**Fast Global Phosphoproteome Profiling of Jurkat T cells by**

**HIFU-TiO2-SCX-LC-MS/MS**

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**ABSTRACT**

We propose a new workflow for fast phosphoproteome profiling. The workflow is based on the use of accelerated in-solution trypsin digestion under an ultrasonic field provided by high-intensity focused ultrasound (HIFU) combined with an inverse strategy based on TiO2 selective phosphopeptide enrichment, fractionation by strong cation exchange chromatography (SCX) and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a high-resolution mass spectrometer. The performance of the method was established for the global phosphoproteome analysis of un-stimulated human Jurkat leukemia T cells (E6.1). Using this accelerated workflow, 15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 different phosphoproteins were efficiently identified with high-throughput and reproducibility in less than 15 h. The functional analysis revealed significant phosphorylation-based networks that are implicated in immune function and tumor development pathways. The present strategy, HIFU-TiO2-SCX-LC-MS/MS, is the fastest analytical method reported to date for generating large-scale phosphoproteomics datasets (<15 h).

**Keywords:** phosphoproteomics, high-intensity focused ultrasound (HIFU), TiO2, strong cation exchange chromatography (SCX), proteomics, mass spectrometry (MS), human Jurkat leukemia T cells

**INTRODUCTION**

Global phosphoproteome profiling is extremely important to obtain answers to many biological questions, such as the control of the activation/inhibition status of specific protein activities, the understanding of different cellular signaling networks, and the diagnosis of several diseases.1-3 Therefore, the dynamic characterization of this post-translational protein modification (PTM) is important for understanding cellular signaling pathways and elucidating numerous disease mechanisms.4,5

To date, substantial efforts have been directed toward the identification and characterization of the maximum number of phosphorylation sites per experiment.6 In conjunction with more powerful data analysis tools, reverse-phase (RP) liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a fundamental analytical method for the high-throughput characterization of thousands of phosphorylation sites.7-12 However, because of the enormous complexity and extended dynamic range associated with eukaryotic cell phosphoproteomes, fractionation and phosphopeptide enrichment methods are crucial steps prior to MS analysis.6,13 Thus, enrichment methods involving either immobilized metal ion affinity chromatography (IMAC)14-16 and/or metal oxide affinity chromatography (MOAC) using titanium dioxide (TiO2) or zirconium dioxide (Zr02) resins17-19 are highly efficient methods for the purification and analysis of the global complex phosphopeptide mixtures generated after the tryptic digestion of cell lysates.

Although both IMAC and MOAC have a considerable capacity to enrich phosphopeptides and have been applied using an one-dimensional approach (IMAC/MOAC-LC-MS/MS) to identify thousands of phosphorylation sites10, sample fractionation is a common requirement for the successful in-depth analysis of phosphoproteomes.20,21 Several chromatographic methods, such as strong cation/anion exchange chromatography (SCX/SAX),22,23 hydrophilic interaction chromatography (HILIC),24 high-pH RP chromatography,25 and electrostatic repulsion hydrophilic interaction chromatography (ERLIC),26 have been used to fractionate complex phosphopeptide mixtures. The most common strategy for the fractionation and purification of phosphopeptides is a two-dimensional setup, in which SCX is followed by phosphopeptide enrichment with IMAC or MOAC.9,11,22,27 Thousands of phosphorylation sites have been identified using the widely accepted SCX-IMAC/MOAC combination approach and high-resolution MS.9,11,21 Nevertheless, considerable efforts requiring hundreds of hours of sample fractionation and MS analyses are needed when using these methodologies. Fast and easy phosphoproteomics methods are indispensable for the study of relevant cellular signaling pathways and for clinical applications.28,29 The recently published EasyPhos method allowed ~20,000 phosphopeptides from a combination of differently stimulated mouse liver cell lines to be characterized in one day.30 However, the tryptic peptide preparation of samples still is one of the most time-consuming steps.

Procedures to enhance the protease activity, such as the application of microwaves,31 immobilized trypsin (as in SMART DigestTM Kits, Thermo Fisher Scientific), high pressure,32 or focalized ultrasound,33 reduce the time needed for trypsin digestion and simplify this step. Thus, the application of only 1-2 minutes of high-intensity focused ultrasound (HIFU) to in-solution tryptic digestions has been reported to yield an efficiency and reproducibility similar to the values obtained using traditional overnight protocols.33,34 Today, HIFU is not only used to accelerate the enzymatic digestion of proteins but also used to rapidly reduce and alkylate cysteines, digest proteins and label peptides with 18O for quantitative proteomics.35 In addition, the ultrasound are used to accelerate the procedures of cell lysis.36

Therefore, in this work, a new strategy for the Fast Global Phosphoproteome Profiling is presented. The proposed methodology is based on the use of (a) cell lysis and protein extraction (time: 45 min), (b) accelerated in-solution tryptic digestion using HIFU (time: 10 min), (c) a single TiO2 phosphopeptide enrichment step (time: 90 min), (d) off-line fractionation of phosphopeptides using SCX (time: 60 min), (e) RP-LC coupled to high-resolution tandem MS (RP-LC-MS/MS) on a LTQ-Orbitrap XL instrument (Thermo Fisher Scientific) (time: 60 min/run) and (f) data analysis using BYONICTM and SEQUEST-HTTM (Thermo Fisher Scientific) (time: 60 min). Each step was individually adjusted to minimize the analysis time.

The present strategy, HIFU-TiO2-SCX-LC-MS/MS, allowed 15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 phosphoproteins from un-stimulated human Jurkat leukemia T cells (E6.1) to be identified in less than 15 h. The functions of relevant phosphorylation-based signaling networks in human Jurkat leukemia T cells were also investigated. To the best of our knowledge, this is the fastest strategy for profiling the global phosphoproteome of any biological tissue or cell line.

**EXPERIMENTAL SECTION**

**Cell culture and cell lysis**

Human Jurkat leukemia T cells (E6.1) were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin G and 100 mg/mL streptomycin (Invitrogen, Thermo Fisher Scientific, San Jose, CA, USA) in a humidified 37°C incubator with 5% CO2. After several weeks of growth, the cells were isolated by centrifugation, and a total of 10E8 un-stimulated T cells were lysed by mechanical disruption for 5 min on ice using a glass potter homogenizer in 1.5 mL of lysis buffer: 100 mM Tris-HCl, 150 mM NaCl, 1% n-Dodecyl-beta-D-maltoside (Pierce, Thermo Fisher Scientific), phosphatase inhibitor cocktail 1 and cocktail 2 (Sigma-Aldrich) and a protease inhibitor cocktail (Sigma-Aldrich). The protein extracts were then centrifuged at 16,000 rpm for 15 min at 4°C (J221-M centrifuge; Beckman, Palo Alto, CA, USA); the supernatants were purified and the proteins were quantified using the bicinchoninic acid (BCA) method (Sigma-Aldrich).

**Accelerated trypsin protein digestion using HIFU**

The protein extracts were subjected to HIFU-assisted trypsin digestion, as previously described.35,37,38 Five milligrams of protein were subjected to in-solution digestion with 100 µg of trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 8, with the simultaneous application of HIFU. A high-intensity ultrasonic probe with a 1-mm tip (Dr. Heilscher, Teltow, Germany) was used to perform the pulsed ultrafast digestion in 3 min at an amplitude of 50%. An additional 100-µg aliquot of trypsin was added to the sample, and the HIFU-assisted digestion was repeated for an additional 3 min. The peptide digests were acidified by the addition of 5% trifluoroacetic acid (TFA) to reduce the pH of the solution to ~2 and cleaned on a SepPak C18 cartridge from Waters (Milford, MA, USA).

**Phosphopeptide enrichment using TiO2**

The phosphopeptide fraction was enriched using a TiO2 packing column, as previously described.39 Five milligrams of dried peptides were reconstituted in 300 µL of a washing solution consisting of 80% acetonitrile (ACN) and 3.5% TFA that was saturated with phthalic acid (100 mg phthalic acid/mL). Then, 3 mg of TiO2 packing (GL Science, Saitama, Japan) was placed in a 1-mL Mobicol spin column (MoBiTec, Gottingen, Germany) and subsequently washed with 300 µL of water, 300 µL of methanol and finally equilibrated with 300 µL of washing solution for at least 10 min (the liquid from each step was removed by centrifugation at 500 g). After removing the washing solution, the peptide solution was added to the Mobicol spin column filled with the blocked TiO2 resin and incubated for 60 min with end-over-end rotation. After the incubation step, the peptide solution was removed by centrifugation, and the resin was thoroughly washed two times each with 300 µL of washing solution, 80% ACN and 0.1% TFA solution, followed by 0.1% TFA. Finally, the phosphopeptides were eluted from the TiO2 resin using two 150-µL aliquots of 0.3 M NH4OH (pH 10.5). After elution, the pH of the pooled eluents was rapidly adjusted to pH 2.7 using 10% TFA, and the phosphopeptides were purified using a C18 Sep-Pak cartridge (Waters Corporation, Milford, MA).

**Phosphopeptide fractionation using SCX**

TiO2-enriched phosphopeptides were further fractionated using SCX chromatography on a cartridge packed with PolySULFOETHYL-A material (The Nest Group Inc. Southborough, MA). Aliquots of peptide solutions (1 mL) were loaded onto a column equilibrated with 30% ACN containing 5 mM KH2PO4, pH 2.7. The buffers used for SCX separation contained 0.05% formic acid, 5 mM KH2PO4 and 30% ACN. Phosphopeptide separation was performed by stepwise elution using the same buffer with increasing KCl concentrations from 0 to 350 mM. Twelve fractions were collected, acidified with formic acid and cleaned on a C18 MicroSpinTM column (The Nest Group) prior to MS analysis.

**LC-MS/MS analysis**

Each SCX fraction was analyzed by LC-MS/MS using a Proxeon EASY-nLC II LC system (Thermo Fisher Scientific) coupled to a LTQ-Orbitrap XL (Thermo Fisher Scientific). Peptides (1 µg) were separated on an RP column (75 μm x 10 cm) packed in-house with C18 resin (Magic C18 AQ 3 μm; Michrom BioResources, Auburn, CA) using 0.1% formic acid in Milli-Q water and 0.1% formic acid in 98% ACN as mobile phases A and B, respectively. A 60-min linear gradient from 5 to 35% B at a flow rate of 300 nL/min was used. The ionization parameters were as follows: 1.95 kV spray voltage and 230°C capillary temperature. Peptides were analyzed in positive ion mode, and complete high-resolution full scans were obtained from 400 to 1600 amu (1 µscan) in the Orbitrap analyzer, followed by four data-dependent collision-induced dissociation (CID) MS/MS scans (1 µscans) using an isolation width of 3 amu and a normalized collision energy of 35%. Fragmented masses were excepted during dynamic exclusion for 30 s after the second fragmentation event, and unassigned charged ions were excluded from the MS/MS analysis.

**MS data processing**

MS/MS spectra were searched using BYONICTM and SEQUEST-HTTM (Proteome Discoverer 2.1 package, Thermo Fisher Scientific)40,41 against the human UniProt/SwissProt protein database (release 2016\_05; 152,493 protein entries). The following constraints were used for the searches: semi-tryptic cleavage with up to two missed cleavage sites and tolerances of 25 ppm for precursor ions and 0.5 Da for MS/MS fragment ions. The variable modifications allowed were methionine oxidation (+15.99492 Da) and phosphorylation (+79.966331 Da) of serine, threonine and tyrosine. Carbamidomethylation (+57.021465 Da) on cysteine was selected as a static modification. The results were subjected to statistical analysis with the Percolator algorithm to ensure that the false discovery rate (FDR) was less than 1%.42

Two technical replicates (n= 2) and three biological replicates (n= 3) were analyzed.

**Functional analysis**

The final list of non-redundant phosphoprotein IDs was submitted to Enrichr (http://amp.pharm.mssm.edu/Enrichr/)43 and to PANTHER programs (http://www.pantherdb.org/)44 for gene ontology (GO) classification and clustering based on four main types of annotations: protein class, molecular function, biological process and cellular components. The corresponding list of Entrez gene symbols was used as the input and the whole human genome was selected as the reference set. Signaling pathways were also investigated using the module Genes2FANs algorithm included in the Enrichr program. A representation of the statistical significance of the analysis was also provided.

The Genes2Networks (G2N) algorithm included in the Expression2Kinase (X2K) software (<http://www.maayanlab.net/X2K/>) was used to connect the input list of phosphoprotein/gene IDs with the background of the mammalian interactome protein networks.45,46 The background of the X2K software contains 24,036 proteins and 389,959 interactions. Using the G2N module, relevant protein kinases were identified and centered to create the protein interaction networks. After entering the list, the program outputted text files and networks files were visualized with the yEd program (yWorks: <http://www.yworks.com/>).

To discover the significant kinases that control these networks, the final list of phosphoproteins/genes was analyzed using the Kinase Enrichment Analysis (KEA) program.47 KEA is a module of the X2K program that provides a computed kinase enrichment probability based on the distribution of the kinase-substrate proportions of the input data and the background of the distribution of the public kinase-substrate databases.48-50 The consolidated dataset contains 14,374 interactions involving 436 kinases. A representation of the statistical significance of the kinase analysis was also provided.

**RESULTS AND DISCUSSION**

**Fast global phosphoproteome profiling strategy**

The goal of the present study was to develop a robust workflow amenable for standardization that allows for very fast global phosphoproteome rofiling of human cell lines. The strategy developed here is summarized in Figure 1 and integrates six main steps: (a) cell lysis and protein extraction (time: 45 min), (b) in-solution trypsin digestion accelerated by HIFU (time: 10 min), (c) a single step of phosphopeptide enrichment using TiO2 (time: 90 min), (d) phosphopeptide fractionation by step elution chromatography using a SCX packing (time: 60 min), (e) LC-MS/MS analysis using a LTQ-Orbitrap XL mass spectrometer (time: 60 min/run), and (f) data analysis using BYONICTM and SEQUEST-HTTM (time: 60 min). Using this strategy, the global phosphoproteome profile for a particular cellular sample was obtained in less than 15 h.

The requirements considered when developing this workflow were simplicity, robustness, high-throughput and reduced analysis time. The future potential for automation was also considered.

Furthermore, the analysis of the biological functions of these phosphorylated proteins from un-stimulated human Jurkat leukemia T cells (E6.1) were also studied using several functional GO and network programs.

The detailed results produced using this HIFU-TiO2-SCX-LC-MS/MS strategy are reported in the subsequent sections.

**Compilation of the phosphoproteome dataset**

In our workflow, large-scale phosphopeptide samples were purified using an inverse strategy (MOAC-SCX) that is faster than the conventional workflows (SCX-MOAC).21,27 The samples were enriched with TiO2 in a single step and then fractionated into 12 SCX fractions. This inverse two-dimensional strategy is more efficient because it avoids the uneven distribution of phosphopeptides during the first dimension in different chromatographic fractions.51 It also dramatically reduces the number of phosphopeptide enrichment steps, since otherwise, it grows depending on the number of fractions collected in the SCX separation. Importantly, the enrichment and fractionation steps were developed to be used on SPE cartridge formats that not only are low cost, but also can be automatable, parallelized in 96 well-plates, and simpler that complex HPLC systems.

After fractionation, the phosphopeptide pools were analyzed using a 60-min linear RP-LC gradient coupled to a high-resolution LTQ-Orbitrap mass spectrometer. Three biological replicates and two technical replicates were analyzed. Thus, thirteen raw LC-MS/MS files were processed per technical replicate: 1 flow-through (F) and 12 SCX fractions. Approximately 128,500 MS/MS events were collected per technical replicate. Raw data are available in the PRIDE proteomeXchange data repository (<https://www.ebi.ac.uk/pride/archive/>).

Peptide sequences were identified using two search engines: BYONICTM and SEQUEST-HTTM (Proteome Discoverer 2.1 software). The estimated FDR for phosphopeptide identification was maintained at less than 1% in all MS/MS analyses to ensure high confidence. Complete lists of unique phosphopeptides, phosphorylation sites, and the corresponding phosphoproteins for each SCX fraction are presented in the tables of the Supporting Table.

Using the present strategy (HIFU-TiO2-SCX-LC-MS/MS), 15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 phosphoproteins from un-stimulated human Jurkat leukemia T cells (E6.1) were identified in less than 15 h.

Figure 2 compares the results obtained using each search engine (BYONICTM *vs* SEQUEST-HTTM). Each program identified several thousands of phosphopeptides, 10,348 and 10,274 phosphopeptides were identified by BYONICTM and SEQUEST-HTTM, respectively (Figure 2a). As expected, the number of phosphoproteins identified in the queried database using both programs overlapped by 83.4% (Figure 2b). A total of 346 phosphoproteins were exclusively identified using BYONICTM, and 180 were exclusively identified using SEQUEST-HTTM. In addition, a total of 15,367 different phosphosites were identified using both programs; 12,157 phosphosites using BYONICTM and 11,477 phosphosites using SEQUEST-HTTM (Figure 2c). BYONICTM is an algorithm more sensitive (more true positives) and specific (fewer false positives) than other search engines as SEQUEST-HTTM.40 Incorporates a substantial amount of chemical knowledge into its fragmentation prediction, such as reduced CID fragmentation on the C-terminal side of proline, major number of variable modifications and string neutral losses from certain modifications.40 Thus, the use of both search engines expands the number of phosphopeptides, phosphosites and phosphoproteins identified and provides evidence for their validity.

Regarding previous data, Table 1 compares the results of the present study and the notable phosphoproteomics results published for human Jurkat leukemia T cells (E6.1) over the past few years. All previous results were collected using human Jurkat leukemia T cells (E6.1) stimulated with different drugs at different time points.52-55 It is well known that the use of drugs to stimulate cells increases the activity of kinases/phosphatases and thus increases the final number of total phosphorylation sites, phosphopeptides and phosphoproteins when all sample conditions are combined. Thus, between 4,346 and 13,476 phosphopeptides were identified in previous studies when all the various stimulation conditions were grouped together.54,55 In contrast, the present work presents the global repository of un-stimulated human Jurkat leukemia T cells determined in a high throughput manner and with high confidence for the first time. A total of 15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 phosphoproteins were obtained from un-stimulated human Jurkat leukemia T cells. These results constitute a global map of the basal phosphorylation status of human Jurkat leukemia T cells, which is interesting and will be useful as a repository for further investigations.

Regarding the time needed to perform the global phosphoproteome profiling, we highlight that all the previous Jurkat phosphoproteomics experiments required more than 48 h to complete the analysis (Table 1). In a recent paper, Humphrey et al. (2015) developed a new phosphoproteomics workflow named EasyPhos that maximizes the coverage of phosphorylation results without requiring fractionation and enables direct phosphopeptide analysis from 96-well plates (DWP wells) (Table 1).30 Using this method, ~20,000 phosphopeptides from different mouse liver cell lines (Hepa 1-6, FL83B and HeLa S3) that had been stimulated with insulin at different time points were identified in just over 24 h.

Importantly, the present work produced a global phosphoproteomics map from un-stimulated human Jurkat leukemia T cells in less than 15 h. This achievement was possible because each step was individually adjusted to minimize the analysis time, particularly due to the application of HIFU during the tryptic digestion (time: 10 min). Because the O-phosphates of phosphoserine, phosphothreonine and phosphotyrosine residues are extremely labile under alkaline conditions (i.e., tryptic digestion at pH 8),56 the acceleration of the time of tryptic digestion using HIFU (10 min) is a good strategy to maintain the number of these phosphosites. Figure S-1 in the Supporting data file, shows the phosphopeptide number identification in a protein sample (1 mg) from Jurkat cells when digested either using HIFU or the overnight protocol. Samples were digested with trypsin, phosphopeptides enriched with TiO2 and analyzed by LC-MSMS in a LTQ-Orbitrap XL mass spectrometer. The results show that the number of phosphopeptides identified using both digestion procedures overlapped by 85.4%, indicating that both digestion strategies are equivalent in terms of number of phosphopeptide identifications but the HIFU strategy is faster than the overnight protocol.

Thus, to the best of our knowledge, the present dataset (15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 unique phosphoproteins) is the largest phosphoproteomics dataset compiled to date for a particular cellular sample in a limited time frame (<15 h).

Because of its simple and rapid nature, parallelization of the method presented here may be applied for the sample preparation. This, together with the use of mass spectrometers of new generation, might increase the number of phosphopeptides identifications and reduce the time of analysis for the dynamic characterization of phosphopeptides. In consequence, the goal of the present work was to present a new and an alternative strategy for the fast identification of phosphopeptides that might be implemented in different equipment and instruments. In addition, the present strategy might be applied to analyze the global phosphoproteome profiles of any control or pathological biological tissue or cell type.

**Distribution of phosphopeptide profiles**

The distribution of the number of non-redundant (unique) phosphopeptides identified by both search engines (BYONICTM and SEQUEST-HTTM) in the different SCX fractions are plotted in Figure 3a. Most phosphopeptides bind to the SCX stationary phase column and mainly elute in the early fractions (0-70 mM of KCl). Based on this observation, SCX fractionation presents a good degree of orthogonally prior to RP and MS analysis.57

The fraction of unique phosphopeptides was plotted relative to the total number of unique peptides identified within each fraction to determine the percentage of phosphopeptide enrichment obtained for each SCX fraction and each search engine (BYONICTM and SEQUEST-HTTM) (Figure 3b). A substantial percentage of phosphopeptide enrichment (77.09%-97.53%) was achieved in the SCX fractions in which most of the phosphopeptides were eluted (0-70 mM of KCl). As shown in Figure 3b, the goals of increasing the selectivity and efficiency of phosphopeptide enrichment were achieved by optimizing the peptide-to-bead ratio and by the addition of a saturated phthalic acid solution to the loading buffer (see the Materials and Methods section).

Phosphopeptides have been reported to be primarily separated by SCX fractionation according to their solution charge status.13 As shown in Figure 4 doubly charged non-redundant phosphopeptides were eluted in the first fractions (0-30 mM), whereas triply and multiply charged non-redundant phosphopeptides were mainly identified in later fractions (40-90 mM). These results are consistent with the number of phosphorylation sites per peptide. Thus, as shown in Figure S-2 in the Supporting Data file, multiply phosphorylated peptides bind weakly to the SCX stationary phase column and mainly elute in the early fractions (0-30 mM), whereas mainly singly phosphorylated peptides were mainly identified in later fractions (40-90 mM). The number of phosphopeptides with a doubly charged state and only one phosphate group gradually decreased as the KCl concentration increased. This decrease results from the repulsion of negatively charged phosphopeptides by the SCX stationary phase.58 Therefore, the phosphoproteome is separated by SCX to enrich the phosphopeptides with different charge status and different number of attached phosphate groups.

As shown Figure S-3a in the Supporting Data file, phosphopeptides with a length lower than 10 amino acid residues were eluted mainly in the first fraction (0 mM), whereas phosphopeptides with 10-15 amino acid residues or 15-20 residues were eluted in the fractions (0-20 mM) and (5-20 mM), respectively. Besides, and according to the grand average of hydropathy (GRAVY) value (Figure S-3b) and isoelectric point (Figure S-3c), the hydrophobic phosphopeptides elute mainly in the first fractions (0-20 mM) and the strong acidic phosphopeptides (pI 3-4) elute mainly in the early fractions.

**Reproducibility of the strategy**

The reproducibility of the strategy was examined in different biological replicates (n=3) and different technical replicates (n=2) (Figure 5). Each of the biological replicates corresponded to un-stimulated human Jurkat T cells cultured for different times. Using the same conditions described in the Materials and Methods section, the same amount of protein per replicate was digested with trypsin with the simultaneous application of HIFU, and the phosphopeptides were enriched using TiO2, fractionated using SCX and then analyzed using LC-MS/MS in a LTQ-Orbitrap instrument. The reproducibility of the two technical replicates was high, with an overlap of 80.6% (Figure 5a). Thus, 1,808 different phosphoproteins were identified in both technical replicates. Regarding the biological replicates, 1,931 different phosphoproteins corresponding to the 61.05% of the data were identified in the three different biological replicates (Figure 5b). Even the reproducibility between pairs of biological replicates (Replicate 1 and Replicate 3) showed that the values of reproducibility achieved values of overlap of greater than 65.29% (2,065 different phosphoproteins). Therefore, we show the good reproducibility of the strategy presented in this work and reveal that the method is robust and produces similar results from end-to-end using different technical and biological replicates.

**Functional and GO analysis**

The final list of non-redundant phosphoproteins IDs was submitted to Enrichr and PANTHER programs for classification and clustering of the biological functions of un-stimulated human Jurkat leukemia T cells (Figure S-4 in the Supporting Data file).

The results of the signaling pathways revealed 10 different significant pathways (Figure 6 and Table S-1 in the Supporting Data). The top-scoring pathway corresponded to proteins involved in the T cell activation pathway (combined score: 13.34). T cell activation regulated by the T cell receptor (TCR) is an essential signal transduction pathway in which T cells recognize antigens produced by foreign pathogens or auto-antigens.59 Defects in this network can cause severe disorders, such as autoimmune diseases and allergies. Human Jurkat leukemia T cells are an immortalized cell line that is commonly used as a cellular model to study T cell signaling pathways.

The PDGF (platelet-derived growth factor) signaling pathway was identified in the second position, with a combined score of 12.2 (Figure 6 and Table S-1 in the Supporting Data). It is implicated in the development of human cancers and malignancies.60 In particular, autocrine PDGF signaling has been implicated in various types of malignancies, such as gliomas and leukemia. Inhibition of the PDGF signaling pathway may improve the efficacy of chemotherapies.61

**Network analysis**

Figure S-5 in the Supporting Data file, shows the global phosphoproteome network for un-stimulated human Jurkat leukemia T cells using the G2N program. This protein-protein interaction network was centered for the top 20 transcription factors and for the top 20 protein kinases. The presence of two potential large subnetworks corresponding to T cell activation and PDGF signaling were also individually plotted in Figures S-6 and S-7 in the Supporting Data file.

Among the list of the top 20 transcription factors that mediate the biological functions of un-stimulated human Jurkat leukemia T cells, we highlight the phosphoproteins MYC, RUNX1 and TCF3 (Figure S-5 and S-6 in the Supporting Data file). Notably, the phosphorylation of several residues of MYC (pT58 and pS62), RUNX1 (pT273 and pS276) TCF3 (pS39), which were also identified in the present study, were previously reported to be involved in the tumorigenesis of T cell leukemia62.

The KEA module included in the X2K software identified the kinases controlling the biological functions of un-stimulated human Jurkat leukemia T cells (Figure S-8 in the Supporting Data file). The top-scoring kinases corresponded to MAPK1 (score: 100.67), MAPK3 (score: 65.49) and MAPK14 (score: 60.87). After the triggering of the TCR in T cells, the MAPK cascade is known to be responsible for T cell activation, inflammation and apoptosis.63 In addition, MAP kinases are commonly deregulated in human cancers, such as childhood acute myeloid leukemia. Inhibition of MAP kinases has emerged as a viable therapeutic strategy to treat patients with excessive inflammation or malignancies.64

GSK3B was identified with a combined score of 47.12. In the context of T cells, GSK3B is responsible of the development of autoimmune diseases because it increases the differentiation of Th17 cells.65 GSK3B inhibitors are currently under study to develop new therapeutic treatments for autoimmune diseases.

HIPK2, CSNK2A1 and CDK4 were identified with combined scores of 34.65, 33.82 and 33.52 respectively. These proteins are involved in the transcriptional regulation of p53-mediated cellular apoptosis and the regulation of the cell cycle during the G1/S transition. Experimental drugs against these kinases are being studied as anti-tumorigeneses treatments.66

Overall, this work presents not only an easy, fast and simple workflow for phosphopeptide analysis, but also a valuable repository of phosphorylation events and significant phosphorylation-based networks, implicated in immune function and tumor development pathways, of un-stimulated human leukemia T cells, that may be very useful for further investigations.

**CONCLUSIONS**

A new strategy for Fast Global Phosphoproteome Profiling is presented in this work. The methodology is based on in-solution trypsin digestion accelerated using HIFU, phosphopeptide enrichment using TiO2, fractionation of phosphopeptides using SCX, and LC-MS/MS analysis by RP chromatography coupled to a high-resolution LTQ-Orbitrap XL mass spectrometer. The simplicity, robustness and speed of the workflow makes it ideal for large scale phosphoproteome analysis, but also opens the door to clinical applications where time to answer is critical. The functional analysis revealed significant phosphorylation-based networks implicated in immune function and tumor development pathways. The methodology reported here, HIFU-TiO2-SCX-LC-MS/MS, is the fastest analytical method and allows the global phosphoproteome profiling to be analyzed with good reproducibility in less than 15 h.

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**DATA DEPOSITION.** The mass spectrometry data and the search engine results presented in this manuscript are deposited in the PRIDE ProteomeXchange Consortium with the identifier PXD006130.

**SUPPORTING INFORMATION AVAILABLE:**

* SUPPORTING TABLE: Complete lists of unique phosphopeptides, phosphorylation sites, and the corresponding phosphoproteins for the complete dataset and for each SCX fraction.
* SUPPORTING DATA:
* Figure S-1: Phosphopeptide number identification in a protein sample (1 mg) from Jurkat cells when digested either using HIFU or the overnight protocol. Samples were digested with trypsin, phosphopeptides enriched with TiO2 and analyzed by LC-MSMS in a LTQ-Orbitrap XL mass spectrometer.
* Figure S-2: Number of phosphorylation sites per peptide for each SCX fraction and each search engine (BYONICTM and SEQUEST HTTM).
* Figure S-3: Peptide length (a), grand average of hydropathy (b) and isoelectric point (c) of phosphopeptides identified per each SCX fraction.
* Figure S-4: Protein class, molecular function, biological process and cellular components categorized by Enrichr and PANTHER.
* Table S-1: Enriched signaling pathways in the phosphoproteome of un-stimulated human Jurkat leukemia T cells according to the Enrichr and PANTHER programs.
* Figure S-5: Global phosphoproteome network for un-stimulated human Jurkat leukemia T cells according to Gene2Networks.
* Figure S-6:T cell activation phosphoproteome network for un-stimulated human Jurkat leukemia T cells according to Gene2Networks.
* Figure S-7: PDGF phosphoproteome network for un-stimulated human Jurkat leukemia T cells according to Gene2Networks.
* Figure S-8:Kinases enriched in the Kinase Enrichment Analysis (KEA) program included in the Expression2Kinase (X2K) software.

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**FIGURE CAPTIONS**

**Figure 1:** Analytical scheme for the fast global phosphoproteome profiling method.

**Figure 2:** Comparison of the results obtained using BYONICTM and SEQUEST-HTTM search engines for the number of phosphopeptides a), for the number of phosphoproteins b) and for the number of phosphosites identified c).

**Figure 3:** a) Distribution of the number of non-redundant phosphopeptides identified in the different SCX fractions. b) Percentage of phosphopeptide enrichment obtained for each SCX fraction and each search engine (BYONICTM and SEQUEST-HTTM).

**Figure 4:** Distribution of the phosphopeptide charge status identified for each SCX fraction and each search engine (BYONICTM and SEQUEST HTTM).

**Figure 5:** Reproducibility of the strategy for two technical replicates (a) and three different biological replicates (b).

**Figure 6:** Enriched signaling pathways in the phosphoproteome of un-stimulated human Jurkat leukemia T cells according to the Enrichr and PANTHER programs.

**Table 1.** Comparison of published phosphoproteomics studies of Human Jurkat leukemia T cells and the present study (Carrera et al.).

|  |  |
| --- | --- |
| **Human Jurkat leukemia T cells (E6.1)** | **Other**  |
| **Reference** | **Mayya, 2009** | **Mertins, 2013** | **Giasanti, 2015** | **Nguyen, 2016** | **Carrera et al.** | **Humphrey, 2015** |
| **Cell line** | Human Jurkat T cells E6.1 | Human Jurkat T cells E6.1 | Human Jurkat T cells E6.1 | Human Jurkat T cells E6.1 | Human Jurkat T cellsE6.1 | Different mouse liver cell lines (Hepa 1-6, FL83B and HeLa S3) |
| **Cell treatment** | Stimulated with anti-CD3at ≠ times | Stimulated with or without Bortezomib or Velcade  | Stimulated with or without PGE  | Stimulated with anti-CD3/anti-CD28at ≠ times | Un-stimulated | Stimulated with insulin at ≠ times |
| **Peptide Digestion** | Trypsin18 hours | Trypsinovernight | Multiple proteases(AspN, Chym., GluC, LysC, and Trypsin) | Trypsin18 hours | HIFU-based tryptic digestion 10 min | TFE-based tryptic digestion18 hours |
| **Enriched Fraction** | SCX-IMAC | High pH RP-IMAC | Ti4+-IMAC | SCX-IMAC-TiO2 | TiO2-SCX | TiO2 in DWP wells  |
| **MS platform** | LTQ (CID) | Q Exactive (HCD) | LTQ-Orbitrap ELITE(CID and ETD) | LTQ-Orbitrap XL (HCD and CID) | LTQ-Orbitrap XL (CID) | Q-Exactive (HCD) |
| **Data analysis** | SEQUEST | MaxQuant | MS-GF+ | SEQUEST+OMSSA+EasyProt | BYONICTM+SEQUEST HTTM | MaxQuant |
| **Phosphopeptides** | **11,708** | **---** | 37,771 (**13,476** trypsin) | **4,346** | **13,029** | ~**20,000** |
| **Phosphosites** | 10,665 | 20,800 | 18,430 | **---** | 15,367 | >10,000 |
| **Phosphoproteins** | 3,084 | **---** | 5,326(3,519 trypsin) | 1,690 | 3,163 | **---** |
| **Time** | >48 hours | >48 hours | >48 hours | >48 hours | **<15 hours** | 24 hours |

**Figure 1**

**Total** Time: **<15h**

TiO2

Phosphopeptides

Functional analysis

**5 mM**

**100 mM**

SCX fractionation

IDs (FDR<1%)

LC-MS/MS

Tryptic digestion accelerated by HIFU

by HIFU

Peptides

Cell culture

Proteins

HIFU-TiO2-SCX-LC-MS/MS

Cell lysis

Isocratic solutions (KCl)

**SEQUEST**

**Byonic**

**Figure 2**

**Figure 3**

a)

b)

**Figure 4**

**Figure 5**

****

**Figure 6**

for TOC only:

