A Bioconductor workflow for processing and analysing spatial proteomics data

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Abstract Spatial proteomics is the systematic study of protein sub-cellular localisation. In this workflow, we describe the analysis of a typical quantitative mass spectrometry-based spatial proteomics experiment using the *MSnbase* and *pRoloc* Bioconductor package suite. To walk the user through the computational pipeline, we use a recently published experiment predicting protein sub-cellular localisation in pluripotent embryonic mouse stem cells. We describe the software infrastructure at hand, importing and processing data, quality control, sub-cellular marker definition and visualisation. We then demonstrate the application and interpretation of statistical learning methods, including novelty detection using semi-supervised learning, classification, clustering and transfer learning and conclude the pipeline with data export. The workflow is aimed at beginners who are familiar with proteomics in general and spatial proteomics in particular.

Introduction

Quantitative mass spectrometry based spatial proteomics involves elaborate, expensive and time consuming experimental procedures and considerable effort is invested in the generation of such data. Multiple research groups have described a variety of approaches to establish high quality proteome-wide datasets. However, data analysis is as critical as data production for reliable and insightful biological interpretation. Here, we walk the reader through a typical pipeline for the analysis of such data using several Bioconductor packages for the R statistical programming environment.

The main package to analyse protein localisation data is *pRoloc*, which offers a set of dedicated functions for the analysis of such data. *pRoloc* itself relies on *MSnbase* to manipulate and process quantitative proteomics data. Many other packages are used by *pRoloc* for clustering, classification and visualisation.

In this workflow, we will describe how to prepare the spatial proteomics data starting from a spreadsheet containing quantitative mass spectrometry data. We will focus on a recent pluripotent mouse embryonic stem cells experiment [1]. These data, as well as additional annotated and pre-formatted datasets from various species are readily available in the *pRolocdata* package.

Installation of Bioconductor package is documented in detail on the Bioconductor installation help page. This procedure is also applicable to any packages, from CRAN as well as GitHub. Once a package has been installed, it needs to be loaded for its functionality to become available in the R session; this is done with the `library` function e.g. to load the *pRoloc* one would type `library("pRoloc")` after installation.

If you have questions about this workflow in particular, or about other Bioconductor packages in general, they are best asked on the Bioconductor support site following the posting guidelines. Questions can be tagged with specific package names or keywords. For more general information about mass spectrometry and proteomics, the readers are invited to read the *RforProteomics* package vignettes and associated papers [2, 3].

Reading and processing spatial proteomics data

The use-case: predicting sub-cellular localisation in pluripotent embryonic mouse stem cells

As a use-case, we analyse a recent high-throughput spatial proteomics dataset from pluripotent mouse embryonic stem cells (E14TG2a) [1]. The data was generated using hyperplexed LOPIT (hyperLOPIT), a state-of-the-art method relying on improved sub-cellular fractionation and more accurate quantitation, leading to more reliable classification of protein localisation across the whole sub-cellular space. The method uses an elaborate sub-cellular fractionation scheme, enabled by the use of Tandem Mass Tag (TMT) [4] 10-plex and application of the MS data acquisition technique named synchronous precursor selection MS^3 (SPS-MS^3) [5], for TMT quantification with high accuracy and precision. Three biological replicates were generated from the E14TG2a experiment, the first was to target low density fractions and the second and third were to emphasis separation of the denser organelles. The intersect of replicates 1 and 2 was treated as a 20-plex dataset for the analysis. As discussed in the manuscript [1], it has been shown that combining replicates across from different gradients can increase spatial resolution [6]. The combination of replicates resulted in 5032 proteins common in both experiments.

These, as well as many other data are directly available as properly structured and annotated computational object from the *pRolocdata* experiment package. In this workflow, we will start with a description of how to generate these ad hoc objects starting from an arbitrary spreadsheets, as produced by many popular third-party applications.

While we focus here on a LOPIT-type dataset, these analyses are relevant for any quantitative spatial proteomics data, irrespective of the fractionation or quantitation (i.e. labelled or label-free) methods.

The infrastructure: `pRoloc` and `MSnbase` packages

To make use of the full functionality of the *pRoloc* software one needs to import their data into R and prepare them as an `MSnSet`. The `MSnSet` is a dedicated data structure for the efficient manipulation and processing of mass spectrometry and proteomics data in R. Figure 1 illustrates a simplified view of the `MSnSet` structure; there exists 3 key sub-parts (termed slots) to such a data object: (1) the `exprs` (short for *expression* data) slot for storing the quantitation data, (2) the `fData` slot (short for *feature*-metadata) for storing the feature meta-data, and finally (3) the `pData` slot (short for *pheno*-metadata, i.e. sample phenotypic data) for storing the sample meta-data.

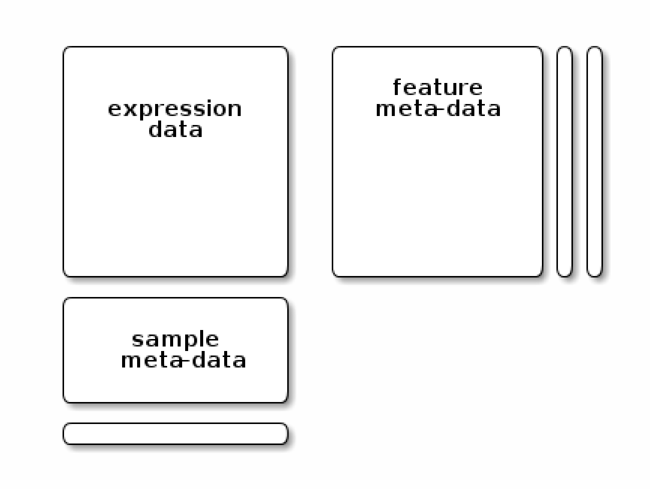


Figure 1. Simplified representation of the `MSnSet` data structure (reproduced with permission from the *MSnbase* vignette)

Feature metadata typically contains general annotation about the proteins (accession numbers, description, ...), information related to the identification search (confidence scores, number of peptides, ...) as well as annotation about know sub-cellular location (see in particular the *Markers* section) and results from data analysis. The sample metadata would, for example, record what labelling ions were used for the respective fraction (when labelled quantitation is used), replicate number, fraction number along the gradient and pooling information.

Another slot of interest is `processingData`, that logs the processing `MSnSet` objects undergo. The processing log can be accessed with the `processingData` function and is displayed under *Processing information* in the textual object summary when an `MSnSet`'s name it typed in the R console.

Importing data

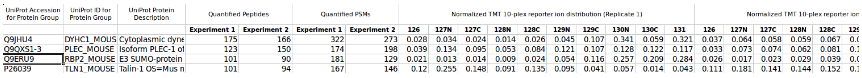
There are a number of ways to import quantitation data and create an `MSnSet` instance. All methods are described in the *MSnbase* input/output capabilities vignette. One suggested simple method is to use the function `readMSnSet2`. The function takes a single spreadsheet filename as input and extracts the columns containing the quantitation data, as identified by the argument `ecol`, to create the expression data, while the other columns in the spreadsheet are appended to the feature meta-data slot. By example, in the code chunk below we read in the `csv` spreadsheet containing the quantitation data from the intersect of replicates 1 and 2 of the mouse map [1], using the `readMSnSet2` function. The data is as available online with the manuscript (see tab 2 of the `xlsx` supplementary data set 1 in [1], which should be exported as a text-based spreadsheet). It is also available as a `csv` in the Bioconductor *pRolocdata* data package, which we make use of below.

To use the `readMSnSet2` function, as a minimum one must specify the file path to the data and which columns of the spreadsheet contain quantitation data. The `getEcols` function exists to help users identify which columns of the spreadsheet contain the quantitation data. In the last line of the code chunk below, we print the file name (not the full path, which will vary from computer to computer).

```
library("MSnbase")
extdatadir <- system.file("extdata", package = "pRolocdata")
csvfile <- dir(extdatadir, full.names = TRUE,
          pattern = "hyperLOPIT-SIData-ms3-rep12-intersect.csv")
basename(csvfile)

## [1] "hyperLOPIT-SIData-ms3-rep12-intersect.csv.gz"
```

Note that the file is compressed (as indicated by the `gz`, for `gzip`, extension), and will be decompressed on-the-fly when read into R.
The spreadsheet that was deposited by the authors contains two headers, with the second header containing information about where the quantitation data is stored.



UniProt Accession for Protein Group	UniProt ID for Protein Group	UniProt Protein Description	Quantified Peptides		Quantified PSMs		Normalized TMT 10-plex reporter ion distribution (Replicate 1)										Normalized TMT 10-plex reporter ion					
			Experiment 1	**Experiment 2**	**Experiment 1**	**Experiment 2**	**126**	**127N**	**127C**	**128N**	**128C**	**129N**	**129C**	**130N**	**130C**	**131**	**126**	**127N**	**127C**	**128N**	**128C**	**12**
Q9JHU4	DYHC1_MOUS	Cytoplasmic dyne	175	166	322	273	0.028	0.034	0.024	0.014	0.026	0.045	0.107	0.341	0.059	0.321	0.037	0.064	0.058	0.059	0.067	0.0
Q9QXS1-3	PLEC_MOUSE	Isoform PLEC-1 of	123	150	174	198	0.039	0.134	0.095	0.053	0.084	0.121	0.107	0.128	0.122	0.117	0.033	0.073	0.074	0.062	0.081	0.1
Q9ERU9	RBP2_MOUSE	E3 SUMO-protein	101	90	181	129	0.021	0.013	0.014	0.009	0.024	0.054	0.116	0.257	0.209	0.284	0.026	0.017	0.023	0.029	0.039	0.0
P26039	TLN1_MOUSE	Talin-1 OS=Mus n	101	94	167	146	0.12	0.255	0.148	0.091	0.135	0.095	0.041	0.057	0.014	0.043	0.111	0.181	0.141	0.144	0.152	0.1

Figure 2. A screenshot of the data in the spreadsheet.

We can display the names of the second header by calling the `getEcols` function with the argument `n = 2` (the default value is `n = 1`), to specify that we wish to display the column names of the second line.

```
getEcols(csvfile, split = ",", n = 2)

##  [1] ""
##  [2] ""
##  [3] ""
##  [4] "Experiment 1"
##  [5] "Experiment 2"
##  [6] "Experiment 1"
##  [7] "Experiment 2"
##  [8] "126"
##  [9] "127N"
## [10] "127C"
## [11] "128N"
## [12] "128C"
## [13] "129N"
## [14] "129C"
## [15] "130N"
## [16] "130C"
## [17] "131"
## [18] "126"
## [19] "127N"
## [20] "127C"
## [21] "128N"
## [22] "128C"
## [23] "129N"
## [24] "129C"
## [25] "130N"
## [26] "130C"
## [27] "131"
## [28] "phenoDisco Input"
## [29] "phenoDisco Output"
## [30] "Curated phenoDisco Output"
## [31] "SVM marker set"
## [32] "SVM classification"
## [33] "SVM score"
## [34] "SVM classification (top quartile)"
## [35] "Final Localization Assignment"
## [36] "First localization evidence?"
## [37] "Curated Organelles"
## [38] "Cytoskeletal Components"
## [39] "Trafficking Proteins"
## [40] "Protein Complexes"
## [41] "Signaling Cascades"
## [42] "Oct4 Interactome"
## [43] "Nanog Interactome"
## [44] "Sox2 Interactome"
## [45] "Cell Surface Proteins"
```

It is now easy for one to identify that the quantitation data, corresponding to the 10 TMT isobaric tags, is located in columns 8 to 27. We now have the two mandatory arguments to `readMSnSet2`, namely the file name (stored in the `csvfile` variable) and the quantitation column indices. In addition to these, it is also possible to pass the optional argument `fnames` to indicate which column to use as the labels by which to identify each protein in the sample. Here, we use `fnames = 1` to use the UniProt identifiers contained in the first (unnamed) column of the spreadsheet. We also need to specify to skip the first line of the file (for the same reason that we used `n = 2` in `getEcols` above) to read the `csv` data and convert it to an `MSnSet` object, named `hl` (for hyperLOPIT).

```
hl <- readMSnSet2(csvfile, ecol = 8:27, fnames = 1, skip = 1)
```

Below, we display a short summary of the data. The data contains 5032 proteins/features common across the 2 biological replicates for the respective 2 x 10-plex reporter tags (20 columns/samples), along with associated feature meta-data such as protein markers, protein description, number of quantified peptides etc (see below).

```
hl

## MSnSet (storageMode: lockedEnvironment)
## assayData: 5032 features, 20 samples
##   element names: exprs
## protocolData: none
## phenoData: none
## featureData
##   featureNames: Q9JHU4 Q9QXS1-3 ... Q9Z2R6 (5032 total)
##   fvarLabels: X X.1 ... Cell.Surface.Proteins (25 total)
##   fvarMetadata: labelDescription
## experimentData: use ’experimentData(object)’
## Annotation:
## - - - Processing information - - -
##  MSnbase version: 2.0.0
```

Below, we examine the quantitative information for first 5 proteins. It is also possible to access specific rows and columns by naming the proteins and TMT tag channels of interest.

```
exprs(hl)[1:5, ]

##             X126 X127N X127C X128N X128C X129N X129C X130N X130C  X131
## Q9JHU4   0.028 0.034 0.024 0.014 0.026 0.045 0.107 0.341 0.059 0.321
## Q9QXS1-3 0.039 0.134 0.095 0.053 0.084 0.121 0.107 0.128 0.122 0.117
## Q9ERU9   0.021 0.013 0.014 0.009 0.024 0.054 0.116 0.257 0.209 0.284
## P26039   0.120 0.255 0.148 0.091 0.135 0.095 0.041 0.057 0.014 0.043
## Q8BTM8   0.055 0.139 0.078 0.050 0.077 0.098 0.093 0.171 0.079 0.160
##          X126.1 X127N.1 X127C.1 X128N.1 X128C.1 X129N.1 X129C.1 X130N.1
## Q9JHU4    0.037   0.064   0.058   0.059   0.067   0.078   0.140   0.208
## Q9QXS1-3  0.033   0.073   0.074   0.062   0.081   0.142   0.190   0.069
## Q9ERU9    0.026   0.017   0.023   0.029   0.039   0.071   0.105   0.171
## P26039    0.111   0.181   0.141   0.144   0.152   0.119   0.075   0.028
## Q8BTM8    0.062   0.108   0.091   0.086   0.099   0.111   0.117   0.095
##          X130C.1 X131.1
## Q9JHU4     0.141  0.147
## Q9QXS1-3   0.151  0.125
## Q9ERU9     0.304  0.215
## P26039     0.017  0.033
## Q8BTM8     0.144  0.087

exprs(hl)[c("Q9ERU9", "Q9Z2R6"), c("X126", "X131.1")]

##         X126 X131.1
## Q9ERU9 0.021  0.215
## Q9Z2R6 0.563  0.000
```

The feature meta-data is stored in the `fData` slot and can be accessed by `fData(hl)`. When using `readMSnSet2`, automatically, everything that is not defined as quantitation data by `ecol` or the feature names by `fnames` is deposited to the `fData` slot.

We see the `fData` contains 25 columns describing information such as the number of peptides, associated markers, machine learning results etc. To identify the feature variable names we can use the function `fvarLabels`. We see that the first 6 feature variable names contain non-discriminatory label names, so we relabel them to help us identify what feature data information is stored in the associated columns.

```
fvarLabels(hl)

##  [1] "X"
##  [2] "X.1"
##  [3] "X.2"
##  [4] "Experiment.1"
##  [5] "Experiment.2"
##  [6] "Experiment.1.1"
##  [7] "Experiment.2.1"
##  [8] "phenoDisco.Input"
##  [9] "phenoDisco.Output"
## [10] "Curated.phenoDisco.Output"
## [11] "SVM.marker.set"
## [12] "SVM.classification"
## [13] "SVM.score"
## [14] "SVM.classification..top.quartile."
## [15] "Final.Localization.Assignment"
## [16] "First.localization.evidence."
## [17] "Curated.Organelles"
## [18] "Cytoskeletal.Components"
## [19] "Trafficking.Proteins"
## [20] "Protein.Complexes"
## [21] "Signaling.Cascades"
## [22] "Oct4.Interactome"
## [23] "Nanog.Interactome"
## [24] "Sox2.Interactome"
## [25] "Cell.Surface.Proteins"

fvarLabels(hl)[1:3] <- c("uniprot.accession", "uniprot.id", "description")
fvarLabels(hl)[4:6] <- paste0("peptides.expt", 1:3)
fData(hl)[1:4, 1:6]

##          uniprot.accession  uniprot.id
## Q9JHU4              Q9JHU4 DYHC1_MOUSE
## Q9QXS1-3          Q9QXS1-3  PLEC_MOUSE
## Q9ERU9              Q9ERU9  RBP2_MOUSE
## P26039              P26039  TLN1_MOUSE
##
## Q9JHU4                                          Cytoplasmic dynein 1 heavy chain 1 OS=Mus
## Q9QXS1-3 Isoform PLEC-1 of Plectin OS=Mus musculus GN=Plec - [PLEC_MOUSE]|Isoform PLEC-1A of P
## Q9ERU9                                                     E3 SUMO-protein ligase RanBP2 OS=Mu
## P26039                                                                         Talin-1 OS=
##          peptides.expt1 peptides.expt2 peptides.expt3
## Q9JHU4              175            166            322
## Q9QXS1-3            123            150            174
## Q9ERU9              101             90            181
## P26039              101             94            167
```

Note that when using the simple `readMSnSet2` procedure, the `pData` slot which is used to store information about the samples/channels is kept empty. It is advised to annotate the channels as well. Below, we annotate the replicate from which the profiles originate and the TMT tag (extracted from the sample/channel names).

```
pData(hl)$Replicate <- rep(1:2, each = 10)
pData(hl)$Tag <- sub("\\.1$", "", sub("^X", "", sampleNames(hl)))
pData(hl)

##         Replicate  Tag
## X126            1  126
## X127N           1 127N
## X127C           1 127C
## X128N           1 128N
## X128C           1 128C
## X129N           1 129N
## X129C           1 129C
## X130N           1 130N
## X130C           1 130C
## X131            1  131
## X126.1          2  126
## X127N.1         2 127N
## X127C.1         2 127C
## X128N.1         2 128N
## X128C.1         2 128C
## X129N.1         2 129N
## X129C.1         2 129C
## X130N.1         2 130N
## X130C.1         2 130C
## X131.1          2  131
```

Throughout this workflow we refer to the different columns that are found in the `exprs` (expression data) slot as channels (short for TMT channels). In the frame of LOPIT and hyperLOPIT these channels consitiue the relative abundance of each protein (along the rows) in the channel of interest. Each TMT channel originates from fractions collected from the density gradient, or a set of pooled fractions or may be a sample originating from an alternative preparation e.g. such as from the chromatin enrichment performed in Christoforou et al [1]. Information about which gradient fractions were used for which tag should also be stored in the sample meta-data `pData` slot.
The sample meta-data that is distributed with the *pRolocdata* package for Christoforou's hyperLOPIT experiment and (as above) the quantitation data file, are located in the `extdata` in the *pRolocdata* package on the hard drive.
In the code chunk below we again use the `dir` function to locate the filepath to the meta-data `csv` file and then read it into R using `read.csv`. We then append the meta-data to the `pData` slot. Information about the gradient fractions used and the associated subcellular fraction densities in each replicate are stored here.

```
expinfo <- dir(extdatadir, full.names = TRUE,
               pattern = "hyperLOPIT-SIData-fraction-info.csv")

fracinfo <- read.csv(expinfo, row.names=1, skip = 2,
                     header = FALSE, stringsAsFactors = FALSE)

pData(hl)$Gradient.Fraction <- c(fracinfo[, 1], fracinfo[, 2])
pData(hl)$Iodixonal.Density <- c(fracinfo[, 4], fracinfo[, 5])
pData(hl)
```

```
##         Replicate  Tag Gradient.Fraction Iodixonal.Density
## X126            1  126           Cytosol               0.0
## X127N           1 127N   1 to 6 (pooled)               6.0
## X127C           1 127C   8 to 9 (pooled)              11.0
## X128N           1 128N 10 to 11 (pooled)              13.3
## X128C           1 128C                12              14.6
## X129N           1 129N                14              17.4
## X129C           1 129C                16              20.1
## X130N           1 130N                18              26.8
## X130C           1 130C         Chromatin                NA
## X131            1  131                19              34.5
## X126.1          2  126           Cytosol               0.0
## X127N.1         2 127N   1 to 6 (pooled)               5.2
## X127C.1         2 127C   7 to 9 (pooled)              10.0
## X128N.1         2 128N 10 to 11 (pooled)              12.5
## X128C.1         2 128C                12              14.0
## X129N.1         2 129N 14 to 15 (pooled)              17.3
## X129C.1         2 129C                17              20.9
## X130N.1         2 130N 18 to 19 (pooled)              24.7
## X130C.1         2 130C         Chromatin                NA
## X131.1          2  131                20              31.9
```

Data processing

Normalisation

There are two aspects related to data normalisation that are relevant to spatial proteomics data processing. The first one focuses on reducing purely technical variation between channels without affecting biological variability (i.e. the shape of the quantitative profiles). This normalisation will depend on the underlying quantitative technology and the experimental design, and will not be addressed in this workflow. The second aspect, and more specific to spatial proteomics data, is scaling all the organelle-specific profiles into a same intensity interval (typically 0 and 1) by, for example, diving each intensity by the sum of the intensities for that quantitative feature. This is not necessary in this example as the intensities for each replicate have already been re-scaled to 1 in Proteome Discoverer. However, if one wanted to do this they would execute the `normalise` function as demonstrated in the below code chunk.

```
hl <- normalise(hl, method = "sum")
```

This transformation of the data assures to cancel the effect of the absolute intensities of the quantitative features along the rows, to focus subsequent analyses on the relative profiles along the sub-cellular channels.

The same `normalise` function (or `normalize`, both spellings are supported) can also be applied in the first case described above. Different normalisation methods such as mean or median scaling, variance stabilisation or quantile normalisation, to cite a few, can be applied to accomodate different needs.

As previously mentioned, before combination, the two replicates in the `hl` data that we read into R were separately normalised by sum (i.e. to 1) across the 10 channels for each replicate respectively. We can verify this by summing each rows for each replicate:

```
summary(rowSums(exprs(hl[, hl$Replicate == 1])))

##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##   0.997   0.999   1.000   1.000   1.001   1.003

summary(rowSums(exprs(hl[, hl$Replicate == 2])))

##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##   0.997   0.999   1.000   1.000   1.001   1.003
```

We see that some features do not add up exactly to 1 due to rounding errors after exporting to intermediate files. These small deviations do not bear any consequences here.

Combining acquisitions

The spreadsheet that was used to create the `hl` MSnSet included the two replicates within one .csv file. We also provide individual replicates in the *pRolocdata* package. Below, we show how to combine `MSnSet` objects and, subsequently, how to filter and handle missing values. We start by loading the *pRolocdata* package and the equivalent replicates using the `data` function.

```
library("pRolocdata")
data(hyperLOPIT2015ms3r1)
data(hyperLOPIT2015ms3r2)
```

At the R prompt, typing

```
pRolocdata()
```

will list the 54 datasets that are available in *pRolocdata*.
Combining data is performed with the `combine` function. This function will inspect the feature and sample names to identify how to combine the data. As we want our replicates to be combined along the columns (same proteins, different sets of channels), we need to assure that the respective sample names differ so they can be identified from one another. The function `updateSampleNames` can be used do this.

```
identical(sampleNames(hyperLOPIT2015ms3r1), sampleNames(hyperLOPIT2015ms3r2))

## [1] TRUE

hyperLOPIT2015ms3r1 <- updateSampleNames(hyperLOPIT2015ms3r1, 1)
hyperLOPIT2015ms3r2 <- updateSampleNames(hyperLOPIT2015ms3r2, 2)
sampleNames(hyperLOPIT2015ms3r1)

##  [1] "X126.1"  "X127N.1" "X127C.1" "X128N.1" "X128C.1" "X129N.1" "X129C.1"
##  [8] "X130N.1" "X130C.1" "X131.1"

sampleNames(hyperLOPIT2015ms3r2)

##  [1] "X126.2"  "X127N.2" "X127C.2" "X128N.2" "X128C.2" "X129N.2" "X129C.2"
##  [8] "X130N.2" "X130C.2" "X131.2"
```

In addition, to matching names, the content of the feature metadata for identical feature annotations must match exactly across the data to be combined. In particular for these data, we expect the same proteins in each replicates to be annotated with the same UniProt entry names and descriptions, but not with the same coverage of number of peptides or peptide-spectrum matches (PSMs).

```
fvarLabels(hyperLOPIT2015ms3r1)

## [1] "EntryName"          "ProteinDescription" "Peptides"
## [4] "PSMs"               "ProteinCoverage"    "markers"

fvarLabels(hyperLOPIT2015ms3r2)

## [1] "EntryName"          "ProteinDescription" "Peptides"
## [4] "PSMs"               "ProteinCoverage"    "markers"
```

Below, we update the replicate specific feature variable names and remove the shared annotation.

```
fvarLabels(hyperLOPIT2015ms3r1)[3:5] <- paste0(fvarLabels(hyperLOPIT2015ms3r1)[3:5], 1)
fvarLabels(hyperLOPIT2015ms3r2)[3:5] <- paste0(fvarLabels(hyperLOPIT2015ms3r2)[3:5], 2)
fData(hyperLOPIT2015ms3r1) <- fData(hyperLOPIT2015ms3r1)[1:5]
fData(hyperLOPIT2015ms3r2) <- fData(hyperLOPIT2015ms3r2)[3:5]
fvarLabels(hyperLOPIT2015ms3r1)

## [1] "EntryName"          "ProteinDescription" "Peptides1"
## [4] "PSMs1"              "ProteinCoverage1"
```

```
fvarLabels(hyperLOPIT2015ms3r2)

## [1] "Peptides2"        "PSMs2"             "ProteinCoverage2"
```

We can now combine the two experiments into a single `MSnSet`:

```
combined <- combine(hyperLOPIT2015ms3r1, hyperLOPIT2015ms3r2)
combined

## MSnSet (storageMode: lockedEnvironment)
## assayData: 6725 features, 20 samples
##   element names: exprs
## protocolData: none
## phenoData
##   sampleNames: X126.1 X127N.1 ... X131.2 (20 total)
##   varLabels: Replicate TMT.Reagent ... Iodixonal.Density (5 total)
##   varMetadata: labelDescription
## featureData
##   featureNames: Q9JHU4 Q9QXS1-3 ... Q9Z2Y3-3 (6725 total)
##   fvarLabels: EntryName ProteinDescription ... ProteinCoverage2 (8
##     total)
##   fvarMetadata: labelDescription
## experimentData: use 'experimentData(object)'
## Annotation:
## - - - Processing information - - -
## Combined [6725,20] and [6268,10] MSnSets Tue Nov  8 14:13:46 2016
##  MSnbase version: 1.21.7
```

More details above combining data are given in the dedicated *Combining MSnSet instances* section of the *MSnbase* tutorial vignette.

Missing data

Missing data are a recurrent issue in mass spectrometry applications, and should be addressed independently of this workflow [7, 8]. In [9], we have described how a high content in missing values in spatial proteomics data and their inappropriate handling leads to a reduction of sub-cellular resolution. Missing data can be imputated using *MSnbase*'s `impute` function. The method underlying the imputation method is then determined by a `methods` parameter. To impute missing values using nearest neighbour imputation, one would

```
hl <- impute(hl, method = "knn")
```

In our particular case, missing values are indicative of protein groups that were not acquired in both replicates (Figure 3).
We prefer to remove proteins that were not assayed in both replicated experiments. This is done with the `filterNA` function that removes features (proteins) that contain more than a certain proportion (default is 0) missing values.

```
combined <- filterNA(combined)
combined

## MSnSet (storageMode: lockedEnvironment)
## assayData: 5032 features, 20 samples
##   element names: exprs
## protocolData: none
## phenoData
##   sampleNames: X126.1 X127N.1 ... X131.2 (20 total)
##   varLabels: Replicate TMT.Reagent ... Iodixonal.Density (5 total)
##   varMetadata: labelDescription
## featureData
##   featureNames: Q9JHU4 Q9QXS1-3 ... Q9Z2R6 (5032 total)
##   fvarLabels: EntryName ProteinDescription ... ProteinCoverage2 (8
image2(is.na(combined), col = c("black", "white"),
       main = "Missing values (white cells) after combining replicates")
```

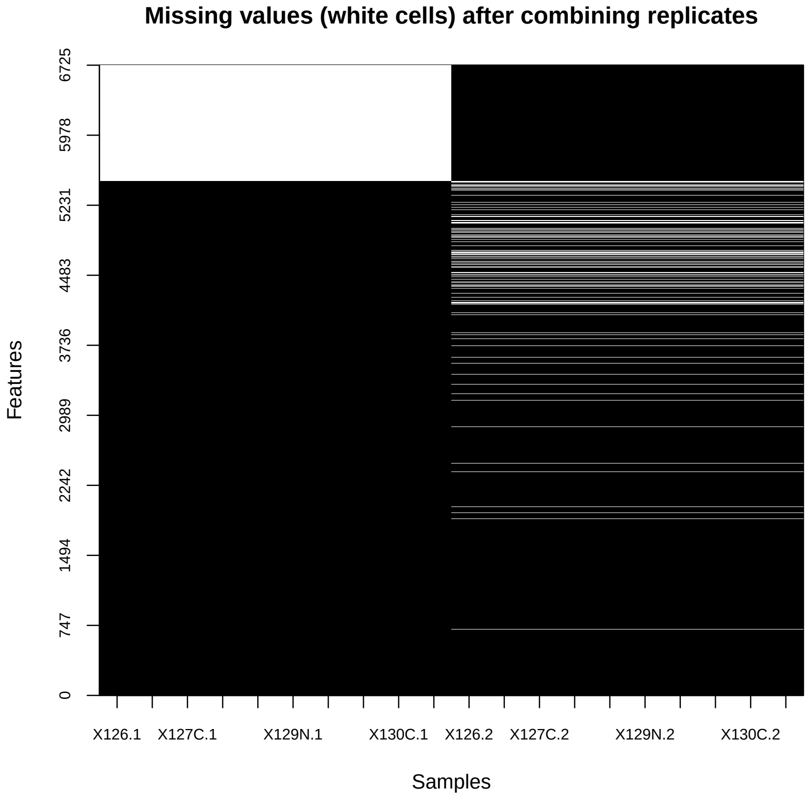


Figure 3. Heatmap of missing values. Note that the features are re-ordered to highlight cluster of proteins with similar numbers of missing values.

```
##     total)
##   fvarMetadata: labelDescription
## experimentData: use 'experimentData(object)'
## Annotation:
## - - - Processing information - - -
## Combined [6725,20] and [6268,10] MSnSets Tue Nov  8 14:13:46 2016
## Subset [6725,20][5032,20] Tue Nov  8 14:13:47 2016
## Removed features with more than 0 NAs: Tue Nov  8 14:13:47 2016
## Dropped featureData's levels Tue Nov  8 14:13:47 2016
##  MSnbase version: 1.21.7
```

When more than 2 datasets are to be combined and too many proteins were not consistently assayed, leading to too many proteins being filtered out, we suggest to implement an ensemble of classifiers voting on protein-sub-cellular niche membership over the output of several experiments (see section *Supervised machine learning* for the description of sub-cellular assignments).

Quality Control

Data quality is routinely examined through visualisation to verify that sub-cellular niches have been separated along the gradient. Based on De Duve's principle [10] proteins that co-localise exhibit similar quantitation profiles across the gradient fractions employed. One approach that has been widely used to visualise and inspect high throughput mass spectrometry-based proteomics data is principal components analysis (PCA). PCA is one of many dimensionality reduction methods, that allows one to effectively summarise multi-dimensional data in to 2 or 3 dimensions to enable visualisation. Very generally, the original continuous multi-dimensional data is transformed into a set of orthogonal components ordered according to the amount of variability that they describe. The `plot2D` method in *pRoloc* allows one

to plot the principal components (PCs) of a dataset against one another, by default the first two components are plotted on the x- and y-axis, respectively (the `dims` argument can be used to plot other PCs). If distinct clusters are observed, we assume that there is organellar separation present in the data. Although, representing the multi-dimensional data along a limited set of PCs does not give us a hard quantitative measure of separation, it is extremely useful summarising complex experimental information in one figure, to get a simplified overview of the data.
In the code chunk below we produce a PCA plot of the mouse stem cell dataset (Figure 4). One point on the plot represents one protein. We can indeed see several distinct protein clusters. We specify `fcol = NULL`, which means not to consider any feature variable to annotate the features (proteins) with colours. We will see later how to use this argument to annotate the PCA plot with prior information about sub-cellular localisation.

```
library("pRoloc")
plot2D(hl, fcol = NULL, col = "black")
plot2D(hl, method = "hexbin")
```

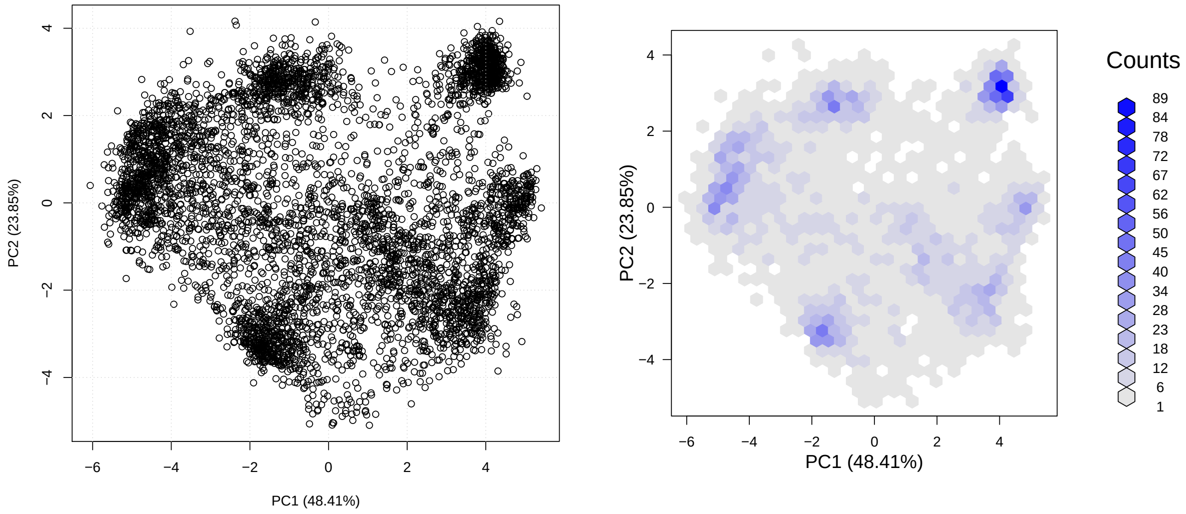


Figure 4. PCA plot of the mouse stem cell data `hl`. Each dot represent a single protein, and cluster of proteins represent proteins residing in the same sub-cellular niche. The figure on the right bins proteins and represent the bins density to highlight the presence of protein clusters.

In the first instance we advise one to visualise their data without any annotation (i.e. with `fcol = NULL`), before proceeding with data annotation. The identification of well resolved clusters in the data, constitutes an unbiased assessment of the data structure, demonstrating the successful separation of sub-cellular clusters.
It is also useful to visualise the relative intensities along the gradient to identify channels displaying particularly low yield. This can be done using the `plotDist` and `boxplot` functions, that plot the protein profiles occupancy along the gradient (we also display the mean channel intensities) and a `boxplot` of the column intensities. In the two plots displayed on figure 5, we re-order the TMT channles to pair corresponding channels in the two replicates (rather than ordering the channels by replicate).

Markers

In the context of spatial proteomics, a marker protein is defined as a well-known resident of a specific sub-cellular niche in a species *and* condition of interest. Applying this to machine learning (ML), and specifically supervised learning, for the task of protein localisation prediction, these markers constitute the labelled training data to use as input to a classification analyses. Defining well-known residents, and obtaining labelled training data for ML analyses can be time consuming, but it is important to define markers that are representative of the multivariate data space and on which a classifier will be trained and generated. *pRoloc* provides a convenience function, `addMarkers`, to directly add markers to a `MSnSet` object, as demonstrated in the code chunk below. These marker sets can be accessed using the `pRolocmarkers()` function. Marker sets are stored as a simple named vector in R, and originate from in-house user-defined sets of markers or from previous published studies [9], which are continuosly updated and integrated.

```
par(mfrow = c(1, 2))
o <- order(hl$Iodixonal.Density)
plotDist(hl[, o], pcol = "#00000010")
lines(colMeans(exprs(hl[, o])), col = "red", type = "b")
boxplot(exprs(hl[, o]), las = 2)
```

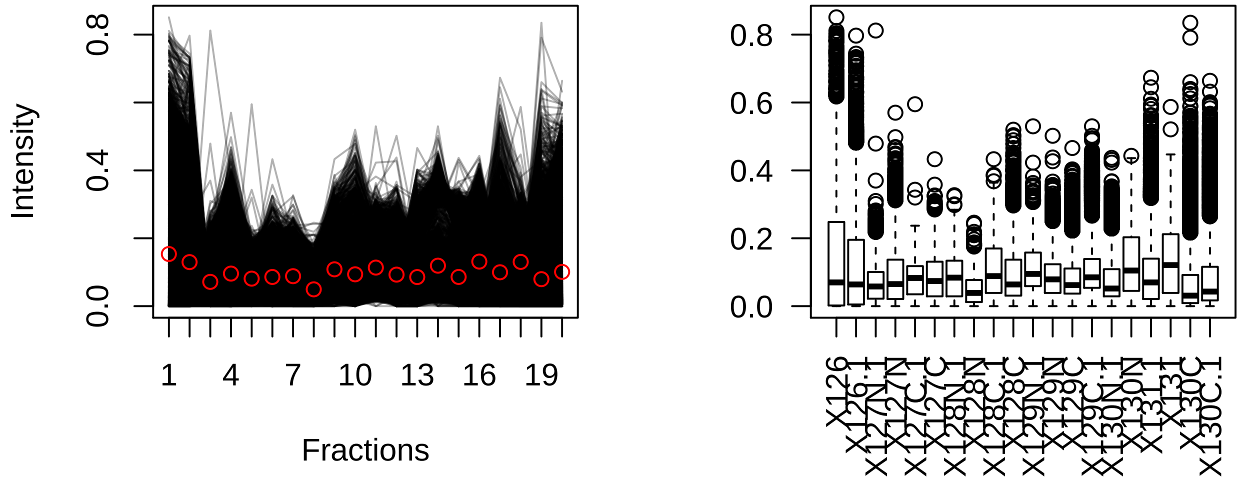


Figure 5. Protein profiles and distribution of channel intensities.

```
## List available marker sets
pRolocmarkers()

## 7 marker lists available:
## Arabidopsis thaliana [atha]:
##  Ids: TAIR, 543 markers
## Drosophila melanogaster [dmel]:
##  Ids: Uniprot, 179 markers
## Gallus gallus [ggal]:
##  Ids: IPI, 102 markers
## Homo sapiens [hsap]:
##  Ids: Uniprot ID, 205 markers
## Mus musculus [mmus]:
##  Ids: Uniprot, 937 markers
## Saccharomyces cerevisiae [scer_sgd]:
##  Ids: SGD, 259 markers
## Saccharomyces cerevisiae [scer_uniprot]:
##  Ids: Uniprot Accession, 259 markers
```

These markers can then be mapped to a `MSnSet`'s `featureNames`. The mouse dataset used here has Uniprot IDs stored as the `featureNames` (see `head(featureNames(hl))`) and the names of the vector of the mouse markers stored in *pRoloc* (`mmus` markers) are also Uniprot IDs (see `head(mrk)` in the code chunk below), so it is straightforward to match names between the markers and the `MSnSet` instance using the `addMarkers` function.

```
## Use mouse markers
mrk <- pRolocmarkers(species = "mmus")
head(mrk)

##                  P26039                 Q6PB66                 P11276
##   "Actin cytoskeleton"        "Mitochondrion" "Extracellular matrix"
##                 Q6PR54                 Q05793                 P19096
##  "Nucleus - Chromatin" "Extracellular matrix"              "Cytosol"

## Add mouse markers
hl <- addMarkers(hl, mrk)

## Markers in data:  937 out of 5032

## organelleMarkers
##            40S Ribosome            60S Ribosome      Actin cytoskeleton
##                      27                      43                      13
##                 Cytosol   Endoplasmic reticulum                Endosome
##                      43                      95                      12
##    Extracellular matrix         Golgi apparatus                Lysosome
##                      10                      27                      33
##           Mitochondrion     Nucleus - Chromatin Nucleus - Non-chromatin
##                     383                      64                      85
##              Peroxisome         Plasma membrane              Proteasome
##                      17                      51                      34
##                 unknown
##                    4095
```

We recommend at least 13 markers per sub-cellular class (see the *Optimisation* section for details about the algorithmic motivation of this number). Markers should be chosen to confidently represent the distribution of genuine residents of a sub-cellular niche. We generally recommend a conservative approach in defining markers to avoid false assignments when assigning sub-cellular localisation of proteins of unknown localisation. A more relaxed definition of markers, i.e. one that broadly or over-confidently defines markers, risks to erroneously assign proteins to a single location, when, in reality, they reside in multiple locations (including the assumed unique location). One can not expect to identify exact boundaries between sub-cellular classes through marker annotation alone; the definition of these boundaries is better handled algorithmically, i.e. after application of the supervised learning algorithm, using the prediction scores (as described in the *Classification* section, in particular Figure 13).

If the protein naming between the marker sets and the `MSnSet` dataset are different e.g. the markers are labelled by Uniprot accession numbers and the dataset entries are labelled by Uniprot entry names, one will have to convert and match the proteins according to the appropriate identifier. Sometimes, we find the equivalent entry name, Uniprot ID or accession number is stored with the data, which makes conversion between identifers relatively straightforward. If this is not the case however, conversion can be performed using *biomaRt*, the Bioconductor annotation resouces or any conversion softwares available online.

It is also possible to use one's own marker list with the `addMarkers` function. The user needs to create a named vector of marker localisation, where the names match some (not necessarily all) of the `MSnSet`'s feature names.

We now visualise these annotations along the PCA plot using the `plot2D` function and then use the `addLegend` function to map the marker classes to the pre-defined colours. We also display the data along the first and seventh PCs using the `dims` argument. Note that in these calls to the `plot2D` function, we have omitted the `fcol` argument and use of the default `"markers"` feature variable to annotated the plot. We choose to display PCs 1 and 7 to illustrate that while upper principal components explain much less variability in the data (2.23% for PC7, as opposed to 48.41% for PC1), we see that the mitochondrial (purple) and peroxisome (dark blue) clusters can be differenciated, despite the apparent overlap in the two first PCs.

The colours have been defined so as to enable to differenciate up to 30 classes. If more are provided, different character symbols (circles, squares, ... and empty and solid symbols) are used. The colours and the default plotting characters (solid dots for the markers and empty circles for the features of unknown localisation) can of course be changed, as described in the `setStockcol` manual page.

As demonstrated in [1] and illustrated in the PCA plot above, the Golgi apparatus proteins (brown) display a dynamic pattern, noting sets of Golgi marker proteins that are distributed amongst other subcellular structures, an observation supported by microscopy. As such, we are going to reset the annotation of Golgi markers to unknown using the `fDataTounknown` function. It is often used to replace empty strings (`""`) or missing values in the markers definition to a common definition of *unknown* localisation.

```
hl <- fDataToUnknown(hl, from = "Golgi apparatus", to = "unknown")
getMarkers(hl)
```

```
par(mfrow = c(1, 2))
plot2D(hl, main = "pRolocmarkers for mouse")
addLegend(hl, cex = .6)
plot2D(hl, dims = c(1, 7), main = "Marker resolution along PC 1 and 7")
```

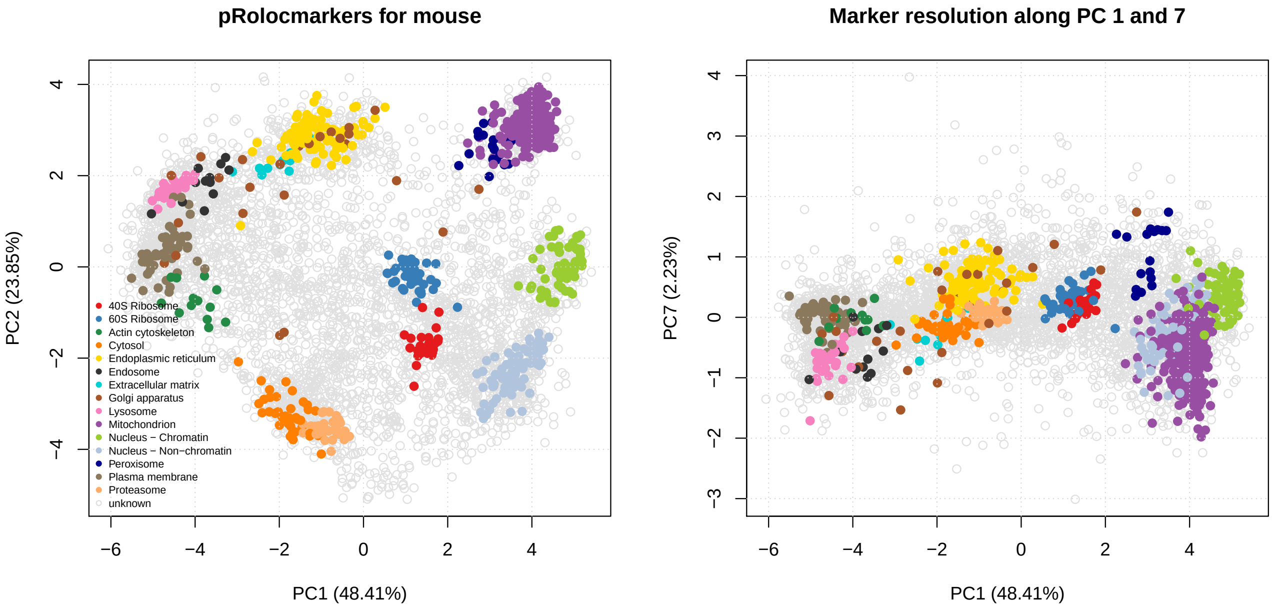


Figure 6. Annotated PCA plots of the `hl` dataset.

```
## organelleMarkers
##            40S Ribosome            60S Ribosome      Actin cytoskeleton
##                      27                      43                      13
##                 Cytosol   Endoplasmic reticulum                Endosome
##                      43                      95                      12
##    Extracellular matrix                Lysosome           Mitochondrion
##                      10                      33                     383
##     Nucleus - Chromatin Nucleus - Non-chromatin              Peroxisome
##                      64                      85                      17
##         Plasma membrane              Proteasome                 unknown
##                      51                      34                    4122
```

In general, the Gene Ontology (GO) [11], and in particular the cellular compartment (CC) namespace are a good starting point for protein annotation and marker definition. It is important to note however that automatic retrieval of sub-cellular localisation information, from *pRoloc* or elsewhere, is only the beginning in defining a marker set for downstream analyses. Expert curation is vital to check that any annotation added is in the correct context for the biological question under investigation.

Another useful visualisation that relies on marker annotation is the representation of the protein profiles occupancy along the gradient using the `plotDist` function. While the PCA plot enables to efficiently visualise the complete dataset and assess the relative separation of different sub-cellular niches, comparing profiles of a few marker clusters is useful to assess how exactly they differ (in terms of peak channels, for example). On figure 7, we plot the profile of the mitochondrial and peroxisome markers to highlight the differences in profiles between these two sets of markers along the 6th and 7th channels, as represented above along the 7th PC on the PCA plot on figure 6.

Finally, in addition to `plot2D`, the `plot3D` function allows to interactively explore a 3-dimensional plot of the data.

Replication

With the aim of maximising the sub-cellular resolution and, consequently, the reliability in protein sub-cellular assignments, we follow the advice in [6] and combine replicated spatial proteomics experiments as described above. Indeed, Trotter et al. have shown a significant improvement in protein–organelle association upon direct combination of single

```
hlo <- hl[, order(hl$Iodixonal.Density)]
plotDist(hlo[fData(hlo)$markers == "Mitochondrion", ],
         pcol = "purple", fractions = "Fraction.No")
title(main = "Marker occupancy profiles along the gradient")
matlines(t(exprs(hlo[fData(hlo)$markers == "Peroxisome", ])),
         lty = 1, col = "darkblue", type = "l")
legend("topleft", c("Mitochondrion", "Peroxisome"),
       lty = 1, col = c("purple", "blue"), bty = "n")
```

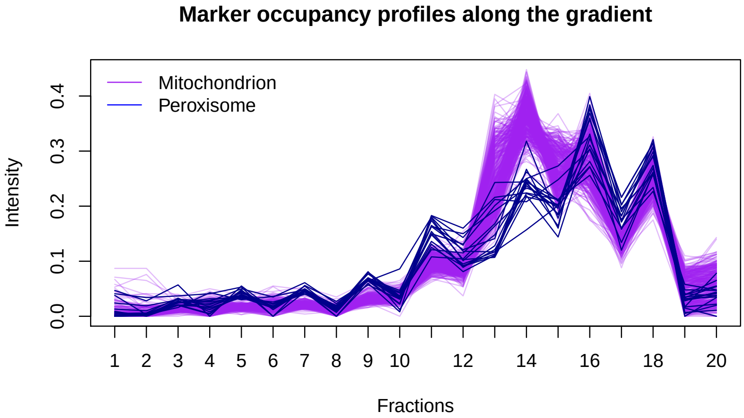


Figure 7. Mitochondrion and peroxisome protein profiles.

experiments, in particular when these resolve different subcellular niches.
Direct comparisons of individual channels in replicated experiments does not provide an adequate, goal-driven assessment of different experiments. Indeed, due to the nature of the experiment and gradient fraction collection, the quantitative channels do not correspond to identical selected fractions along the gradient. As can be seen in the table below (taken from the hl's pData, focusing on channels 7 to 10), different sets of gradient fractions are pooled to obtain enough material and optimise acurate quantitation.

	Replicate	Tag	Gradient.Fraction	Iodixonal.Density
X127N	1	127N	1 to 6 (pooled)	6.00
X127C	1	127C	8 to 9 (pooled)	11.00
X128N	1	128N	10 to 11 (pooled)	13.30
X127C.1	2	127C	7 to 9 (pooled)	10.00
X128N.1	2	128N	10 to 11 (pooled)	12.50

Table 1. Differences in gradient fraction pooling.

The more relevant comparison unit is not a single channel, but rather the complete protein occupancy profiles, which are best visualised as experiment-wide on a PCA plot. As such, we prefer to focus on the direct, qualitative comparison of individual replicate PCA plots, assuring that each displays acceptable sub-cellular resolution. Note that in the code chunk below, we mirror the x-axis to represent the two figures with the same orientation.

```
par(mfrow = c(1, 2))
plot2D(hl[, hl$Replicate == 1], main = "Replicate 1")
plot2D(hl[, hl$Replicate == 2], main = "Replicate 2", mirrorX = TRUE)
```

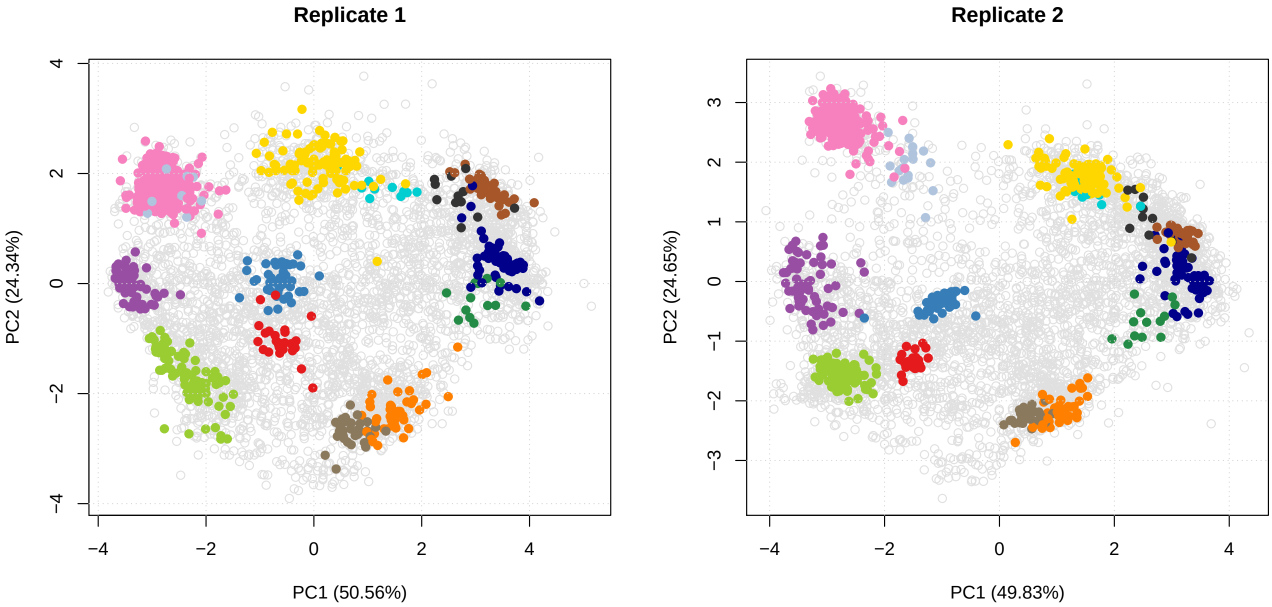
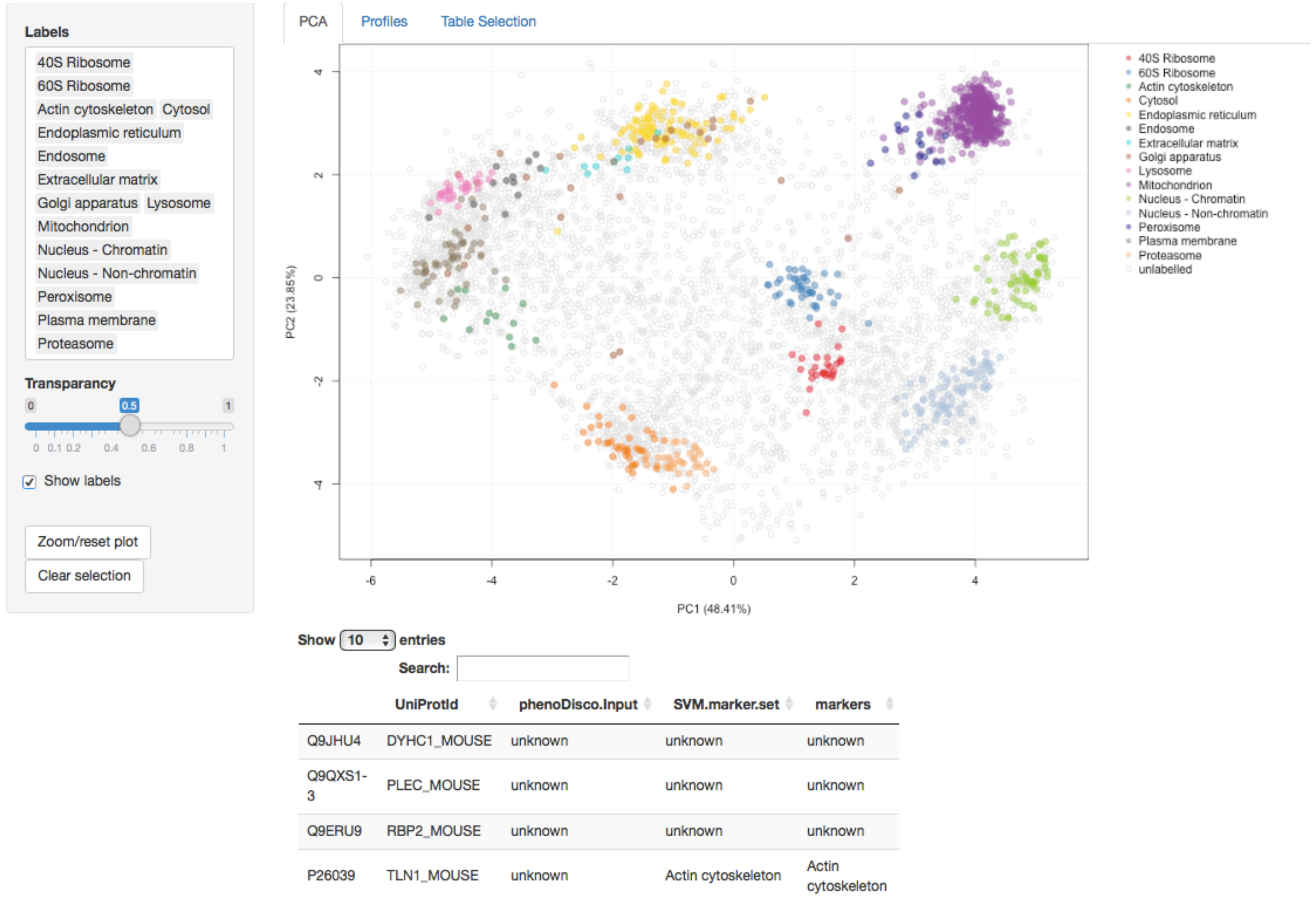


Figure 8. PCA plots of replicates 1 and 2

Interactive visualisation

Visualisation and data exploration is an important aspect of data analyses allowing one to shed light on data structure and patterns of interest. Using the *pRolocGUI* package we can interactively visualise, explore and interrogate quantitative spatial proteomics data. The *pRolocGUI* package is currently under active development and it relies on the `shiny` framework for reactivity and interactivity. The package currently distributes 3 different GUI's (*main* (default), *compare* or *compare*) which are wrapped and launched by the `pRolocVis` function. In the below code chunk we lauch the main app (note, we do not need to specify the argument, `app = "main"` as it is the default).

```
library("pRolocGUI")
pRolocVis(hl)
```



	UniProtId	phenoDisco.Input	SVM.marker.set	markers
Q9JHU4	DYHC1_MOUSE	unknown	unknown	unknown
Q9QXS1-3	PLEC_MOUSE	unknown	unknown	unknown
Q9ERU9	RBP2_MOUSE	unknown	unknown	unknown
P26039	TLN1_MOUSE	unknown	Actin cytoskeleton	Actin cytoskeleton

Figure 9. A screen shot of clickable interface and zoomable PCA plot of the main app in the *pRolocGUI* package.

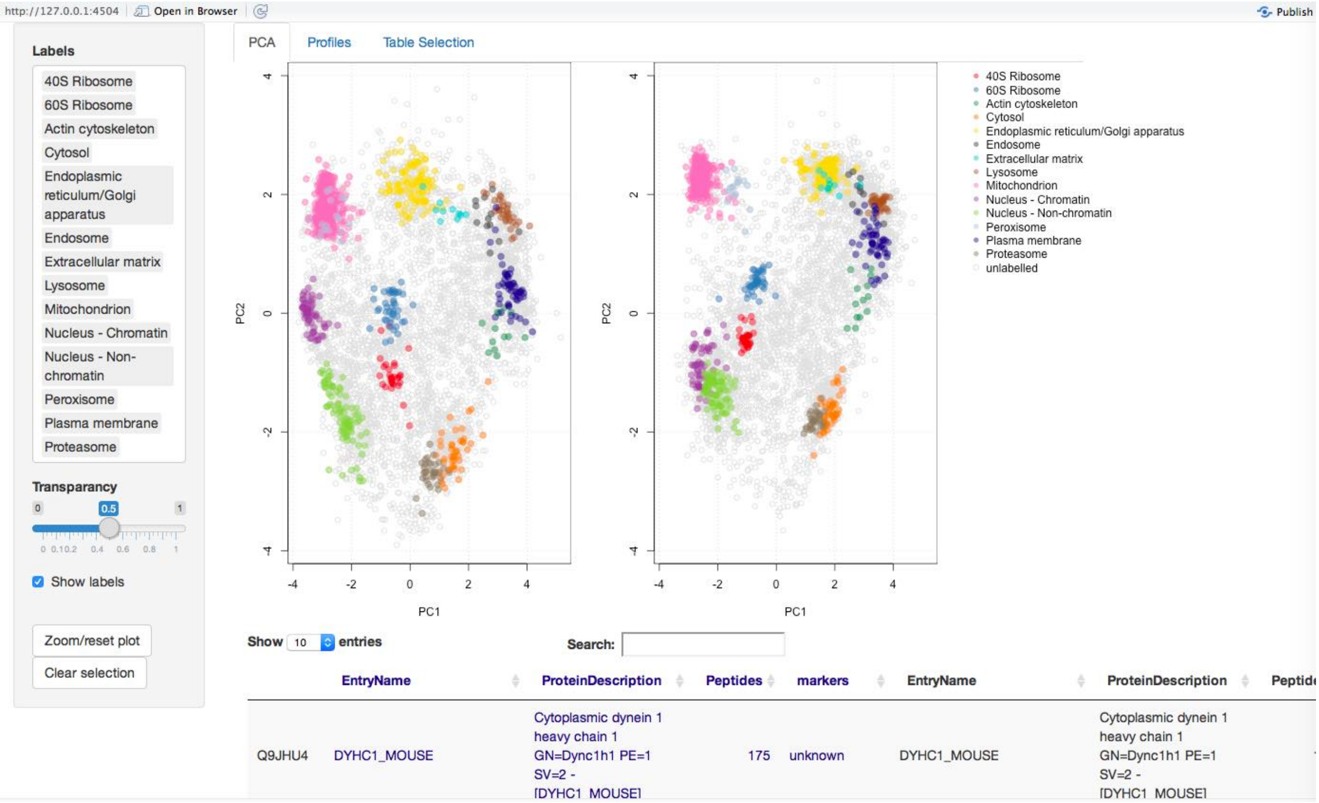
As diplayed in the screenshot above, the *main* application is designed for exploratory data analysis and is divied into 3 tabs: (1) PCA, (2) Profiles and (3) Table selection. The default view upon loading is the PCA tab, which features a clickable interface and zoomable PCA plot with an interactive data table for displaying the quantitation information. Particular proteins of interest can be highlighted using the text search box. There is also an alternate profiles tab for visualisation of the protein profiles, which can be used to examine the patterns of proteins of interest. The Table selection tab provides an interface to control data table column selection. A short animation[1] illustrating the interface is available in the manuscript repository [12].

The *compare* application is useful for examining two replicate experiments, or two experiments from different conditions, treatments etc. The compare application is called by default if the input object to `pRolocVis` is a `MSnSetList` of 2 `MSnSets`, but it can also be specified by calling the argument `app = "compare"`. For example, in the code chunk below we first create a `MSnSetList` of replicates 1 and 2 of the hyperLOPIT data, this is then passed to `pRolocVis`.

```
data(hyperLOPIT2015ms3r1)
data(hyperLOPIT2015ms3r2)
```

[1] `https://github.com/lmsimp/bioc-pRoloc-hyperLOPIT-workflow/blob/master/Figures/pRolocVis_pca.gif`

```
hllst <- MSnSetList(list(hyperLOPIT2015ms3r1, hyperLOPIT2015ms3r2))
pRolocVis(hllst, app = "compare")
```



	EntryName	ProteinDescription	Peptides	markers	EntryName	ProteinDescription	Peptid
Q9JHU4	DYHC1_MOUSE	Cytoplasmic dynein 1 heavy chain 1 GN=Dync1h1 PE=1 SV=2 - [DYHC1_MOUSE]	175	unknown	DYHC1_MOUSE	Cytoplasmic dynein 1 heavy chain 1 GN=Dync1h1 PE=1 SV=2 - [DYHC1_MOUSE]	

Figure 10. The compare application, main panel

The comparison app loads the two PCA plots side-by-side. Only common proteins between the two data sets are displayed. As per the main application, proteins can be searched, identified and highlighted on both PCA plots and in the dedicated profiles tab. One key feature of the compare application is the ability to re-map the second dataset onto the PCA data space of the first (reference) data set (see `?pRolocVis` and the argument `remap = TRUE`). Using the first dataset as the reference set, PCA is carried out on the first dataset and the standard deviations of the principal components (i.e. the square roots of the eigenvalues of the covariance/correlation matrix) and the matrix of variable loadings (i.e. a matrix whose columns contain the eigenvectors) are stored and then used to calculate the principal components of the second dataset. Both datasets are scaled and centered in the usual way. The first dataset appears on the left, and the second re-mapped data appears on the right. The order of the first (the reference data for remapping) and second dataset can be changed through regeneration/re-ordering of the `MSnSetList` object.
The final application *classify*, has been designed to view machine learning classification results according to user-specified thresholds for the assignment of proteins to its sub-cellular location, as discussed later in the subsection *thresholding* in the *supervised machine learning section*.

Novelty Detection

The extraction of sub-cellular protein clusters can be difficult owing to the limited number of marker proteins that exist in databases and elsewhere. Furthermore, given the vast complexity of the cell, automatic annotation retrieval does not always give a full representation of the true sub-cellular diversity in the data. For downstream analyses, such as supervised machine learning, it is desirable to obtain reliable markers that cover as many sub-cellular niches as possible, as these markers are directly used in the training phase of the ML classification. We find that a lack of sub-cellular diversity in the labelled training data leads to prediction errors, as unlabelled instances can only be assigned to a class that exists in the training data [13]. In such scenarios, novelty detection can be useful to identify data-specific sub-cellular groupings such as organelles and protein complexes. The phenotype discovery (phenoDisco) algorithm [13] is one such method and is available in *pRoloc*. It is an iterative semi-supervised learning method that combines the classification of proteins on existing labelled data with the detection of new clusters.
In addition to extracting new phenotypes, novelty detection methods are also useful for confirming the presence of known or postulated clusters in an unbiased fashion. For example, in [1] the `phenoDisco` algorithm was used to con-

firm the data-specific presence of the nucleus and nucleus sub-compartments. In the code chunk below, we demonstrate how to do this analysis, highlighting some of the optional arguments and parameters available for phenotype extraction and give some advice on how to interpret the output.

As the `phenoDisco` algorithm is semi-supervised it uses both labelled (markers) and unlabelled data to explore the data structure and find new sub-cellular data clusters. Thus the first step is to define some input labelled data i.e. markers, that the algorithm will use as input for the supervised learning aspect of the algorithm. As described in [1] we define a set of markers to use as input for the analyses that cover well-known residents from three distinct organelle structures; the mitochondria, plasma membrane and ER, and from three well-known and abundant protein complexes; the proteasome and two ribosomal subunits, 40S and 60S. These input markers are stored in the `featureData` column of `hl` where `fcol = "phenoDisco.Input"`. We can use the convenience accessor function `getMarkers` to print out a table of the markers contained in this marker set. These initial markers were manually curated using information from the UniProt database, the Gene Ontology and the literature.

```r
getMarkers(hl, fcol = "phenoDisco.Input")

## organelleMarkers
##                          40S Ribosome
##                                    26
##                          60S Ribosome
##                                    43
## Endoplasmic reticulum/Golgi apparatus
##                                    76
##                         Mitochondrion
##                                   261
##                       Plasma membrane
##                                    50
##                            Proteasome
##                                    34
##                               unknown
##                                  4542
```

In the code chunk below we show how to run the `phenoDisco` function and return a novelty detection result, according to the specified parameters. The algorithm parameters `times`, `GS` and `p` are passed to the function, along with the `fcol` to tell the algorithm where the input training data is contained.

```r
## As per Christoforou et al (2016),
hl <- phenoDisco(hl, fcol = "phenoDisco.Input", times = 200, GS = 60)
```

Note: We do not evaluate this code chunk in this document as the algorithm is computational intensive and best parallelised over multiple workers. This phenoDisco analysis took 24 hours to complete when parallelised over 40 workers.

The argument `times` indicated the number of times we run unsupervied Gaussian Mixture Modelling before defining a new phenotype cluster. The recommended minimum and default value is 100. In the above code chunk we increase the value to `times = 200` as we have found for larger datasets (e.g. 5000+ proteins) a higher times is requried for convergence. `GS` defines the minimum number of proteins allowed per new data cluster and thus heavily influences what type of new clusters are extracted. For example, if a user is interesed in the detection of small complexes they may wish to use a small `GS = 10`, or `= 20` etc. If they wish to detect larger, more abundant sub-cellular niches a much higher `GS` would be preferable. Specifying a small `GS` can be more time consuming than using a larger `GS`, and there is a trade off between finding interesting small complexes and those that may not be of interest as we find there is a tendancy to find more noise when using a small `GS` compared to using a higher one.

One may also consider increasing the search space for new data clusters by increasing the value of the parameter `G`. This defines the number of GMM components to test and fit; the default is `G = 1:9` (the default value in the *mclust* package [14]). One should note that the decreasing the `GS`, and increasing the values of the arguments `times`, `G` (among other function arguments, see `?phenoDisco`) will heavily influence (increase) the total time taken to run the algorithm. `phenoDisco` supports parallelisation and we strongly suggest you make use of a parallel processing to run these analyses.

The ouput of running the `phenoDisco` algorithm is an `MSnSet` containing the new data clusters, appended to the `featureData` under the name `pd`. The results can be displayed by using the `getMarkers` function. We see that 5 new phenotype data clusters were found.

```
hl

## MSnSet (storageMode: lockedEnvironment)
## assayData: 5032 features, 20 samples
##   element names: exprs
## protocolData: none
## phenoData
##   sampleNames: X126 X127N ... X131.1 (20 total)
##   varLabels: Replicate Tag Gradient.Fraction Iodixonal.Density
##   varMetadata: labelDescription
## featureData
##   featureNames: Q9JHU4 Q9QXS1-3 ... Q9Z2R6 (5032 total)
##   fvarLabels: uniprot.accession uniprot.id ... pd (27 total)
##   fvarMetadata: labelDescription
## experimentData: use 'experimentData(object)'
## Annotation:
## - - - Processing information - - -
## Added markers from  'mrk' marker vector. Tue Nov  8 14:13:49 2016
## Added markers from  'pdres' marker vector. Tue Nov  8 14:13:50 2016
##  MSnbase version: 2.0.0

getMarkers(hl, fcol = "pd")

## organelleMarkers
##                          40S Ribosome
##                                   106
##                          60S Ribosome
##                                    95
## Endoplasmic reticulum/Golgi apparatus
##                                   393
##                          Mitochondrion
##                                   525
##                           Phenotype 1
##                                   300
##                           Phenotype 2
##                                   253
##                           Phenotype 3
##                                   203
##                           Phenotype 4
##                                    74
##                           Phenotype 5
##                                    91
##                       Plasma membrane
##                                   421
##                            Proteasome
##                                    92
##                               unknown
##                                  2479
```

We can plot the results using the `plot2D` function (Figure 11). The five new phenotype data clusters can be extracted and examined. In the code chunk below we write the results to a .csv file. We use the argument `fDataCols` to specify which columns of the `featureData` to write.

```
fData(hl)$pd <- as.character(fData(hl)$pd)
write.exprs(hl, fDataCols = "pd", file = "pd-results.csv", sep = ",")
```

We can also examine the each phenotype intercatively and visualise their protein profiles by using the `pRolocVis` function in the *pRolocGUI* package. We found that phenotype 1 was enriched in nucleus associated proteins, phenotype 2 in chromatin associated proteins, phenotype 3 in cytosolic and phenotypes 4 and 5 in lysosomal and endosomal proteins.

```r
## Re-order the colours for the phenoDisco output
cl <- getMarkerClasses(hl, "pd")
cols <- getStockcol()[seq(cl)]
ind <- grep("Pheno", cl, invert = TRUE)
cols[ind] <- getStockcol()[seq(cl)][1:length(ind)]
cols[-ind] <- getStockcol()[seq(cl)][(length(ind) + 1):length(cl)]

## Plot the input and output
par(mfrow = c(1, 2))
plot2D(hl, fcol = "phenoDisco.Input",
       main = "phenoDisco input markers", col = getStockcol()[1:6])
addLegend(hl, fcol = "phenoDisco.Input", cex = .7)
plot2D(hl, fcol = "pd", main = "phenoDisco output", col = cols)
addLegend(hl, fcol = "pd", cex = .7, col = cols)
```

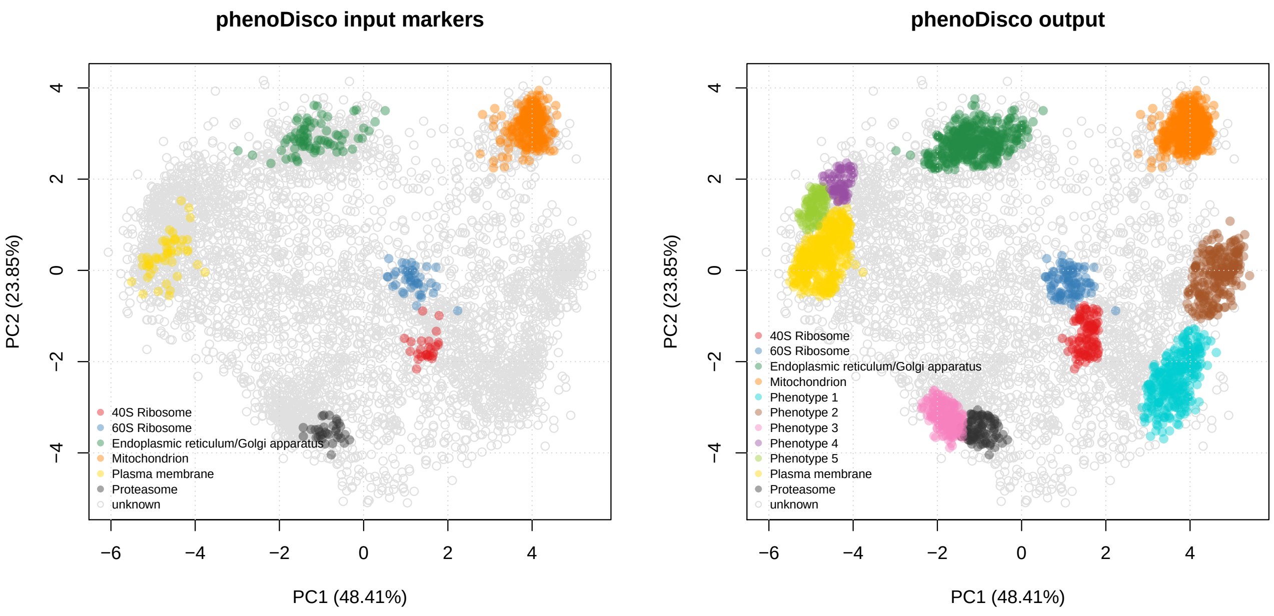


Figure 11. Results of the novelty detection algorithm.

Supervised machine learning

Supervised machine learning, also known as classification, is an essential tool for the assignment of proteins to distinct sub-cellular niches. Using a set of labelled training examples i.e. markers, we can train a machine learning classifier to learn a mapping between the data i.e. the quantitative protein profiles, and a known localisation. The trained classifier can then be used to predict the localisation of a protein of unknown localisation, based on its observed protein profile. To date, this method has been extensively used in spatial quantitative proteomics to assign thousands of proteins to distinct sub-cellular niches [1, 15, 6, 16, 17, 18].

There are several classification algorithms readily available in *pRoloc*, which are documented in the dedicated *pRoloc* machine learning techniques vignette. We find the general tendancy to be that it is not the choice of classifier, but the improper optimisation of the algorithmic parameters, that limits classification accuracy. Before employing any classification algorithm and generating a model on the training data, one must first find the optimal parameters for the algorithm of choice.

Optimisation

In the code chunk below we use a Support Vector Machine (SVM) to learn a classifier on the labelled training data. As previously mentioned, one first needs to train the classifiers parameters before an algorithm can be used to predict the class labels of the proteins with unknown location. One of the most common ways to optimise the parameters of a classifier is to partition the labelled data in to training and testing subsets. In this framework parameters are tested via a grid search using cross-validation on the training partition. The best parameters chosen from the cross-validation stage are then used to build a classifier to predict the class labels of the protein profiles on the test partition. Observed and expected classication results can be compared, and then used to assess how well a given model works by getting an estimate of the classiers ability to achieve a good generalisation i.e. that is given an unknown example predict its class label with high accuracy. In *pRoloc*, algorithmic performance is estimated using stratified 80/20 partitioning for the training/testing subsets respectively, in conjuction with five-fold cross-validation in order to optimise the free parameters via a grid search. This procedure is usually repeated 100 times and then the best parameter(s) are selected upon investigation of classifier accuracy. We recommend a minimum of 13 markers per sub-cellular class for stratified 80/20 partitioning and 5-fold cross-validation; this allows a minimum of 10 examples for parameter optimisation on the training partition i.e. 2 per fold for 5-fold cross-validation, and then 3 for testing the best parameters on the validation set.

Classifier accuracy is estimated using the macro F1 score, i.e. the harmonic mean of precision and recall. In the code chunk below we demonstrate how to optimise the free parameters, `sigma` and `cost`, of a classical SVM classifier with a Gaussian kernel using the function `svmOptimisation`. As the number of labelled instances per class varies from organelle to organelle, we can account for class imbalance by setting specific class weights when generating the SVM model. Below the weights, `w` are set to be inversely proportional to the class frequencies.

```
w <- table(getMarkers(hl, verbose = TRUE))

## organelleMarkers
##           40S Ribosome            60S Ribosome      Actin cytoskeleton
##                     27                      43                      13
##                Cytosol   Endoplasmic reticulum                Endosome
##                     43                      95                      12
##   Extracellular matrix                Lysosome           Mitochondrion
##                     10                      33                     383
##    Nucleus - Chromatin Nucleus - Non-chromatin              Peroxisome
##                     64                      85                      17
##        Plasma membrane              Proteasome                 unknown
##                     51                      34                    4122

w <- 1/w[names(w) != "unknown"]
```

```
## 100 rounds of optimisation with five-fold cross-validation
params <- svmOptimisation(hl, fcol = "markers",
                          times = 100, xval = 5,
                          class.weights = w)
```

As mentioned previously, we rely on the default feature variable `"markers"` to define the class labels and hence can ommit it. To use another feature variables, one need to explicitly specify its name using the `fcol` argument (for example `fcol = "markers2"`).

The output `params` is an object of class `GenRegRes`; a dedicated container for the storage of the design and results from a machine learning optimisation. To assess classifier performance we can examine the macro F1 scores and the most frequently chosen parameters. A high macro F1 score indicates that the marker proteins in the test dataset are consistently and correctly assigned by the the algorithm. Often more than one parameter or set of parameters gives rise to the best generalisation accuracy. As such it is always important to investigate the model parameters and critically assess the best choice. The `f1Count` function counts the number of parameter occurences above a certain F1 value. The best choice may not be as simple as the parameter set that gives rise to the highest macro F1 score. One must be careful to avoid overfitting, and choose parameters that frequently provide high classification accuracy. Below, we see that only a sigma of 0.1 produces macro F1 scores above 0.6, but that a cost of 16 is much more frequently chosen than lower values.

```
f1Count(params, 0.6)

##     4  8 16
## 0.1 1 10 89
```

The parameter optimistion results can also be visualised as a boxplot or heatmap, as shown in figure 12. The `plot` method for `GenRegRes` object shows the respective distributions of the 100 macro F1 scores for the best cost/sigma parameter pairs, and `levelPlot` shows the averaged macro F1 scores, for the full range of parameter values. These figures also indicate that values of 0.1 and 16 for sigma and cost consistently deliver better classification scores.

```
plot(params)
levelPlot(params)
```

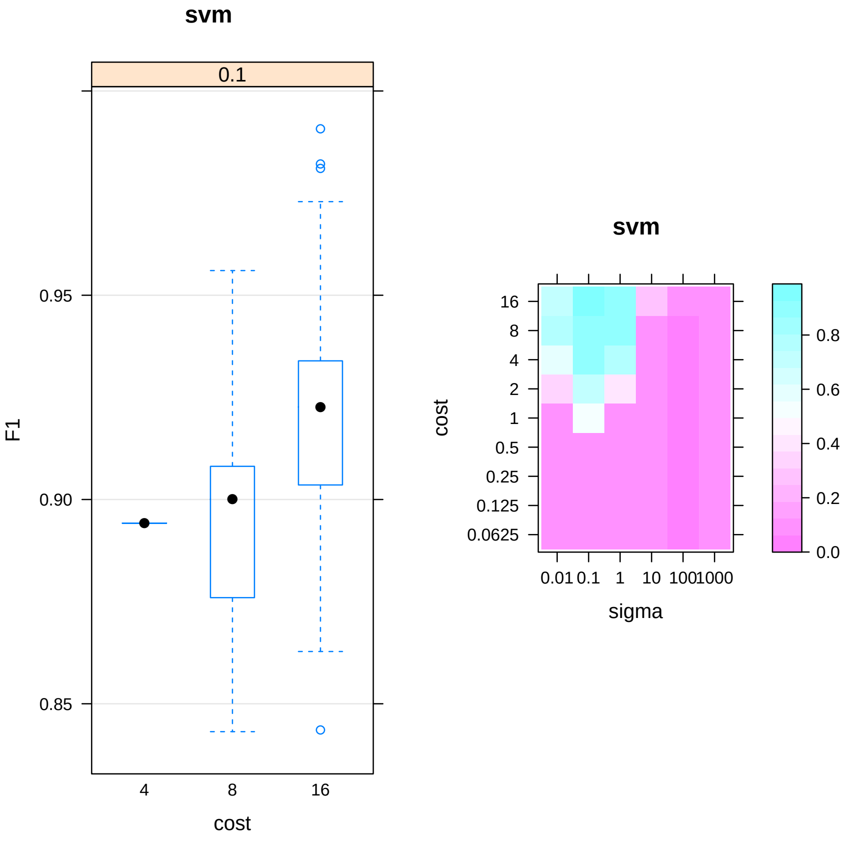


Figure 12. Assessment of the classification model parameter optimisation.

By using the function `getParams` we can extract the best set of parameters. Currently, `getParams` retrieves the first best set is automatically but users are encouraged to critically assess whether this is the most wise choice (which it is, as demonstrated above).

```
(best <- getParams(params))

## sigma  cost
##   0.1  16.0
```

Once we have selected the best parameters we can then use them to build a classifier from the labelled marker proteins.

Classification

We can use the function `svmClassification` to return a classification result for all unlabelled instances in the dataset corresponding to their most likely sub-cellular location. The algorithm parameters are passed to the function, along with the class weights. As above, `fcol` can be ignored as we use the labels defined in the default `"markers"` feature variable.

```
hl <- svmClassification(hl, params, class.weights = w, fcol = "markers")
```

Automatically, the output of the above classification, the organelle predictions and assignment scores, are stored in the `featureData` slot of the `MSnSet`. In this case, they are given the labels `svm` and `svm.scores` for the predictions and scores respectively. The resultant predictions can be visualised using `plot2D`. In the code chunk below `plot2D` is called to generate a PCA plot of the data and `fcol` is used to specify where the new assignments are located e.g. `fcol = "svm"`.

```r
## set point size of each protein to be inversely proportional to the
ptsze <- exp(fData(hl)$svm.scores) - 1
## plot new predictions
plot2D(hl, fcol = "svm", cex = ptsze)
addLegend(hl, fcol = "svm", where = "bottomleft", bty = "n", cex = .5)
```

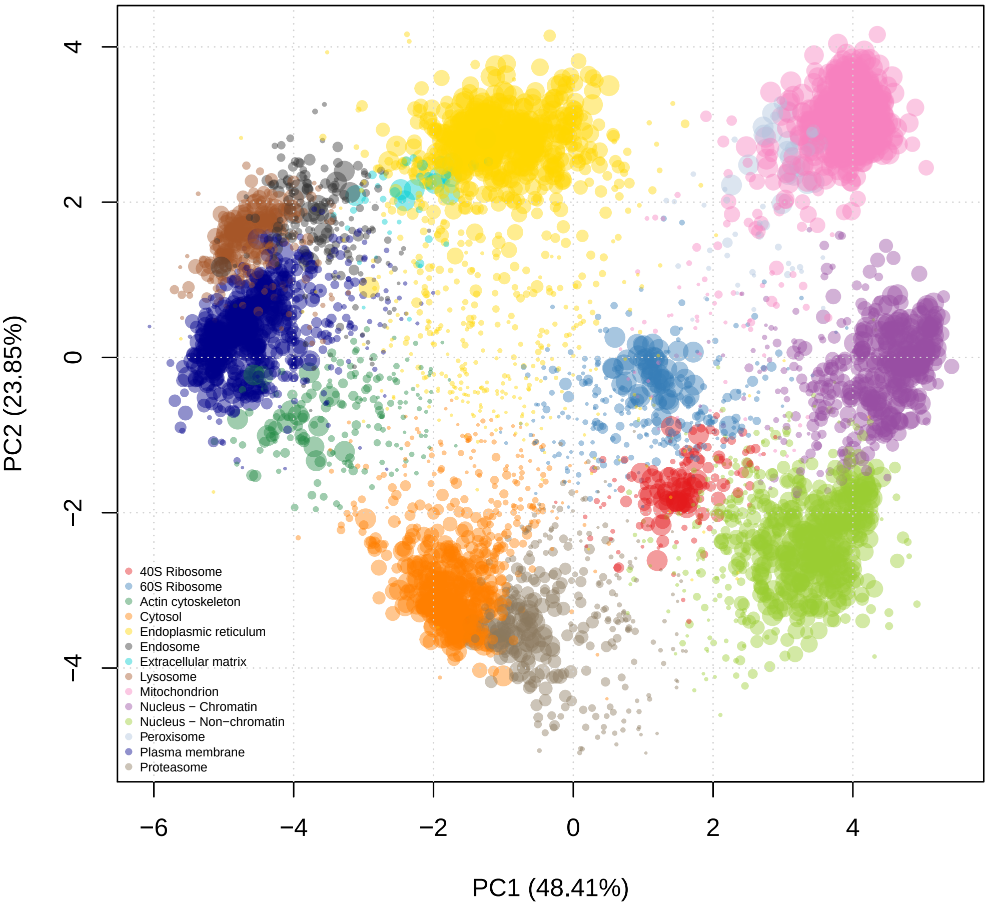


Figure 13. Classification results. Colours indicate class membership and point size are representative or the classification confidence.

Additionally, when calling `plot2D` we can use the `cex` argument to change the size of each point on the plot to be inversely proportional to the SVM score. This gives an initial overview of the high scoring localisations from the SVM predictions.

The adjustment of the point size intuitively confers important information that is more difficult to defined formally, but that we will address in the next section. The classifier (SVM in our case, but this is also valid of other classifiers) defines boundaries based on the labelled marker proteins. These class/organelle boundaries define how non-assigned proteins are classified and with what confidence.

Thresholding

It is common when applying a supervised classification algorithm to set a specific score cutoff on which to define new assignments, below which classifications are kept unknown/unassigned. This is important as in a supervised learning setup, proteins can only be predicted to be localised to one of the sub-cellular niches that appear in the labelled training data. We can not guarantee (and do not expect) that the whole sub-cellular diversity to be represented in the labelled training data as (1) finding markers that represent the whole diversity of the cell is challenging (especially obtaining dual- and multiply-localised protein markers) and (2) many sub-cellular niches contain too few proteins to train on (see above for a motivation of a minimum of 13 markers).

Deciding on a threshold is not trivial as classifier scores are heavily dependent upon the classifier used and different sub-cellular niches can exhibit different score distributions, as highlighted in the boxplot below. We recommend users to set class-specific thresholds. In the code chunk below we display a boxplot of the score distributions per organelle (Figure 14).

```
## First remove the markers
preds <- unknownMSnSet(hl)
## Plot a boxplot of the scores of each organelle
par(oma = c(10.5, 0, 0, 0))
boxplot(svm.scores ~ svm, data = fData(preds),
        ylab = "SVM scores", las = 2)
```

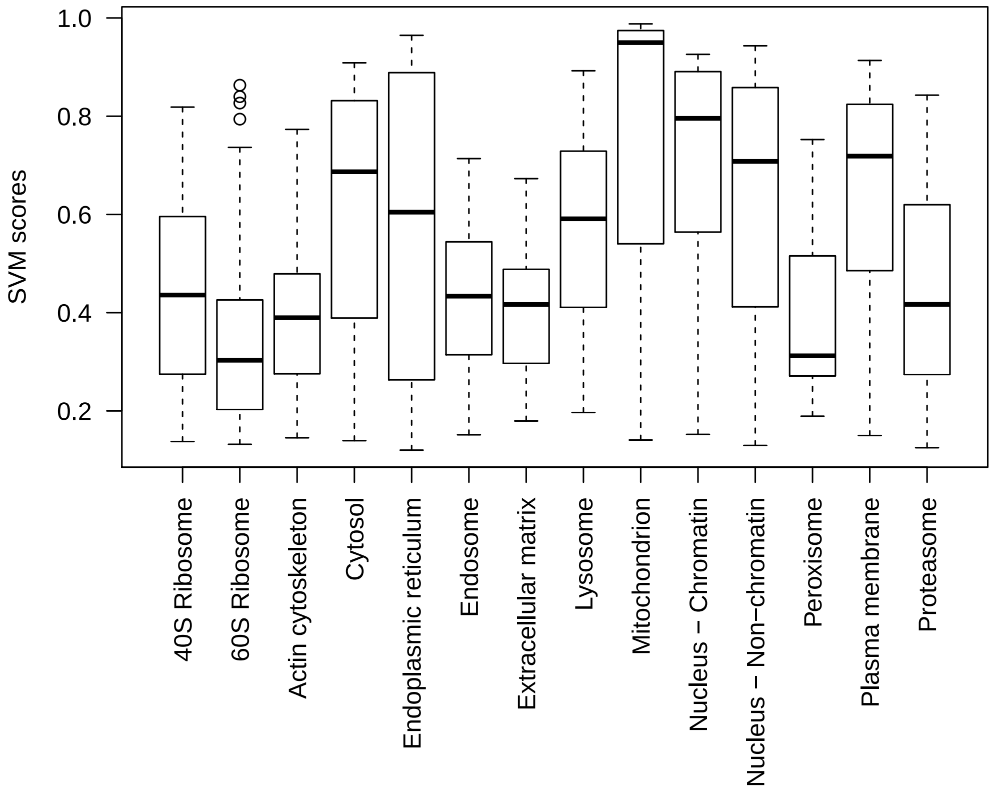


Figure 14. Visualistion of class-specific classification score distribution.

There are many ways to set thresholds and the choice of method will depend on the biological question and experimental design at hand. One viable approach in the frame of the above experimetal design would be to manually set a FDR, say 5%, per organelle. To do this the user would examine the top scoring predictions for each organelle, and then set a threshold at the score at which they achieve 5% of false assignments per organelle. The definintion of a false assignment would depend on the information available, for example, validity or lack of validity for the localisation from another experiment as reported in the literature or a reliable database. If such information is not available, one crude method is to set a threshold per organelle by extracting the median or 3rd quantile score per organelle. For example, in the code chunk below, we use the `orgQuants` function to extract the median organelle scores and then pass these scores to the `getPrediction` function to extract the new localisations that meet this scoring criteria. Any sub-cellular predictions that fall below the specified thresholds are labelled as unknown.

```
ts <- orgQuants(hl, fcol = "svm", scol = "svm.scores", mcol = "markers", t = .5)

##            40S Ribosome            60S Ribosome      Actin cytoskeleton
##               0.4359409               0.3031417               0.3896552
##                 Cytosol   Endoplasmic reticulum                Endosome
##               0.6869014               0.6046754               0.4336399
##     Extracellular matrix                Lysosome           Mitochondrion
##               0.4165647               0.5910830               0.9498244
##      Nucleus - Chromatin Nucleus - Non-chromatin              Peroxisome
##               0.7955788               0.7079722               0.3121358
##         Plasma membrane              Proteasome
##               0.7187306               0.4168615

hl <- getPredictions(hl, fcol = "svm", scol = "svm.scores", mcol = "markers", t = ts)

## ans
##            40S Ribosome            60S Ribosome      Actin cytoskeleton
##                      86                     171                      89
##                 Cytosol   Endoplasmic reticulum                Endosome
##                     294                     477                      99
##     Extracellular matrix                Lysosome           Mitochondrion
##                      27                     123                     522
##      Nucleus - Chromatin Nucleus - Non-chromatin              Peroxisome
##                     230                     342                      38
##         Plasma membrane              Proteasome                 unknown
##                     316                     160                    2058
```

The output of `getPredictons` is the original `MSnSet` dataset with a new feature variable appended to the feature data called `fcol.pred` (i.e. in our case `svm.pred`) containing the prediction results. The results can also be visualied using `plot2D` function (Figure 15) and extracted by retrieving that specific column from the feature metadata using, for example, `fData(hl)$svm.pred`.
There is also a dedicated interactive application to help users examine these distributions in the *pRolocGUI* package (Figure 16). This app can be launched via the `pRolocVis` function and specifying the argument `app = "classify"` along with the relevent `fcol`, `scol` and `mcol` which refer to the columns in the feature data that contain the new assignments, assignment scores and markers respectively (see also `fvarLabels(svmres)`).

```
library("pRolocGUI")
pRolocVis(hl, app = "classify", fcol = "svm", scol = "svm.scores", mcol = "markers")
```

The data is loaded and displayed on a PCA plot and a boxplot is used to display the classifier scores by data class. On the left, there is a sidebar panel with sliders to control the thresholds upon which classifications are made. There are two types of cut-off that the user can choose from: (1) *Quantile* and (2) *User-defined*. By default, when the application is launched quatile scoring is selected and set to 0.5, the median. The class-specific score thresholds that correspond to selecting the desired quantile are shown on as red dots on the boxplot. The assignments on the PCA plot are also updated according to the selected threshold. The quantile threshold can be set by moving the corresponding quantile slider. If one wished to set their own cut-offs the *User-defined* radio button must be selected and then the sliders for defining user-specified scores become active and the scores and highlighted on the boxplot by blue dots. For more information we refer users to the *pRolocGUI* tutorial vignette.

```
plot2D(hl, fcol = "svm.pred")
```

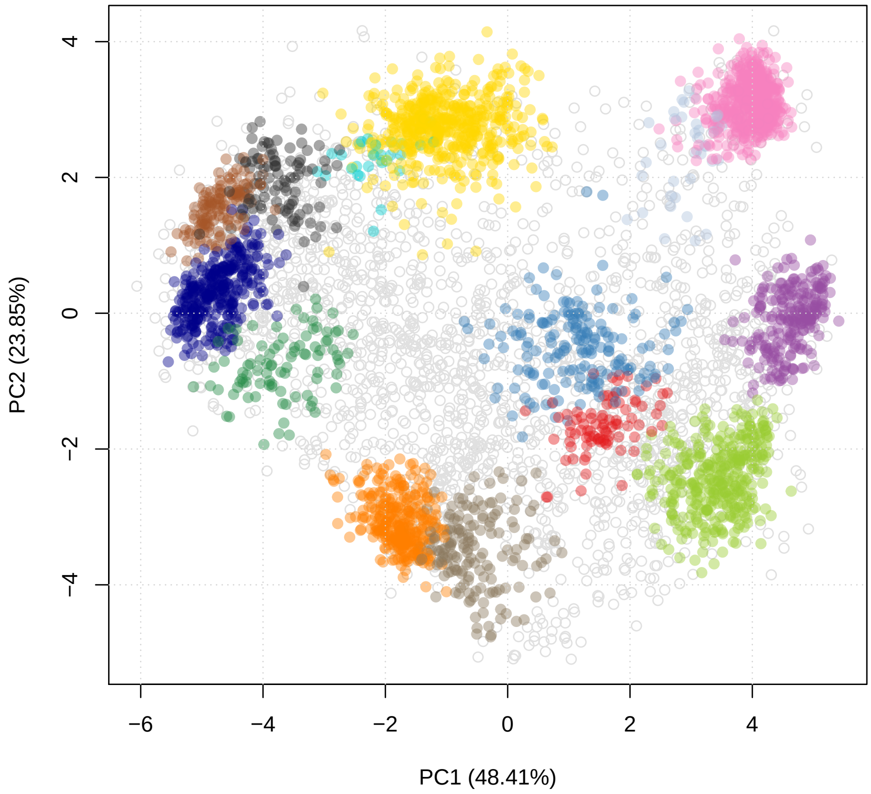
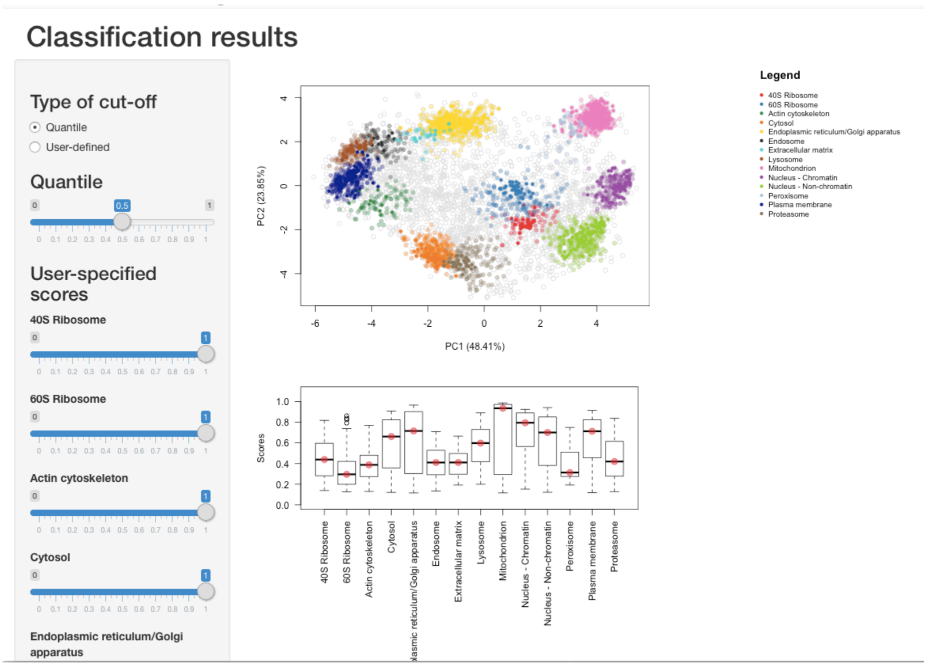


Figure 15. Results of the localisation preductions after thresholding.



Classification results

Type of cut-off

● Quantile

○ User-defined

Quantile

0 — 0.5 — 1

User-specified scores

40S Ribosome: 0 — 1

60S Ribosome: 0 — 1

Actin cytoskeleton: 0 — 1

Cytosol: 0 — 1

Endoplasmic reticulum/Golgi apparatus

Legend

- ● 40S Ribosome
- ● 60S Ribosome
- ● Actin cytoskeleton
- ● Cytosol
- ● Endoplasmic reticulum/Golgi apparatus
- ● Endosome
- ● Extracellular matrix
- ● Lysosome
- ● Mitochondrion
- ● Nucleus - Chromatin
- ● Nucleus - Non-chromatin
- ● Peroxisome
- ● Plasma membrane
- ● Proteasome

Figure 16. The classify application enable the interactive exploration of classification score thresholding.

Transfer learning

In addition to high quality MS-based quantitative proteomics data, there exist a number of other sources of information that are freely available in the public domain that may be useful to assign a protein to its sub-cellular niche. For example, imaging from immunofluorescence microscopy, protein annotations and sequences, and protein-protein interactions among others, represent a rich and vast source of complementary information. We can integrate this auxiliary information with our primary MS-based quantitative data using a paradigm known as transfer learning (TL). The integration of data between different technologies is one of the biggest challenges in computational biology to date and the *pRoloc* package provides functionality to do such analyses. We recently developed two transfer learning algorithms using a k-NN and SVM framework and applied them to the task of protein localisation prediction [19]. In this section we will begin with explaining the concept of transfer learning and then show how to apply this in the frame of spatial proteomics and protein localisation prediction.

In TL one typically has a primary task that one wishes to solve, and some complementary (often heterogeneous) auxiliary information that is related to the primary learning objective, that can be used to help solve the primary goal. For example, here our primary task is to assign proteins to their sub-cellular niche with high generalisation accuracy from data collected from quantitative MS-based experiments. In the example below we extract Gene Ontology (GO) information to use as an auxiliary data source to help solve our task of protein localisation prediction.

Using the functions `setAnnotationParams` and `makeGoSet` we can contruct an auxiliary `MSnSet` of GO terms, from the primary data's features i.e. the protein accession numbers. All the GO terms associated to each accession number are retrieved and used to create a binary matrix where a 1 (0) at position (i,j) indicates that term j has (not) been used to annotate protein i. The GO terms are retrieved from an appropriate repository using the *biomaRt* package. The specific Biomart repository and query will depend on the species under study and the type of identifiers. The first step is to construct the annotation parameters that will enable to perform the query, which is done using `setAnnotationParams`. Typing into the R console `par <- setAnnotationParams()` will present two menus, firstly asking you to identify the species of study, and then what type of identifier you have used to annotate the proteins in your `MSnSet`. It is also possible to pass patterns to match the species e.g. in the code chunk below we pass `"Mus musculus"`, and the identifier type for our data (see `featureNames(hl)`) which is `"Uniprot/Swissprot"`, for the Biomart query.

```
par <- setAnnotationParams(inputs = c("Mus musculus", "UniProt/Swissprot"))
```

Now we have contructed the query parameters we can use the `makeGoSet` function to retrieve and build an auxiliary GO `MSnSet` as described above. By default, the cellular component terms are downloaded, without any filtering on evidence codes. It is also possible to download terms from the molecular function and biological process GO namespaces, and also apply filtering based on evidence codes as desired, see `?makeGoSet` for more details.

```
gocc <- makeGoSet(hl, params = par, namespace = "cellular_component")
```

The function `makeGoSet` uses the *biomaRt* package to query the relevent database (e.g. Ensembl, Uniprot) for GO terms. All GO terms that have been observed for the 5032 proteins in the hyperLOPIT dataset are retieved. Users should note that the number of GO terms retreived is also dependent on the database version queried and thus is always subject to change. We find it is common to see GO terms with only one protein assigned to that term. Such terms do not bring any information for building the classifier and are thus removes using the `filterBinMSnSet` function.

```
gocc <- filterBinMSnSet(hl)
```

Now that we have generated our auxiliary data, we can use the k-NN implementation of transfer learning available in *pRoloc* to integrate this with our primary MS-based quantitative proteomics data using the functions `knntlOptimisation` to estimate the free-parameters for the integration, and `knntlClassification` to do the predictions. We have shown that using transfer learning results in the assignment of proteins to sub-cellular niches with a higher generalisation accuracy than using standard supervised machine learning with a single source of information [19].

TL optimisation

The first step, as with any machine learning algorithm, is to optimise any free paramaters of the classifier. For the k-NN TL classifier there are two sets of parameters that need optimising: the first set are the k's for the primary and auxiliary data sources required for the nearest neighbour calculations for each data source. The second set of parameters (noted by a vector of θ weights) that require optimising are the class weights, one per subcellular niche, that control the proportion of primary and auxiliary data to use for learning. A weight can take any real value number between 0 and 1. A weight of $\theta = 1$ indicates that all weight is given to the primary data (and this implicitly implies that a weight of

$1-\theta = 0$ is given to the auxiliary data), and similarly a weight of $\theta = 0$ implies that all weight is given to the auxiliary data (so 0 is given to the primary source). If we conduct a parameter seach and test weights $\theta = 0, 1/3, 2/3, 1$ for each class, and if we have, for example 10 subcellular niches, this will result in 4^{10} different combinations of parameters to test. The parameter optimisation is therefore time consuming and as such we recommend making use of a computing cluster (code and submissing scripts are also available in the supporting information). The markers in the `hl` dataset contain 14 subcellular classes. If we examine these markers and classes on the PCA plot above we can see that in particular the two ribosomes and two nuclear compartments are highly separated along the first two components, this is also evident from the profiles plot which gives us a good indication that these subcellular niches are well-resolved in the hyperLOPIT dataset. Transfer learning is particularly useful for classes that are not as well separated. We find that subcellular niches that are well-separated under hyperLOPIT and LOPIT obtain a class score of 1 (i.e. use only primary data from transfer learning [19]). Therefore, for the optimisation stage of the analyses we can already infer a subcellular class weight of 1 for these niches and only optimise over the remaining organelles. This can signifciantly cut down optimisation time as by removing these 4 classes from the optimisation (and not the classification) we only have 4^{10} class weight combinations to consider instead of 4^{14} combinations.

In the example below we remove these 4 classes from the marker set, re-run the `knnOptimisation` for each data source and then run the `knntlOptimisation` with the 10 remaining classes. (Note: this is not run live as this the `hl` dataset with 10 classes, 707 markers and 4^{10} combinations of parameters takes around 76 hours to run on the University of Cambridge HPC using 256 workers).

To remove the 4 classes and create a new column of markers in the feature data called `tlmarkers` to use for the analysis:

```r
## create new markers column for tl markers
fData(hl)$tlmarkers <- fData(hl)$markers
fData(gocc)$tlmarkers <- fData(gocc)$markers

## Remove 4 classes
torm  <- c("40S Ribosome", "60S Ribosome",
           "Nucleus - Chromatin",
           "Nucleus - Non-chromatin")
for (i in seq(torm)) {
  hl <- fDataToUnknown(hl, from = torm[i], fcol = "tlmarkers")
  gocc <- fDataToUnknown(gocc, from = torm[i], fcol = "tlmarkers")
}
getMarkerClasses(hl, fcol = "tlmarkers")

##  [1] "Actin cytoskeleton"    "Cytosol"
##  [3] "Endoplasmic reticulum" "Endosome"
##  [5] "Extracellular matrix"  "Lysosome"
##  [7] "Mitochondrion"         "Peroxisome"
##  [9] "Plasma membrane"       "Proteasome"

getMarkerClasses(gocc, fcol = "tlmarkers")

##  [1] "Actin cytoskeleton"    "Cytosol"
##  [3] "Endoplasmic reticulum" "Endosome"
##  [5] "Extracellular matrix"  "Lysosome"
##  [7] "Mitochondrion"         "Peroxisome"
##  [9] "Plasma membrane"       "Proteasome"
```

TL optimisation stage 1 Run `knnOptimisation` to get the best *k*'s for each data source.

```r
## get best k's
kpopt <- knnOptimisation(hl, fcol = "tlmarkers")
kaopt <- knnOptimisation(gocc, fcol = "tlmarkers")
```

From examining the parameter seach plots as described in section *Optimisation*, we find the best *k*'s for both the primary and auxiliary are 3.

TL optimisation stage 2 Run `knntlOptimisation` to get the best transfer learning weights for each sub-cellular class.

```
## Set appropriate parallelisation backend and
## number of workers for the tl
par <- SnowParam(255L, type = "MPI")

## Now peform tl optimisation
tlopt <- knntlOptimisation(hl, gocc, fcol = "tlmarkers",
                           length.out = 4, times = 50,
                           xval = 5, k = c(3, 3),
                           BPPARAM = par)
```

The results of the optimisation can be visalised using the `plot` method for `tlopt` optimisation result:

```
plot(tlopt)
```

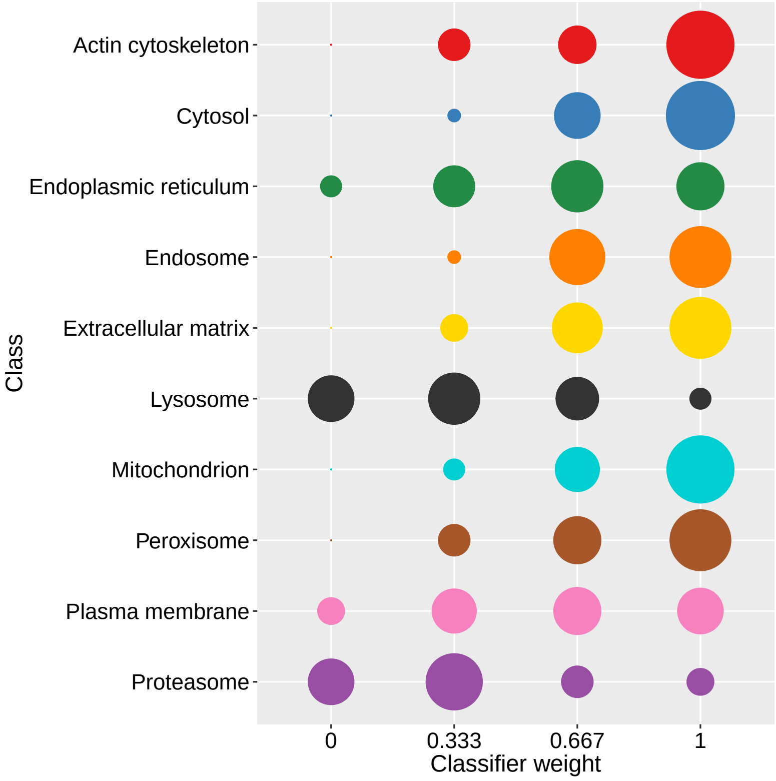


Figure 17. Visualisation of the transfer learning parameter optimisation procedure. Each row displays the frequency of observed weights (along the columns) for a specific sub-cellular class, with large dots representing higher observation frequencies.

TL classification

Looking at the bubble plot displaying the distribution of best weights over the 50 runs we find that for many of the subcellular niches a weight of 1 is most popular (i.e. use only primary hyperLOPIT data in classification), this is unsuprising as we already know the dataset is well resolved for these classes. We see that the most popular weights for the proteasome and lysosome tend to be towards 0, indicating that these niches are well-resolved in the Gene Ontology. This tells us that we would benefit from including auxiliary GO information in our classifier for these subcellular compartments. The plasma membrane weights are relatively equally spread between using hyperLOPIT and GO data. Using the `getParams` function we can return the best weights and then use this as input for the classification.

One of the benefits of the algorithm is the ability to manually select weights for each class. In the optimisation above, for time constraints, we removed the two ribosomal subunits and the two nulcear compartments, and therefore in the code chunk below when we extract the best parameters, these subcellular niches are not included. To include these 4 subcellular niches in the next classification step we must include them in the parameters. We define a weight of 1 for each of these niches, as we know they are well resolved in hyperLOPIT. We then re-order the weights according to `getMarkerClasses` and perform the classification using the function `knntlClassification`.

```
## best parameters for the 10 classes
(bestpar <- getParams(tlopt))

##    Actin cytoskeleton               Cytosol Endoplasmic reticulum
##             1.0000000             1.0000000             0.6666667
##              Endosome  Extracellular matrix              Lysosome
##             1.0000000             1.0000000             0.3333333
##         Mitochondrion            Peroxisome       Plasma membrane
##             1.0000000             1.0000000             0.6666667
##            Proteasome
##             0.3333333

## add weights for classes not included in the optimisation
otherweights <- rep(1, 4)
names(otherweights) <- c("40S Ribosome", "60S Ribosome",
                         "Nucleus - Chromatin",
                         "Nucleus - Non-chromatin")
(bestpar <- c(bestpar, otherweights))

##      Actin cytoskeleton                 Cytosol   Endoplasmic reticulum
##               1.0000000               1.0000000               0.6666667
##                Endosome    Extracellular matrix                Lysosome
##               1.0000000               1.0000000               0.3333333
##           Mitochondrion              Peroxisome         Plasma membrane
##               1.0000000               1.0000000               0.6666667
##              Proteasome            40S Ribosome            60S Ribosome
##               0.3333333               1.0000000               1.0000000
##     Nucleus - Chromatin Nucleus - Non-chromatin
##               1.0000000               1.0000000

## re-order classes
bestpar <- bestpar[getMarkerClasses(hl)]

## Do the classification
hl <- knntlClassification(hl, gocc, bestTheta = bestpar, k = c(3, 3))
```

The results from the classification results and associated scores are appended to the fData slot and named knntl and knntl.scores respectively. Results can be visualised using plot2D, scores assessed and cutoffs calculated using the classify app in pRolocVis, predictions obtained using getPredictions in the same way as demonstrated above for the SVM classifier.

Unsupervised machine learning

In pRoloc there is functionality for unsupervsied machine learning methods. In unsupervised learning, the training data consists of a set of input vectors e.g. protein profiles, ignoring the information about the class label e.g. localisation, other than for annotation purposes. The main goal in unsupervised learning is to uncover groups of similar features within the data, termed clustering. Ordination methods such as principal components analysis (PCA) also fall into the category of unsupervised learning methods, where the data can be projected from a high-dimensional spcae down to two or three dimensions for the purpose of visualisation.

As described and demonstrated already above, PCA is a valuable and powerful method for data visualisation and quality control. Another application uses hierarchical clustering to summarise the relation between marker proteins using he mrkHClust function, where the euclidean distance bewteen average class-specific profiles is used to produce a dendrogramme describing a simple relationship between the sub-cellular classes (Figure 18). The mrkHClust uses the same defaults as all other function, using the markers feature variable to define marker proteins. In the code chunk, we adapt the figure margins to fully display the class names.

We generally find supervised learning more suited to the task of protein localisation prediction in which we use high-quality curated marker proteins to build a classifier, instead of using an entirely unsupervised approach to look for clusters and then look for enrichment of organelles and complexes. In the latter we do not make good use of valuable prior knowledge, and in our experience unsupervised clustering can be extremely difficult due poor estimates of the number of clusters that may appear in the data.

```
par(mar = c(15, 4, 1, 0))
mrkHClust(hl)
```

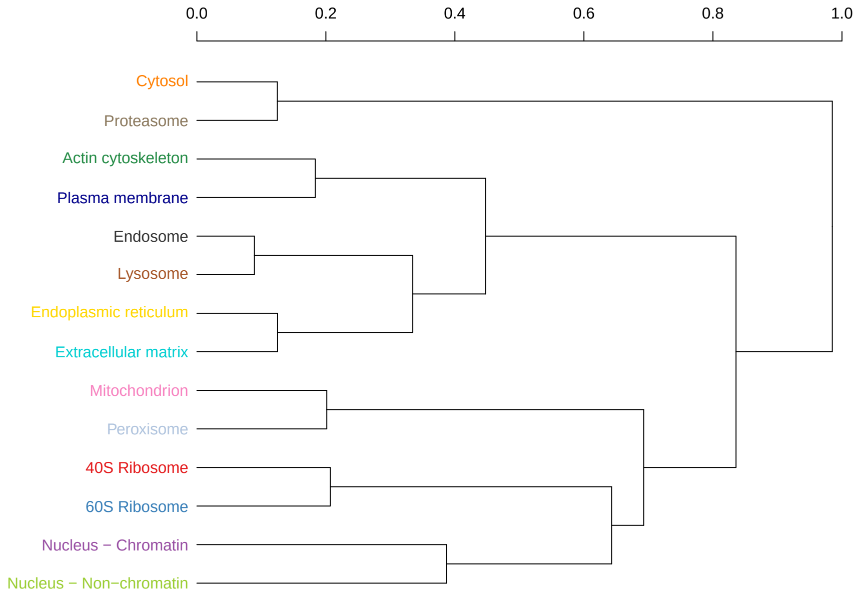


Figure 18. Hierarchical clustering of the average marker profiles summarising the relation between organelles profiles.

Writing and exporting data

An MSnSet can be exported from R using the write.exprs function. This function writes the expression values to a text-based spreadsheet. The fcol argument can be used to specify which featureData columns (as column names, column number or logical) to append to the right of the expression matrix.
In the below code chunk we write the hl object to a csv file. The file argument is used to specify the file path, the sep argument specifies the field separator string, here we use a comma, finally as we want to write all the information in the featureData to the file, as well as the expression data, we specify fvarLabels(hl), that returns all feature variable names, and write the resulting data to the file "hl.csv".

```
write.exprs(hl, file = "hl.csv", sep = ",", fcol = fvarLabels(hl))
```

Exporting to a spreadsheet however looses a lot of important information, such as the processing data, and the sample metadata in the *phenoData* slot. Other objects, such a parameters from the machine learning optimisation, cannot be represented as tabular data. To directly serialise R objects to disk, on can use the standard save function, and later reload the object using save. For example, to save and then re-load the parameters from the SVM optimisation,

```
## To save the parameters as an R object
save(params, file = "svmparams.rda")

## To re-load after saving
load(file = "svmparams.rda")
```

Session information

The function sessionInfo provides a summary of all packages and versions used to generate this document. This enables us to record the exact state of our session that lead to these results. Conversely, if the script stops working or if it returns different results, we are in a position to re-generate the original results using the adequate software versions and retrace changes in the software that lead to failure and/or different results.

```
sessionInfo()

## R version 3.3.2 Patched (2016-11-01 r71616)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Ubuntu 14.04.5 LTS
##
## locale:
##  [1] LC_CTYPE=en_GB.UTF-8       LC_NUMERIC=C
##  [3] LC_TIME=en_GB.UTF-8        LC_COLLATE=en_GB.UTF-8
##  [5] LC_MONETARY=en_GB.UTF-8    LC_MESSAGES=en_GB.UTF-8
##  [7] LC_PAPER=en_GB.UTF-8       LC_NAME=C
##  [9] LC_ADDRESS=C               LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_GB.UTF-8 LC_IDENTIFICATION=C
##
## attached base packages:
## [1] stats4    parallel  methods   stats     graphics  grDevices utils
## [8] datasets  base
##
## other attached packages:
##  [1] pRolocdata_1.12.0    pRoloc_1.14.3        MLInterfaces_1.54.0
##  [4] cluster_2.0.5        annotate_1.52.0      XML_3.98-1.4
##  [7] AnnotationDbi_1.36.0 IRanges_2.8.0        S4Vectors_0.12.0
## [10] MSnbase_2.0.0        ProtGenerics_1.6.0   BiocParallel_1.8.1
## [13] mzR_2.8.0            Rcpp_0.12.7          Biobase_2.34.0
## [16] BiocGenerics_0.20.0  gridExtra_2.2.1      xtable_1.8-2
## [19] BiocStyle_2.2.0      knitr_1.14
##
## loaded via a namespace (and not attached):
##   [1] minqa_1.2.4           colorspace_1.2-7      hwriter_1.3.2
##   [4] class_7.3-14          modeltools_0.2-21     mclust_5.2
##   [7] pls_2.5-0             base64enc_0.1-3       proxy_0.4-16
##  [10] hexbin_1.27.1         MatrixModels_0.4-1    affyio_1.44.0
##  [13] flexmix_2.3-13        mvtnorm_1.0-5         codetools_0.2-15
##  [16] splines_3.3.2         doParallel_1.0.10     impute_1.48.0
##  [19] robustbase_0.92-6     jsonlite_1.1          nloptr_1.0.4
##  [22] caret_6.0-72          pbkrtest_0.4-6        rda_1.0.2-2
##  [25] kernlab_0.9-25        vsn_3.42.3            sfsmisc_1.1-0
##  [28] shiny_0.14.2          sampling_2.7          assertthat_0.1
##  [31] Matrix_1.2-7.1        limma_3.30.2          formatR_1.4
##  [34] htmltools_0.3.5       quantreg_5.29         tools_3.3.2
##  [37] ggvis_0.4.3           gtable_0.2.0          affy_1.52.0
##  [40] reshape2_1.4.2        dplyr_0.5.0           MALDIquant_1.15
##  [43] trimcluster_0.1-2     gdata_2.17.0          preprocessCore_1.36.0
##  [46] nlme_3.1-128          iterators_1.0.8       fpc_2.1-10
##  [49] stringr_1.1.0         lme4_1.1-12           lpSolve_5.6.13
##  [52] mime_0.5              gtools_3.5.0          dendextend_1.3.0
##  [55] DEoptimR_1.0-6        zlibbioc_1.20.0       MASS_7.3-45
##  [58] scales_0.4.0          BiocInstaller_1.24.0  pcaMethods_1.66.0
##  [61] SparseM_1.72          RColorBrewer_1.1-2    ggplot2_2.1.0
##  [64] biomaRt_2.30.0        rpart_4.1-10          stringi_1.1.2
##  [67] RSQLite_1.0.0         highr_0.6             genefilter_1.56.0
##  [70] randomForest_4.6-12   foreach_1.4.3         e1071_1.6-7
##  [73] prabclus_2.2-6        bitops_1.0-6          mzID_1.12.0
##  [76] evaluate_0.10         lattice_0.20-34       htmlwidgets_0.7
##  [79] gbm_2.1.1             plyr_1.8.4            magrittr_1.5
##  [82] R6_2.2.0              DBI_0.5-1             whisker_0.3-2
##  [85] mgcv_1.8-15           survival_2.40-1       RCurl_1.95-4.8
##  [88] nnet_7.3-12           tibble_1.2            msdata_0.14.0
##  [91] car_2.1-3             mlbench_2.1-1         grid_3.3.2
##  [94] FNN_1.1               ModelMetrics_1.1.0    threejs_0.2.2
##  [97] digest_0.6.10         diptest_0.75-7        httpuv_1.3.3
## [100] munsell_0.4.3
```

It is always important to include session information details along with a short reproducible example highlighting the problem or question at hand.
The source of this document, including the code necessary to reproduce the analyses and figures is available in a public manuscript repository on GitHub [12].

Author contributions

All authors developed the software presented in this workflow, and wrote and approved the manuscript.

Competing interests

The authors declare that they have no competing interest.

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References

[1] A Christoforou, CM Mulvey, LM Breckels, PC Hayward, E Geladaki, T Hurrell, T Naake, L Gatto, R Viner, A Martinez Arias, and KS Lilley. A draft map of the mouse pluripotent stem cell spatial proteome. *Nat Commun*, 7:9992, jan 2016. doi: 10.1038/ncomms9992. URL `http://dx.doi.org/10.1038/ncomms9992`.

[2] L Gatto and A Christoforou. Using R and Bioconductor for proteomics data analysis. *Biochim Biophys Acta*, 1844(1 Pt A):42–51, Jan 2014. doi: 10.1016/j.bbapap.2013.04.032.

[3] L Gatto, LM Breckels, T Naake, and S Gibb. Visualization of proteomics data using R and Bioconductor. *PROTEOMICS*, 15(8):1375–1389, 2015. ISSN 1615-9861. doi: 10.1002/pmic.201400392. URL `http://dx.doi.org/10.1002/pmic.201400392`.

[4] A Thompson, J Schäfer, K Kuhn, S Kienle, J Schwarz, G Schmidt, T Neumann, R Johnstone, AK Mohammed, and C Hamon. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.*, 75(8):1895–904, 2003.

[5] GC McAlister, DP Nusinow, MP Jedrychowski, M Wühr, EL Huttlin, BK Erickson, R Rad, W Haas, and SP Gygi. Multinotch ms3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal Chem*, 86(14):7150–8, Jul 2014. doi: 10.1021/ac502040v.

[6] MWB Trotter, PG Sadowski, TPJ Dunkley, AJ Groen, and KS Lilley. Improved sub-cellular resolution via simultaneous analysis of organelle proteomics data across varied experimental conditions. *Proteomics*, 10(23):4213–4219, 2010. ISSN 1615-9861. doi: 10.1002/pmic.201000359. URL `http://dx.doi.org/10.1002/pmic.201000359`.

[7] BJ Webb-Robertson, HK Wiberg, MM Matzke, JN Brown, J Wang, JE McDermott, RD Smith, KD Rodland, TO Metz, JG Pounds, and KM Waters. Review, evaluation, and discussion of the challenges of missing value imputation for mass spectrometry-based label-free global proteomics. *J Proteome Res*, 14(5):1993–2001, May 2015. doi: 10.1021/pr501138h.

[8] C Lazar, L Gatto, M Ferro, C Bruley, and T Burger. Accounting for the multiple natures of missing values in label-free quantitative proteomics data sets to compare imputation strategies. *J Proteome Res*, 15(4):1116–25, Apr 2016. doi: 10.1021/acs.jproteome.5b00981.

[9] L Gatto, L M Breckels, T Burger, DJ Nightingale, AJ Groen, C Campbell, N Nikolovski, CM Mulvey, A Christoforou, M Ferro, and KS Lilley. A foundation for reliable spatial proteomics data analysis. *Mol Cell Proteomics*, 13(8):1937–52, Aug 2014. doi: 10.1074/mcp.M113.036350.

[10] C De Duve and H Beaufay. A short history of tissue fractionation. *J Cell Biol*, 91(3 Pt 2):293s–299s, Dec 1981.

[11] M Ashburner, CA Ball, JA Blake, D Botstein, H Butler, JM Cherry, AP Davis, K Dolinski, SS Dwight, JT Eppig, MA. Harris, DP Hill, L Issel-Tarver, A Kasarskis, S Lewis, JC Matese, JE Richardson, M Ringwald, GM Rubin, and G Sherlock. Gene ontology: tool for the unification of biology. the gene ontology consortium. *Nat Genet*, 25(1):25–9, May 2000. doi: 10.1038/75556.

[12] LM Breckels and L Gatto. A bioconductor workflow for processing and analysing spatial proteomics data. `https://github.com/lmsimp/bioc-pRoloc-hyperLOPIT-workflow`, 2016.

[13] LM Breckels, L Gatto, A Christoforou, AJ Groen, KS Lilley, and MW Trotter. The effect of organelle discovery upon sub-cellular protein localisation. *J Proteomics*, 88:129–40, Aug 2013. doi: 10.1016/j.jprot.2013.02.019.

[14] Chris Fraley, Adrian E. Raftery, Thomas Brendan Murphy, and Luca Scrucca. `mclust` *Version 4 for R: Normal Mixture Modeling for Model-Based Clustering, Classification, and Density Estimation*, 2012.

[15] AJ Groen, G Sancho-Andrés, LM Breckels, L Gatto, F Aniento, and KS Lilley. Identification of trans-golgi network proteins in arabidopsis thaliana root tissue. *J Proteome Res*, 13(2):763–76, Feb 2014. doi: 10.1021/pr4008464.

[16] SL Hall, S Hester, JL Griffin, KS Lilley, and AP Jackson. The organelle proteome of the dt40 lymphocyte cell line. *Mol Cell Proteomics*, 8(6):1295–1305, Jun 2009. doi: 10.1074/mcp.M800394-MCP200. URL `http://dx.doi.org/10.1074/mcp.M800394-MCP200`.

[17] TPJ Dunkley, S Hester, IP Shadforth, J Runions, T Weimar, SL Hanton, JL Griffin, C Bessant, F Brandizzi, C Hawes, RB Watson, P Dupree, and KS Lilley. Mapping the arabidopsis organelle proteome. *Proc Natl Acad Sci USA*, 103(17):6518–6523, Apr 2006. doi: 10.1073/pnas.0506958103. URL `http://dx.doi.org/10.1073/pnas.0506958103`.

[18] DJ Tan, H Dvinge, A Christoforou, P Bertone, A Martinez Arias, and KS Lilley. Mapping organelle proteins and protein complexes in drosophila melanogaster. *J Proteome Res*, 8(6):2667–78, Jun 2009. doi: 10.1021/pr800866n.

[19] LM Breckels, SB Holden, D Wojnar, CM Mulvey, A Christoforou, AJ Groen, MWB Trotter, O Kohlbacher, KS Lilley, and L Gatto. Learning from heterogeneous data sources: An application in spatial proteomics. *PLOS Computational Biology*, 12(5):e1004920, may 2016. doi: 10.1371/journal.pcbi.1004920. URL `http://dx.doi.org/10.1371/journal.pcbi.1004920`.