Comparative Analysis of High-Throughput Metaproteomics Data in Unipept

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Chapter 1

Introduction

Part I

Unipept Desktop

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Chapter 2

Unipept Desktop: a faster, more powerful metaproteomics analysis tool

Abstract Metaproteomics has become an important research tool to study microbial systems, which has resulted in increased metaproteomics data generation. However, efficient tools for processing the acquired data have lagged behind. One widely used tool for metaproteomics data interpretation is Unipept, a web-based tool that provides, amongst others, interactive and insightful visualizations. Due to its web-based implementation, however, the Unipept web application is limited in the amount of data that can be analyzed. In this manuscript we therefore present Unipept Desktop, a desktop application version of Unipept that is designed to drastically increase the throughput and capacity of metaproteomics data analysis. Moreover, it provides a novel comparative analysis pipeline and improves the organization of experimental data into projects, thus addressing the growing need for more performant and versatile analysis tools for metaproteomics data.

2.1 Introduction

Metaproteomics is a relatively young research field that focuses on the study of microbial environments and complex ecosystems, and of the interactions between the organisms involved, through the analysis of the proteins extracted from these environments. Over the past years, the technology to identify proteins from such complex samples has been greatly improved, allowing metaproteomics to transition from relatively small studies to large scale experiments (Rechenberger *et al.*, 2019; Wilmes *et al.*, 2015). The key enabling technologies for this transition are improved mass spectrometers and more powerful proteomics approaches, which have both come a long way since the introduction of metaproteomics analysis in 2004 (Rodríguez-Valera, 2004; Yates, 2019). To allow efficient processing of the resulting increase of acquired data, various dedicated tools have been made available to support metaproteomics data analysis (Muth *et al.*, 2015; Van Den Bossche *et al.*, 2020), but even with this increased bioinformatics support, many challenges still need to be overcome, especially regarding downstream analysis of the obtained identifications (Schiebenhoefer *et al.*, 2019).

Unipept is a leading tool for such downstream metaproteomics data analysis (Herbst *et al.*, 2016) that currently consists of a web application (Gurdeep Singh *et al.*, 2019), a web service, and a command line tool (Verschaffelt *et al.*, 2020). The Unipept web application provides users with the ability to analyze a metaproteomics sample and extract taxonomic and functional information from environmental samples derived from a variety of origins, ranging from the human gut to biogas plants. The Unipept web application provides users with interactive visualizations and allows them to, for example, filter out all functions that are associated with a specific taxon. Due to its web-based nature, however, the size and number of samples that can be analyzed by Unipept are limited. And while it is currently possible to analyze larger data sets using the Unipept CLI, this requires more sophisticated bioinformatics skills and does not provide the interactive link between taxa and functional annotations.

Because of the browser limitations, it can already take a substantial amount of time to process relatively small samples (e.g. containing up to a few thousand identified peptides) using Unipept, depending on the specific search configuration used. These limitations have become an issue, as the advances in metaproteomics have not only increased data set sizes, but have also increased the number of data sets that need to be processed (Zhang and Figeys, 2019).

In order to accommodate this evolution, the throughput of metaproteomics data analyses needs to increase as well, in turn requiring tools that are not constrained in the amount of memory and CPU resources they are allowed to consume. Moreover, analysis results also need to be retained for future reference, ideally in a project-based approach that can group multiple samples, and the corresponding results should be easily shareable with other researchers.

For specific applications, it is also important that all data is processed offline or on-site rather than being sent over the internet. For instance, sensitive medical data is often not allowed to be sent to external services for processing, but must be kept in-house to safeguard patient confidentiality and privacy.

All of the above issues need to be resolved in order to support the growing interest in, and reach of, metaproteomics. We therefore here present the Unipept Desktop Application, a novel cross-platform desktop application designed to specifically overcome these challenges while also retaining the functionality that exists in the current web app.

2.2 Implementation

The Unipept desktop application provides three different types of analyses: *i*) single assay analysis, *ii*) inter-assay comparative analysis, and *iii*) tryptic peptide analysis. The single assay analysis performs a full taxonomic and functional analysis of a single assay and corresponds to the default "metaproteomics analysis" as presented by the Unipept web application. The inter-assay comparative analysis on the other hand, provides the ability to explore similarities and differences between multiple assays. While the comparison of multiple assays was already possible with the Unipept web application, this was only available for a limited number of quite small assays due to strict memory constraints posed by web browsers. The tryptic peptide analysis, lastly, can be used to look up which proteins, taxa and functions are associated with a given peptide.

Unipept Desktop delivers these core functions through a concise user interface (Figure 2.1) that consists of three main parts: the sidebar,

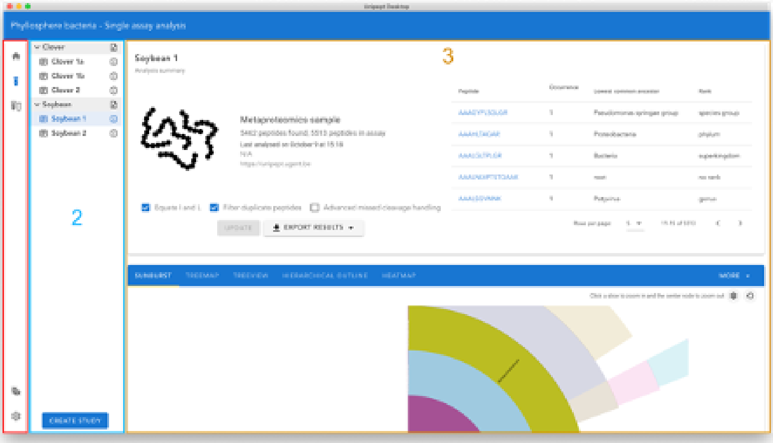
Unipept Desktop
Phyllosphere bacteria - Single assay analysis
1
2
3
Clover
Clover 1a
Clover 1b
Clover 2
Soybean
Soybean 1
Soybean 2
CREATE STUDY
Soybean 1
Analysis summary
Metaproteomics sample
Equate I and L
Filter duplicate peptides
Advanced missed cleavage handling
UPDATE
EXPORT RESULTS
SUNBURST
TREEMAP
TREEVIEW
HIERARCHICAL OUTLINE
HEATMAP
MORE

Figure 2.1: Screenshot of the Unipept Desktop application. The analysis page of the desktop application is depicted here and consists of three main parts: the sidebar that is used to navigate between the different analysis pipelines and functions of the application (1), the project explorer that displays a hierarchical view of the project (2) and the content view that renders analysis results (3).

the project explorer, and the content view. The sidebar on the far left allows the user to navigate between the different analysis pipelines and functions of this application. Directly to the right of the sidebar is the project explorer that allows the user to switch between assays, and to modify the project. The project explorer is only shown when performing single assay or comparative analyses. Assays and studies can be renamed or deleted by right clicking them, after which a context menu opens. Lastly, the content view takes up most of the application's visual space and presents either analysis results or the settings page.

The Unipept Desktop Application also allows offline analysis of data through a choice of the API endpoint in the settings menu. This endpoint, which uses the Unipept API and by default connects to the online Unipept system, can be configured to call any service that supports the Unipept API. By setting up a local instance of the Unipept backend system, the user can thus ensure that all data remains locally. Setting up a local Unipept back-end is possible by cloning the open source Unipept repository on GitHub, but requires advanced technical knowledge. We plan to make the installation process of these custom API endpoints even easier with future releases of Unipept.

Unipept Desktop is powered by the cross-platform Electron framework, which in itself is powered by Chromium browser technology. This means that the application is developed with web-centric technologies, such as the Vue frontend framework and TypeScript, and hence we were able to reuse large parts of the web app's codebase. The choice for the Electron platform was mostly driven by the extensive suite of different functionalities that can be integrated with minimum configuration efforts. Thanks to the Electron platform we can provide an automatic update mechanism, easily generate installation packages for all major platforms (Windows, macOS and Linux), and include automatic crash reporting, amongst others. Once installed, the Unipept Desktop application can thus update fully autonomously in the background, ensuring that users always have the latest functionality and bug fixes installed.

2.2.1 Project-centric analysis

The Unipept Desktop Application has full access to the local filesystem. Hence, it can store an arbitrary amount of data and does not need to worry about strict size limits; this in contrast to web applications that are only allowed to store up to a few megabytes using the local storage API. This allows us to improve upon the organization of data sets by introducing project-based data management capabilities. In accordance with the terminology introduced by the ISA-tab standard for experimental metadata annotation (Sansone *et al.*, 2012), we now refer to a data set derived from a sample as an "assay", while a study is a grouping of multiple, related assays, and a Unipept project represents a collection of such studies.

On the file system, a project is stored in a single folder that contains an SQLite database file, a subfolder for each study and one text file per assay, located in the subfolder of the corresponding study. This folder can be modified outside of the application, using the default file explorer application of your operating system, thus providing maximum flexibility. All changes made to this project folder are automatically detected and imported by the application, granting users the ability to mass import assays and edit project properties with external applications. The application accepts simple text files with one peptide per line. In order to quantify peptide occurrence, a peptide can be included more than once in this file and the "filter duplicate peptides" option should be disabled for the analysis.

Because projects are folder-based, they can contain both the raw input data as well as the analysis results for an assay, making it practical for users to share projects with each other, for instance, in the form of compressed project folders. In addition, previously performed analyses do not need to be recomputed when the application is restarted, as opposed to analyses that were run on the Unipept website, which need to be recomputed every time the website is closed.

2.2.2 Comparative analysis

The Unipept Desktop Application provides both intra-assay and inter-assay comparative analyses that are rendered as heatmap visualizations. The intra-assay comparison can be started from the single assay analysis page by selecting the heatmap tab and provides a wizard to guide users through the set-up process of the comparison (Figure 2). Users are required to select two types of data sources (one for each axis of the heatmap) and indicate which items should be compared. Four different data sources are currently supported: NCBI taxa, GO terms (The Gene Ontology Consortium, 2019), EC numbers and InterPro entries (Finn *et al.*, 2017).

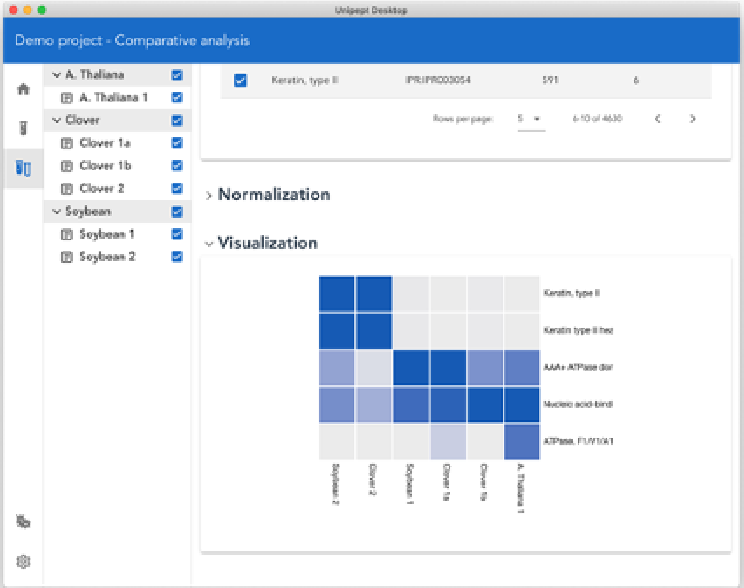
Unipept Desktop
Demo project - Comparative analysis
A. Thaliana
A. Thaliana 1
Clover
Clover 1a
Clover 1b
Clover 2
Soybean
Soybean 1
Soybean 2
Keratin, type II
IPR:IPR003054
591
6
Rows per page:
5
6-10 of 4630
Normalization
Visualization
Keratin, type II
Keratin type II hea
AAA+ ATPase dor
Nucleic acid-bind
ATPase, F1/V1/A1
Soybean 2
Clover 2
Soybean 1
Clover 1a
Clover 1b
A. Thaliana 1

Figure 2.2: Screenshot of the inter-assay comparative analysis pipeline. Note that it is possible to select multiple assays from the project explorer. A heatmap is constructed from the set of items that were selected for comparison at the top of the page.

The inter-assay comparative analysis is designed to visualize differences and similarities in functional or taxonomic composition of multiple assays. Here too, users are presented with a wizard that is similar to the one found in the intra-assay comparison. For inter-assay comparisons, however, the horizontal axis of the heatmap is reserved for the set of selected assays, and users can therefore only select one collection of items that should be compared between the different assays.

Because the number of peptides can drastically differ between multiple assays, three different normalization techniques are provided to the user. The default setting normalizes the heatmap globally, i.e. the minimum and maximum values over the complete grid are computed and all grid values are normalized with respect to these values. The other two normalization techniques also normalize based on minimum and maximum values, but restricted within a row or column, respectively.

It is worth noting that, while the comparative analysis pipeline was originally designed for the Unipept Desktop Application, a slimmed-down version has meanwhile also been integrated into the Unipept web app.

With the advent of the Unipept Desktop Application, users now have a variety of ways in which they can use Unipept. A comparison between the various functionalities offered by these different services is provided in Table 2.1.

2.3 Conclusion

Unipept Desktop is a novel desktop application that extends upon the Unipept web application by eradicating the strict limitations posed by the web-based nature of this application to increase metaproteomics data analysis throughput. Moreover, the Unipept Desktop Application adds new features such as allowing users to structure their data in a hierarchical project-based system, to keep track of their analysis results, and to share or distribute these results very easily. Whereas the Unipept web application is limited to assays with up to 50 000 peptides, the Unipept Desktop Application supports assays containing one million peptides or more. For reference, the desktop app can analyze between 250 and 2000 peptides per second (without advanced missed cleavage handling enabled), depending on the type of assay that’s being analyzed.

	desktop app	web app	CLI	API
visualizations	✓	✓	∼	∼
basic metaproteomics analysis pipeline	✓	✓	✗	✗
tryptic peptide analysis pipeline	✓	✓	✗	✗
comparative analysis	✓	∼	✗	✗
metadata or projects	✓	✗	✗	✗
custom endpoint	✓	✗	✓	✗
store analysis results	✓	✗	∼	✗
process large samples	✓	✗	✓	✓
no command line knowledge required	✓	✓	✗	✗
no installation required	✗	✓	✗	✓

Table 2.1: Comparison of the functionalities provided by the different Unipept services.

In a future release of the Unipept Desktop Application, we plan to provide support for the preparation of custom reference databases and further improve support for offline analysis. This will allow us to gradually evolve to a tool that is not only suitable for metaproteomics data analysis, but also for novel proteogenomics analysis techniques for complex environmental samples.

Our choice for the Electron framework proves to be very valuable as well, as a large portion of Unipept's codebase can thus be shared between the new desktop application and the existing web application. This in turn allows us to easily migrate (a slimmed-down version of) specific desktop features to the web app, and vice versa.

2.4 Availability

The source code for Unipept Desktop is open source and provided under the MIT license as a repository on GitHub: https://github.com/unipept/unipept-desktop. Pre-generated installers for Windows, macOS and Linux (AppImage format) can be downloaded from the release page of our GitHub repository. Installation instructions and documentation for the Unipept Desktop Application can be found on our website: https://unipept.ugent.be/desktop.

2.5 Acknowledgements

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Chapter 3

Support for novel proteogenomics analysis in Unipept

Part II

Other projects

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Chapter 4

MegaGO: a fast yet powerful approach to assess functional Gene Ontology similarity across meta-omics data sets

Abstract The study of microbiomes has gained in importance over the past few years and has led to the emergence of the fields of metagenomics, metatranscriptomics, and metaproteomics. While initially focused on the study of biodiversity within these communities, the emphasis has increasingly shifted to the study of (changes in) the complete set of functions available in these communities. A key tool to study this functional complement of a microbiome is Gene Ontology (GO) term analysis. However, comparing large sets of GO terms is not an easy task due to the deeply branched nature of GO, which limits the utility of exact term matching. To solve this problem, we here present MegaGO, a user-friendly tool that relies on semantic similarity between GO terms to compute the functional similarity between multiple data sets. MegaGO is high performing: Each set can contain thousands of GO terms, and results are calculated in a matter of seconds. MegaGO

is available as a web application at https://megago.ugent.be and is installable via pip as a standalone command line tool and reusable software library. All code is open source under the MIT license and is available at https://github.com/MEGA-GO/.

4.1 Introduction

Microorganisms often live together in a microbial community or microbiome where they create complex functional networks. These microbiomes are therefore commonly studied to reveal both their taxonomic composition as well as their functional repertoire. This is typically achieved by analyzing their gene content using shotgun metagenomics. Whereas this approach allows a detailed investigation of the genomes that are present in such multiorganism samples, it reveals only their functional potential rather than their currently active functions (Jansson and Baker, 2016). To uncover these active functions within a given sample, the characterization of the protein content is often essential (Lohmann *et al.*, 2020).

The growing focus on functional information as a complement to taxonomic information (Louca *et al.*, 2016) is derived from the observation that two taxonomically similar microbial communities could have vastly different functional capacities, whereas taxonomically quite distinct communities could have remarkably similar functions. Whereas the investigation of the active functions is thus increasingly seen as vital to a complete understanding of a microbiome, the identification and comparison of these detected functions remains one of the biggest challenges in the field (Schiebenhoefer *et al.*, 2019).

Several omics tools exist to describe functions in microbial samples, although these tools link functionality to different biological entities such as genes, transcripts, proteins, and peptides (Muth *et al.*, 2015, 2018; Van Den Bossche *et al.*, 2020; Verschaffelt *et al.*, 2020; Gurdeep Singh *et al.*, 2019; Riffle *et al.*, 2018; Schneider *et al.*, 2011; Schiebenhoefer *et al.*, 2020; Huerta-Cepas *et al.*, 2019; Huson *et al.*, 2007). However, most tools are capable of directly or indirectly reporting functional annotations as a set of Gene Ontology (The Gene Ontology Consortium, 2019) (GO) terms, regardless of the biological entities they are assigned to. In October 2020, there were 44264 of these terms in the complete GO tree. GO terms are organized into three independent domains: molecular function, biological process, and cellular component (Ashburner *et al.*, 2000). In each domain, terms are linked into a directed acyclic graph, an excerpt of which is shown in Figure 4.1. In the GO graph, a parent term can have one or more child (e.g., the root node "biological process" is the parent of the children GO:0009987 and GO:0008152), and children can have multiple parents (e.g., the most specific term "translation" has GO:0043043, GO:0034645, and GO:0044267 as parents).

Whereas this highly branched graph structure of GO allows flexible annotation at various levels of detail, it also creates problems when the results from one data set are compared to those of another data set. Indeed, even though two terms may be closely linked in the GO tree and are therefore highly similar (e.g., as parent and child terms or as sibling terms), the typically employed exact term matching will treat these terms as wholly unrelated, as the actual GO terms (and their accession numbers) are not identical. This problem is illustrated in a study by (Sajulga *et al.*, 2020), where a multisample data set was analyzed using several metaproteomics tools. The resulting GO terms were then compared using exact matching. The overlap between the result sets was quantified using the Jaccard index and was found to be low. As previously explained, this low similarity is likely the result of the limitations of the exact term matching approach.

There is thus a clear need for a more sophisticated GO term comparison that takes into account the existing relationships in the full GO tree. However, most existing tools that provide such comparison are based on enrichment analyses (Huang *et al.*, 2009; Waardenberg *et al.*, 2015; Fruzangohar *et al.*, 2013). In such analyses, a list of genes is mapped to GO terms, which are then analyzed for enriched biological phenomena. As a result, to the best of our knowledge, no tools allow the direct comparison of large functional data sets against each other, nor are these able to provide metrics to determine how functionally similar data sets are.

We therefore present MegaGO, a tool for comparing the functional similarity between large sets of GO terms. MegaGO calculates a pairwise similarity score between multiple sets of GO terms for each of the three GO domains and can do so in seconds, even on platforms with limited computational capabilities.

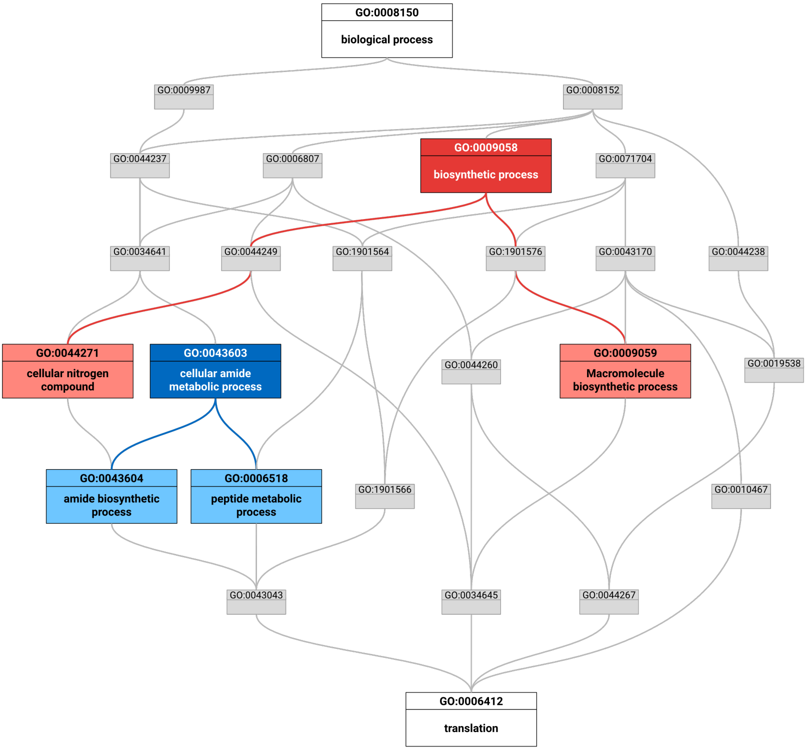
GO:0008150
biological process
GO:0009987
GO:0008152
GO:0044237
GO:0006807
GO:0009058
biosynthetic process
GO:0071704
GO:0034641
GO:0044249
GO:1901564
GO:1901576
GO:0043170
GO:0044238
GO:0044271
cellular nitrogen compound
GO:0043603
cellular amide metabolic process
GO:0044260
GO:0009059
Macromolecule biosynthetic process
GO:0019538
GO:0043604
amide biosynthetic process
GO:0006518
peptide metabolic process
GO:1901566
GO:0010467
GO:0043043
GO:0034645
GO:0044267
GO:0006412
translation

Figure 4.1: Excerpt of the biological process domain of the Gene Ontology showing all parent terms up to the root for "translation" (GO:0006412). The root GO term "biological process" (GO:0008150) has multiple children. The most specific term "translation", in contrast, has multiple parents. When comparing the two terms GO:0044267 and GO:0034645 (portrayed in light red), we find two different lowest common ancestors: GO:0044249 and GO:1901576 (dark red). Only one of these, however, can be the most informative common ancestor (MICA), that is, the common ancestor with the highest information content for the terms in light red. Because an IC of 1.52 is larger than 1.48, the GO:0044249 is the MICA. The terms GO:0043604 and GO:0006518 (in light blue) are more similar than the two terms we previously described and have only one lowest common ancestor, which is also automatically the MICA for these terms: GO:0043603 (in dark blue). IC, information content; *, most informative common ancestor.

4.2 Implementation

To measure the similarity between sets of GO terms, we first need to measure the similarity of two individual terms. We compare two terms using the Lin semantic similarity metric, which can take on a value between 0 and 1 (Supplementary Formula 1a). The Lin semantic similarity is based on the ratio of the information content of the most informative common ancestor (MICA) to the average of the terms' individual information content.

The information content (Supplementary Formula 1b) is computed by estimating the terms' probability of occurrence (Supplementary Formula 1c), including that of all of their children. Term frequencies are estimated based on the manually curated SwissProt database (The UniProt Consortium, 2019). As a result, a high-level GO term such as "biological process" (through its many direct or indirect child terms) will be present in all data sets and thus carries little information. A more specific term such as "translation" (or any of its potential child terms) will occur less frequently and thus will be more informative (Figure 4.1). To finally calculate the similarity of two terms, we compare their information content with that of their shared ancestor that has the highest information content, the MICA. If the information content of the MICA is similar to the terms' individual information content, then the terms are deemed to be similar. The dissimilar terms "peptide biosynthetic process" and "cellular macromolecule biosynthetic" are situated further from their MICA "cellular biosynthetic process" than the similar terms "amide biosynthetic process" and "peptide metabolic process" with their respective MICA "cellular amide metabolic process" (Figure 4.1).

MegaGO, however, can compare not only two terms but also sets of GO terms. More specifically, two sets of GO terms can be compared via the web application, but an unlimited number of sets can be compared via the command line tool. Note that in these sets, duplicate GO terms will be removed so that each GO term will be equally important, regardless of how often it is provided by the user. To compare the sets of GO terms, pairwise term similarities are aggregated using the Best Matching Average (BMA, Supplementary Formula 2) (Schlicker *et al.*, 2006). For each GO term in the first input data set, the BMA finds the GO term with the highest Lin semantic similarity in the second data set and averages the values of these best matches. Moreover, MegaGO calculates the similarity for each of the three domains of the gene ontology (molecular function, biological process, and cellular component), as GO terms from distinct domains do not share parent terms. The general overview of MegaGO is shown in Figure 4.2.

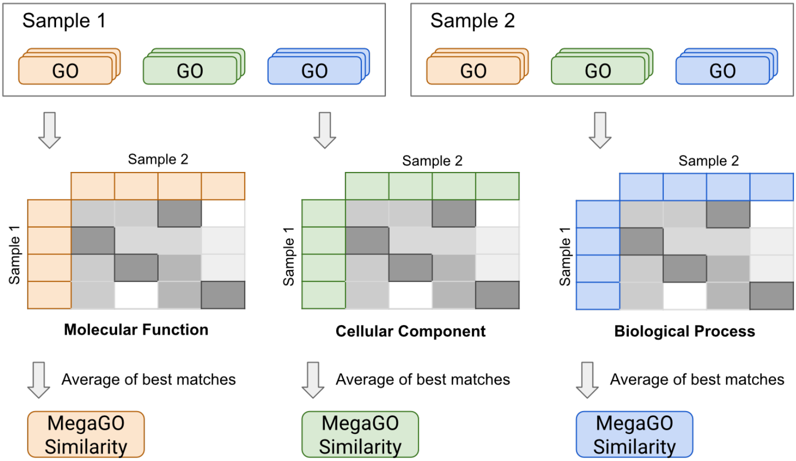
Sample 1
GO
GO
GO
Sample 2
GO
GO
GO
Sample 2
Sample 1
Molecular Function
Sample 2
Sample 1
Cellular Component
Sample 2
Sample 1
Biological Process
Average of best matches
Average of best matches
Average of best matches
MegaGO Similarity
MegaGO Similarity
MegaGO Similarity

Figure 4.2: Overview of MegaGO workflow. The Gene Ontology (GO) terms of each sample set are separated into three GO domains: molecular function, cellular component, and biological process. Each term of each sample set is compared to every term in the other set that is from the same domain. The match with highest similarity for each term is then selected, and the average across all of these best matches is calculated.

MegaGO is implemented in Python, is installable as a Python package from PyPi, and can easily be invoked from the command line. The GOATOOLS (Klopfenstein *et al.*, 2018) library is used to read and process the Gene Ontology and to compute the most informative common ancestor of two GO terms, which are both required to compute the information content value (Supplementary Formula 1, p(go)). GO term counts are recomputed with every update of SwissProt, and a new release is automatically published bimonthly to PyPi, which includes the new data set. Automated testing via GitHub Actions is in place to ensure correctness and reproducibility of the code. In addition, we also developed a user-friendly and easily accessible web application that is available on https://megago.ugent.be. The backend of the web application is developed with the Flask web framework for Python, and the frontend uses Vue. Our web application has been tested on Chromium-based browsers (Chrome, Edge, and Opera) as well as Mozilla Firefox and Safari. The MegaGO application is also available as a Docker-container on Docker Hub (https://hub.docker.com/repository/docker/pverscha/mega-go) and can be started with a single click and without additional configuration requirements. Our Docker container is automatically updated at every change to the underlying MegaGO code. All code is freely available under the permissive open source MIT license on https://github.com/MEGA-GO/. Documentation for our Python script can be found on our Web site: https://megago.ugent.be/help/cli. A guide on how to use the web application is also available: https://megago.ugent.be/help/web.

MegaGO is cross-platform and runs on Windows, macOS, and Linux systems. The system requirements are at least 4 GiB of memory and support for either Python 3.6 (or above) or the Docker runtime.

4.3 Validation

To validate MegaGO, we reprocessed the functional data from (Easterly *et al.*, 2019). This data set consists of 12 paired oral microbiome samples that were cultivated in bioreactors. Each sample was treated with and without sucrose pulsing, hereafter named ws and ns samples, respectively. Each sample contains mass-spectrometry-based proteomics measurements, and all samples were annotated with 1718 GO terms on average. We calculated the pairwise similarity for each of the 300 sample combinations, which took less than 1 min for a single sample pair on the web version of MegaGO. This resulted in a MegaGO similarity score for each of the three GO domains for each sample combination. These similarities were then hierarchically clustered and visualized in a heatmap. All data and intermediate steps of our data analysis are available at https://github.com/MEGA-GO/manuscript-data-analysis/ and can be reproduced with the command line tool using the –heatmap option.

In the heatmap (Figure 4.3), we can observe that the two sample groups cluster together, except for 730ns and 733ns that are clustered in the ws sample group. These two samples were also identified as outliers in

(Easterly *et al.*, 2019) and 733ns was originally also identified as both a taxonomic and functional outlier in (Rudney *et al.*, 2015). Similar results can be observed for the GO domain “molecular function” (Supplementary Figure 1). The MegaGO similarity-based clustering of “cellular component” GO terms (Supplementary Figure 2) has two additional samples clustered outside of their treatment group: 852ws in the ns cluster and 861ns in the ws group. Again, these patterns can also be found in previous analyses: 852ws is placed in direct proximity of the ns samples in the principal component analysis (PCA) of the HOMINGS analysis by Rudney et al., and 861ns is closest to 730ns and 733ns in PCA of Rudney et al.’s taxonomic analysis. Interestingly, subjects 730 and 852 were the only ones without active carious lesions, which could cause their divergence in the similarity analyses.

Results produced by MegaGO are thus in close agreement with prior analyses of the same data, showing that MegaGO offers a valid and very fast approach for comparing the functional composition of samples.

4.4 Conclusions

MegaGO enables the comparison of large sets of GO terms, allowing users to efficiently evaluate multiomics data sets containing thousands of terms. MegaGO separately calculates a similarity for each of the three GO domains (biological process, molecular function, and cellular component). In the current version of MegaGO, quantitative data are not taken into account, thus giving each GO term identical importance in the data set.

MegaGO is compatible with any upstream tool that can provide GO term lists for a data set. Moreover, MegaGO allows the comparison of functional annotations derived from DNA-, RNA-, and protein-based methods as well as combinations thereof.

4.5 Acknowledgements

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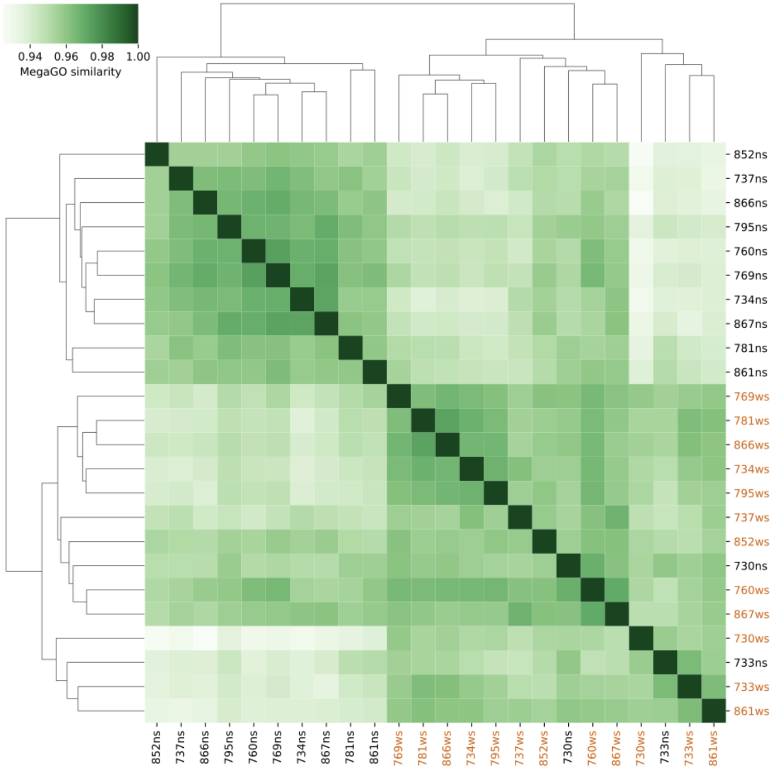

Figure 4.3: Hierarchically clustered heatmap comparing MegaGO similarities for the GO domain "biological process" for each of the samples from (Easterly *et al.*, 2019) Samples that are treated with sucrose pulsing are labeled as "ws" and are displayed in orange.

Berlin, Germany) and Stephan Fuchs (Robert Koch Institute, Berlin, Germany) for their support. P.V., T.V.D.B., L.M., and B.M. acknowledge the Research Foundation - Flanders (FWO) (grants 1164420N, 1S90918N, G042518N, and 12I5220N). L.M. also acknowledges support from the European Union's Horizon 2020 Programme under grant agreement 823839 (H2020-INFRAIA-2018-1). H.S. and B.Y.R. acknowledge support by Deutsche Forschungsgemeinschaft (DFG; grant numbers RE3474/5-1 and RE3474/2-2) and the BMBF-funded de.NBI Cloud within the German Network for Bioinformatics Infrastructure (de.NBI; 031A537B, 031A533A, 031A538A, 031A533B, 031A535A, 031A537C, 031A534A, and 031A532B).

Chapter 5

Unipept CLI 2.0: adding support for visualisations and functional annotations

Abstract Unipept (Mesuere *et al.*, 2012) is a collection of tools developed for fast metaproteomics data analysis. The Unipept ecosystem consists of a web application, an application programming interface (API) as a web service (Mesuere *et al.*, 2016) and a command-line interface (CLI) (Mesuere *et al.*, 2018). The key strengths of Unipept are its speed, its ease-of-use and the extensive use of interactive data visualization in the analysis results. The Unipept database is derived from the UniProt (UniProt, 2019) KB and consists of tryptic peptides linked with taxonomic and functional annotations. Unipept initially launched with support for taxonomic analysis of metaproteomics data in 2012. Version 4.0 (Gurdeep Singh *et al.*, 2019) of the Unipept web application was launched in November 2018 and extended the web interface with support for functional annotations such as Gene Ontology (GO) terms (Ashburner *et al.*, 2000), Enzyme Commission (EC) numbers (Webb, 1992) and InterPro entries (Hunter *et al.*, 2009).

5.1 Introduction

Unipept (Mesuere *et al.*, 2012) is a collection of tools developed for fast metaproteomics data analysis. The Unipept ecosystem consists of a web application, an application programming interface (API) as a web service (Mesuere *et al.*, 2016) and a command-line interface (CLI) (Mesuere *et al.*, 2018). The key strengths of Unipept are its speed, its ease-of-use and the extensive use of interactive data visualization in the analysis results. The Unipept database is derived from the UniProt (The UniProt Consortium, 2019) KB and consists of tryptic peptides linked with taxonomic and functional annotations. Unipept initially launched with support for taxonomic analysis of metaproteomics data in 2012. Version 4.0 (Gurdeep Singh *et al.*, 2019) of the Unipept web application was launched in November 2018 and extended the web interface with support for functional annotations such as Gene Ontology (GO) terms (Ashburner *et al.*, 2000), Enzyme Commission (EC) numbers (Webb, 1992) and InterPro entries (Hunter *et al.*, 2009).

The GO terms are organized into three different domains: 'cellular components', 'molecular functions' and 'biological processes'. Every GO-term is associated with exactly one domain and consists of a name, an identifier and an exact definition. The EC numbers can be used to classify enzymes, based on the chemical reactions that they catalyze. Every EC number consists of four numbers, separated by a dot, yielding a hierarchical classification system with progressively finer enzyme classifications. InterPro is a database that consists of predictive models collected from external databases that can be classified into five different categories. More information about functional annotation in metaproteomics can be found in the study by (Schiebenhoefer *et al.*, 2019).

For each input peptide, Unipept finds all functional annotations associated with all of the UniProt entries in which the peptide occurs. All found functions are listed in order of decreasing number of peptides associated with this function.

In this article, we present several new additions to the Unipept API and CLI which allow third-party applications [such as Galaxy-P (Jagtap *et al.*, 2015)] to integrate the new functional analysis capabilities provided by Unipept.

5.2 Materials and methods

The Unipept API is a high-performance web service that responds in a textual format (JSON) to HTTP-requests from other applications or tools and allows to integrate the services provided by Unipept into other workflows. Unipept's CLI is a Ruby-based application and high-level entry point which allows users to actively query Unipept's database. Compared to the API, users do not need to compile API-requests manually but can rely on the CLI to automatically do so in a parallelized way. In addition, it supports multiple input and output formats such as FASTA and CSV.

The Unipept database and web application were recently expanded to include GO terms, EC numbers and InterPro entries. These new annotations are now also available from newly developed API-endpoints and CLI-functions, providing structured access to this functional information.

Most of the newly developed endpoints support batch retrieval of information for a list of peptides. In this case, the API returns a list of objects where each object in the response corresponds with information associated with one of the input peptides. Every API-endpoint is accompanied by an identically named CLI-function, which provides the user with the ability to import data from or export data to various specifically formatted files. In addition, version 2 of the Unipept CLI introduces the ability to produce interactive visualizations directly from the command line.

Among other information, the Unipept tryptic peptide analysis lists functional annotations associated with a given tryptic peptide. These data are aggregated because a peptide can occur in multiple proteins that each can have multiple functional annotations. For each annotation, we also return the amount of underlying proteins that match with this specific annotation.

Some applications require all known information for a list of tryptic peptides. The 'pept2funct' function is a combination of the preceding three endpoints and returns all functional annotations associated with the given tryptic peptide. 'peptinfo' on the other hand, returns all the available information for one or more tryptic peptides. All functional annotations for this peptide are part of the response, as well as the lowest common ancestor for this peptide. Both functions also support splitting the GO terms and InterPro entries over the respective domains, and naming information can optionally be retrieved.

The ‘taxa2tree’ function constructs a tree from a list of NCBI taxon ids. This tree is an aggregation of the lineages that correspond with the given taxa and can be exported as three distinct output formats: JSON, HTML and as a URL. The HTML and URL representation of a taxonomic tree both provide three interactive data visualizations, albeit with different possibilities. A generated HTML-string first needs to be stored in a file before it can be rendered by a browser and cannot be easily shared with other people but is easily editable. A URL on the other hand is simply a shareable link to an online service that hosts all interactive visualizations.

5.3 Conclusion

Version 2.0 of the Unipept API and CLI is a significant update that provides fast and easy access to the powerful analysis pipeline of Unipept. In addition to the existing taxonomic analysis, it now features multiple functional annotations which will enable users to gain new insights into complex ecosystems. These new features can easily be integrated into third-party tools such as the MetaProteome Analyzer (Muth *et al.*, 2018). Galaxy-P, a highly used workflow integration system, is already successfully making use of the novel analysis functions that are introduced with this new release.

5.4 Funding

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Chapter 6

Unipept Visualizations: an interactive visualization library for biological data

Abstract The Unipept Visualizations library is a JavaScript package to generate interactive visualizations of both hierarchical and non-hierarchical quantitative data. It provides four different visualizations: a sunburst, a treemap, a treeview and a heatmap. Every visualization is fully configurable, supports TypeScript and uses the excellent D3.js library. The Unipept Visualizations library is available for download on NPM: `https://npmjs.com/unipept-visualizations`. All source code is freely available from GitHub under the MIT license: https://github.com/unipept/unipept-visualizations.

6.1 Introduction

Unipept is an ecosystem of software tools for the analysis of metaproteomics datasets that consists of a web application (Gurdeep Singh *et al.*, 2019), a desktop application (Verschaffelt *et al.*, 2021), a command line interface (Verschaffelt *et al.*, 2020) and an application programming interface. It provides taxonomic and functional analysis pipelines for metaproteomics data and highly interactive data visualizations that help interpret the outcome of these analyses.

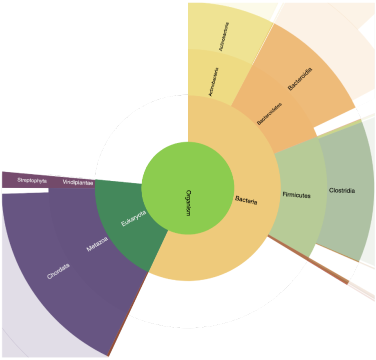
We developed custom visualizations used for Unipept from scratch because existing libraries, such as Krona (Ondov *et al.*, 2011), were lacking essential features or were hard to integrate. They were designed as generic tools to visualize hierarchical quantitative data and can therefore also be used to visualize data from nonproteomics origins. To facilitate reuse of these broadly usable components, we have isolated the visualizations from the main Unipept project and made them available as a standalone package that can easily be reused by other software tools. We released this package under the permissive MIT open-source license, so researchers from other disciplines are free to reuse these visualizations and connect them to their own data sources. Currently, our visualizations are already incorporated in TRAPID 2.0, a web application for the analysis of transcriptomes ((Bucchini *et al.*, 2021) and UMGAP, the Unipept MetaGenomics Pipeline (Van der Jeugt *et al.*, 2022).

6.2 Visualizations

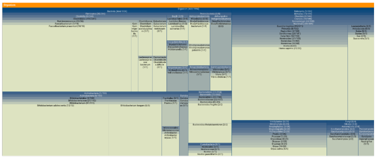
We currently provide four highly interactive data visualizations that are all designed for a specific purpose: a sunburst, a treeview, a treemap and a heatmap. The sunburst (Figure 6.1a), treeview (Figure 6.1d) and treemap (Figure 6.1b) can be used to visualize quantitative hierarchical data and are designed to depict the parent–child relationship of a hierarchy of nodes as clearly as possible, while still incorporating the strength of the relationship between, or the counts associated with, connected nodes. The heatmap (Figure 6.1c), conversely, is not suitable to visualize hierarchical information but displays a magnitude in two dimensions, including optional clustering and dendrogram rendering.

6.2.1 Quantitative hierarchical data visualizations

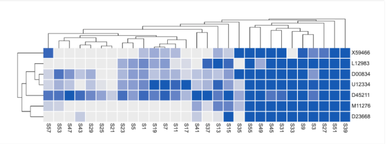
Hierarchical data occurs throughout a variety of bioinformatics disciplines. In the metaproteomics research area alone, many examples of hierarchical data exist, such as the hierarchical structure of the NCBI taxonomy (Schoch *et al.*, 2020), the hierarchy imposed by the enzyme commission numbers and the gene ontology terms (The Gene Ontology Consortium, 2019). In most cases, quantitative data are available for multiple nodes at many levels in the hierarchy. For example, Unipept



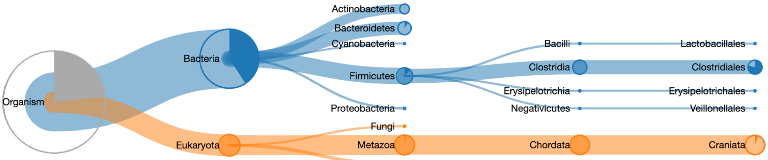
a) Sunburst



b) Treemap



c) Heatmap



d) Treeview

Figure 6.1: Overview of the visualizations currently provided by the Unipept Visualizations library. All examples were generated with default configuration settings, except for the heatmap for which the setting 'dendrogramEnabled' was set to 'true'.

assigns peptide counts to taxa that are scattered around the NCBI taxonomy, including identifications that are highly specific (near leaves of the tree) or lack deep taxonomic resolution (near the root of the tree). Being able to interactively zoom in and out on the hierarchical data enables exploratory analysis.

The three visualizations for hierarchical data provided by our package take input data in the same hierarchical format, making it trivial to switch between the different types of visualization once the input data are formatted correctly.

6.2.2 Quantitative non-hierarchical data visualizations

A heatmap (Figure 6.1c) is a well-known visualization that consists of a two-dimensional grid of cells in which each cell is assigned a specific color from a scale corresponding to its magnitude. The heatmap implementation in our package provides this functionality in an extensively customizable form. Users can reorganize elements, change the color scheme and update label information, among other operations. All values are also automatically normalized to a $[0, 1]$-interval.

As neighboring rows and columns in the input data can have very distinct values, and as this can interfere with reasoning about the heatmap, it is important to group similar values. Our implementation achieves this through hierarchical clustering based on the UPGMA algorithm (Sokal and Michener, 1958). The produced grouping of rows and columns is further clarified by an optional dendrogram that can be plotted alongside each axis of the heatmap.

However, after clustering, it can still occur that two consecutive leaves in a dendrogram are quite dissimilar due to the $2^n - 1$ possible linear orderings that can be derived from a dendrogram (a dendrogram contains $n - 1$ flipping points for which both children can be switched). This can be addressed by reordering the leaves of the tree, as the orientation of the children of all n nodes in a dendrogram can be flipped without affecting the integrity of the dendrogram itself. Our heatmap implementation uses the Modular Leaf Ordering technique (Sakai *et al.*, 2014) to reorder all leaves of the dendrogram such that the distance between consecutive leaves is minimized. This technique is a heuristic that performs very well in comparison to the more resource-intensive Optimal Leaf Ordering (Bar-Joseph *et al.*, 2001) or Gruvaeus–Wainer algorithms (Gruvaeus and Wainer, 1972).

6.3 Implementation

The visualization package has been developed with D3 (Bostock *et al.*, 2011) and TypeScript (Bierman *et al.*, 2014) and every visualization is displayed in the web browser with one of two technologies: SVG or HTML5 canvas. SVG's are easy-to-use and are scalable by nature but often lack necessary performance for complex interactive visualizations. HTML5 canvas, in contrast, provides much better performance using a rasterized image.

Every visualization is presented as a single JavaScript class and provides a full set of configuration options to extend and configure the visualization. New versions of the package will automatically be published on NPM (https://npmjs.org) and GitHub (https://github.com/unipept/unipept-visualizations), so that any project depending on it package can always use the latest version.

We also provide an extensive set of documentation resources that ease the adoption process of our package, as well as a collection of live notebooks (see https://observablehq.com/collection/@unipept/unipept-visualizations). These notebooks provide interactive and editable examples that demonstrate the full potential and guide users through the different configuration options. The code and resources that make up the live notebooks can be modified online and provide a very convenient way to try out the package.

6.4 Funding

This work has been supported by the Research Foundation–Flanders (FWO) [1164420N to P.V.; 12I5220N to B.M.; G042518N to L.M.].

Chapter 7

Other projects

During the course of my career as a PhD student, I have also been working on a lot of different research projects for which I was not the main contributor, but for which I, nonetheless, provided a significant addition. I have selected two of these projects and included them as sections in this chapter.

7.1 Pout2Prot: An efficient tool to create protein (sub)groups from Percolator output files

Abstract In metaproteomics, the study of the collective proteome of microbial communities, the protein inference problem is more challenging than in single-species proteomics. Indeed, a peptide sequence can be present not only in multiple proteins or protein isoforms of the same species, but also in homologous proteins from closely related species. To assign the taxonomy and functions of the microbial species, specialized tools have been developed, such as Prophane. This tool, however, is not directly compatible with post-processing tools such as Percolator. In this manuscript we therefore present Pout2Prot, which takes Percolator Output (.pout) files from multiple experiments and creates protein group and protein subgroup output files (.tsv) that can be used directly with Prophane. We investigated different grouping strategies and compared existing protein grouping tools to develop an advanced protein grouping algorithm that offers a variety of different

approaches, allows grouping for multiple files, and uses a weighted spectral count for protein (sub)groups to reflect abundance. Pout2Prot is available as a web application at https://pout2prot.ugent.be and is installable via pip as a standalone command line tool and reusable software library. All code is open source under the Apache License 2.0 and is available at https://github.com/compomics/pout2prot.

7.1.1 Introduction

In metaproteomics, the study of the collective proteome of whole (microbial) ecosystems, it is important to learn about the taxonomy and functions represented in the community. For this purpose, tools such as Unipept (Verschaffelt *et al.*, 2021) and Prophane (Schiebenhoefer *et al.*, 2020) have been made available to specifically perform downstream annotation of metaproteomic data, while other, more generic tools also provide connections to downstream annotation tools (Schiebenhoefer *et al.*, 2020; Van Den Bossche *et al.*, 2020; Muth *et al.*, 2015). These tools, however, work very differently: while Unipept relies on identified peptides without inferring the corresponding proteins (a peptide-centric approach), Prophane uses protein groups as input (a protein-centric approach). Recently, these two tools were compared in the first multi-lab comparison study in metaproteomics (CAMPI), (Van Den Bossche, Kunath, *et al.*, 2021) which indicated that the choice between these approaches is a matter of user preference.

The process of grouping proteins is unfortunately not as straightforward as it might first appear (Martens and Hermjakob, 2007; Uszkoreit *et al.*, 2015; Audain *et al.*, 2017; Nesvizhskii and Aebersold, 2005). Identified peptide sequences have to be assembled into a list of identified proteins, but when a peptide can be mapped to multiple proteins, this leads to the protein inference problem (Nesvizhskii and Aebersold, 2005). In metaproteomics, this problem is exacerbated due to the presence of homologous proteins from multiple species in its necessarily large protein databases (Schiebenhoefer *et al.*, 2019). Protein grouping is therefore commonly used to generate a more manageable list of identified protein groups that can be used for further downstream analysis. However, different protein grouping algorithms can be chosen, leading to different lists of protein groups from a single set of identified peptides (Martens and Hermjakob, 2007). In the past, many protein grouping methods have been developed, as reviewed in Audain et al., (Audain *et al.*, 2017) but these typically do not interface well with post-processing tools like Percolator, (Käll *et al.*, 2007) which are able to increase the number of peptide-to-spectrum matches (PSMs) due to a better separation of true and false matches (Bouwmeester *et al.*, 2020). Moreover, the common strategy used by these tools is the Occam's razor strategy, which is not always ideal (Van Den Bossche, Kunath, *et al.*, 2021). We here therefore present a new tool, Pout2Prot, which provides users with two relevant protein inference options that are tailored toward metaproteomics use cases: Occam's razor and anti-Occam's razor. Occam's razor is based on the principle of maximum parsimony and provides the smallest set of proteins that explains all observed peptides. Here, however, proteins that are not matched by a unique peptide are discarded, and their associated taxonomy and functions, which might actually be present in the sample, are lost. This algorithm is for example used in the X!TandemPipeline (Langella *et al.*, 2017). On the other hand, anti-Occam's razor is based on the maximal explanatory set of proteins, where any protein that is matched by at least one identified peptide will be included in the reported protein list. This algorithm is used in, for example, MetaProteomeAnalyzer (MPA) (Muth *et al.*, 2015). Unfortunately, there is no simple way to determine a priori which algorithm will be optimal, as this can differ from sample to sample (Muth *et al.*, 2015). These strategies are visually represented in Figure 1.

Figure 1. Protein grouping algorithms Occam's razor (left) and anti-Occam's razor (right). Groups can be based on shared peptide rule (protein groups) or on shared peptide set rule (protein subgroups). This figure also illustrates how PSMs are assigned to protein (sub)groups and shows the weighted PSM count for subgroups. When a PSM is assigned to multiple subgroups, it will be calculated as one divided by the number of subgroups, which can result in fractional PSM counts.

Moreover, as proteins are grouped based on their identified peptides, carefully defined rules are required on when and how to group these proteins. There are two possible approaches here: the first approach consists of grouping all proteins that share one or more identified peptides (i.e., the shared peptide rule), while the second approach consists of only grouping proteins that share the same set (or subset) of identified peptides (i.e., the shared peptide set rule). These two approaches can also be interpreted as grouping at two different levels: the protein group level (based on the shared peptide rule) and the protein subgroup level (based on the shared peptide set rule). These two approaches are also visualized in Figure 1.

Pout2Prot implements all of these approaches: Occam's razor and anti-Occam's razor, and both of these at the protein group and protein subgroup level. During conceptualization and testing, we discovered challenges with the naive description of these algorithms. First, different protein subgroups can have the same peptide and therefore have the same spectrum assigned to them, leading to distorted spectrum counts. Second, when removing proteins using Occam's razor or when assigning subgroups using anti-Occam's razor, "undecidable" cases can occur as illustrated in Figure 2. In these undecidable cases, the naive approach might produce inconsistent results when the algorithm is run multiple times.

Figure 2. Illustration of undecidable cases. Undecidable cases are situations where peptides and proteins are matched in such a way that the naive interpretation of the algorithm cannot make a clear decision. Specifically, this occurs in Occam's razor when one of two or more proteins can be removed to explain the remaining peptides (Figure 2, left), and this occurs in anti-Occam's razor when a protein can be put into a subgroup with two or more other proteins that cannot be subgrouped together (Figure 2, right).

In this manuscript, we describe a new command line tool and web application that can convert .pout files from different experiments into two files containing protein groups and subgroups either as .tsv for direct use with Prophane or as human readable .csv files. Furthermore, we include a file converter that turns Proteome Discoverer output files into the .pout file format. Thus, Pout2Prot enables Percolator (or Proteome Discoverer users) to use Prophane for downstream functional and taxonomic analysis.

7.1.2 Implementation

Pout2Prot is implemented in Python and installable as a Python package from PyPI. It can then be invoked from the command line. We also provide a user-friendly and easily accessible web application of our tool (https://pout2prot.ugent.be). The transpiler Transcrypt (https://www.transcrypt.org/) was used to convert our Python package into JavaScript-compatible code and reuse it in our web application. Protein grouping analysis is efficient and can, consequently, be performed entirely on the user's local machine. Moreover, the web application processes all data locally, so that no data is sent to our servers. This safeguards user data and allows researchers to analyze confidential information more safely.

The detailed implementation of the protein grouping algorithms is visualized in the Supporting Information (Figure S1 and S2) and consists of four sub-algorithms: the creation of protein groups, the removal of proteins using the rule of maximum parsimony, and a subgroup algorithm each for Occam's razor and anti-Occam's razor.

7.1.3 Evaluation

Pout2Prot converts .pout files to protein (sub)group files that can be immediately imported in Prophane for further downstream analysis. This Prophane input file consists of four tab-separated fields: sample category, sample name, protein accessions, and spectrum count. The sample category allows users to divide their experiment in different categories (e.g., "control" and "disease"). If no sample categories are provided, these values will be identical to the sample name, which results in individual quantification by Prophane. The sample name is identical to the name of the .pout file, so each protein (sub)group can be traced back to its origin file. The protein accessions will contain the proteins present in the protein (sub)group, based on the chosen strategy. Finally, the spectrum count contains the weighted spectrum count from all PSMs present in that protein (sub)group, with PSMs present in multiple subgroups counted as fractional values in each subgroup.

7.1.3.1 Qualitative comparison to other tools

To develop a protein grouping algorithm and to truly compare different protein grouping tools, the behavior of the algorithm must be validated against a set of well-defined data, where differences between expected and observed behavior (i.e., the composition of the groups) can be clearly distinguished. During the development of Pout2Prot, it quickly became clear that multiple algorithms can solve certain test cases, but fail at others. This also led to the discovery of the undecidable cases outlined in Figure 2. Therefore, we created 14 test cases (Supporting Information, Figures S3–S16) that capture all possible pitfalls of protein grouping algorithms, and solved those cases by using both Occam's razor and anti-Occam's razor at the protein group and subgroup level. To resolve the issue of undecidability, we propose that no choice should be made at all. For undecidable cases for protein removal (Occam's razor), no protein should be removed, and for undecidable cases of protein subgroups (anti-Occam's razor), the protein in question should remain in its own subgroup.

Table 1 shows the result of the comparison between five protein grouping tools: PIA, Fido (integrated into Percolator), MetaProteomeAnalyzer (MPA), X!TandemPipeline, and Pout2Prot. To run tests with each tool, appropriate input files that reflect the test cases were created manually, and these are all available on the Pout2Prot GitHub repository. If a test case did not produce the expected output, it was investigated more closely to ensure this was not the result of differences between, or potential errors in, these input files. For undecidable cases, it was verified that the random choice behavior could be observed (i.e., multiple analyses, different results). For anti-Occam's razor subgrouping Cases 3 and 10, a difference in behavior was observed for PIA and Fido that can be attributed to a different conception of what a protein group is. Specifically, if a protein's peptide set is a strict subset of another protein's peptide set, PIA and Fido will not group these two proteins, while MPA and Pout2Prot will. Of all the tests that could be run, one resulted in an error: the algorithm for X!TandemPipeline for Case 13. In this case, only one of the six proteins was put into a single group, which leads to a situation where one of the three peptides was not explained by the resulting groups.

Table 1. Comparison of the Outcome of Test Cases for Five Protein Grouping Tools (The 14 test cases were run with the PIA, Fido (Percolator), MetaProteomeAnalyzer (MPA), X!TandemPipeline, and Pout2Prot. Test cases producing the expected outcome are marked as "successful" (green). Otherwise, these are either categorized as "undecidable" (yellow) if a random choice was made in case of undecidability, "incorrect" (red) if the result cannot be explained logically, and as "different approach" for PIA and Fido, because the anti-Occam protein subgrouping approach used here follows different rules (blue). If a tool does not implement a certain grouping method it is marked as "not implemented" (grey).)

While we tried to make a fair comparison, it should be noted that PIA also offers and even recommends another option that falls in between Occam's razor and anti-Occam's razor. This method called SpectrumExtractor uses spectrum level information to determine which proteins should be removed or grouped together. Furthermore, Fido offers an option similar to Occam's razor that operates at the level of the protein database. Percolator and other tools (e.g., Triqler (The and Käll, 2019)) assign probabilities to proteins instead of making a binary choice for each protein. In contrast, Pout2Prot is based on the binary model in which a peptide or protein is either identified or not. This choice is influenced by the fact that a probabilistic approach makes the assignment of taxonomies and functions in metaproteomics very difficult.

7.1.3.2 Performance evaluation

To evaluate the performance of Pout2Prot, we tested it on a metaproteomics data set, derived from the six selected SIHUMIx (Schäpe *et al.*, 2019) data sets used in the Critical Assessment of Metaproteome Investigation (CAMPI) study (Van Den Bossche, Kunath, *et al.*, 2021). Here, we used the X!Tandem (Craig and Beavis, 2004) files available on PRIDE (Perez-Riverol *et al.*, 2019) (PXD023217) to (i) convert these files to Percolator Input (.pin) files with tandem2pin, (ii) process the .pin files with Percolator resulting in Percolator Output (.pout) files, and (iii) convert these .pout files to protein (sub)grouping files with Pout2Prot, once using Occam's razor, once using anti-Occam's razor.

Interestingly, the identification rate (the number of identified spectra divided by the total number of spectra measured) at 1% False Discovery Rate (FDR) increases on average by 7% when using Percolator (Figure 3a, blue bars (X!Tandem) vs red bars (Percolator)). It is important to notice that Pout2Prot takes into account the PSM FDR, not the protein FDR. As expected and described before, the semisupervised machine learning algorithm Percolator is able to increase the number of PSMs due to the better separation of true and false matches (Käll *et al.*, 2007; Bouwmeester *et al.*, 2020). More interestingly, we examined the effect of Percolator on the number of protein groups and subgroups. To establish the number of protein (sub)groups before Percolator analysis, we reanalyzed the publicly available raw files of the selected data sets with MPA, also using X!Tandem with identical search settings. Note here that MPA is only able to group proteins according to the anti-Occam's strategy, so only those numbers were compared in the section below.

Figure 3. (A) Identification rates per sample for X!Tandem and Percolator analyses. Here, the identification rate was defined as the number of identified spectra divided by the total number of spectra measured. (B) Number of protein (sub)groups compared between X!Tandem and Percolator for the anti-Occam's razor strategy, and number of protein (sub)groups using Percolator for the Occam's razor strategy. S03, S05, S07, S08, S11_F1–4, and S14_Rep1 refer to the six SIHUMIx samples.

In Figure 3b, we observe that after Percolator analysis, the number of protein groups per sample increased by 18.5% on average (blue vs red bars) and the number of protein subgroups per sample increased by 25.3% on average (yellow vs green bars). The total number of groups and subgroups across all samples increased more drastically (by 34.7% and 39.9%, respectively) in comparison to the averages per sample. All raw data is available in Supporting Information (Tables S1 and S2).

Furthermore, we also investigated the effect on the number of protein (sub)groups of combining different fractions at different places in the workflow. We combined (i) the Mascot Generic Format (.mgf) files before the X!Tandem search, (ii) before the Percolator search, and (iii) before Pout2Prot protein inference. Since the range for the number of protein (sub)groups constitute a 2–3% difference, the point in the workflow where the different files are combined, is of minimal impact (Supporting Information, Table S3). For completeness, an example result file for taxonomic and functional analysis after processing of Pout2Prot output in Prophane can be found in Supporting Information, Figures S17 and S18). In addition, the time for a Pout2Prot analysis (Occam's razor) for the complete SIHUMIx experiment via the web service was less than 5s.

7.1.4 Conclusion

Pout2Prot enables the conversion of Percolator output (.pout) files to protein group and protein subgroup files, based on either the Occam's razor or anti-Occam's razor strategy, and therefore closes an important gap in the bioinformatic workflow of metaproteomics data analysis. Moreover, Pout2Prot also allows the user to create protein (sub)groups across experiments. The output of Pout2Prot can be imported directly into Prophane, which in turn allows users to perform downstream taxonomic and functional analysis of metaproteomics samples.

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