### Installation

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The OpenSWATH Workflow enables targeted data analysis of data-independent acquisition (DIA) or SWATH-MS proteomic data. The main workflow consists of OpenSWATH, PyProphet, TRIC, IPF and TAPIR. This website provides documentation on installation and application of the tools.
CHAPTER 1

News

Note: 2018-11-07: The Docker image now includes OpenMS 2.4.0 and PyProphet 2.0.1.

Note: 2018-11-07: With the release of OpenMS 2.4.0 and PyProphet 2.0.1, the new OpenSWATH workflow is available in the release branches.

Note: 2018-03-22: We provide an experimental Docker image for the latest development version of the OpenSWATH workflow.

Note: 2017-12-28: The tools of the OpenSWATH Workflow now provide experimental support for new SQLite-based file formats.
The tools and workflows are being developed at the Aebersold Group at IMSB, ETH Zurich, University of Toronto and Columbia University with contributions from others. The core components are implemented as part of the OpenMS framework, the PyProphet, and msproteomicstools distributions.

## 2.1 Docker

### 2.1.1 Overview

Docker is a flexible and popular container management platform. With only few steps, you can obtain a full OpenSWATH installation including all dependencies on Windows, macOS and Linux. The OpenSWATH docker container will continuously be updated to the latest development version.

Users who just want to run the OpenSWATH workflow on their desktops can follow the instructions below to obtain an installation-free, always-updated workflow. It will run with nearly native speed on desktops, clusters and cloud environments.

### 2.1.2 Installing Docker

First, install Docker Community Edition for either Windows, macOS or Linux. On Linux, most current distributions provide this out-of-the-box. After installation, ensure that you assign the number of CPUs and RAM that you would like to use with OpenSWATH (e.g. 4 CPUs, 8GB RAM) and make sure to share your local drives. Please follow the instructions for Windows and macOS.

### 2.1.3 Running OpenSWATH in Docker

Make sure that Docker is up and running in the background. On macOS or Linux, start a Terminal session. On Windows, start PowerShell, CMD or similar. Execute the following steps within the console:
# Download OpenSWATH image (openswath/openswath:0.2.0)
docker pull openswath/openswath:0.2.0

This will download the latest version of the OpenSWATH Docker image and cache it on your machine.

**Note:** Official containers for OpenMS and related tools are available via BioContainers. The image provided here provides compatible release or development versions including related tools in an integrated container.

# Generate tutorial container (osw_tutorial) and log in
docker run --name osw_tutorial --rm -v ~/Desktop/:/data -i -t openswath/openswath:0.2.0

This command will start a container based on the OpenSWATH image and map the local volume ~/Desktop/ (from your desktop) to /data/ (to your container). It will open a Bash command line within the container for you to control the individual components of the workflow. If you want to exit the session, just type `exit` to return to your console.

Within the running container, you can execute all commands as you would in a native environment:

```bash
# Execute OpenSwathWorkflow in docker
OpenSwathWorkflow --help

# Execute PyProphet in docker
pyprophet --help

# Execute TRIC in docker
feature_alignment.py --help
```

All data that will be stored in ~/Desktop, will be available in /data/. For example, we can process and write back the files like this:

```bash
OpenSwathWorkflow \n -in /data/data.mzML \n -tr /data/library.tsv \n -tr_irt /data/iRT_assays.TraML \n -swath_windows_file /data/SWATHwindows_analysis.tsv \n -sort_swath_maps -batchSize 1000 \n -readOptions cacheWorkingInMemory -tempDirectory /tmp/ \n -use_msl_traces \n -mz_extraction_window 50 -ppm \n -mz_correction_function quadratic_regression_delta_ppm \n -TransitionGroupPicker:background_subtraction original \n -RTNormalization:alignmentMethod linear \n -Scoring:stop_report_after_feature 5 \n -out_tsv /data/osw_output.tsv 
```

2.1.4 Software version information

Please find further information at the GitHub repository.
2.2 Binaries

2.2.1 Overview

Precompiled or prepackaged versions of OpenMS, PyProphet and msproteomicstools are readily available for most supported platforms.

2.2.2 OpenMS

OpenSWATH is completely integrated into OpenMS. The current releases can be obtained from GitHub. To make use of the latest developments, consider using the nightly builds for Windows and macOS.

Instructions for compilation and installation can be obtained from the OpenMS documentation.

2.2.3 PyProphet

PyProphet requires Python 2.7 or Python 3. Windows users should install Anaconda. Mac and Linux users should be able to install PyProphet directly from GitHub. It is strongly recommended to install PyProphet within a virtualenv.

```
# Install dependencies
pip install numpy scipy scikit-learn pandas numexpr statsmodels Click matplotlib
→seaborn

# Install PyProphet release version
pip install pyprophet

# Alternative: Install PyProphet development version
pip install git+https://github.com/PyProphet/pyprophet.git@master
```

2.2.4 msproteomicstools & TRIC

msproteomicstools requires Python 2.7 and can be installed through pip. On Microsoft Windows you will first have to install Python (the easiest way to do this is to download Anaconda). You can then install msproteomicstools through PyPI:

```
pip install numpy
pip install msproteomicstools
```

You can alternatively download the msproteomicstools release directly from PyPI. To obtain the latest development version, please download the code from GitHub. If you are using Microsoft Windows and Anaconda, it is possible that BioPython does not properly install and you may have to install it through Anaconda:

```
conda install biopython
```

2.3 Sources

2.3.1 Overview

The source code of OpenMS, PyProphet and msproteomicstools is provided on GitHub under the 3-clause BSD license. Please refer to the individual repositories for information regarding obtaining, compiling and installing the
components.

### 2.3.2 OpenMS

OpenSWATH is fully integrated within OpenMS. Please obtain the latest version of OpenMS from GitHub. Instructions for compilation and installation can be obtained from the OpenMS documentation.

### 2.3.3 PyProphet

Please obtain the latest version of PyProphet from GitHub. The instructions for the installation of the PyProphet Binaries is applicable here as well.

### 2.3.4 msproteomicstools & TRIC

Please obtain the latest version of msproteomicstools from GitHub. The instructions for the installation of the msproteomicstools Binaries is applicable here as well.

### 2.4 Getting Started

**Note:** OpenSWATH is part of the OpenMS project, therefore if you install OpenMS you will get OpenSWATH automatically. Please refer to our installation instructions.

To get started with the DIA or SWATH-MS analysis, we recommend to follow the recently published step-for-step tutorial describing a complete OpenSWATH analysis workflow using OpenSwathWorkflow available from bioRxiv. In addition, you can find in-depth information about the different tools in a SWATH analysis on this page.

#### 2.4.1 Tutorial Data

You can access the tutorial data (a *M. tuberculosis* dataset) used in the 2017 Methods Mol Biol. OpenSWATH tutorial here. The data contains

- 3 mzML instrument data files (centroided)
- 3 WIFF raw instrument data files
- Mtb assay library (for OpenMS 2.1)
- Mtb assay library (for older OpenMS)
- Swath windows file for analysis
- iRT assay file (TraML format)

#### 2.4.2 Questions?

Please address questions directly to the mailing list.

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2.4.3 References

2.5 Generic Transition Lists

2.5.1 Overview

OpenSWATH\textsuperscript{1} supports generic transition lists in tab- or comma-separated value (TSV/CSV) formats. TargetedFileConverter is part of OpenMS\textsuperscript{2} and is available for Windows, macOS and Linux.

Transitions lists from Trans-Proteomic Pipeline and Skyline can be directly converted. Alternatively, it is possible to use the generic transition list format, described below.

2.5.2 Contact and Support

We provide support for TargetedFileConverter using the OpenMS support channels. Please address general questions to the open-ms-general mailing list.

2.5.3 Format

The input to TargetedFileConverter can come either directly from Trans-Proteomic Pipeline or Skyline or the generic transition list format. The columns should be separated either by comma or tab and the files should be saved with Windows or Unix line endings.

Required Columns

The following columns are required:

- PrecursorMz * (float)
- ProductMz * (float; synonyms: FragmentMz)
- LibraryIntensity * (float; synonyms: RelativeFragmentIntensity)
- NormalizedRetentionTime * (float; synonyms: RetentionTime, Tr_recalibrated, iRT, RetentionTime-CalculatorScore) (normalized retention time)

Targeted Proteomics Columns

For targeted proteomics, the following additional columns should be provided:

- ProteinId ** (free text; synonyms: ProteinName)
- PeptideSequence ** (free text, sequence only (no modifications); synonyms: Sequence, StrippedSequence)
- ModifiedPeptideSequence ** (free text, should contain modifications; synonyms: FullUniModPeptide-Name, FullPeptideName, ModifiedSequence)
- PrecursorCharge ** (integer, contains the charge of the precursor; synonyms: Charge)


• **ProductCharge** *(integer, contains the fragment charge; synonyms: FragmentCharge)*
• **FragmentType** *(free text, contains the type of the fragment, e.g. “b” or “y”)*
• **FragmentSeriesNumber** *(integer, e.g. for y7 use “7” here; synonyms: FragmentNumber)*

**Warning:** ModifiedPeptideSequence should contain modifications in UniMod (preferred) (.(UniMod:1)PEPC(UUniMod:4)PEPM(UUniMod:35)PEPR.(UniMod:2)) or TPP (n[43]PEPC[160]PEPM[147]PEPRc[16]) formats. N- and C-terminal modifications are indicated after a pre- or suffix ..

### Targeted Metabolomics Columns

For targeted metabolomics, the following fields are also supported:

• **CompoundName** *(synonyms: CompoundId)*
• **SMILES**
• **SumFormula**

### Grouping Columns

OpenSWATH uses grouped transitions to detect candidate analyte signals. These groups are by default generated based on the input, but can also be manually specified:

• **TransitionGroupId** *(free text, designates the transition group [e.g. peptide] to which this transition belongs; synonyms: TransitionGroupName, transition_group_id)*
• **TransitionId** *(free text, needs to be unique for each transition [in this file]; synonyms: TransitionName, transition_name)*
• **Decoy** *(1: decoy, 0: target, i.e. no decoy; determines whether the transition is a decoy transition or not, synonyms: decoy, isDecoy)*
• **PeptideGroupLabel** *(free text, designates to which peptide label group (as defined in MS:1000893) the peptide belongs to)*
• **DetectingTransition** *(1: use transition to detect peak group, 0: don’t use transition for detection; synonyms: detecting_transition)*
• **IdentifyingTransition** *(1: use transition for peptidoform inference using IPF, 0: don’t use transition for identification; synonyms: identifying_transition)*
• **QuantifyingTransition** *(1: use transition to quantify peak group, 0: don’t use transition for quantification; synonyms: quantifying_transition)*

**Warning:** If you are unsure about these columns, TargetedFileConverter and the downstream tools OpenSwathAssayGenerator and OpenSwathDecoyGenerator will generate them automatically for you.

### Optional Columns

Optionally, the following columns can be specified but they are not actively used by OpenSWATH:
• CollisionEnergy (float; synonyms: CE)
• Annotation (free text, e.g. y7)
• UniprotId (free text; synonyms: UniprotID)
• LabelType (free text, optional description of which label was used, e.g. heavy or light)

Fields indicated with * are strictly required while fields indicated with ** are only required in the specific context (proteomics or metabolomics).

2.5.4 Conversion

Once the generic transition list is generated by the user (or TPP or Skyline), peptide query parameters can be derived by the OpenSWATH tools part of OpenMS. Please follow the instructions on how to install OpenSWATH prior to the next steps.

```
# Import from SpectraST MRM
TargetedFileConverter -in transitionlist.mrm -out transitionlist.TraML

# Import from Skyline or generic transition list format (TSV)
TargetedFileConverter -in transitionlist.tsv -out transitionlist.TraML

# Import from Skyline or generic transition list format (CSV)
TargetedFileConverter -in transitionlist.csv -out transitionlist.TraML
```

2.5.5 References

2.6 Trans-Proteomic Pipeline

2.6.1 Overview

The Trans-Proteomic Pipeline provides a complete workflow to generate spectral libraries suitable for OpenSWATH. Data sets acquired in data-dependent acquisition (DDA) or data-independent acquisition (DIA; preprocessed by DIA-Umpire²) can be searched by spectrum-centric scoring against a reference FASTA database using several supported search engines. After statistical validation using PeptideProphet, iProphet & MAYU, a consensus spectral library is generated by SpectraST, which serves as final input for the OpenMS tool TargetedFileConverter.

2.6.2 Contact and Support

We provide support separately for the TPP, the msproteomicstools and the OpenMS components of the workflow.

2.6.3 Tutorial

A comprehensive tutorial¹ describes the individual steps to generate spectral libraries for SWATH-MS using the Trans-Proteomic Pipeline (TPP). Please follow the tutorial until the generation of SpectraST spectral libraries. If you are

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using DIA data, follow the DIA-Umpire tutorial to generate pseudo-spectra first, which can then be processed using the TPP.

In the last step, import the SpectraST consensus library (format .sptxt or .splib) and convert it to a MRM transition list:

```
# This will generate the file db_assays.mrm
spectrast -cNdb_pqp -cICID-QTOF -cM db_consensus.splib
```

Convert then the MRM transition list to a TraML spectral library and follow the remaining steps in the Generic Transition Lists section.

### 2.6.4 References

### 2.7 Skyline

#### 2.7.1 Overview

Skyline\(^1\) is the most popular analysis software for targeted proteomics. It can be used to export spectral libraries suitable for OpenSWATH.

#### 2.7.2 Contact and Support

Skyline is supported by a very active community via their message board.

For TargetedFileConverter, we provide support via the OpenMS support channels.

#### 2.7.3 Tutorial

The Skyline team provides extensive documentation and tutorials and their website. Please follow their instructions to generate spectral or chromatogram libraries. To export a library for use in OpenSWATH, select File and then Report. You can then import the OpenSWATH.skyr report definition file, which will directly export all necessary columns.

Convert the Skyline transition list to a TraML spectral library and follow the remaining steps in the Generic Transition Lists section.

#### 2.7.4 References

### 2.8 SWATHAtlas

#### 2.8.1 Overview

SWATHAtlas is a repository providing spectral libraries generated from endogeneous samples and synthetic peptides. The libraries are preformatted for several commonly employed targeted data analysis tools, e.g. OpenSWATH, Skyline, Spectronaut and PeakView.

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2.8.2 Contact and Support

The libraries are processed by different tools and we thus provide support via different channels. If you encounter problems with obtaining the libraries, please contact the SWATHAtlas team, e.g. via the TPP Support Group.

For support regarding spectrast2tsv.py, please use the msproteomicstools issue tracker or consider the support channels for the OpenMS components of the workflow.

2.9 Peptide Query Parameter Generation

2.9.1 PQP Generation for OpenSWATH

After importing transitions lists from an upstream workflow (e.g. Trans-Proteomic Pipeline, Skyline or Generic Transition Lists), the transitions can then be optimized using a set of heuristic rules¹:

```
OpenSwathAssayGenerator -in transitionlist.TraML
  -out transitionlist_optimized.TraML
  -swath_windows_file swath64.txt
```

Please note that the SWATH windows file should be of the following format (tab-separated), including header:

```
lower_offset  upper_offset
400    425
424    450
...   ...
```

If necessary, the rules for transition selection can be modified with the following parameters:

```
OpenSwathAssayGenerator <other parameters>
  -min_transitions 6
  -max_transitions 6
  -allowed_fragment_types b,y
  -allowed_fragment_charges 1,2,3,4
  -enable_detection_specific_losses
  -enable_detection_unspecific_losses
  -precursor_mz_threshold 0.025
  -precursor_lower_mz_limit 400
  -precursor_upper_mz_limit 1200
  -product_mz_threshold 0.025
  -product_lower_mz_limit 350
  -product_upper_mz_limit 2000
```

2.9.2 PQP Generation for IPF

If IPF scoring² should be conducted, the following parameters should be considered in addition to the others above:


Unimod contains descriptions of more than 1400 post-translational modifications and represents the standard database. However, many modification types are annotated with residue modifiabilities that go beyond the canonical set (e.g. phosphorylation (S,T,Y,D,H,C,R,K) instead of (S,T,Y)).

For the purpose of site-localization, it is thus very important to provide a modified (restricted) Unimod file to OpenSwathAssayGenerator. This file can be created by editing the file unimod.xml to only the desired modifications and residue modifiabilities. We provide an example for Phosphorylation (S,T,Y), Carbamidomethyl (C), Oxidation (M) and SILAC (R, K). Please note that also usually fixed PTMs like Carbamidomethyl and Oxidation need to be set.

The generation of identification transitions can be adjusted if necessary by the following parameters:

OpenSwathAssayGenerator <other parameters>
-enable_ipf \\
-unimod_file unimod_phospho.xml \\
-max_num_alternative_localizations 10000 \\
-disable_identification_ms2_precursors \\
-disable_identification_specific_losses \\
-enable_identification_unspecific_losses \\
-enable_swath_specificity \\

OpenSwathAssayGenerator excludes peptides that can have too many combinations of alternative site-localization (track the process by setting -debug 10). If 10000 alternative peptidoforms are too few, consider increasing this parameter.

By default, unfragmented precursors are extracted from the SWATH maps and used for scoring by IPF, this can optionally be disabled (-disable_identification_ms2_precursors). Specific losses (e.g. for Phosphorylation) are used by default and improve specificity; unspecific losses are not recommended to use.

In scenarios with extremely small precursor isolation windows (e.g. < 1 Th), -enable_swath_specificity can be used to skip the precursor inference step of IPF. This is not recommended in general.

### 2.9.3 Decoy Generation

Decoys can then be appended using OpenSwathDecoyGenerator:

OpenSwathDecoyGenerator -in transitionlist_optimized.TraML \\
-out transitionlist_optimized_decoys.TraML \\

**Warning:** If you used non-default parameters in OpenSwathAssayGenerator (i.e. -product_mz_threshold, -allowed_fragment_types, -allowed_fragment_charges, -enable_detection_specific_losses or -enable_detection_unspecific_losses), make sure to also specify them for OpenSwathDecoyGenerator. The flag --help will show a list of all options.

You can then convert the TraML to a PQP file:

TargetedFileConverter -in transitionlist_optimized_decoys.TraML \\
-out transitionlist_optimized_decoys.PQP \\

This processed spectral library (including decoys) is the input for OpenSWATH.
2.9.4 References

2.10 OpenSWATH

2.10.1 Overview

OpenSWATH is a proteomics software that allows analysis of LC-MS/MS DIA (data independent acquisition) data using the approach described by Gillet et al. and implemented as part of OpenMS. The original SWATH-MS method uses 32 cycles to iterate through precursor ion windows from 400-426 Da to 1175-1201 Da and at each step acquire 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.

The OpenSwathWorkflow executable is currently the most efficient way of running OpenSWATH and it is available through OpenMS. An extended tutorial describing a complete OpenSWATH analysis workflow using OpenSwathWorkflow was recently published and is also available from bioRxiv with its associated dataset.

The OpenSwathWorkflow implements the OpenSWATH analysis workflow as described in and provides a complete, integrated analysis tool without the need to run multiple tools consecutively.

It executes the following steps in order:

- Reading of the raw input file (provided as mzML, mzXML or sqMass) and RT normalization transition list
- Computing the retention time transformation using RT normalization peptides
- Reading of the transition list
- Extracting the specified transitions
- Scoring the peak groups in the extracted ion chromatograms (XIC)
- Reporting the peak groups and the chromatograms

2.10.2 Contact and Support

We provide support for OpenSWATH using the OpenMS support channels. Please address general questions to the mailing list.

You can contact the authors Hannes Röst and George Rosenberger.

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2.10.3 Input

The input to OpenSwathWorkflow are provided using the following files:

- **in** raw input file (provided as mzML, mzXML or sqMass)
- **tr** transition list (spectral library)
- **tr_irt** an optional transition file containing RT normalization coordinates
- **swath_windows_file** an optional file specifying the *analysis* SWATH windows

**Mass spectrometric data**

The input file *in* is generally a single mzML, mzXML or sqMass file (converted from a raw vendor file format using ProteoWizard).

**Spectral library**

The spectral library *tr* is a spectral library either in .tsv, .TraML or .PQP format (where the TSV or PQP format is recommended). Further information in generating these files can be found in the *Generic Transition Lists* section.

**Retention time normalization**

The retention time normalization peptides are provided using the optional parameter *tr_irt* in TraML format. We suggest to use the *iRTassays.TraML* file provided in the tutorial dataset, if the Biognosys iRT-kit was used during sample preparation.

If the iRT-kit was not used, it is highly recommended to use or generate a set of endogenous peptides for RT normalization. A recent publication\(^5\) provides such a set of CiRT peptides suitable for many eukaryotic samples. The TraML file from the supplementary information can be used as input for *tr_irt*. Since not all CiRT peptides might be found, the flag RTNormalization:estimateBestPeptides should be set to improve initial filtering of poor signals. Further parameters for optimization can be found when invoking OpenSwathWorkflow --help under the RTNormalization section. Those do not require adjustment for most common sample types and LC-MS/MS setups, but might be useful to tweak for specific scenarios.

**SWATH windows definition**

The SWATH windows themselves can either be read from the input files, but it is recommended to provide them explicitly in tab-delimited form. Note that there is a difference between the SWATH window acquisition scheme settings and the SWATH window analysis settings:

The acquisition settings tell the instrument how to acquire the data and how to filter the transitions (see section *Peptide Query Parameter Generation*).

The analysis settings on the other hand specify from which precursor isolation windows to extract the data. Note that the analysis windows should not have any overlap.

We suggest to use the SWATHwindows_analysis.tsv file provided in the tutorial dataset for 32 windows of 25 Da each.

2.10.4 Parameters

Caching of mass spectrometric data

Due to the large size of the files, OpenSwathWorkflow implements a caching strategy where files are cached to disk and then read into memory SWATH-by-SWATH. You can enable this by setting `-readOptions cacheWorkingInMemory -tempDirectory /tmp` where you would need to adjust the temporary directory depending on your platform.

Other potentially useful options you may want to turn on are `batchSize` and `sort_swath_maps`.

Chromatographic parameters

The current parameters are optimized for 2 hour gradients on SCIEX 5600 / 6600 TripleTOF instruments with a peak width of around 30 seconds using iRT peptides. If your chromatography differs, please consider adjusting `-Scoring:TransitionGroupPicker:min_peak_width` to allow for smaller or larger peaks and adjust the `-rt_extraction_window` to use a different extraction window for the retention time.

Mass spectrometric parameters

In m/z domain, consider adjusting `-mz_extraction_window` to your instrument resolution, which can be in Th or ppm (using `-ppm`). In addition to using the iRT peptides for correction of the retention time space, OpenSWATH can also use those peptides to correct the m/z space with the option `-mz_correction_function quadratic_regression_delta_ppm`. For quantification, it can be beneficial to enable background subtraction using `-TransitionGroupPicker:background_subtraction original` as described in the software comparison paper\(^6\).

MS1 and IPF parameters

Furthermore, if you wish to use MS1 information, use the `-use_msl_traces` flag, assuming that your input data contains an MS1 map in addition to the SWATH data. This is generally recommended. If you would like to enable IPF transition-level scoring and your spectral library was generated according to the IPF instructions, you should set the `-enable_uis_scoring` flag.

Example

Therefore, a full run of OpenSWATH may look like this:

```
OpenSwathWorkflow.exe
-in data.mzML -tr library.tsv
-tr_irt iRT_assays.TraML
-swath_windows_file SWATHwindows_analysis.tsv
-sort_swath_maps -batchSize 1000
-readOptions cacheWorkingInMemory -tempDirectory C:\Temp
-use_msl_traces
-mz_extraction_window 50 -ppm
-mz_correction_function quadratic_regression_delta_ppm
-TransitionGroupPicker:background_subtraction original
-RTNormalization:alignmentMethod linear
```

(continues on next page)

Troubleshooting

If you encounter issues with peak picking, try to disable peak filtering by setting
-Scoring:TransitionGroupPicker:compute_peak_quality false which will disable the filtering of peaks by chromatographic quality. Furthermore, you can adjust the smoothing parameters for the peak picking, by adjusting -Scoring:TransitionGroupPicker:PeakPickerMRM:sgolay_frame_length or using a Gaussian smoothing based on your estimated peak width. Adjusting the signal to noise threshold will make the peaks wider or smaller.

2.10.5 Output

The OpenSwathWorkflow produces two types of output:

- identified peaks
- extracted chromatograms

The identified peaks can be stored in tsv format using -out_tsv (recommended), in SQLite format using --out_osw (experimental) or in a featureXML format using -out_features (not recommended).

The extracted chromatograms can be stored in mzML format using out_chrom with an .mzML extension. By default the produced mzML file will be numpress compressed, but can be converted to regular mzML using the OpenMS FileConverter. Alternatively, output can be written in .sqMass format, which is a SQLite-based format (experimental).

2.10.6 Tutorial Data

Availability

To learn OpenSWATH, we suggest to use the M. tuberculosis dataset published alongside the 2017 Methods Mol Biol. OpenSWATH tutorial

4 which is available from the PeptideAtlas raw data repository with accession number PASS00779.

The SWATH-MS Gold Standard and Streptococcus pyogenes data sets (used in the original 2014 Nature Biotechnology publication) are available from the PeptideAtlas raw data repository with accession number PASS00289.

The Skyline results are available from Skyline Panorama Webserver.

Mycobacterium tuberculosis data

- 3 mzML instrument data files (centroided)
- 3 WIFF raw instrument data files
- Mt assay library (for OpenMS 2.1)
- Mt assay library (for older OpenMS)
- Swath windows file for analysis
- iRT assay file (TraML format)
SWATH-MS Gold Standard

- 90 mzXML instrument data files
- 90 WIFF raw instrument data files
- SGS TSV assay library
- SGS TraML assay library
- SGS OpenSWATH results
- SGS Skyline results on Panorama
- SGS manual results

Streptococcus pyogenes

- 4 mzXML instrument data files
- 4 WIFF raw instrument data files
- S. pyo TSV assay library
- S. pyo TraML assay library
- S. pyo OpenSWATH results
- S. pyo summary results

2.10.7 References

2.11 PyProphet

2.11.1 Overview

PyProphet\(^1\) is a reimplementation of the mProphet\(^2\) algorithm for targeted proteomics. It is particularly optimized for analysis of large scale data sets generated by OpenSWATH or DIANA.

This description represents the new SQLite-based workflow that is available since OpenMS 2.4.0 and PyProphet 2.0.1. This version includes the IPF\(^3\) and large-scale data set optimizations\(^4\). You can alternatively follow the instructions for the pyprophet_legacy.

2.11.2 Contact and Support

We provide support for PyProphet on the GitHub repository.
You can contact the authors Uwe Schmitt, Johan Teleman, Hannes Röst and George Rosenberger.

2.11.3 Tutorial

Merging

Generate OSW output files according to section openswath_workflow. PyProphet is then applied to one or several such SQLite-based reports. Several different commands can be run to consecutively do the analysis:

```bash
pyprophet --help
pyprophet merge --help
```

This command provides an overview of all available commands to manipulate OSW input files. Further instructions are available for the individual commands.

```bash
pyprophet merge --out=merged.osw --subsample_ratio=1 *.osw
```

In most scenarios, more than a single DIA / SWATH-MS run was acquired and the samples should be compared qualitatively and/or quantitatively with the OpenSWATH workflow. After individual processing with OpenSWATH and the identical spectral library, the files can be merged by PyProphet.

This command will merge and optionally subsample multiple files. Please note that the experiment-wise context on peptide query-level is applied to merged files, whereas the run-specific context is used with separate OSW files.

If semi-supervised learning is too slow, or the run-specific context is required, create an additional merged file with a smaller subsample_ratio. The model will be stored in the output and can be applied to the full file(s).

Scoring

```bash
pyprophet score --in=merged.osw --level=ms2
```

The main command will conduct semi-supervised learning and error-rate estimation in a fully automated fashion. --help will show the full selection of parameters to adjust the process. The default parameters are recommended for SCIEX TripleTOF 5600/6600 instrument data, but can be adjusted in other scenarios.

When using the IPF extension, the parameter --level can be set to ms2, ms1 or transition. If MS2 and MS2 information should be considered together, --level can alternatively be set to ms1ms2. If MS1 or transition-level data should be scored, the command is executed three times, e.g.:

```bash
pyprophet score --in=merged.osw --level=ms1 \
pyprophet score --in=merged.osw --level=ms2 \
pyprophet score --in=merged.osw --level=transition
```

The scoring steps on MS1 and transition-level have some dependencies on the MS2 peak group signals. The parameter --ipf_max_peakgroup_rank specifies how many peak group candidates should be assessed in IPF. For example, if this parameter is set to 1, only the top scoring peak group will be investigated. In some scenarios, a set of peptide query parameters might detect several peak groups of different peptidoforms that should be independently identified. If the parameter is set to 3, the top 3 peak groups are investigated. Note that for higher values (or very generic applications), it might be a better option to disable the PyProphet assumption of a single best peak group per peptide query. This can be conducted by setting --group_id to feature_id and will change the assumption that all high scoring peak groups are potential peptide signals.
Importantly, PyProphet will store all results in the input OSW files. This can be changed by specifying --out. However, since all steps are non-destructive, this is not necessary.

**IPF**

If IPF should be applied after scoring, the following command can be used:

```bash
pyprophet ipf --in=merged.osw
```

To adjust the IPF-specific parameters, please consult `pyprophet ipf --help`. If MS1 or MS2 precursor data should not be used, e.g. due to poor instrument performance, this can be disabled by setting `--no-ipf_ms1_scoring` and `--no-ipf_ms2_scoring`. The experimental setting `--ipf_grouped_fdr` can be used in case of extremely heterogeneous spectral library, e.g. containing mostly unmodified peptides that are mainly detect and peptidoforms with various potential site-localizations, which are mostly not detectable. This parameter will estimate the FDR independently grouped according to number of site-localizations.

Several thresholds (`--ipf_max_precursor_pep`, `--ipf_max_peakgroup_pep`, `--ipf_max_precursor_peakgroup_pep`, `--ipf_max_transition_pep`) are defined for IPF to exclude very poor signals. When disabled, the error model still works, but sensitivity is reduced. Tweaking of these parameters should only be conducted with a reference data set.

**Contexts & FDR**

To conduct peptide inference in run-specific, experiment-wide and global contexts, the following command can be applied:

```bash
pyprophet peptide --in=merged.osw --context=run-specific \
peptide --in=merged.osw --context=experiment-wide \
peptide --in=merged.osw --context=global
```

This will generate individual PDF reports and store the scores in a non-redundant fashion in the OSW file.

Analogously, this can be conducted on protein-level as well:

```bash
pyprophet protein --in=merged.osw --context=run-specific \
protein --in=merged.osw --context=experiment-wide \
protein --in=merged.osw --context=global
```

**Exporting**

Finally, we can export the results to legacy OpenSWATH TSV report:

```bash
pyprophet export --in=merged.osw --out=legacy.tsv
```

By default, both peptide- and transition-level quantification is reported, which is necessary for requantification or SWATH2stats. If peptide and protein inference in the global context was conducted, the results will be filtered to 1% FDR by default. Further details can be found by `pyprophet export --help`.

**Warning:** By default, IPF results on peptidoform-level will be used if available. This can be disabled by setting `--ipf=disable`. The IPF results require different properties for TRIC. Please ensure that you want to analyze the results in the context of IPF, else, use the `--ipf=disable` or `--ipf=augmented` settings.
Scaling up

When moving to larger data sets that include 10-1000s of runs, the workflow described above might take a lot of time. For such applications, especially when the analysis is run on HPC infrastructure (cloud, cluster, etc.) we have implemented steps that can mostly parallelize on the level of independent runs:

In the first step, we will generate a subsampled classifier that is much faster to learn:

```bash
# Here we recommend to set subsample_rate to 1/N, where N is the number of runs.
# Example for N=10 runs:
pyprophet merge --out=model.osw --subsample_ratio=0.1 *.osw
```

In case of very large data sets, this step can alternatively be parallelized:

```bash
# Here we recommend to set subsample_rate to 1/N, where N is the number of runs.
# Example for N=10 runs:
for run in run_*.osw
    run_subsampled = ${run}s  # generates .osws files
    pyprophet merge --out=$run_subsampled --subsample_ratio=0.1 $run
done
pyprophet merge --out=model.osw *.osws
```

We then learn a classifier on MS1/MS2-level and store the results in `model.osw`:

```bash
pyprophet score --in=model.osw --level=ms1ms2
```

This classifier is then applied to all individual runs in parallel:

```bash
for run in run_*.osw
    pyprophet score --in=$run --apply_weights=model.osw --level=ms1ms2
done
```

We then extract the relevant data for global scoring to generate a tiny file:

```bash
for run in run_*.osw
    run_reduced = ${run}r  # generates .oswr files
    pyprophet reduce --in=$run --out=$run_reduced
done
```

Next, global peptide and protein-level error rate control is conducted by merging the `.oswr` files:

```bash
pyprophet merge --template=model.osw --out=model_global.osw *.oswr
pyprophet peptide --context=global --in=model_global.osw
pyprophet protein --context=global --in=model_global.osw
```

Now we backpropagate the global statistics to the individual runs:

```bash
for run in run_*.osw
    pyprophet backpropagate --in=$run --apply_scores=$run_reduced
done
```
We can then export the results with confidence scores on peptide-query-level (run-specific context), peptide sequence level (global context) and protein level (global context) in parallel:

```bash
for run in run_*\_osw
do
pyprest export --in\=$run
done
```

### 2.11.4 References

### 2.12 Percolator

#### 2.12.1 Overview

Percolator\(^1,2\) is a popular algorithm for statistical validation of shotgun proteomic data. Using the OpenMS TOPP tool `PercolatorAdapter`, Percolator can score the results from OpenSWATH and thus be used instead of PyProphet.

#### 2.12.2 Contact and Support

OpenSWATH support in `PercolatorAdapter` is currently in development and must NOT be used in production environments. We would however be very grateful for testing of the tool and reporting of problems and bugs.

We provide support for `PercolatorAdapter` using the OpenMS support channels. Please address general questions to the open-ms-general mailing list.

Please address any general Percolator inquiries to the Percolator team.

#### 2.12.3 Tutorial

`PercolatorAdapter` is available in the OpenMS development branch. To convert the results of `PercolatorAdapter` to an OpenSWATH TSV report, the SQLite-enabled PyProphet version is required. Please install these versions according to the instructions in the `Binaries` section.

After installation, `PercolatorAdapter` can be run on the OSW results using the following commands:

```bash
# Score on MS2-level
PercolatorAdapter -in\_osw openswath\_results\_osw -out openswath\_results\_osw \ -osw\_level ms2

# Score on MS1-level
PercolatorAdapter -in\_osw openswath\_results\_osw -out openswath\_results\_osw \ -osw\_level ms1

# Score on transition-level
PercolatorAdapter -in\_osw openswath\_results\_osw -out openswath\_results\_osw \ -osw\_level transition
```

---


PyProphet

If IPF should be applied after scoring, the following command can be used:

```
pyprophet ipf --in=merged.osw
```

Finally, we can export the results to a legacy OpenSWATH TSV report:

```
pyprophet export --in=merged.osw --out=legacy.tsv
```

2.12.4 References

2.13 TRIC

2.13.1 Overview

TRIC\(^1\) is an alignment software for targeted proteomics (SRM or SWATH-MS) data. TRIC uses a graph-based alignment strategy based on non-linear retention time correction to integrate information from all available runs. The input consists of a set of csv files derived from a targeted proteomics experiment generated by OpenSWATH (using either mProphet or PyProphet) or generated by Peakview.

There are two basic running modes available. The first one uses a reference-based alignment where a single run is chosen as a reference and all other runs are aligned to it. This is a useful choice for a small number of runs that are chromatographically similar. The second mode generates a guidance tree based on chromatographic similarity of the input runs and uses this tree to align the targeted proteomics runs (the nodes in the tree are runs and the edges are pairwise alignments). Generally this mode is better for a large number of runs or for chromatographically dissimilar samples.

2.13.2 Contact and Support

We provide support for TRIC on the GitHub repository.

You can contact the author Hannes Röst.

2.13.3 Tutorial

After installing TRIC, please familiarize yourself with the TRIC Tutorial. All command line parameters and their effects are explained in the tutorial and the associated tutorial paper (Röst et al). Currently, the recommended parameters for TRIC are:

```
feature_alignment.py
--in file1_input.csv file2_input.csv file3_input.csv
--out aligned.csv
--method LocalMST --realign_method lowess_cython --max_rt_diff 60
--mst:useRTCorrection True --mst:Stdev_multiplier 3.0
--target_fdr 0.01 --max_fdr_quality 0.05
```

An extended tutorial describing a complete OpenSWATH analysis workflow including TRIC was recently published\(^2\) and is also available from bioRxiv.

### 2.13.4 Data

#### Availability

The TRIC Gold Standard, the *Streptococcus pyogenes* data sets and the iPSC datasets are available from the PeptideAtlas raw data repository with accession number PASS00788.

The Skyline results are available from the same repository where a .sky and .sky.view file are provided.

#### TRIC Gold Standard

The TRIC Gold Standard dataset contains a set of manually validated aligned peptides and can be found in the ./ManualValidation folder on the FTP server.

- 16 WIFF raw instrument data files
- 1 Skyline file with manually picked data
- 1 CSV file with the manually picked peaks (Skyline export)
- The TRIC results
- Python script used to compare manual with TRIC data

#### Streptococcus pyogenes

- 16 WIFF raw instrument data files
- 1 Assay library in TraML and CSV format
- 1 iRT library in TraML and CSV format (use instead of default iRT)
- 16 OpenSWATH output files (results/openswath)
- 1 TRIC output file using local MST parameters as described in the paper
- 1 unaligned output matrix (noalign_all_1pcnt.csv)

#### human iPSC

- 8 WIFF raw instrument data files
- 1 Assay library in TraML and CSV format
- OpenSWATH output files
- 1 TRIC output file using local MST parameters as described in the paper

---

2.14 IPF

2.14.1 Overview

IPF (Inference of PeptidoForms)\(^1\) is an extension to the OpenSWATH\(^2\) workflow to increase the specificity of the analysis to the level of peptidoforms (modified peptides with specific site-localization) across multiple runs. IPF is fully implemented as part of OpenMS\(^3\) and PyProphet\(^4\) and compatible with the downstream alignment algorithm TRIC\(^5\).

2.14.2 Contact and Support

We provide support for IPF using the OpenMS support channels. Please address general questions to the open-ms-general mailing list.

You can contact the author George Rosenberger.

2.14.3 Installation

IPF is fully integrated within the tools of the OpenSWATH workflow. Please follow the OpenSWATH, PyProphet and TRIC installation instructions for the latest development branches. The current instructions are written for the new SQLite-based workflow. You can alternatively follow the instructions for the ipf_legacy.

2.14.4 Tutorial

Running IPF requires to modify the parameters of several steps of tools part of the OpenSWATH workflow:

1. **Peptide Query Parameter Generation**

IPF requires a spectral library generated from DDA (or DIA pseudo spectra, e.g. from DIA-Umpire\(^7\)) data. The input can come for example from Trans-Proteomic Pipeline, Skyline or can be provided in the form of Generic Transition Lists. The underlying PSMs do not need to be site-localized, as IPF will assess site-localization independently. However, a site-localized spectral library might provide better peptide query parameters.

---


The first step uses OpenSwathAssayGenerator to append \textit{in silico} identification transitions to the spectral library. The required parameters (including residue modifiability) and considerations are described in the section \textit{Peptide Query Parameter Generation}. The spectral library should also be appended with decoys and converted to a PQP file.

2. Targeted data extraction using OpenSWATH

The next step is conducted using OpenSWATH. Follow the IPF-specific instructions in the section openswath_workflow. Important is to enable MS1 and transition-level scoring by setting the parameters \texttt{--use_msl_traces} and \texttt{--enable_uis_scoring} for OpenSwathWorkflow. Make sure to use the PQP spectral library as input and write an OSW file as output.

3. Statistical validation using PyProphet

PyProphet is then applied to the OpenSWATH results. Follow the IPF-specific instructions in the \textit{PyProphet} section. Export a legacy TSV report for analysis with TRIC.

4. Multi-run alignment using TRIC

TRIC can be applied to the IPF results with the following command:

```
feature_alignment.py --in *_uis_expanded.csv \
--out feature_alignment.csv \ 
--out_matrix feature_alignment_matrix.csv \ 
--file_format openswath \ 
--fdr_cutoff 0.01 \ 
--max_fdr_quality 0.2 \ 
--mst:useRTCorrection True \ 
--mst:Stdev_multiplier 3.0 \ 
--method LocalMST \ 
--max_rt_diff 30 \ 
--alignment_score 0.0001 \ 
--frac_selected 0 \ 
--realign_method lowess_cython \ 
--disable_isotopic_grouping
```

Note that IPF does not report decoys, which is the reason why \texttt{max_fdr_quality} must be set.

2.14.5 Data

Availability

The synthetic phosphopeptide reference mass spectrometry proteomics data is available from PRIDE/ProteomeXchange with the data set identifier PXD004573.

The enriched U2OS phosphopeptide mass spectrometry proteomics data is available from PRIDE/ProteomeXchange with the data set identifier PXD006056.

The 14-3-3\(\beta\) phosphopeptide interactomics mass spectrometry proteomics data is available from PRIDE/ProteomeXchange with the data set identifier PXD006057.

The twin study mass spectrometry proteomics data is available from PRIDE/ProteomeXchange with the data set identifier PXD004574.
2.15 TAPIR

2.15.1 Overview

TAPIR\(^1\) is a visualization software for chromatographic data obtained by mass spectrometry. It provides efficient visualization of high-throughput targeted proteomics experiments.

The TAPIR software is a fast and efficient Python visualization software for chromatograms and peaks identified in targeted proteomics experiments. The input formats are open, community-driven standardized data formats (mzML for raw data storage and TraML encoding the hierarchical relationships between transitions, peptides and proteins).

TAPIR is scalable to proteome-wide targeted proteomics studies (as enabled by SWATH-MS), allowing researchers to visualize high-throughput datasets. The framework integrates well with existing automated analysis pipelines and can be extended beyond targeted proteomics to other types of analyses.

2.15.2 Contact and Support

We provide support for TAPIR on the GitHub repository through GitHub issues or you can contact the author Hannes Röst.

2.15.3 Installation

You can download binaries from here (Mac OS X and 64 bit Microsoft Windows). The source code is available from Github which allows source-based installation. Please follow the instructions found there for manual installation or installation on a Linux system.

Note: for a successful installation on Mac OS X, extract the provided file and drag it into the Applications folder. You may need to allow execution of the software if you see a warning that TAPIR is from an “unidentified developer”. Simply go to System Preferences, click on “Security & Privacy” and in the “General” tab allow the execution of TAPIR.

2.15.4 Tutorial

The TAPIR software is highly flexible and interactive, allowing for investigation of single data traces and data points. Each graph item can be selected and inspected individually, allowing for customization of the visualization and production of publication-quality figures. Data can be exported as an image or in table format and used for further analysis; individual traces can be removed or re-added and all graph settings (such as color, line width, line style etc.) are fully customizable. The implementation relies on guiqwt for these features.


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2.15.5 Data

Availability

You can download a small sample dataset. A larger, real-life dataset can be obtained by downloading these five files (this might take a while since the whole dataset is ca 5 GB)

- Dataset 1 (Cond 1)
- Dataset 2 (Cond 1)
- Dataset 3 (Cond 2)
- Dataset 4 (Cond 2)
- Peak description file

This dataset is retrieved from the original OpenSWATH publication and the two conditions (0% and 10%) refer to the treatment of *S. pyogenes* with human plasma. For each condition, two biological replicates are available.

2.16 SWATH2stats

2.16.1 Overview

SWATH2stats\(^1\) is intended to transform SWATH data from the OpenSWATH software into a format readable by other statistics packages while performing filtering, annotation and FDR estimation.

2.16.2 Contact and Support

You can contact the authors Peter Blattmann and Moritz Heusel.

2.16.3 References