

● Profiling mammalian cell differentiation by MALDI-TOF MS: Developing a highly reproducible and robust sample preparation workflow

In this application note, we present a universal workflow for mammalian cell MALDI-TOF MS analysis in order to distinguish between ground-state naïve and differentiating stem cells.

Abstract

A systematic approach was employed to test parameters such as initial sample handling, matrix choice, and suitability of fixing techniques. The Bruker rapifleX

MALDI PharmaPulse approach allowed for label-free measurement and robust phenotyping of cell differentiation in under one hour from culture to analysis, which is significantly faster and cheaper when compared

with conventional methods. This method has the potential to be automated and can be further expanded towards cellular MALDI-TOF MS screening assays for drug discovery.

Keywords:
rapifleX MALDI
PharmaPulse, cell-based
assay, high-throughput
screening platform,
drug discovery

Introduction

In recent decades, mass spectrometry (MS) has become a widely adopted tool in the field of drug discovery, overcoming the shortcomings of conventional fluorescence label-based technologies. Matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) is one of the most validated MS techniques for high-throughput screening (HTS) as it is tolerant to a number of standard buffer components, and requires small sample quantity and minimal sample clean up. Moreover, recent advancements in instrumentation, such as the speed, sensitivity and robustness of the rapiflex MALDI PharmaPulse, have allowed MALDI-TOF MS to play a greater role in discovering and developing new therapeutics.

Recently, the rapiflex MALDI PharmaPulse has been used in various biochemical assays measuring mass changes upon compound treatment in pharmaceutical research. For example, this technology has been successfully established to identify inhibitors of enzymes transferring post-translational modifications such as ubiquitylation [1,2], phosphorylation [3,4], and methylation [5]. Most of the MALDI-TOF based HTS approaches so far have focused on *in vitro* assays with simple readout, and have been limited to peptide/protein-centric activity assays.

Applying this technology for cellular assays for evaluating compound efficacy affecting a cellular phenotype has been demonstrated on limited applications [6,7]. However, many of these studies list dramatically different experimental procedures which can be problematic for translation of published assays to the pharmaceutical industry. To address the variation in experimental workflows, here we describe a

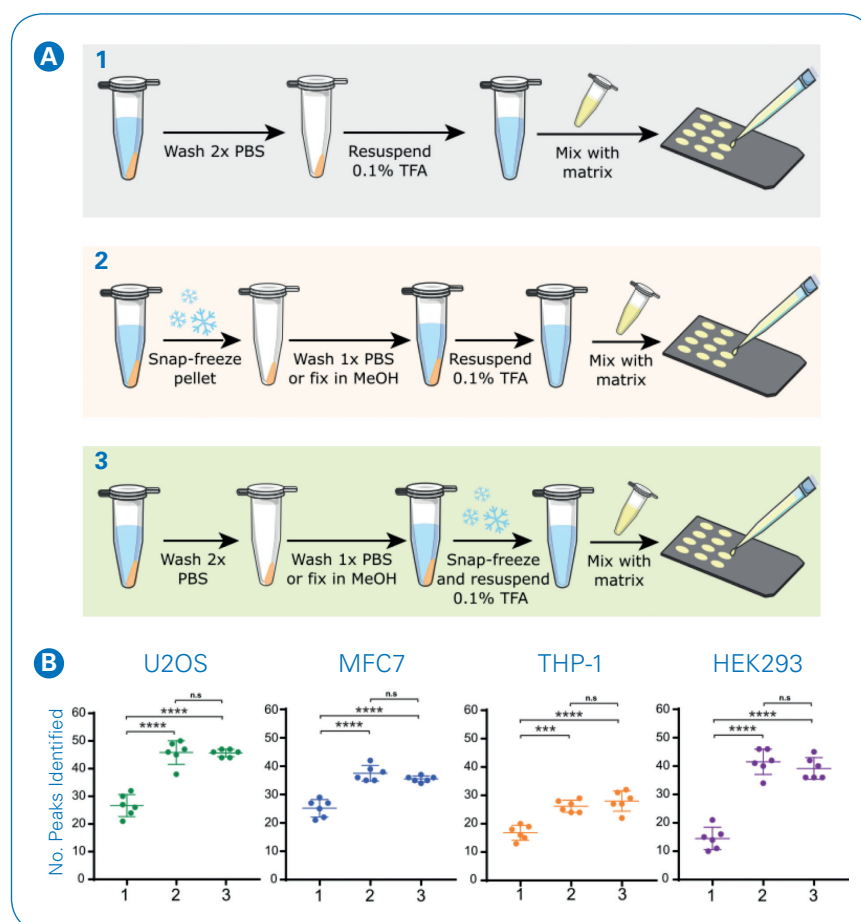


Figure 1: (A) Schematic showing sample preparation optimisation using cell pellets, (B) Number of peaks identified across six technical replicates for each of the experimental workflows shown in (A). Error bars represent standard deviation of six replicates. *** and **** represent $p < 0.001$ and $p < 0.0001$, respectively, Student's t-test.

systematic approach to test various sample preparation parameter such as matrix selection, cell fixing techniques, and sample handling. We have established a sample preparation method that is highly reproducible, sensitive, and robust that would be suitable for expansion to a HTS platform.

Methods

Four human cell lines (HEK293, U2OS, MCF7 and THP-1) were used in order to optimise the sample preparation for whole cell MALDI-TOF MS. Cells were harvested by aliquoting 1 mL (1×10^6 cells/mL) into 1.5 mL microtubes and centrifuging at 300xg,

4°C for 10 minutes. After aspirating the supernatant, the cell pellets were processed in one of three ways, as shown in Figure 1A:

1. Direct analysis where cell pellets were washed twice with PBS, centrifuged (300xg, 4°C for 10 minutes) and resuspended in 0.1% TFA.
2. Cell pellets were snap-frozen on dry ice, then either washed 1x with PBS or fixed in 4% paraformaldehyde solution or methanol on ice. Cell suspensions were then centrifuged (300xg, 4°C for 10 minutes) and resuspended in 0.1% TFA.

Table 1: Measurement parameters

MS conditions rapifleX PharmaPulse MALDI-TOF	
Mass range	2000-20,000 Da
Ion mode	Linear positive
Laser frequency	10 kHz
Laser focus	M5 Smart beam parameter at 45 μm x 45 μm
Accumulated laser shots	10,000 in random walk pattern (complete sample)
Sampling rate	1.25 GS/s
Laser power	Laser power was optimised for individual MALDI matrices in use to yield optimum spectral quality (i.e. number of spectral features; S/N)

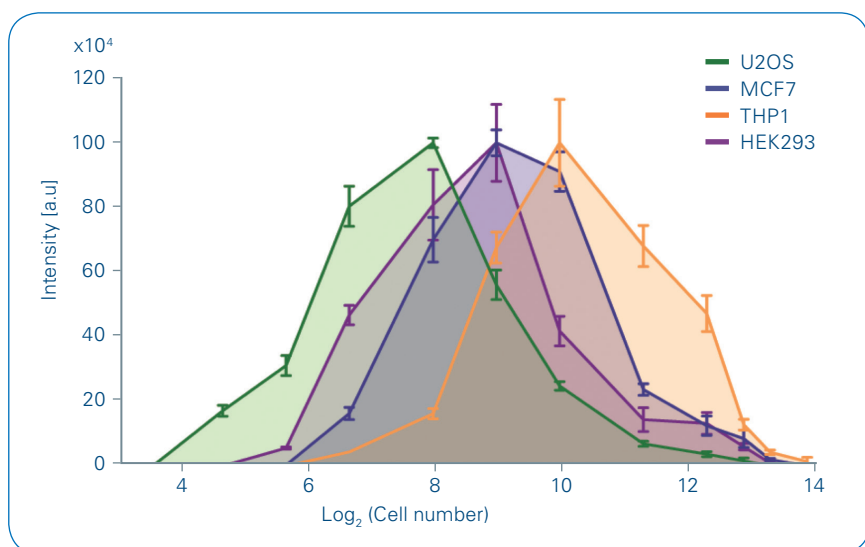


Figure 2: 2D plot of normalised mass spectrum intensity at different cell numbers on target for each of the four cell lines. Plots have maxima indicating optimal cell numbers.

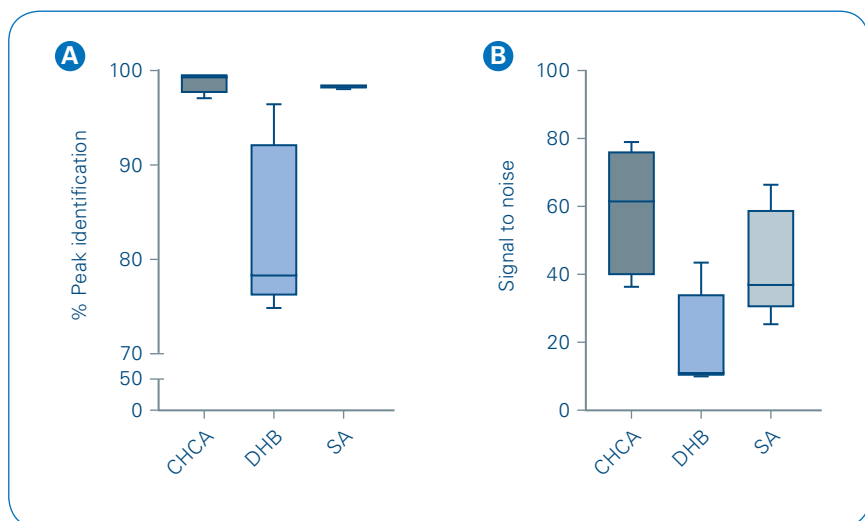


Figure 3: (A) Percent peak identification of the top 5 most intense peaks over a 1536 target for each of the three matrices. (B) Box and whisker plots showing distribution of the signal-to-noise of the top 5 most intense peaks over a 1536 target for each of the three matrices.

3. Cell pellets were washed twice with PBS, centrifuged (300xg, 4°C for 10 minutes). Cell pellets were either washed 1x with PBS or fixed in 4% paraformaldehyde solution or methanol on ice. Cell suspensions were then centrifuged and snap-frozen on dry ice, before being resuspended in 0.1% TFA.

For matrix optimisation, all the matrices (SA, CHCA, and DHB) were prepared in ACN/water (1:1 v/v) supplemented with 0.1% TFA at varying concentration and ratios of matrix solute: 2.5, 10, 20 mg/mL or saturated. For manual deposition, cell suspensions were mixed at a 1:1 ratio with matrix solution and 1 μL was spotted onto a ground steel MALDI target before ambient drying. For automated target spotting, a Mosquito liquid handling robot (TTP Labtech) was used where cell suspension were mixed at a 1:1 ratio with matrix solution before subsequent deposition of 200 nL on a Bruker AnchorChip MALDI target.

The sample plate was subsequently measured on a rapifleX MALDI-TOF mass spectrometer (Bruker Daltonics). For detailed method parameters see Table 1. MALDI-TOF data were processed using the FlexAnalysis 4.0 software and further processed with Perseus [8], and scripts for statistical evaluation.

Results and Discussion

It has been previously reported that freeze-thawing of cell pellets prior to MALDI-TOF MS analysis may have beneficial effects with respect to the number of features identified and overall spectral intensity [9]. This is likely due to the freeze-thaw cycle which permeates the cell membrane. Therefore, freezing before and after a wash with PBS was compared to see whether this process affects the sensitivity and spectra quality

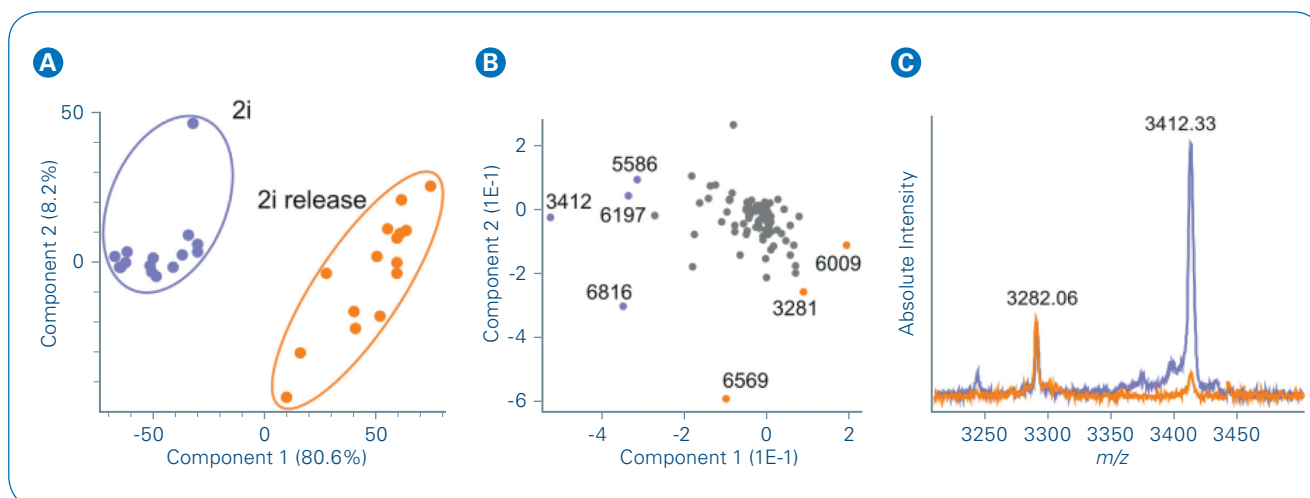


Figure 4: (A) PCA plot of the biological and technical replicates of naïve ground state mESCs compared to differentiating mESCs. (B) Loading plot corresponding to (A) showing the m/z values that contribute most of the separation of the two cellular phenotypes. (C) Selected mass spectral region that shows changes between naïve and differentiating populations of mESCs.

compared with direct analysis. As shown in Figure 1B, both methods of freeze/thawing permeated the cell membrane of about 50-80% of the cells, which led to a significant increase in the number of peaks identified compared to 'intact' cell samples. From this experiment, it was concluded that a freeze/thaw cycle is critical to improve the quality of MALDI-TOF data as it increases the number of features detected. The order in which the freeze/thaw step is performed does not affect the final readout.

Moreover, to determine optimal cell concentration, we spotted 25 to 20,000 cells on target. Surprisingly, there was a narrow window where good spectra could be acquired, with large numbers of cells on-target proving to be detrimental to ionisation (Figure 2). For the used cell lines, an optimum of 50-2000 cells was identified, showing a very high level of sensitivity of the rapifleX mass spectrometer.

Next, different matrices (SA, CHCA, and DHB) were tested to see which matrix is best for MALDI-TOF MS analysis of proteins and peptides. As expected, when each cell line

sample was prepared with either SA, CHCA, or DHB, significantly different mass profiles of the same cell line were observed. Regardless of the matrix concentration, DHB resulted in more variable spectra over technical replicates, while CHCA yielded both informative spectra and more detected peaks at a third of the concentration of DHB and SA. Moreover, in both CHCA and SA, the top five most intense feature were identified in > 98% of spots, showing robustness for HTS, whereas samples spotted with DHB were much more variable (Figure 3A). Interestingly, the signal-to-noise (S/N) ratio (Figure 3B) also varied significantly between the matrix conditions. Samples spotted with CHCA exhibited much greater spectral intensity compared to SA, and an almost 10-fold increase when compared to DHB, as well as a significantly better S/N ratio for these top 5 features. From this data, CHCA was chosen to be the most optimal matrix choice for whole cell analysis due to its superiority across the parameters discussed above and in Heap et al. 2019 [10].

Finally, as a proof-of-concept, the optimised workflow was applied

to distinguish between naïve and differentiating populations using multivariate analysis. The method was validated by the distinct MALDI-TOF MS profiles for naïve ground state mouse embryonic stem cells (mESCs) compared to differentiating mESCs in a pharmacologically controlled system. Using PCA and hierarchical clustering, a subset of peaks were identified to be unique to each condition (Figure 4). This novel sample preparation method enabled a robust, reproducible, and rapid profiling of mammalian cells and is suitable for expansion to a high-throughput platform.

Conclusions

- A systematic study was employed to test initial sample handling, matrix choice, and fixing techniques for MALDI-TOF MS based cell-based assays.
- The rapifleX MALDI-TOF MS allows for label-free and robust measurements for phenotyping cell differentiation of mESCS.
- This method has the potential to be automated and can be further expanded towards MALDI-TOF MS screening assays for drug discovery.



Learn More

You are looking for further Information?
Check out the link or scan the QR code for more details.

www.bruker.com/mpp



References

- [1] Ritorto MS, Ewan R, Perez-Oliva AB, Knebel A, Buhrlage SJ, Wightman M, Kelly SM, Wood NT, Virdee S, Gray NS, Morrice NA, Alessi DR, Trost M (2014). Nature Communications, **5**, 4763.
- [2] De Cesare V, Johnson C, Barlow V, Hastie J, Knebel A, Trost M (2018). Cell Chemical Biology, **25** (9), 1117-1127.e4.
- [3] Winter M, Bretschneider T, Kleiner C, Ries R, Hehn JP, Redemann N, Luippold AH, Bischoff D, Büttner FH. (2018). SLAS DISCOVERY: Advancing Life Sciences R&D, **23** (6), 561-573.
- [4] Heap RE, Hope AG, Pearson L-A, Reyskens KMSE, McElroy SP, Hastie CJ, Porter DW, Arthur JSC, Gray DW, Trost M (2017). SLAS DISCOVERY: Advancing Life Sciences R&D, **22** (10), 1193-1202.
- [5] Guitot K, Drujon T, Burlina F, Sagan S, Beaupierre S, Pamard O, Dodd RH, Guillou C, Bolbach G, Sachon E, Guianvarc'h D (2017). Analytical and Bioanalytical Chemistry, **409** (15), 3767-3777.
- [6] Weigt D, Parrish CA, Krueger JA, Oleykowski CA, Rendina AR, Hopf C (2019). Cell Chemical Biology.
- [7] Winter M, Bretschneider T, Thamm S, Kleiner C, Grabowski D, Chandler S, Ries R, Kley JT, Fowler D, Bartlett C, Binetti R, Broadwater J, Luippold AH, Bischoff D, Büttner FH (2019). SLAS DISCOVERY: Advancing Life Sciences R&D, 2472555219838216.
- [8] Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016). Nature Methods, **13**, 731.
- [9] Chen X, Wo F, Chen J, Tan J, Wang T, Liang X, Wu J (2017). Scientific Reports, **7** (1), 17432.
- [10] Heap RE, Segarra-Fas A, Blain AP, Findlay GM, Trost M (2019). Analyst Oct 22; **144**(21):6371-6381. doi: 10.1039/c9an00771g.

For Research Use Only. Not for Use in Clinical Diagnostic Procedures.



Bruker Daltonics GmbH & Co. KG

Bruker Scientific LLC

Bremen · Germany
Phone +49 (0)421-2205-0

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com