Extending the Separation Space with Trapped Ion Mobility Spectrometry Improves the Accuracy of Isobaric Tag-based Quantitation in Proteomic LC/MS/MS

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Abstract

Two-dimensional separation by nano-LC and trapped ion mobility spectrometry (TIMS) prior to Q/TOF tandem mass spectrometry significantly improves the accuracy of isobaric tag-based quantitation in proteome analysis without the need for additional measurement time for TIMS insertion between LC and Q/TOF MS. The obtained peak capacity of up to 3,300 per hour in LC/TIMS reduced the co-isolation of precursor ions at the quadrupole analyzer, resulting in more accurate ratios of reporter ions derived from isobaric tags in product ion spectra obtained at the TOF analyzer. We also found that TIMS with a narrower quadrupole isolation window could reduce the ratio compression effect at least as effectively as the synchronous precursor selection method using MS3 scans without compromising sensitivity or coverage. Our results suggest that the65 min-gradient LC/TIMS/Q/TOF system is an excellent platform for high-throughput proteomics studies.

Keywords

Trapped ion mobility spectrometry, Tandem mass tag, Ratio compression, High accuracy quantitation

Introduction

Mass spectrometry (MS)-based proteomics is a powerful tool for identifying and quantifying proteins from a wide range of biological samples. Various methodologies are available for quantitative proteomics, including label-free quantitation[1,2] and stable isotope label-based quantitation[3,4], but recently, quantitative proteomics via isobaric chemical tags is attracting increasing attention due to its sample multiplexing capability, high precision and high throughput[5]. Isobaric labeling approaches enable parallel quantitation through monitoring the reporter product ions generated from the isobaric precursor ions of multiplexed samples[6]. However, the accuracy of quantitative data obtained by means of this approach can be adversely affected by contamination with non-targeted precursor ions in the isolation window for the target precursors. Tremendous efforts have already been made to reduce this quantitation ratio distortion. Proton-transfer ion-ion reactions efficiently reduce the charge state of precursor ions, making it possible to remove interfering ions and improve the accuracy of quantitative measurements[7]. Tandem MS

with MS3, including multi-notch MS3 with synchronous precursor selection (SPS) technology, successfully eliminates interfering precursor ions in the analysis of complex samples[8,9]. But, although these approaches can prevent quantitative ratio distortion in isobaric tagging, they have inherent limitations in terms of sensitivity and duty cycle due to the need for selection of product ions in MS2 for further fragmentation. Tandem mass tag (TMT)-based complement reporter ion approaches[10,11] and the EASI-tag[12] based isobaric labeling approach utilize the precursor mass and charge state to reduce background interference and can improve the accuracy of quantitation. However, these approaches suffer from the presence of isotopic peaks of target peptide precursor ion and require a very narrow quadrupole isolation window of around 0.4 Th, which leads to lower sensitivity.

The strategies mentioned above focus mainly on the removal of contaminant ions at the point of selection of precursor ions in MS1. From another perspective, increasing the separation efficiency prior to MS1 is also effective to normalize the distorted quantitative ratios. Extensive pre-fractionation of TMT-labeled peptides by high-pH reversed-phase LC (High-pH RPLC) can reduce the sample complexity prior to LC/MS/MS and restore the distorted ratios in MS2-based isobaric quantitation[13]. However, in such experiments, the advantages of isobaric tags cannot be exploited, because the total analysis time is increased and the throughput is reduced. Ion mobility spectrometry (IMS) is compatible with LC and MS, and enables increased sensitivity and selectivity in MS and an overall increase in peak capacity without increasing the total analysis time[14,15]. So far, LC/IMS/MS experiments using traveling wave ion mobility spectroscopy (TWIMS)[16] and electric field asymmetric ion mobility spectroscopy (FAIMS)[17–19] have demonstrated that it is possible to un-compress the ratio of isobaric tags in quantitative proteome analysis.

The TIMS analyzer developed for TIMS/Q/TOF has two trapping devices in series that alternately trap and release ions. In combination with high-speed MS/MS acquisition by TOF, tandem MS analysis with high sensitivity and high efficiency is possible without discarding ions in TIMS and the quadrupole analyzer, and this is designated as parallel accumulation-serial fragmentation (PASEF) acquisition[20,21]. These features are particularly important for fast, sensitive quantitative proteomics using isobaric labeling, and may be of great benefit. In this paper, we characterize the separation space of 65 min-gradient LC coupled with TIMS and examine the extent to which the LC/TIMS/Q/TOF system can accurately quantitate target precursors of isobarically labeled peptides.

Methods

HeLa and *E. coli* protein digests were prepared as described previously[22,23]. Each digest was labeled with TMT reagents (Thermo Fischer Scientific, Waltham, MA), combined in appropriate ratios and desalted with StageTips[24]. The LC/MS analyses were performed on a timsTOF *Pro* (Bruker, Bremen, Germany) mass spectrometer using a 65 min LC gradient. The experimental procedures are described in detail in the Supporting Information.

Results & Discussion

To characterize the performance of the LC/TIMS/Q/TOF system for TMT-based quantitation in proteomics, we prepared a series of samples based on a two-proteome model[9]. The standard sample was prepared by mixing *E. coli* protein digests with HeLa protein digests after TMT-labeling. The expected TMT 6-plex reporter ion ratios for *E. coli* and HeLa peptides were 10:4:1:1:4:10 and 0:0:0:4:4:4, respectively (Figure 1).

Utilization of separation space prior to Q-TOF mass spectrometry

First, we assessed how much additional separation space can be obtained prior to Q/TOF MS by inserting TIMS after RPLC. If the separation selectivity in both RPLC and TIMS approaches is perfectly orthogonal, the separation space can be maximized. Figure 2 shows the distribution of the extracted monoisotopic peaks using MaxQuant [25,26] (version 1.6.4.0) in the separation space provided by RPLC and TIMS. There was no correlation between the two separation approaches (r^2 = 0.056). The utilization of the RPLC-TIMS two-dimensional separation space was calculated using the density distribution estimated by Perseus software[27]. The calculated utilization rate for the separation space was 34.3%, indicating that more than half of the separation space was unutilized due to the more condensed density distribution of ions with higher charge. Next, the peak capacities of the RPLC and TIMS separations were evaluated. The peak capacities were 197.7 and 48.7 for RPLC and TIMS, respectively, based on a total of 187,247 peptide ions eluted from 20 to 85 min and from 0.6 V･s ･cm^{-2} to 1.5 V･s･cm^{-2}. By combining RPLC and TIMS, a peak capacity of about 3,300 (197.7 x 48.7 x 0.343) could easily be achieved without multiple injections, which are unavoidable in conventional multidimensional LC fractionation.

TIMS separation reduces precursor co-isolation

For isobaric labeling, precursor co-fragmentation can compress reporter ion intensity ratios and thus affect protein quantification. Recent reports on large-scale proteomic analyses of TMT-labeled peptides have indicated that ion mobility separation using TWIMS or FAIMS significantly decreases the extent of co-fragmentation[16–19]. To evaluate the extent of co-fragmentation on LC/TIMS/Q/TOF, we utilized the interference-free index (IFI)[28], which is calculated from the ratio of the reporter ion intensity of an empty channel to that of the sample-containing channel (Figure 3A). For the identified HeLa peptides, IFI values close to 1 indicate that the majority of the ion currents are derived from HeLa peptides and are less contaminated with *E. coli* peptides. In this study, peptides with an IFI greater than 0.95 were defined as interference-free identifications. For the commonly identified HeLa peptides, 19.7%, 55.7%, and 63.4% of the HeLa peptides were classified as interference-free identifications for the analysis using TIMS-off with a standard isolation window at the quadrupole analyzer, TIMS-on with a standard window and TIMS-on with a narrower window, respectively (Figure 3B, Figure S1A). These results indicate that TIMS can significantly reduce precursor co-isolation in the proteome analysis of TMT-labeled samples.

We also analyzed the same samples using the Q/Orbitrap/Ion Trap (Q/OT/IT) instrument (Orbitrap Fusion Lumos) with standard Orbitrap MS2 and SPS-MS3 methods under the same chromatographic conditions. All the identification results were summarized in Figure S2. In this experiment, 24.0%, 43.0% and 59.7% of commonly identified HeLa peptides were classified as no-interferences for Orbitrap-MS2, SPS-MS3, and TIMS/Q/TOF with the narrower isolation window, respectively (Figure 3B, Figure S1A). In this case, SPS-MS3 was not as effective as TIMS/Q/TOF in terms of the reduction of co-isolation events, probably due to inappropriate product ion selection in MS2 for MS3. Recently it was reported that SPS-MS3 combined with real-time database search (RTS) improves the accuracy of quantitation by enabling selection of the proper product ions for MS3[29].

TIMS separation improves TMT quantitative accuracy

To further evaluate the 65 min-gradient LC/TIMS performance for quantitation with isobaric tags, we compared the first three channels and the last three channels of *E. coli* peptides to evaluate the extent to which the HeLa background ions affect the quantitation results. First, we compared the quantitative results with and without TIMS. Figure 4A shows the TMT ion ratios obtained from the analysis of the commonly identified *E. coli* peptides with and without TIMS. MS/MS spectra with no signal in any of the six reporter ion channels were discarded. TMT ratios

(median) with and without TIMS were 10.0 : 4.8 : 1.3 : 1.6 : 4.6 : 11.6 and 10.0 : 5.0 : 1.4 : 2.3 : 6.0 : 12.8, respectively. In other words, the quantitation ratio of TMT channels containing background ions was overestimated by up to 61.3% without TIMS and by up to 19.2% with TIMS, indicating that TIMS is effective for improving the quantitation accuracy. Furthermore, when TIMS was combined with a narrow quadrupole separation window (1.0 Th), the TMT ratio with background ions was overestimated by up to 13.6% (Figure 4A, Figure S1B). Thus, the quantitative accuracy was improved with TIMS compared with that without TIMS, and was improved even more with TIMS using the narrower quadrupole separation window. These trends agreed with the quantification results for the HeLa peptides.

Next, we compared the *E. coli* quantitation results from TIMS/Q/TOF with those from Q/OT/IT using MS2 and SPS-MS3 modes. The quantitation ratios with background ions were overestimated by 70.2%, 19.7%, and 16.2% for commonly identified *E. coli* peptides with Orbitrap-MS2, SPS-MS3 and TIMS/Q/TOF, respectively (Figure 4B, Figure S1B). These results also indicate that TIMS/Q/TOF is at least as effective as SPS-MS3 in reducing the ratio compression effect. A potential benefit of TIMS separation compared to SPS-MS3 is that the additional TIMS separation does not increase the duty cycle owing to the PASEF acquisition scheme, whereas the application of SPS-MS3 methods does result in an increase of the duty cycle even with the intelligent RTS platform. Moreover, TIMS can trap and separate most of the ions from tryptic peptides based on the ion mobility without loss of sensitivity within a single LC run, while differential ion mobility devices such as FAIMS can only work as a filter during capture of the target ions, meaning that FAIMS needs to discard other ions, thus requiring multiple injections to cover all ions. Overall, these results demonstrate that LC/TIMS separation makes the Q/TOF system very attractive for fast, accurate and high-throughput multiplexed proteomics with isobaric tags.

Conclusion

In this study, we characterized the separation space of the orthogonal RPLC/TIMS approaches and examined their application to TMT-based quantitative proteomics. We found that 34.3% of the RPLC/TIMS two-dimensional separation space can be utilized for the analysis of TMT-based multiplexed proteomic samples. We demonstrated that RPLC/TIMS significantly increases the peak capacity prior to Q/TOF tandem MS without increasing the analysis time, which is impossible to achieve with conventional fractionation strategies. TIMS reduces the ratio

compression effect in reporter-ion-based quantitation and could achieve a similar ratio compression reduction to that of the SPS-MS3 method without sacrificing instrument sensitivity or scan speed. We believe isobaric labeling-based quantitation with the LC/TIMS/Q/TOF system has great potential for further development to analyze clinical samples, for which high throughput, high accuracy, and high sensitivity are essential.

Supporting Information

The following supporting information is available free of charge at ACS website http://pubs.acs.org

Materials and methods; detailed experimental procedures

Figure S1. The precursor co-isolation effects evaluated by all identified HeLa and *E. coli* peptides.

Figure S2. An overview of the identification results.

Acknowledgements

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Figure Legends

Figure 1. The standard samples consist of two proteomes. HeLa and *E. coli* proteomic samples were digested, labeled with TMT tags, and mixed at ratios of 0:0:0:4:4:4 (HeLa) and 10:4:1:1:4:10 (*E. coli*).

Figure 2. Orthogonality of peptide separation between RPLC and TIMS. The retention time and ion mobility distribution of monoisotopic peaks detected in full-scan MS are shown. Isotope peaks were aggregated into monoisotopic peaks using MaxQuant software.

Figure 3. The precursor co-isolation effect was evaluated based on commonly identified HeLa peptides. (A) The equation for calculating the interference-free index (IFI). Co-isolation of isobaric precursors leads to a decrease of IFI. (B) The ratios of low interference-containing peptide IDs are shown in 100% stacked bar plots. Peptide IDs with IFI of more than 0.95 were considered as interference-free. IW: quadrupole isolation window.

Figure 4. Compression of TMT ion ratios from *E. coli* peptides by the background HeLa proteome. Commonly identified *E. coli* peptides were used for the analysis. Box plots show the upper quartile, median, and lower quartile for the TMT ion ratios. Outliers were identified using box-plot statistics (threshold: 1.5 x the interquartile range (IQR)). Dashed lines represent expected ratios. IW: quadrupole isolation window. (A) Comparison between TIMS-on and TIMS-off. (B) Comparison among different system settings.

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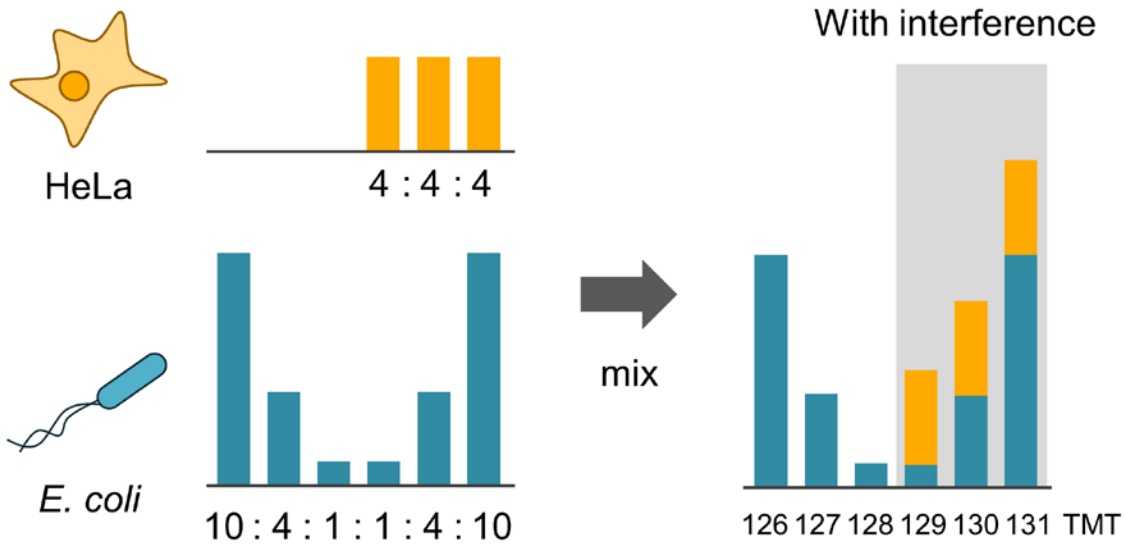
Figure 1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4 : 4 : 4
E. coli
10 : 4 : 1 : 1 : 4 : 10
mix
126 127 128 129 130 131 TMT

Figure 2

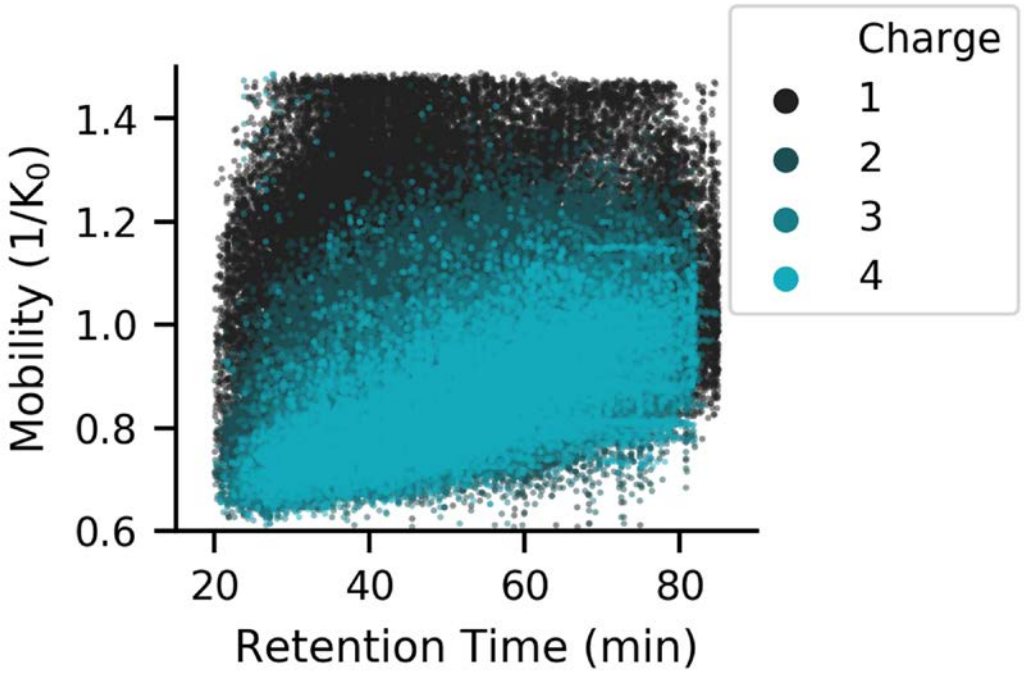

Charge
1
2
3
4
Mobility (1/K0)
1.4
1.2
1.0
0.8
0.6
20
40
60
80
Retention Time (min)

Figure 3

(A)

$$IFI = 1 - \frac{intensity\ (126, empty\ channel)}{averaged\ intensity\ (129, 130\ and\ 131)}$$

(B)

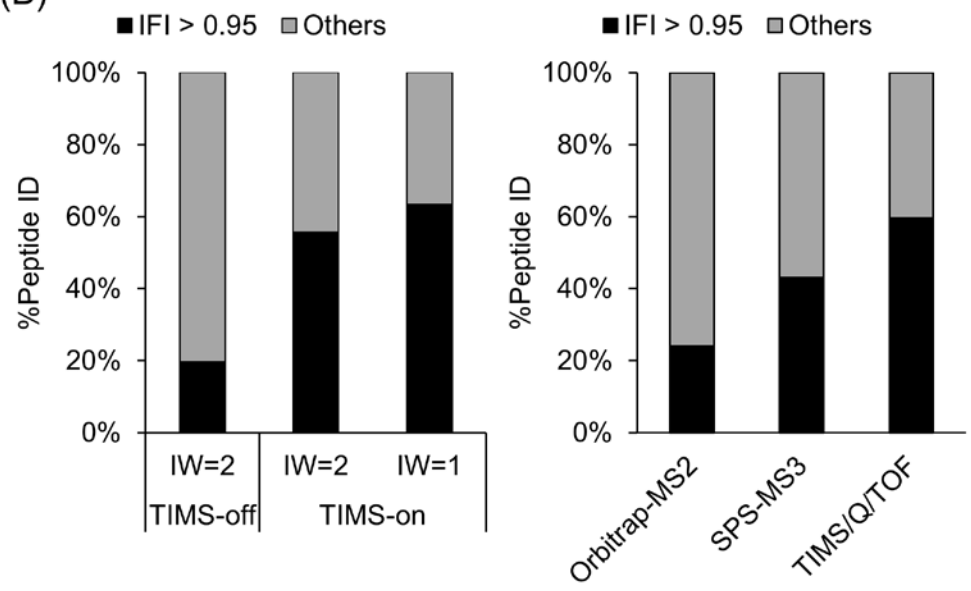
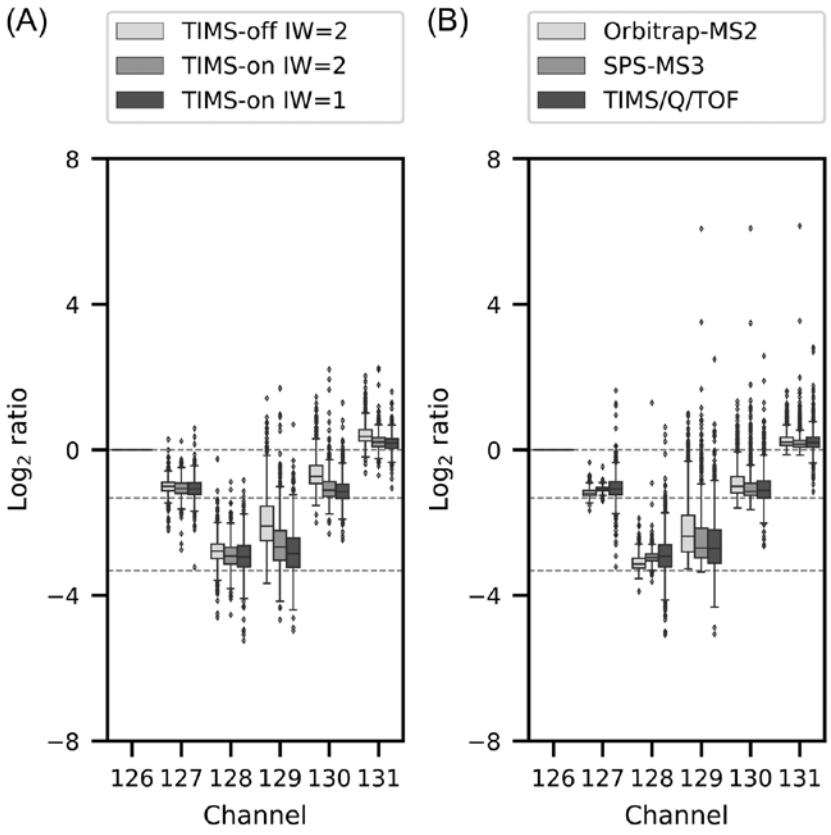
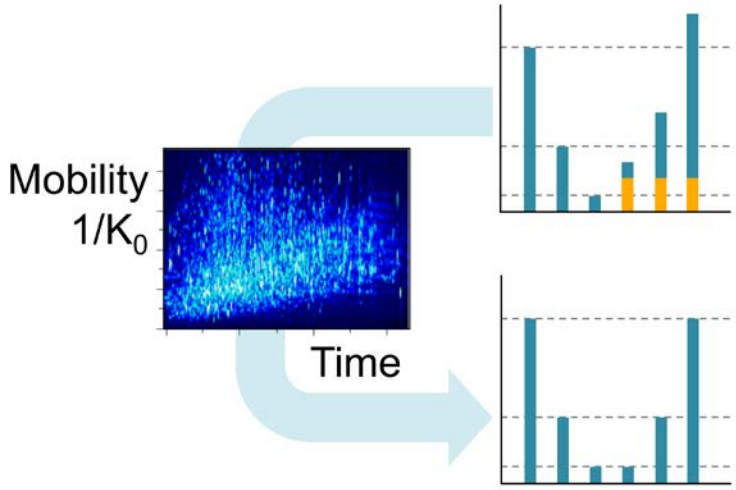


Figure 4


(A)
TIMS-off IW=2
TIMS-on IW=2
TIMS-on IW=1
(B)
Orbitrap-MS2
SPS-MS3
TIMS/Q/TOF
Log2 ratio
8
4
0
−4
−8
126 127 128 129 130 131
Channel

For TOC Only



Supporting Information

Extending the Separation Space with Trapped Ion Mobility Spectrometry Improves the Accuracy of Isobaric Tag-Based Quantitation in Proteomic LC/MS/MS.

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Materials and Methods

Materials

UltraPure™ Tris Buffer and TMT reagents were purchased from Thermo Fischer Scientific (Waltham, MA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Water was purified by a Millipore Milli-Q system (Bedford, MA). All other chemicals and reagents were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan) unless otherwise specified.

Cell Culture

HeLa cells were cultured to 80% confluence in DMEM containing 10% FBS in 10 cm diameter dishes. Cells were washed twice with ice-cold PBS, collected using a cell scraper, and pelleted by centrifugation. *E. coli* strain K-12 BW25113 was grown in LB medium (Nacalai Tesque) with vigorous shaking at 37°C. Cells were collected by centrifugation at mid-log phase.

Protein Digestion and TMT Labeling

Hela cell lysates were digested by means of the phase-transfer surfactant (PTS)-aided trypsin digestion protocol as described previously[1]. *E. coli* lysates were digested after extracting proteins by methanol and chloroform precipitation as described previously[22]. After digestion, the samples were desalted using SDB-XC StageTips[3] and dried. Digested peptides (50 µg) were resuspended in 20 µL 200 mM 2-[4-2(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid (HEPES) pH 7.6, mixed with 0.1 mg of TMT6plex label reagents dissolved in 5 µL acetonitrile, and incubated for 1 h at room temperature. The reaction mixtures were quenched by the addition of hydroxylamine to give a final concentration of 0.33%. After 15 minutes incubation, the samples were acidified with trifluoroacetic acid, diluted to give an acetonitrile concentration below 5%, mixed to obtain the desired ratios, and desalted using SDB-XC StageTips.

LC/TIMS/Q/TOF analysis

NanoLC/TIMS/Q/TOF analyses were performed on a timsTOF Pro (Bruker, Bremen, Germany) connected to an Ultimate 3000 pump (Thermo Fisher Scientific) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptides were separated on self-pulled needle columns (150 mm length, 100 µm ID, 6 µm needle opening) packed with Reprosil-Pur 120 C18-AQ 3 µm reversed-phase material (Dr. Maisch, Ammerbuch, Germany). The injection volume was 5 µL, and the flow rate was 500 nL/min. The separation was achieved by applying a three-step linear gradient of 4−8% ACN in 5 min, 8−32% ACN in 60 min, 32−80% ACN in 5

min and 80% ACN for 10 min in 0.5% acetic acid. The TIMS section was operated with a 100 ms ramp time and a scan range of 0.6-1.5 Vs cm^{-2}. One cycle was composed of 1 MS scan followed by 10 PASEF MS/MS scans. MS and MS/MS spectra were recorded from *m/z* 100 to 1,700. A polygon filter was applied to not select singly charged ions. The quadrupole isolation width was set to 1 or 2 Da. The collision energy was ramped stepwise as a function of increasing ion mobility: 52 eV for 0-19% of the ramp time; 47 eV from 19-38%; 42 eV from 38-57%; 37 eV from 57-76%; and 32 eV for the remainder. For TMT reporter ion detection, TIMS Stepping function was used with the settings of Collision RF as 500 and 1,500, Collision Energy as 125% and 100%, Transfer Time as 25 µs and 60 µs, and Pre Pulse Storage Time as 8 µs and 12 µs, respectively.

LC/Q/Orbitrap/Ion Trap analysis

For comparison with LC/TIMS/Q/TOF, we analyzed the same samples on an Orbitrap Fusion Lumos (Thermo Fisher Scientific) connected to an Ultimate 3000 pump and an HTC-PAL autosampler. MS1 spectra were collected at a resolution of 120,000. Data-dependent Orbitrap (OT) MS2 scans were collected in the Top Speed mode using a cycle time of 3 s between Full MS scans. The quadrupole isolation width was set to 0.7 Da. The Orbitrap was operated at 50,000 resolution, and precursors were fragmented by high-energy collision dissociation (HCD) at a normalized collision energy (NCE) of 38%. In the method with SPS-MS3 analysis, Ion Trap (IT) MS2 spectra were collected at a CID collision energy of 35%. OT MS3 spectra utilized the same Orbitrap parameters as described above, except that the HCD collision energy was increased to 55%. Synchronous-precursor-selection (SPS) was enabled to include 10 MS2 fragment ions in the MS3 scan.

Database searching and data processing

Peptides and proteins were identified through automated database searching using Mascot v2.6 (Matrix Science, London) against the human and *E. coli* concatenated database from UniprotKB/Swiss-Prot release 2018/08 with a precursor mass tolerance of 30 ppm (timsTOF Pro) or 5 ppm (Orbitrap Fusion Lumos), a fragment ion mass tolerance of 0.05 Da (timsTOF Pro), 20 ppm (Orbitrap Fusion Lumos, MS2) or 0.5 Da (Orbitrap Fusion Lumos, SPS-MS3), and strict trypsin/P specificity allowing for up to 2 missed cleavages. Carbamidomethyl (C), TMT6plex (K), and TMT6plex (N-Term) were set as fixed modifications. Methionine oxidation was allowed as a variable modification. Identified peptides were rejected if the Mascot score was below the 95%

confidence limit based on the identity score of each peptide. False discovery rates at a peptide level of less than 1% were estimated by searching against a reversed decoy database. Reporter ion intensities were extracted from mgf files using in-house software. For calculation of peak capacities, retention time and ion mobility information were extracted using MaxQuant[4,5] (version 1.6.4.0).

Data availability

The MS raw data and analysis files have been deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the jPOST partner repository (http:// jpostdb.org)[6] with the data set identifier PXD018650.

References

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Figure S1

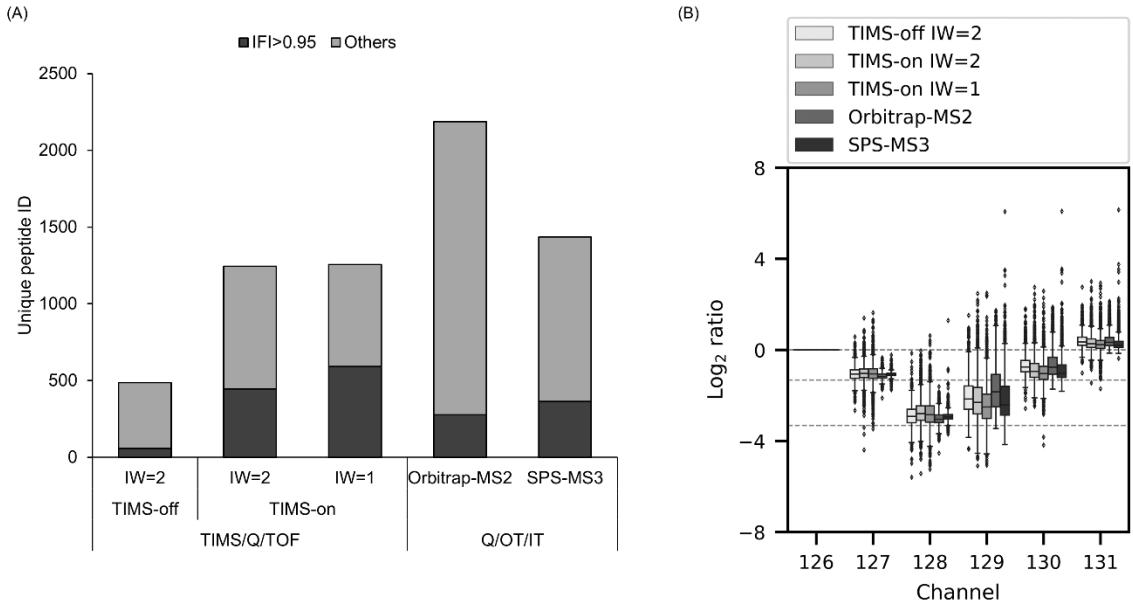

Figure S1. Precursor co-isolation effects were evaluated with all identified HeLa and *E. coli* peptides. (A) The ratios of low interference-containing HeLa peptide IDs are shown in stacked bar plots. The peptide IDs with IFI of more than 0.95 were considered as interference-free. (B) Compression of TMT ion ratios from *E. coli* peptides by background HeLa proteome. Box plots show the upper quartile, median, and lower quartile for the TMT ion ratios. Outliers were identified using box-plot statistics (threshold: 1.5 x the interquartile range (IQR)). Dashed lines represent expected ratios. IW: quadrupole isolation window.

Figure S2

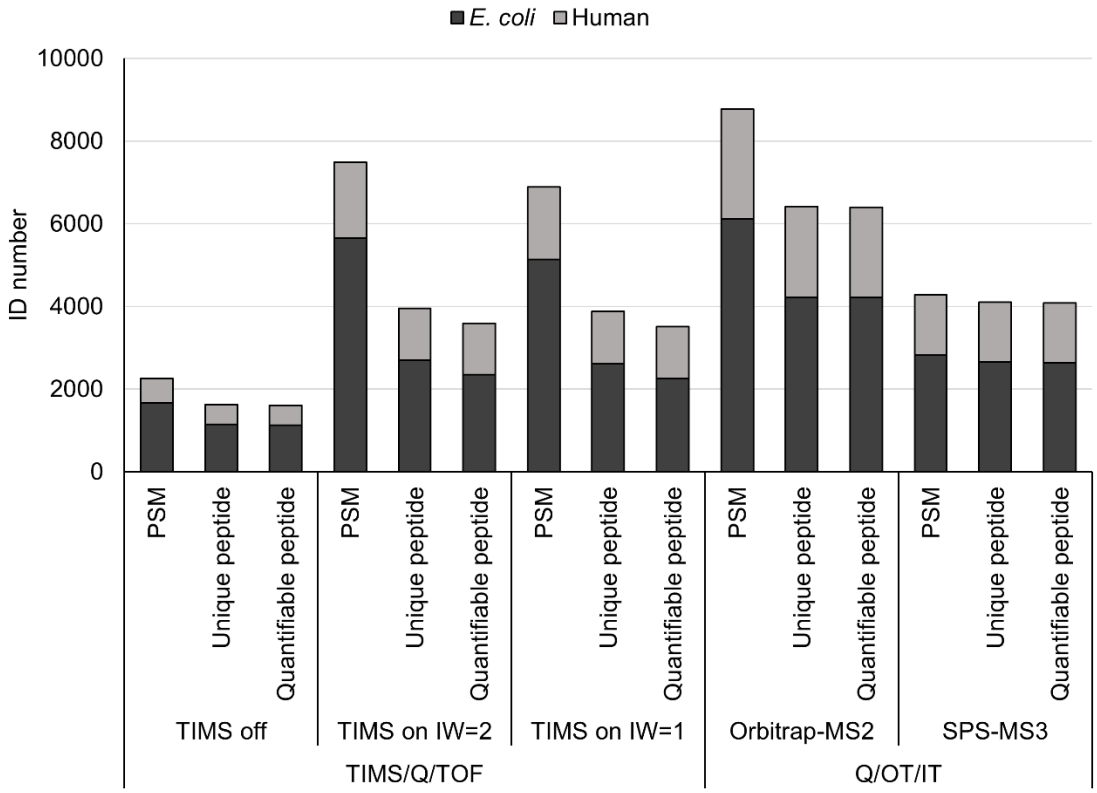

Figure S2. An overview of the identification results. Peptide spectrum match (PSM), unique peptide and quantifiable peptide numbers are shown. *E. coli* peptides with all the six reporter ions and human peptides with all the three reporter ions were considered as quantifiable peptide. IW: quadrupole isolation window.