4D-Proteomics Unveils Post-translational Control of Immune Signaling in HSV-1 Infection

Advanced Lysine Lactylation Profiling Enabled by timsTOF Ultra.

Abstract

Post-translational modifications (PTM) are covalent modifications to proteins that significantly increase proteome diversity, altering protein function, localization, and turnover, among other roles. Viruses exploit certain PTMs to remodel host metabolism, promote replication, and evade immunity. Despite their importance, many PTMs remain underexplored because they occur at low stoichiometry and are difficult to distinguish from higher abundant unmodified peptides. Protein lysine lactylation, a recently recognized PTM induced by the metabolite lactate, exemplifies these challenges. Conventional mass spectrometry approaches lack the separation power, sequencing speed, and sensitivity needed to effectively map lactylation across the proteome.

These barriers are overcome through the implementation of lactyl-peptide enrichment paired with 4D-Proteomics™ using the timsTOF Ultra, which couples trapped ion mobility with PASEF® while providing higher ion transmission, extended dynamic range, and lower limits of detection compared to other MS approaches. Following LC separation, the ion mobility dimension separates isobaric and co-eluting ions to reduce chimeric spectra, while the highly efficient PASEF increases sequencing speed, enabling robust sampling of low-abundance modified peptides with cleaner spectra for identification and modification localization. Using dia-PASEF, over 45,000 sites across more than 6600 proteins were quantified from α-herpesvirus HSV-1-infected fibroblasts, spanning seven orders of magnitude in abundance with high quantitative reproducibility across biological replicates [1]. Lactylation was widespread across cellular compartments and dynamically regulated over infection, affecting proteins in immune signaling, metabolism, and chromatin control. These findings position lactylation as a key viral strategy for immune evasion and demonstrate the value of high-resolution ion mobility-enabled proteomics.

Keywords:
timsTOF Ultra, nanoElute 2, PTMs, lactylation, immune signaling, viral infection

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Introduction

Post-translational modifications rapidly toggle protein functions and serve as an important interface of virus-host interactions. However, their analysis remains among the most technically demanding areas of proteomics, due to sub-stoichiometric abundances. Well-characterized modifications such as phosphorylation [2] and acetylation [3] are comparatively tractable, but atypical PTMs demand extensive optimization of sample preparation and acquisition methods. Moreover, PTM quantification is typically performed at the peptide level, which lacks the signal averaging of global proteomics and amplifies the need for reproducible detection.

Trapped ion mobility spectrometry (TIMS) addresses these analytical barriers by introducing an additional dimension of ion separation in the gas phase with time and space focusing of peptide ions, during which all precursors are assigned collisional cross section (CCS) values. This additional dimension of information, when combined with enrichment and separation of ions in the gas phase, greatly improves both the sensitivity and identification confidence for analyzing low-abundance PTMs. TIMS paired with dia-PASEF further increases throughput and depth of proteome coverage, enabling more reproducible and comprehensive PTM profiling. Additionally, TIMS can facilitate facile separation of larger modifications such as glycans through additional acquisition modes, including glyco-PASEF® [4] and timShift [5].

Here we present the use of the timsTOF Ultra to profile lysine lactylation in host cells infected with the prominent human pathogen, α-herpesvirus HSV-1, which is known to disrupt immune signaling. Comparing wild-type HSV-1 with the *ICP0-RF* mutant, which lacks the ability to suppress immune responses, revealed how lactylation may influence viral modulation of host responses. This approach deepens our understanding of lactylation during infection and showcases the power of high-resolution ion mobility mass spectrometry for uncovering previously inaccessible post-translational modifications in complex systems.

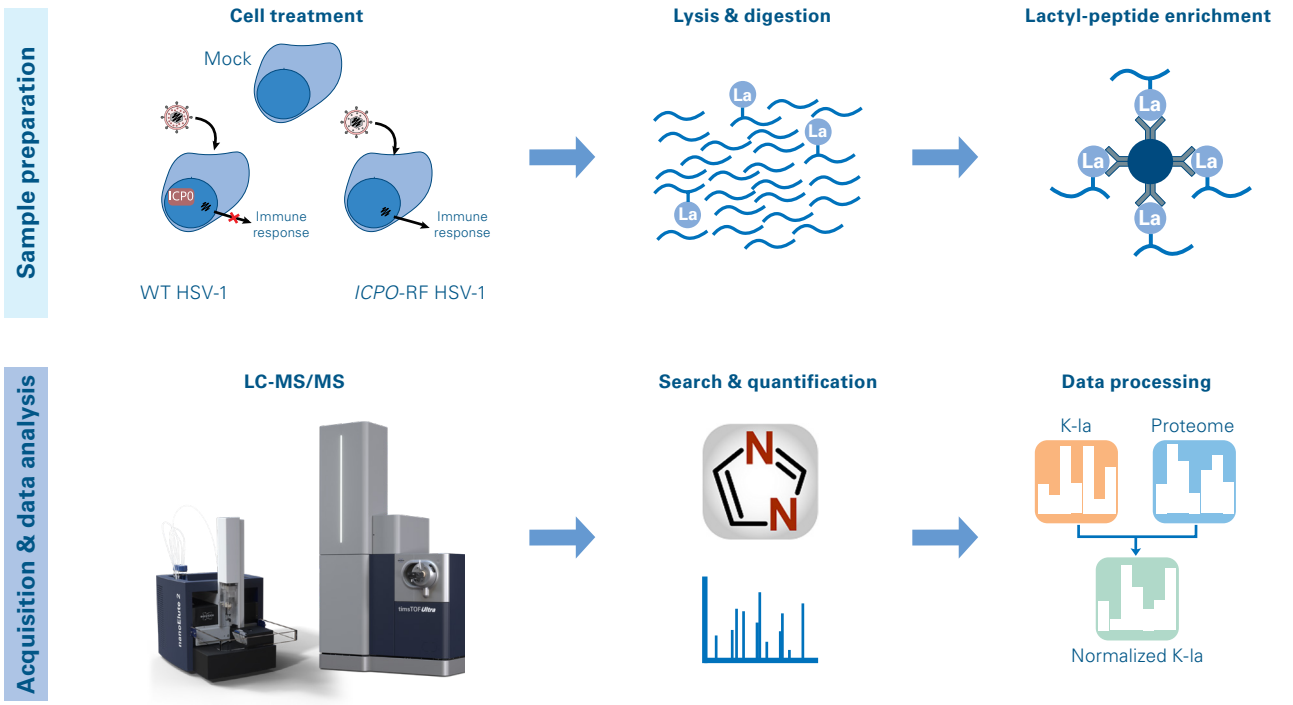

Figure 1. The implementation of 4D-Proteomics enables robust quantification of lysine lactylation sites across the HSV-infected proteome. Workflow for global lactylome sample preparation, acquisition, and data analysis. Both the global proteome and the lysine lactylation-enriched proteome were acquired on a timsTOF Ultra paired to a nanoElute 2, before searching and quantifying with DIA-NN. Following search, the lactyl-lysine sites were normalized to the global proteome before performing downstream statistical analysis. Mock, no virus infection; WT HSV-1, wild type herpes simplex virus 1. *ICP0-RF* HSV-1, mutant herpes simplex virus 1.

Methods

Reduced and alkylated MRC-5 cell proteins were digested with trypsin (Figure 1). Following peptide cleanup, 150 ng was reserved for whole proteome analysis while the remainder of the peptides underwent anti-lactyl-lysine IP (PTM BIO) with agarose beads. Bound peptides were eluted with TFA, desalted on C18 StageTips, dried, and resuspended in 0.1% FA/4% ACN.

Samples were analyzed on a timsTOF Ultra mass spectrometer equipped with a Captive Spray 2 ion source containing a 10 μm emitter. Peptides were resolved using a nanoElute® 2 nLC system with a PepSep® C18 column (1.5 μm by 75 μm by 25 cm). Peptides were separated with a 40-min gradient using a mobile phase composed of 0.1% FA as solvent A and 0.1% FA/99.9% ACN as solvent B. A linear gradient was run, consisting of 3 to 34% buffer B at a flow rate of 200 nL/min.

dia-PASEF was used for all analyses. For lactyl-peptides, TIMS settings included a 100 ms ramp time and a 9.42-Hz ramp rate, while the global proteome included a 50 ms ramp time and 17.80-Hz ramp rate. Both methods include three ion mobility windows per dia-PASEF scan with variable isolation widths adjusted using py_diAID [6], with a total of 16 dia-PASEF scans per cycle. All spectra were acquired within 300 – 1300 *m/z*, with an ion mobility range of 1.45 to 0.60 V cm^{-2}. Collision energy was decreased from 59 eV at $1/K_0$ = 1.6 V cm^{-2} to 20 eV at $1/K_0$ = 0.6 V cm^{-2}.

An experimental lactyl-peptide spectral library was generated from DDA runs on the HSV–infected lactyl-peptide enriched samples using FragPipe 21.1 [7]. This spectral library was used to search with DIA-NN 1.82 beta 278, using the variable modification of 72.011 Da on lysine residues.

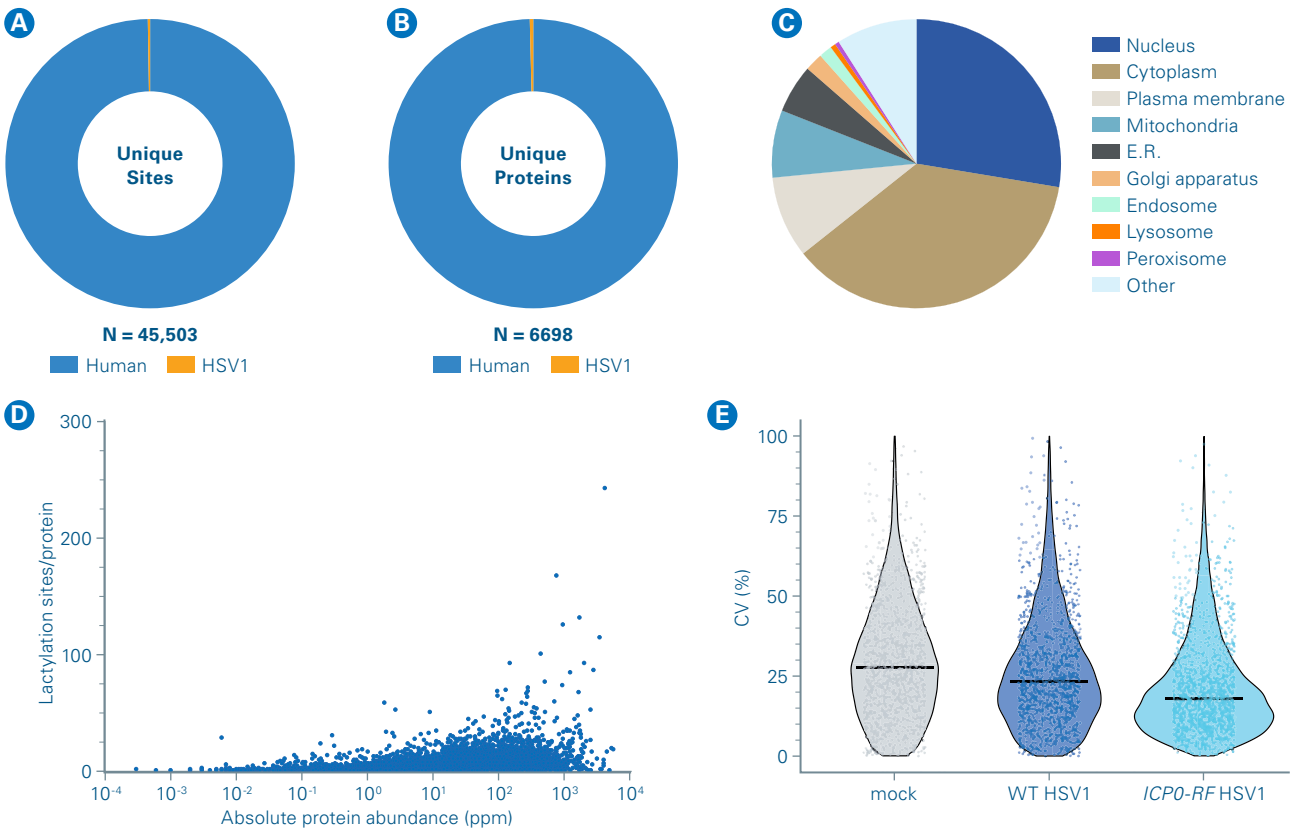

Figure 2. The timsTOF Ultra facilitated deep coverage of the lactyl-lysine proteome, with high sensitivity and precision. (A) Number of unique lysine lactylation sites measured across samples. (B) Number of unique proteins containing quantified lactylation sites. (C) Primary subcellular localization (UniProt) of lactylated proteins across all lactylation sites. (D) Lactylation sites were identified across proteins spanning over 7 orders of magnitude. (E) Lactyl-peptide abundance CVs across three biological replicates across different conditions. Mock, no virus infection; WT HSV-1, wild type herpes simplex virus 1. *ICP0-RF* HSV-1, mutant herpes simplex virus 1.

Results

The paired implementation of affinity purification of lactylated peptides with the 4D-Proteomics resulted in 45,503 unique modification sites quantified across 6698 protein groups (Figure 2A, B). Protein lactylation was observed across all major cellular compartments, with roughly two-thirds of modified proteins localized to the nucleus and cytoplasm, and substantial representation in the mitochondria, endoplasmic reticulum, plasma membrane, and other organelles (Figure 2C). This broad distribution underscores lactylation as a pervasive regulatory layer across the host–virus proteome.

Additionally, when accounting for absolute protein abundances in lung tissue sourced via PaxDb, the timsTOF Ultra facilitated identification and quantification of lactylation on proteins with abundances across seven orders of magnitude (Figure 2D). This sensitivity revealed a significant enrichment of lactylation within intrinsically disordered regions, demonstrating a previously unknown functional role for this PTM. Furthermore, this coverage was accompanied by robust quantitative performance, with an average median CV of 25% across biological replicates (Figure 2E).

Together, these data establish lysine lactylation as a broad and dynamically regulated feature of the host proteome during viral infection (Figure 3A). The extensive modification of nuclear and cytoplasmic proteins, along with enrichment on intrinsically disordered regions, points to lactylation as a versatile regulator of protein networks central to immunity and metabolism. Temporal modulation across infection further implicates lactylation in coordinating host responses, including the dampening of DNA damage repair and antiviral defense pathways (Figure 3B,C). Indeed, the authors of this study demonstrate that the introduction of a K90 lactyl-mimic mutation in the intrinsically disordered region of the host immune factor IFI16 inhibits its ability to induce cytokines, resulting in increased HSV-1 production (Figure 3D-F). More broadly, this extensive profiling of the lactylation landscape facilitated by 4D-Proteomics opens the door for further investigations as to the role of this emerging PTM in immunology and other biological regulation.

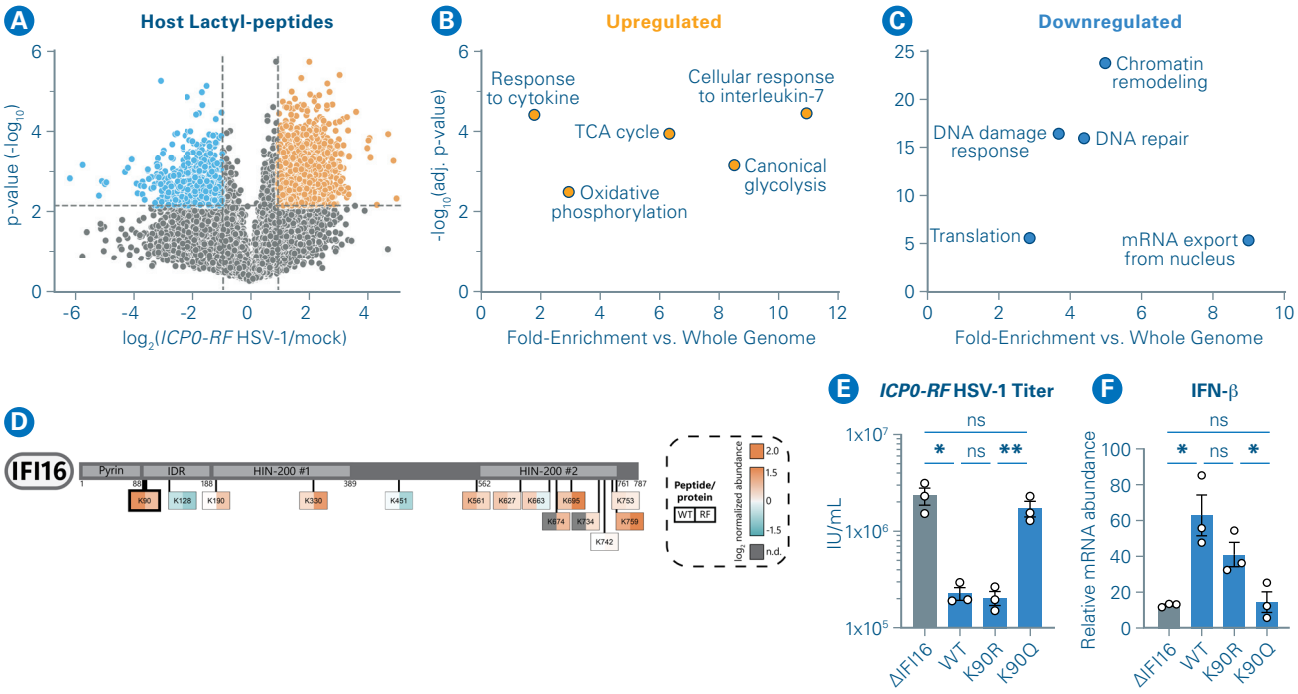

Figure 3. The quantification of lactylation revealed novel roles across biology.
(A) Volcano plot showing host lactyl-peptide abundances (normalized to protein abundance) as $\log_2$ fold-change over mock abundance during *ICP0-RF* HSV-1 infection. Dotted lines show threshold for differentially regulated sites: fold change = 1.5 and P < 0.05. (B) – (C) Scatter plots showing select enriched gene ontology (GO) biological processes among upregulated or downregulated lactyl-peptides using g:Profiler. (D) IFI16 lysine lactylation sites and abundances. (E) IFI16 lactyl-mimic mutation on K90 restores *ICP0-RF* HSV-1 viral replication and (F) inhibits cytokine induction. Two-tailed Student's t test: n.s., not significant, *P < 0.05, **P < 0.01.

Conclusion

- The timsTOF Ultra paired with dia-PASEF enables deeper profiling of lysine lactylation, quantifying over 45,000 sites across more than 6600 unique proteins using five times less input material compared to conventional DIA workflows. The enhanced sensitivity of next-generation instruments—timsTOF Ultra 2 and timsUltra AIP—is expected to further improve these metrics.
- Lactylation sites were detected on proteins spanning seven orders of magnitude in abundance.
- Low coefficient of variation across biological replicates demonstrated high reproducibility of lactyl-peptide quantitation.
- Virus-induced lactate results in lactylation of immune factors, providing a mechanism for virus immune evasion.

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Further reading

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