Minireview

Ubiquitination detection techniques

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Impact Statement

Ubiquitination is crucial for maintaining protein homeostasis in the cell by regulating protein turnover through the ubiquitin–proteasome system. Genetic mutations, and transcriptional or translational failures associated with this system lead to cancers and neurodegenerative diseases. Detection of normal or abnormal ubiquitination is, therefore, important to better understand the molecular mechanisms behind these diseases and to develop treatments. This minireview serves as a resource to compare ubiquitination detection techniques existing in the field developed using numerous technologies and features their applications, benefits, and limitations in a comprehensive manner. This provides insights into the evolution of each technique and its impact on the growth of the field.

Abstract

Ubiquitination is an intricately regulated post-translational modification that involves the covalent attachment of ubiquitin to a substrate protein. The complex dynamic nature of the ubiquitination process regulates diverse cellular functions including targeting proteins for degradation, cell cycle, deoxyribonucleic acid (DNA) damage repair, and numerous cell signaling pathways. Ubiquitination also serves as a crucial mechanism in protein quality control. Dysregulation in ubiquitination could result in lethal disease conditions such as cancers and neurodegenerative diseases. Therefore, the ubiquitination cascade has become an attractive target for therapeutic interventions. Enormous efforts have been made to detect ubiquitination involving different detection techniques to better grasp the underlying molecular mechanisms of ubiquitination. This review discusses a wide range of techniques stretching from the simplest assays to real-time assays. This includes western blotting/immunoblotting, fluorescence assays, chemiluminescence assays, spectrophotometric assays, and nanopore sensing assays. This review compares these applications, and the inherent advantages and limitations.

Keywords: Ubiquitination, detection techniques, spectrophotometric, fluorescence, chemiluminescence

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Introduction

Ubiquitination is a common post-translational modification in eukaryotes that involves the covalent attachment of ubiquitin (Ub), a highly conserved small protein (76 amino acids), to a target substrate protein.[1] This modification causes the generation of discrete cellular signals that regulate various fundamental cellular processes, including targeting misfolded proteins for degradation and maintaining cellular protein quality control.[2–4] Ubiquitination is mediated by an enzymatic cascade involving an E1-ubiquitin-activating enzyme, an E2-ubiquitin-conjugating enzyme, and an E3-ubiquitin ligase enzyme. Ubiquitin is activated first by E1 in an adenosine triphosphate (ATP)-dependent manner. As a result, a thioester bond is formed between the carboxyl terminus of Ub and an active-site cysteine residue of E1. Ub is then transferred from the E1-Ub thioester complex to an active-site cysteine residue of E2, in a thiol exchange reaction to yield the E2-Ub conjugate linked via a thioester bond. Finally, Ub is transferred to the target substrate via an E3, forming an isopeptide bond between the carboxyl-terminal glycine of Ub and an amino group, typically the ε-amino group of a lysine residue, of the target substrate.[5–7] Studies have found several E1 enzymes, more than 35 E2 enzymes, and more than 600 E3 enzymes, encoded by the human genome.[8–10] The transfer of Ub to the target substrate differs, depending on the type of the E3 enzyme. Three types of E3 enzymes have been discovered: really interesting new gene (RING)/U-box, RING-between-RING (RBR), and homology to E6AP C-terminus (HECT). RING ligases bind with E2 and facilitate the transfer of ubiquitin from the E2-Ub conjugate, directly to the target substrate. In contrast, ubiquitin covalently modifies a catalytic cysteine residue of HECT ligases, producing a thioester intermediate. Ub is then transferred from the thioester complex to the target substrate. A hybrid mechanism of RING/HECT ligases is incorporated by RBR ligases. They bind with E2 and transfer Ub from the E2-Ub conjugate to the catalytic cysteine residue of E3 forming a thioester intermediate from which Ub is transferred to the target substrate.[11] Certain E3 enzymes such as mouse double minute 2 (Mdm2), seven in absentia homolog 1 (SIAH1), and tumor necrosis factor (TNF) receptor-associated factor (TRAF) are capable of self-ubiquitination

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Table 1. Different linkage-type facilitates interactions with specific target substrates resulting a specific outcome.

Type of polyubiquitination	Functions
K48	Targets substrates for proteasomal degradation[28,29]
K11	Targets substrates for proteasomal degradation[28,29] Regulation of the cell cycle[30,31]
K6	Mediates DNA damage repair[32]
K27	Controls mitochondrial autophagy[33,34]
K29	Regulation of the cell cycle[35] Takes part in RNA processing and stress responding[5]
K33	Involves in T-cell receptor-mediated signaling pathway[36,37]
K63	Participates in protein–protein interactions and protein trafficking[25,38,39] Plays an important role in regulating NF-κB inflammatory signaling[41,42]
M1-linked	Plays an important role in regulating NF-κB inflammatory signaling[41,42]

(autoubiquitination) which means they can transfer ubiquitin onto lysine residues of the E3.[12]

Ubiquitination is counterbalanced by a process termed deubiquitination, and both are critical for regulating the ubiquitin system. Deubiquitination is carried out by deubiquitinating (DUB) enzymes, and they perform numerous roles within the cell.[13,14] DUB are involved in producing active monoubiquitin forms by processing the inactive ubiquitin precursors which are initially expressed either as fusion proteins encoded with one of the two ribosomal proteins or as linear polyubiquitin chains.[15,16] DUB are also responsible for editing or rescuing ubiquitin–protein conjugates, a process in which ubiquitin is removed from inappropriately targeted substrates or from various adducts formed with small molecule nucleophiles in the cell.[17] In addition, DUB are accountable for the recycling of ubiquitin by associating with the proteasome to process and remove ubiquitin chains before substrate degradation.[15]

Small ubiquitin-like modifier (SUMO) is a small protein that is structurally similar to ubiquitin but is functionally different from ubiquitin[18] and belongs to a class of proteins known as ubiquitin-like (Ubl) proteins.[19] It competes with Ub for modification sites. The attachment of SUMO to a target substrate is referred to as sumoylation, and it follows a conserved enzymatic cascade similar to ubiquitination involving SUMO-specific enzymes, SUMO-activating E1/SUMO-activating E2 (SAE1/SAE2) enzyme, SUMO-conjugating E2 enzyme (Ubc9), and SUMO-ligating E3 enzyme. Sumoylation plays an important role in regulating certain processes such as protein–protein interactions, protein subcellular localization, and deoxyribonucleic acid (DNA) protein binding.[20,21] Besides ubiquitination and sumoylation, processes such as acetylation, phosphorylation, neddylation, and succinylation are involved in the modification of Ub molecules.[22]

The modification of a target substrate can take place by the addition of a single ubiquitin molecule or by the formation of a polyubiquitin chain.[23] The ε-amino group of the seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and the N-terminal amino of methionine (M1) present in ubiquitin can conjugate to another ubiquitin molecule, producing complex polyubiquitin chains with different linkage types.[11] The polyubiquitin chains can be classified into homotypic chains and heterotypic chains. In the homotypic chains, all the ubiquitin molecules are attached via a single common lysine or methionine residue, forming one of eight different chain types. In the heterotypic chains, more than one linkage type is found and can be divided into two types termed mixed/linear chains or branched chains.[23–25] Each linkage type facilitates interactions with specific target substrates resulting in a specific outcome (Table 1).[26] Many of these functions are responsible for the survival and death of the cells. Therefore, any abnormality or failure in ubiquitination can disrupt precisely balanced pathways and lead to fatal conditions such as cancers, neurodegenerative diseases, adaptive and innate immunity-related disorders, and muscle-wasting disorders.[27]

DNA: deoxyribonucleic acid; RNA: ribonucleic acid; NF-κB: nuclear factor kappa B.

The importance of the ubiquitination cascade presents an attractive target for therapeutic interventions. For the past decade, several efforts have been made to discover small molecule inhibitors for ubiquitination cascade enzymes, and some of these potential candidates have produced promising results in the clinical trials for treating certain diseases. Nutlin, which is an inhibitor of RING E3 ligase, has been found to be effective in treating multiple myeloma. Nutlin inhibits the interaction between Mdm2 and p53, preventing the degradation of p53 in cells and reducing tumor growth.[28] MLN4924, a selective, small molecule inhibitor for NEDD8 (ubiquitin-like molecule) activating enzyme (NAE1), has entered phase 1 trials for the treatment of cancers.[29] In the Neddylation pathway, NEDD8 is initially activated by NAE1, then transferred to a NEDD8-conjugating enzyme E2 (UBC12), and later conjugated with a substrate. Cullins are the best-characterized substrates for the neddylation pathway, and they function as a scaffold for cullin–RING ligases (CRL-E3 ligase).[30] The modification of the cullin protein within the CRL is required for activation of its ligase activity. The neddylation pathway controls the activities of CRLs associated with cancer cell growth and survival.[31] The inhibitory activity of MLN4924 restrains CRL mediated turnover of the proteins resulting in human tumor cell apoptosis through disruption of S-phase DNA synthesis.[31] Furthermore, trials are ongoing for bortezomib, an effective proteasome inhibitor used to treat multiple myeloma and mantle lymphoma, to explore its inhibitory activity on the ubiquitination cascade. Despite the development of nutlin, MLN4924, and bortezomib, additional efforts and technologies are needed to further develop and screen small molecule inhibitors of E1, E2, and E3 enzymes. Thus, tremendous efforts have been made in the detection of ubiquitination so as to better understand the underlying molecular mechanisms of this complex pathway and to provide new insights into therapeutic interventions. To date, many ubiquitination detection techniques have been developed (Table 2). This minireview highlights the advancement of different detection techniques in the field, starting from the simplest assays to rapid real-time assays involving cutting-edge methodologies. This minireview explores the techniques used to

Table 2. Summary of detection techniques and their applications.

Detection technique			References
Immunoblotting/western blotting	Ubiquitin antibodies		Hu *et al*.[48]; Rahighi *et al*.[49]; Kulathu *et al*.[50]
	Linkage-specific antibodies	K48 and K63	Kristariyanto *et al*.[51]
		K27	Kristariyanto *et al*.[52]
		K11	Schumacher and Tsomides[53]
		M1	Voytas[54]
		K11/K48 bispecific	Tongaonkar and Madura[55]
	Linkage-specific TUBEs	M1	Selvin[56]
		K63	Szöllosi *et al*.[57]
		K29	Földes-Papp *et al*.[58]
		K48	Shyu and Hu[59]
	Sandwich ELISA		Rahighi *et al*.[49]; Gururaja *et al*.[60]
Radio-activity based assays	Radioactive isotopes		Hu *et al*.[48]; Koszela *et al*.[61]
Fluorescence assays	Fluorescence		Wee *et al*.[62]; Macdonald *et al*.[63]
	FRET		Vijay-Kumar *et al*.[64]; Davydov *et al*.[65]
	TR-FRET		Clegg *et al*.[66]
	Fluorescence polarization		Royer and Scarlata[67]; Toseland[68]
	Fluorescence complementation		Sekar and Periasamy [69]; Bossis *et al*.[70]
	Fluorescence confocal microscopy		Tang *et al*.[71]
CL assays	CL		Gruber *et al*.[73]
	ECL		Macdonald *et al*.[63]
	BL		Carlson *et al*.[77]
	BRET		Klostermeier and Millar[74]
Spectrophotometric assays			Visser *et al*.[75]
NP sensing			Hong *et al*.[76]; Carlson *et al*.[77]

TUBEs: Tandem ubiquitin-binding entities; ELISA: enzyme-linked immunosorbent assay; FRET: fluorescence resonance energy transfer; TR-FRET: time-resolved fluorescence resonance energy transfer; CL: chemiluminescence; NP: nanopore; BRET: bioluminescence resonance energy transfer; BL: bioluminescence; ECL: electrochemiluminescence.

develop each assay, applications of the assay, and benefits and limitations associated with each assay.

Immunoblotting/western blotting

The conventional method of detection for ubiquitination is the use of an immunoblot or a western blot which is based on a specific immuno-interaction between an antibody and an antigen. After performing the ubiquitination assay, the proteins within the reaction mixture are electrophoretically separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a membrane or a blot. After blocking with inert proteins, the membrane or blot is probed with unlabeled primary antibodies that directly bind to the proteins of interest. Unbound primary antibodies are washed away and followed by labeled secondary antibodies that bind to the primary antibodies. Since the ubiquitin modification alters the migration of proteins through the gel by changing the molecular weight and shape of the protein, antibodies are able to recognize the modification thus enabling a researcher to determine whether polyubiquitin chains are attached. The high specificity between the antibody and antigen, and the flexibility to synthesize specific antibodies against the proteins of interest allow the wide application of immunoblots for the detection of ubiquitinated proteins. Over the years, several monoclonal and polyclonal antiubiquitin antibodies have been synthesized, and many differently labeled secondary antibodies have been used to recognize these primary antibodies. Often, the secondary antibodies are labeled with a fluorescent tag, a radiolabeled tag, or coupled to an enzyme which generates a detectable signal via observation of fluorescence, autoradiography, colorimetric change, or CL and is captured by an imaging methodology.[32] Deveraux *et al*.[33] used a radio-iodinated antibody (^{125}I-anti ubiquitin) for the detection of metabolism of ubiquitin conjugates in a mouse carcinoma cell line (t85c cells) which was effective in targeting individual proteins to a certain catabolic pathway. The ubiquitination reactions were setup at different temperatures using a thermolabile E1-activating enzyme, to analyze the high molecular weight ubiquitin conjugates and free ubiquitin in heat-shocked t85c cells. The immunoblot was exposed to autoradiography to detect the ubiquitin conjugation.[33] The study conducted by Takada *et al*.[34] analyzed the intracellular levels of multiubiquitin chains in a reticulocyte lysate under heat shock conditions with the use of immunoblots probed with primary antibody Ub5 F9 against ubiquitin,[34] followed by a secondary antibody labeled with an alkaline phosphatase against the antiubiquitin. This led to a colorimetric detection of multiubiquitin chains after the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.[35]

The commonly used horse-radish peroxidase (HRP)-labeled secondary antibodies result in a detectable CL signal. The study conducted by Baarends *et al*.[36] used antiubiquitin and anti-H2A primary antibodies followed by HRP-labeled secondary antibodies to detect ubiquitinated H2A histones in mouse testis, which was the mostly ubiquitinated histone, since a high turnover of proteins was discovered during mammalian spermatogenesis. Likewise, HRP-labeled

secondary antibodies were used by Yan *et al*.[37] to detect antiubiquitin antibodies that bound to ubiquitinated adeno-associated virus type 2 and 5 (AAV-2 and AAV-5) capsid proteins in HeLa cells. In a study performed to detect ubiquitinated insulin-like growth factor-1 receptor (IGF-1R) by the Mdm2 E3 ligase, Girnita *et al*.[38] used the primary antibodies anti-IGF-1R and anti-His against the poly-histidine tag of ubiquitin and followed by these HRP-labeled secondary antibodies. The study conducted by Yamauchi *et al*.[39] exploring the potential role of TRIM5α protein which belongs to tripartite motif (TRIM) family that mediates species-specific restriction of lentiviruses, these HRP-labeled antibodies against the primary antibodies, anti-RH (RH-tagged ubiquitin) and anti-TRIM5α, distinguished ubiquitinated and non-ubiquitinated TRIM5α protein and discovered that TRIM5α protein functions as an E3-ligase enzyme capable of ubiquitinating itself and other substrates.

Later, several antibodies were synthesized and applied (K48, K63, K27, K11, M1-linked antibodies) to detect specific ubiquitin linkages within polyubiquitin chains as well. Despite the denaturing conditions of SDS-PAGE for western blotting, it is proposed that resistance to denaturation or refolding of ubiquitin on the western blot membrane allows for conformational epitopes to be appropriately presented to antibodies enabling linkage-specific detection.[40] K48 and K63 linkage-specific antibodies were used by Newton *et al*.[41] to gain molecular insights of specific ubiquitin chain editing in a receptor-interacting serine/threonine-protein kinase 1 (RIP1) which led to the stimulation of tumor necrosis factor receptor 1 (TNFR1) in a cascade of steps. The study revealed that ubiquitin chain editing was a mechanism for downregulating signaling by TNFR1.[41] K27 linkage-specific antibodies were used by Yin *et al*.[42] to determine the role of ITCH (E3 ligase) mediated (serine/threonine-protein kinase B-raf) ubiquitination which led to the discovery of signaling between cytokines and the mitogen-activated protein kinase (MAPK) pathway activation in melanoma cells. Min *et al*.[43] used K11 linkage-specific antibodies to determine the role of K11 linkages in mitotic exit. Anaphase-promoting complex/cyclo-some (APC-C) is responsible for controlling the mitotic exit in eukaryotes. APC-C together with UBE2S (E2 enzyme) forms K11-linked polyubiquitin chains on cells arrested during mitotic exit. The study revealed that K11 linkages are crucial for the regulation of the rate of degradation of substrates by APC-C in a co-activator-dependent manner. Linear ubiquitin antibodies (M1-linkage) were utilized by Nakazawa *et al*.[44] to determine their role in the pathogenesis of optineurin (OPTN)-associated amyotrophic lateral sclerosis (ALS). The UBAN domain of OPTN is responsible for binding linear ubiquitin chains. The study found that linear ubiquitination is essential for the regulation of both nuclear factor kappa B (NF-κB) activation and apoptosis which may otherwise result in ALS. A bispecific antibody to detect K11/K48-linked chains was engineered by Yau *et al*.[45] to explore the role of heterotypic ubiquitin chains in protein quality control and discovered that these chains promote rapid proteasomal degradation of aggregated proteins. In addition, these bispecific antibodies identified mitotic regulators and Huntingtin variants as substrates of K11/K48 chains.[45] Recently, Guven *et al*.[46] used antiubiquitin and anti-K48-ubiquitin primary antibodies followed by HRP-labeled secondary antibodies to detect changes in direct inhibitor of apoptosis-binding protein with low pI (Diablo) ubiquitination level, which is a protein that binds inhibitor of apoptosis proteins (IAPs), freeing caspase enzymes that mediate apoptosis. The study allowed the recognition of IAP inhibitors or proteasome inhibitors which displayed E3-ligase activity using this approach.

Even though immunoblots or western blots are widely used for the detection of ubiquitination, there are certain pitfalls associated with them. The technique can be delicate and technically demanding. The assay is time-consuming and labor-intensive due to the involvement of various steps such as blotting the gel, incubation with primary and secondary antibodies separately, and numerous washing steps of the blot. Another major pitfall involved with the immunoblots can be the lack of accuracy in quantitively analyzing ubiquitination levels. To overcome this, sandwich enzyme-linked immunosorbent assay (ELISA) was introduced for the detection and quantification of ubiquitination. The sandwich ELISA technique is a plate-based assay where a primary antibody is used to coat the microtiter well plate. Once the sample with the target antigen is loaded, an enzyme-linked secondary antibody that binds to the antigen is added, and excess secondary antibody is washed away. Next, an enzyme-specific substrate is added which enables the formation of a colored product, and the absorbance is measured by a plate reader. There is no involvement of gels, membranes, or blots. In the abovementioned Takada *et al*.[35] study, a sandwich ELISA was developed for detecting intracellular levels of multiubiquitin chains. Ubiquitin chains were captured on Ub5 F9 primary antibody-coated microtiter plates followed by a peroxidase-conjugated streptavidin secondary antibody. After addition of the 3,3′,5,5′-tetramethylbenzidine substrate, the formation of a colored product enabled quantitative detection by measuring the absorbance of the colored product at 450 nm using a plate reader.[35] Similarly, a sandwich ELISA was established by the previously mentioned Guven *et al*.[46] to quantify total Diablo and total ubiquitinated Diablo. Diablo proteins were captured on anti-Diablo coated plates followed by the addition of antiubiquitin and anti-K48 ubiquitin which were detected using a SULFO tag conjugated secondary antibody. The SULFO tag is an electrochemiluminescent label that couples to the primary amine groups of the protein and produces a chemiluminescent signal read by the plate reader.[46]

Typically, immunoblots or western blots require highly specific primary antibodies against the proteins of interest to achieve high specificity and avoid cross-reactivity. To overcome the need for highly specific ubiquitin antibodies, a number of different ubiquitin-binding proteins have been utilized. Tandem ubiquitin-binding entities (TUBEs) based on ubiquitin-associated domains were developed as an alternative. TUBEs are not only able to detect ubiquitination state of any protein in a more convenient manner but also able to protect polyubiquitinated proteins in cell lysates from proteasomal degradation and DUB.[47] Over time, TUBEs were designed to identify specific linkage types of ubiquitination.[48] Rahighi *et al*.[49] used the UBAN motif (ubiquitin binding in A20 Binding and Inhibitor of NFkB and NF-kB essential modulator [NEMO]) to determine that specific recognition of linear ubiquitin chains (M1-linked) by NEMO is required for NF-kB activation. Kulathu *et al*.[50] recognized

that the Npl4 zinc finger (NZF) domain in the TAK1-binding protein 2 subunit of Transforming growth factor-β (TGF-β)-activated kinase 1 protein kinase specifically binds to K63-linked polyubiquitin chains. NZF1 domain in TRAF-binding domain-containing protein was used by Kristariyanto *et al.*[51] for K29 linkages to study the structural basis, distinct properties, and functions of K29-linked chains in eukaryotes. Kristariyanto *et al.*[51] discovered that tandem motif interacting with ubiquitin (MIU) repeats in deubiquitylating enzyme MINDY (motif interacting with Ub-containing novel DUB family) lysine 48 deubiquitinase 1/Family with sequence similarity 63, member A can be used to recognize K48-linked polyubiquitin chains unveiling new avenues for polyubiquitin chain recognition by ubiquitin-binding domains.[52]

Radioactivity-based assays

The classic well-established radiolabeling technique has also been used for the detection of ubiquitination since it allows radioactive isotopes to detect a protein of interest in both *in vitro* and *in vivo* study conditions. The unstable radioactive isotopes exhibit spontaneous emission of radiation (alpha, beta, and gamma rays) which is typically detected by autoradiography. Autoradiography involves X-ray films for the visualization and quantification of radioactive molecules.[53,54] A radiolabel can be incorporated with a protein using ^{35}S-methionine during protein translation, in the presence of ^{32}P-ATP by enzymatic modification, or by chemical modification of the side chains of amino acids. The use of radiolabeled antibodies such as radio-iodinated antiubiquitin (^{125}I-anti ubiquitin) by Deveraux *et al.*[33] was popular due to the strong emission and facile detection. However, due to toxicity of ^{125}I to the thyroid gland, radio-iodinated protein use is restricted. Subsequently, Tongaonkar *et al.*[55] conducted the ubiquitination reaction with the incorporation of less toxic ^{32}P into ubiquitin. Initially, the thioester linkage formations between the E1-activating enzyme-ubiquitin and E2-ligating enzyme-ubiquitin were determined with the use of ^{32}P. Later, the ubiquitination of histone H2B, one of the histones involved in the structure of eukaryotic chromatin, was detected using autoradiography. The use of ^{32}P was advantageous since it excluded the specific toxicity associated with ^{125}I and the need for purifying ^{35}S radiolabeled protein from cell extracts. In addition, ^{32}P-ubiquitin was useful in investigating mutated ubiquitin variants which could not be achieved by ^{125}I-ubiquitin due to the use of commercial ubiquitin for synthesizing ^{125}I-ubiquitin.[55] Nevertheless, the special handling of the radioactive isotopes, the requirement of a special training in working with radioactive isotopes, the hazardous effects related to radioactive isotopes, and the cost associated with the disposal of the radioactive waste have made the application of radiolabeling less desirable.

Fluorescence assays

Fluorescence technology has drastically improved over the years, and at present, numerous fluorescence techniques such as fluorescence resonance energy transfer (FRET), time-resolved FRET (TR-FRET), confocal fluorescence microscopy, and fluorescence complementation were developed.[56–59] Many of these techniques have been extensively applied for the detection of ubiquitination since they are rapid, versatile, and easy to perform, and they use non-radioactive molecules thus avoiding the challenges of disposal and shelf life inherent to radioactive compounds.[60,61] Wee *et al.*[62] designed a labeled fluorescent-tagged ubiquitin using the amine-reactive dye Oregon-green (Og) succinimidyl ester, which linked exclusively with Lys6 residue of the ubiquitin chain. The labeled ubiquitin was termed as Og-Ub. The transfer of E1-catalyzed ubiquitin to the ubiquitin-conjugating enzyme E2 (Ubc4) was detected by running ubiquitination assay samples on a tris-glycine SDS-PAGE followed by detection of Og-Ub-Ubc4 band using a fluorescence image reader and by reverse-phase High-performance liquid chromatography (HPLC), after loading the sample onto a C18 column following the detection of Og-fluorescence at 520 nm.[62] This method was recommended in studying the mechanisms of different enzymes in the ubiquitination cascade, since it labeled the frequently modified residue in ubiquitin using acetylation reagents[63] and also the most accessible residue in ubiquitin.[63,64] In addition, this can be employed to detect autoubiquitination reactions. However, there is a possibility for the test compounds within a drug screen to interfere with the fluorescence signal.[60,62] Davydov *et al.*[65] developed a fluorescence assay to measure the autoubiquitination of human double minute 2 (HDM2) protein which is an E3 ligase that regulates the tumor suppressor p53. After performing the ubiquitination reaction, HRP was added to the reaction mixture. Later, QuantaBlu fluorescent substrate, diluted in hydrogen peroxide, was mixed with the solution to catalyze the oxidation of the QuantaBlu fluorescent substrate and produce a fluorescent product observable with excitation at 320 nm and emission at 460 nm. The assay displayed excellent reproducibility.[65]

The application of FRET has enabled several assays to detect the ubiquitination of a variety of substrates. FRET involves a donor and an acceptor fluorophore pair and is based on the transfer of excitation energy from the donor to a nearby acceptor. This energy transfer is observed either by measuring the quenching of the fluorescence in the donor or by measuring the increase in the fluorescence of the acceptor.[66–68] The efficiency of FRET relies on the close proximity, within 10 nm, of the FRET pair, and the overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor termed spectral overlap.[69] Consequently, this enables the use of efficient FRET pairs to be fused with proteins of interest to enable detection of protein–protein interactions. Bossis *et al.*[70] developed a FRET-based sumoylation assay by fusing yellow fluorescent protein (YFP) with SUMO (YFP-SUMO) and fusing cyan fluorescent protein (CFP) with the C-terminal domain of Ran GTPase-activating protein, RanGAP (CFP-GAPtail). RanGAP was selected as the model protein since it was the first known protein to be SUMOylated efficiently, and it could be directly modified by Ubc9. The covalent conjugation of YFP-SUMO to the CFP-GAPtail upon sumoylation resulted in a quenching of fluorescence in YFP-SUMO. This was detected by excitation at 485 nm and emission at 527 nm using a fluorescence microplate reader. This approach was effective in identifying and analyzing SUMO-modifying enzymes and substrates. However, the application of the assay was limited to any ubiquitin-like protein and a target substrate

(mono-ubiquitination) in situations for which polyubiquitin-like protein chain formation does not occur.[70]

Gururaja *et al*.[60] established a homogeneous FRET assay system to monitor polyubiquitination using an APC autoubiquitination reaction. APC is an E3 ligase that targets the marked cell cycle protein for degradation.[71] The FRET approach relied on two sets of fluorophore-labeled ubiquitin (Ub) molecules, fluorescein-5-maleimide (F-Ub) as the donor and tetramethyl-rhodamine-6-maleimide (TAMRA-Ub designated as R-Ub) as the acceptor. On polyubiquitination of APC, the fluorescence quenching of F-Ub, when in the proximity of R-Ub during ubiquitin chain assembly, was continuously monitored for kinetics studies by excitation at 485 nm and emission at 520 nm using a fluorescence microplate reader. At the end of the assay, samples were subjected to non-reducing SDS-PAGE or loaded to a gel filtration column attached to a HPLC with a fluorescent detector at 520 nm to further confirm formation of the polyubiquitin chains. The assay allowed direct detection and eliminated several washing steps, shortening the total assay time. This method was suggested to enable monitoring of the kinetics of ubiquitin chain formation by E3-ubiquitin ligases.[60]

FRET can also incorporate the intrinsic fluorescence property of lanthanide elements, such as europium (Eu) and terbium (Tb), to establish lanthanide chelate excitation (LANCE) technology. Boisclair *et al*.[72] developed a plate-based assay to detect autoubiquitination of ubiquitin-ligating enzyme, Rsc using LANCE. Along with the $Eu^{3}+$ (Eu) as the donor, allophycocyanin (aPC) was used as the acceptor. Streptavidin and anti-Glutathione S-transferase (GST) were used as secondary reagents for detection in the assay and were prelabeled with these fluorophores separately. Next, they were attached to the C-terminus of the Rsc protein and ubiquitin, respectively, via biotin and GST tags. During the transfer of ubiquitin from ubiquitin-conjugating enzyme E2 (Ubc4) to Rsc, Eu was in proximity with aPC, causing a quenching of fluorescence in Eu. This was monitored by excitation at 340 nm and emission at 665 nm using a plate reader. The method avoided the need for covalent modification of Rsc and ubiquitin by direct labeling that could have function. However, the major limitation of this assay was the possibility of forming a large molecular complex as a result of indirect labeling that could cause inefficient FRET due to long distances between the donor and acceptor.[72]

The utilization of lanthanide chelates paved the way to combine FRET with time-resolved (TR) measurements developing TR-FRET. Since lanthanide chelates are long-lived donor fluorophores, they allow for a time delay between the excitation and fluorescence detection. This eliminates non-specific short-lived background signals, leading to an increased fluorescence signal compared with normal FRET.[73–76] A terbium (Tb) based LanthaScreen TR-FRET technology was used by Carlson *et al*.[77] to develop a TR-FRET-based assay to detect sumoylation of the RanGAP1 protein. Two different assay formats were designed and named as the antitarget assay format and the interchain assay format. The antitarget assay format utilized Tb/fluorescein FRET pair as the donor and acceptor, respectively. Fluorescein was used to label SUMO (Fl-SUMO), and Tb was used to label GST antibody. This enabled the detection of sumoylation of GST-tagged RanGAP1 using a fluorescence microplate reader. The method allowed the use of a target substrate to be expressed as a fusion protein. The interchain assay format utilized both Fl-SUMO and Tb-SUMO to detect poly-SUMO chain formation on RanGAP1.[77] Martin *et al*.[78] used a TR-FRET approach as well, to monitor the SUMO1-Ubc9 (SUMO-conjugating enzyme) conjugate while adding RanBP2 (SUMO-ligating enzyme). Since this step was essential for the catalysis of final transfer of SUMO to the target substrate, the assay was indirectly detecting the ubiquitination of the target substrate. Enhanced CFP (E-CFP) and Venus-YFP were used as the FRET pair, and these fluorophores were fused to SUMO1 and Ubc9, respectively. On binding of SUMO1-Ubc9, the FRET between two fluorescent tags was monitored by excitation at 400 nm and emission at 530 nm using a microplate fluorometer. This allowed the use of convenient fluorescent tags making the purification process more straightforward and highlighted the application of the assay to monitor multiprotein interactions in the SUMO pathway.[78]

Fluorescence polarization (FP) is another powerful technique that has been used to detect ubiquitination. This fluorescence-based detection technique is widely used to monitor interactions based on the rotational motion of a small fluorescent molecule (tracer) in solution. A free tracer rotates rapidly in solution and produces a low FP signal as the emission of light happens on many different polarization planes and is depolarized compared with the excitatory polarized light. On the contrary, if the tracer is bound to a larger molecule, it rotates slowly in solution and produces a high FP signal as the emission of light is polarized as it is more likely to occur on the same polarization plane as the excitatory polarized light.[79,80] This method allows real-time monitoring of molecular interactions in solution and can provide binding kinetics for molecular interactions. The measurements are non-destructive; thus, the sample can further be used to reanalyze the effects on binding using different parameters such as temperature or pH.[81] Mot *et al*.[82] utilized a FP-based approach to explore the hypothesized ligase activity of the proteolysis1 (PRT1) enzyme in *Arabidopsis thaliana*. PRT1 was identified as a N-end rule pathway E3 ligase. FP was used to monitor the PRT1-mediated ubiquitination in real time by placing fluorescent labels on artificial substrates with different N-termini to characterize the enzyme specificity, activity, and the mechanism. Ubiquitination was further analyzed using in-gel fluorescence scanning. Compared with the traditional detection methods, this approach allowed for the visualization of molecular association and dissociation steps without affecting the system.[82]

Franklin *et al*.[83] developed an assay referred to as UbiReal to monitor the conjugation of ubiquitin in the ubiquitination cascade in real time using FP (Figure 1). The assay produced significant signals in real time for each step in the cascade based on the changes in FP related to each step. The assay was utilized to differentiate between mono- and polyubiquitination as well. The assay was robust, convenient, and less labor-intensive compared with the traditional gel-based assay. Special reagents were not required, and the assay consumed relatively low quantities of reagents. In addition, a specific step in the cascade could be easily

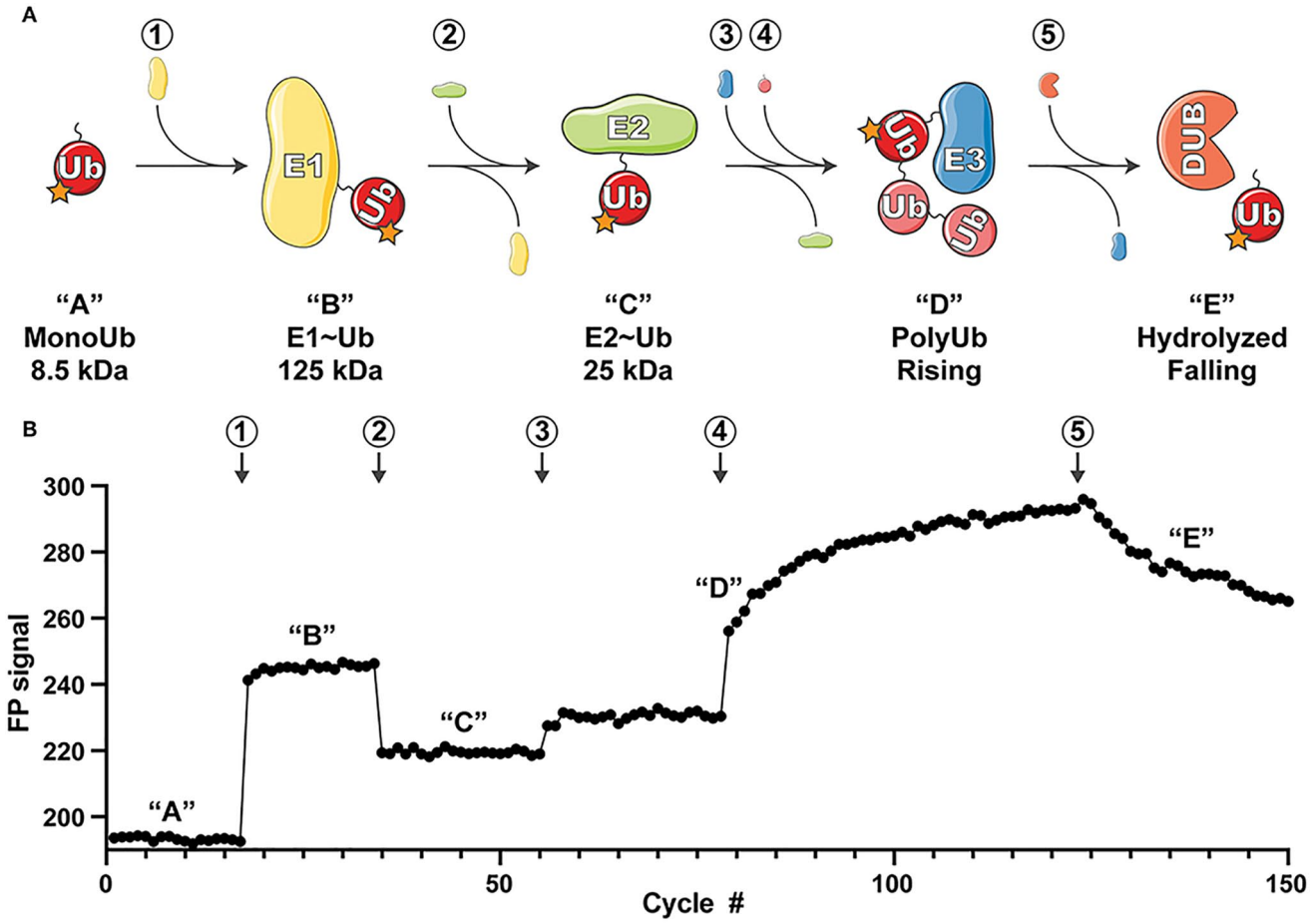

Figure 1. Overview of the UbiReal approach: (A) Schematic representation of the ubiquitin conjugation steps monitored through the UbiReal approach. (B) Data representing the UbiReal approach monitoring the ubiquitin conjugation. A fluorescently labeled ubiquitin was used to track the progression through the cascade involving UBE1 (E1 enzyme), UB2D3 (E2 enzyme), and NleL (E3 enzyme) with significant molecular weights. A large FP signal was produced in the activation step for the conjugation of ubiquitin with UBE with a large molecular weight. Then, the conjugation step involving UBE2D3 with an intermediate molecular weight resulted in a corresponding FP signal. Next, the ligation step involving NleL caused an increase in the FP signal over time due to the addition of unlabeled ubiquitin into the solution, forming a polyubiquitin chain. FP: Fluorescence polarization.
Source: Reprinted from Franklin and Pruneda.[83] Copyright (2019), with permission from Frontiers.

isolated and studied for further analysis, thus, was useful in an instance where any mutation of a protein was to be explored. However, certain limitations were associated with this approach. The approach relies on significant molecular weight differences in each protein involved in each step to produce significant changes in the signal created by FP. The presence of proteins with similar molecular weights may be challenging for producing distinct FP signals for each step. Another limitation of the approach is that the required fluorescent labeling of the protein may hinder certain activities of the protein.[83]

Several assays have been developed on fluorescent complementation to detect ubiquitination as well. Fluorescent complementation is based on the reassembly of two fluorescent protein fragments, each of which is not fluorescent on their own. Once these non-fluorescent fragments are fused with the proteins of interest, if there is any interaction between the proteins, a detectable fluorescence signal is produced by fusion of the fragments of the fluorescent protein.[59,84,85] Lv *et al.*[86] built a fluorescent complementation assay to detect protein ubiquitination in yeast using the two non-fluorescent fusion fragments, the C-terminal fragment (GC) and the N-terminal fragment (GN) of EGFP. The pUbDetec16 plasmid was produced starting with the pY26-TEF/GPD vector and inserting the DNA sequence for GC near the multiple cloning site (MCS) and a DNA sequence encoding for a fusion of GN with the protein product of the ubiquitin encoding Ub13 gene (GN-Ub13). General amino acid permease (GAP1p) protein was selected as the protein of interest since it was known to be ubiquitinated at three amino acid residues: K9, K16, and K76. The wild-type GAP1p was used as the positive control while the triple mutant of GAP1p ($GAPp^{K9R, K16R, K76R}$) was used as the negative control without any ubiquitination sites. The GAP1p gene and $GAPp^{K9R, K16R, K76R}$ were inserted into MCS of pUbDetec16 separately producing pUbDetec16-GAP1p and pUbDetec16-$GAPp^{K9R, K16R, K76R}$. Later, they were transformed into *Saccharomyces cerevisiae* CEN.PK2-1D competent cells, resulting in the co-expression of GAP1p-GC and GN-Ub13. Upon ubiquitination of GAP1p, the two non-fluorescent fusion fragments were fused together forming the fluorescent EGFP. The fluorescence signal was detected only in pUbDetec16-GAP1p transformant indicating the ubiquitination, and no such signal was detected in pUbDetec16-$GAPp^{K9R, K16R, K76R}$. The results

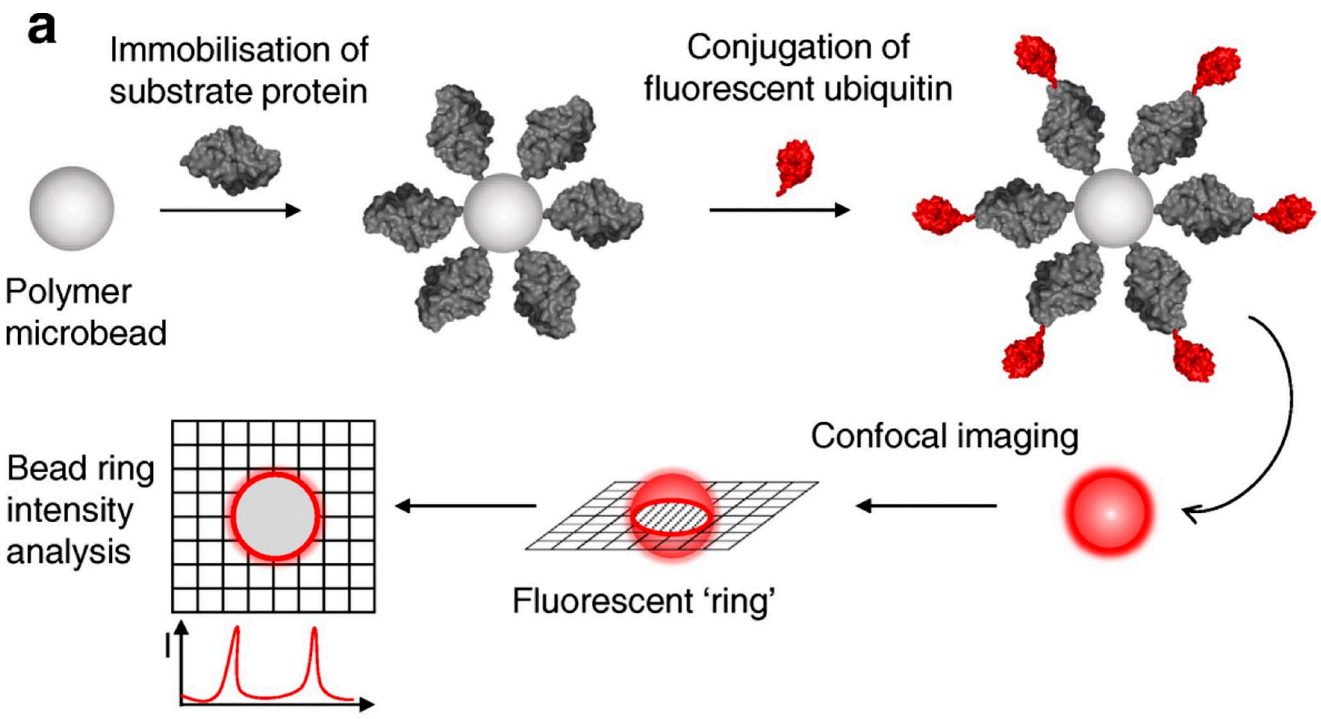

Figure 2. A schematic representation of the overview of fluorescent confocal on-bead assay utilizing ubiquitin-activating enzyme (UBE1), ubiquitin conjugating enzyme (UBE2 L3), E6AP (ubiquitin-ligating enzyme), fluorescently labeled ubiquitin using Cy5 dye (Cy5-ubiquitin), and ATP. Initially, E6AP was immobilized on nickel nitrilotriacetic acid (Ni2 + nitrilotriacetic acid) agarose microbeads and distributed in a microwell plate. Then, the reaction mixture was added, and the reactions were monitored over time. The ubiquitination reaction was monitored by detecting the enzymatic conjugation of labeled ubiquitin onto microbeads, through confocal imaging using an Opera™ High Content Screening System. The conjugation caused the formation of a fluorescent "ring" in the image plane across the microbeads, and the intensity of the fluorescence emission was determined over time. ATP: adenosine triphosphate.
Source: Reprinted from Koszela *et al*.[61] Copyright (2018), with permission from Springer Nature.

obtained by the fluorescent complementation assay were further confirmed by the SDS-PAGE analysis.[86] Similarly, Chen *et al*.[87] designed an ubiquitination-induced fluorescence complementation (UiFC) assay for quantitatively detecting K48 ubiquitin chains *in vitro* and in live cells. The two non-fluorescent fragments, N-terminal and C-terminal of the Venus-YFP, were fused to the ubiquitin-interacting motifs (UIMs) of epsin1 protein, and the resulting proteins were referred to as UiFC-N and UiFC-C. The UIMs of epsin1 protein were known for its high affinity toward polyubiquitin chains compared with monoubiquitin. The *in vitro* assay was plate-based and included different ubiquitination reactions that were carried out for K11, K48, and K63 polyubiquitin chain synthesis. Once the chains were confirmed by immunoblotting using linkage-specific antibodies, they were incubated with UiFC-N and UiFC-C. This allowed the two non-fluorescent fragments of the Venus-YFP to reform the fluorescent complex as a result of the simultaneous binding of UIMs to the polyubiquitin chains. The UiFC fluorescence was measured using a microplate reader, and a greater preference toward K48 chains was displayed by UiFC compared with K11 and K63 chains. Furthermore, it was proposed that these UiFC-N and UiFC could be directly included in ubiquitination reaction from the start as well. The *in vivo* assay was performed using HeLa cells, and the detection was using fluorescence microscopy. The co-localization of UiFC fluorescence (yellow) with the more preferred anti-K48 chain (blue) immunofluorescence led to the quantitative detection of K48 chains in live cells. Furthermore, the use of an Axio Observer fluorescent microscope allowed monitoring of the increase in polyubiquitination under induced stress conditions via time-lapse imaging.[87] Recently, with the emergence of cutting-edge fluorescent confocal microscopy, a defined region within the sample could be exposed to light, compared with the entire sample at once, as in fluorescence microscopy.[88,89] Based on this technique, Koszela *et al*.[61] developed an assay termed ubiquitin–proteasome system-confocal fluorescence nano-scanning (UPS-CONA) to track the ubiquitination cascade using fluorescent confocal imaging, in a real-time manner (Figure 2). This powerful tool can be used to study individual enzymatic activities within the cascade by immobilizing each enzyme separately on the beads in the presence of ubiquitin and the appropriate enzymes in solution and the ubiquitination cascade as a whole by immobilizing a target substrate on beads, in the presence of ubiquitin and all the enzymes in solution. Even though confocal imaging provides high resolution, it may be slow, limiting the application to track fast reactions. In addition, the method is affected by fluorescence bleaching or quenching and is the instrumentation financially costly.[61]

CL assays

CL refers to the production of light by a chemical reaction. The breakdown of an excited state, produced by a chemical reaction, releases energy as photons that can be detected

and quantified. If the excitation event is electromagnetic, the phenomenon is referred to as electrochemiluminescence (ECL), and if it is biological, the phenomenon is referred to as bioluminescence (BL). A typical example for CL is the reaction of luminol in the presence of a metallic catalyst to emit blue light at 425 nm due to oxidation by hydrogen peroxide. CL assays became popular due to their simplicity, high sensitivity, and low background.[90,91] Numerous technologies were built using CL, and several assays were developed using those approaches to detect ubiquitination.[65,92] Based on the CL principle, the amplified luminescent proximity homogeneous assay screen (AlphaScreen) technology was developed which is a bead-based system that uses a cascade of chemical reactions to induce a CL signal. In the presence of an interaction, where the AlphaScreen reagents, the donor bead and the acceptor bead are in proximity; the singlet oxygen species generated by a photosensitizer in the donor bead upon excitation is channeled to react with a thioxene derivative in the acceptor producing a CL signal that further activates a fluorophore in the same bead, emitting light at 520–620 nm. The singlet oxygen species is not detected when there is no interaction between the donor bead and the acceptor bead.[93] This homogeneous, sensitive, and versatile technique offered the advantage of producing reproducible results with minimal quantity of proteins. Rouleau *et al*.[92] utilized AlphaScreen technology to develop a CL-based assay for detecting SUMOylation of a target substrate. The acceptor beads were Ni^2+ coated, and the donor beads were glutathione (GSH) coated. The SUMOylation reaction was performed using E1, E2, His-tagged RanGAP1 E3 ligase, GST-tagged SUMO, and ATP to detect the SUMOylation of RanGAP1. Upon SUMOylation, the SUMO is attached to RanGAP1 resulting in interactions between the acceptor and the donor beads. The Ni^2+-coated acceptor beads interacted with the His-tag of RanGAP1, and the GSH-coated donor beads interacted with the GST tag of SUMO. These donor–acceptor interactions caused the emission of light measured using a plate reader.[92] It was suggested that this method can be used to characterize the activity and kinetics of enzymes involved in SUMOylation as well.

ECL is a unique CL phenomenon where the species generated at the electrode surfaces go through electron transfer chemical reactions producing excited states that emit light. The reaction between tris(2,2′-bipyridine)ruthenium(II) and the co-reactant tripropylamine (($Ru(bpy)_3^2+$/TprA) is a common example of ECL in an aqueous solution. In brief, the oxidation of TPrA at the electrode produces a cation radical of TPrA which rapidly gets converted to a reducing TPrA radical. At the same time, the oxidation of $Ru(bpy)_3^2+$ at the same electrode generates $Ru(bpy)_3^3+$ which is then reduced to an excited $Ru(bpy)_3^2+$ species through the reducing TPrA radical. Decay of the excited $Ru(bpy)_3^2+$ species emits light at 620 nm which can be detected using a photodetector.[94,95] The high versatility, sensitivity, simple setup, and low background noise make ECL assays a good candidate in numerous applications.[96] With the advances in the technique, paramagnetic beads were selected as the carriers for solid-phase ECL measurements, offering the benefit of coating the beads with affinity tags such as streptavidin. The paramagnetic beads-based ECL assay requires two antibody probes targeted against the target antigen of interest: a capture probe (in this case, labeled with biotin) and a detector probe labeled with a ECL label ($Ru(bpy)_3^2+$). Once the magnetic field is placed below the electrode, a sandwich complex is formed between the target and the probes and is captured by the paramagnetic bead. The addition of tri-n-propylamine (TPrA) to the solution with the application of the potential results in the generation of an ECL signal.[94] The ORIGEN analyzer from the ORIGEN technologies is a widely used ECL detector in the field. Davydov *et al*.[65] employed this paramagnetic-based ECL approach to develop an assay to measure the autoubiquitination of human HDM2. The paramagnetic beads were coated with GSH, and the assay captured sandwich complexes formed between the autoubiquitinated GST-tagged HDM2 protein (target antigen) and the two antibody probes: the ECL-labeled (ORIGEN tag) antibody probe and GST-labeled antibody probe. The ORIGEN-M-8-analyzer detected the produced ECL signal at the end of the assay. It was suggested that this assay can be utilized in measuring the activity of other E3-ubiquitin ligases.[65] This assay was more economical with respect to reagent usage and was simple, efficient, and less financially costly since the electrodes were washable and reusable. However, the application of the assay is restricted to *in vitro* studies as the reactions take place only on the electrodes under specific potentials. In addition, the potential toxicity of the $Ru(bpy)_3^2+$ and TPrA is a major drawback of this assay.

BL is a special form of CL where the production and emission of light are triggered by an enzymatic reaction inside a living organism.[97] First discovered in the firefly, it was discovered that luciferase was the enzyme that catalyzed the oxidation of firefly luciferin in the presence of oxygen and ATP-producing oxyluciferin, adenosine monophosphate (AMP), and light. The intensity of light produced correlated to the amount of ATP used. The requirement of ATP for the reaction and the possibility of detection of the produced light using a luminometer highlighted the use of this natural process in developing a classical BL assay to be applied in the laboratory setup.[91,98,99] The same approach was used by Mondal *et al*.[100] to develop a BL assay to monitor the conjugation of ubiquitin and ubiquitin-like proteins. The assay was based on an enzyme-coupled AMP detection system termed as AMP-Glo assay, and it consisted of two main steps. In the first step, the ubiquitination reaction was terminated, and the addition of AMP-Glo reagent 1 depleted the remaining ATP in the reaction mixture. Thereafter, the AMP generated during the ubiquitination reaction was converted to adenosine diphosphate (ADP). Next, an AMP detection solution containing AMP-Glo reagent 2 and kinase Glo-buffer luciferin, and luciferase were added, converting the generated ADP to ATP. The light produced at the end of the assay was a direct measurement of the ATP generated from AMP which was directly proportional to the amount of ubiquitin transferred to E1-activating enzyme. Thus, the generated AMP during the activation step of the cascade was a direct measure of the ubiquitin transferred and enabled the monitoring of the conjugation of ubiquitin and ubiquitin-like proteins. The assay was studied for ubiquitination of Caspase 8/10-associated

RING protein (CARP2) an E3-ubiquitin ligase, and the tumor suppressor, p53, and SUMOylation of RanGAP. This method was recommended for detecting the conjugation of ubiquitin and ubiquitin-like proteins since it permits the use of native ubiquitin and substrates (untagged).[100] However, this assay suffers from low sensitivity when cell extracts are used for the analysis since the presence of free adenine nucleotides in the cell extract may interfere with the BL signal.

Bioluminescence resonance energy transfer (BRET) is another popular cell-based technique for determining molecular interactions. This approach is similar to FRET; however, a bioluminescent donor, usually the luciferase enzyme, is used. Once the enzyme-catalyzed reaction takes place exploiting an appropriate substrate, the acceptor fluorophore will be excited by energy transfer between the donor and the acceptor pair, when in less than 10 nm proximity and have appropriate spectral overlap. Thus, by fusing the proteins of interest with the bioluminescent donor and the acceptor fluorophore, BRET can be employed to measure interactions between the targeted molecules.[101,102] Since BRET involves a bioluminescent donor, it eliminates the disadvantages associated with the FRET technique such as photo-bleaching, auto-fluorescence, and the requirement of excitation of the acceptor fluorophore. In addition, the instrumentation involved with BRET can be simpler and less expensive compared with FRET.[103] Perrory *et al*.[106] used the BRET approach to monitor the ubiquitination of the model protein β-arrestin in real time. β-arrestin is involved in the regulation of G protein-coupled receptor (GPCR) signaling and gets ubiquitinated in response to receptor activation. Class A GPCRs such as the β_2 adrenergic receptor (β_2AR) form a stable complex with β-arrestin while class B GPCRs such as the vasopressin type 2 receptor (V_2R) form a more transient complex.[104,105] This was used to differentiate the sustained and short-lived ubiquitination processes. Renilla luciferase (Rluc) which was used as the bioluminescent donor was fused to the N-terminus of the β-arrestin, and the green fluorescent protein (GFP) was fused to the N-terminus of ubiquitin. As a result, during ubiquitination, the covalent attachment of ubiquitin to β-arrestin will produce a detectable BRET signal when the fused Rluc and GFP groups are brought to close proximity. The assay was initiated by the addition of the Rluc substrate; coelenterazine (DeepBlueC) to the cells co-expressing Myc-tagged V_2R, HA-tagged β_2AR, Rluc-β-arrestin, and GFP-Ub constructs. Upon catalytic degradation of the substrate, emission of blue light occurred at 395 nm, and the energy transfer between Rluc and GFP took place for the ubiquitinated β-arrestin resulting in a re-emission of light which was detected at 510 nm. The results were further confirmed by immunoprecipitation using antibodies for antibody to GFP and the Myc-tag. The BRET assay allowed the detection of ubiquitination of β-arrestin in live cells and to determine the ubiquitination kinetics in real time. The use of living cells provided the advantages of tracking the dynamic nature of the ubiquitination process and eliminated the potential signal variations that can happen from cell lysis, solubilization of the proteins or any purification steps. In addition, this allowed monitoring of different ubiquitination levels in the same cell population at the same time.[106]

Spectrophotometric assays

Spectrophotometry is a widely used analytical approach which is simple, inexpensive, and reproducible. By measuring the changes in the intensity of light absorbed or scattered by a reaction mixture at a certain wavelength range using a spectrophotometer, the progression of an enzymatic reaction is measured. Often, the ultraviolet/visible (UV-Vis) spectroscopy that covers the wavelength range of 180–800 nm is used as the detection method, and if the light falls within the visible range (400–800 nm), the color of the assay can be visualized by eye, and therefore, these assays are referred to as colorimetric assays.[107] Based on this principle, Berndsen and Wolberger[108] developed an indirect colorimetric assay termed molybdenum blue assay which quantified the ubiquitin conjugation by monitoring the pyrophosphate released during the ATP-dependent activation step (first step) of the ubiquitination cascade (Figure 3). Thus, the assay was indirectly detecting the ubiquitination. This assay demonstrated that the production of pyrophosphate by E1 is dependent on the ubiquitin transfer.[108] Since the yeast E2 Ubc13-Mms2 formed polyubiquitin chains in the absence of yeast E3 ligase Rad5, and Rad5 stimulated the rate of formation of polyubiquitin chains by Ubc13-Mms2, the assay was able to determine the rate of unstimulated E1/E2 reaction and the E3 stimulated reaction as well. The kinetics of chain formation determined by the colorimetric assay was further confirmed by a gel-based assay using fluorescently labeled ubiquitin. Samples obtained at different time points were loaded onto an SDS-PAGE, the bands were imaged, and the intensities were measured and later converted to concentrations and fitted to validate the kinetics.[108] This assay offered the advantage of the use of label-free proteins compared with other techniques where labeling is required which may affect the function of enzymes and substrates. However, the use of pyrophosphatase can have a significant ATPase activity, resulting in the generation of phosphate, and leading to a high background signal.

Nanopore sensing

Nanopore (NP) sensing is a relatively new technique for single-molecule analysis based on measuring the temporal changes in the ionic current in the NP. Two chambers filled with electrolytes are divided by an ultrathin membrane and connected via a single nanometer-scale pore. Once a voltage is applied, an electrical potential across the pore is created by dipping the electrodes in the chambers. This generates an electrophoretic force that senses and draws biomolecules to the pore. The translocation of a biomolecule (analyte) via the pore interrupts the steady-state ionic current that flows through the pore and produces detectable modulations in the ionic current. Depending on the size, length, shape, charge, and the interactions of the analyte with the NP wall, the amplitude, duration, and frequency of the modulations are determined.[109,110] Nir *et al*.[111] developed a novel solid-state

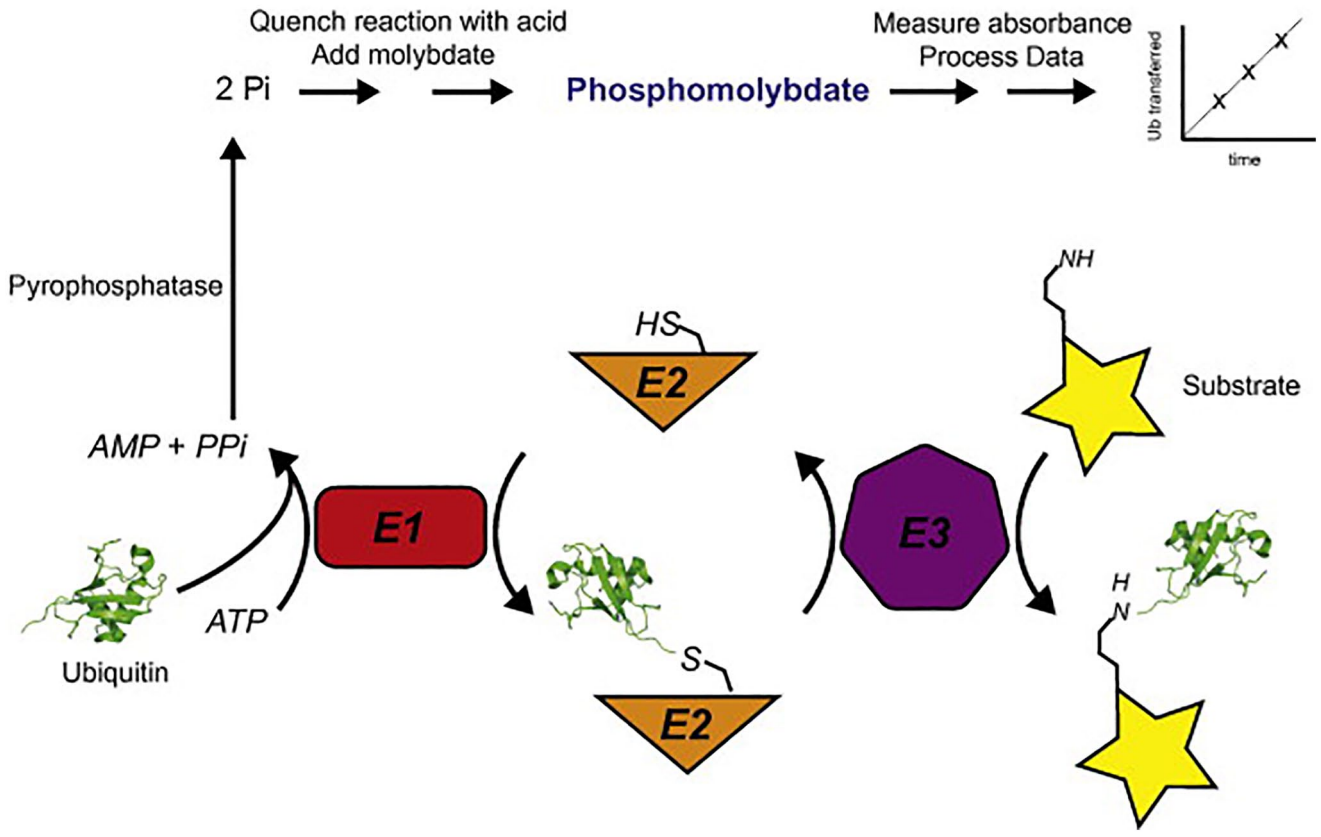

Figure 3. A schematic representation of the molybdenum blue assay. The adenylation of ubiquitin by E1 released pyrophosphate product during the formation of ubiquitin AMP which was then converted to two phosphates via pyrophosphatase. The progression of the reaction was halted, and the phosphomolybdate formation was initiated by the addition of ascorbic acid and ammonium molybdate. Next, the color of the solution was developed using citric acid and sodium arsenite, and the absorbance of phosphomolybdate was measured at 850 nm. Since two phosphate molecules were produced for every ubiquitin transferred, the absorbance was halved and corrected for the background ATP hydrolysis. At the end, the concentration of the phosphate was determined from the corrected absorbance by comparing it to a standard curve. ATP: adenosine triphosphate.
Source: Reprinted from Berndsen and Wolberger.[108] Copyright (2011), with permission from Elsevier.

NP approach for the detection of ubiquitin and for the identification of ubiquitin linkage type at the single-molecule level (Figure 4). To decrease the rate of translocation of the protein through the NP for better analysis, a buffer with a pH closer to the isoelectric point (pI) of the protein was chosen which caused a reduction in the mobility of the protein, allowing more residence time within the NP resulting in better resolution. Using ubiquitin monomers and Lys48 pentamers, the characteristics of single and poly ubiquitinated molecules were analyzed. In addition, this approach was capable of discriminating Lys48 dimers and Lys63 dimers even though they were similar in their molecular weights highlighting its application in single-molecule sensing of differently ubiquitinated substrates. The results obtained by the solid-state NP were validated by a gel-based quantification.[111] Wloka *et al*.[112] established a method to detect the ubiquitination in real time employing an engineered Cytolysin A (ClyA) biological NP. The cis-opening of the pore was larger compared with the trans-opening, trapping the protein for a long time within the pore for better temporal resolution. The self-ubiquitinating yeast E2, Ubc4, was chosen as the model substrate to monitor the ubiquitination reaction. Once the proteins were added to the cis-side of the NP, detectable modulations were observed for the ubiquitination reactions with and without ATP.[112] Compared with existing methods, the NP-sensing approach is rapid, label-free, allow the tracking of simultaneous ubiquitination of different substrates, and less laborious to characterize mono- and polyubiquitin chains. However, the identification of a certain protein in a complex biological sample may be challenging due to the signal from the background proteins.

Conclusions and outlook

Ubiquitination is attained by an enzymatic cascade of steps that regulates various cellular events including proteasomal degradation and many other non-proteolytic functions. The complex and dynamic nature of the ubiquitination process ranging from mono-ubiquitination, multimono-ubiquitination, and multiple types of polyubiquitination enables this exquisite regulation of numerous cellular events. Misregulation of ubiquitination can lead to a wide range of disease conditions. Therefore, the detection of ubiquitination is crucial for conducting comprehensive analyses of the ubiquitination process to better understand the molecular machinery behind this in the hopes of ultimately developing drugs to treat diseases stemming from misregulation of the ubiquitination cascade. With the application of new

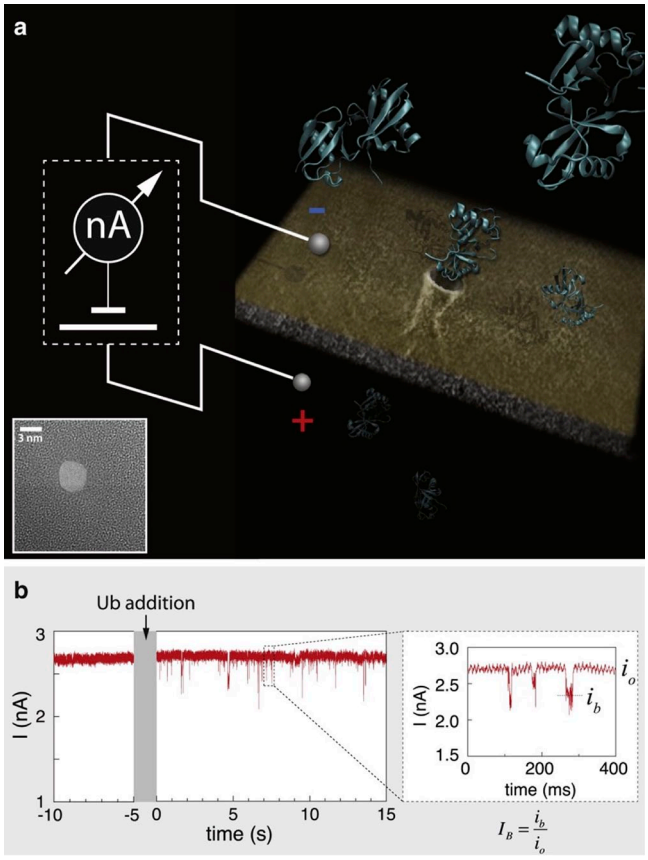

Figure 4. A schematic representation of the passage of di-ubiquitin molecules through the nanopore. The inset for the transmission electron microscopy image displays the solid-state nanopores of 3.5 nm used as single-molecule sensors for ubiquitin and ubiquitin chains.
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technologies, numerous ubiquitination detection techniques have been developed, each with their own benefits and limitations. This minireview outlined the promising ubiquitination detection techniques from established traditional technologies to more recently developed cutting-edge technologies showing that the field continues to develop new avenues for robust, rapid, and versatile detection of ubiquitination.

AUTHORS' CONTRIBUTIONS

All the authors participated in the writing and review of the article.

DECLARATION OF CONFLICTING INTERESTS

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