Journal of Proteomics 253 (2022) 104458

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Review

Emerging trends in developing biosensor techniques to undertake plant phosphoproteomic analysis

Asmat Farooq [a,b], Kaisar Ahmad Bhat [a,c], Rakeeb Ahmad Mir [c], Reetika Mahajan [a], Muslima Nazir [d], Vikas Sharma [b], Sajad Majeed Zargar [a,*]

[a] Proteomics Laboratory, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Shalimar, Kashmir 190025, India
[b] Division of Biochemistry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-J), Chatha, Jammu 180009, India
[c] Department of Biotechnology, School of Biosciences & Biotechnology, BGSB University, Rajouri, India
[d] CORD, University of Kashmir, Hazratbal, Srinagar, Jammu & Kashmir, India

ARTICLE INFO

Keywords:
Biosensors
Phosphoproteomics
Phosphoproteins
Post-translational modifications,

ABSTRACT

Protein modifications particularly phosphorylation is governed by a complex array of mechanisms to attain a functional conformation and regulate important biological processes in organisms during external environmental stimuli and hormone signaling. Phosphoproteomics is a promising field of proteomics for identification of proteins with phosphate groups and their impact on structure, function and localization of proteins. Techniques that allow quantitative detection of proteins and their post-translational modifications (PTMs) have immensely led to understand the structural and functional dynamics of proteins. Biosensor systems are a relatively new biotechnological approach that works on the principle of transforming the interactions of different biological samples *viz* proteins, enzymes, aptamers, nucleic acids and so on into the signals such as electrochemical, colorimetric, optical or magnetic which have been effectively useful in the detection and characterization of phosphoproteins. The focus of our review is to provide a comprehensive account of the critical role and utility of novel biosensors such as, fluorescence based, enrichment based, nanobody based biosensors, as promising technical intercessions to identify phosphoproteins and their influence on structural dynamics of proteins. Furthermore, by studying the innovative phosphoprotein biosensors we will be able to identify the aberrant phosphorylation patterns to precisely diagnose diseases.

1. Introduction

Proteins (or polypeptides) are polymeric chains of amino acids that form a biologically active three-dimensional (3D) structure macromolecule outputs encoded from the genetic material of the cells [1,2]. The native folded structure of proteins is defined by the amino acid sequence of the biosynthesized polypeptide chain [3]. For decades, the prediction of the 3D structure of proteins on the amino acid-based sequences is a challenging topic in computational biophysics, due to its inherent scientific appeal as well as the numerous possible applications such as strong prediction algorithms for protein structure, ranging from genome analysis to protein function discoveries. The issue of the production of amino acid sequences which leads to folding into a specific 3D shape has subsequently gained a lot of interest as a possible direction towards rational protein engineering for biotechnology and medicine. These advances in protein structure characterization and designs have been spurred by technical advancements and a huge growth of biological databases [4]. An important mechanism of protein activation is post-translational modifications (PTMs) such as phosphorylation, glycosylation, acetylation, methylation, ubiquitylation, isoprenylation, SUMOylation (Fig. 1). Among these modifications, phosphorylation is the most common mechanism of PTM, affecting the structure, cellular activity and protein function. The interplay of protein modification with multiple PTMs in prokaryotic and eukaryotic systems contains crucial biological information [5]. It is known that approximately 30–50% of proteins might be phosphorylated at any time. The primary phosphorylation targets in proteins are amino acids, such as, tyrosine (Y), serine (S), threonine (T), histidine (H), aspartate (E), glutamate (D), lysine (K), arginine (R), and cysteine (C) residues [6]. Addition of phosphate group to the proteins induces the structural changes and in-turn regulates the

* Corresponding author.
E-mail address: smzargar@skuastkashmir.ac.in (S.M. Zargar).

https://doi.org/10.1016/j.jprot.2021.104458
Received 21 September 2021; Received in revised form 6 December 2021; Accepted 8 December 2021
Available online 16 December 2021

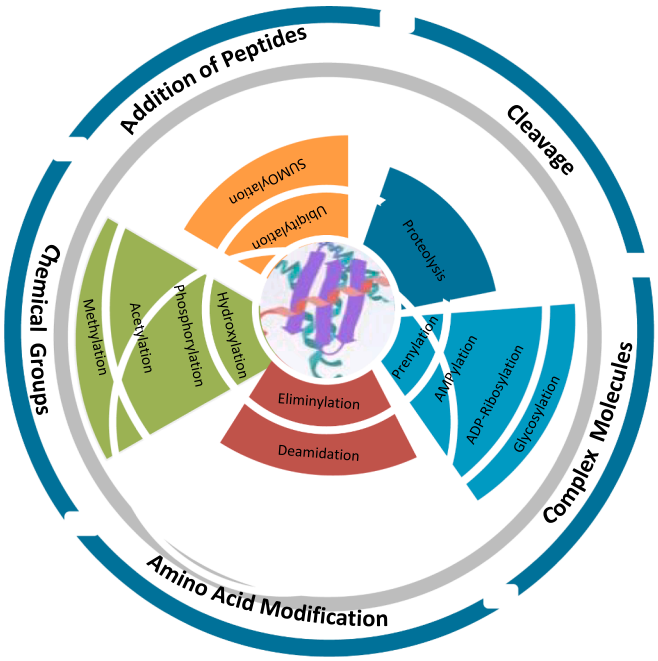

Fig. 1. Schematic representation of different post translational modifications inside the cell.

functions of cells like signal transduction, cellular metabolism, apoptosis, and compartmentalization inside the cells [7]. Signal transduction across mitogen-activated protein kinase (MAPK) cascades in budding yeast, simultaneous stimulus (high salt concentration and pheromones) alters numerous pathways previously considered to be distinct to a particular stimulus. This intra-phosphorylation cross-talk demonstrated that phosphorylation modifications in several pathways interacting on greater levels as compared to previously assumed, demonstrating that the combination of many inputs requires complicated signaling cascade links [8,9].

Phosphoproteomics is a systemic study of protein phosphorylation by identifying phosphoproteins and the phosphorylated amino acid residues through applications of qualitative and quantitative approach [1,2]. Characterization of protein modifications through the phosphoproteomics is an important feature to examine protein structure, localization and functions. Phosphoproteomics helps in finding the regulatory functions of protein phosphorylation and dephosphorylation in molecular networks, as well as the consequences of post-translational modifications on cellular metabolism. Phosphoproteomics has moved attention into screening of possible diseases treatments and plays an important role in comprehension of how phosphorylation participates in converting distinct messages through regular and abnormal physiological responses [10]. Recent biotechnological approaches have lead us towards integrating proteomics with genomics and transcriptomics to explain different protein functions (mainly due to protein phosphorylation) during normal and stress conditions as for most of the protein structures and functions are closely linked [1,11]. Various approaches such as mass spectrometry (MS), Immunoassays, X-ray Diffraction, Electron Microscopy (EM), Nuclear Magnetic Resonance (NMR) and Radioactive ATP assays have been primarily used to identify structural modifications, most importantly with protein phosphorylation [12,13]. However, these techniques or methods are unsuitable due to their high cost, complexity and sensitivity. To overcome the lacuna associated with the existing techniques, innovative biotechnological biosensors for detecting protein phosphorylation and their impact on protein structure are recently introduced. The current review provides updated account on protein phosphorylation and its existing functional role in signal transduction pathways and other cellular roles in higher organisms. The proceeding sections deals with the chronicle of plant phosphoproteomics in different dimensions and majority of this review deals with the basic background of biosensors and their technical applications in detecting and characterizing the phosphoproteins.

2. Protein phosphorylation: a way forward to understand its role in cellular dynamics

Protein phosphorylation is a PTM that is a common occurrence in cells of eukaryotes. During such modifications, phosphate group (PO_3^{-2}) covalently binds to the side chains of amino acid residues mostly serine, threonine and tyrosine [14,15]. Several enzymes and receptors are activated and deactivated *via* phosphorylation/dephosphorylation of proteins by the enzymatic action of specific kinases and phosphatases that are exceptionally significant in many cellular processes such as protein synthesis, cell division, signal transductions, cell growth, development, and senescence [16]. Thereby, this modification acts as molecular regulators of proteins, switching its activity on or off (Fig. 2). Phosphatase behaves in the opposite way as of kinases. The mechanism of action is hydrolysis of phosphoric acid monoesters into a phosphate group and a molecule with a free hydroxyl group. Protein phosphatases when compared to protein kinases, are called passive housekeeping enzymes. These are more difficult to detect because of their distinct composition as compared to protein kinases [17]. As a result, phosphosignaling networks are considered as the center of a wide range of cellular processes. Protein kinases, phosphatases, with their associated targets and phospho-binding proteins, make up the majority of phosphosignaling networks [18]. The known targets of phosphorylation's include various constituents of the cell such as enzymes, structural proteins, cell receptors, ion channels and signaling molecules. For example, the human genome consists of around 568 protein kinases and 156 protein phosphatases that regulate phosphorylation events and thus, plays a key part in biological processes including proliferation, differentiation and cellular death. The kinases may be activated or deactivated in an array of ways, including cis-phosphorylation/autophosphorylation of the kinases, interaction with activator or inhibitor proteins, or observing their localization inside the cells with respect to their substrates [19]. For example, when the external stimulus/ligand binds to an integral membrane protein having kinase action, the signal transduction of phosphorylation pathways are activated and phosphorylates different cytosolic proteins, this cascade prolongs until the particular cellular action is attained [20]. The mutations in the genetic material lead to uncontrolled structural changes in the structures of phosphorylated proteins that involves different signaling pathways and may be responsible for different pathological conditions [1]. Protein phosphorylation is needed for plant cells to recognize as well as respond to different external signals by kinase receptors to various targets. MAPK and PI3K/AKT/mTOR are two of the most critical phosphorylation pathways in higher plants and animals respectively, that play a significant part in the direction of signal transduction and biological processes [21]. Cell survival and proliferation are aided by the PI3K/protein

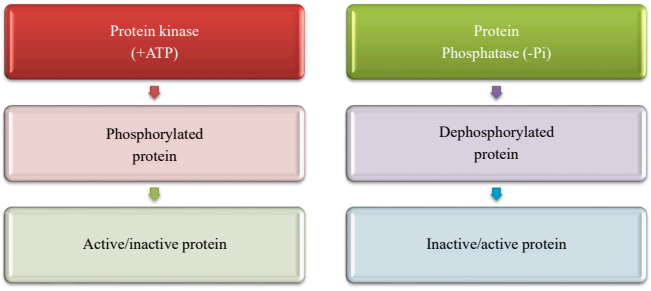

Fig. 2. Phosphorylation/Dephosphorylation activation leads to activation/deactivation of different proteins.

kinase B (PI3K/Akt) pathway. This activates downstream kinases such as mTOR, p70 ribosomal protein S6 kinase 1 (p70S6K1), and 4E-binding protein 1 when the PI3K/Akt pathway is stimulated [10]. MAPK cascades are one of the most frequent pathways for governing cell activity, and they are found in all eukaryotes including plants, fungi, and mammals [22]. MAPKs are present in the cytoplasm and the nucleus where they function in a variety of cellular activities, such as growth, development as well as in stress responses. They are crucial in the transmission of environmental and developmental cues in plants [23–25]. Earlier reports have also confirmed the involvement of phosphorylated proteins in the plant differentiation and development. The protein kinases are responsible for the mechanism of phosphorylation and regulate various processes such as metabolism and different hormonal responses [26]. Differentially expressed, post-translational phosphoproteins were identified from oil palm tissues at various stages such as embryogenic callus, somatic embryo maturation (globular, torpedo and cotyledonary stages) and plantlet stages by LC-MS/MS. A total of 460 proteins were identified which were found to be similar to phosphorylated proteins identified (starch synthase 3, chloroplastic/amyloplastic, cellulose synthase-like protein D1, fructokinase-6, chloroplastic, and the E3 ubiquitin-protein ligase At1g63170-like isoform X2) in the geminated rice embryos and are responsible for metabolism of carbohydrates, protein synthesis or degradation [27]. Various phosphoproteins which were early identified during seed germination in *Arabidopsis*, rapeseed and soybean like GATA transcription factor 26 isoform X2, acyl-CoA-binding domain-containing protein 5-like I soform X1, and mitogen-activated protein kinase YodA-like, are important in the biological mechanisms mostly during embryo germination, seed development [28]. Moreover, phosphoproteins that are involved in the somatic embryogenesis such as transcription factor bHLH68-like, transcription factor bHLH113-like, ethylene-responsive transcription factor LEP-like, and somatic embryogenesis receptor kinase 2-like plays important role in various biological processes, molecular functions and in different cellular components [29]. The proteins identified on the basis of different molecular functions were found higher ratios and were differentiate in proteins, nucleotide, and ion binding, transferase, and kinases. Similarly, the proteins reported that were involved in the cellular components were having highest percentages as membranes, nucleus and the cytoplasm.

3. Plant phosphoproteomics: understanding the technical intercession to phosphoproteins

Phosphoproteomics analysis was often done through various approaches to characterize very complex set of phosphoproteins across sub-cellular compartments and organelles [30]. The characterization of the phosphoproteomic dynamicity to various environment constraints is an effective approach to know and describe a wide range of plant resistance processes. Phosphoproteomics works in identifying phosphorylated proteins, quantifying specific sites of phosphorylation, linking these sites to particular kinases, which are important to biological processes. Although with modern techniques, it is possible to gain knowledge about plant phosphoproteome, still less is known about various protein kinases and the factors, which mediate their specificity in plants as compared to mammals. The need for the recognition of targets for protein kinases to find signaling pathways led to the development of new methods [31]. Majority of plant protein kinases are discovered to take part in crucial functions in stress responses such as salinity, low temperature and pathogen stress. Understanding biochemical reactions that occur during stress reactions can help us to have better insights into the plant biological processes [32,33]. In plants, the main consideration is to identify the particular proteins for phosphorylation and the signaling pathways regulated by such modifications. An effective phosphoproteomic analysis requires the selective enrichment of phosphopeptides, proper peptide identification and quantification detailed analysis of phosphorylation spots. Recent progresses in these fields have been thoroughly examined. The majority of the innovations were initially identified in animal and yeast systems before being introduced to plants [6,30–32]. The kinases cause covalent additions of a phosphate group to serine, threonine, and tyrosine residues in eukaryotes and other amino acids mainly histidine, aspartate, glutamate, lysine, arginine, and cysteine in prokaryotes. This is followed by the removal of the phosphate groups by protein phosphates, which are part of important signaling and regulatory mechanisms. Reversible protein phosphorylation controls a variety of cellular functions, including transmembrane signaling, intracellular signal amplification, and cell cycle regulation [6]. Protein kinases compose of approximately 5.5% of the *Arabidopsis* genome, which is about twice as of human genome. This indicates that phosphorylation events in plants have a high level of precision and are organized in complex networks [37]. The advancements in quantitative phosphoproteomics work in tantum with plant research (Table 1). The growing available phosphoproteomic data worldwide about various species and kingdoms will lead researchers to carry out trans species or cross species studies for characterization of phosphorylation conserved signatures as well as evolutionary adaptations through phosphorylation in studied species. Protein phosphorylation and its biological significance have been studied using a mixture of phosphoprotein/phosphopeptide enrichment methods and scientific advancements in tandem mass spectroscopy. Through the observations of the kinases in the levels of the responses, phosphoprotein enrichment and mass spectrometry techniques can be employed in the finding of substrates in the phosphorylation states. The most commonly used phosphopeptide enrichment system make use of the affinity binding between negatively charged phosphate and positively charged metal ions [34]. Solid Cation Exchange (SCX) is mostly used in tandem with Immobilized Metal Affinity Chromatography (IMAC) for enrichment of phosphopeptides in two steps. SCX-IMAC was used for the first time in plants, and 283 phosphopeptides were identified. Polymer-based Metal-ion Affinity Capture (PolyMAC), a variation of IMAC, has demonstrated high selectivity [38]. Phosphopeptide enrichment using metal dioxides, particularly titanium dioxides (TiO_2) and zirconium dioxides (ZrO_2) is becoming more common. The number of phosphopeptides in biological samples can be greatly improved by serial enrichment with TiO_2 and ZrO_2 [38]. Similarly, protein microarray technologies (antibody microarrays; reverse protein microarrays) can be utilized to detect the phosphoproteins from different complex plant protein samples by using large-scale HT arrays or small-scale customized techniques to detect and quantify numerous PTMs [39].

4. Biosensors and their critical role in phosphoproteomic studies

Biosensors are bioanalytical tools designed to detect analytes quantitatively in a sensitive manner that have broad series of advantages ranging from drug discovery, medical diagnostics, food analysis, agricultural and environmental monitoring, security and defense analysis. The detection of biomolecules, which are either disease markers or drug targets, is one of the major applications of biosensors. Electrochemical biosensors for instance, are useful in identifying protein cancer biomarkers in clinical settings [40,41]. Food analysis, stability, protection and nutritional values can also be analyzed using biosensors [42]. The research area has progressed tremendously from its beginning with clark electrodes to modern-day biosensors. A biosensor works by combining a biological element (antibodies, enzymes, nucleic acids or aptamers), immobilized on a transducer, and connected to a detector to detect the existence of one or more particular analytes, their concentrations and kinetics in a sample [43]. Specificity and selectivity of biosensor is resulted by the catalytic or affinity abilities of the biological element. The signal that originates from the interface between the target analyte and the biological element/sample is converted by a transducer to optical or electrical outputs (Fig. 3). Biosensors are constructive, resolute, precise, cost efficient, and simple to use in contrast to other usual

Table 1
Representation of plant phosphoproteomic studies.

Sample Type	Plant	Proteomic Approach	Method	No. of phosphoproteins	Reference(s)
Cadium Stress	Rice	Total protein	None	86 phosphoproteins	[122]
Cadium Stress	Rice	Total protein	iTRAQ labeling; TiO_2 enrichment and LC-MS/MS	244 proteins	[123]
Developing anthers	Rice	Total protein	TiO_2 MOAC and Nano UHPLC-MS/MS	203 phosphoproteins	[124]
Pistils	Rice	Total protein;	Fe^{3+}-IMAC protein enrichment; HILIC and Nano-LC-MS/MS	588 phosphoproteins	[125]
Brassinosteroid treatment	Rice	Total protein;	TiO_2 MOAC and LC-MS/MS	900 phosphoproteins	[126]
Seeds	Wheat	Total Protein	2-DE, Pro-Q Diamond	14phosphopeptides	[127]
Seedling	Arabidopsis	Total Protein	TiO_2	429 phosphoproteins	[128]
Leaves	Tomato	Total Protein	polyMAC	5471phosphoproteins	[129]
Roots	Arabidopsis	Total Protein	IMAC and TiO_2	2260phosphopeptides	[130]
Microsome	Arabidopsis	Total Protein	TiO_2	1229 phosphopeptides	[33]
Leaves	Cotton	Total Protein	TiO_2	1315 phospeptides	[131]

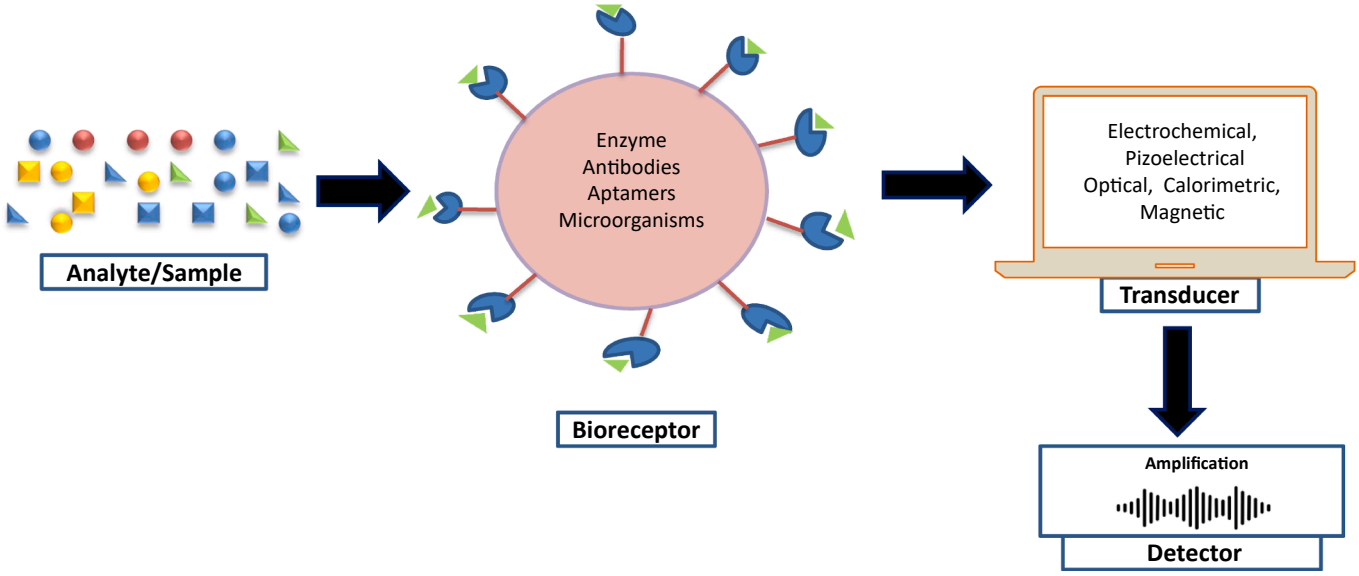

Fig. 3. Working principle of biosensor and its components.

laboratory detection practices because of their portable, reusable, real-time responses, highly specific and selective nature [43]. The biological recognition elements are selected depending on the targets of interest (such as antibodies and aptamers that are preferred for the revealing of bacteria or pathogens, while as enzymes for catalytic results). The immobilization of biological analytes occurs in various ways mainly through adsorption, covalent binding, entrapment and membrane confinement.

4.1. Understanding biosensors

The basic fundamental components of biosensors are a biological element and a sensor element which helps in generating signals accurately to the concentration of an analyte in a reaction to interpret biological or chemical reactions. The first biosensor was an enzyme-based electrode for the quantification of glucose in the sample. Currently remarkable research is done to make the laboratories stable economically for the development of nano size and multiple function biosensors [44]. Nano-materials are mostly applied in biosensors to provide efficient transfer of charged ions between electrode and target, improved electrocatalytic actions, rapid diffusion of the analyte, pre-concentration of analyte molecules at the electrode surface, and anti-fouling capabilities [42,45,46]. Such biosensors are all designed to identify a little quantity of analyte in a complex biological matrix with a tiny sample concentration and no fouling. Several approaches are being employed to maintain the biosensor stable and usable for a longer period of time [45]. The sensor element of the biosensor consists of signal transducing part in the form of electrochemical, colorimetric, magnetic or optical outputs for the selection of different analytes as the important components of the biosensors, the advantages being the quick response abilities, accuracy, portability, reproducibility and stability. The important requirements that must be considered, while designing the highly efficient biosensors are: a) analyte's immobilization should be in indigenous configuration, b) higher convenience of the receptor sites to the specific analytes and c) effective adsorption of the analytes with the surface in use [43]. These requirements are keenly addressed while designing the biosensors.

The effective immobilization of the biological element over the sensor involves various mechanisms including a) physical adsorption, b) self–assembled monolayer (SAM) and c) covalent avidin-biotinylated complex binding. In physical adsorption mechanism coupling occurs *via* hydrophobic forces, Vander Waal's interactions, and ionic interactions for binding between the biological element and sensor. This strategy has been employed to develop enzymatic biosensors [47]. When the biotinylated antibody (Ab) is immobilized on the transducer, it forms Avidin-biotin Ab analyte complex. If the transducer has monolayer assembled and the biological element is attached to the sensor, such type is known as SAM immobilization mechanism (Fig. 4). SAM biosensor was developed for tuberculosis by Costa and co-workers [48]. They developed the electrochemical geno-sensor of mercaptobenzoic acid and magnetite (Fe_3O_4) nanoparticles based on SAM for immobilization of a DNA probe on bare gold electrode. The limit of such geno-sensor was 6 ng/μL. Another genosensor for *Mycobacterium tuberculosis* was developed using Surface Plasmon Resonance (SPR) [49].

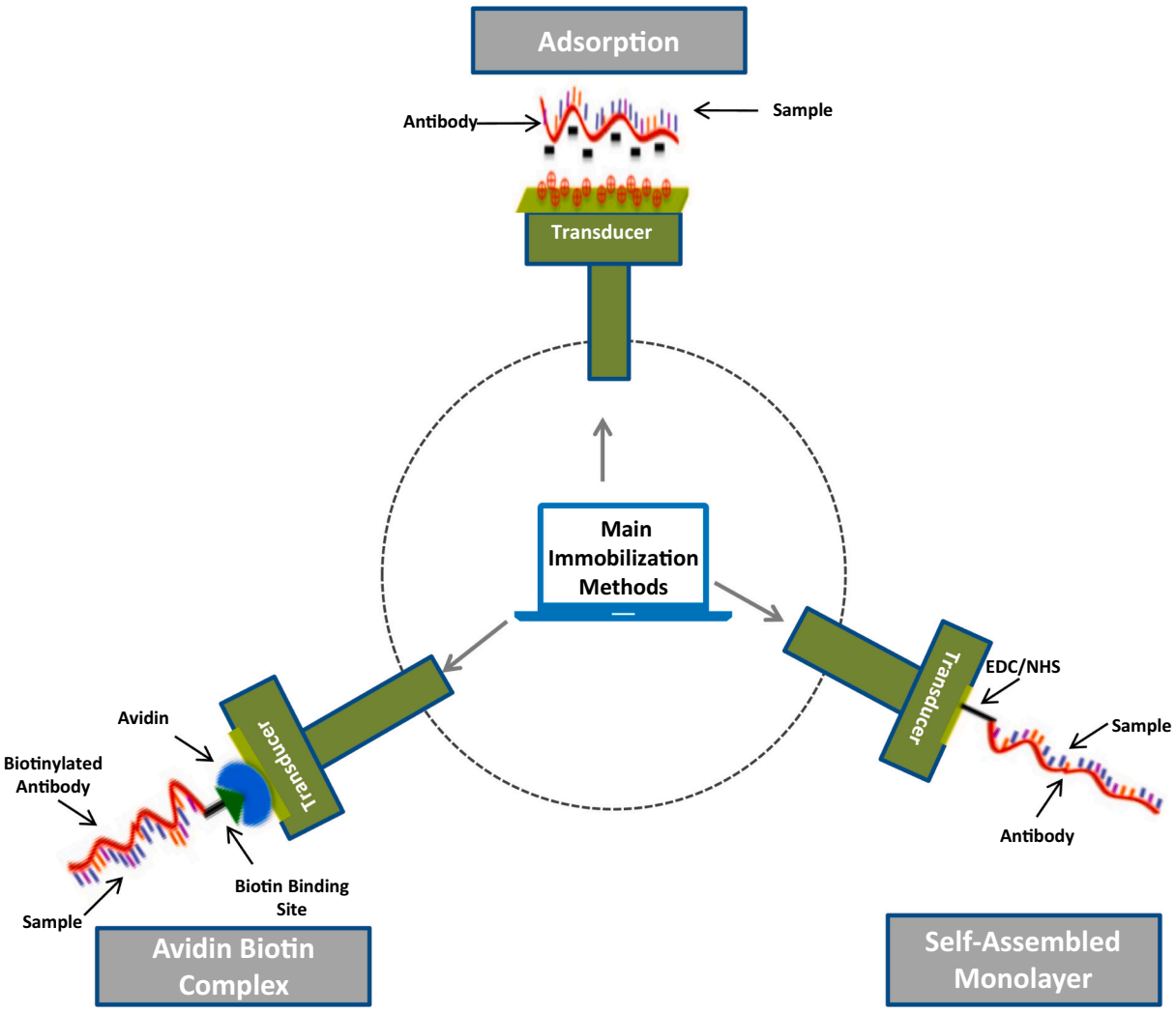

Fig. 4. Different types of immobilization of transducer and the sample.

Biosensors are grouped into various types based on the type of transducer applied or the biological specificity conferring mechanisms (Table 2). The transducer transforms the biomolecule-analyte interaction into quantifiable optical or electrical signal readouts. The most popular biosensors are based on the type of transducer used: (a) electrochemical; (b) optical (including colorimetric); (c) mass based and (d) thermal and energy based. On the basis of biological specificity or recognition, biosensors are classified into five categories depending on the biological recognition element: enzymatic, protein receptor-based, immunosensors, DNA biosensors and whole-cell biosensors [50–53]. The perfect biosensor is highly responsive, selective with a constructive signal to noise ratio, that gives a quantitative response curve over the relative concentration of analyte, a spatially and time resolved readings *in vivo* without disturbing the internal responses of the system, therefore provides the information that is advanced over the other existing approaches.

Table 2
Main classification of biosensors.

Classification	Application (s)	Principle of Operation	Reference (s)
Electrochemical based biosensor	Biomembrane or lipid bilayer, antibodies, enzymes	Amperometric, conductometric, impedance, potentiometric	[132]
Optical based biosensor	Metallic nanoparticles, immunosensors, receptor, nucleic acid, liposome	Chemiluminiscence, fluorescence, surface palsmon resonance	[133,134]
Mass-based biosensor	Coupling, binding extension	Magnetoelectric, piezoelectric	[134]
Thermal and energy based biosensor	Amino acid oxidase, enzyme thermistor, metabolism, optical imaging	Thermometric, fluorescence resonance energy	[135–137]

5. Biosensors for identification of phosphoprotein

Various tools have primarily been utilized for the analysis of structural changes that occurs with different protein modifications (primarily due to phosphorylation). Some of these are described below along with their pros and cons.

5.1. Mass spectrometry

Mass spectrometry (MS) is an analytical tool, which measures the mass to charge ratio (m/z) of gas phase ions and is a vital tool for proteomics research for characterizing proteomes, to develop new biomarkers and to understand the cell and molecular regulator systems. MS is the primary bioanalytical approach for a thorough investigation of post-translation protein changes which is necessary for the dynamic

phosphoproteome profiling and quantification [54]. Various ionization methods are applied for the quantifying of phosphopeptides and phosphoproteins by MS approach. They include Fast Atom Bombardment (FAB), Electro Spray Ionization (ESI), Plasma Desorption and Matrix Assisted Laser Desorption Ionization (MALDI). The ESI-MS and MALDI time-of-flight (TOF) have enhanced the sensitivity/specificity of the mass detection of peptides at the femto-mole level and addition of phosphate to an amino acid can be determined by sequencing the peptide using Collision Induced Dissociation Mass Spectrometry (CID-MS). In recent decades, MALDI-TOF/TOF method has been widely used to identify proteins that are responsible to increase the tolerance of plants under different kinds of abiotic stresses such as salinity, moisture and water-deficit stresses [55–57]. The mass spectrometric method is therefore, directional, quick and potential method for phosphoprotein analysis. Rice phosphoproteomic experiments have made significant advances in phosphoproteomics strategies such as phosphoprotein recognition, phoshopeptide enrichment, MS based characterization, and phosphorylation quantification over the last two decades and thus providing tremendous opportunities to classify phosphoproteins and phosphosites [35,58]. In biological systems, two-dimensional gel electrophoresis (2DE) is one of the flexible and commonly applied technique for proteome studies and phosphorylation changes. It generates a graphic design of all proteins which allows for directional detection of possible variations in protein isoforms and PTMs that were not expected by gene analysis [59]. Moreover, thousands of proteins are detected from a complex mixtures by using isoelectric focusing (IEF) and SDS-polyacrylamide gel electrophoresis [59]. The reports of tyrosine phosphorylated (pY) plant proteins are becoming progressively more widespread, and there have been several efforts to explain the pY-proteomics of various organisms. The application of targeted mass spectrometry techniques has been promising in identification and quantification of pY. Targeted techniques such as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) are two of the most important techniques for the analysis of particular phosphosites. In these approaches, a triple quadrupole instrument is necessary to detect the specific precursors and product ions charge by mass ratio values of the target peptides for the quantification of phosphorylated and unphosphorylated. Parallel Reaction Monitoring (PRM) is an important approach related to the single and multiple reaction monitoring and is recently employed to identify, separate and detect the particular precursors and product ions of target protein. This approach is now more specific than Orbitrap because the later is used for high-resolution mass separation and the identification of low-resolution electron multiplier. In addition to this, PRM is extremely responsive and fast as it enables the identification of whole fragment ions in single scan for the target precursor ions in comparison to the selected reaction monitoring or multiple reaction monitoring that characteristically identifies fewer fragment ions in a sequence. The method is more helpful in identifying low abundance phosphopeptides due to its higher mass accuracy as enable the different phosphosites confidently. Synthetic heavy isotope-labeled standards such as absolute quantification (AQUA) of peptides can be coupled to these targeted approaches, and enables absolute quantitation [60]. Despite the fact that experimental methods for MS-based phosphoproteomics data acquirement have advanced appreciably in recent years, resulting in a steadily increasing number of visible phosphosites. There are still a number of drawbacks for investigating signaling mechanisms and phosphopeptides using phosphoproteomics data which ultimately paved way for other advanced biotechnological approaches to follow. Some of these limitations include the lack of observed phosphopeptides as strong/ sharp peaks particularly in the presence of non-phosphorylated proteins due to ionic suppression and the binding efficiency of phoshopeptide to columns because of hydrophilic nature. Another difficulty associated with them is the production of too small or too large peptide fragments as these are not observed in mass spectrum. Phosphopetides are negatively charged and thus electrospray becomes difficult as it is performed in positive mode. Recognition of phosphorylated serine, threonine or tyrosine residues using MS is not generally possible. Phosphoserine and phosphothreonine residues are labile (for example, β-elimination), while phosphotyrosine residues are comparatively extra stable. This necessarily leads to diverse MS techniques for the analysis [61].

5.2. Immuno-based tools

Standard *in situ* protein detection techniques for immune-fluorescence and immune-histochemistry include the use of only one primary protein antibody. However, phosphospecific antibodies typically detect the nearly similar phosphorylated epitopes of the targets. The generation of protein phosphorylation specific antibodies (PSSAs) made *in-situ* study of phosphoproteins possible leading to glimpses of various protein phosphorylation reactions in different complex cellular structures as well as tissues and high exact identification of phosphorylated proteins using Duolink (that works on the principle of *in situ* proximity ligation assay). *In situ* proximity ligation assay provides a strong yet easy way to quantitatively observe post-translation changes, such as phosphorylation of proteins with outstanding specificity and sensitivity in fixed cells and tissue by employing Duolink-components. Moreover, covalently transformed proteins may as well be detected *in vivo* using the Duolink dual recognition format [62]. Monoclonal antibodies raised against neurofilament proteins became the first PSSAs. Their identification as PSSAs was an unexpected finding. The PSSAs generated using synthetic tyrosine-containing phosphopeptides were also used for observing the activation of erbB-2 [61]. PSSAs for almost 300 types of phosphoproteins as well as phosphorylation sites are now commercially available. About 90% of phosphorylation in proteins occurs at the amino acids mostly in serine and threonine. Therefore, it is necessary to identify and quantify such sites. Site-specific phosphorylated amino acid antibodies can be helpful in quantifying such signaling transductions and mapping the phosphorylation sites. Characterization of such sites through chemical modifications or mass spectroscopy in the very early developmental stages is important as well as promising. The antibody that is to be used is predicted and identified with bioinformatics data such as algorithm Scan-site that establishes the ordered data of proteins associated with anticipation of antibody cross reactivities. Understanding the epitope sequences will allow the formation of specific matrix to predict the sites where the phosphorylated protein will interact with the specific antibody. The main benefit of using this method is its ability to generate antibodies that distinguishes sub-group kinase substrates or the sites of modifications such as methylation, acetylation and proteolytic cleavages [61]. This analysis is an advantageous upon the use of amino acids that may be modified and used as immunogens, because using the modified amino acids in the perspective of varied peptides tends to select the antibodies, which are widely reactive. Such incorporation provided increased contact surface and binding energy of antibody-antigen interactions for higher affinity. These two effects generate antibodies that focus on constant residues and are widely resistant at various positions. The strength of immune-based tools lies in their higher sensitivity, multiplex capabilities, quantitative outputs while as the weakness of these techniques are long-drawn-out antibody generation, antibody cross-reactions, inadequate accessibility of phosphorous-specific antibody, requirement for exterior functionalization [61].

5.3. Nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) spectroscopy is another approach used to detect protein structure at micro levels mainly in the solution phase. The mechanism of NMR was first of all described independently by Bloch; Purcell and coworkers [63,64]. The principle of NMR is that the nucleus of atoms is composed of protons and neutrons (except hydrogen nucleus, consists of proton only). When a couple of protons or neutrons exist in the nucleus, they are aligned in a way so that

their spins are terminated out. Therefore, a net spin subsists for a nucleus alone if it has an unpaired/odd proton or neutrons or both and alone such nuclei are NMR reactive or observant. Several nuclei of biological interest are accessible for study, mainly hydrogen (H), phosphorus (P), sodium (Na) and carbon (C). The first in-cell NMR principle was explained and demonstrated by Serber and Co-workers [65] in *Escherichia coli* cells, where the name "in-cell NMR" was applied to explain high resolution NMR to acquire structural information of a macromolecule. For example, a protein within living cells as to "*in vivo* NMR" which refers to the assessment of small molecules inside the living organisms. *In-vivo* NMR is normally connected with lower sensitivity and loss of signal and the requisite protein amount is considerably higher than the endogenous levels [61,66]. The organization of the Tau protein and functions were investigated with high resolution NMR-spectroscopy. The NMR analytical process allowed Tau phosphorylation sites to be identified, all modifications were visualized in a particular experiment and the degree of the incorporation of phosphates was also quantified [67]. The functional protein kinases have been studied using NMR. However, these investigations have found that conformational changes and dynamics in the regulatory function of kinases play a crucial role [68].

5.4. Electron microscopy

Electrons are also utilized to detect protein structural changes. Proteins scatter electrons approximately ten thousand times more powerfully than X-rays and these electrons are accelerated inside electric fields quite a hundred thousand volts to wavelengths which are shorter than the distance between the atoms in protein structure [69,70]. The addition of cryo-technology with electron microscopy (EM) leads to precise structural study of bigger membrane phosphoproteins. Such EM helps in studying conformational changes and interactions, which are mostly short-lived and complex to separate and steady in their biochemistry. Cryo-EM is used to determine the ribosome configuration to obtain the knowledge on the protein biosynthesis and its regulation, spliceosome, photosystem II–light harvesting super complex [71] and ATP synthases [72,73], the exosome [74], chaperone heat-shock protein [75] and the serine/threonine protein kinase mTOR. It also helps in studying the rearrangement of protein subunits and binding to diverse partners and substrates throughout their gathering and functional cycles. The disadvantage of EM technique is the time required for the generation of samples, difficult to obtain images of tilted samples, very low noise to signal ratio and requirement of high-vacuum environment [76].

5.5. X-ray crystallography

X-ray crystallography (XRC) is an experimental detection of the atomic and molecular composition of a protein, in which the crystal configuration causes incident radiations to be scattered into several directions. By observing the angles and intensities of these diffracted rays, a crystallographer can generate 3D images of the number of electrons inside the protein crystalline configuration. Therefore, micro-level analysis of structural characteristics for large number of proteins is promising [77]. The discovery of X-ray crystallography almost 100 years before by Von Laue has led to the foundation for imaging 3D crystal structures. In biological sciences, various structures such as entire cellular structure, viral structures, structure of organelles and various necessary protein species are not able to crystallize and therefore, such structures are not viewed through X-ray crystallography. To overcome these limitations, other approaches such as nuclear magnetic resonance (NMR) or crystallography electron microscopy can be used [78]. The two processes of X-ray diffraction microscopy are constantly under development in which first is based upon the X-ray diffraction [79] and second is Bragg's diffraction by the crystalline sample structure to identify the shape and strain effect in the nanocrystal []. The disadvantages of XRC are that it is restricted to solid samples only, not favorable for complex physiological systems, requires large quantity of sample, extended investigation time period, harmful to sample because of radiations and phase troubles all through the investigation which leads to small signal to noise fractions [1].

6. Emerging biosensor tools for phosphoproteome analysis

Consequently, it is necessary to understand the changes in protein structure through new techniques and approaches. Thus, it is important to gain a deeper understanding of how new advanced biosensors can identify protein structural changes. In the past three decades, considerable attention has been devoted to biosensor research to be used for numerous purposes with remarkable progress. Based on publications on biosensors and phosphoproteome detection using the same in public domain by using specific keyword-based search on PubMed (https://pubmed.ncbi.nlm.nih.gov); an increased trend in research is being observed (Fig. 5). The different biosensor tools that are utilized includes Foster or Fluorescence Resonance Energy Transfer (FRET) based, Nanobody based, Enrichment based, Kinase Activity based and Translocation based biosensors (Fig. 6).

6.1. Forster or fluorescence based biosensors

Fluorescent technology offers great sensitivity, flexible design, great performance and autonomous operation. It recently emerged as a unique identifier for studies linked to protein kinases. These biosensors have been established to make possible the live-cell revelation of protein configurational changes caused by phosphorylation. Long-range dipole–dipole interactions are used in fluorescence resonance energy transfer (FRET) biosensors to pass ATPs from a donor fluorophore to the recipient fluorophore in a non-radiative fashion through vanderwaal's interactions and a genetic switch with a fluorescent protein duo capable for FRET which enables observation of the conformation of the switch by the alteration in fluorescence [81,82]. Fluorescence biosensors are engineered for enabling real time observation of protein conformational changes essentially because of phosphorylation. To identify structural modifications associated with an enzyme that causes phosphorylation, a fluorescence-based technique was designed for *in vitro* and *in vivo* investigation of modifications of aurora kinase A (AURKA) which is the most important protein kinase feature of different abnormal cell divisions and the process of commencement through the cell cycle [1]. The two fluorophores-donors enhanced GFP (EGFP) and acceptor-mCherry/

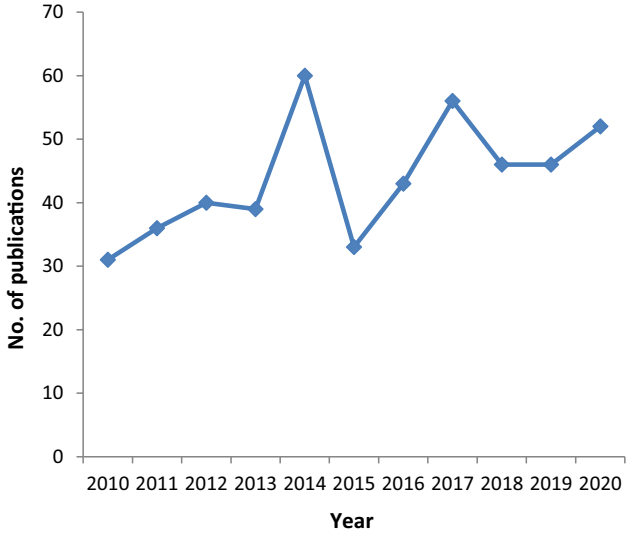

Fig. 5. Number of publications using phosphorylated biosensors retrieved from PUBMED (protein and phosphorylated biosensors). Consulted in May 2021.

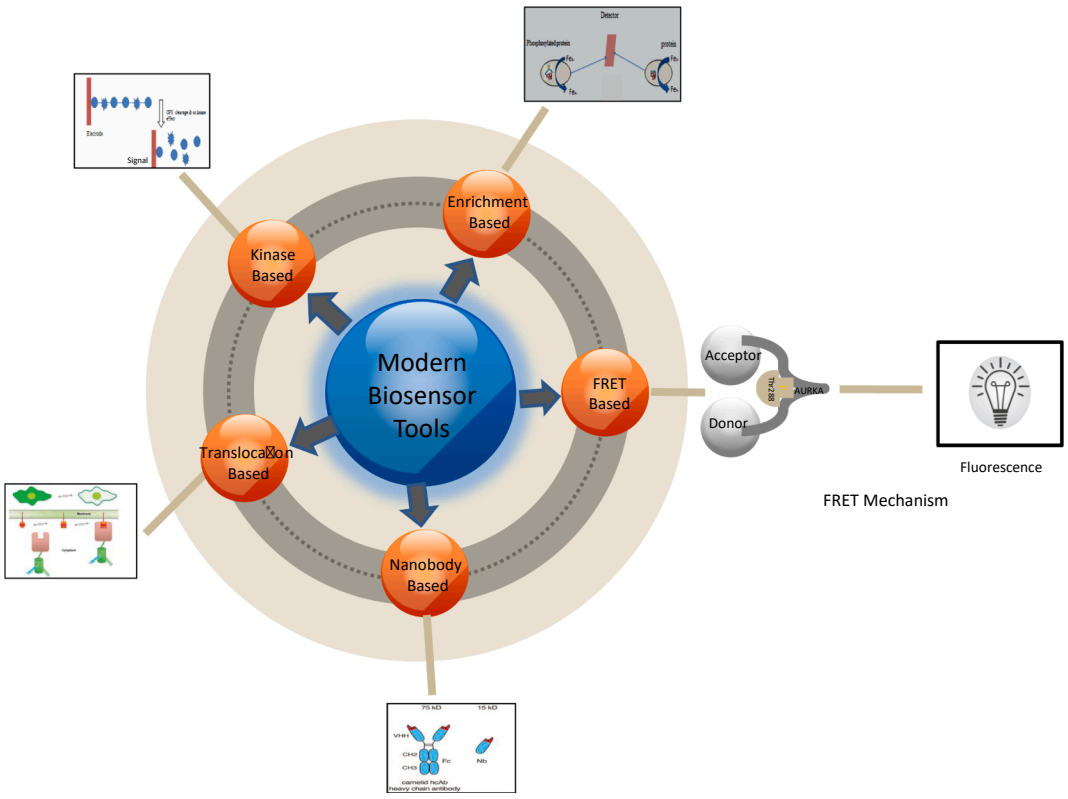

Fig. 6. Major types of modern biosensor tools for phosphoproteome studies with their general mechanism.

Red fluorescence protein (monomeric RFP), were labeled at the -NH_3 and -COOH ends respectively to the protein-AURKA. When AURKA is phosphorylated at the amino acid (Thr288), the catalytic sub-unit experiences alterations, the donor and acceptor fluorophores come in close proximity and fluorescence is observed therefore, helps in FRET identification (Fig. 6). The change in fluorescence efficiencies defines the distances between the two fluorophore pairs (donor and acceptor fluorophores) and is related to the levels of phosphorylation induced conformational changes in protein AURKA. Therefore, Fluorescence resonance energy transfer efficiencies offer promising knowledge about active modifications of AURKA structure and its functions [1]. FRET is distance and orientation dependent, along with fast drop in effectiveness at nano-scale spaces smaller than the scatter limit of light and is a functional biophysical property for sense proximity on a nanoscale to be visualized as co-localization. The common design for a kinase/s-messenger biosensor involves a sensor unit, which sense the targeted changes and a reporter unit to observe the sensor unit's state. This high throughput fluorescence-based biosensor approach is currently applied and is verified for large number of other proteins in plants. In such approaches various tags such as CLIP (20 kDa), Halotags (33 kDa) were covalently bound with both donor and acceptor fluorescent pairs to bring them into considerable proximities (less than or equal to 10 nm) which is favorable for FRET measurements when specifically attached with the target proteins resulting in higher contrast and higher temporal resolution imaging [83]. Wang and Co-workers in 2015, designed one quantum dot (QD) based nanosensor. On the surface of streptavidin-functionalized QDs, phosphorylated and biotinylated peptides were assembled in its design after catalyzing with protein kinase which yield FRET signal formed between QDs and peptide [25].

6.2. *Nanobody based biosensors*

Protein kinase assays have made substantial development from the last few decades. A variety of techniques for protein kinase action analysis and inhibitor screening have been published to date, including radiometric, colorimetric, electrochemical, fluorescent, and other techniques. New intriguing results in creating protein kinase biosensors with improved precision, specificity, and sensitivity have recently been achievable due to various types of nano-materials, therefore, thankful to the advent of nanotechnology. These are used in a variety of applications because of their unique physical structures and exceptional optical, electrical, catalytic, and magnetic capabilities [40,84–86]. Nanobodies are defined as the small (12-15 kDa) recombinant, antigen specific and invariable single-domain fragments having the fully potential antigen-binding capacity of the original heavy chain–only antibodies that naturally occur in Camelids [87,88]. Nanobodies are encoded therefore by single exon and these single-domain antibodies can be used as biosensors to track conformational properties of their targets inside a living cell [89]. Nanobodies, unlike typical antibodies, can penetrate the small clefts and voids of protein molecules (such as kinase active sites) allowing them to detect small structure changes with greater sensitivity. Nanobodies also have a higher affinity for target antigen epitopes, a short half-life, and are less toxic to cells under physiological conditions [90]. The specific and selective nanobodies should be for specific target proteins, which is necessary for expanding various biosensor applications. The various present techniques such as chromatography or surface plasmon resonance are not useful in the purpose of binding interactions between nanobodies and target protein. For solving these limitations, an engineered yeast-display nanobody library (containing at-least 13,108 unique full-length nanobody clones) was just now formed in which the identification process involves incubation of antigens along with fluorescent tag and a newly formed yeast library [91]. The structurally specific nanobody was identified by analyzing their binding abilities with specific antigens such as applied in G-protein coupled receptors (GPCR) by utilizing magnetic activated cell sorting (MACS) or fluorescence activated cell sorting (FACS) [1]. As the cytoplasm of the cells is reducing in nature and therefore, these nanobodies possess limited disulfide bonds which make them stable in such environment [92]. The

application of nanobody-based biosensors in different experiments, has also established the role of endocytosis on the signaling by the receptors and in the endosomal signaling pathways. Using such biosensors has been also helpful in understanding the receptor activation and their signal transduction such as in plasma membrane, endoplasmic reticulum, golgi bodies and other compartments. A nanobody biosensor (Nb37-GFP) was employed for recognition of Gαs (a guanine nucleotide free form) from GTP for different substrate and hormones that involves phosphorylation cascades in endosomes [93]. Nanobody binding to particular sites of the proteins was found to be affecting the structures of that targeted protein [94]. The selection of bound clones was done and then increased in copy number in the typical yeast culture media. The small nanobodies are also applicable in studying permeability characteristics of cells to analyze protein conformational changes within the living cells. Lin and Co-workers [87] detailed the essential phosphor-recognition processes while developing protein kinase detection techniques. These include the phospho-specific recognizing proteins, metal chelation, ATP analog labels, phosphorylation product measurement, and many more. Metal chelation (*e.g.*, Zr^{4+}, Zn^{2+}, Ti^{4+}, and Cu^{2+}) is the most common method for phosphorus recognition. However, these methods are linked with different limitations, which include recognition, efficiency and stability issues. Therefore, research and development of nanobodies will be advantageous in fulfilling the various expectations of nanobody, based biosensors. Furthermore, the self-assembly of alkyl phosphates on the surfaces of metal oxide nanoparticles mostly SnO_2, TiO_2, Al_3O_4, ZrO_2, and ZnO has been demonstrated. As a result, these metal oxide nanoparticles may be used to concentrate phosphopeptides [95].

6.3. Enrichment based biosensors

The enrichment strategies are mainly done either at the levels of phosphoproteins or at the levels of phosphopeptides. Most enrichment strategies involve chemical modifications, affinity chromatography (having affinities for peptides or proteins, which contain negatively charged phosphate groups on a positively charged matrix) or immunoprecipitation by utilizing phospho-specific antibodies. The phosphopeptide enrichment strategies start with protein extraction. As the total protein extracts from a given tissue is generally complex, therefore, multi-step protein extraction is important leading to higher proteome fractions (such as three fraction protein extraction applied for *Arabidopsis thaliana* pollen). In the first step of protein extraction, a Tris-based extraction buffer is used to get salt-soluble fractions and with different steps, protein is extracted. The peptide mixture, including modified peptides (for example phosphorylated and glycosylated ones) is enriched for phosphor-peptides. The enhanced or enriched fraction is consequently MS-analyzed. A second purification step utilizes different selection matrices, such as Fe^{3+} IMAC (Immobilized Metal ion Affinity Chromatography) or TiO_2-MOAC (Metal Oxide Affinity Chromatography) for removing the left over non-phosphorylated peptides earlier to Mass Spectrometry analysis. Also Zr-IMAC magnetic microparticles is used in the phosphoproteomic enrichment processes and performs at the same, if not better, than the IMAC and MOAC procedures [96]. A recent study found, single-stranded (ss) DNA aptamers to specifically bound to phosphorylated peptide epitopes of Tau protein as an alternative to antibodies using the enrichment method [97]. A method based on protein chip immunosensor was developed for increased sensitivity for detection of *Listeria spp.* It provides 10 fold increase in sensitivity compared to bacteria immunosensing coupled to FRET detection [98]. Traditional aggregation of nano-particles has been used as enrichment substrate and indicators to various analytes of agriculture and food samples like proteins, cells, nucleic acids and metal ions due to its ease of modification, simplicity of operation, and high applicability [99–101]. On the basis of pre-concentration principle, various enrichment methods such as material-based enrichment techniques, electric-based enrichment techniques [102], and bio-organism-based enrichment techniques [103] are proposed to pre-concentrate low-abundant analytes [104]. The biosensor incorporated enrichment method (where low-abundant samples are incorporated with the biosensor investigative devices) is an established general approach to enhance the sensitivity and precision of the analysis of phosphoproteins in the analyte. The first two techniques are used analytically because of greater utilization in the field of agriculture. In material based enrichment method the enriched samples are adsorbed or particularly attached to the sorbent [105]. There are three types of material-based enrichment approaches *viz*, magnetic beads, absorbent equipment and aggregated nanoparticle. Magnetic beads consist of magnetic material like iron oxides, oxides of cobalt, manganese *etc.* The most commonly used is iron oxide that acts as absorbent of biosensor integrated analysis because of its large surface area, cost effectiveness, modification friendly and greater efficiency. Mesoporous nanomaterials, which have 3Dstructures, huge surface area, and multiple nano-scale pore channels, are mostly used in the enrichment of phosphopeptides [106]. Ordered mesoporous carbon composites with metallic zirconia nanoparticles were produced and demonstrated with good hydrophilicity for phosphopeptides selective enrichment [85]. Functionalized metal organic frameworks (MOFs) have recently been studied for phosphopeptides specific enrichment [107,108]. Dual-functionalized MOFs, for instance, have been produced [109], which combines the affinities of immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC). MOAC was provided by the Zr—O cluster, whereas IMAC was provided by immobilized Ti^{4+}. For phoshopeptide, it demonstrated a strong binding capacity as well as excellent post-enrichment recovery [110].

6.4. Kinase activity based biosensors

Protein kinases regulate almost every aspect of cellular activity. These critical signaling nodes combine several route inputs to control complicated physiological processes and help in detecting various abnormalities. The fluorescence protein-based biosensor generations have reformed kinase signaling research to allow precise kinase action measurements inside living cells. Kinase action imaging is rapidly being utilized to decipher the genetic control of protein kinase A (PKA), an archetypal kinase which orchestrates several physiological processes by integrating many pathways [111]. The kinase-based biosensors depend on phosphorylation caused by kinase enzyme, which leads to the protein conformational changes and results in the formation of various signal readouts such as electrochemical or optical or magnetic outputs. The net phosphorylation levels are detected by measuring directly or by measuring kinase action during phosphorylation [1]. Wang and Co-workers in 2017, devised an easy and more sensitive electrochemical biosensor to detect phosphorylation activity of PKA in which positively charged electrochemical probe $[Ru(NH_3)5Cl]^{2+}$ is altered to contact the changed peptides or modified electrodes (because of compact formation) and self-assembly monolayer which is positively charged sample peptide [112]. When the phosphorylation was done, SAM was loosened and facilitated the probe permeability because of the interactions between the phosphate and probe. On that basis, 'on' biosensor signal was made-up and had achieved the purpose of tyrosine kinase activity assay which was based upon the changes of tyrosine residues earlier and later on phosphorylation of the residues. Another well-organized technique for examining protein kinase catalytic action and screen inhibitor is highly advantageous to kinase-related drug discovery, premature diseases identifications and remedial effects. A simple example of kinase catalyzed based biosensor is detection of the action of casein kinase II (CK2) based on phosphorylation modification against enzyme carboxypeptidase Y (CPY) digestion triggered signal amplifications, in which CK2 catalyzed phosphorylation event prevents the substrate peptides from the digestion of CPY and results in a weak electrochemical signal. While in absence of phosphorylation, the substrate peptide is cleaved by CPY and a strong electrochemical signal is attained [113]. A

new and easy electrochemical approach for kinase action assay and inhibitor selection was designed, based on phosphorylation protection against CPY digestion, where the $Fe(CN)_6^{3-/4-}$ were established as the electrochemical probe, and the peptide rich in negatively charged aspartic acid was utilized as substrate peptide [105]. A biosensor with good sensitivity for detecting kinase activity and degree of phosphorylation was developed using electrochemically mediated polymerization. This method depends on phosphorylating the substrate with the kinase of interest, followed by attaching polymerization initiator in the form of alkyl halide to the phosphorylated sites and then attachment of the electroactive polymer through electrochemically mediated atom transfer radical polymerization (eATRP) [114]. These kinase-based biosensors though being less expensive than current methods and independent of the need for phosphospecific antibodies, have many drawbacks, as these are limited to *in vitro* conditions only and these are usually substrate-specific and not suitable for common usage. Furthermore, kinase-based sensors are presently limited to the use of synthesized peptides as proof of concept, and their viability for application in real-sample analysis is still to be determined [1].

6.5. Translocation based biosensors

Kinase Translocator Reporters (KTRs) analogous to kinase-based biosensors are genetically encoded fluorescent reporters that transform kinase activity into a nucleo-cytoplasmic shuttling event. Translocation-based biosensors use spatial movement to detect protein structural change events. Localization changes are generally carried *via* phosphorylation in living cells, and thus KTRs exploits this process to assist kinase reporter development [115]. Transport bio-sensors (TB) may be applied for mapping and quantifying the activity of nuclear import and export signals (NLS/NES) [116], evaluate transport co-factors, and discover substances that interfere with nucleo-cytoplasmic transport [117]. During expression in eukaryotic cells, it frequently shuttles between the nucleus and the cytoplasm, but it preferentially localizes to the cytoplasm due to the different strengths of the transport signals (NES > NLS). In the expression constructs, NLS-/NES-sequences are interchangeable, allowing for quick assessment of potential transport signals. Briefly, a KTR is an engineered construct in which a kinase substrate is merged to a nuclear localization signal (NLS) and nuclear export signal (NES) either of which may be phosphorylatable as well as to a fluorescent protein to carry microscopy-based detection of its localization [115,118]. An unphosphorylated kinase translocator reporters enters the nucleus using its nuclear localizing signal (through bonding with importin). Inside the nucleus, the export signal being phosphorylated and experiences a structural change which allows the kinase translocator to break its bond with the importin and exit from the nucleus and form a fresh bonding to the exportin protein [118]. Therefore, the reporter is transferred inside and outside of the nucleus that mainly depends on structural modifications because of kinase action. These reporters have been promising for simultaneously measuring several forms of kinases (such as JNK and ERK) in cells by epifluorescence microscopy [1]. Protein translocation assays have the possibilities to be used for lead series analysis, chemical library primary screening, and even gene silencing studies. However, reliable and trustworthy biological readout systems with a higher signal-to-noise ratio are indispensable for any relevant applications of high-content and high-throughput cell-based assays [119]. Research observations have suggested that the efficiency of KTRs is more as compared to the fluorescence based reporters to identify the unphosphorylated states of the proteins [118]. In fluorescence based biosensors, the phosphorylated protein forms a compact configuration due to which, it is not available to dephosphorylated enzymes but in case of KTRs which easily and constantly change between these two forms of transport from nucleus to cytoplasm and *vice versa* as phosphorylation and dephosphorylation affects the transport of the protein *e.g.*, an element of MAPK protein family namely ERK5 (an extracellular signal regulated kinase 5) regulates the shuttling and localization of signaling cascade between nucleus and cytoplasm inside the cell. Such shuttling of ERK5 is CRM1 dependent and is found to be regulated by nuclear localization export process. NES as mentioned earlier is a small leucine-rich sequence of amino acids that interacts with the export receptor CRM1/Exportin1. These results suggested an important significance of dependence of control mechanism by phosphorylated protein shuttling. Therefore, the only purpose of kinase-based biosensors depends on the kinase activity that makes it more advantageous and purposeful than other biosensors and other existing tools for identification and characterization.

6.5.1. Protein structural changes induced by aberrant phosphoproteins

The conformation changes in the protein structure due to the outer or inner disturbances strongly affect the protein functions and therefore, effect cellular metabolism. An important regulator of phosphorylated serine or threonine to undergo dephosphorylation is Phosphoprotein Phosphatase-1 (PP1) which regulates number of signaling pathways inside cell. Such aberrant phosphoprotein enzyme has been reported to be involved in a variety of pathological conditions. The phosphorylated threonine which is not dephosphorylated due to the aberrant phosphatase Phosphoprotein inhibits the functions by making protein complexes instead of directly inhibiting the structures or substrate competitions [117]. Therefore, equilibrium between the protein kinases and phosphatases is important to regulate the various biological processes. An important cellular process -autophagy depends upon the phosphorylation and it was reported that the autophosphorylation of Atg1 protein is a “regulatory switch” which decides the beginning of the process [122]. A recent proteomics approach is recently developed known as Limited proteolysis-coupled mass spectrometry (LiP-MS) which identifies the complex conformation changes on proteomics wider scales. The extract of proteins of interest, undergo first nonspecific double enzyme digest step in their natural conditions which is followed by the complete digest with particular sequence specific enzyme treatment (trypsin) in denaturing environments. Such enzyme digestions create conformation specific peptides which are open to Mass spectroscopy studies. Subsequently, shotgun/targeted mass spectroscopy and unlabeled quantification is used to identify and measure structure/conformation dependent digested phosphopeptide sequences directly in the protein extracts. The various applications of LiP-MS involve the detection of induced protein conformational changes, drug target identification, identifications of disease-associated protein conformation changes along with the investigation of various aggregates straight from the biological samples [123]. Therefore, Mass spectroscopy approaches have been promising in the identification of various structural modifications due to the different post translational modifications and the resolution of dissociation constants due to the protein-ligand interactions [124].

7. Conclusion and future perspectives

The development of several modern techniques significantly aids in the quantification of phosphorylation profile and their stress-induced modifications in plants. These rapid, sensitive and simple techniques for studying phosphoproteome studies are critically important for understanding different signal communication pathways and structural conformations important for investigating cellular dynamics. Various techniques for understanding different PTMs, particularly phosphorylation and protein kinase activity detection have been developed in the past years. Mostly *in vitro* techniques proved to be successful such as mass spectrometry, immune-based techniques, nuclear magnetic resonance, X-ray crystallography techniques and so on which led to the discovery of new nodes and limits in signaling as well as regulatory pathways which are dependent on phosphorylation. Even though several innovative insights have been attained from quantitative phosphoproteomics, some progress is needed to comprehensively improve total PTM proteome. Currently, modern biosensor tools based

phosphoproteomic technologies like FRET, Nanobody, electrochemical, kinase and translocation-based biosensors have been established as an exciting addition to already existing biosensors to discover new phosphorylation sites and signaling pathways. These biosensors have the ability to undertake protein structural analysis more efficiently and inexpensively. As a result, more long-lasting, sensitive, specific, and biocompatible biosensors for phosphoprotein/phosphopeptide or phosphate from complex sample matrices are required, as well as novel potential bioreceptor/transducer as probes to observe PTM regulations. As large data set accumulates, informatic tools will be indispensable, (like informatics has reported phosphorylation probabilities to be frequent at the ends of protein sequences). Researchers not only provided new insights into the complex phosphorylation regulatory networks in plants, but also important information for future functional observations of protein phosphorylation in plant growth and crop development under different abiotic and biotic stresses. The phosphoproteomics techniques are still evolving and far from optimal. Further improvements are important for comprehensive analysis of the phosphoproteome. Additionally, to reduce the effects of interferences, practical and efficient separation procedures must be created. Apart from the aforementioned considerations, developing portable, cost-effective, and high-throughput analytical methods remains difficult due to the requirement for multidisciplinary expert collaboration. Challenges for the future include generating an approach that ensures that all sites of phosphorylation are characterized and precisely quantified. In this context, improvement of enrichment strategies and quantification of phosphorylation are the main aims towards the future.

Declaration of Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgements

SMZ acknowledge Honble Vice Chancellor SKUAST-K (Prof. Nazir Ahmad Ganai) for the support and guidance in undertaking advanced research in plant biotechnological aspects.

References

[1] Ahmed, Phosphoprotein biosensors for monitoring pathological protein structural changes, Trends Biotechnol. 38 (2019) 519–531.
[2] J. Li, C. Silva-Sanchez, T. Zhang, S. Chen, H. Li, Phosphoproteomics technologies and applications in plant biology research, Front. Plant Sci. 6 (2015), https://doi.org/10.3389/fpls.2015.00430.
[3] D. Balchin, M. Hayer-Hartl, F.U. Hartl, In vivo aspects of protein folding and quality control, Science. 353 (2016) aac4354, https://doi.org/10.1126/science.aac4354.
[4] B. Kuhlman, P. Bradley, Advances in protein structure prediction and design, Nat Rev Mol Cell Biol. 20 (11) (2019) 681–697.
[5] P. Beltrao, P. Bork, N.J. Krogan, N. Van, Evolution and functional cross-talk of protein post-translational modifications, Mol. Syst. Biol. 22 (2013) 9,714.
[6] I.L. Batalha, C.R. Lowe, A.C. Roque, Platforms for enrichment of phosphorylated proteins and peptides in proteomics, Trends Biotechnol. 30 (2012) 100–110.
[7] P.S.L. de Oliveira, F.A.N. Ferraz, D.A. Pena, D.T. Pramio, F.A. Morais, D. Schechtman, Revisiting protein kinase-substrate interactions: toward therapeutic development, Sci. Signal. 9 (2016) re3, https://doi.org/10.1126/scisignal.aad4016.
[8] E.B. Dammer, A.K. Lee, D.M. Duong, M. Gearing, J.J. Lah, A.I. Levey, N. T. Seyfried, Quantitative phosphoproteomics of Alzheimer's disease reveals cross-talk between kinases and small heat shock proteins, Proteomics. 15 (2015) 508–519.
[9] S. Vaga, M. Bernardo-Faura, T. Cokelaer, A. Maiolica, C.A. Barnes, L.C. Gillet, B. Hegemann, F. Drogen, H. Sharifian, E. Klipp, M. Peter, J. Saez-Rodriguez, R. Aebersold, Mol, Syst Biol. 10 (2014) 767,265.
[10] C. López-Camarillo, E.A. Ocampo, M.L. Casamichana, C. Pérez-Plasencia, E. Álvarez-Sánchez, L.A. Marchat, Protein kinases and transcription factors activation in response to UV-radiation of skin: implications for carcinogenesis, Int. J. Mol. Sci. 13 (2011) 142–172, https://doi.org/10.3390/ijms13010142.
[11] P. Mertins, D.R. Mani, K.V. Ruggles, M.A. Gillette, K.R. Clauser, P. Wang, X. Wang, J.W. Qiao, S. Cao, F. Petralia, E. Kawaler, F. Mundt, K. Krug, Z. Tu, J. T. Lei, M.L. Gatza, M. Wilkerson, C.M. Perou, V. Yellapantula, K. Huang, C. Lin, M.D. McLellan, P. Yan, S.R. Davies, R.R. Townsend, S.J. Skates, J. Wang, B. Zhang, C.R. Kinsinger, M. Mesri, H. Rodriguez, L. Ding, A.G. Paulovich, D. Fenyö, M.J. Ellis, S.A. Carr, Proteogenomics connects somatic mutations to signalling in breast cancer, Nature 534 (2016) 55–62, https://doi.org/10.1038/nature18003.
[12] M. Ivan, M. Boris, Impact of phosphoproteomicsonstudies of bacterial physiology, FEMS Microbiol. Rev. 36 (4) (2012) 877–892.
[13] L. Yu, R. Mark, Chance, integrating phosphoproteomics in systems biology, Comput. Struct. Biotechnol. J. 10 (2014) 17.
[14] X. Li, M. Wilmanns, J. Thornton, M. Köhn, Elucidating human phosphatase-substrate networks, Sci. Signal. 14 (2013) 275.
[15] F. Sacco, L. Perfetto, L. Castagnoli, G. Cesareni, Human phosphatase interactome: an intricate family portrait, FEBS Lett. 586 (2012) 2732–2739.
[16] K.L. McCance, S.E. Huether, Biologic basis for disease in adults and children, in: V.L. Brashers, N.S. Rote (Eds.), Pathophysiology, 7th ed., Elsevier, 2014.
[17] Y. Liu, M.R. Chance, Integrating phosphoproteomics in systems biology, Comput Struct Biotechnol J. 10 (17) (2014) 90–97.
[18] Z. Liu, Y. Wang, Y. Xue, Phosphoproteomics-based network medicine, FEBS J. 280 (2013) 5696–5704.
[19] F. Ardito, M. Giuliani, D. Perrone, G. Troiano, L.L. Muzio, The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review), Int. J. Mol. Med. 40 (2017) 271–280, https://doi.org/10.3892/ijmm.2017.3036.
[20] P. Jagodzik, Małgorzata Tajdel-Zielinska, Agata Ciesla, Małgorzata Marczak, Agnieszka Ludwikow, Mitogen-activated protein kinase cascades in plant hormone signaling, Front. Plant Sci. 9 (2018) 1387.
[21] F. Xu, L. Na, Y. Li, L. Chen, Roles of the PI3K/AKT/mTOR signalling pathways in neurodegenerative diseases and tumours, Cell Biosci. 10 (2020) 54, https://doi.org/10.1186/s13578-020-00416-0.
[22] C. Xu, R. Liu, Q. Zhang, X. Chen, Y. Qian, W. Fang, The diversification of evolutionarily conserved MAPK cascades correlates with the evolution of fungal species and development of lifestyles, Genome Biol. Evol. 9 (2017) 311–322, https://doi.org/10.1093/gbe/evw051.
[23] A. Danquah, A. Zélicourt, M. Boudsocq, J. Neubauer, F.N. Frei Dit, N. Leonhardt, Identification and characterization of an ABA-activated MAP kinase cascade in Arabidopsis thaliana, Plant J. 82 (2015) 232–244.
[24] A.H. Sheikh, B. Raghuram, S.K. Jalmi, D.P. Wankhede, P. Singh, A.K. Sinha, Interaction between two rice mitogen activated protein kinases and its possible role in plant defense, BMC Plant Biol. 13 (2013).
[25] Z. Wang, C. Jia, J. Li, B. Xu, Z. Jin, Identification of six mitogen-activated protein kinase (MAPK) genes in banana (Musa acuminata L. AAA group, cv. Cavendish) under infection of Fusarium Oxysporum f. sp cubense Tropical Race 4, Acta Physiol. Plant. 37 (2015) 115, https://doi.org/10.1007/s11738-015-1868-x.
[26] P. Wang, C.-C. Hsu, Y. Du, P. Zhu, C. Zhao, X. Fu, C. Zhang, J.S. Paez, A.P. Macho, W.A. Tao, J.-K. Zhu, Mapping proteome-wide targets of protein kinases in plant stress responses, Proc. Natl. Acad. Sci. 117 (2020) 3270–3280, https://doi.org/10.1073/pnas.1919901117.
[27] C. Han, P. Yang, K. Sakata, S. Komatsu, Quantitative proteomics reveals the role of protein phosphorylation in rice embryos during early stages of germination, J. Proteome Res. 13 (2014) 1766–1782, https://doi.org/10.1021/pr401295c.
[28] L.J. Meyer, J. Gao, D. Xu, J.J. Thelen, Phosphoproteomic analysis of seed maturation in arabidopsis, rapeseed, and soybean, Plant Physiol. 159 (2012) 517–528, https://doi.org/10.1104/pp.111.191700.
[29] S. Aroonluk, S. Roytrakul, C. Jantasuriyarat, Identification and characterization of phosphoproteins in somatic embryogenesis acquisition during oil palm tissue culture, Plants 9 (2019) 36, https://doi.org/10.3390/plants9010036.
[30] Y. Guo, Q. Xu, D. Canzio, J. Shou, J. Li, D.U. Gorkin, I. Jung, H. Wu, Y. Zhai, Y. Tang, Y. Lu, Y. Wu, Z. Jia, W. Li, M.Q. Zhang, B. Ren, A.R. Krainer, T. Maniatis, Q. Wu, CRISPR inversion of CTCF sites alters genome topology and enhancer/promoter function, Cell. 162 (2015) 900–910, https://doi.org/10.1016/j.cell.2015.07.038.
[31] S.K. Jha, S. Malik, M. Sharma, A. Pandey, G.K. Pandey, Recent advances in substrate identification of protein kinases in plants and their role in stress management, Curr Genomics. 18 (2017) 523–541.
[32] P. Chen, R. Li, R. Zhou, Comparative phosphoproteomic analysis reveals differentially phosphorylated proteins regulate anther and pollen development in kenaf cytoplasmic male sterility line, Amino Acids 50 (2018) 841–862, https://doi.org/10.1007/s00726-018-2564-0.
[33] K.E. Stecker, B.B. Minkoff, M.R. Sussman, Phosphoproteomic analyses reveal early signaling events in the osmotic stress response, Plant Physiol. 165 (2014) 1171–1187, https://doi.org/10.1104/pp.114.238816.
[34] J. Fíla, D. Honys, Enrichment techniques employed in phosphoproteomics, Amino Acids 43 (2012) 1025–1047, https://doi.org/10.1007/s00726-011-1111-z.
[35] K.G. Kline-Jonakin, G.A. Barrett-Wilt, M.R. Sussman, Quantitative plant phosphoproteomics, Curr. Opin. Plant Biol. 14 (2011) 507–511, https://doi.org/10.1016/j.pbi.2011.06.008.
[37] B. Schulze, T. Mentzel, A.K. Jehle, K. Mueller, S. Beeler, T. Boller, G. Felix, D. Chinchilla, Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1, J. Biol. Chem. 285 (2010) 9444–9451, https://doi.org/10.1074/jbc.M109.096842.
[38] F. Yang, M.N. Melo-Braga, M.R. Larsen, H.J.L. Jørgensen, G. Palmisano, Battle through signaling between wheat and the fungal pathogen septoria tritici revealed by proteomics and phosphoproteomics, Mol. Cell. Proteomics 12 (2013) 2497–2508, https://doi.org/10.1074/mcp.M113.027532.
[39] C.D. Moore, O.Z. Ajala, H. Zhu, Applications in high-content functional protein microarrays, Curr. Opin. Chem. Biol. 30 (2016) 21–27, https://doi.org/10.1016/j.cbpa.2015.10.013.

[40] N. Formisano, P. Jolly, N. Bhalla, M. Cromhout, S.P. Flanagan, R. Fogel, Optimisation of an electrochemical impedance spectroscopy aptasensor by exploiting quartz crystal microbalance with dissipation signals, Sens. Actuators B 220 (2015) 369–375, https://doi.org/10.1016/j.snb.2015.05.049.

[41] P. Jolly, N. Formisano, P. Estrela, DNA aptamer-based detection of prostate cancer, Chem 69 (2015) 77–89.

[42] T.K. Sharma, R. Ramanathan, R. Rakwal, G.K. Agrawal, V. Bansal, Moving forward in plant food safety and security through nanobiosensors: adopt or adapt biomedical technologies? Proteomics. 15 (2015) 1680–1692, https://doi.org/10.1002/pmic.201400503.

[43] G. Luka, A. Ahmadi, H. Najjaran, E. Alocilja, M. DeRosa, K. Wolthers, A. Malki, H. Aziz, A. Althani, M. Hoorfar, Microfluidics integrated biosensors: a leading technology towards lab-on-a-Chip and Sensing applications, Sensors. 15 (2015) 30011–30031, https://doi.org/10.3390/s151229783.

[44] J. Chao, D. Zhu, Y. Zhang, L. Wang, C. Fan, DNA nanotechnology-enabled biosensors, Biosens. Bioelectron. 76 (2016) 68–79, https://doi.org/10.1016/j.bios.2015.07.007.

[45] B. Purohit, P.R. Vernekar, N.P. Shetti, P. Chandra, Biosensor nanoengineering: design, operation, and implementation for biomolecular analysis, Sens. Int. 1 (2020), 100040, https://doi.org/10.1016/j.sintl.2020.100040.

[46] K.R. Vinothkumar, R. Henderson, Single particle electron cryomicroscopy: trends, issues and future perspective, Q. Rev. Biophys. 49 (2016), e13, https://doi.org/10.1017/S0033583516000068.

[47] A. Sassolas, L.J. Blum, B.D. Leca-Bouvier, Immobilization strategies to develop enzymatic biosensors, Biotechnol. Adv. 30 (2012) 489–511, https://doi.org/10.1016/j.biotechadv.2011.09.003.

[48] M.P. Costa, C.A.S. Andrade, R.A. Montenegro, F.L. Melo, M.D.L. Oliveira, Self-assembled monolayers of mercaptobenzoic acid and magnetite nanoparticles as an efficient support for development of tuberculosis genosensor, J. Colloid Interface Sci. 433 (2014) 141–148, https://doi.org/10.1016/j.jcis.2014.07.014.

[49] V. Rodovalho, L. Alves, A. Castro, J.M. Madurro, A. Brito-Madurro, A. Santos, Biosensors applied to diagnosis of infectious diseases – an update, Austin J Biosens Bioelectron. 1 (2015) 1015.

[50] H.A. Alhadrami, R. Chinnappan, S. Eissa, A.A. Rahamn, M. Zourob, High affinity truncated DNA aptamers for the development of fluorescence based progesterone biosensors, Anal. Biochem. 525 (2017) 78–84, https://doi.org/10.1016/j.ab.2017.02.014.

[51] S.H.A. Hassan, S.W. Van Ginkel, M.A.M. Hussein, R. Abskharon, S.-E. Oh, Toxicity assessment using different bioassays and microbial biosensors, Environ. Int. 92–93 (2016) 106–118, https://doi.org/10.1016/j.envint.2016.03.003.

[52] W. He, S. Yuan, W.-H. Zhong, Md.A. Siddikee, C.-C. Dai, Application of genetically engineered microbial whole-cell biosensors for combined chemosensing, Appl. Microbiol. Biotechnol. 100 (2016) 1109–1119, https://doi.org/10.1007/s00253-015-7160-6.

[53] S.M. Yoo, S.Y. Lee, Optical biosensors for the detection of pathogenic microorganisms, Trends Biotechnol. 34 (2016) 7–25, https://doi.org/10.1016/j.tibtech.2015.09.012.

[54] C.Y.X. Chan, M.A. Gritsenko, R.D. Smith, W.-J. Qian, The current state of the art of quantitative phosphoproteomics and its applications to diabetes research, Expert Rev. Proteomics. 13 (2016) 421–433, https://doi.org/10.1586/14789450.2016.1164604.

[55] K. Jamshidi Goharrizi, A. Baghizadeh, M. Kalantar, F. Fatehi, Assessment of changes in some biochemical traits and proteomic profile of UCB-1 pistachio rootstock leaf under salinity stress, J. Plant Growth Regul. 39 (2020) 608–630.

[56] M. Nazari, S.S. Moosavi, M. Maleki, K. Jamshidi Goharrizi, Chloroplastic acyl carrier protein synthase I and chloroplastic 20 kDa chaperonin proteins are involved in wheat (Triticum aestivum) in response to moisture stress, J. Plant Interact. 15 (2020) 180–187.

[57] K. Jamshidi Goharrizi, F. Fatehi, M. Nazari, F. Salehi, M. Maleki, Assessment of changes in the content of sulforaphane and expression levels of CYP79F1 and myrosinase genes and proteomic profile of Lepidium draba plant under water-deficit stress induced by polyethylene glycol, Acta Physiol. Plant. 42 (2020) 1–18.

[58] G.K. Agrawal, R. Rakwal, Rice proteomics: a move toward expanded proteome coverage to comparative and functional proteomics uncovers the mysteries of rice and plant biology, PROTEOMICS. 11 (2011) 1630–1649, https://doi.org/10.1002/pmic.201000696.

[59] C. Silva-Sanchez, H. Li, S. Chen, Recent advances and challenges in plant phosphoproteomics, PROTEOMICS. 15 (2015) 1127–1141, https://doi.org/10.1002/pmic.201400410.

[60] N. Ahsan, R.S. Wilson, R.S.P. Rao, F. Salvato, M. Sabila, H. Ullah, J.A. Miernyk, Mass spectrometry-based identification of Phospho-Tyr in plant proteomics, J. Proteome Res. 19 (2020) 561–571, https://doi.org/10.1021/acs.jproteome.9b00550.

[61] M. Ahmed, K.M. Koo, P.N. Mainwaring, L.G. Carrascosa, M. Trau, Phosphoprotein biosensors for monitoring pathological protein structural changes, Trends Biotechnol. 38 (2020) 519–531, https://doi.org/10.1016/j.tibtech.2019.11.006.

[62] M. Gullberg, A.-C. Andersson, Highly specific detection of phosphorylated proteins by Duolink, Nat. Methods 6 (2009) vii–viii, https://doi.org/10.1038/nmeth.f.267.

[63] F. Bloch, Nuclear induction, Phys. Today 3 (1950) 22–25, https://doi.org/10.1063/1.3066970.

[64] E.M. Purcell, R.V. Pound, N. Bloembergen, Nuclear magnetic resonance absorption in hydrogen gas, Phys. Rev. 70 (1946) 986–987, https://doi.org/10.1103/PhysRev.70.986.

[65] Z. Serber, A.T. Keatinge-Clay, R. Ledwidge, A.E. Kelly, S.M. Miller, V. Dötsch, High-resolution macromolecular NMR spectroscopy inside living cells, J. Am. Chem. Soc. 123 (2001) 2446–2447, https://doi.org/10.1021/ja0057528.

[66] E. Luchinat, L. Banci, A unique tool for cellular structural biology: in-cell NMR, J. Biol. Chem. 291 (2016) 3776–3784, https://doi.org/10.1074/jbc.R115.643247.

[67] C. Danis, C. Despres, L.M. Bessa, I. Malki, H. Merzougui, I. Huvent, H. Qi, G. Lippens, F.-X. Cantrelle, R. Schneider, X. Hanoulle, C. Smet-Nocca, I. Landrieu, Nuclear magnetic resonance spectroscopy for the identification of multiple Phosphorylations of intrinsically disordered proteins, J. Vis. Exp. (2016) 55001, https://doi.org/10.3791/55001.

[68] Y. Xiao, J.C. Liddle, A. Pardi, N.G. Ahn, Dynamics of protein kinases: insights from nuclear magnetic resonance, Acc. Chem. Res. 48 (2015) 1106–1114, https://doi.org/10.1021/acs.accounts.5b00001.

[69] X. Bai, G. McMullan, S.H.W. Scheres, How cryo-EM is revolutionizing structural biology, Trends Biochem. Sci. 40 (2015) 49–57, https://doi.org/10.1016/j.tibs.2014.10.005.

[70] R. Fernandez-Leiro, S.H.W. Scheres, Unravelling biological macromolecules with cryo-electron microscopy, Nature. 537 (2016) 339–346, https://doi.org/10.1038/nature19948.

[71] X. Wei, X. Su, P. Cao, X. Liu, W. Chang, M. Li, Z. Liu, Structure of spinach photosystem II–LHCII supercomplex at 3.2 Å resolution, Nature 534 (2016) 69–74.

[72] M. Allegretti, N. Klusch, D.J. Mills, J. Vonck, W. Kühlbrandt, K.M. Davies, Horizontal membrane-intrinsic α-helices in the stator a-subunit of an F-type ATP synthase, Nature. 521 (2015) 237–240.

[73] J. Zhao, S. Benlekbir, J.L. Rubinstein, Electron cryomicroscopy observation of rotational states in a eukaryotic V-ATPase, Nature. 521 (2015) 241–245.

[74] J.J. Liu, C.Y. Niu, Y. Wu, D. Tan, Y. Wang, M.D. Ye, H.W. Wang, CryoEM structure of yeast cytoplasmic exosome complex, Cell Res. 26 (2016) 822–837.

[75] K.A. Verba, R.Y.-R. Wang, A. Arakawa, Y. Liu, M. Shirouzu, S. Yokoyama, D. A. Agard, Atomic structure of Hsp90:Cdc37:Cdk4 reveals Hsp90 regulates kinase via dramatic unfolding, Science. 352 (2016) 1542–1547, https://doi.org/10.1126/science.aaf5023.

[76] S. Subramaniam, W. Kühlbrandt, R. Henderson, CryoEM at IUCrJ: a new era, IUCrJ. 3 (2016) 3–7, https://doi.org/10.1107/S2052252515023738.

[77] H. Zhang, C. Diao, S. Liu, S. Jiang, F. Shi, X. Jing, X-ray diffraction and Raman scattering investigations on Ba, J. Alloys Compd. (2014) 717–723. Mg (1− x)/3ZrxTa2 (1− x)/3.

[78] A. Merk, A. Bartesaghi, S. Banerjee, V. Falconieri, P. Rao, M.I. Davis, R. Pragani, M.B. Boxer, L.A. Earl, J.L.S. Milne, S. Subramaniam, Breaking Cryo-EM resolution barriers to facilitate drug discovery, Cell. 165 (2016) 1698–1707, https://doi.org/10.1016/j.cell.2016.05.040.

[79] G. McMullan, S. Chen, R. Henderson, A.R. Faruqi, Detective quantum efficiency of electron area detectors in electron microscopy, Ultramicroscopy. 109 (2009) 1126–1143, https://doi.org/10.1016/j.ultramic.2009.04.002.

[81] L. Liu, F. He, Y. Yu, Y. Wang, Application of FRET biosensors in mechanobiology and mechanopharmacological screening, Front. Bioeng. Biotechnol. 8 (2020) 1299.

[82] L. Oldach, J. Zhang, Encoded fluorescent biosensors for live-cell visualization of protein phosphorylation, Chem. Biol. 21 (2014) 186–197.

[83] P. Scholler, D. Moreno-Delgado, N. Lecat-Guillet, E. Doumazane, C. Monnier, F. Charrier-Savournin, L. Fabre, C. Chouvet, S. Soldevila, L. Lamarque, G. Donsimoni, T. Roux, J.M. Zwier, E. Trinquet, P. Rondard, J.P. Pin, HTS-compatible FRET-based conformational sensors clarify membrane receptor activation, in, Nat. Chem. Biol. p. 4 (2017) 372–380.

[84] A. Pandey, A.N. Nikam, B.S. Padya, S. Kulkarni, G. Fernandes, A.B. Shreya, S. Mutalik, Surface architectured black phosphorous nanoconstructs based smart and versatile platform for cancer theranostics, Coord. Chem. Rev. 435 (2021), 213826.

[85] L. Zhang, Z. Xiong, Y. Chen, L. Peng, B. Yu, X. Gao, R. Zhang, L. Zhang, W. Zhang, Soft-template synthesis of hydrophilic metallic zirconia nanoparticle-incorporated ordered mesoporous carbon composite and its application in phosphopeptide enrichment, RSC Adv. 6 (2016) 30014–30020, https://doi.org/10.1039/C6RA00326E.

[86] A. Review, Nanomaterials applied in graphene-based electrochemical biosensors, Sens. Mater. (2015) 1, https://doi.org/10.18494/SAM.2015.1059.

[87] W. Liu, H. Song, Q. Chen, J. Yu, M. Xian, R. Nian, D. Feng, Recent advances in the selection and identification of antigen-specific nanobodies, Mol. Immunol. 96 (2018) 37–47.

[88] S. Muyldermans, Nanobodies: natural single-domain antibodies, Annu. Rev. Biochem. 82 (2013) 775–797.

[89] E. Pardon, T. Laeremans, S. Triest, S.G.F. Rasmussen, A. Wohlkönig, A. Ruf, S. Muyldermans, W.G.J. Hol, B.K. Kobilka, J. Steyaert, A general protocol for the generation of Nanobodies for structural biology, Nat. Protoc. 9 (2014) 674–693, https://doi.org/10.1038/nprot.2014.039.

[90] D. Nevoltris, B. Lombard, G. Mathis, Patrick Chames, Daniel Baty, Conformational nanobodies reveal tethered epidermal growth factor receptor involved in EGFR/ErbB2 predimers, ACS Nano 9 (2015) 1388–1399.

[91] C. McMahon, A.S. Baier, R. Pascolutti, M. Wegrecki, S. Zheng, J.X. Ong, A. C. Kruse, Yeast surface display platform for rapid discovery of conformationally selective nanobodies, Nat. Struct. Mol. Biol. 25 (2018) 289–296.

[92] A.L.J. Marschall, S. Dübel, T. Böldicke, Recent advances with ER targeted Intrabodies, Adv. Exp. Med. Biol. 917 (2016) 77–93, https://doi.org/10.1007/978-3-319-32805-8_5.

[93] R. Irannejad, J.C. Tomshine, J.R. Tomshine, M. Chevalier, J.P. Mahoney, J. Steyaert, M. Zastrow, Conformational biosensors reveal GPCR signalling from endosomes, Nature 495 (2013) 534–538.
[94] F. El-Turk, F.N. Newby, E. De Genst, T. Guilliams, T. Sprules, A. Mittermaier, C. M. Dobson, M. Vendruscolo, Structural effects of two camelid nanobodies directed to distinct C-terminal epitopes on α-synuclein, Biochemistry 55 (2016) 3116–3122, https://doi.org/10.1021/acs.biochem.6b00149.
[95] H. Lin, K. Yuan, C. Deng, Preparation of a TiO2-NH2 modified MALDI plate for on-plate simultaneous enrichment of phosphopeptides and glycopeptides, Talanta. 175 (2017) 427–434, https://doi.org/10.1016/j.talanta.2017.07.078.
[96] I.A. Diez, I. Govender, P. Naicker, S. Stoychev, J. Jordaan, O. Jensen, Zirconium (IV)-IMAC for phosphopeptide enrichment in phosphoproteomics, Preprint published in, BioRxiv 20 (2020) 453–462, https://doi.org/10.1021/acs.jproteome.0c00508 (34pp).
[97] I.T. Teng, X. Li, H.A. Yadikar, Z. Yang, L. Li, Y. Lyu, X. Pan, K.K. Wang, W. Tan, Identification and characterization of DNA aptamers specific for phosphorylation epitopes of tau protein, J. Am. Chem. Soc. 140 (2018) 14314–14323.
[98] F. Cimaglia, E. De, E. Lorenzis, V. Mezzolla, F. Rossi, P. Poltronieri, Detection of L. monocytogenes in enrichment cultures by immunoseparation and immunosensors, IEEE Sensors J. 16 (19) (2016) 7045–7052.
[99] M. García-Aranda, M. Redondo, Protein kinase targets in breast cancer, Int. J. Mol. Sci. 18 (2017) 2543.
[100] X.L. Luo, X. Xie, Y.C. Meng, T.L. Sun, J.S. Ding, W.H. Zhou, Ligands dissociation induced gold nanoparticles aggregation for colorimetric Al3þdetection, Anal. Chim Acta. 1087 (76) (2019) 85.
[101] X. Li, Z. Wu, Xiaodong Zhou, J. Hu, Colorimetric response of peptide modified gold nanoparticles: an original assay for ultrasensitive silver detection, Biosens. Bioelectron. 92 (2017) 496–501, https://doi.org/10.1016/j.bios.2016.10.075.
[102] E. Borberg, M. Zverzhinetsky, A. Krivitsky, A. Kosloff, O. Heifler, G. Degabli, H. P. Soroka, R.S. Fainaro, L. Burstein, S. Reuveni, H. Diamant, V. Krivitsky, F. Patolsky, Light-controlled selective collection-and-release of biomolecules by an on-Chip nanostructured device, Nano Lett. 19 (2019) 5868–5878, https://doi.org/10.1021/acs.nanolett.9b01323.
[103] A.I. Solovyev, M. Kostejn, G. Kuncova, P. Dostalek, J. Rohovec, T. Navratil, Preconcentration and detection of mercury with bioluminescent bioreporter E. coli ARL1, Appl, Microbiol Biotechnol. 99 (2015) (20,8793 8802).
[104] Y. Lu, Q. Yang, J. Wu, Recent advances in biosensor-integrated enrichment methods for preconcentrating and detecting the low-abundant analytes in agriculture and food samples, TrAC Trends Anal. Chem. 128 (2020), 115914, https://doi.org/10.1016/j.trac.2020.115914.
[105] X.-S. Li, B.-F. Yuan, Y.-Q. Feng, Recent advances in phosphopeptide enrichment: strategies and techniques, TrAC, Trends Anal. Chem. 78 (2016) 70–83, https://doi.org/10.1016/j.trac.2015.11.001.
[106] M. Han, Z. Li, Mesoporous metal oxide nanoparticles for selective enrichment of phosphopeptides from complex sample matrices, Anal. Methods 8 (2016) 7747–7754, https://doi.org/10.1039/C6AY02069K.
[107] X. Qi, C. Chang, X. Xu, Y. Zhang, Y. Bai, H. Liu, Magnetization of 3-dimentional homochiral metal-organic frameworks for efficient and highly selective capture of phosphopeptides, J. Chromatogr. A 1468 (2016) 49–54, https://doi.org/10.1016/j.chroma.2016.09.046.
[108] Y.Q. Xie, C.H. Deng, Highly efficient enrichment of phosphopeptides by a magnetic lanthanide metal-organic framework, Talanta 159 (2016) 1–6.
[109] J. Zhou, Y. Liang, X. He, L. Chen, Y. Zhang, Dual-functionalized magnetic metal–organic framework for highly specific enrichment of phosphopeptides, ACS Sustain. Chem. Eng. 5 (2017) 11413–11421, https://doi.org/10.1021/acssuschemeng.7b02521.
[110] Z. Yan, P. Deng, Y. Liu, Recent advances in protein kinase activity analysis based on nanomaterials, Int. J. Mol. Sci. 20 (2019) 1440, https://doi.org/10.3390/ijms20061440.
[111] E.C. Greenwald, S. Mehta, J. Zhang, Encoded fluorescent biosensors illuminate the spatiotemporal regulation of signaling networks, Chem. Rev. 118 (2018) 11707–11794.
[112] Z. Wang, Z. Yan, F. Wang, J. Cai, L. Guo, J. Su, Y. Liu, Highly sensitive photoelectrochemical biosensor for kinase activity detection and inhibition based on the surface defect recognition and multiple signal amplification of metal-organic frameworks, Biosens. Bioelectron. 97 (2017) 107–114, https://doi.org/10.1016/j.bios.2017.05.011.
[113] H. Yin, X. Wang, Y. Guo, Y. Zhou, S. Ai, Electrochemical detection of protein kinase activity based on carboxypeptidase Y digestion triggered signal amplification, Biosens. Bioelectron. 66 (2015) 77–83, https://doi.org/10.1016/j.bios.2014.11.014.
[114] Q. Hu, Q. Wang, C. Jiang, J. Zhang, J. Kong, X. Zhang, Electrochemically mediated polymerization for highly sensitive detection of protein kinase activity, Biosens. Bioelectron. 110 (2018) 52–57, https://doi.org/10.1016/j.bios.2018.03.030.
[115] T. Kudo, S. Jeknić, D.N. Macklin, S. Akhter, J.J. Hughey, S. Regot, M.W. Covert, Live-cell measurements of kinase activity in single cells using translocation reporters, Nat. Protoc. 13 (2018) 155–169, https://doi.org/10.1038/nprot.2017.128.
[116] P. Johnston, Y. Hua, D. Camarco, T.Y. Shun, J.S. Lazo, J.R. Grandis, High-contentpSTAT3/1 imaging assays to screen for selec-tive inhibitors of STAT3 pathway activation inhead and neck cancer cell lines, Assay Drug Dev Technol. 12 (2014) 55–79.
[117] Y. Hua, D.P. Camarco, C.J. Strock, P.A. Johnston, High content positional biosensor assay to screen for compounds that prevent or disrupt androgen receptor and transcription intermediary factor 2 protein-protein interactions, Methods Mol. Biol. 1683 (2018) 211–227.
[118] S. Regot, J.J. Hughey, B.T. Bajar, S. Carrasco, M.W. Covert, High-sensitivity measurements of multiple kinase activities in live single cells, Cell. 157 (2014) 1724–1734, https://doi.org/10.1016/j.cell.2014.04.039.
[119] C.J. Fuller, A.F. Straight, Image analysis benchmarking methods for high-content screen design, J. Microsc. 238 (2) (2010) 145–161.
[122] R. Torggler, D. Papinski, C. Kraft, Assays to monitor autophagy in Saccharomyces cerevisiae, Cells. 6 (2017) 23, https://doi.org/10.3390/cells6030023.
[123] S. Schopper, A. Kahraman, P. Leuenberger, Y. Feng, I. Piazza, O. Müller, P. J. Boersema, P. Picotti, Measuring protein structural changes on a proteome-wide scale using limited proteolysis-coupled mass spectrometry, Nat. Protoc. 12 (2017) 2391–2410, https://doi.org/10.1038/nprot.2017.100.
[124] Y. Feng, G. De Franceschi, A. Kahraman, M. Soste, A. Melnik, P.J. Boersema, P. P. de Laureto, Y. Nikolaev, A.P. Oliveira, P. Picotti, Global analysis of protein structural changes in complex proteomes, Nat. Biotechnol. 32 (2014) 1036–1044, https://doi.org/10.1038/nbt.2999.