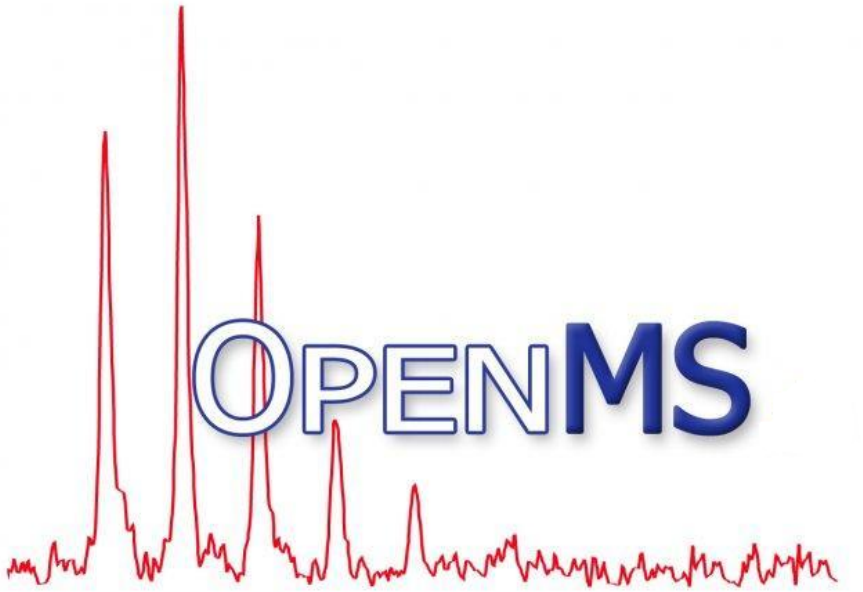

OpenMS Tutorial

The OpenMS Developers

Mathias Walzer, Timo Sachsenberg, Fabian Aicheler,
George Rosenberger, Hannes Roest,
Marc Rurik, Stephan Aiche, Johannes Veit,
Knut Reinert, and Oliver Kohlbacher

Contents

1 General remarks

- This handout will guide you through an introductory tutorial for the OpenMS/TOPP software package [1].
- OpenMS [2] is a versatile open-source library for mass spectrometry data analysis. Based on this library, we offer a collection of command-line tools ready to be used by end users. These so-called TOPP tools (short for "The OpenMS Proteomics Pipeline") [3] can be understood as small building blocks of arbitrary complex data analysis workflows.
- In order to facilitate workflow construction, OpenMS was integrated into KNIME [4], the Konstanz Information Miner, an open-source integration platform providing a powerful and flexible workflow system combined with advanced data analytics, visualisation, and report capabilities. Raw MS data as well as the results of data processing using TOPP can be visualized using TOPPView [5].
- In this hands-on tutorial session, you will become familiar with some of the basic functionalities of OpenMS/TOPP, TOPPView, and KNIME and learn how to use a selection of TOPP tools used in the tutorial workflows.
- All data referenced in this tutorial can be found in the 🗀 `Example_Data` folder that came with this tutorial.

2 Getting started

2.1 Data conversion

Each MS instrument vendor has one or more formats for storing the acquired data. Converting these data into an open format (preferably mzML) is the very first step when you want to work with open-source mass spectrometry software. A freely available conversion tool is ProteoWizard. The OpenMS installation package for Windows automatically installs ProteoWizard, so you do not need to download and install it separately.

Please note that due to restrictions from the instrument vendors, file format conversion for most formats is only possible on Windows systems, so exporting from the acquisition PC connected to the instrument is usually the most convenient option. All files used in this tutorial have already been converted to mzML by us, so you do not need to do it yourself.

2.2 Data visualization using `TOPPView`

Visualizing the data is the first step in quality control, an essential tool in understanding the data, and of course an essential step in pipeline development. OpenMS provides a convenient viewer for some of the data: `TOPPView`.

We will guide you through some of the basic features of `TOPPView`. Please familiarize yourself with the key controls and visualization methods. We will make use of these later throughout the tutorial. Let's start with a first look at one of the files of our tutorial data set:

- Start `TOPPView` (see Start-Menu or Applications on MacOS)
- Go to File > Open File, navigate to the directory where you copied the contents of the USB stick to, and select `OpenMS` ▸ `small` ▸ `velos005614.mzML` . This file contains a reduced LC-MS map (only a selected RT and m/z range was extracted using the TOPP tool FileFilter) of a label-free measurement of the human platelet proteome recorded on an Orbitrap velos. The other two mzML files contain technical replicates of this experiment. First, we want to obtain a global view on the whole LC-MS map - the default option *Map view 2D* is the correct one and we can click the ok button.
- Play around.

- Three basic modes allow you to interact with the displayed data: scrolling, zooming and measuring:
  - Scroll mode
    * Is activated by default (though each loaded spectra file is displayed zoomed out first, so you do not need to scroll).
    * Allows you to browse your data by moving around in RT and m/z range.
    * When zommed in, to scroll the spectra map, click-drag on the current view.
    * Arrow keys can be used to scroll the view as well.
  - Zoom mode
    * Zooming into the data: either mark an area in the current view with your mouse while holding the left mouse button plus the ctrl key to zoom to this area or use your mouse wheel to zoom in and out.
    * All previous zoom levels are stored in a zoom history. The zoom history can be traversed using ctrl + + or ctrl + - or the mouse wheel (scroll up and down).
    * Pressing the Backspace key zooms out to show the full LC-MS map (and also resets the zoom history).
  - Measure mode
    * It is activated using the ⇧ key.
    * Press the left mouse button down while a peak is selected and drag the mouse to another peak to measure the distance between peaks.
    * This mode is implemented in the 1D and 2D mode only.
- Right click on your 2D map and select Switch to 3D view and examine your data in 3D mode
- Go back to the 2D view. In 2D mode, visualize your data in different normalization modes, use linear, percentage and log-view (icons on the upper left tool bar).

> **Note:** On *Apple OS X*, due to a bug in one of the external libraries used by

> OpenMS, you will see a small window of the 3D mode when switching to 2D. Close the 3D tab in order to get rid of it.

- In `TOPPView` you can also execute TOPP tools. Go to Tools » Apply tool (whole layer) and choose a TOPP tool (e.g., FileInfo) and inspect the results.

2.3 Introduction to KNIME / OpenMS

Using OpenMS in combination with KNIME you can create, edit, open, save, and run workflows combining TOPP tools with the powerful data analysis capabilities of KNIME. Workflows can be created conveniently in a graphical user interface. The parameters of all involved tools can be edited within the application and are also saved as part of the workflow. Furthermore, KNIME interactively performs validity checks during the workflow editing process, in order to make it more difficult to create an invalid workflow.

Throughout most of the parts of this tutorial you will use KNIME to create and execute workflows. This first step is to make yourself familiar with KNIME.

2.3.1 Install OpenMS using KNIME

Before we can start with the tutorial we need to install all the required extensions for KNIME.

First, we install some additional extensions that are required by our OpenMS nodes or used in the Tutorials e.g. for visualization.

1. Click on Help » Install New Software...
2. From the Work with: drop down list select the http://update.knime.org/analytics-platform/2.12
3. Now select the following plugins from the *KNIME & Extensions* category
    - KNIME Base Chemistry Types & Nodes
    - KNIME Chemistry Add-Ons
    - KNIME File Handling Nodes
    - KNIME Interactive R Statistics Integration

- KNIME Math Expression (JEP)
- KNIME Report Designer
- KNIME SVG Support
- KNIME XLS Support
- KNIME XML-Processing

4. And the following plugin from the *Marvin Chemistry Extensions (donated by Infocom & Chemaxon)* category
    - ChemAxon/Infocom Marvin Extensions Feature
5. Follow the instructions and after a restart of KNIME the dependencies will be installed.

You are now ready to install the OpenMS nodes.

1. Open KNIME.
2. Click on Help > Install New Software...
3. From the Work with: drop down list select the http://tech.knime.org/update/community-contributions/truste
4. Select the **OpenMS** nodes in the category "KNIME Community Contributions - Bioinformatics & NGS" and click Next.
5. Follow the instructions and after a restart of KNIME the OpenMS nodes will be available under "Community Nodes".

2.3.2 KNIME Concepts

A **workflow** is a sequence of computational steps applied to a single or multiple input data sets to process and analyse the data. In KNIME such workflows are implemented graphically by combining so-called **nodes**. A node represents a single analysis step in a workflow. Nodes have input and output **ports** where the data enters the node or the results are provided for other nodes after processing, respectively. KNIME distinguishes between different port types, representing different types of data. The most common representation of

data in KNIME are tables (similar to an excel sheet). Ports that accept tables are marked with a small triangle. For OpenMS we use a different port type, so called **file ports**, representing complete files. Those ports are marked by a small grey box. Dark grey boxes represent mandatory inputs and light grey boxes optional inputs.

A typical OpenMS workflow in KNIME can be divided in two conceptually different parts:

- Nodes for signal and data processing, filtering and data reduction. Here, files are passed between nodes. Execution times of the individual steps are longer as the main computational steps are performed.
- Downstream statistical analysis and visualization. Here, tables are passed between nodes.

Between file based processing and table based analysis a conversion node typically performs the conversion from OpenMS results into KNIME tables.

Nodes can have three different states, indicated by the small traffic light below the node.

- Inactive, failed, and not yet fully configured nodes are marked red.
- Configured but not yet executed nodes are marked yellow.
- Successfully executed nodes are marked green.

If the node execution failed the node will switch to the red state.

Most nodes will be configured as soon as all input ports are connected. For some nodes additional parameters have to be provided that cannot be either guessed from the data or filled with sensible defaults. In this case, of if you want to customise the default configuration, you can open the configuration dialog of a node with a double-click on the node. For OpenMS you will see a configuration dialog like the one shown in Figure 1.

> **Note:** OpenMS distinguishes between normal parameters and advanced parameters. Advanced parameters are by default hidden from the users since they should only rarely be customised. In case you want to have a look at the parameters or need to customise them in one of the tutorials you can show them by clicking on

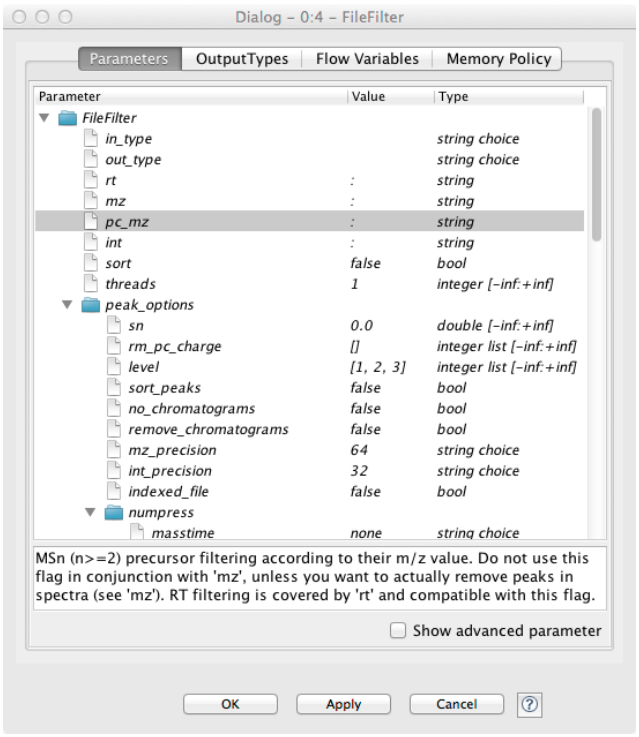

Figure 1: Node configuration dialog of an OpenMS node.

> the checkbox Show advanced parameter in the lower part of the dialog.

The dialog shows the individual parameters, their current value and type, and, in the lower part of the dialog, the documentation for the currently selected parameter.

2.3.3 Overview of the graphical user interface

The graphical user interface (GUI) of KNIME consists of different components or so called panels that are shown in Figure 2. We will shortly introduce the individual panels and their purpose below.

Workflow Editor The workflow editor is the central part of the KNIME GUI. Here you assemble the workflow by adding nodes from the Node Repository via ”drag & drop”. Nodes can be connected by clicking on the output port of one node and releasing the mouse at the desired input port of the next node.

Workflow Explorer Shows a list of available workflows (also called workflow projects).

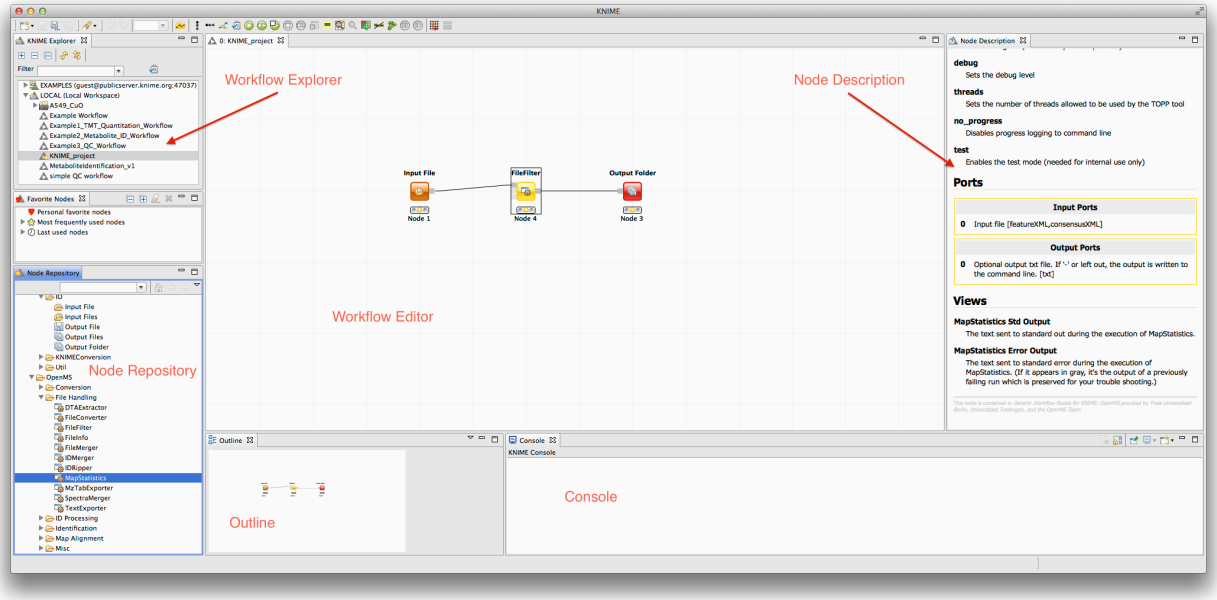

Figure 2: The KNIME workbench.

You can open a workflow by double clicking it. A new workflow can be created with a right-click in the Workflow Explorer followed by selecting New KNIME Workflow....

Node Repository Shows all nodes that are available in your KNIME installation. Every plugin you install will provide new nodes that can be found here. The OpenMS nodes can be found in Community Nodes > OpenMS. Nodes for managing files (e.g., Input Files or Output Folders) can be found in Community Nodes > GenericKnimeNodes. You can search the node repository by typing the node name into the small text box in the upper part of the node repository.

Outline The Outline panel contains a small overview of the complete workflow. While of limited use when working on a small workflow, this feature is very helpful as soon as the workflows get bigger.

Console In the console panel warning and error messages are shown. This panel will provide helpful information if one of the nodes failed or shows an warnings sign.

Node Description As soon as a node is selected, the Node Description window will show the documentation of the node including documentation for all its parameters. For

OpenMS nodes you will also find a link to the tool page in the online documentation.

2.3.4 Creating workflows

Workflows can easily be created by a right click in the Workflow Explorer followed by clicking on New KNIME Workflow....

2.3.5 Sharing workflows

To be able to share a workflow with others KNIME supports the import and export of complete workflows. To export a workflow select it in the Workflow Explorer and select File > Export KNIME Workflow.... KNIME will export workflows as a zip file containing all the information on nodes, their connections, and their configuration. Those zip files can again be imported by selecting File > Import KNIME Workflow....

> **Note:** For your convenience we added all workflows discussed in this tutorial to the `Workflows` folder. If you want to check your own workflow by comparing it to the solution or got stuck, simply import the full workflow from the corresponding zip file.

2.3.6 Duplicating workflows

During the tutorial a lot of the workflows will be created based on the workflow from a previous task. To keep the intermediate workflows we suggest you create copies of your workflows so you can see the progress. To create a copy of your workflow follow the following steps

- Right click on the workflow you want to create a copy of in the Workflow Explorer and select Copy.
- Right click again somewhere on the workflow explorer and select Paste.
- This will create a workflow with same name as the one you copied with a (2) appended.

- To distinguish them later on you can easily rename the workflows in the Workflow Explorer by right clicking on the workflow and selecting Rename.

  > **Note:** To rename a workflow it has to be closed.

2.3.7 A minimal workflow

Let us now start with the creation of our very first, very simple workflow. As a first step, we will gather some basic information about the data set before starting the actual development of a data analysis workflow.

- Create a new workflow.
- Add an `Input File` node and an `Output Folder` node (to be found in Community Nodes > GenericKnimeNodes > IO and a `FileInfo` node (to be found in the category Community Nodes > OpenMS > File Handling) to the workflow.
- Connect the `Input File` node to the `FileInfo` node, and the first output port of the `FileInfo` node to the `Output Folder` node.

  > **Note:** In case you are unsure about which node port to use, hovering the cursor over the port in question will display the port name and what kind of input it expects.

  The complete workflow is shown in Figure 3. FileInfo can produce two different kinds of output files.

- All nodes are still marked red, since we are missing an actual input file. Double-click the Input File node and select Browse. In the file system browser select `OpenMS` ▸ `tiny` ▸ `velos005614.mzML` and click Open. Afterwards close the dialog by clicking Ok.

  > **Note:** Make sure to use the "tiny" version this time, not "small", for the sake of faster workflow execution.

- The `Input File` node and the `FileInfo` node should now have switched to yellow, but the `Output Folder` node is still red. Double-click on the `Output Folder` node and click on Browse to select an output directory for the generated data.

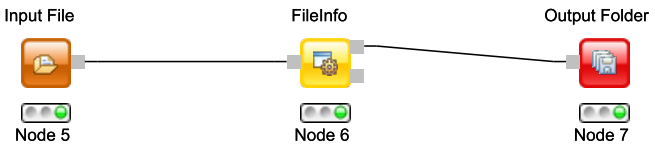

Figure 3: A minimal workflow calling FileInfo on a single file.

- Great! Your first workflow is now ready to be run. Press ⇧ + F7 to execute the complete workflow. You can also right click on any node of your workflow and select Execute from the context menu.
- The traffic lights tell you about the current status of all nodes in your workflow. Currently running tools show either a progress in percent or a moving blue bar, nodes waiting for data show the small word "queued", and successfully executed ones become green. If something goes wrong (e.g., a tool crashes), the light will become red.
- In order to inspect the results, you can just right-click the **`Output Folder`** node and select View: Open the output folder. You can then open the text file and inspect its contents. You will find some basic information of the data contained in the mzML file, e.g., the total number of spectra and peaks, the RT and m/z range, and how many MS1 and MS2 spectra the file contains.

Workflows are typically constructed to process a large number of files automatically. As a simple example, consider you would like to gather this information for more than one file. We will now modify the workflow to compute the same information on three different files and then write the output files to a folder.

- We start from the previous workflow.
- First we need to replace our single input file with multiple files. Therefore we add the **`Input Files`** node from the category Community Nodes › GenericKnimeNodes › IO.
- To select the files we double-click on the **`Input Files`** node and click on Add. In the filesystem browser we select all three files from the directory 🗀 **`OpenMS`** ▸ **`tiny`**. And close the dialog with Ok.

Figure 4: A minimal workflow calling FileInfo on multiple files in a loop.

- We now add two more nodes: the `ZipLoopStart` and the `ZipLoopEnd` node from the category Community Nodes > GenericKnimeNodes > Flow.
- Afterwards we connect the `Input Files` node to the first port of the `ZipLoopStart` node, the first port of the `ZipLoopStart` node to the `FileInfo` node, the first output port of the `FileInfo` node to the first input port of the `ZipLoopEnd` node, and the first output port of the `ZipLoopEnd` node to the `Output Folder` node (, not `Output File`). The complete workflow is shown in Figure 4
- The workflow is already complete. Simply execute the workflow and inspect the output as before.

In case you had trouble to understand what ZipLoopStart and ZipLoopEnd do - here is a brief explanation:

- The `Input Files` node passes a list of files to the `ZipLoopStart` node.
- The `ZipLoopStart` node takes the files as input, but passes the single files sequentially (that is: one after the other) to the next node.
- The `ZipLoopEnd` collects the single files that arrive at its input port. After all files have been processed, the collected files are passed again as file list to the next node that follows.

3 Label-free quantification

3.1 Introduction

In this chapter, we will build a workflow with OpenMS / KNIME to quantify a label-free experiment. Label-free quantification is a method aiming to compare the relative amounts of proteins or peptides in two or more samples. We will start from the minimal workflow from the last chapter and, step-by-step, build a label-free quantitation workflow.

3.2 Peptide Identification

As a start, we will extend the minimal workflow so that it performs a peptide identification using the OMSSA [6] search engine. Since OpenMS version 1.10, OMSSA is included in the OpenMS installation, so you do not need to download and install it yourself.

- Let's start by replacing the input files in our `Input Files` node by the three mzML files in 🗀 `OpenMS` ▸ `lfq` ▸ `lfq_spikein_dilution_1-3.mzML`. This is a reduced toy dataset where each of the three runs contains a constant background of *S. pyogenes* peptides as well as human spike-in peptides in different concentrations.[7]
- Instead of FileInfo, we want to perform OMSSA identification, so we simply replace the `FileInfo` node with the `OMSSAAdapter` node Community Nodes » OpenMS » Identification, and we are almost done. Just make sure you have connected the `ZipLoopStart` node with the `in` port of the `OMSSAAdapter` node.
- OMSSA, like most mass spectrometry identification engines, relies on searching the input spectra against sequence databases. Thus, we need to introduce a search database input. As we want to use the same search database for all of our input files, we can just add a single `Input File` node to the workflow and connect it directly with the `OMSSAAdapter database` port. KNIME will automatically reuse this Input node each time a new ZipLoop iteration is started. In order to specify the database, select 🗀 `OpenMS` ▸ `FASTA` ▸ `s_pyo_sf370_potato_human_target_decoy_with_contaminants.fasta`, and we have a very basic peptide identification workflow.

> **Note:** You might also want to save your new identification workflow under a different name. Have a look at Section 2.3.6 for information on how to create copies of workflows.

- The result of a single OMSSA run is basically a number of peptide-spectrum-matches (PSM) with a score each, and these will be stored in an idXML file. Now we can run the pipeline and after execution is finished, we can have a first look at the results: just open the input files folder with a file browser and from there open an mzML file in `TOPPView`.

- Here, you can annotate this spectrum data file with the peptide identification results. Choose Tools > Annotate with identification from the menu and select the idXML file that `OMSSAAdapter` generated (it is located within the output directory that you specified when starting the pipeline).

- On the right, select the tab Identification view. Using this view, you can see all identified peptides and browse the corresponding MS2 spectra.

> **Note:** Opening the output file of `OMSSAAdapter` (the idXML file) directly is also possible, but the direct visualization of an idXML file is less useful.

- The search results stored in the idXML file can also be read back into a KNIME table for inspection and subsequent analyses: Add a `TextExporter` Community Nodes > OpenMS > File Handling node to your workflow and connect the output port of your `OMSSAAdapter` (the same port your `ZipLoopEnd` is connected to) to its input port. This tool will convert the idXML file to a more human-readable text file which can also be read into a KNIME table using the `IDTextReader` node. Add an `IDTextReader` node Community Nodes > OpenMS > Conversion after `TextExporter` and execute it. Now you can right-click `IDTextReader` and select ID Table to browse your peptide identifications.

- From here, you can use all the tools KNIME offers for analyzing the data in this table. As a simple example, you could add a `Histogram` Data Views node after `IDTextReader`, double-click it, select *peptide_charge* as binning column, hit OK, and execute it. Right-clicking and selecting View: Histogram view will open a plot showing the charge state distribution of your identifications.

In the next step, we will tweak the parameters of OMSSA to better reflect the instrument's accuracy. Also, we will extend our pipeline with a false discovery rate (FDR) filter to retain only those identifications that will yield an FDR of < 1 %.

- Open the configuration dialog of `OMSSAAdapter`. The dataset was recorded using an LTQ Orbitrap XL mass spectrometer, so we can set the precursor mass tolerance to a smaller value, say 10 ppm. Set *precursor_ mass_ tolerance* to 10 and *precursor_mass_tolerance_unit_ppm* to *true*.

  > **Note:** Whenever you change the configuration of a node the node as well as all its successors will be reset to the Configured state.

- Set *max_precursor_charge* to 5, in order to also search for peptides with charges up to 5.

- Add Carbamidomethyl (C) as fixed modification and Oxidation (M) as variable modification.

  > **Note:** To add a modification click on the empty value field in the configuration dialog to open the list editor dialog. In the new dialog click Add. Then select the newly added modification to open the drop down list where you can select the correct modification.

- A common step in analyis is to search not only against a regular protein database, but to also search against a decoy database for FDR estimation. The fasta file we used before already contains such a decoy database. For OpenMS to know which OMSSA PSM came from which part of the file (i.e. target versus decoy), we have to index the results. Therefore extend the workflow with a `PeptideIndexer` node Community Nodes > OpenMS > ID Processing. This node needs the idXML as input as well as the database file.

  > **Note:** You can direct the files of an `Input File` node to more than just one destination port.

- The decoys in the database are prefixed with "REV_", so we have to set *decoy_string* to *REV_* and *prefix* to *true* in the configuration dialog of `PeptideIndexer`.

- Now we can go for the FDR estimation, which the `FalseDiscoveryRate` node will calculate for us Community Nodes > OpenMS > ID Processing. As we have a combined search database and thus only one idXML per mzML we will only use the *in* port of the `FalseDiscoveryRate` node.
- In order to set the FDR level to 1%, we need an `IDFilter` node from Community Nodes > OpenMS > ID Processing. Configuring its parameter *score* → *pep* to 0.01 will do the trick. The FDR calculations (embedded in the idXML) from the `FalseDiscoveryRate` node will go into the *in* port of the `IDFilter` node.
- Execute your workflow and inspect the results using `IDTextReader` like you did before. How many peptides did you identify at this FDR threshold?

> **Note:** The finished identification workflow is now sufficiently complex that we might want to encapsulate it in a Meta node. For this, select all nodes inside the ZipLoop (including the `Input File` node) and right-click to select Collapse into Meta node and name it ID. Meta nodes are useful when you construct even larger workflows and want to keep an overview.

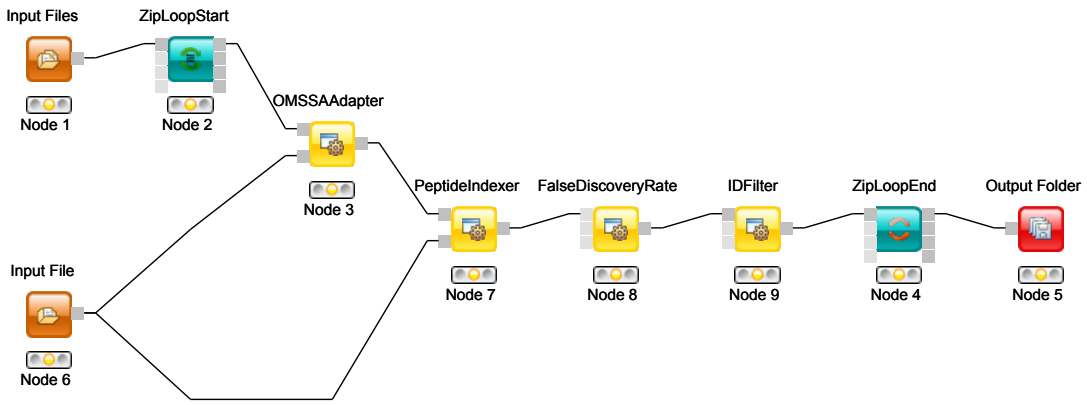

Figure 5: OMSSA ID pipeline including FDR filtering

3.2.1 Bonus task: identification using several search engines

> **Note:** If you are ahead of the tutorial or later on, you can further improve your FDR identification workflow by a so-called consensus identification using several search engines. Otherwise, just continue with section 3.3.

It has become widely accepted that the parallel usage of different search engines can increase peptide identification rates in shotgun proteomics experiments. The ConsensusID algorithm is based on the calculation of posterior error probabilities (PEP) and a combination of the normalized scores by considering missing peptide sequences.

- Next to the `OMSSAAdapter` add a `XTandemAdapter` Community Nodes › OpenMS › Identification node and set its parameters and ports analogously to the `OMSSAAdapter`.
- To calculate the PEP, introduce each a `IDPosteriorErrorProbability` Community Nodes › OpenMS › ID Processing node to the output of each ID engine adapter node. This will calculate the PEP to each hit and output an updated idXML.
- To create a consensus we must first merge these two files with a `FileMerger` node Community Nodes › GenericKnimeNodes › Flow so we can then merge the corresponding IDs with a `IDMerger` Community Nodes › OpenMS › File Handling.
- Now we can create a consensus identification with the `ConsensusID` Community Nodes › OpenMS › ID Processing node. We can connect this to the `PeptideIndexer` and go along with our existing FDR filtering.

  > **Note:** By default, X!Tandem takes additional enzyme cutting rules into consideration (besides the specified tryptic digest). Thus you have to set PeptideIndexer's *enzyme* → *specificity* parameter to `semi` to accept X!Tandems semi tryptic identifications as well.

3.3 Quantification

Now that we have successfully constructed a peptide identification pipeline, we can add quantification capabilities to our workflow.

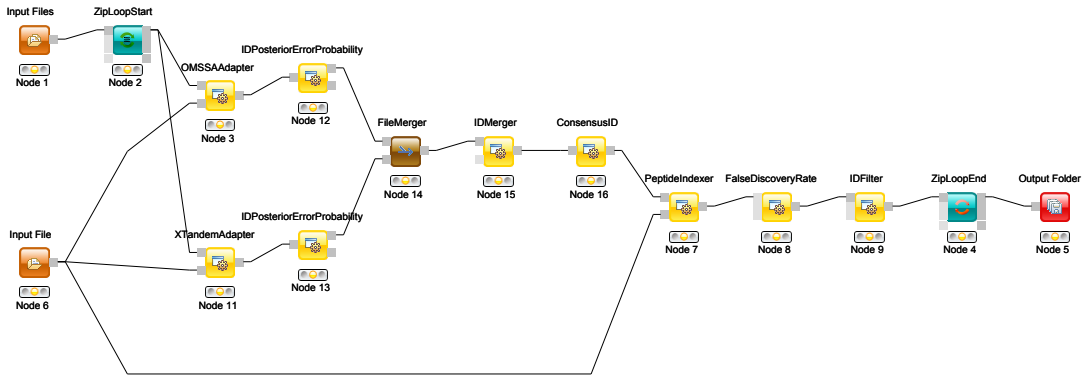

Figure 6: Complete consensus identification workflow

- Add a `FeatureFinderCentroided` node Community Nodes › OpenMS › Quantitation which gets input from the first output port of the `ZipLoopStart` node. Also, add an `IDMapper` node Community Nodes › OpenMS › ID Processing which gets input from the `FeatureFinderCentroided` node and the ID Meta node (or `IDFilter` node if you haven't used the Meta node). The output of the `IDMapper` is then connected to the `ZipLoopEnd` node.

- `FeatureFinderCentroided` finds and quantifies peptide ion signals contained in the MS1 data. It reduces the entire signal, i.e., all peaks explained by one and the same peptide ion signal, to a single peak at the maximum of the chromatographic elution profile of the monoisotopic mass trace of this peptide ion and assigns an overall intensity.

- `FeatureFinderCentroided` produces a featureXML file as ouput, containing only quantitative information of so-far unidentified peptide signals. In order to annotate these with the corresponding ID information, we need the `IDMapper` node.

- Run your pipeline and inspect the results of the `IDMapper` node in TOPPView.

- In order to assess how well the feature finding worked, you can project the features contained in the featureXML file on the raw data contained in the mzML file. In TOPPView choose File › Open file and select the mzML file corresponding to your featureXML file in 🗀 `OpenMS` ▸ `lfq`. In the dialog that pops up, select Open in › New layer. Zoom in until you see boxes (found features) around the peptide signals in the raw data.

> **Note:** The RT range is very narrow. Thus, select the full RT range and zoom only into the m/z dimension by holding down CTRL (CMD on the Mac) and repeatedly dragging a narrow box from the very left to the very right.

- You can see which features were annotated with a peptide identification by first selecting the featureXML file in the `Layers` window on the upper right side and then clicking on the icon with the letters A, B and C on the upper icon bar. Now, click on the small triangle next to that icon and select `Peptide identification`.

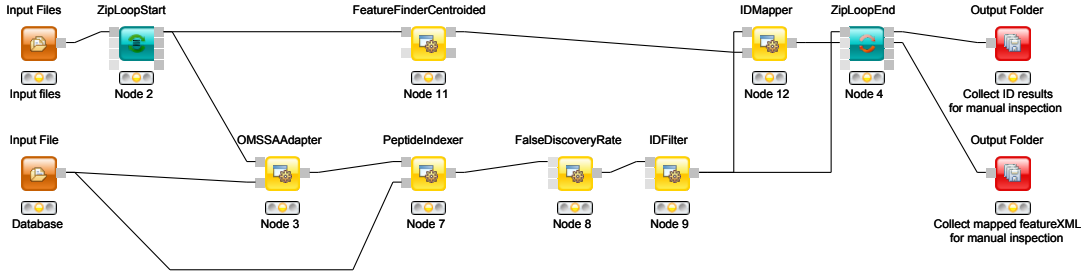

Figure 7: Extended workflow featuring peptide identification and quantification

3.4 Combining quantitative information across several label-free experiments

So far, we successfully performed peptide identification as well as quantification on individual LC-MS runs. For differential label-free analyses, however, we need to identify and quantify corresponding signals in different experiments and link them together to compare their intensities. Thus, we will now run our pipeline on all three available input files and extend it a bit further, so that it is able to find and link features across several runs.

- To find features across several maps, we first have to align them to correct for retention time shifts between the different label-free measurements. With the `MapAlignerPoseClustering` Community Nodes > OpenMS > Map Alignment, we can align corresponding peptide signals to each other as closely as possible by applying a transformation in the RT dimension.

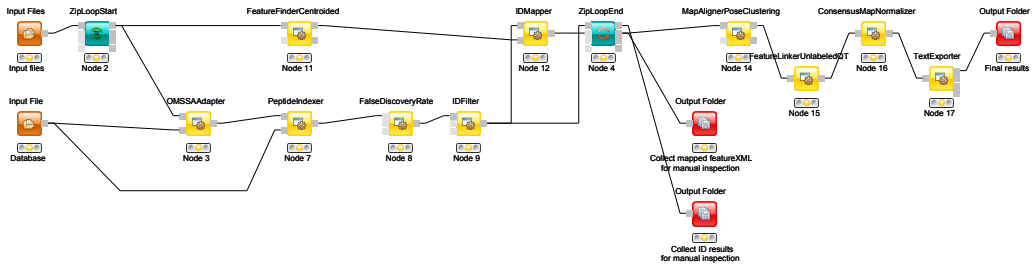

Figure 8: Complete identification and label-free quantification workflow

> **Note:** `MapAlignerPoseClustering` consumes several featureXML files and its output should still be several featureXML files containing the same features, but with the transformed RT values. In its configuration dialog, make sure that *OutputTypes* is set to featureXML.

- With the `FeatureLinkerUnlabeledQT` node Community Nodes › OpenMS › Map Alignment, we can then perform the actual linking of corresponding features. Its output is a consensusXML file containing linked groups of corresponding features across the different experiments.

- Since the overall intensities can vary a lot between different measurements (for example, because the amount of injected analytes was different), we apply the `ConsensusMapNormalizer` Community Nodes › OpenMS › Map Alignment as a last processing step. Configure its parameters with setting *algorithm_type* to `median`. It will then normalize the maps in such a way that the median intensity of all input maps is equal.

- Finally, we export the resulting normalized consensusXML file to a csv format using `TextExporter`. Connect its out port to a new `Output Folder` node.

> **Note:** You can specify the desired column separation character in the parameter settings (by default, it is set to " " (a space)). The output file of `TextExporter` can also be opened with external tools, e.g., Microsoft Excel, for downstream statistical analyses.

3.4.1 Basic data analysis in KNIME

For downstream analysis of the quantification results within the KNIME environment, you can use the `ConsensusTextReader` node Community Nodes > OpenMS > Conversion instead of the `Output Folder` node to convert the output into a KNIME table (indicated by an arrow as output port). After running the node you can view the KNIME table by right clicking on the `ConsensusTextReader` and selecting Consensus Table. Every row in this table corresponds to a so-called consensus feature, i.e., a peptide signal quantified across several runs. The first couple of columns describe the consensus feature as a whole (average RT and m/z across the maps, charge, etc.). The remaining columns describe the exact positions and intensities of the quantified features separately for all input samples (e.g., intensity_0 is the intensity of the feature in the first input file). The last 11 columns contain information on peptide identification.

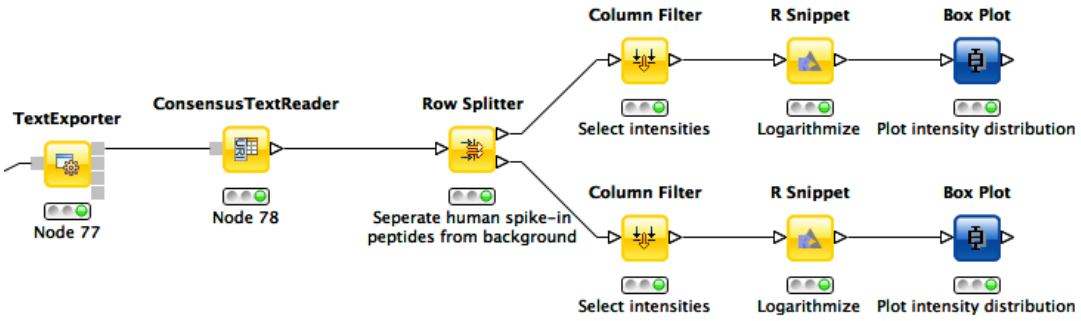

Figure 9: Simple KNIME data analysis example for LFQ

- Now, let's say we want to plot the log intensity distributions of the human spike-in peptides for all input files. In addition, we will plot the intensity distributions of the background peptides.
- As shown in Fig. 9, add a `Row Splitter` node Data Manipulation > Row > Filter after `ConsensusTextReader`. Double-click it to configure. The human spike-in peptides have accessions starting with "hum". Thus, set the column to test to *accessions*, select pattern matching as matching criterion, enter *hum** into the corresponding text field, and check the *contains wild cards* box. Press OK and execute the node.

- Row splitter produces two output tables: the first one contains all rows from the input table matching the filter criterion, and the second table contains all other rows. You can inspect the tables by right-clicking and selecting *Filtered* and *Filtered Out*. The former table should now contain only peptides with a human accession, whereas the latter should contain all remaining peptides (including unidentified ones).

- Now, since we only want to plot intensities, we can add a **Column Filter** node Data Manipulation > Column > Filter, connect its input port to the *Filtered* output port of the **Row Filter**, and open its configuration dialog. We could either manually select the columns we want to keep, or, more elegantly, select *Wildcard/Regex Selection* and enter *intensity_?* as the pattern. KNIME will interactively show you which columns your pattern applies to while you're typing.

- Since we want to plot log intensities, we will now compute the log of all intensity values in our table. The easiest way to do this in KNIME is a small piece of R code. Add an **R Snippet** node R after **Column Filter** and double-click to configure. In the *R Script* text editor, enter the following code:

```
x <- knime.in       # store copy of input table in x
x[x == 0] <- NA     # replace all zeros by NA (= missing value)
x <- log10(x)       # compute log of all values
knime.out <- x      # write result to output table
```

- Now we are ready to plot! Add a **Box Plot** node Data Views after the **R Snippet** node, execute it, and open its view. If everything went well, you should see a significant fold change of your human peptide intensities across the three runs.

- In order to verify that the concentration of background peptides is constant in all three runs, you can just copy and paste the three nodes after **Row Splitter** and connect the duplicated **Column Filter** to the second output port (*Filtered Out*) of **Row Splitter**, as shown in Fig. 9. Execute and open the view of your second **Box Plot**.

- That's it! You have constructed an entire identification and label-free quantification workflow including a simple data analysis using KNIME!

> **Note:** For further inspiration you might want to take a look at the more advanced KNIME data analysis examples in the metabolomics tutorial.

4 Metabolomics

4.1 Introduction

Quantitation and identification of chemical compounds are basic tasks in metabolomic studies. In this tutorial session we construct a UPLC-MS based, label-free quantitation and identification workflow. Following quantitation and identification we then perform statistical downstream analysis to detect quantitation values that differ significantly between two conditions. This approach can, for example, be used to detect biomarkers. Here, we use two spike-in conditions of a dilution series (0.5 mg/l and 10.0 mg/l, male blood background, measured in triplicates) comprising seven isotopically labeled compounds. Goal of this tutorial is to detect and quantify these differential spike-in compounds against the complex background.

4.2 Quantifying metabolites across several experiments

For the quantification of the metabolites we choose a similar approach to the one used for peptides based on one of OpenMS' feature finder.

- Create a new workflow called for instance "Metabolomics".
- Add a `Input Files` node and configure it with all mzML files from 🗀 `Metabolomics ▸ datasets`.
- Add a `ZipLoopStart` node and connect the `Input Files` node to the first port of the `ZipLoopStart` node.
- Add a `FeatureFinderMetabo` node (from Community Nodes › OpenMS › Quantitation) and connect the first output port of the `ZipLoopStart` to the `FeatureFinderMetabo`.
- For an optimal result adjust the following settings. Please note that some of these are advanced parameters.

parameter	value
algorithm → *common* → *chrom_fwhm*	8.0
algorithm → *mtd* → *trace_termination_criterion*	sample_rate
algorithm → *mtd* → *min_trace_length*	3.0
algorithm → *mtd* → *max_trace_length*	600.0
algorithm → *epd* → *width_filtering*	off

- Add a `ZipLoopEnd` node and connect the output of the `FeatureFinderMetabo` to the first port of the `ZipLoopEnd` node.
- After the `ZipLoopEnd` node add a `MapAlignerPoseClustering` node (Community Nodes › OpenMS › Map Alignment), set its Output Type to featureXML, and adjust the following settings

parameter	value
algorithm → *max_num_peaks_considered*	−1
algorithm → *superimposer* → *mz_pair_max_distance*	0.005
algorithm → *superimposer* → *num_used_points*	10000
algorithm → *pairfinder* → *distance_RT* → *max_difference*	20.0
algorithm → *pairfinder* → *distance_MZ* → *max_difference*	20.0
algorithm → *pairfinder* → *distance_MZ* → *unit*	*ppm*

- After the `MapAlignerPoseClustering` add a `FeatureLinkerUnlabeledQT` (Community Nodes › OpenMS › Map Alignment) and adjust the following settings

parameter	value
algorithm → *distance_RT* → *max_difference*	40.0
algorithm → *distance_MZ* → *max_difference*	20.0
algorithm → *distance_MZ* → *unit*	ppm

- After the `FeatureLinkerUnlabeledQT` add a `TextExporter` node (Community Nodes › OpenMS › File Handling).
- Add an `Output Folder` node and configure it with an output directory where you want to store the resulting files.

- Run the pipeline and inspect the output.

You should find a single, tab-separated file containing the information on where metabolites were found and with which intensities. You can also add `Output Folder` nodes at different stages of the workflow and inspect the intermediate results (e.g., identified metabolite features for each input map). The complete workflow can be seen in Figure 10. In the following section we will try to identify those metabolites.

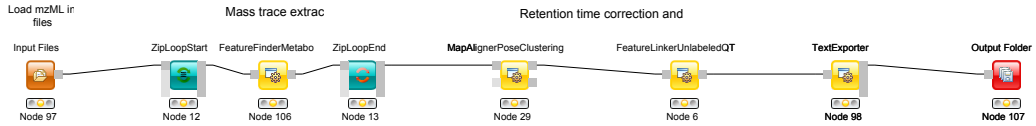

Figure 10: Label-free quantification workflow for metabolites

4.3 Identifying metabolites in LC-MS/MS samples

At the current state we found several metabolites in the individual maps but so far don't know what they are. To identify metabolites OpenMS provides the `AccurateMassSearch` node which searches observed masses against the Human Metabolome Database (HMDB)[8, 9, 10]. We start with the workflow from the previous section (see Figure 10).

- Add a `FileConverter` node and connect the output of the `FeatureLinkerUnlabeledQT` to the incoming port.
- Open the Configure dialog of the `FileConverter` and select the tab "OutputTypes". In the drop down list for FileConverter.1.out select "featureXML".
- Add an `AccurateMassSearch` node and connect the output of the `FileConverter` to the first port of the `AccurateMassSearch`.
- Add four `Input File` nodes and configure them with the following files
  - `Metabolomics▸databases▸PositiveAdducts.tsv`
    This file specifies the list of adducts that are considered in the positive mode. Each line contains the formula and charge of an adduct separated by a semicolon (e.g. M+H;1+). The mass of the adduct is calculated automatically.

  - Metabolomics ▸ databases ▸ NegativeAdducts.tsv
    This file specifies the list of adducts that are considered in the negative mode analogous to the positive mode.
  - Metabolomics ▸ databases ▸ HMDBMappingFile.tsv
    This file contains information from a metabolite database in this case from HMDB. It has three tab-separated columns mass, formula, and identifier. This allows for an efficient search by mass.
  - Metabolomics ▸ databases ▸ HMDB2StructMapping.tsv
    This file contains additional information about the identifiers in the mapping file. It has four tab-separated columns that contain the identifier, name, SMILES, and INCHI. These will be included in the result file. The identifiers in this file must match the identifiers in the HMDBMappingFile.tsv.

- In the same order as they are given above connect them to the remaining input ports of the AccurateMassSearch node.
- Add an Output Folder node and connect the first output port of the AccurateMassSearch node to the output folder.

The result of the AccurateMassSearch node is in the mzTab format [11] so you can easily open it in a text editor or import it into Excel or KNIME, which we will do in the next section. The complete workflow from this section is shown in Figure 11.

4.4 Convert your data into a KNIME table

The result from the TextExporter node as well as the result from the AccurateMassSearch node are files while standard KNIME nodes display and processes only KNIME tables. To convert these files into KNIME tables we need two different nodes. For the AccurateMassSearch results we use the SmallMoleculeMzTabReader node (Community Nodes › OpenMS › Conversion › mzTab), for the result of the TextExporter we use the ConsensusTextReader (Community Nodes › OpenMS › Conversion).

When executed, both nodes will import the OpenMS files and provide access to the data as KNIME tables. You can now easily combine both tables using the Joiner node

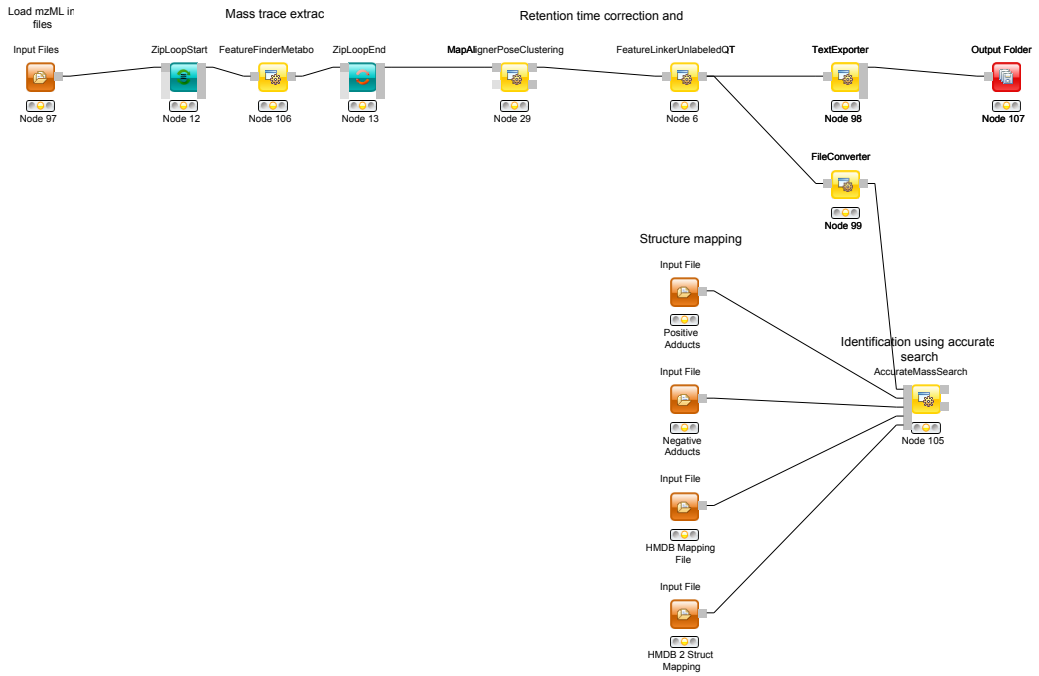

Figure 11: Label-free quantification and identification workflow for metabolites

(Data Manipulation » Column » Split & Combine) and configuring it to match the m/z and retention time values of the respective tables. The full workflow is shown in Figure 12.

4.4.1 Bonus task: Visualising data

Now that you have your data in KNIME you should try to get a feeling for the capabilities of KNIME.

Task

☑ Check out the `Molecule Type Cast` node to render the structural formula contained in the result table.

Task

☑ Have a look at the `Column Filter` node to reduce the table to the interesting columns, e.g., only the Ids, chemical formula, and intensities.

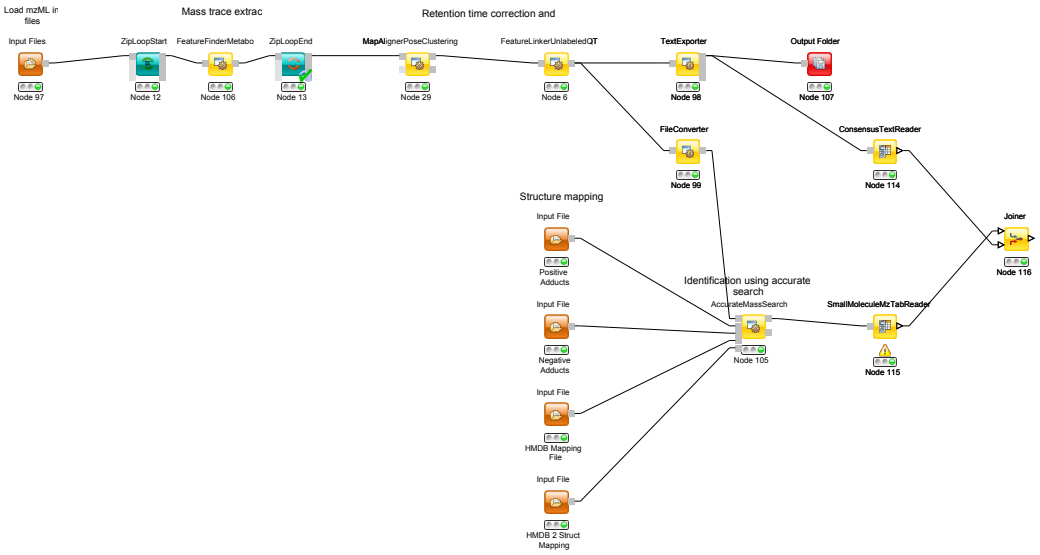

Figure 12: Label-free quantification and identification workflow for metabolites that loads the results into KNIME and joins the tables.

Task

Try to compute and visualise the m/z and retention time error of the different elements of the consensus features.

4.5 Downstream data analysis and reporting

In this part of the metabolomics session we take a look at more advanced downstream analysis and the use of the statistical programming language R. As laid out in the introduction we try to detect a set of spike-in compounds against a complex blood background. As there are many ways to perform this type of analysis we provide a complete workflow.

Task

Import the workflow from `Workflows▸metabolite_analysis.zip` in KNIME:
File ▸ Import KNIME Workflow...

The section below will guide you in your understanding of the different parts of the workflow. Once you understood the workflow you should play around and be creative.

Maybe create a novel visualization in KNIME or R? Do some more elaborate statistical analysis? Feel free to experiment and show us your results if you like. Note that some basic R knowledge is required to fully understand the processing in `R Snippet` nodes.

4.5.1 Data preparation ID

This part is analogous to what you did for the simple metabolomics pipeline.

4.5.2 Data preparation Quant

The first part is identical to what you did for the simple metabolomics pipeline. Additionally, we convert zero intensities into NA values and remove all rows that contain at least one NA value from the analysis. We do this using a very simple `R Snippet` and subsequent `Missing Value filter` node.

Task

Inspect the `R Snippet` by double-clicking on it. The KNIME table that is passed to an `R Snippet` node is available in R as a data.frame named knime.in. The result of this node will be read from the data.frame knime.out after the script finishes. Try to understand and evaluate parts of the script (Eval Selection). In this dialog you can also print intermediary results using for example the R command head() or cat() to the Console pane.

4.5.3 Statistical analysis

After we linked features across all maps, we want to identify features that are significantly deregulated between the two conditions. We will first scale and normalize the data, then perform a t-test, and finally correct the obtained p-values for multiple testing using Benjamini-Hochberg. All of these steps will be carried out in individual `R Snippet` nodes.

- Double-click on the first `R Snippet` node labeled "log scaling" to open the `R Snippet` dialog. In the middle you will see a short R script that performs the log scaling. To perform the log scaling we use a so-called regular expression (grepl) to select all columns containing the intensities in the six maps and take the log_2 logarithm.

- The output of the log scaling node is also used to draw a boxplot that can be used to examine the structure of the data. Since we only want to plot the intensities in the different maps (and not m/z or rt) we first use a `Column Filter` node to keep only the columns that contain the intensities. We connect the resulting table to a `Box Plot` node which draws one box for every column in the input table. Right-click and select View: Box Plot.

- The median normalization is performed in a similar way to the log scaling. First we calculate the median intensity for each intensity column, then we subtract the median from every intensity.

- Open the `Box Plot` connected to the normalization node and compare it to the box plot connected to the log scaling node to examine the effect of the median normalization.

- To perform the t-test we defined the two groups we want to compare. Then we call the t-test for every consensus feature unless it has missing values. Finally we save the p-values and fold-changes in two new columns named p-value and FC.

- The `Numeric Row Splitter` is used to filter less interesting parts of the data. In this case we only keep columns where the fold-change is ≥ 2.

- We adjust the p-values for multiple testing using Benjamini-Hochberg and keep all consensus features with a q-value ≤ 0.01 (i.e. we target a false-discovery rate of 1%).

4.5.4 Data preparation for Reporting

Following the identification, quantification and statistical analysis our data is merged and formatted for reporting. First we want to discard our normalized and logarithmized intensity values in favor of the original ones. To this end we first remove the intensity columns (`Column Filter`) and add the original intensities back (`Joiner`). Note that we use an *Inner Join* [1]. Combining ID and Quantification table into a single table is again achieved using a `Joiner` node.

[1] *Inner Join* is a technical term that describes how database tables are merged.

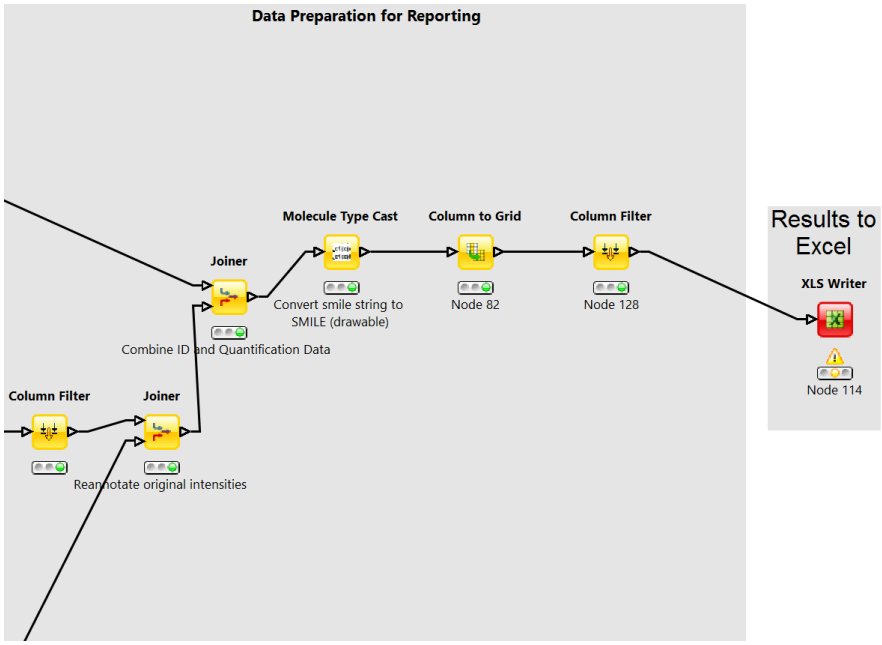

Figure 13: Data preparation for reporting

Question

What happens if we use an *Left Outer Join*, *Right Outer Join* or *Full Outer Join* instead of the *Inner Join*?

Task

Inspect the output of the join operation and after the Molecule Type Cast.

While all relevant information is now contained in our table the presentation could be improved. Currently, we have several rows corresponding to a single consensus feature (=linked feature) but with different, alternative identifications. It would be more convenient to have only one row for each consensus feature with all accurate mass identifications added as additional columns. To achieve we use the `Column to Grid` node that flattens several rows with the same consensus number into a single one. Note that we have to specify the maximum number of columns in the grid so we set this to a large value (e.g. 100). We finally select only the columns we are interested in with the last `Column Filter` and export the data to an Excel file (`XLS Writer`).

5 OpenSWATH

5.1 Introduction

OpenSWATH [12] is a module of OpenMS that allows analysis of LC-MS/MS DIA (data independent acquisition) data using the approach described by Gillet *et al.* [13]. The DIA approach described there uses 32 cycles to iterate through precursor ion windows from 400-426 Da to 1175-1201 Da and at each step acquires a complete, multiplexed fragment ion spectrum of all precursors present in that window. After 32 fragmentations (or 3.2 seconds), the cycle is restarted and the first window (400-426 Da) is fragmented again, thus delivering complete "snapshots" of all fragments of a specific window every 3.2 seconds.

The analysis approach described by Gillet et al. extracts ion traces of specific fragment ions from all MS2 spectra that have the same precursor isolation window, thus generating data that is very similar to SRM traces.

5.2 Installation of OpenSWATH

OpenSWATH has been fully integrated since OpenMS 1.10 (`http://open-ms.sourceforge.net` [3, 2, 14]).

5.3 Installation of mProphet

mProphet (`http://www.mprophet.org/`) [15] is available as standalone script in 🗀 `External_Tools` ▸ `mProphet`. R (`http://www.r-project.org/`) and the package MASS (`http://cran.r-project.org/web/packages/MASS/`) are further required to execute mProphet. Please obtain a version for either Windows, Mac or Linux directly from CRAN.

pyprophet, a much faster reimplementation of the mProphet algorithm is available from PyPI (`https://pypi.python.org/pypi/pyprophet/`). The usage of pyprophet instead of mProphet is suggested for large-scale applications, but the installation requires more dependencies and therefore, for this tutorial the application of mProphet is described.

5.4 Generating the Assay Library

5.4.1 Generating TraML from transition lists

OpenSWATH requires the assay libraries to be supplied in the TraML format [16]. To enable manual editing of transition lists, the TOPP tool `ConvertTSVToTraML` is available that uses tab separated files as input. Example datasets are provided in 🗀 `OpenSWATH` ▸ `assay`. Please note that the transition lists need to be named `.csv`.

The header of the transition list contains the following variables (with example values in brackets):

`PrecursorMz`
: The mass-to-charge (m/z) of the precursor ion. (728.88)

`ProductMz`
: The mass-to-charge (m/z) of the product or fragment ion. (924.539)

`Tr_recalibrated`
: The **normalized** retention time (or iRT) [17] of the peptide. (26.5)

`transition_name`
: A **unique** identifier for the transition.
(AQUA4SWATH_HMLangeA_ADSTGTLVITDPTR(UniMod:267)/2_y8)

`CE`
: The collision energy that should be used for the acquisition. (27)
`Optional` (not used by OpenSWATH)

`LibraryIntensity`
: The relative intensity of the transition. (3305.3)

`transition_group_id`
: A **unique** identifier for the transition group.
(AQUA4SWATH_HMLangeA_ADSTGTLVITDPTR(UniMod:267)/2)

`decoy`
: A binary value whether the transition is target or decoy (target:0, decoy:1). (0)

PeptideSequence

The unmodified peptide sequence. (ADSTGTLVITDPTR)

ProteinName

A unique identifier for the protein. (AQUA4SWATH_HMLangeA)

Annotation

The fragment ion annotation. (y8)

`Optional` (not used by OpenSWATH)

FullUniModPeptideName

The peptide sequence with UniMod modifications. (ADSTGTLVITDPTR(UniMod:267))

MissedCleavages

The number of missed cleavages during enzymatic digestion. (0)

`Optional` (not used by OpenSWATH)

Replicates

The number of replicates. (0)

`Optional` (not used by OpenSWATH)

NrModifications

The number of modifications. (0)

`Optional` (not used by OpenSWATH)

PrecursorCharge

The precursor ion charge. (2)

GroupLabel

The stable isotope label. (light)

`Optional` (not used by OpenSWATH)

UniprotID

The Uniprot ID of the protein. ()

`Optional` (not used by OpenSWATH)

To convert transitions lists to TraML, use `ConvertTSVToTraML`:

Linux or Mac

On the Terminal:

```
ConvertTSVToTraML -in OpenSWATH_SGS_AssayLibrary.csv -out OpenSWATH_SGS_AssayLibrary.↩
    TraML
```

Windows

On the TOPP command line:

```
ConvertTSVToTraML.exe -in OpenSWATH_SGS_AssayLibrary.csv -out OpenSWATH_SGS_AssayLibrary↩
    .TraML
```

5.4.2 Appending decoys to a TraML

To append decoys to a TraML, the TOPP tool `OpenSwathDecoyGenerator` can be used:

Linux or Mac

On the Terminal:

```
OpenSwathDecoyGenerator -in OpenSWATH_SGS_AssayLibrary.TraML -out ↩
    OpenSWATH_SGS_AssayLibrary_with_Decoys.TraML -min_transitions 3 -max_transitions 6 -↩
    method shuffle -append -exclude_similar
```

Windows

On the TOPP command line:

```
OpenSwathDecoyGenerator.exe -in OpenSWATH_SGS_AssayLibrary.TraML -out ↩
    OpenSWATH_SGS_AssayLibrary_with_Decoys.TraML -min_transitions 3 -max_transitions 6 -↩
    method shuffle -append -exclude_similar
```

5.5 OpenSWATH KNIME

An example KNIME workflow for OpenSWATH is supplied in 🗀 `Workflows` (Figure 14). The example dataset can be used for this workflow (filenames in brackets):

1. Open Workflows ▸ OpenSWATH.zip in KNIME: File » Import KNIME Workflow...
2. Select the normalized retention time (iRT) assay library in TraML format by double-clicking on node Input File » iRT Assay Library.
   (OpenSWATH ▸ assay ▸ OpenSWATH_iRT_AssayLibrary.TraML)
3. Select the SWATH MS data in mzML format as input by double-clicking on node Input File » SWATH-MS files.
   (OpenSWATH ▸ data ▸ split_napedro_L120420_010_SW-*.nf.pp.mzML)
4. Select the target peptide assay library in TraML format as input by double-clicking on node Input Files » Assay Library.
   (OpenSWATH ▸ assay ▸ OpenSWATH_SGS_AssayLibrary.TraML)
5. Set the output destination by double-clicking on node Output File.
6. Run the workflow.

The resulting output can be found at your selected path, which will be used as input for mProphet. Execute the script on the Terminal (Linux or Mac) or cmd.exe (Windows) in OpenSWATH ▸ result:

```
R --slave --args bin_dir=../../../External_Tools/mProphet/ mquest=OpenSWATH_output.csv workflow=↩
    LABEL_FREE num_xval=5 run_log=FALSE write_classifier=1 write_all_pg=1 < ../../../↩
    External_Tools/mProphet/mProphet.R
```

The main output will be called
OpenSWATH ▸ result ▸ mProphet_all_peakgroups.xls
with statistical information available in
OpenSWATH ▸ result ▸ mProphet.pdf.

Please note that due to the semi-supervised machine learning approach of mProphet the results differ slightly when mProphet is executed several times.

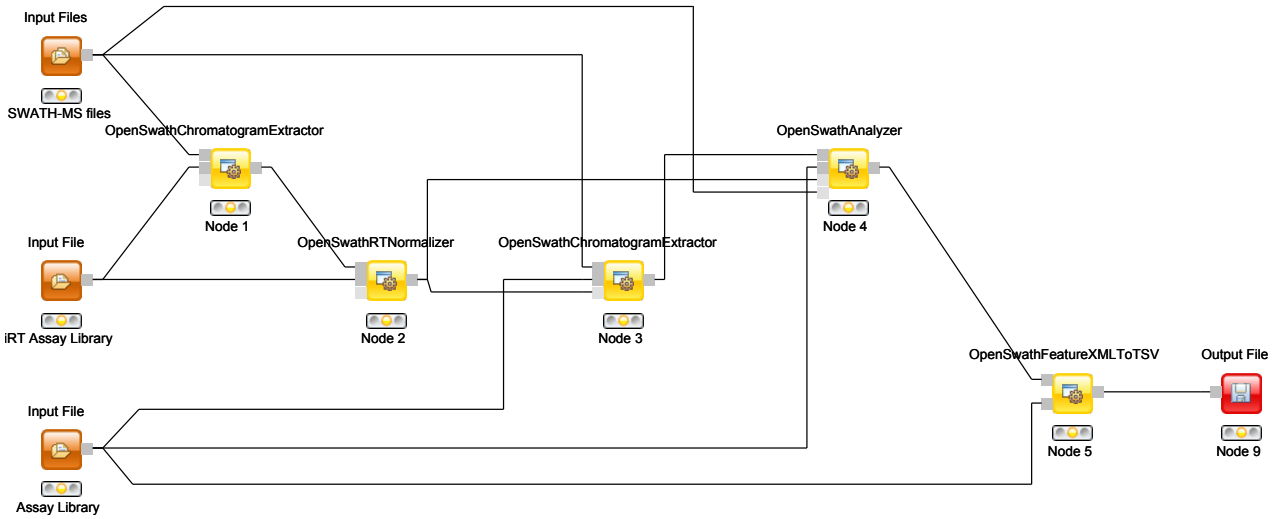

Figure 14: OpenSWATH KNIME Workflow.

5.6 Example dataset

This sample dataset is part of the larger SWATH MS Gold Standard (SGS) dataset which is described in the publication of Roest et al. [12]. It contains one of 90 SWATH MS runs with significant data reduction (peak picking of the raw, profile data) to make file transfer and working with it easier.

5.7 Real-life applications

SWATH-MS datasets are huge, several gigabyte per run. Especially when complex samples in combination with large assay libraries are analyzed, the TOPP tool based workflow requires much computational resources. For this reason, an integrated tool (`OpenSwathWorkflow`) combining all the steps in a single executable has been developed. It is shipped with OpenMS/develop and will be shipped with OpenMS 1.12. Instructions on how to use this tool can be found on `http://www.openswath.org`.

References

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