Package ‘"proteoQ"’

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Introduction to proteoQ

Chemical labeling using tandem mass tag (TMT) has been commonly applied in mass spectrometry (MS)-based quantification of proteins and peptides. The `proteoQ` tool is designed to aid automated and reproducible analysis of proteomics data. It interacts with an `Excel` spread sheet for dynamic sample selections, aesthetics controls and statistical modelings. The arrangement allows users to put data manipulation behind the scene and apply metadata to openly address biological questions using various informatic tools. In addition, the entire workflow is documented and can be conveniently reproduced upon revisiting.

The tool currently processes the peptide spectrum matches (PSM) tables from Mascot searches for 6-, 10- or 11-plex TMT experiments. Peptide and protein results are then produced with users' selection of parameters in data filtration, alignment and normalization. The package further offers a suite of tools and functionalities in statistics, informatics and data visualization by creating 'wrappers' around published R routines.

Installation

To install this package, start R (version "3.6") and enter:

```
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")
BiocManager::install(c("Biobase", "Mfuzz", "limma"))

if (!requireNamespace("devtools", quietly = TRUE))
    install.packages("devtools")
devtools::install_github("qzhang503/proteoQ")
```

Part I — Data normalization

In this section I illustrate the following applications of `proteoQ`:

- Summarization of PSM data to peptide and protein reports.

- Visualization of quality metrics in peptide and protein data.
- Re-normalization of data in part or in full

The data set I use in this section corresponds to the proteomics data from Mertins et al. (2018). In the study, two different breast cancer subtypes, triple negative (WHIM2) and luminal (WHIM16), from patient-derived xenograft (PDX) models were assessed by three independent laboratories. At each site, lysates from WHIM2 and WHIM16 were each split and labeled with 10-plex TMT at equal sample sizes and repeated on a different day. This results in a total of 60 samples labeled under six 10-plex TMT experiments. The samples under each 10-plex TMT were fractionated by off-line, high pH reversed-phase (Hp-RP) chromatography, followed by `LC/MS` analysis. The raw PSM results from Mascot searches are stored in a companion R package, `proteoQDA`, and are accessbile through the following installation:

```
devtools::install_github("qzhang503/proteoQDA")
```

1.1 Set up the experiments

We first set up a working directory:

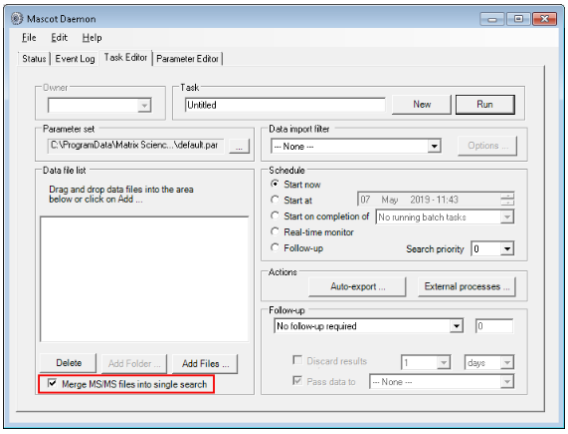
```
dat_dir <- "c:\\The\\First\\Example"
```

The workflow begins with PSM table(s) in a `csv` format from the Mascot search engine. When exporting PSM results, I typically set the option of `Include sub-set protein hits` to `0` with my opinionated choice in satisfying the principle of parsimony. The options of `Header` and `Peptide quantitation` should be checked to include the search parameters and quantitative values, respectively. The filename(s) of the exports will be taken as is.[1]

[1]The default file names begin with letter `F`, followed by six digits and ends with `.csv` in file name extension.

Scheme 1. Mascot export.

The same peptide sequence under different PSM files can be assigned to different protein IDs when inferring proteins from peptides using algorithms such as greedy set cover. To avoid such ambiguity in protein inference, I typically enable the option of `Merge MS/MS files into single search` in Mascot Daemon. If the option is disabled, peptide sequences that have been assigned to multiple protein IDs will be removed for now when constructing peptide reports.



Scheme 2 Mascot daemon.

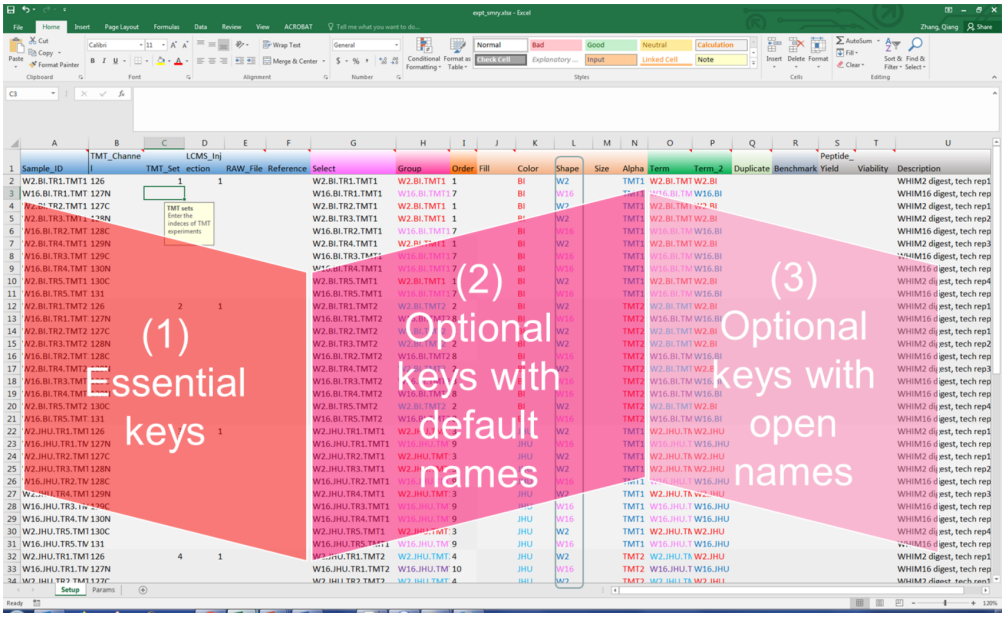
The merged search may become cumbersome with growing data sets. In this example, I combined the MS peak lists from the Hp-RP fractions within the same 10-plex TMT experiment, but not the lists across experiments. This results in a total of six pieces of PSM results in `Mascot` exports. To get us started, we go ahead and copy the PSM files that we have prepared in `proteoQDA` over to the working directory:

```
library(proteoQDA)
cptac_csv_1(dat_dir)
```

The workflow involves an `Excel` template containing the metadata of multiplex experiment numbers, including TMT channels, LC/MS injection indices, sample IDs, reference channels, `RAW` MS data file names and addditional fields from the users. The default file name for the experimental summary is `expt_smry.xlsx`. If samples were fractionated off-line prior to `LC/MS`, a second `Excel` template will also be filled out to link multiple `RAW` MS file names that are associated to the same sample IDs. The default file name for the fractionation summary is `frac_smry.xlsx`.[2] Unless otherwise mentioned, we will assume these default file names throughtout the document.

Columns in the `expt_smry.xlsx` are approximately divided into the following three tiers: (1) `essential`, (2) `optional default` and (3) `optional open`. We supply the required information of the TMT experiments under the essential columns. The optional default columns serve as the fields for convenient lookups in sample selection, grouping, ordering, aesthetics etc. For instance, the program will by default look for values under the `Color` column if no instruction was given in the color coding of a PCA plot. The optional open fields on the other hand allow us to define our own analysis and aesthetics: we may openly define multiple columns of contrasts at different levels of granularity for uses in linear modeling. Description of the column keys can be found from the help document by entering `?proteoQ::load_expts` from a `R` console.

[2]To extract the names of RAW files under a `raw_dir` folder: `extract_raws(raw_dir)`



Scheme 3 The layout of `expt_smry.xlsx`.

We next copy over a pre-compiled `expt_smry.xlsx` and a `frac_smry.xlsx` to the working directory:

```
cptac_expt_1(dat_dir)
cptac_frac_1(dat_dir)
```

We now have all the pieces that are required by `proteoQ` in place. Let's have a quick glance at the `expt_smry.xlsx` file. We note that no reference channels were indicated under the column `Reference`. With `proteoQ`, the `log2FC` of each species in a given sample is calculated either (a) in relative to the reference(s) within each multiplex TMT experiment or (b) to the mean of all samples in the same experiment if reference(s) are absent. Hence, the later approach will be employed to the examplary data set that we are working with. In this special case, the `mean(log2FC)` for a given species in each TMT experiment is averaged from five `WHIM2` and five `WHIM16` samples, which is biologically equivalent across TMT experiments.

As a final step of the setup, we will load the experimental summary and some precomputed results:

```
library(proteoQ)
load_expts()
```

1.2 Summarize PSMs to peptides and proteins

Process PSMs — In this section, I demonstrate the summarisation of PSM data to peptides and proteins. We start by processing PSM data from `Mascot` outputs:

```
# PSM reports
normPSM(
  rptr_intco = 1000,
  rm_craps = FALSE,
  rm_krts = FALSE,
  rm_outliers = FALSE,
  plot_violins = TRUE
)
```

```
# or accept the default parameters
normPSM()
```

PSM outliers will be assessed at a basis of per peptide and per sample at `rm_outliers = TRUE`, which can be a slow process for large data sets. To circumvent repeated efforts in the assessment of PSM outliers, we may set `rm_outliers = FALSE` and `plot_violins = TRUE` when first executing `normPSM()`. We then visually inspect the violin plots of reporter-ion intensity. Empirically, PSMs with reporter-ion intensity less than 1,000 are trimmed and samples with median intensity that is 2/3 or less to the average of majority samples are removed from further analysis.[3]

Summarize PSMs to peptides — We next summarise PSM to peptides.

```
# peptide reports
normPep(
  id = pep_seq,
  method_psm_pep = median,
  method_align = MGKernel,
  range_log2r = c(5, 95),
  range_int = c(5, 95),
  n_comp = 3,
  seed = 749662,
  maxit = 200,
  epsilon = 1e-05
)
```

At `id = pep_seq_mod`, peptide sequences that are different in variable modificaitons will be treated as different species. The `log2FC` of peptide data will be aligned by median centering across samples by default. If `method_align = MGKernel` is chosen, `log2FC` will be aligned under the assumption of multiple Gaussian kernels.[4] The parameter `n_comp` defines the number of Gaussian kernels and `seed` set a seed for reproducible fittings. The parameters `range_log2r` and `range_int` define the range of `log2FC` and the range of reporter-ion intensity, respectively, for use in the scaling of standard deviation across samples.

Let's compare the `log2FC` profiles with and without scaling normalization:[5]

```
# without scaling
pepHist(
  scale_log2r = FALSE,
  ncol = 10
)

# with scaling
pepHist(
  scale_log2r = TRUE,
  ncol = 10
)
```

By default, the above calls will look for none void entries under column `Select` in `expt_smry.xlsx`. This will results in histogram plots with 60 panels in each, which may not be easy to explore as a whole. In stead,

[3] The sample removal and PSM re-processing can be achieved by deleting the corresponding entries under the column `Sample_ID` in `expt_smry.xlsx`, followed by the re-load of the experiment, `load_expts()`, and the re-execution of `normPSM()` with desired parameters.

[4] Density kernel estimates can occasionally capture spikes in the profiles of log2FC for data alignment. Users will need to inspect the alignment of ratio histograms and may optimize the data normalization with different combinations of tuning parameters before proceeding to the next steps.

[5] `normPep()` will report log2FC results both before and after the scaling of standard deviations.

we will break the plots down by their data origins. We begin with modifying the `expt_smry.xlsx` file by adding the columns `BI`, `JHU` and `PNNL`. Each of the new columns includes sample entries that are tied to their laboratory origins.

https://www.youtube.com/embed/3B5et8VY3hE

We now are ready to plot histograms for each subset of data.[6] In this document, we only display the plots using the `BI` subset:

```
# without scaling
pepHist(
  scale_log2r = FALSE,
  col_select = BI,
  filename = Hist_BI_N.png,
  ncol = 5
)

# with scaling
pepHist(
  scale_log2r = TRUE,
  col_select = BI,
  filename = Hist_BI_Z.png,
  ncol = 5
)
```

NB: We interactively told `pepHist()` that we are interested in sample entries under the newly created `BI` column. Behind the scene, the interactions are facilitated by `openxlsx` via the readind and writing of the `Setup` workbook in `expt_smry.xlsx`. We also supply a file name, assuming that we want to keep the earlierly generated plots with default file names of `Peptide_Histogram_N.png` and `Peptide_Histogram_Z.png`.

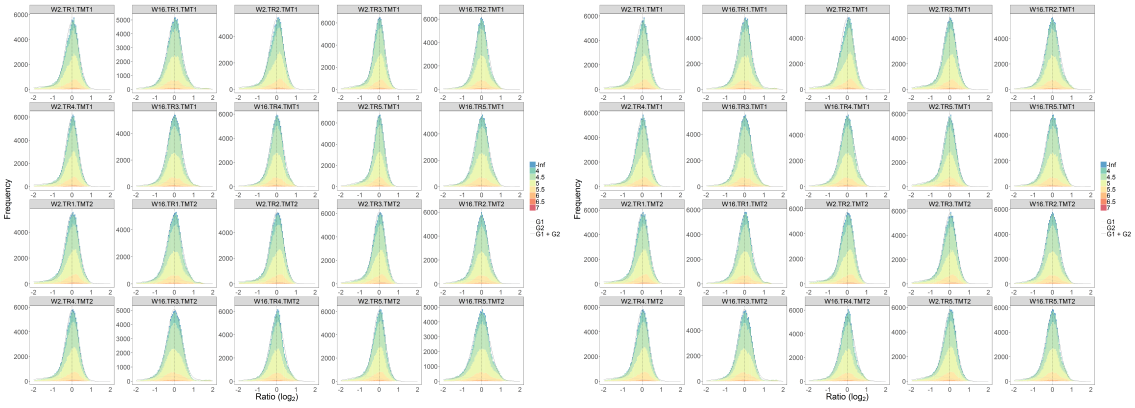


Figure 1. Histograms of peptide log2FC. Left: `scale_log2r = FALSE`; right, `scale_log2r = TRUE`.

As expected, the widths of `log2FC` profiles become more consistent after the scaling normalization. However, such adjustment may cause artifacts when the standard deviaiton across samples are genuinely different. I typically test `scale_log2r` at both `TRUE` and `FALSE`, then make a choice in data scaling together with my a priori knowledge of the characteristics of both samples and references.[7] I will use the same data set to illustrate the impacts of references in scaling normalization in Lab 3.1. Alignment of `log2FC` against housekeeping or normalizer protein(s) is also available. This seems suitable when sometime the quantities of proteins of interest are different across samples where the assumption of constitutive expression for the vast majority of proteins may not hold.

[6] System files will be automatically updated from the modified `expt_smry.xlsx`

[7] The default is `scale_log2r = TRUE` throughout the package. When calling functions involved parameter `scale_log2r`, users can specify explicitly `scale_log2r = FALSE` or define its value under the global environment.

Summarize peptides to proteins — We then summarise peptides to proteins using a two-component Gaussian kernel.[8]

```
# protein reports
normPrn(
  id = gene,
  method_pep_prn = median,
  method_align = MGKernel,
  range_log2r = c(5, 95),
  range_int = c(5, 95),
  n_comp = 2,
  seed = 749662,
  fasta = "C:\\Results\\DB\\Refseq\\RefSeq_HM_Frozen_20130727.fasta",
  maxit = 200,
  epsilon = 1e-05
)
```

Similar to the peptide summary, we inspect the alignment and the scaling of ratio profiles:

```
# without scaling
prnHist(
  scale_log2r = FALSE,
  ncol = 10
)

# with scaling
prnHist(
  scale_log2r = TRUE,
  ncol = 10
)
```

NB: At this point, we might have reach a consensus on the choice of scaling normalization. If so, it may be plausible to set the value of `scale_log2r` under the Global environment, which is typically the R console that we are interacting with.

```
# if agree
scale_log2r <- TRUE

# or if disagree
scale_logr <- FALSE
```

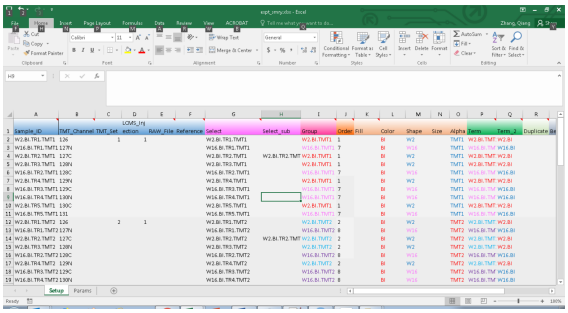
In this way, we can skip the repetitive setting of `scale_log2r` in our workflow from this point on, and more importantly, prevent ourselves from peppering the settings of **TRUE** or **FALSE** from calls to calls.

1.3 Data renormalization against a sample subset

A multi-Gaussian kernel can fail capturing the `log2FC` profiles for a subset of samples. This is less an issue with a small number of samples. Using a trial-and-error approach, we can start over with a new combination of parameters, such as a different `seed`, and/or a different range of `scale_log2r` et al. However, the one-size-fit-all attempt may remain inadequate when the number of samples is relatively large. The

[8]Prameter `fasta` is solely used for the calculation of protein percent coverage. Precomputed data will be used if no `fasta` database is provided.

`proteoQ` allow users to *focus* fit aganist selected samples. This is the job of argument `col_refit`. Let's say we want to re-fit the `log2FC` for samples `W2.BI.TR2.TMT1` and `W2.BI.TR2.TMT2`. We simply add a column, which I named it `Select_sub`, to `expt_smry.xlsx` with the sample entries for re-fit being indicated under the column:



Scheme 4 Partial refit.

We then execute the following codes with argument `col_fit` being linked to the newly created column:

```
normPep(
  id = pep_seq,
  method_psm_pep = median,
  method_align = MGKernel,
  range_log2r = c(5, 95),
  range_int = c(5, 95),
  n_comp = 3,
  col_refit = Select_sub,
  seed = 749662,
  maxit = 200,
  epsilon = 1e-05,
)
```

Part II — Basic informatics

In this section I illustrate the following applications of `proteoQ`:

- Basic informatic analysis against peptide and protein data.
- Linear modeling using contrast fits

2.1 MDS and PCA plots

We first visualize MDS, PCA and Euclidean distance against the peptide data. We start with metric MDS for peptide data:

```
# all data sets
pepMDS(
  show_ids = FALSE
)
```

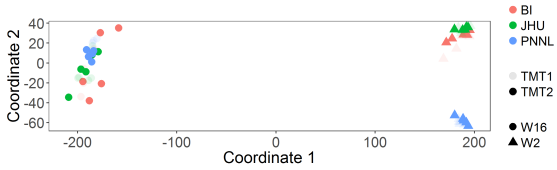


Figure 2A MDS of peptide `log2FC`.

It is clear that the WHIM2 and WHIM16 samples are well separated by the Euclidean distance of `log2FC` (**Figure 2A**). We next take the `JHU` data subset as an example to explore batch effects in the proteomic sample handling:

```
# `JHU` subset
pepMDS(
  col_select = JHU,
  filename = MDS_JHU.png,
  show_ids = FALSE
)
```

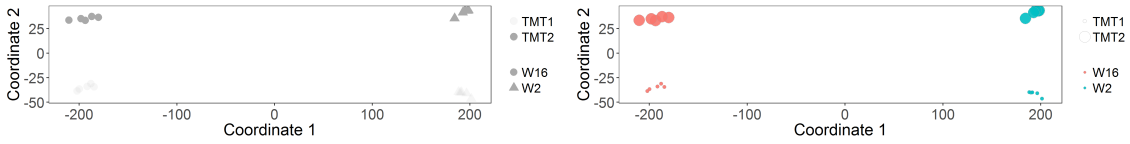


Figure 2B-2C. MDS of peptide `log2FC` for the `JHU` subset. Left: original aesthetics; right, modefied aesthetics.

We immediately spot that all samples are coded with the same color (**Figure 2B**). This is not a surprise as the values under column `expt_smry.xlsx::Color` are exclusively `JHU` for the `JHU` subset. For similar reasons, the two different batches of `TMT1` and `TMT2` are distinguished by transparency, which is governed by column `expt_smry.xlsx::Alpha`. We may wish to modify the aesthetics using different keys: e.g., color coding by WHIMs and size coding by batches, without the recourse of writing new R scripts. One solution is to link the attributes and sample IDs by creating additional columns in `expt_smry.xlsx`. In this example, we have had coincidentally prepared the column `Shape` and `Alpha` to code WHIMs and batches, respectively, for the `JHU` subset. Therefore, we can recycle them directly to make a new plot (**Figure 2C**):

```
# `JHU` subset
pepMDS(
  col_select = JHU,
  col_fill = Shape, # WHIMs
  col_size = Alpha, # batches
  filename = MDS_JHU_new_aes.png,
  show_ids = FALSE
)
```

Accordingly, the `prnMDS` performs `MDS` for protein data. For `PCA` analysis, the corresponding functions are `pepPCA` and `prnPCA` for peptide and protein data, respectively.

While MDS approximates Euclidean distances at a low dimensional space. Sometime it may be useful to have an accurate view of the distance matrix. Functions pepEucDist and prnEucDist plot the heat maps of Euclidean distance matrix for peptides and proteins, respectively. They are wrappers of pheatmap and inherit many parameters therein. Supposed that we are interested in visualizing the distance matrix for the JHU subset:

```
# `JHU` subset
pepEucDist(
  col_select = JHU,
  annot_cols = c("Shape", "Alpha"),
  annot_colnames = c("WHIM", "Batch"),

  # parameters from `pheatmap`
  display_numbers = TRUE,
  number_color = "grey30",
  number_format = "%.1f",

  clustering_distance_rows = "euclidean",
  clustering_distance_cols = "euclidean",

  fontsize = 16,
  fontsize_row = 20,
  fontsize_col = 20,
  fontsize_number = 8,

  cluster_rows = TRUE,
  show_rownames = TRUE,
  show_colnames = TRUE,
  border_color = "grey60",
  cellwidth = 24,
  cellheight = 24,
  width = 14,
  height = 12,

  filename = EucDist_JHU.png
)
```

Parameter annot_cols defines the tracks to be displayed on the top of distrance-matrix plots. In this example, we have choosen expt_smry.xlsx::Shape and expt_smry.xlsx::Alpha, which encodes the WHIM subtypes and the batch numbers, respectively. Parameter annot_colnames allows us to rename the tracks from Shape and Alpha to WHIM and Batch, respectively, for better intuition. We can alternatively add columns WHIM and Batch if we choose not to recycle columns Shape and Alpha.

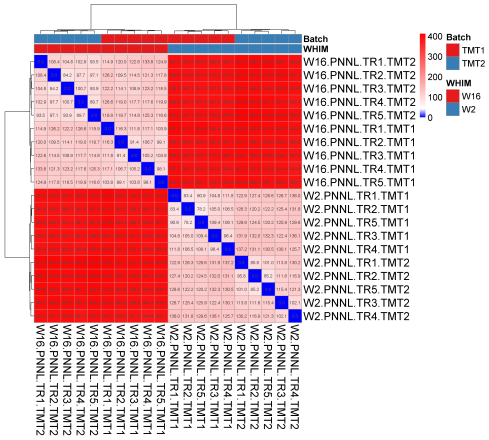


Figure 2D. EucDist of peptide `log2FC`.

2.2 Correlation plots

In this section, we visualize the batch effects through correlation plots. The `proteoQ` tool currently limits itself to a maximum of 44 samples for a correlation plot. In the document, we will perform correlation analysis against the `PNNL` data subset. By default, samples will be arranged diagnoally from upper left to bottom right by the row order of `expt_smry.xlsx::Select`. We have learned from the earlier `MDS` analysis that the batch effects are smaller than the differences between `W2` and `W16`. We may wish to put the `TMT1` and `TMT2` groups adjacient to each other for visualization of more nuance batch effects, followed by the correlational comparison of WHIM subtypes. We can achieve this by supervising sample IDs at a customized order. In the `expt_smry.xlsx`, I have prepared an `Order` column where samples within the `JHU` subset were arranged in the descending order of `W2.TMT1`, `W2.TMT2`, `W16.TMT1` and `W16.TMT2`. Now we tell the program to look for the `Order` column for sample arrangement:

```
# peptide correlation
pepCorr(
  col_select = PNNL,
  col_order = Order,
  filename = PNNL.png,

  use_log10 = TRUE,
  scale_log2r = TRUE,
  min_int = 3.5,
  max_int = 6.5,
  min_log2r = -2,
  max_log2r = 2,
  width = 24,
  height = 24
)

# protein correlation
prnCorr(
  col_select = PNNL,
  col_order = Order,
  filename = PNNL.png,

  use_log10 = TRUE,
```

```
  scale_log2r = TRUE,
  min_int = 3.5,
  max_int = 6.5,
  min_log2r = -2,
  max_log2r = 2,
  width = 24,
  height = 24,
)
```

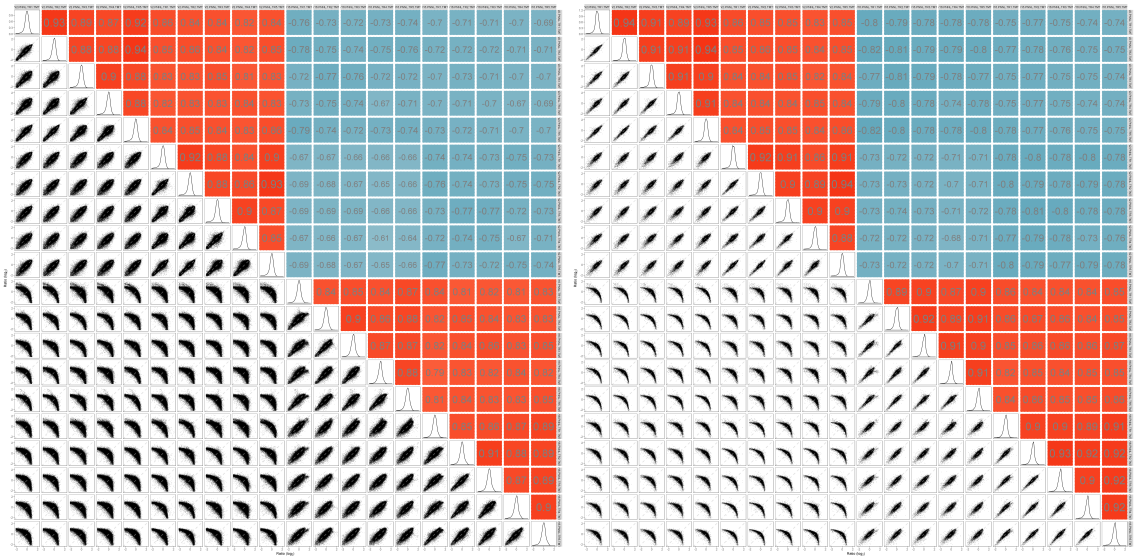


Figure 3A-3B. Correlation of `log2FC` for the `PNNL` subset. Left: peptide; right, protein.

2.3 Imputation of NA values

The following performs the imputation of peptide and protein data. More information can be found from `mice`.

```
# peptides
pepImp(m = 2, maxit = 2)

# proteins
prnImp(m = 5, maxit = 5)
```

2.4 Heat map

The following performs heat map visualization against protein data. Detailed description of the arguments can be found from `pheatmap`.

```
# protein heat maps
prnHM(
  xmin = -1,
  xmax = 1,
  x_margin = 0.1,
  annot_cols = c("Group", "Color", "Alpha", "Shape"),
```

```
  annot_colnames = c("Group", "Lab", "Batch", "WHIM"),
  cluster_rows = TRUE,
  cutree_rows = 10,
  show_rownames = FALSE,
  show_colnames = TRUE,
  fontsize_row = 3,
  cellwidth = 14,
  width = 18,
  height = 12,
)
```

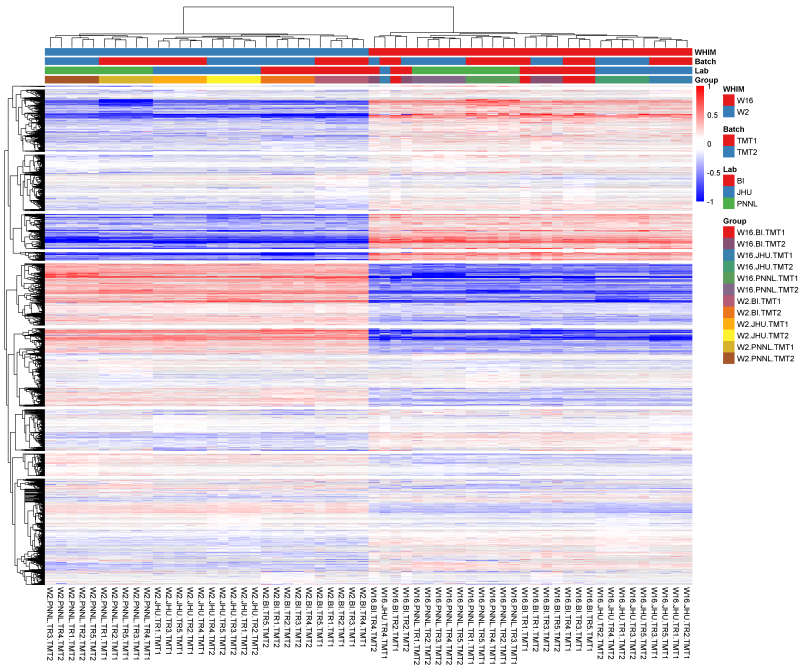


Figure 4. Heat map visualization of protein `log2FC` at `scale_log2r = TRUE`.

2.5 Significance tests and volcano plot visualization

In this section, we perform the significance analysis of peptide and protein data. The approach of contrast fit is used here (Chambers, J. M. (1992) Linear models; `limma`, Gordon Smith). We first define the contrast groups for significance tests. For this purpose, I have devided the samples by their WHIM subtypes, laboratory locations and batch numbers. This ends up with entries of `W2.BI.TMT1`, `W2.BI.TMT2` etc. under the `expt_smry.xlsx::Term` column. The interactive environment between the Excel file and the proteoQ tool allows us to enter more columns of contrasts when needed. For instance, we might also be interested in a more course comparison of inter-laboratory differences without batch effects. The corresponding contrasts of `W2.BI`, `W2.BI` etc. can be found under a pre-made column, `Term_2`. Having these columns in hand, we are now ready to perform significance tests for peptides and protein species. In the demo, we will analyze protein data and perform volcano plot visualization:

```
# significance tests of protein log2FC
prnSig(
  impute_na = FALSE,
  W2_bat = ~ Term["(W2.BI.TMT2-W2.BI.TMT1)", "(W2.JHU.TMT2-W2.JHU.TMT1)", "(W2.PNNL.TMT2-W2.PNNL.TMT1)"]
  W2_loc = ~ Term_2["W2.BI-W2.JHU", "W2.BI-W2.PNNL", "W2.JHU-W2.PNNL"], # location effects
```

```
)

# volcano plots
prnVol()
```

Note that we have informed the `prnSig` function to look for contrasts under columns `Term` and `Term_2`, followed by the cotrast pairs in square brackets. Pairs of contrasts are separated by commas.

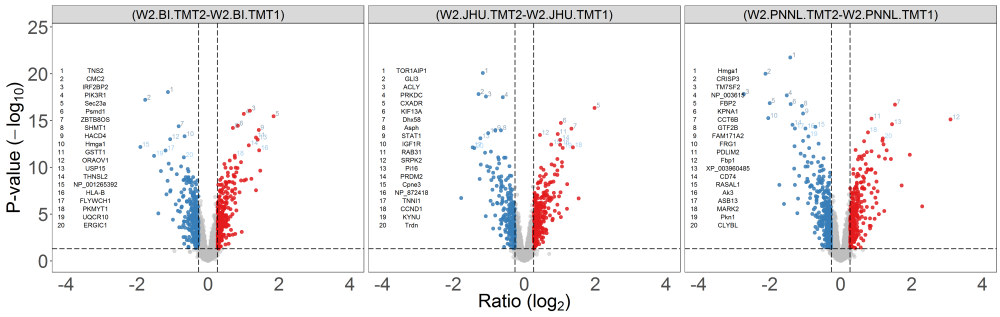


Figure 5A. Volcano plots of protein `log2FC` between two batches.

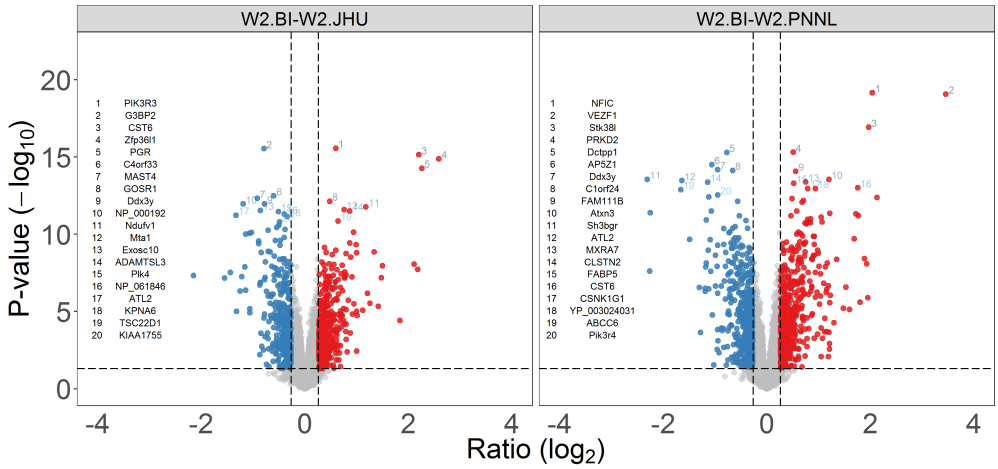


Figure 5B. Volcano plots of protein `log2FC` between locations.

2.6 Gene sets in volcano plots

There are a handful of tools for gene set enrichement analysis, such as GSEA, GSVA, gage, to name a few. It may be intuitive as well if we can visualize the enrichment of gene sets under the context of volcano plots. Currently, the `preoteoQ` takes a naive approach to visualize the *asymmetricity* of protein probability *p*-values under volcano plots. In the analysis of Gene Set Probability Asymmetricity (GSPA), the significance `pVals` of proteins obtained from linear modeling are taken, followed by the calculation of the geometric mean of `pVals` for the groups of up- or down-regulated proteins within a gene set, as well as the corresponding mean `log2FC`. The quotient of the two `pVals` is then taken to represent the significance of enrichment and the delta of the two `log2FC` for use as the fold change of enrichment. The arguments `pval_cutoff` and `logFC_cutoff` allow us to filter out low impact genes.

```
prnGSPA(
  impute_na = FALSE,
  pval_cutoff = 5E-2,
  logFC_cutoff = log2(1.2),
  gset_nm = c("go_sets", "kegg_sets"),
)
```

To visualize the distribution of protein `log2FC` and `pVal` within gene sets:

```
gspaMap(
  show_labels = TRUE,
  pval_cutoff = 1E-2,
  logFC_cutoff = log2(1.2),
  show_sig = pVal,
  yco = 0.01,
)
```

This will produce the volcano plots of proteins within gene sets that have passed our selection criteria. Here, we show one of the examples:

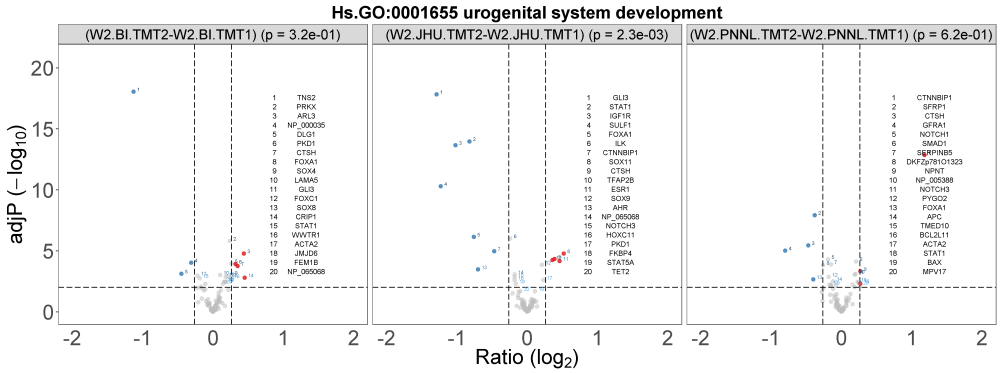


Figure 6. An example of volcano plots of protein `log2FC` under a gene set.

2.7 Trend Analysis

The following performs the trend analysis against protein expressions. More information can be found from `Mfuzz`.

```
# soft clustering of protein data
anal_prnTrend(
  scale_log2r = TRUE,
  n_clust = 6
)

# visualization
plot_prnTrend()
```

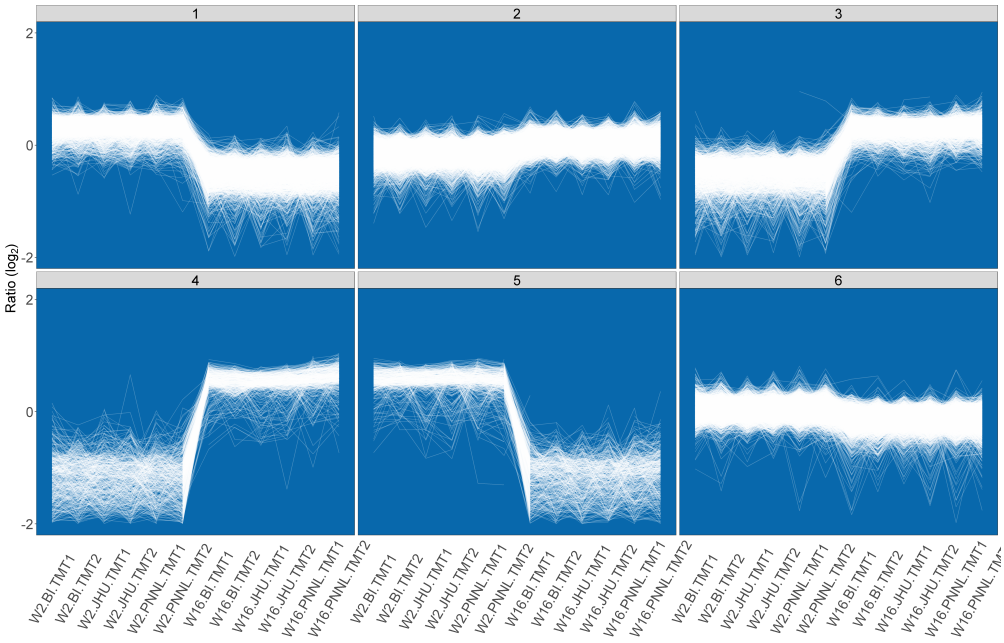


Figure 7. Trend analysis of protein `log2FC`.

2.8 NMF Analysis

The following performs the NMF analysis against protein data. More details can be found from `NMF`.

```
# load library
library(NMF)

# NMF analysis
anal_prnNMF(
  # col_group = Group, # optional a priori knowledge of sample groups
  scale_log2r = TRUE,
  r = 6,
  nrun = 200
)

# consensus heat map
plot_prnNMFCon(
  r = 6,
  annot_cols = c("Color", "Alpha", "Shape"),
  annot_colnames = c("Lab", "Batch", "WHIM"),
  width = 10,
  height = 10
)

# coefficient heat map
plot_prnNMFCoef(
  r = 6,
  annot_cols = c("Color", "Alpha", "Shape"),
  annot_colnames = c("Lab", "Batch", "WHIM"),
  width = 10,
```

```
  height = 10
)

# metagene heat map(s)
plot_metaNMF(
  r = 6,
  annot_cols = c("Color", "Alpha", "Shape"),
  annot_colnames = c("Lab", "Batch", "WHIM"),

  fontsize = 8,
  fontsize_col = 5
)
```

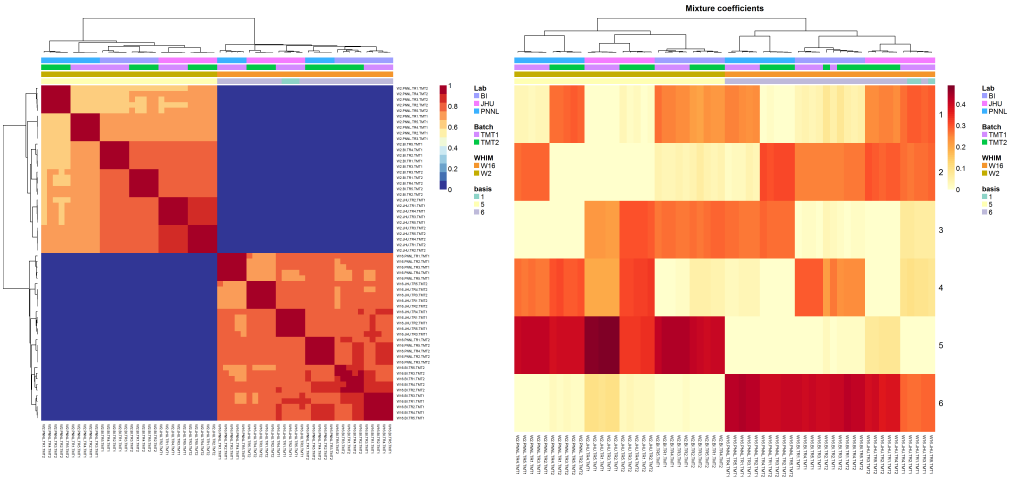


Figure 8A-8B. NMF analysis of protein `log2FC`. Left: concensus; right: coefficients.

Part III — Labs

3.1. Choices of references

In this lab, we explore the effects of reference choices on data normalization. We first copy data over to the file directory specified by `temp_dir`, followed by PSM, peptide normalization and histogram visualization of peptide `log2FC`.

```
# directory setup
temp_dir <- "c:\\The\\W2_ref\\Example"
library(proteoQDA)
cptac_csv_1(temp_dir)
cptac_expt_ref_w2(temp_dir)
cptac_frac_1(temp_dir)

# analysis
library(proteoQ)
load_expts(temp_dir, expt_smry_ref_w2.xlsx)

normPSM()
```

```
normPep(
  id = pep_seq,
  method_psm_pep = median,
  method_align = MGKernel,
  range_log2r = c(5, 95),
  range_int = c(5, 95),
  n_comp = 3,
  seed = 911,
  maxit = 200,
  epsilon = 1e-05
)

# visualization
pepHist(
  scale_log2r = FALSE,
  ncol = 9
)
```

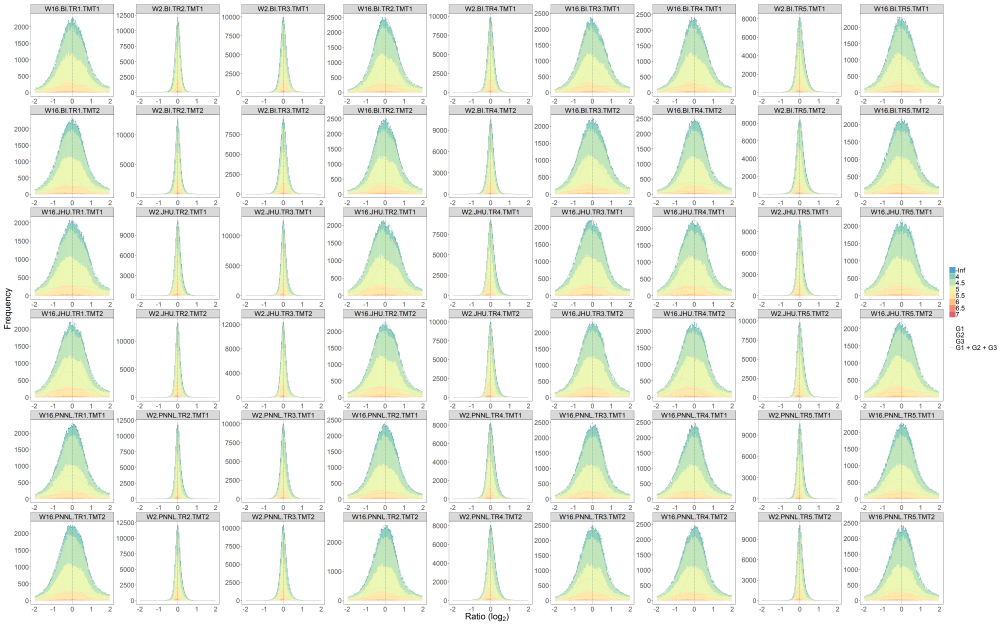


Figure S1A. Histograms of peptide `log2FC` with a WHIM2 reference.

Notice that in the above histograms the `log2FC` profiles of `WHIM2` samples are much narrower than those of `WHIM16` (**Figure S1A**). This will occur when a reference is more similar to one group of sample(s) than the other. In our case, the reference is one of `WHIM2`. The difference in the breadth of `log2FC` profiles between the `WHIM16` and the `WHIM2` groups is likely due to the genuine difference in their proteomes. If the above argument is valid, a scaling normalize would moderate, and thus bias, the quantitative difference in proteomes between `WHIM2` and `WHIM16`.

We alternatively seek a "center-of-mass" representation for uses as references. We select one `WHIM2` and one `WHIM16` from each 10-plex TMT. The `proteoQ` tool will average the signals from designated references. Thefore, the derived reference can be viewed as a mid point of the `WHIM2` and the `WHIM16` proteomes. We next perform analogously the data summary and histogram visualization. With the new reference, we have achieved `log2FC` profiles that are more comparable in breadth between `WHIM2` and `WHIM16` samples. With the new reference, a scaling normalization may be suitable for later steps.

```r
# directory setup
temp_dir_2 <- "c:\\The\\W2_W16_ref\\Example"
library(proteoQDA)
cptac_csv_1(temp_dir_2)
expt_smry_ref_w2_w16(temp_dir_2)
cptac_frac_1(temp_dir_2)

# experiment upload
library(proteoQ)
load_expts(temp_dir_2, expt_smry_ref_w2_w16.xlsx)

# PSM normalization
normPSM()

# peptide normalization
normPep(
  id = pep_seq,
  method_psm_pep = median,
  method_align = MGKernel,
  range_log2r = c(5, 95),
  range_int = c(5, 95),
  n_comp = 3,
  seed = 911,
  maxit = 200,
  epsilon = 1e-05,
)

# visualization
pepHist(
  scale_log2r = FALSE,
  ncol = 8
)
```

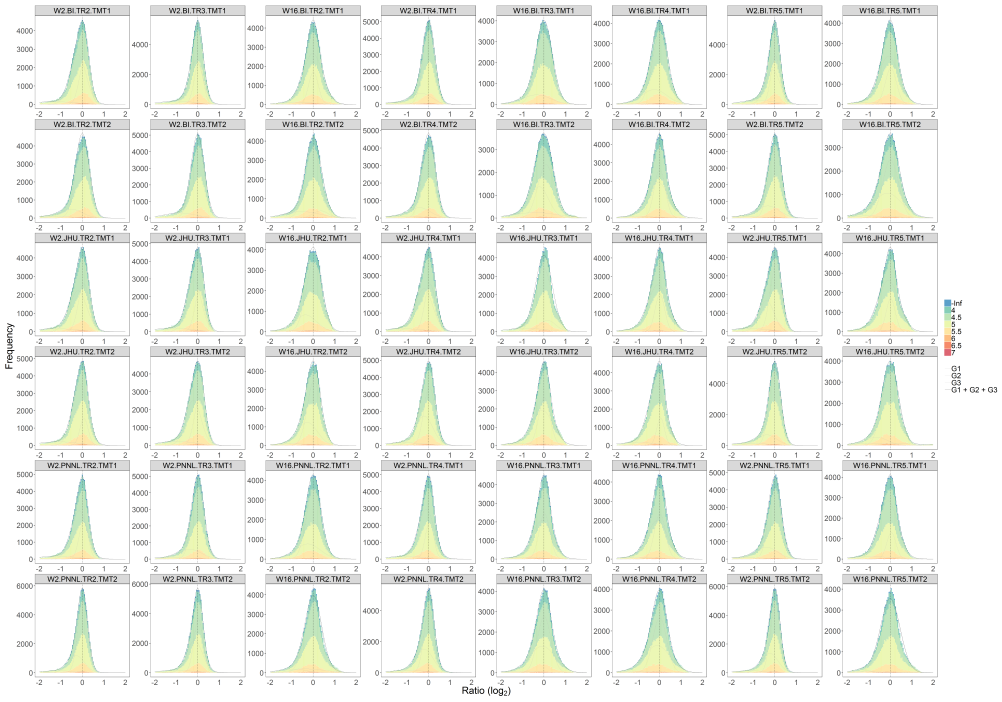


Figure S1B. Histograms of peptide `log2FC` with a combined WHIM2 and WHIM16 reference.

3.2. Peptide subsets

In addition to the global proteomes, the CPTAC publication contains phosphopeptide data from the same samples.(2018) In this lab, we will explore the stoichiometry of phosphopeptide subsets in relative to the combined data sets of `global + phospho` peptides. We first performed a search aganist the combined data. The search results are available in `proteoQDA`. We next copy the result files over, followed by the analysis and visualization of the `BI` subset:

```
# directory setup
temp_phospho_dir <- "c:\\The\\Phosphopeptide\\Example"
library(proteoQDA)
cptac_csv_2(temp_phospho_dir)
cptac_expt_2(temp_phospho_dir)
cptac_frac_2(temp_phospho_dir)

# experiment upload
library(proteoQ)
load_expts(temp_phospho_dir, expt_smry.xlsx)

# PSM normalization
normPSM()

# peptide normalization
normPep(
  id = pep_seq_mod, # peptides with different variable modifications
  method_psm_pep = median,
  method_align = MGKernel,
  range_log2r = c(5, 95),
  range_int = c(5, 95),
```

```
  n_comp = 3,
  seed = 749662,
  maxit = 200,
  epsilon = 1e-05
)

# histograms for all peptides
pepHist(
  col_select = BI,
  scale_log2r = TRUE,
  ncol = 4,
  filename = "BI_all_peptides.png"
)

# histograms for phosphopeptide subsets
pepHist(
  col_select = BI,
  scale_log2r = TRUE,
  pep_pattern = "sty",
  ncol = 4,
  filename = "BI_pSTY.png"
)
```

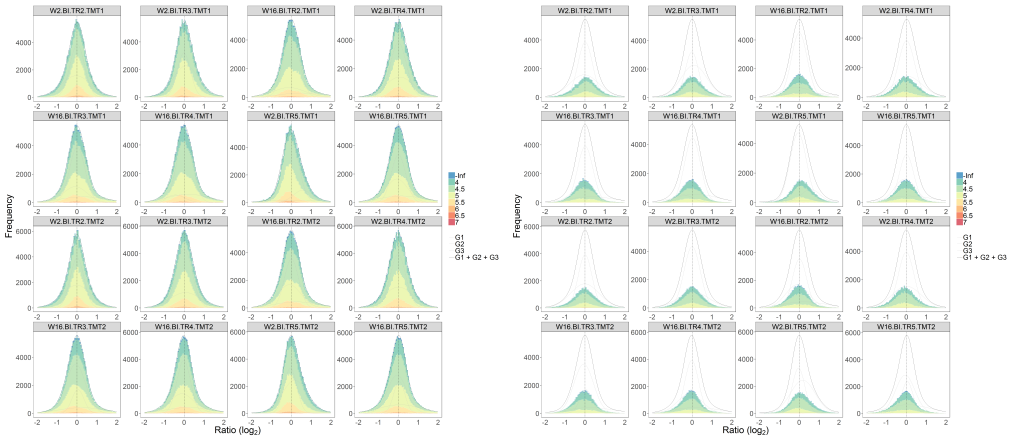


Figure S2A-S2B. Histogram visualization of peptide `log2FC`. Left: global + phospho; right: phospho only.

Ideally, the profiles of the `log2FC` between the `phospho` subsets and the overall data would either align at the maximum density or perhaps offset by similar distance among replicated samples. In this example, the alginment at maximum density seems to be case. The observation raises the possibility of measuring the stoichiometry of phosphoproteomes in relative to global data across sample types or conditions.

NB: I used underscore to denote N-terminal acetylation. The R language will throw an error if we attemp to use `pep_pattern = _` to subset peptides with N-terminal acetylation. In this case, we will need to quote the underscore: `pep_pattern = "_"`.

3.3 Random effects

Single random effect — In proteomic studies involved multiple multiplex `TMT` experiments, the limited multiplicity of isobaric tags requires sample parting into subgroups. Measures in `log2FC` are then obtained within each subgroup by comparing to common reference materials, followed by data bridging across

experiments. This setup violates the independence assumption in statistical sampling as the measures of log2FC are batched by TMT experiments. In this lab, we will use the CPTAC data to test the statistical significance in protein abundance between the WHIM2 and the WHIM16 subtypes, by first taking the batch effects into account. We will use mixed-effects models to explore the random effects that were introduced by the data stitching. In case that you would like to find out more about mixed-effects models in R, I found the online tutorial a helpful resource.

We start off by copying over the expt_smry.xlsx file, which contains a newly created column, Term_3, for terms to be used in the statistical tests of WHIM2 and WHIM16. We also copy over the protein results from Part I of the vignette and carry out the signficance tests with and without random effects.

```
# directory setup
temp_raneff_dir <- "c:\\The\\Random_effects\\Example"
library(proteoQDA)
cptac_prn_1(temp_raneff_dir)
cptac_expt_3(temp_raneff_dir)
cptac_frac_3(temp_raneff_dir)

# analysis
library(proteoQ)
load_expts(temp_raneff_dir, expt_smry.xlsx)

prnSig(
  impute_na = FALSE,
  W2_vs_W16_fix = ~ Term_3["W16-W2"], # fixed effect only
  W2_vs_W16_mix = ~ Term_3["W16-W2"] + (1|TMT_Set), # one fixed and one random effects
)

# volcano plots
prnVol()
```

In the formula linked to argument W2_vs_W16_mix, the random effect (1|TMT_Set) is an addition to the fix effect Term_3["W16-W2"]. The syntax (1|TMT_Set) indicates the TMT_Set term to be parsed as a random effect. The name of the term is again a column key in expt_smry.xlsx. In this example, the TMT batches are documented under the column TMT_Set and can be applied directly to our formula. Upon the completion of the protein signficance tests, we can analyze analogously the gene set enrichment against these new formulas by calling functions prnGSPA and gspaMAP.

Multiple random effects — In this section, we will test the statistical significance in protein abundance changes between the WHIM2 and the WHIM16 subtypes, by taking additively both the TMT batch effects and the laboratory effects into account. At the time of writing the document, I don't yet know how to handle multiple random effects using limma. Alternatively, I use lmerTest to do the work.

Missing values can frequently fail random-effects modeling with more complex error structures and need additional cares. One workaround is to simply restrict ourselves to entries that are complete in cases. This would lead to a number of proteins not measurable in their statistical significance. Alternatively, we may seek to fill in missing values using techniques such as multivariate imputation. At present, I use the mice function from the R package "mice" in prnImp() and pepImp() for the imputation of protein and peptide data, respectively. To impute protein data:

```
prnImp(m = 5, maxit = 5)
```

We further note that the laboratory differences are coded under columns Color in expt_smry.xlsx. We then test the statistical difference between WHIM2 and WHIM16 aganist the following three models:

```
prnSig(
  impute_na = TRUE,
  method = lm,
  W2_vs_W16_fix = ~ Term_3["W16-W2"], # one fixed effect
  W2_vs_W16_mix = ~ Term_3["W16-W2"] + (1|TMT_Set), # one fixed and one random effect
  W2_vs_W16_mix_2 = ~ Term_3["W16-W2"] + (1|TMT_Set) + (1|Color), # one fixed and two random effects
)

# correlation plots
df <- read.csv(file.path(temp_raneff_dir, "Protein\\Model\\Protein_pVals.txt"),
    check.names = FALSE, header = TRUE, sep = "\t") %>%
  dplyr::select(grep("pVal\\s+", names(.))) %>%
  `colnames<-`(c("none", "one", "two")) %>%
  dplyr::mutate_all(~ -log10(.x)) %>%
  GGally::ggpairs(df, columnLabels = as.character(names(df)), labeller = label_wrap_gen(10), title = ""
    xlab = expression("pVal ("*-log[10]*")"), ylab = expression("pVal ("*-log[10]*")"))
```

The correlation plots indicate that the random effects of batches and laboratory locations are much smaller than the fixed effect of the biological differences of `WHIM2` and `WHIM16`.

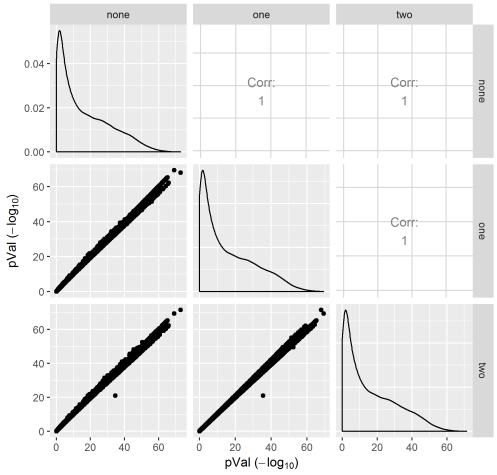


Figure S3. Pearson r of protein significance p-values.

References

Philipp, Martins. 2018. "Reproducible Workflow for Multiplexed Deep-Scale Proteome and Phosphoproteome Analysis of Tumor Tissues by Liquid Chromatography-Mass Spectrometry." *Nature Protocols* 13 (7): 1632–61. https://doi.org/10.1038/s41596-018-0006-9.