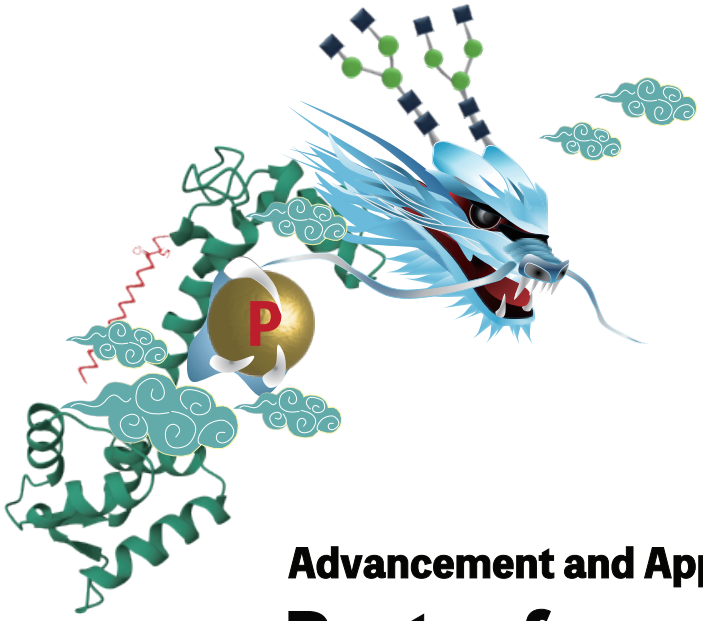
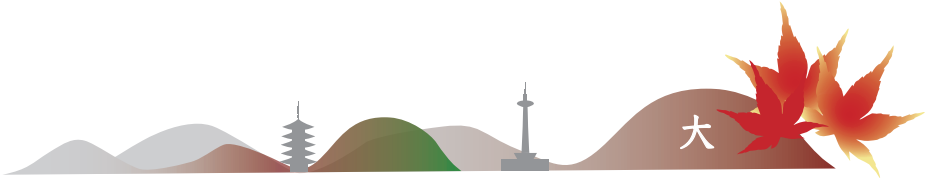


Advancement and Application of Methods for

Proteoform-Centric Proteomics

2024 Japanese–German Research Symposium

November 16 & 17
Kyoto, Japan



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Dear Colleagues,

We would like to welcome you to the 2024 Japanese–German Research Symposium “Advancement and Application of Methods for Proteoform-Centric Proteomics”. This symposium brings together leading Japanese and German researchers in the field of proteoform research who will be presenting their latest research results.

Proteoform is a term used to describe the variety of protein chemical structures produced from a single gene in living organisms. Since the presence of proteoforms causes diversity in the physiological functions of proteins, the importance analyzing proteoforms has become increasingly more recognized in recent years. The detection of the vast number of proteoforms, estimated to be several million in humans, however, is an extremely difficult technical challenge. The scientific challenges involved in understanding proteoforms in their entirety require highly interdisciplinary research, ranging from analytical chemistry and biochemistry to bioinformatics and engineering, and offer high potential for innovation. The technologies we develop will have a strong impact on our understanding of the molecular processes of various life phenomena and can be applied to all areas of the life sciences, including basic biology, biomedicine and biotechnology. In particular, detailed understanding of proteoforms will provide crucial insights into disease mechanisms and the development of biomarkers.

The speakers at this symposium have different approaches to analyzing proteoforms, a frontier in proteomics research, giving us a great opportunity to learn about various innovative research methodologies in one place. The discussions that will take place during the two-day symposium aim to promote the fusion of different analytical methods, perhaps even the birth of a new proteoform analysis methodology.

This symposium has been designed to appeal not only to proteomics researchers but also to participants who are interested in learning more about proteoforms. Lectures here require no prior knowledge and will be presented in an accessible style that is welcoming to both experts and newcomers alike. Our hope is that attendees will find this seminar to be a one-of-a-kind opportunity to learn the appeal of proteoform-centric biology and be inspired to start proteoform research in their own laboratories.

We wish all participants of the symposium a successful meeting and are looking forward for lively discussions about this emerging research field.

Best wishes,

Nobuaki Takemori, Ph.D.
Ehime University

Andreas Tholey, Ph.D.
Christian-Albrechts-Universität Kiel

A Message from Dr. Paul O Danis, CEO of CTDP

CTDP
Consortium for Top-Down Proteomics

Bringing Proteoforms to Life[sm]

November 16, 2024

Dear Symposium Attendees,

On behalf of the Consortium for Top-Down Proteomics, we would like to welcome you to the 2024 Japanese–German Research Symposium, *Advancement and Application of Methods for Proteoform-Centric Proteomics*. We also congratulate the Organizers for putting this important event together. With the continued advancements in the various technologies, our abilities to unravel the intricacies and complexities of proteoform-specific biology are increasing dramatically.

The Symposium will serve two very important missions: first is the presentation of exciting developments in new methods for analysis, separation, and data reduction as well as applications across a wide array of challenges. Critical discussions around these topics will forge new ideas and create new important relationships between the engaged participants. Secondly, this will help to build a strong bridge to the proteomics community of Japan and foster increased collaboration and cooperation with partners around the world.

Together this will move the field forward towards our common goals of improving the understanding of the molecular basis of biology, leading to improved outcomes and solutions for healthcare and the environment. We are grateful for the energy and ideas that have launched this event, and we wish you all a very exciting, productive, and fulfilling meeting.

Regards,

Paul O. Danis, Ph.D.

CEO

SCHEDULE

Saturday, Nov 16

12:00–13:00 Registration

13:00–13:20 **Welcome and Opening Remarks**

13:20–15:00 **Session 1: Top-Down Proteomics, Part I**

Top-down proteomics and proteoforms – a versatile playground for analytical chemistry
Andreas Tholey, PhD (Christian-Albrechts-Universität Kiel)

Truncated Proteoforms in Top-Down Proteomics Studies
Philipp T Kaulich, PhD (Christian-Albrechts-Universität Kiel)

Image-Guided 3D Laser Sampling for Subsequent Deep Omics: Background, Methodology and Applications in Cancer Research
Jan Hahn, PhD (University Medical Center Hamburg-Eppendorf)

Via infrared-laser based sampling towards the original composition of proteoforms in tissues
Hartmut Schlüter, PhD (University Medical Center Hamburg-Eppendorf)

15:00–15:20 Coffee Break

15:20–16:35 **Session 2: Proteoform Analysis with Bottom-Up Approach**

Elucidation of regulatory mechanisms of protein kinases based on kinome profiling
Naoyuki Sugiyama, PhD (National Cerebral and Cardiovascular Center)

Defining proteoforms through post-translational modification proteomics
Kazuya Tsumagari, PhD (RIKEN)

Significance of Protein Terminus Excavation as a Trace of RNA Splicing, Translation and Proteolysis
Yasushi Ishihama, PhD (Kyoto University)

16:35–16:40 Short Break

16:40–17:10 **Session 3: Lightning Talks by Japanese Student Participants**

Large-scale advanced design and evaluation of kinase-specific artificial substrate peptides
Liang Junqi (Kyoto University)

Ultrahigh-speed, high-sensitivity and high-depth proteomics by capillary-flow LC/MS/MS
Ayana Tomioka (Kyoto University)

17:10–17:25 Break

17:25–18:40 **Session 4: Proteofom Analysis in Clinical/Biological Studies**

The role and the potential of posttranslational modifications of collagen to enhance bone health and repair
Sabine Fuchs, PhD (University Medical Center Schleswig-Holstein)

The protein levels of post-transcriptionally regulated genes determine stem cell fate into three germ layers
Mio Iwasaki, PhD (Kyoto University)

In Variatate Concordia: Can single-molecule DNA and RNA sequencing in stem cell systems inform proteoform mapping?
Franz-Josef Müller, MD, PhD (University Hospital Schleswig-Holstein)

18:40–19:00 Break

19:00–20:30 **Welcome Reception**

Sunday, Nov 17

9:00–9:15 Registration

9:15–10:30 **Session 5: Top-Down Proteomics, Part II**

Characterization of proteoforms of intact proteins by one and two-dimensional CE-MS techniques
Christian Neusüß, PhD (Aalen University)

Computational methods in top-down proteomics to address challenges in proteoform analysis
Kyowon Jeong, PhD (University of Tübingen)

OpenMS - An open-source software suite for (top-down) proteomics
Oliver Kohlbacher, PhD (University of Tübingen)

10:30–10:45 Coffee Break

10:45–12:00 **Session 6: Promising Technologies for Proteoform Analysis**

Proteomics with cell-free synthesized peptides/proteins
Yoshihiro Shimizu, PhD (RIKEN)

Development of tapping-mode scanning probe electrospray ionization for mass spectrometry imaging of tissues and cells
Yoichi Otsuka, PhD (Osaka University)

Multi-dimensional vibrational circular dichroism as targeted for biological samples
Hisako Sato, PhD (Ehime University)

12:00–12:30 **A Word from JSPS and DFG**

12:30–13:30 Lunch

13:30–14:45 **Session 7: Top-Down/Bottom-Up Peptidomics**

Biomedical applications of peptidomics, a subdivision of proteoform profiling
Kazuki Sasaki, MD, PhD (Tochigi Cancer Center)

Ion mobility mass spectrometry for immunopeptidomics in cancer immunotherapy
Yuriko Minegishi, PhD (Japanese Foundation for Cancer Research)

Peptidomic analyses of plasma and tissues based on high-yield peptide extraction method
Yoshio Kodera, PhD (Kitasato University)

14:45–15:00 Coffee Break

15:00–16:15 **Session 8: Structural and Single Molecule Analysis Techniques for Proteoform Analysis**

Nanofluidic and Lab on a Chip devices for single molecule analysis
Irene Fernandez-Cuesta, PhD (University of Hamburg)

High-precision proteoform quantification reveals phosphorylation kinetics of AMP-activated protein kinase
Boris Krichel, PhD (Univ. Lübeck & Centre for Structural Systems Biology)

Connecting proteoforms to higher-order structure by combining native and top-down MS
Frederik Lermyte, PhD (Technical University of Darmstadt)

16:15–16:30 Break

16:30–17:20 **Session 9: Gel-Based Proteoform Analysis**

Gel-Based Human Proteoform Atlas: Mapping of Human Proteoforms using SDS Polyacrylamide Gel Electrophoresis
Nobuaki Takemori, PhD (Ehime University)

Development of Auto-2D: a fully automated two-dimensional electrophoresis system, and application for the top-down proteomics to identify proteoforms in tumor samples
Norie Araki, PhD (Kumamoto University)

17:20–17:30 **Final Remarks**

Monday, Nov 18*

* The events on this day are limited to symposium speakers.

9:00–11:00 Meeting (ROHM Theatre Kyoto, Conference Room I)

11:30–12:45 Excursion 1: Shojin Ryori Experience (vegetarian Buddhist cuisine)

13:30–17:30 Excursion 2: Tour of Shimadzu Corporation

ABSTRACTS

Session 1: Top-Down Proteomics, Part I

Top-down proteomics and proteoforms – a versatile playground for analytical chemistry

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Proteoforms, which are all protein species being generated out of a gene by different biological processes at the stage of transcription (e.g., alternative splicing) or by protein modifications introduced after protein translation (posttranslational modifications), are the functional active units in living systems.

Presently, the field of proteomics is dominated by the bottom-up (BUP) approach, which is based on the analysis at peptide level followed by protein inference. However, this approach is accompanied by a loss of information about proteoforms, which creates an urgent demand for a paradigm shift in the discipline to decipher the full functional repertoire in living systems. This encompasses all levels of proteome analysis, including proteoform identification and quantification, and studies aiming to investigate proteoform functions and their involvement in biological processes, e.g., by determination of proteoform activities, interactions, or structural features.

Intact proteoform analysis, i.e., top-down proteomics (TDP), is a logical step forward to reach these goals. However, despite significant advancements made in recent years, TDP is still challenging due to a number of factors at all levels of proteome analysis, which requires the improvement of existing and the development of novel analytical approaches.

The talks will highlight the major challenges encountered with intact proteoform analytics and will exemplarily show routes to overcome the present limitations [1-3], including approaches to obtain proteoform-level information in biological materials with limited availability [4]. In particular, the potential of an integrated use of TDP, middle-down proteomics, BUP, and terminomics approaches [5] will be discussed.

References

[1] Kaulich PT, Cassidy L, Winkels K, Tholey A (2022). Improved Identification of Proteoforms in Top-Down Proteomics Using FAIMS with Internal CV Stepping. ***Anal Chem***, 94: 3600-3607. doi: 10.1021/acs.analchem.1c05123

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[3] Kaulich PT, Jeong K, Kohlbacher O, Tholey A (2024). Influence of Different Sample Preparation Approaches on Proteoform Identification by Top-Down Proteomics. ***Nat Methods***, in press. doi: 10.1038/s41592-024-02481-6

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[5] Winkels K, Koudelka T, Kaulich PT, Leippe M, Tholey A (2022). Validation of Top-Down Proteomics Data by Bottom-Up-Based N-Terminomics Reveals Pitfalls in Top-Down-Based Terminomics Workflows. ***J Proteome Res***, 21: 2185-2196. doi: 10.1021/acs.jproteome.2c00277.

Session 1: Top-Down Proteomics, Part I

Truncated Proteoforms in Top-Down Proteomics Studies

Philipp T. Kaulich, Andreas Tholey

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Top-down proteomics (TDP) offers a powerful approach to identifying intact proteoforms, inherently providing information about protein modifications [1]. Truncated proteoforms are among the most frequently observed modifications in TDP studies. [2,3] These truncations can originate, for example, from proteolytic processing, splicing events, or as artifacts introduced during sample preparation.

Here, we selected over eighty datasets from more than forty different TDP studies published over the past decade, spanning various organisms, sample preparation approaches, data acquisitions, and data analysis, and investigated the reported proteoforms.

On average, approximately 70% of the reported proteoforms were truncated, of which only 20% were deposited in the UniProt database. Detailed examination of the truncation sites revealed substantial differences between the proteoform N- and C-terminus and across the various datasets. Despite these variations, some studies exhibited similar cleavage patterns, such as preferential cleavage C-terminal to lysine and arginine residues or between aspartate-proline bonds. Moreover, our analysis showed that certain proteoform termini of a given protein were consistently reported across multiple independent TDP studies, suggesting potential biological relevance. We evaluated the impact of factors like sample type, sample preparation, data acquisition and analysis, and protein structure on the occurrence of truncated proteoforms. Some observations pointed to artifacts introduced during sample preparation (e.g., heating) [3] or mass spectrometric analysis (e.g., in-source fragmentation).

In conclusion, this study provides a comprehensive overview of truncated proteoforms identified in TDP studies, highlighting both methodological influences and potential biological significance. Furthermore, the results can serve as a resource for other scientists to investigate the termini from a protein of interest.

References

[1] Winkels K, Koudelka T, Kaulich PT, Leippe M, Tholey, A. (2022). Validation of Top-Down Proteomics Data by Bottom-Up-Based N-Terminomics Reveals Pitfalls in Top-Down-Based Terminomics Workflows. ***J. Proteome Res.***, 21 (9), 2185–2196. https://doi.org/10.1021/acs.jproteome.2c00277.

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Session 1: Top-Down Proteomics, Part I

Image-Guided 3D Laser Sampling for Subsequent Deep Omics: Background, Methodology and Applications in Cancer Research

Ayham Moustafa, Manuela Moritz, Mohamed E.Elsesy, Wael Y. Mansour, Hartmut Schlüter, Jan Hahn

Section Mass Spectrometry and Proteomics; University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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Ultrashort pulsed infrared lasers can sample and homogenize biological tissue through desorption by impulsive vibrational excitation. Lasers tuned to 2940 nm with pulse widths in the picosecond or nanosecond range transform irradiated tissue into aerosol without damaging adjacent structures. This soft, cold vaporization process solubilizes tissue molecules, including proteins, lipids, metabolites, and mRNA, preserving them for omics analysis.

We present an early-stage prototype of an image-guided laser ablation system, 3D-MiTi-LAb, for precise 3D sampling of tissues and biological samples [1]. The system uses pre-recorded image data to guide a nanosecond infrared laser (NIRL). A reflected light microscope and optical coherence tomography (OCT) system register images to the 3D ablation coordinates, enabling precise aiming with 20 µm resolution (lateral and axial). We analyzed the collected samples using differential quantitative bottom-up proteomics [2], shotgun lipidomics [3], and qPCR, confirming the feasibility of NIRL tissue sampling with subsequent omics analysis.

In cancer research applications, we created 3D maps of spheroids and organoids in Matrigel with OCT before laser ablation. Prostate cancer cell lines VCaP and DU145, and brain rhabdoid tumor-derived cell lines BT16 and BT16+lin28A, were cultured in Matrigel to form spheroids/organoids in 3D. Differential quantitative proteomics analyzed the homogenized aerosol, quantifying 5051 proteins. Additionally, we quantified 3414 proteins from formalin-fixed prostate cancer patient tissues immunohistochemically stained against AMACR, distinguishing between AMACR-positive and negative patterns.

References

[1] Hahn J, Moritz M, Walter A, Wieck T, Moustafa A, Mansour WY, Harms C, Stenzel P, Heeren J, Haider M-T, Lange T, Schlüter H (2024). *Multimodal Image-Guided Laser Ablation System for Precise 3D Tissue Sampling for Subsequent Analysis of Biomolecules in Cancer Research*. Proc. of SPIE Vol. **12846**, 128460C-1.

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Session 1: Top-Down Proteomics, Part I

Via infrared-laser based sampling towards the original composition of proteoforms in tissues

Manuela Moritz, Jan Hahn & Hartmut Schlüter

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Mechanical homogenization is the common method for solubilizing proteins present in solid tissues, e.g. for subsequent proteomics. During mechanical homogenization compartments of the cells in tissues like lysosomes are broken, thereby releasing their contents comprising a large variety of enzymes. The released enzymes are acting on other proteins in the homogenate resulting in the conversion of original proteoforms into new artificial proteoforms. With a nano-second infrared laser (NIRL) or a picosecond-infrared laser (PIRL) a very fast sampling and simultaneously homogenization of tissues is possible. Furthermore, the process is very soft enabling the solubilization of proteins without any fragmentation [1]. The process of sampling and homogenization of tissues is based on the adsorption of energy of water molecules in the tissues by its irradiation with NIRL or PIRL, resulting in an explosion of the tissue thus converting the irradiated part of the tissue into an aerosol. The aerosol is containing all tissue molecules in a solubilized form. Since this process is very fast the conversion of proteins by solubilized enzymes is minimized, increasing significantly the opportunity to obtain a view on the original composition of proteoforms in tissues prior to sampling and homogenization [2]. The current system of the infrared-lased based sampling and homogenization technology is comprising an imaging system including an optical coherence tomography (OCT) and a light- and fluorescence microscope. With the imaging systems 3-dimensional maps of the tissues are obtained by which targets are identified. Selected targets are then irradiated with the laser and resulting aerosols condensed.

References

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Session 2: Proteoform Analysis with Bottom-Up Approach

Elucidation of regulatory mechanisms of protein kinases based on kinome profiling

Naoyuki Sugiyama [1,2]

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Protein phosphorylation plays important roles in the regulation of protein activity and molecular recognition, and is one of the essential post-translational modifications for understanding intracellular signal transduction. Due to innovations in various technologies and analytical methods in phosphoproteome analysis, it is now possible to analyze more than tens of thousands of phosphorylation sites simultaneously. However, current phosphoproteomics methods have some technical limitations, and there are challenges to effectively utilize large-scale phosphoproteome data.

In particular, direct observation of phosphoproteins in the intact state is an important issue for understanding functional diversity due to co-occurring effects of phosphate groups, other post-translational modifications, and splicing within the same protein.

In this presentation, I will discuss our recent progress in the bottom-up approach to phosphoproteome analysis, including the large-scale identification of *in vitro* substrates of human protein kinases using quantitative phosphoproteomic approach and the development of novel methods to analyze protein expression levels and phosphorylation sites with low phosphorylation levels. In addition, the prediction of the responsible kinase for each phosphorylation site based on a kinase substrate model and the development of the method for measuring kinome activity using artificial substrate peptide library will also be presented. In addition, future prospects for intact phosphoproteome analysis will be discussed.

Session 2: Proteoform Analysis with Bottom-Up Approach

Defining proteoforms through post-translational modification proteomics

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One of the factors that complicate the proteome is post-translational modifications (PTMs) at the N- and C-termini of proteins or on the side chains of specific amino acid residues. Many post-translational modifications are tightly regulated by enzymes, such as phosphorylation on serine, threonine, and tyrosine residues, which dynamically change in response to various stimuli and regulate protein function. On the other hand, there are also irreversible proteoform changes, such as processing by proteases [1], and PTMs that occur non-enzymatically in response to the intracellular environment, such as some lysine acetylation [2]. Thus, post-translational modifications are important in defining proteoforms because they exponentially increase proteoforms of the gene products. Bottom-up proteomics, which measures short peptide fragments, is particularly useful for the analysis of PTMs in terms of identification and quantification of modified site. In this presentation, we will present our recent researches for analyzing post-translational modifications, including lipid modifications [3].

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Session 2: Proteoform Analysis with Bottom-Up Approach

Significance of Protein Terminus Excavation as a Trace of RNA Splicing, Translation and Proteolysis

Yasushi Ishihama[1,2]

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One of the fundamental questions that proteomics must address include the extent to which various perturbations in the genome, transcriptome and translatome are reflected in the proteome, and the extent to which protein lifetimes are regulated by perturbations in the genome and transcriptome. To solve this problem, protein fragment measurement by conventional shotgun proteomics is not sufficient and requires the analysis of proteoforms, the various molecular forms found in the translated product of a single gene, including gene mutation products, spliced RNA transcripts, single amino acid polymorphisms, post-translational modifications, non-cannonically translated products and proteolytic processing products.

Our laboratory has recently developed CHAMP methods (CHromatographic AMplification of Protein N, C-terminal peptides) [1-4], chromatographic approaches for isolating N- and C-terminal peptides of proteins that recognizes the charged position of the target peptide by ion-exchange chromatography or ligand-exchange chromatography. Using CHAMP methods, the isolation of protein terminal peptides can be achieved with >95% selectivity in only two steps: protease digestion and chromatographic separation. By matching a customized transcriptome database with terminal proteomic LC/MS/MS data, large-scale proteoform analyses of different protein termini are possible. In this talk, I will report on the current status and challenges of protein terminomics for the elucidation of human proteoforms, especially for the excavation of protein termini as traces of RNA splicing, translation and proteolysis.

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Session 3: Lightning Talks by Japanese Student Participants

Large-scale advanced design and evaluation of kinase-specific artificial substrate peptides

Liang Junqi[1], Uehara Mao[1], Nakazono Junna[1], Sakamoto Dai[1], Sugiyama Naoyuki[1,2], Ishihama Yasushi[1,3]

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Protein phosphorylation, mediated by protein kinases, is a major and essential post-translational modification (PTM) that plays a critical role in intracellular signal transduction across various cellular processes [1]. Interrogation of kinase activities has become crucial because their activity changes are related to diseases. The complexity of kinase-substrate relationships presents a significant challenge for estimating large scale kinase (kinome) activity from phosphoproteome profiling [2]. Recent efforts have used substrate peptides that are thought to be selectively phosphorylated by specific kinases, allowing for the direct measurement of individual kinase activities. However, the selectivity of these substrates is often inadequate [3]. Therefore, there is a need to develop a method capable of designing substrate peptides with both high selectivity and sensitivity for kinome profiling.

In this study, PWM (Position Weight Matrix)-based substrate peptide design was employed to develop highly sensitive and highly selective substrate peptides for human serine/threonine protein kinases (STKs). With PWMs constructed from an *in vitro* database from our lab [4], up to 5^9 candidate substrate peptides were *in silico* generated for each kinase, and target substrates for each kinase were selected among candidates. We also evaluated different methods of *in silico* prediction of substrate peptides. Experimental evaluation showed that the PWM-based method for substrate design has potential in predicting substrate peptides with high sensitivity and high selectivity to their target kinases. Combining all methods, 291 substrate peptides were successfully designed with high sensitivity and selectivity for 76 human protein kinases.

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Session 3: Lightning Talks by Japanese Student Participants

Ultrahigh-speed, high-sensitivity and high-depth proteomics by capillary-flow LC/MS/MS

Ayana Tomioka[1], Ryota Tomioka[1], Eisuke Kanao[1,2], Kosuke Ogata[1], Naoyuki Sugiyama[1,3], Yasushi Ishihama[1,2]

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Bottom-up proteomics is an excellent method for obtaining proteome profiles of protein expression, post-translational modifications and structural changes. In recent years, proteomics research, which requires the measurement of a large number of samples, such as spatial proteomics, biomarker discovery and single-cell analysis, has increasingly attracted attention. However, previous studies aimed at high-throughput measurements have limited throughput to about 400 samples per day (SPD) and the number of identified protein groups to about 3,000 to 4,000. This is because increasing the LC flow rate for higher speed tends to reduce detection sensitivity. Here, we developed ultrahigh-speed proteome analysis method, that completes measurements within 1.4 minutes per sample, which achieves a throughput of 1000 SPD. To evaluate the sensitivity of this method, we measured three synthetic peptides by conventional nanoLC/MS/MS and this method and found that the peptide peak height was comparable in each method. Next, we evaluated the performance of this method using a dilution series of HeLa digest; LC/MS/MS measurements were performed using diaPASEF and injection amount was varied from 10 ng to 1,000 ng. As a result of condition optimization, more than 3,000 protein groups could be identified from a 100 ng sample. Furthermore, even when using samples as small as 10 ng, approximately 2,000 protein groups were successfully identified, confirming that the method has both high throughput and high sensitivity.

References

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Session 4: Proteofom Analysis in Clinical/Biological Studies

The role and the potential of posttranslational modifications of collagen to enhance bone health and repair

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Bone tissue undergoes highly dynamic remodelling processes during bone development and repair, mechanical stimulation or physiological bone turn over. A series of proteins including matrix proteins such as osteopontin and bone sialoprotein (BSP), growth factors (BMP), transcription factor (Runx2) are involved in these highly orchestrated processes, partly guided by posttranslational modification of these proteins. In this context collagen type 1 is the major structural protein in bone tissue maintaining bone function and integrity via interaction with bone cells, protein complexes or mineral components of the bone. Posttranslational modification of collagen actively influences the mineralization process, as well as the stiffness of the bone matrix, thus supporting bone functionality and repair. In bone regeneration and bone surgery, collagen matrices, hydrogels or scaffolds are widely used to fill bone defects. In this context the modification of collagens or its derivatives introducing targeted chemical modifications would enable to create collagens with improved or new functions. This talk will give an overview of posttranslational modifications in bone repair and discuss options to use such modifications in therapeutical approaches.

Session 4: Proteofom Analysis in Clinical/Biological Studies

The protein levels of post-transcriptionally regulated genes determine stem cell fate into three germ layers

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Diamond-Blackfan anemia (DBA) is a congenital bone marrow failure syndrome characterized by a significant decrease in red blood cells and physical abnormalities, including craniofacial malformations. DBA results from heterozygous mutations in ribosomal protein (RP) genes, with over 20 causative genetic mutations identified to date. However, approximately 40% of the patients do not exhibit these mutations, suggesting the presence of other causative genes or mechanisms.

We have previously conducted large-scale trans-omics analyses of human induced pluripotent stem cells (hiPSCs) and somatic cells [1]. While mRNA levels remained constant between these two cell types, the analysis revealed 228 genes with significantly higher protein levels in hiPSCs, including nearly all the reported DBA-causing genes. We generated hiPSCs from patients with high-frequency mutations in DBA genes, such as RPS19, RPL11, and RPL5. DBA-hiPSCs showed defective differentiation potential in the early mesoderm. Furthermore, although the protein levels of RPs in DBA-hiPSCs were comparable to those in healthy hiPSCs, they decreased during mesodermal differentiation. These findings suggest that DBA genes undergo different quantity control between hiPSCs and differentiated cells. Importantly, this regulation occurs post-transcriptionally, and not during transcription. These data also imply abnormalities in the regulation of protein levels (proteostasis) during differentiation and development in patients with DBA. This study proposes a novel mechanism for the onset of DBA through the regulation of proteostasis and leads to a better understanding of common principles underlying mutation-independent DBA pathogenesis.

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Session 4: Proteofom Analysis in Clinical/Biological Studies

In Variatate Concordia: Can single-molecule DNA and RNA sequencing in stem cell systems inform proteoform mapping?

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Studying proteoforms—the diverse structural variations of proteins resulting from alternative splicing, RNA editing, and post-translational modifications—is crucial for understanding disease-specific molecular mechanisms and remains a significant objective in proteomics. Recent advances in single-molecule sequencing technologies, such as Oxford Nanopore Technologies Direct RNA Sequencing, enable more detailed analysis of transcriptomic complexity at the isoform level, offering new potential for top-down and bottom-up proteomic studies. Despite these advancements, single-molecule sequencing has not yet been fully integrated into proteoform research. Building on recent findings showing that integrating ONT-DRS with other long-read sequencing platforms enhances proteoform detection, we propose a conceptual framework for applying this approach to stem cell systems and human preclinical studies.

Direct RNA sequencing could enable high-resolution isoform mapping in stem cell-derived models, potentially uncovering novel proteoform diversity associated with cell differentiation and disease-specific states. Cross-referencing such datasets with mass spectrometry (MS) data would improve proteoform databases, enhancing the sensitivity and specificity of proteomic analyses. As these technologies advance, their combined application could create a sophisticated proteogenomic workflow capable of identifying previously uncharacterized proteoforms in complex human disorders.

Moreover, characterizing disease-associated proteoforms could lay the groundwork for training artificial intelligence (AI) models incorporating epigenetic signals from single-molecule DNA sequencing. Building on our recent work with MethyLYZR, an AI model for rapid brain tumor classification based on CpG methylation signatures, we envision an integrative approach combining proteoform data with epigenetic profiling at the loci encoding these proteins. Utilizing CpG methylation data as an additional feature in AI frameworks could lead to robust diagnostic tools capable of distinguishing subtle disease states with high precision, especially in oncology. For example, methylation markers associated with specific proteoforms could assist in differentiating between brain tumor subtypes in real-time diagnostic workflows.

This conceptual framework underscores the translational potential of integrating single-molecule RNA and DNA sequencing data with proteomic and AI technologies to advance disease diagnostics.

Session 5: Top-Down Proteomics, Part II

Characterization of proteoforms of intact proteins by one and two-dimensional CE-MS techniques

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Electromigrative techniques are powerful tools for the separation of intact proteins and their proteoforms. Mass spectrometry is an ever-evolving information-rich technology with strong improvements especially on the intact protein level in recent years. The combination of both techniques contributes significantly to the detailed characterization of proteins. However, CE-MS is still restricted by the sensitivity and ease-of-use of the interface in conjunction with low injection volumes limiting its application for biological samples. Various solutions will be presented here overcoming these shortcomings.

Initially, the power of CE-MS for the characterization of proteoforms will be presented applying the *nanoCEasy* interface [1]. Efficient separation of proteins and proteoforms depends strongly on the applied capillary coating. Very recently we developed efficient coatings enabling finetuning the EOF [2] and, thus, increase the separation efficiency for proteins of certain mobility. Here, first results on the application for protein separation from biological samples will be presented.

nanoLC-CE-MS is a promising tool for targeted protein and proteoform analysis in biological samples. Initially a heart-cut nanoLC-CE-MS was setup and the performance regarding improved sensitivity as well as separation of proteoforms was demonstrated [3]. Due to the increased loadability of the nanoLC, the nanoLC-CZE-MS setup exhibits a 280-fold increased concentration sensitivity compared to CZE-MS alone. The combination of high sensitivity and orthogonal selectivity enables the detailed characterisation of intact proteoforms at physiologically relevant concentrations. The very recent approach towards selective comprehensive analysis in a novel online nanoLC-CE-MS configuration will be discussed along with potential future applications of nanoLC-CE-MS.

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Session 5: Top-Down Proteomics, Part II

Computational methods in top-down proteomics to address challenges in proteoform analysis

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Top-Down Proteomics (TDP) has emerged as the dominant method for elucidating the intricacies of proteoform diversity, providing insights crucial for understanding biological processes. With development ranging from sample preparation to instrumentation, there has been a notable increase in research endeavors adopting and developing different TDP protocols that suit the objectives of the studies. Moreover, the information density within TDP data sets have grown dramatically, and more TDP data sets are being deposited in the public repository like PRIDE. However, due to distinct characteristics (e.g., complexity of ion signals), the computational tools used in the well-established field of bottom-up proteomics (BUP) cannot be readily adopted for TDP; dedicated methods are still demanded for the data analysis, data acquisition, and signal processing.

In this presentation, unique characteristics of the TDP datasets mentioned above will be discussed. Then our contribution to the field of computational TDP, which include various computational methods such as deconvolution [1], data acquisition [2], and quantification [3], will be presented, focusing on a concise overview of the main concepts and core results of each method. Finally, the current project for the proteoform identification and characterization method that push the boundaries of the existing search engines will be discussed.

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Session 5: Top-Down Proteomics, Part II

OpenMS - An open-source software suite for (top-down) proteomics

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Efficient and reproducible analysis of mass spectrometry data remains a major challenge in proteomics, in particular with growing volumes of data and increasing sensitivity of instruments. In particular in top-down proteomics, there is still a significant lack of tools for data processing.

With OpenMS we have been developing a platform-independent open-source software package for mass spectrometry data for 20 years. OpenMS offers a wide range of tools that can be combined into complex and reproducible data analysis workflows.

With the FLASH suite of tools, OpenMS has started to add tools specifically tailed to top-down mass spectrometry. We will introduce FLASHQuant for fast and reliable proteoform quantification and FLASHViewer for interactive visualization and exploration of complex mass spectrometry data.

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Session 6: Promising Technologies for Proteoform Analysis

Proteomics with cell-free synthesized peptides/proteins

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Whether using a top-down or bottom-up approach, a key challenge in high-sensitivity analysis of proteins and peptides via mass spectrometry is the inability to amplify these target molecules. To address this issue, creating substances with the same chemical structure as the target molecules allows us to extract extensive information from exact masses, retention times in liquid chromatography (LC), the intensity of primary ions, and fragment patterns from MS/MS analysis. We have developed high-sensitivity quantitative methods for target proteins and peptides based on a cell-free peptide (or protein) synthesis system [1]. This method was further optimized for efficiency through multiplexing [2], resulting in high-throughput, high-sensitivity quantitative systems, which can be used in applications such as cancer marker discovery [3]. In this presentation, I will introduce our method and discuss its application in both top-down and bottom-up proteomics fields.

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Session 6: Promising Technologies for Proteoform Analysis

Development of Tapping-mode Scanning Probe Electrospray Ionization for Mass Spectrometry Imaging of Tissues and Cells

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Analytical techniques that can precisely measure the chemical state of cells that form biological tissues and acquire characteristic values that can precisely specify disease states are important for the advancement of disease diagnosis and treatment. Mass spectrometry imaging (MSI) can visualize the distribution of multiple molecules within a solid sample in a single measurement and is particularly effective for visualizing the cellular components of biological tissues [1].

In this presentation, we introduce the development of a technology called “t-SPESI” (tapping-mode scanning probe electrospray ionization) that extracts and ionizes local regions of biological tissue using extremely small amounts of solvent (from pL to sub pL). t-SPESI is a unique technology that rapidly extracts and ionizes sample components by allowing the charged solvent to come into contact with the sample through a capillary probe that oscillates at high speed [2-4]. Recently, by developing elemental technology to control probe oscillation [5] and solvent flow, it has become possible to perform high-precision MSI of diseased human and mouse tissues and single cells.

We introduce examples of lipid imaging of diseased tissue and cells to date and hope to report the progress of our recent research on MSI of proteins. In the future, we will continue to develop advanced t-SPESI technology and conduct research that “observes, learns from, and utilizes” biological tissue, and we will strive to discover cellular changes in diseased tissue and present new information that can be applied to medical and pharmaceutical research.

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Session 6: Promising Technologies for Proteoform Analysis

Multi-dimensional vibrational circular dichroism as targeted for biological samples

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This lecture presents our recent works on vibrational circular dichroism spectroscopy (VCD) [1]. VCD is a chiro-optical method detecting molecular chirality in the infrared region. The method is applicable to compounds with no electronic transition in the UV-vis region. One drawback of VCD is the weakness of a signal. It limits application to stable homogenous solution samples. To overcome the difficulty, we found the enhancement of signals in case of "supramolecular chirality", the chirality organized by a number of molecules, whose scale extends over molecular chirality [1-3].

As an extension of VCD measurements, we recently constructed a microscopic VCD instrument. The system employs a quantum cascade later (QCL) as an intense IR source. It is denoted as the multi-dimensional VCD (MultiD-VCD) spectrometer. VCD signals are collected along the multi-axes of wavenumber, positional coordinates and time. It enables us to scan VCD signals two dimensionally at the spatial resolution of 100 μm [4,5]. A light source can be switched between a thermal light and QCL, choosing the measurement mode between FT-VCD (thermal light source) and QCL-VCD, respectively.

Recently the QCL-VCD system has widen its targets to the scope of biological samples. For examples, the two-dimensional patterns of protein secondary structures are obtained for the wings of various kinds insects [6-8]. It is revealed that a wing comprises the heterogenous distribution of protein domains in various secondary structures. The results give us a clue to the origin of flying ability as well as the evolutionally development in the insect world.

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Session 7: Top-Down/Bottom-Up Peptidomics

Biomedical applications of peptidomics, a subdivision of proteoform profiling

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Limited proteolysis, or proteolytic processing at specific sites, refers to a biochemical event that expands proteome diversity by generating smaller functional segments. The resultant polypeptides, representing an integral part of the proteoform, are investigated in their intact form. Biomedically relevant mammalian peptides include hormones, neuropeptides, anti-microbial peptides and HLA-associated peptides, with the smallest one being Thyrotropin Releasing Hormone (TRH), a 3-amino-acid peptide.

Molecular endocrinologists were aware at the end of the 1990s that mass spectrometry is the method of choice to characterize bioactive peptides, as exemplified by TRH harboring two post-translational modifications in such a short sequence. Indeed, a pioneering study was published in 1997 profiling endogenous peptides in human hemofiltrate by Dr. Forssmann and his colleagues at the then Niedersächsisches Institut für Peptid-Forschung, Germany [1]. Four years later, the term peptidomics emerged in the original papers by Dutch and Belgian entomologists investigating neuropeptides in insect endocrine glands [2] [3]. Our study focused on secretory vesicles was the first to demonstrate that peptidomics helps to identify novel bioactive peptides [5], followed by subsequent research [6-9, 11]. We also showed that a peptidomic approach is able to uncover potential cancer markers [4] and identify ectodomain cleavage sites of transmembrane proteins [10].

Peptidomics is expected to eluciate the entire repertoire of smaller polypeptides, but secondary, degradative proteolysis blurs the identification of the original processing products, especially those without a particular conformation. Top-notch, high-throughput mass spectrometers could resolve the issue that has plagued peptidomics researchers.

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Session 7: Top-Down/Bottom-Up Peptidomics

Ion mobility mass spectrometry for immunopeptidomics in cancer immunotherapy

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With the rapid progress in cancer immunotherapy such as CAR-T cells, bispecific antibodies, adoptive T cell therapies and cancer vaccines, reliable information about optimal antigens to benefit from these therapies is required. The identities of these antigens is so-called immunopeptides, that are derived from a variety of proteoforms, including canonical and non-canonical ORF-derived translation products that sometimes carry PTMs. Immunopeptidomics, analyzing the immunopeptides by mass spectrometry (MS), is currently the only technology that can directly identify the immunopeptides actually presented on tumor cells. And it has therefore attracted great interest in the fields such as infection, autoimmune diseases and cancer immunotherapy. The identification of delicate analytes such as immunopeptides has been greatly improved due to recent developments in MS instrumentation. In particular, the ion separation systems based on shape-resolved ion mobility have become one of the leading key technologies in immunopeptidomics [1].

We have previously established the immunopeptidomics by differential ion mobility (DIM)-MS and conducted analyses to identify immunopeptides directly from biopsy-sized human colorectal tissue and reported the identification of somatic mutation-carrying neoantigens including oncogenic KRAS mutation-carrying antigen [2]. Another ion mobility technology, called trapped ion mobility mass spectrometry (tims), facilitated identification of immunopeptides both from canonical- and noncanonical-ORF derived translation products with more sequence variations, that are crucial for a better understanding of the full figure of the individual immunopeptidome for precision medicine.

In this seminar, we would like to introduce our latest challenges in establishing inclusive-immunopeptidomics by ion mobility-assisted MS.

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Session 7: Top-Down/Bottom-Up Peptidomics

Peptidomic analyses of plasma and tissues based on high-yield peptide extraction method.

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Endogenous peptides in plasma play very important roles in the regulation of homeostasis in the body. However, they are much more difficult to analyze and identify than proteins. The differential solubilization (DS) method that we developed enables high-efficiency extraction of peptides from serum/plasma, including peptides bound to carrier protein such as albumin [1]. Based on DS method, we successfully identified more than 11,000 peptides from plasma including well-known bioactive peptides such as insulin B chain, manserin, hepcidin, motilin, salusin-β, neurotensin, and somatomedin-B. From this 11,000 peptide library, we selected about 150 peptides derived from secreted protein and attempted to search for bioactive peptides. After investigating the response of various cultured cells and in vivo bioactivity, we discovered five bioactive peptides [2-4].

Next, we improved the DS method optimized for small amounts of tissue, and conducted peptide analysis of approximately 0.5 mg of hypothalamic tissue taken from frozen sections of cryopreserved brains. As a result, we identified 1,535 peptides derived from 297 proteins and detected 35 known peptide hormones [5]. Furthermore, we have recently applied this DS method to liver, large intestine, small intestine, pancreas, islets.

Peptidomic analysis technology based on the DS method are expected to accelerate research on novel bioactive peptides and disease-related peptides in body fluids and tissues.

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Session 8: Structural & Single Molecule Analysis Techniques for Proteoform Analysis

Nanofluidic and Lab on a Chip devices for single molecule analysis

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Lab-on-a-chip devices are miniaturized platforms capable of performing multiple tasks that are routinarily done in labs, in a small chip, just a few centimeters in size. Some of these tasks include liquids mixing, enzymatic digestions, separation, sensing, etc. In this work, we will present micro and nanofluidic devices [1], their fabrication and how they can be used for sample collection, purification and preparation on-chip and examples of how they can be used for single molecule analysis combined with in-line optical sensing in real time. These devices, which are fully transparent and made of polymer, contain micro and nanofluidic elements, like nanochannels, and are integrated with electrodes and other active sensing components, like plasmonic antennas [2] or suspended structures [3]. These devices are especially interesting for the handling of very small liquid volumes, since they just need a few nanoliters of material. In addition, the reactions are faster, and more reproducible than those performed with larger liquid volumes in the lab.

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High-precision Proteoform Quantification Reveals Phosphorylation Kinetics of AMP-activated Protein Kinase

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AMP-activated kinase complex constitutes the regulatory hub of the cell energy metabolism. The activity of AMPK is facilitated through sophisticated accumulation of molecular events creating multiple active proteoforms and conformers of AMPK complex. Despite being inherently connected to AMPKs function, its proteoform diversity has not been systematically characterized. Here we report the first study on kinetics of phosphorylation which regulates AMPK activity utilizing a hybrid mass spectrometry approach.

Full-length heterotrimeric AMPK- α1β1γ1 complex has been expressed in *E.coli*. AMPK was post-translationally modified *in vitro* with Calcium-Calmodulin dependent kinase kinase complex (CaMKK) and Protein Phosphatase 2A in presence of nucleotides, inhibitors, and activators. Samples were taken from different conditions and timepoints. Total subunit phosphorylation was determined using intact mass RPLC MS via C4 in-house packed columns and a Bruker Impact II Q-ToF. Site specific phosphorylation kinetics was determined using data-dependent acquisition (DDA)-PASEF mode on a timsToFpro. Top-down MS analysis was performed using a TriVersa Nanomate system coupled with solariX XR 12-Tesla Fourier Transform Ion Cyclotron Resonance mass spectrometer (FTICR-MS). For data analysis, a proteoforms simulator was implemented via the R-studio Shiny App.

In this work, we present a unique strategy for high-precision proteoform quantitation, revealing multiple site-specific enzyme reactions in parallel and thereby vastly extending the knowledge about intramolecular activation of AMPK complex. Our hybrid MS approach can easily be expanded to other central metabolic regulators.

Session 8: Structural and Single Molecule Analysis Techniques for Proteoform Analysis

Connecting proteoforms to higher-order structure by combining native and top-down MS

Frederik Lermyte

Technical University of Darmstadt

Session 9: Gel-Based Proteoform Analysis

Gel-Based Human Proteoform Atlas: Mapping of Human Proteoforms using SDS Polyacrylamide Gel Electrophoresis

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Sample prefractionation prior to MS analysis is essential to increase the number of proteoforms detectable in top-down proteomics. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a simple, reproducible, and high-resolution protein separation technique that has been used in many protein experiments in biochemistry and molecular biology. Until recently, however, its use as a sample fractionation method in top-down proteomics has been hampered by the difficulty of extracting intact proteins from polyacrylamide gel matrices. Passively Eluting Proteins from Polyacrylamide gels as Intact species for Mass Spectromtery (PEPPI-MS), which we first reported in 2020 [Ref.1], is a method that enables highly efficient and rapid extraction of intact proteins from gels. With the advent of PEPPI, SDS-PAGE has finally become a powerful tool for pre-fractionating proteoforms in top-down proteomics. Molecular weight-based fractionation of proteoforms by PEPPI is highly compatible with other fractionation methods; for example, the GeLC-FAIMS workflow, which was created by integrating ion mobility-LC mass spectrometry and PEPPI, has successfully accelerated in-depth top-down proteomics and middle-down proteomics [Ref.2–4]. In this talk, I will be presenting our recent progress towards high-throughput and automated PEPPI and discuss the possibility of constructing a high-resolution human proteoform atlas by integrating SDS-PAGE and various proteoform analysis techniques.

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Session 9: Gel-Based Proteoform Analysis

Development of Auto-2D: a fully automated two-dimensional electrophoresis system, and application for the top-down proteomics to identify the proteoform in tumors

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With the advancement of mass spectrometry, large-scale bottom-up proteomics has become common. However, it is still extremely difficult to elucidate in detail of the proteoforms and their changes, with various post-translational modification and splicing sites in biological proteins. The two-dimensional electrophoresis (2D-PAGE), which had been widely used in the early years of proteomic field, is still the most useful method for the separation of nearly 10,000 proteins from biological tissues, cells, and body fluids, etc, at once. However, 2D-PAGE has some difficulties to perform because it requires skilled techniques to expand proteins quantitatively with perfect reproducibility, and it is time-consuming. In this context, we have previously succeeded in developing a fully automated 2D electrophoresis system (Auto2D), which is simple and easy to use with high resolution, sensitivity, and reproducibility in a short time[1.2], and proposed to apply it to proteoform analysis under top-down proteomics, which has recently attracted attention.

In this symposium, using Auto-2D, some of the successful analyses of tumor samples such as tumor tissues and cells, cancer stem cells, and cancer patient sera will be introduced[3,4,5]. In particular, studies focusing on the analysis/profiling of changes in the proteoforms such as phosphorylation, glycosylation, proteolysis, and alternative splicing, associating to the mechanizm of cancer formation, malignancy and drug resistance will be presented. In addition, how the proteoforms identified by this method could be useful for the development of tumor-targeted drugs and clinical markers, as well as the future perspectives in top-down proteomics will be discussed.

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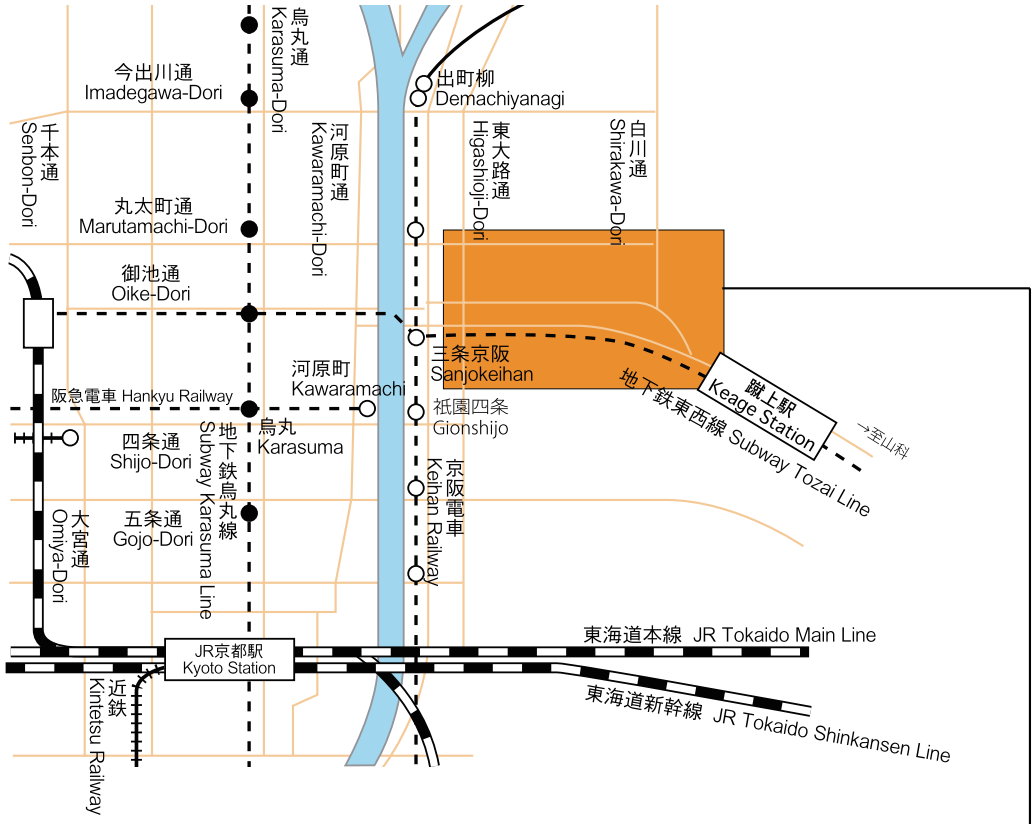
Venue Information



GUIDE MAP

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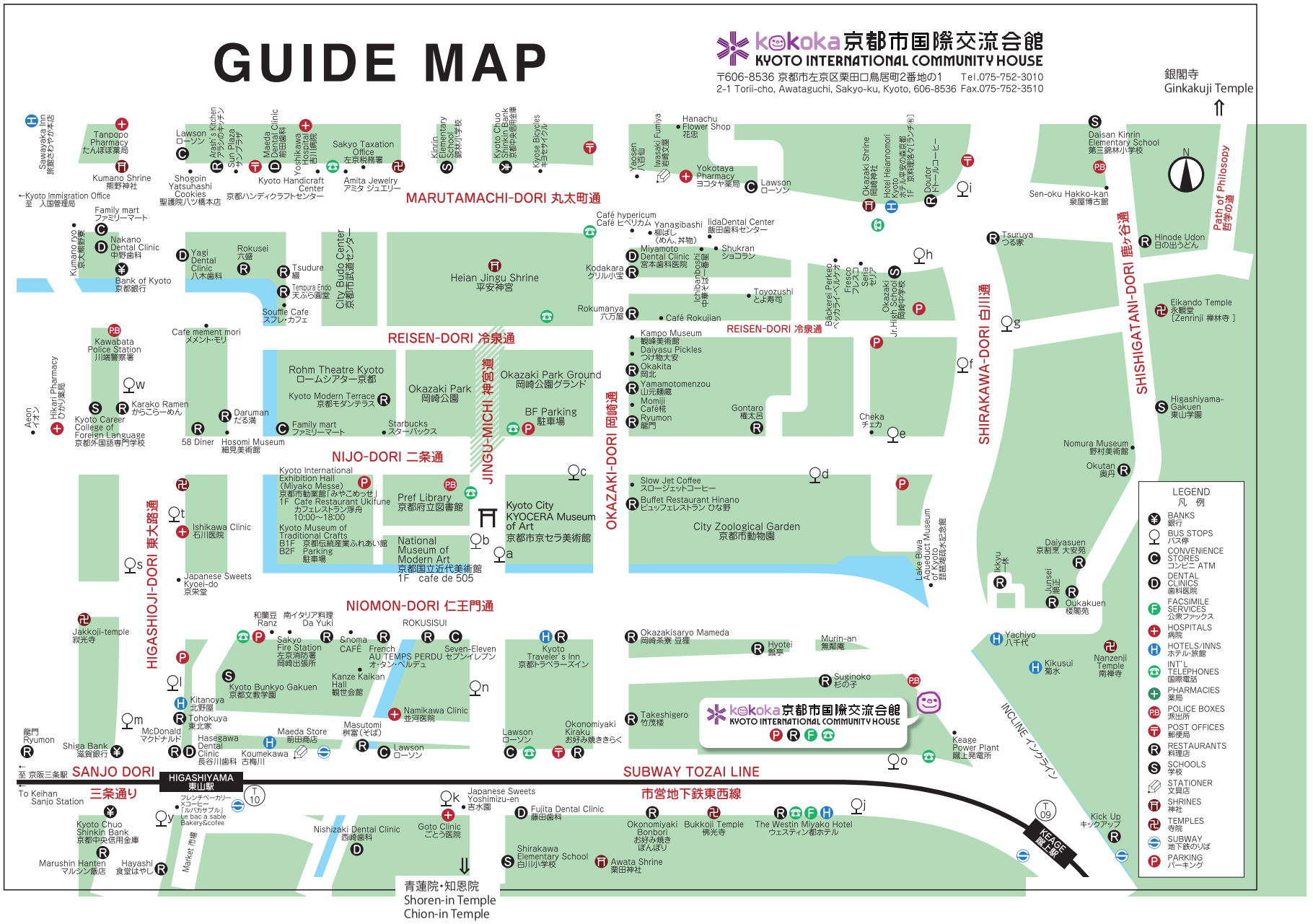

General Information

Registration

The venue for this symposium is the Kyoto International Community House (KOKOKA). The registration table can be found in front of the special conference room on the 2nd floor of KOKOKA. Registration will open at noon on the 16th and will remain open for the duration of the symposium.

Venue

All symposium lectures will be held in the Special Conference Room on the 2nd floor of KOKOKA.

Inquiries

If you have any questions or requests, please contact us at the e-mail address below:

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Upcoming Event

プロテオミクスにおけるプロテオフォーム解析手法の最前線とその応用