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ACCELERATED ARTICLE

New targeted approaches for the quantification of data-independent acquisition mass spectrometry

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The use of data-independent acquisition (DIA) approaches for the reproducible and precise quantification of complex protein samples has increased in the last years. The protein information arising from DIA analysis is stored in digital protein maps (DIA maps) that can be interrogated in a targeted way by using ad hoc or publically available peptide spectral libraries generated on the same sample species as for the generation of the DIA maps. The restricted availability of certain difficult-to-obtain human tissues (i.e., brain) together with the caveats of using spectral libraries generated under variable experimental conditions limits the potential of DIA. Therefore, DIA workflows would benefit from high-quality and extended spectral libraries that could be generated without the need of using valuable samples for library production. We describe here two new targeted approaches, using either classical data-dependent acquisition repositories (not specifically built for DIA) or ad hoc mouse spectral libraries, which enable the profiling of human brain DIA data set. The comparison of our results to both the most extended publically available human spectral library and to a state-of-the-art untargeted method supports the use of these new strategies to improve future DIA profiling efforts.

Keywords:
Data-independent acquisition / Spectral libraries

Additional supporting information may be found in the online version of this article at the publisher's web-site

The changes in the proteome underlying the regulation of biological processes is of major interest in biomedical research and relies on the ability of proteomic methods to generate quantitative, reproducible, and comprehensive data sets. To date, data-dependent acquisition (DDA) mass spectrometry (MS) methods have been extensively used. In DDA, a subset of peptide precursors is selected from a survey scan (MS1) for subsequent fragmentation and acquisition of a fragment-ion spectrum (MS2). Their identification together with the quantitative information results in the discovery of differences in protein expression. However, the selection of

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Abbreviations: **DDA**, data-dependent acquisition; **ddHI**, dd human library; **DIA**, data-independent acquisition; **ddMdHI**, DDA-rpository (dd) mouse-derived human library; **MdHI**, mouse-derived human library; **PFC**, prefrontal cortex; **PHI**, pan-human spectral library; **RT**, retention time; **SC**, spinal cord

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Significance of the study

A lot of interest has been generated around DIA methods applied to discovery-based proteomics, as they promise to overcome the well-known stochastic nature of classical DDA schemes. However, new analytical solutions are needed in order to increase the analyzed landscape of the DIA maps. In this study, we present a targeted analytical strategy to increase the quantitative information recovered from human DIA maps by using either the extended sources stored in classical DDA repositories or ad hoc mouse spectral libraries. To the best of our knowledge, this is the first demonstration of the possibility to unlock years of DDA research for the analysis of DIA data sets, which we expect will have an important impact in the growing field of DIA proteomics.

peptide precursors is stochastic favoring the collection of high-abundant/intense peptides. As a result, roughly 27% of all detectable peptides can be identified in a single run [1, 2], and 30% of the selected peptides can vary between replicates [3]. As an alternative approach, pioneer work [4–8] showed the potential of data-independent acquisition (DIA) methods where all peptide ions (in a given m/z range and above the detection limit of the LC-MS instrument) of a complex sample are fragmented independently of their intensity, theoretically enabling the identification and quantification of all peptide precursors. However, DIA-MS2 spectra stored in the form of DIA maps are composed of fragmented ions belonging to different peptide precursors. This chimeric nature of the MS2 spectra is a bottleneck for identification using classical database search strategies.

The most widely used analytical solution of DIA methods is the use of reference spectral libraries for the targeted extraction of quantitative information of the peptides included in these libraries [9] using tools such as Spectronaut, OpenSWATH, Skyline, or PeakView. While this type of targeted approaches reaches excellent reproducibility [10], the analysis is restricted to a subset of the comprehensive peptide information stored in DIA maps because reference spectral libraries are limited in their size as they are generated by DDA proteomics on the same sample species. Public repositories containing species-specific spectral libraries (i.e., peptide atlas) aim to solve this bottleneck; however, the limited availability of certain tissue samples together with the caveats of using spectral libraries generated under variable experimental conditions [11, 12] prevents DIA to achieve its full potential.

Based on the correspondence between the peptide fragmentation pattern and the peptide sequence [13], we sought to improve the targeted analysis of human DIA maps by developing two novel and complementary strategies. On the one hand, we used classical DDA repositories as means of human reference spectral libraries. On the other hand, we generated ad hoc spectral libraries using mouse tissues, a widely available and closely related animal model. Our results demonstrate our approaches having an improved performance in analyzing human DIA maps when compared to the most extended pan-human spectral library (PHl) [14] and to a recent untargeted approach [15].

First, we isolated regions of spinal cord (SC) and prefrontal cortex (PFC) from human and mouse (Fig. 1A). Our selection was guided by the important roles of these tissues in human pathophysiology and their low availability compared to other frequently used human matrices (e.g., plasma). Following, we constructed extended mouse and human ad hoc libraries (Ml and Hl, respectively) using a combined strategy. In order to reach the most comprehensive coverage possible of the expressed proteome, we use multiple injections and long chromatography gradients, as well as three tissue fractions for each of the tissues: whole cell, membrane enriched, and cytosol enriched (Supporting Information Figs. 1 and 2). Finally, we created a mouse-derived human library (MdHl) by selecting only peptides displaying 100% identity with their human counterparts as annotated in Uniprot (of the 43 956 mouse peptides 26 953 overlap with human peptides).

As an additional strategy, we aimed to construct high-quality spectral libraries using the currently most extended data sets from classical DDA repositories of mouse and human cortex [16–18]. This relies on normalized retention times (RTs) and on the ability to predict accurate RTs for peptides detected in different experimental conditions. For that, we applied a high-precision iRT algorithm [11] to all peptides from the mentioned data sets that resulted in two DDA-repository (dd) spectral libraries: a dd mouse-derived human library (ddMdHl) and a dd human library (ddHl).

Comparison of the four generated libraries with PHl revealed a significant amount of newly incorporated proteins (Fig. 1B and Supporting Information Table 1). A closer look revealed that 223 proteins of the Hl are not present in the PHl but are included in the three other libraries of which a significant amount are involved in physiological functions assumed by PFC and SC (e.g., transmission of nerve impulse; Supporting Information Fig. 3).

Next, we evaluated the ability of the generated libraries to profile differences in protein expression in human tissues. To this end, we obtained DIA maps from three biological replicates of whole cell lysates of human SC and PFC (Fig. 1). Our results indicate that Hl profiled the highest number of unique peptides and proteins in both tissues and in all of the three biological replicates (Fig. 2, Supporting Information Table 2 and Supporting Information Fig. 4A–C), including also the highest number of peptides representing

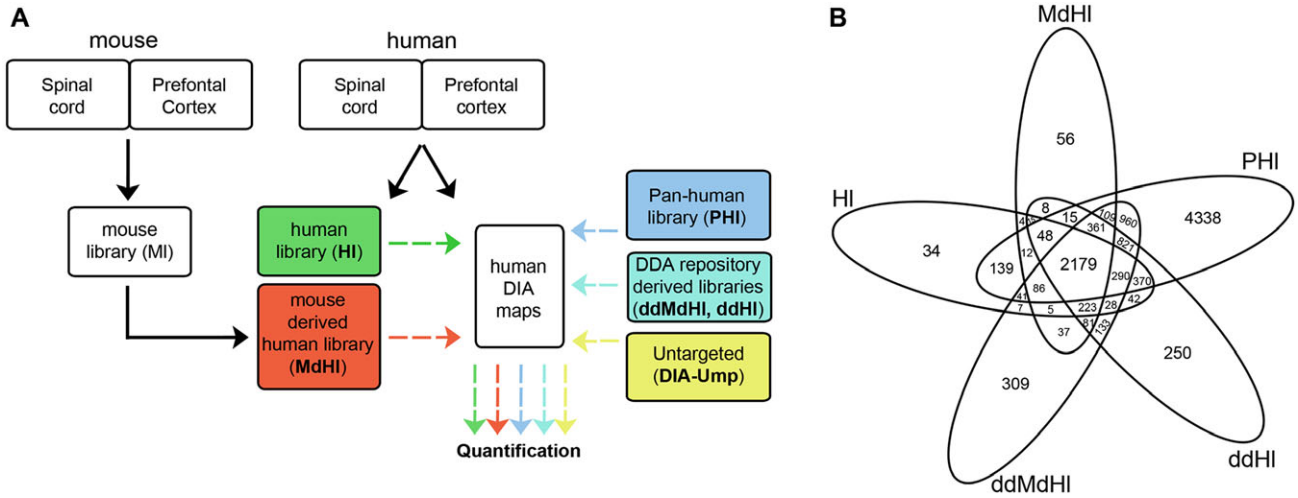

Figure 1. Experimental workflow. (A) *Ad hoc* and DDA-repository-derived (dd) human- (H) and mouse-derived (Md) peptide spectral libraries (HI, MdHI, ddHI, and ddMdHI, respectively) were used for the quantification of DIA maps (human DIA maps) obtained from human PFC and SC. Their performance was compared to the pan-human peptide spectral library (PHI) and to DIA-Umpire (DIA-Ump). (B) Venn diagram showing the number of proteins in each of the five libraries.

each protein (Supporting Information Fig. 4D). Importantly, ddHl, ddMdHl, and MdHl outperformed PHl (114% more peptides for ddHl in PFC; Fig. 2 and Supporting Information Table 2) with comparable coefficients of variation (Supporting Information Fig. 5). The improvements shown here with interspecies resources (mouse) differ conceptually from previously published DDA database search strategies [19] as our approach does not rely on modifying the size of the database search space or on modifying the DIA map search space in order to influence the probability of positive peptide identification.

Our data partially confirm but also demonstrate the potential of the strategy to overcome limitations when aiming to use spectral data obtained in different experimental conditions (i.e., different mass spectrometers that tend to produce different charge states of a given peptide). A closer look at the results show that the MdHl (generated using the same QExactive mass spectrometer as for the DIA maps) was able to extract complete quantitative information for 893 proteins more than the PHl (generated using a TripleTOF 5600+ mass spectrometer) in human PFC (Supporting Information Table 2), although 506 of those proteins were also represented in the PHl. A deeper analysis revealed that 56% of these proteins were represented by different peptides in the PHl spectral library, whereas in the remaining 44% different charge states, RT and fragment ion selections were apparent. These differences go in line with the reported differences in spectral transferability between different instruments [20]. On the other hand, we believe that the tissue similarity plays an important role: ddHl that is derived from the analysis of human frontal cortex (Supporting Information Table 1) is able to extract information of 32 more proteins than MdHl (Supporting Information Table 2), although the latter library was produced using exactly the same instrumentation as for the human DIA data sets. Further, ddHl extracted 925 proteins more than PHl (Supporting Information Table 2) that did not include any brain tissue for its generation [14]. In summary, our results show that although ad hoc in-house spectral

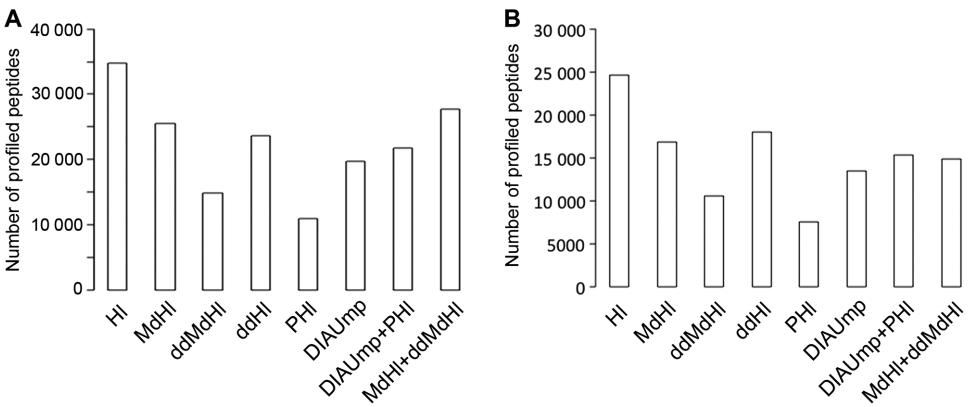

Figure 2. Performance of each library in human PFC and SC. (A) Number of peptides profiled by each of the spectral libraries in the human PFC DIA data set and (B) in the human SC DIA data set.

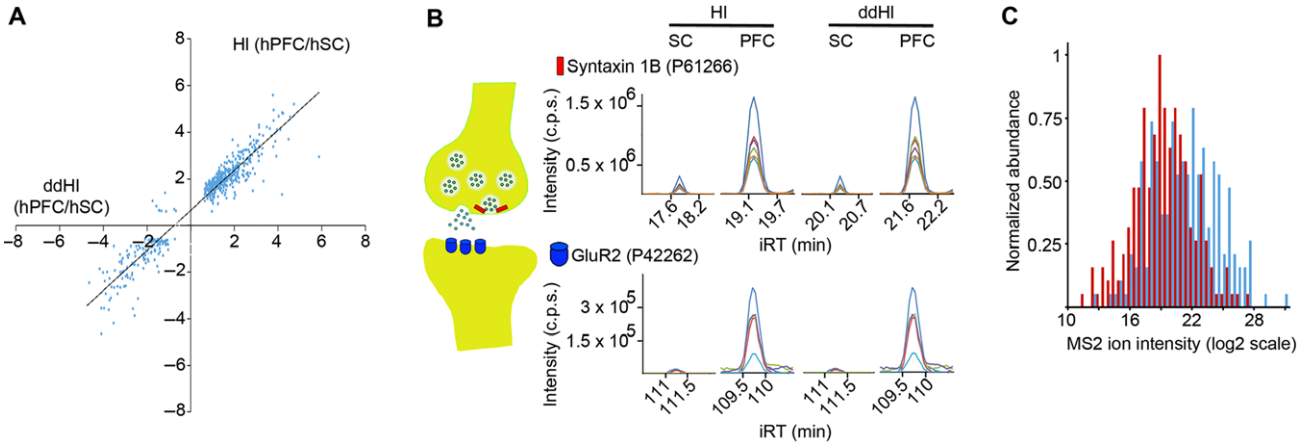

Figure 3. Quantification properties of each of the libraries. (A) Ratio of expression (hPFC/hSC) for 525 proteins calculated based on HI and ddHI. Slope of regression 0.86 with a $R^2 = 0.91$. (B) Examples of expression differences between the two human tissues for the presynaptic protein Syntaxin 1B and the postsynaptic protein GluR2 upon use of HI and ddHI. (C) Distribution of the 204 fragment ion intensities identified uniquely by DIA-Umpire (red bars), or 204 randomly selected fragments shared with at least one of the five spectral libraries (blue bars) from the human PFC DIA data set analysis.

libraries, generated from the same species and with the same instrumentation, continue as the most efficient choice, there is a big potential of applying high-precision iRT algorithms [11] to extended data sets obtained from classical DDA repositories (not specifically built for DIA).

Subsequently, we evaluated whether the peptide assays included in all these libraries harbor not only proteotypic but also quantitative properties [21]. As shown in Fig. 3A, the differences in abundance detected by ddHl, in comparison to Hl, showed remarkable consistency—525 proteins were commonly identified with ddHl and Hl to be differentially expressed, including important synaptic proteins (Fig. 3B). Comparable results were obtained for ddMdHl and MdHl (Supporting Information Fig. 6).

Recent computational efforts have been developed to analyze DIA data sets without the need for spectral libraries [15, 22, 23]—referred to as untargeted approaches. Our data show that library-based targeted extraction using Hl, MdHl, and ddHl outperformed the untargeted strategy of DIA-Umpire in terms of number of extracted peptides and proteins (Fig. 2, Supporting Information Table 2 and Supporting Information Figs. 4 and 5). An alternative for the analysis of our human DIA data sets without relying in the use of ad hoc Hl or MdHl would be to use DIA-Umpire to generate a complementary library and combine it with the identifications when using the publically available PHl. Our results indicate that the identifications reached by ddHl or MdHl alone or MdHl grouped with ddMdHl still outperformed a combination of DIA-Umpire and PHl (Fig. 2, Supporting Information Table 2 and Supporting Information Fig. 4).

The detection improvements over DIA-Umpire were comparable to or even bigger than the ones reached by latest untargeted solutions when similar conditions were compared: While MdHl alone and in combination with ddMdHl showed an improvement of 29% and 40% (Supporting Information Table 2), MSSPLIT-DIA [22] showed an approximate 25% improvement using a spectral library generated from the same sample. On the other hand, Group-DIA [23] did not show any improvement in peptide identifications when three DIA runs were considered (a similar number of runs analyzed to construct the DIA maps in our study).

Interestingly, 204 of the peptides detected by DIA-Umpire were not present in any of the five other libraries. Further analysis showed that the intensities of the fragment ions exclusively profiled by DIA-Umpire were significantly lower in comparison to the ones commonly detected with the other five libraries (two sample *t*-test, two-tailed, $P < 0.0001$; similarity of the variance was confirmed using *F* test, $P = 0.13$; Fig. 3C). These results suggest that future integrative strategies would benefit from the combination of targeted and untargeted approaches in order to increase the analysis depth of DIA data sets.

DIA methods are in a prime position for proteomic-based biomarker discovery efforts. Several strategies are under development toward the goal to improve the amount of reliable peptide identifications from DIA maps, including computational [15, 22, 23] and technological efforts [24]. The novel strategies presented here add new possibilities to these efforts that can be easily implemented in future studies, unlocking extended classical DDA repositories [25] for comprehensive profiling of human samples by DIA.

Experimental procedures

Human and mouse tissue

Native, unfixed, snap-frozen CNS tissue of PFC and SC of three nonneurological patients was obtained from the

archives of the Institute of Neuropathology, University Medical Center, Göttingen (UMG), Germany. Postmortem tissue sampled for diagnostic purposes was used for the present study in conformity with the rules and regulations of the ethics committee of the UMG, reference number 6/5/16. Similar regions from three male 12–14 weeks old wild-type C57Bl/6J mice were obtained. No randomization or blinding was used in this study. All mouse experiments are approved by the IACUC of the Max Planck Institute of Experimental Medicine (MPIEM).

Sample preparation for MS acquisition

The frozen tissue was homogenized with help of a glass/Teflon homogenizer in 4% SDS lysis buffer (4% SDS in 100 mM Tris, 10 mM DTT, 5% glycerol, complete protease inhibitor cocktail, Roche, pH 7.5) and by shearing with a 25G needle. The homogenate was incubated for 10 min at 70°C, followed by centrifugation at 10 000 × *g* for 5 min for removal of cell debris at room temperature (note: all centrifugation steps in this study were done at room temperature, except otherwise mentioned). The supernatant equals the whole cell lysate. Following this, acetone precipitation of the proteins was done by the addition of 5× volume precooled acetone (Roth) and incubation for 2 h at –20°C. The precipitated proteins were centrifuged at 14 000 × *g* for 30 min, washed with ice-cold 80% ethanol (AppliedChem), and centrifuged again at 14 000 × *g* for 30 min. The air-dried proteins were resuspended under constant agitation in 2% SDS lysis buffer. For generation of the membrane and cytosolic fractions, the whole cell lysate was subjected to ultracentrifugation at 100 000 × *g* at 18°C for 1 h (TLA 100.3 rotor, Beckman Coulter). The pellet was dissolved in 4% SDS lysis buffer. The supernatant (cytosol) and dissolved pellet (membrane fraction) were subjected to acetone precipitation as described above.

The samples in 2% SDS were prepared for mass spectrometric acquisition using the FASP protocol with 30k Vivacon 500 spinfilters [26] (Sartorius). The peptides were desalted using C18 MacroSpin columns from The Nest Group according to manufacturer's instructions. After drying, the peptides were resuspended in 1% ACN and 0.1% formic acid. The Biognosys' HRM Calibration Kit was added to all of the samples according to manufacturer's instructions (required for the DIA analysis using Biognosys' Spectronaut). All the three biological replicates for each of the four tissues (two in human and two in mouse) were processed in parallel.

Mass spectrometric acquisition

Two micrograms of the samples was analyzed on a self-made analytical column (75 μm × 30 cm) packed with 3 μm Magic C18AQ medium (Bruker) at 50°C, using an Easy-nLC connected to a Q Exactive mass spectrometer (Thermo Scientific). The peptides were separated by a 2 h segmented gradient from 0 to 9% ACN in 3 min, next to 28% ACN in 102 min, to 36% ACN in 12 min, and to 44% in 3 min, with 0.1% formic acid at 300 nL/min. Then, a linear increase followed to 90% ACN in 2 min and 90% ACN was run for 8 min. For DDA acquisition, the "fast" method from Kelstrup [27] was used with the alteration described in Bruderer et al. [28]. In order to produce the most extended spectral libraries possible, we have analyzed ten independent samples for each of the tissues.

The HRM DIA method was performed using one technical replicate for each of the three biological replicates, and as described in Bruderer et al. with the following modification: the survey scan was recorded at 70 000 resolution in order to reach superior data quality through the introduction of MS1-based scores. The raw mass spectrometric DIA data and the spectral libraries were stored at the public repository PeptideAtlas (http://www.peptideatlas. org, No. PASS00782, the username is PASS00782 and the password is NN4585e).

MaxQuant data analysis

The DDA spectra were processed with the MaxQuant Version 1.5.1.2 analysis software [29]. The spectra were searched by the Andromeda search engine [30]. The minimal peptide length was set to 6. Search criteria included carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine and acetyl (protein N-terminus) as variable modifications. The mass tolerance for the precursor was 4.5 ppm and for the fragment ions was 20 ppm. The human DDA files were searched against a human UniProtKB/Swiss-Prot fasta database (state 11.12.2014, 20 199 entries), the contaminants of MaxQuant and the Biognosys iRT peptide sequences [31] (11 entries). The murine DDA files were searched against a mouse UniProtKB/Swiss-Prot fasta database (state 11.12.2014, 16 697 entries), the contaminants of MaxQuant and the iRT peptide sequences.

Spectral library generation

To generate the spectral libraries, DDA spectra were analyzed as described above and a spectral library was generated using the spectral library generation functionality of Spectronaut (details can be found in the Supporting Information).

Spectronaut analysis

The DIA data were analyzed with Spectronaut 8, a mass spectrometer vendor independent software from Biognosys following default settings (details can be found in the Supporting Information).

DIA-Umpire analysis

Additionally, the DIA data were analyzed using the DIA-Umpire workflow as described in the DIA-Umpire manuscript [15] (details can be found in the Supporting Information).

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The authors R.B. and L.R. are employees of Biognosys AG (Switzerland). Spectronaut is a trademark of Biognosys AG. D.G.V. received a research award from Biognosys AG that was not used for the current study. The rest of the authors declare no competing financial interests.

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