

FINDING NEW INTERACTING PROTEINS BY A SIMPLE AND POWERFUL YEAST 2 HYBRID TECHNIQUE

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Protein-protein interactions are essential to cellular mechanisms at all levels in biologically responsive systems. Intracellular protein-protein interactions occur in the formation of multi-protein complexes, which trigger multiple cellular events. These interactions can be narrowed down into the binding of two proteins. One to one protein interaction is effectively studied by using yeast two hybrid screenings. A key of this technique is that in most eukaryotic transcription factors, the activating domains and binding domains are modular and function in close proximity to each other with out direct binding. By using genetically engineered strain of yeast in which the biosynthesis of certain nutrients is lacking and plasmids, which are engineered to produce a protein product in which activation domain or DNA-binding domain is fused onto a protein, one to one protein interaction can be determined. This powerful technique is established in the late 1980s and since then many variations has been reported.

In this summer school, not only a basic premise and technical procedures of yeast 2 hybrid screening method but also different applications and strengths/weaknesses are discussed.

ROLE OF UBIQUITIN-LIKE DOMAIN IN REGULATION OF SIGNALING

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Ubiquitin (Ub) is a small protein modifier that regulates many biological processes, including gene transcription, cell-cycle progression, DNA repair, apoptosis, virus budding and receptor endocytosis. Ub can be conjugated to target proteins either as a monomer or as Ub chains that vary in length and linkage type. In addition to Ub itself, many ubiquitin-like (Ubl) proteins have been identified, including small Ub-like modifier (SUMO), interferon stimulated gene 15 (ISG15), autophagy 8 (ATG 8) and neural precursor cell expressed, developmentally down regulated 8 (NEDD8). Ubls have a similar structural-fold to Ub, but use specific conjugation machineries and are recognized by distinct Ubl-binding domains. Ubls are implicated in the regulation of many cellular processes such as gene transcription, signal transduction, autophagy and cell-cycle control. The conserved ubiquitin β -grasp fold has had numerous functions during evolution and is used, for example, as a protein domain integrated in the coding sequence of several cellular proteins. These ubiquitin-like domains (ULDs) adopt a Ub-like fold within the tertiary structure of the host protein. ULDs can intrinsically regulate the conformational status of the host protein, as well as participate in the reorganization of protein complexes. In this talk, I would like to discuss how ULD regulates down-stream signaling by giving a few examples of ULD-containing proteins.

MASS SPECTROMETRY – BASED PROTEOMICS: PRINCIPLES AND APPLICATIONS

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Proteomics is defined as a global approach to study the structure and function of all proteins in the cell (the proteome). Due to the high structural complexity of proteins, and the fact that their function is mediated by the amino acid sequence and more than 200 different posttranslational modifications, the global analysis of proteome presents an extremely challenging task. Among several strategies to investigate proteome biology, quantitative mass spectrometry (MS) has evolved as a method of choice for global protein detection, structural characterization, assessment of protein interactions and dynamics.

In modern quantitative MS-based proteomics, protein identification and quantitation are achieved at the peptide, rather than the protein level. In a typical experiment, cell lines or tissues are differentially labeled by stable isotopes, mixed together, lysed and digested by a protease (usually trypsin). The resulting complex peptide mixtures are further fractionated, separated by nano-HPLC, electrosprayed and analyzed in mass spectrometer. Stable isotopes introduce specific mass labels that enable quantitation directly from the peptide signals in mass spectra. Peptide masses are recorded at high (sub-ppm) mass accuracy, and peptides are further fragmented to provide primary structure information (4 This approach has a much higher analytical capacity than "classical" protein-based approaches, largely based on 2D PAGE separation and subsequent MS identification of protein spots. Importantly, most proteins in a sample are usually represented by more than one peptide, leading to more opportunities for identification and quantitation, and therefore to more comprehensive and accurate results.

Combined with recent advances in MS data processing and quantitation software, MS-based proteomics is currently capable of quantifying expression changes of >5,000 proteins in a single experiment, and is rapidly approaching quantitation depth of "message-based" gene expression assays, such as microarrays. However, MS -based proteomics is a diverse and rapidly evolving field, currently consisting of several technological platforms for protein detection, quantification and sample preparation, and it is often difficult to recognize the proper platform for a specific analytical problem. Here I will present the state-of-the art in MS -based proteomics, introduce and compare the existing technologies, and argue that not all of them are up to the task of performing a system-wide and in-depth quantitative analysis of the proteome.

Literature:

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QUANTITATIVE PHOSPHOPROTEOMICS APPLIED TO ELUCIDATION OF SIGNAL TRANSDUCTION PATHWAYS

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Advances in phosphopeptide enrichment, mass spectrometry and data processing have revolutionized phosphoproteomics and enabled deep insights into molecular processes underlying signal transduction. Global and phosphorylation site-specific studies are commonly performed using at least two orthogonal phosphopeptide enrichment steps, such as strong cation exchange and TiO₂ chromatography, followed by fast and accurate MS and MS/MS measurements.

We have recently developed a general mass spectrometric approach for identification and quantitation of phosphorylation sites as a function of stimulus, time, and subcellular location, and applied it to study phosphoproteome dynamics in HeLa cells upon stimulation with epidermal growth factor (EGF). For this purpose cells were differentially labeled using stable isotope labeling with amino acids in cell culture (SILAC) and incubated with EGF for 0, 1, 5, 10, 15 and 20 minutes. Treated cells were pooled, lysed and divided in nuclear and cytosolic fractions. Protein extracts were digested with trypsin and resulting peptide mixtures were subjected to two stages of phosphopeptide enrichment, using strong cation exchange and TiO₂ chromatography. Enriched phosphopeptides were separated on nano-HPLC and measured on LTQ-FT ICR and LTQ-Orbitrap mass spectrometers.

We have detected more than 6,600 phosphorylation sites on more than 2,200 proteins and recorded them in the Phosida database (www.phosida.com). Fourteen percent of phosphorylation sites were modulated at least 2-fold by EGF, and these were classified by their temporal profiles. A majority of proteins contained multiple phosphorylation sites showing different kinetics, suggesting that they serve as platforms for integrating signals. In addition to protein kinase cascades, the targets of reversible phosphorylation included ubiquitin ligases, guanine nucleotide exchange factors, and at least 46 different transcriptional regulators. This generic approach can be used to study almost any signaling pathway in the cell culture and its applications in other areas, such as Ser/Thr/Tyr phosphorylation signaling in prokaryotes, will be presented and discussed.

The dynamic phosphoproteome provides a missing link in an integrative view of cellular regulation and mass spectrometry-based proteomics is currently the only technological platform that can study dynamics of phosphorylation events during signal transduction at a global level.

Literature:

Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. *PNAS*, 2004. 101, 12130-12135

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INSIDE NATURE

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The biology team at Nature receive on average 120-150 new manuscript submissions per week of which only about 10-12 can be published. My presentation will outline who the editors in the biology team are, how we decide which papers will be published, how we manage the review process and how authors and reviewers interact with the editors. I will also outline how we work with the other sections of the journal, such as the News & Views editors and the journalist responsible for our news pages. Other kind of editorial jobs at Nature and other Nature journals will be discussed. Furthermore, I will highlight issues related to fraud in science and how the recent high-profile cases have influenced the publication process.

PROTEIN ARRAYS I: GENERAL INTRODUCTION

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Microarrays have revolutionised the detection of biomolecules by introducing the highly parallel incubation of sample on a multitude of miniaturised probes. With DNA microarrays firmly established, development moves rapidly forward to protein microarrays for detection and analysis of proteins and their functions in large (eventually proteome-wide) sets. To date, protein microarrays typically consist of hundreds to thousands of features immobilised on a solid support. Their advantages include speed, high sensitivity and economical use of samples and reagents. A general distinction can be made between (i) capture arrays, (ii) functional protein arrays and (iii) reverse arrays. In capture arrays, specific protein binding molecules (usually antibodies) are used to detect target molecules in complex samples such as blood plasma. Applications include biomarker discovery and the analysis of protein phosphorylation patterns. Functional protein arrays consist of arrayed proteins other than binders and are used to carry out screening of protein-protein, protein-DNA or protein-drug interactions. Reverse arrays are arrays of immobilised complex samples, e.g. patient sera or cell lysates. Compared to nucleic acids, proteins are difficult to obtain, chemically heterogeneous, rather unstable, and their functionality often depends critically on the integrity of their structure. Therefore protein array technology is faced with bottlenecks for supply of proteins and challenges for their immobilisation and functional storage prior to use.

PROTEIN ARRAYS II: IN-SITU ARRAYING BY CELL-FREE PROTEIN EXPRESSION

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In-situ or on-chip protein array methods use cell-free expression systems to produce proteins directly onto an immobilising surface from co-distributed or pre-arrayed DNA or RNA, enabling protein arrays to be created on demand. These methods address three main issues in protein array technology: (i) efficient protein expression and availability, (ii) functional protein immobilisation and purification in a single step and (iii) protein on-chip stability over time. By simultaneously expressing and immobilising many proteins in parallel on the chip surface, the laborious and often costly processes of DNA cloning, expression and separate protein purification are avoided. Methods are PISA (protein in situ array), NAPPA (nucleic acid programmable protein array) and DAPA (DNA array to protein array). DAPA allows the printing of replicate protein microarrays directly from a DNA microarray template. At least 20 copies of a protein microarray can be obtained from a single DNA microarray, while requiring only "macro" handling steps. Therefore, DAPA has the potential to bring protein microarray systems to laboratory environments lacking the equipment for microarray production.

Literature:

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FORCE FIELD BASED METHODS FOR COMPUTATIONAL STUDY OF MACROMOLECULES

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Revealing of the life secrets requires a complex approach to biological problems utilizing both the experimental and the theoretical methods.

Recognition between biologically important molecules is a result of balance between different molecular properties: shape, charge distribution, entropy and dynamics. In order to investigate the molecular properties and correlate the structural parameters with biological activity we use various molecular modelling methods in our research, as well as statistical and data processing approaches. However, these investigations depend on the existence of the reliable 3D structure of macromolecules. For this purpose the X-ray diffraction is the most widely used methods. Follow neutron diffraction, NMR, microscopic and spectroscopic methods.

Development of computers, their relatively low price and plethora of available software resulted in wide application of molecular modelling in almost all biosciences. In the lecture I'll explain difference between quantum mechanical and empirical methods and outline a general procedure that could be used in study of proteins and their complexes. The most often used techniques of molecular modelling will be outlined: protein homology modelling, geometry optimisation, building 3D structure of small molecules, ligand docking, protein flexibility investigation by molecular dynamics (MD) simulations and Normal modes analysis (NMA). Also I'll explain the basic idea of hybrid, QM/MM method and its applicability in modelling enzymatic reactions. Finally, I'll give a possible scenario for modelling a virtual (*in silico*) experiment and how to use it as a template for the true one. For this purpose the main idea of 3D-QSAR (3-dimensional quantitative structure activity relationship) analysis will be explained, as well as importance of skill in models interpretation, predicting biological response and finding the modification that would change this response.

The connection with the laboratory will be highlighted through a comparison of selected experimental properties (X-ray and neutron diffraction, electron microscopy, and NMR, IR, and UV spectra) with those that can be obtained through modeling. In addition, attention will be given to the productive use of known results through the interaction with databases of, for example, macromolecular structures or the properties of small molecules.

Molecular mechanics, molecular dynamics and Monte Carlo methods (as well as NMA and majority of 3D QSAR investigations) are 'the force field based approaches'. A force field is characterised by a set of atomic parameters and the equation for the potential energy of a system, such is for example the following one:

$$U = \sum \frac{1}{2} k_b (b - b_0)^2 + \sum \frac{1}{2} k_k (a - a_0)^2 + \sum \frac{1}{2} k_t (\tau - \tau_0)^2 + \phi - \tau [1 + \cos(n \sum_{ij} [q_i q_j / R_{ij} + (A_{ik} / R_{ik}^{12} - B_{ik} / R_{ik}^6))]]$$

The first three terms in this expression are so called bonding energy terms (bond, valence angle and dihedral angle terms) and the last two terms describe electrostatic (Coulombic) and van der Waals interactions. The atomic parameters (bond lengths, angles, partial atomic charges, van der Waals radiuses) and constants, k (in the first three terms) are determined experimentally and by the high level quantum mechanical (mostly *ab initio*) calculations.

MD is used to simulate molecules in their natural conditions, for example proteins interacting in water with certain concentration of ions added at defined temperature, T . The basis for MD simulations is the ergodic hypothesis which says that statistical (volume) ensemble averages are equal to time averages of the system, if the system is

simulated long enough. Dynamics of a particle i , in such system is described by the

$$-\frac{dU_i}{dt} = m_i \frac{d^2 r}{dt^2}$$

Newton's equation of motion:

The time step in MD simulations is determined by the vibrational frequency of the particles in the system and in the all atoms MD simulations it is most often 1 fs, 10^{-15} s. Monte Carlo (MC) methods are sometimes used as an alternative to MD simulations to search conformational space of molecules and possible binding modes of a ligand. To achieve reliable results the repeated random sampling is accomplished and the results are grouped according to the spatial similarity and the potential energy. Using the Metropolis MC approach it is possible to determine the Boltzmann average of a property of the system.

Normal mode analysis (NMA) is based on analysis of stable protein conformations close to a minimum of potential energy which is approximated by the harmonic potential:

$V(\mathbf{r}) = \frac{1}{2} (\mathbf{r} - \mathbf{r}^0) K(\mathbf{r}^0) (\mathbf{r} - \mathbf{r}^0)$, where \mathbf{r}^0 denotes a $3N$ dimensional vector describing stable protein conformation and \mathbf{r} the slightly distorted one. In NMA each amino acid

residue is often represented with a single point (either C_α or center of mass coordinates). The vibrational mode frequencies are determined by solving the differential equation of motion. For the purpose of revealing possible protein conformations the most interesting are the low frequency mode because they usually describe large rearrangements of protein domains.

Finally, the 3D QSAR analysis utilizes different statistical methods to correlate values based on the 3D structure of molecules with the experimentally determined molecular activity.

Literature:

Andrew R. Leach 'Molecular Modelling: Principles and Applications'

PROTEINS *IN SILICO* - THREE CASE-STUDIES

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In this lecture I'll give an overview of our most interesting results achieved for different proteins and their complexes namely:

1. The specific interactions in the complexes between Ras protein and its effectors Raf and RalGDS
2. Dynamic investigation of proteins and their complexes with small molecules represented on the example of the ABP1- auxin (IAA) complexes.
3. Study of the enzyme reactions - our research on *Burkholderia cepacia* lipase enantioselectivity and perspective of computational importance in tailoring new enzymes.

1. Majority of the signal transduction and regulatory processes in cell are based on protein-protein interaction, and they are very sensitive to protein mutations. Herein I shall give an overview of our theoretical studies accomplished for complexes between H-Ras and its effectors, proteins Raf and RalGDS.¹

Ras is a small guanosine triphosphate (GTP)-binding protein that serves as a switch in the mitogen-activating protein kinase pathway (MAPK). In its active, *on* conformation (GTP bound), Ras interacts with Raf, a Ser/Thr specific protein kinase, an immediate downstream target of Ras in MAPK. Another protein that Ras interacts with is Ral guanine nucleotide dissociation stimulator (RalGDS). This interaction is connected with the control of cytoskeletal rearrangements. Consequences of certain Ras mutations are uncontrolled growth and division of cells, and the mutated Ras proteins are frequently found in diverse human tumours. It is clear that knowledge about interactions between Ras proteins (Ras, Rap...) and their effectors is important from both biological and medicinal point of view. In the study we found that the interactions between Ras and its effectors Raf and RalGDS are electrostatically driven. Also we derived 3D QSAR models for predicting binding affinities of mutant proteins and determined the potential binding places for small molecules that would either prevent any effector binding or would help to increase selectivity of Ras toward particular effector.

2. Auxins are the first identified plant hormones. They regulate plant growth and affect many processes in the plant cell. Auxin Binding Protein 1 (ABP 1) has been known for decades as the protein that binds auxin-related compounds with high affinity. In order to understand molecular mechanism of auxin binding to ABP1 we accomplished a series of molecular dynamics (MD) simulations of ABP1 and it's complexes with auxin-related compounds.² The results pointed to the shift in population of two most common ABP1 conformations induced by binding of an auxin molecule. Considering assumed orientation of ABP1 to the membrane these two conformations differently interact with membrane proteins. Using random expulsion molecular dynamics (REMD) simulations we spotted possible routes by which auxin-related compounds enter and/or leave the ABP1 active site. One of these routes leads into the membrane and the others to the lumen of endoplasmic reticulum or cytosol, depending on the ABP1 location.

3. Beside indispensable role lipase have as biocatalyst in living organisms they are also very interesting for biotechnology and pharmaceutical industry, mostly because of their wide substrate selectivity and high enantioselectivity. In this respect the most important are microbial lipase such is lipase from bacteria *Burkholderia cepacia*. From biotechnological point of view it is interesting because of its ability to catalyze the secondary alcohols with high enantioselectivity.³

We used two different approaches: 3D QSAR^{4,5} and the quantum mechanical (QM)^{6,7} to investigate the high enantioselectivity of *Burkholderia cepacia* lipase (BCL) toward secondary alcohols. First, for the series of the secondary alcohol esters we identified their possible binding modes in the BCL active site and derived the 3D QSAR model for predicting BCL enantioselectivity towards secondary alcohol. Afterwards, using

the quantum chemistry methods, we studied chemical transformation of the secondary alcohol (*R,S*)-1-phenoxy-2-hydroxybutane (**1**) and its ester (**E1**). The experimentally determined enantiomeric ratio for **1** is extremely high, $E > 200$.

Starting from the four covalent complexes with different orientation of the substrate (two determined by molecular modelling,⁴ and two derived from the X-ray structure of the BCL-inhibitor complex^{3,7}), we modelled the ester (*S* and *R* – **E1**) hydrolysis and the alcohol (*S* and *R* – **1**) esterification using quantum mechanical⁶ (QM) and the hybrid QM/MM⁷ methods. The calculations revealed that ester release is possible from all four covalent complexes. Alcohol release from the BCL-**E1** complex in which the *S*-substrate is bound in the same manner as the *S*-inhibitor in the crystal structure however is not possible. The results proved that the productive binding modes of the secondary alcohol enantiomers were correctly predicted by molecular modelling and gave confirmation of the reliability of our 3D QSAR model.

Literature:

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OVERVIEW: FLUORESCENCE MICROSCOPY METHODS FOR MOLECULAR IMAGING

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Fluorescence microscopy is one of the most commonly used tools in biomedical research. Through technologies like confocal microscopy, much information can be collected on the intracellular localization of proteins. By analyzing the three-dimensional distribution of proteins or by observing the changes in localization in in-vivo timelapse sequences, much can be understood about the function of a protein of interest. The power and potential of such approaches will be discussed in the first part of the seminar. The direct study of proteins at the molecular level is however not possible, as the resolving power of light microscopy is limited to details at the level of 200-300 nm, which corresponds to the size of intracellular organelles. Several approaches have been developed to collect information about molecular kinetics or the direct interactions of molecules on the light microscope. These include Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Correlation Spectroscopy (FCS) for the study of molecular dynamics and Fluorescence Resonance Energy Transfer (FRET) for the detection of molecular interactions. The second part of the seminar will give a short introduction into these techniques.

A CLOSER LOOK: PHOTBLEACHING TECHNIQUES: AN IN-DEPTH LOOK

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Photobleaching, as well as the related method of photoactivation, is one of the most fundamental tools in the study of molecules in the light microscope. In this seminar, two of the most commonly used approaches will be presented in detail.

In Fluorescence Recovery after Photobleaching (FRAP) a small area of a sample is bleached and the recovery of the fluorescence signal by the exchange of bleached and non-bleached molecules is observed. From the speed of recovery, conclusions can be drawn on the molecular dynamics. In Fluorescence Resonance Energy Transfer (FRET) detection, the specific photobleaching of either the donor or the acceptor fluorophore is frequently used as a tool to visualize FRET interactions. Considerations for FRAP and FRET photobleaching methods, including possible pitfalls, will be discussed and, for FRET, alternative methods will be presented.

Computer room session:

The main aim of the practical session is to learn how to analyze FRAP and FRET data. For this, example datasets will be provided and the analysis will be performed in ImageJ. In a first part, the diversity of microscope control softwares will shortly be explored in the Leica softwares LCS lite, LASAF lite and the Zeiss Image Browser. Data from all these softwares can however be imported into the image processing software ImageJ and the import of distinct microscopy dataformats into ImageJ for further analysis will be performed. In a second part, FRAP datasets will be quantitatively analyzed to demonstrate the steps required to measure, normalize and analyze such data. These data will then be exported to Excel. In a third part, FRET acceptor photobleaching datasets will be analyzed by comparing the images before and after photobleaching and by calculating the apparent FRET efficiency.

Literature:

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GUEST LECTURERS

HOW TO CRYSTALLIZE A PROTEIN: STRUCTURAL STUDIES OF PIN DOMAINS OF HUMAN NMD PROTEINS

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X-ray crystallography is essentially a form of very high-resolution microscopy. It enables us to visualize protein structures at the atomic level and enhances our understanding of protein function. Specifically, we can study how proteins interact with other molecules, how they undergo conformational changes, and how they perform catalysis in the case of enzymes. Technological advancement has enabled us to tackle bigger and more challenging problems but the basic principles remain the same. We shall discuss these principles on the example of human nonsense mediated mRNA decay (NMD) proteins. NMD is a conserved, eukaryotic-specific quality control mechanism that mediates rapid degradation of transcripts bearing premature translation termination codons. This mechanism provides protection against potentially harmful effects of unproductively processed mRNAs and the synthesis of C-terminally truncated peptides. The central component of this mechanism is UPF1 (SMG2). The activity of UPF1 is regulated through cycles of phosphorylation and dephosphorylation.

SMG6 and SMG5 are essential factors in NMD. These proteins have been implicated in the dephosphorylation of UPF1. Both SMG5 and SMG6 have been predicted to contain a C-terminal PIN (P_{II}T N-terminus) domain, present in proteins with ribonuclease activity. Structural analysis has showed that although these proteins share a similar overall fold related to ribonucleases of the RNase H family, they have local differences at the putative active site. SMG6 has the canonical triad of acidic residues that are crucial in RNase H for nuclease activity, while SMG5 lacks these key catalytic residues. The structural differences are reflected at the functional level; suggesting that the NMD machinery has intrinsic nuclease activity that is likely to contribute to the rapid decay of mRNAs that terminate translation prematurely.

A FORAY INTO INTERDISCIPLINARY SCIENCE: ENHANCING THE ANALYTICAL POWER OF SDS-PAGE USING MACHINE LEARNING ALGORITHMS

Fran Supek

Division of electronics, Rudjer Boskovic Institute, Croatia

My lecture will attempt to demonstrate how a complex protein mixture originating from plant tissue can be reliably 'fingerprinted' by running conventional one-dimensional SDS-PAGE in bulk and analyzing gel banding patterns using machine learning methods. An unsupervised approach to filter noise and systemic biases (principal component analysis) was coupled to state-of-the-art supervised methods for classification (support vector machines) and attribute ranking (ReliefF) to improve tissue discrimination, visualization and recognition of important gel regions.

Literature:

Supek F, Peharec P, Krsnik-Rasol M, Smuc T. Enhanced analytical power of SDS-PAGE using machine learning algorithms. *Proteomics* 2008 Jan; 8(1):28-31