APPLICATION NOTE

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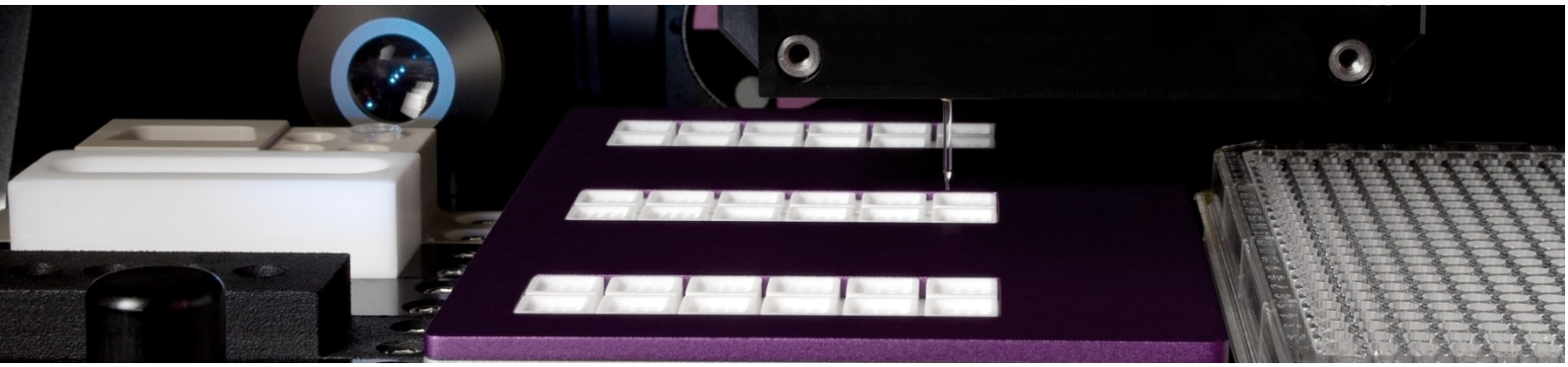

proteoCHIP & cellenONE taking multiplexed single cell proteomics sample preparation to the next level

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Abstract

Single-cell proteomics has the potential to transform biomedical research as it will become essential to complement single cell transcriptomics and genomics approaches. Recent advances in mass spectrometry have enabled the emergence of several sample preparation methods for undertaking single-cell proteomics analyses. However, an automated and loss-less sample preparation workflow for single cell proteomics is still lacking. Here, by combining the cellenONE® single cell isolation and picoliter dispensing platform with a dedicated consumable (proteoCHIP 12*16), a new sample preparation workflow for multiplexed single cell proteomics analyses was developed. Through miniaturization and automation, the workflow resulted in unprecedented sensitivity (up to 1500 proteins per run) but more importantly allowed the first demonstration of only multiplexed single cell analyses (no abundant carrier required) with outstanding reporter ion signal to noise ratio. By combining automated sample preparation and superior data quality using a commercially available platform and consumables, this complete solution has the potential to help the widespread implementation of single cell proteomics methods in biological studies.

Introduction

Advances in mass spectrometry (MS)-based proteomics have driven proteome identification and quantification of different tissues or cells (1,2). New technical capabilities enable the continuous progress of more sensitive MS-based methods but throughput, measurement variability and most importantly sample preparation still lack behind. Significant achievements have been made using label-free single cell proteomics (4), however, these approaches are limited in throughput as single cells are analyzed individually, one after the other.

Methods allowing to multiplex samples using isobaric peptide labelling (e.g. tandem mass tags – TMT) are continuously improved and extended (5). However, only recently they were implemented to increase throughput and total peptide input in single cell studies.

SCoPE-MS (Single Cell ProtEomics by Mass Spectrometry) was the first method describing the analysis of multiplexed FACS sorted single cells processed in a 96-well plate. This method then combined 8 TMT-labelled single cells with a carrier channel containing 200 cells to maximize peptide identifications (6). Another method nanoPOTS (nanodroplet Processing in One pot for Trace Samples) based on micropatterned microscope slides was initially used to process label-free cells (3, 8). The latest implementation of this platform named nested nanoPOTS (N2) was developed using cellenONE to allow drastic reduction in sample volume and multiplexing but still used a carrier channel equivalent to 100 cells (7). The use of such carrier to overcome adsorptive losses and increase ion counts was studied by several authors, demonstrating that the number of cells should be carefully balanced and typically maintained under 200 cells not to impact biological conclusions (9, 10).

To overcome the above-described limitations, a highly versatile and automated workflow for multiplexed single cell proteomics sample preparation has been developed by the Research Institute of Molecular Pathology in Vienna (IMP) in cooperation with Cellenion and is summarized here (11). The workflow is based on the proteoCHIP 12*16, a newly developed consumable containing twelve times sixteen nanowells for processing up to 192 single cells per chip in conjunction with the cellenONE®. Importantly, the workflow developed overcomes all manual sample handling steps and allows direct interfacing with a standard autosampler to maximize peptide recovery throughout the sample preparation.

Materials and methods

proteoCHIP 12*16:

The proteoCHIP 12*16 (Figure 1, left panel) is composed of two parts each in a microscope slide footprint: the nanowell part (Figure 1, left panel, bottom), containing twelve sets of sixteen wells and the funnel part (Figure 1, left panel, top) designed for pooling each set of sixteen wells and allowing direct interfacing with a HPLC autosampler for injection. The proteoCHIP 12*16 is fabricated from a carefully selected material which has been shown to improve peptide recovery compared to traditional plastic or glassware (12,13). The proteoCHIP 12*16 is designed to miniaturize sample reaction in sub microliter and prevent cross contamination between each well. In order to prevent evaporation during lysis and digestion each well of the proteoCHIP 12*16 is preloaded with oil.

The proteoCHIP 12*16 holder has been developed for cellenONE® (Figure 1, right panel), it accommodates up to 3 proteoCHIPs to allows simultaneous processing of up to 576 single cells per run (Figure 1, left panel).

Figure 1. Pictures of the proteoCHIP 12*16 and the cellenONE® single cell isolation and picoliter dispensing platform. (left panel) proteoCHIP 12*16. Three nanowell part (bottom) placed in their dedicated holder. One funnel part (top) used for pooling each sets of multiplexed sample and direct injection into the autosampler. (right panel) Illustration of the cellenONE®.

Sample preparation workflow

The entire sample preparation workflow (Figure 2) is performed inside the cellenONE®. The first step consists in dispensing a master mix for enzymatic lysis and digestion in each well of the proteoCHIP 12*16 (40nL/well). In the second step, image-based single-cell isolation is used to dispense single cells in the master mix contained in each well. Then, lysis and digestion are initiated by setting the temperature of the deck at 50°C for 2 hours. After incubation, 60nL of buffer were added to each well prior to TMT labels (11mM in acetonitrile, 100nL/well,) addition to their respective wells, these were left to react for 1 hour at room temperature and finally quenched using a mixture of hydroxylamine and hydrochloric acid (0.5% and 0.08% v/v, respectively) for 15 minutes at room temperature. Finally, the funnel part is placed upside down onto the nanowell part, which is then flipped and transferred to a dedicated centrifuge holder (Figure 2B) prior to benchtop centrifugation. Subsequently, the funnel part contains each pooled sample and is transferred onto the autosampler holder (Figure 2C) for direct injection into the HPLC.

This workflow is described in detail in Hartlmayr, D. and Ctortecka C., et al. (11).

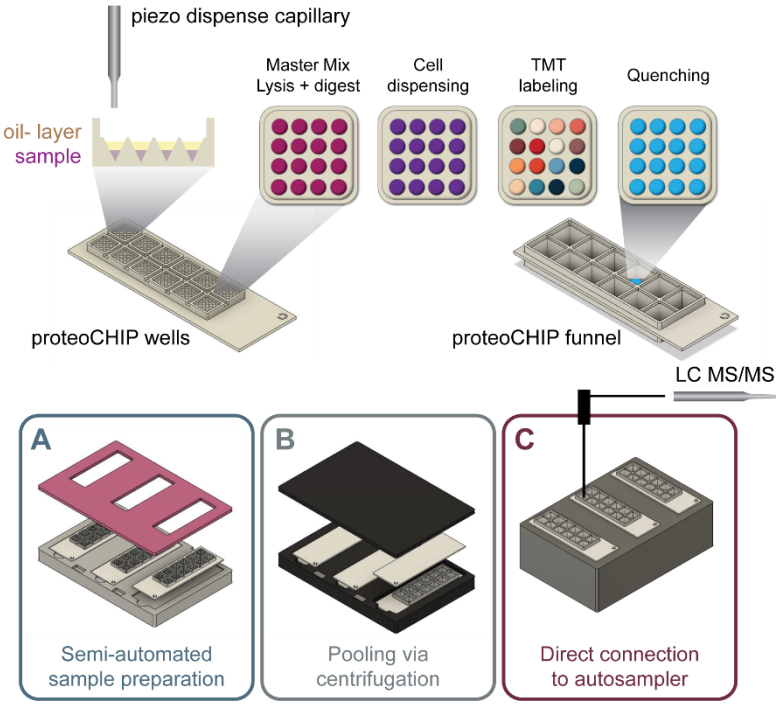

Figure 2. Illustration of the proteoCHIP 12*16 workflow. (a) (Top) Successive dispensing steps for each set of 16 single cells. (Bottom), A. proteoCHIP holder used in cellenONE, B. holder used for pooling of each sample set in proteoCHIP funnels using centrifugation, C. holder for direct interfacing of proteoCHIP funnels with an HPLC autosampler.

Figure taken from: Hartlmayr, D. and Ctortecka C., et al. "An automated workflow for label-free and multiplexed single cell proteomics sample preparation at unprecedented sensitivity." bioRxiv (2021).

Results and discussion

Establishment of an automated sample preparation workflow for single cell proteomics

A new protocol for automated sample preparation prior to multiplexed single cells proteomic analyses was optimised using the newly developed proteoCHIP 12*16 in combination with cellenONE® (11). This workflow allowed removal of all manual pipetting steps which are a major source of peptide loss and drastically reduced sample volumes which is essential to increase the efficiency of sample preparation.

Outstanding protein identification with reduced carrier

By reducing the number of cells used in the carrier channel from 200 cells to only 20 cells, optimal ion statistics and quantification accuracy was achieved (10). This optimized workflow resulted in the identification of around 1000 proteins per single HeLa cell using both TMT 10-plex and TMTpro (Figure 3a).

Unseen reporter ion signal-to-noise ratio without a carrier

To further optimize the efficiency of this workflow, the signal-to-noise ratios (S/N ratio) of the reporter ions were measured for both a reduced carrier (20 cells) and in absence of such channel. We observed higher S/N ratios than previously reported samples preparations (7,8), but more importantly, this was further enhanced when the carrier channel was completely removed while this protocol maintained good protein identification (800 to 900 proteins). This is the first report of a multiplexed sample preparation without the need of a carrier to improve peptide identifications by increasing ion counts.

In summary, the workflow introduced here, yielded high protein identification while a reduction in the carrier ratio or even its removal provided the best reporter ion S/N ratio reported for a multiplexed single cell proteomic experiment, yet.

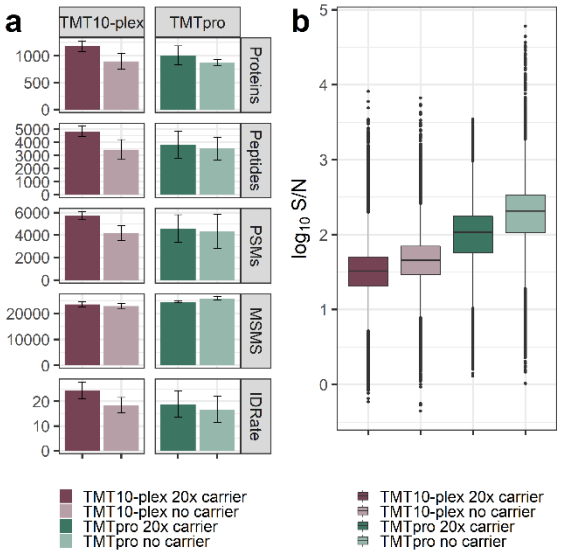

Figure 3 Application of the proteoCHIP for single cell proteomics sample preparation with TMT10-plex and TMTpro reagents at different carrier compositions. (a) Number of identified proteins, peptides, PSMs, all MS/MS scans and the ID rate for TMT10-plex (red) and TMTpro (green). Error bars represent median absolute deviation. (b) Log_{10} S/N of all single cell reporter ions at indicated condition over five replicates. Figure adapted from: Hartlmayr, D. and Ctortecka C., et al. "An automated workflow for label-free and multiplexed single cell proteomics sample preparation at unprecedented sensitivity." bioRxiv (2021).

Conclusion and future direction

A miniaturized and automated single cell proteomics sample preparation workflow using the cellenONE® and the proteoCHIP 12*16 has been demonstrated for multiplexed analysis of single cells in proteomic applications.

The optimized protocol and design of the proteoCHIP 12*16:

- drastically reduced digest volumes, limited chemical noise, and increased the efficiency of the sample preparation
- allowed automatic pooling of multiplexed samples and direct interfacing with standard autosampler for LC-MS/MS analysis, reducing peptide losses and additional variance
- allowed for complete removal of carrier channel or use of highly reduced carrier with only 20 cells, which resulted in increased quantitative accuracy
- could be implemented with most isobaric labeling reagents

The method presented here is the first complete workflow allowing automated sample preparation for multiplexed single cell proteomics analyses. By removing manual pipetting steps, by miniaturizing reaction volumes, and offering direct HPLC interfacing, it provided comparable protein identification while demonstrating outstanding S/N ratio. Being based on commercial solutions, this workflow has all the necessary ingredients to establish itself as a reference for all research groups wanting to implement single cell proteomic analyses in their laboratories.

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