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Ultra-sensitive LC-MS workflow for in-depth label-free analysis of single mammalian cells with nanodroplet sample processing

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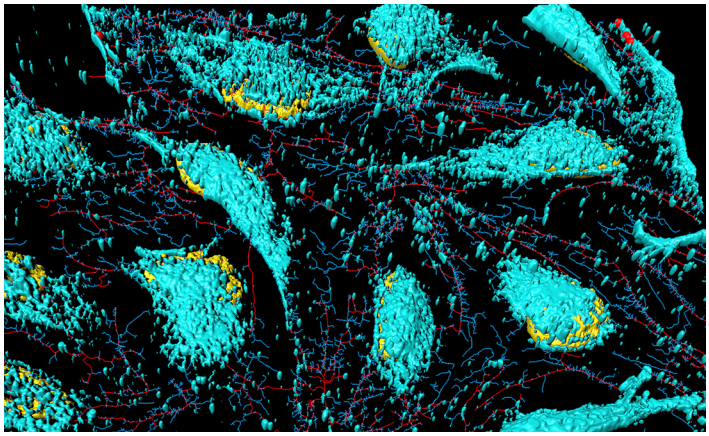
Abstract

In the last decade, single-cell RNA sequencing has advanced our understanding of transcript heterogeneity. Currently, there are strong efforts to enable single-cell proteomic analysis using mass spectrometry (MS)-based workflows. While the analysis of single-cell-sized aliquots from bulk-prepared tryptic digests has been demonstrated, only recently have label-free strategies been reported for profiling hundreds of proteins from single mammalian cells. Further development in sample processing, separations, MS and data analysis seems necessary to realize single-cell proteomics with greater depth of coverage and quantitative accuracy. Here we introduce an improved LC separation coupled to the new Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometers with a Thermo Scientific™ FAIMS Pro™ interface to increase proteome coverage for single mammalian cells.



Materials and methods

Single cells were isolated and processed on nanoPOTS (nanodroplet Processing in One-pot for Trace Samples) platform.[1] Solid phase extraction (SPE) and 20 µm i.d. analytical column were used for peptide trapping and separation.[2] A Thermo Scientific™ UltiMate™ 3000 RSLCnano system coupled to an Orbitrap Eclipse Tribrid MS with a FAIMS Pro interface were used for this ultra-sensitive workflow. Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.4 software with 2-steps SEQUEST search parameter including tryptic and semi tryptic search and percolator was used between each search to calculate the false discovery rate (FDR) and only those spectra with q-values lower than 0.01 were sent to the subsequent search filter and MaxQuant software was used for match between runs to estimate proteins in the blank sample run.

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Single viable cultured HeLa cells were imaged using an inverted microscope incorporated into the nanoPOTS platform, Figure 1. A glass capillary tip was used to aspirate individual targeted cells and dispense them into nanoPOTS nano-wells for proteomics sample processing. Processing samples in lower than typical digestion volume minimizes the surface samples are in contact with, thereby, helping to avoid sample loss in low amount of protein samples in a single-cell level, Table 1.

The three steps of tryptic peptide sample loading, and LC setup are shown in Figure 2; this workflow provides direct introduction of tryptic digested single-cell proteins to the analytical column for peptide separation on trap followed up with an ultra-low nanoflow rate LC-MS analysis.[2]

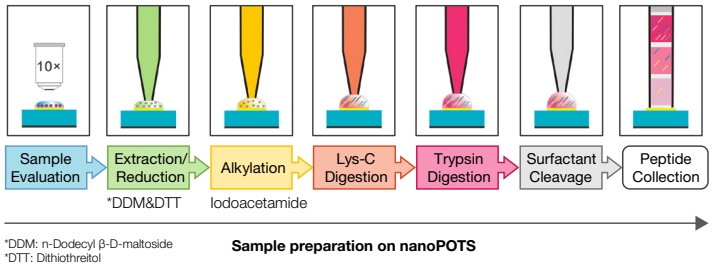

Figure 1. Protein digestion with nanoPOTS.

Table 1. Miniaturizing the digestion volume.

	Tube/Micro Well Method	nanoPOTS Method
Reaction Volume	100 µL	200 nL
Surface	127 mm^2	0.8 mm^2
Digestion Kinetics	Low	High

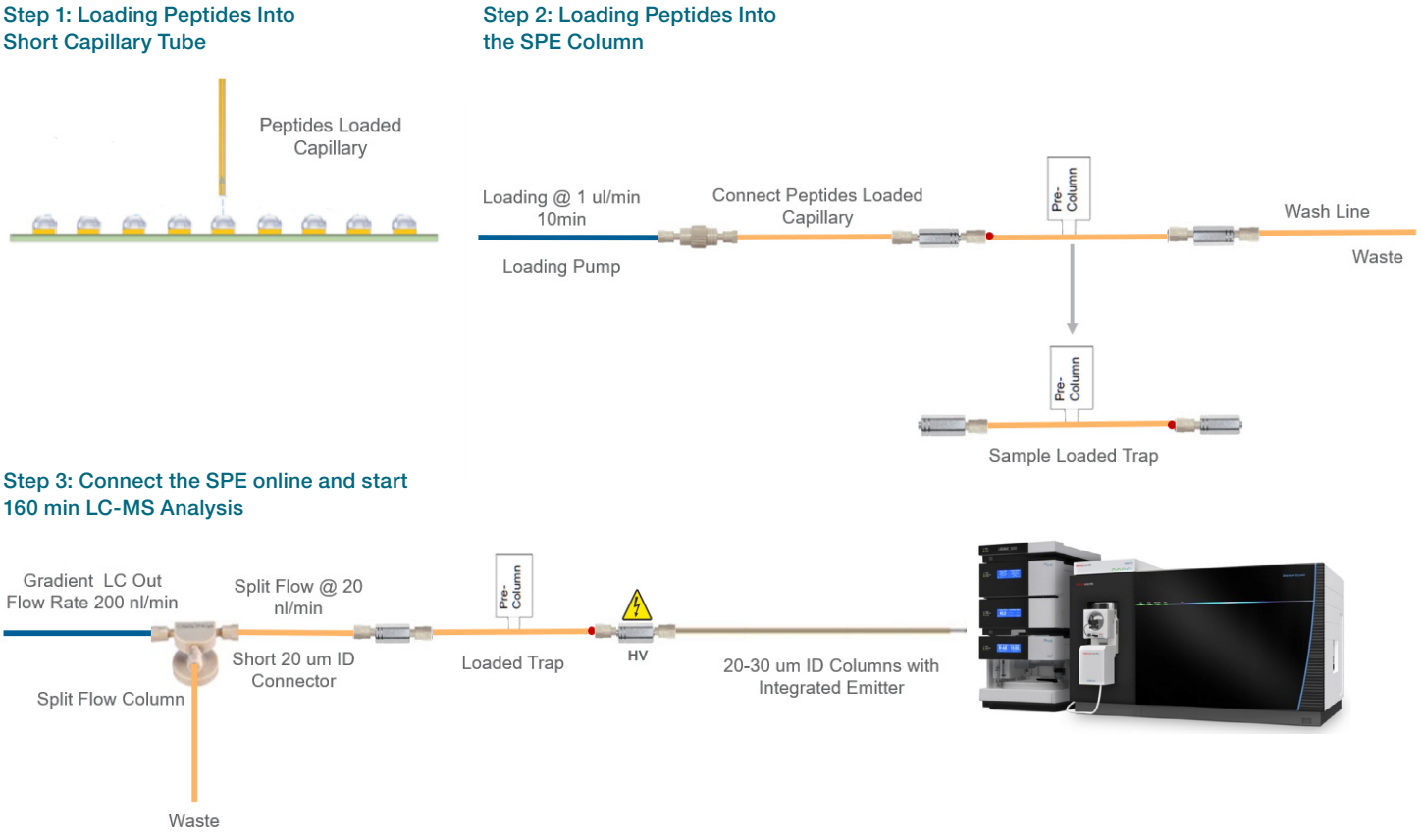

Figure 2. Ultra-sensitive low nanoflow LC-MS workflow for single-cell analysis.

To maximize sensitivity, and improve protein/peptide coverage, we evaluated high-field asymmetric waveform ion mobility spectrometry (FAIMS) for single-cell proteomics analysis. Reducing chemical noise and matrix interferences to increase the sensitivity[3] required to analyze single-cell proteome is one of the major bottle neck in the field of single-cell proteomics. To address this challenge, we evaluated FAIMS Pro interface, the next generation differential ion mobility, for single-cell analysis to see if the FAIMS Pro interface could reduce the chemical background and enhance signal to noise of peptides from single-cell proteome digest. FAIMS, offers separation based on a combination of factors, such as charge state, shape, conformation, and size of gas phase ions (Figure 3). It had previously shown to improve dynamic range and peak capacity, which are particularly beneficial in proteomics application of bulk samples.[3] Here we have investigated its benefit for low sample input applications like single-cell proteomics analysis.

Results

As a benchmark, we explored protein identification and coverage from single HeLa cells without utilizing FAIMS, Figure 4. The data shows the unique peptides and protein groups identified from isolated HeLa cells with MS analysis on an Orbitrap Eclipse Tribrid MS with HCD fragmentation (Orbitrap detection).

After analyzing single-cell proteome with nanoPOTS sample preparation protocol, we noticed peptide suppression by singly charged chemical backgrounds like PEGS. FAIMS Pro interface was used to evaluate enhanced peptide/protein coverage, Figure 5. The FAIMS Pro method was further optimized by evaluating fragmentation schemes, Figure 6.

The use of FAIMS Pro Interface and ultra-sensitive Orbitrap Eclipse Tribrid MS LC-MS data acquisition for single-cell analysis provides performance gains required for improved protein coverage in a label-free proteomics workflow. ~3000 peptides and ~830 protein groups were identified by MS/MS from a single HeLa cell alone, and match between runs identifications increased identifications to ~1300 protein groups and ~5800 peptide groups. This is the first example of >1000 proteins being identified from single mammalian cells with a label-free proteomics approach. This maximum recovery LC-MS workflow provide analytical sensitivity needed for analyzing any sample limited proteomics project with unmatched sensitivity to analyze single-cell proteome.

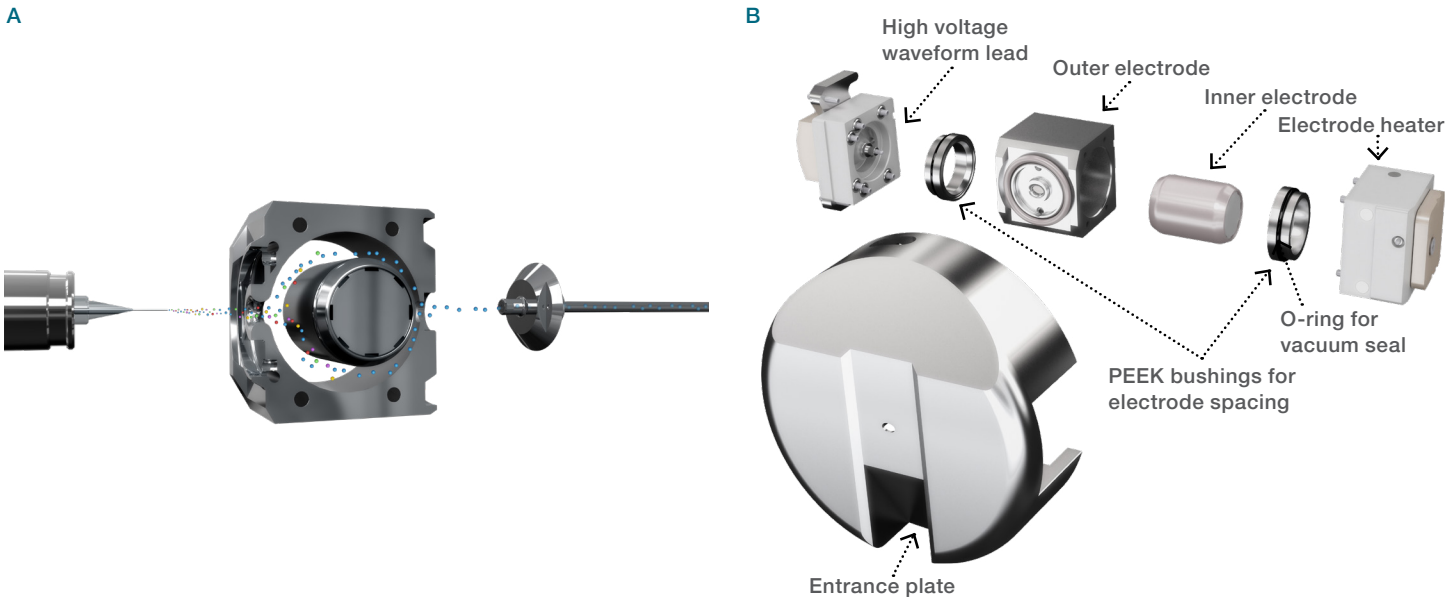

Figure 3. FAIMS Pro interface. The FAIMS Pro interface offers separation based on a combination of factors, such as charge state, shape, conformation, and size of gas phase ions[3]. It also improves dynamic range and peak capacity, which are particularly beneficial in proteomics applications like single-cell analysis. Side view of ion spray source, FAIMS electrodes and transfer tube to the mass spectrometer (A), and schematic overview of the FAIMS Pro interface; electrode assembly with 1.5 mm gap between outer and inner electrode (B) which is easy to clean is shown here.

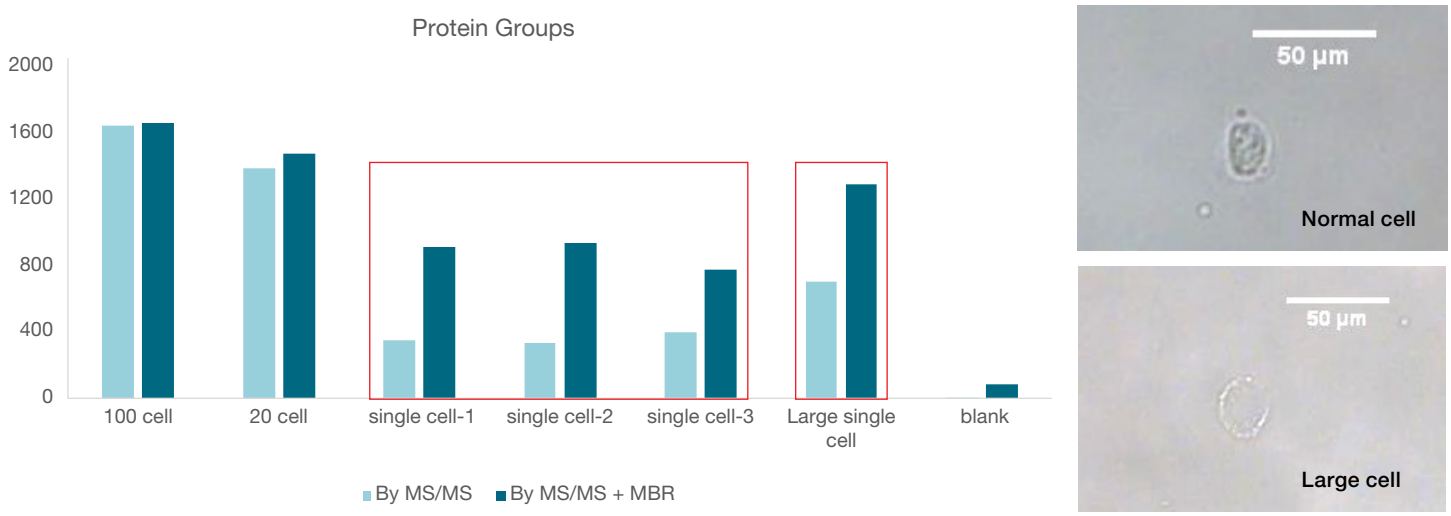

Figure 4. Protein identification from single HeLa cells. Isolated single HeLa cells, (uniform and large), pooled HeLa cells 20 and 100 cells were digested and analyzed along with blank injection. Protein group identifications reported are based on MaxQuant with MS/MS spectra and >1% FDR. MaxQuant match between run (MBR) was against 20 and 100 identified spectra to estimate method carry over after 100 cell sample analysis from analysis of proteins identified blank sample. On average ~400 protein groups were identified from single HeLa cells with MS/MS and ~1000 with MBR with ~2× more proteins identified from large HeLa cell analyzed without FAIMS Pro interface.. The protein coverage is 2× more than previously reported protein coverage with Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer.

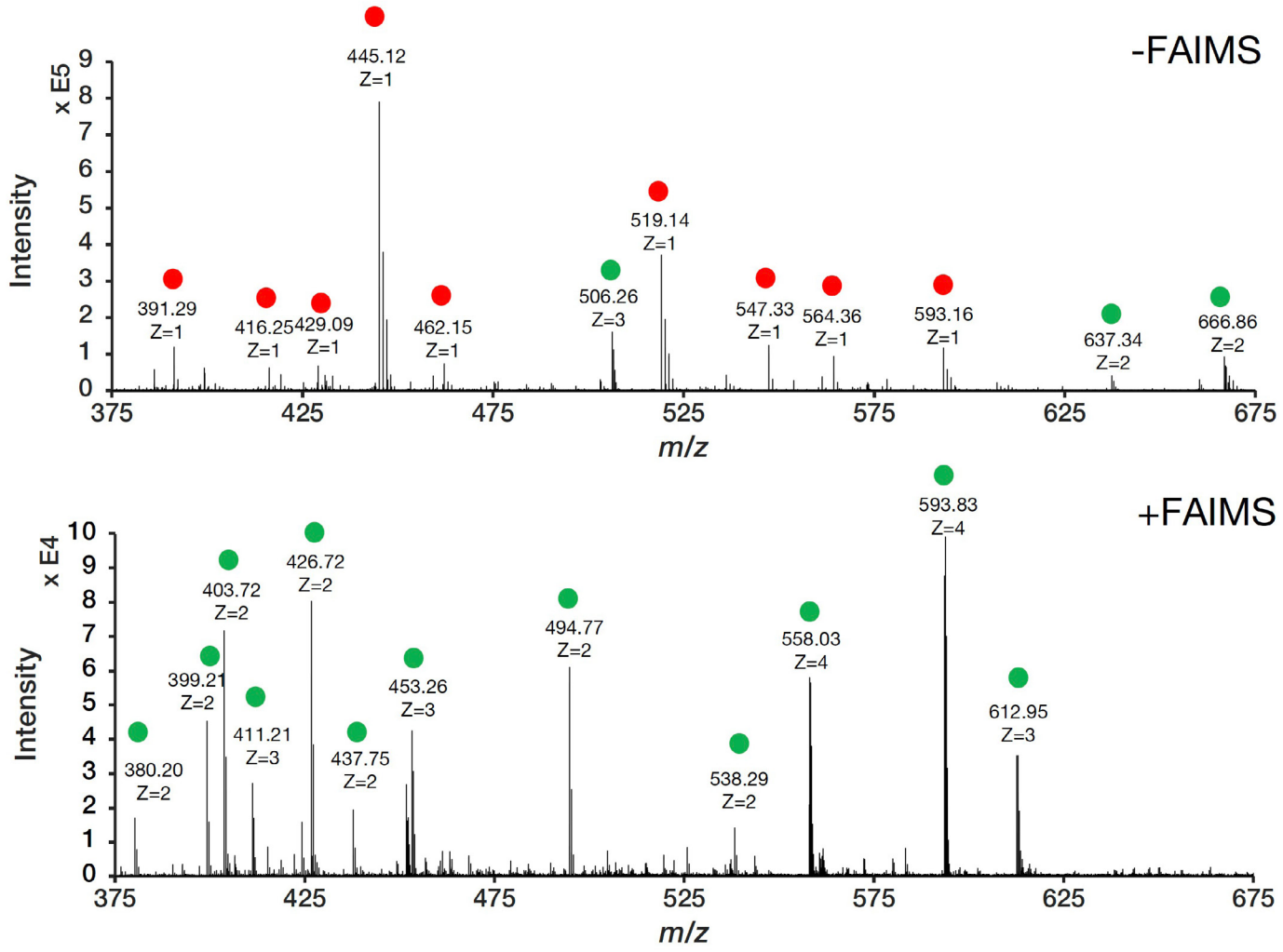

Figure 5. Proteome coverage enhancement with FAIMS Pro interface. FAIMS Pro Interface enhances peptide/ proteome coverage in singe-cell level by removing +1 charged chemical noise ions (red dots) which are suppress multiply charged peptides ions (green dots) from single cell, allowing lower abundant peptides to be detected by MS.

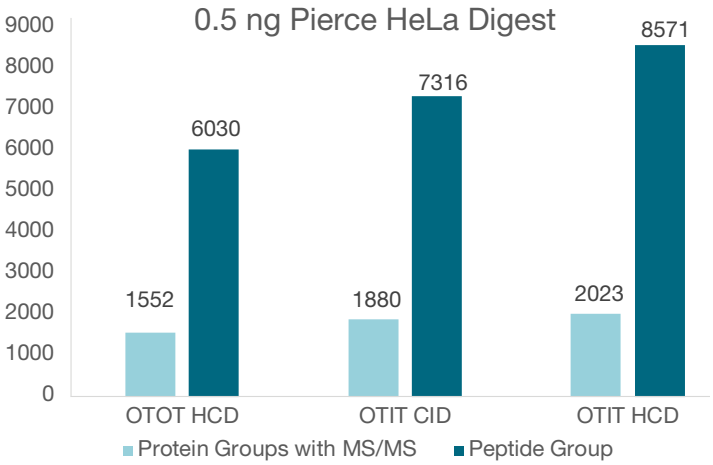

Figure 6. FAIMS Pro method performance. The performance of this ultra-sensitive LC-MS workflow was evaluated and optimized using 0.5 ng aliquots of Thermo Scientific™ Pierce™ HeLa Protein Digest Standard. MS and HCD fragmentation detected within Orbitrap mass analyzer shown to provide sensitivity and selectivity required for single-cell proteomics analysis. With a 2 hr gradient and two CV (compensation voltage) switching, 2023 protein groups and 8571 peptide group were identified from only 0.5 ng HeLa digest with FDR rate of 1% or better.

Table 2. LFQ performance enhancement with optimized workflow.

	Initial workflow	Optimized method
Sample Preparation	nanoPOTS	nanoPOTS
Separation	30 µm i.d. column	20 µm i.d. column
Mass Spectrometer	Orbitrap Fusion Lumos Tribrid MS	Orbitrap Eclipse Tribrid MS + FAIMS Pro interface
Data Analysis	MaxQuant	Proteome Discoverer software
Single HeLa Cell Protein Group ID by MS/MS (MS/MS+MBR)	211 (669)	829 (1300)
100 HeLa Cells Protein Group ID by MS/MS	2109	3067

Conclusions

- The nanoPOTS nanodroplet processing platform dramatically enhance proteomic sample processing and analysis for small cell populations and single cells.
- Our ultra-sensitive low nanoflow LC-MS method using FAIMS Pro interface and Orbitrap Eclipse Tribrid mass spectrometer in trap sensitivity has significantly improved single-cell proteome coverage.
- This MS-based proteomics workflow has become a valuable tool for label-free single-cell proteomics analysis.

References

1. Zhu, Y. et. al. Nat. Commun. 9, 882, (2018)
2. Kelly, R.T. et. al. Anal. Chem. 92,3, 2665-2671 (2020)
3. Schweppe D.K. et. al. Anal. Chem. 91,6, 4010-4016 (2019)

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