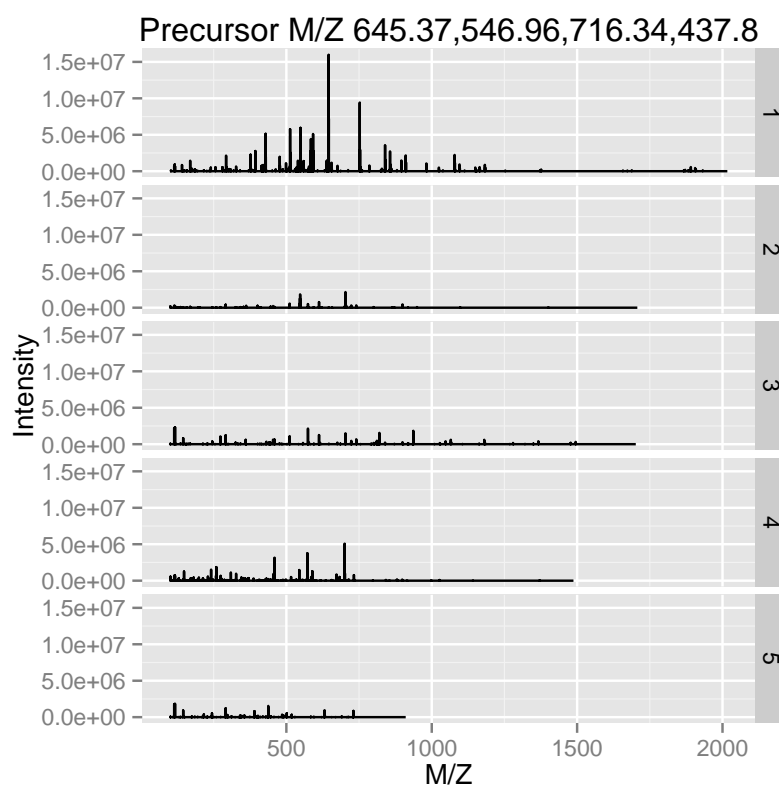
Object of class "MSnExp"
Object size in memory: 0.2 Mb
- - - Spectra data - - -
MS level(s): 2
Number of MS1 acquisitions: 1
Number of MSn scans: 5
Number of precursor ions: 5
4 unique MZs
Precursor MZ's: 437.8 - 716.34
MSn M/Z range: 100 2017
MSn retention times: 25:1 - 25:2 minutes
- - - Processing information - - -
Data loaded: Mon Jul  8 11:04:54 2013
MSnbase version: 1.8.0
- - - Meta data - - -
phenoData
  rowNames: 1
  varLabels: sampleNames fileNumbers
```

```
varMetadata: labelDescription
Loaded from:
  dummyiTRAQ.mzXML
protocolData: none
featureData
  featureNames: X1.1 X2.1 ... X5.1 (5 total)
  fvarLabels: spectrum
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'

> ## Extract a single spectrum
> raw[[3]]

Object of class "Spectrum2"
Precursor: 645.4
Retention time: 25:2
Charge: 2
MSn level: 2
Peaks count: 2125
Total ion count: 150838188
```

```
> plot(raw, full = TRUE)
```



```
> plot(raw[[3]], full = TRUE, reporters = iTRAQ4)
```

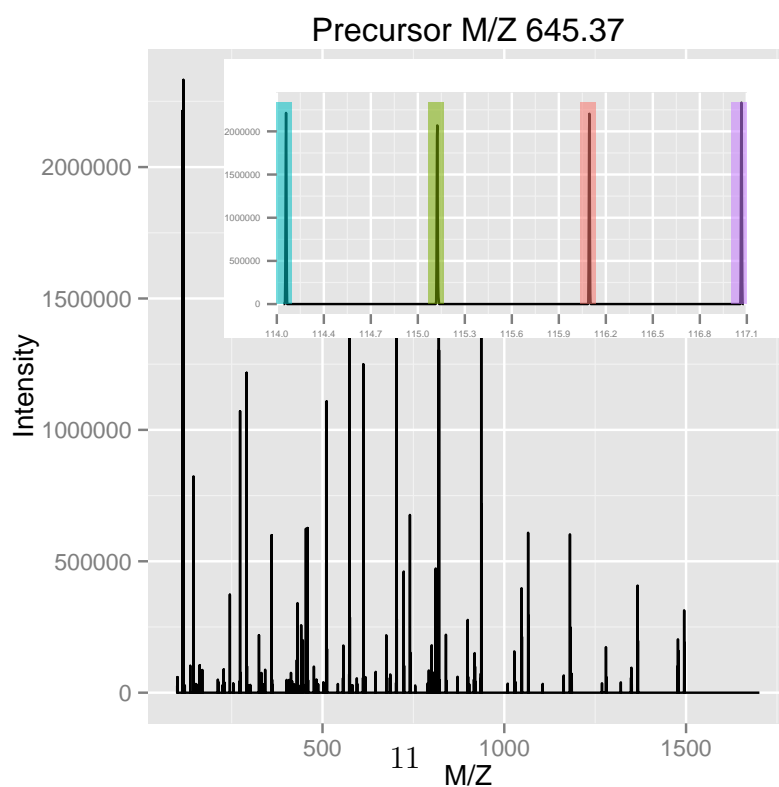


Figure 1: The plot method can be used on experiments, i.e. spectrum collections (left), or individual spectra (right).

3.1 mgf read/write support

Read and write support for data in the `mgf`¹⁵ and `mzTab`¹⁶ formats are available via the `readMgfData/writeMgfData` and `readMzTabData/writeMzTabData` functions, respectively. An example for the latter is shown in the next section.

4 Quantitative proteomics

As an running example throughout this document, we will use a TMT 6-plex data set, PXD000001 to illustrate quantitative data processing. The code chunk below first downloads this data file from the ProteomeXchange server using the `getPXD000001mzTab` function from the **RforProteomics** package.

4.1 The mzTab format

The first code chunk downloads the data, reads it into R and generates an `MSnSet` instance and then calculates protein intensities by summing the peptide quantitation data. Figure 2 illustrates the intensities for 5 proteins.

```
> ## Downloads the experiment
> mztab <- getPXD000001mzTab()
> mztab ## the mzTab file name

[1] "./F063721.dat-mztab.txt"

> ## Load mzTab peptide data
> qnt <- readMzTabData(mztab, what = "PEP")

Detected a metadata section
Detected a peptide section

> sampleNames(qnt) <- reporterNames(TMT6)
> head(exprs(qnt))

TMT6.126 TMT6.127 TMT6.128 TMT6.129 TMT6.130 TMT6.131
1 10630132 11238708 12424917 10997763 9928972 10398534
```

¹⁵http://www.matrixscience.com/help/data_file_help.html#GEN

¹⁶<https://code.google.com/p/mztab/>

```

2 11105690 12403253 13160903 12229367 11061660 10131218
3 1183431 1322371 1599088 1243715 1306602 1159064
4 5384958 5508454 6883086 6136023 5626680 5213771
5 18033537 17926487 21052620 19810368 17381162 17268329
6 9873585 10299931 11142071 10258214 9664315 9518271

> ## combine into proteins
> ## - using the 'accession' feature meta data
> ## - sum the peptide intensities
> protqnt <- combineFeatures(qnt,
+                             groupBy = fData(qnt)$accession,
+                             fun = sum)

```

Combined 1528 features into 404 using user-defined function

```

> qntS <- normalise(qnt, "sum")
> qntV <- normalise(qntS, "vs")
> qntV2 <- normalise(qnt, "vs")
>
> acc <- c("P00489", "P00924", "P02769", "P62894", "ECA")
>
> idx <- sapply(acc, grep, fData(qnt)$accession)
> idx2 <- sapply(idx, head, 3)
> small <- qntS[unlist(idx2), ]
>
> idx3 <- sapply(idx, head, 10)
> medium <- qntV[unlist(idx3), ]
>
> m <- exprs(medium)
> colnames(m) <- c("126", "127", "128", "129", "130",
+                  "131")
> rownames(m) <- fData(medium)$accession
> rownames(m)[grep("CYC", rownames(m))] <- "CYT"
> rownames(m)[grep("ENO", rownames(m))] <- "ENO"
> rownames(m)[grep("ALB", rownames(m))] <- "BSA"

```

```

> cls <- brewer.pal(5, "Set1")
> matplot(t(tail(exprs(protqnt), n = 5)), type = "b",
+         lty = 1, col = cls,
+         ylab = "Protein intensity (summed peptides)",
+         xlab = "TMT reporters")
> legend("topright", tail(featureNames(protqnt), n=5),
+       lty = 1, bty = "n", cex = .8, col = cls)

```

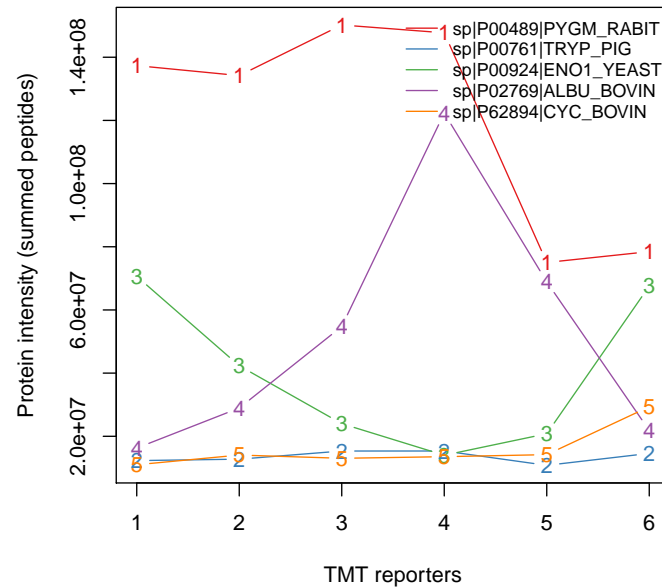


Figure 2: Protein quantitation data.

```

> rownames(m)[grep("PYGM", rownames(m))] <- "PHO"
> rownames(m)[grep("ECA", rownames(m))] <- "Background"
>
> cls <- c(brewer.pal(length(unique(rownames(m))) - 1,
+   "Set1"), "grey")
> names(cls) <- unique(rownames(m))
> wbcpl <- colorRampPalette(c("white", "darkblue"))(256)

```

```
> heatmap(m, col = wbccl, RowSideColors = cls[rownames(m)])
```

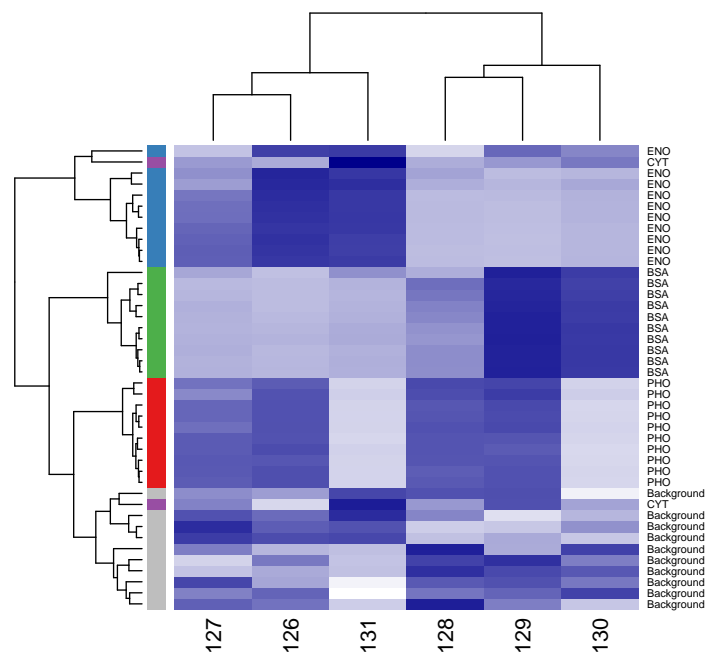


Figure 3: A heatmap.

```

> dfr <- data.frame(exprs(small),
+                   Protein = as.character(fData(small)$accession),
+                   Feature = featureNames(small),
+                   stringsAsFactors = FALSE)
> colnames(dfr) <- c("126", "127", "128", "129", "130", "131",
+                   "Protein", "Feature")
> dfr$Protein[dfr$Protein == "sp|P00924|ENO1_YEAST"] <- "ENO"
> dfr$Protein[dfr$Protein == "sp|P62894|CYC_BOVIN"] <- "CYT"
> dfr$Protein[dfr$Protein == "sp|P02769|ALBU_BOVIN"] <- "BSA"
> dfr$Protein[dfr$Protein == "sp|P00489|PYGM_RABIT"] <- "PHO"
> dfr$Protein[grep("ECA", dfr$Protein)] <- "Background"
> dfr2 <- melt(dfr)

```

Using Protein, Feature as id variables

```

> ggplot(aes(x = variable, y = value, colour = Protein),
+         data = dfr2) +
+   geom_point() +
+   geom_line(aes(group=as.factor(Feature)), alpha = 0.5) +
+   facet_grid(. ~ Protein) + theme(legend.position="none") +
+   labs(x = "Reporters", y = "Normalised intensity")

```

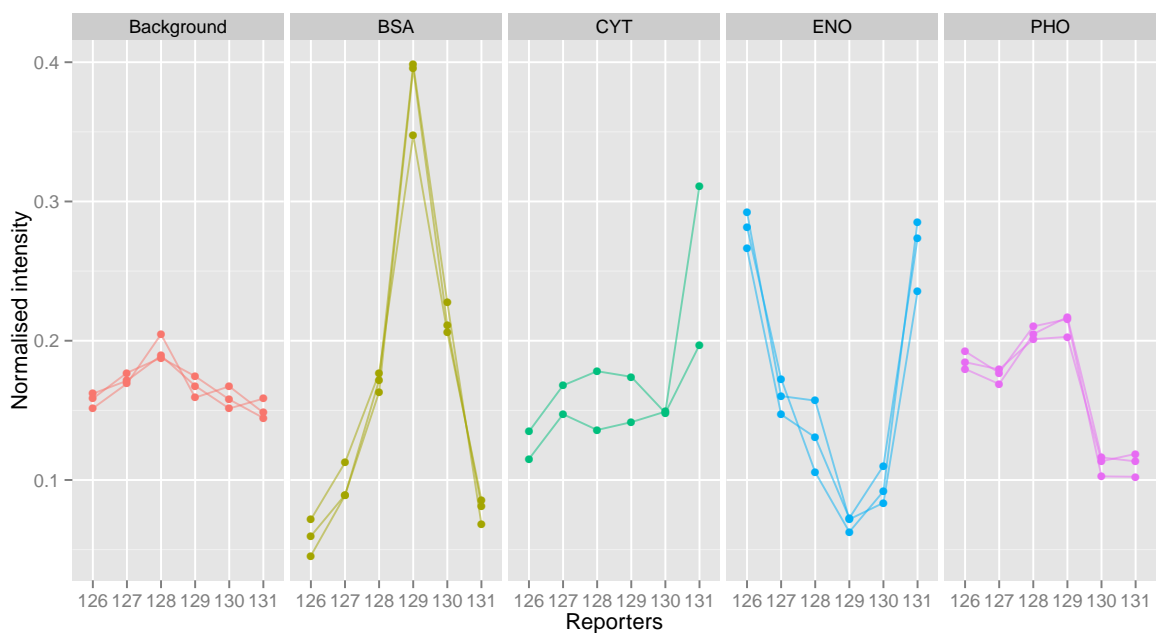


Figure 4: Spikes plot using **ggplot2**.

4.2 Working with raw data

```
> mzxml <- getPXD000001mzXML()
> rawms <- readMSData(mzxml, centroided = TRUE, verbose = FALSE)
> qntms <- quantify(rawms, reporters = TMT7, method = "max",
+   verbose = FALSE, parallel = FALSE)
>
> d <- data.frame(Signal = rowSums(exprs(qntms)[, 1:6]),
+   Incomplete = exprs(qntms)[, 7])
> d <- log(d)
> cls <- rep("#00000050", nrow(qnt))
> pch <- rep(1, nrow(qnt))
> cls[grep("P02769", fData(qnt)$accession)] <- "gold4" ## BSA
> cls[grep("P00924", fData(qnt)$accession)] <- "dodgerblue" ## ENO
> cls[grep("P62894", fData(qnt)$accession)] <- "springgreen4" ## CYT
> cls[grep("P00489", fData(qnt)$accession)] <- "darkorchid2" ## PHO
> pch[grep("P02769", fData(qnt)$accession)] <- 19
> pch[grep("P00924", fData(qnt)$accession)] <- 19
> pch[grep("P62894", fData(qnt)$accession)] <- 19
> pch[grep("P00489", fData(qnt)$accession)] <- 19
```

```
> mzp <- plotMzDelta(rawms, reporters = TMT6, verbose = FALSE) +
+   ggtitle("")
```

Scale for 'x' is already present. Adding another scale for 'x', which will replace the existing scale.

```
> mzp
```

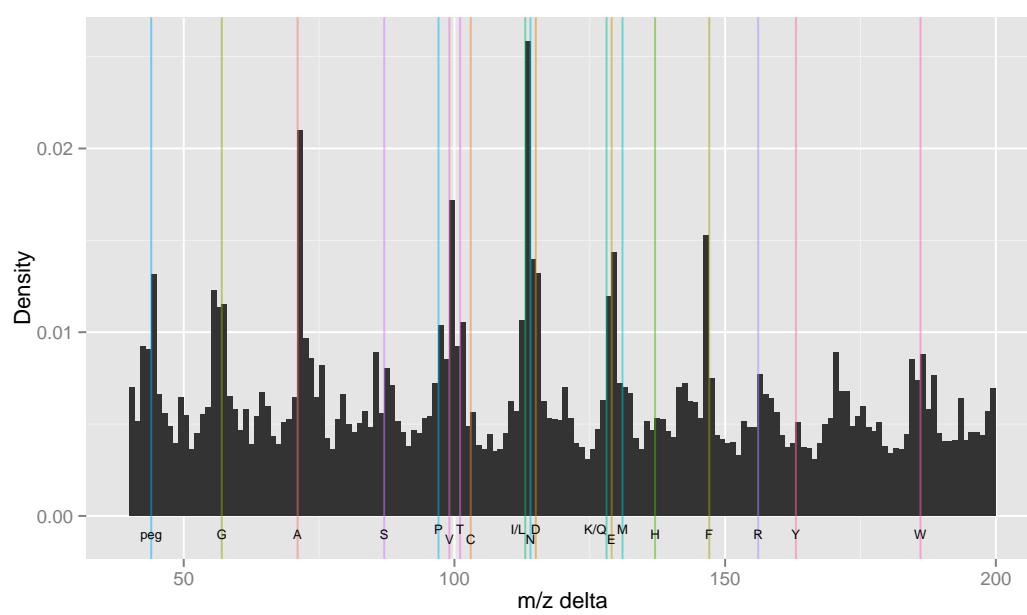


Figure 5: A m/z delta plot.

```

> plot(Signal ~ Incomplete, data = d,
+       xlab = expression(Incomplete~dissociation),
+       ylab = expression(Sum~of~reporters~intensities),
+       pch = 19,
+       col = "#4582B380")
> grid()
> abline(0, 1, lty = "dotted")
> abline(lm(Signal ~ Incomplete, data = d), col = "darkblue")

```

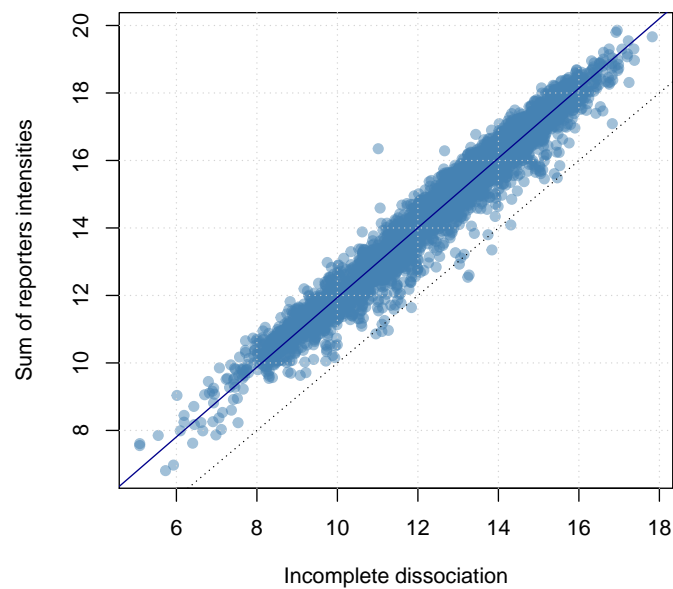


Figure 6: Incomplete dissociation.

```
> MAplot(qnt[, c(4, 2)], cex = 0.9, col = cls, pch = pch,
+       show.statistics = FALSE)
```

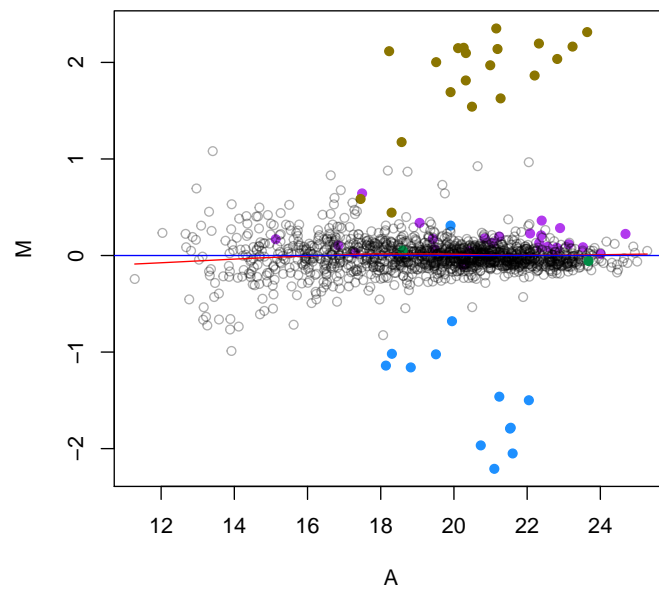


Figure 7: MAplot on an `MSnSet` instance.

4.3 The MALDIquant package

This section illustrates some of **MALDIquant**'s data processing capabilities [11]. The code is taken from the `processing-peaks.R` script downloaded from the package homepage¹⁷.

Loading the data

```
> ## load packages
> library("MALDIquant")
> library("MALDIquantForeign")
> ## getting test data
> datapath <-
+   file.path(system.file("Examples",
+                           package = "readBrukerFlexData"),
+             "2010_05_19_Gibb_C8_A1")
> dir(datapath)

[1] "O_A1" "O_A2"

> sA1 <- importBrukerFlex(datapath, verbose=FALSE)
> # in the following we use only the first spectrum
> s <- sA1[[1]]
>
> summary(mass(s))

  Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
  1000   2370   4330   4720   6870  10000

> summary(intensity(s))

  Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
     4    180   1560   2840   4660  32600

> head(as.matrix(s))
```

¹⁷<http://strimmerlab.org/software/malDIquant/>

	mass	intensity
[1,]	999.9	11278
[2,]	1000.1	11350
[3,]	1000.3	10879
[4,]	1000.5	10684
[5,]	1000.7	10740
[6,]	1000.9	10947

```
> plot(s)
```

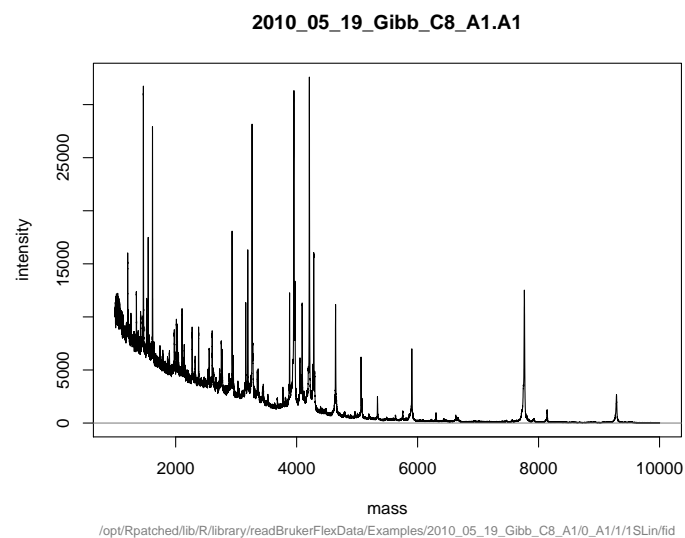


Figure 8: Spectrum plotting in **MALDIquant**.

Preprocessing

```
> ## sqrt transform (for variance stabilization)
> s2 <- transformIntensity(s, fun = sqrt)
> s2
```

```
S4 class type           : MassSpectrum
Number of m/z values    : 22431
Range of m/z values     : 999.939 - 10001.925
Range of intensity values: 2e+00 - 1.805e+02
```

```

Memory usage      : 360.039 KiB
Name              : 2010_05_19_Gibb_C8_A1.A1
File             : /opt/Rpatched/lib/R/library/readBrukerFlexData/Examples/20

>
> ## smoothing - 5 point moving average
> s3 <- transformIntensity(s2, movingAverage, halfWindowSize = 2)
> s3

S4 class type      : MassSpectrum
Number of m/z values : 22431
Range of m/z values : 999.939 - 10001.925
Range of intensity values: 3.606e+00 - 1.792e+02
Memory usage      : 360.039 KiB
Name              : 2010_05_19_Gibb_C8_A1.A1
File             : /opt/Rpatched/lib/R/library/readBrukerFlexData/Examples/20

>
> ## baseline subtraction
> s4 <- removeBaseline(s3, method = "SNIP")
> s4

S4 class type      : MassSpectrum
Number of m/z values : 22431
Range of m/z values : 999.939 - 10001.925
Range of intensity values: 0e+00 - 1.404e+02
Memory usage      : 360.039 KiB
Name              : 2010_05_19_Gibb_C8_A1.A1
File             : /opt/Rpatched/lib/R/library/readBrukerFlexData/Examples/20

```

Peak picking

```

> ## peak picking
> p <- detectPeaks(s4)
> length(p) # 181

```

```
[1] 186
```

```
> peak.data <- as.matrix(p) # extract peak information
```

```
> par(mfrow = c(2, 3))
> xl <- range(mass(s))
> # use same xlim on all plots for better
> # comparison
> plot(s, sub = "", main = "1: raw", xlim = xl)
> plot(s2, sub = "", main = "2: variance stabilisation",
+      xlim = xl)
> plot(s3, sub = "", main = "3: smoothing", xlim = xl)
> plot(s4, sub = "", main = "4: base line correction",
+      xlim = xl)
> plot(s4, sub = "", main = "5: peak detection", xlim = xl)
> points(p)
> top20 <- intensity(p) %in% sort(intensity(p), decreasing = TRUE)[1:20]
> labelPeaks(p, index = top20, underline = TRUE)
> plot(p, sub = "", main = "6: peak plot", xlim = xl)
> labelPeaks(p, index = top20, underline = TRUE)
```

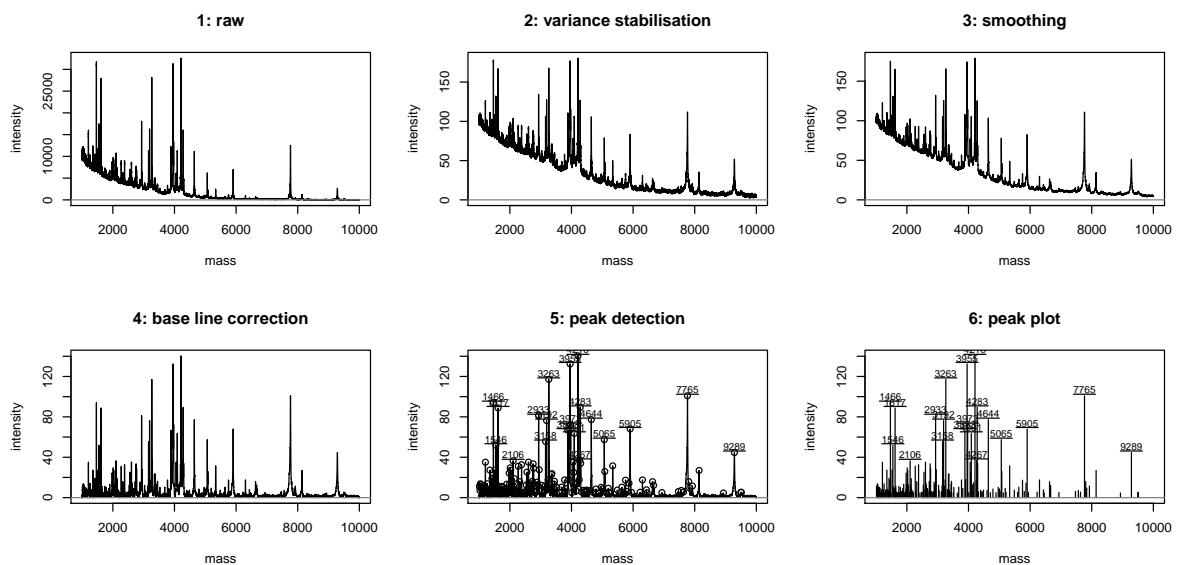


Figure 9: Spectrum plotting in MALDIquant.

4.4 Working with peptide sequences

```
> library(IPPD)
> library(BRAIN)
> atoms <- getAtomsFromSeq("SIVPSGASTGVHEALEMR")
> unlist(atoms)

  C   H   N   O   S
77 129  23  27   1

>
> library(Rdisop)
> pepmol <- getMolecule(paste0(names(atoms),
+                               unlist(atoms),
+                               collapse = ""))
> pepmol

$formula
[1] "C77H129N23O27S"

$score
[1] 1

$exactmass
[1] 1840

$charge
[1] 0

$parity
[1] "e"

$valid
[1] "Valid"

$DBE
```

```

[1] 25

$isotopes
$isotopes[[1]]
      [,1]      [,2]      [,3]      [,4]      [,5]
[1,] 1839.9149 1840.9177 1841.9197 1.843e+03 1.844e+03
[2,]   0.3427   0.3353   0.1961 8.474e-02 2.953e-02
      [,6]      [,7]      [,8]      [,9]     [,10]
[1,] 1.845e+03 1.846e+03 1.847e+03 1.848e+03 1.849e+03
[2,] 8.692e-03 2.226e-03 5.066e-04 1.040e-04 1.950e-05

>
> ##
> library(OrgMassSpecR)
> data(itraqdata)
>
> simplottest <-
+   itraqdata[featureNames(itraqdata) %in% paste0("X", 46:47)]
> sim <- SpectrumSimilarity(as(simplottest[[1]], "data.frame"),
+                           as(simplottest[[2]], "data.frame"),
+                           top.lab = "itraqdata[['X46']]",
+                           bottom.lab = "itraqdata[['X47']]",
+                           b = 25)

      mz intensity.top intensity.bottom
1  114.1              0              44
2  114.1              0              53
3  114.1              0              43
4  115.1              0              25
5  364.7             25              0
6  374.2              0              39
7  374.2              0              45
8  374.2              0              35
9  388.2              0              35
10 388.3              0              75
11 388.3              0             100

```

12	388.3	0	90
13	388.3	35	53
14	388.3	100	53
15	388.3	90	53
16	388.3	53	53
17	388.3	75	53
18	414.3	31	0
19	414.3	27	0
20	487.3	0	33
21	487.3	0	37
22	487.3	0	28
23	603.3	42	0
24	603.4	55	0
25	603.4	48	0
26	603.4	27	0
27	615.3	0	28
28	615.3	0	56
29	615.4	0	70
30	615.4	0	59
31	615.4	26	32
32	615.4	44	32
33	615.4	56	32
34	615.4	47	32
35	702.4	27	0
36	702.4	30	0
37	728.4	0	28
38	728.5	64	29
39	728.5	64	29
40	728.5	42	29
41	728.5	42	29
42	803.4	30	0
43	803.5	38	0
44	803.5	32	0
45	827.5	28	0
46	827.5	35	0

```

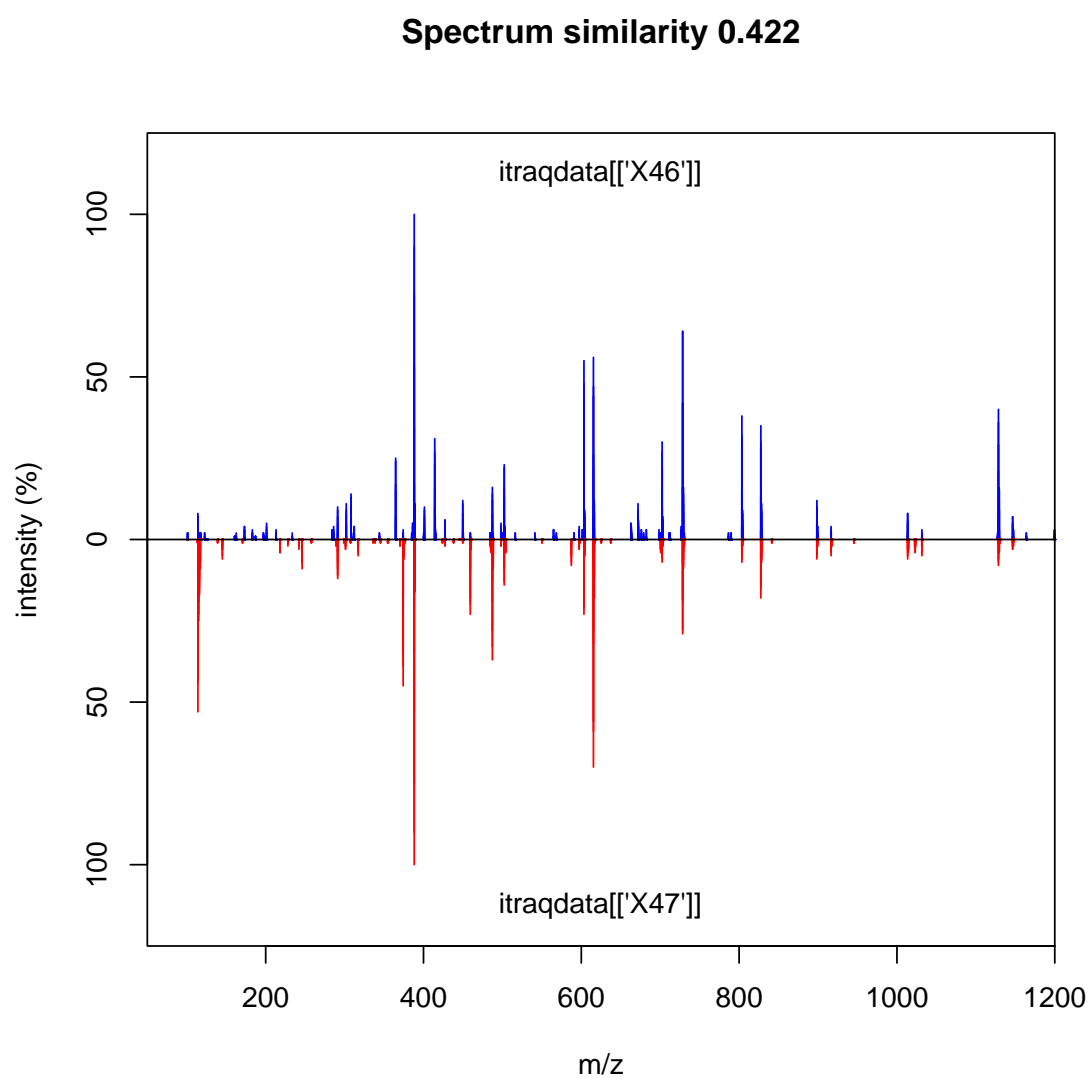
47  827.5      30      0
48 1128.6      36      0
49 1128.6      40      0
50 1128.7      29      0

```

```

> title(main = paste("Spectrum similarity", round(sim, 3)))

```



```

>
> MonoisotopicMass(formula = list(C = 2, O = 1, H=6))
[1] 46.04

```

```

> molecule <- getMolecule("C2H5OH")
> molecule$exactmass

[1] 46.04

> ## x11()
> ## plot(t(.pepmol$isotopes[[1]]), type = "h")
>
> ## x <- IsotopicDistribution(formula = list(C = 2, O = 1, H=6))
> ## t(molecule$isotopes[[1]])
> ## par(mfrow = c(2,1))
> ## plot(t(molecule$isotopes[[1]]), type = "h")
> ## plot(x[, c(1,3)], type = "h")
>
> ## data(myo500)
> ## masses <- c(147.053, 148.056)
> ## intensities <- c(93, 5.8)
> ## molecules <- decomposeIsotopes(masses, intensities)
>
> ## experimental eno peptides
> exppep <-
+   as.character(fData(qnt[grep("ENO", fData(qnt)[, 2]), ])[, 1]) ## 13
> minlength <- min(nchar(exppep))
>
> eno <- download.file("http://www.uniprot.org/uniprot/P00924.fasta",
+                       destfile = "P00924.fasta")
> eno <- paste(readLines("P00924.fasta")[-1], collapse = "")
> enopep <- Digest(eno, missed = 1)
> nrow(enopep) ## 103

[1] 103

> sum(nchar(enopep$peptide) >= minlength) ## 68

[1] 68

> pepcnt <- enopep[enopep[, 1] %in% exppep, ]
> nrow(pepcnt) ## 13

[1] 13

```

The following code chunks demonstrate how to use the **cleaver** package for in-silico cleavage of polypeptides, e.g. cleaving of *Gastric juice peptide 1 (P01358)* using *Trypsin*:

```
> library(cleaver)
> cleave("LAAGKVEDSD", enzym = "trypsin")

$LAAGKVEDSD
[1] "LAAGK" "VEDSD"
```

Sometimes cleavage is not perfect and the enzym miss some cleavage positions:

```
> ## miss one cleavage position
> cleave("LAAGKVEDSD", enzym = "trypsin", missedCleavages = 1)

$LAAGKVEDSD
[1] "LAAGKVEDSD"

>
> ## miss zero or one cleavage positions
> cleave("LAAGKVEDSD", enzym = "trypsin", missedCleavages = 0:1)

$LAAGKVEDSD
[1] "LAAGK"      "VEDSD"      "LAAGKVEDSD"
```

```
> ## example code to generate an Texshade image to
> ## be included directly in a Latex document or R
> ## vignette
>
> ## seq1file <- 'seq1.tex'
> ## cat('\begin{texshade}{Figures/P00924.fasta}
> ## \setsize{numbering}{footnotesize}
> ## \setsize{residues}{footnotesize}
> ## \residuesperline*{70}
> ## \shadingmode{functional} \hideconsensus
> ## \vspace{1mm} \hidenames
> ## \noblockskip\n', file = seq1file) tmp <-
```

```

> ## sapply(1:nrow(pepcnt), function(i) { col <-
> ## ifelse((i %% 2) == 0, 'Blue', 'RoyalBlue')
> ## cat('\shaderegion{1}{', pepcnt$start[i],
> ## '...', pepcnt$stop[i], '}{White}{', col,
> ## '}\n', file = seq1file, append = TRUE) })
> ## cat('\end{texshade} \caption{Visualising
> ## observed peptides for the Yeast enolase
> ## protein. Peptides are shaded in blue and
> ## black. The last peptide is a mis-cleavage and
> ## overlaps with
> ## \texttt{IEEELGDNAVFA GENFHHGDK}.)}
> ## \label{fig:seq} \end{center}
> ## \end{figure}\n\n', file = seq1file,
> ## append = TRUE)

```

¹⁵N incorporation

```

> ## 15N example
> ## incorporation rates from 0, 0.1, ..., 0.9, 0.95, 1
> incrate <- c(seq(0, 0.9, 0.1), 0.95, 1)
> inc <- lapply(incrate, function(inc)
+               IsotopicDistributionN("YEVQGEVFTKPQLWP", inc))
> par(mfrow = c(4,3))
> for (i in 1:length(inc))
+   plot(inc[[i]][, c(1, 3)], xlim = c(1823, 1848),
+        type = "h",
+        main = paste0("15N incorporation at ", incrate[i]*100, "%"))

```

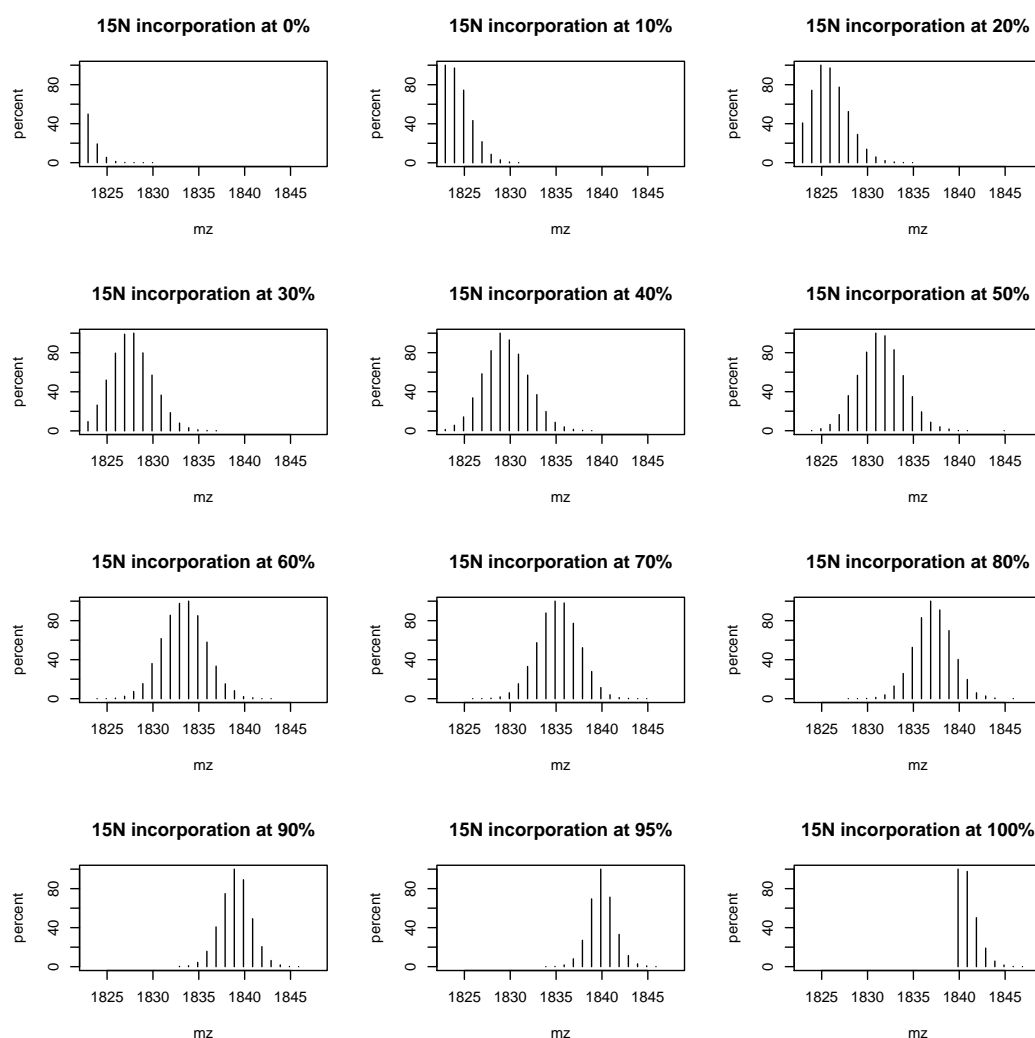


Figure 10: Isotopic envelope for the YEVQGEVFTKPQLWP peptide at different ^{15}N incorporation rates.

4.5 The isobar package

The **isobar** package [3] provides methods for the statistical analysis of isobarically tagged MS² experiments.

```
> library(isobar)
>
> ## Prepare the PXD000001 data for isobar analysis
> .ions <- exprs(qnt)
> .mass <- matrix(mz(TMT6), nrow(qnt), byrow=TRUE, ncol = 6)
> colnames(.ions) <- colnames(.mass) <-
+   reporterTagNames(new("TMT6plexSpectra"))
> rownames(.ions) <- rownames(.mass) <-
+   paste(fData(qnt)$accession, fData(qnt)$sequence, sep = ".")
> pgtbl <- data.frame(spectrum = rownames(.ions),
+                     peptide = fData(qnt)$sequence,
+                     modif = ":",
+                     start.pos = 1,
+                     protein = fData(qnt)$accession,
+                     accession = fData(qnt)$accession)
> x <- new("TMT6plexSpectra", pgtbl, .ions, .mass)
> featureData(x)$proteins <- as.character(fData(qnt)$accession)
>
> x <- correctIsotopeImpurities(x) ## using identity matrix here

LOG: isotopeImpurities.corrected: TRUE

> x <- normalize(x, per.file = FALSE)

LOG: is.normalized: TRUE
LOG: normalization.multiplicative.factor channel 126: 0.8905
LOG: normalization.multiplicative.factor channel 127: 0.9288
LOG: normalization.multiplicative.factor channel 128: 1
LOG: normalization.multiplicative.factor channel 129: 0.949
LOG: normalization.multiplicative.factor channel 130: 0.8677
LOG: normalization.multiplicative.factor channel 131: 0.8965

> ## spikes
```

```

> spks <- c(protein.g(proteinGroup(x), "P00489"),
+           protein.g(proteinGroup(x), "P00924"),
+           protein.g(proteinGroup(x), "P02769"),
+           protein.g(proteinGroup(x), "P62894"))
>
> cls2 <- rep("#00000040", nrow(x))
> pch2 <- rep(1, nrow(x))
> cls2[grepl("P02769", featureNames(x))] <- "gold4" ## BSA
> cls2[grepl("P00924", featureNames(x))] <- "dodgerblue" ## ENO
> cls2[grepl("P62894", featureNames(x))] <- "springgreen4" ## CYT
> cls2[grepl("P00489", featureNames(x))] <- "darkorchid2" ## PHO
> pch2[grepl("P02769", featureNames(x))] <- 19
> pch2[grepl("P00924", featureNames(x))] <- 19
> pch2[grepl("P62894", featureNames(x))] <- 19
> pch2[grepl("P00489", featureNames(x))] <- 19
>
> nm <- NoiseModel(x)

[1] 0.07346 941.45023 2.82447

> ib.background <- subsetIBSpectra(x, protein=spks,
+                                 direction = "exclude")
> nm.background <- NoiseModel(ib.background)

[1] 0.01346 2.85121 0.84631

> ib.spks <- subsetIBSpectra(x, protein = spks,
+                             direction="include",
+                             specificity="reporter-specific")
> nm.spks <- NoiseModel(ib.spks, one.to.one=FALSE, pool=TRUE)

4 proteins with more than 10 spectra, taking top 50.
[1] 1.000e-10 5.829e+00 6.610e-01

>
> ratios <- 10^estimateRatio(x, nm,
+                             channel1="127", channel2="129",

```

```

+             protein = spks,
+             combine = FALSE)[, "lratio"]
>
> res <- estimateRatio(x, nm,
+             channel1="127", channel2="129",
+             protein = unique(fData(x)$proteins),
+             combine = FALSE,
+             sign.level = 0.01)[, c(1, 2, 6, 8)]
> res <- as.data.frame(res)
> res$lratio <- -(res$lratio)
>
> cls3 <- rep("#00000050", nrow(res))
> pch3 <- rep(1, nrow(res))
> cls3[grepl("P02769", rownames(res))] <- "gold4" ## BSA
> cls3[grepl("P00924", rownames(res))] <- "dodgerblue" ## ENO
> cls3[grepl("P62894", rownames(res))] <- "springgreen4" ## CYT
> cls3[grepl("P00489", rownames(res))] <- "darkorchid2" ## PHO
> pch3[grepl("P02769", rownames(res))] <- 19
> pch3[grepl("P00924", rownames(res))] <- 19
> pch3[grepl("P62894", rownames(res))] <- 19
> pch3[grepl("P00489", rownames(res))] <- 19
>
> rat.exp <- c(PHO = 2/2,
+             ENO = 5/1,
+             BSA = 2.5/10,
+             CYT = 1/1)

```

```

> par(mfrow = c(1, 2))
> maplot(x, noise.model = c(nm.background, nm.spks, nm),
+       channel1 = "127", channel2 = "129", pch = 19, col = cls2,
+       main = "Spectra MA plot")
> abline(h = 1, lty = "dashed", col = "grey")
> legend("topright", c("BSA", "ENO", "CYT", "PHO"), pch = 19,
+       col = c("gold4", "dodgerblue", "springgreen4",
+       "darkorchid2"), bty = "n", cex = 0.7)
> plot(res$lratio, -log10(res$p.value.rat), col = cls3,
+       pch = pch3, xlab = expression(log[10] ~ fold -
+       change), ylab = expression(-log[10] ~ p - value),
+       main = "Protein volcano plot", xlim = c(-0.7, 0.7))
> grid()
> abline(h = -log10(0.01), lty = "dotted")
> abline(v = log10(c(2, 0.5)), lty = "dotted")
> abline(v = -0.003, col = "springgreen4", lty = "dashed",
+       lwd = 2)
> abline(v = 0.003, col = "darkorchid2", lty = "dashed",
+       lwd = 2)
> abline(v = log10(5), col = "dodgerblue", lty = "dashed",
+       lwd = 2)
> abline(v = log10(0.25), col = "gold4", lty = "dashed",
+       lwd = 2)
> points(res[spks, "lratio"], -log10(res[spks, "p.value.rat"]),
+       col = c("darkorchid2", "dodgerblue", "gold4", "springgreen4"),
+       pch = 19)

```

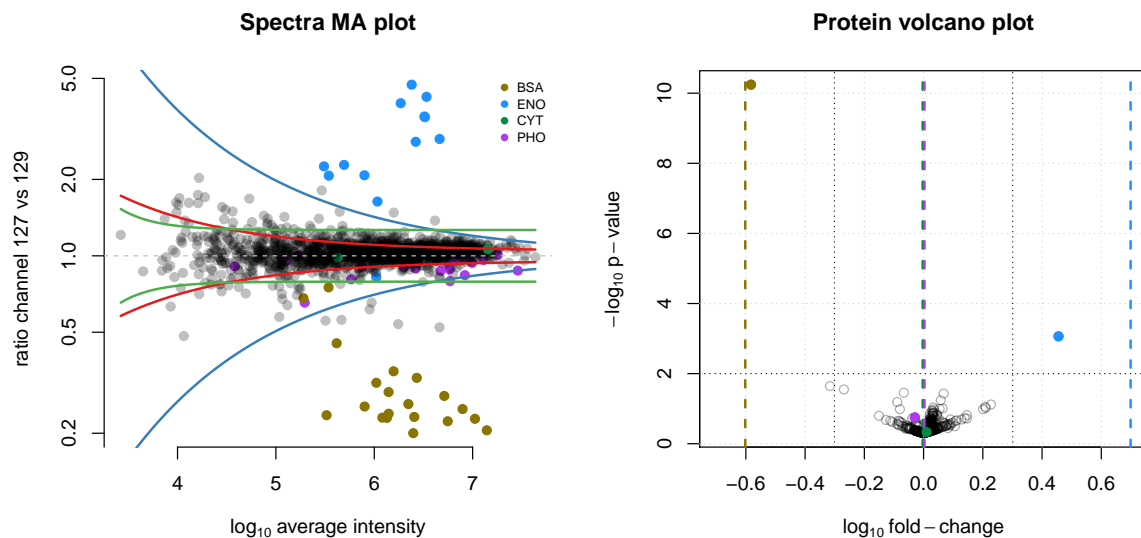


Figure 11: Result from the **isobar** pipeline.

4.6 The synapter package

The **synapter** [2] package comes with a detailed vignette that describes how to prepare the MS^E data and then process it in R. Several interfaces are available provided the user with maximum control, easy batch processing capabilities or a graphical user interface. The conversion into **MSnSet** instances and filter and combination thereof as well as statistical analysis are also described.

```
> ## open the synapter vignette
> library("synapter")
> synapterGuide()
```

5 MS^2 spectra identification

A recent addition to Bioconductor 2.12 is the **rTANDEM** package, that provides a direct interface to the X!Tandem software [5]. A typical **rTANDEM** pipeline comprises

1. Prepare the input data.
2. Run the search.
3. Import the search results and extract the peptides and proteins

Using example code/data from the **rTANDEM** vignette/package, these steps are executed as described below.

5.1 Preparation of the input data

```
> library(rTANDEM)
> taxonomy <- rTTaxo(taxon = "yeast",
+                   format = "peptide",
+                   URL = system.file(
+                       "extdata/fasta/scd.fasta.pro",
+                       package="rTANDEM"))
> param <- rTParam()
> param <- setParamValue(param,
```

```

+             'protein', 'taxon',
+             value="yeast")
> param <- setParamValue(param, 'list path',
+             'taxonomy information', taxonomy)
> param <- setParamValue(param,
+             'list path', 'default parameters',
+             value = system.file(
+             "extdata/default_input.xml",
+             package="rTANDEM"))
> param <- setParamValue(param, 'spectrum', 'path',
+             value = system.file(
+             "extdata/test_spectra.mgf",
+             package="rTANDEM"))
> param <- setParamValue(param, 'output', 'xsl path',
+             value = system.file(
+             "extdata/tandem-input-style.xsl",
+             package="rTANDEM"))
> param <- setParamValue(param, 'output', 'path',
+             value = paste(getwd(),
+             "output.xml", sep="/"))

```

5.2 Performing the search

The analysis is run using the `tandem` function (see also the `rtandem` function), which returns the results data file path (only the file name is displayed below).

```

> resultPath <- tandem(param)

Loading spectra
(mgf). loaded.
Spectra matching criteria = 242
Starting threads . started.
Computing models:
testin sequences modelled = 5 ks
Model refinement:

```

```

partial cleavage ..... done.
unanticipated cleavage ..... done.
modified N-terminus ..... done.
finishing refinement ... done.
Creating report:
initial calculations ..... done.
sorting ..... done.
finding repeats ..... done.
evaluating results ..... done.
calculating expectations ..... done.
writing results ..... done.

Valid models = 31
Unique models = 30
Estimated false positives = 1 +/- 1

> basename(resultPath)

[1] "output.2013_07_08_11_06_36.t.xml"

```

5.3 Import and analyse results

```

> res <- GetResultsFromXML(resultPath)
> ## the inferred proteins
> proteins <- GetProteins(res, log.expect = -1.3, min.peptides = 2)
> proteins[, -(4:5), with = FALSE]

```

	uid	expect.value	label	description	num.peptides
1:	576	-19.4	YCR012W	YCR012W	4
2:	2281	-14.0	YGR234W	YGR234W	3
3:	1811	-8.3	YFR053C	YFR053C	2
4:	4	-6.5	YAL005C	YAL005C	2
5:	3517	-6.5	YLL024C	YLL024C	2

```

> ## the identified peptides for YFR053C

```

```
> peptides <- GetPeptides(protein.uid = 1811, results = res,
+   expect = 0.05)
> peptides[, c(1:4, 9, 10:16), with = FALSE]
```

	pep.id	prot.uid	spectrum.id	spectrum.mh	tandem.score
1:	102.1.1	1811	102	942.5	31.9
2:	250.1.1	1811	250	1212.6	35.0

	mh	delta	peak.count	missed.cleavages
1:	942.5	-0.0220	NA	0
2:	1212.6	0.0079	NA	0

	start.position	end.position	sequence
1:	166	173	INEGILQR
2:	437	447	DIYGTGDASK

More details are provided in the vignette available with `(vignette("rTANDEM"))`, for instance the extraction of degenerated peptides, i.e. peptides found in multiple proteins.

6 Annotation

In this section, we briefly present some Bioconductor annotation infrastructure.

We start with the **hpar** package, an interface to the *Human Protein Atlas* [14, 15], to retrieve subcellular localisation information for the ENSG00000002746 ensemble gene.

```
> id <- "ENSG00000002746"
> library("hpar")
> getHpa(id, "SubcellularLoc")
```

	Gene	Main.location
24	ENSG00000002746	Nucleus but not nucleoli;Cytoplasm

	Other.location	Expression.type	Reliability
24		APE	High

Below, we make use of the human annotation package **org.Hs.eg.db** and the Gene Ontology annotation package **GO.db** to retrieve the same information as above.


```

> library(org.Hs.eg.db)
> library(GO.db)
> ans <- select(org.Hs.eg.db, keys = id, cols = c("ENSEMBL",
+       "GO", "ONTOLOGY"), keytype = "ENSEMBL")
> ans <- ans[ans$ONTOLOGY == "CC", ]
> ans

```

	ENSEMBL	GO	EVIDENCE	ONTOLOGY
2	ENSG000000002746	GO:0005634	IDA	CC
3	ENSG000000002746	GO:0005737	IDA	CC

```

> sapply(as.list(GOTERM[ans$GO]), slot, "Term")

GO:0005634  GO:0005737
  "nucleus" "cytoplasm"

```

Finally, this information can also be retrieved from on-line databases using the **biomaRt** package [7].

```

> library("biomaRt")
> ensembl <- useMart("ensembl", dataset = "hsapiens_gene_ensembl")
> efilter <- "ensembl_gene_id"
> eattr <- c("go_id", "name_1006", "namespace_1003")
> bmres <- getBM(attributes = eattr, filters = efilter,
+       values = id, mart = ensembl)
> bmres[bmres$namespace_1003 == "cellular_component",
+       "name_1006"]

[1] "nucleus"    "cytoplasm"

```

7 Other packages

7.1 Bioconductor packages

This section provides a complete list of packages available in the relevant Bioconductor version 2.13 (as of July 8, 2013) *biocView*¹⁸ categories. Tables 1, 2 and 3 represent the packages for the Proteomics (37 packages), MassSpectrometry (21 packages) and MassSpectrometryData (6 experiment packages) categories.

Package	Title
ASEB	Predict Acetylated Lysine Sites
BRAIN	Baffling Recursive Algorithm for Isotope distributionN calculations
CellNOptR	Training of boolean logic models of signalling networks using prior knowledge networks and perturbation data.
ChemmineR	Cheminformatics of Drug-like Small Molecule Data
cisPath	Visualization and manage of the protein-protein interaction networks.
cleaver	Cleavage of polypeptide sequences
clippda	A package for the clinical proteomic profiling data analysis
CNORdt	Add-on to CellNOptR: Discretized time treatments
CNORfeeder	Integration of CellNOptR to add missing links
CNORode	ODE add-on to CellNOptR
deltaGseg	deltaGseg
eiR	Accelerated similarity searching of small molecules
fmcsR	Flexible Maximum Common Substructure (FMCS) Searching
GraphPAC	Identification of Mutational Clusters in Proteins via a Graph Theoretical Approach.
hpar	Human Protein Atlas in R
iPAC	Identification of Protein Amino acid Clustering
IPPD	Isotopic peak pattern deconvolution for Protein Mass Spectrometry by template matching
isobar	Analysis and quantitation of isobarically tagged MSMS proteomics data
LPEadj	A correction of the local pooled error (LPE) method to replace the asymptotic variance adjustment with an unbiased adjustment based on sample size.
MassSpecWavelet	Mass spectrum processing by wavelet-based algorithms
MSnbase	MSnbase: Base Functions and Classes for MS-based Proteomics
mzR	parser for netCDF, mzXML, mzData and mzML files (mass spectrometry data)
PAnnBuilder	Protein annotation data package builder
pathview	a tool set for pathway based data integration and visualization
PCpheno	Phenotypes and cellular organizational units
plgem	Detect differential expression in microarray and proteomics datasets with the Power Law Global Error Model (PLGEM)
PLPE	Local Pooled Error Test for Differential Expression with Paired High-throughput Data
ppiStats	Protein-Protein Interaction Statistical Package
PROcess	Ciphergen SELDI-TOF Processing
procoil	Prediction of Oligomerization of Coiled Coil Proteins
prot2D	Statistical Tools for volume data from 2D Gel Electrophoresis
RCASPAR	A package for survival time prediction based on a piecewise baseline hazard Cox regression model.
RpsiXML	R interface to PSI-MI 2.5 files
rTANDEM	Encapsulate X!Tandem in R.
ScISI	In Silico Interactome
SLGI	Synthetic Lethal Genetic Interaction
synapter	Label-free data analysis pipeline for optimal identification and quantitation

Table 1: Packages available under the Proteomics *biocViews* category.

¹⁸<http://www.bioconductor.org/packages/devel/BiocViews.html>

Package	Title
apComplex	Estimate protein complex membership using AP-MS protein data
BRAIN	Baffling Recursive Algorithm for Isotope distribution calculations
CAMERA	Collection of annotation related methods for mass spectrometry data
flagme	Analysis of Metabolomics GC/MS Data
gaga	GaGa hierarchical model for high-throughput data analysis
iontree	Data management and analysis of ion trees from ion-trap mass spectrometry
isobar	Analysis and quantitation of isobarically tagged MSMS proteomics data
MassArray	Analytical Tools for MassArray Data
MassSpecWavelet	Mass spectrum processing by wavelet-based algorithms
MSnbase	MSnbase: Base Functions and Classes for MS-based Proteomics
mzR	parser for netCDF, mzXML, mzData and mzML files (mass spectrometry data)
PAPi	Predict metabolic pathway activity based on metabolomics data
PROcess	Ciphergen SELDI-TOF Processing
Rdisop	Decomposition of Isotopic Patterns
Risa	Converting experimental metadata from ISA-tab into Bioconductor data structures
RMassBank	Workflow to process tandem MS files and build MassBank records
rols	An R interface to the Ontology Lookup Service
rTANDEM	Encapsulate X!Tandem in R.
synapter	Label-free data analysis pipeline for optimal identification and quantitation
TargetSearch	A package for the analysis of GC-MS metabolite profiling data.
xcms	LC/MS and GC/MS Data Analysis

Table 2: Packages available under the MassSpectrometry *biocViews* category.

Package	Title
faahKO	Saghatelian et al. (2004) FAAH knockout LC/MS data
gcspikelite	Spike-in data for GC/MS data and methods within flagme
msdata	Various Mass Spectrometry raw data example files
RforProteomics	Companion package to the 'Using R and Bioconductor for proteomics data analysis' publication
RMassBankData	Test dataset for RMassBank
synapterdata	Data accompanying the synapter package

Table 3: Experimental Packages available under the MassSpectrometryData *biocViews* category.

7.2 The Chemometrics and Computational Physics CRAN Task View

The CRAN task view on Chemometrics and Computational Physics¹⁹ lists 70 packages, including a set of packages for mass spectrometry and proteomics, some of which are illustrated in this document. The most relevant (non Bioconductor) packages are summarised below.

MALDIquant provides tools for quantitative analysis of MALDI-TOF mass spectrometry data, with support for baseline correction, peak detection and plotting of mass spectra

(<http://cran.r-project.org/web/packages/MALDIquant/index.html>).

OrgMassSpecR is for organic/biological mass spectrometry, with a focus on graphical display, quantification using stable isotope dilution, and protein hydrogen/deuterium

¹⁹<http://cran.r-project.org/web/views/ChemPhys.html>

exchange experiments

(<http://cran.r-project.org/web/packages/OrgMassSpecR/index.html>).

FTICRMS provides functions for Analyzing Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry Data

(<http://cran.r-project.org/web/packages/FTICRMS/index.html>).

titan provides a GUI to analyze mass spectrometric data on the relative abundance of two substances from a titration series

(<http://cran.r-project.org/web/packages/titan/index.html>).

7.3 Other CRAN packages

Finally, **digeR**²⁰, which is available on CRAN but not listed in the Chemometrics and Computational Physics Task View, provides a GUI interface for analysing 2D DIGE data. It allows to perform correlation analysis, score plot, classification, feature selection and power analysis for 2D DIGE experiment data.

Suggestions for additional R packages are welcome and will be added to the vignette. Please send suggestions and possibly a short description and/or a example utilisation with code to lg390@cam.ac.uk. The only requirement is that the package must be available on an official package channel (CRAN, Bioconductor, R-forge, Omegahat), i.e. not only available through a personal web page.

²⁰<http://cran.r-project.org/web/packages/digeR/index.html>

8 Session information

All software and respective versions used in this document, as returned by `sessionInfo()` are detailed below.

- R version 3.0.1 Patched (2013-06-16 r62969), x86_64-unknown-linux-gnu
- Base packages: base, datasets, graphics, grDevices, methods, parallel, stats, utils
- Other packages: AnnotationDbi 1.22.6, Biobase 2.20.1, BiocGenerics 0.6.0, biomaRt 2.16.0, Biostrings 2.28.0, bitops 1.0-5, BRAIN 1.6.0, cleaver 0.99.2, data.table 1.8.8, DBI 0.2-7, digest 0.6.3, ggplot2 0.9.3.1, GO.db 2.9.0, hpar 1.2.0, IPPD 1.8.0, IRanges 1.18.1, isobar 1.6.2, knitr 1.2, lattice 0.20-15, MALDIquant 1.7, MALDIquantForeign 0.4, MASS 7.3-27, Matrix 1.0-12, msdata 0.1.13, MSnbase 1.8.0, mzR 1.6.1, org.Hs.eg.db 2.9.0, OrgMassSpecR 0.3-12, plyr 1.8, PolynomF 0.94, RColorBrewer 1.0-5, Rcpp 0.10.4, RcppClassic 0.9.3, Rdisop 1.20.0, reshape2 1.2.2, RforProteomics 1.0.7, rols 1.2.1, RSQLite 0.11.4, rTANDEM 1.0.0, XML 3.98-1.1, xtable 1.7-1
- Loaded via a namespace (and not attached): affy 1.38.1, affyio 1.28.0, base64enc 0.1-1, BiocInstaller 1.10.2, codetools 0.2-8, colorspace 1.2-2, dichromat 2.0-0, distr 2.4, downloader 0.3, evaluate 0.4.4, formatR 0.8, grid 3.0.1, gtable 0.1.2, impute 1.34.0, labeling 0.2, limma 3.16.5, munsell 0.4, preprocessCore 1.22.0, proto 0.3-10, RCurl 1.95-4.1, readBrukerFlexData 1.6.3, readMzXmlData 2.7, R.methodsS3 1.4.4, R.oo 1.13.9, R.utils 1.25.2, scales 0.2.3, sfsmisc 1.0-23, SSOAP 0.8-0, startupmsg 0.8, stats4 3.0.1, stringr 0.6.2, tools 3.0.1, vsn 3.28.0, XMLSchema 0.7-2, zlibbioc 1.6.0

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