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A tool for the automated, targeted analysis of data-independent acquisition MS-data: OpenSWATH

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To the Editor:

LC-MS/MS-based proteomics is the method of choice for large-scale identification and quantification of proteins in a sample1. Several LC-MS/MS methods have been developed that differ in their objectives and performance profiles [2]. Among these, shotgun proteomics (also referred to as discovery proteomics) using data-dependent acquisition (DDA) and targeted proteomics using selected reaction monitoring (SRM, also referred to as multiple reaction monitoring, MRM) have been widely adopted. Alternatively, some mass spectrometers can also be operated in data-independent acquisition (DIA) mode [9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. In DIA mode, the instrument fragments all precursors generated from a sample that are within a predetermined *m/z* and retention time range. Usually, the instrument cycles through the precursor ion *m/z* range in segments of specified width, at each cycle producing a highly multiplexed fragment ion spectrum. Multiple DIA methods have been described with different instrument types and setups, duty cycles and window widths. Methods such as MS^E fragment all precursors [11], whereas others, such as PAcIFIC, use precursor selection windows as small as 2.5 Da [12] (see ref [22] for a recent overview). Analysis of DIA data has thus far been done manually. In this correspondence, we describe software for automated DIA analysis, benchmark it against manual analysis of >30,000 chromatograms from 342 synthesized peptides, and use it to analyze the proteome of *Streptococcus pyogenes*.

DIA methods offer several potential advantages over shotgun proteomics and SRM. Specifically, data acquired in DIA mode is continuous in time *and* fragment-ion intensity for all recorded spectra, thus increasing the dimensionality of the data relative to shotgun proteomics, in which full fragment ion intensity scans are recorded only at selected time points (MS/MS spectra), or SRM, in which continuous time profiles are acquired but only for selected fragment

ions (ion chromatograms) [3, 1, 4, 5, 6]. Thus, DIA methods produce a complete record in two dimensions of the fragment-ion spectra of all precursors generated from a sample. By acquiring time-resolved data of all fragment ions, DIA has the potential to overcome some of the limitations of the current proteomic methods and to combine the high throughput of shotgun proteomics with the high reproducibility of SRM.

However, DIA data has historically been more difficult to analyze than shotgun or SRM data. To limit the time needed for data analysis and the amount of sample required, one typically uses larger precursor-isolation windows than in shotgun proteomics or SRM [22]. This leads to highly complex, composite fragment-ion spectra from multiple precursors and thus to a loss of the direct relationship between a precursor and its fragment ions, making subsequent data analysis nontrivial. To date, DIA data have been analyzed by one of two strategies. In the first, fragment ion spectra [10, 12 or pseudo fragment ion spectra (which are computationally reconstructed from the complex datasets [14, 15, 17, 18, 19]) are searched by methods developed for data-dependent acquisition. In these approaches, a proteomics search engine compares experimental spectra to theoretical spectra generated by an *in silico* tryptic digest of a proteome, assuming that the fragment ion spectrum is derived from a single precursor. These approaches suffer from the high complexity of the data and the fact that errors in the generation of pseudo-spectra will propagate through the analysis workflow.

Recently, we have proposed an alternative, fundamentally different DIA data analysis approach, which is derived from methods for analyzing SRM-based targeted proteomics data. We implemented it in a method called SWATH-MS [22]. In SWATH-MS, precursor ions from sequential segments of 25 mass units are concurrently fragmented and the resulting composite fragment ions are recorded at high mass accuracy in a time of flight (TOF) analyzer. In our

targeted data analysis strategy, extracted ion chromatograms (XIC) of the most intense transitions of a targeted peptide are generated from all corresponding MS2 spectra, producing chromatographic data that are similar to SRM traces (Fig. 1a). This approach reduces the complexity of the data substantially, facilitating data analysis while retaining the complete fragment ion information of all precursors. So far, such data analysis was performed semi-manually and to our knowledge, no automated workflow has been published. However, certain specialized software, such as Skyline[31] and PeakView (AB SCIEX), can visualize the XIC data, making manual analysis possible. Automation of this process is critical, however, because in a single typical SWATH-MS dataset tens of thousands of peptides are identified.

Here we present OpenSWATH, an open source (Modified BSD Licence) software that allows targeted analysis of DIA data in an automated fashion at high throughput. OpenSWATH is cross-platform software, written in C++, that relies only on open data formats, allowing it to analyze DIA data from multiple instrument vendors (Supplementary Note 1) [23]. The algorithm can be summarized in the following five steps (Fig. 1 and Supplementary Notes 2–4).

1) Data Conversion: OpenSWATH takes as input the acquired SWATH-MS data and an assay library. These are converted to suitable open file formats (mzML and TraML [24, 25]). The assay library contains precursor and fragment ion *m/z* values (transitions) as well as relative fragment-ion intensities and normalized peptide retention times. Decoy assays are appended to the target assay library using the OpenSwathDecoyGenerator for later classification and error rate estimation.

2) Retention time alignment: Each run is aligned against a previously determined normalized retention-time space using reference peptides whose mappings to the normalized space are known (e.g. spiked-in peptides), as described previously [26]. Outlier detection is subsequently

applied to remove wrongly assigned reference peptides and to evaluate the quality of the alignment.

3) Chromatogram extraction: Using the *m/z* and retention time information from the assay library, the workflow extracts an ion chromatogram from the corresponding MS2 map, producing integrated fragment-ion counts versus retention time data. The extraction function (Top-hat or Bartlett) and *m/z* window-width can be specified to account for the instrument-specific MS2 resolution.

4) Peak group scoring: The core algorithm identifies 'peak groups' (that is, positions in the chromatograms where individual fragment traces co-elute), and scores them using multiple, orthogonal scores (Supplementary Note 2.4). These scores are based on the elution profiles of the fragment ions, the correspondence of the peak group with the expected retention time and fragment ion intensity from the assay library, as well as the properties of the full MS2 spectrum at the chromatographic peak apex.

5) Statistical analysis: The separation between true and false signal is achieved using a set of decoy assays that were scored exactly the same way as the target assays. The false discovery rate (FDR) can then be estimated, for example by the *mProphet* algorithm [27]. If multiple runs are present, a peak group alignment can be performed to annotate signals that could not be confidently assigned using data from a single run alone, as described previously for data-dependent aquisition and SRM data [28].

To validate and benchmark our SWATH-MS data analysis algorithms, we created a 'gold standard' dataset of known composition (termed SGS for SWATH-MS Gold Standard), consisting of 422 chemically synthesized, stable isotope–labeled standard (SIS) peptides [29, 30]. To simulate differently abundant peptides in proteomic backgrounds of varying complexity, we added the peptides in ten dilution steps at final concentrations ranging from 0.058 fmol/μL to

30.0 fmol/μL into three different backgrounds (water or trypsinized whole-cell protein extracts from *Homo sapiens* or *Saccharomyces cerevisiae*, normalized to 1 μg of total protein, Supplementary Note 5). To explore the lower end of the dynamic range, we specifically chose to study the influence of background complexity on ion suppression and signal-to-noise (see below and Supplementary Note 5.4). These samples were measured on the AB SCIEX TripleTOF 5600 System in DIA mode as described previously [22]. Using an assay library for 342 peptides (not all 422 peptides generated high quality fragment ion spectra), the 30,780 chromatograms were extracted in Skyline [31] and manually analyzed to determine the true peak group (if present). In parallel, the same data were processed with OpenSWATH and results were compared to those generated by the manual analysis.

To assess the identification accuracy of OpenSWATH, we calculated the pseudo-receiver operator characteristics (ROC) using the best peak group per chromatogram and computed an AUC >0.9 (Fig. 2a). At a fixed FDR of 5% (as computed by *mProphet* [27]), the software could achieve a recall of 87.5% and a precision of 94.3%. Furthermore, we noticed that the misidentification rate (i.e. cases where the highest scoring peak group is not the correct peak group) is below 0.7%. Thus, most of the false identifications were caused by peak groups that were not confidently assigned by manual curation, rather than by misidentification by OpenSWATH. Furthermore, we found a good correspondence between the estimated FDR and the true, manually determined false positive rate (with a slight underestimation of 0.9% at 1% FDR, Fig. 2b), indicating that OpenSWATH can identify peptides with high precision and that it supports the accurate selection of the desired false positive rate. However, accurate error rate estimations critically depend on a suitable decoy strategy[32] (Supplementary Note 1.5). Similar to methods for SRM data analysis, OpenSWATH uses the sum of the integrated chromatographic

fragment ion peak areas of SWATH-MS data to quantify peptides. When analyzing the coefficients of variation (CV) of quantified signals reported in all technical replicates, we consistently found mean CVs below 20% (Fig. 2c). By normalizing the intensities of each peptide signal to the intensity of the most concentrated run (1x dilution) we could evaluate the quantification accuracy achieved by the software over large fold changes (Fig. 2d). Because our goal was to study quantification accuracy, we did not include misidentified peptides in our analysis. We found that the manually determined changes between subsequent dilution steps (water: 2.35±1.0, yeast: 2.03±0.45, human: 2.11±0.53, mean fold change ± standard deviation) matched closely with the changes determined using OpenSWATH (water: 2.62±1.43, yeast: 2.02±0.44, human: 1.96±0.39). From this we computed a deviation from the theoretical value of 31.2%, 1.0% and 2.0% and a coefficient of variation of 54.6%, 21.9% and 20.2% for the OpenSWATH quantification (respectively for the three backgrounds, outliers removed), suggesting that OpenSWATH quantification is suitable for obtaining relative quantification values for differentially abundant peptides. The quantification in water is less accurate and precise than in the yeast and human backgrounds, because without a matrix, the spiked-in SIS peptides were prone to surface adsorption during sample preparation (Supplementary Note 5.4)

We next explored the performance of OpenSWATH in identifying and quantifying peptides from a full tryptic digest of a *S. pyogenes* microbial sample. To study proteomic changes that occur upon vascular invasion of the pathogen, we grew *S. pyogenes* (strain SF370) in 0% and 10% human plasma in biological duplicates and analyzed the samples in SWATH-MS mode on an AB SCIEX TripleTOF 5600 System. First, we created a spectral library of *S. pyogenes* by combining the measurements of 10 fractions of the *S. pyogenes* proteome in data-dependent aquisition (shotgun) mode on the same instrument, providing an extensive coverage of the

expressed *S. pyogenes* proteome, with 1322 proteins (out of 1905 ORFs) mapping to 20,027 proteotypic peptide precursors at 1% peptide-spectrum match FDR (Fig. 3a).

Using OpenSWATH, we identified and quantified 927 proteins (out of 1322 targeted proteins) of *S. pyogenes* consistently in each of the four LC-MS/MS runs at 1% FDR. Out of these, 767 proteins were quantified by more than one peptide per protein. Thus, we achieved >70% coverage of the expressed proteome spanning more than three orders of dynamic range in estimated protein ion count (Fig. 3b) in a single injection. The results from these analyses surpassed previous shotgun proteomics and SRM approaches in terms of number of quantified proteins at 1% FDR (765 proteins were quantified in an extensive SRM study with multiple injections per sample and 523 proteins were identified in a shotgun proteomics study with 98.92% overlap with our data, see Supplementary Note 1.4) [33, 28]. The fraction of the assay library which could not be detected may be partially explained by the fact that not all proteins were expressed under the conditions studied and that these proteins have also rarely been identified in earlier studies (nearly 80% were never identified in PeptideAtlas [34]).

OpenSWATH identified 82 proteins, which showed significant ($P < 0.05$ in an multiple testing-corrected ANOVA test) differences in abundance between the two conditions in two biological replicates (Fig. 3c,d, see Supplementary Note 7 for a complete list). Ten out of 13 proteins associated with fatty acid biosynthesis are significantly ($P < 0.05$) downregulated, consistent with results of previous studies on *S. pyogenes* [33]. As expected, we also found several known virulence factors to be upregulated (e.g. HasA, HasB, Slo, SpeC and CovR) [35, 36]. Additionally, we observed significant ($P < 0.05$) downregulation of an ABC transporter complex for inorganic phosphate import (PstB1, PstB2 and PstS), as well as significant ($P < 0.05$) upregulation of six proteins involved in pyrimidine biosynthesis (PyrF, PyrD, PyrE, PyrB, PyrR and Upp). Although these results agree with previous observations on *S. pyogenes*, they

also provide the first indications that the Pst system is involved in responding to human plasma in *S. pyogenes*. In conclusion, our results derived from SWATH-MS datasets analyzed with OpenSWATH are consistent with many previous suppositions about bacterial virulence but additionally are able to provide the foundation for new hypotheses (Supplementary Note 7).

By combining the most advanced DIA technology with a software capable of analyzing the resulting complex datasets, we were able to substantially scale-up the targeted proteomic approach described previously[22] and show that targeted analysis of DIA data facilitates high-throughput analysis of microbial whole-cell lysates, as demonstrated on the example of *S. pyogenes*. Using the SGS validation dataset, we further demonstrate high sensitivity of the method and software for identification and quantification. Our open source software is available as standalone executable at http://www.openswath.org (Supplementary Source Code File 1). The OpenSWATH algorithms are provided as C++ software library (allowing integration of our algorithms into a multitude of popular proteomics software such as TOPP[37] or Skyline[31]) and are also packaged within OpenMS [37], which will make targeted DIA data analysis immediately accessible to a large research community. Owing to the nature of DIA data, which contain a complete record of all fragment ions of a biological sample, reanalysis of a dataset is possible completely *in silico*, allowing researchers to re-query data with their specific hypothesis in mind. With the availability of fast DIA-capable instruments, assay libraries, available in proteome-wide coverage owing to large-scale peptide synthesis efforts and now, an automated software for DIA targeted data analysis, all pieces for a successful and more widespread use of this powerful technology are now available.

AUTHOR CONTRIBUTIONS

H.R. & G.R. designed, implemented and executed the C++ code and the analysis workflow. H.R acquired and analyzed the *S. pyogenes* data. H.R. & G.R. & L.M. & R.A. wrote the manuscript. G.R. & L.G. provided the SGS sample. P.N. & L.G. & B.C. provided critical input on the project. H.R. & G.R. & P.N. analyzed the SGS dataset manually. L.G. & S.M. & O.S. performed all the measurements and provided important feedback. W.W. did code review, assisted in software design and provided the visualization of the example peptide data. B.C. performed testing of the software. J.M. performed the biological experiments. L.M. & R.A. designed and supervised the study.

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Competing financial interests

J.M. is co-founder and board member of Biognosys AG. J.M. and R.A. hold shares of Biognosys AG, which operates in the field covered by the article. The research group of R.A. is supported by AB SCIEX by providing access to prototype instrumentation.

Abbreviations

The following abbreviations were used: ANOVA, analysis of variance AUC, Area under the (ROC) curve; CV, coefficient of variation; DDA, data-dependent acquisition; DIA, data-independent acquisition; FDR, false discovery rate: $FDR=FP/(TP+FP)$; m/z, mass-over-charge; PAcIFIC, Precursor Acquisition Independent From Ion Count; ROC, Receiver operating characteristic; RT, (chromatographic) retention time; SIS, stable isotope-labeled standard; SRM, selected reaction monitoring; TOF, time of flight; XIC, extracted ion chromatogram

Supplementary information and Data

Mass spectrometry data are available at the PeptideAtlas raw data repository (PASS00289).

We further provide the following supplementary information and data:

- Supplementary Notes
- Supplementary Table 1 with fold changes of all quantified proteins of *S. pyogenes*
- Supplementary Table 2 with all SIS peptide sequences in the SGS samples
- Data File 3 with all manual and OpenSWATH results for the SGS dataset
- Data File 4 Assay library for the SGS SIS peptides
- Data File 5 Assay library for the *S. pyogenes* peptides
- Data File 6 ini file for the OpenSWATH TOPPTools
- Data File 7 OpenSWATH TOPPAS workflow for OpenMS 1.10
- Data File 8 KNIME Workflow for OpenSWATH
- Source Code File 1 Complete source code of the described software

Figures

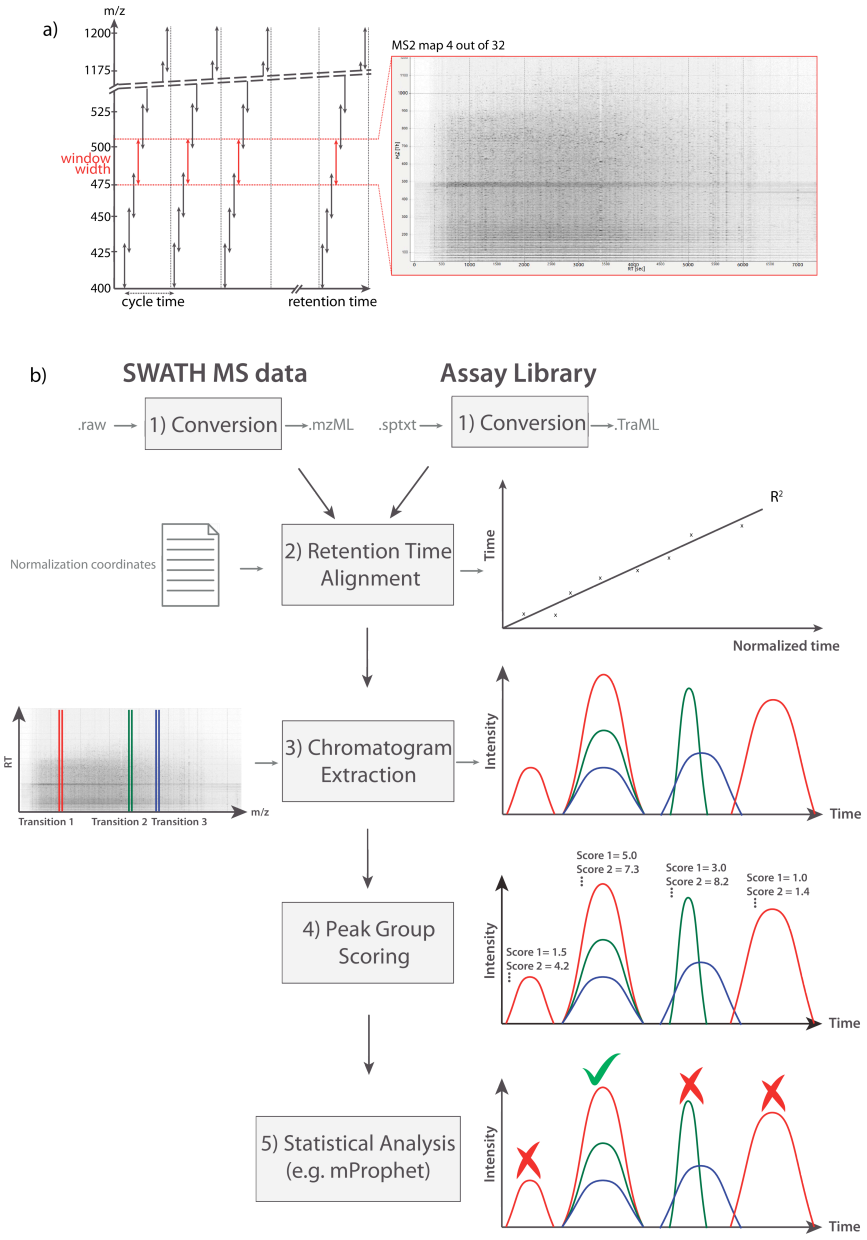

Figure 1 SWATH-MS data-independent acquisition and OpenSWATH analysis. **(a)** The DIA method used here consists of sequential acquisition of fragment ion spectra with overlapping

precursor isolation windows. Here, a swath window width of 25 Da is depicted which allows stepping through a mass range of 400 - 1200 *m/z* in 32 individual steps. If all fragment ion spectra of the same isolation window are aligned, a MS2 map (so-called swath) is obtained (right side, swath 4 out of 32 is schematically shown). Figure adapted from ref. [22]. (**b)** The individual steps performed by the OpenSWATH software are illustrated for a peptide precursor with three transitions: red, green and blue. 1) Data conversion, 2) Retention time alignment, 3) Chromatogram extraction, 4) Peak group scoring, 5) Statistical Analysis to estimate an FDR (false discovery rate). See main text for a more detailed explanation of the workflow. 22

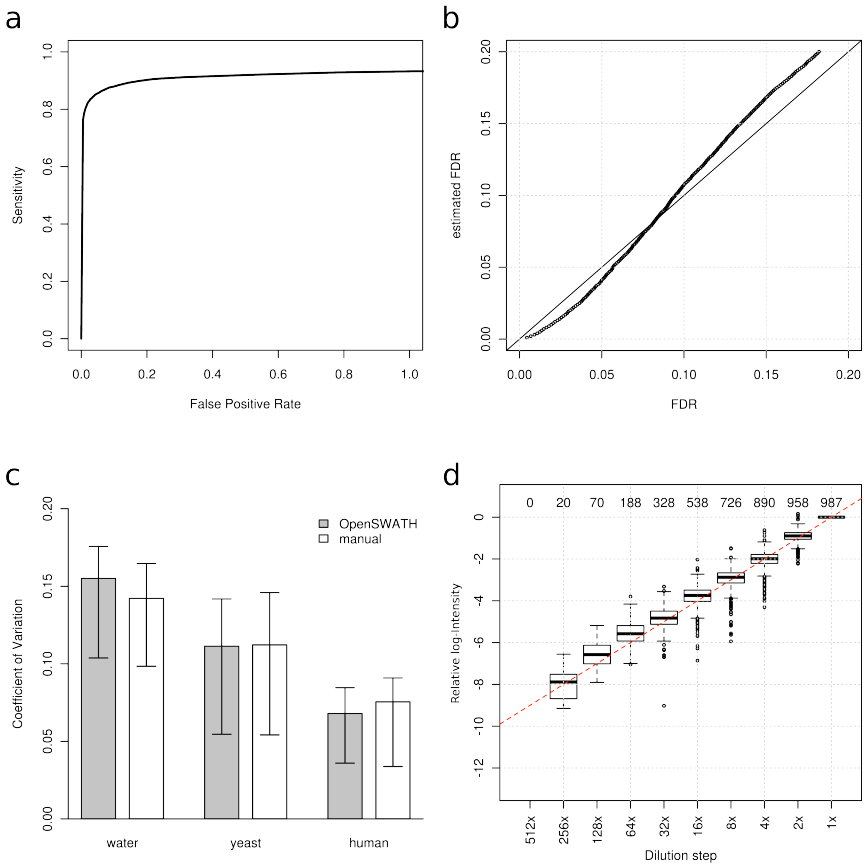

Figure 2 Identification and quantification accuracy of OpenSWATH on the SGS dataset. Four-hundred twenty-two peptides were spiked into three different proteomic backgrounds in a 10-step dilution series to produce a 'gold standard' dataset. (**a)** Pseudo-ROC curve showing sensitivity (recall of true signals) versus the false positive rate, achieving an AUC >0.9 using OpenSWATH. Because misidentified peaks cannot be recovered, even at high score cutoff values, a sensitivity of 1.0 cannot be reached. (**b)** The estimated FDR (by mProphet) versus manually curated, true FDR on the SGS dataset. The continuous line at 45 degrees shows the optimal values. (**c)** Coefficients of variation (CVs) across the three technical replicates are below 20% CV (no significant difference between OpenSWATH and manual quantification for yeast and human backgrounds using the Mann-Whitney test). (**d)** Peptide intensities quantified by OpenSWATH for all ten dilution steps, normalized to the most intense concentration shown for the yeast proteomic background. The red dashed line indicates the ideal values (two-fold difference to the next dilution). The number of peaks considered is given on the top. For panels c and d, only peptides that were detectable above a cutoff of 1 % FDR were analyzed and only true positives were considered. For panel c, only peptides present in all triplicates were analyzed.

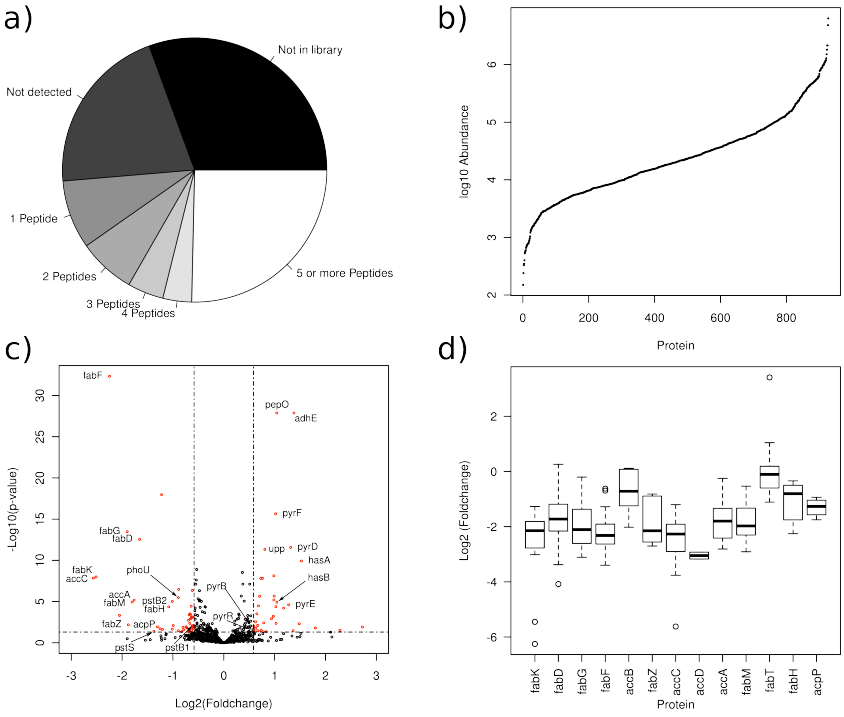
a)
Not in library
Not detected
1 Peptide
2 Peptides
3 Peptides
4 Peptides
5 or more Peptides
b)
log10 Abundance
Protein
c)
-Log10(p-value)
Log2(Foldchange)
fabF
pepO
adhE
pyrF
fabG
fabD
upp
pyrD
hasA
fabK
accC
phoU
hasB
accA
fabM
pstB2
fabH
pyrB
pyrE
fabZ
acpP
pyrR
pstS
pstB1
d)
Log2 (Foldchange)
Protein
fabK
fabD
fabG
fabF
accB
fabZ
accC
accD
accA
fabM
fabT
fabH
acpP

Figure 3 *Streptococcus pyogenes* exposed to human plasma (0% plasma *vs.* 10% plasma). Analysis of two biological replicates with OpenSWATH at 1% assay FDR yields over 900 proteins and 6000 peptides consistently quantified over four SWATH-MS runs. (**a**) Proteome coverage of *S. pyogenes*: of 1905 annotated ORFs, 1322 were detected using mass spectrometry after extensive fractionation (constituting the assay library) and 927 could be detected consistently in each of four unfractionated samples using SWATH-MS. (**b**) Protein abundances of *S. pyogenes* as detected by SWATH-MS estimated by the ion count of the most intense peptide. (**c**) Volcano plot (log-fold change *vs.* log-p-value) of protein expression determined by ANOVA analysis on two biological replicates. Red dots indicates fold changes above 1.5 fold and a Benjamini & Hochberg corrected p-value below 0.05. (**d**) The fold-changes of all 13 proteins involved in fatty acid biosynthesis (FAB) in *S. pyogenes*, in the same order as they appear on their respective operons. All proteins are significantly upregulated except accD, accB and fabT (where fabT is a transcriptional repressor and not expected to be upregulated).

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