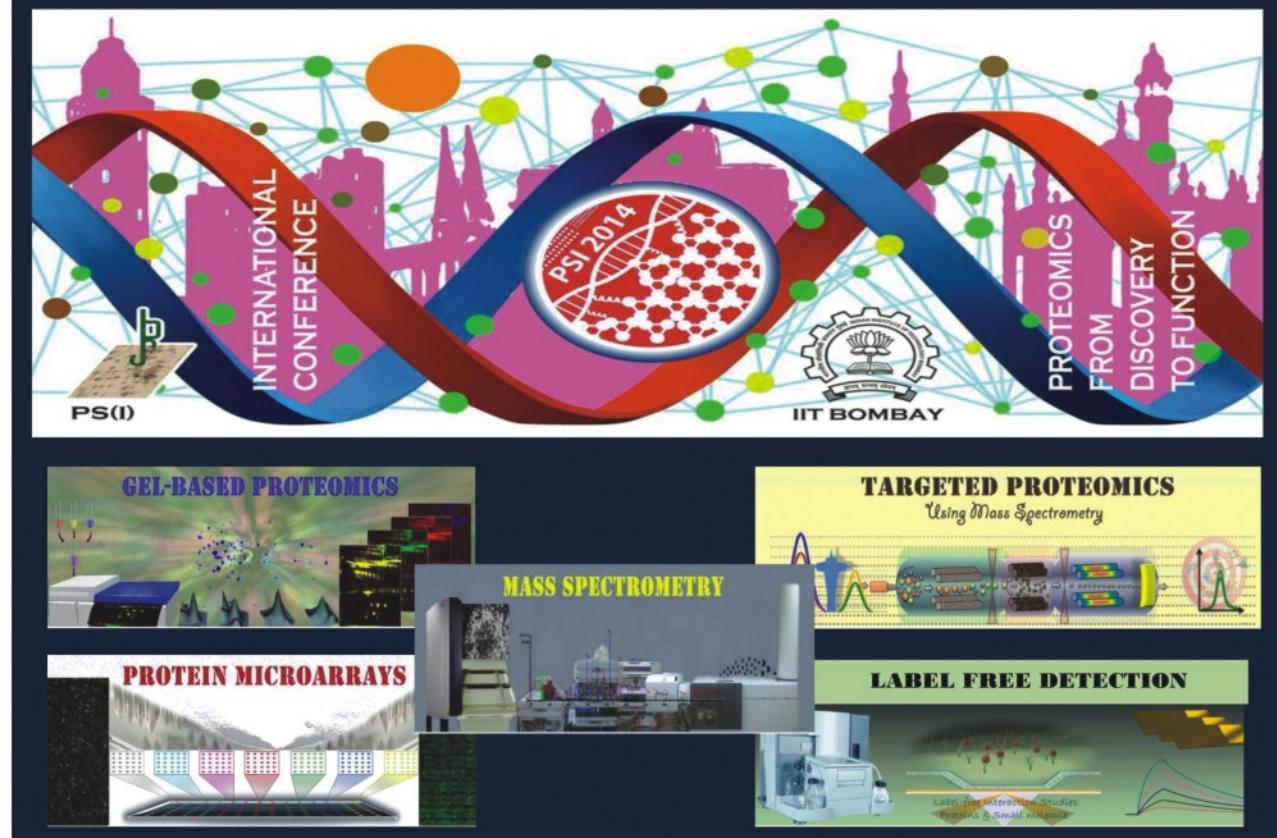


Journal of
PROTEINS AND PROTEOMICS

Special Issue:
 High-throughput Proteomic Technologies
 for Clinical Applications

Guest Editor: Dr. Sanjeeva Srivastava

WORKSHOP PROCEEDINGS



Co-editors: Drs. Sandipan Ray, Veenita Shah, Prasad Phapale, Rekha Jain, Panga Jaipal Reddy, Kishore Gollapalli

PSI 6TH ANNUAL MEETING
Proteomics from Discovery to Function
INTERNATIONAL PROTEOMICS CONFERENCE
DECEMBER 6-11, 2014-IIT BOMBAY, MUMBAI

PROTEOMICS SOCIETY, INDIA PS(I)
 6th Annual Meeting of
PROTEOMICS WORKSHOPS
 10th-11th DECEMBER, 2014
 VENUE: INDIAN INSTITUTE OF TECHNOLOGY (IIT) BOMBAY, MUMBAI

GEL-BASED PROTEOMICS
 Learning Objectives

- Introduction to gel-based proteomics
- 2-DE workflow theory and hands-on session
- 2-DIGE labeling and Workflow
- Training in 2-DE data analysis
- Training in 2D-GE data analysis
- New advancements in gel-based proteomics
- Group discussions

MASS SPECTROMETRY
 Learning Objectives

- Introduction to mass spectrometry
- Sample preparation-in-gel and in-solution digestion
- MALDI-TOF/TOF analysis for PMF and MS/MS
- LC-MS/MS analysis
- Quantitative proteomic analysis
- MS data analysis
- New advancements in mass spectrometry
- Group discussions

TARGETED PROTEOMICS
 Learning Objectives

- Introduction to hypothesis driven proteomics measurement for validation
- Enhance understanding from exploratory proteomic to targeted
- Basic knowledge of skyline to implement targeted experiments
- Introduction to key concepts of targeted proteomics experiments
- Creating & using libraries of prior empirical measurements
- Absolute qualification versus detection differences
- Statistics of study design & avoiding statistical pitfalls
- Basic understanding of MS stats

PROTEIN MICROARRAYS
 Learning Objectives

- Introduction to protein microarrays
- Different types of protein microarray platforms
- Application of protein microarrays for protein interactions autoantibody screening
- Hands-on sessions for printing arrays
- Hands-on sessions for microarray assays
- Training in data analysis & interpretation
- New advancements in microarray technology
- Group discussions

LABEL-FREE DETECTION
 Learning Objectives

- Introduction to Surface Plasmon Resonance & its applications
- Sensor surface properties & surface preparation strategies
- Interaction analysis for Protein-protein & Protein-small molecule studies
- Hands-on sessions for immobilization, binding & kinetics assays
- Training in data analysis & interpretation
- Group discussions

Welcome to Mumbai

Platinum Sponsors
 Thermo SCIENTIFIC
 YASHRAJ BIOTECHNOLOGIES
 AB SCIEX
 ELSEVIER

PRINCIPAL SPONSORS
 Agilent Technologies
 Waters
 THE SCIENCE OF WHAT'S POSSIBLE™

Diamond Sponsors
 BIO-RAD
 BRUKER
 BIA
 Cambridge Isotope Laboratories, Inc.
 PROTEOMICS CONSULTANTS

ALPCORD NETWORK
 TRAVEL & CONFERENCES MANAGEMENT COMPANY

TOURISM PARTNER
 THE LEELA

MEDIA PARTNER
 THE ANALYTICAL SCIENTIST

PUBLISHING PARTNER
 WizIQ

HOSPITALITY PARTNER
 THE NEW BOMBAY DESIGN

Editor-in-Chief : Dr. Suman Kundu

Department of Biochemistry, University of Delhi South Campus
Benito Juarez Road, New Delhi-110021, India; E-mail : jppindia@gmail.com

Founder Editors

Prof. Arvind M. Kayastha

School of Biotechnology
Banaras Hindu University
Varanasi - 221005, India

Prof. Vikash Kumar Dubey

Department of Biotechnology
Indian Institute of Technology Guwahati
Assam-781039, India

Prof. Michael Blaber

Department of Biomedical Sciences
College of Medicine, Florida State University
Tallahassee, FL 32306, USA

Dr. Monica Sundd

National Institute of Immunology
Aruna Asaf Ali Marg
New Delhi -110067, India

Editorial Board

Dr. Himangshu S. Bose

Mercer University School of Medicine & Memorial
Health University, Medical Center, 4700 Waters Avenue
Savannah, GA 31404, USA

Dr. Rizwan Hasan Khan

Interdisciplinary Biotechnology Unit
Aligarh Muslim University, Aligarh-202 002
India

Dr. Gülsah Sanlı

Chemistry Department, Faculty of Science
Izmir Institute of Technology
Gülbahçe/ Urla/ Izmir, Turkey 35430

Dr. Debasish Mukhopadhyay

Structural Genomics Division
Saha Institute of Nuclear Physics
1/AF, Bidhannagar, Kolkata 700 064, India

Dr. Amal Kanti Bera

Department of Biotechnology, Indian Institute of
Technology Madras, Chennai 600036, India

Dr. Kalpana Bhargava

Defence Institute of Physiological and
Allied Sciences (DIPAS)
Defence Research and Development Organization (DRDO)
Lucknow Road, Timarpur, Delhi- 110054, India

International Advisory Board

Abhijit Chakraborty, Saha Institute of Nuclear Physics, India

Aragula Guru Rao, Iowa State University, USA

B. Jayaram, Indian Institute of Technology Delhi, India

Faizan Ahmad, Jamia Millia Islamia, India

Jibin K. Dattagupta, Saha Institute of Nuclear Physics, India

Maurizio Brunori, Sapienza – University of Rome, Italy

Michael I. Oshtrakh, Ural Federal University, Russian Federation

Niranjan Charabarty, National Institute of Plant Genome
Research, India

Rajiv Bhat, Jawaharlal Nehru University, India

Shantanu Sengupta, Institute of Genomics and Integrative
Biology, India

Subhra Chakrabarty, National Institute of Plant Genome
Research, India

Tapan K. Chaudhuri, Indian Institute of Technology Delhi, India

Tej P. Singh, All India Institute of Medical Sciences, India

Associate Board Members

Dr. Alo Nag

Department of Biochemistry
University of Delhi South Campus
Benito Juarez Road, New Delhi-110021, India

Dr. Md. Imtiyaz Hassan

Centre for Interdisciplinary Research in Basic Sciences
Jamia Millia Islamia, Jamia Nagar
New Delhi - 10025, India

Annual Subscription: ₹ 3000 (India) US\$ 125 (Foreign)

Price for Single Issue: ₹ 2000/-



INTERNATIONAL SCIENCE PRESS

F-2562, Ansal's Palam Vihar, Gurgaon, Haryana, INDIA, Phone: 91-124-2365193

E-mail: internationalsciences_press@yahoo.co.in; internationalsciencespress@gmail.com

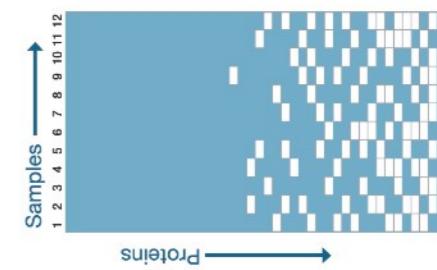
TripleTOF® 6600 + SWATH™ 2.0

Maximizing productivity in targeted proteomics

SWATH™ 2.0 data completeness

Capture more critical data faster, so you can uncover new protein relationships.

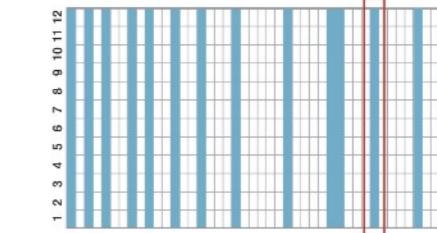
The TripleTOF® 6600 enables unrivaled coverage of your proteome in a single analysis with greater dynamic range and enhanced data quality.



Conventional Proteomics Strategy:
Data Dependent Acquisition (DDA)
Broad coverage, with large variability



SWATH™ Strategy:
Data Independent Acquisition
BEST OF ALL STRATEGIES
Exceptional multiplexing, with
completeness that rivals MRM



Targeted Proteomics Strategy
(MRM)
Narrow focus, with excellent
data completeness



Targeted Proteomics Strategy
(MRM)
Narrow focus, with excellent
data completeness

Solving the challenge of missing values in proteomics research

CONTENTS

1. Preamble by Dr. Sanjeeva Srivastava	
2. Module I- Gel Based Proteomics	25
3. Module II- Mass Spectrometry	61
4. Module III- Protein Microarrays	79
5. Module IV- Label Free Detection	97
6. Module V- Targeted Proteomics	107
7. Module VI- Characterization of Biosimilars and Biotherapeutics	111
Index	125

PREAMBLE

The proteome is defined as set of proteins encoded by the genome and proteomics is the study of the proteome, which includes study of all proteins expressed in any given cell at a given time, the set of all protein isoforms, post-translational modifications (PTMs) and their interactions. The last two decades have seen a surge in proteomics-based research in the field of healthcare and translational research. In spite of the enormous complexity associated with protein-based studies, technologies like gel-based, mass spectrometry, microarray and label-free technologies have been instrumental in this rise of proteomics in generating large-scale data sets for biomarkers, protein-protein interactions and protein profiles in different diseases.

The technologies which have emerged as key facilitators of advancement in proteomics, involve a great deal of instrumentation for innovation. It is therefore important for young researchers to be well-trained in these technologies and know the practical constraints governing these technologies. To address a biological question various proteomic technologies could be employed, but which one is the most appropriate to address a given problem requires good understanding of the pros and cons of various technologies. Such knowledge would help the researchers to make a sound choice of platform to test their hypothesis and design their experiments accordingly. Knowledge of suitable quality control measures and precautions is also of substantial importance in such cases. However, what is probably of key essence in such a setting is the training required to handle these instruments for generation of data from various samples, data interpretation, analysis and establishing resources for data management and storage; which requires dedicated training in context of proteomics-based research.

To this end, the post-conference (6th PSI meeting and International Proteomics Conference) proteomics workshop courses are structured with an aim to train the participants in different core areas, which are currently being used to revolutionize proteomics-based research to enable scientists to make informed choices regarding selection of platforms for their study. We have scheduled six hands-on workshops – gel-based proteomics, mass spectrometry, protein microarrays, label-free detection, targeted proteomics and biosimilars & proteomics; and while we intended that all the participants to get exposure of all technologies as much as participants requested us, but due to the time constrain and limited numbers we have made parallel training sessions for these workshops.

2-DE has evolved tremendously and became one of the extremely popular platforms for the protein separation and differential protein expression analysis. The relatively simplistic set-up and instrumentation involved makes the gel-based proteomics popular across all proteomic establishments. The gel-based proteomics workshop aims to provide training on Two Dimensional Electrophoresis (2-DE) and Difference in-gel electrophoresis (DIGE). Since gel-based proteomics also requires protein identification using mass spectrometry, we have also included a short hands-on session on MALDI-TOF/TOF analysis. Further, data analysis, data interpretation and downstream analysis of samples will also be covered in this workshop.

Mass Spectrometry (MS) is one of the central analytical techniques for protein research. The last decade has seen immense progress in proteomics research, which has been a result of both, advances in existing mass spectrometers and of entirely novel concepts and novel combinations of existing hybrid instruments. Although Gel-based and Mass Spectrometry-based Proteomics aids in showing trends of relative proteins, but by using isotopic and isobaric tags, it is possible to determine the relative abundance of proteins more accurately. Quantitative proteomic techniques like iTRAQ, TMT and SILAC have become very popular off late and have several advantages in comparison to traditional proteomics techniques. The mass spectrometry workshop is structured to provide the participants with a basic knowledge of the principles involved in various quantitative proteomics platforms, hands on training on iTRAQ labeling, off-gel fractionation, sample cleanup for LC-MS, Peptide mass fingerprinting analysis, MS/MS ion search and quantitative proteomics data analysis.

Apart from the discovery and quantitative proteomics, targeted proteomics is emerging as a promising tool for proteomics researchers with interest in quantifying specific proteins in complex mixtures. This workshop would primarily focus on Single Reaction Monitoring (SRM), Parallel Reaction Monitoring (PRM), Absolute Protein

Quantification, platforms like Skyline, experimental design and quality control, data analysis and downstream processing of data. Participants will get training in Skyline software, which has become the software standard in targeted proteomics and an essential tool in bridging between discovery and targeted proteomics. Additionally, hands-on sessions on MS will be conducted briefly to demonstrate the experimental data generated from targeted proteomics experiments.

Proteins are the actual effector molecules that carry out majority of the functions in the cell and most drug targets are present at protein level. Therefore, proteomic studies are extremely important for better understanding of physiology of living systems. Protein microarray platform comprised of thousands of discrete proteins and allow multiplex proteins analysis in a single experiment and is a promising tool to understand the complex biological processes. The microarray workshop broadly overviews various platforms like genomic, protein and tissue microarrays with more emphasis on the experimental set-up of protein microarrays with cell free expression based platforms like Nucleic Acid Protein Programmable Array (NAPPA) as an example. Data processing and analysis in addition to pathway analysis tools would be discussed in detail.

Label-free detection technologies have been used for detection, monitoring and characterization of biomolecular interactions. Surface plasmon resonance (SPR), Bio-layer interferometry (BLI) and Nanomaterial-based techniques have overcome the drawbacks of interference caused by the tagging in labeling techniques. The molecule under study being "label-free" restricts any modulation in its confirmation, maintaining its native structure. This module would emphasize how label-free platforms could be employed to study protein-protein and protein-small molecule interactions. The basic principles along the intricacies of experimental set up with hands-on training along with data analysis would be accomplished in this session.

Biosimilar medicines are now becoming a reality globally and there exists an incredible opportunity for the biopharmaceutical sector to capitalize on what is set to become the fastest growing sector of pharmaceutical industry. Several proteomic tools have offered powerful solutions to challenges in biosimilar characterization. The focused hands-on workshops on Quantitative and Targeted proteomics, Label-free Interaction biosensors and Gel and biochromatography-based tools for Biosimilar Characterization have been scheduled in this workshop to provide an overview of the various solutions that proteomics can offer to the Biopharmaceutical Industry.

I had an opportunity to teach 3 proteomics courses in Cold Spring Harbor Laboratory (CSHL), New York during 2007 and 2008. This experience was very rewarding and highly motivated me to schedule trainings and hands-on workshops in India. I would like to mention that we have established an excellent infrastructure for quantitative and targeted proteomics at IIT Bombay, which includes different instrumentations and software for gel-based proteomics, MS-based, Microarrays and Label-free detection technologies. We have both expertise and infrastructure for conducting these parallel training programs on multiple high-throughput proteomics technologies, which are extremely promising for translational proteomics research.

These hands-on workshops will be moderated by distinguished scientists and experts from academia and industry. I am grateful to all the moderators, instructors, industry experts and TAs who have worked very hard to design these workshops. These endeavors are aimed to train the young researchers in cutting-edge proteomic technologies and we are very optimistic that the participants will have a very stimulating experience.

Sincerely yours,
Dr. Sanjeeva Srivastava
Convener, 6th PSI Meeting & International Conference
IIT Bombay, Mumbai

Development of Different e-Learning Proteomics Resources in India

Sandipan Ray and Sanjeeva Srivastava*

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India

*Correspondence: Dr. Sanjeeva Srivastava, Department of Biosciences and Bioengineering, IIT Bombay, Mumbai 400 076, India; E-mail: sanjeeva@iitb.ac.in

Distance education and E-/open learning endeavours are certainly advantageous for the resource-limited developing countries, where the numbers of potential learners are much higher than the number of well-experienced teachers and educational institutes capable of providing the required infrastructures for basic and advanced scientific education. As a result, to accomplish the rising stipulate, internet-based teaching programmes; specifically massive open online courses (MOOCs) are coming forward, and providing effective platforms alternative to conventional class-room teaching. E-learning and virtual labs are rapidly changing the culture of education in developing countries [1]. We would also like to mention that in recent years; India is playing an increasingly significant role in development of high quality, open source web-based educational materials organized as scientific courses for global distribution. In 2012 the Ministry of Human Resource Development (MHRD), Government of India launched a comprehensive Virtual Lab Project, which is a collection of 91 virtual laboratories containing hundreds of experiments in nine disciplines of science and engineering.

In recent years, proteomics and related disciplines have been incorporated as imperative parts of academic course curriculums across the globe due to its increasing impact on clinical and industrial research. Since development of various expensive and sophisticated proteomics facilities in academic settings remains challenging due to the shrinking educational budgets, primarily in the low and middle income countries; various e-Learning and open-learning programs are gaining popularity. To this end, our research group at the Indian Institute of Technology Bombay has developed Static Virtual Proteomics Lab and Clinical Proteomics Remote Triggered Virtual Laboratories, and other related e-Learning resources for proteomics with an ambition to disseminate high-quality educational contents. It is the opening initiative from a developing country for the establishment of a web-based learning platform in proteomics and best to our

knowledge; hitherto there is no existing virtual lab dedicated exclusively to online proteomics education. Our Static Virtual Proteomics Lab (<http://iitb.vlab.co.in/?sub=41&brch=118>) endeavor has been included as a tutorial article under the International Proteomics Tutorial Programme (IPTP 14) conducted by HUPO and EuPA [2]. It contains three modules; an overview of gel-based proteomics (module I), MALDI-TOF MS (module II) and bioinformatics (module III), each with their own set of experiments [3]. After getting immense success in development of a static Virtual Proteomics Lab, as a continuation we have established a Clinical Proteomics Remote Triggered Virtual Laboratory (<http://iitb.vlab.co.in/?sub=41&brch=237>) to create a highly realistic virtual environment that can provide the learners a firsthand experience of performing different proteomic technologies commonly used in clinical proteomics research. Additionally, in separate web-based courses; National Programme on Technology Enhanced Learning (NPTEL) and Open Source Courseware Animations Repository (OSCAR) we have provided basic working principles and comprehensive details of different advanced proteomics technologies including SILAC, iTRAQ, ICAT, protein microarrays, SPR, and nanoproteomics approaches using videos, animations and interactive simulations. We are expecting that these new e-Learning resources in proteomics will serve as extremely valuable platforms for students and researchers from different disciplines of proteomics at a global level.

References

- [1] Srivastava S, Özdemir V, Ray S, Panga JR, Noronha S, et al. Online education: E-learning booster in developing world. *Nature* 2013; 501(7467): 316.
- [2] Ray S, Koshy NR, Reddy PJ, Srivastava S. Virtual Labs in Proteomics: New E-Learning Tools. *J Proteomics* 2012; 75: 2515-25.
- [3] Ray S, Koshy NR, Diwakar S, Nair B, Srivastava S. Sakshat Labs: India's virtual proteomics initiative. *PLoS Biol* 2012; 10(7): e1001353.



Amersham™ Western Blotting system

The new standard in Western blotting

The fully integrated Amersham WB system is designed to provide you with consistent, quantifiable data every sample, every time by minimizing assay variability. Normalization of the data, combined with a standardized and monitored process every step of the way enable you to get more out of each blot. With less repeat experiments and controls you will feel confident that you have the results to move forward.

Visit www.gelifesciences.com/artofwesternblotting for more information.

GE works.



Standardized for reproducibility

- Fully integrated system
- High quality images
- Optimized reagents
- Optimized protocols
- Automated data analysis

...and all monitored remotely through a Smart App

Quantitative

- Total protein normalization
- Fluorescent multiplexing
- Integrated laser scanner

To know more, write in to supportdesk.india@ge.com

PRINCIPAL SPONSOR

Amersham | Biacore | AKTA | Whatman | Cytell | Xuri

GE and GE monogram are trademarks of General Electric Company.
Amersham, Biacore, AKTA, Whatman, Cytell and Xuri are trademarks of General Electric Company or one of its subsidiaries.
© 2014 General Electric Company - All right reserved.
29-1175-30 AA 08/2014

GEL-BASED PROTEOMICS WORKSHOP

Venue: Seminar Room 3, Ground Floor, VMCC and CRNTS, IIT Bombay

Day 1 (10th Dec, 2014): Classical 2DE and MALDI TOF/TOF MS Analysis

Instructors

Dr. Jens R. Coorssen (*University of Western Sydney, Australia*)

Dr. Sixue Chen (*University of Florida, USA*)

Dr. Rukmini Govekar (*Advanced Centre for Treatment, Research and Education in Cancer, India*)

IITB Coordinator

Dr. Sandipan Ray (*Indian Institute of Technology Bombay, India*) [sandipaniitbombay@gmail.com]

9:00 – 9:15 AM	Overview of gel-based proteomics
Lab session	Dr. Sandipan Ray <i>Indian Institute of Technology Bombay, India</i>
9:15 – 10:15 AM	Overview of 2DE work-flow Rehydration of IPG strips by the participants Isoelectric focusing (1 st Dimension) by the participants (from already rehydrated strips)
10:15 – 10:30 AM	Tea Break
10:30 AM – 12:00	MALDI TOF/TOF analysis for identification of proteins [Lecture & live demonstration]
12:00 – 1:00 PM	Analysis of 2D gels for identification of differentially expressed proteins
1:00 – 2:00 PM	Lunch Break
2:00 - 2:45 PM	Quantitative top-down proteomics: A high resolution 2D gel-based approach for the analysis of biomolecular mechanisms Dr. Jens R. Coorssen <i>University of Western Sydney, Australia</i>
2:45 – 3:30 PM	2-DE workflow, reagent requirement and sample preparation guidance for gel-based proteomics Speaker Dr. Sandipan Ray <i>Indian Institute of Technology Bombay, India</i>
3:30 – 4:00 PM	Application of gel-based proteomics to study cancer pathobiology Speaker Dr. Rukmini Govekar <i>Advanced Centre for Treatment, Research and Education in Cancer, India</i>
4:00 – 4:30 PM	Application of gel-based proteomics to study plant stress responses Speaker Dr. Sixue Chen <i>University of Florida, USA</i>
4:30 – 4:45 PM	Tea Break
4:45 - 6:45 PM	Equilibration and SDS-PAGE separation (2nd Dimension) of proteins
6:45 - 7:30 PM	Staining and scanning of 2D gels

Day 2 (11th Dec, 2014): Difference gel electrophoresis (DIGE)

8:15 - 9:30 AM	Cy-Dye labeling of protein samples for differential proteomics analysis
9:30 - 10:30 AM	SDS-PAGE separation of Cy-Dye labeled samples
10:15 - 10:45 AM	Tea Break

JPP 4

10:45 - 12:15 PM	Scanning and image acquisition of DIGE gels - group of 10 people at a time (CRNTS)- Interaction with instructors and discussion
12:15 - 12:30 PM	Change of venue
12:30 - 1:00 PM	Quiz and Assignment 1 (2-DE and MALDI TOF-MS)
1:00 - 2:00 PM	Lunch Break
2:00 - 3:00 PM	2D-DIGE data analysis
3:00 - 3:30 PM	Quiz and Assignment 2 (2D-DIGE)
3:30 - 4:00 PM	Mixed mode separation of proteins and antibodies in biopharmaceutical purification process
Speaker	<i>Mr. K Y V Ramana</i> <i>Pall Life Sciences</i>
4:00 - 4:15 PM	Tea Break
4:15 - 5:00 PM	Chromatographic kinetic fingerprint measurement of protein digestion using monolithic trypsin column
Speaker	<i>Dr. Aleš Štrancar</i> <i>BIA Separations</i>
5:00 - 5:30 PM	Question-answer session & concluding remarks

INTRODUCED AT ASMS 2013

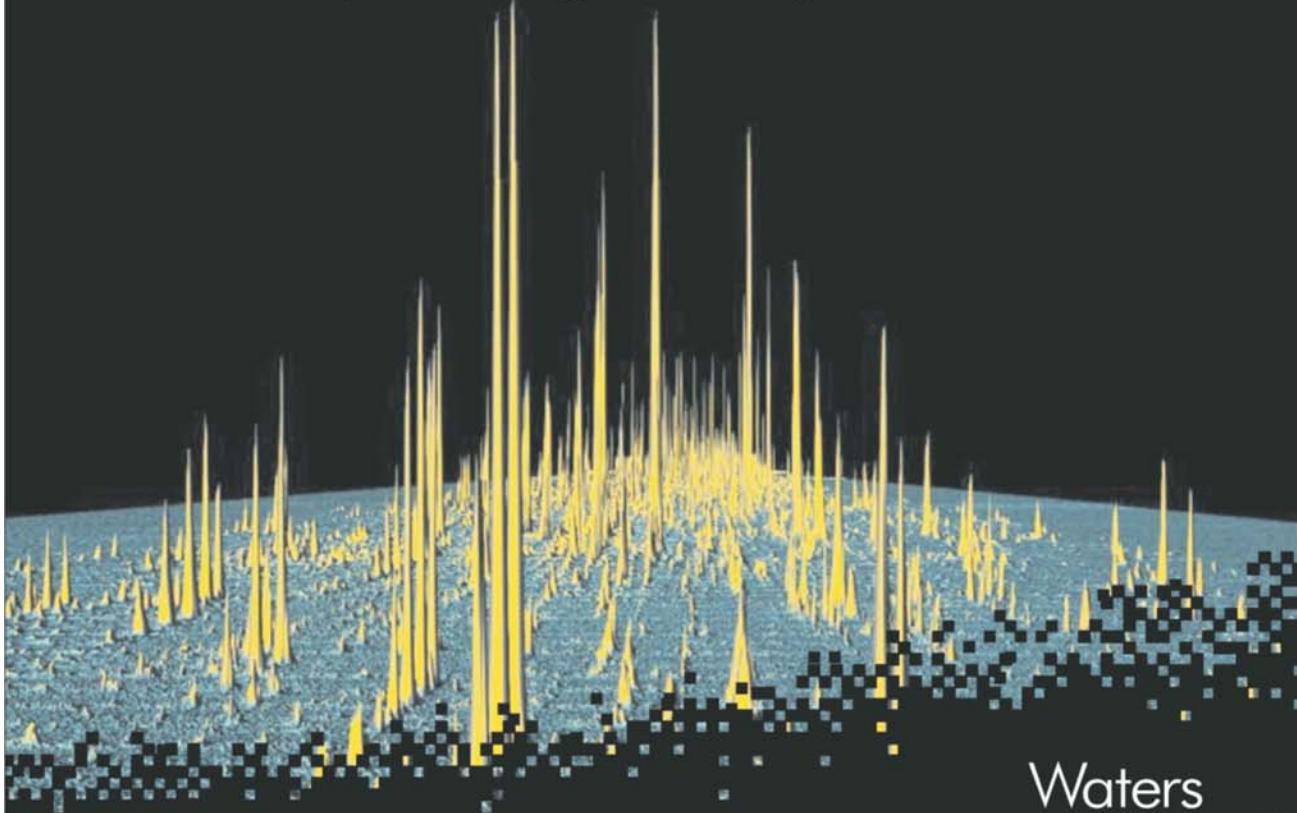
waters.com

THERE ARE
**THREE DIMENSIONS
 OF RESOLUTION.**

SYNAPT® G2-Si IS THE ONLY WAY TO SEE THEM ALL.

CHROMATOGRAPHY. MOBILITY. MASS. Sometimes mass resolution and chromatography are not enough.

With high-efficiency T-Wave™ ion mobility solutions, you can routinely access an additional dimension of separation, based on molecular size and shape. That's the power of molecular collision cross section and SYNAPT HDMS,™ delivering significant enhancements in peak capacity, specificity, sensitivity and structural insight. The proof is undeniable.

See the proof behind the technology that redefines resolution, visit waters.com/SYNAPT

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters (India) Pvt. Ltd.
 36A, 2nd Phase, Peenya Industrial Area, Bangalore - 560 058. Tel: 080-49292200-03 (3 lines)
 Fax: 080-49292204. E-mail: Waters_India@waters.com

Mumbai : Tel : 022-25170127, Fax : 022-25170616, E-mail : Office_Mumbai@waters.com
 New Delhi : Tel : 011 - 40194100 - 4149, Fax : 011 - 40194115, E-mail: Office_Newdelhi@waters.com
 Kolkata : Tel : 033-22828338, Fax: 033-22828384 , E-mail: Office_Calcutta@waters.com
 Hyderabad : Tel : 040-27901532, Telefax : 040-27901586, E-mail: Office_Hyderabad@waters.com
 Chandigarh : Tel : 0172-2623149, Telefax : 0172-2623145, E-mail: Office_Chandigarh@waters.com
 Ahmedabad : Tel : 079-26930122, Fax : 079-2693246, E-mail: Office_Ahmedabad@waters.com

PRINCIPAL SPONSOR

Pharmaceutical & Life Sciences | Food | Environmental | Clinical | Chemical Materials

©2013 Waters Corporation. Waters, SYNAPT, T-Wave, HDMS and The Science of What's Possible are trademarks of Waters Corporation.

Agilent Technologies is proud to be partner of 6th Proteomics Society Of India
Conference @ IIT Bombay, Mumbai

One Stop Solution for OMICs

ATTAIN SUPERIOR

MASS SPEC PERFORMANCE

Confidence means attaining superior analytical performance, 24/7 reliability, maximum uptime and productivity—everything you expect from the leader in mass spectrometry. Across your MS platforms, Agilent provides one single, consistent user interface. After 40+ years of innovation and experience with the industry's largest installed base, Agilent's best-in-class portfolio continues to push the boundaries of MS technology, delivering market-leading solutions.

Learn more about Agilent mass spec technology at:
www.agilent.com/chem/MSperformance

The Measure of Confidence



Scan the QR code for
more information about
Agilent's LC/MS,
GC/MS, ICP-MS solutions
and MassHunter Software



© Agilent Technologies, Inc. 2013

PRINCIPAL SPONSOR



Agilent Technologies

MASS SPECTROMETRY WORKSHOP

Venue: Seminar Room 13, First Floor, VMCC and CRNTS, IIT Bombay

Instructors

- Dr. Philip C. Andrews** (*University of Michigan, USA*)
- Dr. David C. Muddiman** (*University of North Carolina, USA*)
- Dr. Rapole Srikanth** (*National Centre for Cell Science, India*)
- Dr. Mayuri N. Gandhi** (*Indian Institute of Technology Bombay, India*)
- IITB coordinator**
- Mr. Kishore Gollapalli** (*kishore.aprjc@gmail.com*)

Day 1 (10th Dec, 2014): Quantitative proteomic analysis

8:30 – 09:00 AM	Quantitative proteomic analysis using SILAC
Speaker	Dr. Harsha Gowda <i>Institute of Bioinformatics, India</i>
09:00 – 10:00 AM	Quantitative proteomic analysis using iTRAQ (iTRAQ tutorial)
Lab Session	Dr. Rapole Srikanth <i>National Centre for Cell Science, India</i>
10:00 – 10:15 AM	Tea Break
10:15 – 12:00 PM	Off-gel fractionation hands on session
Lab Session	
12:00 –12:30 PM	Desalting and concentrating peptides
Lab Session	
12:30 –01:00 PM	Quantitative mass spectrometry imaging (QMSI) by a novel IR-MALDESI approach
Speaker	Dr. David C. Muddiman <i>University of North Carolina, USA</i>
01:00 –02:00 PM	Lunch Break
02:00 –07:40 PM	Experimental modules (I-IV)
04:40 –05:00 PM	Tea break
Module 1	Conceptual guide to mass-spectrometry based protein discovery Mr. Ashish Pargaonkar/ Dr. Ravi Krovidi <i>Agilent Technologies</i>
02:00 – 03:20 PM	Group-I (Venue: 110B & CRNTS seminar room-1)
03:20 – 04:40 PM	Group-II (Venue: 110B & CRNTS seminar room-1)
Module 2	Label-free expression/quantitative proteomics using HRMS Dr. Prasanna Rajagopal/ Dr. Rajiv Bharadwaj <i>Waters</i>
02:00 – 03:20 PM	Group-II (Venue: CRNTS seminar room-2)
03:20 – 04:40 PM	Group-I (Venue: CRNTS seminar room-2)
Module 3	TMT multiplexing quantitative proteomics using high resolution mass spectrometry Mr. Santosh Renuse <i>Thermo Fisher Scientific</i>
05:00 – 06:20 PM	Group-I (Venue: CRNTS seminar room-1)
06:20 – 07:40 PM	Group-II (Venue: CRNTS seminar room-1)

Module 4	Application of capillary electrophoresis and triple TOF MS in quantitative proteomics
	Dr. Dipankar Malakar/ Dr. Annu Uppal <i>AB SCIEX</i>
05:00 – 06:20 PM	Group-II (Venue: CRNTS seminar room-2)
06:20 – 07:40 PM	Group-I (Venue: CRNTS seminar room-2)

Day 2 (11th Dec, 2014): Mass spectrometry data analysis and interpretation

08:30 – 09:15 AM	Advancements in the field of mass spectrometry
Speaker	Dr. Philip C. Andrews <i>University of Michigan, USA</i>
09:15 – 10:00 AM	Complementarity of label-free and labeled quantitative proteomics approaches
Speaker	Dr. Catherine E. Costello <i>Boston University School of Medicine, USA</i>
10:00 – 10:15 AM	Tea Break
10:15 – 10:45 PM	Protein identification using MASCOT search engine
Lab Session	
10:45 – 12:00 PM	Quantitative proteomics data analysis
Lab Session	
12:00 – 01:00 PM	Quiz and Assignments
01:00 – 02:00 PM	Lunch Break
02:00 – 03:30 PM	High throughput MALDI-imaging for novel biomarkers: Clinical perspectives
Lab Session	
Speaker	Dr. Sivaramaiah Nallapeta <i>Bruker Daltonics</i>
03:30 – 04:00 PM	Sequence analysis using MALDI-TOF/TOF
Lab Session	Dr. Sivaramaiah Nallapeta <i>Bruker Daltonics</i>
04:00 – 04:30 PM	SILAC based quantitative proteomic analysis
Lab Session	Santosh Renuse <i>Thermo Fisher Scientific</i>
04:30 – 05:00 PM	Targeted phosphoproteomics analysis of immunoaffinity enriched tyrosine phosphorylation in mouse tissues
	Dr. Ravi Krovidi <i>Agilent Technologies</i>
05:00 – 05:15 PM	Tea Break
05:15 – 06:15 PM	Chromatographic kinetic fingerprint measurement of protein digestion using monolithic trypsin column
Lab Session	<i>BIA Separations</i>
06:15 – 06:45 PM	Discussion & concluding remarks



An ISO 13485 : 2003 & ISO 9001 : 2008 certified and FDA (I) licensed unit

Your Perfect Partner for Antigens & Antibodies.

Global SUPPLIER for Worlds' IVD Companies and Leading Bulk Distributors and Resellers for:

► *Human Native antigens*

► *Recombinant antigens*

► *Monoclonal antibodies*

► *Calibrator and controls*



PLATINUM SPONSOR

www.yashraj.com

"What I do with my Octet HTX time? Ride."

Epitope binning studies in days, not weeks.

Are your antibody epitope binning experiments just getting bigger and bigger? The new Octet HTX system lets you get them done in days, not weeks. Start a 32 x 32 cross-competition matrix in the morning, then analyze your data with a few mouse clicks before you leave the lab for the day. Can your ELISA or SPR system do that?

Lauren gets out of the lab more often now to ride. What will you do with your extra time?



fortéBIO
A Division of Pall Life Sciences

fortebio.com | pallindiamarketing@pall.com

PALL Life Sciences

PLATINUM SPONSOR

Fast. Accurate. EASY.

PROTEIN MICROARRAY WORKSHOP

Venue: Seminar Room 14, First Floor, VMCC and BSBE, IIT Bombay

Day 1 (10th Dec, 2014): (i) Auto-antibody profiling (ii) Genomic microarray analysis

Instructors

Dr. Manuel Fuentes (*University of Salamanca, Spain*)

Dr. Santosh Noronha (*Indian Institute of Technology Bombay, India*)

Dr. Sudha Rao (*Genotypic Technology, India*)

Dr. Sanjay Navani (*Lab Surgpath, HPR project, Uppsala University*)

IIT Coordinator

Rekha Jain (*Email ID: rekha.jain.iitb@gmail.com*)

8:00 - 8:30 AM	An overview of the auto-antibody profiling experiment Labwork - Blocking of protein array chip Speaker: Rekha Jain <i>Indian Institute of Technology Bombay, India</i>
8:30 - 9:30 AM	High throughput analysis: Microarrays for DNA and proteins Speaker: Dr. Sudha Rao <i>Genotypic Technology, India</i>
9:30 - 10:15 AM	Washing and serum incubation (2 hr) Lab Session
10:15 - 10:30 AM	Coffee Break
10:30 - 11:30 AM	DNA Microarray-A powerful tool for biomedical Research Speaker: Dr. Anuj Gupta <i>Agilent Technologies, Genomics</i>
12:15 AM - 1:00	Washing and Secondary antibody incubation Lab Session
1:00 - 2:00 PM	Lunch Break
2:00 - 2:45 PM	Washing and drying of slides Lab Session
2:45 - 3:00 PM	Change of Venue
3:00 - 5:00 PM	Slide Scanning, printing for Group I and II and tutorial for Group III and IV Lab Session
5:00 - 5:15 PM	Tea Break
5:15 - 7:15 PM	Slide Scanning, printing for Group III and IV and tutorial for Group I and II Lab Session

Day 2 (11th Dec, 2014): (i) Data analysis (ii) *In vitro* transcription-translation (IVTT)

8:45 - 9:45 AM	Tips and troubleshooting for building protein array and an overview of NAPPA microarray Speaker: Dr. Manuel Fuentes <i>University of Salamanca, Spain</i>
----------------	--

JPP 12

9:45 - 10:15 AM	Preparation of NAPPA mix
	Lab Session
10:15 - 10:30 AM	Tea Break
10:30 - 11:15 AM	Demonstration of microarray data analysis (preliminary; creating GPR files) and tutorial on data acquisition and processing
	Speaker Mr. Narendra Goud <i>Indian Institute of Technology Bombay, India</i>
11:15 - 12:00 PM	Microarray statistical data analysis
	Speaker Dr. Santosh Noronha <i>Indian Institute of Technology Bombay, India</i>
12:00 - 1:00 PM	Assignment
1:00 - 2:00 PM	Lunch Break
2:00 - 2:30 PM	IVTT experiment set up
	Lab Session
2:30 - 3:30 PM	Pathway analysis using Metacore: Tutorial and demonstration
	Speaker Mr. Faiz Abzur <i>Thomos Reuters, India</i>
4:00 - 4:30 PM	IVTT results demonstration
	Lab Session
4:30 - 4:45 PM	Tea Break
4:45 - 5:45 PM	Applications of tissue microarray
	Speaker Dr. Sanjay Navani <i>Lab Surgpath, HPR project, Uppsala University</i>
5:45 - 6:30 PM	Interactive session with speakers
6:30 – 7:00 PM	Concluding talk

ANSWERS FOR SCIENCE. KNOWLEDGE FOR LIFE.

Nothing hides from
SWATH™

PROTEINS AND PEPTIDES BEWARE



SWATH™ Acquisition 2.0 with variable windows is here.

The new **AB SCIEX TripleTOF® 6600 System with SWATH™ 2.0** captures virtually every detectable peptide and protein in every run, with MRM-quality quantitation and sample-to-sample reproducibility that accelerate discovery.

Maximize sample information for a more complete view of the proteome. Quantify thousands of proteins across hundreds of samples with almost no method development. Archive a digital record of every proteome you can re-interrogate at any time. Only the TripleTOF® 6600 combines increased dynamic range with the high speed, sensitivity and resolution to enable SWATH™ 2.0, unlocking the full power of data independent acquisition.

The next-generation proteomics platform has arrived.
To learn more, visit absciex.com/swath+6600

PLATINUM SPONSOR AB SCIEX

Thermo
SCIENTIFIC

A Thermo Fisher Scientific Brand

More Proteins. More accurately. Faster than ever.

Biology is complex and understanding it is a big challenge. Identify and quantify more proteins and complexities such as PTMs faster and more accurately with our new portfolio of LC-MS instruments, sample prep solutions and software. HRAM solutions using Thermo Scientific™ Orbitrap™ MS quantifies all detectable proteins and peptides with high specificity and fewer false positives, while triple quadrupole MS delivers SRM sensitivity and speed to detect targeted proteins more quickly. Join us in meeting today's challenges. Together we'll transform proteomics.

Quantitation transformed.

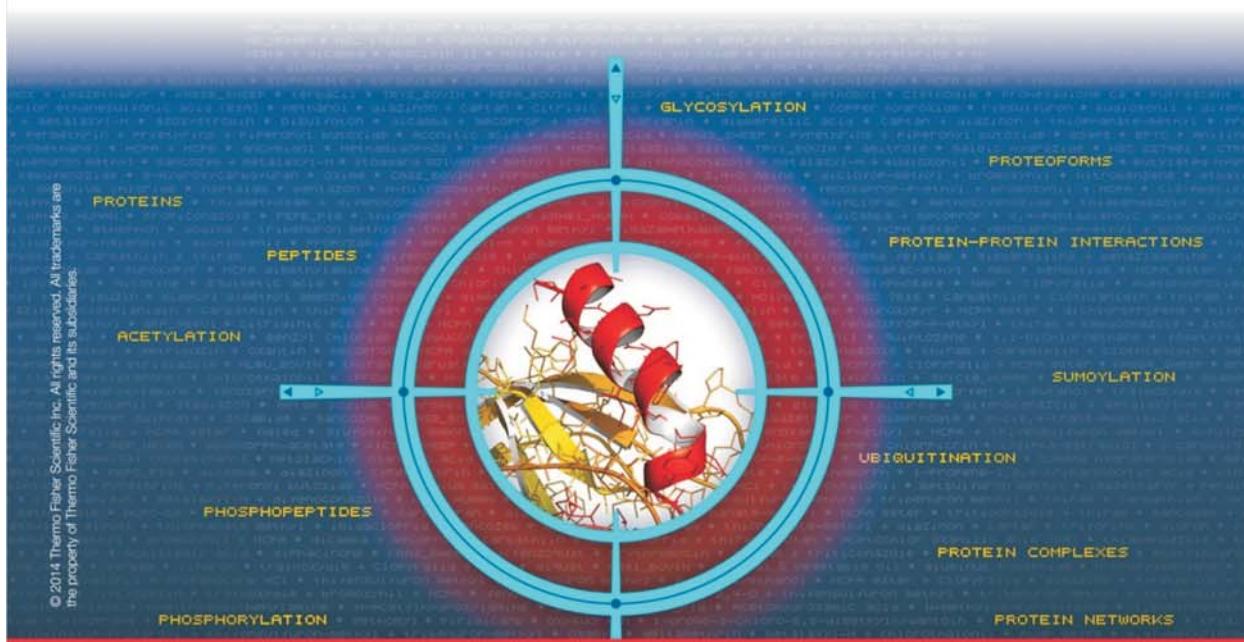
- Discover more at thermoscientific.com/quan-transformed

For more information, please contact:

Thermo Fisher Scientific India Pvt. Ltd.

102,104, C Wing, Delphi, Hiranandani Business Park | Powai, Mumbai 400076 India

Tel: +91-22-6742 9494 Email ID: analyze.in@thermofisher.com



Thermo Scientific™ Orbitrap Fusion™ MS
Unprecedented depth of analysis and throughput for biological discovery



Thermo Scientific™ Q Exactive™ HF MS
Screen and quantify known and unknown targets with HRAM Orbitrap technology



Thermo Scientific™ TSQ Quantiva™ MS
Leading SRM sensitivity and speed in a triple quadrupole MS/MS

PLATINUM SPONSOR

Label-free Proteomics Workshop

Venue: Seminar Room 5, Ground Floor, VMCC, IIT Bombay

Day 1: 10th December

Instructors: Dr. Ed Nice (*Monash University, Australia*), Dr. Madhurarekha (*Sandor Proteomics Pvt. Ltd., India*), Dr. Sharmistha Dey (*All India Institute of Medical Sciences, New Delhi, India*), IITB Coordinator - Dr. Veenita Shah, Indian Institute of Technology (IIT), Bombay, India

8:45 - 9:30 AM	Plenary Lecture An introduction to SPR technologies and their application
Speaker	Dr. Ed Nice <i>Monash University, Australia</i>
9:30 - 10:00 AM	Invited Lecture-1 Assay design and reagents for application development for SPR studies
Speaker	Dr. Anette Persson <i>GE Healthcare Life Sciences, Sweden</i>
10:00 - 10:30 AM	Invited Lecture-2 Mechanistic and structural insights from SPR/BLI studies of protein: ligand interactions
Speaker	Dr. R. Varadarajan <i>Indian Institute of Science, Bangalore</i>
10:30 - 01:00 PM	Binding and kinetics assay to study the interaction between β 2-microglobulin and anti- β 2-microglobulin using SPR (Group I)
10:30 - 01:00 PM	Characterization of PSA-Anti PSA interactions using bio-layer interferometry (Group II)
10:30 - 01:00 PM	Kinetics of different β -lactamase mutants with α -lactamase inhibitor protein (Group III)
1:00 - 02:00 PM	Lunch Break
02:00 - 02:30 PM	Invited Lecture-3 Application of SPR in antibody screening and characterization
Speaker	Dr. Rajesh Saha <i>Bio Rad Laboratories (India) Pvt Ltd</i>
02:30- 05:00 PM	Binding assay to study the interaction between α 2-microglobulin and anti- β 2-microglobulin using SPR (Group II)
02:30 - 05:00 PM	Characterization of PSA-Anti PSA interactions using bio-layer interferometry (Group III)
02:30 - 05:00 PM	Kinetics of different β -lactamase mutants with β -lactamase inhibitor protein (Group I)
05:00 - 07:30 PM	Binding assay to study the interaction between β 2-microglobulin and anti- β 2-microglobulin using SPR (Group III)
05:00 - 07:30 PM	Characterization of PSA-Anti PSA interactions using bio-layer interferometry (Group I)
05:00 - 07:30 PM	Kinetics of different β -lactamase mutants with β -lactamase inhibitor protein (Group II)

Day 2: 11th December

Instructors: **Dr. Ed Nice (Monash University, Australia), Dr. Madhurarekha (Sandor Proteomics Pvt. Ltd., India), Dr. Sharmistha Dey (All India Institute of Medical Sciences, New Delhi, India), Dr. Veenita Shah, Indian Institute of Technology (IIT), Bombay, India**

09:00 - 09:30 AM	Kinetics assay for β 2M and anti- β 2M interaction using SPR
09:30 - 11:30 AM	Binding and kinetics assay data analysis and interpretation
11:30 - 11:45 AM	Tea Break
11:45 - 01:00 PM	Participants' evaluation (Quizzes/Assignments)
01:00 - 02:00 PM	Lunch Break
02:00 - 02:30 PM	Invited Lecture-1 Promising serum protein marker for early detection of Alzheimer's disease
Speaker	Dr. Sharmistha Dey <i>All India Institute of Medical Sciences, New Delhi, India</i>
02:30 -03:00 PM	Invited Lecture-2 Label-free interaction systems for routine academic research
Speaker	Dr. Sriram Kumarswamy <i>Pall ForteBio, USA</i>
03:00 – 03:30 PM	Invited Lecture-3 Label-free interaction applications using SPR
Speaker	Dr. Madhurarekha <i>Sandor Proteomics Pvt. Ltd., India</i>
03:30 - 04:00 PM	Invited Lecture-4 Development of binding kinetics assay using a simple dip & read BLI-based octet platform
Speaker	Dr. Vishal Kamat <i>Regeneron Pharmaceuticals, USA</i>
04:00 – 05:00 PM	Discussion on data analysis & troubleshooting
05:00 - 05:30 PM	Certificate Distribution



Productivity and Versatility – all day, every day

Your Success with impact II UHR-QTOF system

- **High Dynamic Range** – 5 orders of magnitude gives you deepest insight into your sample
- **One-shot plug & play acquisition** – Ensuring qualitative and quantitative results in one LC run with fastest time-to-success
- Spectral accuracy with **Instant Expertise™** from small molecules up to intact proteins
- **Isotopic fidelity** for definitive molecular formulae determination

For more information please visit www.bruker.com

For research use only. Not for use in diagnostic procedures.

Innovation with Integrity

DIAMOND SPONSOR

LC-MS/MS

Bruker Daltonics India Pvt. Ltd.

L-15/500, Mahipalpur Extension,
New Delhi - 110037, India
Tel: +91-11-26786141 · Fax: +91-11-26786141
www.bruker.com

IMAGING // CHEMIDOC™ TOUCH IMAGING SYSTEM

Introducing the New ChemiDoc™ Touch

ChemiDoc™ Touch Imaging System



Sensitivity in detection,
power in quantitation.

Simple, precise, and flexible, the **ChemiDoc™ Touch Imaging System** is a complete solution for gel and western blot imaging.

- Equal to film in sensitivity and resolution
- Superior to film in signal-to-noise ratio and linear dynamic range
- Highly intuitive software and interface optimise data gathering

With this seamless integration of high-quality imaging and quantitative tools, the path from experiment to usable data has never been so clear or streamlined.

Research. Together.

To find your local sales office, visit www.bio-rad.com/contact/
In India, call us directly at +91 124 4029 300

Visit us at www.bio-rad.com

BIO-RAD

DIAMOND SPONSOR

TARGETED PROTEOMICS WORKSHOP

Venue: Seminar Room 13, First Floor, VMCC and CRNTS, IIT Bombay

Day 1 (10th Dec, 2014)

- Instructors:** Mr. Brendan MacLean (*University of Washington, USA*)
Dr. Mahesh J Kulkarni (*National Chemical Laboratory, India*)
Dr. Christina Ludwig (*ETH Zurich, Switzerland*)
Ms. Meena Choi (*Purdue University, USA*)
- IIT Coordinator:** Mr. Panga Jaipal Reddy (jaipalpanga@gmail.com)

8.00-8.10 AM Speaker	Opening remarks Mr. Brendan MacLean <i>University of Washington, USA</i>
8:10-9.10 AM Speaker	Lecture: Moving from discovery to targeted proteomics – focus on SRM Dr. Christina Ludwig <i>ETH Zurich, Switzerland</i>
9:10 – 10:00 AM Speaker	Introduction to Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
10:00 – 10:15 AM	Tea Break
10:15 – 11:30 AM Speaker	Tutorial: Targeted method editing in Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
11:30 – 1:00 PM Speaker	Tutorial: Targeted method refinement in Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
1:00 – 2:00 PM	Lunch break
2:00 – 3:00 PM Speaker	Lecture: Protein quantification using SRM Dr. Christina Ludwig <i>ETH Zurich, Switzerland</i>
3:00 – 4:45 PM Speaker	Tutorial: Data analysis in Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
4.45 -5.00 PM	Tea Break

Hands-on sessions (5:00-7:00 PM)

- Module-I**
- Group-1 (5:00-6:00 PM)** Leap from discovery phase to validation phase: Quantitative proteomics workflows by Dr. Taegen Clary (*Agilent Technologies, USA*)
- Group-2 (6:00-7:00 PM)** Workflow demonstration for quantitative approach using standard protein model/ therapeutic protein by Dr. Ashish Pargaonkar (*Agilent Technologies, India*)

Module-2

Group-2	Analysis of proteins and peptides for targeted proteomics using Progenesis QI Dr. Paul Goulding (<i>Waters India Pvt. Ltd</i>) Progenesis QI for proteomics
Group-1 (6:00-7:00 PM)	Dr. Rajiv Bharadwaj (<i>Waters India Pvt. Ltd</i>)

Day 2 (11th Dec. 2014)

8:00 – 8.30 AM	Lecture: Mass spectrometry and targeted proteomics Dr. Mahesh J Kulkarni <i>National Chemical Laboratory, India</i>
8:30 – 9.30 AM	Lecture: Statistical considerations for experimental design and data analysis Ms. Meena Choi <i>Purdue University, USA</i>
9:30 – 10:30 AM	Lecture: Using MS stats for quality control and fold change assessment Ms. Meena Choi <i>Purdue University, USA</i>
10:30 – 10:45 AM	Tea Break
10:45 – 11:15 AM	Lecture: Overview and keys to success in processing DDA data with Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
11:15 – 1:00 PM	Tutorial: Label-free quantitative analysis of DDA data with Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
1:00 – 2:00 PM	Lunch Break (Targeted proteomics using HR-XIC- Mr. Michael Andersen- <i>Bruker Daltonics</i>)
2:00 – 3:30 PM	Tutorial: Parallel reaction monitoring (PRM) with Skyline Mr. Brendan MacLean <i>University of Washington, USA</i>
3:30 – 4:30 PM	Quiz and Assignment
4:30 – 4:45 PM	Tea Break

Hands-on sessions (4:45-6:45 PM)

Module III

Group-1 (4:45-5:45 PM)	Next Gen Proteomics & Quantitative Biology Dr. Dipankar Malakar (<i>ABSCIEX, India</i>)
Group-2 (5:45-6:45 PM)	SWATH 2.0 & MRM^{HR} workflow demonstration Dr. Annu Uppal & Dr. Dipankar Malakar (<i>ABSCIEX, India</i>)

Module IV

Group-2 (4:45-5:45 PM)	Validation of Macrophage capping protein (CAPG) in synovial fluid of arthritis patients using multiple reaction monitoring Mr. Santosh Renuse (<i>Thermo Fisher, India</i>)
Group-1 (5:45-6:45 PM)	Targeted proteomics workflow demonstration Mr. Sangram Pattanaik (<i>Thermo Fisher, India</i>)

Are you looking for?

commitment
flexibility
speed
know-how
support
care^{quality}
productivity
expertise
dedication
solutions
reproducibility
simplicity
optimization



Enjoy all inclusive
treatment with



sales@biaseparations.com
tel: +386 59 699 500
www.biaseparations.com

DIAMOND SPONSOR

Leaders in Monolith
Chromatography

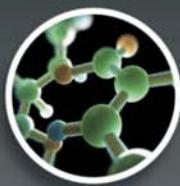


Cambridge Isotope
Laboratories, Inc.
isotope.com



Promochem
A Distributor of CIL Products

Enriching Scientific Discovery



SILAC and SILAM

Heavy-labeled amino acids and MouseExpress® Feed and Tissue for quantitative proteomics tissue analysis



INLIGHT™ Glycan Tagging Kit

For the quantification of N-linked glycans by mass spectrometry



Stable Isotope Dimethyl Labeling

For fast and inexpensive quantitative proteomic labeling by mass spectrometry



Heavy-Labeled MS Protein Standard

Human ApoA-1 and Human IGF-1 for accurate quantitation using a bottom-up proteomic workflow



LGC Promochem India
Private Limited

Model Export Bhavan, 14th Cross, IV Phase
Peenya Industrial Area, Bangalore-560 058, India
Tel: +91-80-6701-2049 | Email: cilsales@lgcpromochem.in
Cell: +91-9480691598 | Web: www.lgcstandards.com

DIAMOND SPONSOR

BIOSIMILAR CHARACTERIZATION WORKSHOP

Venue: VMCC - Seminar Room 11 and CRNTS, IIT Bombay

Day 1 (9th Dec, 2014)

Instructors:	Dr. Robert Moritz; Institute for Systems Biology, USA Dr. Mayuri Gandhi; IIT Bombay Mr. Brendan MacLean; University of Washington, USA Dr. Christina Ludwig; ETH Zurich, Switzerland
IITB Coordinator:	Dr. Prasad Phapale (biosimilarproteomicsintconf@gmail.com)

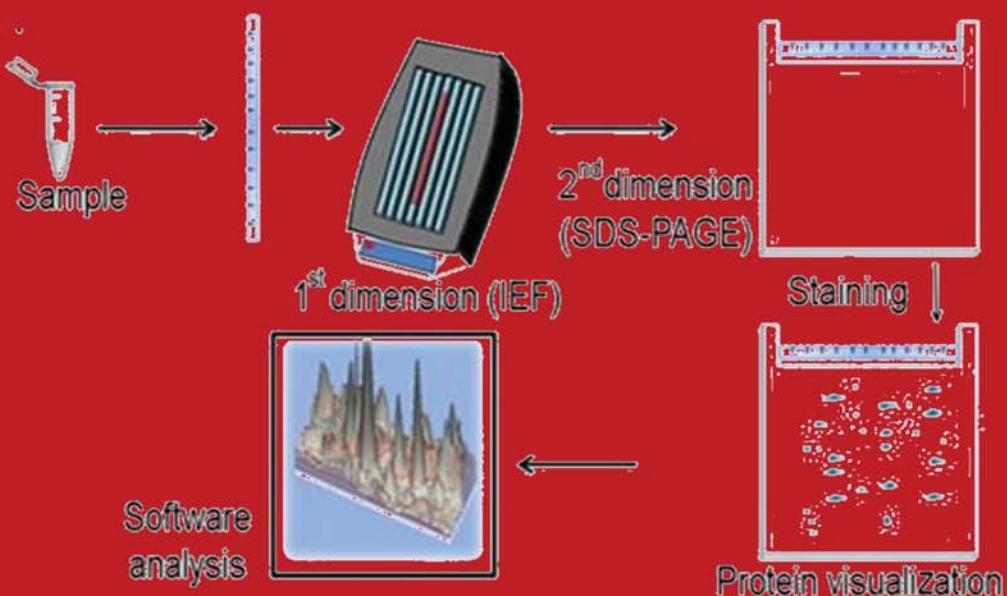
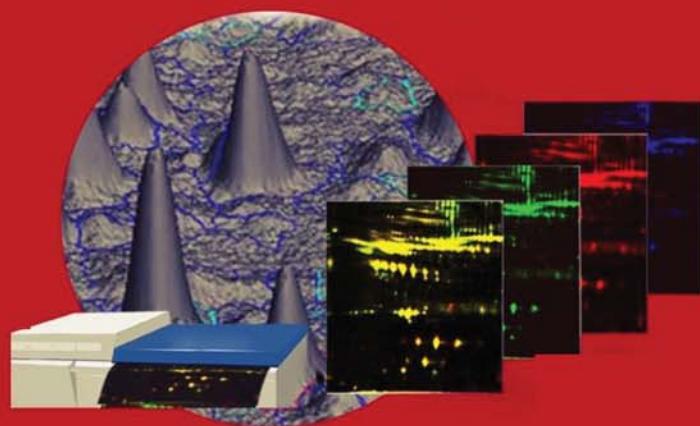
Module 1	Discovery Proteomics for Biosimilar characterization
8:00 – 9:15 AM	Higher order biosimilar characterization using High Resolution MS
Lab session	Taegen Clary and Ashish Pargaonkar <i>Agilent Technologies</i>
Module 2	Biosimilar characterization using Targeted Proteomics
9:15 – 10:30 AM	Approach for peptide quantification and its applicability
Lab session	Taegen Clary, Ashish Paragaonkar and Saurabh Nagpal <i>Agilent Technologies</i>
10:30 – 10:45 AM	Tea Break
Module 3	Biosimilar characterization using HDMS
10:45 – 12:00 PM	The characterization of biopharmaceuticals and biosimilars by on-line LC/MS
Lab Demo	Dr. Rajiv Bharadwaj <i>Waters India Pvt Ltd</i>
Module 4	Biosimilars characterization using high-end capillary electrophoresis (UV, PDA, LIF and CE-ESI-MS)
12:00 – 12:15 PM	Introduction on CE and advances in CE for biosimilars characterization
Speakers	Abel Shalom B, Dr. Prashant Dour and Aalhad Abhyankar <i>ABSciex</i>
12:15 – 12:45 PM	CE instrument hardware and work flow
Lab Demo	Abel Shalom B, Dr. Prashant Dour and Aalhad Abhyankar <i>ABSciex</i>
12:45 to 1:15 PM	Regulatory requirement for biosimilars vs CE contribution for regulatory and monographs
Lab Demo	Abel Shalom B, Dr. Prashant Dour and Dr. Dipankar Malakar <i>ABSciex</i>
1:15 – 2:00 PM	Luncheon Lecture: Biopharmaceutical characterization using MALDI-TOF/TOF
Speaker	Dr. Bob Galvin <i>Bruker Daltonics</i>

Lecture and Demos

2:00 – 2:20 PM	Run Data Display with data analysis and data interpretation (CE-MS)
Speakers	Abel Shalom B, Dr. Prashant Dour <i>ABSciex</i>

2:20 – 2:40PM	Role of ion mobility mass spectrometry for the characterization of biopharmaceuticals
Speaker	Dr. Rajiv Bharadwaj <i>Waters India Pvt Ltd</i>
2:40 – 3:00 PM	Discovery proteomics for biosimilar characterization
Speaker	Dr. Taegen Clary <i>Agilent Technologies, USA</i>
3:00 – 3:20 PM	Demo on Intact mass and top down analysis using MALDI-TOF/TOF
Speaker	Dr. Bob Galvin <i>Bruker Daltonics</i>
3:20 –3:35 PM	Tea break
	Functional characterization of biosimilars
3:35 –5:35 PM	Biosimilar functional characterization: Lab Demo for label-free interaction studies
Lab and Lectures	Dr. Sriram Kumaraswamy and Dr. Veenita Shah <i>Pall Corporation, GE Healthcare and IIT Bombay</i>
5:35 – 7:00 PM	Gel-based & Chromatographic separation technologies
Lab and Lectures	<i>BIA Separations and IIT Bombay</i>

GEL-BASED PROTEOMICS (Module I)



Gel-based Proteomics

Preface

Sandipan Ray

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India

During the first decade of the twenty first century the field of proteomics has witnessed tremendous applications of gel-based proteomics approaches. Even now, gel-based proteomics coupled with mass spectrometry is the major backbone of most published proteomic studies. Gel-based technological approaches are routinely used in proteomics research for separation, characterization as well as quantification of proteins in versatile range of biological samples. Gel-based proteomics includes 1D SDS-PAGE, native PAGE, 2-DE and advanced 2D-DIGE, which are often used in combination with MS-based proteomics for global/differential proteomic analysis. Major challenges associated with gel-based proteomics includes; poor reproducibility, limited sensitivity and dynamic range (10^3 - 10^4), less coverage of complex proteome, low-throughput, biasness in analysis process, long experimental process and high level of dependence on performer's technical skill. The poor reproducibility of classical 2-DE owing to the gel-to-gel variations has been partially resolved by the introduction of DIGE, where the test and control samples are processed in a same gel (Unlu et al., 1997). In recent years, the detection approaches for gel-based proteomic techniques have also improved immensely to detect low-abundance protein biomarkers in different biological fluids. Apart from the traditional CBB or silver staining, more sensitive and superior staining agents reagents (post-electrophoresis Epicoccone fluorescent dyes like Lightning Fast and Deep purple as well as pre electrophoresis fluorescent dyes such as CyDyes have been introduced to increase the dynamic range and coverage in gel-based proteomics (Miller et al., 2006). Activity-based protein profiling using gel-based proteomics through the introduction of enzyme-targeting probes allowed the researchers to get information regarding protein activity apart from relative abundance (Huang et al., 2003).

Owing to the rapid advancement in state-of-the art proteomics technologies, continuous expansion of our scientific understanding, and challenges associated with gel-based proteomics research, it has become essential to keep up with current trends and advances in proteomics and metabolomics research. Economic crunches and perpetually limited educational/research resources often make it difficult for most of the institutes to afford the sophisticated laboratory set-ups required for cutting-edge integrated proteomics research. Even well-funded institutions often limit

their infrastructure to what they require for research purposes, and access to advanced labs is most frequently restricted to authorized research personnel. Additionally, analysis and management of large datasets obtained from the high-throughput proteomics research is also a formidable challenge and requires well-experienced personnel. Consequently, there is an urgent need for dissemination of the knowledge/experience gained by the expertise of these fields to the young researchers and students. Therefore, such training event will be extremely useful for young students and researchers working/planning to conduct research in similar fields.

This workshop majorly aims to equip young researchers with the basic know-hows of the gel-based proteomics platforms that include hands-on training on protein extraction from several biological samples, isoelectric focusing (IEF_1st dimension), 2nd dimension (SDS-PAGE), gel staining, scanning, and analysis, and different steps of advanced 2D-DIGE technology. The workshop also would acquaint the participants with various facets of post gel electrophoresis work-flow like spot identification, land-marking proteins, spot cutting for MALDI (Matrix assisted Laser Desorption Ionization) TOF (time-of-flight) analysis. The participants would also be trained to analyze the outcome of 2-DE and 2D-DIGE gels for differential proteomics analysis. We intend to accommodate approximately 40 selected trainees and provide them comprehensive laboratory and lecture courses with a focus on cutting-edge gel-based proteomic technologies. A group of instructors from different countries having extensive expertise in various basic and advanced gel-based proteomics technologies, data analysis and interpretation, and scientific writing will conduct the course. All in all this would be a blend of practical learning and skill developing platform for anyone who wishes to start venturing into proteomics.

Instructors and Speakers

Dr. Jens R. Coorssen

University of Western, Sydney, Australia



Talk Title: Quantitative top-down proteomics: A high-resolution 2D gel-based approach for the analysis of bimolecular mechanisms

Biography: Jens trained in Canada (BSc, MSc, PhD) with subsequent Fellowships at the Max Planck Institute for Medical Research (Heidelberg) and the National Institutes of Health (USA). Following a NIH Visiting Scientist appointment he was an Associate Professor in the Faculty of Medicine and the Hotchkiss Brain Institute, University of Calgary. In 2008 he was

appointed Foundation Professor of Molecular Physiology at the new School of Medicine, University of Western Sydney. In 2009 he was asked to head the nascent UWS Molecular Medicine Research Group. He is an Honorary Professor in the School of Medicine at the University of Sydney, adjunct Professor in the Faculty of Medicine (University of Calgary) and a Foreign Associate of ICORD / the Blusson Spinal Cord Centre (British Columbia). His group had refined and defined new approaches for high-resolution proteomic analyses using 2D gel electrophoresis.

Dr. Sixue Chen

Proteomics Facility, University of Florida, Gainesville, FL 32611, United States

Talk Title: Application of gel-based proteomics to study plant stress responses



Biography: Dr. Sixue Chen's areas of expertise fall in Biochemistry, Plant Metabolism, Functional Genomics, Proteomics, Metabolomics, and Mass Spectrometry. He learned mass spectrometry when he was collaborating with a chemist at the Danish Royal Veterinary and Agricultural University 16 years ago. Dr. Chen carried out a lot of small molecule work at that time. Since joining University of Pennsylvania in 2001, he has worked on many different projects using proteomics and mass spectrometry. Dr. Chen has accumulated experience with different separation and fractionation technologies (e.g., 2-DE), different HPLC instruments including the nano-flow ultra-performance LC, as well as mass spectrometers. During his tenure as the Proteomics Facility Director at the Danforth Center in Missouri, USA, Dr. Chen has developed a high throughput protein sequencing technology using mass spectrometry. Dr. Chen has successfully administered projects, trained students and scientists, collaborated with other researchers, and produced more than 100 peer-reviewed publications. As a result of the valuable previous experience, Dr. Chen is aware of the importance of collaboration and implementing powerful proteomics technologies in solving challenging scientific problems. At University of Florida, Dr. Chen has established three major research projects; all were funded by the US National Science Foundation. Plant guard cell hormone signaling and glucosinolate metabolism were used as model systems. Based on findings from large-scale "omics" studies, many novel, testable hypotheses have been derived. Another major component of Dr. Chen's research program has been hypothesis driven and testing, i.e., characterizing molecular, biochemical and physiological functions of specific genes and proteins. The integration of hypothesis generation and hypothesis driven research

will ultimately lead to a holistic view of cellular networks and processes in higher organisms.

Dr. Rukmini Govekar

Scientific officer, Advanced Centre for Treatment, Research and Education in Cancer, ACTREC Mumbai, India



Talk Title: Application of gel-based proteomics to study cancer pathobiology

Biography: Dr. Rukmini Govekar has done her PhD from Cancer Research Institute, Mumbai in 1991 in the field of environmental carcinogenesis. She has been the scientific officer at ACTREC since 1994. She is currently working as a Principle Investigator at ACTREC. Rukmini's Lab focuses on understanding the biology of BCR/ABL-induced leukaemia using proteomics approach. Additionally she is in-charge of mass spectrometry facility at ACTREC.

Dr. Sandipan Ray

Proteomics Lab, Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India



Talk Title: 2-DE workflow, reagent requirement and sample preparation guidance for gel-based proteomics

Biography: Sandipan Ray received his M.Sc. degree in Biotechnology from the University of Calcutta, India in 2009. During his PhD career from Indian Institute of Technology Bombay, he has published more than twenty peer-reviewed research articles and reviews in the field of clinical proteomics and emerging proteomics technologies. He is an active member of the American Society of Tropical Medicine and Hygiene (ASTMH), ASTMH Committee on Global Health (ACGH), Human Proteome Organization (HUPO), US-HUPO, Proteomics Society India (PSI) and Society of Biological Chemists (SBC) and many other national and international communities. He is a regular reviewer of OMICS: A Journal of Integrative Biology. His current research interests include proteomic analysis of different tropical infectious diseases and human cancers to decipher disease pathogenesis and identify surrogate protein markers. He is actively involved in the development of Virtual Proteomics Lab and other related E-Learning resources at IIT Bombay. He has participated and presented his research work in many international conferences including Human Proteome Organization (HUPO) 11th Annual World Congress,

Boston, Massachusetts, USA (2012), US HUPO 8th Annual Conference, San Francisco, CA, USA (2012), International Scientific Meetings- “Recent Developments in Malaria Research”; International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India (2009 and 2013). He is also recipient of quite a few international and national awards including Congress Student Travel Stipend Award: Human Proteome Organization (HUPO) 11th Annual World Congress (2012), International Travel Support Award: Science and Engineering Research Board, Department of Science & Technology (DST), Government of India, Student Travel Support Award: US HUPO 8th Annual Conference and many others.

Dr. Aleš Strancar

BIA Separations



Talk Title: Chromatographic kinetic fingerprint measurement of protein digestion using monolithic trypsin column

Biography: Ales Strancar, the CEO of the BIA Separations and one of the main inventors of the CIM Convective Interaction Media® (new generation of chromatographic support). Ales Strancar is author or co-author of more than 70 scientific papers dealing with separation and purification technologies. He is a co-author of 5 granted USA patents and their foreign equivalents in the field of biomolecule separations and purification. As well he is a co-author of several book chapters dealing with novel chromatography technologies for biomolecule separation. Ales Strancar holds a position of Assistant Professor at Biotechnical Faculty of University of Ljubljana.

Mr. K Y V Ramana

Pall Life Sciences



Talk Title: Mixed mode separation of Proteins and antibodies in biopharmaceutical purification process

Biography: Mr. K Y V Ramana is a Bio Process Specialist Leader of Pall Corporation, Providing technical support on Pall life sciences products to Biotech and Vaccine research and manufacturing. Worked at Shantha Biotech Ltd, a unit of Sanofi Ltd and Syngene International Limited, a unit of Biocon Ltd. Graduated and masters in Life Sciences from recognized university of Andhra Pradesh, India.

Teaching Assistants for the course

IIT Bombay Represntives

Mr. Sandip Kumar Patel: Integrated PhD student , Proteomics Lab, IIT Bombay

Mr. Vipin Kumar : Integrated PhD student, Proteomics Lab, IIT Bombay

Ms. Shuvolina Mukherjee : PhD scholar IIT Bombay

Ms. Amruta Bhave : Junior research fellow, IIT Bombay

Industry Representatives

(i) Bio-Rad

Mr. Mukesh Poddar, Mr. Karthik Banuchander, Mr. Vipan Manhas, Mr. Osama Shakeel Sait, Dr. Senthil Karunakaran

(ii) GE Healthcare

Dr. Srinivasarao Chennareddy

Dr. Shubhendu Seal

Abstracts

Quantitative top-down proteomics: A high resolution 2D gel-based approach for the analysis of bimolecular mechanisms

Jens R. Coorsen

Foundation Chair of Molecular Physiology, School of Medicine, University of Western Sydney, NSW, Australia

Abstract: Two-dimensional gel electrophoresis (2DE) remains widely regarded as a gold-standard for proteomic analyses. Despite ‘issues’ with the method being routinely noted in the review literature, there has been little to substantiate these claims and many do not seem plausible when the 2DE technique is considered from the perspective of its underlying chemistry and that of proteins. As (or perhaps because) gel-based proteomics is a mature technology, factors contributing to possible reductions in performance are known and thus it has been possible to optimize analyses by targeted refinement of 2DE. I will review my group’s efforts to quantitatively improve every stage of 2DE analysis, from sample preparation and protein extraction, to in-gel spot ‘fractionation’ for improved overall protein resolution, through to improved in-gel protein staining and detection approaches for quantitative, markedly enhanced proteome coverage. As the overall objective of this work is to provide optimal analyses of a range of sample types, often with a focus to understanding physiological mechanisms, I will also briefly review our application of this refined 2DE protocol in investigations of tissues relevant to

basic and clinical sciences, including examples of biomedical and agricultural importance. I will also present recent data highlighting the real resolving power of this 2DE approach relative to other widely used assessment techniques. Thus, by developing and applying 'deep' or iterative imaging approaches we have quantitatively established that the actual resolution and coverage of proteins within a given 2DE analysis is much more substantial than has ever previously been realized. Processing and mass spectrometric analyses of these newly detected protein species confirms that the refined 2DE protocol we have developed enables access even to proteins known from other studies to be of extremely low abundance. Using mouse brain as a model, we have shown that we can readily resolve, image, and identify >>3000 proteins with high confidence; we anticipate that fine-tuning of this and other developing imaging approaches will yield even further enhancements in proteome coverage. Essentially, quantitative, high resolution proteomic analyses are now widely available at a fraction of the cost of other approaches; as an aside, we have developed simple, quantitative lipidomic analyses in parallel. Thus, 2DE is a rigorous, high-resolution technique for large-scale proteomic analyses, particularly the dissection of molecular mechanisms. The importance of this in terms of resolving different protein species/isoforms, post-transnationally modified proteins, having pI and molecular weight information concurrently, as well as being able to analyze multiple samples in parallel, cannot be underestimated. Nevertheless, considering the complexity of native proteomes, it must be remembered that there is no panacea, only pros and cons in all experimental methods. Critical, quantitative methodological evaluation and re-evaluation will always lie at the core of the most effective proteomic analyses.

Application of gel-based proteomics to study plant stress responses

Dr. Sixue Chen

Proteomics Facility, University of Florida, Gainesville, FL 32611, United States

Abstract: Plants are sessile organisms that are constantly challenged by biotic and abiotic stresses. Oxidative stress as part of most of the stresses cause fast protein redox modifications to turn on/off important cellular biochemical processes so that plants can respond efficiently to environmental changes. Here 2D gel based proteomics was applied to capture the potential thiol switches involved in jasmonate signaling, which is essential for plant growth, development and defense. Methyl jasmonate (MeJA) treatment led to enhanced production of hydrogen peroxide, indicating oxidative stress. With monobromobimane (mBBr) labeling to capture oxidized

sulphydryl groups and 2D gel separation, many protein spots that displayed significant redox and/or total protein expression changes were identified. By comparative analysis of mBBr and SyproRuby gel images, many proteins that were redox responsive and proteins that displayed abundance changes in response to MeJA were determined. Interestingly, stress and defense proteins constitute a large group that responded to MeJA. In addition, many cysteine residues involved in the disulfide dynamics were mapped based on tandem MS data. Protein redox regulation is increasingly recognized as an important switch of protein activities in yeast, bacteria, mammals and plants. Identification of redox proteins and their cysteine residues involved in the redox regulation allows for a deeper understanding of molecular mechanisms underlying organismal response and tolerance to environmental stresses.

2-DE workflow, reagent requirement and sample preparation guidance for serum for gel-based proteomics analysis

Sandipan Ray

Proteomics Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India

Abstract: 2-DE is a protein profiling technique that carries out separation of proteins on the basis of two parameters- isoelectric points (1st dimension) and relative molecular mass (2nd dimension). 2-DE DIGE is an advanced form of 2-DE that allows simultaneous analysis of multiple samples on a single gel by carrying out differential labeling of each sample using three different cyanine dyes (Cy2, Cy3 and Cy 5). After sample collection, protein extraction and processing involve multiple steps, often including depletion of high-abundance proteins and pre-fractionation prior to the actual proteomic analysis due to the complexity of serum/plasma samples and the wide dynamic range of protein concentration. Pre-analytical variations introduced during sample collection, handling and storage process, are challenging for obtaining consistency in findings. In addition, the complexity of biological sample, a very wide dynamic range of protein concentrations (10^{10} - 10^{12}), the presence of high-abundance proteins masking low-abundance marker proteins, high levels of salts and other interfering compounds, insufficient sensitivity of the detection technology and low-throughput are the major obstacles for discovery of blood biomarkers.

In order to circumvent the challenges associated with the serum sample, and to obtain a comprehensive coverage of serum proteome on gels, multiple processing steps are required prior to the proteomic analysis. In this study firstly we evaluated different protein extraction protocols from published papers to

remove high abundant proteins and obtain maximum coverage of the serum proteome. We also performed a comparative study of different sample processing strategies in various combinations to obtain best resolution and maximum spot number. Effect of sample processing on the 2-DE profile of human serum was evaluated on the basis of number of spots resolved on the gel as well as quality of the gel.

Two Dimensional-Gel Electrophoresis (2-DE)

Preamble

Gel-based proteomics is one of the versatile fields of proteomics, which has provided us with tools that can be used for protein separation, characterization as well as quantification. The goal of proteomic studies is to detect altered protein expression and modifications associated with disease or to find molecular targets for biomarkers and therapy. Gel-based proteomics includes techniques like one-dimensional SDS-PAGE or Native PAGE, 2-DE and DIGE. The proteins that are differentially expressed can be further identified using techniques like Mass Spectrometry. Gel-based proteomics mainly exploits the principle of electrophoresis; and provides information about protein properties such as molecular weight and isoelectric point. In this workshop an overview of the different gel-based techniques and their working principles will be provided.

Important terms in brief

- **Gel-based Proteomics:** High-throughput techniques for separation of proteins from complex mixtures using polyacrylamide gels.
- **Electrophoresis:** The separation of charged molecules under the influence of an applied electric field.
- **IEF:** Iso-electric Focusing: Separation of proteins based on their isoelectric points (pI).
- **IPG:** Immobilized pH Gradient
- **SDS-PAGE:** Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis, which brings about further separation based on their relative molecular mass.
- **2-DE:** Two Dimensional Gel Electrophoresis is a protein separation technique that carries out separation using isoelectric focusing in first dimension, followed by SDS-PAGE in the second dimension.
- **2D DIGE:** 2D Difference in Gel Electrophoresis is an advanced form of 2DE that allows simultaneous

analysis of test and control samples on a single gel by carrying out differential labelling of each sample. This minimizes gel-to-gel variations and enables easy processing of large number of samples

Protein Extraction

Proteomics aims to study and characterize proteins by involving functional elucidation, their interaction with other biomolecules and their physiological significance in the system. Hence for proteomic analysis of any sample, the first and by far the foremost important factor is protein extraction and sample preparation. The amount of information that can be gathered from the proteomic data hugely depends on the quality and quantity of sample. The techniques involved in proteomics are extremely sensitive to interfering compounds and hence they need to be eliminated before any proteomic analysis can be done. The type of sample to be processed determines the protocol that should be used for preparing the sample.

Learning Objectives

1. To understand the principle of protein extraction from various biological sources.
2. To learn the various steps involved in obtaining proteins from various types of samples.

Extraction protocols from different samples

I) BACTERIAL SAMPLES

- **PROCEDURE FOR BACTERIAL CULTURE**
 1. Prepare 2% LB media.
 2. Autoclave the media and tips at 15lb pressure, 15min, 121°C.
 3. Inoculate 100 µL fresh cultures into LB tubes.
 4. Grow the bacteria for 6-8 hrs at 37°C up to OD 0.8-1.0.
 5. After required growth, growth is stopped by centrifugation of LB tubes at 8,000g for 15min at 4°C.
- **PROTEIN EXTRACTION FROM BACTERIAL SAMPLES**
 1. Obtain 20ml bacterial culture and centrifuge it at 8000 g to get pellet.
 2. Discard supernatant and reconstitute the pellet in Phosphate Buffer (1X).
 3. Wash the pellet thrice with 1X PBS. Each time centrifuge @ 7000 rpm for 5 minute at 4°C.
 4. Dissolve pellet in 1 ml phosphate buffer having protease inhibitor and 2 mg/ml Lysozyme enzyme. Incubate at RT for 30 minute

5. Sonicate for a total of 2 minute with 30 seconds pulse, 4 cycles at 40% amplitude.
6. Centrifuge at 12,000 g for 5 min at 4°C. Obtain supernatant add 1 ml trizol reagent and 200ul chloroform. Shake vigorously for 15 sec and incubate for 15 minute at RT.
7. Centrifuge at 12,000g for 15 minute discard supernatant which is aqueous and transparent containing RNA.
8. To the bottom layer, add 300 µL 95 % (v/v) ethanol, centrifuge at 5,000g for 5 min at 4°C to remove DNA.
9. To the supernatant, add 4 volumes of chilled acetone and incubate for 6 hours at – 20°C.
10. Centrifuge at 12,000g for 5 minute to obtain protein pellet.
11. The pellet is then washed 4 times with Guanidium-HCl in 95% ethanol. Intermittent sonication may be required for effective pellet re-suspension.
12. The obtained pellet is then washed twice with acetone to get rid of any salts or other interfering agents from the pellet.
13. Air dry the pellet and dissolve it in appropriate amount of Rehydration Buffer.
14. Quantify using 2D Quant Kit or Bradford's reagent.

(II) CEREBORSPINAL FLUID SAMPLE (CSF)

• PROTEIN PRECIPITATION

1. CSF samples are taken out of -80° refrigerator and thawed completely on ice.
 2. 500µL of CSF sample is taken in a 1.5mL eppendorf tube and sonicated on ice. [Sonication parameters: Pulse ON for 5 sec, OFF for 15 sec, 8 cycles, 20% amplitude].
- CAUTION:** Please make sure to discard all the tips separately that are used to directly pipette the CSF samples. Discard in wrapped foils in Biohazard bags.
3. The sonicated sample is divided into two 15.mL tubes with each tube containing 250 µL sample.
 4. Add 1200 µL of ice-cold acetone to both the tubes, vortex for 10 sec and incubate at -20°C for 3 hours with short vortexing after every hour.
 5. After 3 hours, centrifuge the samples at 18000g for 30 minute at 4°C.
 6. From each tube, transfer the supernatant in 3 1.5 mL tubes such that every tube has 400 µL supernatant and the remaining supernatant is the original tube containing the pellet.

7. Add 1000 µL of ice-cold acetone to all the tubes, vortex (all the tubes except the ones containing the pellets) and incubate at -20°C O/N (approx. 16 hours).
8. On the next day, centrifuge the samples at 18000g for 30 min at 4°C.
9. Discard the supernatant completely.
10. Pool all the pellets in a total of 200 µL of RHB [Add 100 µL of RHB to the first tube, dissolve the pellet completely and transfer this buffer to the next tube containing the pellet and dissolve the pellet completely and repeat this is all the tubes. Finally collect the RHB containing all the dissolved pellets in the final tube. Repeat this again with 100 µL of fresh RHB in all tubes to dissolve any remaining proteins sticking to the tube walls and pool this in the same final tube containing previous buffer to make a total of 200 µL of mixture].
11. The samples are then proceeded for desalting.

• DESALTING OF THE SAMPLE [USING DESALTING KIT FROM GE]

1. The protein mixture collected above is divided into two 1.5 mL tubes containing 100 µL mixture each.
2. Add 300 µL of precipitant to both the tubes, vortex for 20 sec and incubate on ice for 15 minute
3. Add 300 µL of co-precipitant, vortex and for 20 sec and centrifuge at 15000g for 5 min at 4°C.
4. Remove the supernatant.
5. Give a brief spin to collect any remaining supernatant and discard it.
6. Without disturbing the pellet, add 40 µL of co-precipitant and incubate on ice for 5 minute
7. Centrifuge at 15000g for 5 min at 4°C.
8. Discard the supernatant.
9. Add 40 µL of DW to the pellet and disperse the pellet by tapping/vortexing.
10. Add 1ml of chilled wash buffer and 5 µL of wash additive to each tube and vortex for 30 sec every 10 minute for 1 hour.
11. Centrifuge at 18000g for 10 minute at 4°C.
12. Remove the supernatant completely and allow the pellet to dry for max 3-5 minute
13. Re-dissolve the pellet in each tube in 50 µL of RHB completely and pool them in a single tube to make 100 µL of protein extract.
14. Quantify using 2D Quant Kit or Bradford's reagent.
15. The extracted proteins are stored in -20°C till further use.

(III) BRAIN TISSUE SAMPLE

1. Take out the brain tissue sample from -80°C freezer and place it in a petri dish and cut it with a surgical blade. Petri dish should be placed on dry ice (if not present then pour little amount of liquid nitrogen on to the ice and keep the petri dish on it).
2. Take 50 mg of the brain tissue and add 0.5 ml of PBS to it and sonicate using following parameters. Amplitude 30%, Pulse 10 sec., gap 10 sec. time 2:30 minute or 15 Cycles. (NOTE: Sonication should be done by keeping the sample on the ice.)
3. Centrifuge the sonicated samples at 12,000g for 10 minute at 4°C.
4. Collect the supernatant in a new Eppendorf tube and discard the pellet in a Bio-Hazard dust bin.
5. To the supernatant add 1 ml of Trizol reagent and mix gently. Then add 200μl of chloroform (CHCl_3) and mix the components vigorously (10-15 sec.) until the sample turns into milky white.
6. Then incubate the sample at room temperature for 5 minute for the phase separation.
7. After phase separation centrifuge the samples at 14,000 rpm for 15 minute at 4°C, which results in the formation of 3 different layers in the Eppendorf tube.
8. The top layer contains RNA and the middle layer contains DNA and bottom pink colored organic layer contains both proteins and DNA.
9. Discard the top and the middle layers and to the bottom layer add 300 μL of absolute ethanol (for 1ml of Trizol reagent) and mix the sample gently until it becomes homogeneous mixture. Incubate at room temperature for 2-3 minute and then centrifuge at 2000 rpm for 15 minute at 4°C to pellet down the DNA.
10. Transfer the pink colored supernatant into a new Eppendorf tube and add 4 parts of chilled acetone and incubate at -80°C for 2 hours.
11. Centrifuge the sample at 14,000 rpm for 10 minute at 4°C. Discard the supernatant.
12. Wash the protein pellet with 0.3% Guanidinium hydrogen chloride in 95% ethanol for 3-4 times to remove the phenol. After the first addition of 0.3% Guanidinium hydrogen chloride in 95% ethanol a hard pellet is formed. Sonicate the pellet to bring it in suspension. In each washing step centrifuge the sample at 12,000 rpm for 10 minute at 4°C.
13. After 2 washes with 0.3% Guanidinium-HCl in ethanol wash the protein pellet with chilled acetone for 3-4 times to remove any interfering agents like salts from the protein sample.

14. After the acetone washes allow the protein pellet to air dry and add 200-250μl of rehydration buffer.
15. Quantify the protein using 2D Quant Kit or Bradford's reagent. The extracted proteins are stored in -20°C till further use.

(IV) YEAST CELLS

1. 5mL YPD media is inoculated with single colony of required strain taken from YPD plate (1% yeast extract from Hi Media, 2% peptone from Hi Media, 2% dextrose from SRL and 2% agar from Hi Media) stored at 4°C.
2. After inoculation tube is incubated at 30°C, 200rpm overnight.
3. Next morning, 100mL ofYPD (1% yeast extract, 2% peptone, 2% dextrose) is inoculated with overnight grown culture such that initial $\text{OD}_{600} \sim 0.200$.
4. After inoculation flask is incubated at 30°C, 200rpm till OD_{600} reaches to 1.000 (approximately after 4 hours of incubation).
5. Cells are pelleted down by centrifugation at 5000rpm for 5min at 4°C.
6. Pellet is washed twice with cold distilled water and centrifuged at 5000rpm for 5min at 4°C.
7. Pellet is again dispersed in cold distilled water and cell suspension is transferred into 2mL tubes used in glass bead beater.
8. Tube is centrifuged as above and supernatant is discarded. Finally pellet is re-suspended in 800μL of homogenization buffer (50mM Tris-chloride from sigma, 1mM EDTA, from GE, 0.2% DTT from sigma).
9. Cells are lysed by using bead beater operated as 6 cycles of 1min each with beads equal to half the volume of tube. In between every cycle tube is kept on ice for 3minute
10. Whole cell lysate is centrifuged at 12000rpm for 5minute at 4°C. Approx. 600μL of supernatant is collected into fresh autoclaved 2mL Eppendorf tube without disturbing glass beads.
11. 1mL of Trizol reagent is added to tube having supernatant and vortex for 30sec. 200μL of chloroform is added and tube is vortexed for 30 sec and left at RT for 15minute
12. Tube is further centrifuged at 12000rpm for 15min at 4°C. Upper transparent layer having RNA is discarded.
13. To remaining bottom layer 300μL of cold ethanol is added and tube is vortexed for 30sec and left at RT for 15minute

14. Tube is then centrifuged as in previous step.
15. Supernatant is collected into fresh 2mL autoclaved Eppendorf tube without disturbing DNA pellet.
16. Protein is precipitated by adding 1.5mL of chilled acetone with 0.2% DTT to every 500µL of collected supernatant.
17. Tube is left at RT for 15 min and then centrifuged at 12000rpm, 4°C for 5minute
18. Supernatant is discarded and pellet is broken apart by using clean glass rod.
19. Protein pellet is washed thrice with 0.3M Guanidinium chloride in 95% ethanol.
20. Finally protein pellet is washed thrice with chilled acetone with 0.2% DTT.
21. Pellet is air dried and resuspended in rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS).
22. Protein is further quantified by 2DE Quant Kit (from GE) or by Bradford method.
23. The extracted proteins are stored in -20°C till further use.

(V) SERUM SAMPLE

DILUTION AND SONICATION

1. Thaw the serum sample by placing it on ice for 10 minute
2. Transfer 250ul of the serum to a fresh tube and dilute it with 750 ul of Phosphate Buffer.
3. Vortex it for 30 seconds.
4. Sonicate the sample with 5 seconds pulse and 30 seconds gap for 8 cycles at 20% amplitude.
5. Prepare 2 aliquots of the suspension, of 500 µL each.

• ACTIVATION OF COLUMN

1. Mix the resin and slurry thoroughly by inversion of tubes. Break the valve on the lower end of the column. Loosen the screw cap on the column and place the column in a 2 ml Eppendorf tube. Centrifuge for 30 seconds at RT at 100g. This ensures collection of liquid in the lower tube and formation of a white bed.
2. The liquid is discarded and the column is equilibrated by addition of 400 ul PBS pH.
3. This is followed by centrifugation at 800g at RT for 30 seconds. This step is repeated one more time to ensure proper column equilibration.
4. Discard the used tubes and transfer the activated columns to a fresh vial.

• SAMPLE LOADING

1. Add 250 µL of the sample to the activated column which is in a fresh vial.
2. Incubate on ice for 10 mins and then centrifuge at 800g at RT for 30 seconds.
3. Do not discard the vial contents. For Elution, Add 100 µL PBS to the column and centrifuge at 800g at RT for 30 seconds. Repeat this step one more time. Transfer column to a new vial and do not discard the old vial.
4. Add more 250 µL of the sample to the column and repeat steps 2 and 3.
5. Mix both the depleted aliquots, label them properly and store them at -20°C.

• TCA-ACETONE PRECIPITATION

1. Thaw the depleted aliquots on ice for 10 min
2. Split the total 1.6 ml aliquot into eight 200 µL aliquots.
3. Add 800 µL of chilled acetone to all the aliquots and incubate at -20°C for 2 hours.
4. Centrifuge at 15000g for 20 min at 4°C.
5. Collect the supernatants and distribute them into 500 µL each aliquots and mark them as A for the pellet and B1 and B2 for the supernatants.
6. Wash A with 1 ml acetone and vortex it for 30 sec and store at -20°C for 15 minute
7. Add 1 ml acetone to B1 and B2 and incubate at -20°C for 30 minute
8. Centrifuge A at 15000g for 20 min at 4°C, remove the supernatant and dry the pellet at RT for 5 minute
9. Centrifuge B1 and B2 at 15000g for 20 min at 4°C and remove the supernatant.

10. Wash A with 1 ml acetone and vortex it for 30 sec and store at -20°C for 30 minute
11. Centrifuge A at 15000g for 10 min at 4°C, remove the supernatant and dry the pellet at RT for 5 minute
12. Reconstitute A and B with 50 µL Rehydration buffer and mix all.
13. Label and store at -20°C.

• DESALTING USING 2D-CLEAN UP KIT

1. Prepare aliquots of 120 µL of each sample in 5 tubes
2. Vortex for 30 sec and incubate for 15 min on ice

3. Centrifuge at 14000g for 15 min at 18°C and discard supernatant.
4. Add 40 µL of co-precipitant and incubate on ice for 5 minute
5. Centrifuge at 14000g for 15 min at 18°C and discard supernatant.
6. Dissolve in 25 µL distilled water and vortex briefly.
7. Wash with 1 ml wash buffer and 5 µL wash additive.
8. Incubate at -20°C for 1 hr, vortex every 10 min for 30 sec.
9. Centrifuge at 14000g for 15 min at 18°C and discard supernatant.
10. Dry the pellet and reconstitute in 700 µL rehydration buffer.
11. Label and store at -20°C.

(VI) ALGAL SAMPLE

1. Weigh about 100mg of algal lyophilized powder. Soak it in approximately 1 ml of distilled water and leave it overnight.
2. The wet mass is further used as a starting material for protein extraction.
3. Pellet down to remove the water by centrifugation at 10000 rpm for 5 minute
4. Discard supernatant and reconstitute the pellet in Phosphate Buffer (1X).
5. Wash the pellet thrice with 1X PBS. Each time centrifuge @ 7000 rpm for 5 minute at 4°C. The supernatant is discarded each time.
6. Dissolve pellet in 1 ml phosphate buffer having protease inhibitor and 2 mg/ml Lysozyme enzyme. Incubate at RT for 30 minute(Optional step to aid lysis)
7. Sonicate for a total of 5 mins, with 5 seconds pulse, 4 cycles at 40% amplitude.
8. Collect the cell lysate and centrifuge at 12,000 g for 5 min at 4°C. Obtain supernatant add 1 ml trizol reagent and 200ul chloroform. Shake vigorously for 15 sec and incubate for 5 min at RT.
9. Centrifuge at 12,000g for 15 minute Discard supernatant which is aqueous and transparent containing RNA.
10. To the bottom layer, add 300 ul 95 % (v/v) ethanol, centrifuge at 5,000g for 5 min at 4°C to remove DNA.
11. To the supernatant, add 4 volumes of chilled acetone and incubate for minimum 6 hrs and maximum overnight at – 20°C.

12. Centrifuge at 12,000g for 5 min to obtain protein pellet.
13. The pellet is then washed 4 times with Guanidium-HCl in 95% ethanol. Intermittent sonication may be required for effective pellet resuspension.
14. The obtained pellet is then washed twice with acetone to get rid of any salts or other interfering agents from the pellet.
15. Air dry the pellet and dissolve it in appropriate amount of Rehydration Buffer.
16. Quantify using 2D Quant Kit or Bradford's reagent.

Principle of 2DE

Two-dimensional gel electrophoresis (2-D electrophoresis) is widely used technique for the analysis of complex protein mixtures. This method separate proteins in two steps, the first-dimension is isoelectric focusing, which separates proteins according to their isoelectric points; the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (as we have studied in our previous lectures). In this way, complex mixtures consisted of thousands of different proteins can be separated.

This technique has an excellent resolving power, and today, it is possible to visualize over 10,000 spots corresponding to over 1,000 proteins, multiple spots containing different molecular forms of the same protein, on a single 2-DE gel. Due to the pivotal problem of protein solubility, the overwhelming majority of electrophoretic protein separations is made under denaturing conditions. Two types of reagents are used in 2-DE buffers to ensure protein solubility and denaturation. The first type, chaotropes (e.g. urea, thiourea and CHAPS) used at multimolar concentrations, is able to unfold proteins by weakening noncovalent bonds (hydrophobic interactions, hydrogen bonds) between proteins. The second one is ionic detergents, in which SDS (sodium dodecyl sulfate) is the archetype. It is made of a long and flexible hydrocarbon chain linked to an ionic polar head. The detergent molecules will bind through their hydrophobic hydrocarbon tail to hydrophobic amino acids. This binding favors amino acid-detergent interactions over amino acid-amino acid interactions, thereby promoting denaturation. Moreover, nonionic or zwitterionic detergents such as Triton X-100 are also used for protein solubilisation, since IEF requires low ion concentration in the sample. The detection method postgel migration is achieved either by the use of visible stains such as silver and Coomassie or fluorescent stains such as Sypro Ruby,

Learning Objectives

- A. Introduction to 2 Dimensional gel electrophoresis&its applications
- B. Work flow of 2DE
- C. Rehydration of IPG strip
- D. Isoelectric focusing of Rehydrated IPG strip (1st dimension)
- E. 2nd dimensional SDS-PAGE
- F. Staining and destaining
- G. Image analysis using IMP7 software

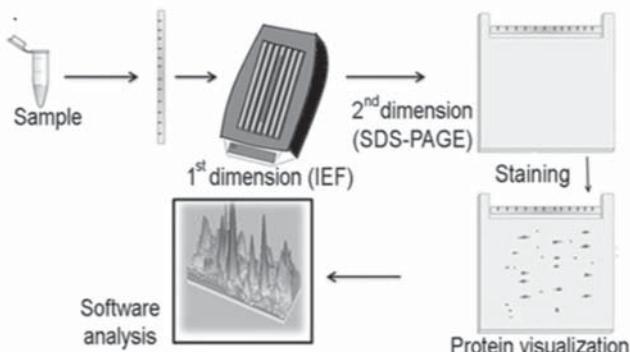


Figure 1.1: Overall work flow of Two-dimensional gel electrophoresis (2DE)

Experimental Set-up

DAY 1

Rehydration of IPG strips

IPG strips are put into rehydration solution according to the strip length.

STRIP LENGTH (in cms)	QUANTITY OF REHYDRATION SOLUTION TO BE USED (in μ l)
7	125
11	200
18	350
24	450

1. Add rehydration solution (containing 1% (v/v) IPG buffer and 40mM DTT) to the protein sample to make up the required volume according to the strip length.
2. Wash the re-swelling tray with non-ionic detergents and then with D/W. Dry it out completely with lint-free tissues. Level the re-swelling tray.
3. Pipette the prepared samples in individual channels of the re-swelling tray.

4. Hold IPG strip with forceps from edges and remove plastic cover from strip starting at + end.
5. Place the strip in channel with gel side down and + end of strip should be against the sloped end of the channel.
6. Overlay each strip with 3 ml of cover fluid oil after 1 hr to prevent evaporation and urea crystallization. Slide the lid on to the tray.
7. Place 1ml of cover fluid oil in channels which don't have strips Min 10-12hrs rehydration is required but it can be 15-18 hrs for 18cm/24cm strip.

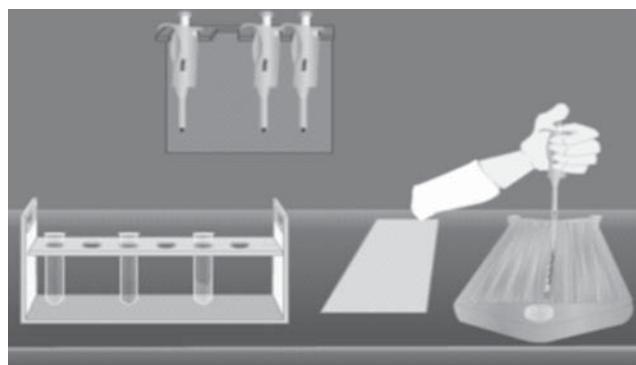


Figure 1.2: Rehydration of IPG strips

DAY 2: Isoelectric focusing of Rehydrated IPG strip

1. Level the IPGphor unit.
2. Switch on IPGphor and system.
3. Place the manifold on to the unit.
4. Add cover fluid (Fresh oil in the actual lanes wherein strip is placed and 2 lanes adjacent to it, and Used oil in the remaining lanes), evenly in the 12 manifold channels.
6. Remove the IPG strip from re-swelling tray, drain excess oil on to the tissue paper and place in manifold channel. Gel side should be up.
7. Place moistened electrode wicks/tissue paper wicks which are pre-wet with D/W; on to the two edges of the strip contacting the gel (important for conductivity and also absorb salts).
8. Over these wicks position the respective electrodes, close the lid and start IEF according to set protocol (according to the sample type and strip length).

DAY3: 2nd dimensional SDS-PAGE electrophoresis

1. Set the gel-casting unit and check for any leakage.
2. Prepare the cocktail for 12.5% SDS-PAGE.

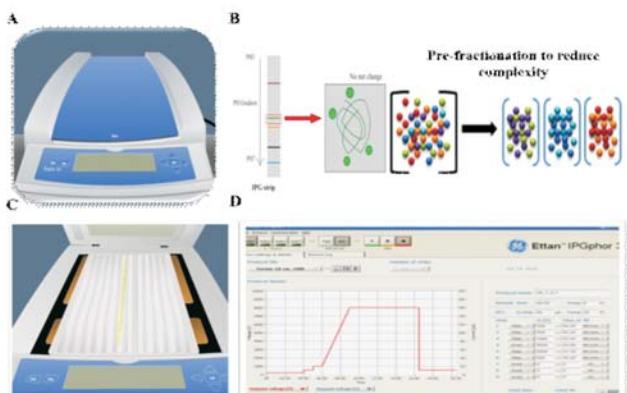


Figure 1.3: A.IPG Phorinstrument for performing IEF. B. Mechanism of the first dimensional separation C. Positioning of the IEF plate with IPG strip on the IPG Phor instrument. D. Glimpse of the voltage gradient of the IEF.

3. Pour the gel and leave it for polymerization.
4. Spray 0.1% SDS solution on the top of the gel surface.
5. Check the gel surface and then start the equilibration process
6. 1st Equilibration: IPG strips were equilibrated with equilibration buffer (6 M Urea, 75 mMTris-HCl pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue) containing DTT (10mg/ml) for 15 min on roller mixer.
7. 2nd Equilibration: The IPG strips were incubated with second buffer containing Iodoacetamide (25mg/ml) for 15min on roller mixer.
8. After 2nd equilibration wash the strip with1x SDS electrophoresis buffer.
9. Place the strip on top of the polymerized gel carefully. Then seal it with melted agarose.
10. Fill the buffer tank with 1X running buffer (stock soln. 10X), upto the demarcation on the running unit.

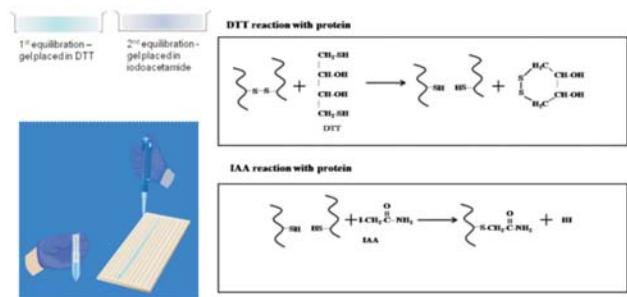


Figure 1.4: Equilibration steps; 1st equilibration involves treatment of the IPG strip with DTT in order to break the disulphide bonds so as to denature the protein before separation in the 2nd dimension. The 2nd equilibration step is done to alkylate the protein moieties so that the protein remains in the denatured condition for the run on SDS-PAGE

11. Place the unit in buffer tank appropriately.
12. Fill the inner buffer tank with 2X running buffer (above the lower limit).
13. Connect the electrodes. Set the Voltage and switch on power supply.
14. When the dye front comes out from lower end, switch off power supply.
15. Remove and keep the glass plates in DW. Remove the gel carefully from glass plate units and keep in staining solution (coomassie blue) and allow it to stain for 4-6 hrs (keeping on a horizontal shaker).
16. After proper staining place the gel in destaining solution. Keep small pieces of folded tissue papers at the two edges of the tray to absorb stains.
17. When the gel is destained completely, wash the gels in DW for 1 hr and scan it using LabScan software.
18. Save the gel image with proper labelling in .Mel and .Tiff formats.

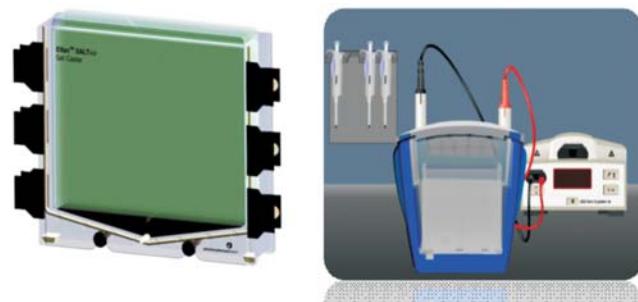


Figure 1.5: A. Gel casting cassette for 2nd dimension separation. B. Complete running unit for 2nd dimensional gel electrophoresis

IMAGE ANALYSIS USING IMP7 SOFTWARE

STAGE 1 OF IMAGE ANALYSIS

1. The scanned images which are saved in the .mel or .tiff format are first imported to the image pool of the IMP7 image analysis software from the source folder.
2. It is important to keep in mind that all the images which are to be analyzed are of the same dimensions and the contrast & brightness conditions are also more or less same. This should be done prior to importing the images.
3. Within the IMP7 software, a new project is created with the required label.
4. A new workspace is then created in the project and all the images (control as well as treated; for all the reps under study) are considered in the workspace for analysis.

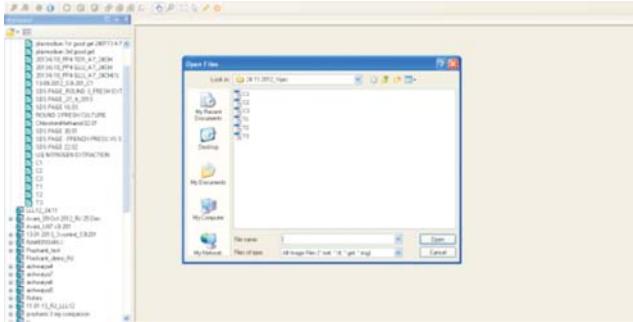


Figure 1.6: Screenshot of IMP7 software showing the procedure for uploading new gel image

5. Now a match set which comprises of all the gel images (control as well as treated; for all the reps under study) is created.
6. This is followed by manual detection of spots; wherein the spot boundaries are individually marked in each of the gel images. The contrast and zooming features can be adjusted for better resolution of spot boundaries.
7. The spots can also be chosen to be detected automatically by the software.



Figure 1.7: Screenshot of IMP7 software showing the procedure for spot matching

STAGE 2 OF IMAGE ANALYSIS

1. Once all the spots are manually detected in each of the gels, the next step of matching gels can be carried out. Ensure that the spots that are detected are approximately equal in number in each of the gels. (+/- 100 permissible).
2. Create Match classes and then include gel images in the respective classes of 'Control' and 'Treated'.
3. Display images belonging to one of the two classes. For instance, take up 'Control' class. It has the gel images with spots that were manually detected. The first gel is taken as the 'Reference gel', when the individual gels are being matched amongst themselves.

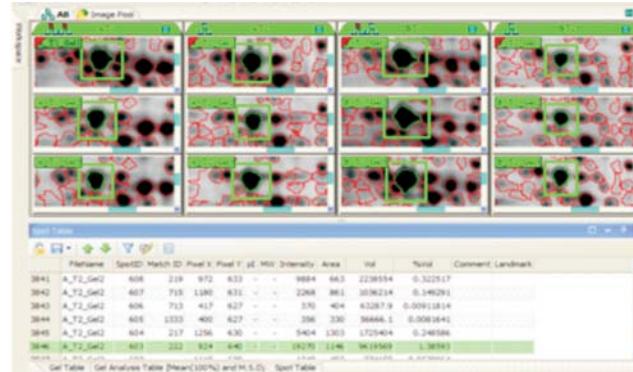


Figure 1.8: Screenshot of IMP7 software showing the procedure for identification of spots.

4. Alternatively, all the gels can be considered in a single match class and then be compared. Here too, the first gel will be taken as 'Reference gel', by default. This can be changed by simply dragging the required gel in the first position in the order of the created class.
5. Define Landmarks (i.e. spots that are prominent and common to each of the gels that are being compared). Ensure that the entire gel area is covered when you are defining landmarks. Optimally 4-5 landmarks are sufficient for analysis.
6. The gels are now ready to be matched within the said class.
7. Click on 'Match gels' icon on the toolbar. It will now display the matched gels and the number of total matches that are identified. The Match count includes the spots which are present in all the gels under consideration as well as the spots which are present in at least 3 out of the 6 gels under comparison.
8. From the Match Analysis Table, the ANOVA values for each of the matches can be retrieved. The matches with ANOVA value <0.05 are considered to be significant. These spots are then

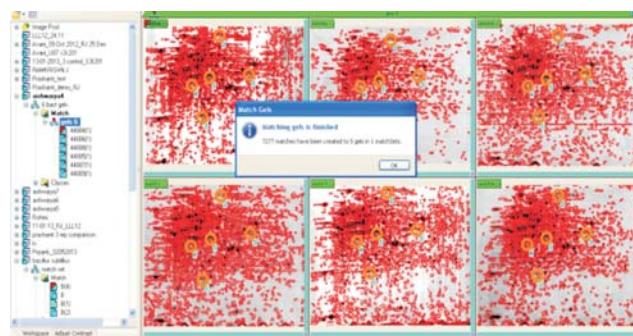


Figure 1.9: Screenshot of IMP7 software showing the procedure for 'landmarking' and aligning the spots that are present in all the gels

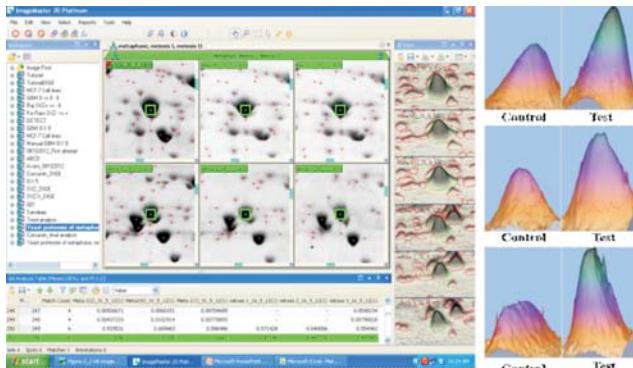


Figure 1.10: Screenshot of IMP7 software showing the procedure for identifying the spots and visualization in 3D view.

checked if they are matched properly or not. The software may miss out pre-marked spots and hence there might be loss of significant spots. In that case manually checking of the 3D views of the spots to understand the fidelity of matches is essential.

9. Subsequently, if the spots are missed out in any of the gels, it could be compared and marked. Add new match and look for the change in ANOVA value. If the value still continues to be <0.05 , then it is a confirmed match. Calculate Standard Deviation from Mean and Standard Error.
10. Then calculate the Ratio, of (Avg. of Treated intensities)/ (Avg. of Control Intensities). Correspondingly, note down the trends i.e. whether down regulated or unregulated. For unregulated ones, reciprocal ratio should be taken.

Two Dimensional- Difference In Gel Electrophoresis (2D-DIGE)

Learning Objectives

- A. Introduction to Gel based proteomics and its applications.
- B. To understand the principle of 2D-DIGE.
- C. Work flow of 2D-DIGE.
- D. Sample preparation and Cy Dyes labelling.
- E. Rehydration of IPG strip.
- F. Isoelectric focusing of Rehydrated IPG strip (1st dimension).
- G. 2nd dimensional SDS-PAGE.
- H. Scanning of DIGE gels using Typhoon FLA9500 scanner.
- I. Image analysis using Decyder software (DIA and BVA module)

Principle of 2D-DIGE

Two Dimensional-Differential Gel electrophoresis (2D-DIGE) is an improvement over conventional two dimensional gel electrophoresis (2DE) and involves the use of fluorescent dyes called CyDyes for labeling protein samples. This technique has greatly helped in improving the sensitivity, accuracy and reproducibility of 2DE in terms of quantitation.

CyDyes are fluorescent labels with similar mass and charge, pH insensitive and photostable with each label emitting a distinct, highly sensitive signal. Minimal labeling of the samples using CyDyes ensures that each visualized protein has a single dye molecule effectively labeling only 3% of the total protein in each sample. During labeling, the NHS-ester reactive group of CyDyes binds to the α amino group of Lysine resulting in addition of mass by 500 Daltons and replacement of the positive charge on Lysine with the positive charge of the CyDye thereby maintaining the pI of the protein.

The labeling of samples using CyDyes in 2D-DIGE allows comparison of samples from two different conditions across large datasets, not possible using 2DE due to gel-to-gel variation. Also, the reduced number of gels required for comparison of proteomes using 2D-DIGE saves a lot of time and energy thereby making analysis very easy.

Experimental Set-up

DAY 1

Sample preparation and labeling:

PART 1. Reconstitution of CyDyes (Performed only when a fresh DIGE kit is used)

1. Use 99.8% anhydrous Dimethylformamide (DMF) for reconstitution of the CyDyes. The DMF must be anhydrous and should not be contaminated with water. To prevent contamination with water make small aliquots (1mL) of DMF in amber colored microcentrifuge tubes (DMF is light sensitive) and use a fresh tube for every reconstitution.
2. Keep the CyDye kit at room temperature for 10 mins before reconstitution.
3. Centrifuge the tubes briefly to ensure that CyDye minimal dyes solid powders are at the bottom of the tubes.
4. 15 μ L DMF to 5 nmol/L of dye. The stock solution of Cy2 will have a deep yellow, Cy3 a deep red, and Cy5 a deep blue color.
5. Replace the cap on the dye microcentrifuge tube and vortex vigorously for 30 seconds.

6. Centrifuge the microcentrifuge tubes for 30 seconds at 12,000 g.
7. Final concentration of CyDye minimal dyes will be 333 pmole/ μ L.
8. Store the reconstituted CyDye at -80°C. This working concentration can be stored for 1 month time.
8. Incubate for 10 minutes on ice in the dark.
9. Mix the labelled samples and dilute with rehydration buffer for rehydration of IPG strip

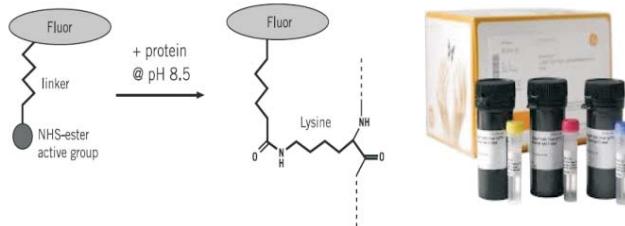


Figure 1.11: A. CyDyesbind to lysine residues of proteins
B. CyDye DIGE kit

PART 2. CyDye labeling of protein samples

1. Add a volume of sample equivalent to 50 μ g protein to a micro centrifuge tube. Prepare an internal standard by mixing the equal amounts of all samples to be analyzed in the experiment.
2. Before labeling adjust the pH of each sample to 8.5 using 100 mM NaOH.
3. Add 1 μ L of diluted CyDye to the microcentrifuge tube containing the 50 μ g protein sample (**~333 CyDye pmol per 50 μ g protein**). Samples (test and control) should be labeled with Cy3 and Cy5. Label the internal standard with Cy2. Dye-swapping should be performed while labeling the test and control samples for eliminating any type of dye effects.
4. Vortex gently for 30 seconds and centrifuge the microcentrifuge tubes for 30 seconds.
5. Leave the micro centrifuge tubes on ice for 30 minutes in the dark.
6. After incubation, add 1 μ L of 10 mmol/L lysine to stop the reaction.
7. Vortex gently for 30 seconds and centrifuge the micro centrifuge tubes for 30 seconds.

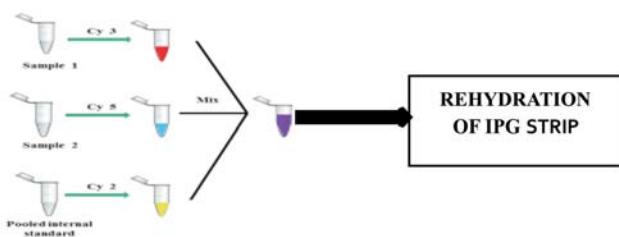


Figure 1.12: Labeling of strategy of samples with CyDyes for DIGE.

DAY 1

Rehydration of IPG strip: Procedure same as previous.

DAY 2

Isoelectric focussing of Rehydrated IPG strip: Procedure same as previous.

DAY 3

2nd dimensional SDS-PAGE: Procedure same as previous.

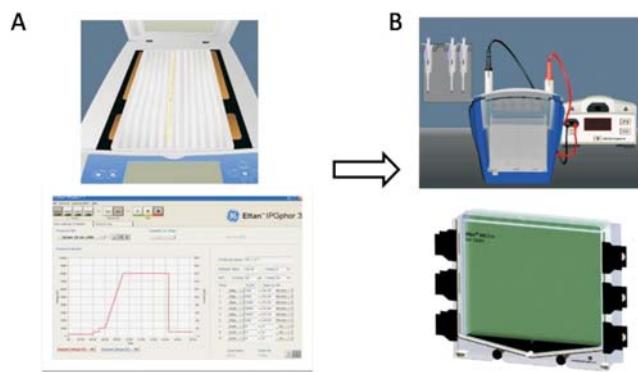


Figure 1.13: Isoelectric focusing of Rehydrated IPG strip.
B. 2nd dimensional SDS-PAGE

Scanning of DIGE gels using Typhoon FLA9500 scanner

1. The gels, post the 2nd dimensional PAGE can be done with or without the glass plates. The glass plates are then cleaned with lint-free tissues, to ensure that they are free of common contaminants like dust, hair, fibers, etc.
2. The cleaned glass plates are then placed in the tray (ETTAN DALT stage) in a proper orientation. They are clamped to keep them in place.
3. The Typhoon scanner is then turned on and the front panel is opened to insert the DIGE stage.
4. Note: The front panel of the scanner is not opened until the orange colored light stops blinking.
5. The tray is inserted into the scanner in the right direction and the front panel is then closed. Proper placing is ensured when a click sound is heard.
6. The instrument is now ready to scan your gels.

Using the software to scan the gels

1. Click on the Typhoon FLA9500 icon on the desktop. Then a window is opened which has multiple options.
2. Click on 2D-DIGE icon which opens another window that has multiple options for scanning of the DIGE gels.
3. Enter details like name of the gels, date of the scan, etc. and the destination folder as per the user's choice.
4. Select the ETTAN DALT stage as a platform for scanning.
5. The scanning area is now displayed on the window. If only one gel is to be scanned, then right click on the second gel and delete it.
6. Note: At a time, 2 gels can be scanned.
7. Set the appropriate wavelengths for the 3 different dyes, namely Cy2, Cy3 and Cy5.
8. Now select Cy2 as 'standard' by checking the corresponding box. Set the PMT for all the three dyes as 500 and resolution as 100µm.
9. If the aim is to simply look for presence or absence of spots, then go for Preview scan else proceed to scanning.
10. A window depicting the status of scanning is displayed.
11. After completion of the scan at all the 3 individual wavelengths, a beep sound is heard.
12. Post this, click on 'Return', which opens the scanning window. If you wish to re-scan your gels, repeat the above procedure or else close the window.
13. Now, your gel images will be saved in the '.gel' and '.tiff' formats in the mentioned destination folder.



Figure 1.14: Scanning of the gel on different wavelengths and subsequent gel image generation.

Image analysis using Decyder software

Steps for using the Decyder software for analysis of gels are as follows:

PART 1: Image Loading

The first step in DIGE analysis is loading the relevant images in the software. For this, the Image Loader option on the first screen is selected. The .tiff files and .GEL files obtained from Typhoon scanner are used for acquiring the images into the software. All 3 images – Cy2, Cy3, Cy5 – for one gel are selected and loaded. This image loading can be done for as many biological replicates as needed for the analysis.

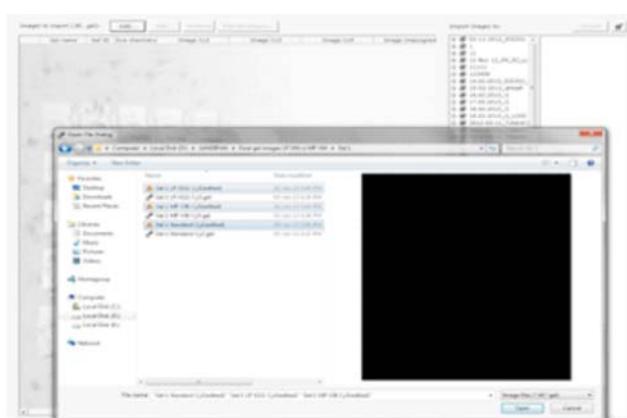


Figure 1.15: Uploading of the gel images on the DeCyder software

PART 2: Image Editing

The imported crude images need to be edited for correct juxtaposition and further analysis of gels. All the imported gel images are selected and the 'Edit Gel Images' tab is selected. This view shows the Cy2 images i.e. Standards of all the gels. The Cy2 images can be cropped and rotated as required in this window. The changes made to Cy2 gels are extrapolated to the Cy3 and Cy5 gels as well i.e. the other gels are cropped using the same dimensions. All the changes are saved, and the window is closed.

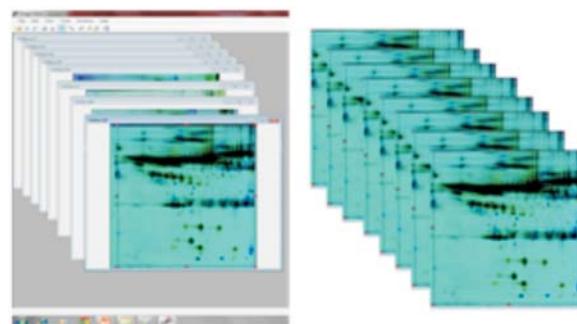


Figure 1.16: Editing of the gel images on the DeCyder software.

PART 3. Image Import

The New Project icon at the right corner of the screen is clicked, and new file is created and named. The cropped images are imported in this folder.

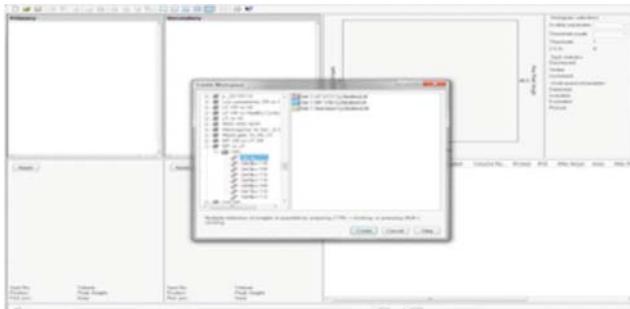


Figure 1.17: Creating of ‘group’ of the various experimental criteria on DeCyder

PART 4. Image Analysis

DIA – Differential In-Gel Analysis

For analysis of the Cy2, Cy3 and Cy5 of one set the DIA is used. For this, a new DIA Workspace is created, and relevant images are imported into the workspace. The estimated number of spots are added, and the images are processed. After processing, the DIA gives 3D views of all the spots, and the Max Slope and Max Volume for all. The Max Slope for a real protein spot is between 1to1.5. Exclude Filters can be set up to exclude certain areas, because there is no distinction between real protein spots and dust particles.

Biological Variation Analysis – BVA

The BVA is used for analyzing the Cy2, Cy3, Cy5 gels of more than one biological replicates – e.g. to compare different patients samples etc. The Batch Processor also helps in comparison of a large number of gels simultaneously. For this, all the gels to be analysed are selected. The DIA and BVA batchlist are created, and run. After this process, one of the Cy2 gels is selected as the Master gel, and the other gels are matched against this gel.

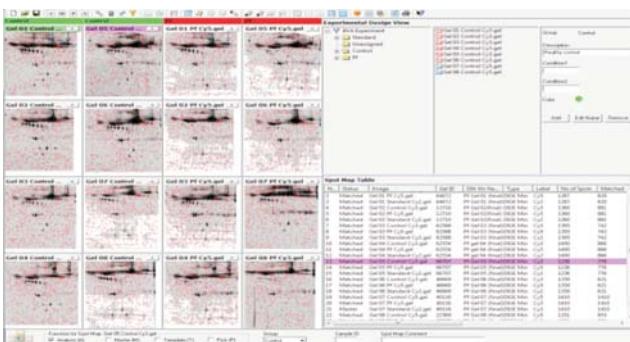


Figure 1.18: Screenshot of the overall gel view with their analysis tables as seen in Decyder

A new BVA Workspace are created. Two groups are made – control and test, and the gels are sorted appropriately. BVA has 4 modes:

Spot Map Mode

Only the Standard gels are shown in this mode.

Match Mode

All the gels can be matched in this mode. There is an option for adding new matches, creating new spots, merging some spots and splitting the spots which are demarcated by the software. Then, a match set can be created which incorporates all the changes. Thus, this helps in accurate matching of the gels. The checking has to be done manually, and spots may be added or changed accordingly. This tool is especially helpful for marking those spots which are actually preset, but have not been detected by the software.

Protein Mode

The protein mode shows the statistical values for all the gels. It also shows the number of gels in which that spot has appeared. The values of the selected protein parameters: Student’s T-test, One-way ANOVA, Two-way ANOVA and the fold change are displayed for each and every spot, along with the spot number on the Master gel. This mode helps in refining our data by giving statistical parameters as to which spots are to be selected, and which to be discarded.

Appearance Mode

Appearance Mode gives the comparison of peak heights etc. for every component.

3D View

The 3D view for each spot can be seen, and compared with one another. This tool is especially helpful to compare the spots chosen by the software, and to detect spots where improper matching has been done. This mode is also useful to detect the authenticity of a spot – whether it is a protein spot or a dust particle.

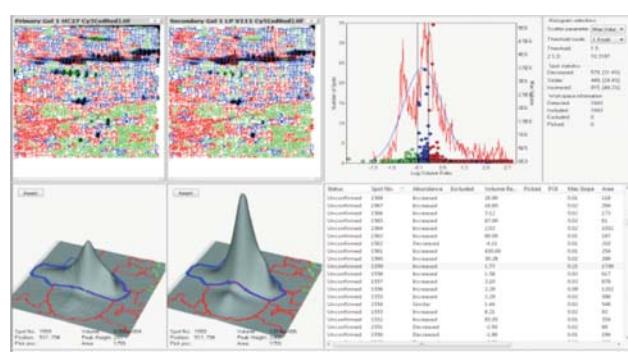


Figure 1.19: Editing of the gel images on the DeCyder software

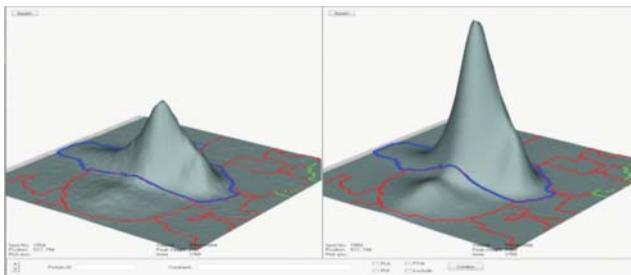


Figure 1.20: 3D view of same protein spot in two different gels

Graph View The Graph View shows the relative abundance of the protein in each gel. This is done for individual spots. The graph view plots the values of the Control samples, and their average, as well as the Experimental group and the average. The graph view also enables us to easily know whether a particular spot has been upregulated or downregulated.

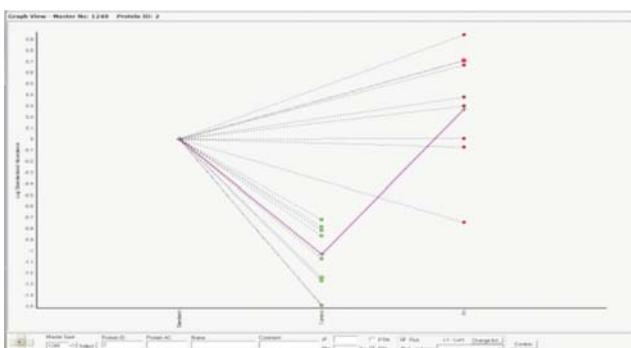


Figure 1.21: Graphical view of expression pattern of a single protein in all conditions

Two-dimensional electrophoresis followed by mass spectrometry analysis using MALDI-TOF/TOF for protein identification is widely used approach for the gel-based proteomics. Therefore, we intend to cover MALDI-TOF/TOF mass spectrometry also in gel-based proteomics workshop.

Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF)

Learning Objectives

1. Principle
 - a. Bruker Autoflex - Technical specifications
 - b. Calibration- Significance & types
 - c. Matrixes used in MALDI - different types
 - d. Software used for analysis - In brief
2. Applications of MALDI
3. Experimental Set-up
 - a. General (for all sample types) working protocol

- b. In-gel digestion protocol for peptide samples - MS/MS for Peptide Mass Fingerprint.
 - c. Workflow of Mass Spectrometry
4. Demo Experiment / Hands-on-experience
 - a. Objective 1: Mass identification of CGA protein.
 - b. Objective 2: MS & MS/MS analysis of known sample - BSA.
 - c. Objective 3: MS of unknown sample.

Principle

In MALDI, matrix plays an important role of being the Ion source as it absorbs energy from the laser upon bombardment & transfers the acquired energy to the sample molecules. The sample molecules then undergoes desorption/ionization. The ionization of the molecules with laser bombardment forms a cloud of ions in between the target plate P1 & P2. Due to reduction of voltage of the P2 plate which is higher in position to P1 (In Bruker Autoflex speed instrument which shows vertical set up), the ions moves on the P2 plate & further these ions moves to the analyzer (TOF in this case), gets separated based on mass & charge and finally gets detected by the detector. Based on the detection of the ionized samples by the detector data acquisition is performed which thereby generates the spectrum with help of specific software. The entire set up is in vacuum.

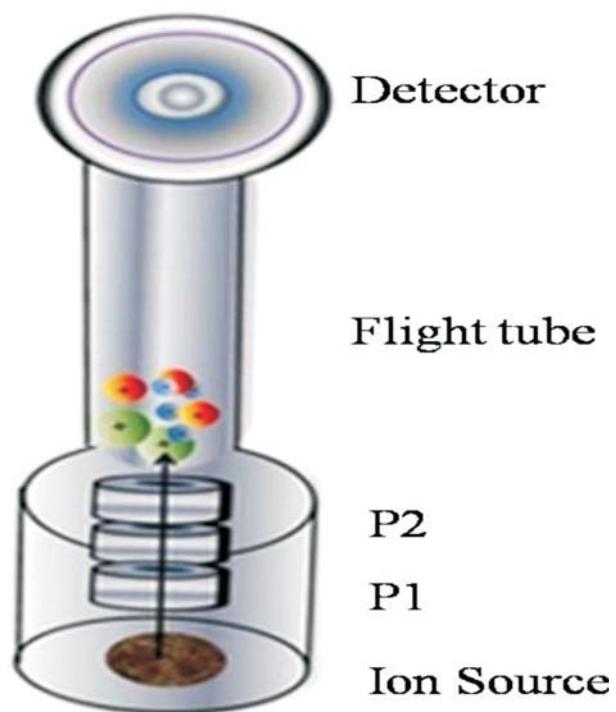


Figure 1.22: Components of MALDI-TOF/TOF

A) Bruker Autoflex - Technical Specifications

- 1) Laser technology - 355 nm frequency tripled Nd:YAG Smart Beam laser
- 2) Laser focus diameter - 10 μm enables high spatial resolution imaging without pixel overlap.
- 3) Resolving power - Wide mass range resolving power up to 26,000.
- 4) Two Modes of operation - Linear mode for labile molecules or anything bigger than 5kDa & reflectron mode for small molecules and peptides.

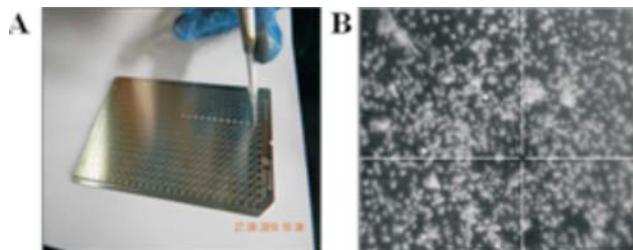


Figure 1.23: (A) MALDI Target plat (B) Crystal of CHCA matrix

- 5) Ion source cleaning - Laser based self-cleaning
- 6) Target plate - 384 position target plate (~1 μL spot size).
- 7) Specificity of Ionization - Can analyze both positive & negative ions in the same spot.

(B) Calibration- Significance & types

Calibration of the instrument prior any analysis on MALDI is mandatory as it shows significant changes in mass if analysis is done without calibration. The calibration standards are mixture of known standard samples & are categorized based on the type of sample, the mass range they can be used for & the make. The most commonly used calibrants are:

1) Peptide calibrant –

For peptides & other molecules in this range / 757.39 - 3147.47 Da

2) Protein standard I –

For small proteins & other molecules in this range / 5.73-16.9 kDa

3) Protein standard II –

For large proteins & other molecules in this range / 22.3 - 44.6 kDa

4) Fullerite mixture –

For Polymers / 720 - 1008Da

(C) Matrixes used in MALDI

Matrix selection is also play very crucial for sample analysis and it based on the laser wavelength used.

Matrix have low mass to be sublimable and strong absorbance at lower wavelength.

(D) Softwares used for analysis

The commonly used softwares for MALDI are **Auto Control** used for data acquisition, **Flex Analysis** for analyzing data and assigning mass and **Biotools** which is an interface between MALDI data and protein sequence databases for protein identification.

Table
Different types of matrix and their applications

Name of the matrix	Preparation	Excitation wavelength (nm)	Applications
α-cyano-4-hydroxycinnamic acid (α-cyano)	50% Acetonitrile, 0.1% TFA in extra pure water	337, 355	Peptides less than 5000 Da, lipids and nucleic acids
Sinapinic acid	30-50% acetonitrile, 0.1% TFA in deionized water	337,355,266	Peptides and proteins higher than 5000 Da and sometimes also used
2,5-Dihydroxybenzoic acid (DHB)	50% acetonitrile or 50% any one of the solvent such as chloroform, acetone, methanol along with water	337, 355, 266	Small molecules and peptides which are not ionized by other matrices
Trihydroxyacetophenone (THAP)	Acetonitrile along with ammonium citrate were used for making matrix with extra pure water	337, 355	Used for small nucleotides and also used for phosphorylation studies of proteins
Picolinic acid	Dissolved in ethanol and water for matrix preparation	266	Generally used for nucleotides

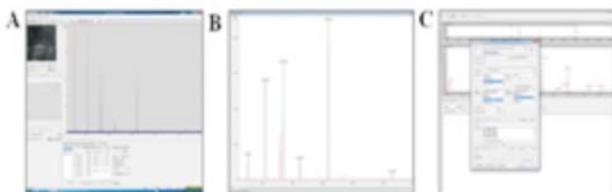


Figure 1.24: Software used for protein identification: A) Flex Control: Data acquisition, B) Flex Analysis: Data analysis, C) Biotoools: Interface

2. Applications of MALDI

MALDI is used in various application of proteome research, analysis of synthesized compound, molar mass and molar mass distribution and identification of micro-organism.

MS/MS or Tandem mass spectrometry

Tandem mass spectrometry, also known as MS/MS or MS2, involves multiple steps of mass

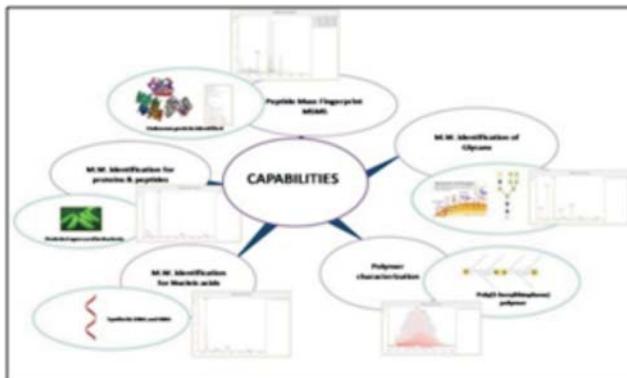


Figure 1.25: Different application of MALDI

spectrometry selection, with some form of fragmentation occurring in between the stages. Generally two analyzers *viz.* TOF are used in Bruker Auto flex speed. Based on the data from the MS spectra, precursor ions or parent ions are selected and are further fragmented to get MS/MS spectra. Using the MS/MS spectra through the bio tools, protein identification is done using data from various protein sequence databases.

3. Experimental set up

A) General (for all sample types) working protocol

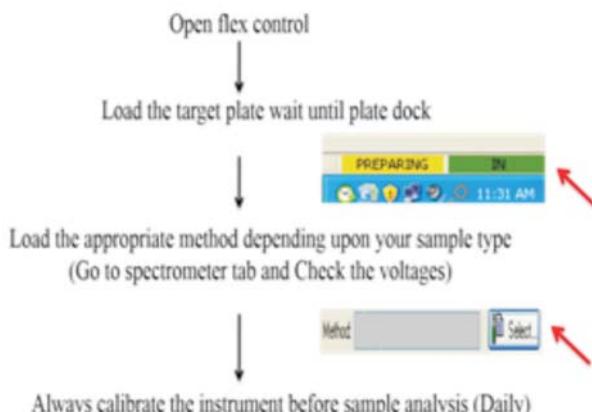
- 1) Mix sample & matrix solutions in a suitable ratio based on the sample type.
- 2) Spot the sample on a target plate i.e. put 1-2 μ L of sample on a specific well on the target plate.
- 3) Let the mixture dry of its own & the mixture co-crystallizes upon drying.
- 4) Place the target plate in the plate holder & insert in MALDI instrument in the plate chamber.
- 5) Shoot with laser on the crystals (in the well of the target).
- 6) Collect, analyze & detect ions using specific software.

B) Matrix Preparation

1. The matrix is prepared in 30-50% TA solution (0.1% TFA and Acetonitrile), with a concentration of 0.75 mg/mL CHCA matrix. The matrix once prepared, can be used maximum for a period of 10 days, as the components undergo oxidation with time. Also, it is important to keep in mind that acetonitrile evaporates easily.
2. The sample that is recovered by ZipTip procedure is used for spotting. Mix 1 μ L of sample with 1 μ L of matrix and spot it on the designated spot on the MALDI plate. After the spot has dried out completely, then the plate is placed inside the instrument and the spots are analyzed as follows.

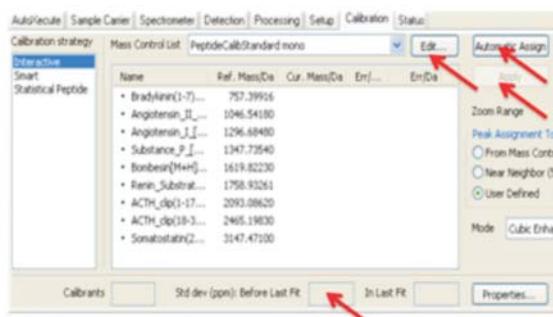
(C) Work Flow for MALDI ULTRAFLEX

A. FLEX CONTROL



B. CALIBRATION

1. Spectrum is acquired from the spotted calibrants.
2. Do automatic assign/manual depending on the quality of spectrum acquired in calibration tab
3. Always check standard deviation before applying calibration.

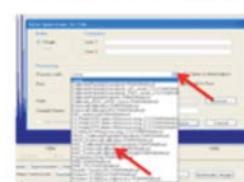


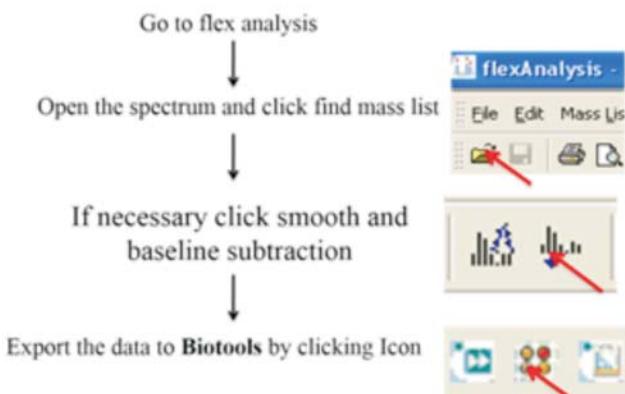
C. Sample Analysis

Go to sample spot and start acquiring spectra by adjusting laser intensities

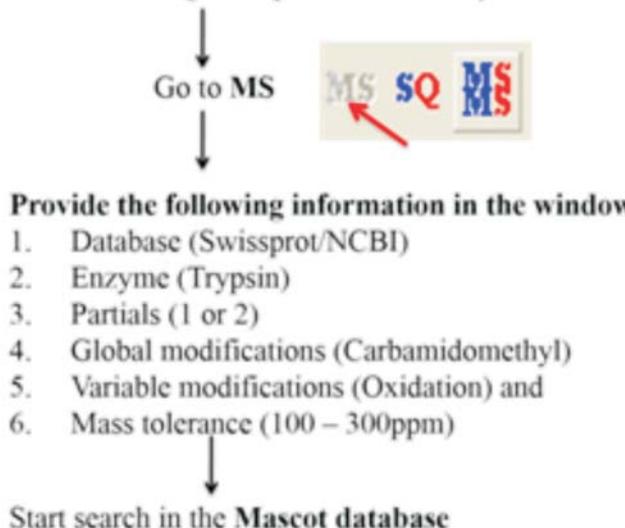
Add 3-4 good intense spectrums to get the average spectrum of the sample (Choose the "display sum buffer" icon in the tool bar)

Save the spectrum with appropriate process method

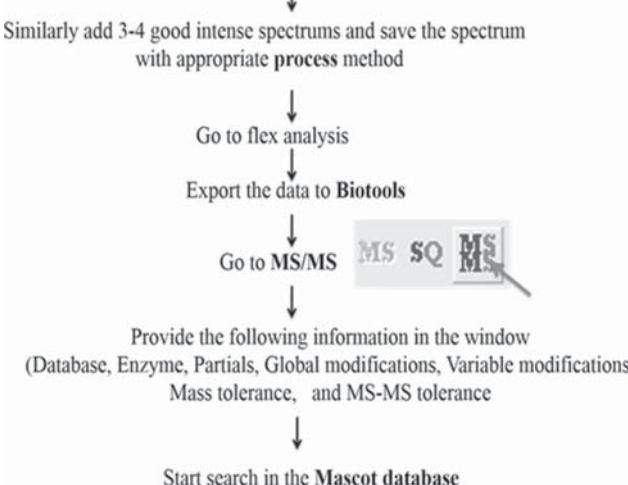
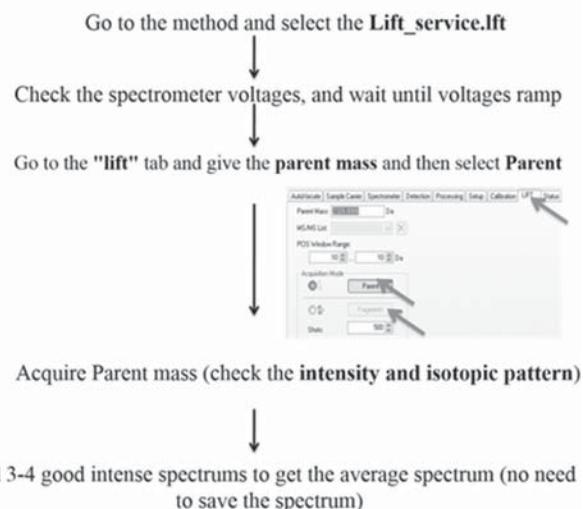


D. Flex Analysis**E. Biotoools**

Loaded Spectrum can be labeled manually by selecting label peaks if necessary



Press fragments and acquire spectra

**F. MALDI -TOF/TOF****4. Demo Experiment / Hands-on-experience**

- Objective 1:** Mass identification of CGA protein.
- Objective 2:** MS & MS/MS analysis of known sample - BSA.
- Objective 3:** MS of unknown sample.

Mass identification of CGA protein

Aim: To check the mass of CGA protein using MS spectra.

Matrix used: Sinapinic acid

Method used: LP 5-20 kDa

Protocol

- Matrix prepared by dissolving Sinapinic acid in TA 50 solution (50% ACN solution mixed in 0.1%TFA solution in the ratio 1:1) to make it a saturated solution.
- Sample prepared by digestion of protein by Trypsin (In-gel digestion) obtained from gel, followed by Zip-tipping for desalting & speed vac. for concentrating.
- Mix sample & matrix solutions in 1:2 ratio i.e. take 2 µL of sample & 4 µL of matrix in an eppendorf tube and mix thoroughly.
- Spot the sample on a target plate by putting 2 µL of sample on a well on the target plate. Note the well number.
- Let the mixture dry of its own & the mixture co-crystallizes upon drying.
- Place the target plate in the plate holder & insert in MALDI instrument in the plate chamber.
- Shoot with laser on the crystals (in the well of the target).

8. Acquire data using Flex control & Flex analysis softwares.
9. The spectra will have intensity on Y-axis & m/z on X-axis. The m/z value refers to the mass of the protein under observation.

Results

Mass observed (write as observed)-

Spectra (roughly to be drawn after observation)

MS & MS/MS analysis of known sample - BSA

Aim: To do the MS & MS/MS analysis of known sample - BSA (**Bovine Serum albumin**)

Matrix used: HCCA (α - cyano-4-hydroxy cinnamic acid).

Method used: RP-750-3500 Da

Protocol

1. Matrix prepared by dissolving HCCA in TA 50 solution (50% ACN solution mixed in 0.1%TFA solution in the ratio 1:1) to make it a saturated solution.
2. Mix sample & matrix solutions in 1:2 ratio i.e. take 2 μ L of sample & 4 μ L of matrix in an eppendorf tube and mix thoroughly.
3. Spot the sample on a target plate by putting 2 μ L of sample on a well on the target plate. Note the well number.
4. Let the mixture dry of its own & the mixture co-crystallizes upon drying.
5. Place the target plate in the plate holder & insert in MALDI instrument in the plate chamber.
6. Shoot with laser on the crystals (in the well of the target).
7. Acquire data using Flex control & Flex analysis softwares for MS and using Biotools check for the protein identification.
8. For MS/MS, based on MS spectra select a parent peak which is further fragmented to get daughter peaks thereby generating MS/MS spectra.
9. Select the method LIFT/LIFT for MS/MS analysis
10. Acquire the data of few high intensity peaks (Minimum 7-8 peak) for MS/MS using Flex control & Flex analysis softwares.
11. The data of masses generated by the MS/MS spectra will be further used to get Peptide Mass Fingerprint using Biotools software.

Results

Mass observed (write as observed)-Spectra (roughly to be drawn after observation) –

MS analysis of unknown sample

Aim: To do the MS analysis of known sample

Matrix used: HCCA (α - cyano-4-hydroxy cinnamic acid).

Method used: RP-750-3500-Da

Protocol

1. Matrix prepared by dissolving HCCA in TA 50 solution (50% ACN solution mixed in 0.1%TFA solution in the ratio 1:1) to make it a saturated solution.
2. Mix sample & matrix solutions in 1:2 ratio i.e. take 2 μ L of sample & 4 μ L of matrix in an eppendorf tube and mix thoroughly.
3. Spot the sample on a target plate by putting 2 μ L of sample on a well on the target plate. Note the well number.
4. Let the mixture dry of its own & the mixture co-crystallizes upon drying.
5. Place the target plate in the plate holder & insert in MALDI instrument in the plate chamber.
6. Shoot with laser on the crystals (in the well of the target).
7. Acquire data using Flex control & Flex analysis softwares for MS and using Biotools check for the protein identification.

Results

Mass observed (write as observed)-Spectra (roughly to be drawn after observation) –

References & further Reading

1. Raymond, S., Aurell, B. Two-Dimensional Gel Electrophoresis. *Science*. 1962; 138:152-153.
2. Reddy, P. J., Rao, A. A., Malhotra, D., Sharma, S., Kumar, R. et al., A simple protein extraction method for proteomic analysis of diverse biological specimens. *Current Proteomics*, 2013; 10:298-311.
3. Ray S, Koshy NR, Reddy PJ, Srivastava S. Virtual Labs in Proteomics: New E-Learning Tools. *J Proteomics*.2012; 75: 2515-2525.
4. Rabilloud T. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. *Proteomics*.2002; 1:3-10.
5. Dowsey A, Dunn M, Yang G. Automated image alignment for 2d gel electrophoresis in a high-throughput proteomics pipeline. *Bioinformatics*. 2008; 24:950–957.
6. R. Westermeier, H. Schickle, Arch. Physiol. Biochem. 2009; 115, 279-285.
7. Wittmann-Liebold B, Graack HR, Pohl T. Two dimensional gel electrophoresis as tool for proteomics studies in combination with protein

- identification by mass spectrometry. *Proteomics*. 2006; 6:4688-4703.
8. Lilley KS, Razzaq A, Dupree P. Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. *CurrOpinChem Biol.* 2002; 6:46–50.
 9. Gorg A, Weiss W and Dunn MJ: Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 2004; 4: 3665-3685.
 10. Unlu M, Morgan ME, Minden JS: Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 1997; 18: 2071–2077.
 11. Minden JS, Dowd SR, Meyer HE, Stuhler K. Difference gelectrophoresis. *Electrophoresis* 2009; 30: S156–S161.
 12. Timms JF, Cramer R. Difference gel electrophoresis. *Proteomics*. 2008;8:4886-97
 13. Sapra R. The use of difference in-gel electrophoresis for quantitation of protein expression. *Methods Mol Biol.* 2009; 492:93-112.
 14. Tannu NS, Hemby SE. Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. *Nat Protoc.* 2006; 1:1732–1742.
 15. Kondo T, Hirohashi S. Application of highly sensitive fluorescent dyes (CyDye DIGE Fluor saturation dyes) to laser microdissection and two-dimensional difference gel electrophoresis (2D-DIGE) for cancer proteomics. *Nat Protoc.* 2006; 1:2940-56.
 16. Viswanathan S, Unlü M, Minden JS. Two-dimensional difference gel electrophoresis. *Nat Protoc.* 2006; 1:1351-8.
 17. Rabilloud T, Lelong C. Two-dimensional gel electrophoresis in proteomics: a tutorial. *J Proteomics*. 2011; 74:1829-41.
 18. Righetti PG, Sebastian R, Citterio A. Capillary electrophoresis and isoelectric focusing in peptide and protein analysis. *Proteomics*.2013; 13: 325-40.
 19. Jones AR, Gibson F. An update on data standards for gel electrophoresis. *Proteomics*. 2007; 1:35-40.
 20. Abdallah CD-GE, Renaut J, Sergeant K. Gel-based and gel-free quantitative proteomics approaches at a glance. *Int J Plant Genomics*. 2012; ID 494572.

e-Learning Resources

We have developed various free e-learning resources which can be accessed in the following URLs for better understanding of the above modules:

<http://nptel.ac.in/courses/102101040/>

<http://nptel.ac.in/courses/102101007/>

<http://iitb.vlab.co.in/?sub=41&brch=118&sim=375&cnt=1>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=383&cnt=1>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=412&cnt=1>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=413&cnt=1360>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=414&cnt=1>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=416&cnt=1>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=630&cnt=1>
<http://iitb.vlab.co.in/?sub=41&brch=118&sim=655&cnt=1>
<http://ekalavya.iitb.ac.in/oscarHome.do>

Application Note 1

Application of Gel-based Proteomics to Investigate Malaria Pathogenesis and Identify Surrogate Protein Markers of Infection

Sandipan Ray, Sandip K. Patel and Sanjeeva Srivastava

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, E-mail: sanjeeva@iitb.ac.in

Malaria is by far the world's most significant tropical infectious disease with estimated 207 million malaria cases worldwide in 2012, leading to 6,27,000 deaths [1]. According to the recent WHO statistics; about 100 countries in the world are considered malarious and 3.3 billion people i.e. nearly half of the world's population, are at risk of malaria. India significantly contributes to the global malaria burden and has the largest population in the world at risk of malaria [2]. Despite worldwide initiatives, emerging drug resistance in different species of *Plasmodium* and paucity of information about the exact underlying mechanism of the disease pathogenesis are hindering the management and eradication of malaria. *Plasmodium falciparum* (*Pf*) infection represents the major cause of malaria associated mortality worldwide. Although *Plasmodium vivax* (*Pv*) infection is historically regarded as benign, recently the severe and fatal incidents of *vivax* malaria reported from different regions of the world, rendered this clinical paradigm deceptive. Mechanisms that trigger the transition of uncomplicated malaria into severe-complicated manifestations are largely obscure, particularly in case of *Pv* infection [3].

The spectacular advancements, achieved in the preceding decade with the completion the genome

sequence of different species of *Plasmodium* and its insect and vertebrate hosts [4], have propelled the growth of proteomics into different arenas of malaria research. Serum proteomics has attracted considerable interest for biomarker discovery since serum can be collected less invasively and it is an ideal biological sample that contains an archive of information due to the presence of variety of proteins released by diseased tissues. The rapid alterations in expression pattern of various serum proteins in response to external stimulus and correlation between the serum protein levels and pathogenic infections is extremely valuable and of particular interest from a clinical and diagnostic perspective. Over the last decade diagnostic applications of serum proteomics have steadily been growing [5]. Multifarious interaction between the human and malaria parasites provokes complex immune cascades in the host [6], whilst *Plasmodium* develops versatile mechanisms to escape their host defense machinery [7]. Ultimate clinical outcome of malaria is influenced by different host, parasite, environmental and social factors [8]. Plasmodial infections significantly affect different vital physiological processes and as a consequence modulate the host's proteome. Once infected, a continuous interaction between the pathogen and the host immune system initiates a complex immune response to prevent the pathogenic infection and growth through multiple anti-parasitic effector functions, including inhibition of invasion and cytoadherence, antibody-dependent cytotoxicity and cellular inhibition. Blood biomarkers for pathogenic infections can be used for early diagnosis, discrimination between closely related infections with similar clinical manifestations as well as aid in scrutinizing disease progression, response to therapy, and predicting outcomes. Additionally, investigation of the pathogen induced alterations in host proteome under diseased conditions can provide valuable information regarding disease pathogenesis and host immune responses. Proteomic analysis of serum/plasma has been extensively performed for detection of surrogate proteins markers for cancers and different types of other human diseases. However, very few studies have been conducted to identify surrogate protein markers for malaria, particularly for *Pv* infection. A few studies have been conducted to identify serum/plasma biomarkers of malaria. In a recent study Armah et al., have demonstrated an co-relation between cerebral malaria (a deadly complication of *Pf* infection) associated mortality with elevated serum and CSF levels of apoptotic factors (IP-10, IL-1ra, sTNFR1, sTNFR2, sFas) as well as reduced serum and CSF levels of neuroprotective angiogenic growth factors (PDGFbb) [9], while in another study plasma superoxide dismutase (SOD-1) level has been identified as a surrogate marker of *Pv* malaria severity [10]. Kim and co-workers have performed serum proteome analysis of *vivax* malaria patients in Korean population

and reported alteration in serum levels of haptoglobin and serum amyloid A [11]. In another recent interesting work, Kassa et al., have reported quite a few interesting inflammation-related serum biomarkers for malaria and demonstrated their interactions with malarial-related molecules is hemozoin (HZ), which is a dark-brown crystal formed by the parasite and released into the host during the burst of infected red blood cells [12]. Very recently, Bachmann et al., have reported elevated muscle proteins in plasma of children with cerebral malaria by affinity proteomics screening using antibody suspension bead arrays [13].

Our research group at Indian Institute of Technology Bombay, Mumbai is also performing comprehensive proteomic analysis of serum samples from patients suffering from *vivax* and *falciparum* malaria using different proteomic and immunological technologies to analyze alterations in the human serum proteome as a result of plasmodial infections to obtain mechanistic insights about disease pathogenesis, host immune response, and identification of protein markers of infection and disease severity [14,15]. This multicentric study was conducted by involving malaria patients and controls from both the urban and the rural populations from three different malaria endemic regions of India; Mumbai, Kolkata and Bikaner. Recruitment of the subjects for this multidisciplinary study was carried out during 2010 to 2013 at multiple hospitals including Seth GS Medical College & KEM Hospital-Mumbai, Grant Government Medical College and Sir JJ Group of Hospitals-Mumbai, PD Hinduja National Hospital & Medical Research Centre-Mumbai, Medical College Hospital - Kolkata and Malaria Research Center- Bikaner, with the approval of the institutional ethics committee of each study site. Serum samples from *vivax* and *falciparum* malaria patients and healthy controls were analyzed using different gel-based (2-DE and 2D-DIGE) proteomics approaches, and results were validated by employing immunoassay-based approaches (Figure 1 and 2). Functional pathway analysis involving the identified differentially expressed proteins revealed the modulation of different vital physiological pathways, including lipid metabolism and transport, acute phase response signaling, chemokine and cytokine signaling, complement cascades and blood coagulation in malaria [14,15]. Specificity of the identified serum markers for malaria was evaluated by analysis of two other infectious diseases resulting in fevers; dengue fever and leptospirosis [16,17]. A panel of identified proteins consisting of six candidates; Serum amyloid A (SAA), Hemopexin (HPX), Apolipoprotein E (Apo E), Haptoglobin (HP), Retinol-binding protein 4 (RBP4) and Apolipoprotein A-I (Apo A-I) provided excellent discrimination of malaria from healthy community controls and other different infectious diseases [18]. Analysis of longitudinal cohorts (early febrile, defervescence and convalescent stages) indicated that

majority of the proteins were maximally elevated in malaria patients during the early febrile phase of the infection. Identified proteins like SAA, Apo A-I and Apo E which exhibited a sequential alteration in their expression levels in different severity levels of malaria, could serve as potential predictive markers for disease severity. While candidates like HP, RBP4 and Ceruloplasmin, which exhibited cyclic alterations in their expression levels with the progression of the disease, can be utilized as possible markers for

monitoring therapeutic interventions. The excellent discrimination among the malaria, controls and other infectious disease groups obtained by various prediction models on the basis of differentially expressed serum proteins, identified in this study testifies the potential of this analytical approach for the discrimination of *vivax* and *falciparum* malaria as well as other infectious and non-infectious human diseases on the basis of host proteomic alterations.

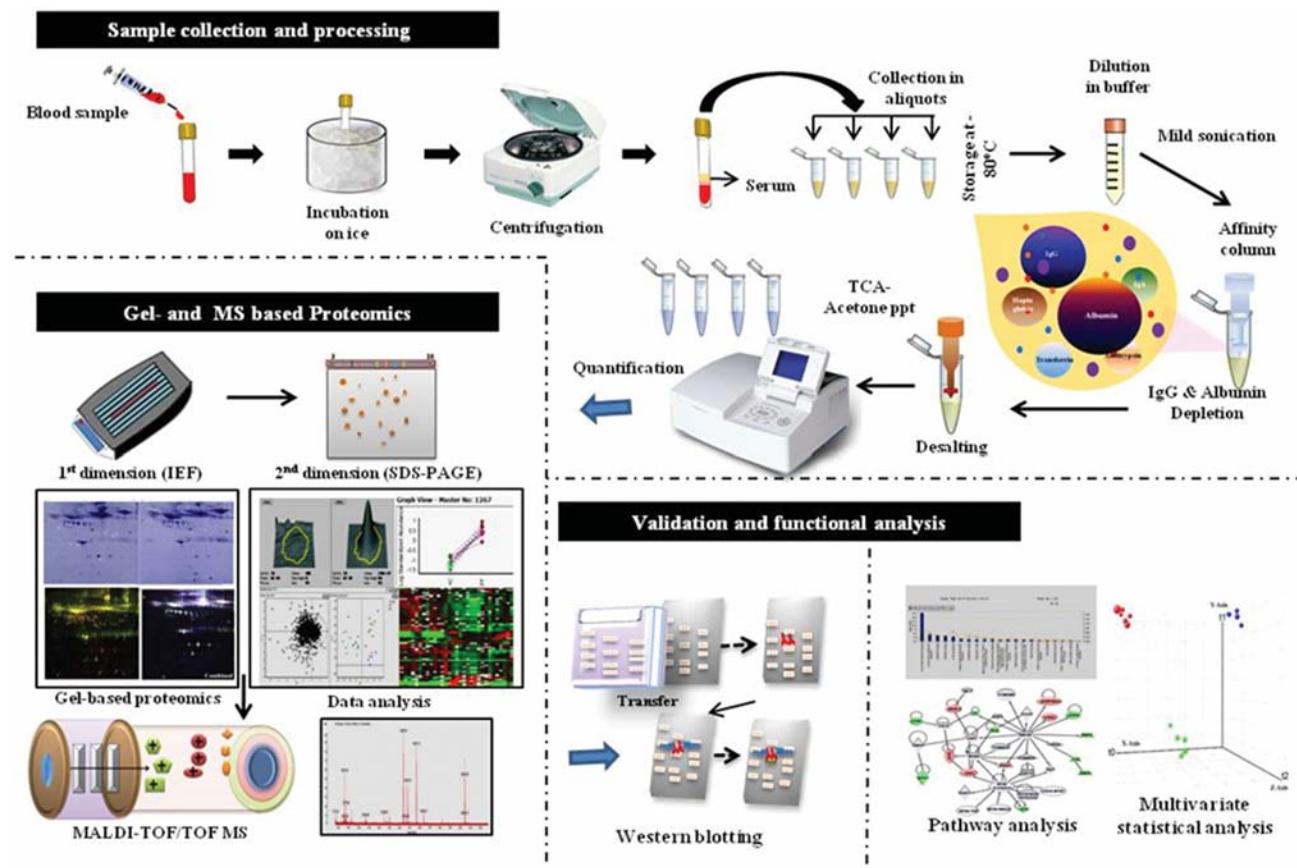


Figure 1.26: Schematic representation of experimental strategy for proteomic analysis of alterations in the human serum proteome in malaria

Comparative proteomic analysis of *falciparum* and *vivax* malaria, other infectious diseases (dengue fever and leptospirosis) and healthy controls was performed by using two complementary quantitative proteomic approaches; 2D-DIGE along with MALDI-TOF/TOF MS; and results were validated by employing ELISA and western blotting. Individual performance of a few

selected differentially expressed proteins was analyzed using receiver operating characteristic (ROC) curves. Identified differentially expressed proteins in malaria were subjected to functional pathway analysis for understanding their biological contexts, involvement in various physiological pathways and association with disease pathophysiology (modified from Ref no. [18]).

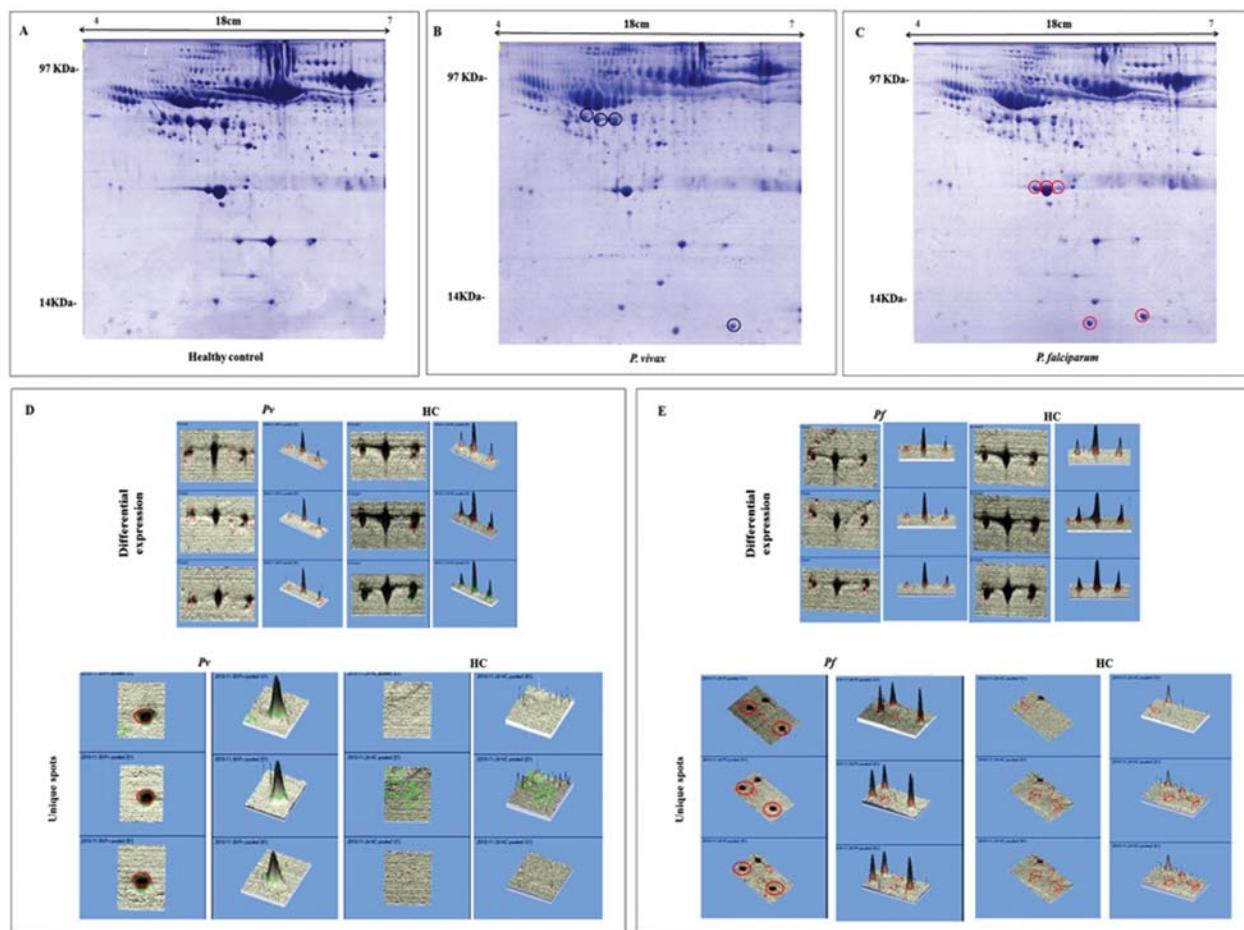


Figure 1.27: Representative 2D gels of pooled serum from healthy control subjects (A), vivax malaria (B) and falciparum malaria (C) patients. Each individual was analyzed in triplicate. Graphical representation of some differentially expressed proteins (D and E).

References

1. World Malaria Report, World Health Organization 2013. ISBN: 9 789241 56469 4 http://www.who.int/malaria/publications/world_malaria_report_2013/en/
2. Shah NK, Dhillon GPS, Dash AP, Arora U, et al. Antimalarial drug resistance of Plasmodium *falciparum* in India: changes over time and space. Lancet Infect Dis. 2011, 11, 57-64.
3. Rogerson SJ, Carter R. Severe *vivax* malaria: newly recognised or rediscovered. PLoS Med. 2008, 5, e136.
4. Gardner, M. J., Hall, N., Fung, E., White, O. et al., Genome sequence of the human malaria parasite Plasmodium *falciparum*. Nature 2002, 419, 498-511.
5. Ray S, Reddy PJ, Jain R, Gollapalli K, et al. Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. Proteomics 2011, 11, 2139-61.
6. Hisaeda H. et al. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat Med. 2004, 10(1), 29-30.
7. Olszewski KL, Morrisey JM, Wilinski D, Burns JM, et al. Host-parasite interactions revealed by Plasmodium *falciparum* metabolomics. Cell Host Microbe. 2009, 5, 191-9.
8. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature 2002, 415, 673-9.
9. Armah HB, Wilson NO, Sarfo BY, Powell MD, et al. Cerebrospinal fluid and serum biomarkers of cerebral malaria mortality in Ghanaian children. Malaria J 2007, 6, 147.
10. Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, et al. Plasma Superoxide Dismutase-1 as a surrogate marker of *vivax* malaria severity. PLoS Negl Trop Dis. 2010, 4, e650.
11. Bahk YY, Na B, Cho S, Kim J, et al. Proteomic analysis of Haptoglobin and Amyloid A protein levels in patients with *vivax* malaria. Korean J Parasitol. 2010, 48, 203-11.
12. Kassa FA, Shio MT, Bellemare M-J, Faye B, et al. New Inflammation-Related Biomarkers during Malaria Infection. PLoS ONE 2011, 6, e26495.

13. Bachmann J, Burté F, Pramana S, Conte I, et al. Affinity proteomics reveals elevated muscle proteins in plasma of children with cerebral malaria. PLoS Pathog. 2014, 17, 10, e1004038.
14. Ray S, Renu D, Srivastava R, Gollapalli K, et al. Proteomic investigation of *falciparum* and *vivax* malaria for identification of surrogate protein markers. PLoS One 2012, 7, e41751.
15. Ray S, Kamath KS, Srivastava R, Raghu D, et al. Serum proteome analysis of *vivax* malaria: An insight into the disease pathogenesis and host immune response. J Proteomics. 2012, 75, 3063-80.
16. Ray S, Srivastava R, Tripathi K, Vaibhav V, Patankar S, Srivastava S. Serum proteome changes in dengue virus infected patients from a dengue endemic area of India: Towards new molecular targets? Omics 2012, 16, 527-36.
17. Srivastava R, Ray S, Vaibhav V, Gollapalli K, Jhaveri T, et al., Serum profiling of leptospirosis patients to investigate proteomic alterations. J Proteomics. 2012, 76, 56-68.
18. Ray S, Patel S, Kumar V, Damahe J. Srivastava S. Differential expression of serum/plasma proteins in various infectious diseases: Specific or nonspecific signatures. Proteomics Clin. Appl. 2014, 8, 53-72.

Application Note 2

Application of Gel-based Proteomics for Detection of Phosphoproteins in Sera of Malaria Patients

Vipin Kumar, Sandipan Ray and Sanjeeva Srivastava

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, E-mail: sanjeeva@iitb.ac.in

Phosphoproteins play important roles in cellular activity such as signaling and regulation of protein function [1]. There are many techniques to identify phosphoproteins from complex proteome mixtures including ELISA-based detection approaches which involve use of antibodies, radioactivity-based involving the use of on radioisotopes or staining of proteins with dyes like Pro-Q-Diamond dye (Pro-Q-DPS). ELISA and radioactivity-based approaches are expensive and can't detect phosphoproteins separated in a PAGE. To this end, Pro-Q-DPS can detect phosphoproteins within the SDS-Polyacrylamide gel (SDS-PAGE gel). The sensitivity of Pro-Q-DPS is 1-16 ng range. Lipids also contain phosphate group to which Pro-Q-DPS usually binds. It is very important to ensure that the sample being used is lipid free so that only the proteins in the sample get stained. Wessel et al. described a protocol for delipidation of protein which is compatible for serum sample [2].

In this study we intended to reveal the presence of phosphoproteins in the serum samples of patients suffering from severe and non-severe falciparum malaria. For revealing the presence of phosphoproteins in the malaria and control sera, the 2D gels were stained using a Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies, Molecular Probes, CA, USA), while the total proteome was visualized by staining the same gels with SYPRO® Ruby Protein Gel Stain (Life Technologies, Molecular Probes, CA, USA) following the manufacturer's protocol.

Methodology

For PQD staining the sample should be lipid free, otherwise it can interfere the detection of the phosphorylated proteins. For delipidation of the serum 0.4mL of methanol (4 times) was added to 0.1 ml of samples, samples were vortexed and then centrifuged at 9000g for 10seconds. 0.1mL of chloroform was then added to the tube, vortexed and centrifuged at 9000g for 10s following which 0.3ml of D/W water was added and centrifuged at 9000g for 10seconds. The upper phase was removed and discarded carefully. 0.3ml of methanol was now added to the lower phase and the protein was precipitated by centrifugation at 9000g for 2 minutes. The supernatant was removed while the pellet was air-dried and finally dissolved in rehydration buffer.

For detection of the phosphoproteins, the gels were incubated in fixation solution (50% methanol + 10% acetic acid + 40% Milli-Q water) for 30 minutes and kept on shaker. This step was repeated one more time. Then the gels were washed thrice in Milli-Q water for 10 minutes following which the gel was incubated in PRO-Q-DPS (1:3 dilution in Milli-Q water) for 90 minutes with gentle agitation. After this, the gels were washed three times using destaining solution (50 mL of 1M sodium acetate (pH 4.0) + 750 mL milli-Q-water + 200 ml acetonitrile) for 30 mins each. Then the gels were stained by using Sypro Ruby stain for overall proteome detection. There is no need of fixation since we have already fixed the proteins. After that, the gels

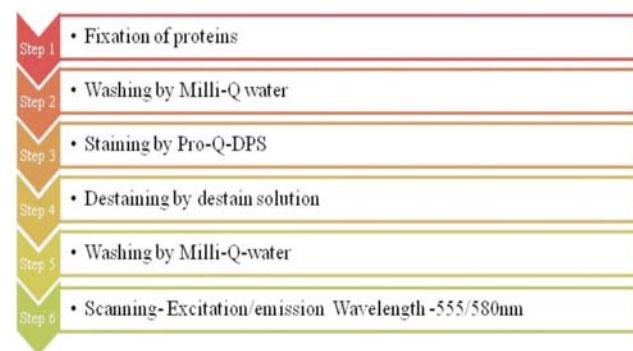


Figure 1.28: Workflow of phospho staining of 2D gels using Pro-Q-Diamond

were stained with the Sypro Ruby (1:3 diluted) stain for overnight. Then the gels were washed by using the wash solution (10% methanol + 7% acetic acid + 83% of milli-Q-water) for 30 minute Finally, the gels were rinsed twice with Milli-Q water and scanned by using typhoon 9200 (parameters: PMT_750, pixels_100 μ m, excitation/emission_ 450/610nm).

Results

Comparison of healthy control and severe *falciparum* malaria serum phosphoproteome using Pro-Q-Diamond

The serum proteome of 27 severe *falciparum* patients was compared to 45 healthy controls by using classical 2-DE stained by Pro-Q-Diamond. The whole experiment was carried out in three biological replicates. Each biological replicate included 9 severe *falciparum* malaria patients and 15 healthy controls. There were 5 significant spots observed in case of *falciparum* malaria whereas there were no corresponding spot at the corresponding position in healthy controls. However, spots at the corresponding positions were present in healthy control gel stained by Sypro Ruby (Figure 2 and 3), indicating that, these proteins were found to be non-phosphorylated in

healthy control whereas phosphorylated in *falciparum* patients.

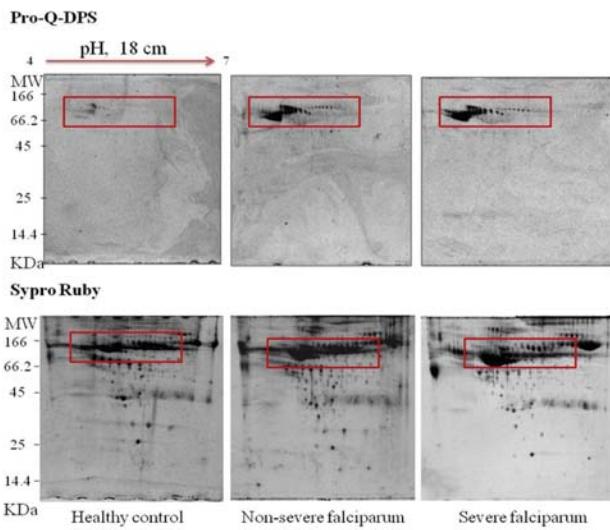


Figure 1.29: Detection of phospho proteins in healthy subjects and malaria patients sera. Phosphoproteins in the malaria and control sera were detected by staining the 2D gels using a Pro-Q® Diamond Phosphoprotein Gel Stain, while the total proteome was visualized by staining the same gels with SYPRO Ruby Protein Gel Stain.

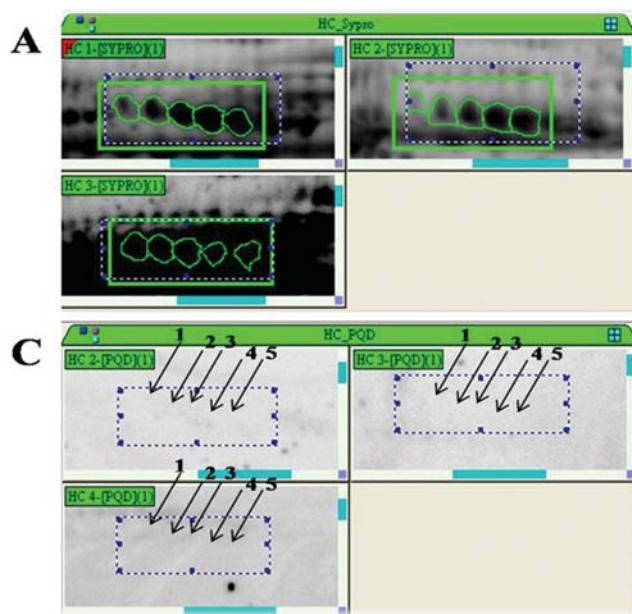


Figure 1.30: Phosphoproteome pattern in serum of HC and SFM patients. A) HC gel stained by Sypro Ruby B) SFM gel stained by Sypro Ruby C) HC gel stained by Pro-Q-DPS D) SFM gel stained by Pro-Q-DPS. [HC: healthy control, SFM: Severe falciparum malaria]

Comparison of non-severe and severe *falciparum* phosphoproteome using Pro-Diamond

The serum proteome of 27 severe *falciparum* patients compared with 42 non-severe *falciparum* malaria patients by using classical 2-DE stained by Pro-Q-

Diamond. The whole experiment was carried out in three biological replicates. Each biological replicate subjects 9 severe *falciparum* patients and 14 non-severe *falciparum* malaria. There was no significant difference of phosphoproteome between non-severe and severe *falciparum* malaria.

Discussion

In this study, we identified some proteins on the Pro-Q-Diamond stained 2D gels, which are phosphorylated in *falciparum* patients as compared to that of healthy controls. But, there were no significant differences in phosphorylation level of proteins in case of severe and non-severe *falciparum* malaria. The identification of these spots revealed by in-gel digestion then followed by mass spectrometry.

The expression level of hemopexin was found to be up-regulated in case of severe *falciparum* malaria with respect to healthy control. The binding strength of hemopexin with hemoproteins is very high [3] It forms the complex with heme proteins and induces intracellular antioxidant activities. Since during the malaria infection, hemoglobin degradation produces globin free heme and certain hemoproteins, which can act as active triggers of low-density lipoprotein (LDL) peroxidation [4] Hemopexin also plays a very important role in protecting apolipoprotein B and LDL from oxidation.

References

- Schulenberg B, Goodman N, Aggeler R, Capaldi A, Patton F. Characterization of dynamic and steady-state protein phosphorylation using a fluorescent phosphoprotein-gel stain and mass spectrometry. *Electrophoresis* 2004; 25, 2526-2532.
- Wessel D, Flugge I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 1984; 138, 141-143.
- Thurnham I, Singkamani R. The acute phase response and vitamin A status in malaria. *Trans. R. Soc. Trop. Med. Hyg.* 1991; 85, 194-199.
- Das S, Thurnham I, Das B. Plasma alpha-tocopherol, retinol, and carotenoids in children with falciparum malaria. *Am. J. Clin. Nutr.* 1996; 64, 94-100.

Application Note 3

Application of Gel-based Proteomics in Investigation of *Plasmodium falciparum* 3D7 proteome

Komal Pawar, Prashanti Patel, Sandipan Ray and Sanjeeva Srivastava

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, E-mail: sanjeeva@iitb.ac.in

This study was aimed to characterize and analyse the *P. falciparum* proteome for profile several proteins. Also the intent was to map the proteome of the parasite indifferent stages of *P. falciparum* life cycle.

Methodology: The study was done using several proteomics approach including 2D-DIGE and Mass spectrometry.

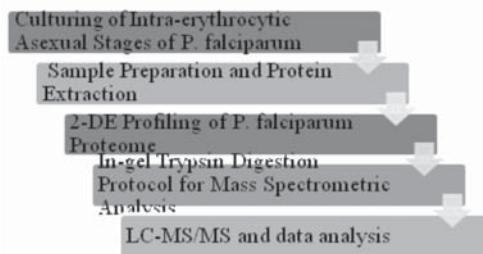


Figure 1.31: Overall workflow utilizing multiple proteomics approaches

Selective results

Optimization of Protein Extraction Protocol for 2-D Electrophoresis of *P. falciparum*

Proteome analysis of *P. falciparum* comes with large number of technical difficulties since many of the *Plasmodial* proteins are comparatively insoluble and highly charged (Smit S. et al., 2009). Thus it is essential to optimize the lysis buffer composition to ensure higher solubility of the proteins. But, at the same time care should be taken for the presence of excessive salts, detergents, or other contaminants which can greatly influence the electrophoretic separation of proteins in gel-based techniques and such impurities should be removed or minimized during sample processing.

Different protein extraction protocols were tested to obtain the optimum results which includes TCA-acetone precipitation, SDS treatment followed by sonication, trizol treatment. However, optimal results were only obtained when the samples were processed with 2D-Cleanup Kit (GE Healthcare)

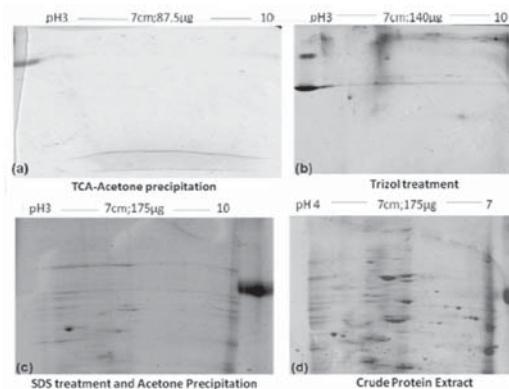


Figure 1.32: Comparison of protein extraction protocols tested during optimization. There were many issues in sample preparation as seen on the gels. Yield of protein with TCA acetone precipitation (a) was very less and thus proteins were not detected by coomassie blue stain. Also, with Trizol treated (b) and SDS treated sample (c), significant streaking and gaps were observed. Among these, crude protein extract (d) gave better result and thus was used for further experiments.

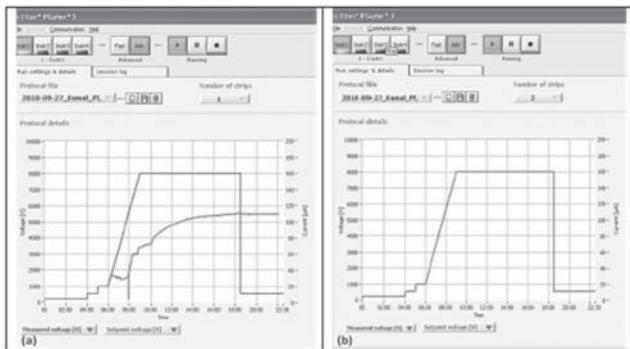


Figure 1.33: Effect of desalting on IEF. There was significant improvement in IEF after desalting the sample (b). Desalting helps in cleaning the sample and helps to reach higher voltage which could not be reached with crude sample (a).

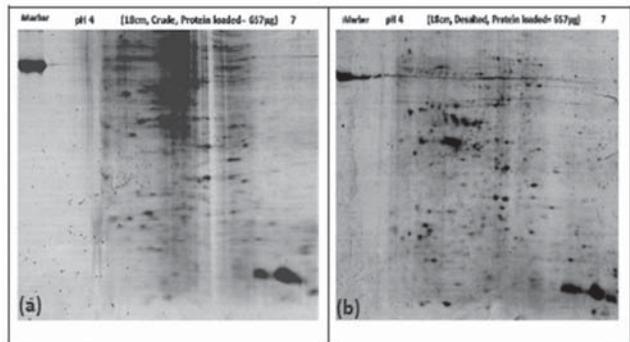


Figure 1.34: Effect of desalting on protein separation and gel quality. With crude sample (a), proteins were poorly resolved because of improper IEF. While desalted sample (b) had far better separation on the gel.

Optimization of IEF Protocol

The proteomic analysis of *Plasmodium falciparum* is extremely difficult due to the complexity of the parasite proteome (Smit, S et al., 2009). As a result, there was need to optimize IEF protocol for better separation of proteins in first dimension. An additional step of 200V for 4hrs was incorporated in the beginning of IEF and the total Vhs was increased from 16800Vh to 27550 Vh (for 7cm IPG strip, corresponding improvements were made for respective strip lengths). These modifications considerably improved separation of proteins and also reduced both horizontal and vertical streaking.

Determination of Effect of pH Range of IPG Strip

Proteome profiling of *P. falciparum* strain 3D7 was performed using immobilized pH gradient (IPG) strips of pH ranges 4-7 and 3-10.

Determination of the Effect of IPG Strip Length

Optimization of protein extraction protocol was followed by selection of optimum length of IPG strip

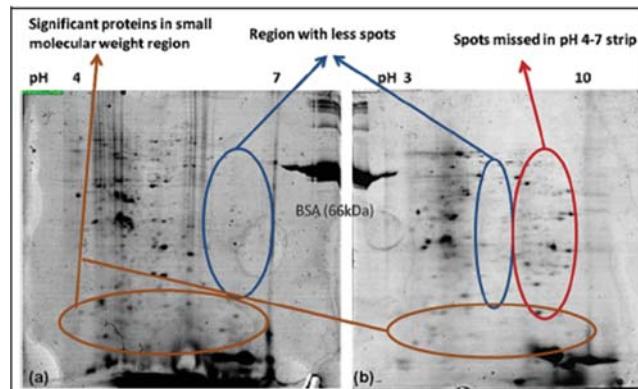


Figure 1.35: Effect of pH range on proteome profile of *P. falciparum*. Using IPG strip of pH range 4-7(a), better separation was observed but alkaline proteins were resolved using pH range 3-10. (b).

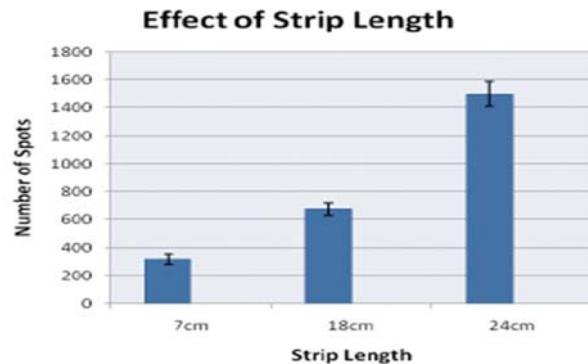


Figure 1.36: Graphical representation of effect of strip length on number of spots visualized in 2D gels. Data is represented as mean \pm SEM, where n=3.

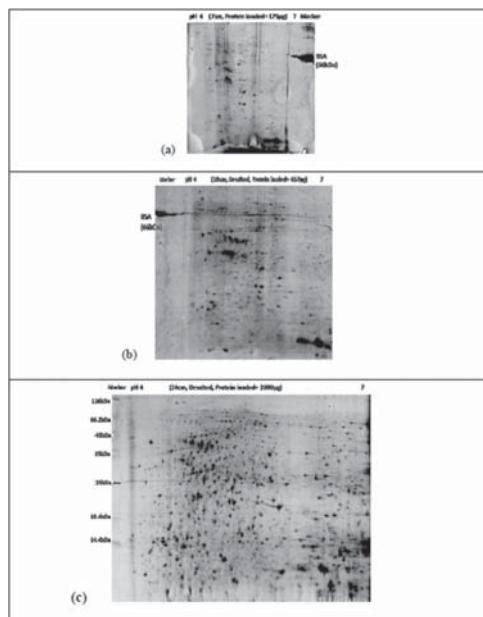


Figure 1.37 : Effect of strip length on 2-DE profile of *P. falciparum*. IPG strips of pH range 4-7 and length 7cm (a), 18cm (b) and 24cm (c) were used to determine the optimum length to study proteome of *P. falciparum*.

length to achieve maximum coverage and best resolution. IPG strips of length 7cm, 18cm and 24cm were used to check the coverage and resolution. It is obvious that longer strip lengths will give better resolution. Nonetheless, in case of some samples, 18cm strip provides sufficient resolution; in that case, 24cm strip is not required. For *P. falciparum*, it was observed that, 24cm strip gave best separation of proteins. The rest of the experiment was done using 24cm strip.

Discussion

The above mentioned optimizations helped in gaining insight into the parasite proteome and also strengthened the prominence of gel based tools in such studies.

Optimization for 2-DE of *Plasmodium falciparum* strain 3D7

2-DE has ability to resolve the proteome at any particular time, which is distinctly advantageous for multistage organism like *P. falciparum*. Among the proteome investigation techniques, 2-DE has attracted significant attention (Wang, P. et al., 2009) due to many advantages such as, information on protein size, quantity, isoforms with different pIs, and good resolution of abundant proteins (Lopez, M. F. et al., 2000). The first step for the current study was to optimize the protocol for 2-DE of *P. falciparum* to achieve maximum coverage of the proteome.

Optimization of Protein Extraction Protocol

Proteins of *Plasmodium* are notoriously insoluble, comparatively large, non-homologous and highly charged (Birkholtz et al., 2008). Thus for the maximum solubility of the proteins, composition of lysis buffer is extremely crucial. Lysis buffer modified from Nirmalanet al. is able to solubilize a large portion of *Plasmoidal*proteins (Smit et al., 2009). In this study, use of 5 times less saponin (for lysing RBCs), and increased washing steps helped in efficient removal of hemoglobin and other human proteins. Also, shorter sonication cycles with prolonged cooling time in-between cycles helped in maintaining the integrity of proteins giving them very less chance to degrade. Although, the proteome coverage was improved but there were difficulties in both IEF and resolution of proteins. This was overcome by cleaning the sample with 2D Clean-Up Kit (GE Healthcare) which drastically improved the IEF and gel quality in terms of separation of proteins.

Conditions for 2-DE

One of the crucial steps for 2-DE is the IEF. This is the first step where proteins are separated according to their pIs. IEF settings were modified than those

previously used in literature. There was considerable improvement in IEF by addition of a step at the beginning of IEF (200V for 4hrs) which helped in removing all the salts and impurities which hampered the IEF process previously. Also, increasing the total Vh from 16800 to 27550Vh (for a 7cm strip, and respective changes for larger strips) helped in better separation of proteins which, were otherwise not resolved and showed significant streaking in the gel.

Also, the protein profiles were checked using 4-7 and 3-10 pH range IPG strips. Interestingly, it was observed that there were many alkaline proteins which were separated using IPG strip of pH range 3-10, but, better separation of proteins was observed using pH 4-7 strip. Narrow range IPG strips can be used for maximum separation and full coverage of proteins. With pH range, strip length is also essential for optimum separation of proteins. In some cases, smaller lengths of strips (18cm, 11cm, etc.) are enough to get a well resolved proteome profile, but in case of complex samples like *Plasmodium*, higher strip lengths are essential. It was observed that, 24cm IPG strip offered best results in terms of resolution of proteins.

Conclusion

The above work provides as a fine case study where gel based proteomic techniques played a crucial role in deciphering the proteome of a clinically significant organism and the various technicalities involved .It also has several optimisation steps that can serve as a case study for anyone who is set for using the platform of 2DE, 2D-DIGE for their questions. Protein extraction procedures, IEF conditions, strip length and other parameters do influence the results and hence needs to be optimised in a case specific manner.

References

1. Cao, J. H., and A. P. Geballe. 1995. Translational inhibition by a human cytomegalovirus upstream open reading frame despite inefficient utilization of its AUG codon. *J. Virol.* 69:1030-1036.
2. NirmalanNiroshini, Paul F. G. Sims and John E. Hyde. 2004. Quantitative proteomics of thehuman malaria parasite *Plasmodium falciparum* and its application to studies of developmentand inhibition. *Molecular Microbiology*, 52(4), 1187–1199.
3. Smit, S; Stoychev, S; Louw, A.I; Birkholtz, L.M. 2009. Proteomic Profiling of Plasmodiumfalciparum through Improved, Semiquantitative Two-Dimensional Gel Electrophoresis.Journal of Proteome Research 9:2170–2181.
4. Wang, P.; Bouwman, F. G.; Mariman, E. C. M. Generally detected proteins in comparativeproteomics - A matter of cellular stress response. *Proteomics* 2009, 9, 1–12.

5. Wang, P.; Bouwman, F. G.; Mariman, E. C. M. Generally detected proteins in comparativeproteomics - A matter of cellular stress response. *Proteomics* 2009, 9, 1–12.

Application Note: 04

Bacterial proteomics: Protocol standardization for gel-based proteomics

Panga Jaipal Reddy, Saurabh Kumar and Sanjeeva Srivastava*

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, *Email: sanjeeva@iitb.ac.in

Proteomic analysis can unravel the functional genomic information; hence it has evolved as an advanced discipline in the post genomic era, providing insights into the regulation of complex biological systems [1]. Two-dimensional electrophoresis (2-DE) is one of the powerful proteomic tools, which is used to study high-throughput protein expression profile as a function of biological variation. Though many gel-free technologies have evolved over the last decade, 2-DE continues to be largely preferred due to its ease, low-cost, multi-sample analysis and quantification capabilities. 2-DE and mass spectrometric identification work together for comprehensive proteome analysis [2]. Besides its advancement, 2-DE has some inherited problems such as low proteome coverage and poor reproducibility. Cyanine labels were used to tag the proteins before resolving on 2D-PAGE and it is called as differential in gel electrophoresis (DIGE).

B. subtilis is a gram positive model organism having ~4200 ORFs coded for various proteins and DNA microarray experiments revealed expression of ~2500 genes under standard conditions. The comprehensive proteome of *B. subtilis* under different environmental conditions were available in literature [3]. Totarol is a natural diterpenoid has ability to restrain the growth of *B. subtilis* by targeting the cell division machinery and leads to the filamentous morphology. In present study, we have performed differential expression analysis of *B. subtilis* under totarol treatment at two different time intervals. For any proteomic experiment, the protein extraction protocol is the crucial and we optimized our own protocol from literature [4].

Methodology

B. subtilis was grown in Luria Broth (LB) overnight from the glycerol stock and re-inoculated into fresh culture and grown further. Totarol (Industrial Research Limited, New Zealand) (1.5 uM) was added to the culture after reaching the OD to 0.2 and the samples were collected at 20 min and 60 min treatment. The

untreated control was prepared simultaneously treated with only DMSO. The cultures were harvested and washed with PBS buffer (pH-7.4) for 4 times for protein extraction.

Protocol -I: Bacterial pellet was re-suspended in extraction buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4 and 5 mM MgCl₂) having protease inhibitor cocktail. Bacterial lysis was carried out using sonication (30 cycles for 3 times at 40% amplitude). To the lysate, 44 U Benzonase was added and incubated at RT for 30 min to remove the DNA. Centrifuge the mixture to remove cell debris and to the supernatant 4 volumes of chilled TCA-acetone with 20mM DTT was added and keep at -20°C for 4 hrs. Protein pellet was collected after centrifugation and re-dissolved in rehydration buffer (8 M urea, 2% CHAPS, 1% IPG buffer and BPB).

Protocol-II: Bacterial pellet is re-suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) having protease inhibitor cocktail. Bacterial lysis is carried out by addition of 2% SDS followed by heating the sample at 95 C for 5 minute Centrifuge the mixture and collect the supernatant.

Protocol-III: Bacterial pellet is re-suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) having protease inhibitor cocktail. Bacterial lysis is carried out by addition of 1 mg/ml lysozyme and incubates at RT for 30 minute Further bacterial lysis is carried out using sonication (30 cycles for 3 times at 40% amplitude). Centrifuge the mixture and collect the supernatant.

To the supernatant obtained from both protocol-II and Protocol-III was processed separately using TRIzol method. To the supernatant, TRIzol (1 mL for 20mL culture) and chloroform (200 μ L for 1 mL TRIzol) was added and shake vigorously for 15 sec. Centrifuge the mixture at 12,000 rpm for 15 min at 4 C and to the bottom pink layer, add ethanol (300 μ L for 1 mL TRIzol) to precipitate DNA. To the supernatant after DNA removal, 4 volumes of chilled acetone was added and incubated at -20°C for 30 minute Protein pellet is washed with 0.3 M guanidium isothiocyanate in 95% ethanol for 4 times to remove residual phenol. Protein pellet was dissolved in rehydration buffer (8 M urea, 2M thiourea, 2% CHAPS, 1% IPG buffer and BPB).

Protein extracted from Protocol-III was used for rehydration of IPG strips having different lengths (7, 11, 18 and 24 cm linear IPG strips with 4-7 pH range). The amount of protein loaded on IPG strips depends on length of the strip and we used 120 μ g for 7 cm, 200 μ g for 11 cm, 400 μ g for 18 cm and 600 μ g for 24 cm. Passive rehydration was performed for 12 hrs for 7 and 11 cm strips and 14 hrs for 18 and 24 cm strips. The focusing was performed using IPGphor and the IEF parameters were also standardized for different strip

lengths. Overall voltage applied is 27,000 V/12 h for 7 cm, 44,000 V/ 12 h for 11 cm, 80,000 V/17 h for 18 cm and 93,000 V/18 h for 24 cm IPG strip.

The focused IPG strips were subjected to 1st equilibration solution (8M urea, 1.5 mM tris-HCl (pH 8.8), glycerol, SDS and DTT and BPB) and 2nd equilibration solution (8M urea, 1.5 mM tris-HCl (pH 8.8), glycerol, SDS, IAA and BPB) for 15 each and resolve the proteins on 12.5% SDS-PAGE. The protein spots were visualized using PhastGelTM Blue R (GE healthcare) and gels were scanned using LabScan software from GE life science.

Protein extracted from control, 20 min totarol treated and 60 min totarol treated using Protocol-III was used for CyDye labeling. The control protein (60 µg) was labeled with Cy3 (400 pmol) and totarol treated protein (60 µg) was labeled with Cy5 (400 pmol) and internal control having equal amount of control (30 µg) and totarol (30 µg) treated protein was labeled with Cy2 (400 pmol) for 1 hr in dark. The unlabeled dyes were quenched with 10 mM lysine. Prior to labeling, the pH of the protein adjusted to 8.5 using 100 mM NaOH. The labeled proteins were pooled and rehydrated on 24 cm IPG strip of pH 4-7. The isoelectric focusing (IEF) and second dimension was performed similarly as mentioned above for 24 cm IPG for conventional 2-DE. The labeling was performed separately for both 20 min and 60 min totarol treated samples. The gel images were obtained using Typhoon FLA9500 scanner (GE healthcare) and analysed using DeCyder software 7.0 (GE healthcare).

Differential expressed proteins obtained from DIGE analysis were excised from preparative gels and subjected to in-gel digestion as reported by Reddy et al. The protein identification was performed using 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Framingham, MA) linked with 4000 series explorer software (v.3.5.3) and the data was analyzed using MASCOT version 2.1 (<http://www.martixscience.com>) search engine. The analysis parameters: Enzyme-trypsin, single missed cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification.

Result and Discussion

Gel based proteomics is one of the best approach to attain comprehensive proteome analysis. Though microbial proteome is less complex, protein extraction protocol is essential to get the good proteome coverage. Three different sample processing protocols have been implicated for bacterial protein extraction. Comparison of three protocols has revealed that protocol-I have given 100 ± 10 spots, protocol-II has given 120 ± 10 and protocol-III has given 150 ± 10 protein spots on 7 cm 2-DE gel. Protocol-III has showed good spot number,

resolution and quality among the other protocols tried (Figure 1 (I)).

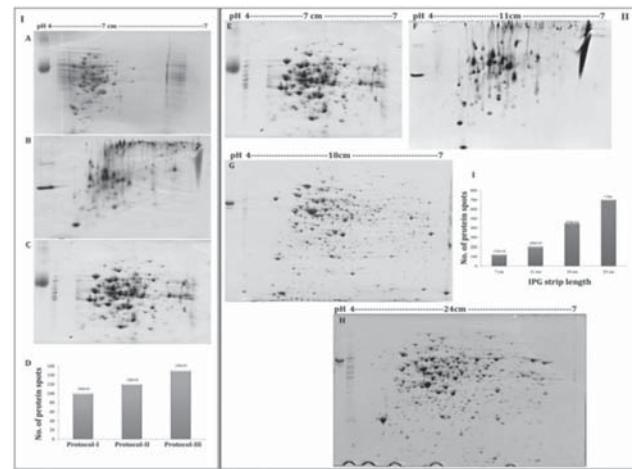


Figure 1.38: (I) Selection of protein extraction protocol for bacterial proteomics using 7 cm IPG strip (pH 4-7) by loading 120 µg proteins. [A] Extraction buffer-Benzonase and TCA-acetone precipitation [B] 2% SDS and TRIzol reagent for protein extraction [C] Lysozyme and TRIzol reagent for protein extraction [D] the bar graph showing the protein spot number. (II) Examination of the effect of strip length on the 2-DE profile of bacterial proteome using lysozyme and TRIzol protein extraction. [E] 7 cm, [F] 11 cm [G] 18cm strip [H] 24 cm IPG strips (pH 4-7) were used in the experiment [I] the bar graph showing the protein spot number in different strip length.

We have separated the bacterial proteome extracted using protocol-III on different IPG strip lengths (7, 11, 18 and 24 cm, linear 4-7 pH). As we expected, the number of protein spot, resolution and quality of the gels improved significantly as the strip length increased. The number protein spots identified in 7 cm, 11 and 18 cm were ~150, ~200 and ~450 spots respectively and better coverage has been obtained using 24 cm strips with more than 1000 spots. We concluded that 24 cm IPG strips with pH 4-7 will give better results with protocol-III and the same protocol is used for differential proteome analysis of *B. subtilis* under totarol treatment (Figure 1 (II)).

2D-DIGE images obtained from 20 min and 60 min totarol treated samples has showed very good reproducibility, resolution and coverage. The DIGE images were analyzed using DeCyder software (GE healthcare) by creating separate projects for both 20 min and 60 min totarol treated samples. Differential in gel analysis (DIA) has showed nearly 2000 protein spots across all the gel images with good reproducibility. Protocol-III has given high quality data with negligible streaking and getting 2000 spots on DIGE is well accepted. Protocol-III doesn't require any additional clean-up steps where many literatures reported 2-DE protein extraction protocols were associated with clean-up steps. Biological variation

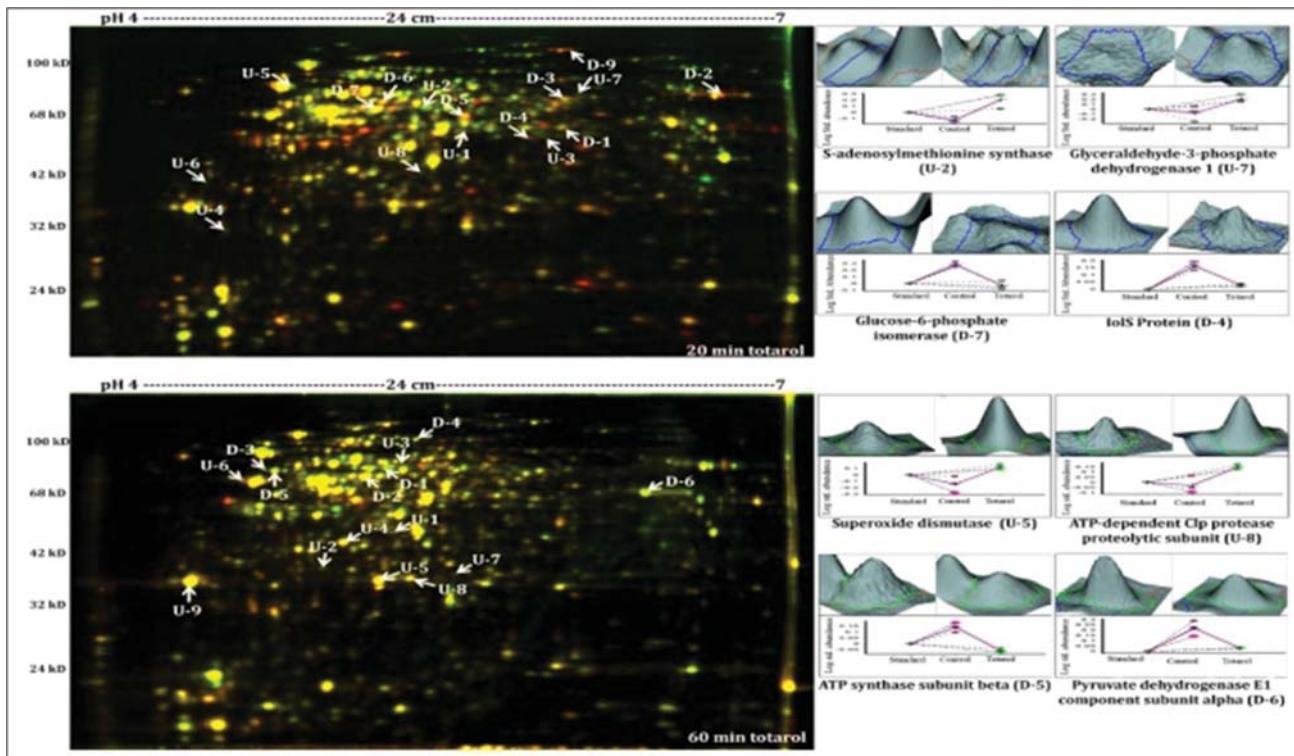


Figure 1.39: Representative combined DIGE gel images for 20 min and 60 min totarol treated *B. subtilis* proteome. 3D views of the selected differentially expressed proteins ($p < 0.05$) identified in DIGE and its BVA graphs. Data is represented as mean \pm SE (where $n = 3$).

analysis (BVA) has highlighted differential expression of 16 (8 up regulated and 8 down regulated) and 15 proteins spots (6 down regulated and 9 up regulated) in 20 and 60 min totarol treated samples with statistical significance. The entire differential expressed protein spots in 20 and 60 min has been identified using MALDI-TOF/TOF analysis. Most of the differential expressed is involved in central metabolism for energy generation (Table 1).

The quality of proteomic experiment is purely depends on protein extraction. In gel-based proteomics, the protein extraction method should be very important because of the isoelectric focusing (IEF) is very sensitive to many interfering agents. If the extraction method doesn't remove these, interfering agents, the quality of the output will be terrible. Protocol-III is the ideal protocol where it will remove the most important interfering agents such as DNA, RNA, lipids and polysaccharides because of phenol, chloroform, ethanol etc. The acetone precipitation and guandium-HCl washing will remove the unwanted salts. The complete protocol end up with pure protein with no interfering agents and give no chance to additional clean-up steps. Here we presented the feasibility of protocol-III for *B. subtilis* comprehensive proteome profile using 2-DE and DIGE with good resolution and reproducibility.

Table 1
Details of identification of differential expressed proteins

S. No	UNIPROT ID	Name of the protein	Score
20 min totarol			
D-1	P94521	Putative aminopeptidase ysdC	228
D-2	P21879	Inosine-5'-monophosphate dehydrogenase	283
D-3	P94391	1-pyrroline-5-carboxylate dehydrogenase 2	823
D-4	P46336	IolS Protein	878
D-5	Q08352	Alanine dehydrogenase	1020
D-6	P18157	Glycerol kinase	637
D-7	P80860	Glucose-6-phosphate isomerase	704
D-8	P23129	2-oxoglutarate dehydrogenase E1 component	703
U-1	P09124	Glyceraldehyde-3-phosphate dehydrogenase 1	318
U-2	P54419	S-adenosylmethionine synthase	460
U-3	P37253	Ketol-acid reductoisomerase	1240
U-4	P80864	Probable thiol peroxidase	524
U-5	P17820	Chaperone protein dnaK	304
U-6	O32114	Diaminopimelate epimerase	109
U-7	P09124	Glyceraldehyde-3-phosphate dehydrogenase 1	238
U-8	P42976	Dihydronicotinate reductase	154
60 min totarol			
D-1	P37808	ATP synthase subunit alpha	360
D-2	P80860	Glucose-6-phosphate isomerase	704

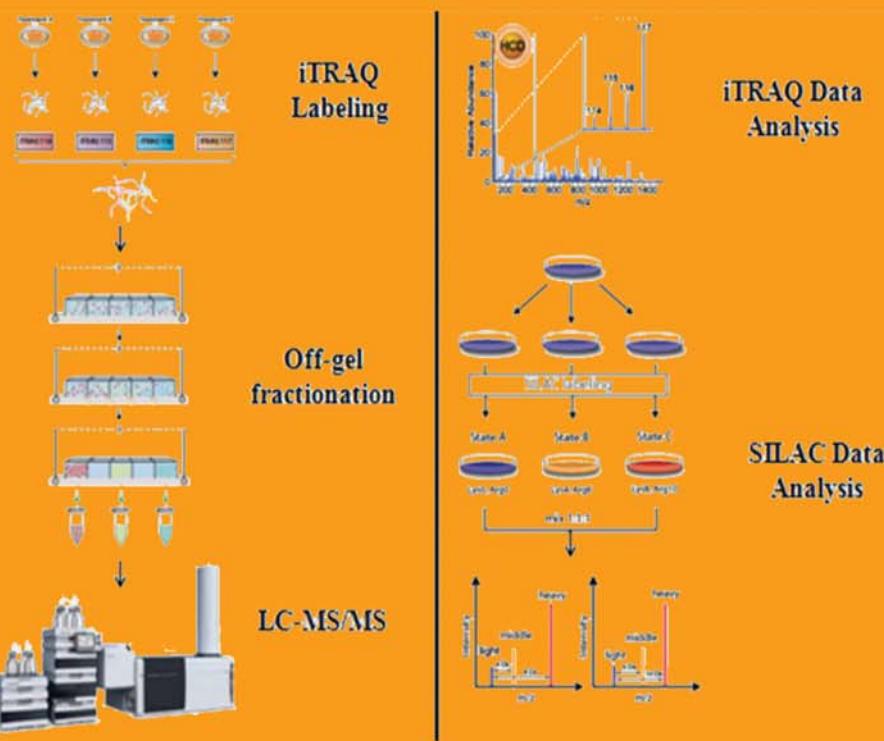
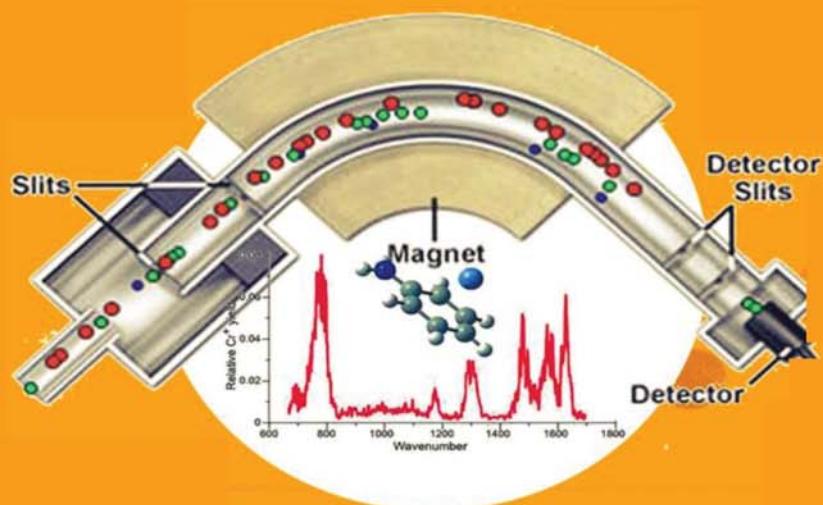
D-3	P37809	ATP synthase subunit beta	954
D-4	O07631	GTP-binding protein TypA/ BipA homolog	377
D-5	P37809	ATP synthase subunit beta	535
D-6	P21881	Pyruvate dehydrogenase E1 component subunit alpha	666
U-1	P13243	Probable fructose-bisphosphate aldolase	70
U-2	P39121	Deoxyribose-phosphate aldolase	55
U-3	O32162	FeS cluster assembly protein suffB	368
U-4	P27876	Triosephosphate isomerase	564
U-5	P54375	Superoxide dismutase [Mn]	326
U-6	P37869	Enolase	494
U-7	O31749	Uridylate kinase	112
U-8	P80244	ATP-dependent Clp protease proteolytic subunit	171
U-9	P80239	Alkyl hydroperoxide reductase subunit C	280

References

- Fields S. Proteomics. *Proteomics in genomeland*. *Science* 2001;291(5507):1221-4.
- Curreem SO, Watt RM, Lau SK, Woo PC. Two-dimensional gel electrophoresis in bacterial proteomics. *Protein Cell* 2012;3(5):346-63.
- Büttner K, Bernhardt J, Scharf C et al. A comprehensive two-dimensional map of cytosolic proteins of *Bacillus subtilis*. *Electrophoresis* 2001;22(14):2908-35.
- Jaiswal R, Beuria TK, Mohan R, Mahajan SK, Panda D. Totarol inhibits bacterial cytokinesis 1. Fields S. *Proteomics. Proteomics in genomeland*. *Science* 2001;291(5507):1221-4.

MASS SPECTROMETRY

(Module II)



Mass Spectrometry

Preface

Kishore Gollapalli¹, Saicharan Ghantasala¹ and Srikanth Rapole²

Indian Institute of Technology Bombay, Mumbai¹ and National Centre for Cell Science, Pune²

Conventional proteomic techniques such as 2DE, 2D-DIGE played an important role in the early part of proteomics research during the 1960s-70s. With the advent of GC-MS a large number of compounds could be studied by exploiting the separation ability of GC and detection ability of MS. However, the major shortcoming of this technique was that it could be applied only for volatile compounds and could not be used for studies pertaining to biological compounds. The emergence of LC-MS in the early 1990s paved way for a revolution in the field of proteomics and has in the last decade emerged as the most advanced and superior platform in the field of proteomics owing to its diverse applications.

LC-MS is a hyphenated technique that combines the separation ability of HPLC with the superior detection ability of a mass spectrometer. A typical LC-MS instrument comprises of a liquid column chromatography system to separate sample components before they enter the mass spectrometer where the ions separate in their gaseous phase based on differences in m/z (mass to charge) ratio. The mass spectrometer in turn consists of an ionization source, an analyzer, a detector and a software computing system. The different commercially available LC-MS systems differ from each other in their use of each of the above mentioned components and techniques.

Irrespective of the separation system, i.e., HPLC, capillary LC or flow injection there is always a need to generate gas phase ions by eliminating the solvent before components enter a mass spectrometer. Commercially available instruments employ either atmospheric pressure ionization (API) technique or electron impact ionization (EI) to eliminate the solvent and generating gas phase ions. While the API technique involves generation of ions and elimination of the solvent simultaneously at atmospheric pressure, the EI technique involves separate steps for elimination of solvent and generation of ions.

Different sources for ionization include the electro spray ionization (ESI), the atmospheric pressure chemical ionization (APCI) and the atmospheric pressure photo ionization (APPI). Depending on the sample type, different ionization sources offer different levels of performance.

The analyzer forms the most important component of the instrument and is responsible for separation of

the various ions resulting in better coverage. The quadrupole analyzer is the most common of all known analyzers and employs two pairs of metallic rods with one set of rods at a positive electrical potential while the other at a negative electrical potential. These analyzers can either be single quadrupole or triple quadrupole configuration and vary greatly in their applications and capability.

TOF analyzer involves a time offlight tube through which charged ions accelerate in the presence of an applied voltage. The velocities of various ions and the time taken to reach the detector depend on their m/z ratio. This analyzer acquires spectra extremely quickly and offers high sensitivity and mass accuracy in determining molecular formulas of small molecules.

Ion trap analyzers trap ions in a 3 dimensional space by employing hyperbolic electrodes using radio and static frequency. The sequential ejection of the ions on the basis of m/z ratio results in a mass spectrum. This analyzer also allows isolation of a specific ion and ejecting all the others besides allowing fragmentation and isolation of ions several times before generating the final mass spectrum.

Now-a-days hybrid mass analyzers are in use, which have great advantages over the individual mass analyzers.

Recent advancements in technology have resulted in enhancing the capabilities of LC-MS in proteomics. The various applications of LC-MS technology include-

- The bottom-up method for protein sequencing remains the most basic use of LC-MS and involves analysis of peptides obtained by enzymatic digestion against a protein database for peptide mass fingerprinting. This method however, cannot be used to study a protein in its native state since post translational modifications are not likely to be maintained during dissociation at peptide level.
- Quantitative LC-MS proteomics: Quantification of proteins helps in better differentiating and understanding the roles of different proteins in various diseased and normal conditions. LC-MS protein quantification falls in two categories: differential labeling or label free. Differential labeling can involve two main categories- those using chemical derivatisation or enzymatic modification of proteins or peptides and those involving incorporation of isotope-labeled amino acids *in vivo*. iTRAQ labeling, proteolytic ¹⁸O labeling, ICAT labeling and SILAC are a few commonly employed chemical labeling techniques for effective quantitation using LC-MS. One major shortcoming of quantitation using labels is that it can be applied only to a

few specimens, which can be a problem during statistical analysis of large number of clinical samples. Also, the methods such as protein derivatisation can be expensive and are likely to result in chemical variability thereby giving inaccurate results. Label free quantitation using LC-MS helps overcomes the shortcomings of quantitation using differential labels and is broadly classified into methods based on evaluation of ion currents and those based on spectral counting.

- Protein profiling of sub cellular organelles and protein complexes to give a picture of molecular functions.
- Quantification of proteins with various post translational modifications such as glycosylation, phosphorylation has led to understanding and deciphering various pathways in many diseases. Related approaches that quantify protein modifications such as acetylation or methylation are also likely to be developed thereby providing valuable biological insights.

The use of LC-MS has resulted in the emergence of activitomic approaches that have helped quantify various enzymatic activities. This has given rise to development of lipidomics, metabolomics and phosphoproteomics which have greatly improved our understanding of cancers which were beyond our scope until a few years back.

One of the most recent application of LC-MS is in the top-down proteomics where intact proteins are analyzed using high resolution MS and dissociated subsequently by a tandem mass spectrometer. This method provides almost 100% coverage for an entire protein sequence besides providing valuable information on posttranslational modifications since they remains largely intact even during dissociation.

Instructors and Speakers

David C. Muddiman

North Carolina State University, Raleigh, NC 27695, United States



Talk Title: Quantitative mass spectrometry imaging (QMSI) by a novel IR-MALDESI approach

Biography: David C. Muddiman is currently a Distinguished Professor of Chemistry and Founder and Director of the W.M. Keck FT-ICR Mass Spectrometry Laboratory at North Carolina State University in Raleigh, NC. Prior to moving his research group to North Carolina State University, David was a Professor of Biochemistry and Molecular Biology and Founder and Director of the Mayo Proteomics Research Center at the Mayo Clinic College of Medicine in Rochester, MN. Prior to his appointment at the Mayo Clinic, David was an Associate Professor of Chemistry at Virginia Commonwealth University where he began his academic career as an assistant professor in 1997 with an adjunct appointment in the Department of Biochemistry and Molecular Biophysics where he was also a member of the Massey Cancer Center. David was born in Long Beach, CA in 1967 but spent most of his formative years in a small town in Pennsylvania. David received his B.S. in chemistry from Gannon University (Erie, PA) in 1990 and his Ph.D. in Analytical Chemistry from the University of Pittsburgh in 1995 under the auspices of David M. Hercules. He then was a Department of Energy Postdoctoral Fellow at Pacific Northwest National Laboratory in the Environmental Molecular Sciences Laboratory working with Richard D. Smith from 1995-1997. Importantly, David had gained significant experience in the area of surface science during his graduate work with Professor Hercules and then electrospray ionization and Fourier transform ion cyclotron resonance mass spectrometry with Dr. Smith. He currently serves on the Editorial Advisory Board of *Mass Spectrometry Reviews*, *Molecular and Cellular Proteomics*, *Rapid Communications in Mass Spectrometry*, and the *Journal of Chromatography B*. David is also an Associate Editor for the *Encyclopedia of Analytical Chemistry*. He also serves on the advisory board of the NIH Funded Complex Carbohydrate Research Center, University of Georgia, National Science Foundation FT-ICR Mass Spectrometry Laboratory, National High Magnetic Field Laboratory, Florida State University and the Yale/NIDA Neuroproteomics Center, Yale University. He is currently a member of the ASMS Board of Directors (elected) and is the Treasurer and member of Executive Board of US-HUPO (elected). His group has presented over 500 invited lectures and presentations at national and international meetings

include 17 plenary/keynote lectures. His group has published over 200 peer-reviewed papers and has received four US patents. He is the recipient of the 2010 Biemann Medal, American Society for Mass Spectrometry, 2009 NCSU Alumni Outstanding Research Award, the 2004 ACS Arthur F. Findeis Award, the 1999 American Society for Mass Spectrometry Research Award, and the 1990-91 Safford Award, University of Pittsburgh, for Excellence in Teaching.

Dr. Philip Andrews

Medical School Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA



Talk Title:

Biography: Dr. Philip Andrews is a professor at Medical School Department of Biological Chemistry, University of Michigan. My laboratory has worked on protein structure by mass spectrometry computational methods extensively over the past 20 years and we have developed a number of new techniques for protein analysis with our main emphases being on post-translational modifications and protein interactions. I was trained in protein chemistry and enzymology at Purdue University and during a subsequent post-doctoral stint I applied my training to the application of mass spectrometry to protein structure. At the University of Michigan I expanded this experience to the large scale analysis of proteins by mass spectrometry. Computational and Bioinformatics research has always been a major component of my laboratory and we have extensive experience and infrastructure in the analysis and management of large proteomics data sets, in the management of complex projects, and development of online resources. My laboratory is primarily focused on the analysis of protein interactions of membrane proteins and the interactions of large complexes. We also work on phosphoproteome, general PTM analysis and pathway mapping. Many of our projects are collaborative in nature, underscoring our experience in bringing complex collaborative projects to successful conclusions. My laboratory has collaborated on a broad range of projects that involve identification of protein interactions and quantification of their changes. I also have extensive experience in proteomics, bioinformatics, and the management, analysis, and dissemination of large data sets in multi-institutional projects.

Rapole Srikanth

National Centre for Cell Science, NCCS Complex, Pune University Campus, Ganeshkhind, Maharashtra, Pune- 411007, India,

**Talk Title: Quantitative proteomic analysis using iTRAQ method**

Biography: Dr. Rapole Srikanth completed his master's degree in organic chemistry from Devi Ahilya University. He did his PhD in analytical chemistry from Indian Institute of Chemical technology (IICT), Hyderabad where he developed new mass spectrometry methods for analysing beta-carbo-peptides. He did his post-doc from University of Massachusetts (UMASS) on metal protein interactions and protein-protein interactions to understand the mechanism of protein aggregation and amyloid formation. After that, he worked as proteomics and mass spectrometry lab director for two years at University of Connecticut. Currently, he is working as a scientist at NCCS, Pune. His main research interest is to quantitatively identify the protein signatures involving in human diseases including cancer using state-of-the-art and highly sensitive mass spectrometry-based proteomic approaches. In addition, his group is also working to identify and quantify key metabolites and lipids associated with human diseases using mass spectrometry and bioinformatics. He is an active member of Indian society for mass spectrometry, American society for mass spectrometry and proteomics society of India. He has been published more than 40 publications in reputed international journals. He has received best paper award from CSIR in physical sciences. Recently, he has received DBT-Rapid grant for young investigators award.

Dr. Harsha Gowda

Institute of Bioinformatics, Discoverer Building, 7th Floor, International Tech Park, Pattandur Agrahara, Whitefield, Bengaluru, Karnataka 560066, India

**Talk Title: Quantitative proteomic analysis using SILAC**

Biography: Dr. Harsha Gowda did his Ph.D. at the Institute of Bioinformatics, Bangalore. During his Ph.D., he worked in Dr. Akhilesh Pandey's laboratory at Johns Hopkins University, USA on proteomic profiling of pancreatic cancers where his work involved proteomic approaches to study signaling pathways activated in pancreatic cancers and identification of novel biomarkers. In addition, he has worked as a visiting scientist in Dr. Gary Siuzdak's laboratory at

Scripps Center for Metabolomics and Mass Spectrometry, USA. He is a recipient of Wellcome Trust-DBT Early Career Fellowship which is awarded for the most promising young researchers in India. He is a reviewer for several international journals including *Journal of Proteome Research*, *Proteomics*, *Journal of Proteomics*, *Molecular Biosystems* and *International Journal of Cancer* and an Editorial Board member of *Journal of Proteomics*. At IOB, he is employing cutting-edge technologies in genomics, proteomics and metabolomics to investigate biomarkers and therapeutic targets for various cancers.

Catherine E. Costello

Boston University School of Medicine, Mass Spectrometry Resource, 670 Albany Street, Rm 511, Boston, MA 02118-2646, USA.

**Talk Title: Fundamental aspects of mass spectrometry**

Biography: Dr. Catherine E. Costello is a William Fairfield Warren Distinguished University Professor at Boston Univ. (BU), with appointments in Biochemistry, Biophysics and Chemistry. She earned her AB at Emmanuel College, Boston, and her MS and PhD at Georgetown Univ., Washington, DC and was a postdoctoral fellow and Senior Research Scientist at the Mass. Inst. of Technology. She is the Founding Director of the BU School of Medicine Center for Biomedical Mass Spectrometry. Her research addresses the development and application of MS-based methods for investigation of protein post-translational modifications, protein folding disorders, cardiovascular and infectious diseases, glycobiology and bioactive lipids. She has authored more than 320 scientific papers. She is Vice President of the International MS Foundation (IMSF) and a member of the Board of the US-Human Proteome Organization (HUPO). She is an American Chemical Society (ACS) Fellow, an ACS Councilor and current chair of its Northeastern Section. She has been President of the International HUPO and the American Society for MS. She serves on several editorial and advisory boards. She has received the Award for Discovery in Proteomics (HUPO), the Thomson Medal (IMSF) and the Field and Franklin Award (ACS).

Mayuri Gandhi

Centre for Research in Nanotechnology & Science, Indian Institute of Technology Bombay, Powai, Mumbai 400076, Maharashtra, India.

**Talk Title:**

Biography: Dr. Mayuri Gandhi is a research scientist in Centre for Research in Nanotechnology & Science, at Indian Institute of Technology Bombay, India. She did her master's from Mumbai University, India, and Ph.D. from Indian Institute of Technology Bombay, Mumbai, India. She did her Ph.D. in "Separation of Toxic Metal Pollutants from Environment by Extraction with Cryptands". She had experience in working with many sophisticated instruments like High resolution Liquid Chromatography Mass Spectrometer (HRLCMSMS), 300 MHz Nuclear Magnetic Resonance (NMR), Gas Chromatography Mass Spectrometer (GCMS), Inductively Coupled Plasma Atomic Emission (ICP-AES), Fourier Transform Infrared Spectrometer-Imaging (FT-IR & FTIR Imaging), Nanosecond Fluorescence Spectrometer (NSFS), Electron Spin Resonance Spectrometer (ESR), and Laser Raman Spectrometer (LRS).

Learning Objectives

- Introduction to mass spectrometry based proteomics
- Principles of in-gel and in-solution digestion
- iTRAQ labeling
- Principle of offgel fractionation
- Sample cleanup for LC-MS
- Peptide mass fingerprinting analysis
- MS/MS ion search
- iTRAQ data analysis

2.1. Pre-processing and quality control of samples: key for proteomic applications

Saicharan Ghantasala and Kishore Gollapalli

Indian Institute of Technology Bombay, Mumbai, India

The success of any proteomics experiment lies in the protein extraction method being followed and also the accuracy of the quantification method. Once the proteins are extracted and dissolved in buffer, the choice of the quantification method used depends largely on the buffer used. For example, Bradford assay is not compatible with extracts dissolved in rehydration buffer with more than 4 M urea and hence can result in inaccurate quantification values.

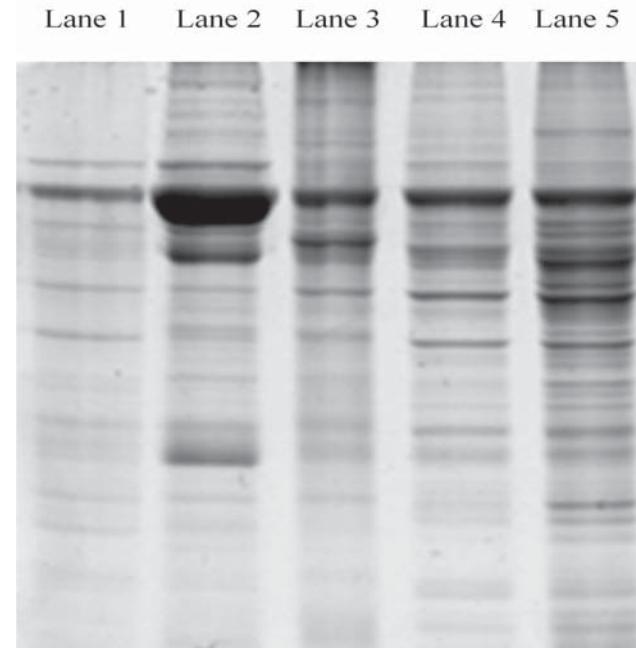


Figure 2.1: 12% SDS-PAGE image of different protein samples indicating the different possible variations that can affect the outcome of the experiment

SDS-PAGE profile of proteins helps in deciding the quality of samples and whether they are fit for further downstream processing. The gel image above (Figure 2.1) is the perfect example indicating how different variations either during extraction or quantification can be understood based on the profile. Lane 1 indicates a case of over quantification of the sample as a result of which the actual amount of sample loaded is quite less which is evident from the faint bands seen in the gel. Lane 2 indicates under quantification of the sample as a result of which the actual amount of protein loaded is much higher as seen in the top section of the lane. Lane 3 indicates that the protein extraction was not proper since only the high abundant bands are visible while the low abundant ones are hardly seen. Lane 4 indicates the variation among the clinical samples evident from the profile. Lane 5 indicates the result of good protein extraction evident from the bands seen.

Though SDS-PAGE gives a good idea of the quality of proteins extracted, approx. 10-15 µg of sample needs to be loaded for observing profile of proteins. In order to save such large amounts of protein from getting used up, instruments such as bioanalyser can be of great use since it is very accurate and uses relatively less amount of protein sample.

2.2. In-gel digestion protocol for mass spectrometry analysis:

Wash 1.5 ml snapcap microcentrifuge tubes with 500 µl methanol and 500 µl milli-Q water rinses prior to use.

Cut the protein bands into small pieces using razor/surgical blade and transfer into microcentrifuge tubes.

Materials

- 25mM Ammonium bicarbonate solution (25mM ABC)
- 50mM Ammonium bicarbonate solution (50mM ABC)
- 100mM Ammonium bicarbonate solution (100mM ABC)
- Solution A: Mixture of acetonitrile and 25mM ammonium bicarbonate in 2:1 ratio.
- 10mM Dithiothreitol (10mM DTT)
- 100mM Iodoacetamide (100mM IAA)
- Mass spec. grade trypsin (Conc. 100ng/ μ l)

Procedure

Rehydration and dehydration of gel pieces for destaining

- Rehydrate the gel pieces by adding 100 μ l of 25mM ABC and vortexing for 5 min.
- Remove supernatant and add 100 μ l of Solution-A and vortex for 5 min.
- Discard the supernatant and repeat the above two steps (addition of 25mM ABC and Solution-A two more times).

Note: Continue the washing steps until the gel pieces turns into transparent.

Reduction/Alkylation (Optional, if protein contains disulphide bonds only)

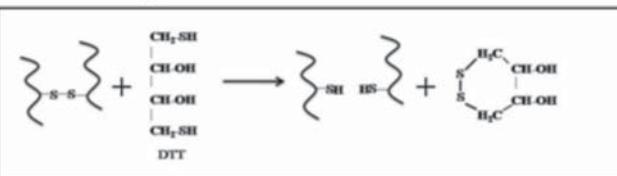
- After washing steps rehydrate gel pieces in 50 μ l of 10mM Dithiothreitol (DTT).
- Incubate the gel pieces at 56°C for 1hr.
- Then discard the supernatant and add 100 μ l of 25mM ABC, vortex for 5 min. and discard the solution.
- Add 50 μ l of 100mM Iodoacetamide and incubate at room temperature in dark conditions for 30min. (Refer figure 2.2).
- Discard the solution and wash the gel pieces twice with 25mM ABC and Solution-A alternatively.
- After washing the gel pieces, allow them to air dry.

Trypsin digestion

- Add 400ng of trypsin to gel pieces in each microcentrifuge tube and incubate on ice for 30 min. which allows the gel pieces to absorb the trypsin.

- After 30min. add 50 μ l of 50mM ABC to the gel pieces and incubate at 37°C for 16hr.

DTT reaction with protein



IAA reaction with protein

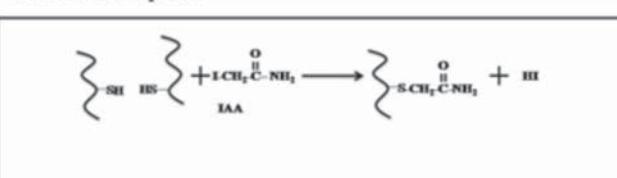
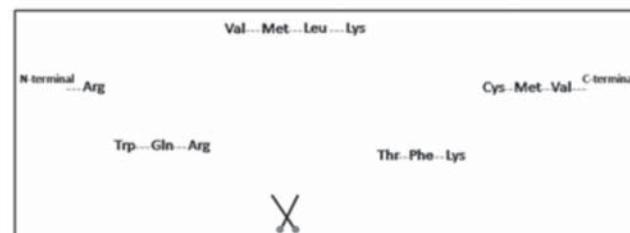
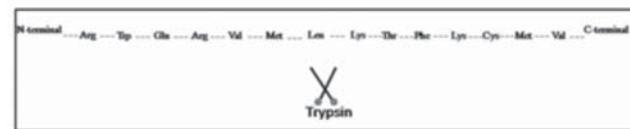


Figure 2.2: Reduction and alkylation of the proteins

Trypsin digestion



Peptide Extraction

- Collect the peptide solution from the overnight incubated gel pieces with trypsin.
- Add 100 μ l of extraction sol.1 containing 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) to the gel pieces and vortex for 10min. followed by collection of the peptide solution.
- Then add 60% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) to the gel pieces, sonicate for 1 min (2 sec pulse, 1 sec gap, 25% amplitude) and vortex for 10 min. followed by the collection of extracted peptide solution.
- Finally add 80% ACN and 0.1% TFA to the gel pieces and vortex for 10 min. followed by collection of peptide solution.
- Speed vac. the peptide extract to reduce the volume of the peptide sample, followed by concentrating the peptides using ziptipping process.

2.3. In-solution digestion

- Take 5-100 μ g of protein sample and add **1 μ l of denaturing solution** containing 2% SDS vortex and centrifuge the sample for 30 sec.
- Add **2 μ l of 50mM Reducing reagent** {tris-(2-carboxyethyl)phosphine (TCEP)} to the protein sample, vortex and spin the sample.
- Incubate the sample at 60°C for 1 hour. Spin the sample.
- Add **1 μ l of cysteine blocking reagent** (100mM Iodoacetamide) vortex and spin followed by incubation at room temperature for 10 min.
- Reconstitute the Trypsin in proper buffer (0.5M Triethyl ammonium bicarbonate buffer)
- Add Trypsin to the protein sample (1:20 or 1:50 ratio of Trypsin: protein) and incubate overnight (16 hrs.) at 37°C.

2.4. iTRAQ labeling

- iTRAQ reagents should be reconstituted in 70 μ l of ethanol.
- The reconstituted iTRAQ reagents were added to the respective in-solution digested peptide samples.
- The iTRAQ reagent was allowed to bind to the peptides by incubating at the room temperature for 1 hour.
- After completion of labeling process the labeling reaction was stopped by the addition of 100 μ l of MilliQ water to the sample vials.
- After quenching the reaction the labeled samples were pooled and subjected to offgel fractionation
- iTRAQ labeling mechanism is given in figure 2.4.

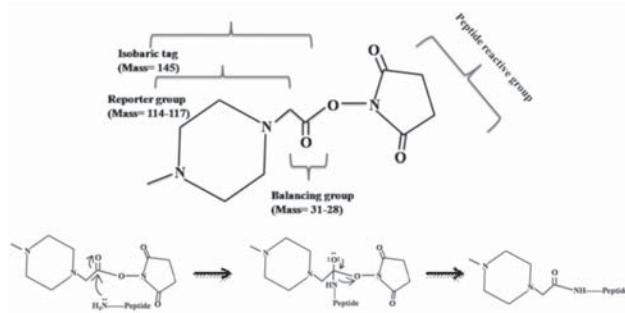


Figure 2.4: iTRAQ labeling mechanism

2.5. Offgel fractionation

- The pooled iTRAQ labeled peptide sample volume was made up to 3.6 ml using MilliQ water.
- High resolution IPG strips of pH 3-10NL (24 cm length) were subjected to rehydration by the

addition of 50 μ l of MilliQ water to the cups on the IPG strip. Passive rehydration was performed for 30 min. followed by the addition of 150 μ l of labeled peptide samples to each cup.

- After addition of peptide sample, the opening of the cup was sealed with a sealer.
- The peptide samples were allowed to separate based on their pI with a maximum voltage of 5000V.

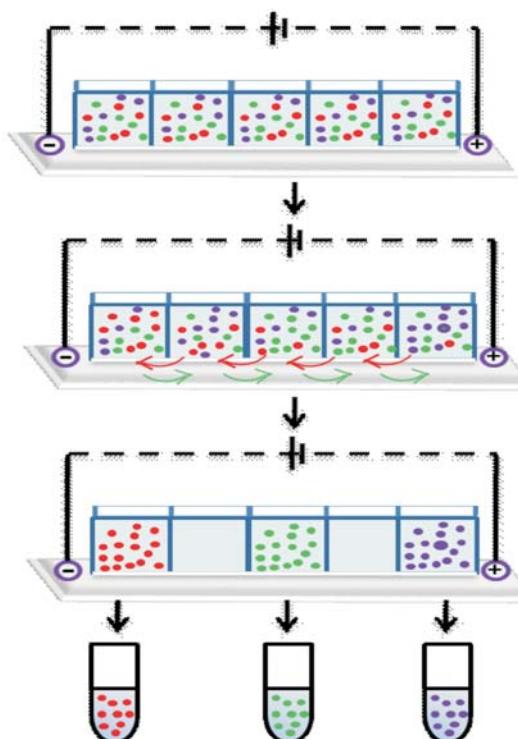


Figure 2.5: Offgel fractionation: Separation of the peptides takes place based on their pI.

2.6. Zip-tipping (Desalting and peptide concentration):

I. Materials

- Wetting solution (100% HPLC-grade acetonitrile)
- Washing solution (0.1% TFA in MilliQ water)
- Elution solution (50% acetonitrile in 0.1% TFA)

II. Procedure

- Equilibration:** Aspirate 10 μ l of wetting solution into Ziptip and dispense to waste. Repeat this process 3 times. Then aspirate washing solution into Ziptip and dispense to waste. Repeat this process 3 times.
- Peptide binding & washing the salts:** Aspirate the protein/peptide sample using ZipTips and dispense in the same vial. Repeat this times. Then

wash the salts/interfering agents by aspirating 10 μ l of washing solution and dispensing to waste.

Note: A 5% methanol in 0.1% TFA/water wash can improve desalting efficiency.

- **Elution of peptides:** Dispense 10 μ l of elution solution into clean 0.5 ml microcentrifuge tube using a standard pipette tip. Aspirate and dispense the elution solution through ZipTip at least 8-10 times without introducing air bubbles.

2.7. Peptide mass fingerprint analysis (PMF Analysis)

- Bovine serum protein extract has been subjected to SDS-PAGE. One of the bands (about 71kDa) has been submitted to in-gel digestion (trypsin digestion) process followed by MALDI-TOF MS measurement.
- After acquisition of MS data for the given in-gel digested protein sample, save the MS data file as .mgf file (Mascot generic format). Go to the Mascot website (www.matrixscience.com). This takes you to the Matrix Science homepage, which hosts



The screenshot shows the Matrix Science homepage with the 'Access Mascot Server' link highlighted.

Access Mascot Server

You are welcome to submit searches to this free Mascot Server. Searches of MS/MS data are limited to 1200 spectra and some functions, such as no enzyme restrictions, are unavailable. Automated searching of batches of files is not permitted. If you want to automate search submission, perform large searches, search additional sequence databases, or customize modifications, quantitation methods, etc., you'll need to license your own, in-house copy of Mascot Server.

Peptide Mass Fingerprint
The experimental data is a list of peptide mass values from the digestion of a protein by a specific enzyme such as trypsin.
[Perform search](#) | [Example of results report](#) | [Tutorial](#)

Sequence Query
One or more peptide mass values associated with information such as partial or ambiguous sequence strings, amino acid composition information, MS/MS fragment ion masses, etc. A super-set of a sequence tag query.
[Perform search](#) | [Example of results report](#) | [More information](#)

MS/MS Ions Search
Identification based on raw MS/MS data from one or more peptides.

MASCOT Peptide Mass Fingerprint

Your name: kishore Email: kishore_apro@gmail.com
Search title:
Database(s): SwissProt NCBI nr contaminants dRAP Enzyme: Trypsin Allow up to: 1 missed cleavages
Taxonomy: All entries Fixed modifications: none selected Variable modifications: none selected Display all modifications: Acetyl (K), Acetyl (Protein N-termini), Amidated (C-term), Amidated (Protein C-term), Ammonium-loss (N-term C), Biotin (K), Biotinylation, Carbamidomethyl (C), Carbamyl (K), Carbamyl (N-term)
protein mass: kDa Mass values: * MH+ * M_r - H⁺ Peptide tol.: ± 1.2 Da Monoisotopic: Average Data input: * Data file: Choose File No file chosen * Query Report top: AUTO hits Decoy: Start Search... Reset Form

Mascot. Click the 'Mascot' link on the top of the form to go to the Mascot home page. Select the Peptide Mass Fingerprint Search Form. This search uses MALDI-TOF spectra of peptide ion masses from a protein digest to predict the protein sequence.

Fill in the fields as follows:

Your name: Write your name.

Email: Your real email address. Your Mascot results will be emailed to your email ID if your internet is disconnected.

Search Title: Use 'Example-1' or some other proper title.

Database: This selects the database for Mascot to search against. Choose SwissProt database.

Taxonomy: Allows the searches to be restricted to a specific species or group of species. If you don't know the source of the protein then use 'All Species'.

Enzyme: The enzyme used to digest the protein into peptides should be mentioned. Choose 'Trypsin'.

Missed Cleavages: In the trypsin digestion process, the protein sample is partially digested, resulting in peptides with intact cleavage sites. With increase in the number of missed cleavages the probability of identifying missed cleavages in the partially digested sample increases. Select '1' allowed missed cleavage site.

Fixed modifications: If the protein sample is treated with any chemical reagent, which results in the modification of peptide N- and C-terminii, etc. that should be mentioned in the fixed modifications. This sample has been treated with iodoacetamide, so in the fixed modifications box, select 'Carbamidomethyl (C)'.

Variable Modifications: Unlike the fixed modifications, where Mascot considers only the modified masses; unmodified masses are equally considered in the variable modification. Generally oxidation of methionine is common in the proteins. So select oxidation of methionine (Oxidation (M)).

Protein Mass: Leave it blank, if you don't know the mass of the protein. If you know the molecular mass of the protein from the SDS-PAGE experiments then enter that value in the given box.

Peptide Tolerance: This parameter shows the ability of the mass spectrometer to precisely report the sample peptide masses. Use a tolerance of +/- 1.2 Da (Default value).

Mass Values: In generally, the solvents used for running the peptide samples on mass spec. are acidic solvents. So the peptides attain +ve charges. So select MH+.

Monoisotopic or Average: The monoisotopic mass is the mass of the most abundant isotope. The average mass is the abundance weighted mass of all isotopic components. Various isotope peaks of peptides can be readily resolved by the modern mass spectrometers, so choose Monoisotopic.

Data File: The mass spec. data file contains ion peak table. Click on browse, and then select the data file1.txt.

Decoy: This database contains reversed/randomized sequences from which we don't expect any true matches. The matches obtained from the comparison of decoy database will be false positives and this will be helpful in the determination of false discovery rates (FDR).

Report top: The number of hits (with top score) which should be displayed. Keep it Auto (default).

MASCOT Peptide Mass Fingerprint

Your name: kishore Email: kishore.aprjc@gmail.com

Search title: Example-1

Database(s): SwissProt, NCBI nr, contaminants, dRAP

Enzyme: Trypsin, Allow up to 1 missed cleavages

Taxonomy: All entries

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M), Nitric Oxide (NO), Phospho (ST)

Protein mass: kDa, Peptide tol. ± 1.2 Da

Mass values: MH*, M_n, M-H*

Monoisotopic Average

Data file: Choose File: Vipin Kumar_A.txt

Query:

Data input:

Decoy Report top: AUTO hits

Start Search ... Reset Form

©2014 Matrix Science | Links | Site map | ?

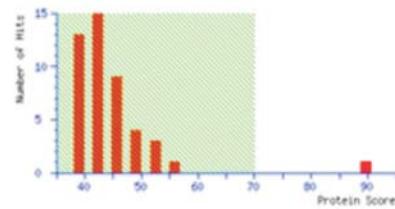
- After filling the PMF form click on start search.
- Only one protein was found to be significant with $p < 0.05$. The hits present in the green colour box are statistically insignificant.
- At the right hand side of the “Format as” field there is a drop down option, using which change “Concise Protein Summary” to “Protein Summary” and then click on “Format As” button. A new window can be seen at the bottom of the browser (see below) which contains the details of peptides identified from the top scoring protein.

Mascot Search Results

User: kishore
Email: kishore.aprjc@gmail.com
Search title: Example-1
MS data file: Vipin Kumar_A.txt
Database: SwissProt 2014_10 (546790 sequences; 194613039 residues)
Timestamp: 23 NOV 2014 at 15:33:18 GMT
Top Score: 90 for ALBU_BOVIN, Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Mascot Score Histogram

Protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event.
Protein scores greater than 70 are significant ($p < 0.05$).



Concise Protein Summary Report

Accession	Mass	Score	Description
ALBU_BOVIN	71244	90	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
RUN_SACEN	48702	56	Probable dual-specificity RNA methyltransferase RIMN OS=Saccharopolyspora erythraea

Accession	Mass	Score	Description
ALBU_BOVIN	71244	90	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
RUN_SACEN	48702	56	Probable dual-specificity RNA methyltransferase RIMN OS=Saccharopolyspora erythraea

Re-Search All Search Unmatched

Accession	Mass	Score	Description
ALBU_BOVIN	71244	90	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Index

Accession Mass Score Description

1. ALBU_BOVIN 71244 90 Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

2. RUN_SACEN 48702 56 Probable dual-specificity RNA methyltransferase RIMN OS=Saccharopolyspora erythraea

Re-Search All Search Unmatched

Results List

Accession	Mass	Score	Expect	Hits
ALBU_BOVIN	71244	90	0.0006	42
				Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
				Observed Mr(expt) Mr(calc) Delta Start End Miss Peptide
507.8951	506.8978	507.2441	-0.3563	229 - 232 0 K.FGSLR.A
545.2352	544.2279	544.3333	-0.1054	101 - 105 0 K.VASLR.E
649.2731	648.2658	648.3265	-0.0607	205 - 209 0 K.IETIHR.E
649.9770	648.9697	648.3265	0.6433	205 - 209 0 K.IETIHR.E
655.9994	654.9921	655.3402	-0.3481	24 - 28 1 R.RDTHK.S
665.3231	664.3158	664.3214	-0.0056	205 - 209 0 K.IETIHR.E + Oxidation (H)
665.9591	664.9518	664.3697	0.5821	156 - 160 1 K.KHVKY.V
689.3273	688.3200	688.3656	-0.0457	236 - 241 0 K.AHSVAR.L
711.3228	710.3147	711.3664	-1.0517	29 - 34 0 K.SEIAGR.F
712.3351	711.3278	711.3664	-0.0386	29 - 34 0 K.SEIAGR.F
927.5041	926.4969	926.4861	0.0107	161 - 167 0 K.VLYEIAHR.R
968.4475	959.4402	959.5400	-0.0998	210 - 218 1 R.EKVLAASSAR.Q
975.5373	972.5301	973.4505	-0.9204	37 - 44 0 K.DLGEHEPK.G
987.4335	986.4262	986.5297	-0.1035	29 - 36 1 K.SEIAGR.K.D
1138.5128	1137.5055	1137.4907	0.0148	499 - 507 0 K.CCTESLNR.I.R
1161.6571	1162.6488	1162.6234	0.0264	66 - 75 0 K.LVNELTPEAK.T
1166.5069	1165.4996	1165.4856	0.0140	466 - 468 0 R.CCTKPESE.R.H
1193.6233	1192.6169	1192.5949	0.0211	25 - 34 1 R.DTHKSEIAHR.F
1248.6429	1248.6357	1248.6139	0.0218	35 - 44 1 R.FKDQGEHEPK.G
1283.7328	1282.7255	1282.7034	0.0222	361 - 371 0 R.HPEYAVSVRL.L
1293.5992	1292.5919	1293.6969	-1.1049	246 - 256 1 K.FPKAEFVEVT.K.L
1305.7379	1304.7306	1304.7088	0.0218	402 - 412 0 K.HLVDEPQNLK.Q

- Click on the accession number of any one of the proteins which displays sequence coverage and the complete sequence of the protein with the peptides identified in red colour.

MATRIX SCIENCE MASCOT Search Results

Protein View: ALBU_BOVIN

Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Database: SwissProt
Score: 90
Expect: 0.0006
Nominal mass (M_r): 71244
Calculated pI: 5.62
Taxonomy: Bos taurus

Sequence similarity is available as [an NCBI BLAST search of ALBU_BOVIN against nr](#).

Search parameters

MS data file: Vipin Kumar_A.txt
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass values searched: 100
Mass values matched: 42

Protein sequence coverage: 56%

Matched peptides shown in bold red.

```
1 MNQTFITFLL LNLSVAYRN VNFADTHKSE IANRFKFDLGE ERFKGLVILIA
51 SNQVLQQCPF DNHRYLNKL TEFAMTCAVS ENHAGCEKSL HTLNDELCNK
101 VASQLEKETGKN HADCEKNEP ERNECELSHN KNDSPDLPKNK PNPTLNCDF
151 KNADCEKNWNKN HADCEKNEP YNEYAPELLVY ANKNGVYTGN CQNACEWNGAC
201 LNLPIENTRHEN KNYVIAENAKMPN YNEYAPELLVY ANSLAKANGVN YNLGNCFPKNA
251 FNEVYFTKLVN LNTKVHECHN QNDLNECNADN ANQNYVNICDNN QNTNSSNFLKN
301 CNCDNPKLNK HNIAENKEVNEDN INPENNPLPNT DNAENDKVN KNYNCANDAFL
351 GNSTLNVEYSRN HNPEYAVNSVNLL RNLANKENATLN ENECCNAKDNFN ACNYNVNTDNKL
401 KNHNYDNEPNHL INQNCCNQNPFN LNGNYNQNALN INYNTANRNYNQN VNETNPLNEVN
451 RNINQNYNRCN CNTRNESNEPN CNTENYNSLNL NNLCNTMNEN FNUNERNTTNCO
501 TNESNLYNHRN PNTANTYN VNPANKENLFN TNPANTCNIPN TNERNQNQTN
551 ANLVNELNKEN HNKN KNATENQNTVN MNENNYNAVN KNCANDCNMEN FNAENOKNLVN
601 SNTNALAN
```

Unformatted sequence string: 607 residues (for pasting into other applications).

Sort peptides by: Residue Number Increasing Mass Decreasing Mass
 Show predicted peptides also.

2.8. MS/MS Ions Search

- Protein extracts from *Arabidopsis thaliana* were subjected to SDS-PAGE followed by excision of a protein band (around 15kDa) from the SDS-polyacrylamide gel and further subjected to in-gel digestion. In-gel digested protein samples were analyzed using ESI-Quad-TOF instrument to find out the identity of the protein.
- Go to the Mascot Home and select the MS/MS Ion Search Form.
- Fill the form:

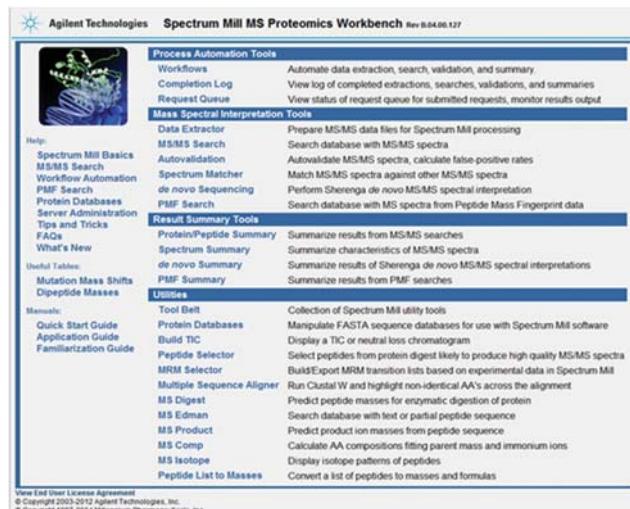
- Fill the name, email, search title, database, enzyme, missed cleavages, taxonomy, fixed modifications, variable modifications, monoisotopic/ average mass and peptide tolerance as mentioned above in the PMF analysis.
- In MS/MS ion search we find some new fields/ parameters along with the fields which we can see in the PMF analysis
- MS/MS tolerance:** It depends on the sensitivity of the instrument. This is the error window for the MS/MS fragment ion masses. Keep it 0.6 Da (default value)
- Peptide charge:** This depends on the ionization source. Here we used ESI (Electron Spray Ionization) method. So the peptides will get multiple charges and hence a peptide charge of +2, +3 and +4 should be selected. If MALDI ionization method is used then the peptides will get single positive charge. Then we have to select 1+ as the peptide charge.
- Quantitation:** If the peptides/ proteins are labeled with some mass tags or stable isotopes, which are used for quantitation of the proteins/ peptides, then we can select that particular labeling method to get the quantitation details.
- Data file:** Select the data file from your computer.
- Data format:** Use Mascot generic format.
- Precursor:** If you know the precursor ion mass then enter that value. Otherwise leave it blank.
- Instrument:** Select the mass spectrometer used for acquiring the data. We used ESI-Quad-TOF for data acquisition. So select ESI-Quad-TOF from the drop down options.
- Error tolerant:** When this button is checked, a standard, first pass search is performed using the search parameters specified in the form. From the results of the first pass search, all of the database entries that contain one or more peptide matches with scores at or above the homology threshold, (or identity threshold if there is no homology threshold), are selected for an error tolerant, second pass search. At the completion of the second pass search, a single report is generated, combining the results from both passes.
- After filling the form click on start search.
- The protein hits which appear outside the green colour box are statistically significant hits.
- The details of the identified protein hits are given below the graph, which is plotted between protein score and number of hits.
- For each protein, the first line contains the accession number (linked to the corresponding

subunit IV B, chloroplastic, which are having very close molecular masses (14,958 and 15,188 respectively). This shows that the protein band which was cut and used for in-gel digestion was having both the proteins. Because of this reason we got good protein scores for both the proteins.

2.9. iTRAQ data analysis using SpectrumMill software:

Procedure

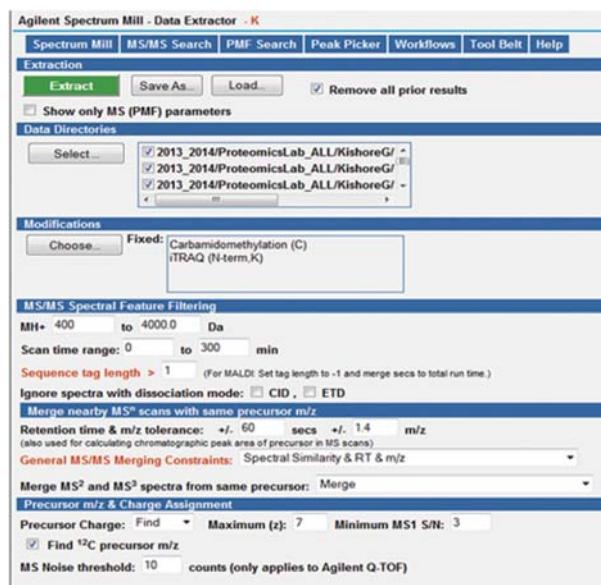
- Double click on the Spectrum Mill software icon on the desktop.



- Click on Data Extractor

Data Extractor

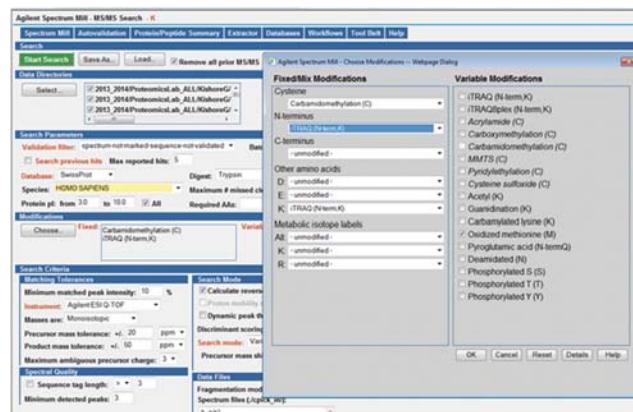
- Click on the select option present below the data directories to select the MS/MS data files.



- Click on the Choose option given below the Modifications
- Select Carbamidomethylation on cysteine and iTRAQ at the N-terminal of lysine {iTRAQ(N-term, K)} as fixed modifications.
- Remaining parameters like MS/MS scan range, scan time etc. can be varied based on the user need.

MS/MS Search

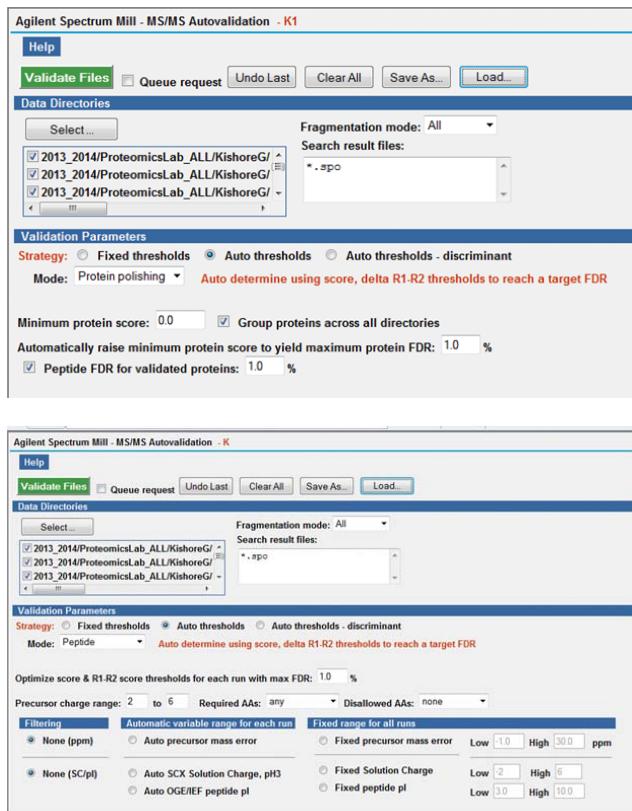
- Beside the database option there is a dropdown option using which we can select the database of our interest (eg. SwissProt, NCBIInr etc.).
- In the same way select the enzyme used for the protein digestion (eg. Trypsin, chymotrypsin).
- Select the species from which the protein samples were extracted (eg. *Homo sapiens*, *Saccharomyces cerevisiae* etc.). If source of the protein sample is not known, then select "All" in the "Species" option. This search will take longer time as the software matches the given spectra with all the entries given in a database.
- Select the maximum allowed missed cleavages 1 or 2.
- Select Carbamidomethylation and iTRAQ (N-term, K) as fixed modifications and oxidation of methionine as variable modification.
- Precursor mass tolerance and product mass tolerance values depend on the instrument sensitivity. With decrease in these values the stringency of the search increases.



Autovalidation

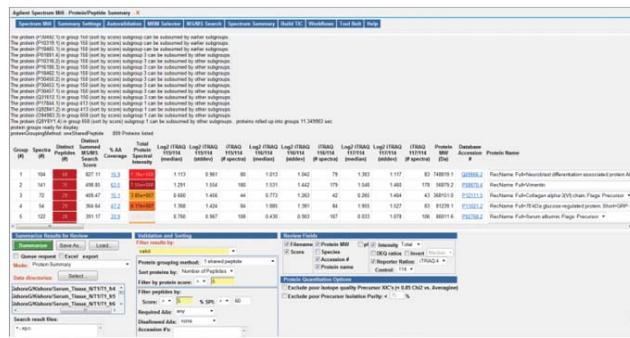
- Validation should be done at both protein and peptide level.
- In the validation parameters under strategy select Auto thresholds.
- In the mode select Peptide.
- Maximum FDR should be 1%.
- Precursor charge range 2 to 6.

- Keep default values for the remaining parameters.
 - Then click on “Validate Files” option at the top.
 - After validating the spectra at peptide level, validate them at protein level.
 - Now change the mode to protein by selecting “Protein polishing” under the drop down options.
 - Minimum protein score can be kept from 0-15 based on the quality of the data.
 - Maximum FDR should be 1%.
 - Peptide FDR for the validated proteins should be 1%.



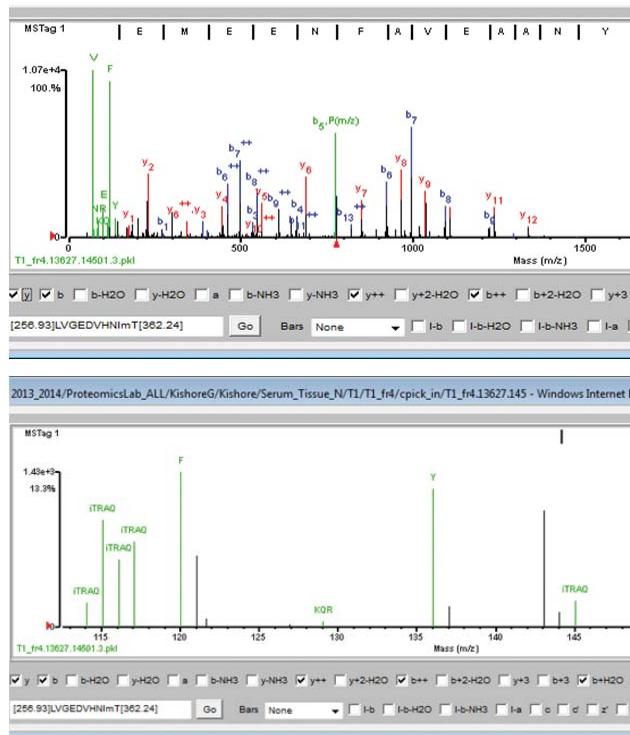
Protein/peptide summary

- Under “Mode” option select protein summary.
 - Under results filter select “valid” option to get validated protein information.
 - Sort proteins by number of peptides/score.
 - Filter by protein score > 5
 - Filter by peptide score > 5 and SPI > 60%.
 - Under “Review Fields” check the boxes in front of File name, Score, Protein MW, Accession #, Protein name, Intensity (Total), Reporter Ratio.
 - Under Reporter Ratios select iTRAQ4 (if you are using iTRAQ 4 plexing)
 - The iTRAQ reagent used for labeling the control sample should be mentioned.



Calculation of relative abundance of proteins

- Software generates log₂ ratios of all the reporter ions with respect to the reporter ion which was used for labeling the control sample.
 - These log₂ values can be converted to absolute values using 2^{n} formula. Where n= log₂ ratio of the reporter ions.
 - In order to view spectra for the peptides of an identified protein in the present analysis, go to peptide summary mode and enter the accession number of the protein in the given box at the bottom panel, then click on summarize.
 - We will get information about all identified peptides of the selected protein, and then click on one of the peptides % SPI value, which results in the pop up of another window showing the spectra for the selected peptide.
 - Zoom in the 110 to 140 m/z region to see the reporter ion intensities.



- In order to know details about the sequence determination of a given peptide, click on the file name of the peptide, which opens a new window at the bottom pane, showing the details of b-ions & y-ions used for sequence deduction.

bacterial protein samples respectively. After adding the iTRAQ reagents to respective in-solution digested protein samples, the samples were incubated at room temperature for 1 hour, followed by quenching the labeling reaction by adding 100 μ l of Milli Q water and kept at room temperature for 30 minutes. After 30 minutes the labeled samples were pooled and subjected to strong cation exchange chromatography. The SCX fractions were desalted using C18 Zip-tips. The desalted SCX fractions were subjected to LC-MALDI-TOF/TOF analysis. MS data acquisition was done in the positive ion mode in the mass range of 800-4000 m/z. Instrument was calibrated to 50 ppm accuracy using 4700 Proteomics Analyzer Standard Kit containing 6 peptide mixture. Precursor ion selection parameters are as follows: minimum S/N ratio, 60; maximum precursors per spot, 18. All MS/MS spectra were acquired with 2 kV collision energy by accumulation of 2500 laser shots. The MS/MS data was analyzed using GPS Explorer software (AB SCIEX) interfaced with MACOT search engine. The MASCOT analysis (MS/MS ion search) parameters are as follows: taxonomy: *B. subtilis* with following parameters: MS tolerance, 75ppm; MS/MS tolerance, 0.4Da; Enzyme used, Trypsin; Fixed modification, carbamidomethyl; Variable modification, oxidation (methionine), Deamidation (N,Q).

Application note 1

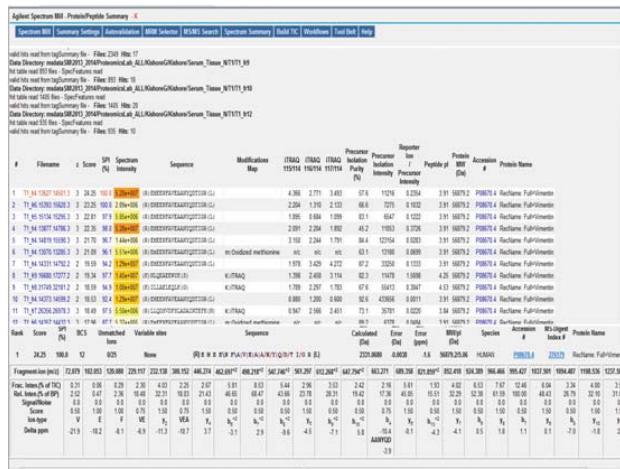
Study of quantitative proteomic alterations in *B. subtilis* on treatment with totarol, an antibacterial drug. Quantitative proteome analysis of *B. subtilis* after treatment with natural terpene.

Panga Jaipal Reddy, Kishore Gollapalli and Sanjeeva Srivastava*

*Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, *Email: sanjeeva@iitb.ac.in*

Treatment of bacteria with an antibacterial agent results in the alteration of bacterial proteome. Quantitative proteomic analysis of control and drug treated bacterial proteome reveals the drug targets and the mechanism of action of the drug. In the current study using iTRAQ based quantitative proteomic analysis, we identified probable molecular targets for the totarol in *B. subtilis*.

Methodology: The bacterial protein extraction was performed using TRIzol reagent following the manufacturer's protocol. Bacterial cultures were subjected to totarol treatment for different time intervals like 20 minutes, 60 minutes and 120 minutes. The bacterial protein extracts were dissolved in rehydration buffer, which contains Urea, CHAPS and other detergents. Prior to iTRAQ labeling the bacterial protein samples were buffer exchanged with 0.5M TEAB buffer. The buffer exchanged protein samples were quantified using Bradford reagent and 50 µg of protein from control, 20 minutes, 60 minutes and 120 minutes drug treated bacterial samples were subjected to iTRAQ labeling. iTRAQ reagent reagent 114, 115, 116 and 117 were used to label tryptic digests of control, 20 minutes, 60 minutes and 120 minutes totarol treated



Results and Discussion: Total 307 *B. subtilis* proteins were identified in our current study. Out of the 307 proteins only 204 proteins were identified with least 2 peptides for identification. Various ribosome associated proteins like 50S ribosomal protein L13 and 30S ribosomal protein S4 etc. found to be negatively correlated with increase in the time period of drug treatment. Proteins like Protein LiaH, Transcription elongation factor GreA, Catalase etc. were found to be positively correlated with increase in the time of drug treatment. Representative mass spectra for a *B. subtilis* peptide is given in the figure 2.6.

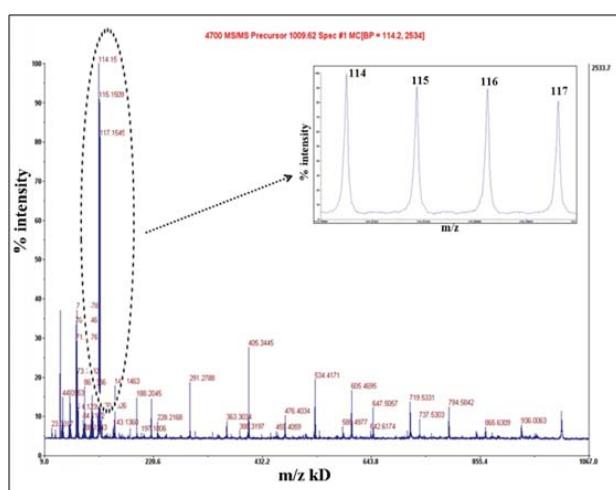


Figure 2.6: Representative mass spectra for one of the *B. subtilis* peptides (Reporter ion intensities were given in the inset).

Application note 2

Investigation of proteomic alterations in Glioma tumor tissues containing wild type and mutant IDH 1 genes using iTRAQ based quantitative approach.

Kishore Gollapalli, Saicharan Ghantasala and Sanjeeva Srivastava*

*Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, *E-mail: sanjeeva@iitb.ac.in*

Gliomas with mutations in the IDH1 gene have different clinical outcomes as compared to gliomas with IDH1 wild type gene. To understand the differences between the two subpopulations of Gliomas, we used iTRAQ based quantitative LC-MS/MS approach for proteins from glioma tumor tissues.

Methodology: Protein extraction from glioma tissues of patients with and without IDH1 gene mutation was done using the TRIzol protocol as per manufacturer's instructions. The obtained protein pellet was dissolved in rehydration buffer containing Urea, CHAPS and other detergents. The dissolved protein pellets were now subjected to buffer exchange using 0.5M TEAB buffer to make them LC-MS compatible. The buffer exchanged samples were then quantified using Bradford reagent and 50 µg of each of buffer exchanged samples was subjected to reduction, alkylation followed by in-solution digestion. iTRAQ labeling for the samples was then performed following the manufacturer's protocol. Tryptic digests from Glioma patients with wild type IDH1 protein were labeled with iTRAQ labels 114 and 116, while protein samples from Glioma patients with mutant IDH1 protein were labeled with iTRAQ labels 115 and 117. The iTRAQ labeled samples were pooled together and subjected to offgel fractionation. The different fractions obtained after offgel fractionation were then collected in separate tubes and zip-tipped to remove any salts present which could interfere with the LC-MS run. The fractions obtained after offgel fractionation were subjected to LC-MS/MS, where the peptides were enriched on the C18 enrichment column and separated on a 75 µm x 43mm analytical/ separation column in the protein chip (Agilent HPLC-Chip: G4240-62001ZORBAX 300SB-C18) using a gradient mobile phase consisting of two different solvents 0.1% formic acid solution (Solvent A) and 90% acetonitrile (Solvent B) at a flow rate of 0.5µl/min. The following gradient method was used for the separation of peptide on the chip over a period of 50 min.: 0-3 min., 5-20% solvent B; 3-45 min., 20-45% solvent B; 45-50 min., 45-95% solvent B; nitrogen gas was maintained at 250°C with a flow rate of 9 L/min. The mass range in the MS was 100-3200 (m/z), with a MS Scan rate of 5 spectra/sec. Total 15 high intense peptide ions (peaks) having

charge ≥ 2 from MS were selected for MS/MS. In MS/MS mode, the mass range was 50-3200 (m/z), with a MS/MS scan rate of 5 spectra/sec. The data was acquired in centroid mode. The MS/MS data was further analyzed using Spectrum mill software (Agilent Technologies). The mass spectrometry data was searched against SwissProt database using *Homosapiens* as taxonomy; carbamidomethylation (C) & iTRAQ (N-term, K) as fixed modifications and Oxidation of methionine as variable modification; precursor and product mass tolerance were 20 and 50 ppm respectively.

Results and Discussion

A total of 657 proteins were found to be differentially expressed among the two subpopulations. Of these 657 proteins, 233 proteins were identified to have at least 2 peptides with FDR set to 1%. Proteins with roles in oxidative stress response, VEGF signaling pathway and ATP synthesis were found to have significantly altered expression levels among the two subpopulations of gliomas. A few proteins with significant difference in expression included GFAP, BASP-1, Endophilin A1 and LDH. Representative mass spectra for one of the BASP1 protein with the reporter ion intensities have been given in the figure 2.7.

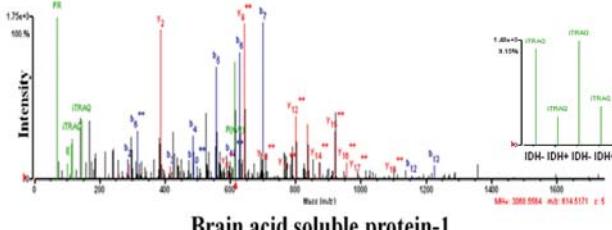


Figure 2.7: Representative iTRAQ spectra for BASP1- a significantly altered protein in the glioma tissue proteomic analysis.

Application note 3

Identification of metastatic candidate markers in Triple negative breast cancer using 2-DE and MALDI-TOF/TOF: A tissue culture based approach

Debasish Paul^{1,y}, Raju Dabhi^{1,y}, Kishore Gollapalli², Manas Santra¹ and Srikanth Rapole^{1*}

¹National Centre for Cell Science, Ganeshkhind, Pune 411007, Maharashtra, India.

²Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India, *E-mail: rsrikanth@nccs.res.in.

Biomarker identification is one of the most important field of investigation because of their diagnostic and prognostic values. Cancer is one of the deadliest diseases and triple negative breast (TNBC) cancer is the most difficult cancer to diagnose, because of the lack of appropriate biomarkers. Due to the

technological advances of mass spectrometry based proteomics in the last era, now we have the potential to look into the clinical problems which were unreachable till last decade. This study provides such a platform and identified differentially expressed proteins in MDA-MB-231(TNBC) compared to non-metastatic cell line MCF-7 using two-dimensional electrophoresis (2-DE) in combination with mass spectrometry. This study is a preliminary one and further investigations on the differentially expressed proteins and their mechanisms may be of scientific and clinical importance.

Methodology: Breast cancer cell line MCF-7 is grown in DMEM medium and MDA-MB-231 in RPMI as monolayer supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humid, 5% CO₂ atmosphere. Protein extraction was done from both the cell lines and subjected to classical two dimensional gel electrophoresis. The gels were stained coomassie brilliant blue for overnight followed by destaining followed by scanning and analysis of the gel images using Image Master 2D Platinum software (GE Healthcare). Significantly altered proteins were excised from the gel and subjected to in-gel digestion followed by ziptipping and MALDI-TOF/TOF analysis. The mass spectrometry data was analyzed using GPS Explorer Software.

Results and Discussion: Using 2-DE proteomic analysis, we compared protein expression patterns between non-metastatic cell line (MCF-7) and metastatic TNBC cell line (MDA-MB-231) (Figure 2.8). The 2-DE data revealed consistent protein profiles for each cell line. We have identified total 71 proteins and 19 out of them showed significant ($p<0.05$) differential expression. The change of the protein level was analyzed using -1.2 fold for down-regulation ad 1.2 fold for up-regulation cut off. Out of the 19 differentially expressed proteins 11 were up regulated and 8 were down regulated. PANTHER analysis revealed involvement of differentially expressed proteins in biological functions of metabolic processes, cellular processes and developmental processes (Figure 2.9). Concerning major biological pathways include glycolysis, integrin signaling pathway, chemokine and cytokine signaling pathways.

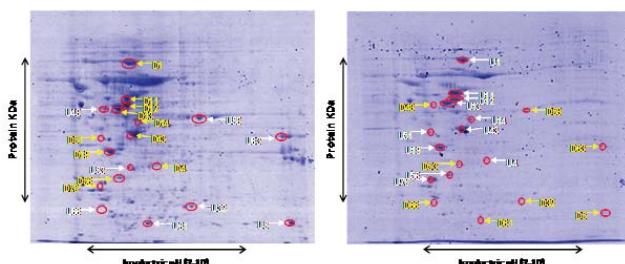


Figure 2.8: 2-DE images of MCF-7 (Left) and MDA-MB-231 (Right) with marked differentially expressed proteins.

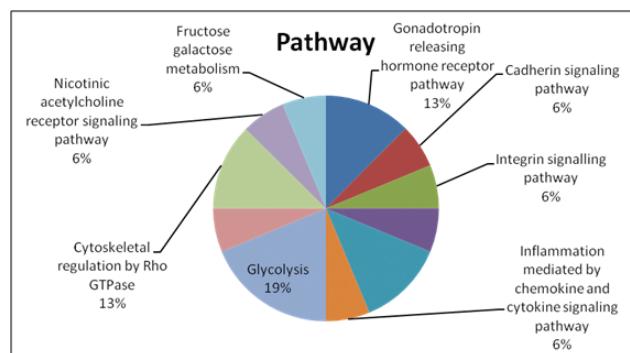
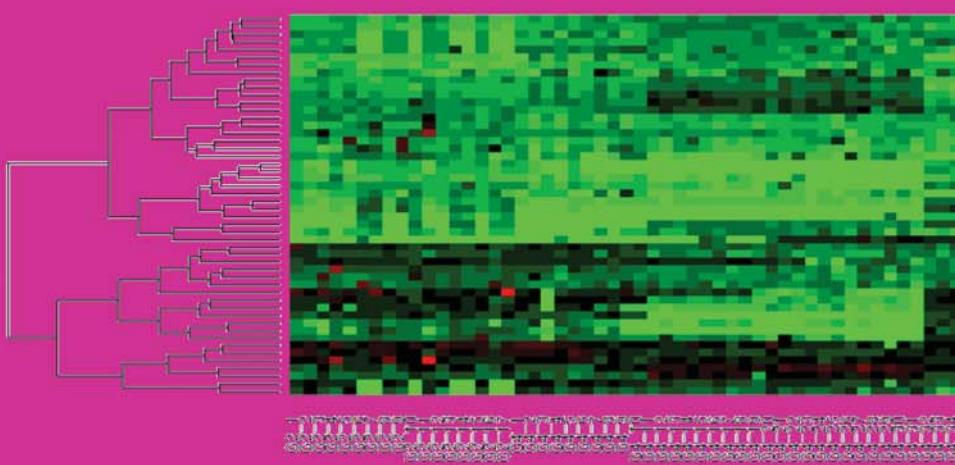
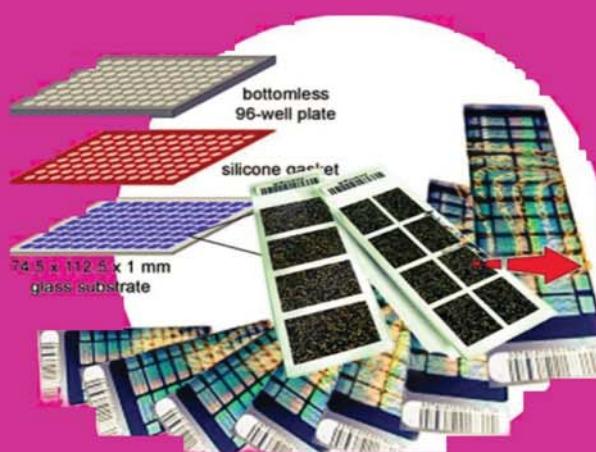


Figure 2.9: Pie charts showing the association of the identified proteins in various biological pathways.

References

- Williamson LN, Bartlett MG. Quantitative liquid chromatography/time-of-flight mass spectrometry. Biomedical Chromatography 2007; 21:567-76.
- Bristow AWT. Accurate mass measurement for the determination of elemental formula-a tutorial. Mass Spectrometry Reviews 2006;25:99-111.
- Payne AH, Glish GL. Tandem mass spectrometry in quadrupole ion trap and ion cyclotron resonance mass spectrometers. Methods in Enzymology 2005; 402:109-48.
- Chen G, Pramanik BN. Application of LC/MS to proteomics studies: current status and future prospects. Drug Discov Today. 2009;14:465-71.
- Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev. 2009;30:19-34.
- McLafferty FW1, Breuker K, Jin M, Han X, Infusini G, Jiang H, et al. Top-down MS, a powerful complement to the high capabilities of proteolysis proteomics. FEBS J. 2007; 274:6256-68.
- Xie F, Liu T, Qian WJ, Petyuk VA, Smith RD. Liquid chromatography-mass spectrometry-based quantitative proteomics. J Biol Chem. 2011;286:25443-9.
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006; 1: 2856-60.
- Cutillas, Pedro R., Timms, John F. LC-MS/MS in proteomics. Methods and applications. Vol. 658; Humana Press/ Springer.
- <http://www.abrf.org/Committees/Education/Activities/Mascot-exercise-ABRF1.pdf>
- http://biomass.fudan.edu.cn/st/ICAT.ashx#ITRAQ_4
- <http://www.chem.agilent.com/Library/brochures/5990-5596EN.pdf>
- <http://ccc.chem.pitt.edu/wipf/Waters%20LC-MS%20primer.pdf>

PROTEIN MICROARRAYS (Module III)



Protein Microarrays

Preface

Rekha Jain, Nikita Gahoi, ApurvaAtak, NarendraGoud and Shabarni Gupta

Indian Institute of Technology Bombay, Mumbai, India

The success of DNA microarrays for gene expression profiling has translated development of protein microarrays for simultaneous analysis of hundreds to thousands of proteins. Protein microarrays consist of small quantities of immobilized proteins printed onto solid supports such as glass slides and have exhibited immense potential for classical and functional proteome analysis. The lack of a PCR-equivalent amplification process for proteins, the wide range of hybridization chemistries and the need to maintain structural integrity for protein function studies, make the process of construction of protein microarrays a considerably greater challenge compared to DNA microarrays. Besides these obstacles, microarray enables simultaneous screening of thousands of proteins using minimal amount of biological samples and reagents. It allows proteome level analysis and thus is a promising tool to understand complex biological processes.

Protein microarray is broadly classified as Forward-Phase protein microarrays (FPPAs) and reverse-phase protein microarrays (RPPAs). In FPPAs, the affinity between the two interacting partners is known and the immobilized proteins are either antigens or antibodies. It has wide applications in clinical and basic research for example, disease diagnosis, autoantibody screening, protein-ligand interactions. In RPPA, entire cell lysate or biological fluids are imprinted on slides and probed with specific antibody. Such an array is extremely useful when sample size is a limitation.

In conventional protein microarray, expression and purification of thousands of recombinant proteins is a major hurdle. Therefore, cell-free expression is an alternative, where proteins are synthesized from their corresponding DNA templates directly on the microarray surface. Protein arrays generated by this technique have shown great potential in eliminating the drawbacks of traditional cell-based methods.

In this workshop, one such cell-free expression based technique that is Nucleic Acid Programmable Protein Arrays (NAPPA) will be discussed, where DNA or NAPPA mix would be printed on aminosilane coated glass slide using microarrayer. *Invitro* transcription translation (IVTT) of proteins will be performed using rabbit reticulocytelysate (RRL) since it contains the complete transcription and translation machinery. This workshop would demonstrate production of luciferase protein by using luciferase gene cloned in an expression

vector using IVTT mix. In addition to this, other applications of protein arrays like autoantibody profiling using diseased serum samples and tissue microarrays would be demonstrated. We would also overview certain core platforms for microarray data analysis in this workshop.

Speaker Biography

Speaker: Dr. Manuel Fuentes

Lab Surpath, HPR project, India



Biography: Manuel Fuentes graduated in Chemistry and Biochemistry from the University of Salamanca (Spain). After his Masters in Biotechnology at University of Bielefeld (Germany), he joined Biocatalysis Department at National Spanish Research Council (Madrid, Spain) for his PhD work, entitled “Design and development of conjugation and immobilization methods of biomolecules for diagnostic methods useful in Genomics and Proteomics”.

Later, he moved to Harvard Institute of Proteomics at Harvard Medical School (Boston, EE.UU.), where he worked on biomarker and drug discovery in tumor and autoimmune pathologies by using a combination of high-throughput label-free proteomics approaches. In 2009, he joined as a scientist at Cancer Research Center, University of Salamanca, where his research focused on biomarker and drug discovery in hematological diseases, with a focus on personalized medicine. Manuel Fuentes is a co-author of 80 peer-reviewed papers (ISI web of Knowledge) in international journals, 9 licensed international patents, 10 book chapters, and has given more than 50 invited lectures in several national and international meetings.

Dr. Sudha Narayan Rao

Genotypic Technology, India



Biography: Dr. Sudha Rao is the Founder of DhitiOmics Technologies Pvt Ltd and Co-Founder and Executive Director of the Genotypic Technology where she is involved in multiple operations of the Company including the Microarray and Next Generations labs, Bioinformatics and Microarray design units, Enterprise resource planning, HR and Administration of the company, collaborations and new products.

Dr. Sudha Rao completed her graduation and post graduation from the University of Mysore followed by Ph.D. in Biotechnology from Madurai Kamaraj

University, Madurai. She completed her post doctoral studies in neurobiology at Cornell Medical School. After that she worked at Quark Biotech in Israel as a consultant where she was involved in identification of genetic suppressor elements. She has contributed several hundred RNA profiles to the RIN database, developed and implemented HR policies and Performance Management Systems in the Organization, implemented information management systems including implementation of ERP solution SAP, the first such for a Genomics company in the World which won the SAP Ace Award in 2010. She has to her credit several publications in peer-reviewed journals and is actively involved in collaborative research in Genomics. Dr. SudhaRao spends her leisure time in travelling, listening to music and art.

Speaker: Santosh Naronha

Indian Institute of technology, Bombay



Biography: Dr. SantoshNaronha completed his Bachelor's in chemical engineering from I.I.T Madras in 1990 and pursued his PhD from the University of Maryland, Baltimore County in 1996. He has worked as a Visiting Fellow at NIH, Bethesda and is currently an assistant professor at IIT Bombay.

His areas of interest include the large scale production and purification of recombinant proteins which involves various aspects such as the creation of genetic constructs, modification of host strains, optimization of fermentation and process control techniques, development of biosensors for online estimation of metabolites, and development of novel techniques for product recovery from high cell density cultures. An associated interest is the development of process techniques for large scale production of DNA vaccines. These vaccine candidates are currently the most promising methods of controlling diseases such as tuberculosis. Dr. SantoshNaronha is also involved in the application of geometrical mathematical techniques to protein structure analysis.

Speaker: Mr. Faiz Abuzar

Thomson Reuters, India



Biography: Mr. FaizAbuzar completed his post graduation in Biochemistry from the Aligarh University and has several years of experience in Academic & Government research ecosystem, in Business Development, New Business Strategy & Consultative sales role. He was involved in positioning of Information

(Databases/Intelligent Information) and helping clients in bridging information gap crucial for novel, patentable research. Mr. FaizAbuzar is the currently working account manager at Thomson Reuter, A&G India.

Speaker: Dr. Sanjay Navani

Site Director, The Human Protein Atlas (HPA) Project, India, Lab Surgpath, Mumbai (India)



Biography: The Swedish Human Protein Atlas (HPA) program has been set up to allow for a systematic exploration of the human proteome using Antibody-Based Proteomics.

This is accomplished by combining high-throughput generation of affinity-purified (mono-specific) antibodies with protein profiling in a multitude of tissues/cell types assembled in tissue microarrays. The vision is to systematically generate quality assured antibodies to all non-redundant human proteins, and to use these reagents to functionally explore human proteins, protein variants and protein interactions. The data is publically available and presented as high resolution images of immuno-histochemically stained tissues and cell lines (www.proteinatlas.org). Available proteins can be reached through searches for specific genes or by browsing individual chromosomes.

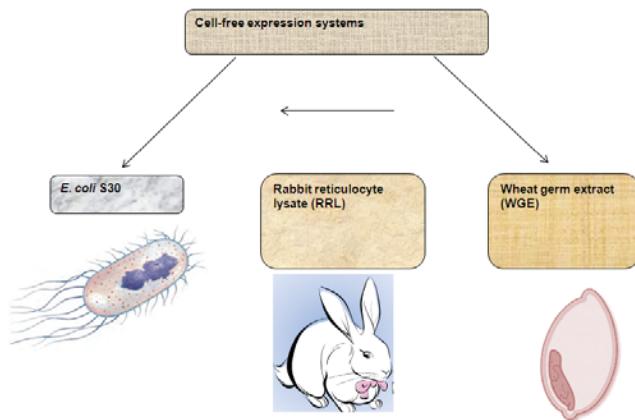
Protein microarrays: A versatile platform from biomarker screening to protein-protein interactions

Rekha Jain

Indian Institute of Technology Bombay, Mumbai, India

A microarray is a planar substrate such as a piece of glass on which different biomolecules are affixed at separate locations in an ordered manner thus forming a microscopic array that allows the simultaneous study of a large number of biomolecules in a high throughput manner. For studying gene expression in different organisms, a cDNA microarray may be used in which PCR amplified and purified DNA fragments or oligonucleotides are printed onto a planar substrate to form an array. cDNA prepared through reverse transcription from different conditions, for example, infected and uninfected cells are then used to hybridize the array for expression analysis. Similarly, protein microarrays involve the immobilization of proteins on the chip and can be used for various applications such as biomarker discovery, autoantibody detection, and cytokine detection, identification of protein-protein interaction and substrates of protein kinases, identification of targets of biologically active small molecules and post-translational modifications (PTMs) analysis. The nature of biomolecule to be printed on the chip depends on any of these biological objectives. The slides are usually subjected to amines, aldehyde or epoxy treatments to aid in the immobilization of protein molecules on slide while aminosilane coating is used for DNA immobilization. The printed chips are then incubated with a probe linked to a label that can in turn be used to detect the probes bound to the appropriate target molecules on the chip. The antigen-antibody interaction can be detected by various techniques, most of them involving a different secondary antibody. The protein microarray is then scanned using a microarray scanner that allows detection of the fluorescently labeled proteins or antibodies. The output from this scanner is received by a software after which the data can be analyzed.

Initially, traditional cell based methods were used to make protein microarrays. This however required cloning of every gene followed by protein expression and purification, a process extremely time consuming and cumbersome. Protein purity, maintenance of protein folding, and functionality during the purification and immobilization steps are some of the major challenges associated with cell based protein array approach therefore it has been replaced by cell free protein expression [MacBeath G et al. 2000]. Cell free protein expression is the expression of proteins from template DNA, which is inserted in expression plasmids that contains the genetic information such as promoter, translational initiator, gene of interest,



translational terminator etc, in the correct orientation and reading frame for protein synthesis. The addition of a crude cell lysate which contains cellular machinery such as enzymes, ribosomes, tRNA etc., and exogenously added co-factors - nucleotides, ATP, salts, essential amino acids, required for *in vitro* transcription and translation (IVTT). This system also allows protein folding and post-translational modifications to produce structurally and functionally mature proteins. There are different types of cell-free expression systems (Figure 3.1, Table 3.1).

Figure 3.1: Different types of cell-free expression systems used for *in vitro* protein synthesis starting from DNA templates. These systems contain all the necessary components and machinery for transcription and translation. Some factors such as energy generating components, essential amino acids etc. need to be added to the system for successful protein synthesis.

Table 3.1
Different methods of cell-free protein synthesis, merits and demerits are tabulated.

<i>Method</i>	<i>Advantages</i>	<i>Disadvantages</i>
(i) Protein <i>in situ</i> array (PISA)	<ul style="list-style-type: none"> • Protein purification not required • Rapid, single step process • Specific protein attachment • Formation of soluble proteins • Overcomes common problems (Protein insolubility, degradation and aggregation issues) during protein expression in prokaryotic systems 	<ul style="list-style-type: none"> • Possible loss of function during immobilization • Not cost effective as a relatively high volume of cell-free lysate required • Hexa-histidine tag may interfere with proper protein folding
(ii) Nucleic Acid Programmable Protein Array (NAPPA)	<ul style="list-style-type: none"> • No need to express and purify protein separately • Expression in mammalian milieu (natural folding) • Proteins produced just-in-time for assay • Shelf-life not an issue • Access to all cloned cDNAs • Retains functionality of traditional protein arrays • Arrays stable on bench until activated 	<ul style="list-style-type: none"> • Cloning procedure required, Pure protein array not produced as expressed protein remains co-localized to cDNA • Peptide tags may lead to steric effects blocking important binding domains • Functionality of proteins
(iii) Multiple Spotting Technique (MIST)	<ul style="list-style-type: none"> • Unpurified DNA products used as template • Very high-density protein arrays generated • Reusable DNA template array • Pure protein array generated • DNA template array can be stored for long durations 	<ul style="list-style-type: none"> • Loss of signal intensity with prolonged incubation time • Non-specific protein binding • Time consuming process • Broadening of spots due to diffusion • Not ascertained if multimeric proteins assemble effectively • Time consuming process • Possible loss of function on binding to Halotag • HT application will require optimization of printing
(iv) DNA Array to protein array (DAPA)		
(v) Halo-link protein array	<ul style="list-style-type: none"> • Strong covalent bond between protein and ligand • No material loss during washing • Oriented capture of protein • No non-specific adsorption • Easy quantification • No need for a microarrayer 	

Autoantibody Profiling using Protein Microarrays

Nikita Gahoi, ApurvaAtak, ManubhaiKP and Shabarni Gupta

Indian Institute of Technology Bombay, Mumbai, India

A microarray experimental design includes five major steps: A biological question, sample preparation, a biochemical reaction, detection and data analysis.

Objective: Auto-antibody detection in sera of glioma patients.

Requirements: Serum from glioma patients and healthy individuals, Human proteome array, superblock, Anti-GST antibody, Cy5 linked anti-HuIgG, Alexafluor555 anti-Rabbit Ab, TBST wash buffer, centrifuge for slide drying, shaker, small boxes, etc.

Principle: Formation of auto-antibodies has been reported in several cancers in addition to auto-immune diseases [Zaenker P et al. 2013]. In cancer, auto-antibodies are generated against either tumor derived proteins which are absent or barely detectable in normal tissue or in response to post-translationally modified proteins [Brichory FM et al. 2001]. These auto-antibodies appear in the blood stream many years before the onset of disease in many cases and thus can serve as useful bio-markers for early detection of cancer. Protein arrays provide a high-throughput platform where several thousands of features corresponding to thousands of proteins along with positive and negative controls are imprinted on slide and offers rapid and efficient auto-antibody detection by serum screening.

Procedure: Serum screening is performed using following procedure.

(i) Blocking: For blocking, the slides are incubated with 3%BSA in Superblock with gentle shaking for 1 hour at room temperature.

Purpose of blocking: To avoid any non-specific binding, the activated surface of the slide, where proteins are not imprinted must be blocked.

(ii) Washing: After blocking the slides are rinsed thoroughly (3x5 min) using 1xTBST on a magnetic stirrer at room temperature.

The purpose of washing: The extra blocking reagent adhered to the slide should not interfere with the protein and antibody (Ab) interaction and hence must be removed completely.

(iii) Drying: To remove the TBST from slides a quick rinse with distilled water is given followed by centrifugation at 900rpm for 2 min.

(iv) Primary Ab incubation: The slides are then incubated with 1xTBST containing 1:500 dilution of

serum, 1:5000 dilution of Rabbit Anti-GST Ab and 2% BSA for 2 hours with gentle shaking at room temperature.

Why a combination of Serum and Anti-GST Ab are used as primary Ab?

Here serum acts as a source of Primary Ab i.e., the autoantibody produced against the human proteins in response to disease. Anti-GST Ab is used as all the immobilized proteins are GST-tagged, and will subsequently help in the quality check of the slide.

(v) Washing: After primary Ab incubation the slides are rinsed thoroughly (4x5 min) using 1xTBST on a magnetic stirrer at room temperature.

Purpose of washing

Here washing removes any non-specific Abs that is present. This step is crucial as any non-specific Ab will further bind to the secondary Ab leading to signal amplification and false positive results. Therefore, washing should be stringent.

(vi) Drying: To remove the TBST from slides a quick rinse with distilled water is given followed by centrifugation at 900rpm for 2 min.

(vii) Secondary Ab incubation: The slides are then incubated with 1:1000 dilution of Cy5-labeled Anti-human IgG and 1:5000 dilution of Cy3-labeled Anti-Rabbit Ab and 2% BSA present in 1xTBST for 45 min 1hour in dark with gentle shaking at room temperature.

Why a combination of secondary Abs is used?

This leads to a dual channel data, where Cy5-labeled Anti-human IgG binds to antibodies of human origin (autoantibodies) and is scanned at 635 nm while Alexafluor 555 anti-Rabbit Ab binds to the Anti-GST Ab which is scanned at 532 nm. Therefore, both quality check and Autoantibody screening could be performed simultaneously.

(viii) Washing: Once the secondary Ab incubation is done the slides are rinsed thoroughly (4x5 min) using 1xTBST on a magnetic stirrer at room temperature in dark.

Purpose of washing

Here washing removes any extra non-specifically bound Abs. Improper washing may lead to high background, therefore proper washing should be done.

(ix) Drying: To remove the TBST from slides a quick rinse with distilled water is given followed by centrifugation at 900rpm for 2 min.

NOTE: A thorough washing is achieved by flipping the slides and changing the position of the slides during washing.

(x) Scanning: The final step of the experiment is the scanning of the processed slide where the slide is scanned in two wavelengths that is 635 nm (red channel) to screen for auto-antibodies and 532 nm (green channel) for quality check of the slide. Scanner should be switched on at-least 20 min prior to scanning, this allows the lasers to stabilize. A procedure for scanning is given below (Figure 3.2):

1. Insert slide into the slide holder space in an inverted form i.e., the imprinted protein spots should be facing down.
2. Open GenePix Pro7 software and then go to laser settings and adjust the wavelengths at which slides should be scanned (depends on the conjugated fluorophore used for detection).
3. Go to Hardware Settings and see the preview at different PMT gain values and select the optimum PMT value where minimum numbers of spots (positive controls) are saturated while most of the low intensity interactions are also captured.
4. Scan the slide at optimum PMT value.
5. Once scanning is complete, the image should be saved in .tiff format in respective folder.

(xi) Data acquisition: The .tiff images obtained should be further processed to obtain the signal intensities of all the imprinted protein spots on the slide. For this, each type of slide has their respective

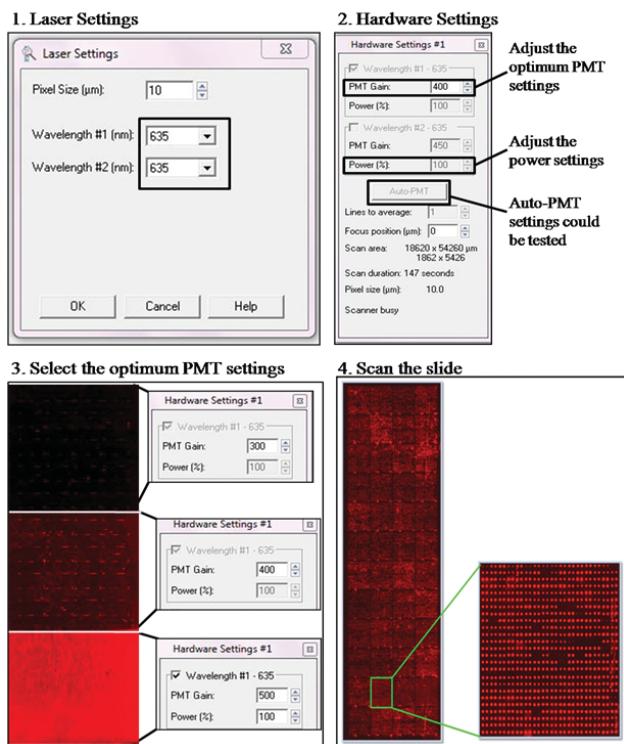


Figure 3.2: Optimization of Scanning Parameters

.GAL files which represents the name and exact location of protein imprinted on the slide. Data (Signal intensity) for each spots is acquired from the processed slide using following steps (Figure 3.3):

1. Open the particular .tiff image in GenePix Software.
2. Then open the .gal file, this file is in the form of grids containing the location of all protein spots arrayed on the chip.
3. First adjust the grid over the slide, and then align blocks followed by individual spots over the slide. If needed adjust the diameter and background signal intensities to obtain the true positive signal intensities only. Once all the features are aligned properly save the settings in .gps format.
4. Finally, save all the signal intensities in the form of .gpr files.

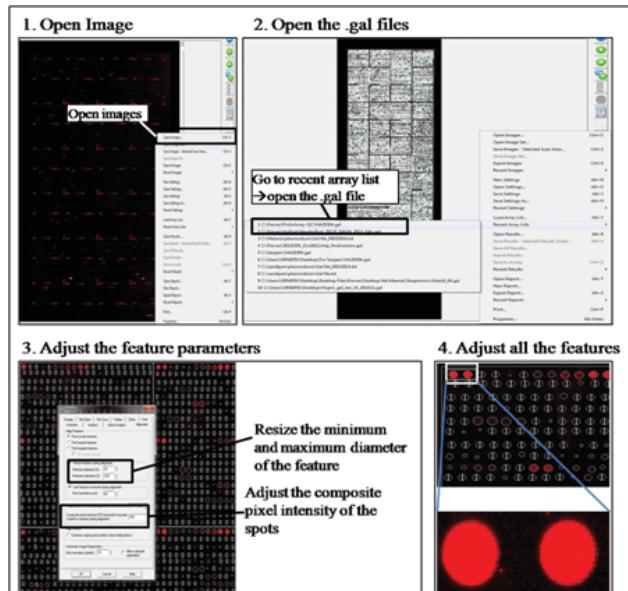


Figure 3.3: Steps for data acquisition

Microarray data analysis

NarendraGoud, Nikita Gahoi, ApurvaAtak, Rekha Jain and Shabarni Gupta

Indian Institute of Technology Bombay, Mumbai, India

Various software and programming tools like R, Perl, Python, BRBarry tools etc. can be used for processing the data generated from microarray experiment. After scanning, the spot intensities of each slide are stored as a .gpr, .chp, .tab or .txt file. Basically these are text files generated from different platforms used for scanning with different custom headers and can be read by using various programming tools and applications.

Various steps involved in the data analysis are as follows:

- **Quality assessment of raw data:** The raw data assessment helps us to have an overview of the quality of the data. Usually the spots are printed in duplicates and the plot of these intensities gives us an idea about the reproducibility. The raw signal intensities of all proteins in all the analyzed samples can also be plotted to get an insight about the preprocessing techniques to be used and also to compare the effects of these preprocessing techniques on the data (Figure 3.4).

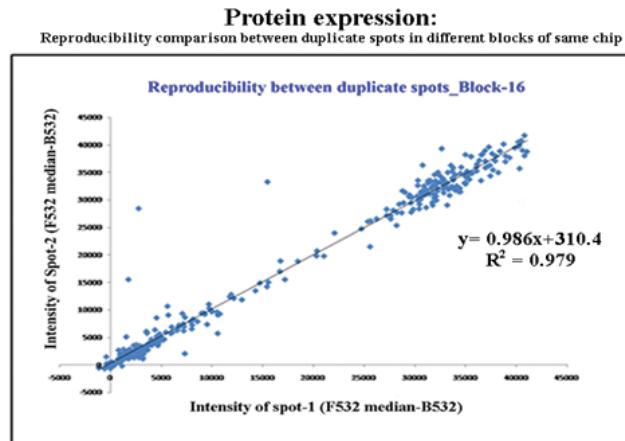
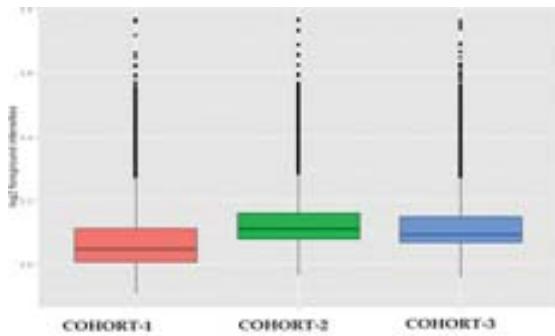


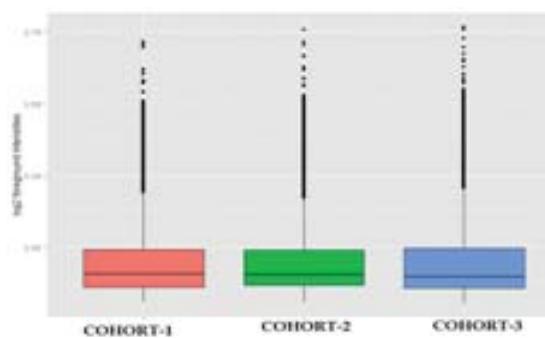
Figure 3.4: The scatter plot of the foreground intensities of the duplicate spots of different blocks in a particular microarray chip

- **Preprocessing:** The raw data is background corrected to account for the local variations (local background near the spots) of the slides. The aim of background correction is to get the true value of the intensity of a particular spot. The background corrected data is sometimes normalized to reduce the variation arising between the chips due to day to day variations, manufacturing variability and other technical variations. The objective of normalization is to make the expression values of the non-differentially expressed proteins similar (Figure 3.5).

- **Differential expression:** After preprocessing the next step is to identify the differentially expressed proteins (Figure 3.6). Different statistical tests are performed on the preprocessed data and ranks are assigned to each protein based on these tests. Using various empirical criteria (like fold change cutoff, *p*-value cutoff) on these proteins we get the set of differentially expressed proteins which may be further analyzed.
- **Gene set enrichment analysis (GSEA):** A GSEA can be performed on the set of differentially expressed proteins to get an overview of the various signaling pathways and biological processes that get altered in specific experimental conditions or diseased states (described later).



Raw Data



Preprocessed data

Figure 3.5: The box plots of the raw data, background corrected and normalized data

- **Further analysis:** The set of differentially expressed proteins can be further analyzed to get an economical set of proteins by using approaches like correspondence analysis. Support vector machines can also be modeled to differentiate the sub classes of data.

Pathway analysis: Gene set enrichment analysis can be performed on statistically significant

differentially expressed proteins using various bioinformatic tools as described below (Figure 3.7).

(i) Gene set enrichment analysis (GSEA) using GeneTrail: Gene set enrichment analysis (GSEA) can be performed using following steps.

1. Go to GeneTrail homepage (<http://genetrail.bioinf.uni-sb.de/>). Click on 'GeneTrail'

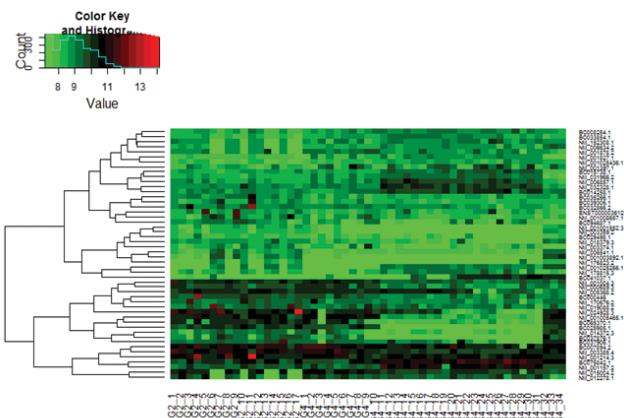


Figure 3.6: The heat map of the differentially expressed proteins in a comparison of the healthy and diseased cohorts

2. Select ‘Gene Set Enrichment Analysis’. Click on ‘Proceed’.
3. Select the organism name. We selected Homo sapiens. Click on ‘Proceed’.
4. Select the Identifier type from the dropdown menu. We have selected Gene Symbol here. Click ‘Proceed’.
5. Choose the input file to be uploaded in .txt format. Click on ‘upload’ and then click ‘Proceed’. Choose the other parameters and click on ‘Submit’.
6. You can view the results page as HTML page or download the pdf file for it. The output file consists of results for all the analysis types as per the parameters selected in step 5.



Abstract

We present a comprehensive and efficient gene set analysis tool, called **GeneTrail** that offers a rich functionality and is easy to use. Our web-based application facilitates the statistical evaluation of high-throughput genomic or proteomic data sets with respect to enrichment of functional categories. GeneTrail covers a wide variety of biological categories and pathways, among others KEGG, TRANSPATH, TRANSFAC, and GO. Our web server provides two common statistical approaches, “Over-Representation Analysis” (ORA) comparing a reference set of genes to a test set, and “Gene Set Enrichment Analysis” (GSEA) scoring sorted lists of genes. Besides other newly developed features, GeneTrail’s statistics module includes a novel dynamic-programming algorithm that improves the p-value computation of GSEA methods considerably.



Selection of enrichment analysis method:

Select type of analysis: ?

Over- / Under-representation Analysis
 Gene Set Enrichment Analysis



Step 1/5:



Select organism ?

Homo sapiens
 Mus musculus
 Rattus norvegicus
 Staphylococcus aureus N315
 Corynebacterium glutamicum ATCC 13032
 Arabidopsis thaliana
 Caenorhabditis elegans
 Drosophila melanogaster
 Aspergillus fumigatus Af293
 Danio rerio
 Sosic scrofa



Step 2/5:



Select identifier type: ?

Gene Symbol

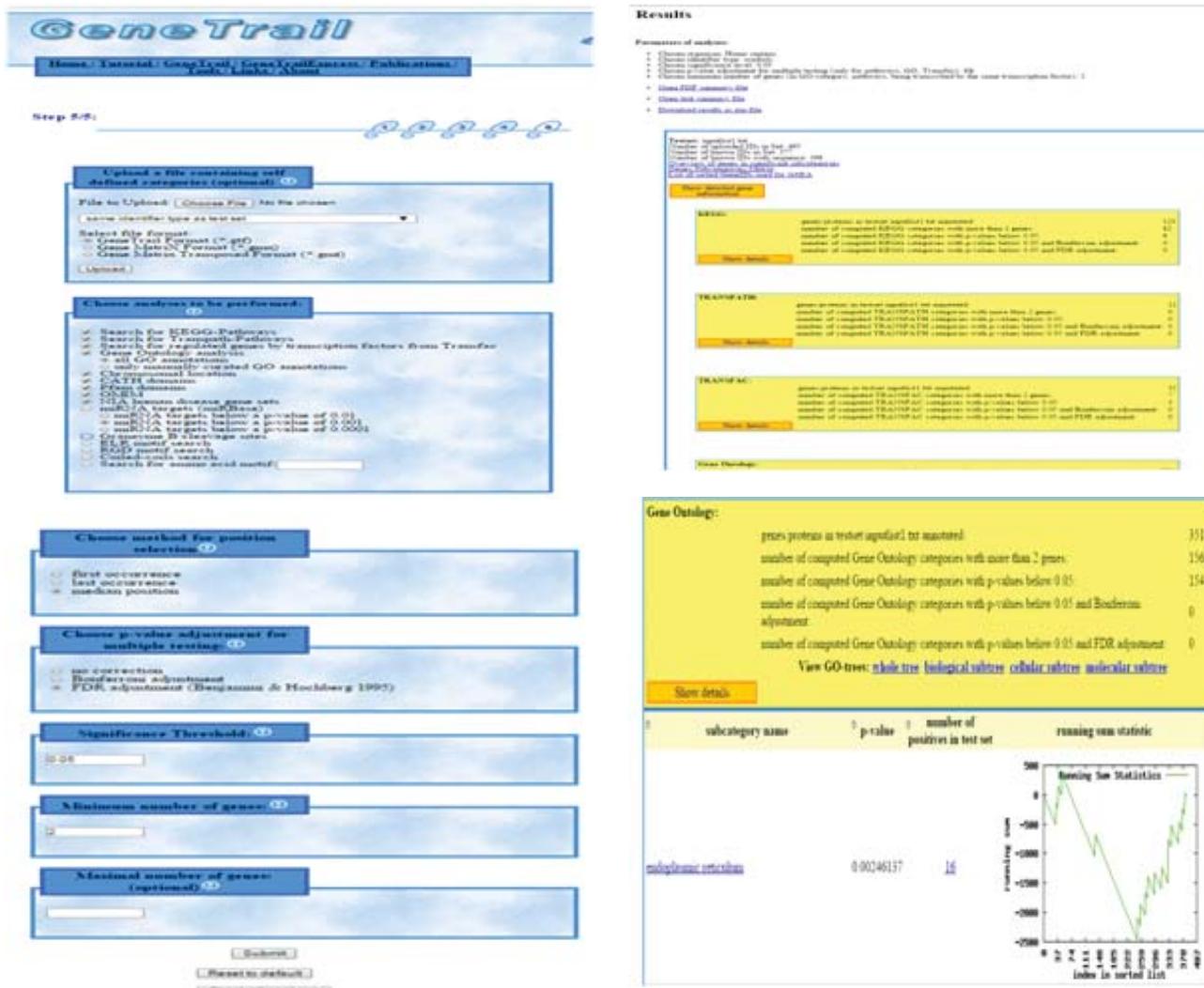


Figure 3.7: Steps for Gene Set Enrichment Analysis using GeneTrail Software

(ii) The Database for Annotation, Visualization and Integrated Discovery (DAVID): Gene set enrichment analysis (GSEA) using DAVID can be performed using following steps.

1. Go to the homepage of ‘DAVID’ (<http://david.abcc.ncifcrf.gov/>). Click on ‘Gene Functional Classification’.
2. Upload the input file as .txt file or directly copy paste it in the space provided. We have entered an input file of Entrez IDs. Select the Identifier type as ‘Entrez Gene ID’ since our input file contains Entrez IDs. Select the list type as ‘Gene List’. Click on ‘Submit List’.
3. Select the organism as ‘Homo sapiens’. If you click on ‘Show Gene List’ it displays list of all the input lists that it has processed successfully. You can download this file for further use if needed.⁴ For an immediate

idea of the results, click on + tab provided next to the results categories. For combined view of the results, click on the tabs provided at the bottom of the page.

Printing DNA (NAPPA mix) on aminosilane coated glass slides

(i) Preparation of NAPPA mix

- (a) Aminosilane coating on glass slides: Glass slides are coated with aminosilane by placing them in 2% solution of aminosilane solution in acetone for 5min on a rocking shaker. Slides are then rinsed with acetone followed by milliQ water. After drying, slides are stored in a box at room temperature.
- (b) Bacterial culture and DNA prep: Selected clones are cultured in LB containing ampicillin (100µg/



ml) and plasmid DNA for those clones are prepared and quantified. Plasmid DNAs are dissolved in NAPPA master mix which can be printed on the slide. Master mix contains BSA (3.6mg/ml), BS³ cross-linker (1.25mg/ml) and polyclonal anti-GST antibody (50μg/ml).

(ii) Printing

Several physical and chemical parameters affect the quality of printing. These include type of pins, humidity, hydrophobicity of slide surface, proper washing between transfer of different liquids, viscosity and surface tension of the liquid to be printed.

- **Pins geometry and surface chemistry of pins:** Pins are available in a wide range of tip and channel sizes which determine spot diameter and loading volume. Stealth SMP3 microarray spotting pins have an uptake volume of 0.25 μ l and a delivery volume of 600 μ l, providing up to 400 spots per loading.

- **Washing of pins:** Thorough pin cleaning is extremely important for successful arraying, to avoid cross contamination especially for protein applications. Due to the compositions and differing viscosity of various spotting solutions used for protein arrays, minimizing the amount of carry-over between spots is a critical step for successful protein arraying. By varying the number of cycles through the wash stations, it is possible to print proteins on a required surface without any carry-over. Sonication is required to increase the effectiveness of the wash cycles. Mostly printers include a built-in sonication station for improved cleaning, ideal for printing especially for protein microarrays.

Microarray Printer

Microarray printers are available with user friendly softwares which allow users to set custom parameters required for printing of microarray slides. Users need to define various parameters such as type of source plate (which contains samples), pins cleaning protocol, sample sequence, etc (Figure 3.8 and 3.9).

Parts of microarray printer

- a) Robotic arm with print head
 - b) Washing station with sonication platform
 - c) Blotting platform
 - d) Platform to accommodate slides
 - e) Humidity control
 - f) Temperature control
 - g) Place to keep source plates

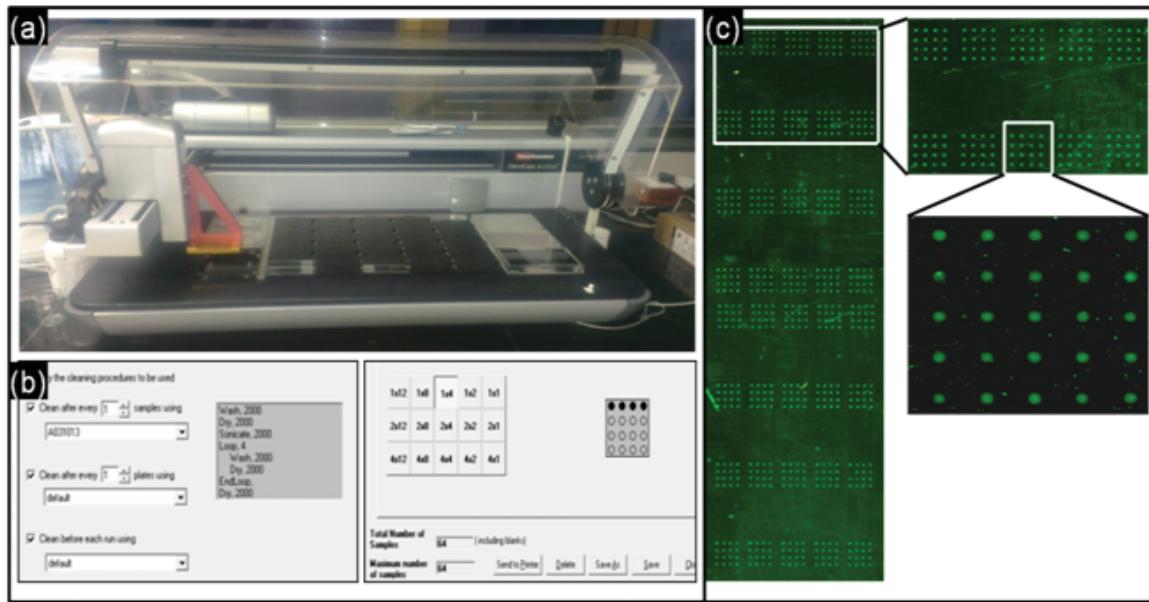


Figure 3.8: (a) Microarray printer (b) Printing parameters (c) Printed slides

Protocol

- (1) First select the type of sample plate as 96-well plate or 384 well plates.
- (2) Enter details for number of pins and position which user wants to use for printing.
- (3) Set the value of origin offset.
- (4) Array design: It depends on the number of spots and pattern which users want to print on slide.

- (5) Next enter the value for dipping time.
- (6) Enter details for protocol of cleaning the pins which is a crucial step for successful printing.
- (7) Set the number of dots for blotting to remove extra sample stuck on edges of pins.
- (8) Enter details for sample sequence which helps in making .gal file.

(1) Sample plate

Parameters for Run Method

Method Name: A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Select the plate type you intend to use from the list below:

default - 384
384 well

Number of Plates: 1

Total Number of Samples: 300 (including blanks)

Maximum number of samples: 300

Send to Printer | Delete | Save As... | Save | Done

(2) Pins configuration

Parameters for Run Method

Method Name: A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Select the pin configuration you wish to use with this method:

1x12 | 1x8 | 1x4 | 1x2 | 1x1
2x12 | 2x8 | 2x4 | 2x2 | 2x1
4x12 | 4x8 | 4x4 | 4x2 | 4x1

Total Number of Samples: 300 (including blanks)

Maximum number of samples: 300

Send to Printer | Delete | Save As... | Save | Done

(3) Origin Offset

Parameters for Run Method

Method Name A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Specify the amount to offset the origin from the upper-right corner of the slide.

Total Number of Samples 300 (including blanks)

Maximum number of samples 300

Send to Printer Delete Save As Save Close

(4) Array Design

Parameters for Run Method

Method Name A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Dot Spacing (microns)	Horizontal 400	Vertical 400	Number of dots per sample 1
Number of dots per subarray 5	$\frac{1}{5}$	$\frac{1}{5}$	25 dots per subarray
Maximum 6	6	6	25 / 1 = 25 samples per subarray

Array Spacing (microns)	Horizontal 600	Vertical 600	<input checked="" type="checkbox"/> Different samples for each array
Number of arrays 1	$\frac{1}{1}$	$\frac{1}{1}$	<input type="checkbox"/> Same samples for each array (replicate array)
Maximum 3	3	3	

Total Number of Samples 300 (including blanks)

Maximum number of samples 300

Send to Printer Delete Save As Save Close

(5) Dipping

Parameters for Run Method

Method Name A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Choose the frequency and duration of dips from the sample plates.

Number of dips 2

Dip Time 2000 (Milliseconds)

Redip after every 1 (Seconds)

Print Time 400 (Milliseconds)

Total Number of Samples 300 (including blanks)

Maximum number of samples 300

Send to Printer Delete Save As Save Close

(7) Blotting

Parameters for Run Method

Method Name A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Check for Blotting with this Method

Enter the number of dots you wish to blot for each sample

Number of blotting dots 1

Spacing (microns) 400

Contact Time (ms) 0

Total Number of Samples 300 (including blank:)

Maximum number of samples 300

Send to Printer Delete Save As Save Close

(6) Cleaning settings

Parameters for Run Method

Method Name A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Specify the cleaning procedures to be used.

Clean after every 1 samples using A031013

Clean after every 1 plates using default

Clean before each run using default

Wash, 2000
Dry, 2000
Sonicate, 2000
Loop, 4
Wash, 2000
Dry, 2000
EndLoop
Dry, 2000

Total Number of Samples 300 (including blanks)

Maximum number of samples 300

Send to Printer Delete Save As Save Close

(8) Sample sequence

Parameters for Run Method

Method Name A031013

Sample Plates | Pin Config | Origin Offset | Array Design | Dipping | Cleaning | Blotting | Sample Sequence

Horizontal Fill
 Vertical Fill

Sample Sequence

30 N1-N12
33 N1-N12

Block 32

Insert Blank Append >> Remove Append Blank Remove All

Total Number of Samples 300 (including blank:)

Maximum number of samples 300

Send to Printer Delete Save As Save Close

Figure 3.9: Workflow for printing DNA or protein using OmniGrid Accent microarray printer

In-vitro transcription and translation (IVTT); NAPPA technology

Objective: Demonstration of *in-vitro* transcription and translation (IVTT)

Requirements: Plasmid DNA which contains target gene (pANT7 vector), rabbit reticulocytelysate system (promega), luminometer, incubator, etc.

Principle: Cell free protein expression was first demonstrated by He M and Taussig MJ in 2001 where they showed Protein *in situ* array (PISA). Nucleic acid programmable protein array (NAPPA) was developed by Labaer and colleagues (Ramachandran et al. 2004). NAPPA combines recombinant cloning technologies with cell-free protein expression. In this method, cDNA encoding a fusion of protein of interest with a tag (usually glutathione-S-transferase, GST) is expressed in plasmids by recombinant cloning. These cDNA corresponding to specific genes/proteins are then immobilized on amidosilane coated glass slides. The array is activated by adding rabbit reticulocytelysate (RRL) supplemented with T7 polymerase, RNase inhibitors and essential amino acids. Following transcription and translation the newly synthesized protein fused to the GST tag is then captured by the anti-tag (GST) antibodies attached to the array (Figure. 3.10).

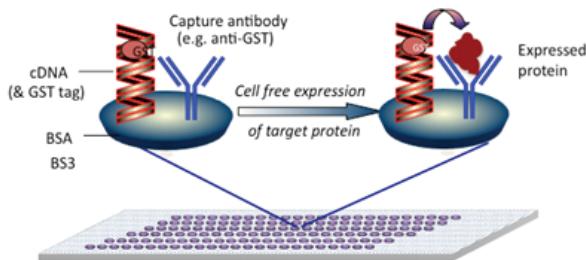


Figure 3.10: NAPPA protein microarray where protein of interest is tagged with GST and expressed using IVTT. The expressed protein is captured on slide coated with anti-GST antibody

Procedure: *In-vitro* transcription and translation (IVTT) is performed using following procedure.

- (i) Three IVTT reaction systems are set up using nuclease free microfuge tips, tubes and water as follows:
- (ii) The above systems are incubated at 30 degrees Celsius for 3hrs.
- (iii) Proteins can be expected to be produced after this incubation period and can be verified using western blot.
- (iv) Once standardized, these DNA samples can be printed on ligand coated slides and can be

flooded with IVTT mix for NAPPA based experiments.

Table 3.2
Details of reaction set-up of IVTT reaction

System	No DNA control (μ l)	Positive control (μ l)	Test reaction (μ l)
IVTT Mix	40	40	40
Methionine	1	1	1
DNA cloned in pANT7 vector	0	-	2
Luciferase DNA	0	2	-
Nuclease free water	9	7volume made up to 50 μ l	

Precautions: Following precautions should be taken into consideration.

- Nuclease free reagents and plastic ware must be used throughout the experiment to avoid DNA or RNA degradation during the process.
- One must ensure that the temperatures do not fluctuate by more than a degree during the incubations.
- The IVTT Mix must not be thawed more than twice as its activity would substantially diminish and would result in non-functional reactions.
- To ensure optimal IVTT, a luciferase positive control reaction is set up with luciferase gene cloned in a vector. The production of luciferase is checked using a luminometer using the no DNA control as a blank. If the IVTT mixture is functional and the reaction conditions are optimal, luminescence readings show a stark rise in the positive control reactions as compared to the blank.

Application note 3a

Data analysis pipeline in Protein Microarrays using R-programming

Saket Choudhary¹, Narendra Goud², Shabarni Gupta², Parvez Syed², Santosh Noronha¹, Sanjeeva Srivastava^{2*}

*are equal contributors in this work

¹Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India

²Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India

*Correspondence: Dr. Sanjeeva Srivastava, Department of Biosciences and Bioengineering, IIT Bombay, Mumbai 400 076, India; E-mail: sanjeeva@iitb.ac.in

Introduction

Over the last couple of decades, Microarrays have emerged as a resourceful technique for measuring extent of differential expression in a high-throughput manner on genomics and proteomics based platforms [1,2]. These technologies have been harnessed extensively to yield major breakthrough in the field of biomarker research. The sophistication in such experimental set-ups extends beyond performing the experiments. Data analysis of microarrays has a complex pipeline which involves a series of computationally demanding techniques along with sound scientific judgments which helps make data interpretation an unbiased endeavor.

This article is a case study of microarray data analysis using representative data for autoantibody profiling of serum samples from patients with low grade (cohort I) and high grade (cohort II) brain tumor on Human Proteome Array chips (HuProt™) which describes a pipeline established to identify differentially expressed proteins in these cohorts using protein microarray platforms.

Methodology

In this study, serum samples from 18 patients comprising Cohort I and 18 patients comprising Cohort II were used to identify differential autoantibody profiles in both cohorts using HuProt chips containing ~17000 unique full-length proteins printed in duplicates.

After the assay, the processed microarrays were scanned with GenePix 4000B Microarray Scanner (Molecular Devices). GenePix Pro 7 (Molecular Devices) was used for image processing and data acquisition and the data was stored in form of .gpr(gene pix results) file.

The data analysis was done using R programming language [3]. We used the limma^[4] package from the

Bioconductor^[5] package for the data analysis. The following steps describe the pipeline utilized:

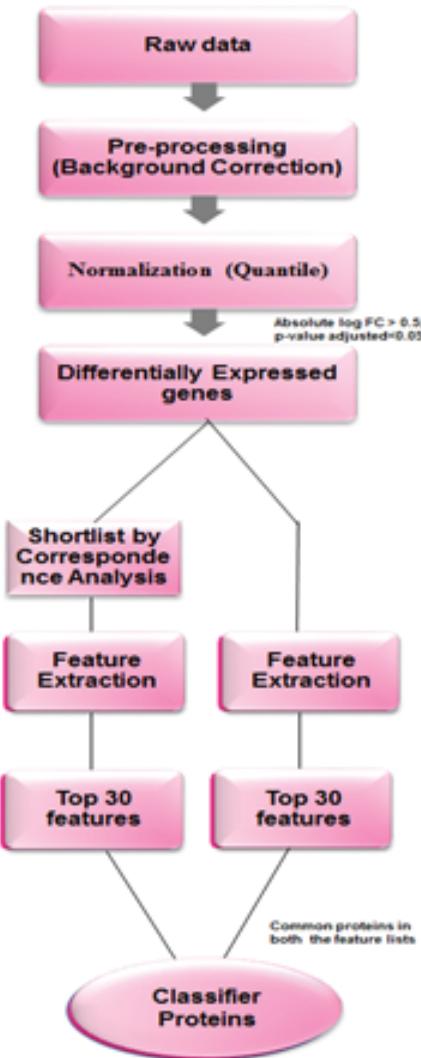


Figure 3.a.1: Schematic representation of data analysis strategy

- The raw data was background corrected by ‘normexp-by-control’ method which uses only the negative controls. In order to reduce the variability of the log ratios the final adjusted values were given an offset of 100. The “nec” method from the limma package was used to perform the background correction [6,7].
- The background corrected data was then quantile normalized using the quantile normalization function.
- The differentially expressed proteins were then selected from the preprocessed data (background corrected and normalized).
- A moderated t-test was used to select the differentially expressed proteins, using a null

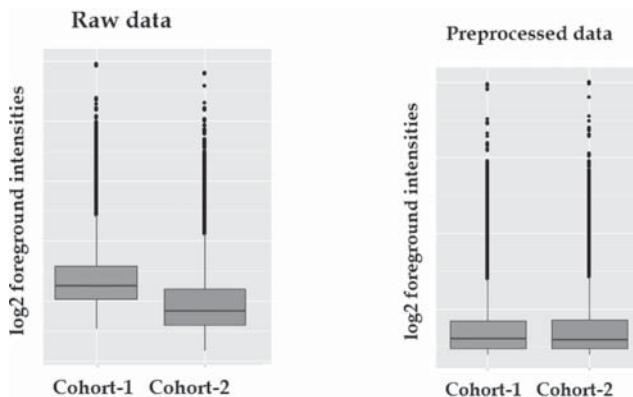


Figure 3.a.2 highlights the distribution of data across all slides pertaining to each group i.e. Raw data and data after Normalization.

hypothesis that the proteins are not differentially expressed. ‘Limma’ package was used to perform these tests.

- The differentially expressed proteins are selected using the criteria of absolute log fold change^[8] greater than 0.5 and an adjusted p value less than 0.05, this criteria is to focus on the proteins which is statistically significant and also a high fold change. To look for the implications of these proteins on tumourigenesis gene set enrichment analysis can be performed on the set of differentially expressed proteins to look for the pathways involved or other functional annotation studies depending on the focus of the experiment.
- The stringency of the selection of differentially expressed proteins can be varied depending on the nature of data obtained. We standardized the above mentioned stringency criteria with regard to adjusted p-value and Fold change. On the basis of our set criteria, the differentially expressed proteins were large.
- So as to get a compact set of proteins these differentially expressed proteins were further analyzed. Support Vector Machines (SVM)^[9] models are one of the popular classifier techniques used for multivariate statistical analysis. A Linear SVM was used to deduce a list of 30 proteins from the set of differentially expressed proteins.
- Correspondence analysis^[10] is also a multivariate technique to summarize the multi dimension to two dimensions. Another set of 30 proteins was deduced by performing correspondence analysis on the set of differentially expressed proteins and then an SVM on the proteins obtained from Correspondence analysis.
- To get a more robust set of classifiers which could differentiate between the two cohorts efficiently, a panel of 18 classifier proteins were selected which

are common in the first set of 30 proteins as well as the second set of 30 proteins.

- The sensitivity, specificity, AUC and classification rate was calculated using recursive feature elimination after SVM and the panel including the minimum number of proteins with highest sensitivity, specificity, AUC and classification accuracy was chosen as a panel of classifier protein to distinguish two diseased cohorts.

Results and Discussion

The raw data generated after prepossessing (background correction and normalization) was median centered and was used for further analysis. The proteins which showed a log₂ fold change greater than 0.5 or less than -0.5 and had an adjusted p-value less than 0.05 were considered as differentially expressed while comparing both cohorts.

A list of 42 proteins was found to be differentially expressed in the comparison of Cohort I as compared to Cohort II. Among the differentially expressed proteins 24 were up regulated and 18 were down regulated in Cohort I as compared to Cohort II. The heat maps were generated using the differentially expressed proteins. As the list of differentially expressed proteins were large and to get a compact set of proteins these differentially expressed proteins were further analyzed. A list of 30 proteins was deduced by applying SVM on the set of differentially expressed proteins. Similarly another set of 30 proteins was deduced by performing correspondence analysis on the set of differentially expressed proteins and then an SVM on the proteins obtained from Correspondence analysis. A panel of classifier proteins was selected as the common proteins present in the first set of 30 proteins as well as the second set of 30 proteins. 18 proteins were identified which could differentiate Cohort I and Cohort II with 78% sensitivity and 76% specificity with Area Under Curve (AUC) value of 0.95 and classification accuracy of 77.5%. This panel

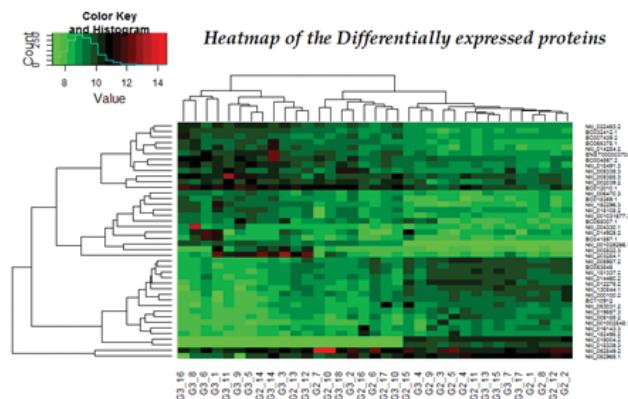


Figure 3.a.3 represents heat-maps of the 42 differentially expressed proteins

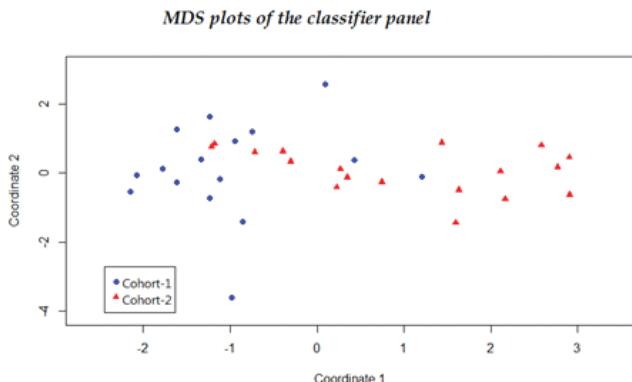


Figure 3.a.4 represents non-metric MDS lot used to visualize the separation between the two cohorts.

included 11 up-regulated and 7 down regulated proteins. A non-metric Multidimensional Scaling Plot^[11] was used to visualize the separation between the two cohorts.

Challenges with microarray data analysis:

Due to intermediate steps involved in assay the spot intensities recorded by the software are prone background noise and bias. This may be due to different reasons:

- Manufacturing defects resulting in variation of chips.
- Chips may show an overall difference in intensities due to variation in the amount of dye used.
- Day to day variations like change in temperature, humidity etc.^[12]
- Gene specific dye bias^[13]: These errors can be reduced by using a good replicable design. A good biological replicable design may be achieved by using a large number of subjects, though this might result in high variation, but the results tend to be less biased.

References

1. Jens Sobek, Kerstin Bartscherer, Anette Jacob, Jvrg D Hoheisel, and Philipp Angenendt. Microarray technology as a universal tool for high-throughput analysis of biological systems. Combinatorial chemistry & high throughput screening, 9(5):365{380, 2006. 50
2. Allison DB1, Cui X, Page GP, Sabripour M., Microarray data analysis: from disarray to consolidation and consensus.Nat Rev Genet. 2006 Jan;7(1):55-65.
3. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.
4. Smyth,G.K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, Article3 (2004).
5. Gentleman,R.C. et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).
6. Wei Shi, Alicia Oshlack, and Gordon K Smyth. Optimizing the noise versus bias trade-off for illumina whole genome expression beadchips. Nucleic acids research, 38(22):e204{e204, 2010. 54
7. Jeremy D Silver, Matthew E Ritchie, and Gordon K Smyth. Microarray background correction: maximum likelihood estimation for the normal-exponential convolution. *Biostatistics (Oxford, England)*, 10(2):352{63, April 2009. ISSN 1468-4357. doi: 10.1093/biostatistics/kxn042. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2648902&tool=pmcentrez&rendertype=abstract>. 55
8. Daniela Witten and Robert Tibshirani. A comparison of fold-change and the t-statistic for microarray data analysis. Department of Statistics, Stanford University technical report, 2007. 61
9. Yuchun Tang, Yan-Qing Zhang, Zhen Huang, Xiaohua Hu, and Yichuan Zhao. Recursive fuzzy granulation for gene subsets extraction and cancer classification. *Information Technology in Biomedicine, IEEE Transactions on*, 12(6):723{730, 2008. 83}
10. JP Benzecri. coll., 1973. L'analyse des donnees. Tome I: La taxinomie. Tome II: L'analyse des correspondances, 1973. 74
11. Borg, I., Groenen, P. (2005). *Modern Multidimensional Scaling: theory and applications* (2nd ed.). New York: Springer-Verlag. pp. 207–212. ISBN 0-387-94845-7.
12. Ruixiao Lu, Geun-Cheol Lee, Michael Shultz, Chris Dardick, Kihong Jung, Jirapa Phetsom, Yi Jia, Robert H Rice, Zelanna Goldberg, Patrick S Schnable, et al. Assessing probe-specific dye and slide biases in two-color microarray data. *BMC bioinformatics*, 9(1):314, 2008. 52
13. Marie-Laure Martin-Magniette, Julie Aubert, Eric Cabannes, and Jean-Jacques Daudin. Evaluation of the gene-specific dye bias in cdna microarray experiments. *Bioinformatics*, 21(9):1995{2000, 2005. 52.

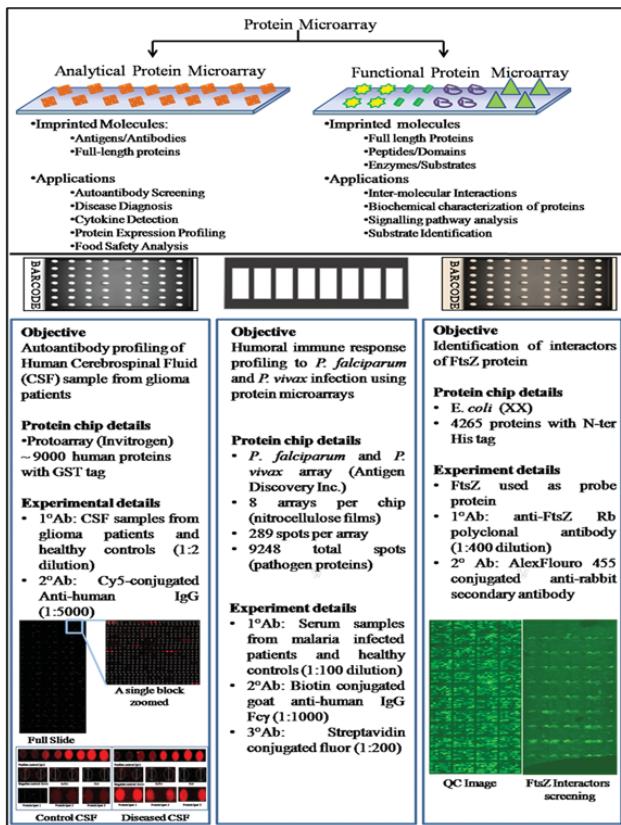
Application Note

Protein Microarrays for Clinical Applications

Rekha Jain, Apoorva Venkatesh and Panga Jaipal Reddy

Indian Institute of Technology Bombay, Mumbai, India

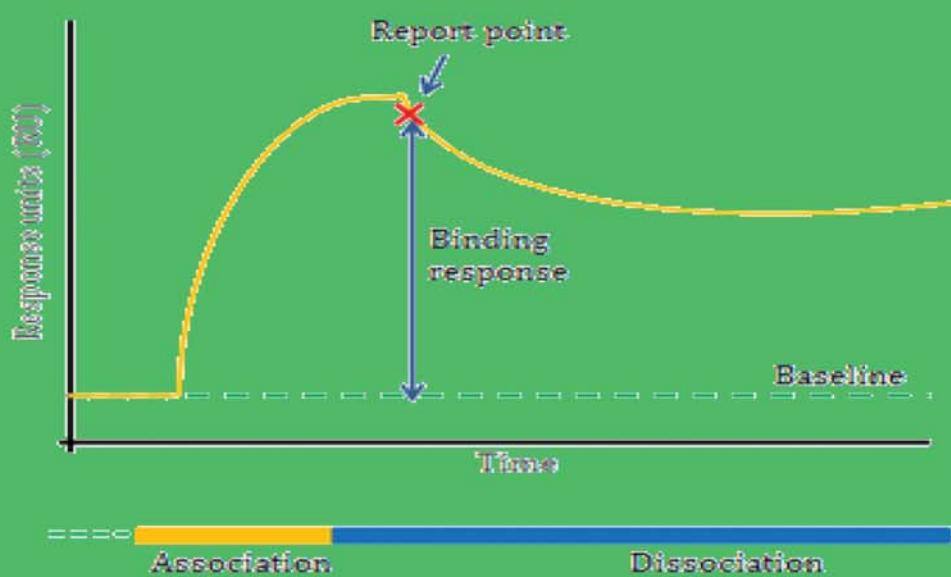
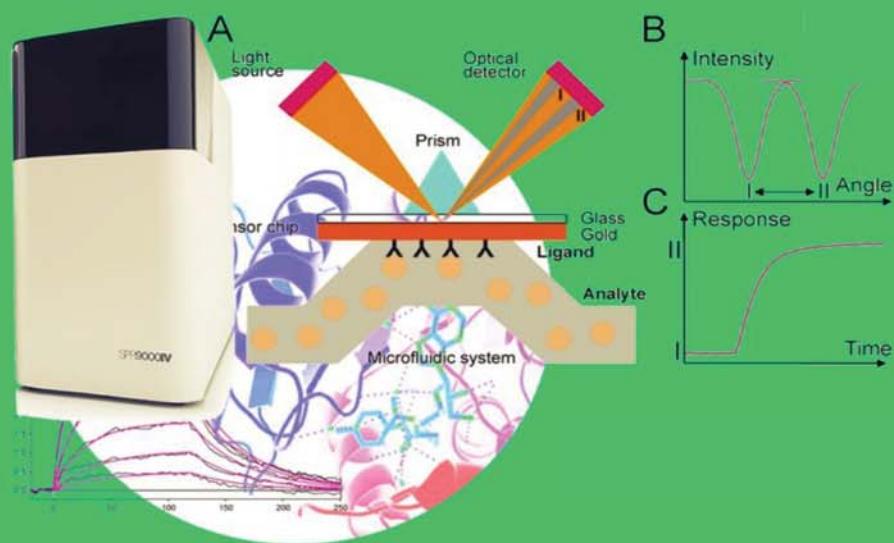
Protein microarray has several analytical and functional applications in clinical and industrial areas. A pictorial representation of various applications of Protein microarray is given below.



Key references

- MacBeath G and Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science*. 2000;289:1760-3.
- Zaenker P1, Ziman MR. Serologic autoantibodies as diagnostic cancer biomarkers—a review. *Cancer Epidemiol Biomarkers Prev*. 2013;22:2161-81.
- Brichory FM, Misek DE, Yim AM, et al. An immunerresponse manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *Proc Natl Acad Sci U S A*, 2001;98:9824-9.
- He M., Taussig, M. J., Single step generation of protein arrays from DNA by cell-free expression and *in situ* immobilisation (PISA method). *Nucleic Acids Res*. 2001, 29, e73.
- Ramachandran N, Hainsworth E, Bhullar, B, Eisenstein S. et al., Self-assembling protein microarrays. *Science* 2004,305, 86–90.
- Mark Schena. *Microarray Analysis*. Wiley-Liss Press, 2003.
- <http://genetrail.bioinf.uni-sb.de/enrichment-analysis.php?js=1&cc=1>
- <http://david.abcc.ncifcrf.gov/>

LABEL FREE DETECTION (Module IV)



Label-free Proteomics

Preface

Veenita Shah¹, Samridhi Sharma¹ and Ed Nice²

¹Indian Institute of Technology Bombay, Mumbai, India

²Monash University, Australia

Label-free detection technologies have become imperative in the field of proteomics for detection, monitoring and characterization of biomolecular interactions. These technologies are gaining acceptance over the traditional labeling systems due to their high sensitivity, accuracy and speed for high-throughput cellular and biochemical assays [1]. Such label-free techniques rely on the measurement of some inherent properties of the query molecules (e.g., mass and dielectric property) and allow direct, real-time detection of biomolecules in a high-throughput manner. To eliminate the interference caused by the tagging in labeling techniques, various label-free approaches, including surface plasmon resonance (SPR)-based techniques (SPR and SPR imaging [SPRI]), nanomaterial-based techniques, nanohole arrays and interferometric assays have emerged [2]. These methods avoid any tethering to the biomolecule of the interrogation thereby preventing any conformational restriction and change in the functions of the molecule. These methods of detection play an important role for characterizing cellular signaling mechanisms and biological interactions of various proteins. This workshop focuses on performing the binding and kinetics assays in order to study the protein-protein interaction using techniques like surface Plasmon resonance (SPR) and bio-layer interferometry (BLI).

Surface plasmon resonance (SPR) is a biosensor technology that enables label-free and real-time measurement of biomolecular interactions. An SPR experiment involves immobilization of a ligand (e.g. protein or nucleic acid) on the functionalized surface of a sensor chip followed by passing of the other interacting molecule called analyte (protein or small molecule) over the chip surface to investigate the binding affinity and kinetics between the two molecules [3]. SPR is an optical phenomenon that occurs in thin conducting films between media of different refractive index, when p-polarized light is reflected off a thin metal film (gold surface on the sensor chip) at a certain wavelength and angle under the condition of total internal reflection (Fig. 4.1). At a certain angle of incidence and energy, the incident light excited plasmons in the gold film [4]. The total internal reflection field generates an evanescent wave in the gold film across the interface into the medium of lower refractive index, i.e. the molecules in contact with the chip surface. The excited surface plasmons are very sensitive to the refractive index change at the surface of the gold film. Changes in the solute concentration

at the sensor chip surface cause changes in the refractive index of the solution that can be measured as an SPR response. The change of the incident angle required for SPR is defined as SPR response in the unit of response unit (RU).

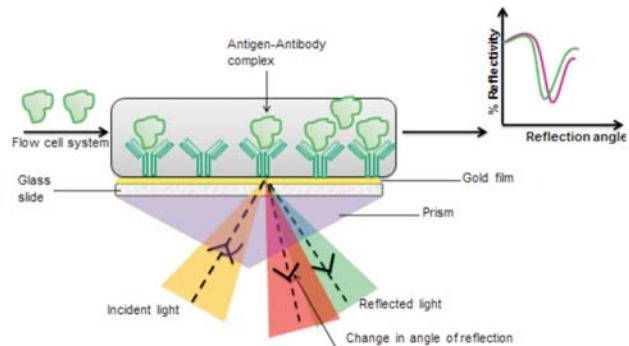


Figure 4.1: Surface plasmon resonance

The binding of the analyte to the ligand is traced in real time by following the change in SPR signal over time, referred as sensorgram. The binding response increases as analyte passes over the sensor chip and associates with ligand followed by a decrease in response as the analyte injection is stopped, and the binding complex dissociates[5]. The sensorgram is fitted to a suitable kinetic model for determination of kinetic parameters such as the association rate constant (k_a , $M^{-1} s^{-1}$), the dissociation rate constant (k_d , s^{-1}) and the equilibrium constant (K_D , M). The different SPR-based platforms available are used for diagnostic purposes, drug-discovery studies and structural biology applications.

Kinetic data is crucial in characterizing an interaction between two molecules to allow thorough understanding of binding mechanism. Interactions with the same affinity can have markedly different association and dissociation rates, as shown in Figure 4.2. A small molecule with high affinity (low K_D value)

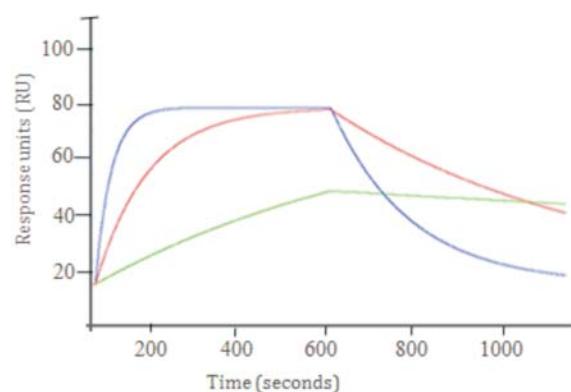


Figure 4.2: Sensorgram plots showing the response (RU) versus time for three different interactions with the same affinity but markedly different association and dissociation rate constants.

for a protein target may be a poor drug *in vivo* if it also has a very high dissociation rate. In addition, characterizing interactions between biomolecules is fundamental to knowing their molecular functions and structure activity relationships.

A wide variety of applications can be performed using label-free detection techniques, including specificity, affinity, kinetics, qualitative ranking, concentration analysis and thermodynamics of binding. This information can in turn be applied in pharmaceutical validations, antibody-antigen interactions, immunogenicity testing and development of therapeutics.

SPEAKERS

Dr. Ed Nice

Monash University, Australia

Talk Title: An introduction to SPR technologies and their application.

Biography: Professor Edouard (Ed) Nice is the head of Clinical Biomarker Discovery and Validation and former Director of the Monoclonal Antibody Technology Facility (MATF) at Monash University Clayton campus. He is also a visiting Professor at Sichuan University/West China Hospital. His scientific research has largely been focused on the development of new techniques (including micro-preparative HPLC, biosensor analysis and proteomics) for the micro-isolation, purification, characterization and analysis of protein and peptide growth factors, their receptors and associated signaling molecules with the aim of understanding the role of these molecules in neoplasia (in particular colorectal cancer (CRC)). Many of these studies have been aimed at the discovery and characterization of potential CRC biomarkers for early detection and surveillance of the disease. To this end, he has pioneered the field of Faecal Proteomics, and the role of quantitative mass spectrometry (using the technique of multiple reaction monitoring (MRM)) for the development and validation of sensitive and specific multiplexed assays for use with panels of CRC biomarkers. This platform technology can be readily translated to other cancers, or other pathologies (e.g. cardiovascular disease, diabetes, and neuropathology). He has published more than 200 peer-reviewed manuscripts in high-ranking international journals and special journals related to his fields of interest. These articles have been cited >8500 times, with an average number of citations per article of 37.85. Sixteen articles have more > 100 citations. They have an h-index of 50. He has had various successful collaborations with both industry and academia, and holds a number of patents in his field. Ed is on the editorial board of a number of

scientific journals including Expert Review of Proteomics, MCP, Journal of Proteomics, Clinical Proteomics, Analytical Biochemistry, Biomedical Chromatography, Current Signal Transduction Therapy and Growth Factors. He was a founding member of the Australian Peptide Association, co-chair and treasurer of the HUPO 2010 meeting in Sydney, and is currently one of the leaders of HUPO Chair 7 initiative, is a co-chair of the Cancer HPP initiative and a member of the HUPO antibody and financial committee.

Abstract: Since the introduction of commercial instrumentation in the early 1990's, surface plasmon resonance (SPR) has rapidly become one of the methods of choice for the quantitative analysis of protein-protein interactions. It allows sensitive, label free, real time monitoring of biomolecular interactions, based on changes in refractive index (due to changes in mass) at the sensor surface following binding. In this presentation, I will discuss the basic theories of SPR and the related SPR imaging technologies, and briefly introduce some of the commercial instrumentation. I will present important practical considerations including immobilization strategies and signal amplification. Finally, I will overview a number of recent interesting applications in the field, including those relating to proteomics research, high-throughput monoclonal antibody development and drug discovery.

Dr. Anette Persson

GE Healthcare Life Sciences, Sweden

Talk Title: Assay design and reagents for application development for SPR studies.

Biography: Dr. Persson has worked with label-free interactions since the earliest years. With a background in biochemistry and clinical chemistry at Uppsala University, she came to Pharmacia Biosensor in the late 1980's, a few years before the commercial launch of the first Biacore instrument. After ten years in the system department, working with application development with a focus on the integration of sensor surfaces, flow system, and application needs in the first biosensor instruments, Dr. Persson moved to a position as European Application Specialist with a supporting role for both existing users and sales to new customers. Since 1997, Dr. Persson has held a position of Manager in the Knowledge and Training group with responsibility for developing and providing training courses and application support.

Abstract: There are several parameters involved in how to get your surface plasmon resonance-based assay for biomolecular interactions to work properly. This presentation highlights the importance of different

immobilization techniques and immobilization levels as the first step in designing an assay. Buffers can be a key to success, a crucial factor when designing experiments. Case studies will show the outcome of different choices.

Dr. R. Varadarajan

Molecular Biophysics Unit, Indian Institute of Science, Bangalore

Talk Title: Mechanistic and structural insights from SPR/BLI studies of protein:ligand interactions.

Biography: The research work in Dr. Varadarajan's lab focuses on the general area of protein structure and folding. A wide variety of spectroscopic, calorimetric, crystallographic, molecular biological and computational tools are used to obtain information on protein structure, stability and function. The lab is also interested in rationally modulating protein stability through mutation, understanding residue-specific contributions to protein stability, improving methods for disulfide design and developing screens for identifying stabilized mutants of proteins. New methods for the design of temperature sensitive mutants of globular proteins have been developed. The lab is also interested in the informational content of large mutant data sets and has recently shown that these can be used to guide protein structure predictions. Another focus of research involves protein engineering studies of the viral envelope protein from the AIDS virus, HIV-1 and its cellular receptor CD4. The goal of this work is to produce stable, well folded molecules of HIV gp120/gp41 immunogens for eventual use as part of an AIDS vaccine as well the design of inhibitors of viral entry into the cell. Similar approaches are also being used to design immunogens intended to produce neutralizing antibodies against influenza virus, another re-emerging global health threat.

Abstract: We describe studies of protein:ligand binding in different systems using both SPR and BLI. These systems include the *E. coli* toxin CcdB, designed fragments of the surface proteins of HIV-1 and influenza. CcdB data provide mechanistic insights into the binding of this protein to two cognate ligands, CcdA and DNA gyrase. In the case of HIV-1 and influenza, binding of various designed fragments of the respective surface proteins to various antibodies was characterized. Binding data were used to prioritize immunogens for further testing. In the case of influenza, some of the designed immunogens successfully protected mice from pathogenic, heterologous challenge.

Dr. Rajesh Saha

Bio Rad Laboratories (India) Pvt Ltd

Talk Title: Application of SPR in antibody screening and characterization

Biography: Dr. Rajesh Sahais a Senior Field Application Specialist in Bio-Rad, and is based in Kolkata. He has worked for a period of ten years in the field of tRNA-protein interaction under the guidance of Professor Siddhartha Roy in Bose Institute and IICB, the two Premier Research Institutes of Kolkata. After completion of his Ph.D. and two years of post-doctoral research, he joined Merck Millipore in 2011 as an Application Specialist, based in Hyderabad. In Merck Millipore, he worked for almost one year, gathering experiences on technologies like Luminex and Flow Cytometry. Thereafter, he joined Bio-Rad, on November 2011, with new job responsibilities as a part of our sales support team. In Bio-Rad, he is primarily responsible for providing Application Support for Proteon XPR36 for the entire country in addition to providing application support to regional sales team for all the other instrument and consumables.

Abstract: Surface plasmon resonance (SPR) biosensors analyze biomolecular interactions in a label-free and real-time manner. The ProteOn XPR36 protein interaction array system is second generation SPR platform for analyzing label-free bimolecular interactions. It features a 6 x 6 interaction array to provide versatility in experimental design, high productivity, and high data quality. Monoclonal antibodies are an essential tool in the development of immunoassays used in basic biomedical research. These antibodies are central to bio therapeutic strategies, directed toward the diagnosis and treatment of diseases. Generating real time kinetics and affinity data accurately and rapidly is a real challenging and formidable task. The high throughput Proteon XPR36 has really simplified this daunting task. This novel system is ideally suited for various antibody characterization and development protocols. In the context of large, broad and very complex studies like hybridoma screening and ranking data are compared with epitope mapping, specificity analysis, cross reactivity determination or other study, all advantages of the Proteon system are multiplied and thus is a boon for the biomedical research.

Dr. Sharmistha Dey

All India Institute of Medical Sciences, New Delhi, India

Talk Title: Promising serum protein marker for early detection of Alzheimer's disease.

Biography: Dr. Sharmistha Dey is an Assistant Professor in the department of Biophysics All India

Institute of Medical Sciences (AIIMS), New Delhi, India. Her research has been in both basic and applied implications, related to common public health problems. Her field of research is development of protein biomarker for different diseases and design study by *in vitro* and *in vivo* of new biological molecules as therapeutic agents. Her team has developed some protein marker for oral cancer, pancreatic cancer and breast cancer and age related diseases like Alzheimer disease and frailty. She is the recipient of AIIMS Excellence award – 2012 for outstanding published research work. Also, she has been a guest scientist in the Department of Chemistry, University of Newcastle, U.K. She is a life member of Indian Biophysical Society Life member of European Peptide Society, Indian Peptide Society and Indian Science Congress.

Abstract: Sirtuin (SIRT) pathway has a crucial role in Alzheimer's disease (AD). The present study evaluated the alterations in serum sirtuin1 (SIRT1) concentration in healthy individuals (young and old) and patients with AD and mild cognitive impairment (MCI). Blood samples were collected from 40 AD and 9 MCI patients (as cases) and 22 young healthy adults and 22 healthy elderly individuals (as controls). Serum SIRT1 was estimated by surface plasmon resonance (SPR), western blot and enzyme-linked immunosorbent assay (ELISA). A significant ($p<0.0001$) decline in SIRT1 concentration was observed in patients with AD (2.27 ± 0.46 ng/ μ L) and MCI (3.64 ± 0.15 ng/ μ L) compared to healthy elderly individuals (4.82 ± 0.4 ng/ μ L). The serum SIRT1 concentration in healthy elderly was also significantly lower ($p<0.0001$) compared to young healthy controls (8.16 ± 0.87 ng/ μ L). This study, first of its kind, has demonstrated, decline in serum concentration of SIRT1 in healthy individuals as they age. In patients with AD and MCI, the decline was even more pronounced, which provides an opportunity to develop this protein as a predictive marker of AD in early stages with suitable cut off values.

Dr. Sriram Kumaraswamy

Pall ForteBio LLC

Talk Title: Label-free interaction systems for routine academic research.

Biography: Dr. Sriram Kumaraswamy is Senior Director of Strategic Applications for Pall ForteBio, a division of Pall Life Sciences. He has over 12 years of experience in the biotechnology industry serving in various positions involving research and development, product management and marketing, and technical applications development. In his current role, Sriram leads applications development at Pall ForteBio. Previously, Sriram led the development and commercialization of new products at Axela Biosensors

and at QTL Biosystems. Sriram has a Ph. D. in physical organic chemistry from the University of California at Berkeley studying high energy organic compounds. He did his post-doctoral work in optical materials and technology laboratory at the University of Arizona. Dr. Sriram has published several articles in peer-reviewed journals, and is author of several patents.

Abstract: Bio-layer interferometry (BLI) is a label-free technology for measuring biomolecular and small molecule interactions. It is an optical technique that analyzes the interference pattern of white light reflected from biosensor surfaces to provide interaction affinity, on-rates and off-rates, and protein and virus particle concentration measurements. The unique detection principle employed by BLI allows the specific measurement of target molecules in crude samples such as serum, cell lysate and cell culture fluids. BLI systems are simple to operate and enable routine use of label-free assays in academic research laboratories. This presentation will provide an introduction to BLI technology, instruments range from small personal-use systems to high throughput models, their applications and comparison to surface plasmon resonance (SPR).

Dr. Madhurarekha

Sandor Proteomics Pvt. Ltd., India

Talk Title: Label-free interaction applications using SPR

Biography: Dr. Madhurarekha Ch, is in charge of instrumentation facility at CDFD and heading the proteomics facility at Sandor. The CDFD facility involves instruments like FACS, Confocal, X-Ray, CD, DNA Sequencers, D-HPLC, HPLC, SPR, RT PCR, LC-MALDI TOF TOF and GC-MS for samples analysis by outsourcing and services. She has 12+ years in proteomics research and CRO operations. She has also worked as senior scientist in Dr. Reddy's lab where her research work was focused on proteomics approaches for biomarkers for inflammation, CAD, obesity, diabetes, toxico proteomics, protein-drug interactions, PTMs and biotherapeutics. She has also worked as research scientist in the Department of Animal Sciences University of Hyderabad. Dr. Madhurarekha has received many awards for her excellent contribution in the field of proteomics.

Abstract: Surface plasmon resonance (SPR) has become an important optical biosensing technology in the areas of biochemistry, biology, and medical sciences because of its real-time, label-free and non-invasive nature which finds applications in health science research, drug discovery, clinical diagnosis and environmental and agricultural monitoring. SPR allows for the qualitative and quantitative measurements of biomolecular interactions in real-time. The presentation will include methodology for

experimentation for several applications of SPR in academic and industrial research.

Dr. Vishal Kamat

Regeneron Pharmaceuticals, USA

Talk Title: Kinetic Studies of Bio-molecular Interactions Using a Simple Dip & Read BLI-Based Octet Platform

Biography: Dr. Kamat has 8+ years of experience studying protein-protein interactions using multiple real-time, label-free biosensor platforms. After the completion of his Ph.D. on the combined use of monoclonal antibodies for the synergistic inhibition of tumor growth, he took a position as a post-doctoral scientist in the HTS Biomolecular Center, at Regeneron Pharmaceuticals. Currently, he is working as a scientist supporting Regeneron's drug discovery pipeline. He utilizes his expertise in label-free biomolecular studies to characterize therapeutic antibodies for clinical development. He also supports pre-clinical development by devising novel label-free assays to characterize anti-drug antibodies. He also contributes in the writing of reports utilized in both IP and regulatory filings.

Abstract: The study of protein-protein interaction has always been in the forefront of drug discovery. Different real-time label-free biosensors are currently available to study various aspects of protein binding. Kinetics profile of monoclonal antibodies binding to their target is one of the key factors that guide the selection of lead therapeutic antibodies. So, accurate measurement of antibody binding kinetics is important. Various factors that could influence binding kinetics measurements and optimization of these parameters for accurate kinetics and affinity characterization of monoclonal antibodies will be presented. Comparison of binding kinetics profile for monoclonal antibodies against multiple targets measured using surface plasmon resonance (SPR) and bio-layer interferometry (BLI) will be also be presented.

Invited Lecture: Mr. Pradeep Narayan

Thermo Fisher Scientific, Bangalore

Talk Title: Recombinant antibodies with increased affinity, sensitivity and specificity

Biography: Mr. Narayan's background spans the segments of recombinant monoclonal antibody screening and characterization for research and therapeutic purpose. With his specific interest in protein-protein interaction, he has leveraged it to develop affinity based screening methods to select the

best monoclonal antibody candidate and complete characterization methods. Prior to being a part of Thermo Fisher Scientific community as a scientist, he has served as a research scientist in Biocon research limited on developing cell-based assays to demonstrate mechanism of action of therapeutic monoclonal antibodies and small molecules. He has extensively worked on different label-free technologies like SPR and BLI to characterize protein-protein interaction. Mr. Narayan is a post-graduate in bio-technology from Bangalore University, and is currently serving as scientist in Thermo Fisher Scientific.

Abstract: The current market calls for high quality detection antibodies that exhibit reduced background in functional assays. In this regard, the ABfinity™ technology was utilized to develop recombinant secondary antibodies that performed better than currently promoted secondary antibodies. Extensive functional screenings at preliminary stages, which include affinity ranking via bio-layer interferometry technology, have facilitated a rigorous selection process. This, in turn, assists the selection of a highly specific and sensitive recombinant detector antibody at early stages of development.

Objective: To perform the binding and kinetics assay to study the interaction between $\beta 2$ -microglobulin and anti- $\beta 2$ -microglobulin using SPR.

Overview: In this interaction study, one of the interactants is immobilized on the carboxy-methylated sensor chip surface while the other is passed over that surface in solution. The ligand (anti- $\beta 2$ -microglobulin) refers to the immobilized component and the interactant present in solution, injected over the surface, is referred as analyte ($\beta 2$ -microglobulin). The response is measured in resonance units (RU) and is proportional to the molecular mass on the surface. For an interactant of a given mass, therefore, the response is proportional to the number of molecules at the surface. A sensorgram is generated during the course of analysis which is a plot of response against time, showing the progress of interaction (Fig. 4.3).

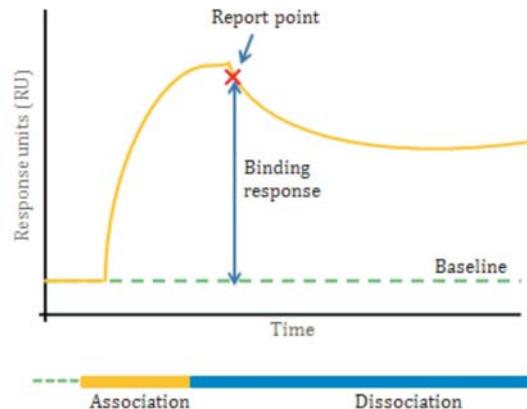


Figure 4.3: Sensorgram

DETAILED PROTOCOL

1. Insert CM5 sensor chip into the chip loader of the instrument. Prime the system with buffer and run the normalize procedure to calibrate the detector unit.
 2. To immobilize the anti- α 2-microglobulin antibody on the CM5 sensor chip, select the immobilization procedure in the wizard and specify the immobilization parameters, including flow cell (fc2, fc1) information, ligand name (anti- α 2-microglobulin), contact time (420s), flow rate (10 μ L/min), method (amine coupling) and temperature (25 °C). The option Aim for immobilization gives you possibility to specify a certain immobilization level that you want to target.
 3. The right hand part of the rack positions dialog box shows the minimum volumes of samples required for immobilization as well as rack positions assigned for each vial. The left hand part shows the rack with used positions marked.
 4. Prepare your samples and place them in the sample and reagent rack according to the rack position table.
 - a. Dilute anti- α 2-microglobulin antibody by mixing 6 μ L of ligand stock solution (1mg/mL) with 194 μ L 10mM sodium acetate, pH 5.0.
 - b. Aliquot 100 μ L of EDC (N-ethyl-N-(dimethylaminopropyl)-carbodiimide, 75mg/mL) and NHS (N-hydroxysuccinimide, 11.5 mg/mL), 150 μ L of 1M Ethanolamine and an empty capped vial for EDC+NHS
 - c. Place all the capped vials in the correct position in the rack and insert it.
 5. Start to begin the immobilization procedure.
 6. For the binding assay, choose binding analysis in the assay folder and specify the parameters such as flow path (fc 4-3), chip type (CM5), start-up conditions, contact time (180 s), dissociation time (0 s), and regeneration solution (10mM glycine-HCl pH 2.5), flow rate (10 μ L/min) and stabilization period (5 s), sample information and temperature settings (25 °C).
 7. Review the cycle run list to see the summary of the cycles to be run. Prepare your samples checking the volumes required in the program table.
 8. Prepare 200 μ L of the α 2-microglobulin solution by mixing 2 μ L of the analyte stock solution with 198 μ L HBS-EP+ (10 mM HEPES pH 7.4, 150 mM NaCl, 3mM EDTA, 0.05% (v/v) P20) to make 85 nM concentration of protein. Pipette 20 μ L of diluted analyte solution to another tube and mix with 180 μ L HBS-EP+ to prepare 8.5nM analyte solution. Prepare 10 mM glycine pH 2.5 of regeneration solution
 9. Cap all the vials containing samples and reagents and place them in the correct positions in the correct rack. Start to begin the binding assay.
 10. Perform the data analysis using T200 evaluation software.
 11. For kinetics, the ligand is first immobilized at a lower immobilization level on the sensor chip surface. A multi-cycle kinetic is then performed using the Kinetics/Affinity wizard.
 12. The immobilization level required for the ligand is calculated based on the following formula, where R_{max} is the maximum binding response (RU), R_L is the immobilization level, S_m is the Stoichiometric ratio (number of binding sites per ligand) and MW is the molecular weight.
- $R_{max} = analyteMW \cdot ligandMW \cdot R_l \cdot S_m$
13. The immobilization procedure was followed as described earlier using the Aim for Immobilization option specifying the target level of immobilization (calculated using the above equation: 1200 RU).
 14. For kinetics assay, choose kinetics analysis in the assay folder and specify the parameters such as flow path (fc 4-3), chip type (CM5), start-up conditions, contact time (120 s), dissociation time (300 s), regeneration solution (10 mM glycine-HCl, pH 2.5), flow rate (30 μ L/min) and stabilization period (0 s), sample information and temperature settings (25 °C).
 15. Prepare a range of dilution series of analyte (containing a minimum of 5 different concentrations) with at least one sample concentration in duplicate, and including three zero concentration samples. Prepare 600 μ L of the β 2-microglobulin solution by mixing 2.26 μ L of the analyte stock solution with 598 μ L HBS-EP+ (10 mM HEPES pH 7.4, 150 mM NaCl, 3mM EDTA, 0.05% (v/v) P20) to make 32nM(β 2M1) working solution. Perform four serial 2-fold dilutions of the antigen solution: add 300 μ L of 32nM β 2M1 diluted analyte solution to another tube and mix with 300 μ L HBS-EP+ to prepare 16nM (β 2M2) analyte solution. Mix gently. Repeat dilution step to make 8 nM (β 2M3), 4 nM (β 2M4), and 2nM (β 2M5). Aliquot required volume of 10 mM glycine pH 2.5 for regeneration.
 16. Aliquot all samples and reagents in 7mm vials, cap all the vials and place them in the correct positions in the correct rack. Start to begin the kinetics assay.

17. Data was collected using the Biacore control software, and analysis was performed with T200 evaluation software using 1:1 binding model. Inspect the sensorgrams. The black curves superimposed on top of the sensorgrams represent the calculated curves. The quality control tab gives a brief overview of the reliability of results. The report window displays the fitted parameters. Review the values in the table. The U value is an estimate of the uniqueness of the calculated values for rate constants and R_{\max} . The residual plot shows the difference in RU between each data point for the experimental curves and the calculated curves. The shape and distribution of the residuals indicate how well the data fit to the chosen model. Examples of binding data and kinetics data are shown in Figure 4.4 and Figure 4.5.

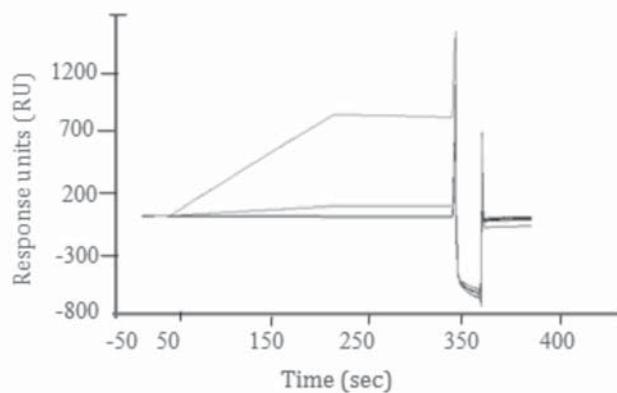


Figure 4.4: Binding Data

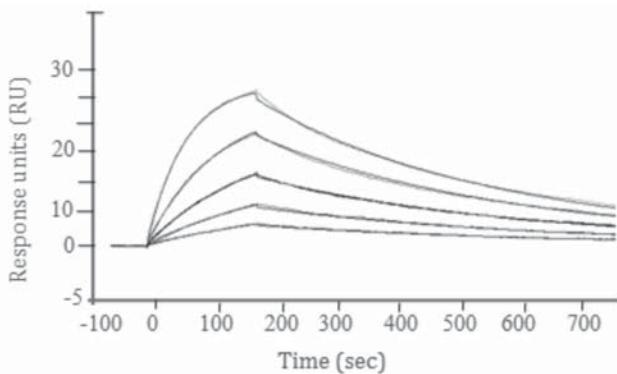


Figure 4.5: Kinetics Data

SPR-BASED PROTEIN INTERACTION ARRAY SYSTEM

SPR-based biosensors are also available in a protein interaction array format for high-throughput purposes. Simultaneous analysis of up to six ligands and six analytes can be performed generating a 6X6 interaction array. Such systems can increase the level of flexibility of experimental design and troubleshooting capabilities in shorter time period with improved efficiency.

Example: To study the kinetics of different protein mutants with an inhibitor protein.

Brief: Five mutant proteins are bound to a sensor chip in parallel channels; with one channel left blank (Fig. 4.6). The inhibitor protein dilution series is then injected into five orthogonal rows, with buffer used in one row as a standard. Six sensorgrams are produced simultaneously; enabling comparison of the binding kinetics for each mutant protein. The system can measure the interaction kinetics of the protein/inhibitor pair by injection of analytes in six channels. Each of the 36 interactions generates a sensorgram of each interaction spot for detailed kinetic analysis. The kinetics data is analyzed using a suitable model such as langmuir 1:1 model. Example of fitted data is shown in Figure 4.7.

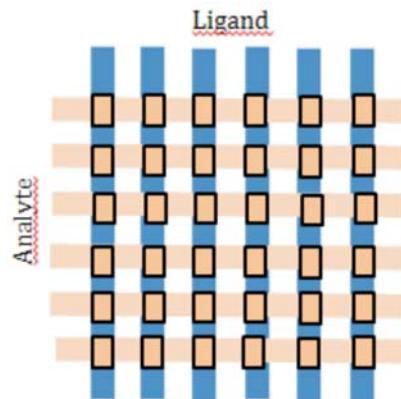


Figure 4.6: SPR-based 6X6 interaction array

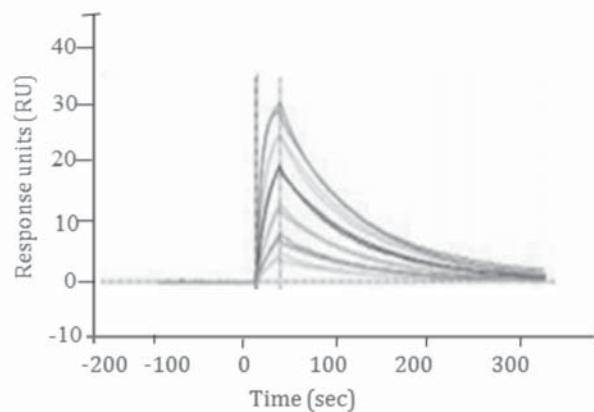


Figure 4.7: Kinetics analysis

BIO-LAYER INTERFEROMETRY

Bio-layer interferometry (BLI) is another label-free technology for measuring biomolecular interactions in real time monitoring the binding specificity, kinetics and concentration analysis with precision and accuracy [6]. It is an optical technique that analyzes the interference produced from light reflections of a single

source from two surfaces: a layer of immobilized protein on the biosensor and an internal reference layer (Fig. 4.8). The association of target molecules on the biosensor is measured due to a shift in the interference pattern that can be measured in real-time.

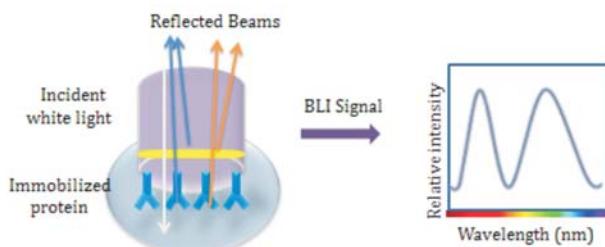


Figure 4.8A

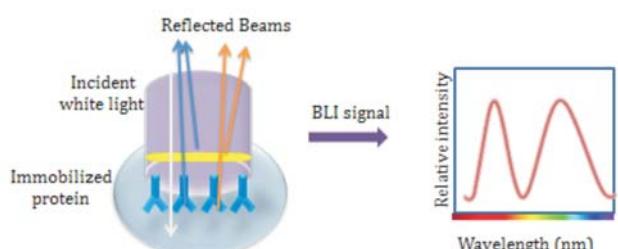


Figure 4.8B

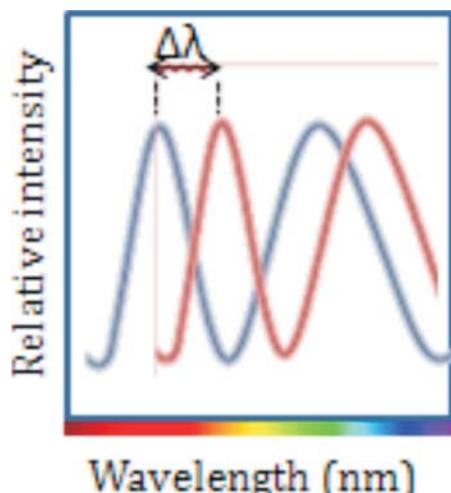


Figure 4.9

The binding between the analyte molecule and ligand immobilized on the biosensor results in increase in the optical thickness of the biosensor tip which is responsible for the wavelength shift (Fig. 4.9). This shift in wavelength is a direct measure of the change in the optical thickness of the biological layer ($\Delta\epsilon$). The response profile is generated from the interference pattern in response to the association/dissociation of molecules to/from the biosensor. However the

interference pattern is independent of the change in the refractive index of the surrounding medium, alterations in the flow rate and unbound molecules, which makes BLI an ideal phenomenon for performing protein-protein binding, quantification, affinity and kinetics even for the crude samples.

Example: Characterization of antigen-antibody interactions using bio-layer interferometry.

Brief: Biotinylated antibody ligand is first immobilized onto Streptavidin biosensors, followed by association and dissociation steps performed using different concentrations of the antigen and a zero-analyte reference sample (Fig. 4.10). Kinetic data is analyzed using analysis software using reference subtraction and global 1:1 fitting to obtain kinetic and affinity constants for the antigen/antibody pair (Fig. 4.11).

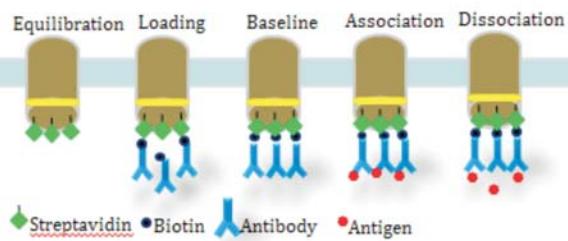


Figure 4.10: Kinetics assay using streptavidin biosensors and antigen-antibody binding pair

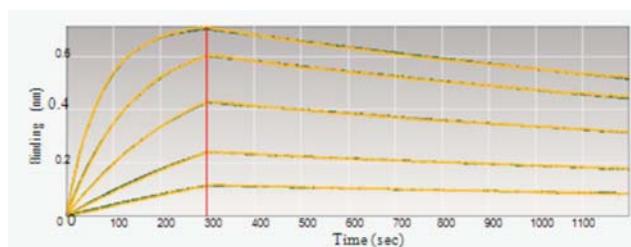


Figure 4.11: Analyzed data for antigen-antibody kinetics assay

APPLICATIONS OF LABEL-FREE TECHNOLOGIES

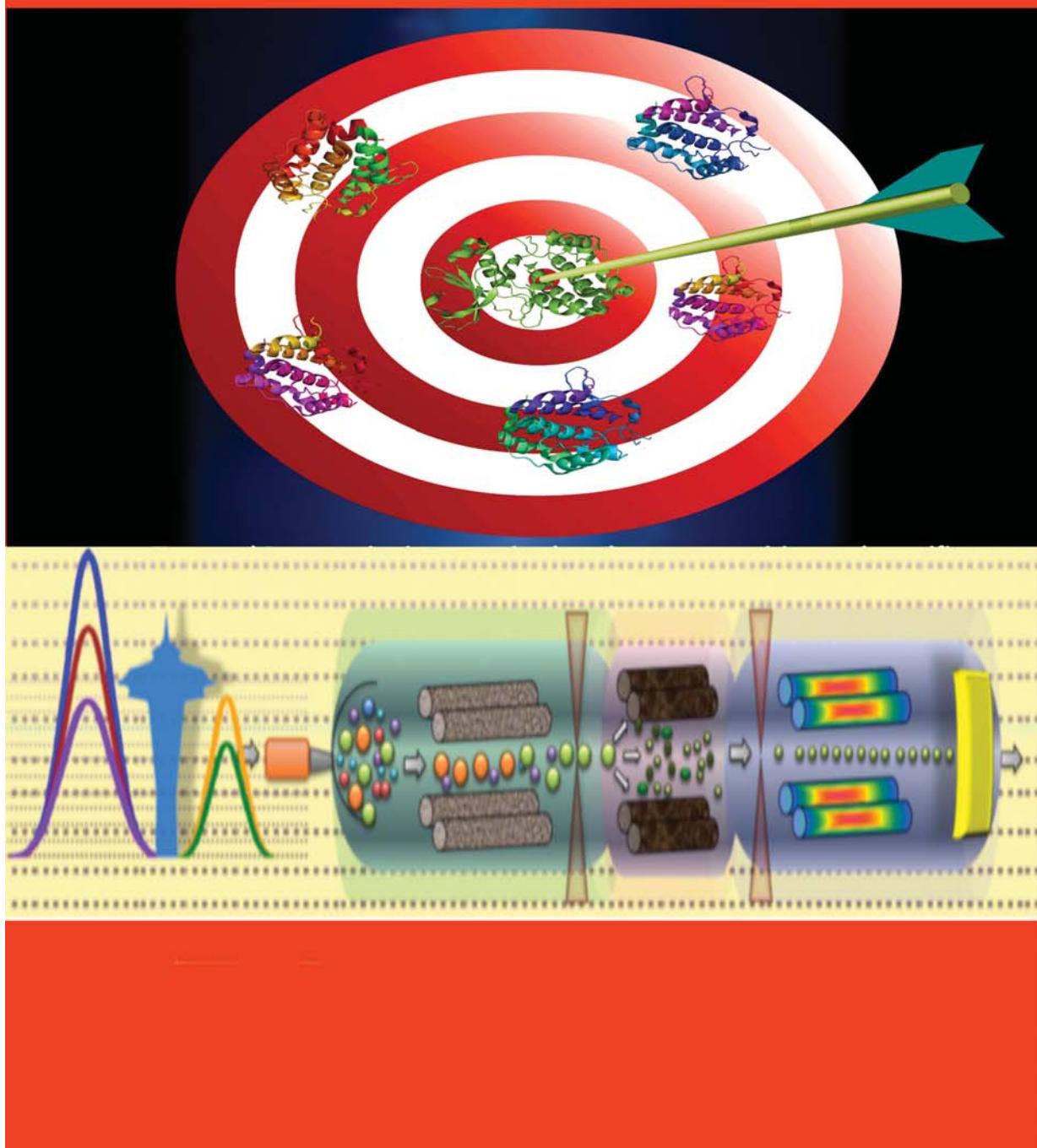
- Real-time analysis and characterization of protein-protein and other biomolecular interactions, including binding affinity and kinetics.
- Efficient screening of potential drug molecules and inhibitors of tumor targets.
- Quantitative analysis of food matrices and milk proteins.
- Characterization of anti-drug antibodies during the clinical development process.

- Measurement of low abundance biomarkers in complex sample matrix.
- Concentration analysis in development of therapeutic proteins.

References

- [1] Qavi AJ, Washburn AL, Byeon J-Y, Bailey RC. Label-Free Technologies for Quantitative Multiparameter Biological Analysis. *Analytical and bioanalytical chemistry* 2009;394:121-35.
- [2] Shiau AK, Massari ME, Ozbal CC. Back to basics: label-free technologies for small molecule screening. *Comb Chem High Throughput Screen* 2008; 11:231-7.
- [3] Boozer C, Kim G, Cong S, Guan H, Londergan T. Looking towards label-free biomolecular interaction analysis in a high-throughput format: a review of new surface plasmon resonance technologies. *Current Opinion in Biotechnology* 2006; 17:400-5.
- [4] Englebienne P, Hoonacker AV, Verhas M. Surface plasmon resonance: principles, methods and applications in biomedical sciences. *Spectroscopy* 2003;17: 255–73.
- [5] Pattnaik P. Surface plasmon resonance: applications in understanding receptor-ligand interaction. *Appl Biochem Biotechnol* 2005;126:79-92.
- [6] Concepcion J, Witte K, Wartchow C, Choo S, Yao D, Persson H, Wei J, Li P, Heidecker B, Ma W, Varma R, Zhao LS, Perillat D, Carricato G, Recknor M, Du K, Ho H, Ellis T, Gamez J, Howes M, Phi-Wilson J, Lockard S, Zuk R, Tan H. Label-free detection of biomolecular interactions using BioLayer interferometry for kinetic characterization. *Comb Chem High Throughput Screen*. 2009; 12:791-800.
- [7] Nice EC, Catimel B. Instrumental biosensors: new perspectives for the analysis of biomolecular interactions. *Bioessays* 1999; 21:339 – 51.
- [8] Domagala T, Konstantopoulos N, Smyth F, Jorissen RN, Fabri L, Geleick D, Lax I, Schlessinger J, Sawyer W, Howlett GJ, Burgess AW, Nice EC. Stoichiometry, Kinetic and Binding Analysis of the Interaction between Epidermal Growth Factor (EGF) and the Extracellular Domain of the EGF Receptor. *Growth Factors*, 2000;18:11 – 29
- [9] Catimel B, Rothacker J, Nice E. The use of biosensors for micro-affinity purification: an integrated approach to proteomics. *J Biochem Biophys Methods*, 2001; 49:289-312.
- [10] Hiep, HM, Endo, T., Kerman, K., Chikae, M. et al., A localized surface Plasmon resonance based immunosensor for the detection of casein in milk. *Sci. Technol. Adv. Mat.* 2007, 8, 331–338.
- [11] Ray S, Mehta G, Srivastava S. Label-free detection techniques for protein microarrays: prospects, merits and challenges. *Proteomics* 2010; 10:731–48.
- [12] Karlsson R. SPR for molecular interaction analysis: a review of emerging application areas. *J Mol Recognit* 2004;17:151-61.
- [13] Dasilva N, DÁez P, Matarraz S, GonzÁlez-GonzÁlez M, Paradinas S, Orfao A, et al. Biomarker Discovery by Novel Sensors Based on Nanoproteomics Approaches. *Sensors* 2012;12:2284-308.
- [14] Daghestani HN, Day BW. Theory and Applications of Surface Plasmon Resonance, Resonant Mirror, Resonant Waveguide Grating, and Dual Polarization Interferometry Biosensors. *Sensors (Basel, Switzerland)* 2010;10:9630-46.
- [15] Kumar R, Mohan N, Upadhyay AD, Singh AP, Sahu V, Dwivedi S, et al. Identification of serum sirtuins as novel noninvasive protein markers for frailty. *Aging Cell* 2014 13:975-80.
- [16] Situ C, Mooney MH, Elliott CT, Buijs J. Advances in surface plasmon resonance biosensor technology towards high-throughput, food-safety analysis. *TrAC Trends in Analytical Chemistry* 2010;29:1305-15.
- [17] Balducci C, Beeg M, Stravalaci M, Bastone A, Sclip A, Biasini E, et al. Synthetic amyloid-beta oligomers impair long-term memory independently of cellular prion protein. *ProcNatlAcadSci* 2010;107: 2295-300.
- [18] Wu C-M, Lin L-Y. Immobilization of metallothionein as a sensitive biosensor chip for the detection of metal ions by surface plasmon resonance. *Biosensors and Bioelectronics* 2004;20:864-71.
- [19] GE Healthcare: <http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-IN/brands/biacore/>
- [20] ForteBio, Pall Life Sciences: <http://www.fortebio.com/bli-technology.html>
- [21] Bio-Rad: <http://www.bio-rad.com/en-in/product/protein-xpr36-protein-interaction-array-system>

TARGETED PROTEOMICS (Module V)



Targeted Proteomics

Preface

Brendan MacLean¹, Christina Ludwig², Meena Choi³, Panga Jaipal Reddy⁴ and Mahesh Kulkarni⁵

University of Washington, USA¹; ETH Zurich, Switzerland²; Purdue University, USA³; Indian Institute of Technology Bombay⁴; National Chemical Laboratory, India⁵

The study of a complete set of proteins in a biological system at a particular time frame is called proteomics. Many research groups across the globe have published thousands of papers on reference proteome maps, but the complete proteome is still an unattained dream with conventional proteomic techniques. Mass spectrometers have become the primary instruments for measuring proteins in complex mixtures. Over the last two decades, researchers all over the world have grappled with the question of how to measure the whole proteome of a biological system. Liquid chromatogram-MS/MS based workflows have played a vital role in exploring the proteome profiles of various biological systems including *Homo sapiens*. Current protein coverage for any biological system is still far from complete, due to bias in mass spectrometry against low abundant proteins, and the wide variety of potential post-translational modifications. Nor can it be said that even detectable proteins can all be accurately quantified at one time with controlled error rates. While the proteomics field will certainly continue seeking to expand whole proteome coverage, "targeted proteomics" has become an important tool for reliable quantification of subsets of proteins within the whole proteome, over large multi-sample studies. For questions where the proteins of interest are not known *a priori*, discovery proteomics will remain a critical tool. Further, discovery proteomic data obtained from shotgun proteomics and stored in public databases can be used as important information for targeted proteomics experiments.

Although targeted proteomics is a recent application of mass spectrometry, it is evolving rapidly as a powerful tool to quantify proteins of interest in complex biological samples. Selected reaction monitoring (SRM) has become popular in targeted proteomics in last decade for quantifying proteins of interest. In SRM experiments, parameters such as (i) LC fractionation need to be done prior to MS analysis, (ii) peptides need to be select from proteins of interest, (iii) fragment ions need to be selected from the peptides for quantification and (iv) instrument parameters such as collision energy may need to be tuned for optimal fragmentation of all the selected peptides. Triple quadrupole (QqQ) tandem mass spectrometry is used for SRM analysis where the first quadrupole (Q1) operates in radio frequency mode to select the specific

mass to charge (m/z) for a peptide precursor. The inert gases in collision cell (q2) further fragments (transitions) the selected peptide ion. The last quadrupole (Q3) operates in radio frequency mode to select the specific fragmented ions (transitions) for intensity measurement. Operation of tandem mass spectrometry in radio frequency mode will enhance the reproducibility, specificity and sensitivity of detection and quantification.

In SRM, tryptic digested peptides are analyzed for quantification rather than intact proteins. Caution is required in selecting the precursor (peptide) for SRM quantification due to differences in physiochemical properties of peptides because of amino acid composition. Important features to be considered for SRM analysis include, (i) selected peptides should be unique to the targeted proteins, (ii) selected peptides should not be liable to modifications (unless modified states themselves are targeted), (iii) the selected peptides should be highly expressed in mass spectrometry conditions for the targeted proteins, (iv) the selected fragment ions should be highly expressed in mass spectrometry conditions for the targeted peptides, (v) reference peptides or proteins should be synthesized for absolute quantification of peptides in biological samples, and (vi) instrument parameters may be tuned for optimal fragmentation of all the selected peptides. The optimization of each parameter mentioned above for every peptide separately is time consuming and costly. Software for targeted method development is essential to the study of even a handful of proteins. Skyline has become the software standard in targeted proteomics, and an essential tool in bridging between discovery and targeted proteomics. Skyline is now in use by many research groups across the globe on different mass spectrometry platforms for large-scale targeted proteomic experiments.

Skyline: the tool for targeted proteomics

Brendan MacLean

University of Washington, USA

Establishing selected reaction monitoring (SRM - also referred to as multiple reaction monitoring - MRM) assays has great clinical importance in diagnosis and may provide a mass spectrometry replacement of a western blot (antibody limitation). Shotgun proteomics has provided a base of prior knowledge for targeted proteomics. If tandem mass spectra are not available for targeted peptides, Skyline can present the spectra by accessing the publicly available repositories from Global Proteome Machine (GPM), National Institute of Standards (NIST), the Institute for Systems Biology (ISB), and the MacCoss Lab. In addition, Skyline can build new spectral libraries using peptide search outputs from Mascot, SEQUEST, X!Tandem, Spectrum

Mill, Protein Pilot and many other standard formats. These spectral libraries can include post-translational modifications not frequently represented in public libraries. Skyline can also help in the selection of the peptides for targeted proteomics by considering the information found in spectral libraries and the background proteome. Skyline can also build libraries from prior targeted experiments, called chromatogram libraries, which can store relative peptide expression information. Add to this libraries for normalized retention time (iRT) and optimized collision energy and a complete set of prior knowledge of peptide physiochemical properties may be stored for re-use in targeted experiments using Skyline. Targeted method files exported from Skyline can be imported to the instruments of all major mass spectrometer vendors such as AB SCIEX, Agilent, Thermo Fisher, Shimadzu and Waters. Creation of targeted methods in Skyline is critical to performing SRM experiments for targeted proteome analysis.

The absolute quantification of selected proteins using SRM is of great interest in the clinical setting. For this purpose, peptide absolute quantification can be performed using single point calibration or multiple point calibration. In single point calibration, a matching “heavy” stable isotope labeled standard peptide is spiked into the sample for each analyte peptide to be measured. Peptide quantities are calculated by comparing each analyte peptide with its heavy labeled standard, assuming a slope of 1 and a y-intercept of zero. Single point calibration also requires the assumption that both heavy and light peptides are within the linear range. In the case of multiple point calibration, a calibration curve can be made by measuring the signal intensity of multiple standard injections at known concentrations. The abundance of an analyte peptide can be calculated by comparing its measured intensity with the linear range of the calibration curve. Here there is no assumption of slope and the linear range is usually known from the calibration. Despite these advantages, single point calibration remains attractive due to its simplicity.

The Skyline software presented in this workshop provides extensive support for working with stable isotope dilution standards, and processing SRM data for quantitative experiments. It also supports exporting results to custom reports in Excel CSV format. These reports can act as direct inputs to statistical tools integrated with Skyline as External Tools. External tools like MSstats for grouped study statistics and QuaSAR for calibration curve analysis can be installed to the Skyline Tools menu and run with a point-and-click interface. Skyline and its External Tools provide support for many complete targeted method design and data analysis workflows.

Instructors and Speakers

Targeted method design and data analysis workflows.

Brendan MacLean

University of Washington, USA



Talk Title: Introduction to Skyline and Targeted proteomics

Biography: Mr. Brendan worked at Microsoft for 8 years in the 1990s where he was a lead developer and development manager for the Visual C++/Developer Studio Project. Since leaving Microsoft, Brendan has been the Vice President of Engineering for Westside Corporation, Director of Engineering for BEA Systems, Inc., Sr. Software Engineer at the Fred Hutchinson Cancer Research Center, and a founding partner of LabKey Software. In this last position he was one of the key programmers responsible for the Computational Proteomics Analysis System (CPAS), made significant contributions to the development of X!Tandem and the Trans Proteomic Pipeline, and created the LabKey Enterprise Pipeline. Since August, 2008 he has worked as a Sr. Software Engineer within the MacCoss lab and been responsible for all aspects of design, development and support in creating the Skyline Targeted Proteomics Environment and its growing worldwide user community.

Christina Ludwig

Institute of Molecular Systems Biology, ETH Zürich, Switzerland



Talk Title: Moving from discovery to targeted proteomics – focus on SRM

Biography: Christina Ludwig, Ph.D., has studied Chemistry at the Philipps-University in Marburg (Germany) and performed her PhD in the field of chemical protein engineering in the research group of Professor Henning Mootz at the Technical University Dortmund (Germany). In March 2009 Christina joined the laboratory of Professor Ruedi Aebersold at the ETH Zürich (Switzerland) as a postdoctoral fellow.

Her current research interests are focused on technical development projects related to targeted mass spectrometry, including Selected Reaction Monitoring (SRM-MS) and SWATH-MS. She is involved in projects aiming for the specific identification and quantification of post-translational modifications and the determination of phosphorylation stoichiometries. Her second field of interest focuses on the estimation of absolute protein abundances from label-free targeted mass spectrometric data.

Together with two of her colleagues Christina has initiated, planned and organized a one-week targeted proteomics course at the ETH Zürich in 2013, which due to its great success has become an established annual course of her institution. Further, she has been invited as lecturer to numerous courses and proteomic summer schools all around Europe over the last few years.

Mahesh J Kulkarni

Proteomics Facility, Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Homi Bhabha Road, Pune, 411008, India

Talk Title: Mass spectrometry and targeted proteomics

Biography: Dr. Mahesh Kulkarni is a scientist at CSIR-National Chemical Laboratory Pune. He obtained his Ph.D. from University of Agricultural Sciences Bangalore. His area of research is Chemical Proteomics, Mass spectrometry, Diabetes and Aging. He was post Doctoral fellow at CCMB, Hyderabad and Genome Institute of Singapore, Singapore.



Ms. Meena Choi

Department of Statistics, Purdue University, 250 N. University Street, West Lafayette, IN, USA 47907

Talk Title: Statistical considerations for experimental design and data analysis



Biography: Meena Choi completed her M.S in applied statistics from the Purdue University, USA in 2011. She is doing her doctoral research at Purdue University, West Lafayette, USA under supervision of Dr. Olga Vitek. She is actively participated in conducting workshops on “Selected Reaction Monitoring”. ETH Zurich, Switzerland, 2013 and “Statistical methods for quantitative proteomics: design of experiments and interpretation of results”. Annual Conference of the US Human Proteome Organization (US HUPO), Baltimore, 2013. She is the lead developer of “MSstats” used for statistical protein quantification in label-free and label based LC-MS data and also involved in developing “SRMstats” used for statistical protein quantification in SRM experiments.

Mr. Panga Jaipal Reddy

Department Biosciences and Bioengineering, Proteomics Laboratory, Indian Institute of Technology Bombay, Powai, Mumbai, India-400076



Biography: Panga Jaipal Reddy obtained his B.Sc. Degree from Osmania University and completed his Masters in Biochemistry from the University of Pune, India in 2008. Presently, he is working as a senior research fellow in Department of Biosciences and Bioengineering, IIT Bombay, India. He is the author of few scientific publications in reputed journals. He has participated in the development of Virtual Proteomics Laboratory, Clinical proteomics remote triggering virtual laboratory, *Open Source Courseware Animations Repository (OSCAR)* and National Programme on Technology Enhanced Learning (NPTEL) at IIT Bombay. His current research interests include understanding the regulation of Z-ring assembly and identification of drug targets using proteomics. He has publication in various peer-reviewed journals like Journal of Proteomics, Proteomics and Expert reviews of Proteomics. He has been involved as a instructor in several workshops on “gel-based proteomics” and “mass spectrometry proteomics” held at IIT Bombay.

Learning Objectives

- Introduction to hypothesis driven proteomics measurement for validation
- Enhance understanding from exploratory proteomics to targeted
- Basic knowledge of skyline to implement targeted experiments
- Introduction to key concepts of targeted proteomics experiments
- Creating and using libraries of prior empirical measurements
- Absolute qualification versus detecting differences
- Statistics of study design & avoiding statistical pitfalls
- Basic understanding of MS stats

Note: handouts and tutorial material will be provided during the targeted proteomics workshop.

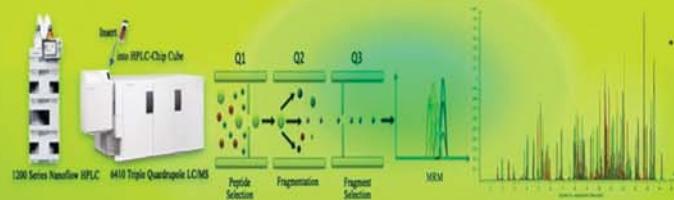
CHARACTERIZATION OF BIOSIMILARS & BIOTHERAPEUTICS

Module VI

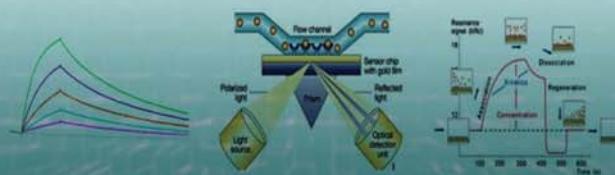
HIGH RESOLUTION MASS SPEC BASED BIOSIMILAR CHARACTERIZATION



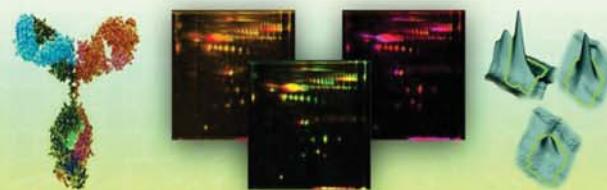
TARGETED PROTEOMICS FOR BIOSIMILAR QUANTIFICATION



FUNCTIONAL CHARACTERIZATION



GEL-BASED PROTEOMICS & BIOCHROMATOGRAPHY



Biosimilars and Proteomics

Preface

Prasad Phapale and Sanjeeva Srivastava

Indian Institute of Technology Bombay

A biosimilar product is developed to have structural, physiological and biological properties similar to one that has previously been licensed. Biosimilar development involves optimization of a process to provide a well similar product as that of innovator molecule. An array of comparability strategies should be planned to prove similarity to the innovator molecule. Any changes in the manufacturing process, biological sources and scale ups may affect quality attributes of biosimilarity which should be controlled and monitored throughout the product development. To have such quality control and demonstrate biosimilarity at each stage of development is the most critical factor during regulatory submissions and commercialization. To address this challenge the biopharma industry needs reliable analytical tools and methods to comply the molecular biosimilarity specifications set by regulatory bodies.

Biosimilar medicines are now becoming a reality globally and there exists an incredible opportunity for Indian Biopharma to capitalize on what is set to become the fastest growing sector of pharmaceutical industry. However, there are several unmet challenges from its development to commercialization. Biosimilar development begins with extensive structural and functional characterization, which underpins all further product development activities. As regulatory bodies are now setting to define clear rules; the extensive characterization of Biotherapeutics becomes critical to ensure the patient's safety and poses great challenge. The Mass Spectrometry and Label-free interaction biosensors based proteomic tools offers powerful solutions to address challenges in biosimilar characterization. The advances in proteomic technology can greatly assist biopharma professionals to tackle development and characterization challenges for biologics.

Developing on this theme of integrating proteomic and biopharma community we have announced a special track on "Proteomics perspective on Development and Characterization of Biosimilars and Biotherapeutics" along with focused Hands-on 'Workshop on Biosimilar Characterization' at IIT Bombay. The real-time data acquisition and analysis demonstrations at 'state-of-the-art' high-resolution mass spec proteomics facility of the IIT Bombay will add truly stimulating experience for the biopharma professionals. The experts from proteomic community worldwide will train Indian biopharma professionals on biosimilar characterization using advanced mass spectrometry and label-free interaction proteomic tools.

Instructors and Speakers

Dr. Robert Moritz

Institute for Systems Biology, USA



Biography: Dr. Robert Moritz, a native of Australia, joined the ISB faculty in mid-2008 as Associate Professor and Director of Proteomics. Dr. Moritz began his work in 1983 in the Joint Protein Structure laboratory of Prof. Richard J. Simpson (JPSL-Ludwig Institute for Cancer Research, and The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). During his 25 years at JPSL, Dr. Moritz designed and implemented a number of technologies currently used in many proteomics laboratories across the globe. Examples include technologies such as the development of micro-chromatography for proteomics from the late 1980's to its current day implementation, a micro-fractionation technique widely used by many laboratories worldwide. His collaborative research into cytokine biochemistry, protein-receptor chemistry and cellular biochemistry culminated in the novel identification of a number of proteins (e.g., IL-6, IL-9 A33 ligand, DIABLO, as well as several others), their interacting partners, and 3-dimensional structures of their cell surface receptors important in human health concerns such as cancer and inflammation. During his time at JPSL, Dr. Moritz progressed through the ranks whilst obtaining his Bachelor's degree in Biochemistry with first-class Honors, and his Ph.D., from the University of Melbourne. Dr. Moritz has brought wide-ranging skills and expertise to ISB, much of it drawn from his Australian experience. There, in 2005, he conceptualized a shared proteomics high-performance computing system, organized a consortium of proteomic scientists from all states in Australia, and proposed a computational system specifically for proteomics data analysis for all Australian researchers to access. For this work, in 2006, he was awarded an enabling grant from the Australian National Health and Medical Research Council worth AUS\$2M. With that award, he established a bioinformatics center in Australia that enabled proteomic researchers anywhere in the country to analyze mass spectrometry data. It was the first effort on a national scale to bring proteomic data analysis and algorithms to any researcher in the whole country without the need for them to build their own bioinformatics group. In late 2006, the Australian Proteomics Computational Facility (APCF, www.apcf.edu.au) was inaugurated, and Dr. Moritz remains as Director of the APCF. The dedicated proteomics data analysis facility is equipped with a 1000 CPU high-performance computing cluster, and full-time software engineers for the continued development of proteomics algorithms and data validation. This facility serves all researchers in Australia and others regardless of their global

geographical location. He is continuing that work at ISB by expanding the ISB proteomics centre into a national facility with online tools for data analysis.

Application of mass spectrometry in the characterization of biosimilars

Dr. Taegen Clary

*Life Sciences and Diagnostics Group,
Agilent Technologies*



Taegen Clary is currently the Director of Marketing for the Pharmaceutical Segment at Agilent Technologies, managing global segment management teams and global and regional marketing program managers focused on the pharma and biopharma markets. Taegen joined Agilent in 2007 as a product manager in the Chemistry and Supplies Division, managing the bio-chromatography portfolio. In 2010, Taegen joined Life Sciences Group Segment Marketing organization as the Biopharmaceutical Segment Manager. Prior to Agilent, Taegen worked at Bio-Rad Laboratories, managing the laboratory scale bio-chromatography portfolio. He also worked in the pharmacology department at Berlex Biosciences, now a part of Bayer Healthcare, analyzing both small molecule drugs and biomarkers using LC/MS. Taegen has a Bachelor's of Science in Biotechnology from UC Davis and an MBA from St. Mary's College of California.

Dr. Sriram Kumaraswamy

Senior Director, Pall ForteBio LLC



Sriram Kumaraswamy is senior director, strategic marketing and applications for Pall ForteBio LLC. The research scientist turned marketing professional leads the development and implementation of commercial strategy to grow Pall's process analytical technology portfolio with a cross-functional team of marketers and scientists. Sriram has responsibility for product management, outbound marketing, and applications development and technical support functions at Pall ForteBio.

Previously, he conducted research and development at start-up technology ventures to successfully develop and commercialize label-free and fluorescence-based instrument and assay platforms that supported drug discovery research. Sriram has four patents and several publications on fluorescent assay techniques and has written book chapters on label-free biolayer interferometry. He conducted post-doctoral research on semi-conducting materials at the University of Arizona, and doctoral research in synthetic and physical organic chemistry at the University of California at Berkeley. Sriram is

passionate about technical innovation and commercialization.

Dr. Prashant Dour

Senior Application Specialist, SCIEX Separation

Prashant is a Senior Application Specialist at SCIEX Separation, a part of AB SCIEX. He studied Life Science at Rani Durgavati University in Jabalpur, India and earned a Ph.D in 2006. During the eight years I was involved in the research & development and application support in different organizations. After PhD in 2006 he joined Indian Council of Medical Research Jabalpur and worked on hepatitis, tuberculosis and HIV. Prior to current position, he was Application Specialist at Qiagen, India. He joined Beckman Coulter in 2013 and continuing working in Beckman and AB SCIEX in application support of capillary electrophoresis. In current role he is responsible to support and develop new method for capillary electrophoresis.

Dr. Mark A. McDowall

Waters Corporation, United Kingdom



Dr Mark McDowall has a long association with Waters Corporation and currently associated as business development consultant focusing on mass spectrometry in India. His academics research was focused on 'primitive' LC/MS technologies to the analysis of peptides and pharmaceutical products and mechanism of thermospray ionisation. He was involved in mass spectrometry operations and management, strategic development of Pharmaceutical Discovery & Life Science business and Scientific Advisory Board in his Waters tenure.

Dr. Prasad Phapale

Dr. Prasad is one of the lead coordinator for this biosimilar workshop. He has done his doctorate from South Korea and post-doctorate from NCL, Pune. He has good experience of working on mass spec technologies for Metabolomics, Proteomics and Lipidomics. Before joining IIT Bombay he has worked in several pharmaceutical companies as research scientist.



Dr. Brendan MacLean;

University of Washington, USA

Dr. Christina Ludwig;

ETH Zurich, Switzerland

Dr. Mayuri Gandhi;

IIT Bombay, Biographies are already given in Mass spectrometry and Targeted Proteomics workshop sections.

Biosimilar characterization and regulatory aspects

Prasad Phapale

Indian Institute of Technology Bombay

Recently several leading regulatory agencies including Indian DCGI have published guidelines for Biologics product development including test procedures and acceptance criteria for biosimilar products. The leading guidelines followed worldwide and what is supposed to be bible of biosimilar characterization is 'ICH Topic Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products'. This guide for industry lists number of structural, physicochemical and biological tests and suggest analytical methods which are required by regulatory authorities for the characterization of monoclonal antibodies.

Summary of test procedures proposed by ICH Topic Q6B "Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products"

I) Structural characterization

1. Amino acid sequence
2. Amino acid composition
3. Terminal amino acid sequence
4. Peptide map
5. Sulphydryl group(s) and disulfide bridges
6. Carbohydrate structure- Glycan analysis

II) Physicochemical properties

1. Molecular weight or size
2. Isoform pattern
3. Extinction coefficient
4. Electrophoretic pattern
5. Liquid Chromatographic pattern
6. Spectroscopic profiles

III) Impurity Profiling – Process related and Product related impurities

General analytical tools which can be used to evaluate above properties

1. Molecular weight: Intact protein analysis by MALDI-MS and ESI-MS
2. Amino acid sequence and modifications: LC-MS/MS, HRMS, CE-MS, tryptic digestion, chromatography, peptide mapping, de-novo sequencing
3. Glycosylation and carbohydrate structure: enzymatic digestion, peptide mapping, CE, MS

4. Protein Folding: HRMS based S-S bridge mapping, calorimetry, ion mobility MS, NMR, circular dichroism, fourier transform spectroscopy, fluorescence
5. PEGylation & isomers: chromatography, peptide mapping
6. Aggregation: Analytical ultracentrifugation, size-exclusion chromatography, field flow fractionation, light scattering, microscopy
7. Proteolysis: 1-D and 2-D gel electrophoresis, chromatography, MS
8. Impurity profiling: targeted proteomics, Triple quad LC-MS/MS, MRM mode, immunoassays, metal & solvents analysis
9. Variants in size, charge, hydrophobicity: chromatography; gel & capillary electrophoresis, light scattering, IM-MS, UV

The above ICH guidelines recommend the manufacturer to establish well-characterized in-house reference materials, which will serve quality check for further production batches. The guide also advocates use of new proteomics and analytical advances in technology for characterization of biologics and proving biosimilarity.

We have structured our workshop keeping the essence ICH Q6B guides. The 'workshop on biosimilar characterization' is having four distinct sessions focusing on each proteomic methodology with specific objectives of utilizing those techniques for mAb characterization.

Typical mAb structure

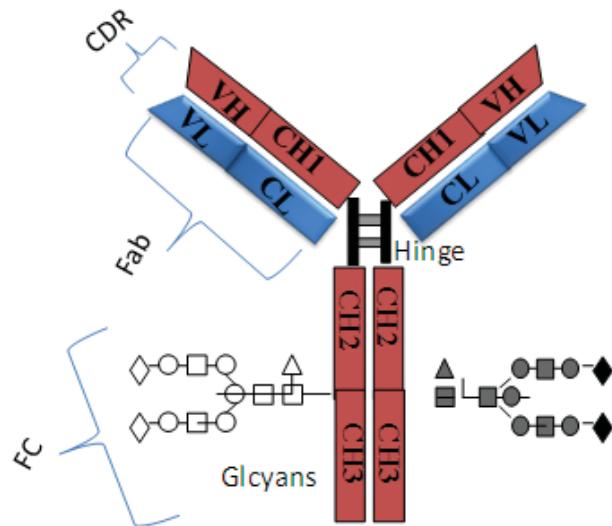


Figure 6.1: Typical structure of mAb protein.

Typical structure of antibody mAb (molecular mass ~150 to 170 kDa) has 3 distinguished regions (from Figure 6.1): two antigen-binding (Fabs) fragments and the crystallizable (Fc) fragment which are linked together by the hinge region. Each of the two Fabs has one light chain and one heavy chain with VH and CH1 domains. The Fc region, involved in the dimerization between the two heavy chains (brown color), and two Fab regions (light chain shown as blue color). They are result from the interaction between one light and one heavy chain coupled by disulfide bridges. The two Fabs are connected with the Fc by hinge region which inter-chain disulfide bridges between the two heavy chains. Glycosylation occurs in the CH2 domain and variable domains (VL, variable light; VH, variable heavy). (CDR, complementarity determining regions) contains the antigen-binding site and determines antibody specificity.

Sessions

- I. High resolution mass spec based characterization of biosimilar mAbs
- II. Targeted proteomics for quantification of mAb and their impurities using triple quad LC-MS/MS
- III. Functional characterization of biologics using SPR and Bio-Layer Interferometry technologies
- IV. Gel-based and biochromatography analysis of mAbs

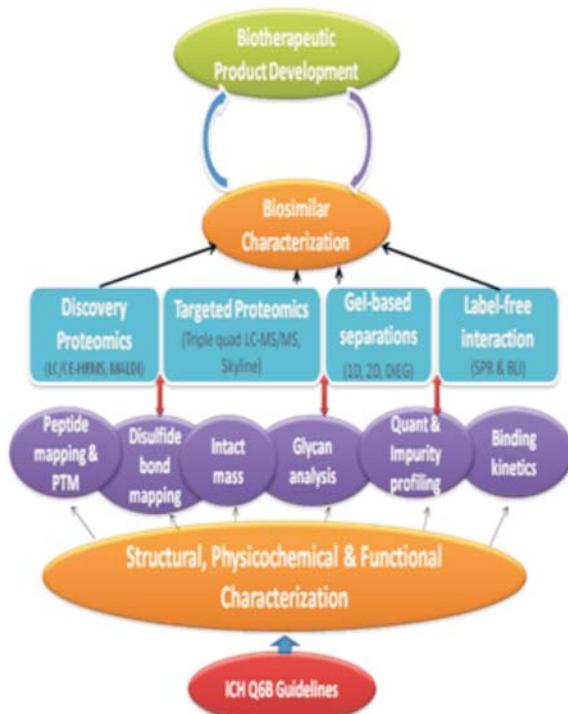


Figure 6.2: Overall structure of workshop based on biosimilar characterization recommendations from ICH Q6B

Session I: High resolution mass spectrometry (HRMS) based tools for biosimilar characterization

Learning Objectives

1. Introduction to HR-LC-MS/MS based biosimilar mAb analysis and data interpretation
2. Enzymatic digestion and LC-MS/MS work-flow
3. Peptide mapping
4. Glycomic analysis
5. Mapping of disulfide bridges
6. Intact protein mass determination
7. Complementary HRMS tools for characterization: Capillary electrophoresis and Ion mobility

1. Introduction

Mass spectrometry (MS) is one of the key analytical techniques to determine primary amino acid sequence variants, mapping disulfide bridges, glycan analysis as well as quantification of monoclonal antibodies (mAbs) and their related impurities. This will support in assessing biosimilarity and batch to batch variability with reference monoclonal antibodies (mAbs). The next generation of high-resolution mass spectrometers and improved MS-based informatics tools substantially improves the standards of biosimilar mAbs from structural and physicochemical characterization to commercialize these biological products into highly regulated markets.

2. Enzymatic digestion and LC-MS/MS work-flow

Please refer to earlier protocols in Module 2

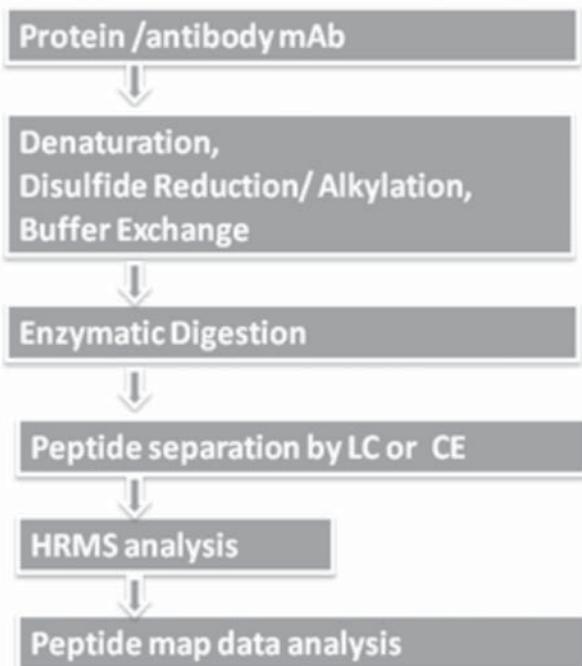
3. Peptide Mapping

Comprehensive protein sequencing is critical for the quality control and comparability of biotherapeutics. Peptide mapping is the common analytical technique for characterizing biologic therapeutic proteins. This is a vital technique for studying the primary structure of biologics. During the course of biosimilar development peptide mapping is used for the preliminary proof of structural characterization. This tool is also employed for batch-to-batch quality testing during bioprocess development and pre-clinical trials. Peptide mapping is now become more advanced and rapid tool for monitoring the recombinant cell stability, process development and biocompatibility with reference standard. Use of high resolution mass spec (HRMS) offers comprehensive solutions for the highly accurate determination of protein sequence, identification of post translational modifications, and routine peptide fingerprinting for QC check by hyphenating with liquid chromatography (LC), nanoLC and capillary electrophoresis (CE) systems.

"Please follow the link for information on enzymatic and chemical agents for protein digestion http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/peptideMapping.pdf"

The protein mAb is first enzymatically digested into smaller peptides using single or multiple proteases. Several enzymatic and chemical modifiers can be used (Table 1) as recommended in European Pharmacopoeia to obtain better sequence coverage. The peptide mixture is then separated by reversed phase chromatography and detected by mass spectrometry. HRMS detection provides highly accurate masses (< 10 ppm) of the peptides with their abundance followed by their selective fragmentation which greatly enhances the data content for peptide mapping. LC-MS/MS analysis provides the additional dimension of separation by m/z for chromatographically co-eluting peptides which increase speed and specificity of analysis. Further, tandem mass spectrometry data with fragment ion (MS/MS) information of peptides establish the evidence for the amino acid sequence for peptides and their post-translational modifications (PTM) such as glycosylation, phosphorylation, oxidation, and deamidation. For workshop we will provide hands-on analysis of mAb on Agilent 6550 iFunnel Q-TOF LC/MS system along with spectrum mill and MMP software.

Workflow for peptide mapping



4. mAb-Glyco Analysis

Glycosylation is a post-translation event which makes complex sugar (glycan) structures to specific amino acid

sequences. Depending on expression system different glycan structures are formed. The N-linked glycosylation is a critical and complex post-translational modification for protein mAb. Hence its monitoring and measurement throughout development and manufacture of drug glycoproteins is essential. The difference in glycosylation pattern can influence safety, efficacy, potency and pharmacokinetics of therapeutic protein and so the analysis of these patterns is an imperative part of therapeutic glycoproteins characterization particularly mAbs. Due to polar nature of glycans the hydrophilic interaction chromatography (HILIC) separation is preferred method over reversed phase for glycan analysis. The HILIC/LC coupled to HRMS provides key mass and structural information for glycan analysis. The glycomics analysis should provide: carbohydrate content such as neutral sugars, amino sugars and sialic acids, carbohydrate chains structure, the oligosaccharide pattern, linkage and glycosylation sites on the protein chain.

Brief protocol for mAb Glyco chip using Protocol Agilent 6550 iFunnel Q-TOF LC/MS system

Hardware Modifications

Install 8 µl injection loop for proper operation of mAb Glyco chip.

Configure Chip-Cube to "backflush" mode.

Yellow Capillary (PEEK capillary, 25 µm, 105 cm) from sampler to chip cube port 5.

Buffer Used

Capillary Pump (A1): Deglycosylation Buffer (Add 3% ACN)

Capillary Pump (B1): Not Used

Nano Pump (A1): Water + 0.1% FA

Nano Pump (B1): ACN + 0.1% FA

Do not run any other solvent in the cap pump apart from deglycosylation buffer, for safer side you can keep channel B1 of cap pump in deglycosylation buffer and purge.

Qualitative Analysis

Load the method "mAb_Glycan_DataAnalysis.m".

In the Method Explorer, go to Find Compounds, Find by Molecular Feature and Mass Filter and define the file "mAb_Glyco_AM_PCD.cdb" as database.

In the Method Explorer, go to Identify Compounds, search Database and Database and define the file "mAb_Glyco_AM_PCD.cdb".

Workflow for glycan analysis

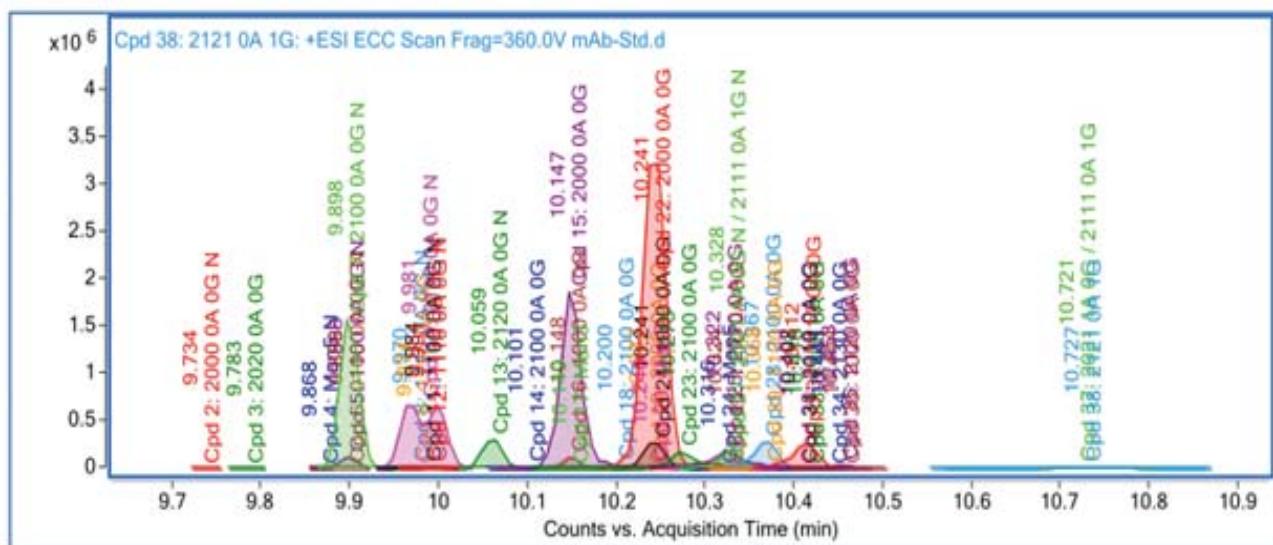
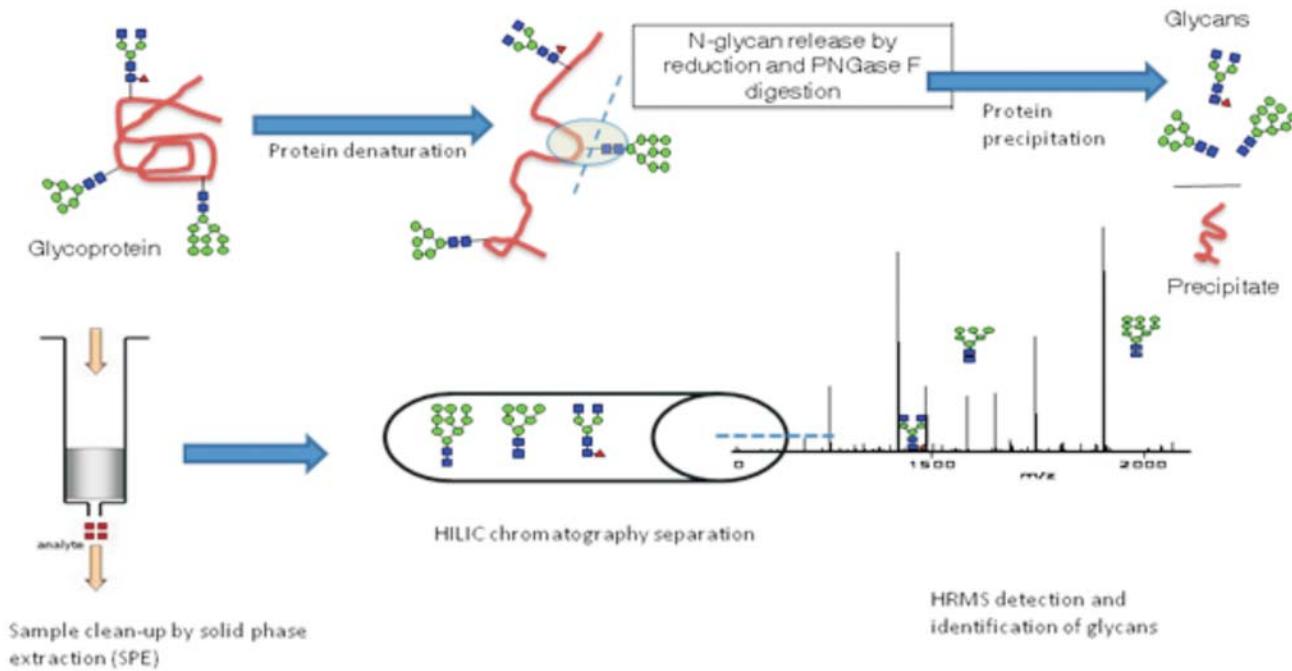


Figure 6.3: Above chromatogram shows all the compounds identified after database search

5. Disulfide bond mapping

The recombinant monoclonal IgG therapeutic antibodies contain highly conserved disulfide bond structure which is a uniform and homogeneous structural feature. As shown in figure the light and heavy chain of the IgG is connected by a disulfide bond between the last cysteine residue of the light chain and the fifth cysteine residue of the heavy chain (intra-chain

disulfide bonds). However inter-chain disulfide bond is linked by the last cysteine residue of the light chain and the third cysteine residue of the heavy chain. The level of exposure between intra-chain and inter-chain disulfide bonds is different.

Variations in disulfide bond arrangements may affect efficacy and stability of biotherapeutic antibodies and is necessary part of regulatory submissions.

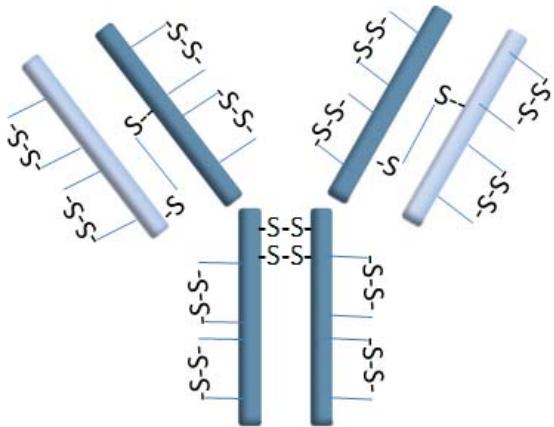
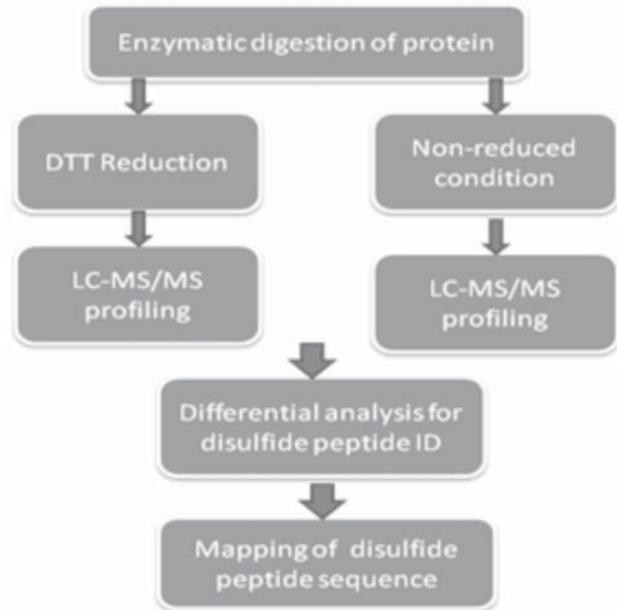


Figure 6.4: Schematic representation of Intra- and inter-chain disulfide bridges in IgG.

Workflow for disulfide bond mapping:



6. Intact mass determination using MALDI-TOF

The Matrix assisted laser desorption technique combined with a MS detector (MALDI-MS) is an indispensable tool in analysis of proteins, biomolecules and antibodies and is the most rapid MS method of analysis available. The technique involves mixing of the analyte into a matrix spotted on target plate (~ 1-2 μ L) and radiated for absorption/desorption by specialized laser radiation for ionization of the analyte. A laser is used to generate ions of high molecular weight proteins and polymers. Analyte is embedded to crystallize aromatic matrix which causes the large molecules to ionize instead of decomposing after transfer of energy from laser beam. Transfers of large amount of laser energy absorbed ionize analytes to gas phase.

Ions formed then accelerated into a flight-tube in the mass spectrometer and are subjected to a high electrical field. A typical sample preparation involves using about 10^{-6} M solution of the analyte mixed with 0.1 M solution of the matrix in suitable solvent. The typical matrices used are DHB (dihydroxybenzoic acid), DNA (1, 5-Diaminonaphthalene) and DHAP (2, 5-Dihydroxyacetophenone). Detailed sequence information for antibodies subunits can be determined using MALDI N- and C-terminal top-down sequencing (TDS) analysis. Please refer to Module 2 for top-down sequencing protocols.

Intact mass analysis of antibodies is routinely performed in biopharma labs for rapid confirmation of mass and glycosylation profile. Further Antibody-antigen complexes (mAbAg) can be analysed using MALDI-TOF-MS. Some scientists also used intact MALDI-MS analysis for determination trisulfide bonds analysis under non-reduced conditions where appear as additional peaks adjacent to the main peaks.

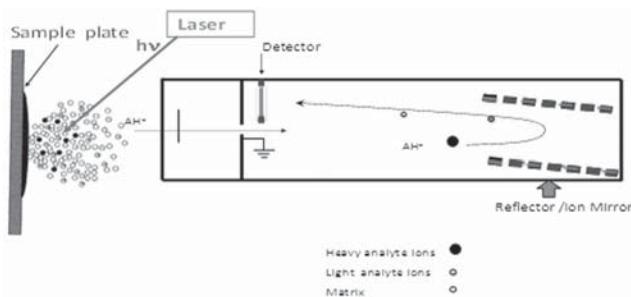


Figure 6.5: Schematic representation of MALDI-TOF

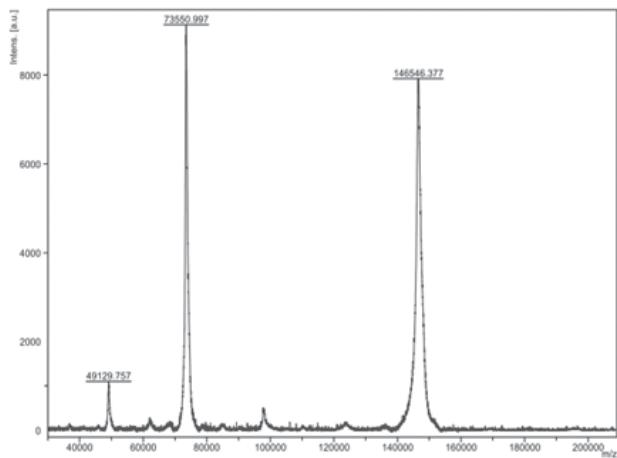


Figure 6.6: Intact mass determination of Rituximab using Bruker MALDI-TOF/TOF

7. A) Complementary tools: Biosimilars Characterization using High-end Capillary Electrophoresis (UV, PDA, LIF and CE-ESI-MS)

Agenda: Title	People Involved
Introduction on CE and advances in CE for Biosimilars Characterization-	Abel Shalom B, Dr. Prashant Dour and Aalhad Abhyankar
a. Introduction. b. Advantages and Principle. c. List of Application at par with Biosimilars. Characterization. d. Types of Detector.	
CE instrument Hardware and work flow -	Abel Shalom B, Dr. Prashant Dour and Aalhad Abhyankar
a. Explanation on the Hardware. b. Cartridge and Sample Preparation. c. Instrument preparation for run.	
Regulatory requirement for Biosimilars Vs CE Contribution for regulatory and Monographs:	Abel Shalom B, Dr. Prashant Dour and Dr. Dipankar Malakar
a. Molecular Wt. Determination – SDS Molecular wt. Kit. b. Purity assessment – IgG Purity and heterogeneity kit. c. Identification and quantification of charge variants – cIEF kit and CZE methods. d. nGlycan profiling - nCHO carbohydrate analysis Kit. e. Miscellaneous Applications. (Peptide map, chiral analysis, metabolomics etc.,) f. CE-ESI-MS	
Run Data Display with data analysis and data interpretation.	Abel Shalom B, Dr. Prashant Dour

Title: Characterization and Quality Control of Biomolecules Using Capillary Electrophoresis

Abel Shalom, Prashant Dour

Sciex Separations a division of AB SCIEX, Gurgaon India

Abstract: The PA 800 plus Pharmaceutical Analysis System provides a comprehensive, automated, and quantitative solution for the characterization and analysis of proteins. Innovative system design ensures dependable operation and durability. The PA 800 plus application menu includes SDS-gel molecular weight analysis, charge heterogeneity analysis utilizing advanced capillary isoelectric focusing technology, and carbohydrate profiling for the assessment of glycoprotein micro heterogeneity.

In CE, the separation is based on charge, size and frictional force and it offers fast separations with exceptional efficiency. CE has proved to be an efficient and versatile approach for physicochemical characterization of bioactive molecules and resolution for charged substances such as biomolecules, low molecular weight basic or acidic drugs and ions.

In the biopharmaceutical industry today, CE-SDS is applied at all stages of the pharmaceutical development process, including high-throughput process development, structural isoform analysis, carbohydrate occupancy, and more common molecular size variant analysis for characterization and release.

In the present study CE-SDS method shows the good linearity of SDS-MW size standard and separation of impurities in IgG sample in reduced and non-reduced form. The cIEF method differentiates the charge variant of protein molecule. The Capillary electrophoresis (CE) technology has been successfully used to separate major IgG N-linked oligosaccharides G0, G1, and G2 structures from one another.

Title: Rapid Characterization of Biologics using a CESI 8000 – AB SCIEX TripleTOF® 5600+ System

Abel Shalom, Prashant Dour, Dipankar Malakar

Sciex Separations a division of AB SCIEX, Gurgaon India

Abstract: Monoclonal antibodies (mAbs) make up an important class of biotherapeutics undergoing significant growth in the pharmaceutical industry today. Currently, more than 30 mAbs have been approved for use in treatment of a number of indications ranging from various forms of cancer to autoimmune and infectious diseases. Therapeutic pipelines for mAbs and mAb-like molecules like bi-specific antibodies, single chain variable fragments (scFv), and antibody drug conjugates (ADC) are expanding. Due to approaching innovator patent expirations, a growing number of mAb biosimilar and bio-better products are also in development. Consequently, there has been a shift towards more comprehensive characterization of both innovator mAbs as well as the alternatives since changes in primary amino acid sequences, quality attribute modifications, and/or such post translational modifications as glycosylation may impact therapeutic efficacy, bioavailability, and biosafety.

Capillary electrophoresis (CE) technology using absorbance- or fluorescence-based detection methods has long been applied in the biopharmaceutical industry for the characterization of mAb purity and heterogeneity in the forms of capillary zone electrophoresis (CZE), capillary isoelectric focusing (cIEF) and capillary SDS gel electrophoresis (CE-SDS). The exceptionally high separation power of capillary electrophoresis (CE) in conjunction with the sample identification capability of mass spectrometry fulfills Level-3 characterization requirements necessary to reveal mAb modifications and degradations. Some important attributes including primary sequence, presence of degradation hotspots like oxidation, deamidation, isomerization, cyclization, and post-translational modifications like glycosylation are only detected, localized, and quantified by peptide analysis.

To facilitate this work, SCIEX Separations has integrated capillary electrophoresis (CE) and electrospray ionization (ESI) into a single dynamic process, called CESI. This process was first described by Moini et al., and further refined by Beckman Coulter

to create the CESI 8000 High Performance Separation –ESI module. In this technical note we describe comprehensive characterization of a representative monoclonal antibody Trastuzumab (Herceptin), illustrating the benefits of CESI coupled with high resolution mass spectrometry. Small and large peptides in the range of 3 – 65 amino acids have been separated, identified with 100% sequence coverage and quantified, including degradative hotspots such as asparagine-deamidation, methionine-oxidation, glutamic-acid-cyclization, and C-terminal lysine heterogeneity using a single separation of only 100 fmol of trypsin protease-digested sample. The low-flow rate of the system (~20 nL/min) maximized ionization efficiency and dramatically reduced ion suppression.

Case Study: Comprehensive Characterization of Trastuzumab and a Candidate Biosimilar using a Single-Injection Peptide Mapping Approach with CESI-MS

Prashant Dour

SCIEX Separation, A division of AB SCIEX,

Abstract: Monoclonal antibodies (mAbs) are highly complex proteins that display a wide range of microheterogeneity that requires multiple analytical methods for full structure assessment and quality control. As a consequence, the characterization of mAbs on different levels is particularly product - and time - consuming. This work presents the characterization of trastuzumab sequence using sheathless capillary electrophoresis (referred as CESI) – tandem mass spectrometry (CESI MS/MS). Using only 100 fmoles of sample, this bottom-up proteomic-like approach, CESI-MS/MS provided 100% sequence coverage for both heavy and light chain via peptide fragment fingerprinting (PFF) identification. The same highly sensitive analysis enabled precise characterization of the post-translational hot spots (glycosylation, deamidation, oxidation and aspartic acid isomerization) of both Trastuzumab and a candidate biosimilar. This workflow allowed a significant time saving compared with conventional LC-MS approaches, requiring only a single tryptic digest and one sample injection per sample.

7B) Complementary tools: HDMS and On-Line LC/MS

High Definition MS Strategies for the Development of Novel Biopharmaceuticals &Characterization of Biosimilars.

Mark A McDowell.

Waters Corporation, Wilmslow, Cheshire, SK9 4AX, United Kingdom.

High Definition Mass Spectrometry (HDMS) is defined as the on-line combination of Ion Mobility (IM)

separation and high resolution Time-Of-Flight (TOF) Mass Spectrometry (MS). Ion Mobility separates ionized molecules (e.g. proteins/peptides) on the basis of their size-to-charge ratio. Conceptually IM separation can thus be likened to *'gas phase electrophoresis'*. Moreover IM separation is very fast, typically taking only 10-100 milliseconds per analysis, and is thus readily performed in concert with on-line HPLC separation.

After calibration (e.g. with well-characterized peptide/protein standards) an HDMS instrument is able to determine the Collision Cross-Section (CCS), in square Angstroms, of any protein in its native (or modified) state. CCS is a reproducible and precise physicochemical property of an ionized protein that can be used diagnostically. CCS data provide an insight to tertiary protein structure that complements Hydrogen/Deuterium Exchange (H/DX) MS data. The CCS of a biopharmaceutical can provide an orthogonal metric for product characterization and/or the detection of protein misfolding.

IM separation is compatible with both Collision Induced Dissociation (CID) and Electron Transfer Dissociation (ETD). We have successfully combined these techniques for the top/middle down characterization of biopharmaceuticals and biosimilars.

Title: The Characterization of Biopharmaceuticals and Biosimilars by On-Line LC/MS.

Rajiv Bharadwaj

Waters India (Pvt.) Limited, Peenya II Phase, Peenya, Bengaluru, Karnataka, India

Abstract: This workshop will provide an introduction to the Waters Biopharmaceutical Platform Solution that integrates Ultra Performance Liquid Chromatography (UPLC) and high resolution Mass Spectrometry (MS) with *'GxP ready'* software. We will discuss and demonstrate the utility of this system for applications including: intact protein mass analysis, peptide mapping, released glycan analysis and host cell protein profiling.

This analytical platform enables a biopharmaceutical laboratory to comprehensively and routinely acquire, process, and report LC and MS characterization data throughout a biotherapeutic's development lifecycle. From bioseparations to LC/MS characterization, this Biopharmaceutical Platform provides detailed workflows that enable results to be readily generated & shared from discovery to product development, to quality control (GxP) analysis.

- UNIFI software merges LC and MS data in an intuitive environment that encompasses data processing, visualization, reporting, and configurable compliance tools

- GlycoBase Database of glucose unit (GU) values is integrated, as a library, to streamline the analysis of glycoproteins, including the identification of glycan retention times and correlated glycan structures.
- Integrates Q-ToF MS and optical detectors within a networked laboratory workgroup.
- May be deployed within a regulated and/or non-regulated laboratory environment to enable high resolution bioseparations and high performance mass analysis from biopharmaceutical product development to routine biosimilar quality control.
- Advanced data mining and comparison capabilities to enhance the understanding of results with the ability to aggregate and manage information effectively.

A ‘round table’ discussion addressing the participants most commonly encountered practical challenges in biopharmaceutical analysis (by LC/MS) will conclude this workshop.

Session II: Triple quad LC-MS/MS based Targeted Proteomics

Learning Objective

- Setting targeted multiple reaction monitoring (MRM)
- Absolute quantitation of protein therapeutics and process impurities
- Use of skyline software for target peptide selection
- Impurity profiling

Biopharmaceuticals comprise of large and complex protein biomolecules. This makes their quantification more challenging due to higher molecular weight proteins and presence of host protein impurities of even complex nature. This challenge becomes much bigger if working with serum and plasma due to abundance of variety of complex proteins and other biomolecules.

Use triple quad LC-MS/MS instrument in multiple reaction monitoring (MRM) mode allows a highly sensitive and selective targeted quantitation of protein/peptide abundances in complex biological samples. MRM mass spec has commonly been used for the analysis of small molecules. In contrast to discovery proteomics mass spec which detect all proteins in a untargeted manner, MRM is highly specific (targeted), and by tuning mass spec according to target peptides of interest the sensitivity can be several fold improved than other full scan methods.

The different scan types other than MRM, as shown in Figure 6.7 such as precursor ion scan, neutral loss scan can be used for quantification of specific moieties release from peptides (eg; glycans, glutathione).

During MRM scan the first mass analyzer (Q1) is set to only transmit (filter) the parent weight of a protein or selected peptide, the collision energy is set to produce a characteristic fragment of that peptide ion in collision cell (Q2). The third mass analyzer (Q3), is set to transmit (select for filter) this characteristic and unique peptide fragment only. Therefore, MRM mode only selectively detects single fragment which improves its sensitivity remarkably with higher reproducibility. This makes quantification of mAb and its impurities possible with great confidence. Further the labeled peptide internal standards can be used to track any analytical error and to normalize the instrumental sources of response variation.

The selection of unique peptide for quantification is critical step and please refers to Module 5 for protocols on selection of targeted peptide.

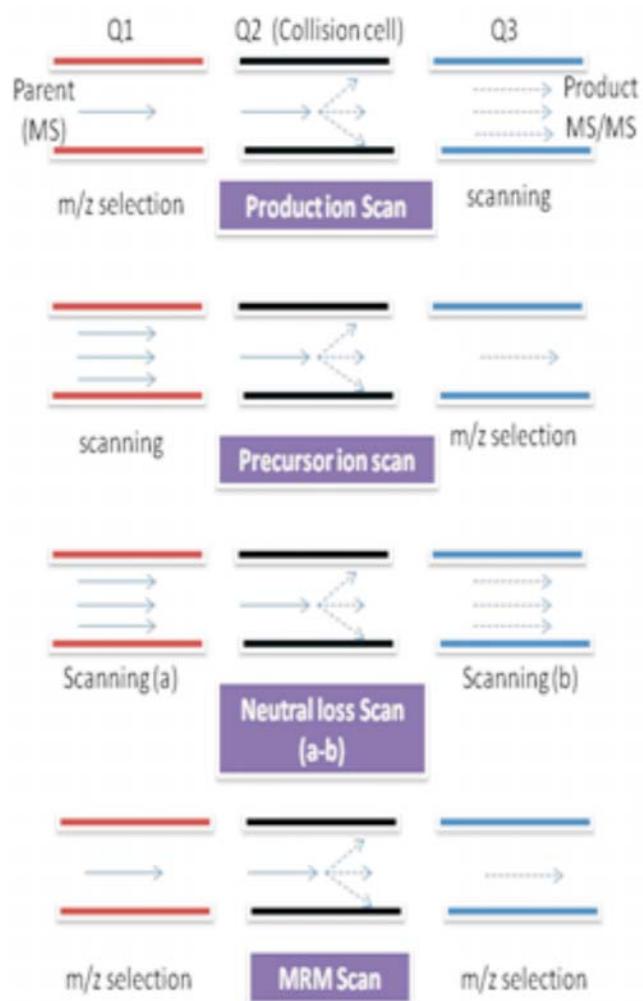


Figure 6.7: Different scan modes used in Triple quad LC-MS/MS analysis for quantification of several targeted peptide or chemical moieties.

The on-site demo of Agilent Triple Quad LC-MS/MS 6490 system will be give to all workshop participants.

Session III: Functional Characterization using Label-free interaction proteomic tools

1. Surface Plasmon Resonance (SPR)

Please refer to Module 4 for detailed protocols.

2. Bio-Layer Interferometry technology

Detailed Agenda for Label free characterization using Bio-Layer Interferometry technology:

Please refere to Module 4 for theory of this technology

Agenda

Introduction to BLI Technology (+Video): 15 minutes

Basic Quantitation Assay (using a biosensor to capture analyte directly) and Regeneration of Biosensors: 30 minutes

Real-time Data Analysis: 15 minutes

Binding Kinetics demonstration using Herceptin antibody and its binding partner: 45 minutes

Real-time data analysis: 15 minutes

Sample details

Herceptin Antibody

Herceptin Binding Partner

10X Kinetics Buffer

Amine Coupling or Streptavidin or Anti-Human IgG Fc Capture (AHC) Biosensors

96-well microplates: black, flat bottom, polypropylene microplates (Greiner Bio-One)

Instrument details and data analysis

Instrument Model Name: Octet Red96

Make: ForteBio a division of Pall Corporation

Octet Red Instrument Serial Number: FB-50082

Octet Instrument Power Supply controller: Serial No: 00490

Instrument computer Work station along with Moniter Serial Number: X16-96076, Human IgG calibrator 18-1030, Protein A biosensor: 18-5010

Session IV: Gel-based& Chromatographic separation technologies

For gel based protocols please refer to Module 1.

Advanced Chromatographic separation technologies

Title: Introduction of PAT to Improve the Efficiency and Robustness of Biosimilars & Biotherapeutics Manufacturing

Aleš Štrancar, BIA Separations

While Process Analytical Technology (PAT) has been used for decades to ensure process reproducibility, applying PAT to biopharmaceutical manufacturing is much more complex due to the complexity of biomolecules and the batch to batch variability resulting from slight environmental changes during production.

During the development of an up- and/or downstream process of the target biomolecules, it is essential to have a fast, accurate and reliable analytical method for determining the quantity and purity as the product is intended for human use. Therefore highly efficient and selective columns are needed.

HPLC columns that would be able to handle samples from different feed streams and determine the amount of the target molecule and impurity profile in nearly real time, would mean a step forward when it comes to implementing in-process control in process development and PAT into novel bioprocesses.

In this presentation, examples of PAT in pharmaceutical manufacturing that takes advantage of the unique properties of specially designed monolithic HPLC columns, supplied by Agilent or by BIA Separations, to provide rapid, actionable information will be presented.

Further Readings

1. ICH Q6B guidelines on test procedures for biologics; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf
2. Guidelines on Similar Biologics:Regulatory Requirements for Marketing Authorization in India <http://dbtbiosafety.nic.in/Files%5CCDSCO-DBTSimilarBiologicsfinal.pdf>
3. ICH Q5E guidelines for Comparability of Biologics; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5E/Step4/Q5E_Guideline.pdf
4. USFDA Biosimilar guidelines for scientific considerations; <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM291128.pdf>.
5. US FDA biosimilar guidelines overview; <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm291232.htm>
6. EMA comment on Q6b; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002824.pdf
7. Steven A Berkowitz, John R Engen, Jeffrey R Mazzeo, and Graham B Jones. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov; 11(7): 527–540.

8. Joy Concepcion et al. Label-Free Detection of Biomolecular Interactions Using BioLayer Interferometry for Kinetic Characterization. Combinatorial Chemistry & High Throughput Screening, 2009, 12, 791-800.
9. Alain Beck, Sarah Sanglier-Cianfeirani, and Alain Van Dorsselaer. Biosimilar, Biobetter, and Next Generation Antibody Characterization by Mass Spectrometry. Anal. Chem., 2012, 84 (11), pp 4637–4646.
10. Cédric Atmanene et al. Extending Mass Spectrometry Contribution to Therapeutic Monoclonal Antibody Lead Optimization: Characterization of Immune Complexes Using Non-covalent ESI-MS. Anal. Chem., 2009, 81 (15), pp 6364–6373.

INDEX

- Abhyankar, Aalhad, 23, 119
Abzur, Faiz, 12, 81
Andrews, Philip, 7, 8, 64
Atak, Apurva, 80, 84
Banuchander, Karthik, 28
Bharadwaj, Rajiv, 7, 20, 23, 24, 120
Bhave, Amruta, 28
Costello, Catherine, 8, 65
Chen, Sixue, 3, 27, 29
Chennareddy, Srinivasrao, 28
Choi, Meena, 19, 20, 108, 110
Choudhary, Saket, 93
Clary, Taegen, 19, 23, 24, 113
Coorsen, Jens, 3, 26, 28
Dabhi, Raju, 76
Dey, Sharmistha, 16, 100
Dour, Prashant, 23, 113, 118, 119, 120
Fuentes, Manuel, 11, 80
Gahoi, Nikita, 80, 84
Galvin, Bob, 23, 24
Gandhi, Mayuri, 7, 23, 66, 113
Ghantasala, Saicharan, 66, 76
Gollapalli, Kishore, 7, 50, 51, 62, 66, 75, 76
Goud, Narendra, 12, 80, 85, 93
Goulding, Paul, 20
Govekar, Rukmini, 3, 27
Gowda, Harsha, 7, 65
Gupta, Anuj, 11
Gupta, Shabarni, 80, 84, 93
Jain, Rekha, 11, 50, 80, 82, 85, 96
Kamat, Vishal, 16, 102
Karunakaran, Senthil, 28
Kulkarni, Mahesh, 19, 20, 108, 110
Kumar, Saurabh, 56
Kumar, Vipin, 28, 51
Kumarswamy, Sriram, 16, 24, 101, 113
Krovvidi, Ravi, 7, 8
Ludwig, Christina, 19, 23, 108, 109, 113
MacLean, Brendan, 19, 20, 23, 108, 109, 113
Madhurarekha, 15, 16, 101
Malakar, Dipankar, 8, 20, 23, 118, 119
Manubhai, KP, 84
Manhas, Vipan, 28
McDowall, Mark, 113, 120
Moritz, Robert, 23, 112
Muddiman, David, 7, 64
Mukerjee, Shuvolina, 28
Nagpal, Saurabh, 23
Nallapeta, Sivaramaiah, 8
Narayan, Pradeep, 102
Navani, Sanjay, 11, 12, 81
Nice, Ed15, 16, 98, 99
Noronha, Santosh, 11, 12, 81, 93
Pargaonkar, Ashish, 7, 19, 23
Patel, Prashanti, 53
Patel, Sandip, 28, 47
Pattanaik, Sangram, 20
Paul, Debasish, 76
Pawar, Komal, 53
Phapale, Prasad, 23, 112, 114
Persson, Anette, 15, 99
Poddar, Mukesh, 28
Rajgopal, Prasanna, 7
Ramanna, K Y V, 4, 28
Rao, Sudha, 11, 80
Ray, Sandipan, 1, 3, 26, 27, 29, 46, 47, 50, 51, 53, 106
Reddy, Panga Jaipal, 19, 56, 75, 96, 108, 110
Renuse, Santosh, 7, 8, 20
Saha, Rajesh, 15, 100
Santra, Manas, 76
Sait, Osama Shakeel, 28
Seal, Shubhendu, 28
Shah, Vineeta, 15, 16, 24, 98
Shalom, Abel, 23, 118, 119
Sharma, Samridhi, 98
Srikanth, Rapole, 7, 62, 65, 76
Srivastava, Sanjeeva, 1, 46, 47, 51, 53, 56, 75, 76, 93, 106, 112
Štrancar, Ales, 4, 28, 122
Syed, Parvez, 93
Uppal, Annu, 8, 20
Varadarajan, R, 15, 100
Venkatesh, Apoorva, 96