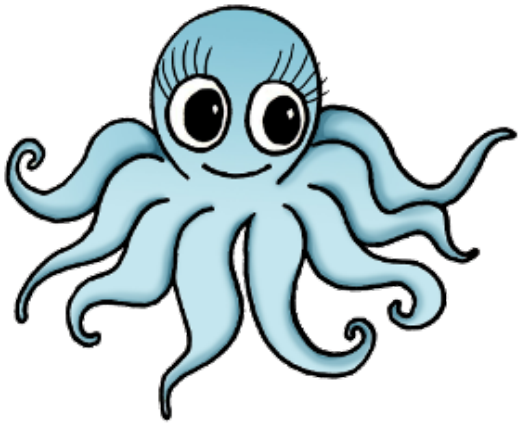
PIA – Protein Inference Algorithms
Tutorial



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https://github.com/mpc-bioinformatics/pia

Version 06/02/2017, 16:05:21.

Contents

1 Introduction

1.1 PIA – Protein Inference algorithms

PIA is an open source toolbox for MS based protein inference and identification analysis. As in bottom-up MS proteomics actually peptides are identified, but most often the entities of interest are proteins, the protein content of an analysed sample must be constructed from the knowledge of the contained peptides. This step is known as "protein inference" and is the heart of PIA. Furthermore, PIA can be used to inspect, analyse, perform quality checks on and filter identified peptide spectrum matches (PSMs) and peptides. This can be performed either via a web frontend, KNIME nodes or the command line. While the latter method is intended for advanced scripting and the command line can now also be downloaded as a Docker image, we will mainly discuss the usage of KNIME nodes in this tutorial.

1.2 Prerequisites

All data and workflows can be downloaded in the tutorial repository at `https://github.com/julianu/pia-tutorial`.

- Knowledge of mass spectrometry based bottom-up peptide identification
- Basic knowledge of KNIME and constructing workflows with KNIME
- You should be familiar with the OpenMS spectrum identification using KNIME (if not, please refer to the OpenMS tutorials at `http://www.openms.de/tutorials/`)
- The tutorial was tested using the stable OpenMS 2.1.0 nodes and KNIME 3.3.1
- For parts of the tutorial you need the R nodes of KNIME

1.3 Version

This tutorial was created on 06/02/2017 at 16:05:21.

2 Installation of PIA KNIME nodes

If not yet done, you first need to download and install KNIME to your system (`https://www.knime.org/downloads/overview`). The easiest way to work with PIA inside KNIME is to install the "**KNIME Analytics Platform + all free extensions**", which comes with many nice nodes and functions, including the PIA nodes.

If you did install KNIME without all free extensions, or upgraded from an older version, you can install the nodes from the community contributions repository. For this start KNIME and go to **HELP > INSTALL NEW SOFTWARE...**. Select the community contributions repository under the **WORK WITH** drop down menu, it should have th eaddress `http://update.knime.org/community-contributions/trusted/3.3`. The PIA nodes can be found in the **BIOINFORMATICS & NGS** group or simply by searching for them. Select the PIA nodes, click next, accept the license and restart KNIME after the installation is finished.

If all went well, you will see the PIA octopus on the splash screen of KNIME (together with all the other icons) and you will find the PIA nodes inside the **COMMUNITY NODES** in the Node Repository (usually left bottom side of screen). This tutorial also needs the OpenMS and R nodes to be installed. These need to be installed manually as well, if you did not install the "KNIME Analytics Platform + all free extensions" as recommended above. You should also already be familiar with the OpenMS spectrum identification using KNIME.

3 Running PIA in KNIME

First download the workflows and data for the tutorial at `https://github.com/julianu/pia-tutorial`.

3.1 First workflow

First, we will run a minimal workflow identifying spectra in a mzML file with the search engine X!Tandem and using PIA for the protein inference and also analysis of the identified PSMs and peptides.

Import and open the workflow **PIA_FIRST_ANALYSIS** from the provided workflows into your KNIME workspace. Please select the file **LFQ_SPIKEIN_DILUTION_1.MZML** as the analysed mzML file (upper **INPUT FILE**) and the FASTA file **S_PYO_SF370_POTATO_HUMAN_TARGET_DECOY_WITH_CONTAMINANTS.FASTA** as database for spectrum identification. We used X!Tandem for spectrum identification in this workflow. If you like, you can change it to any other supported software in KNIME, the basic configuration settings are:

- 10 ppm precursor tolerance
- 0.4 Da fragment tolerance
- tryptic digestion with up to 2 missed cleavages
- fixed Carbamidomethyl of C and variable Oxidation of M

The **PEPTIDEINDEXER** is needed to add the protein / accession information to the identified peptide spectrum matches (PSMs). For the tutorial data, use "REV_" as the decoy string and prefix as decoy position and make sure that Trypsin is set as enzyme with none as specificity. After adjusting the **INPUT FILE** nodes (and maybe the search node), run the workflow until the **PIA COMPILER**.

This node takes a list of files. Note, that the ports from OpenMS need to be converted into a URI file list. You could alternatively use a **LIST FILES** node to select the files containing prior performed spectrum identifications in any supported format like Mascot Dat, X!Tandem's XML files, Proteome Discoverer's MSF files or any mzIdentML file, and use these files as input for the compiler. This node is necessary to structure the data before a PIA analysis. It must be performed only once per set of identification files. The **PIA COMPILER** has as input settings only a compilation name, which can be chosen freely. The number of files passed to PIA is not limited, though processing more files needs more

main memory. Each file in the compilation gets a FileID, which you can explore with some additional information of the merge looking at the **VIEW: SUMMARY** after runing the **PIA COMPILER**. These IDs can also be used for filtering and advanced settings later on.

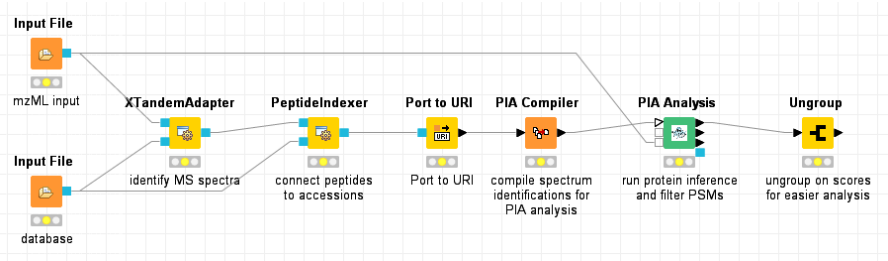

Figure 1: A small protein analysis workflow using X!Tandem for spectrum identification and PIA for protein inference.

The first connected port is for the PIA compilation, directly coming from the compiler node. If you should have a saved compilation, you could also use this and the second input port. The first port with a suitable configured file will be processed. The third input port is used to pass spectrum data to PIA, which can later be used to visualise automatically annotated spectra. If you don't want to use the spectrum viewer, you should not connect anything to this port, as the matching of the PSMs to their spectra might take some time.

3.1.1 General Settings

After running the compiler, open the settings of the **PIA ANALYSIS** node. You will find four tabs for the settings: one general and one for each of the levels of analysis (i.e. PSMs, peptides and proteins). If you connected the compiler node directly to the analysis node, select the column containing the PIA XML file (there should mostly be only one named "gzipped PIA XML file"). You can also set, whether PIA should fail if no decoys were found in the analysis, whether PSM sets should be created and whether modifications should be considered to distinguish peptides (see Figure 2). Creating PSM sets should always be used, if the same spectrum file was analysed with different search engines. This option will then combine the results of multiple searches, otherwise it can be deselected. Mostly, a peptide and it's scoring influence for the proteins should only be described by its sequence. If you have stable modifications, though, you can select to distinguish peptides by sequences and modifications. The **PIA ANALYSIS** node allows to export the analysis into several file formats. The level of the export (PSM, peptides and protein) as well as the format can be selected appropriately.

The analyses at the PSM and peptide level of PIA will be performed only for the input

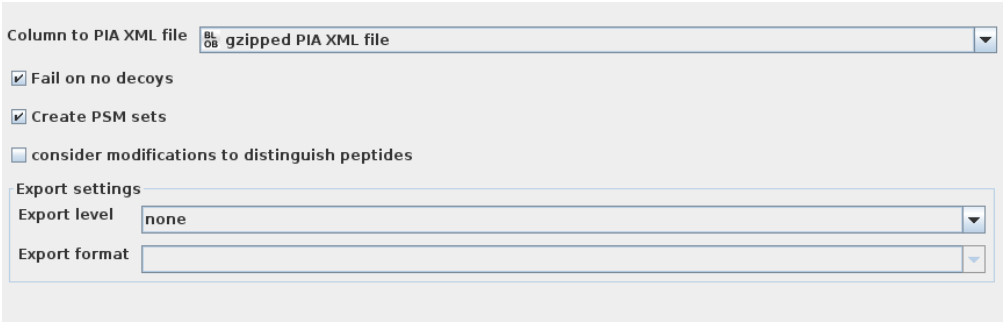

Figure 2: The general settings dialog of the **PIA ANALYSIS** node.

file given by the FileID in the respective settings tab. Usually, the IDs start with 1 and are sorted by the order of the given input files. A special case is the combination of all runs (either with PSM sets or without), also called overview, which always has the ID 0. The 0 for FileID is the default for the PSM and peptide level analyses.

3.1.2 PSM Settings

Now, have a look at the PSMs settings of the **PIA ANALYSIS**. The first setting is the just mentioned file ID, which is 0 in the example and thus reflects the combination of all results (Note: we have only one file in the example, but the "Combined FDRScore" will only be calculated on file 0. For one single input file, though, it is identical to the normal FDRScore). Next you can choose to calculate the false discovery rate (FDR), and thus also FDRScore and q-value, for all input files. The FDRScore [2] smoothes the FDR q-values in an analysis and thus facilitates a better discrimination of identifications instead of using the FDR q-values alone. If PSM sets will be created, also the Combined FDRScore can be calculated, which furthermore allows the combination of search results from multiple MS runs as well as identifications from different search engines.

A very important step is the selection of how decoys are distinguished from target identifications. PIA allows to use regular expressions for this, which are applied to the accessions. In the example in Figure 3, "REV.*" is set as regular expression. So each accession starting with the string "REV" (and all its peptides) will be assigned to be decoys and all not matching accessions to be targets. Alternatively, if "by search engine" is selected, identifications must be annotated in the input files as targets and decoys. This is e.g. the case if in Mascot the "Decoy" option is selected for an MS/MS search. For better compatibility though, the usage of a target-decoy database and the assignment by regular expressions is recommended.

Some search engines report more than one identification per spectrum. In the next option, you can choose to either use all these identifications or only the one with the best

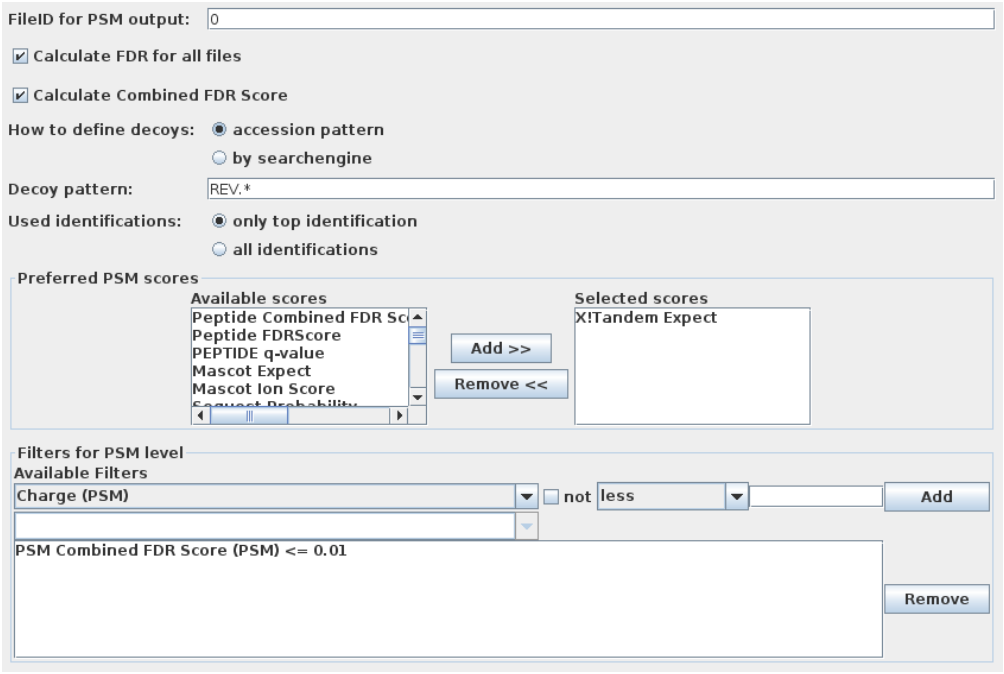

Figure 3: The PSMs settings dialog of the **PIA ANALYSIS** node.

score for FDR analysis and all following steps. Finally, you can select which scores are used for FDR estimation. If a search engine reports multiple score (e.g. X!Tandem's Hyperscore and Expect), you can choose the preferred score here. If no score was chosen, PIA will use the main score of the search engine (or, if this was not given, just any score).

All the settings up to this point are used for the FDR calculation (except for the output file ID) and influence the peptide and protein reports as well. The filters afflict only the PSM level and its report. Here you can chose from the available filters and set the parameters accordingly. For score filters it is necessary to select the according score as well (below the filters selection). After selecting a filter and setting the parameters, don't forget to click **ADD**. The currently activated filters will be shown in the list. In the example workflow, a filter for the "Combined FDRScore $<= 0.01$" is selected. Keep in mind, that all filters have to be fulfilled for a PSM to pass the filters.

3.1.3 Peptides Settings

Next, select the peptides settings. All settings here are only for the peptide export and do not afflict the protein inference. Therefore, you can also turn the peptide inference off and will get an empty report. This might be required, if you need to save time and main memory during the analysis. The selection of the file ID has the same meaning as on the PSM level

(exporting only information of this file, or 0 for the combination of all results). Also the filters are used in the same way. An exception are the PSM level filters: with these, the PSMs which are actually used to create peptides, are filtered. In the example workflow and in Figure 4, only PSMs with "Combined FDR Score <= 0.01" are inferred to peptides.

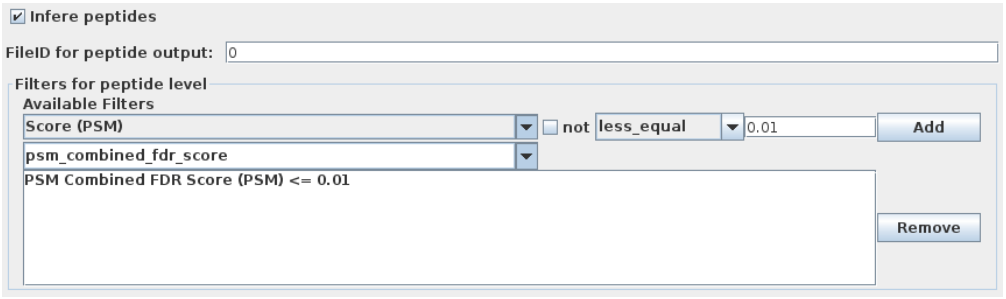

Figure 4: The peptides settings dialog of the **PIA ANALYSIS** node.

3.1.4 Protein Inference and Protein Settings

Finally, take a look at the proteins settings. Also the protein inference can be turned off, if you are only interested in analyses on the PSM or peptide level. First, you have to chose an inference method. PIA provides you with three different methods (for a more thorough explanation of these methods, please refer to [1])

- **Occam's Razor** is based on the parsimony principle and returns the smallest set of proteins, which explain all identified peptides. This is a very widely used strategy for protein inference.
- **Spectrum Extractor** is the recommended method. It is also based on the parsimonious approach, but assigns a spectrum to only one peptide. This peptide is selected in such a way, that it increases the score of the most probable, possible protein group.
- **Report All** This is actually no real inference, but reports all possible protein groups, based on the data. Use this with caution and only when you know, what you are doing!

After selecting the inference method, you can apply a variety of filters, which should be applied on PSM, peptide and protein level and directly afflict the results of the inference. You should almost always filter on the FDR, either using the FDRScore or the Combined FDR Score, usually on a value of about 0.01. But you could also set filters which make a protein to require at least two peptides to be valid, and many more filters.

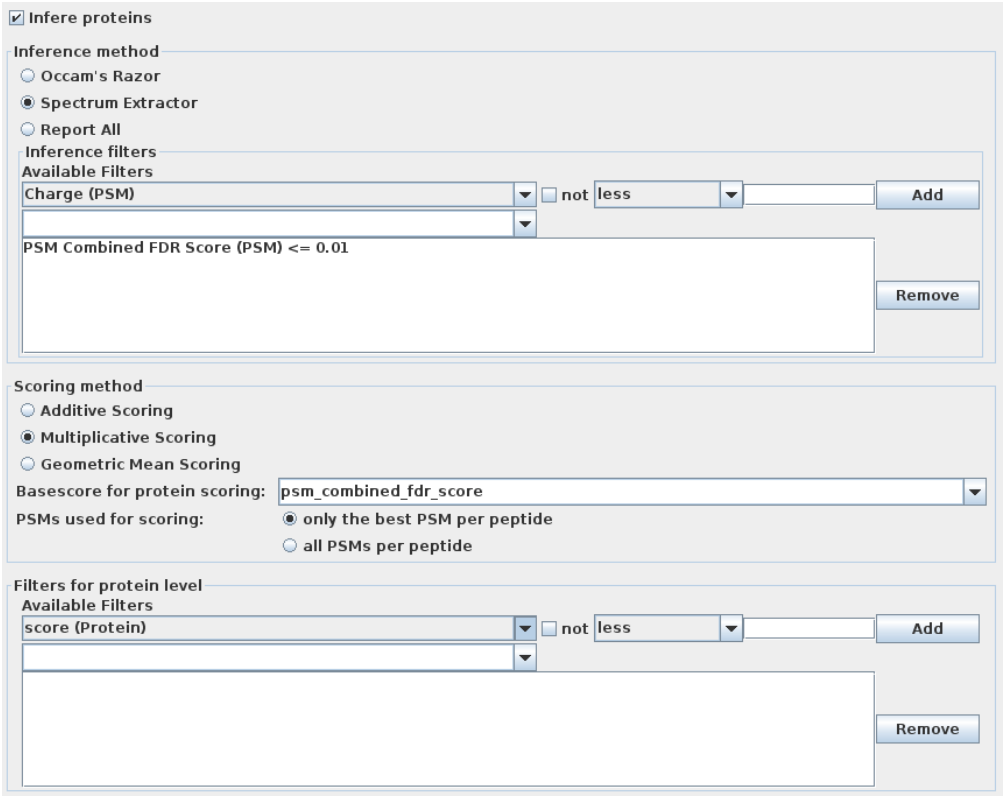

Figure 5: The proteins settings dialog of the **PIA ANALYSIS** node.

Next, you need to select the scoring method. Here, you should use "additive scoring" only if your base score has a "higher score is better" probability, like e.g. the Mascot Ion Score or X!Tandem's Hyperscore. Otherwise, use one of the other two scorings. The "multiplicative scoring" takes the number of identified peptides into account, in the way that the final protein score is usually better with more distinct peptides. The "geometric mean scoring" on the other hand calculates the mean of all peptide scores.

Finally, you need to select the base score and whether only the best PSM (recommended) or all PSMs of a peptide should be used for scoring. In our example, the Combined FDRScore is used. (Note: As base score you can only select scores on the PSM level, as the peptides will be generated during the inference according to your applied filters.)

The protein report can also be filtered in the same way as the PSMs and peptides report. Be aware, that this is significantly different to setting an inference filter on protein level: filters applied for the inference make it possible to not even create a protein group. Filters on the protein report afflict only what is reported.

3.1.5 A word on filters

Though PIA provides you with many filters on each of the PSM, peptide and protein level, you can also apply these filters later in the workflow. For this, you can simply apply an appropriate **Row Filter** node after running the **PIA Analysis**. But also keep in mind that setting the inference filters on the protein level are behaving very differently, as explained above.

3.2 Looking at the Results

Now run the **PIA Analysis** node. This can, depending on the loaded data, take some time. The example data should be processed in a few moments though. The node has four output ports: the first two are the (filtered) reports on the PSM and peptide level for the selected input file, the third is the protein level report and the last is a file port for the exported data, if any was created as set in the general settings.

3.2.1 Analysis Viewer

To explore the results of the analysis, right click on the **PIA Analysis** node after the execution finished and select the **View: PIA Results Analysis**. In the top left corner you will see all the inferred protein groups. PIA always works with protein groups, even if such a group might contain only one accession. All the accessions in one group have exactly the same evidence, i.e. the same PSMs and peptides, and cannot be distinguished on the given data and applied inference settings. The score is calculated using the selected base-score and scoring method. A higher score is always better (for base scores with "lower score better" a log value is used for transformations). If the complete protein sequences were provided, the coverages for the proteins are calculated. Furthermore, the number of assigned spectra, PSMs and peptides are given. If the FDR was calculated, also the decoy status and the FDR q-value will be given.

For the currently selected protein group, the assigned (not filtered) peptides are listed with their information in the middle of the window. On the bottom left, all PSM sets of the selected peptide are listed and on the bottom right finally the individual PSMs are given. If the spectrum file was given, you can view the annotated spectrum when clicking on the button by the PSM (see also PSM Results).

On the top right you will see a directed graph showing the relations between accessions of the currently selected protein group and its peptides and PSMs. In the example workflow, take a look at the groups "J7MBF9,Q99XR9" (it has a score of 52.74) and "Q9A0M0" (score of 20.40). The accessions of the selected group are coloured in dark green. Accessions of

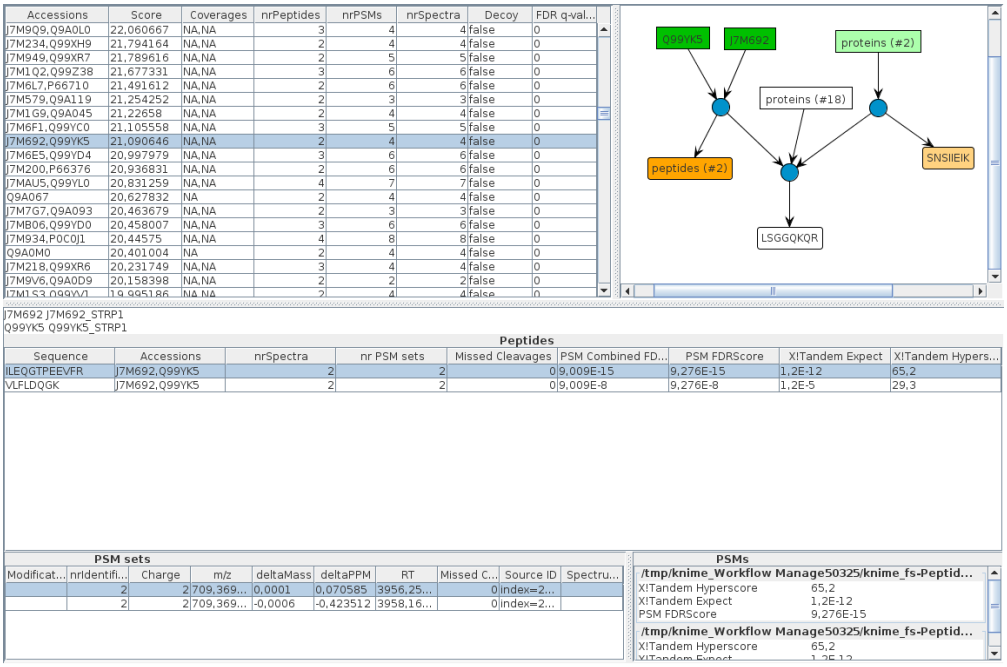

Figure 6: The PIA analysis viewer allows an intuitive exploration of the data.

(not reported) sub-groups are given in light-green without border and accessions reported in other groups in light green with black border. Peptides are coloured in orange (dark for the selected group, light for other groups in the same way as accessions). The blue circles are drawn only to construct a correct tree. Nodes which hold multiple items, can be expanded by double clicking on them, as can peptides to show their PSMs. All items can be re-arranged by drag-and-drop.

3.2.2 PSM Results

To look at the PSM level report, right click on the node and select **0: PSM RESULTS**. in this table you have almost all available information for the PSMs, like the amino acid sequence, a list of accessions, modifications, precursor charge, m/z value, the mass error, etc. Also, you have three columns for the scores (scores, score names and score shorts), which are lists each. Here, the score on a given position in the list corresponds to the score name and its short (which is an abbreviated name) on the same positions in the list. This makes it possible to export multiple scores in one row. In our example, we have only the Combined FDR score, though.

For easier analysis, the scores can be ungrouped using the **UNGROUP** node on the PSM report, which is the last step in the workflow. Take a quick look at the **UNGROUP**'s settings

and verify, that it is set to ungroup on the three score columns (and not the accessions). Run the node and look at the results: the score columns contain now individual names and numbers, which can be used for further sorting and filtering.

An alternative way to look at all reported PSMs (and not the sets) is using the "PSM Spectrum Viewer". Here you can see the PSMs together with the automatically annotated spectra (using [3]). This only works, if you connected the identified spectrum file to the third PIA input port.

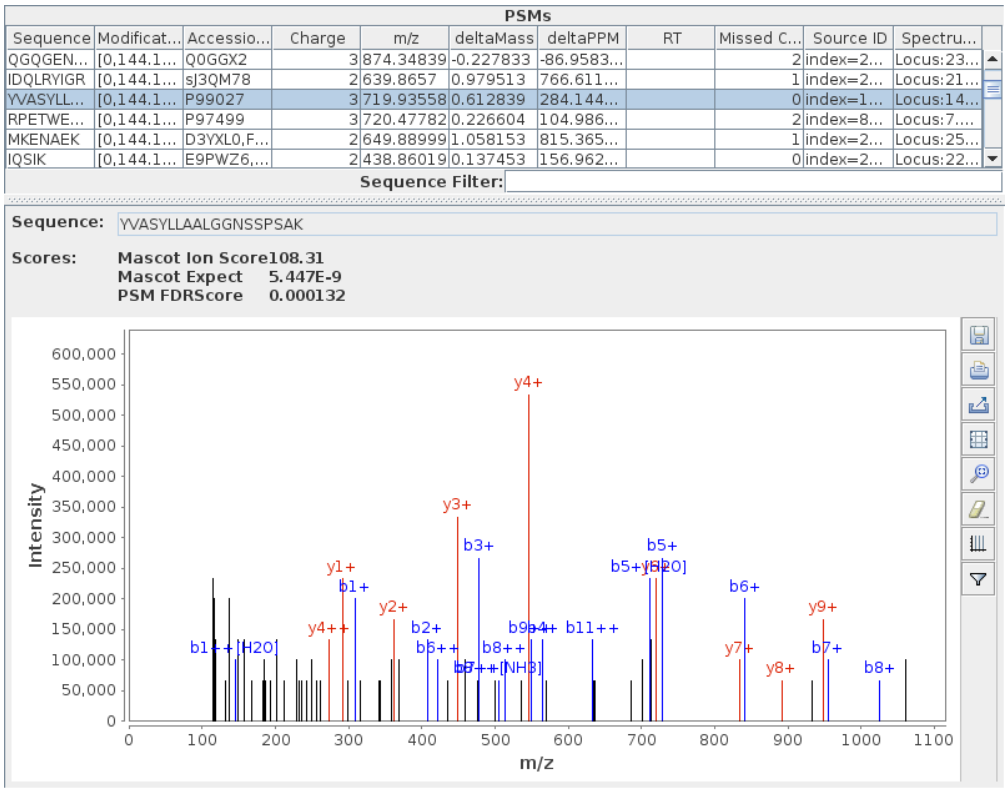

Figure 7: The PSM spectrum viewer showing an automatically annotated spectrum.

3.2.3 Peptide Results

The peptide results are given in the same way as the PSM results. The peptides are inferred either with or without taking modifications into account.

3.2.4 Protein Results

The created protein table on the third port holds almost the same information as the table in the Analysis Viewer. Node, that the accessions are lists again, as PIA always reports protein

groups. Additionally, each group has a clusterID, which represents the connected set or tree in the PIA intermediate structure. Elements in such a cluster are connected by their relations of accessions and PSMs, as can be seen in the Analysis Viewer on the top right. This does not mean, that reported protein groups with the same cluster ID share a peptide, but they are in the same component (a tree in the top right region of the **ANALYSIS VIEWER**) and can be connected by other (even not reported) accessions.

All of these tables can easily be processed with default KNIME nodes. This facilitates filtering, sorting, plotting etc. But also an analysis with R or Python can be created without much effort.

3.2.5 The Export File Port

On this port the created export file can be found. This could be either used for storage (e.g. when creating an mzIdentML file) but also as input for OpenMS's **PROTEINQUANTIFIER**, if an idXML file was exported.

3.3 Tasks

- Find the protein group "J7MBF9,Q99XR9" in the Analysis Viewer (it has a score of 52.74). Try to understand, why this protein was reported, but no group with J7M5J8 was reported.
- Now find find the protein group "J7M692,Q99YK5" (Score 21.09). Why was this group and also "J7MBA7,Q9A1H1" (Score 4.65) reported?
- If you change the PSM level report to report the PSMs of the first file in the **PIA ANALYSIS** node, you will have multiple scores in the PSM report. Ungroup for the scores, to get one row for each score. But then you have each PSM thrice, so you need to filter using the **ROW FILTER** and test on the score name or short, to only get the score you want to work with.

4 Comparing PIA and Fido

PIA is only one way for protein inference. It uses a deterministic model and is mostly based on parsimonious approaches. Another approach is Fido [4], which is integrated by OpenMS into KNIME. We will make a short comparison on a small example in another workflow.

Import and open the workflow **PIA_AND_FIDO**. This workflow contains the same parts as the workflow of the prior tutorial and additionally a Fido protein inference and a comparative **JOINER**. First, you need to adjust the **INPUT FILE** nodes to the mzML and the FASTA file, then you can run the workflow. The Fido metanode performs a default OpenMS Fido protein inference and reads the protein data back to a KNIME table from the created idXML file. Afterwards, the proteins are filtered on 1% protein FDR and no longer needed columns are removed. To facilitate the joining of the tables, the accessions are converted from lists to strings in the **COLUMN RENAME** nodes.

Finally, the results are joined on the accessions, i.e. same reports are combined into one row in the table. To look at the joined data, right click on the node and select **JOINED TABLE**. Here you can see, that PIA and Fido reported almost the same protein groups. Only the rankings, based on PIA's score and Fido's Protein Probability, differ. Scroll to the bottom of the list. Here are several groups only reported by Fido (the ones having "?" marking missing values). These still have high Protein Probabilities, which is due to Fido's way of reporting sub-groups: PIA does not report any sub-groups, while Fido with the default settings does.

4.1 Tasks

- Fido reports the group "J7M5J8,Q9A086", while PIA does not (see first task in prior section). Why?
- Play around with PIA's settings and observe, how the overlap of the inference algorithms change.
- Change the workflow in such a way, that you can compare two PIA analyses with different settings. Improve it to compare three or more analyses.

5 Advanced and real life usage for PIA

5.1 PIA and OpenMS Protein Quantification

PIA can be used for protein inference on quantitative data combined with OpenMS. First you need to run a feature detection, map IDs, align and normalise the data to create a featureXML, as explained in the OpenMS tutorial. Then, you can use PIA to create the inference input for the **ProteinQuantifier**. For this, you just need to select idXML and protein level as export in the general settings and run the **PIA Analysis** node. Take care, to adjust your settings correctly!

There is one small problem at the moment though: OpenMS always parses an accession in a FASTA file up to the first space (in UniProt e.g. "sp|Q6GZX3|002L_FRG3G"), while PIA tries to only report the accession (in the example "Q6GZX3"). To avoid errors in the protein group assignment, you should adjust your FASTA file to contain an alphanumerical accession followed by a space and then the description in the protein headers. In the example, this would be ">Q6GZX3 002L_FRG3G Uncharacterized protein 002L..." instead of the normal UniProt entry ">sp|Q6GZX3|002L_FRG3G Uncharacterized protein 002L..."

You can try to use PIA in the tutorials provided by OpenMS, which use Fido for protein inference before quantification.

5.2 Web Frontend and Docker

The web frontend, which we did not discuss, can be tested at `https://github.com/BioDocker/containers/tree/master/pia-web-server/1.1.0-SNAPSHOT`.

If you want to run your own installation and are accustomed to use Docker, you can find a Dockerfile to install PIA running in Apache Tomcat at `https://github.com/BioDocker/containers/tree/master/pia-web-server/1.1.0-SNAPSHOT`.

References

[1] Uszkoreit et al., *PIA: An Intuitive Protein Inference Engine with a Web-Based User Interface.*, J Proteome Res, 2015.

[2] Jones et al., *Improving sensitivity in proteome studies by analysis of false discovery rates for multiple search engines.*, Proteomics, 2009.

[3] Perez-Riverol et al., *ms-data-core-api: an open-source, metadata-oriented library for computational proteomics.*, Bioinformatics, 2015.

[4] Serang et al., *Efficient marginalization to compute protein posterior probabilities from shotgun mass spectrometry data*, J. Proteome Res., 2010.