Extending the sensitivity, consistency and depth of single-cell proteomics

Simultaneous maximization of sensitivity, data completeness and throughput in mass-spectrometry proteomics often necessitates trade-offs. To mitigate these trade-offs, we introduce a prioritization algorithm that achieves high sensitivity and data completeness while maximizing throughput. With prioritized single-cell proteomics (pSCoPE), we consistently and accurately quantify proteins and their post-translational modifications in single macrophages and link them to endocytic activity.

This is a summary of:
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The question

Many biological systems comprise cells with diverse functions and molecular compositions[1]. The study of this diversity requires single-cell protein analysis and has motivated the development of methods for single-cell sample preparation[2] and mass-spectrometry (MS) analysis[1]. The data from these methods indicate that MS can detect tens of thousands of peptide-like features from individual cells. However, methods that isolate, fragment and analyse one peptide-like feature at a time lack sufficient time to analyse all features[3]. These limitations prompt the questions of how we can allocate limited instrument time to optimally serve biological investigations, and how MS can consistently quantify proteins of interest in every sample (a single cell) while simultaneously maximizing the number of quantified proteins per cell. These questions arose while we were investigating macrophage polarization (the mechanisms that drive macrophages towards diverse functional phenotypes spanning a functional spectrum from pro-inflammatory to anti-inflammatory), and we sought to find a general solution.

The solution

Our solution is based on explicitly assigning a priority to each peptide and then allocating the limited MS time according to these priorities. This approach (prioritized single-cell proteomics (pSCoPE)) uses precise estimates of peptide elution times[4] to ensure efficient isolation and fragmentation of high-priority peptides; fragmentation and secondary MS scans of the peptide fragments are required for confident sequence identification and quantification[3]. The high consistency of peptide isolation across all samples results in over twofold higher data completeness (fraction of quantified datapoints in the data matrix) for pSCoPE than for controlled shotgun analysis, which selects peptides for analysis on the basis of their abundance. The gains are most prominent for peptides that are often missed by shotgun analysis and enabled us to achieve 93% data completeness across all quantified proteins and single cells.

This high consistency of protein quantification is not at the expense of decreased proteome coverage, because the prioritization logic supplies peptides to be fragmented and quantified at all times, thereby using 100% of the available instrument time. As all supplied peptide ions are preselected to be identifiable, prioritized analysis resulted in confident sequence assignments to 84% of all fragmented peptides, compared with 40% for shotgun analysis. This improvement in sequence identification doubled the number of proteins quantified per single cell.

Prioritization enabled both extension of the dynamic range towards less abundant proteins (Fig. 1a) and high quantitative accuracy (Fig. 1b). The extended dynamic range is accomplished by prioritizing peptides from proteins of biological interest, which can have low abundance. We demonstrate that such prioritized peptides are fragmented with 98% efficiency even when many proteins are prioritized. The high accuracy of protein quantification (Fig. 1b) is achieved by sampling peptides close to the apexes of their elution peaks, where the signal is highest. This apex sampling is enabled by calibrating elution times using MaxQuant.Live[4]. Because these strategies can be applied to post-translational modifications, we were able to prioritize and quantify peptides with post-translational modifications. Specifically, we quantified the single-cell levels of proteolytically cleaved peptides associated with inflammatory states. These measurements indicated substantial gradients in the levels of many proteins, including those involved in proton transport and phagosome maturation. We found that these molecular gradients are coupled to endocytic activity, demonstrating a link between molecular and functional macrophage polarization.

Future directions

We have demonstrated the utility of prioritization for quantifying proteolytically cleaved peptides, and this utility can be generalized to various post-translational modifications, such as phosphorylation. Furthermore, the analysis can be extended to protein analysis of diverse samples (for example, tissues), rather than only single cells as in our study. Such extensions are facilitated by the freely available protocols and software at https://scp.slavovlab.net/pSCoPE.

The benefits of prioritized proteomics include substantially increasing the likelihood of quantifying proteins of interest (in particular those with low abundance), reducing missing data and quantifying more proteins. These benefits facilitate the estimation of protein covariation, which reflects many biological processes[2,5], and are particularly important for building quantitative models of protein interactions and signaling networks[5]. Such models may infer biological mechanisms by making few assumptions, but they require accurate and complete measurements[5]. Prioritized proteomics is a step toward making such measurements.

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EXPERT OPINION

"Huffman et al. establish a new strategy to improve the efficiency of single-cell proteomics using prioritized mass spectrometric methods (pSCoPE). pSCoPE aims to address three current challenges in the field: abundance bias, stochastic data-dependent acquisition ion selection, and inefficient peptide sequencing. The authors use prioritized subsets of target peptide lists to adapt scan parameters in real time with data acquisition as a robust method for single-cell molecular phenotyping." **Devin K. Schweppe, University of Washington, Seattle, WA, USA.**

FIGURE

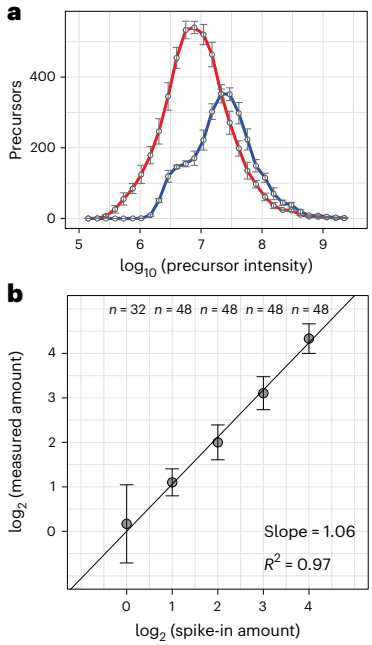

Fig. 1 | Prioritization increases the sensitivity and depth of accurate protein quantification in single cells. a, Prioritized analysis (red) increased the dynamic range and the number of quantified peptides compared with shotgun analysis (blue) with matched parameters. **b**, Comparison between peptides spiked-in as positive controls at single-cell levels and their measured ratios. The agreement indicates high quantitative accuracy; the regression slope and goodness of fit (R^2) are displayed. Data points represent mean ± s.d.

BEHIND THE PAPER

The potential of directed MS methods to increase the sensitivity of single-cell proteomics was evident from the outset[1,3] and motivated one of the first projects in my new laboratory in 2016. However, our initial experiments resulted in modest gains. The gains increased by including real-time alignment of retention times with MaxQuant.Live[4], but we still faced a frustrating and disappointing trade-off between high data completeness and high proteome coverage. For more than a year, we fluctuated between high data completeness with low proteome coverage and low data completeness with high proteome coverage. Then we resolved this trade-off by introducing the prioritization logic, which marked a major turning point for the methodology. When combined with efficient sample preparation[2] and improvements in MaxQuant.Live, prioritization resulted in a robust method that was ready to be used for investigating connections between molecular and functional polarization in primary macrophages. **N.S.**

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FROM THE EDITOR

"Prioritized SCoPE (pSCoPE) is a strategy to improve the consistency, sensitivity, and depth of protein quantification in single-cell proteomics experiments by prioritizing the selection of peptides of interest using real-time retention-time alignment. I think the approach will help to address some very real limitations of current single-cell proteomics protocols." **Arunima Singh, Senior Editor, *Nature Methods*.**