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The pursuit of ultrasensitive phosphoproteomics to unravel signalling in rare cells

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Abstract

Mass spectrometry-based phosphoproteomic workflows enable routine study of signalling dynamics in contexts where cells are abundant. However, the analysis of cell signalling governed by phosphorylation requires an additional enrichment step which poses a significant challenge when dealing with limited material. The development of sensitive techniques that allow phosphoproteomic analysis of a few hundred cells is increasingly enabling researchers to disambiguate the complexity of protein signalling networks within heterogeneous cell populations. The imminent task at hand is to apply these techniques in research contexts guided by the available biological material to address complex questions about cellular function. Examples range from characterising differential treatment responses in distinct cell populations to investigating rare cell types from primary patient material or *in vivo* models. To achieve this, adapted protocols need to consider appropriate isolation of specific cells, simplified sample processing to avoid losses, labelling and multiplexing, and optimised analytical methodologies. Here, we discuss these aspects of the workflow, highlighting how innovations from low-input and single-cell proteomics can be adapted to drive low-input phosphoproteomics forward.

Introduction

Cellular heterogeneity is an inherent property of all tissues. This phenotypic diversity is essential for the normal functioning of biological systems as well as the origin of dysfunction in many diseases. Phenotypic heterogeneity is the result of cell specialisation, which occurs through proteome remodelling and regulation of physiological processes. It is becoming increasingly evident that distinct subsets of cells are responsible for many disease outcomes, including drug responsiveness, therapy resistance, cancer origins and metastasis, and infection [1-6]. These cellular behaviours are largely governed by signalling networks dependent on protein abundance, localisation and activity, mediated by post-translational modifications (PTMs). Of specific interest, phosphorylation events carried out by kinases and phosphatases trigger downstream signalling cascades. While proteomic analysis of rare cell types has yielded significant insights into the post-transcriptional mechanisms governing cell diversity [7-12], phosphoproteomics is required to provide a functional snapshot of signalling dynamics [13-17].

With the widespread adoption of next-generation sequencing technologies, research focused on understanding the mechanisms of cell diversity has relied heavily on DNA and RNA sequencing approaches. The ability to amplify genetic material provides a significant advantage over proteomic methods for the study of cell populations present at low frequencies. Indeed, these techniques have enabled the identification of transcriptional programs governing heterogeneity and cell fate decisions [4,6]. While

amplification does improve the sensitivity of sequencing methods, it also introduces biases and stochastic artefacts, leading to the unequal amplification of different molecules [18,19]. Proteomics enables the direct measurement of protein abundances and their post-translational modifications, providing a more accurate reflection of cellular processes. Studying the proteome is challenging owing to the wide dynamic range of protein abundances and myriad of proteoforms [20,21]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the sole unbiased technique capable of quantitatively analysing full proteomes [22,23]. Traditional proteomic approaches rely on multiple sample processing steps, including centrifugation, pipetting, desalting, sonication, and transfer of samples between multiple reaction tubes [24]. Chemical detergents or chaotropic denaturants are used to extract proteins which need to be removed by downstream desalting or buffer exchange methods before LC-MS/MS analysis [25]. Due to sample losses at every stage of processing, these methods require protein from millions of cells to achieve comprehensive proteome coverage, thus limiting the scope of the biological questions that can be addressed using these traditional techniques. That being said, low-input proteomic methods are advancing rapidly, enabling comprehensive quantification of proteins in rare cells, thus overcoming the limitation of using mRNA reads as an inaccurate proxy for protein expression [26-30]. These new low-input methods have been developed to overcome the limitations of traditional workflows that are only suitable for high-input samples. These challenges include, but are not limited to, cell isolation and recovery, sample losses during multi-step workflows, sub-optimal chemical reaction kinetics, and reduced quantification accuracy associated with sample complexity and dynamic range [31-33].

Despite progress in low-input sample preparation and acquisition methods, our ability to comprehensively profile post-translational modifications (PTMs) that govern signalling networks has remained limited to higher-input, bulk biological samples [34,35]. Owing to the substoichiometric nature of phosphorylated proteoforms, additional enrichment strategies are required to isolate phosphorylated peptides prior to LC-MS/MS analysis to reduce sample complexity and improve their detectability [36,37]. Given that phosphorylated proteins are heavily masked by the total proteome, the challenges associated with isolating and preparing low cell numbers for proteomic analysis are compounded when studying phosphorylation events [38] **(Fig. 1)**. Innovative approaches are needed to overcome these limitations and maximise their potential impact on scientific advancements.

To date, the field has broadly focused on methodological developments across different aspects of the phosphoproteomic workflow, including sample preparation and enrichment, advanced chromatography techniques, MS instrumentation and computational analysis [39-45]. Indeed, these leaps have demonstrated that it is possible to achieve sensitive and quantitative phosphoproteomics on low-input samples **(Fig. 2)**. However, the next challenge is to consolidate these advances and improve widespread adoption in application-driven proteomic research laboratories. Custom hardware modifications are generally not accessible for most laboratories, so sample preparation improvements should be prioritised to maximise the analytical power of state-of-the-art mass spectrometers. This requires the development of simple, accessible, cost-effective workflows which can be robustly integrated with existing cell isolation techniques and applied to a wide range of biologically relevant systems and models. Here, we review current trends in low-input phosphoproteomic approaches with applications for studying cell populations of interest. Our discussion includes

practical guidelines for each stage of the sample preparation workflow, covering essential aspects such as cell isolation, lysis and digestion, multiplexing, phosphopeptide enrichment, fractionation and MS acquisition methods.

Cell isolation

Cell isolation techniques are essential for studying specific cell populations from heterogeneous samples. Laser capture microdissection (LCM) and fluorescence-activated cell sorting (FACS) are the most commonly employed methods for single-cell isolation. Magnetic-activated cell sorting (MACS) is an alternative approach to FACS and LCM, offering rapid antibody-based enrichment of cell populations **(Fig. 3)**. All these techniques are capable of yielding purified cells of a specific subtype, but the choice between these methods depends on the sample complexity and desired level of precision, with each method offering its own advantages and limitations [46]. These should be considered in the context of the biological question being addressed when designing and optimising low-input phosphoproteomic workflows. While these methods are widely used for low-input and single-cell proteomics, integrating them with phosphoproteomics is more challenging because labile phosphorylation must be preserved during isolation and processing. Adapting strategies from low-input and single-cell proteomics will improve compatibility with cell isolation techniques and expand the scope of biological questions accessible to phosphoproteomic analysis.

MACS employs magnetic nanoparticles conjugated with antibodies to isolate specific cells, providing a simpler and more cost-effective alternative to FACS [47]. MACS (Magnetic-Activated Cell Sorting) is typically used for isolating and purifying cells from the hematopoietic system due to the availability of well-characterised cell surface markers, scalability and efficiency. Although MACS is effective for purifying large quantities of cells, it is less frequently used for isolating rare cell populations, particularly when sample material is scarce [48]. While it is possible to combine low-input proteomic sample preparation techniques with MACS, this approach is seldom reported in the literature and will not be a focus of this review.

LCM allows precise isolation of individual cells or small clusters from tissue sections using a microscope and laser-based microdissection system, making it ideal for preserving spatial information within tissues [49]. The coupling of LCM to LC-MS/MS has been described extensively, whereby a laser is directed towards a predefined region in a processed tissue section, resulting in the selective detachment of the cells from the surrounding tissue [50-57]. Depending on the LCM system used, the captured cells may be catapulted into a collection tube using a defocused laser pulse or directly collected onto a capture cap or adhesive cap for subsequent retrieval [53]. With recent advances in automated sample preparation procedures and computational methods, it is now possible to perform unbiased ultrasensitive spatial proteomics on low-cell numbers, and even single cells, from fixed tissue [53,54,56]. Notably, Makhmut *et al*. describe an integrated workflow combining LCM with an automated robotic system for end-to-end sample processing [58]. Using this method, they were able to identify ~2,000 proteins from 4,000 μm^2 regions of immune and epithelial cells. Other technologies, such as the Single Cellome™ System SS2000, are being employed to expand the use of laser microdissection to live cells with the use of confocal microscopy. Thus far, this approach has only been applied to lipidomics and metabolomics, but it shows great promise for future phosphoproteomic analyses of specific cell types [59,60].

FACS utilises fluorescent labels and flow cytometry to separate cells using high-speed streams of charged droplets based on morphological parameters and the expression of extracellular and intracellular proteins [48]. FACS is widely used for live-cell isolation due to its high specificity, low technical requirements, throughput, and suitability for various cell types [61]. FACS has already been paired with proteomic workflows; however, adoption for low-input phosphoproteomics has been limited. This is partly due to the multi-step processing involved, which results in significant sample loss. During FACS sorting, cells are suspended in a sheath fluid (usually phosphate-buffered saline (PBS)) to maintain osmotic pressure and preserve cell viability. When sorting thousands of cells, the sheath fluid collected post-sorting represents a major challenge for low-input sample preparation. Removing the buffer can lead to sample loss, while processing the sample in the buffer can impair lysis and digestion due to poor reaction kinetics. Attempts have been made to overcome these limitations, the most common of which is arguably centrifugation followed by removal of the supernatant, with the option of additional PBS washes and lyophilisation. Following the isolation of FACS-sorted cells, both Sielaff *et al*., and Alfredsson *et al*., carried out a wash step with PBS before lysis, which was effective at removing most unwanted contaminants, such as residual BSA (Bovine Serum Albumin) and EDTA (Ethylenediaminetetraacetic acid) present in the FACS buffer [62,63]. Amon *et al*., successfully used this method to quantify >5,000 protein groups from 25,000 sorted hematopoietic stem and progenitor cells [29]. Indeed, they noted that leaving 50 µL on top of the pellet was critical for the reproducibility of material recovery to avoid losing nonadherent pelleted cells. Other groups have demonstrated the efficiency of both acetone precipitation and filter-based buffer exchange techniques for FACS-compatible proteomics [64,65]. In addition to the practical challenges of preparing FACS-sorted cells for phosphoproteomics, the process of sorting itself can perturb the phosphoproteome. This is most problematic in acute stimulation signalling experiments, where the sorting duration exceeds the treatment window. For more stable phenotypes, such as defined cell states or differentiation stages, FACS remains a practical option, albeit with the aforementioned limitations. To overcome these challenges, lower-stress sorting alternatives are emerging that avoid the high voltages and pressures of conventional FACS instruments and may better preserve endogenous phosphorylation events. CellenONE® leverages piezo-acoustic droplet generation technology and real-time imaging for gentle, low-volume isolation of single cells. Imaging-based cell isolation methods are generally slower than laser-based techniques due to the limitation of generating quality images of fast-flowing cells [66]. Hence, the cellenONE® device is better suited for handling small quantities of cells (<1,000), while FACS is more appropriate for high-throughput applications, comfortably sorting thousands of cells per second. The cellenONE® system has been widely applied to single-cell proteomics, demonstrating marked improvements in protein recovery, sensitivity and quantification accuracy [67-72]. However, its potential for low-input phosphoproteomics has yet to be explored.

Cell isolation techniques are required for studying rare cell populations, so their impact on sample preparation should not be overlooked when optimising downstream phosphoproteomic workflows. Simulating low-input conditions by aliquoting bulk digests is valuable for technical optimisation but does not recapitulate the real conditions required for isolating biologically relevant samples. Consequently, end-to-end phosphoproteomic protocols that incorporate cell isolation techniques are needed.

Sample lysis and digestion

Preparing isolated cells for phosphoproteomics requires multistep processing, which starts with protein extraction and digestion **(Fig. 4A)**. Maximising protein recovery is especially important for low-input samples where the available material is already limited. Optimising cell lysis and protein digestion to prevent excess sample loss can significantly improve the quality and robustness of data acquisition [73]. Detergent- or chaotropic-based lysis buffers are the most widely used for bottom-up proteomics, but applying traditional high-input protein extraction protocols to FACS-compatible low-input workflows is not trivial. Maintaining optimal lysis conditions requires removing FACS buffer by either pelleting or filtering, which risks excessive and irreproducible sample loss. Conversely, prioritising sample recovery and persevering the supernatant after FACS dilutes the lysis buffer and increases the reaction volume, resulting in less efficient lysis and digestion.

Several studies have successfully combined FACS with urea-based lysis methods for proteomic analysis, but these techniques have not yet been applied to low-input phosphoproteomics. Amon *et al*. isolated and analysed 25,000 hematopoietic stem and progenitor cells [29]. To generate reproducible, high-quality data, they specify the importance of using low protein-binding tubes to minimise the loss of hydrophobic peptides and small volumes (10 µL) for lysis and digestion to improve reaction efficiencies. Sielaff *et al*. FACS-sorted 25,000 bone marrow-derived macrophages and compared three cleanup techniques: filter-aided sample preparation (FASP), single-pot solid-phase-enhanced sample preparation (SP3), and a commercial kit based on the in-StageTip (iST) method [62]. They showed that SP3 and iST methods provided the highest proteome coverage. The poor performance observed in the FASP samples was attributed to a bias toward peptides with lower molecular weights, suggesting that larger peptides were partially retained on the membrane.

Others have combined LCM or FACS with detergent-based lysis buffers, with successful sample recovery and proteomic analysis down to 100 cells [65,74-77]. Of note, Friedrich *et al*. optimised protein extraction from formalin-fixed paraffin-embedded (FFPE) tissue samples and showed that sodium dodecyl sulphate (SDS) followed by SP3 cleanup outperforms sodium deoxycholate (SDC) and Rapigest lysis methods, in terms of total identified proteins [75]. Similarly, Muller *et al*. automated the SP3 protocol using a liquid-handling robot [76]. Using this method, they were able to reproducibly quantify 500–1,000 proteins from an input of 100–1,000 manually counted and isolated HeLa cells and then applied this approach to FFPE tissue samples. More recently, Üresin *et al*. were able to vastly improve on previous studies by identifying ~7,000 proteins from 500 haematopoietic cells. These cells were FACS-sorted from murine bone marrow directly into detergent-based lysis buffer prior to digestion and finally desalting using the Evotip Pure™ tips [77]. A major success of this study is the use of off-the-shelf consumables to prepare and analyse biologically relevant FACS-sorted samples. This approach underscores the advancements in rare cell proteomics, demonstrating the ability to achieve deep coverage and yield novel insights from limited cellular material.

The single-cell proteomics field has broadly abandoned traditional detergents and chaotropic denaturants in favour of water-based buffers in combination with freeze-

heat cycles to induce hypotonic lysis [73,78]. These methods have proven successful in single-cell and carrier sample preparations (hundreds or thousands of cells) by minimising sample losses whilst effectively extracting protein. This approach has been successfully applied to a FACS-based proteomic approach for analysing 5-200 cells [79] and for phosphoproteomic analysis of 1,000 cells [80]. Hence, this appears to be a promising lysis strategy for low-input (phospho)proteomics. Beyond water lysis, many low-input and single-cell proteomics workflows now include low concentrations of MS-compatible non-ionic detergents, most commonly n-dodecyl-β-D-maltoside (DDM) [68,81,82]. The principal gain from DDM appears to be the mitigation of peptide and protein adsorption to plastic and glass rather than improved lysis efficiency. Consistent with this, Muneer *et al.* evaluated SDC, urea and RapiGest for microscale phosphoproteomics and used DDM mainly to passivate vial surfaces, not as the primary lytic reagent. For microscale phosphoproteomics, they showed that precoating tubes with DDM resulted in the recovery of significantly more phosphopeptides, especially longer and multiply phosphorylated peptides (Muneer et al., 2024). Likewise, Johnston *et al.* and Tsai *et al.* reported that high-temperature treatment alone is sufficient for cell disruption, but that adding DDM further increases recoveries by reducing surface losses [68,82]. Given that the removal of DDM can be challenging, detergent selection and concentration should be chosen based on the biological question and peptide clean-up strategy.

These insights from single-cell and low-input proteomics highlight a shift toward miniaturised, integrated lysis-digestion workflows and away from traditional chaotropic or detergent-based buffers, reducing losses and improving sensitivity [73,79,83]. Applying these streamlined approaches to low-input phosphoproteomics will be key to achieving reproducible, deep coverage from limited samples.

Labelling

Labelling methods enable multiplexing, which offers several advantages over sequential analysis of individual samples. It can improve throughput, data completeness, and workflow consistency by enabling multiple samples to be analysed in a single run. Perhaps most importantly, multiplexing reduces technical variability associated with sample preparation steps post-labelling, which is particularly relevant when studying dynamic modifications such as phosphorylation [84,85]. Labelling methods, can be performed at the cellular, protein or peptide level, depending on the experimental design and mechanism of incorporation for isotopes [30]. The most common approaches can be categorised broadly as either isobaric or non-isobaric. Isobaric methods include tandem mass tags (TMT) [86] and isobaric tags for relative and absolute quantitation (iTRAQ) [87], while non-isobaric methods include stable isotope labelling by amino acids in cell culture (SILAC) [88], mass differential tags for relative and absolute quantification (mTRAQ) [89], and dimethyl labelling [90]. The choice between these methods depends on the desired quantification method and the multiplexing capacity required for the study. Non-isobaric stable isotope labelling introduces mass differences at the MS1 level by incorporating distinct isotopes into peptides or proteins within individual samples, enabling relative quantification by comparing peak intensities of peptides with mass differences corresponding to the degree of isotope incorporation. In contrast, isobaric labelling uses chemical tags with the same nominal masses (but not the same isotopic composition) that generate the ‘same’ MS1 signal but distinct reporter ions upon fragmentation, enabling quantification at the MSn level.

TMT reagents, the most widely used isobaric labels, enable proteome-wide relative quantification of multiple samples in parallel [91-94]. The TMT tag is made up of three components: a reactive group that interacts with the primary amines of peptides, a reporter group for quantification, and a mass balance related to the reporter group to keep the overall mass of the tag the 'same' between different channels [86]. Post-labelling, tagged samples can be multiplexed and analysed in a single run, or fractionated to obtain deeper coverage of the proteome (see section 'Chromatography and Fractionation') [92]. Following the MS1 scan of tagged peptides, fragmentation generates TMT reporter ions along with peptide fragment ions. The intensities of the reporter ions can be used for relative quantification, while the peptide fragmentation pattern can be used for sequence identification. The use of the MS2 scan to both accommodate quantification and identification requires increased signal and, thus, consideration for acquisition parameters. The reporter ion ratios provide information about protein expression levels within each sample.

TMT is often the preferred method for low-input phosphoproteomics due to its increased multiplexing capacity, application to a range of biological samples and improved quantitative precision compared to other labelling techniques [41,95-97]. Multiplexing also allows incorporation of additional "carrier" channels to boost the MS1 signal, enabling subsequent MS2 triggering [98]. The use of carrier channels has shown promise in the field of single-cell proteomics [26,78,99], where throughput and protein amount are limiting. A further advantage of TMT is the near absence of missing values within a single plex; however, integration of multiple plexes introduces batch effects and missing values, requiring careful experimental design and normalisation strategies [100]. Despite these advantages, isobaric labelling can suffer from ratio compression caused by co-fragmenting and co-eluting peptides, which introduce contaminating reporter ions [101-103]. This leads to an underestimation of true fold changes, as the majority of the proteome is not differentially regulated [104]. The use of carrier channels can exacerbate this issue, as highly abundant peptides in the carrier dominate the isolation window, further contaminating reporter ion signals from low-abundance samples [105]. Acceptable carrier ratios have been reported ranging from <20x to <200x depending on the instrument parameters, sample type and input amount [106-109].

Recent advances have expanded TMT multiplexing from 18 to 35 channels by combining 18 non-deuterated and 17 deuterated tags [110]. However, this approach requires substantially higher resolving power or advanced processing methods to resolve the closely spaced reporter ions, and bridge normalisation to correct for deuterium isotope effects [110]. Importantly, higher multiplexing increases demands for MS2 signal in order to accommodate the near doubling of reporter channels. For low-input phosphoproteomic applications, where peptide abundances are already limiting, this can exacerbate ratio compression and reduce quantitative accuracy. To date, the trade-offs between increased multiplexing and reduced ion counts per channel have not been systematically assessed for low-input phosphoproteomics. Accurate quantitative data can be obtained with multiplexed TMT experiments using conservative carrier ratios or optimising instrument parameters and post-analysis filtering [109].

Standard TMT labelling protocols typically require 25-100 µg input material and are generally performed in-solution by adding the TMT reagent to concentrated, purified

peptides [111]. This sample preparation format is incompatible with low-input samples due to the low substrate concentration, which results in reduced labelling efficiency. This can be partially overcome by reducing the reaction volume and increasing the amount of TMT, but this can result in excess TMT reagent and overlabelling which can inhibit peptide ionisation and increase sample complexity [112,113]. Performing TMT labelling on-column instead of in-solution enhances reaction kinetics and reduces sample loss of low input samples [40,79,113]. On-column labelling has the potential to streamline low-input sample preparation workflows by combining labelling with desalting to limit sample loss and concentrate peptides. A handful of studies have already demonstrated the success of this method, with some highlighting its application for low-input phosphoproteomics. The first description of an on-column TMT method was presented by Böhm *et al*., where they used 3 mg of protein material to show their novel C18-based labelling approach yielded a ~20% increase in peptide identifications while reducing over-labelling caused by side reactions [113]. Another approach to reduce overlabelling is to quench the reaction with hydroxylamine; however, Demyanenko *et al.* recently showed this is insufficient to remove over-labelled peptides. Instead, they propose methylamine as an effective alternative, lowering the proportion of over-labelled peptides to <1% without impacting labelling efficiency [114]. This step can be readily incorporated into existing TMT-based, low-input phosphoproteomic workflows to improve identification rates and quantitative precision.

Park *et al*., recently published their one-STAGE tip method for TMT labelling of low-input amounts [79]. Using a single STAGE-tip, without the need for sample transfer, they combined the on-column TMT-labelling method described by Myers *et al*., with Minimal ProteOmic sample Preparation (mPOP) lysis developed by Specht *et al.* [64,73]. Interestingly, the reaction conditions vary between Park *et al.* and Ogata *et al.,* who both present low-input on-column TMT labelling methods. Park *et al*., used HEPES buffer pH 8, while Ogata *et al*., used 50 mM phosphate buffer pH 6.5 [40,79]. Given that optimal TMT labelling occurs in basic conditions, while optimal C18 peptide binding occurs at acidic pH, there is a trade-off that needs to be considered and optimised. Ogata *et al*., compared their on-column TMT-labelling method at different pH values, ranging from 4.5 to 8.5 and showed that the best fully-labelling rate was achieved at 6.5 [40]. While increasing the pH above 6.5 can reduce the labelling time, as described by Park *et al*., it may also lead to additional peptide loss, which should be considered when working with low cell numbers. More recently, we demonstrated that on-tip TMT labelling outperforms in-solution methods, almost doubling the number of peptide identifications for 1,000 cells. This method was further combined with downstream phosphopeptide enrichment, enabling nanogram-scale phosphoproteomic analysis [80].

Phosphopeptide enrichment

Phosphoenrichment can be performed at the protein level, but it is more commonly carried out at the peptide level because phosphopeptides have simpler chemical properties and structures, which make them easier to separate. These methods have been reviewed in detail elsewhere [115]. Currently, the most popular enrichment methods rely on affinity-based chromatography, such as Immobilised Metal Ion Affinity Chromatography (IMAC) or Metal Oxide Affinity Chromatography (MOAC) **(Fig. 4C)**. Both these methods exploit the ability of metal ions or metal oxides to interact with phosphate groups with high specificity and affinity. IMAC utilises metal ions like Fe3+, Ga3+, Ti4+, or Zr4+ chelated to solid supports, while MOAC employs metal oxides,

predominantly TiO2 and ZrO2. These materials are available in various formats, including columns and beads, each offering unique advantages for different experimental needs [116-118]. Phosphoproteomic protocols using these enrichment materials have typically been optimised for hundreds of micrograms of starting material, and are not suitable for low cell numbers. Recent work has aimed to address the problems associated with high volumes, poor reaction efficiencies and binding selectivity and specificity. Bortel et al. focused on systematically optimising experimental parameters such as loading and elution buffer compositions, bead ratios and binding time [119]. By including sequential enrichment and stepwise addition of beads, they were able to increase the depth of coverage by 20% [119]. More recently, Muneer *et al.* developed the iPhosChip – a custom microfluidic chip incorporating a miniaturised TiO_2 column, enabling phosphoproteomic analysis of nanoscale samples, down to a single cell [120]. As well as miniaturisation, automated phosphopeptide enrichment has been shown to improve reproducibility and minimise losses compared to manual processing, with robotic platforms enabling precise handling of sub-microgram samples [121]. In addition, controlling the temperature by cooling the column during the desalting step markedly improves the retention of hydrophilic phosphopeptides and could be readily integrated into automated workflows [122].

While it is evident that multiplexing is a promising option for low-input phosphoproteomics, it is still not clear how labelling should be combined with phosphopeptide enrichment to achieve the best results. Tsai *et al*. developed a tandem tip-based workflow for low-input phosphoproteomics [41]. Their approach combined TMT labelling of sorted cells followed by IMAC phosphoenrichment. A major success of this study was the use of FACS to validate the workflow with sorted samples. Despite previous evidence that on-tip TMT labelling can outperform in-solution labelling for low-input samples, they opted to use an in-solution method followed by tandem C18-IMAC-C18 enrichment and desalting. Using this approach, they were able to quantify ~600 phosphopeptides from 100 FACS-sorted cells. Using an alternative workflow, Ogata *et al.* used titanium oxide chromatography for phosphopeptide enrichment, followed by TMT labelling [40]. We built upon this work by performing phosphoenrichment after labelling, which leverages the throughput and reproducibility benefits achieved by multiplexing [80].

Together, these studies highlight the advances in phosphoproteomic enrichment techniques enabling the analysis of nanoscale samples. With methods now available for both labelled and label-free workflows, ranging from commercially available beads to miniaturised microfluidic columns, there is a broad toolkit to suit diverse experimental needs. Ongoing miniaturisation and automation of enrichment platforms, such as low-temperature microcolumns and microfluidic chips, are further enhancing sensitivity and expanding the scope of biological questions that phosphoproteomics can address.

Sample separation and fractionation

Multidimensional separation techniques have been developed to address the challenge of high complexity and dynamic range, as well as the need to measure low abundance analytes in proteomic samples [123,124] **(Fig. 4D)**. While reverse-phase liquid chromatography coupled to mass spectrometry is used ubiquitously, additional orthogonal separation approaches can dramatically improve peptide separation and

thus increase the sampled proteome depth [125]. While the benefits of additional fractionation techniques are obvious for high-input samples, the trade-offs between peptide separation, peak height and sample losses associated with additional processing steps are less apparent for low-input samples, where spectrum complexity is reduced [95]. A handful of studies offer comparisons of the available fractionation techniques, providing valuable insights into optimal strategies for low-input phosphoproteomic samples.

The use of commercially available generic chromatography hardware demonstrates that it is indeed possible to generate ultrasensitive biologically relevant proteomic data from standard LC systems [95,126]. Having said that, many groups have demonstrated impressive gains in proteome coverage by employing specialist LC strategies for proteomic analysis of low-input samples. The Evosep ONE LC system is showing great promise for streamlining sample preparation of low-input samples by combining on-tip desalting with LC separation. A recent study by Ye *et al*., used a one-pot sample preparation workflow for streamlined lysis, digestion and desalting of low-cell numbers down to 20 HeLa cells and single oocytes [71]. This method reproducibly identified > 9,000 proteins from ~1000 HeLa cells in a high-throughput manner. However, low sample volumes (5 µL) are required to prevent excess dilution of the lysis and digestion buffer, which limits the number of compatible cell isolation techniques. Using a reconfigured Evosep One LC system for the first dimension and a repurposed 3D printer as a fraction collector, Kitano *et al*., observed a two-fold gain in phosphosite identifications compared to a single-shot workflow for phosphoproteomic analysis of 50 µg HeLa [126]. While this approach has not yet been applied to low-input FACS-sorted samples, it shows promise for future high-recovery fractionation techniques. As an alternative to the C18-based Evosep ONE, the highly-ordered nature of micropillar array columns (µPAC) offers superior stability and increased sensitivity compared to traditional nanoflow HPLC columns. Stadlmann *et al*., highlighted these performance improvements using 10ng HeLa digest samples, demonstrating that precursor ion intensities typically doubled when employing µPAC cartridges and yielded approximately 20% more peptide identifications [127]. µPAC columns have already been adopted for single-cell proteomics and will undoubtedly aid in the advancement of ultrasensitive phosphoproteomics [128].

2D ZIC-HILIC (Zwitterionic Hydrophilic Interaction Liquid Chromatography) developed by Di Palma *et al*., significantly outperformed standard 1D reverse phase nanoLC–MS [129]. Their method led to the identification of twenty-fold more unique peptides assigned to almost ten times more proteins from a fraction corresponding to 5,000 cells from an original sample of 30,000 FACS-sorted stem cells. Dou *et al*., developed the nanoFAC (nanoflow Fractionation and Automated Concatenation) 2D RPLC platform, which demonstrated a >300% increase in peptide identification for 100–1000 ng of HeLa tryptic digest (equivalent to ~500–5,000 cells) [130]. Furthermore, by integrating with phosphopeptide enrichment, the nanoFAC 2D RPLC platform identified ~20,000 phosphopeptides from 100µg of MCF-7 cell lysate. Scaling down this method for low-input phosphoproteomic analysis of rare cells would be an exciting development. Academic collaboration or commercial development of these columns would make advanced multidimensional fractionation methods more accessible to non-specialist groups. In the study by Koenig *et al*., , two distinct high-pH reversed-phase fractionation methods, stage-tip fractionation (STF) and microflow (MF), were compared while reducing the peptide input amount from 12.5 to 1 µg per sample [95].

Their findings suggest that, for input amounts exceeding 5 µg per sample, the combination of TMT labelling followed by MF fractionation and phosphoenrichment yields the most extensive phosphoproteome coverage. Conversely, STF is optimal for input amounts less than 5 µg per sample. In this context, it appears that prioritising low-surface area techniques to reduce sample loss outperforms traditionally superior fractionation methods used for high-input samples.

In the interest of reducing analysis time and improving throughput, many of the pioneering groups in the field of single-cell proteomics have opted to use online gas phase fractionation methods (e.g. High field asymmetric waveform ion mobility (FAIMS), trapped ion mobility spectrometry (TIMS)) rather than offline liquid fractionation or tandem chromatography techniques [69,78,128,131]. Selective filtering of ions, based on their mobility, enables better separation of isomeric and closely related species, thus increasing signal-to-noise ratios, allowing for the detection of low-abundance proteins, which is particularly important for low-input samples [39,132,133]. Yang *et al.*, were able to achieve sensitive phosphoproteomic analysis of low-input samples by using Parallel Accumulation and Serial Fragmentation (PASEF) in conjunction with timsTOF instruments to leverage ion mobility separation and prioritised data acquisition [132]. Using this approach, they were able to explore the spatial dynamics of the mouse brain by systematically analysing distinct regions. While their biological insights were derived from 100 µg samples, their findings demonstrated the technique's ability to handle samples as low as 10 µg [132]. This breakthrough highlights the substantial potential of ion mobility approaches for low-input phosphoproteomics and represents a promising direction for future research.

Mass spectrometry acquisition

The two data acquisition methods for bottom-up discovery proteomics are data-independent acquisition (DIA) and data-dependent acquisition (DDA) **(Fig. 4E)**. DDA selects precursor ions based on real-time abundance, typically choosing the most intense ions for fragmentation, while DIA simultaneously fragments all precursor ions within a predefined mass range DDA selects precursor ions based on real-time abundance, typically choosing the most intense ions for fragmentation [134-139]. In the context of phosphoproteomics, the acquisition strategy not only impacts the depth and accuracy of peptide identification and quantification, but also the confidence with which phosphorylation modifications can be localised to specific residues. These acquisition methods can be combined with different quantitative approaches, either label-free quantification (LFQ) or label-based techniques [140,141]. Isobaric labelling methods rely on MS^n-based quantification, meaning they can only be used in conjunction with DDA due to the non-selective nature of precursor fragmentation performed during DIA. The current incompatibility of DIA and isobaric labels means there are trade-offs to consider when designing low-input phosphoproteomic experiments. Isobaric labelling has the advantage of increasing the total number of cells per injection by multiplexing, which improves throughput and provides more starting material for phosphopeptide enrichment. Conversely, DIA can overcome the limitation of stochasticity associated with DDA, thus improving sensitivity and coverage [142]. While DIA can increase proteome depth, the added spectral complexity makes phosphosite assignment more challenging. In low-input samples, the reduced abundance of site-determining fragment ions further exacerbates this limitation, meaning the increased coverage offered by DIA can come at the expense of confident site localisation.

The recently published LFQ-DIA methods for single-cell proteomics highlighted the possibility of DIA for low-input proteomics [128,143,144]. Importantly, these studies demonstrate the power of library-free DIA methods, which are necessary for the analysis of low cell numbers, given the limited sample availability for generating spectral libraries. DIA has yielded significant gains in peptide identifications for both high-input phosphoproteomics [34,145,146] and single-cell proteomics [27,71,128,143,144]. More recently, LFQ-DIA has been applied to low microgram-scale phosphoproteomics [43,81,119]. By minimising the total processing time, and reducing liquid volumes and transfer steps, the µPhos method developed by Oliinyk *et al*., enabled the identification of ~6,500 well-localised phosphopeptides from ~5 µg HeLa. Using a narrow-window DIA approach with optimised sequential phosphoenrichment, Bortel *et al.* were able to identify of ~8,000 well-localised phosphopeptides from ~3 µg of HeLa. Muneer *et al*. developed SOP-Phos, a one-pot workflow for microscale DIA phosphoproteomics. Using this method they were able to identify ~8,000 well-localised phosphopeptides from ~5 µg starting material. Together, these studies highlight the benefits of using automated liquid handling platforms and microscaled sample preparation methods to achieve deep phosphoproteomic profiling of low cell numbers using DIA. Below ~1 µg of material, single-shot phosphoproteomic DIA analysis remains increasingly challenging **(Fig. 2)**, especially when accurate site localisation is required for biological interpretation and functional downstream analysis (Searle et al., 2019). While DIA typically yields increased peptide identifications compared to DDA, it often produces a lower proportion of confidently localised sites due to the increased spectral complexity, a challenge that is further exacerbated in low-input workflows, where the intensity of diagnostic fragment ions is reduced (Muneer et al., 2024, Kitata et al., 2021). Benchmarking studies have shown that experiment-matched libraries, generated from comparable sample types and input amounts, deliver the deepest coverage and highest localisation confidence (Muneer et al., 2025, Bekker-Jensen et al., 2020). While this is not always feasible when sample amount is limited, libraries built from related but more abundant samples may be a practical alternative, though this approach requires further validation. Advances in scoring algorithms have significantly improved site assignment: traditional approaches rely on predefined statistical models (Bekker-Jensen et al., 2020, Olsen et al., 2006, Taus et al., 2011, Fermin et al., 2013), whereas recent artificial intelligence-based methods leverage predictive modelling of fragment intensities and retention times to achieve higher localisation accuracy [147,148]. Overall, careful optimisation of acquisition parameters, spectral library design, and scoring remains critical to ensure that the increased depth afforded by DIA translates into data suitable for biologically meaningful and reproducible functional analyses.

In parallel, there has been progress in unifying multiplexing and DIA in order to increase sample throughput while preserving proteome coverage and quantification accuracy. Groups have used stable isotope labelling methods to improve the throughput of DIA proteomics by multiplexing up to five channels [27,149,150]. Unlike isobaric mass tags, stable isotope labels have mass differences which are maintained throughout fragmentation, enabling quantification using the MS1 spectra [90]. This overcomes the limitations associated with simultaneous fragmentation in DIA and allows for distinction between peptides from different samples, even in the complex fragmentation patterns of DIA. In addition, MS1 quantification offers more reliable quantification when low-intensity fragment ions are insufficient for MS2 quantification

[128]. These advantages have previously been demonstrated for single-cell proteomics and could be similarly beneficial for low-input phosphoproteomics [128]. Multiplexed DIA has the potential to overcome the limitations associated with both LFQ-DIA and multiplexed DDA to yield significant improvements in phosphoproteome coverage of low-input samples.

The prevalence of missing values is a major challenge for low-input proteomics. This is partly due to the MS2 selection criteria of shotgun DDA approaches, which prioritise higher abundant precursor ions, either by selecting a predefined number (N) of the most intense precursor ions in each cycle, or selecting the most intense ions in a fixed time window. This results in the sequencing of different precursors across samples, leading to missing values. This can be largely overcome by DIA and, to some extent, TMT for samples in the same plex, but other technologies are also emerging to address this issue [93,140]. MaxQuant.Live is a hybrid method aiming to unify targeted and shotgun proteomics using a global targeting approach whereby an arbitrary number of precursors of interest are detected in real-time, followed by standard fragmentation or advanced peptide-specific analysis [151,152]. This approach could be particularly advantageous for low-input proteomic (and specifically phosphoproteomic) approaches, where data completeness and reproducibility are major challenges [151,152]. Indeed, Huffman *et al*. further developed the real-time retention-time alignment concept from MaxQuant.Live and introduced a prioritisation hierarchy to temporally define the order of peptide analysis [70]. This approach was showcased in the context of single-cell analysis, where they were able to demonstrate improved sensitivity, completeness and increased proteome coverage over twofold. In addition, Martínez-Val *et al*. introduced Hybrid-DIA, a data acquisition strategy that integrates targeted and discovery approaches to achieve improved coverage of known signalling pathways [153]. Given the high dynamic range and stochastic and transient nature of PTMs, these approaches could be even more advantageous in the context of low-input phosphoproteomics, especially for hypothesis-driven biological questions where there is pre-existing knowledge of proteins of interest.

Summary

This review highlights the rapidly advancing landscape of low-input phosphoproteomic sample preparation and analysis. Although recent developments have led to optimised methods for efficient labelling and multiplexing, phosphopeptide enrichment, fractionation, and high-sensitivity MS acquisition, examples of these workflows being utilised to tackle interesting biological questions remain limited. This is partly due to insufficient integration with existing cell isolation technologies, which pose significant challenges to conventional phosphoproteomic sample preparation protocols. While diluted bulk lysates are useful for technical optimisation experiments, low-input workflows should be validated with appropriate samples that are representative of relevant biological models. Such validation is crucial for encouraging widespread adoption beyond specialist laboratories, ensuring the maximum impact is achieved from these technical developments.

The evidence presented herein suggests vast promise for solid-phase TMT labelling. This approach outperforms in-solution labelling for both low- and high-input samples by reducing the rate of overlabelling, reducing the amount of TMT reagent required and streamlining the overall workflow by combining desalting with labelling, leading to

improved compatibility with existing cell isolation methods. With the wide range of C18-based columns and tips available commercially, this approach can be applied to any sample amount. For samples less than 1 µg, multiplexing still appears to be advantageous, compared to single-shot DIA **(Fig. 2)**. Conversely, samples greater than 1 µg would likely benefit from a DIA method, depending on the robustness and throughput capabilities of the sample preparation workflow.

The development of ultrasensitive low-input phosphoproteomic methodologies is essential for addressing complex biological questions where cell numbers are limited. This problem is particularly acute in areas such as developmental and stem cell biology, origins and evolution of disease, and research involving model organisms, where specific cell types are available in low quantities. Leveraging phosphoproteomic technologies in these contexts will offer novel insights into cell signalling that were not previously possible with next-generation sequencing technologies. Looking forward, further progress will rely on building upon the rapid advances made in single-cell proteomics, particularly in miniaturisation and automation, to streamline phosphoproteomic workflows. Improved integration with cell isolation techniques and validation in biologically relevant models will be key to translating these technical advances into impactful discoveries, enabling deeper mapping of signalling in primary tissue samples and rare cell populations.

Figure legends

Fig. 1 Existing challenges of low-input phosphoproteomic sample preparation. A, Multiple sample processing steps result in sample loss and poor reproducibility. **B,** The input sample amount will determine the extent of sample loss, which is exacerbated during the analysis of proteoforms. **C,** Traditional lysis methods are optimised for large amounts of protein, which can result in low protein concentration and poor reaction efficiencies in low-input samples.

Fig. 2 Advances in low-input phosphoproteomics. An overview of the existing methods available for low-input (≤10 µg per sample) phosphoproteomics. To compare across studies, 1 µg is equivalent to 10,000 cells. Where phosphopeptide data were available with a >0.75 localisation probability filter applied, these figures are shown. For papers where well-localised phosphopeptide information was not reported, searched data were downloaded from PRIDE [154], a localisation probability cut-off of >0.75 was applied, and duplicates were removed. Where replicates were available, the average was calculated, and the best results per input amount were selected irrespective of fractionation method, search parameters or instruments used.

Fig. 3 Cell isolation techniques for different sample types. Many sample types are of interest for phosphoproteomic analysis, including *in vitro* and *in vivo* models, primary patient samples, organoids and tissue sections. These samples can be dissociated into a single-cell suspension and isolated using either Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS), Magnetic-Activated Cell Sorting (MACS), or image-based sorters (i.e. cellenONE®). Alternatively, cells of interest can be extracted from tissue sections using Laser Capture Microdissection (LCM).

Fig. 4 Phosphoproteomic sample preparation and data acquisition. A, Intact cells can be lysed using a range of chemical or mechanical methods. Proteolysis can be

performed under different conditions to improve digestion efficiency depending on the input sample type and protein amount. **B,** Optional isobaric labelling can be carried out at the peptide level using traditional in-solution or more recent solid-phase methods. **C,** IMAC (Immobilised Metal Affinity Chromatography) or MOAC (Metal Oxide Affinity Chromatography) phosphopeptide enrichment methods can be performed under different conditions depending on the input sample amount. **D,** Prior to LC-MS/MS analysis, sample fractionation techniques can be used to improve separation and depth of phosphoproteome coverage. Ion mobility methods can separate ions based on their charge states, while chromatography can be used to separate peptides based on their physicochemical properties. **E,** Two main MS acquisition methods are available for discovery-based phosphoproteomics. Data Dependent Acquisition (DDA) is compatible with isobaric labelling and performs fragmentation of selected MS1 precursors for subsequent MS2 analysis. Data Independent Acquisition (DIA) executes fragmentation of all precursors in a given *m/z* window, and the complex MS2 spectra are deconvoluted post-acquisition.

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E.J.G. and S.S. wrote the review manuscript.

Competing Interest

The authors declare no competing interests.