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PAcIFIC goes faster, quantitative and accurate

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Abstract

Data-dependent precursor ion selection is widely used in shotgun proteomics to profile the protein components of complex samples. Although very popular, this bottom-up method presents major drawbacks in terms of detectable dynamic range. Recently, we demonstrated the superior performance of a data-independent method we termed Peptide Acquisition Independent From Ion Count (PAcIFIC). Here, we report a faster, accurate, multiplexed and quantitative PAcIFIC method. Our results show that the time needed to perform such analysis can be decreased by 33% to 66% using modern ion trap instruments and that high mass accuracy can be applied to such a strategy. Quantification capability is demonstrated on protein standards and a whole bacterial cell lysate using isobaric tagging. Finally, we confirm in yeast the dynamic range capabilities of such a method where proteins down to less than 50 copies per cell can be monitored without sample pre-fractionation.

Introduction

Mass spectrometry (MS) has become an essential and powerful tool for the identification of proteins from complex matrices [1]. Traditional mass spectrometry uses techniques such as matrix assisted laser desorption ionization time-of-flight MS for simple mixture analysis [2, 3] and more widely LC-MS/MS for more complex mixture analysis [4, 5]. During this LC-MS/MS process on complex proteomic samples, peptides eluting from the column and entering the mass spectrometer by electrospray ionization are selected for fragmentation based on their intensity using a so-called data-dependant acquisition (DDA). Precursor ions are selected for fragmentation in a serial manner (highest to lowest intensities) and added to an exclusion list for a limited period of time to allow for the selection of precursors with lower intensities. While used widely among the proteomic community, this method suffers from some well known limitations. Namely, with increasing sample complexity, the mass spectrometer cannot fragment in a serial fashion all peptides eluting during a short period of time; ~ 10 to 15 seconds being a realistic baseline time available for a chromatographic peak during nanocapillary separations. Thus, not all precursor ions can be selected for fragmentation while present in the gas phase and therefore these peptides and potentially their parent proteins will not be identified. This single instrument shortcoming is compounded by peptide ionization efficiency and dynamic range effects [6] that remain less well defined than the DDA shortcoming. Additionally, as previously demonstrated by Chait and colleagues for phosphopeptide analysis dynamic range may be extended by use of a hypothesis-driven strategy where expected precursor masses are deduced in silico and the

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tandem mass spectrometry experiment set to target these species where precursor ions are not detectable [7]. Such an approach has therefore to be considered as data-independent acquisition (DIA) in the sense that no precursor ion signal is necessary to trigger a product ion spectrum. Other similar strategies have historically been used with larger precursor ion isolation windows requiring the use of specific algorithms to reconstruct the precursor-ion/fragment-ion lineage [8-12].

Recently, we demonstrated the utility of a DIA strategy called Precursor Acquisition Independent From Ion Count (PAcIFIC) [13] that systematically interrogates all *m/z* values within a given *m/z* range. This PAcIFIC method acquires tandem mass spectra every 1.5 *m/z* regardless of whether a precursor ion signal is present or not. As demonstrated precursor ion scans are not required because of the narrow *m/z* ranges that automatically define the precursor range allowing standard search engines to provide peptide sequence matches. Additionally, we showed that compared to DDA strategies this DIA PAcIFIC strategy easily doubles protein identifications as well as increases individual protein sequence coverage by approx. 10%. As shown by Chait for phosphopeptides and our initial work on serum, dynamic range is also increased by at least one order of magnitude because the DIA nature of this strategy is not biased by presence or absence of precursor ion signals. This allows a significant portion of peptide identifications to be made from peptides with no detectable precursor ion which we refer to as “orphan” peptides.

While these qualitative advantages are beneficial, quantitation was lacking in our original publication on PAcIFIC because of the lack of precursor ion acquisitions. However, as we demonstrate in a recent manuscript (Hengel et al., in review J Proteome Research) spectral counting is a viable option in the absence of MS1 scans. Still, this and all other label-free strategies suffer from the need to complete several replicates for each state to be compared [14] which in the case of PAcIFIC will take several days to cycle through an entire 1000 u. The obvious solution is to conduct quantification directly from the acquired tandem mass spectra, e.g. using isobaric tags such as iTRAQ [15] or TMT [16]. As we demonstrate herein this approach provides for standard stable isotope type quantification as well as multiplexing of the PAcIFIC approach.

Here we describe: **i)** the optimization of the PAcIFIC strategy to decrease acquisition time from the original five days to only three days on an LTQ XL (Thermo) or less than two days on the new LTQ Velos (Thermo) without affecting peptide identification rates, **ii)** the development of a quantitative PAcIFIC method (qPAcIFIC) using isobaric tagging and a combination of CID-PQD scans on a standard mixture of 14 proteins as well as on a complex total bacterial cell lysate, and **iii)** the combination of PAcIFIC with high resolution - high mass accuracy precursor ion scans (aPAcIFIC) that increase stringency during database searches and account for accidental CID events (also defined as mixed precursor CID or co-fragmenting precursor ions) [9, 17, 18] as well as allowing better characterization of the rate of so called “orphan” peptides.

Experimental Procedures

Materials

All reagents and solvents were of the highest available purity. The following proteins were purchased from Sigma-Aldrich (St.Louis, MO, USA): bovine carbonic anhydrase (C3934), chicken lysozyme (L4631), chicken egg albumin (A7642), bovine serum albumin (A7517), bovine milk beta-lactoglobulin (L4756), bovine liver catalase (C1345), bovine milk alpha-casein (C6780), bovine milk beta-casein (C6905), bovine pancreas insulin (I5500), horse heart myoglobin (M1882), bovine apo-transferrin (T1428), aspergillus oryzae alpha-amylase (10065) and horse cytochrome C (C2867). Human transferrin was purchased from Fortune

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biological Inc. (Gaithersburg, MD, USA). Each protein was prepared in a 1 mg/ml stock solution.

14-proteins standard preparation

Two separate solutions (15 and 16) with varying concentrations were prepared using the 14 protein stock solutions as described in Table S-1. 85.6 μg (¼ of solution 15) and respectively 86.4 μg (¼ of solution 16) were digested as follows. Both solutions were dried, resuspended in 100 μl 100 mM ammonium bicarbonate containing 6M urea and sonicated for 10 min. Proteins were then reduced for 45 min at 37 °C by adding 10 μl of 45 mM DTT and further alkylated with 10 μl 100 mM iodoacetamide for another 45 min at room temperature in the dark. Solutions were further diluted with 300 μl 100 mM ammonium bicarbonate and digested overnight at room temperature with 2 μg of trypsin (Promega, Madison, WI, USA). Both protein digested solutions were finally desalted using MacroSpin C18 columns (30-300 μg capacity, SMMSS18V, The Nest Group, Southborough, MA, USA) according to the manufacturer's protocol. Eluates were dried and stored at -80°C for further isobaric tagging.

P. aeruginosa sample preparation

P.aeruginosa bacterial cells were cultured and prepared as previously described [19]. 330 μl (3 μlg/μll) of protein extract was added to 120 mg of urea to reach 6M. Proteins were reduced for 60 min at 37°C by adding 5 μl of DTT 200 mM and further alkylated for another 60 min at room temperature in the dark with 45 μl iodoacetamide 200 mM. Proteins were diluted with 610 μl of ammonium bicarbonate 100 mM. 20 μg of trypsin (Promega, Madison, WI, USA) was finally added and proteins digested overnight at room temperature. 250 μg aliquots were further prepared and desalted using MacroSpin C18 columns (30-300 μg capacity, SMMSS18V, The Nest Group, Southborough, MA, USA) according to the manufacturer's protocol. Eluates were stored at -80°C for further use.

S. cerevisiae sample prepapration

Wild-type yeast cells were grown in YPD (2% yeast extract, 1% peptone, 2% glucose) to log phase. Yeast cells were quickly pelleted, washed, and immediately lysed in a buffer containing 7.4 mL/L β–mercaptoethanol and 7.4 g/L of NaOH while incubated at 4 °C for 10 minutes. The proteins were precipitated by using 10% trichloroacetic acid (v/v) and washed twice with ice-cold acetone. Proteins were denatured by 6 M urea in 50 mM NH_4HCO_3 and reduced for 1 hour at 37 °C with 5 mM tris(2-carboxyethyl)phosphine. Alkylation of cystein residues was performed with 30 mM iodoacetamide, for 1 hr in the dark, followed by reduction with dithiolthreitol, final concentration 30 mM, and incubated for 1 hr. Volume was then increased eightfold with 50 mM ammonium bicarbonate to dilute urea and incubated overnight at 37 °C with sequencing grade trypsin (50:1 protein:trypsin ratio). The resultant peptides were desalted as described in *P. aeruginosa* sample preparation.

Isobaric tagging

Both the 14 protein mixture (i) and the *P. aeruginosa* (ii) samples were labeled with a Tandem Mass Tag (TMT) 2-plex kit (Thermo Scientific, Rockford, IL, USA). (i) Isobaric tagging of the 14 protein mixture was done as follows. Both digests from solution 15 and 16 (see Table S-1) were resuspended in 110 μl of 100 mM TEAB and sonicated for 10 min. After sonication, TMT tags 126 and 127 were dissolved in 41 μl acetonitrile and added to each respective tube. Incubation proceeded for 1 hour at room temperature. Both reactions were then quenched with 5% hydroxylamine for 15 min, pooled 1:1, freeze dried and stored at -80 C for further mass spectrometry analysis. (ii) Isobaric tagging of the *P. aeruginosa*

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was done as follows. Twice 100 μg of digested desalted peptides were resuspended in 110 μl of 100 mM TEAB and sonicated for 10 min. Labeling was identical to (i).

LC-MS configuration

Nano LC separation was performed with a nanoACQUITY system (Waters, Milford, MA, USA) interfaced to the following mass spectrometers from Thermo Scientific (San Jose, CA, USA) depending on the application: (i) an LTQ XL ion trap, (ii) an LTQ Velos ion trap, or (iii) an LTQ Orbitrap XL. Peptides were trapped on a homemade 100 μm i.d. × 20 mm long precolumn packed with 200 Å (5 μm) Magic C18 particles (C18AQ; Michrom), separated on a gravity-pulled 75 μm i.d. × 150 mm long analytical column packed with 100 Å (5μm) Magic C18 particles (C18AQ; Michrom) and analyzed in positive ion mode. For each LC-MS/MS analysis, an estimated amount of 0.5 μg of peptides was loaded on the precolumn at 4 μL/min in water/acetonitrile (95/5) with 0.1% (v/v) formic acid. Peptides were eluted using an acetonitrile gradient flowing at 250 nL/min using mobile phase consisting of the following: A, water, 0.1% formic acid; B, acetonitrile, 0.1% formic acid. The gradient program was as follows: 0 min, A (95%), B (5%); 60 min, A (65%), B (35%); 65 min, A (15%), B (85%); 70 min, A (85%), B (15%); 75-95 min, A (95%), B (5%).

For further details on each optimization method for PAcIFIC, qPAcIFIC and aPAcIFIC and their related data pre-processing, database search and post-processing procedures, see the experimental section in Supporting information.

Results and discussion

Minimizing the time required for a full PAcIFIC analysis

The original PAcIFIC strategy [13] used a DIA tandem mass spectrometry (MS2) cycle consisting of 10 events incremented by 1.5 u from one *m/z* channel to the next scan event (i.e. 400.0, 401.5, 403.0, etc.). This process required approximately 67 LC-MSMS injections, each covering 15 m/z, or five days (67 × 100min run) on an LTQ XL ion trap to accomplish a full range analysis from 400-1400 u.

In order to explore how best to minimize the time required without a decrease in peptide identifications, we performed several experiments in which three essential parameters were modified: **i)** number of MS2 scan events per cycle, **ii)** precursor ion isolation width, and **(iii)** *m/z* channel increment. For this optimization the same *P. aeruginosa* digest used in the original publication was used. A DIA MS2 scan being on average 0.3 seconds on an LTQ XL ion trap, only a limited number of scan events can be programmed which should not exceed the average chromatographic peak retention time (approx. 10-15 seconds). Ideally, at least two to three of these cycles should be performed in order to obtain a tandem mass spectrum on a given ion at the highest possible base peak intensity to yield at least one high quality tandem mass spectrum for that ion. For optimization purposes eight different combinations of the previously mentioned parameters were conducted in duplicate on 10 different *m/z* ranges known to be the most abundant in peptide hits with identical starting *m/z* value (565, 610, 655, 700, 745, 790, 835, 880, 925 and 970). The number of MS2 scan events per cycle tested were: **i)** 10 (experiment A), 15 (experiment B) and 20 (experiment C) with an isolation width of 2.5 and a channel increment of 1.5 *m/z*, ii) 10 (experiment F) and 15 (experiment G) with an isolation width of 2.5 and a channel increment of 2.0, **iii)** 10 with 2.5 isolation width and 2.5 channel increment (experiment H), **iv)** 10 with 3.0 isolation width and 2.5 channel increment (experiment I), and finally **v)** 10 with 3.0 isolation width and 3.0 channel increment (experiment J) (See Table 1 and Figure S-1 for more details). Figure 1 summarizes the result of this first experiment. As illustrated, comparable protein and peptide hits can be achieved with a combination of 15 MS2 spectra, an isolation width

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of 2.5 *m/z* and a channel increment of 1.5 *m/z* (experiment B) compared to the previously published cycle of 10 (experiment A). This combination represents a 33% percent improvement in analysis time by going from 4.7 days to 3.1 days (45 × 100min run). Only a slight decrease in the number of peptide identifications was observed when using 20 MS2 scan events (experiment C), but such losses may be acceptable should time be limited and in which case only 2.4 days (34 × 100min run) are necessary to cover an identical *m/z* range. Interestingly, increasing the channel increment from 1.5 to a higher value resulted in a drastic loss in peptide identifications. We believe this is because an overlap of the precursor ion isolation width for CID between each *m/z* channel of at least 33% is optimum for matching peptide isotopic envelopes and therefore maximizing the number of high quality tandem mass spectra. This overlap prevents isotopic distributions being split between the PAcIFIC *m/z* channels that decrease the quality of resulting tandem mass spectra, a problem exacerbated by the use of isolation waveforms that truncate on either side of the selected value. Finally, a dual linear ion trap, the LTQ Velos [20], was introduced that is capable of almost double the scanning rate of the LTQ XL ion trap. Preliminary results suggest that on average 0.2 seconds are necessary to acquire a data-independent MS2 spectrum which translates to a PAcIFIC cycle of 25 MS2 spectra (See SI Result and Figure S-2 for more details). This change in rate decreases the time needed for a full PAcIFIC experiment over 1000 u to ~ 1.5 days (22 × 100min run).

Quantitative PAcIFIC (qPAcIFIC)

We have shown in the previous section that the PAcIFIC method could be drastically optimized in terms of speed and that less than three days of instrument time are necessary to perform such thorough analysis on a regular ion trap. However, such analysis lacks the quantitative capacity and throughput that is required for modern proteomic experiments. While we have recently shown that spectral counting may be used with standard PAcIFIC data (Hengel et al., in review at J Proteome Research), this remains unsatisfying in that data acquisition is serial and some biochemical experiments [21] require use of stable isotopes to track the history of a given sample type. In order to meet these two requirements we investigated the coupling of our PAcIFIC strategy with the stable isotope isobaric tagging approach offered by the Tandem Mass Tag (TMT) method [16]. In order to circumvent the one-third *m/z* cut-off limit inherent to ion trap instruments, reporter ions could not be recorded in regular CID spectrum and so a supplementary PQD spectral scan was added to the PAcIFIC DIA strategy that recorded TMT reporter ions in the low *m/z* region. Figure 2 illustrates the acquisition process **(A)** as well as data processing **(B)** used for the TMT-based qPAcIFIC strategy. In order to keep the duty cycle to a reasonable time (i.e. 5.4 seconds on average) using an optimized 15 scan event cycle as previously discussed, PQD spectra were acquired only on a limited range corresponding to the region where the isobaric tags are expected (121-132 *m/z*) to drastically reduce scanning time (see Figure S-3A and S-3B). The information contained in these pairs of CID-PQD spectra is then combined into a composite tandem mass spectrum that can be used for database search as well as quantification.

As a proof of principle, we conducted a TMT qPAcIFIC experiment on a mixture of 14 protein standards. Two separate solutions with varying concentrations of each protein were prepared with theoretical ratios spanning from 1:10 to 10:1 and respectively labeled with TMT 126 and 127 tags before being pooled and analyzed in duplicate. Figure 3A shows the results obtained from this experiment after Sequest search and quantification with Libra (TPP, ISB, Seattle). To serve as a control, a separate analysis was conducted using a DDA strategy where the three most intense ions were selected for CID and PQD fragmentation. As expected, experimental ratios were in accordance with theoretical ones. Our DIA approach yielded similar results in terms of ratio to a regular DDA strategy therefore validating that the shifts (or ratio compression) observed between experimental and

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theoretical values (especially at higher ratios) could not be attributed simply to our new experimental qPAcIFIC design.

While precise quantification at higher ratio may be desirable, most biologists are interested in proteins showing subtle changes in abundance and will corroborate MS results with other experiments. Therefore, an important metric for any quantification strategy remains the standard deviation that one can expect from an experiment where all ratios are supposed to be equal to 1:1. In order to answer that question, a total cell lysate of *P. aeruginosa* was split into two identical protein amounts (100 μg) that were separately digested and then labeled with TMT 126 and 127 tags, respectively, before being pooled and analyzed. From this experiment all identified proteins are expected to be present at a 1:1 ratio and any deviation from this value provides a measurement of the accuracy of the strategy. Figure 3B shows the results obtained from this experiment using stringent peptide filtering and quantification. In order to consider a protein as quantified, at least two or more peptides with probability higher than 0.99 had to be present. Moreover, intensity for both reporter ions had to be higher than 500 in ion intensity ‘counts’, a value considered as very conservative. With such filtering criteria, a total of 1396 proteins with two or more peptides and probability ≥ 0.99 were indentified out of which 755 (55%) could be quantified using 500 ‘counts’ as the intensity threshold. The average and median ratio obtained were 1.01 and 1.00 respectively with a standard deviation of only 0.15. These results clearly suggest that even when conservative filtering is applied, our qPAcIFIC approach allows for accurate quantification over a broad number of proteins within a complex sample such as the bacterium *P. aeruginosa*. However, if a less conservative approach is used allowing for quantification of single hits with probability higher than 0.99 and an intensity threshold set to only 20 ‘counts’, then out of 2234 proteins indentified 2033 (91%) can be quantified with an average and median ratio of 1.04 and 1.0 respectively with a standard deviation of 0.33. While the standard deviation increases to a value that renders differentiation of true subtle protein abundance changes more difficult, it does however show that this strategy is very effective at identifying and quantifying a large number of proteins as well as multiplexing the PAcIFIC strategy.

PAcIFIC at high mass accuracy (aPAcIFIC)

As part of our original PAcIFIC analysis [13], we detected a class of peptides for which no precursor ions could be detected which we referred to as “orphan” peptides. These peptides were identified solely based on the quality of their tandem mass spectra and were, as per Chait's earlier reports, a result of selecting ions from *m/z* channels in the absence of precursor ions. In addition to this category of orphan peptides, detectable because of intrinsically higher signal to noise for their tandem mass spectra than precursor spectra, we also investigated whether there was a difference in occurrence of accidental CID events (i.e. co-fragmenting peptides) between our PAcIFIC DIA and standard DDA protocols [18]. Thus, in order to determine the extent to which these two categories occurred in our optimized PAcIFIC strategy, we developed a modified PAcIFIC method in which high mass accuracy precursor ion spectra were acquired. Additionally, having previously shown PAcIFIC to be capable of detecting proteins an order of magnitude lower in abundance in human serum than standard DDA methods, we wished to better define the detectable dynamic range of PAcIFIC. To investigate these ideas we analyzed a *Saccharomyces cerevesiae* whole cell lysate using a modified PAcIFIC method that provided for acquisition of precursor ion scans at high mass accuracy while still maintaining the basic PAcIFIC protocol. Specifically, a precursor ion spectrum was acquired in the Orbitrap mass analyzer simultaneously with acquisition of five DIA product ion spectra in the LTQ. This provided three precursor ion spectra equally distributed among 15 DIA product ion spectra during one PAcIFIC cycle (see Figure S-4). Because of the parallel nature of the LTQ-Orbitrap mass spectrometer,

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these precursor ion spectra could be acquired without affecting the overall duty cycle of the PAcIFIC analysis that we optimized for 15 DIA product ion spectra. After the analysis, these precursor ion spectra were deconvoluted with Hardklor [17] to extract a list of all possible isotopic features. An in-house program was further used to correct the precursor mass of the DIA product ion spectra (i.e. corresponding to the center of the window) to a possible precursor mass identified in the deconvoluted list of isotopic features using the following conditions: **i)** a precursor mass tolerance set to ± 1.25 *m/z* and **ii)** a retention time tolerance of ± 15 seconds. In the case of multiple choices as could occur from co-fragmenting peptides, only the three best precursors were selected based on their Hardklor detection score and a separate DTA file created with their respective precursor ion masses. All precursor ion mass corrected DTAs were searched against the appropriate database using high mass accuracy (±10 ppm) while the uncorrected DTAs were searched using a 3.75 Da precursor tolerance used in the standard PAcIFIC database search (see Figure S-4). The dataset was further statistically validated using PeptideProphet and an FDR of less than 1% used as the cut-off.

From this type data acquisition and subsequent data analysis two main benefits are derived. First, the number of orphans can be precisely estimated from such a PAcIFIC strategy. Indeed, peptides indentified by a product ion spectrum for which no precursor ion mass could be detected in any of the precursor ion spectra and for which no other precursor corrected spectrum matches the same peptide sequence can be considered as being an "orphan" peptide. When estimated for this yeast dataset, 18% of the peptide IDs were accounted for as "orphan" peptides. Second, the number of "accidental" CIDs or co-fragmenting peptides can be estimated by allowing the same product ion spectrum to be written more than once with different precursor ion masses. Thus, by searching the dataset at 10 ppm, in some cases a single product ion spectrum may match two different peptide sequences. In this yeast dataset, this accounted for approx. 8% of the peptide IDs. It has to be mentioned that this number of accidental CID events is only valid for the peptide portion for which a precursor ion is observed and not for the "orphan" peptides where, for the moment, accounting for co-fragmenting peptide is not possible. This number is in good agreement with our previously reported dataset on the bacterium *Pseudomonas aeruginosa* [13, 19].

Finally, we have previously shown that a high dynamic range ($1E^7$) could be achieved with this PAcIFIC technique on a plasma sample [13] which was an order of magnitude better (at the same FDR) as other published shotgun proteomic methods. Weissmann and colleagues [22] reported on protein abundance in *Saccharomyces cerevesiae* estimating that 80% of the proteome is translated during normal growth conditions with a dynamic range from fewer than 50 to more than 1E6 protein molecules per cell. We therefore compared our list of proteins (FDR < 0.5% and ≥2 unique peptides per protein) to their list of protein abundance. Figure 4 shows that our PAcIFIC strategy is capable of identifying proteins over the entire dynamic range expressed by yeast under standard growth conditions and that the relative distribution of proteins identified, based on abundance, follows a trend similar to the Weissmann dataset. This clearly indicates that our PAcIFIC strategy has a dynamic range of detection that is not biased toward high abundance proteins, but instead detects proteins easily down to 50 copies/cell. This attribute of PAcIFIC is mainly due to the DIA nature of the technique which conducts CID regardless of the signal intensity in the selected *m/z* channel. Thus, peptides with low or no precursor ion signal are not discriminated as is the case with DDA methods. Finally, we note that our PAcIFIC proteomic discovery strategy achieves similar results to popular targeted multiple reaction monitoring (MRM) strategies such as presented by Aebersold and colleagues [23] who successfully measured proteins down to 50 copies per cell. In fact, the PAcIFIC method can be described as a pseudo-MRM strategy that has the benefit of specificity over MRM methods because all fragment ions for

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each channel are acquired. Additionally, PAcIFIC may be used to define empirically all relevant MRM transitions for a given sample of interest.

Concluding remarks

We presented a strategy that optimized PAcIFIC acquisitions to less than two days on the fastest scanning ion trap available currently (LTQ-Velos) or only three days on prior generation ion traps. We have also demonstrated how PAcIFIC may be multiplexed and quantitative. Additionally, a modified strategy using high mass accuracy precursor ion scans allowed the number of “orphan” peptides and co-fragmenting peptides to be estimated. Finally, when applied to analysis of a yeast whole cell lysate we have shown that PAcIFIC detects proteins across the entire measurable dynamic range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Aebersold R, Mann M. Nature. 2003; 422:198–207. [PubMed: 12634793]
2. Mann M, Hojrup P, Roepstorff P. Biol Mass Spectrom. 1993; 22:338–345. [PubMed: 8329463]
3. Strupat K, Karas M, Hillenkamp F, Eckerskorn C, Lottspeich F. Analytical Chemistry. 1994; 66:464–470.
4. McCormack AL, Schieltz DM, Goode B, Yang S, Barnes G, Drubin D, Yates JR 3rd. Anal Chem. 1997; 69:767–776. [PubMed: 9043199]
5. Washburn MP, Wolters D, Yates JR 3rd. Nat Biotechnol. 2001; 19:242–247. [PubMed: 11231557]
6. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M. Mol Cell Proteomics. 2005; 4:1265–1272. [PubMed: 15958392]
7. Chang EJ, Archambault V, McLachlin DT, Krutchinsky AN, Chait BT. Anal Chem. 2004; 76:4472–4483. [PubMed: 15283590]
8. Bateman RH, Carruthers R, Hoyes JB, Jones C, Langridge JI, Millar A, Vissers JP. J Am Soc Mass Spectrom. 2002; 13:792–803. [PubMed: 12148804]
9. Geromanos SJ, Vissers JP, Silva JC, Dorschel CA, Li GZ, Gorenstein MV, Bateman RH, Langridge JI. Proteomics. 2009; 9:1683–1695. [PubMed: 19294628]
10. Masselon C, Anderson GA, Harkewicz R, Bruce JE, Pasa-Tolic L, Smith RD. Anal Chem. 2000; 72:1918–1924. [PubMed: 10784162]
11. Purvine S, Eppel JT, Yi EC, Goodlett DR. Proteomics. 2003; 3:847–850. [PubMed: 12833507]
12. Venable JD, Dong MQ, Wohlschlegel J, Dillin A, Yates JR. Nat Methods. 2004; 1:39–45. [PubMed: 15782151]
13. Panchaud A, Scherl A, Shaffer SA, von Haller PD, Kulasekara HD, Miller SI, Goodlett DR. Anal Chem. 2009; 81:6481–6488. [PubMed: 19572557]
14. Foss EJ, Radulovic D, Shaffer SA, Ruderfer DM, Bedalov A, Goodlett DR, Kruglyak L. Nat Genet. 2007; 39:1369–1375. [PubMed: 17952072]
15. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Mol Cell Proteomics. 2004; 3:1154–1169. [PubMed: 15385600]

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16. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. Anal Chem. 2003; 75:1895–1904. [PubMed: 12713048]
17. Hoopmann MR, Finney GL, MacCoss MJ. Anal Chem. 2007; 79:5620–5632. [PubMed: 17580982]
18. Scherl A, Tsai YS, Shaffer SA, Goodlett DR. Proteomics. 2008; 8:2791–2797. [PubMed: 18655048]
19. Scherl A, Shaffer SA, Taylor GK, Kulasekara HD, Miller SI, Goodlett DR. Anal Chem. 2008; 80:1182–1191. [PubMed: 18211032]
20. Second TP, Blethrow JD, Schwartz JC, Merrihew GE, MacCoss MJ, Swaney DL, Russell JD, Coon JJ, Zabrouskov V. Anal Chem. 2009; 81:7757–7765. [PubMed: 19689114]
21. Ranish JA, Hahn S, Lu Y, Yi EC, Li XJ, Eng J, Aebersold R. Nat Genet. 2004; 36:707–713. [PubMed: 15220919]
22. Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS. Nature. 2003; 425:737–741. [PubMed: 14562106]
23. Picotti P, Bodenmiller B, Mueller LN, Domon B, Aebersold R. Cell. 2009; 138:795–806. [PubMed: 19664813]

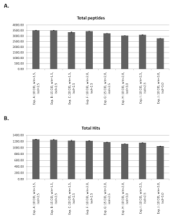

Figure 1.
Comparison of the different optimization processes for PAcIFIC in terms of total number of peptides (A) and proteins (B) identified. See Supplementary Figure 1 for more details of each optimization conditions.

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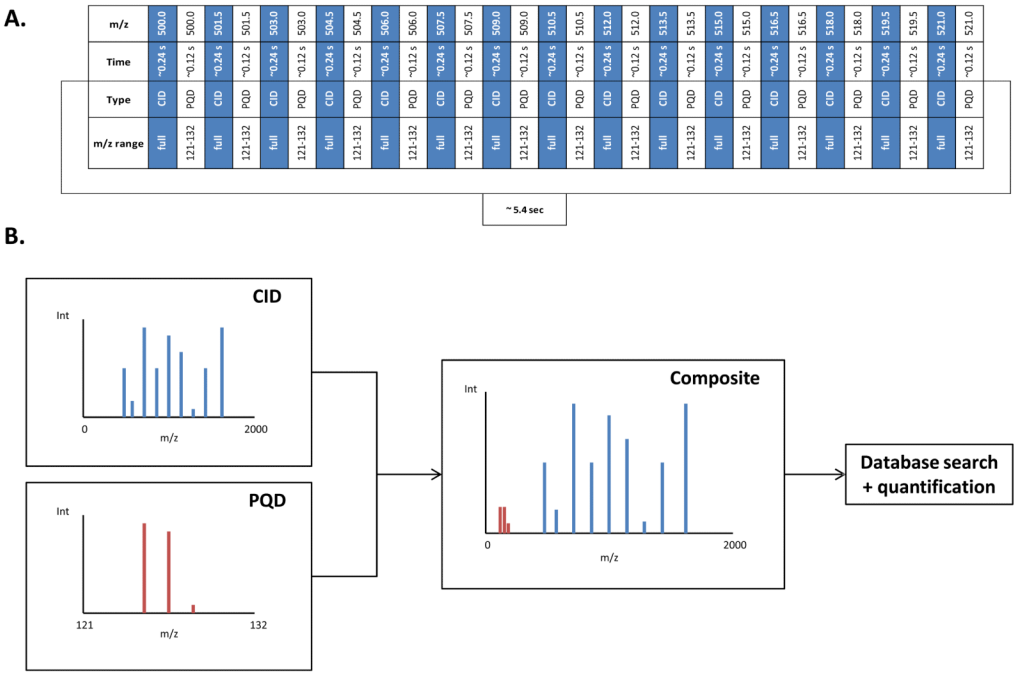

Figure 2.

Description of the quantitative PAcIFIC acquisition. (A) In between each regular CID scan used for identification, a narrow PQD scan is acquired only in the m/z range corresponding to the reporter ions for faster scanning time. (B) Both CID and PQD scans are merged to create a composite spectrum that is further used for database searching and quantification.

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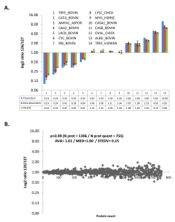

Figure 3.

Quantitative PAcIFIC experiments. (A) Protein standards ratio distribution. Two protein mixtures with different protein concentration but equal total protein amount were labeled with TMT 126 and 127 respectively. The final pooled mixture was analyzed using a classical data-dependent method (red) as well as PAcIFIC (green) for comparison. Ratios obtained for each method are compared to the theoretical ratios (blue). (B) Pseudomonas aeruginosa protein ratio distribution. Two identical protein aliquots were labeled separately with TMT 126 and 127 respectively before being combined and analyzed by our quantitative PAcIFIC method. A total of 1396 proteins were identified at FDR < 0.5% with two or more peptides of which 755 could be quantified with two or more peptides yielding to an average ratio of 1.01 with a standard deviation of 0.15.

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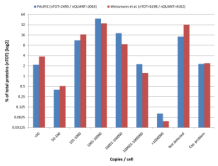

Figure 4.

Dynamic range achieved by PAcIFIC in yeast. PAcIFIC was applied to a total yeast cell lysate and identified proteins - with two or more peptides per protein - compared to the quantitative dataset published by Weissmann and colleagues. Frequencies of proteins identified are calculated for several bins corresponding to different abundance ranges (copies/cell) as measured in the Weissmann dataset. A similar trend is observed in terms of protein abundance categories with proteins identified with less than 50 copies per cell clearly highlighting the dynamic range and sensitivity achieved by our data-independent PAcIFIC method. nTOT: total number of protein identified; nQUANT: total proteins with quantitative information; Exp. Problem: defined by Weissmann et al. as a detected band by western blot with an experimental quantification problem.

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Table 1

Summary table of all PAcIFIC optimization experiments conducted with a glossary of the main parameters described.

Experiment	Nb. of CID channel	Channel increment [m/z]	Isolation width [m/z]	m/z range covered	Instrument(s) used	channel overlap [%]	Nb. of injections needed to cover a range of 400-1400 m/z *(time equivalent in days)*
A	10	1.5	2.5	15.0	LTQ XL	40	67 (4.7)
B	15	1.5	2.5	22.5	LTQ XL, LTQ Velos	40	45 (3.1)
C	20	1.5	2.5	30.0	LTQ XL, LTQ Velos	40	34 (2.4)
D	25	1.5	2.5	37.5	LTQ XL, LTQ Velos	40	27 (1.9)
E	30	1.5	2.5	45.0	LTQ XL, LTQ Velos	40	22 (1.5)
F	10	2	2.5	20.0	LTQ XL	20	50 (3.5)
G	15	2	2.5	30.0	LTQ XL	20	34 (2.4)
H	10	2.5	3	25.0	LTQ XL	16	40 (2.8)
I	10	2.5	2.5	25.0	LTQ XL	0	40 (2.8)
J	10	3	3	30.0	LTQ XL	0	34 (2.4)

Glossary:
Scan event: corresponds to one scanning event within the MS instrument. Can be a survey scan or product ion scan.
Acquisition cycle: a single LC-MSMS injection during which a defined number of scan events are performed.
CID Channel: one product ion spectrum with a defeined data-independent precursor mass.
Channel increment: precursor mass m/z difference used to switch from one CID channel to the adjacent one.
Isolation width: defines the size of the window that will be used to isolate a specific precursor ion.
Channel overlap: measures how much overlap we have from one CID channel to the next one. It is defined as 1-(channel increment/isolation width).

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