Review

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Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial

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Abstract

Many research questions in fields such as personalized medicine, drug screens or systems biology depend on obtaining consistent and quantitatively accurate proteomics data from many samples. SWATH-MS is a specific variant of data-independent acquisition (DIA) methods and is emerging as a technology that combines deep proteome coverage capabilities with quantitative consistency and accuracy. In a SWATH-MS measurement, all ionized peptides of a given sample that fall within a specified mass range are fragmented in a systematic and unbiased fashion using rather large precursor isolation windows. To analyse SWATH-MS data, a strategy based on peptide-centric scoring has been established, which typically requires prior knowledge about the chromatographic and mass spectrometric behaviour of peptides of interest in the form of spectral libraries and peptide query parameters. This tutorial provides guidelines on how to set up and plan a SWATH-MS experiment, how to perform the mass spectrometric measurement and how to analyse SWATH-MS data using peptide-centric scoring. Furthermore, concepts on how to improve SWATH-MS data acquisition, potential trade-offs of parameter settings and alternative data analysis strategies are discussed.

Keywords data-independent acquisition; mass spectrometry; quantitative proteomics; SWATH-MS; systems biology

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Introduction

Over the last decades, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become the technology of choice for the high-throughput characterization of proteins and proteomes (Aebersold & Mann, 2016). Recent developments in the field have moved beyond enumerating the proteins, peptides or post-translational modifications detected in one or few samples towards delivering high quality and consistent quantification in large-scale projects that comprise 100s of samples. Especially in areas such as personalized medicine, biomarker research, drug screens, genetic association studies or systems biology, large numbers of individuals, conditions and perturbations need to be investigated to draw meaningful biological conclusions. For this purpose, the large data matrices generated must be as reproducible, complete and accurate as possible. In order to address these needs, several different proteomic strategies have been developed over the last years.

An emerging strategy, and the focus of this tutorial, is sequential window acquisition of all theoretical mass spectra (SWATH-MS), which was described by Gillet *et al* (2012). For a detailed introduction into the history, the basic principles as well as the general advantages and limitations of SWATH-MS (also summarized in Table 1), we refer to the Appendix. Briefly, for a SWATH-MS measurement, typically non-labelled protein samples are digested with trypsin and the resulting peptides are analysed by liquid chromatography coupled to a tandem mass spectrometer operating in the so-called data-independent acquisition (DIA) mode. In this mode, all ionized compounds of a given sample that fall within a specified mass range are fragmented in a systematic and unbiased fashion. Figure 1A–C shows the DIA scheme described as the initial implementation of SWATH-MS (Gillet *et al*, 2012), using 32 consecutive, slightly overlapping precursor isolation windows, with a width of 25 *m/z* each. Depending on sample complexity, this acquisition scheme will lead to the co-fragmentation of many co-eluting peptides concurrently selected in the precursor ion window and ultimately to highly multiplexed and complex fragment ion spectra (Fig 1D). To deal with this complexity, Gillet *et al* proposed a novel data analysis strategy based on peptide-centric scoring, which relies on querying chromatographic and mass spectrometric coordinates of the proteins and peptides of interest in form of so-called peptide query parameters (PQPs). PQPs are typically derived from previously generated spectral libraries.

In addition to the described SWATH-MS method, a wealth of other DIA schemes and alternative data analysis strategies has been

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Table 1. Advantages and limitations of SWATH-MS in comparison with data-dependent (DDA) and targeted (SRM, PRM) proteomics.

	Data independent acquisition-based SWATH-MS	**Data-dependent acquisition (DDA)**	**Targeted acquisition (SRM and PRM)**
Ease of data acquisition	** Easy, requires definition of mass range to cover, precursor isolation window width and number of MS2 scans per cycle	*** Easiest, default setup on most mass spectrometers, requires definition of TopN method, MS2 trigger threshold and dynamic exclusion time	* Hardest, requires generation and optimization of targeted peptide assays for data acquisition
Ease of data analysis	* Currently hardest, requires peptide query parameters, sophisticated software tools, large informatics resources	*** Currently easiest, multitude of pipelines available	** Currently easy, several software tools developed for manual and automated analysis
Breadth of protein and peptide detection/multiplexing	*** 10,000s of peptides per MS injection quantifiable	*** 10,000s of peptides per MS injection quantifiable	* 10s - 100s peptides per MS injection quantifiable
Selectivity/sensitivity/dynamic quantification range	** 4 orders of magnitude per MS injection	** 4 orders of magnitude per MS injection	*** 4–5 orders of magnitude per MS injection
Reproducibility/data consistency	*** High, due to peptide-centric scoring analysis	* Low, due to stochastic sampling in DDA	*** High, due to targeted data acquisition
Retrospective targeting (using chromatogram extraction)	*** Possible on MS1 and MS2 level	** Possible on MS1 level only	* Not possible due to targeted data acquisition

*Least optimal performance.
**Medium performance.
***Best performance.

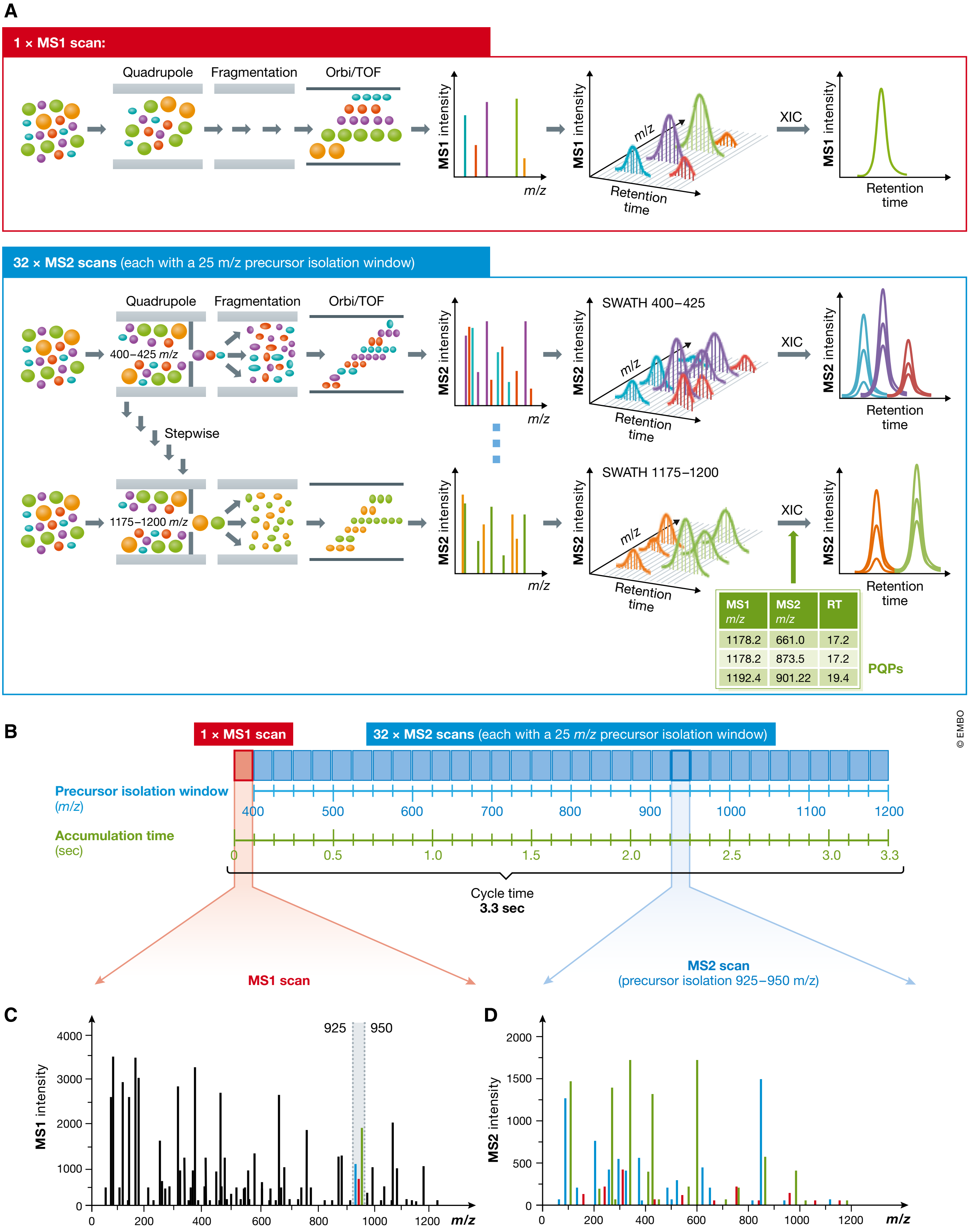
established. For an overview, see Appendix Fig S1, as well as recent reviews (Chapman *et al*, 2014; Bilbao *et al*, 2015). While the term “SWATH” became a registered trademark of SCIEX in the context of Q-TOF instrumentation, the company Biognosys trademarked the name “Hyper Reaction Monitoring” (HRM) for an analogous mode of data acquisition on Orbitrap instrumentation (Box 1). Throughout this tutorial, we only use the term “SWATH-MS”, independently from the underlying instrument type, and the generic term “DIA” when referring to the breadth of all data-independent acquisition strategies.

When is SWATH-MS the method of choice for my proteomic study?

The major advantage of SWATH-MS is that it supports quantitative analyses of peptides covering 1,000s of proteins with a high quantitative consistency and accuracy. It is ideally suited for projects that entail a large number of samples and that require accurate and reproducible quantification for the major fraction of the expressed proteome or peptidome in each sample. Typical projects that require exactly these properties include for example biomarker studies (Liu *et al*, 2014; Muntel *et al*, 2015; Kulkarni *et al*, 2016; Ortea *et al*, 2016), genetic association studies (Liu *et al*, 2015; Okada *et al*, 2016; Williams *et al*, 2016), clinical drug/perturbation studies (preprint: Litichevskiy *et al*, 2018; Tan *et al*, 2017; Keam *et al*, 2018) or exploratory basic research (Collins *et al*, 2013; Lambert *et al*, 2013; Parker *et al*, 2015a; Schubert *et al*, 2015b). SWATH-MS is also particularly well suited for studies that need fast analyses using LC gradient lengths below 60 min (Vowinckel *et al*, 2018). Proteome coverages of ~50% of the MS-detectable proteome have been achieved in complex mammalian samples in a single-shot analysis (Bruderer *et al*, 2017; Kelstrup *et al*, 2018). A current drawback

Figure 1. Principle of sequentially windowed data-independent acquisition in SWATH-MS.

(A) SWATH-MS measurements are performed on fast scanning hybrid mass spectrometers, typically employing a quadrupole as first mass analyser and a TOF or Orbitrap as second mass analyser. In SWATH-MS mode, typically a single precursor ion (MS1) spectrum is recorded, followed by a series of fragment ion (MS2) spectra with wide precursor isolation windows (for example 25 *m/z*). Through repeated cycling of consecutive precursor isolation windows over a defined mass range, a comprehensive data set is recorded, which includes continuous information on all detectable fragment and precursor ions. Hence, extracted ion chromatograms can be generated on MS2 as well as MS1 level. For the analysis of SWATH-MS data, a peptide-centric scoring strategy can be employed, which requires prior knowledge about the chromatographic and mass spectrometric behaviour of all queried peptides in form of peptide query parameters (PQPs). (B) The SWATH-MS data acquisition scheme described by Gillet *et al* (2012) for a Q-TOF mass spectrometer uses 32 MS2 scans with defined increments of 25 *m/z*, starting at 400 *m/z* and ending at 1,200 *m/z*. One full MS1 scan is recorded at the beginning. By applying an acquisition time of 100 ms per scan, a total cycle time of ~3.3 s is achieved. (C) The MS1 full scan detects all peptide precursors eluting at a given time point. For example, in the mass range from 925 to 950 *m/z*, three co-eluting peptide species are detected (green, red and blue). (D) The corresponding MS2 scan with a precursor isolation window of 925–950 *m/z* represents a mixed MS2 spectrum with fragments of all three peptide species.



MS1 *m/z*	MS2 *m/z*	RT
1178.2	661.0	17.2
1178.2	873.5	17.2
1192.4	901.22	19.4

Figure 1.

Box 1: Definitions of frequently used terms in the context of SWATH-MS

Term	Definition
DIA	Data-Independent Acquisition (DIA)—here, we use DIA as an umbrella term for mass spectrometric acquisition methods that continuously acquire fragment ion (MS2) spectra in an unbiased fashion, without requiring the detection of peptide precursor ions in an MS1 survey scan (as in DDA) nor prior knowledge about peptide precursor *m/z* values (as in SRM and PRM). Specific acquisition methods of the DIA family include for example SWATH-MS, Shotgun-CID, MS^{E}, XDIA, MSX, AIF FT-ARM and others
SWATH-MS	Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS)—represents a specific variant of DIA, performed on hybrid full-scan instruments (preferably Q-TOF and Q-Orbitrap). The term SWATH is a registered trademark of SCIEX. In SWATH-MS data acquisition, successive pre-defined ranges of precursor *m/z* values are isolated and subjected to co-fragmentation (Gillet *et al*, 2012). Peptide-centric scoring of SWATH-MS data can be performed for example by using prior knowledge in form of a spectral library
HRM	Hyper Reaction Monitoring (HRM)—Synonym for SWATH-MS. The term has been implemented in the context of data acquisition on Orbitrap mass analysers and is a registered trademark of Biognosys
Peptide-centric scoring	A data query strategy which starts with a pre-defined list of target peptides and tests whether those peptides are detectable in the data with a certain confidence. It can be applied to individual MS2 spectra or to extracted ion chromatograms (XICs). For a peptide-centric scoring analysis, peptide query parameters need to be readily available. Typically, data acquired in SRM or PRM mode are analysed by peptide-centric scoring, but also data acquired by DIA methods, such as SWATH-MS, can be analysed in this way. An equivalent term also used in the literature is "targeted data extraction"
Spectrum-centric scoring	A data analysis type which aims at finding the peptide sequence(s) from a user-specified proteome that explain(s) a given MS2 spectrum best. It is typically applied in the context of discovery-driven proteomics with data acquired by data-dependent acquisition (DDA), but also data acquired by different DIA methods can be analysed in this way.
Peptide query parameters (PQPs)	Compendium of parameters required for peptide identification by peptide-centric scoring. PQPs are stored in a table format and include (i) optimal (proteotypic) peptides to target for a given protein, (ii) chromatographic elution times of those peptides on the applied chromatography setup, (iii) most intense fragment ions (typically four to six) generated under the applied fragmentation conditions, (iv) charge state(s) of precursors and fragment ions and (v) relative ion intensity of all selected fragments. PQPs can be derived from previous discovery-driven experiments, from which all peptide identifications are summarized in form of a spectral library
(Targeted) Peptide assay	Synonym for peptide query parameters (PQPs). Term mainly used in the context of SRM, where different tier levels (1–3) of analytical assay validation have been defined (Carr *et al*, 2014)
Spectral library	Compendium of MS2 spectra confidently assigned to a specific peptide sequence, typically acquired by discovery-driven proteomics using data-dependent acquisition. In case, several MS2 spectra refer to the same peptide sequence either the best scoring spectrum or an average consensus spectrum gets reported. Peptide retention time information can also be stored in a spectral library file and normalized retention times can be generated through retention time re-alignment using reference peptides. Alternatively, spectral libraries can be generated from deconvoluted pseudo-MS/MS spectra directly from DIA data (DIA-Umpire; Tsou *et al*, 2015)
Transition	Pair of a precursor and one corresponding fragment ion *m/z* value. Mainly used in the context of SRM
Targeted proteomics	Umbrella term for mass spectrometric methods that aim at quantifying a list of pre-defined proteins, peptides or PTM-peptides of interest. In the two classical targeted proteomic approaches, SRM and PRM, the data acquisition itself is performed in a targeted fashion. However, also data acquired "untargeted", using for example DIA measurements, can be analysed using a peptide-centric data analysis strategy, which classifies SWATH-MS as targeted proteomic approaches

of SWATH-MS compared to the classical targeted proteomic approaches (SRM or PRM) is that peptide quantification with SWATH-MS is still three- to 10-fold less sensitive (Gillet *et al*, 2012; Liu *et al*, 2013; Schmidlin *et al*, 2016). Hence, targeted data acquisition remains the better option for projects that involve quantification of particularly low-abundant proteins and peptides with maximal accuracy. A further drawback of SWATH-MS in comparison with DDA-based methods is the required upfront effort on experimental or *in silico* spectral library and PQP generation and optimization (Table 1).

In the context of very large-scale quantitative proteomic analyses, two alternative mass spectrometric strategies are currently used successfully in the field in addition to SWATH-MS. The first is the classical label-free DDA proteomics workflow, where quantification is based on precursor ion (MS1) intensities or spectral counts, and which can possibly be combined with peptide fractionation techniques to improve proteome coverage (Lawrence *et al*, 2015; Geyer *et al*, 2016; Frejno *et al*, 2017). An important improvement for the application of MS1 quantification to DDA data sets was the development of analysis tools that allow the transfer of peptide identifications between samples and thereby improve the completeness of the quantitative data matrix (Prakash *et al*, 2006; Mueller *et al*, 2007; Cox *et al*, 2014). However, even when using these tools, the number of missing values in DDA data sets still remains higher than for data acquired in SWATH-MS mode, especially for peptides and proteins in the low concentration range (Bruderer *et al*, 2015, 2017; Kelstrup *et al*, 2018). While direct comparisons of an optimal label-free MS1/DDA workflow versus a SWATH-MS workflow are challenging, several papers have demonstrated that when the same sample is injected under the same conditions using the same mass spectrometer operated once in DDA and once in SWATH-MS mode, SWATH-MS outperforms DDA in terms of detectable peptides and associated proteins as well as measurement reproducibility (Bruderer *et al*, 2015; Kelstrup *et al*, 2018).

A second popular strategy for large-scale quantitative proteomics relies on isobaric labelling, using, for example, tandem mass tags (TMT) (Thompson *et al*, 2003) or isobaric tags for relative and absolute quantitation (iTRAQ) (Unwin *et al*, 2005). Frequently, isobaric labelling is followed by an extensive peptide fractionation procedure. The resulting fractions are then analysed individually by DDA mass spectrometry (Chick *et al*, 2016; Roumeliotis *et al*, 2017). With state-of-the-art TMT reagents 10 (McAlister *et al*, 2012) or 11, samples can be mixed and analysed simultaneously, leading to minimal sample preparation biases and highly consistent and complete data matrices within a set of multiplexed samples. While early implementations of this method suffered from quantitative ratio compression due to interferences in reporter ions from co-eluting and co-fragmenting peptides, this has been addressed to some extent by using optimized data acquisition and analysis methods (Ting *et al*, 2011; Savitski *et al*, 2013; McAlister *et al*, 2014; Ahrne *et al*, 2016; O'Brien *et al*, 2018; Sonnett *et al*, 2018). Labelling samples with isobaric tags can be an optimal workflow for comparative analysis of medium-sized projects; however, if 100s of samples need to be analysed, the issues of data incompleteness and batch effects can become apparent again across sets of multiplexed samples. To date, studies that directly compare isobaric tagging with SWATH-MS are missing.

The overall intent of this tutorial is to guide readers towards performing their own SWATH-MS measurements. We give guidelines on how to set up and plan a SWATH-MS experiment, how to perform the mass spectrometric measurement using data-independent acquisition and how to analyse SWATH-MS data using peptide-centric scoring. Furthermore, concepts on how to improve SWATH-MS data acquisition, potential trade-offs of parameter settings and alternative data analysis strategies are discussed.

Setting up and planning a SWATH-MS experiment

If a SWATH-MS study progresses towards the analysis of 100s and eventually 1,000s of samples, particular attention should be paid to the feasibility of producing comparable data of good quality, both longitudinally on a single instrument, as well as across multiple instruments of the same type, and conceivably across different instrument platforms. Encouraging progress has been made demonstrating the comparability of SWATH-MS data generated between laboratories using standard samples (Collins *et al*, 2017).

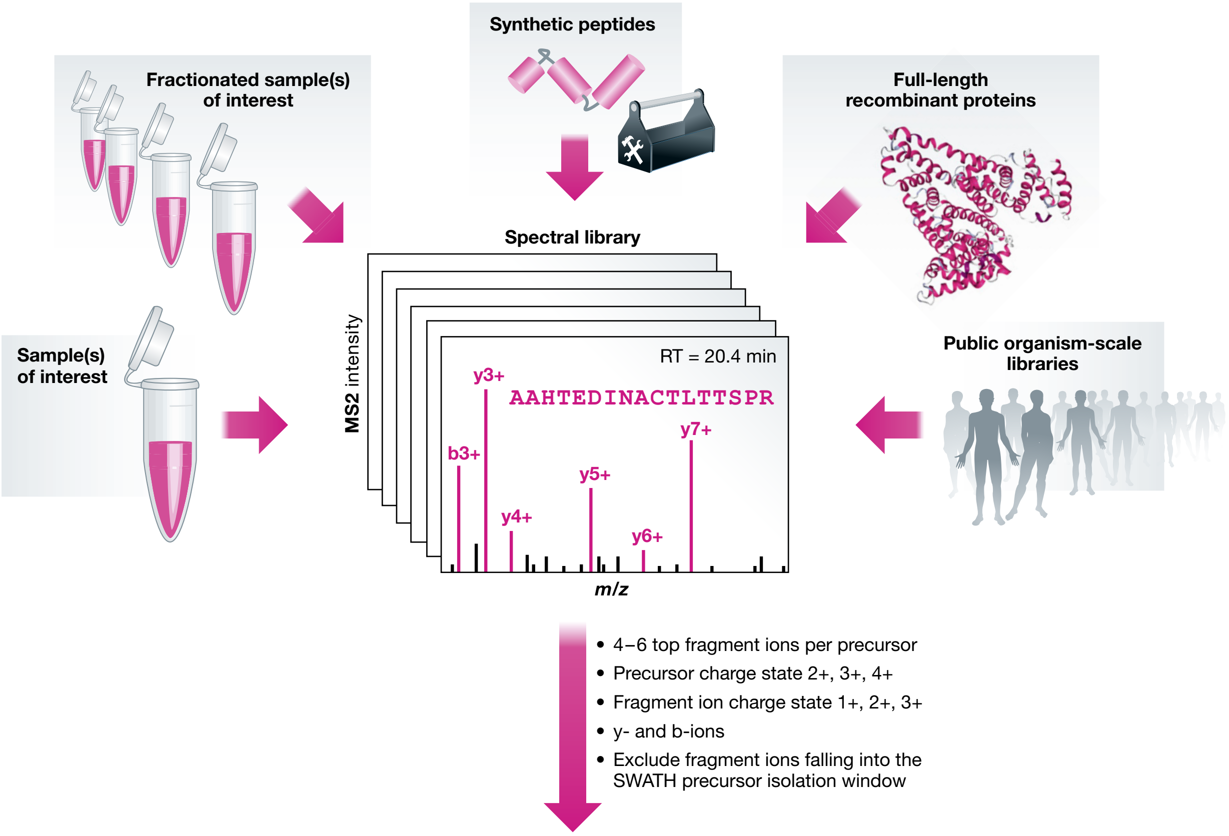
Cumulative instrument contamination during a measurement series, caused for example by contaminants such as lipids, polymers or detergents, is a major concern and extra care must be taken to produce samples that are mostly devoid of such contaminants. Contaminants are an especially important issue in the context of SWATH-MS, because we observed faster and more severe instrument performance loss (such as charging effects and sensitivity issues) in SWATH-MS mode than in DDA or PRM mode on the same instrument. One possible explanation for this observation is that the instrument operated in SWATH-MS mode has a substantially higher ion flux in the fragment ion scans (which compose > 90% of the data acquisition time), which in turn means that also more sample contaminants and impurities might enter and contaminate the instrument, leading to a faster performance loss. Therefore, monitoring the performance of the mass spectrometer and maintaining it at an acceptable level are an important prerequisite. Recent efforts have been undertaken to develop software tools that enable systematic tracking of instrument performance and that can be applied also to SWATH-MS data (Rudnick *et al*, 2010; Wang *et al*, 2014; Bereman *et al*, 2016; Chiva *et al*, 2018).

When planning a label-free large-scale proteomic experiment, statistical considerations for the experimental design, such as group size, biological and technical variability or achievable sensitivity and selectivity, should be taken into account (Krzywinski & Altman, 2014b). We suggest that particular attention should be paid to proper randomization and blocking (Krzywinski & Altman, 2014a) of a sufficient number of biological and technical sample replicates (Blainey *et al*, 2014) to ensure optimal statistical power (Krzywinski & Altman, 2013) during the downstream data analysis process.

Prior knowledge required for peptide-centric scoring and spectral libraries

The underlying concept behind SWATH-MS is that empirically derived prior knowledge of the mass spectrometric and chromatographic behaviour of peptides of interest can be used to selectively extract peptide-specific information from highly convoluted SWATH data in a targeted fashion (Gillet *et al*, 2012). This required prior knowledge is referred to as "peptide query parameters" (PQPs). It is worth noting that "prior" in this context indicates that PQPs should be available as a prerequisite before data analysis is undertaken, while the actual acquisition of the SWATH-MS data itself does not depend on the availability of PQPs.

What kind of information do PQPs contain? In detail, the information includes (i) the peptide sequence(s) to monitor for a given protein, (ii) the dominant precursor ion *m/z* value(s) of the peptide(s) and thus the charge state distribution, (iii) the four to six most intense fragment ion *m/z* values for the peptide(s) under the applied fragmentation conditions, (iv) information about the expected fragmentation pattern under the applied conditions, i.e. the relative fragment ion intensities, and v) the expected retention time of the peptide(s) and thus the associated fragment ion signals, ideally normalized to a reference. PQPs can commonly be obtained from a spectral library [or potentially chromatogram library (Sharma *et al*, 2014)] and are stored in a table format as shown in Fig 2. Computational pipelines integrating all steps of spectral library generation and PQP extraction have been developed that simplify and standardize this process and are available for example within Skyline (Egertson *et al*, 2015), PeakView (SCIEX), Spectronaut Pulsar (further referred to as Spectronaut) (Bruderer *et al*, 2015) (Biognosys) and the Trans-Proteomic Pipeline (TPP) (Deutsch *et al*, 2010). Further tools to prepare and convert [specL (Panse *et al*, 2015), Fraggle/Tramler/Franklin (Teleman *et al*, 2017)] or extend [SwathXtend (Wu *et al*, 2016)] sets of peptide query parameters are also available. Particularly large spectral libraries can be optimized and constrained by using MSPLIT-DIA (Wang *et al*, 2015).


Synthetic peptides
Fractionated sample(s) of interest
Full-length recombinant proteins
Spectral library
Sample(s) of interest
Public organism-scale libraries
MS2 intensity
RT = 20.4 min
AAHTEDINACTLTTSPR
y3+
b3+
y4+
y5+
y6+
y7+
m/z
• 4–6 top fragment ions per precursor
• Precursor charge state 2+, 3+, 4+
• Fragment ion charge state 1+, 2+, 3+
• y- and b-ions
• Exclude fragment ions falling into the SWATH precursor isolation window

Peptide query parameters (PQPs)
for target peptide AAHTEDINACTLTTSPR

Protein	Peptide sequence	Precursor ion m/z	Fragment ion m/z	Precursor charge	Fragment charge	Fragment ion type	Fragment relative intensity	Normalized retention time
Raf1	AAHTEDINACTLTTSPR	929.4416	775.4308	2	1	y7	70	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	674.3832	2	1	y6	10	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	561.2991	2	1	y5	40	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	460.2514	2	1	y4	20	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	359.2037	2	1	y3	100	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	280.1404	2	1	b3	60	45.3

Figure 2. What are peptide query parameters (PQPs) and where do these parameters come from?
PQPs contain information about the chromatographic and mass spectrometric behaviour of a given peptide, as exemplified here for the peptide AAHTEDINACTLTTSPR. Various different input sample types can be used for the purpose of PQP generation. Typically, those samples are analysed in initial DDA measurements, and the results are summarized in the form of one or several spectral library files. From the spectral library file(s), the relevant PQPs are extracted by filtering the identified peptide coordinates using the indicated criteria. PQPs contain information about: the underlying protein, peptide sequence, precursor *m/z*, fragment *m/z*, precursor and fragment charge, fragment ion type, expected relative fragment ion intensities and normalized retention time (retention time relative to a set of reference peptides, iRT).

For further details on how to create spectral libraries and PQPs from DDA data, we refer to a recent protocol paper (Schubert *et al*, 2015a). In the following paragraphs, we will discuss the types and sources of information that can be used to generate spectral libraries from which PQPs can be extracted.

An alternative to acquiring DDA runs for spectral library generation represents spectrum-centric scoring of the DIA data. While such algorithms have been proposed early in the development of DIA acquisition schemes, recently developed algorithms such as DIA-Umpire (Tsou *et al*, 2015), Group-DIA (Li *et al*, 2015) and Spectronaut Pulsar (Biognosys) make specifically use of the improved data quality of the latest generation instruments. Generally, these algorithms generate a cumulative spectral library of a related set of samples and achieve similar coverage as DDA runs of unfractionated samples.

Representative sample types for spectral library generation

Here, one has to choose between endogenous sources of peptides from the samples of interest, synthetic analogues of those peptides or recombinant full-length proteins (Fig 2). The majority of

SWATH-MS studies to date have used a side-by-side characterization of the samples of interest by DDA analysis for generation of spectral libraries. These libraries frequently include a sample fractionation step prior to DDA analysis, which can be beneficial from a sensitivity perspective for post-acquisition peptide queries (Rosenberger *et al*, 2014; Zi *et al*, 2014; Selevsek *et al*, 2015). This is because the sensitivity and coverage of single-shot DDA analysis are frequently lower than that of SWATH-MS data (Bruderer *et al*, 2015; Kelstrup *et al*, 2018). Therefore, the SWATH-MS data would not be fully covered by a "single shot" DDA spectral library generation strategy. The strategy focusing on repeated DDA analysis of non-fractionated samples is sometimes favoured because it is straightforward to implement; however, primarily for library completeness and quality reasons, other sources of peptides can be considered.

Chemically synthesized peptides have long been proposed as a source of prior knowledge, in particular, with respect to the development of SRM assays (Kuster *et al*, 2005; Picotti *et al*, 2010), and large-scale efforts to synthesize and measure peptides for several organisms on a proteome-wide scale have been reported (Picotti *et al*, 2013; Schubert *et al*, 2013; Kusebauch *et al*, 2016; Zolg *et al*, 2017). This approach has several advantages: (i) all proteins can be represented in the library, irrespective of whether they have been previously observed, (ii) high-quality MS2 spectra and derived PQPs can be generated, because the synthetic peptides can be analysed at very high concentrations and represent ground truth, and (iii) the error rate in the spectral library generated should be close to zero. In some cases, the selection of which peptides to synthesize for a given protein has been driven by prior empirical observation. However, since there has been so far no species with absolute complete proteome coverage by DDA methods, sets of proteotypic peptides for each protein have also been computationally predicted (Mallick *et al*, 2007). Since computational prediction methods have turned out to be less reliable than expected (Searle *et al*, 2015), empirical peptide selection is still preferred over computational prediction. More recent efforts to create synthetic proteomes for general purposes in proteomics have extended to a very large scale (> 330,000 human peptides) by synthesizing much larger numbers of peptides per protein and by also including PTMs and common sequence variants (www.proteometools.org; Zolg *et al*, 2017). A useful extension of this approach is to create full-length proteins by recombinant methods or *in vitro* transcription/translation systems. In this way, the most suitable peptides per protein for analysis can be determined empirically (Stergachis *et al*, 2011; Matsumoto *et al*, 2017). While those large-scale synthetic peptide and protein MS resources have not yet been fully exploited for SWATH-MS analysis, it seems likely that they will be useful resources going forward. Hybrid libraries consisting of endogenous samples and synthetic peptides to increase coverage may also be an attractive option and have proved useful in the case of an organism-scale library for *Mycobacterium tuberculosis* (Schubert *et al*, 2015b).

Comprehensive versus sample-specific spectral libraries

A natural extension of the ideas discussed above is to attempt to characterize peptides by DDA from all proteins in a given species to generate a comprehensive "off the shelf", species-specific library for general use, obviating the need to generate experiment- or sample-specific libraries. These libraries could include data from endogenous samples and/or synthetic proteomes. Presently, such organism-scale spectral libraries are publicly available for *S. pyogenes* (Karlsson *et al*, 2012), *S. cerevisiae* (Picotti *et al*, 2013; Selevsek *et al*, 2015), *M. tuberculosis* (Schubert *et al*, 2013, 2015b), *H. sapiens* (Rosenberger *et al*, 2014; Kusebauch *et al*, 2016; Zolg *et al*, 2017), phyllosphere-colonizing bacteria (*M. exotorquens*, *P. syringae* and *S. melonis*) (Muller *et al*, 2016) and *E. coli* (Ludwig *et al*, in preparation). Further, the number of public repositories for various types of MS data that could conceivably also be used as prior knowledge in the analysis of SWATH-MS data continues to increase (Craig *et al*, 2004; Martens *et al*, 2005; Deutsch *et al*, 2008; Picotti *et al*, 2008; Sharma *et al*, 2014; Whiteaker *et al*, 2014; Wilhelm *et al*, 2014; Zolg *et al*, 2017). These include SWATHAtlas (www.swathatlas.org), which focuses on SWATH-MS and provides spectral libraries in formats directly compatible with most software tools for peptide-centric analysis of SWATH-MS data.

An important consideration for the use of such public libraries will be portability of information between instrument types and between laboratories. For example, efforts have been made to evaluate the effect of using DDA spectra generated on different instrument types as prior knowledge (Toprak *et al*, 2014). Although the best comparability is achieved when fragment ion spectra are generated on the same instrument platform, the data also indicated that fragment ion spectra acquired from instruments using "beam type" collision-induced dissociation (e.g. QqQ, QqTOF and Orbitrap operated in HCD mode) produce spectra that are sufficiently comparable for their effective use as prior knowledge in peptide-centric analysis (de Graaf *et al*, 2011; Zolg *et al*, 2017). The portability of chromatographic information between laboratories has been advanced through the use of normalized retention times (Norbeck *et al*, 2005) such as indexed retention time (iRT) (Escher *et al*, 2012). The accuracy of such methods is generally tolerant to changes in gradient length and column dimensions; however, larger errors will result from changes in other factors that may significantly affect the peptide elution order such as mobile/stationary phases or column temperature. An inter-laboratory evaluation study recently showed that an organism-scale spectral library could effectively be used to analyse SWATH-MS data generated from 11 different laboratories worldwide (Collins *et al*, 2017).

Another important issue to consider when working with very large-scale spectral library resources and querying very high numbers of peptides in many samples measured by SWATH-MS is appropriate error rate control. In such analyses, it is common that a significant fraction of queried peptides from the library are actually not present in the samples of interest at a detectable level and, as such, represent channels in which false positives may appear and accumulate if many runs are analysed. If SWATH extraction results are summarized at the inferred protein level, the error rate can be further inflated by such false positives (Reiter *et al*, 2009; Rosenberger *et al*, 2017a). Therefore, it is important to control false discovery rates at the protein level, rather or in addition to the peptide level and to compute false discovery rates globally for a complete experiment rather than at the per-file level. Rosenberger *et al* (2017a) recently established statistical concepts derived from discovery proteomics that can be applied to appropriately control the error rate of detected peptides and inferred proteins in SWATH-MS.

In conclusion, using organism-scale "off the shelf" libraries might be convenient from the perspective of reducing the effort of creating project specific libraries, but their use may eventually result in some loss of statistical power due to the additional multiple testing burden and necessary correction that comes from querying such a large number of peptides. Alternatively, researchers need not necessarily query all of the peptides in a spectral library, but can also focus on just a subset of proteins that are of interest for their specific biological question.

Which and how many peptides should be queried for?—From targeted to discovery uses of SWATH-MS

In the initial implementation of SWATH-MS, the primary goal was to use peptide-centric scoring based on prior knowledge combined with sequentially windowed data-independent acquisition (Gillet *et al*, 2012) to develop an approach that generates data resembling those from classical targeted proteomics approaches such as SRM but at a largely extended scale of analytes. Hence, a discrete set of peptides from proteins related to a particular biological question was targeted for quantification. However, it was soon realized that SWATH-MS data sets offer the opportunity to characterize complex samples in a more comprehensive manner by extracting and scoring ion chromatograms for a maximum number of peptides, by using sample-specific or combined libraries. Over time, the number of peptides that were queried in comprehensive SWATH-MS studies has grown significantly to the point that, with the appropriate error-rate control (Rosenberger *et al*, 2017a), it is now possible and reasonable to query for tens to hundreds of thousands of peptides derived from organism-scale spectral libraries in a fashion that resembles discovery-based proteomics much more than targeted proteomics in its scope (Bruderer *et al*, 2017).

The choice of whether to choose a targeted approach and extract peptides for only a smaller set of proteins of interest, or whether to extract data for all peptides contained in a large-scale spectral library, will depend on the specificity of the hypothesis that underlies the targeted approach, i.e. how useful the set of proteins being measured is for answering a given biological question. If a biological hypothesis can be addressed with a selected set of target proteins, then increasing the number of queries by including peptides from a large-scale library will increase the multiple testing burden. If the hypothesis cannot be addressed by few selected proteins and requires a data-driven investigation, such as exemplified by classification problems, then extracting a maximum number of peptides from a large-scale library will likely provide the most informative result, despite the increased number of statistical tests that need to be performed.

The possibility that specific peptide queries might result in false-positive signals arising from contaminating peptide species that are similar to the target peptide in their mass spectrometric and chromatographic parameter space has been a concern of peptide-centric scoring. This might be the case for peptides with a sequence that is similar to that of the target peptide, or for peptides for which the precursor mass difference between target and mismatch can be explained by a modification of the common backbone sequence. A recent study comparing standard database searching of DDA data with open modification searches of the same data found that this scenario occurs rather infrequently (Kong *et al*, 2017). The open modification search assigned a given fragment ion spectrum to a modified peptide sequence rather than to the unmodified peptide sequence originally assigned by the standard database search only for ca. 1% of the identified peptides. This suggests that false-positive identifications in SWATH-MS data due to unanticipated modified peptides might also occur rather infrequently, but nevertheless, close attention in ongoing algorithmic development should be paid to the likelihood of matching-related peptide species (Rost *et al*, 2012). Data analysis methods have now been developed that address the problem of related "peptidoforms" in SWATH datasets (Rosenberger *et al*, 2017b).

Overall, the high degree of fragment ion data completeness, both in the mass and chromatographic dimension, constitutes a main strength of data acquired by SWATH-MS and related DIA approaches, as it offers the opportunity of applying diverse and flexible data analysis strategies. It also equally supports different experimental strategies, including targeted quantification of selected analytes and discovery-driven studies of all detectable analytes, provided that the types of possible scoring errors are recognized and appropriately controlled.

Performing a SWATH-MS measurement

Setting up liquid chromatography for SWATH-MS

The LC setup used for SWATH-MS measurements does not differ from that used for targeted or DDA setups. Typically, peptide mixtures of 0.5–2 μg total peptide mass are separated by injection onto a nano-HPLC system equipped with a 15–50-cm-long column packed with reversed phase particles (e.g. 3 or 1.8 μm C18 material), operated at 300 nl/min flow rate and eluted with a solvent gradient of increasing organic composition. Note that ~5 μl/min microflow HPLC systems are also compatible with SWATH-MS, though at the cost of a three- to fivefold loss of sensitivity that can be compensated by injecting three to five times more peptide amount on column (Bruderer *et al*, 2017; Vowinckel *et al*, 2018).

Peptide-centric analysis of SWATH-MS data depends on a sufficient number of data points (typically in the range of 10) collected over the elution profile of a peptide peak to allow the accurate reconstruction of the respective chromatographic peak (Fig 3C). The 2–4 s cycle time required by the current windowed SWATH-MS methods to cycle through the precursor isolation range, together with the required number of measurements over the peak indicates that the average chromatographic peak width in SWATH-MS measurements should not fall below 20–40 s. This is in contrast to single-window DIA methods, which only cycle between a single MS1 and a single MS2 scan and which can accommodate the 2–3 s-wide chromatographic peaks produced by higher performance LC setups [for example AIF (Geiger *et al*, 2010) or MS^E (Silva *et al*, 2006)]. As MS instruments improve in scanning speed, narrower chromatographic peaks may increasingly become compatible with windowed SWATH-MS methods.

Interestingly, SWATH-MS copes well with shortening of the HPLC gradient length. While the number of acquired MS2 spectra and the number of peptides identified decrease proportionally with the gradient length in DDA mode, the deterministic nature of the MS2 sampling in SWATH-MS mode attenuates the attrition in number of peptides identified for shorter separation gradients (Fig 3A). The loss in the number of peptide identifications at shorter gradients in SWATH-MS mode seems, therefore, to be related to the increase in precursor co-elution and ensuing potential ion suppression and co-fragmentation effects rather than to

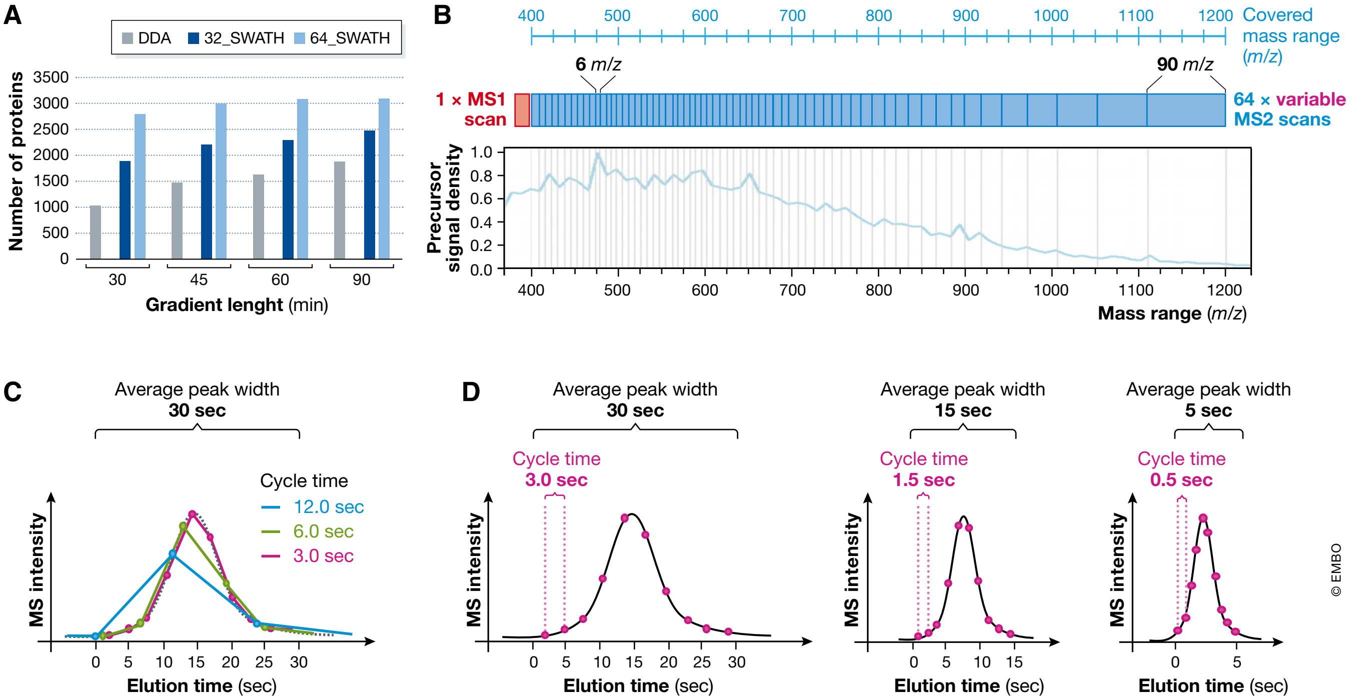


Figure 3. Setting up the optimal SWATH-MS data acquisition scheme.

(A) Effect of liquid chromatography gradient length on the number of identified proteins for technical triplicate injections of a trypsin-digested HEK cell lysate acquired either in DDA (grey bars), SWATH 32 fixed windows (dark blue bars) or SWATH 64 variable windows (light blue bars) on a Q-TOF instrument. (B) To improve precursor selectivity in SWATH-MS, an acquisition scheme using variable precursor isolation window widths can be used to partition the precursor density equally across all isolation windows. (C) Ten recorded data points are considered necessary for accurate reconstruction of a chromatographic peak. For a peak width of 30 s a cycle time of 3 s leads to 10 recorded data points (red), which allows appropriate reconstruction of the actual peak shape (grey dashed line). Longer cycle times, for example 6 s (green) or 12 s (blue), lead to under sampling and the correct peak shape can no longer be optimally reconstructed. (D) If the average peak width is reduced from 30 (left panel) to 15 (middle panel) or even 5 s (right panel), the cycle time needs to be decreased accordingly from 3 to 1.5 and 0.5 s, in order to maintain 10 data points over the elution profile.

undersampling. As MS instrumentation continues to improve, it is likely that SWATH-MS will reach higher selectivity and identification rates under short gradient regimens compatible with relatively high sample throughput.

In summary, we advise a 2-h nano-HPLC gradient for the acquisition of high-quality spectral libraries (Schubert *et al*, 2015a), while the use of shorter nano- (or micro-) HPLC gradients to acquire SWATH-MS data, for example in the range of 30–60 min, still provides results with good selectivity and proteome coverage at a significantly higher sample throughput.

Setting up the SWATH-MS data acquisition method

Finding an optimally balanced method

The optimal setup of precursor ion (MS1) and fragment ion (MS2) scans in a SWATH-MS acquisition scheme depends on a range of considerations, including the expected mass range for analytes of interest, underlying sample complexity, available mass spectrometer type with its specific resolution and scanning speed, the LC setup and its expected average chromatographic peak width, as well as the desired measurement selectivity and sensitivity. In practice, the following instrument parameters need to be considered: (i) the precursor *m/z* range to cover, (ii) the widths and number of precursor isolation windows, (iii) the fragment and precursor ion accumulation/scanning time and resolution, (iv) the chromatographic cycle time and (v) the number of injections per sample. In the following section, each of these parameters is discussed in detail. The experience of the authors is primarily focussed on Q-TOF instrument configurations, and our recommendations in that respect are based on empirical knowledge, optimization and simulation (Rost *et al*, 2012). However, as the quadrupole-Orbitrap is increasingly popular for SWATH-MS analysis, we also refer to successfully published methods on that configuration. A detailed overview of acquisition parameters for Q-TOF as well as Q-Orbitrap instruments is provided in Table EV1. Those parameters can serve as a starting point for the generation of SWATH-MS data of good quality; however, further optimization might allow an increase in the level of quantifiable proteins and peptides. Of final note, optimal SWATH-MS parameters for Q-TOF as well as Q-Orbitrap instruments are still subject to adaptations and might change significantly in the future.

Precursor *m/z* range to cover The precursor *m/z* range covered in a SWATH-MS measurement is defined by the adjacent set of precursor isolation windows that the instrument cycles through across the chromatographic separation. Ideally, this mass range should cover as completely as possible the *m/z* space of the proteins or peptides of interest. Analytes outside the specified range are not monitored and can therefore neither be detected nor quantified. If the aim is to analyse a significant portion of the proteome and if trypsin is used as proteolytic enzyme during the sample preparation, the most peptide-rich region typically spans 400–1,200 *m/z* (Gillet *et al*, 2012). The range can be further reduced to 500–900 *m/z* (Egertson *et al*, 2015) depending on instrument and analyte constraints with a minimal attrition of quantified proteins. If other proteases than trypsin are used, or if SWATH-MS is applied to analytes other than peptides (e.g. metabolites), the precursor *m/z* range needs to be adapted accordingly. Coordinates for *m/z* ranges successfully used in selected publications on Q-TOF and Q-Orbitrap instrument are given in Table EV1.

Precursor isolation window placement The precursor isolation window width defines the range of peptide precursor masses that are co-isolated and co-fragmented in a given MS2 scan. Hence, the isolation window width directly influences the selectivity and the dynamic range of the measurement and in turn the sensitivity of peptide detection. It is probably the parameter that varies the most between various DIA methods, ranging from one isolation window of 800 *m/z* to hundred windows of 2 *m/z* (Appendix Fig S1).

Whereas the use of wider isolation windows allows the mass spectrometer to cycle faster through the predetermined precursor *m/z* range, it results in a higher number of peptide precursors being co-fragmented, more strongly convoluted MS2 spectra and lower sensitivity due to a limited intra-scan dynamic range. Conversely, using narrower isolation windows reduces the number of co-fragmented precursors and signal interference, but limits other parameters such as the covered precursor *m/z* range per injection or the number of data points recorded over the chromatographic peak profile.

Note that the precursor isolation window width does not need to be identical for all MS2 scans. Rather, variable window widths (Zhang *et al*, 2015) can be used to optimize the number or intensity distribution of precursor ion signals between isolation windows (Fig 3B). Such optimal variable window patterns can be applied on a per-sample basis or identically across multiple samples. Automated tools are available to facilitate such placement based on empirical data ("SWATH Variable Window Calculator" provided by SCIEX). In practice, we observed that the fine adjustment of the isolation window widths does not drastically influence the peptide identification or quantification results. For practical reasons, we therefore generated a "universal 64 variable window" acquisition scheme for TOF instruments (Table EV1) that seem compatible with most complex tryptic digests from various organisms ranging in complexity from bacteria to human. This precursor isolation scheme was devised to split ~50,000 experimental human tryptic precursor signals from a human cell line in 64 equal bins that vary from 5.9 *m/z* width for the range [472.4–478.3], where the density of precursor ion signals is the highest, up to 90.9 *m/z* width for the range [1,109.6–1,200.5]. The gain achieved by moving from 32 fixed to 64 variable windows has been demonstrated both qualitatively and quantitatively (Navarro *et al*, 2016). Bruderer *et al* optimized a SWATH-MS acquisition method for the Q-Exactive with 19 variable windows (Bruderer *et al*, 2015) and the Q-Exactive HF with 24 variable windows (Bruderer *et al*, 2017), whereas Kelstrup *et al* (Kelstrup *et al*, 2018) published a method with 70 windows for the latest and fastest Q-Exactive HF-X instrument (Table EV1).

Recently, alternative DIA acquisition methods have reported the use of deterministic but differential isolation windows during LC separation and a peptide's elution profile (Egertson *et al*, 2013; Moseley *et al*, 2018). However, their analysis requires specific deconvolution tools to decipher, post-acquisition, the contribution of the differential interferences arising during the peptide co-isolation process across the various isolation schemes.

Fragment and precursor ion accumulation time/resolving power For Q-TOF instruments, the MS acquisition or accumulation time defines how long the mass spectrometer accumulates ion signals for a given MS scan. With longer accumulation times, the signal-to-noise ratio for the acquired spectrum increases. Conversely, longer accumulation times increase the time the instrument requires to cycle through the series of MS scans. Therefore, the accumulation time should be chosen in conjunction with the number of precursor isolation windows (Fig 3C).

For Orbitrap mass analysers, the MS acquisition is subdivided into two parallelized time-constrained processes. First, the "injection time" is the time required to collect in the ion trap the desired number of charged species designated by the automatic gain control (AGC) parameter. Second, the "scan time" is the time required by the Orbitrap to record the mass spectrum depending on the set resolution. On new generation instruments (Q-Exactive and Fusion/Lumos), these two processes are parallelized, and thus, the more time-consuming step determines the cycle time. Typically, the maximal injection time is set according to the required scan time. Recent publications have reported methods with 19 (Bruderer *et al*, 2015) (Q-Exactive) and 24 variable precursor isolation windows (Bruderer *et al*, 2017) (Q-Exactive HF) at a resolution of 30,000 (at Full Width Half Maximum) or 70 windows (Kelstrup *et al*, 2018) (Q-Exactive HF-X) with fixed width of 9 *m/z* at a resolution of 15,000 (Table EV1). With the rapidly evolving specifications of these instruments, further optimization of the acquisition settings can be expected.

In summary, for Q-TOF instruments coupled to a nano-LC setup that results in an average chromatographic peak width in the range of ca. 30 s, we recommend to use an MS2 accumulation time in the range of 50 ms for each of 64 variable MS2 acquisition windows. This adds up to a total cycle time of ~3.3 s. On the Q-Exactive HF instrument, the use of 24 variable windows at 30,000 resolution has been described recently, leading to a similar cycle time (Bruderer *et al*, 2017). However, due to the added complexity of balancing automatic gain control, scan time and duty cycle effects on Orbitrap instrumentation, further optimization for given instrument configurations and sample types should be considered. We also recommend the inclusion of a 250 ms MS1 scan on Q-TOF instruments before each cycle of MS2 scans. Similarly, the inclusion of one or several MS1 scans is recommended for Orbitrap instruments. Information from the MS1 scan is increasingly being used by recent DIA analysis software, either to confirm the precursor mass of the peptide of interest upon peptide-centric targeted chromatogram extraction or to aid in the deconvolution of co-eluting fragment ion traces with spectrum-centric analysis tools.

Chromatographic cycle time In SWATH-MS, the "cycle time", also referred to as "duty cycle" or "sampling rate", corresponds to the sum of the accumulation times set for the MS1 and MS2 scan series (plus any instrument overhead time which is usually negligible). Hence, the cycle time defines how often along the chromatographic elution profile of a peptide the same ion gets recorded. The cycle time is critical for any chromatography-based quantification approach. Generally, around 10 acquired data points are considered necessary to reconstruct chromatographic peak shapes and to perform accurate quantification (Fig 3C) (Matthews & Hayes, 1976; Lange *et al*, 2008). However, the available time to collect 10 data points throughout an average chromatographic peak width can vary substantially, depending on the specific chromatographic setup. In an experiment with an average peak width (at base) in the range of 30 s (typical peak width in a 3 μm C18 particles nano-HPLC setup with gradient length in the range of 1–2 h), a cycle time of 3 s is appropriate. However, if a higher resolution chromatographic system is employed, the average peptide elution peak width might be significantly shorter, for example 5 s. In such cases, the cycle time must be decreased, e.g. to 0.5 s, to ensure the constant recording of 10 data points over the elution profile (Fig 3D).

Number of injections per sample For large-scale proteomic studies that include 100s of samples, a single injection per sample is strongly preferable to maintain sample throughput and quantification consistency. However, for smaller scale projects, multiple injections per sample (*N*), each covering a different *m/z* range, might be an attractive alternative to improve selectivity and sensitivity of the SWATH-MS measurement (Panchaud *et al*, 2009; Ting *et al*, 2017). This allows the reduction in precursor isolation window width by a factor of *N*, while ensuring that a large precursor *m/z* range can be covered.

Setting the collision energy in SWATH-MS

Whereas for DDA, SRM and PRM acquisition methods, the collision energy can be set individually on a per-precursor basis (based on precursor *m/z* value and charge state), or even optimized for a specific transition (SRM), the collision energy in SWATH-MS can only be set on a per-isolation window basis. In other words, if two peptides with different charge states are selected in the same precursor isolation window, they will be fragmented with the same collision energy, which is likely to be suboptimal for at least one of the charge states. However, this suboptimal peptide fragmentation can be compensated for, to some degree, as long as the measured relative fragment ion intensities are similar to those in the spectral library used to query the data. This can be achieved by acquiring DDA fragment ion spectra for spectral library generation with a charge-state-independent collision energy equation, i.e. a collision energy varying with the precursor *m/z* but identical for all charge states. Note that this strategy may come at the cost of lost identifications in the DDA analysis due to suboptimal fragmentation of higher charge state peptides. This suboptimal fragmentation might be partially rescued by ramping the collision energy within a SWATH window, for example from −15 to +15 V, over the course of MS2 accumulation in both DDA and SWATH-MS mode.

In summary, for Q-TOF instruments, we recommend to set a collision energy proportional to the *m/z* of a 2+ charged precursor for DDA library generation and to apply the same collision energy of a theoretical 2+ precursor located in the centre of the precursor isolation window for SWATH-MS. Further, we recommend to systematically assess the fragmentation similarity between instruments and external public spectral libraries. Software tools that support such comparative analysis are readily available (Toprak *et al*, 2014). If significant differences between the fragmentation pattern of a spectral library and a SWATH-MS measurement are identified, it might be worth to optimize the used collision energy equation in the SWATH-MS measurements until a maximal similarity to the spectral library is found. This will ensure optimal portability of the peptide query parameters across instruments and laboratories.

Should adjacent precursor isolation windows be overlapping?

In the original SWATH-MS publication, we suggested to acquire consecutive SWATH windows with a 1 *m/z* precursor isolation *m/z* overlap (Fig 4A and Table EV1) (Gillet *et al*, 2012). This was implemented for two reasons: first, precursor ion isolation of the quadrupole mass analyser does not work with 100% efficiency over the whole mass range, but is compromised at the borders of the isolation windows (Fig 4B). Therefore, a small *m/z* overlap on both window edges compensates to some degree signal losses due to not perfectly square-shaped ion transmission efficiencies and ensures that precursors with *m/z* located at the border of the isolation window suffer as little as possible from signal attrition. Second, overlapping windows ensure maximal transfer of the complete isotopic pattern of precursors that would otherwise be split between two consecutive isolation windows for precursor masses close to a window edge (compare Fig 4C and D). Importantly, unless the entire precursor isotopic envelope gets isolated, the fragment ions will show a distorted isotopic pattern, which will potentially result in lower scoring of this peptide at data analysis stage (see section "Automated peak group scoring"). The SWATH-MS schemes published to date with 1 *m/z* window overlaps do not completely account for the problems mentioned above in the most extreme cases and, as such, slightly larger overlaps could be considered in future schemes. Note that, even if the precursor isotopic distribution is split between two consecutive isolation windows (as shown in Fig 4C), *label-free* quantification of that peptide using fragment ion intensities will still be accurate across runs, as long as the window geometries are reproducible and consistent between scans and injections (Egertson *et al*, 2015). A problem may occur, though, in the case of labelled SILAC-type experiments when one of the precursor isotopic distribution (e.g. the light form) would be split but the heavy is not. In this case, the light-to-heavy monoisotopic fragment ion ratios would be distorted due to the fact that part of the intensity of the monoisotopic peak at the fragment ion level is arising from the non-monoisotopic peaks in the precursor isotopic envelope.

Another possibility to minimize the impact of imperfect ion transmission efficiency is to purposely position the isolation window edges at masses where peptide *m/z* values are unlikely to occur as described by Egertson *et al* (2013). Such theoretically optimal window placement can be realized easily using the Skyline software (Egertson *et al*, 2015). Though this does not *per se* account for the signal attrition at the window borders, nor prevents precursor isotopes to split in different windows, it ensures that the monoisotopic precursor mass does not fall exactly on a window border.

In summary, the 1 *m/z* window overlapping suggested by Gillet *et al* (2012) causes a small loss in terms of selectivity, because the effectively measured windows are slightly broader. However, this is justified by positive effects like minimized signal losses for peptides

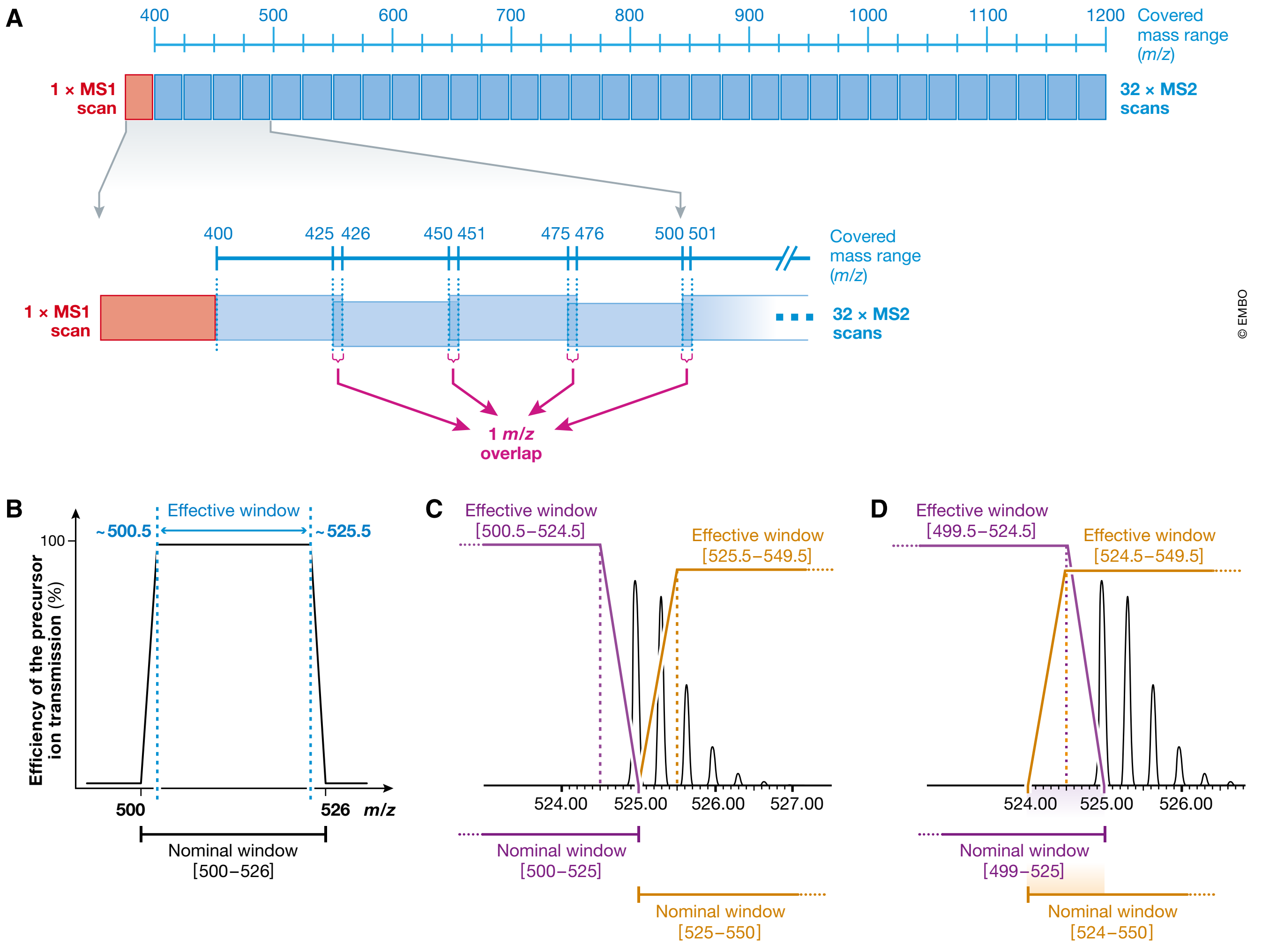


Figure 4. The rationale for using slightly overlapping precursor isolation windows.

(A) In the first implementation of SWATH-MS, a 1 m/z overlap between adjacent SWATH windows was used to compensate the inefficient ion transmission of the quadrupole at the edges of the precursor isolation window (B) and to limit the effect of precursor isotope splitting between windows (C and D). (B) Schematic representation of the efficiency of ion transmission with a state-of-the-art quadrupole mass analyser filtering for the [500–526] m/z range. (C) Theoretical isotopic distribution of the doubly charged peptide MLSYPITIGSLLHK (m/z = 524.965). If non-overlapping nominal windows are used [(500–525) and (525–550)], the isotopic profile is split between both windows and falls within the inefficient ion transmission range of the quadrupole [effective windows from ca. (500.5–524.5) and (525.5–549.5)]. Even if the window edge is placed at a mass where no precursor mass is supposed to occur (for example 525.1), the issue of inefficient ion transmission and loss of parts of the isotopic envelope would remain. (D) With overlapping nominal windows [(499–525) and (524–550)], most of the precursor isotopic pattern will be transmitted within the effective ion transmission range of the quadrupole [effective windows from ca. (499.5–524.5) and (524.5–549.5)].

falling at the sides of the isolation windows and more complete transfer of the isotopic envelope. If no window overlap is implemented, it is highly recommendable to perform optimal window placement as suggested by Egertson *et al* (2013).

Advanced DIA acquisition schemes with improved precursor selectivity

The selectivity of SWATH-MS is a direct function of the precursor isolation window width. Further, selectivity directly affects the achievable dynamic range and sensitivity of a SWATH-MS measurement. Recently, novel acquisition methods have been proposed to improve the selectivity of SWATH-MS (Fig 5).

Multiplexed DIA (MSX) In 2013, the MacCoss group reported the development of MSX (Egertson *et al*, 2013), a multiplexed DIA scheme. Instead of recording MS2 spectra for precursors selected in systematic SWATH windows of 20 m/z width, MSX records at each cycle fragment ions for peptides selected from different multiple *non-contiguous* isolation windows (e.g. five non-contiguous isolation windows of 4 m/z, Fig 5A). For the MSX setup, the overall absolute selectivity per spectrum is theoretically the same as for a 20 m/z wide SWATH-MS method. However, in MSX, a given peptide precursor will randomly co-fragment with different species in each cycle, resulting in different interferences over the elution profile of a peptide. In its current implementation, MSX requires an instrument with multiplexed trapping capabilities (ruling out Q-TOFs).

Offset isolation windows intra-run In the offset window approach, the precursor isolation window boundaries are shifted back and

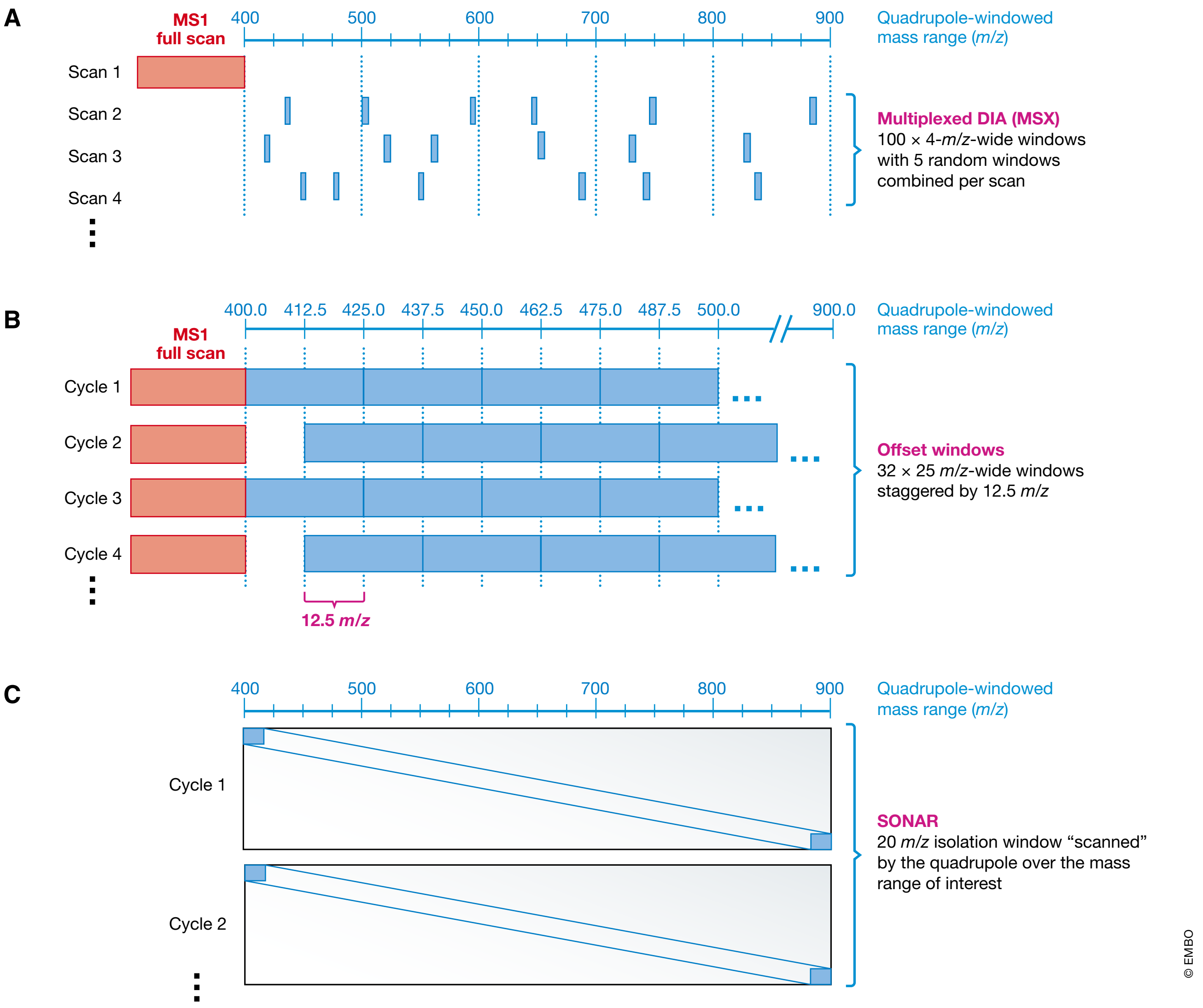

A
MS1 full scan
400
500
600
700
800
900
Quadrupole-windowed mass range (m/z)
Scan 1
Scan 2
Scan 3
Scan 4
Multiplexed DIA (MSX)
100 × 4-m/z-wide windows with 5 random windows combined per scan
B
400.0
412.5
425.0
437.5
450.0
462.5
475.0
487.5
500.0
900.0
Quadrupole-windowed mass range (m/z)
MS1 full scan
Cycle 1
Cycle 2
Cycle 3
Cycle 4
Offset windows
32 × 25 m/z-wide windows staggered by 12.5 m/z
12.5 m/z
C
400
500
600
700
800
900
Quadrupole-windowed mass range (m/z)
Cycle 1
Cycle 2
SONAR
20 m/z isolation window "scanned" by the quadrupole over the mass range of interest
© EMBO

Figure 5. Advanced DIA acquisition schemes with improved precursor selectivity.
(A) Multiplexed DIA (MSX) (Egertson *et al*, 2013) can be used to improve data selectivity by isolating and co-fragmenting at each cycle different non-contiguous precursor mass regions. (B) In the offset-windowed DIA approach, the precursor isolation window boundaries are offset by a discrete mass between consecutive cycles. For example, 25 *m/z* isolation windows get shifted by ± 12.5 *m/z* in each cycle. (C) In the "scanning quadrupole" isolation approach, termed SONAR (Moseley *et al*, 2018), the instrument continuously scans a wide precursor isolation window through the entire precursor mass range of interest, for example by scanning a mass range from 500 to 900 *m/z* using 200 × 20 *m/z* wide windows swiped with an 2 *m/z* increment.

forth between consecutive cycles in a way that the isolation windows overlap by a significant proportion (e.g. 25 *m/z* isolation windows overlapping by 12.5 *m/z*; Fig 5B). Similarly to MSX, the offset acquisition scheme results in differential precursor ion co-isolation and co-fragmentation in every second cycle. Therefore, interferences that originate from species co-selected in the differently overlapping part of the isolation window can be partitioned away and mathematically removed. This approach shows improved selectivity and lower limit of quantification compared to the standard non-overlapping approach, without extra cost related to cycle time, accumulation time, covered mass range or resolving power (https://skyline.gs.washington.edu/labkey/files/home/software/Skyline/2013-ASMS-Overlapped-DIA.pdf). However, the method still requires the use of an automated algorithm which pre-empts easy direct raw data inspection or direct raw fragment ion extraction prior to data deconvolution. Unlike MSX, this method is also possible on non-trapping instruments such as Q-TOFs.

Scanning quadrupole isolation The latest advancement of SWATH-MS was recently published under the name "SONAR" (Waters) by Moseley *et al* (2018). Instead of "stepping" through consecutive isolation windows cyclically one after the other, the quadrupole constantly "scans", for example, a 24 *m/z* wide isolation window through the entire precursor mass range of interest (Fig 5C). In other words, while with the original SWATH-MS method, a given precursor will conceptually be present in only one isolation window and absent from all the others, with the "scanning windows" one can see fragment ion signals appearing and disappearing accordingly to the precursor's isotopes entrance and exit from the scanning window. This essentially adds a fourth dimension on top of retention time, mass and intensity to the SWATH-MS data. The method will require significant modifications of the existing peptide-centric analysis tools, but intuitively, this extra dimension will allow to access another level of selectivity.

Peptide-centric scoring of SWATH-MS data

Over the past 20 years, a wide variety of strategies have been developed to analyse DIA data. These strategies can be classified into two groups, depending on whether they are based on querying the data in a spectrum-centric or in a peptide-centric manner (Ting *et al*, 2015). In this tutorial, we focus on the chromatogram-based peptide-centric scoring analysis workflow for SWATH-MS data illustrated in Fig 6 and discuss specific considerations for each step. For alternative analysis strategies, we refer to recent reviews (Chapman *et al*, 2014; Bilbao *et al*, 2015).

Several software tools have been developed that support all or most steps of the workflow shown in Fig 6. These include for example OpenSWATH (Rost *et al*, 2014) (part of OpenMS, Rost *et al*, 2016b; Sturm *et al*, 2008), Skyline (MacLean *et al*, 2010), PeakView (SCIEX) and Spectronaut (Bruderer *et al*, 2015) (Biognosys). A recent comparative study (Navarro *et al*, 2016) using a ground truth data set has demonstrated remarkable agreement for the qualitative and quantitative results produced by those peptide-centric scoring tools as well as by the spectrum-centric scoring algorithm DIA-Umpire (Tsou *et al*, 2015) that was also included in the study.

Peptide query parameter (PQP) definition

The first step in a peptide-centric analysis is the definition of PQPs. While recently developed algorithms can score data without the need for empirically derived PQPs (Tsou *et al*, 2015; Ting *et al*, 2017), and thereby make the availability of prior knowledge in form of spectral libraries obsolete, empirical PQPs derived from experimental fragment ion spectra can still provide higher sensitivity, especially when working with rather large precursor isolation windows (> 5 *m/z*) during data acquisition. Depending on the sample complexity, several high-quality peak groups can be found for a given queried peptide. To quantitatively describe the frequency of this phenomenon, decoy peptides are used, which are expected to closely model the properties of the target peptides in terms of their chromatographic and mass spectrometric parameter space, but which should not be present in the sample. The decoy PQPs are generated *in silico* from the target peptide list by reversing or shuffling the amino acid sequence (Fig 6A). Decoy peptides are crucial for controlling the error rate of peptide detection and protein inference in SWATH-MS data, because they can be used to estimate the effect of random co-elution of fragment ion chromatograms in the underlying data (Rost *et al*, 2014). It is generally recommended to use the same number of fragment ions per peptide for both targets and decoys to not introduce any biases during peak scoring, because peptide queries with six co-eluting fragment ions can have different score distributions than queries with only three fragment ions.

Chromatogram extraction

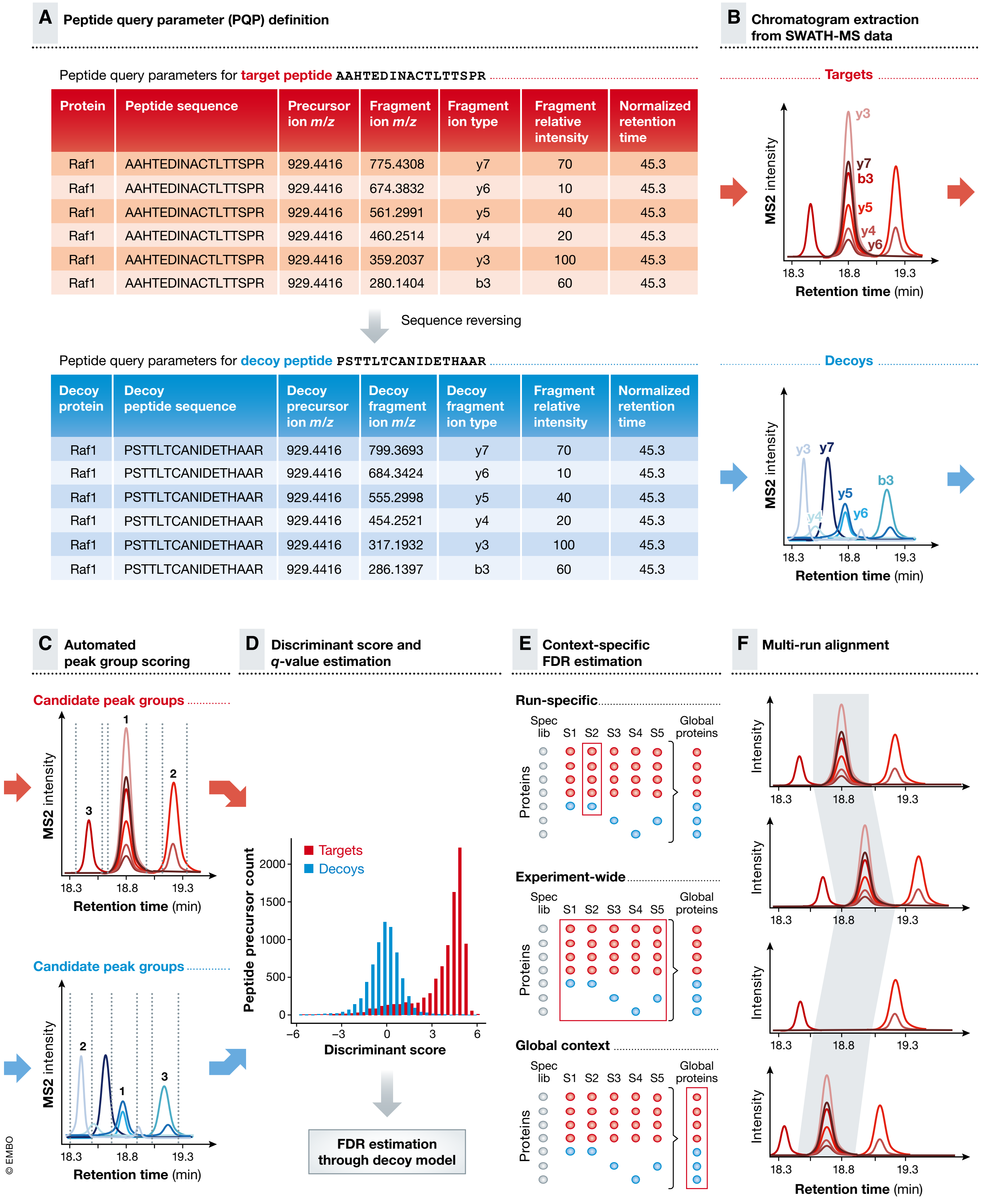
The next step of a peptide-centric data analysis strategy is to use the defined PQPs to extract precursor and fragment ion chromatograms for the peptides of interest. As a peptide elutes from the column, the signal intensity of its queried fragment ions will vary synchronously following a usually Gaussian-like peptide elution profile, forming a chromatographic "peak group" that will be used to assess the peptide detection and to estimate its quantity (Fig 6B). Fragment ion chromatograms are extracted in the same manner for the target and decoy peptides. In essence, this chromatogram extraction step transforms, and thus reduces the multiplexed SWATH-MS2 spectra, to a data structure highly similar to SRM or PRM data.

To substantially improve the selectivity of peptide-centric SWATH-MS data analysis, the chromatogram extraction is usually not conducted throughout the whole chromatographic gradient length but only in a retention time window centred around the expected elution time for the queried peptide (similar to the scheduled SRM strategy). A set of shared endogenous (Parker *et al*, 2015b; Bruderer *et al*, 2016) or synthetic spike-in peptides (Escher *et al*, 2012) can be used to mediate transformation of the previously empirically determined elution times and to restrict the size of retention time extraction window as much as possible. The specific elution times of such reference peptides serve as beacons to define a normalized retention time (such as in the iRT approach; Escher *et al*, 2012) to which the elution times of query peptides can be aligned. The functionality to automatically perform a normalized indexed retention time alignment is implemented in many popular SWATH-MS data analysis tools (e.g. OpenSWATH, Skyline, PeakView and Spectronaut).

The mass tolerance or width of the ion extraction also directly impacts the selectivity of the chromatographic trace signals and thus the derived identification scores and abundance estimates. In general, an extraction width of half of the mass spectrometric peak width provides a good compromise between maximising the fraction

Figure 6. Principle of peptide-centric scoring of SWATH-MS data.
(A) Peptide-centric scoring begins with a set of peptide query parameters (PQPs), which represent retention time, precursor ion masses, fragment ion masses and fragment ion signal intensity coordinates for the target peptides (red table). PQPs are also required for decoy peptides and are generated, for example, by reversing the amino acid sequence of target peptides, while keeping the terminal amino acid (blue table). Decoy peptides are used to assess the chance that peptides which are expected to be absent in the sample may also be detected by chance. (B) Extracted ion chromatograms (XICs) are generated based on PQPs from the continuously acquired SWATH-MS2 spectra for target and decoy peptides. This results in a transformed and reduced data structure similar to data generated by targeted proteomics (SRM or PRM). (C) Fragment ion chromatograms are grouped according to their peptide association and "peak groups" with defined peak boundaries in the retention time dimension are selected. (D) For both target and decoy peak groups, a range of chromatogram- and spectrum-based scores are computed and combined to a discriminant score by a semi-supervised learning approach. The false discovery rate (FDR) of a set of detected peptides can be estimated by statistical modelling of the score distributions of target and decoy peptides. (E) For large-scale SWATH-MS analyses, error rate control should not only be performed on the peptide level, but should be extended to the protein level. Further, in large experiments including many samples, it might not be sufficient to conduct error rate control individually per run ("run-specific" context), but better on an "experiment-wide" scale. The "global" context considers only the best scoring detected peak groups, peptides or inferred proteins over all runs. (F) A multi-run alignment allows to correct or reinforce confidence in peak detection by leveraging the chromatographic time consistency and transfer of detection confidence across runs.

▶



Peptide query parameters for target peptide AAHTEDINACTLTTSPR

Protein	Peptide sequence	Precursor ion *m/z*	Fragment ion *m/z*	Fragment ion type	Fragment relative intensity	Normalized retention time
Raf1	AAHTEDINACTLTTSPR	929.4416	775.4308	y7	70	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	674.3832	y6	10	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	561.2991	y5	40	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	460.2514	y4	20	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	359.2037	y3	100	45.3
Raf1	AAHTEDINACTLTTSPR	929.4416	280.1404	b3	60	45.3

Peptide query parameters for decoy peptide PSTTLTCANIDETHAAR

Decoy protein	Decoy peptide sequence	Decoy precursor ion *m/z*	Decoy fragment ion *m/z*	Decoy fragment ion type	Fragment relative intensity	Normalized retention time
Raf1	PSTTLTCANIDETHAAR	929.4416	799.3693	y7	70	45.3
Raf1	PSTTLTCANIDETHAAR	929.4416	684.3424	y6	10	45.3
Raf1	PSTTLTCANIDETHAAR	929.4416	555.2998	y5	40	45.3
Raf1	PSTTLTCANIDETHAAR	929.4416	454.2521	y4	20	45.3
Raf1	PSTTLTCANIDETHAAR	929.4416	317.1932	y3	100	45.3
Raf1	PSTTLTCANIDETHAAR	929.4416	286.1397	b3	60	45.3

Figure 6.

of recovered ion signals while maintaining a reasonable selectivity of the extracted data. In practice, we suggest applying an extraction width of ~50 ppm or less for MS2 measurements performed with a resolution of 15,000.

Chromatographic extraction can be performed on profile (raw) or centroided MS2 data level. Centroided MS2 data are significantly smaller in size and were shown to perform similarly to profile data in terms of identification depth and quantification accuracy (Navarro *et al*, 2016). The optimal extraction window width for centroided data may be less than that of the same data extracted in profile mode, because in this case the extraction window width can be optimized with respect to the mass error distribution. However special attention should be given to the choice of centroiding algorithm used. We have observed that they can dramatically affect the data in terms of mass accuracy, signal-to-noise levels and relative fragment ion intensity (e.g. depending on whether the centroiding is done on peak height or peak area) (Toprak *et al*, 2014). Accurate centroiding is especially challenging for SWATH-MS data due to the high level of co-fragmenting peptides and the high level of overlapping fragment ion signals in the MS2 spectra. Such algorithm-specific artefacts will affect the scoring of the fragment ion peak groups as well as the statistics of peptide detection. It is thus recommended to follow the instrument vendor and algorithm developer instructions for the choice of centroiding algorithm.

Finally, if the SWATH-MS data acquisition scheme includes MS1 scans within a chromatography-compatible cycle time, precursor ion chromatograms can also be extracted from the MS1 data and scored in combination with the fragment ion peak group to enhance confidence in the peptide detection.

Automated peak group scoring

In the next step of the workflow, an algorithm defines the left and right borders of one or several potential chromatographic peak groups and scores each of these candidate peptide signals individually (Fig 6C). This is done independently for the extracted target as well as decoy chromatographic traces. During this scoring process, a variety of individual scores is computed (Reiter *et al*, 2011). Those can be categorized into five types: (i) scores related to chromatographic performance, (ii) scores correlating the measured data to the information from the spectral library, (iii) scores correlating the data to external or internal isotope-labelled standards (if available), (iv) scores taking into account the high resolution and accurate mass of the MS2 scans and (v) scores that exploit information from the precursor ion (MS1) scans. All or most of those individual scores have been implemented in one way or another into the SWATH-MS software tools OpenSWATH, Skyline, PeakView and Spectronaut, which all automatically combine them into a final discriminant score.

Discriminant score and q-value estimation

By applying the individual scores described before to extracted ion chromatograms of target and decoy peptides from SWATH-MS data, individual score distributions can be computed. Ideally, in these distributions, “true positive” target and “false positive” decoy peptides are clearly separable. However, depending on experiment-specific factors such as sample complexity, instrument performance and SWATH-MS method setup, some scores might perform better than others, while a single individual score is generally not discriminative enough to ascertain the detection and correct quantification of a given peptide. Therefore, it is beneficial to combine the individual scores into a single discriminant score in a way that allows the most sensitive recall of the peptide queries (Fig 6D). This goal can be achieved by using a semi-supervised learning algorithm (Kall *et al*, 2007) that converges iteratively towards the most optimal set of weighted individual score combinations to separate targets and decoys. The peak group with the highest discriminant score (rank 1, Fig 6C) is then usually considered as the most likely detected peak group for a set of queried peptides, which may eventually be revised upon across-run alignment.

Several requirements need to be fulfilled in order to allow successful learning and proper statistical modelling of SWATH-MS data. First, a sufficient number of high scoring true positive peptides are necessary for the semi-supervised algorithm to learn the scoring characteristics of true detection events and to achieve a sensible combination of individual weight-feature scores. Second, the decoy peptides must appropriately represent peptides that are not detectable in the sample.

In general, we highly recommend to inspect target and decoy score distribution plots that most SWATH-MS analysis tools report to verify that (i) assumptions made with regard to the decoy distributions (e.g. normality) are not violated, (ii) the target scores appear approximately as a bimodal distribution when analysing complex samples with medium to large spectral libraries and (iii) the decoy distribution matches the true-negative part of the bimodal target distribution both in terms of apex, shape and width (Fig 6D). In scenarios, where the spectral library overlaps to a very high degree (> 90%) with the detected precursor ions of the sample, the true-negative target distribution might be very small, thus rendering criterion (iii) difficult to judge (Reiter *et al*, 2011). If one or more of those conditions are not fulfilled, it is likely that either the machine learning or the q-value estimation step has failed or are biased, which could result in misleading statistics and inappropriate error rate control.

Context-specific error rate estimation

The above described strategies are commonly applied to individual runs separately. However, under certain circumstances, this might introduce biases, especially when large numbers of peptides are queried across data sets consisting of a large number of heterogeneous samples, or when spectral libraries are used that were assembled from different sample types (e.g. obtained from public repositories) or contain a large number of true-negative peptides and proteins for the tested samples. We therefore suggest to adapt the analysis strategy according to requirements of the specific data set as described recently by Rosenberger *et al* (2017a). Briefly, to ensure comparable scoring and statistics throughout the complete data set, the semi-supervised machine learning step should be conducted in an experiment-wide fashion on all the candidate peak groups for all runs at once (Fig 6E). For practical reasons (file size and memory restrictions), this step can also be performed on a representative selection of peptide queries randomly subsampled through each sample of the data set. Then, the same score weights are applied to all the candidate peak groups throughout all the runs of the data set (Rosenberger *et al*, 2017a).

While a 1% peptide FDR threshold on a per run basis (referred to as “run-specific” context) might be sufficient for small sample sizes analysed with sample-specific libraries, accumulation of false positives will occur when conducting large numbers of queries across large sample sets. Similar as for DDA database search strategies, more stringent score cut-offs become necessary on large data sets to account for multiple hypothesis testing. We therefore recommend to also use target and decoy score distributions to conduct error rate control at the protein level in addition to the peptide level (Rosenberger *et al*, 2017a). Further, we suggest assessing the FDR in an "experiment-wide" context where q-values are estimated over all samples using a single score distribution, as well as in a "global" context, where only the best scoring instance of a given peptide or inferred protein from all samples in the experiment is used. We recommend that the list of peptide queries and inferred proteins identified in the "global" context at 1% FDR can be used to filter the resulting data from the "experiment-wide" context to prevent accumulation of false positives in experiments with many samples (Fig 6E; Rosenberger *et al*, 2017a). The above described context-specific error estimation has been implemented in PyProphet (Rosenberger *et al*, 2017a), a component of the OpenSWATH workflow, and Spectronaut (Bruderer *et al*, 2017).

Across-run alignment and transfer of identification confidence

A main assumption of peptide-centric scoring is that only the best scoring peak group per peptide represents the true peptide signal. This may however not always be the case, for example when the peptide abundance drops below the limit of detection in one or several runs of a sample cohort and a candidate peak group that does not originate from the target peptide achieves a better score. Frequently, this wrong peak group is detected at a different retention time than the true peptide, resulting in a false value in the final peptide quantification matrix. By re-emphasizing the consistency of the retention time information across samples and aligning the peak boundaries of the peak group from the run where the peptide was most confidently identified, it is possible to detect and amend those false peak group ranking mistakes (Fig 6F). This process sometimes re-ranks the peak groups or may rescue some peak groups at the expected retention time that would otherwise not have passed the 1% peptide FDR threshold in those specific runs. An algorithm for multi-run retention time alignment is TRIC (TRansfer of Identification Confidence), a component of the OpenSWATH workflow (Rost *et al*, 2016a).

In general, multi-run alignment is similar to what has already been developed and applied successfully for the analysis of DDA data (Prakash *et al*, 2006; Mueller *et al*, 2007; Cox *et al*, 2014). However, while in DDA peptide identification and quantification are often inferred across runs based solely on retention time and precursor *m/z*, in SWATH-MS the full information of all fragment ions can be used as part of the alignment strategy and to control the error rate.

Peptide and protein quantification

Just like any other bottom-up LC-MS/MS proteomic methods, SWATH-MS provides quantification data on peptide level and peptide quantities are typically computed by summing or averaging the integrated peak area of several fragment ions. It is not necessary to use all of the queried fragment ions per peptide to perform accurate quantification. Rather only high-quality and interference-free fragment traces should be selected and for such a task automated algorithms have been developed (Keller *et al*, 2015; Teleman *et al*, 2015). Additionally, MS1-based quantification on the precursor extraction ion chromatograms may provide orthogonal quantification information (Rardin *et al*, 2015). In many instances, however, the lower limit of quantification (LLOQ) appears to be more strongly impaired at the precursor than at the fragment ion level (Gillet *et al*, 2012; Egertson *et al*, 2013; Collins *et al*, 2017). This is due to the lower selectivity and the higher dynamic range of detection required in full MS1 scans compared to that of windowed-isolation MS2 scans.

To infer the abundance of a protein from a SWATH-MS measurement, the measured intensities of one or many peptides need to be aggregated into a final protein intensity value per sample. For this purpose, various strategies have been developed, for example (i) summing or averaging the n-most intense peptides per protein (Silva *et al*, 2006; Ludwig *et al*, 2012), (ii) summing up all peptide intensities per sample, regardless of whether they occur across samples or not (iBAQ; Schwanhausser *et al*, 2011), or (iii) considering only those peptides per protein that occur in the two samples to be compared (LFQ; Cox *et al*, 2014). Optionally, those aggregated protein intensity values can be further normalized by a factor representative of the detectability of that protein, for example protein length (Zybailov *et al*, 2006), number of theoretical peptides generated (Schwanhausser *et al*, 2011) or a computed peptide detection probability score (Lu *et al*, 2007). Missing values can be either recovered using the re-quantification values provided from the across-run re-alignment analysis (Rost *et al*, 2016a) or using statistical imputation as provided by other tools (Karpievitch *et al*, 2012). Compared to SRM or PRM, which commonly only use a few measured targeted peptides for protein inference, and to DDA, which may not identify and quantify the same peptides per protein through large sample sets, SWATH-MS peptide-centric analysis intrinsically offers a more consistent and complete choice in peptides for protein quantification.

Several statistical tools have been developed to assess differential peptide or protein abundance between experimental groups and to compute the relative fold change in peptide or protein quantification starting from fragment ion level data (Chang *et al*, 2012). For most consistent relative protein quantification comparisons, we recommend to systematically use the same, and throughout the complete SWATH-MS data set the most robust, fragments and peptides per protein. For the selection of these fragments and peptides, information such as conservation of the relative fragment ion signal intensities per peptide and the relative peptide intensities per protein can be leveraged across the whole data set, as MSstats (Choi *et al*, 2014) or mapDIA (Teo *et al*, 2015) do for fold-change statistics computation.

Post-translational modifications

SWATH-MS peptide-centric scoring is directly compatible with measuring peptides carrying post-translational modifications (PTMs). However, analysis of modified peptides by SWATH-MS presents additional challenges that stem from the often high similarity of MS2 spectra arising from related peptide species, including the non-modified peptide, modified peptides with the same backbone carrying the same modification at different sites or peptides with the same backbone carrying (near) isobaric modifications. Such peptides may be co-isolated in the same SWATH-MS window and produce multiple high scoring peak groups.

As in DDA analysis, specific strategies have been developed to confidently identify or detect modified peptides in DIA and

SWATH-MS data sets. The most straightforward way is to directly use PQPs of the modified peptide versions, for example from spectral libraries that were acquired from DDA analyses of PTM-enriched samples or with open modification search strategies (Na & Paek, 2015). An alternative strategy relies on using non-modified PQPs to query for modified peptides and focuses on additional high scoring peak groups. Those additional peak groups may be detected in the same isolation window as the non-modified peptides or in other isolation windows. Such strategies have been implemented in MSPLIT-DIA (Wang *et al*, 2015) and SWATHProphet$^{\mathrm{PTM}}$ (Keller *et al*, 2016) and were shown to work most successfully when the peptide fragmentation shows enough similarity up to the site of the modification (Toprak *et al*, 2014).

A recently introduced algorithm termed "Inference of PeptidoForms" (IPF) (Rosenberger *et al*, 2017b) extends the standard OpenSWATH workflow to modified peptides. IPF uses a two-stage workflow in which the first pass resembles a standard OpenSWATH analysis whereby typically, about six fragment ions from DDA- or DIA-derived spectral libraries are extracted and scored to detect high-quality peak groups. These transitions need to be specific for the targeted peptide sequence and types and numbers of modifications; however, they do not need to be specific for positional isomers. In a second step, IPF uses XICs extracted both for precursor signals in MS1 and theoretically predicted fragment ions that can differentiate positional isomers. This information is then integrated using a Bayesian hierarchical model leading to a single peptidoform confidence score for each detected peak group. The site localization is thus independently reassessed, even if the underlying peptidoform is not present in the spectral library.

In summary, PTM-SWATH analyses are applicable to total cellular lysates as well as to samples enriched for specific types of modifications, such as phospho-enriched samples. In recent years, several different PTM-types have been successfully studied with SWATH-MS (Krautkramer *et al*, 2015; Sidoli *et al*, 2015; Lawrence *et al*, 2016; Rosenberger *et al*, 2017b). Particularly in experiments involving many experimental conditions or replicates, similar improvements in terms of consistent detection and quantification can be expected for modified and unmodified peptides.

Outlook

Since the first implementations of DIA in the early 2000s (Masselon *et al*, 2000; Purvine *et al*, 2003; Venable *et al*, 2004), numerous improvements in terms of data acquisition speed, mass accuracy and resolution have taken place on the instrument side. Also, since the first description of a peptide-centric data analysis workflow (Gillet *et al*, 2012), substantial progress has been achieved in terms of spectral library generation (Schubert *et al*, 2015a), automated data analysis pipelines (Rost *et al*, 2014, 2016a; Keller *et al*, 2015) and statistical control in peptide and protein error rates (Rosenberger *et al*, 2017a). The excellent reproducibility and accuracy of SWATH-MS data acquisition have been proven in a large study comparing data acquired in different laboratories worldwide (Collins *et al*, 2017). Also, the robustness of peptide-centric data analysis tools has been demonstrated recently by comparing five major software tools currently used for peptide-centric scoring of SWATH-MS data (Navarro *et al*, 2016). The development of SWATH-MS laid the foundation for novel large-scale biological studies, such as the study of mitochondrial links to liver metabolism in a mouse reference population (Williams *et al*, 2016), the study of phosphoproteomic and chromatin signature in response to drug treatments in cancer cell lines (preprint: Litichevskiy *et al*, 2018) or the contribution of heritability and environment to different traits in pairs of monozygotic and dizygotic twins (Liu *et al*, 2015). Throughout this tutorial, we have documented the advancements and referenced established workflows, software tools and analysis pipelines that will hopefully support and facilitate SWATH-MS experiments by a large user community in the years to come.

Although most published SWATH-MS studies to date used spectral libraries generated by DDA, novel approaches that do not rely on libraries were developed. Spectrum-centric scoring approaches such as DIA-Umpire (Tsou *et al*, 2015) or Group-DIA (Li *et al*, 2015) generate pseudo-MS2 spectra directly from DIA data and subject them to conventional database search algorithms. Peptide-centric library-free analysis tools such as FT-ARM (Weisbrod *et al*, 2012) or PECAN (Ting *et al*, 2017) use theoretical fragment ion predictions to query and score the multiplexed MS2 spectra of SWATH-MS data. Interestingly, tools that rely on prior knowledge in the form of spectral libraries seem to deal better with lower selectivity data than library-free tools (Navarro *et al*, 2016; Ting *et al*, 2017). This finding suggests that, as measurement selectivity improves with better instruments (smaller precursor isolation windows, higher peak capacity chromatography, scanning quadrupoles, ion mobility separations, etc.), prior knowledge for data analysis might become less important. On the other hand, prior knowledge in the form of organism-wide deep proteome data sets from endogenous samples or synthetic proteomics is likely to increase substantially during the same time period. This might spur the development of novel analysis methods that can better leverage such libraries.

In the future, we expect that mass spectrometers will continue to improve in sensitivity and scanning speed. Hence, it is foreseeable that the time necessary to acquire high-quality precursor and fragment ion spectra will decrease, allowing for higher number of narrower isolation windows and enabling to approach the 1–3 *m/z* isolation width commonly used during DDA acquisition and PRM. We speculate that increasing instrument sensitivity and speed will reach a threshold where ultimately the benefits of all currently employed data acquisition methods can be achieved with a single "super" method. The question then will no longer be whether one should do a DDA, DIA or targeted experiment, but whether to use a peptide-centric or spectrum-centric data analysis strategy. It seems unlikely that a purely spectrum-centric approach, where each consecutive MS2 scan is subjected to a database search algorithm independently, would be most effective, because the rich information in the chromatographic dimension and prior information about the fragmentation pattern would not be leveraged. As such, it seems more likely that either peptide-centric approaches as described in detail in this tutorial or hybrid methods between spectrum- and peptide-centric analyses best exploit the highly comprehensive and selective data sets in the future.

Other future-oriented features of DIA in general, and SWATH-MS in particular, are the simplicity of the underlying data acquisition method and the suitability for high-throughput proteomics. Already today ~50% of the MS-detectable proteome can be reproducibly measured by SWATH-MS at a relatively fast time scale (< 1 h per sample). This progress will hopefully allow to further democratize access to proteomics data in the future and to perform novel types of cross study comparisons, which are goals that the proteomics community had difficulties achieving in the past.

Expanded View for this article is available online.

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Conflict of interest

The authors declare that they have no conflict of interest. RA is a shareholder in the company Biognosys which operates in the field of research covered by this article.

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