BRUKER

Professor Yasushi Ishihama
Laboratory of Molecular and Cellular BioAnalysis, Kyoto University

Customer Insights

● Accelerating phosphoproteomics research with trapped ion mobility mass spectrometry (TIMS)

Innovation with Integrity

Accelerating phosphoproteomics research with trapped ion mobility mass spectrometry (TIMS)

Researchers at Kyoto University are advancing clinical proteomics research with high-throughput powered by 4D-Proteomics TIMS technology on Bruker's timsTOF Pro.

Working with Bruker
Professor Yasushi Ishihama, Laboratory of Molecular and Cellular BioAnalysis, Kyoto University, uses advanced mass spectrometry technology to drive his proteomics research:

"Trapped ion mobility mass spectrometry (TIMS) with the timsTOF Pro is very attractive for us, in terms of its robustness and incredibly high-throughput capabilities."

Laboratory of Molecular and Cellular BioAnalysis, Kyoto University

The Graduate School of Pharmaceutical Sciences, Kyoto University, Japan, was established in 1953 and encompasses the Division of Pharmaceutical Sciences, the Division of Biomedical Sciences, and the Division of Bioinformatics and Chemical Genomics. Within the Division of Pharmaceutical Sciences, the Laboratory of Molecular & Cellular BioAnalysis (MCBA) was set up in October 2010 by Professor Yasushi Ishihama. The laboratory's work focuses on proteomic research based on separation chemistry and mass spectrometry (MS), and its main research theme is to measure and analyze both total proteomes and individual proteins at the molecular and cellular level. The MCBA laboratory uses molecular and cellular analysis assisted by separation and analytical sciences, and is particularly focused on developing proteomic methodologies based on MS, micro-separation sciences, and bioinformatics. The laboratory is also developing practical applications for the analysis of cellular functions and drug discovery.

The MCBA laboratory is centered on students, with four new students joining each year. Currently, 12 graduate students, three Ph.D. students, and one post-doctoral researcher are carrying out research at the laboratory, in addition to two permanent employees.

MCBA research activity

Since 2001, Prof. Ishihama has focused on proteome analyses based on nano liquid chromatography mass spectrometry (nanoLC-MS) technologies. So far, he and his colleagues have developed several proteomics tools such as nanoLC electrospray ionization (-ESI) needle columns, StageTip mini-columns for sample preparation, a culture derived isotope tag (CDIT) approach for absolute quantitation, and phosphopeptide enrichment approaches based on immobilized metal affinity capture (IMAC). These technologies have been applied to the malaria proteome, the chaperonin GroEL interactome, the mouse brain proteome, and a target discovery study for an antitumor natural product.

After moving to Keio University in 2006, Prof. Ishihama concentrated on two main topics, phosphoproteomics and membrane proteomics, and developed hydroxy acid-modified metal oxide chromatography (HAMMOC) for phospho-peptide enrichment and phase transfer surfactant (PTS)-aided protease digestion for membrane proteome studies, respectively. Based on these techniques, his group carried out several phospho- and/or membrane proteomics projects on human cancer, malaria, plants and bacteria. His group, together with RIKEN Plant Science Center, reported the first large-scale plant phosphoproteome analysis including tyrosine phosphorylation [1].

Currently, Prof. Ishihama's group at Kyoto University is focusing on technology development for nanoLC-MS systems with meter-long capillary columns, as well as phosphoproteomics-based cell signaling network analysis. The MCBA laboratory emphasizes the importance of elucidating cellular functions through the measurement of biomolecules based on analytical chemistry, specifically conducting research on five key topics:

- Development of novel analytical technologies for proteomics
- Human proteome analysis based on single-shot LC-MS systems
- Elucidation of intracellular phosphorylation network analysis
- Quantitative clinical proteome analysis of tissue samples
- Studies on molecular targeting drug discovery based on phosphoproteomics.

He is also interested in computational analysis for large-scale proteomics data in collaboration with researchers at the Graduate School of Informatics, Kyoto University. In addition, he has developed the proteome repository/database, known as jPOST, together with five other research institutions in Japan, to standardize and share proteome data around the world as a partner of the global standard ProteomeXchange consortium. Unlike genomic and transcriptomic research, the measurement technologies for proteomics are still evolving, and the complete analysis of a proteome has not yet been achieved. The end goal of proteomics studies is not just to

identify all the proteins that can be expressed, but also to uncover cellular protein events such as (1) protein expression/degradation, (2) protein localization, (3) protein interaction, (4) protein post-translational modifications (PTM), and (5) protein processing/splicing.

Attending HUPO Proteomics Standards Initiative meeting 2019 in Cape Town, South Africa

The MCBA laboratory is aiming to develop novel approaches to tackle the technical barriers and to explore proteomic research for clarifying biological problems. In cellular signal transduction networks, reversible phosphorylation is one of the key events in transducing a signal into the nucleus to control gene expression. Approximately 30% of human proteins were previously estimated to be phosphorylated, but the group developed a highly selective enrichment method for phosphopeptides and, when applied to proteome-wide acquisition of cellular phosphorylation status, revealed that at least 70% of human proteins are phosphorylated [2, 3]. Now, Prof. Ishihama aims to connect the kinases with their substrates to reveal the entire picture of the signaling network, using experimental and computational approaches.

The laboratory's phosphoproteomics methods have been employed to carry out in vivo phosphoproteome profiling of kinase-targeting drugs, which would facilitate drug discovery and development for cancer therapy, as well as exploring the functional analysis of newly discovered phosphorylated molecules. Prof. Ishihama comments on the journey that led him to proteomics:

"I worked in a pharmaceutical company for 14 years after I completed my studies. I then moved back to academia in 2006, at a time when proteomics was just emerging, so it was a great time to enter the field. There have been many technological improvements and developments, but still new technology is needed to drive the field. That's where my background in analytical chemistry is useful and can be applied to life science."

Four new members join the MCBA laboratory in April 2019

Introducing TIMS to phosphoproteomics

Developments in high resolution mass spectrometers and specific enrichment of phosphorylated peptides tailored for the global analysis of protein phosphorylation represent powerful tools for molecular and cellular biologists studying signal transduction pathways. Despite these advances, identifying PTMs remains significantly more challenging compared with unmodified peptides, as they often occur at low abundances and the differences in protein phosphorylation span several orders of magnitude, driving the need for instruments with higher sensitivity and increased peak capacities.

Deep characterization and quantitative analysis of proteins and PTMs are critical to understanding signaling pathways and abnormal disease states. The sophistication of modern instrumentation enables the identification of tens of thousands of phosphopeptides in a single-shot LC-MS run, but the percentage occurring as positional isomers is unknown. The combination of ion mobility spectrometry (IMS) with MS is a well-established technique that has shown considerable potential for improving peptide identification, providing structural information that is complementary to LC and MS. IMS-MS separates ions based on differences in their shape (IMS) and mass (MS), delivering information on the three-dimensional (3D) structure of an ion. This added ability to separate ions by differences in conformation makes it possible to separate isobaric and isomeric species, such as phosphopeptide positional isomers (i.e. peptides that differ only by the residue that is phosphorylated), which are not easily distinguished by MS techniques alone [4].

The commercialization of trapped ion mobility spectrometry (TIMS) in 2016 built on the advances in IMS technology made over previous years. TIMS enables the interrogation and manipulation of mobility separated ion populations in the gas-phase, with high efficiency, duty cycle and high resolving power in millisecond-second time-scales, and with the possibility to measure collisional cross section (CCS) using first principles that can be further utilized for structural assignments [5], in CCS-aware workflows. The addition of TIMS provides a 4th dimension that is complementary to the previously used mass, intensity and retention time dimensions, resulting in 4D-Protoemics methods. TIMS is most often coupled with a time-of-flight (TOF) mass analyzer to capitalize on its high-speed capabilities. Prof. Ishihama introduced Bruker's timsTOF Pro to the MCBA laboratory in May 2018, which integrates TIMS with ultra-high-resolution quadrupole TOF (QTOF) technology. The timsTOF Pro uses parallel accumulation – serial fragmentation (PASEF) to provide high speed
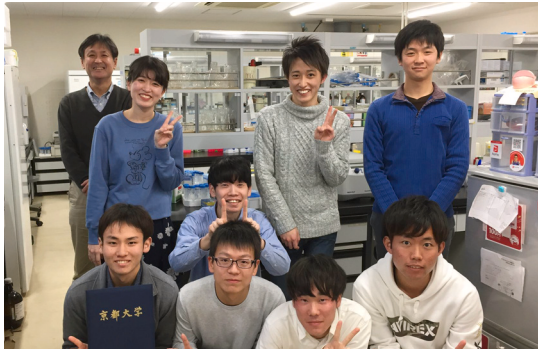
Professor Ishihama with members to be graduated in March 2019, in the lab bench room

and increased sensitivity, delivering effective MS/MS acquisition rates to reach new depths in phosphoproteomics.

Clinical metaproteomics research

The high speed and sensitivity of the PASEF acquisition mode facilitates high throughput on a TIMS-QTOF MS system, which is particularly important for Prof. Ishihama's research as his laboratory collects numerous clinical samples, primarily fecal, from the nearby Kyoto University Hospital to conduct metaproteomics studies:

"*This is a slightly new direction for us. We look at the metagenome to analyze the proteome profile from humans and from bacteria and assess the relationships between the two. We aim to investigate the interaction between humans and bacteria at the proteome level to uncover potential biomarkers of disease.*

We're very interested to see how the timsTOF Pro, with its unique feature of trapped ion mobility, will help us develop this new methodology,"

comments Prof. Ishihama.

A fractionation approach is often used to obtain deeper proteome coverage, but this method is time-consuming and if many clinical samples are analyzed, the time frame is not realistic. The MCBA laboratory uses rapid nanoLC combined with the timsTOF Pro to analyze one sample per minute. The combination of 4D-Proteomics with the instrument's robustness and high sensitivity makes the timsTOF Pro an ideal tool for analyzing clinical samples for research purposes.

The benefit of combining TIMS with PASEF for 4D-Proteomics in the clinical research setting is the greater phosphoproteome coverage obtainable without large sample volumes. High quality results can be produced from < 200 ng sample load, reducing sample preparation time and cost, as well as MS maintenance frequency.

Protein kinase profiling

As well as metaproteomics, Prof. Ishihama's phosphoproteomics research has implications in drug discovery, particularly for phosphorylations related to cancer. By implementing the one-minute gradient with the timsTOF Pro, the laboratory can investigate the mode of action of kinase inhibitors (Figure 1). Kinase networks are important for cellular signal transduction, by their catalysis of reversible protein phosphorylation, and kinase-mediated phosphorylation signals are known to cause or drive the progression of diseases such as cancer. Many drugs that inhibit a specific kinase or kinases have been developed for kinase-targeting therapy and, to date, more than 19,000 kinase inhibitors targeting ~260 protein kinases have been reported [6], and ~30 small-molecular kinase inhibitors have been approved for clinical use by the United States Food and Drug Administration (US FDA) [7].
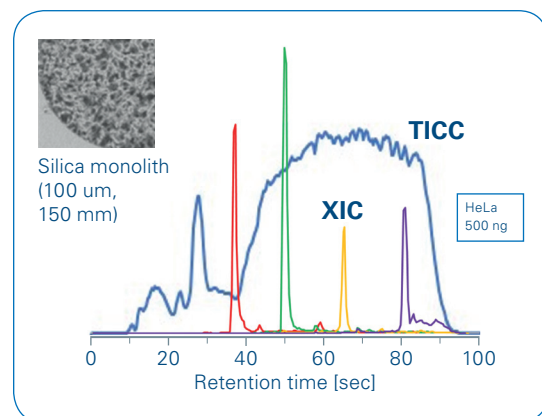

Figure 1: Capillary LC with timsTOF Pro. 1-minute gradient, 100 sec/run, 864 runs/day. TICC=total ion current chromatogram; XIC = extracted-ion chromatogram.

For the past 20 years, identification of phosphorylated proteins based on MS has been used in many studies aimed at large-scale analysis of cellular signaling. Building on Prof. Ishihama's work on the kinome with LC-MS/MS [8, 9], his laboratory is now achieving throughput levels only possible with the timsTOF Pro.

Graduate School of Pharmaceutical Sciences, Kyoto University

Relationship with Bruker

Prof. Ishihama was first exposed to Bruker technology during his post-doctoral studies at the University of Southern Denmark in Odense, supervised by leading proteomics researcher Prof. Matthias Mann. Since Prof. Mann became a director of the Max Planck Institute of Biochemistry in 2005, Prof. Ishihama has maintained this connection and benefits from an exchange of knowledge on technical innovations in the field. Prof. Ishihama describes how his partnership with Bruker has developed:

"I first used an ion trap instrument when I was in Denmark about 18 years ago. At that time, Bruker had just launched its new ion trap instrument, but the sensitivity was not high enough, so I discussed with their experts in Bremen how to improve the sensitivity.

This has been a very valuable exchange."

Prof. Ishihama also has a good relationship with Bruker personnel in Japan, where he obtained useful information prior to the purchase of the timsTOF Pro.

Continuing cutting-edge research

It is clear that developments in MS-based proteomics, such as TIMS enabled 4D-Proteomics, have allowed researchers to gain deeper insights into the molecular and cellular functionality of the human body. Prof. Ishihama comments on where he sees the field advancing:

"I think one of the most exciting developments in proteomics is data independent acquisition (DIA). There are no missing values and it's suitable for computational science, which is more versatile now and big data can be used relatively easily.

I think DIA could be like next generation sequencing (NGS) for proteomics – a proteome-focusing next generation sequencer could be a big trend in the next few years."

DIA is a relatively recently developed MS acquisition technique where, unlike data dependent acquisition (DDA), MS^2 scans are acquired in a continuous and unbiased manner for all precursor ions falling within a specific mass range. The combination of NGS with proteomics is becoming more widely used in multi-omics fields such as proteogenomics, which is finding application in clinical research, particularly precision oncology [10].

The current coverage of the proteome is far from 100% and, although technology such as 4D-Proteomics on the timsTOF Pro takes large steps in throughput and sensitivity, researchers are continually looking for improvements. The increased versatility of computational proteomics, in combination with software and hardware developments, is another important future direction for proteomics. Prof. Ishihama's group is collaborating with the informatics department to train students to extract as much information as possible from the large amount of data obtained from high throughput analyses on the timsTOF Pro.

For more information about the Laboratory of Molecular and Cellular BioAnalysis, please visit http://www.pharm.kyoto-u.ac.jp/seizai/index_e.html.

For more information on Bruker's timsTOF Pro, please visit https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/o-tof/timstof-pro.html.

References

[1] Sugiyama N, Nakagami H, Mochida K, Daudi A, Tomita M, Shirasu K, Ishihama Y (2008). *Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in Arabidopsis*. Mol Syst Biol, **4**:193.

[2] Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, Gnad F, Cox J, Jensen TS, Nigg EA, Brunak S, Mann M (2010). *Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis*. Sci Signal, **3**(104):ra3.

[3] Sharma K, D'Souza RCJ, Tyanova S, Schaab C, Wiśniewski JR, Cox J and Mann M (2014). *Ultradeep Human Phosphoproteome Reveals a Distinct Regulatory Nature of Tyr and Ser/Thr-Based Signaling*. Cell Reports, **8**(5):1583-1594.

[4] Glover MS, Dilger JM, Acton MD, Arnold RJ, Radivojac P, and Clemmer DE (2018). *Examining the Influence of Phosphorylation on Peptide Ion Structure by Ion Mobility Spectrometry-Mass Spectrometry*, J Am Soc Mass Spectrom, **25**(5): 786-794.

[5] Fernandez-Lima F (2016). *Trapped Ion Mobility Spectrometry: past, present and future trends*, Int. J. Ion Mobil. Spec, **19**:65–67.

[6] Hu Y, Furtmann N and Bajorath J (2015). *Current compound coverage of the kinome*, J Med Chem, **58**(1):30-40.

[7] Wu P, Nielsen TE and Clausen MH (2016). *Small-molecule kinase inhibitors: an analysis of FDA-approved drugs*, Drug Discovery Today, **21**(1):5-10.

[8] Sugiyama N and Ishihama Y (2016). *Large-scale profiling of protein-kinases for cellular signaling studies by mass spectrometry and other techniques*, Journal of Pharmaceutical and Biomedical Analysis, **130**: 264-272.

[9] Sugiyama N, Imamura H and Ishihama Y (2019). *Large-scale Discovery of Substrates of the Human Kinome*, Scientific Reports, **9**:10503.

[10] Ang MY, Low TY, Lee PY, Nazarie WFWM, Guryev V and Jamal R (2019). *Proteogenomics: From next-generation sequencing (NGS) and mass spectrometry-based proteomics to precision medicine*, Clinica Chimica Acta, **498**:38-46.

First instrument in Japan (May, 2018): The Japanese room is not high enough for the tall German timsTOF Pro

Customer Insights

Accelerating phosphoproteomics research with trapped ion mobility mass spectrometry (TIMS)

Researchers at Kyoto University are advancing clinical proteomics research with high-throughput powered by 4D-Proteomics TIMS technology on Bruker's timsTOF Pro.

About the Laboratory of Molecular and Cellular BioAnalysis

Established in October 2010, the Laboratory of Molecular and Cellular BioAnalysis (MCBA), is part of the Graduate School of Pharmaceutical Sciences at Kyoto University. Led by Professor Yasushi Ishihama, the MCBA laboratory aims to uncover the function of each individual protein and the proteome, by measuring and analyzing samples at molecular and cellular levels. The laboratory particularly focuses on developing proteomic methodologies based on mass spectrometry, microseparation sciences and bioinformatics, and is challenging practical applications to analysis of cellular functions and drug discoveries. The MCBA laboratory is progressing toward development of novel technology for proteomics and application to cellular biology, drug discovery and clinical study.

About Bruker Corporation

Bruker is enabling scientists to make breakthrough discoveries and develop new applications that improve the quality of human life. Bruker's high-performance scientific instruments and high-value analytical and diagnostic solutions enable scientists to explore life and materials at molecular, cellular and microscopic levels. In close cooperation with our customers, Bruker is enabling innovation, improved productivity and customer success in life science molecular research, in applied and pharma applications, in microscopy and nanoanalysis, and in industrial applications, as well as in cell biology, preclinical imaging, clinical phenomics and proteomics research and clinical microbiology.

For more information, please visit: **www.bruker.com**

Bruker Daltonik GmbH

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

Bruker Scientific LLC

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

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