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Quantitative accuracy and precision in multiplexed single-cell proteomics.

Authors:
Claudia Ctortecka¹,*, Karel Stejskal¹,²,³, Gabriela Krššáková¹,²,³, Sasha Mendjan² and Karl Mechtler¹,²,³,*.

Affiliations:

¹ Research Institute of Molecular Pathology (IMP), Vienna BioCenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria.
² Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna BioCenter (VBC), Dr. Bohr-Gasse 3, 1030 Vienna, Austria.
³ The Gregor Mendel Institute of Molecular Plant Biology of the Austrian Academy of Sciences (GMI), Vienna BioCenter (VBC), Dr. Bohr-Gasse 3, 1030 Vienna, Austria.

* Correspondence:
Claudia Ctortecka
Claudia.ctortecka@imp.ac.at
Karl Mechtler
Karl.mechtler@imp.ac.at
Research Institute of Molecular Pathology (IMP)
Campus Vienna Biocenter 1,
1030 Vienna,
Austria
Abstract

Single-cell proteomics workflows have considerably improved in sensitivity and reproducibility to characterize yet unknown biological phenomena. With the emergence of multiplexed single-cell proteomics, studies increasingly present single-cell measurements in conjunction with an abundant congruent carrier to improve precursor selection and enhance identifications. While these extreme carrier spikes are often >100-times more abundant than the investigated samples, undoubtedly the total ion current increases, but quantitative accuracy possibly is affected. We here focus on narrowly titrated carrier spikes (i.e., <20x) and assess their elimination for comparable sensitivity at superior accuracy. We find that subtle changes in the carrier ratio can severely impact measurement variability and describe alternative multiplexing strategies to evaluate data quality. Lastly, we demonstrate elevated replicate overlap while preserving acquisition throughput at improved quantitative accuracy with DIA-TMT and discuss optimized experimental designs for multiplexed proteomics of trace samples. This comprehensive benchmarking gives an overview of currently available techniques and guides conceptualizing the optimal single-cell proteomics experiment.
Introduction

Single-cell proteomics has been demonstrated as a viable complement to single-cell transcriptomics studies with striking sensitivity. Those single-cell analyses of presumed homogenous cell populations have attributed biological variability at both the transcriptome and the proteome levels.\(^1,2\) Previously, most protein analyses with single-cell resolution have been antibody-based or were limited to large cells such as oocytes.\(^3,4\) More recent technological innovations now allow for the hypothesis-free proteome analysis of single mammalian cells.\(^5-7\) First of such aimed at overcoming the limited sensitivity of available mass spectrometers through isobaric labeling. Isobaric labels use their identical mass with different isobaric distribution, allowing the simultaneous analysis of multiple samples and their quantification upon fragmentation within one MS\(^n\) scan. SCoPE-MS (Single Cell ProtEomics by Mass Spectrometry) combines TMT-multiplexed single cells with a 200-cell congruent carrier sample.\(^8\) The highly abundant carrier overcomes adsorptive losses before MS analysis, boosts the peptide signals during MS1 scans, therefore, increasing the signal to noise ratio (S/N) of the peptide precursor and serves fragment ions for identification. Following the initial publication, such congruent carrier spikes were employed to improve the triggering of peptides of interest at varying ratios from 25x up to 500x.\(^7,9,10\)

While an abundant carrier improves peptide identifications by increasing ion counts, selecting appropriate acquisition parameters and carrier compositions are crucial to preserving quantitative accuracy.\(^11-13\) Such imbalanced levels of multiplexed carrier samples with ratios of 200 or higher were demonstrated to possibly impact biological conclusions.\(^9,11,13,14\) The effects of extreme ratios on ion suppression\(^15\), ratio compression\(^16,17\) and quantification accuracy\(^9\) were previously described for standard and trace samples. The latter was addressed by increasing the number of ions sampled from each precursor\(^12,18,19\), constrained by the injection time (IT) and the automatic gain control (AGC) target. However, increasing the total number of ions sampled per precursor capitalizes the ions originating from the carrier within imbalanced samples.\(^13,20\) Additionally, the thereof lengthened cycle times reduce the number of MS/MS scans within one analytical run, inflating missing data between replicates due to precursor stochasticity.\(^21\)

Recently, Cheung and colleagues evaluated the ‘carrier proteome’ effects for trace samples proposing a maximum level of a congruent carrier (~20x) for optimal ion statistics and quantification accuracy.\(^13\) They and others thoroughly discuss the need for appropriate MS acquisition parameters and S/N filtering when performing MS-based single-cell proteomics experiments.\(^12,13\) Based on this, SCPCompanion, a tool for quality control and post-acquisition filtering of multiplexed carrier experiments, was introduced.\(^13\) However, it remains unclear which levels of excess carrier provide the optimal balance between sensitivity and accuracy.
or whether even drastically reduced ratios impair quantitative precision. Therefore, we demonstrate the applicability and confirm the need for S/N filtering for improved data quality in various multiplexed experimental designs. Moreover, with alternative multiplexing or acquisition strategies, we discuss the impact of precursor co-isolation and precursor stochasticity in the analysis of trace samples. This study aims to outline the advantages of available experimental setups and compile critical aspects, including identification rates, measurement accuracy, acquisition variability, and missing quantitative data in single-cell proteomics experiments.

Results
To directly compare diverse multiplexing strategies for ultra-low input samples, we performed labeling of HeLa digest in bulk. We combined 150 pg peptide input per TMT-label, which we will from now on refer to as 'single cell', with carrier titrations. Based on previous findings concerning accurate ratio reporting, we performed TMT10-plex experiments with a maximum carrier of 10x. As similar studies for the sixteen channel TMTpro are lacking, but several studies demonstrated quantitative implications of a >20x carrier, we extended this titration to 20x. Additionally, we evaluated a ‘dual carrier’ with the carrier distributed across two TMT channels to reduce extreme ratios but still boost ions. To overcome isobaric interference of the carrier sample, we did not include adjacent channels in the quantitative evaluations. TMT10-plex experiments ranged from 1.5 ng to 4.2 ng total peptide input for the no carrier and dual 10x carrier samples, respectively. Similarly, the sixteen plex TMTpro experiments span from 2.4 ng to 5.1 ng total peptide input for the no-carrier or the 20x/ dual 10x carrier samples, respectively (Fig. 1a). For SCoPE-MS and SCoPE2 (SCoPE) experiments, we adapted the respective acquisition parameters and experimental setup (detailed in the methods section), yielding a final peptide amount of 31.2 ng or 16.8 ng for SCoPE2 samples (Fig. 1a). SCoPE2 is described to result in about 1,000 proteins from real single-cell measurements. Adopting their experimental setup, we reproducibly identify ~1,300 proteins from diluted bulk samples and are therefore confident to reflect previously published protocols accurately.
Figure 1: Characterization of TMT multiplexed carrier titrations. (a) Graphical illustration of experimental setups and carrier compositions. (b) Identified proteins, peptide groups, PSMs, number of MS/MS scans, ID-rates, (c) median summed MS/MS intensity and (d) delta between expected to the acquired carrier to 'single cell' channel ratio across all MS/MS scans or only PSMs for SCoPE (brown), TMT10-plex (purple) and TMTpro (blue) samples at indicated carrier spikes. Median and median absolute deviation (mad) is shown.

Abundant carrier spikes enhance protein identifications but suffer from ratio compression.

We identified around 1,000 proteins for SCoPE-MS (1,167 protein groups, 31.2 ng), SCoPE2 (1,296 protein groups, 16.8 ng) and TMTpro 15-20x experiments (1,071 protein groups, 4.35 ng; 1,134 protein groups, 5.1 ng, respectively). Interestingly, SCoPE-MS experiments with a 200x carrier and 250 ms max IT yielded 30 % more MS/MS scans than SCoPE2 with 100x carrier and 300 ms max. IT (Fig. 1b). Nevertheless, the 50 % identification rate of SCoPE2 outperformed all other experimental setups presented in this study. In detail, TMTpro 10-20x samples triggered over 16,000 scans low intensity MS/MS scans with only 20 % identification rate and finally 15 % less protein identifications compared to SCoPE2 (Fig. 1b-c). Similarly, the 60 % increased peptide amount of respective TMTpro and TMT10-plex samples yields more intense MS/MS scans, resulting in superior identification rates (Fig. 1b). Additionally, the larger TMTpro reagents (419 Da) require only 30 % normalized collision energy (% NCE) compared to 34 % for the TMT10-plex tag (344 Da) to fragment the tag efficiently. Surprisingly,
although we injected similar peptide amounts, average MS/MS scans and identification rates declined in split-carrier experiments for TMT10-plex and TMTpro. Taken together, due to the increased peptide amount per injection, extreme carrier spikes result in high-intensity MS/MS spectra but only in conjunction with increased AGC targets and max. It serves the necessary fragment ions for enhanced protein identifications (Fig. 1b-c).

To investigate the quantitative accuracy of the multiplexed measurements, we first included all MS/MS scans with reporter ion (RI) signals and determined the delta of expected to acquired RI intensities. Strikingly, we observed that the ‘single cell’ signal in SCoPE-MS experiments was compressed by close to 50 %, but the carrier reduction of SCoPE2 drastically improved ratio compression to only 10 %. Next, we only considered identified scans and observed that the most abundant MS/MS scans provide sufficient fragment ions for identification but exhibit strong ratio compression (Fig. 1d; Supplemental Fig. 1). Further, only 29 % and 53 % of all MS/MS were within the carrier range (±/- 50 % of expected ratio) for SCoPE-MS and SCoPE2, respectively. This is in stark contrast to most balanced TMT10-plex and TMTpro experiments with more than 80 % of all MS/MS scans within the expected carrier ratio (Supplemental Fig. 1). We speculate that high-intensity MS/MS spectra exhibit elevated noise levels, which impacts measurement accuracy in the presence of an abundant carrier.

Considering the abundant carrier spike within the RI cluster, we dissected intensity profiles of individual channels and observed isobaric interference in SCoPE experiments, as expected (Fig. 2a-b). Therefore, they exclude the adjacent channel for single cells but establish an empty or reference channel for quality control and normalization.7,8,14 Lower carrier ratios did not exhibit isobaric interference, but adjacent channels were nevertheless excluded from subsequent analysis (Fig. 2c-f). To investigate whether measurement variation and signal intensity parallels, we correlated the coefficient of variation (CV) between ‘single cell’ RIs within one MS/MS scan to the average RI S/N. For this, we combined three technical replicates, removed all MS/MS scans with the only carrier or only missing RIs and determined the % CV. In our experimental setup, all ‘single cell’ channels are distributed equimolar, theoretically resulting in 0 % CV. As expected, most MS/MS scans with low S/N and multiple missing quantitative values show high variance (Fig. 2g-l). Despite enhanced average MS/MS intensity in SCoPE experiments, the mean ‘single cell’ S/N is lower than balanced TMT10-plex and TMTpro experiments (Fig. 1c; Fig. 2g-l). While SCoPE-MS experiments presented a decreased median of 25 % CV compared to 30 % in SCoPE2, the latter indicates a trend towards small % CV in high S/N MS/MS scans (Fig. 2g-h). Further, the 200x carrier in SCoPE-MS experiments leads to a detrimental suppression of the ‘single cell’ RIs giving rise to almost 75 % missing data (Fig. 2g, m). In contrast, the reduced 100x carrier yielded higher ‘single
cell' S/N, resulting in over 90 or 75 % of all MS/MS scans with at least one or two RIs, respectively (Fig. 2h, m).

Figure 2: Measurement stability, RI variability and quantification accuracy at various carrier ratios. RI intensity distributions based on all MS/MS scans for (a-b) SCoPE, (c-d) TMT10-plex and (e-f) TMTpro experiments at indicated carrier spikes. Percent CV across 'single cell' channels and log₁₀ mean RI S/N for (g-h) SCoPE, (i-j) TMT10-plex and (k-l) TMTpro samples at given carrier ratios. The horizontal solid line and dashed lines indicate median S/N across all MS/MS scans or post-S/N filtering, respectively. The vertical blue line specifies the S/N filter cut-off. Colors reflect the number of missing 'single cell' RIs per MS/MS scan. Percent missing quantitative data in (m) SCoPE, TMT10-plex and (n) TMTpro carrier titrations per MS/MS scan.

Based on analogous observations, S/N filtering was introduced via SCPCompanion, which we applied to our datasets, reducing the number of MS/MS scans and removing almost all scans with missing RIs. In detail, the SCoPE2 dataset with a minimum RI S/N of 12.6 eliminated over 96 % of all MS/MS scans but improved the median CV by 5 %. Similarly, from TMTpro, no carrier and 10x samples over 90 % of all MS/MS scans were removed, but median CV was enhanced by 10 % (Fig. 2h-l). In most experimental setups but especially across the limited carrier TMTpro samples, we observed a trend of low % CV in high S/N MS/MS scans (Fig. 2g-l). While identifications and measurement stability of TMTpro >10x and SCoPE2 experiments are comparable, ratio compression and quantitative inaccuracy due to the 100x carrier suggest
limiting the carrier to a maximum of 20x in combination with appropriate S/N filters (Fig. 1b; Fig. 2b, f, h, l). Further, the increased multiplexing capacities and consequently higher input of TMTpro samples yield enhanced protein identifications, with less missing data and superior quantitative accuracy than TMT10-plex experiments (Fig. 1b; Fig. 2i-n).

An alternative labeling strategy reveals frequent precursor co-isolation.

Based on these findings, we aimed to preserve an abundant carrier's advantages but remove the extreme ratio from the RI cluster for improved quantification accuracy. For this, in anticipation that the targeted quantitation of only ‘single cell’ derived peptides would greatly reduce the impact of inter-channel ratio compression, we made use of the defined mass difference provided by differential labeling of carrier and sample peptides with TMTzero (224.152 amu) and TMT10-plex (229.162 amu) reagent, respectively. We digested the samples with Lys-C to label peptides on the C- and the N-termini, increasing the mass difference between the carrier and the ‘single cell’ channels. We combine TMT10-plex labeled ‘single cell’ peptide input with an abundant TMTzero carrier at varying ratios starting with an equivalent of the ten TMT10-plex labeled cells (i.e., 1to1; 1.5 ng) up to 200 times the ‘single cell’ peptide input (i.e., 1to20; 30 ng; Fig. 3a).

Like inter-channel experiments, an abundant TMTzero carrier repeatedly increased high-intensity MS/MS scans, but the protein identifications declined with elevated carrier ratios (Fig. 3b-c). The TMTzero approach stemming from the different mass of the TMT10-plex and the TMTzero tag discrimination of ‘single cell’ versus carrier identifications is feasible. The 126 channel (i.e., fragment mass of TMTzero) was excluded from the subsequent analysis to overcome isobaric interference of mixed spectra, allowing to estimate co-isolation of a carrier precursor by the presence of a RI signal with 126.128 Da. This enables to estimate the frequency of only ‘single cell’, carrier or mixed MS/MS scans across the carrier titration. Interestingly, the relative frequency of co-isolating ‘single cell’ and carrier precursor increases with a 5x carrier but decreases at ≥10x carrier ratio (Fig. 3d). Based on this, we speculate that the 20 % reduced ID-rate across increasing TMTzero carrier is partly due to the reduced number of TMT10-plex precursors, owing to the mass-based segregation of carrier and sample peptide ion species in MS (Fig. 3b). Further, we observed that extreme congruent carrier spikes increase the chance of isolating only a carrier precursor 5-fold compared to balanced experiments (Fig. 3d). These findings indicate that in conjunction with an abundant carrier, it is likely that most PSMs correspond to a carrier-derived identification rather than the single cells. Consequently, the carrier must equally represent all single-cell precursors for accurate acquisition, which could be challenging for heterogeneous samples.
Figure 3: Measurement variability with inter-carrier spikes using TMT<sub>zero</sub>. (a) Graphical illustration of the TMT<sub>zero</sub> triggering strategy (b) Protein groups, peptide groups, PSMs, MS/MS scans, ID rates and (c) median summed MS/MS intensity of TMT<sub>zero</sub> at indicated carrier spikes. (d) Percent median frequency of MS/MS scans across 'single cell' RIs (sc only), only carrier RI (carrier only) and co-isolation of carrier and 'single cell' precursors within one MS/MS scan (both) at indicated carrier spikes. Median and mad are shown. (e-f) RI intensity distribution across all MS/MS scans in TMT<sub>zero</sub> experiments. Colors indicate different TMT channels.

Despite low protein identifications, we evaluated measurement stability and quantification accuracy of the TMT<sub>zero</sub> experimental setups. Corroborating earlier observations with similar inter-channel carrier ratios, we observed stable 'single cell' signal but elevated isobaric interference in the 126 and adjacent channels (Fig. 3e-f). While the median CV below 25 % across all MS/MS scans in TMT<sub>zero</sub> 1x experiments decreased to only 13 % after S/N filtering, a ≥10x carrier spike resulted in frequent missing values and up to 30 % CV (Supplemental Fig. 2a-c). We conclude that removing the carrier from the multiplexed 'single cells' via TMT<sub>zero</sub> elevates 'single cell' RI S/N but at extreme ratios impairs protein identifications and measurement accuracy (Fig. 3b, Supplemental Fig. 2a-c). Moreover, the mass difference between 'single cells' and carrier revealed close to 50 % co-isolation and up to 60 % carrier-only quantitative data in imbalanced ultra-low input samples (Fig. 3c). This suggests that a congruent carrier indeed improves MS/MS triggering and serves fragment ions; however, the identical features do not discern between solely a carrier or a single cell PSM. Consequently, we speculate that all multiplexed ultra-low input experiments suffer a similar frequency of co-isolation and convoluted RI clusters.
Intentional co-isolation reduces missing data in ultra-low input samples.

TMT<sub>zero</sub> experiments allowed to estimate unintentional co-isolation, RI convolution and its impact on MS/MS-based quantification accuracy (Fig. 3c-f; Supplemental Fig. 2a-c), as previously discussed by many.\textsuperscript{13,17,22–28} Additionally, we and others found detrimental amounts of missing data in multi-batch data-dependent proteomics experiments (Fig. 2m-n).\textsuperscript{21,29–32} This is most prominently addressed via data-independent acquisition (DIA), which our group recently extended to multiplexed samples.\textsuperscript{32} While co-isolation is non-negotiable with our 5 Th DIA-TMT method, the prescheduled acquisition strategy theoretically generates no-missing data across multiple analytical runs (Fig. 4a). In detail, our small window DIA-TMT method allows us to uniformly acquire both signal and noise in an unbiased and reproducible manner. The stochastic nature of data-dependent acquisition (DDA) methods, especially in analyzing ultra-low input samples, generates detrimental amounts of missing data. Consequently, this demands most quantitative data be computationally generated across large sample cohorts. However, the obvious application of any single-cell technology to characterize tissues or cellular subpopulations requires quantitative profiles of hundreds or thousands of samples, which is facilitated via our sensitive DIA-TMT strategy.\textsuperscript{32}

Accordingly, we evaluated the quantification accuracy and reproducibility of DIA-TMT in conjunction with the TMTpro carrier titrations. Interestingly, the DIA-TMT samples yielded slightly increased protein identifications at similar PSMs than corresponding DDA measurements (Fig. 4b). We speculate that this is because of the decreased cycle time and optimized fragmentation due to the stepped collision energy providing optimal fragmentation for co-isolated precursors with different charge states. Further, the measurement variance of no carrier samples is comparable to DDA experiments but increases in combination with a congruent carrier spike (Fig. 2e-f; Fig. 4c-d). While we observed similar overall RI intensities for DDA and DIA measurements, median RI S/N levels increased by 40 \% for the latter.
Figure 4: Impact of intentional co-isolation on ‘single cell’ variability and accuracy. (a) Graphical illustration of DIA-TMT acquisition strategies with carrier titrations. (b) Protein groups, peptide groups and MS/MS scans of DIA-TMT samples at indicated carrier spikes. Median and mad is shown. (c-d) RI intensity distributions across all MS/MS scans and (e-f) percent CV against log_{10} mean RI S/N for DIA-TMT samples at indicated carrier spikes. The horizontal solid line and dashed lines indicate median S/N across all MS/MS scans or post-S/N filtering, respectively. The vertical blue line indicates the S/N filter cut-off. Colors indicate the number of missing single-cell RIs. Replicate overlap of (g) DDA and corresponding (h) DIA-TMT samples based on unique peptides.

Interestingly, despite some distorted scans at high RI S/N, the DIA-TMT strategy presented close to 20 % CV between ‘single cells’ across all carrier titrations. As previously discussed, S/N filtering further decreased the median CV to around 10 %, corresponding to the lowest ‘single cell’ variation across all experiments (Fig. 4e-f). Lastly, to directly compare replicate overlaps in DDA and DIA, we intersected unique peptide identifications. DIA-TMT resulted in a 25 % replicate overlap increase in contrast to corresponding DDA samples across all carrier titrations (Fig. 4g-h). With low measurement variance, exceptional accuracy and close to 90% replicate overlap, DIA-TMT demonstrates its potential to overcome missing data at comparable quantitative accuracy in ultra-low input samples.
Discussion

We dissect different multiplexing strategies at extensive carrier titrations to investigate the impact on identification rates, reproducibility, quantification accuracy and measurement interference. Interestingly, we observe almost a linear increase in protein identifications across the low carrier titrations for both isobaric reagents. Moreover, we find that congruent carrier spikes, effectively contribute ions to the ‘single cells’ and consequently increase MS/MS intensities. Already a small carrier (<20x) improves ID-rates, which eventually plateaus at ≥100x ratio for 60-minute gradients. Interestingly, the SCoPE2 acquisition parameters and the 50 % carrier decrease compared to SCoPE-MS, already reduced ion suppression, increased ID rates, and improved measurement accuracy (Fig. 1b, d; Fig. 2a-b, g-h; Fig. 5a-c; Supplemental Fig. 1). While even lower carrier spikes on average resulted in less intense MS/MS scans and lower ID rates, with less extreme RI ratios we observed no ratio compression, less measurement variability, and predominantly fewer missing values (Fig. 1b-c; Fig. 2i-j; Fig. 5a-c; Supplemental Fig. 1). However, even non-stringent S/N filtering often eliminated around 90 % of MS/MS scans, suggesting that the RI S/N of such ultra-low input samples is suboptimal across experimental setups (Fig. 2g-l). This is especially concerning as diluted bulk digests utilized in this study contain less chemical background than real single-cell samples.13,33

Moreover, the no-carrier samples demonstrated outstanding measurement accuracy and reduced variability, especially in TMTpro samples (Fig. 2c, e, h, j; Fig. 5b-c). Despite comparable peptide input and total ion current of 10x TMT10-plex or 5x TMTpro samples, the latter yielded 25 % more MS/MS scans and protein identifications. Due to similar ‘single cell’ CVs with both isobaric reagents, we speculate that the global identification-increase with TMTpro results from different fragmentation patterns (Fig. 1b). This further provided a major advantage to overcome detrimental precursor stochasticity and improve reproducibility with our multiplexed DIA strategy. Consequentially, the DIA-TMT acquisition of TMTpro samples provided comparable protein identifications, superior data completeness resulting in close to 100 % replicate overlap at reduced measurement variance (Fig. 4c-h; Fig. 5a-d).
Figure 5: Cumulative comparison of measurement accuracy, variance, and reproducibility across all experimental setups. (a) Identified proteins groups, (b) delta carrier to ‘single cell’ intensities (optimal: 0), (c) percent of MS/MS scans with ‘single cell’ RI within +/- 50% of expected carrier ratio and (d) percent replicate overlap across triplicates based on unique peptides for SCoPE (brown), TMT10-plex (purple), TMTpro (blue), TMTzero (turquoise) and DIA-TMT (red). Bar graphs display median and error bars indicate mad.

The alternative triggering via the TMTzero approach confirmed that abundant carrier spikes dominate low abundant ‘single cell’ MS/MS spectra, even if segregated from the RI cluster (Fig. 3c-d). Importantly, such co-isolated MS/MS spectra may comprise mainly of carrier b- and y-ions while the presence of any RI signal is used for quantification of ‘single cells’.\textsuperscript{13,14} Even though high TMTzero RI intensities indicate that the acquisition strategy might overestimate the prevalence of mixed spectra, already at ≥50x carrier spikes most MS/MS scans either comprise only carrier or co-isolated precursors (Fig. 3d-f). While background and possible contaminations are reduced to a minimum in our bulk dilutions, this might affect the biological interpretation of real single-cell samples. Contrarily, the intentional co-isolation in a prescheduled acquisition scheme using DIA-TMT successfully defines cell-types, underrepresented single protein knockouts, and presents less quantitative variance at theoretically no missing data (Fig. 4e-f).\textsuperscript{32} The need for quantitative data imputation in standard single cell DDA data is highly elevated compared to standard input, while the absence of technical replicates challenges reliability.\textsuperscript{12,21,34–36} Despite the intentional co-isolation in DIA-TMT, eliminating precursor stochasticity drastically improved accuracy and sensitivity (Fig. 4b-f).\textsuperscript{32} Hence we speculate that large proportions computationally generated quantitative data
introduced by reduced replicate overlap in combination with precursor co-isolation and extreme carrier ratios are particularly error prone.

We present a comprehensive overview of currently available multiplexed single-cell experimental proteomics setups considering protein identifications, measurement variance, quantitative accuracy, and missing data. We find that specific experimental questions require individual prioritization of parameters when designing ultra-low input or single-cell experiments. Based on these findings, we conclude that low carrier spikes (i.e., ≤20x) in conjunction with sensitive instrument setup are pivotal for accurate single-cell proteomics analysis and thus any biological interpretation (Fig. 5a-d). With more sensitive instrumentation and dedicated experimental approaches single-cell proteomics has achieved remarkable proteome depth and throughput. Nevertheless, many parameters such as cell state, sample preparation, chromatography and ultimately the acquisition style impact data quality and especially the meaningfulness. We are confident that efficient sample preparation workflows, novel instrumentation and tightly controlled computational approaches will drive biological applications and further demonstrate the impact of hypothesis-free proteome measurements at single-cell resolution.
Methods

Sample preparation

Cells were pelleted, washed with phosphate-buffered saline (PBS) and stored at -80 °C until further processing. Cell pellets were lysed using a methanol:chloroform:water solution (4:1:3), sonicated and dried to completeness in a speed-vac concentrator. The dry protein pellets were then resuspended in 8M urea in 10 mM HCl. Prior to alkylation with iodoacetamide (40 mM, 30 min at room temperature (RT), in the dark), the samples were adjusted to 200 mM Tris/HCl pH 8.0 and reduced using dithiothreitol (50 mM, 37 °C, 30 min). The reduced and alkylated samples were diluted to a final concentration of 4 M urea in 100 mM Tris/HCl pH 8 and digested with endoproteinase LysC (Wako, enzyme:protein = 1:100) for 3 hours at 37°C, if indicated. Tryptic samples were subsequently diluted to 2 M urea in 100 mM Tris/HCl pH 8 and digested with trypsin (Promega, enzyme:protein = 1:100) over night at 37 °C. After proteolytic degradation, the samples were adjusted to pH 2 using 10% trifluoroacetic acid (TFA) and desalted using C18 solid-phase extraction cartridges (SPE, C18 Sep-pak, 200 mg Waters) eluted with 40% acetonitrile (ACN) in 0.1% TFA. SPE eluate volume was reduced using a vacuum centrifuge and labeled according to manufacturer's instructions. Briefly, samples were labeled in 100 mM TEAB and 10% ACN for 1 hour at RT. Unreacted TMT reagent was quenched with 5% hydroxylamine/HCl for 20 minutes at RT and subsequently mixed corresponding to each sample pool.

LC MS/MS analysis

Samples were measured on a Orbitrap Exploris 480 Mass Spectrometer (Thermo Fisher Scientific) with a reversed phase Dionex UltiMate 3000 high-performance liquid chromatography (HPLC) RSLCnano System coupled via a Nanospray Flex ion source equipped with FAIMS Pro (Thermo Fisher Scientific). Chromatographic separation was performed on nanoEase M/Z Peptide BEH C18 Column (130Å, 1.7 µm, 75 µm x 150 mm, Waters, Germany) developing a two-step solvent gradient ranging from 1.2 to 30% over 90 min and 30 to 48% ACN in 0.08% formic acid within 20 min, at a flow rate of 250 nL/min. For all experiments FAIMS was operated at a constant compensation voltage of -50 V.

SCoPE-MS and SCoPE2 acquisition strategies were performed as published, with small adaptations to the mass spectrometer used in this study. For SCoPE-MS samples briefly, full MS data were acquired in the range of 395–1,800 m/z at a resolution of 60,000. The maximum AGC and IT was set to 3e6 and automatic inject time. Top 20 multiply charged precursor ions (2-3) with a minimum intensity of 2e4 were isolated for higher-energy collisional dissociation (HCD) MS/MS using a 1 Th wide isolation window. Precursors were accumulated until they
either reached an AGC target value of $10^5$, or a maximum IT of 250 ms. MS/MS data were generated with a NCE of 34, at a resolution of 60,000, with the first mass fixed to 110 m/z. Upon first fragmentation precursor ions were dynamically excluded (dynEx) for 20 seconds. For SCoPE2 samples briefly, full MS data were acquired in a range of 450-1,600 m/z at a resolution of 60,000 with a maximum AGC target of $30^6$ and automatic IT. Top 7 multiply charged precursors (2-4) with a minimum intensity of 2e4 were isolated for HCD fragmentation using a 0.7 Th isolation window with 0.3 Th offset. Selected precursors were accumulated for maximum AGC target of $10^5$ or 300 ms IT. MS/MS scans were acquired at NCE 34, at a resolution of 60,000, with the first mass fixed to 110 m/z and a dynEx of 30 s.

Full MS data of multiplexed carrier experiments were acquired in a range of 375-1,200 m/z with a maximum AGC target of $30^6$ and automatic inject time at 120,000 resolution. Top 10 multiply charged precursors (2-5) over a minimum intensity of 5e3 were isolated using a 0.7 Th isolation window. MS/MS scans were acquired at a resolution of 60,000 at a fixed first mass of 110 m/z with a maximum AGC target of $10^5$ or IT of 118 ms. Previously isolated precursors were subsequently excluded from fragmentation with dynEx of 120 s. TMT10-plex precursors were fragmented at an NCE of 34 and TMTpro at an NCE of 32.

TMT$_{zero}$ experiments were acquired with a MS1 resolution of 60,000 over a scan range of 375-1,200 m/z with a maximum AGC target of $30^6$ and automatic IT. Multiply charged precursors (2-3) over a minimum intensity of 2e4 were selected using a ‘targeted mass-difference method’ for 3 sec cycle time. For this, the delta masses of 5.0105 Da or 10.0209 Da were selected for MS/MS. Within the targeted mass-differences method, only precursors were selected for triggering whenever the partner intensity matched relative to the most intense precursor, with a mass tolerance of 10 ppm. Targeted precursors were isolated with a 0.7 Th isolation window at an AGC target of $10^5$, for maximum 250 ms IT. Selected precursors were fragmented at an NCE of 34 with MS/MS scan resolution of 45,000 and subsequently excluded with a dynEx of 100 s.

DIA-TMT experiments were acquired in the range of 400–800 m/z at a resolution of 45,000. The AGC was set to $20^5$ and the maximum IT was automatically determined for each scan. DIA windows were acquired with a 5 Th isolation windows and 1 Th overlap with the first mass fixed to 120 m/z. This corresponds to 80 DIA windows a cycle time of 6 seconds. TMT10-plex samples were fragmented with a stepped NCE of 30, 37.5, 45 and TMTpro samples with a stepped NCE of 25, 30, 40.
Data analysis

RI quantification was performed within the Proteome Discoverer environment (version 2.3.0.484) using the in-house developed, freely available PD node “IMP-Hyperplex” (pd-nodes.org) to extract the intensity and S/N values for all a RIs at a mass tolerance of 10 ppm. The software extracts raw RI intensities or S/N values from respective spectra for quantification. Quality control of raw data was performed using RawTools. Venn diagrams were generated using BioVenn.

Peptide identification was performed using the standard parameters in SpectroMine™ 2.0 against the human reference proteome sequence database (UniProt; version: 2018-11-26 accessed April 2019). Briefly, we performed a specific tryptic search with maximum two missed cleavages limiting peptides to 7-52 amino acids. We included carbamidomethylation on cysteins, TMT or TMTpro on lysine and all N-terms as fixed modifications. As variable modifications, acetylation on protein N-terms and methionine oxidation were included. By default, SpectroMine™ performs ideal mass tolerance calculations at MS and MS/MS levels and mass calibration for each feature. Subsequently, identifications were filtered for 1% FDR on PSM, peptide and protein-group level for further processing.

TMT spectral libraries were generated from the DDA files with above indicated parameters based on the highest input and customized using a script provided by Oliver Bernhard from Biognosys and available on GitHub (ctorteckac/DIA-TMT). Libraries were searched with Spectronaut™ performing mass tolerance calculations and spectra matching based on extensive mass calibration. The most intense peak within the previously defined mass tolerance is then selected and matched with a minimum of three matching fragment ions per MS/MS scan. Retention time alignments is based on iRT Reference Strategy with minimum \( R^2 \) 0.8. ‘Mutated’ decoys with scrambled sequences are filtered for 1% FDR on precursor and protein levels.
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Author Contribution

C.C. designed the study, prepared the samples, performed the data analysis, and wrote the manuscript. K.S., G.K. and C.C. acquired samples and maintained equipment. S.M. and K.M. supervised the study. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data Availability

All mass spectrometry-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier [PXD027912]

Reviewer account details:

Username: reviewer_pxd027912@ebi.ac.uk
Password: G1jNd1XX