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Enzyme-based protein tagging systems for site-specific labeling of proteins in living cells

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Abstract

Various protein labeling methods based on the specific interactions between genetically encoded tags and synthetic probes have been proposed to complement fluorescent protein-based labeling. In particular, labeling methods based on enzyme reactions have been intensively developed by taking advantage of the highly specific interactions between enzymes and their substrates. In this approach, the peptides or proteins are genetically attached to the target proteins as a tag, and the various labels are then incorporated into the tags by enzyme reactions with the substrates carrying those labels. On the other hand, we have been developing an enzyme-based protein labeling system distinct from the existing ones. In our system, the substrate protein is attached to the target proteins as a tag, and the labels are incorporated into the tag by post-translational modification with an enzyme carrying those labels followed by tight complexation between the enzyme and the substrate protein. In this review, I summarize the enzyme-based protein labeling systems with a focus on several typical methods and then describe our labeling system based on tight complexation between the enzyme and the substrate protein.

Introduction

Fluorescence imaging of proteins in living cells is an indispensable approach for elucidating their functions, movement, and localization in the native environments. For imaging of proteins in living cells, the desired proteins must be labeled specifically, and various protein labeling methods have been developed for this purpose. Among them, methods with fluorescent proteins have been widely used for their simplicity and specificity, where fluorescent proteins are genetically attached to the target proteins by introducing the genes of the fusion proteins into the cells. The utilization of fluorescent proteins as labels has started from the studies with green fluorescent protein (GFP) from the *Aequorea vitoria* jellyfish [1]; after that, numerous variants of GFP and fluorescent proteins from different species have become available for labeling, which have superior fluorescent properties and cover a wide spectral range [2-4]. Therefore, the utility of fluorescent proteins as labels has been increasingly enhanced.

However, labeling with fluorescent proteins cannot be applied to all protein analyses because of the great diversity of structures and functions of protein molecules. Thus, different types of labeling methods have also been developed to complement fluorescent protein-based labeling. Above all, the labeling methods have been intensively developed which combine genetically encoded tags with synthetic fluorescent probes, where the target proteins are fused to the peptide or protein tags to which fluorescent probes are attached by specific molecular interactions or enzymatic reactions [5-10]. The pioneering approach is based on the peptide tag containing a tetracysteine sequence (TC tag), which was reported by Tsien *et al.* in 1998 [11]. In this system, the target proteins are fused to the TC tag, which are specifically labeled by the reactions with biarsenical compounds such as FlAsH and ReAsH [12,13]. Similar approaches have also been developed by combining an oligo-histidine tag or oligo-aspartate tag with Ni^{2+}- and Zn^{2+}-fluorophore complexes [14-17]. Although these systems have the advantage of using short peptides as a tag, they have a limitation in terms of the specificity of labeling, due to the

nonspecific binding of probes to the cellular components.

Thus, to improve the specificity of labeling, various methods have been developed by exploiting the enzymes with a high recognition ability for their substrates (Fig. 1) [5-10]. Here, the labels are attached to the genetically encoded tags through the enzymatic reactions with their substrates carrying those labels. Most of these enzyme-based labeling methods can be classified into two groups (Figs. 1a and 1b). In one group, the target proteins are fused to the peptide tags, and the labels are incorporated into the tags by the post-translational modification reactions with enzymes (Fig. 1a). In the other group, the enzymes are genetically attached to the target proteins as a tag, and the labels are incorporated into the tags by the self-modification reactions of the enzymes (Fig. 1b). These enzyme-based labeling methods enable highly specific labeling of the proteins and are applied to the labeling of proteins in living cells; some of these methods have already been made commercially available. However, in these methods, it is generally difficult to attach multiple copies of the labels to the target proteins.

To expand the utility of the enzyme-based labeling system, we have been developing an original labeling system that does not fit into either of the two groups described above (Fig. 1c) [18-20]. Here, a substrate protein is genetically attached to the target proteins as a tag, and the labels are attached to the tag by the post-translational reaction of the enzyme carrying those labels followed by tight complexation between the enzyme and the substrate protein. By utilizing this labeling system, we succeeded in the fluorescent detection of the target proteins on a solid support [18,19] or in living cells [18,20]. In this method, multiple copies of labels can be attached to the target proteins by the reaction with the enzyme carrying those multiple labels. In addition, this system can be applied to the labeling of a cellular organelle by controlling the localization of the fluorescent protein in the cells [21]. In this review, I summarize the enzyme-based protein labeling systems focusing on several typical methods and then describe the development of our labeling system based on tight complexation between the

enzyme and the substrate protein.

Protein labeling based on post-translational modification to the peptide-tags

Among the labeling methods based on post-translational modification to the peptide-tags [5,6,9] (Fig. 1a), I focus on those exploiting sortase, phosphopantetheinyltransferase (PPTase), and lipoic acid ligase (LplA). Sortase is a transpeptidase found in many Gram-positive bacteria. In particular, Sortase A (SrtA) from *Stapylococcus aureus* is widely used for protein labeling, which specifically recognizes the short peptide sequence LPXTG (where X is any amino acid) [22]. SrtA cleaves the amide bond between threonine and glycine on the recognition site to produce a thioester bond between the carboxyl group of threonine and an enzyme-derived cysteine. This intermediate undergoes a nucleophilic attack by an amino group of an oligoglycine substrate to produce a new amino bond between the two substrates. In the typical protein labeling with SrtA, the LPXTG motif is genetically attached to the target proteins as a tag, and various labels are incorporated into the tag by the enzymatic reaction of SrtA with the oligoglycine substrates carrying the labels (Fig. 2a).

The use of SrtA-mediated labeling in living cells was first reported in 2007 by Popp and co-workers [23]. They selected human CD40L, a type II membrane protein, as a target protein. CD40L fused to a C-terminal LPETG tag was expressed in mammalian cells and was labeled with oligoglycine substrates carrying biotin or tetramethylrhodamine (TMR) by SrtA added to the medium. Labeling of CD40L on the surface of the cells was clearly demonstrated by blotting and fluorescence imaging analysis. In the same manner, Tanaka and co-workers succeeded in labeling of osteoclast differentiation factor (ODF) fused to a C-terminal LPETG tag with oligoglycine substrates carrying probes on the cell surface [24]. They also succeeded in protein-protein conjugation on the cell surface, where ODF fused to a C-terminal LPETG tag was ligated to GFP carrying a pentaglycine substrate on its N-terminus. Recently, SrtA-mediated labeling was exploited for identifying receptor-ligand interactions between immune cells [25],

and was also applied to *in viv*o protein labeling in *Escherichia coli* [26] and *Caenorhabditis elegans* [27] by employing Ca^{2+}-independent SrtA.

Labeling with an enzyme PPTase is also one of the typical methods based on the post-translational modification to the peptide-tags [5,6,9]. PPTase catalyzes the transfer of a phosphopantetheinyl group derived from coenzyme A (CoA) to a serine residue of the peptidyl carrier protein (PCP) or acyl carrier protein (ACP) domains of several synthetases [28-30]. Surfactin phosphopantetheinyl transferase (Sfp) from *Bacillus subtilis* and acyl carrier protein synthase (AcpS) from *Escherichia coli* are widely exploited as PPTases in this labeling strategy owing to their promiscuity with respect to the CoA substrate; Sfp and AcpS recognize a wide range of CoA derivatives carrying various labels as substrates [28-30]. In the labeling, PCP or ACP domains are fused to the target proteins as a tag, and the labels are attached to the fusion proteins by Sfp- or AcpS-mediated reactions with CoA derivatives as substrates (Fig. 2b). Based on this strategy, membrane proteins fused to PCP or ACP were successfully labeled on the living cells with fluorescent dyes or biotin followed by Streptavidin-coated quantum dots [30,31].

The size of PCP and ACP comprising 75-80 amino acids is relatively large compared to the small peptide tags used in other labeling approaches. Thus, Walsh and coworkers identified an 11-residue peptide as a substrate for Sfp from a genome library of *B. subtilis* by phage display; that peptide had the sequence DSEFIASKLA and was denoted as ybbR [32]. They demonstrated that ybbR can be fused to either the N- or C-terminus of a target protein or inserted into a flexible loop region of a target protein as a tag. The same group further identified the 12-residue peptides S6 (GDSLSWLLRLLN) and A1 (GDSLDMLEWSLM) from a phage-display peptide library as efficient substrates for Sfp and AcpS, respectively [33]. The specificity of the S6 tag to Sfp is orthogonal to that of the A1 tag to AspS, and by taking advantage of this property, multicolor imaging of two different proteins can be conducted in the living cells through Sfp- and AcpS-catalyzed modifications. In fact, the S6 and A1 tags were fused to epidermal growth

factor receptor (EGFR) and transferrin receptor 1 (TfR1), respectively, and the cells expressing those two fusion proteins were treated sequentially with AcpS in the presence of the Alexa 488-modified CoA and then with Sfp in the presence of the Texas red-modified CoA; as a result, the distribution of the two labeled receptors was clearly visualized on the same cell by fluorescence microscopy [33].

The LplA is also one of the typical enzymes exploited in the labeling strategies based on the peptide-tags [5,6,9]. LplA catalyzes the covalent attachment of lipoic acid to a specific lysine residue of metabolic enzymes in an ATP-dependent manner [34]. Ting and co-workers designed an azidoalkanoic acid which can be used as a substrate for *E. coli* LplA in place of lipoic acid [35]. They also identified a 22-residue peptide substrate for *E. coli* LplA by rational design based on the natural protein substrates of LplA; the peptide was named LAP (LplA acceptor peptide) [35]. In their labeling strategy, LAP is genetically attached to a target protein as a tag, and an alkyl azide is incorporated into the LAP tag by the LplA-mediated reaction with an azidoalkanoic acid as a substrate (Fig. 2c). Subsequently, the alkyl azide moiety is derivatized with a cyclooctyne-fluorophore conjugate via strain-promoted alkyne-azide cycloaddition (SPAAC). Based on this strategy, they successfully labeled a low-density lipoprotein receptor on the surface of the living cells [35]. Then, they further minimized the LAP tag to a 13-residue peptide by *in vitro* selection with a yeast phage display [36], which has been used as a standard tag in an LplA-mediated labeling thereafter.

In order to expand their system to protein labeling inside living cells, Ting's group then created a mutant of LplA recognizing a 7-hydroxycoumarin derivative as a substrate in place of lipoic acid through structure-guided mutagenesis [37]. The cells expressing the mutant of LplA and a target protein fused to the LAP tag were treated with the 7-hydroxycoumarin substrate, and following washout of the excess substrate, they successfully observed the proper distribution of each target protein in the cells by monitoring fluorescence from the substrate.

They also succeeded in labeling of cellular proteins by the two-step labeling procedures based on SPAAC [38] and Diels-Alder cycloaddition [39]. LplA-mediated labeling was also exploited for incorporation of the label into internal sites of a protein [40].

Overall, an advantage of these labeling systems based on the peptide-tags is the small size of the tags fused to the target proteins. In general, short peptides are desirable as a tag to minimize the adverse effects on the physiological roles of the proteins to be analyzed. However, the application of these systems in living cells is mainly limited to the cell surface labeling. In order to conduct the labeling inside living cells, enzymes and the substrates carrying labels need to be introduced into the cells, and it is also necessary to suppress background enzymatic reactions by endogenous components. As mentioned above, in the labeling system with LplA, the labeling inside mammalian living cells was achieved by an elaborate improvement of the systems.

Protein labeling based on the enzyme-tags

In the labeling systems using enzymes as a tag (Fig. 1b), those enabling the labeling inside living cells have been developed by sophisticated engineering of the enzymes and substrates [7,8,10]. Here, I focus on the labeling systems using O^6-alkylguanine-DNA alkyltransferase (AGT) and haloalkane dehalogenase (HLD) as a tag. AGT is a human repair protein, which irreversibly transfers the alkyl group from O^6-alkylguanine-DNA to a cysteine residue in its active site. As the substrate specificity of AGT is relatively low, AGT can react with O^6-benzylguanine (BG) derivatives modified at the para position of the benzyl group, leading to the transfer of the modified benzyl group to its reactive cysteine residue. Johnsson and co-workers applied this enzyme reaction to protein labeling [41,42]; in their system, AGT is genetically attached to the target proteins as a tag, which is modified with BG derivatives carrying various labels through the self-modification reaction of AGT (Fig. 3a). They improved

AGT by mutagenesis based on the directed evolution and finally obtained a mutant of AGT (20 kDa) with 52-fold higher activity toward the BG derivatives compared with the wild-type AGT [43]; its mutant also shows low affinity to DNA and is more resistant to oxidation. This mutant of AGT was named SNAP-tag, and has been applied to labeling of various proteins inside and on the surface of the living cells.

They further developed another variant of AGT which recognizes benzyl-cytosine as the substrate and which they named CLIP-tag [44]. The SNAP-tag and the CLIP-tag exhibit substrate specificities orthogonal to each other, and thus these tags can be used to label the different target proteins simultaneously in living cells. The SNAP-tag labeling was also exploited for the introduction of labels in super-resolution imaging. For example, β-tubulin fused to the SNAP-tag was labeled with a BG derivative carrying a photoswitchable probe in mammalian cells, and the filamentary structures of microtubules were observed with a resolution below the diffraction limit by stochastic optical reconstruction microscopy [45]; similarly, SNAP-tag labeling was successfully exploited in stimulated emission depletion microscopy [46]. The SNAP-tag labeling was also applied to imaging of subcellular structures in the zebrafish embryo [47] and in the tissues of mice [48].

The protein labeling with an enzyme reaction by HLD is another system based on the self-modification reaction of the tag. The HLD removes halides from aliphatic hydrocarbons in a two-step reaction: first, the ester bond is formed between an aspartate residue in the active site and the hydrocarbon substrate via a nucleophilic displacement mechanism, and then the ester bond is hydrolyzed to yield the alcohol as the final product. A mutation to the conserved histidine residue responsible for the hydrolysis of the intermediate ester is known to lead to a trapped intermediate, where the aliphatic substrate remains to be attached to the aspartate in the enzyme [49]. By applying this mutation to the HLD from *Rhodococcs rhodochrous*, Los and co-workers developed a protein labeling system [50,51], where a target protein fused to the

mutated HLD is labeled with the aliphatic chloride carrying various labels through the partial reaction of the HLD (Fig. 3b). They designed and optimized the linker of the aliphatic chloride suitable for incorporation of labels based on the crystal structure of the HLD. They also conducted mutagenesis to the His-mutant to improve the reaction rate; they finally identified a variant of the HLD (33 kDa) having a kinetics superior to that of the original His-mutant by over 4 orders of magnitude: they named this variant HaloTag [50].

To demonstrate the usefulness of the HaloTag system as a labeling method in living cells, they expressed a nuclear factor p65 fused to HaloTag in mammalian cells, and labeled the fusion protein with the aliphatic chloride carrying TMR [50]. As a result, they successfully observed the translocation of the p65 fusion protein from the cytosol to the nucleus upon stimulation of the cells with TNF-α. They also succeeded in monitoring the TNF-α-dependent degradation of IκB by labeling it with the HaloTag system [50]. By taking advantage of its specificity and flexibility, the HaloTag system was exploited for the protein labeling in the super-resolution imaging [52,53] and for staining the tumor nodules in mice [54]. The HaloTag system was also applied to the protein degradation system; in this application, the hydrophobic molecule was attached to the target proteins fused to HaloTag, which were then degraded by the proteasome through the cellular quality control mechanism [55].

As a whole, the advantage of the labeling systems based on enzyme-tags is that the various types of labels can be attached to the target proteins inside living cells by virtue of their high specificity of labeling. On the other hand, the large size of the tags is generally considered to be a disadvantage, because large tags could exert a more significant effect on the structures, functions, and localization of the target proteins. Nevertheless, these labeling systems have already been applied to various systems, including those dealing with tissues and animals as mentioned above.

A labeling system based on biotinylation from the archaeon *Sulfolobus tokodaii*

We have been developing an enzyme-mediated labeling system which is not categorized into the two groups mentioned above (Fig. 1c and Fig. 4) [18-20]. In our labeling strategy, biotinylation from the archaeon *Sulfolobus tokodaii* is utilized as an enzyme reaction. In this biotinylation, biotin protein ligase (BPL) catalyzes the attachment of biotin to a specific lysine residue of its substrate protein, biotin carboxyl carrier protein (BCCP), in a two-step reaction:

$$\text{Biotin} + \text{ATP} \rightarrow \text{Biotinyl-AMP} + \text{PPi} \quad (1)$$

$$\text{Biotinyl-AMP} + \text{apo-BCCP} \rightarrow \text{holo-BCCP} + \text{AMP} \quad (2)$$

First, biotinyl-AMP is produced by the reaction of biotin with ATP, and then, biotin is transferred to the lysine residue of BCCP (apo-BCCP), resulting in production of the biotinylated BCCP (holo-BCCP). Although biotinylation is ubiquitous in nature, we focused on the biotinylation from *S. tokodaii* [56,57]. One of the notable features of *S. tokodaii* biotinylation is that the enzyme BPL forms an extremely stable complex with its product, holo-BCCP [58]. We applied this unique enzyme reaction to protein labeling by genetically attaching BCCP to the target proteins as a tag [18-20]. The resulting fusion proteins were labeled by biotinylation with BPL carrying fluorophores (Figs. 4a and 4b).

To construct such a labeling system, we first prepared the truncated BCCP. From the previous studies on biotinylation from other organisms, it is known that BCCP is composed of N- and C-terminal domains, and that the N-terminal domain of BCCP is not responsible for biotinylation. Here, *S. tokodaii* BCCP is composed of 169 amino acid residues, and the region of the N-terminal 100 residues is deduced to be the N-terminal domain from a comparison of the primary sequence of *S. tokodaii* BCCP with those of other organisms. Therefore, we prepared a truncated BCCP which lacks the N-terminal 100 residues [58]. The truncated BCCP

(69 aa) was confirmed to retain the substrate activity for biotinylation. In addition, the binding affinity of holo-BCCP with BPL was found to be enhanced by deletion of the N-terminal domain; the dissociation constant for the complex of the holo form of the truncated BCCP with BPL was estimated to be 1.2 nM based on surface plasmon resonance measurement with a BPL-modified sensor chip [58]. The reaction rate of biotinylation was also found to be high enough to be used for labeling; the rate constant for the reaction between the truncated BCCP and BPL was estimated to be $2.5 \pm 0.3 \times 10^4$ s^{-1} M^{-1} by blotting analysis of the products [18]. Furthermore, from the model structure of the complex of *S. tokodaii* BPL and BCCP, it was inferred that the N- and C- termini of the truncated BCCP are located on the opposite side of the lysine residue to be biotinylated (Fig. 4c), and thus it was considered that the target proteins can be fused to both termini of BCCP without interfering with the biotinylation reaction. Based on these facts, we utilized this truncated BCCP as a tag in the labeling system based on *S. tokodaii* biotinylation.

Next, to construct a labeling system with BPL carrying synthetic fluorophores (Fig. 4a), we modified BPL on its cysteine residues with maleimide derivatives of fluorescent dyes. *S. tokodaii* BPL (233 aa) carries three cysteine residues (Cys3, Cys204, and Cys219), and from the model structure of the complex, it was deduced that these cysteine residues are not responsible for biotinylation, and thus are amenable to modification (Fig. 4c). To enhance the sensitivity of the labeling system, we constructed a mutated BPL with an additional cysteine residue [18]. Specifically, Arg152 located on the opposite side of the binding interface with BCCP was converted to a cysteine residue (Fig. 4c). This mutant of BPL was modified with maleimide derivatives of fluorescein or DyLight547; the extent of labeling was estimated to be 3.6 dyes per BPL molecule on average. As expected, the modified BPLs were found to retain the enzymatic activity and high binding affinity to holo-BCCP [18], and thus these modified BPLs were used for labeling of the target proteins.

Fluorescence labeling of proteins in living cells based on *S. tokodaii* biotinylation

To assess the feasibility of the *S. tokodaii* biotinylation-based labeling system in living cells, we first conducted labeling of a membrane protein on the cell surface by using BPL modified with synthetic fluorophores [18]. Specifically, bradykinin B2 receptor (B2R) was selected as a target membrane protein. BCCP was fused to the N-terminus of B2R, which is exposed to the outer side of the plasma membrane. The resulting fusion protein (BCCP-B2R) (Fig. 4d) was expressed in mammalian cells, and labeled with BPL carrying multiple copies of fluorescein or DyLight547. After washing, the cells were observed by confocal microscopy. As a result, fluorescence from each dye was clearly observed on the surface of the cells, showing the specific labeling of BCCP-B2R with BPL carrying fluorescent dyes. In addition, we succeeded in monitoring the internalization of the labeled BCCP-B2R upon addition of its agonist, bradykinin.

Next, we applied *S. tokodaii* biotinylation to the labeling of proteins inside living cells. Here, BPL carrying GFP on its N-terminus (GFP-BPL) was used as a fluorescent probe, which was expressed in the cells by introducing its expression plasmid by DNA transfection (Fig. 4b). With GFP-BPL, B2R carrying BCCP on its C-terminus (B2R-BCCP) (Fig. 4d) was labeled on the inner side of the plasma membrane [18]. In the cells expressing both fusion proteins, fluorescence from GFP was clearly observed along the periphery of the cells by confocal microscopy. On the other hand, fluorescence was observed from the whole area of the cells, when B2R not carrying BCCP was coexpressed with GFP-BPL. From these results, it was found that the target protein can be labeled through association of BPL with BCCP inside the cells.

To further assess the characteristics of labeling with BPL fused to fluorescent protein, the cytoskeletal proteins, β-actin and α-tubulin, were selected as target proteins and labeled in the living cells [20]. The β-actin carrying BCCP on its N-terminus (BCCP-Actin) (Fig. 4d) was coexpressed with GFP-BPL, and the cells expressing both fusion proteins were observed by confocal microscopy. As expected, filamentary structures derived from the actin filaments were

clearly observed in the cells (Fig. 5). This result indicates that BCCP-Actin was labeled by GFP-BPL and the labeled BCCP-Actin molecules were successfully incorporated into the actin filaments. We also succeeded in observation of the actin filaments by labeling BCCP-Actin with BPL carrying a red fluorescent protein, DsRed, as a fluorescent probe, demonstrating that this labeling system allows for attaching different types of fluorescent proteins to a target protein without reconstructing the expression system for that protein. This is regarded as a practical advantage over the conventional labeling system with fluorescent proteins, in which fluorescent proteins are directly attached to the target proteins.

We also attempted the labeling of β-actin carrying BCCP on its C-terminus (Actin-BCCP) (Fig. 4d) with GFP-BPL. In this case, filamentary structures were not observed in the fluorescence image, but granular spots were observed in the cytosol [20]. Same behavior was observed in the cells expressing a β-actin fusion protein in which GFP is directly attached to the C-terminus of β-actin. These results indicate that attaching the protein to the C-terminus of β-actin inhibits the formation of actin filaments. This behavior seems to be reasonable, considering the fact that the C-terminus of β-actin is located on the binding interface between the β-actin molecules in actin filaments [59].

We next attempted labeling of another cytoskeletal protein, α-tubulin, with our labeling system [20]. Thus, α-tubulin carrying BCCP on its N-terminus (BCCP-Tubulin) (Fig. 4d) was coexpressed with GFP-BPL, and the cells expressing both fusion proteins were observed by confocal microscopy. As a result, filamentary structures were only partially observed in the fluorescence image, indicating that BCCP-Tubulin complexed with GFP-BPL was not effectively integrated into the microtubules. We inferred that this is derived from the fact that the N-terminus of α-tubulin is located on the inner face of a microtubule and thus the BCCP moiety of BCCP-Tubulin is displayed on the inner face when it is integrated into a microtubule. In this case, integration of BCCP-Tubulin complexed with GFP-BPL into a microtubule might

be impeded due to the steric hindrance.

Thus, we attempted labeling of α-tubulin carrying BCCP on its C-terminus (Tubulin-BCCP) (Fig. 4d); the C-terminus of α-tubulin is located on the outer surface of the microtubule. In the cells coexpressing Tubulin-BCCP and GFP-BPL, fine filamentary structures derived from the microtubules were clearly observed [20], showing that Tubulin-BCCP was labeled by GFP-BPL and that the labeled Tubulin-BCCP molecules were successfully incorporated into the microtubules. On the other hand, in the cells expressing α-tubulin directly fused to GFP on its C-terminus, filamentary structures were scarcely observed [20]; the same result was observed in the previous study [60]. This demonstrates that our approach has an advantage over the conventional labeling method with fluorescent proteins. The origin of this advantage has not yet been clarified, but we speculate that the BCCP moiety functions as a rigid spacer between GFP-BPL and α-tubulin to avoid the steric hindrance that accompanies the formation of microtubules.

Fluorescence labeling of the nuclear envelope based on *S. tokodaii* biotinylation

Protein labeling based on *S. tokodaii* was recently applied to labeling of the nuclear envelop (NE) in living cells [21]. Labeling of the NE is mostly conducted with the inner nuclear membrane (INM) proteins fused to fluorescent proteins as markers [61-63]. However, in these methods, the INM proteins interact with various components in the nucleus, and as a result, expression of the INM proteins-markers could perturb the functions of the nucleus. Thus, we developed a labeling method for the NE that does not rely on the INM proteins, by exploiting *S. tokodaii* biotinylation. Here, labeling of the NE is accomplished by trapping GFP on the INM based on the interaction between BPL and BCCP (Fig. 6).

Specifically, BPL was fused to the C-terminus of a single transmembrane domain (TM) of the human platelet-derived growth factor receptor (Fig. 6a). The resulting fusion protein, TM-

BPL, was expressed in mammalian cells, with the BPL moiety being exposed to the surface of the membrane facing the cytoplasm or nucleoplasm. On the other hand, BCCP was fused to the N-terminus of GFP carrying the nuclear localization signal (NLS) (Fig. 6a), and the resulting fusion protein, BCCP-GFP-NLS, was coexpressed with TM-BPL in mammalian cells. In the cells expressing both fusion proteins, BCCP-GFP-NLS is transferred into the nucleus and it is then trapped on the INM through the complexation between BPL and BCCP moieties via biotinylation, resulting in the selective labeling of the NE (Fig. 6b). With this method, we succeeded in observing the breakdown and reformation of the NE during mitosis [21]. In addition, the difference in timing of the formation of the NE and the nuclear lamina was clearly visualized by simultaneous labeling of both components.

In the NE labeling based on *S. tokodaii* biotinylation, a characteristic of the molecular transport in the periphery of the nuclear pore complex (NPC) is utilized to enhance the selectivity of the labeling (Fig. 6c). While the soluble proteins move into the nucleus through the central pore of the NPC, the membrane proteins move through the narrow channel at the boundary between the NPC and the nuclear membrane. Here, there is a size constraint in the transport of the membrane proteins; if the size of domains in the cytoplasmic/nucleoplasmic side of the membrane proteins exceeds 60 kDa, they cannot pass through this channel [64,65]. In this sense, TM-BPL can pass through the channel because the molecular size of BPL is 27 kDa. However, when TM-BPL is complexed with BCCP-GFP-NLS on the INM, its entire size in the nucleoplasmic side exceeds 60 kDa and thus the complex cannot pass through the channel, which results in the retention and concentration of the label on the INM.

One mechanism remains to be clarified in this NE labeling system: the relocalization of the GFP signal on the newly formed NE during cell division. Time-lapse imaging of the cells during mitosis revealed that the complex of BCCP-GPF-NLS with TM-BPL formed on the INM of the original cells is relocalized to the INM of the daughter cells; this behavior cannot be

explained by the mechanism mentioned above. We envisage that this fact contains important knowledge regarding the mechanism of the NE reformation, and a further investigation is now underway.

Conclusion

In this review, I summarized the enzyme-based methods of protein-labeling, which have been developed to complement the labeling methods using fluorescent proteins. As described above, most of the enzyme-based labeling methods can be classified into two groups: the methods based on peptide-tags and those based on enzyme-tags. Both approaches allow for attachment of various synthetic labels to the target proteins by taking advantage of the highly specific interactions between the enzymes and their substrates. The advantage of the former methods is that the short peptides are used as a tag; however, the application to living cells is mostly limited to the cellular surface. On the other hand, the latter methods enable labeling of proteins inside the living cells. In spite of the disadvantage of the large size of the tags, at present, these are considered to be the most practical methods to complement the fluorescent protein-based techniques.

I also summarized the labeling method based on *S. tokodaii* biotinylation. In this method, the substrate protein and the enzyme carrying fluorophores are utilized as the tag and fluorescent probes, respectively. Thus, this method is regarded as distinct from the other enzyme-based labeling methods. One of the characteristics of this method is that multiple copies of fluorophores can be attached to the target protein by using BPL modified with the synthetic fluorescent dyes, resulting in highly sensitive fluorescence detection; in most of the enzyme-based labeling methods, only a single fluorophore is attached to the target protein. The method based on *S. tokodaii* biotinylation also enables the labeling of proteins inside living cells by using BPL fused to fluorescent proteins as a probe. In this approach, different types of

fluorescent proteins can be attached to the target proteins without reconstructing the expression systems for those target proteins. In addition, the timing of labeling could be controlled in principle because fluorescent proteins are not directly attached to the target proteins. By taking advantage of this property, we are now constructing a labeling system in which the timing of labeling can be controlled by adjusting the expression of the fluorescent probes in the cells.

The *S. tokodaii* biotinylation can also be applied to labeling of a cellular organelle by using it to control the localization of the fluorescent protein, as exemplified in labeling of the NE. From this point of view, an approach which combines *S. tokodaii* biotinylation with fluorescent proteins would be considered a functional extension of the fluorescent protein-based labeling. In the future, we anticipate that such an approach will be used for the labeling of different cellular components and the exploration of various cellular mechanisms.

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Figure legends

Fig. 1. Protein labeling based on the enzymatic reactions. (a) A target protein is fused to a peptide, and a substrate carrying an appropriate label is attached to the peptide tag through the post-translational modification reaction with the enzyme. (b) A target protein is fused to an enzyme, and the substrate carrying an appropriate label is attached to the tag through the self-modification reaction of the enzyme. (c) A target protein is fused to a substrate protein, and the enzyme carrying an appropriate label is attached to the protein tag through complexation between the enzyme and the substrate protein following the post-translational modification reaction.

Fig. 2. Protein labeling based on post-translation modification to the peptide tags. (a) A target protein is fused to a pentapeptide LPXTG, which is labeled with a modified oligoglycine peptide by an enzyme reaction with SrtA. (b) A target protein is fused to PCP, ACP or a peptide tag, which is labeled with a coenzyme A derivative by an enzyme reaction with PPTase (Sfp or AcpS). (c) A target protein is fused to the LAP-tag, which is labeled with an alkylazide by an enzyme reaction with LplA, and the alkyl azide moiety is then derivatized with a cyclooctyne-fluorophore conjugate.

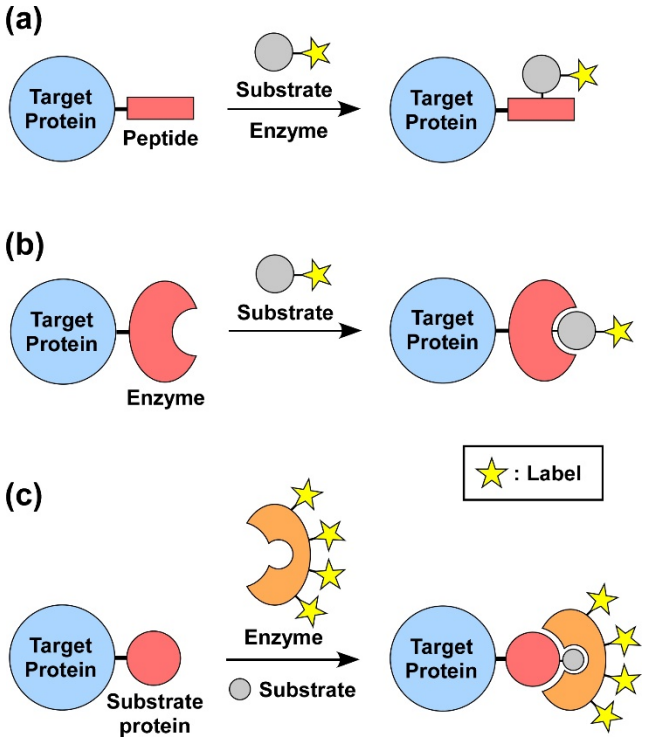
Fig. 3. Protein labeling based on the enzyme-tags. (a) A target protein is fused to the mutated AGT (SNAP-tag or CLIP-tag), which is labeled with a benzyl guanine (BG) or benzyl cytosine (BC) derivative via the self-modification reaction of the SNAP-tag or the CLIP-tag. (b) A target protein is fused to the mutated HLD (HaloTag), which is labeled with a modified aliphatic chloride via the self-modification reaction of the HaloTag.

Fig. 4. A labeling system based on *S. tokodaii* biotinylation. (a) A target protein fused to BCCP is labeled by BPL modified with synthetic fluorophores through biotinylation. (b) A target protein fused to BCCP is labeled by BPL fused to a fluorescent protein coexpressed in the cell through biotinylation. (c) A model structure of *S. tokodaii* BPL complexed with the truncated BCCP lacking the N-terminal 100 amino acid residues [18]. BPL and BCCP are shown in green and blue, respectively. Biotin, cysteine residues, Arg152 of BPL, and Lys135 of BCCP to be biotinylated are shown with space-filling models. N and C represent the N- and C-termini of the truncated BCCP, respectively. (d) Domain structures of the target proteins fused to BCCP.

Fig. 5. A typical fluorescence image of cells coexpressing BCCP-Actin and GFP-BPL. HeLa cells were transfected with the expression plasmids for BCCP-Actin and GFP-BPL. Twenty-four hours after transfection, the cells were imaged by confocal laser scanning microscopy. The left and right panels represent a fluorescence image derived from GFP and a differential interference contrast image of cells, respectively. Scale bars represent 10 μm.

Fig. 6. Fluorescence labeling of the NE based on *S. tokodaii* biotinylation. (a) Domain structures of TM-BPL and BCCP-GFP-NLS. (b) Schematic illustration of the cell coexpressing TM-BPL and BCCP-GFP-NLS. TM-BPL is distributed in the membrane network of the cell including the INM. BCCP-GFP-NLS is trapped on the INM through the complexation between BPL and BCCP moieties. ER and ONM represent the endoplasmic reticulum and the outer nuclear membrane, respectively. (c) Movement of the membrane protein in the periphery of the NPC. TM-BPL can travel between the INM and the ONM when it is not complexed with BCCP-GFP-NLS; however, upon complexation with BCCP-GFP-NLS on the INM, it cannot move to the ONM because of the size constraint of the channel at the boundary between the nuclear membrane and the NPC.

Fig. 1. S. Sueda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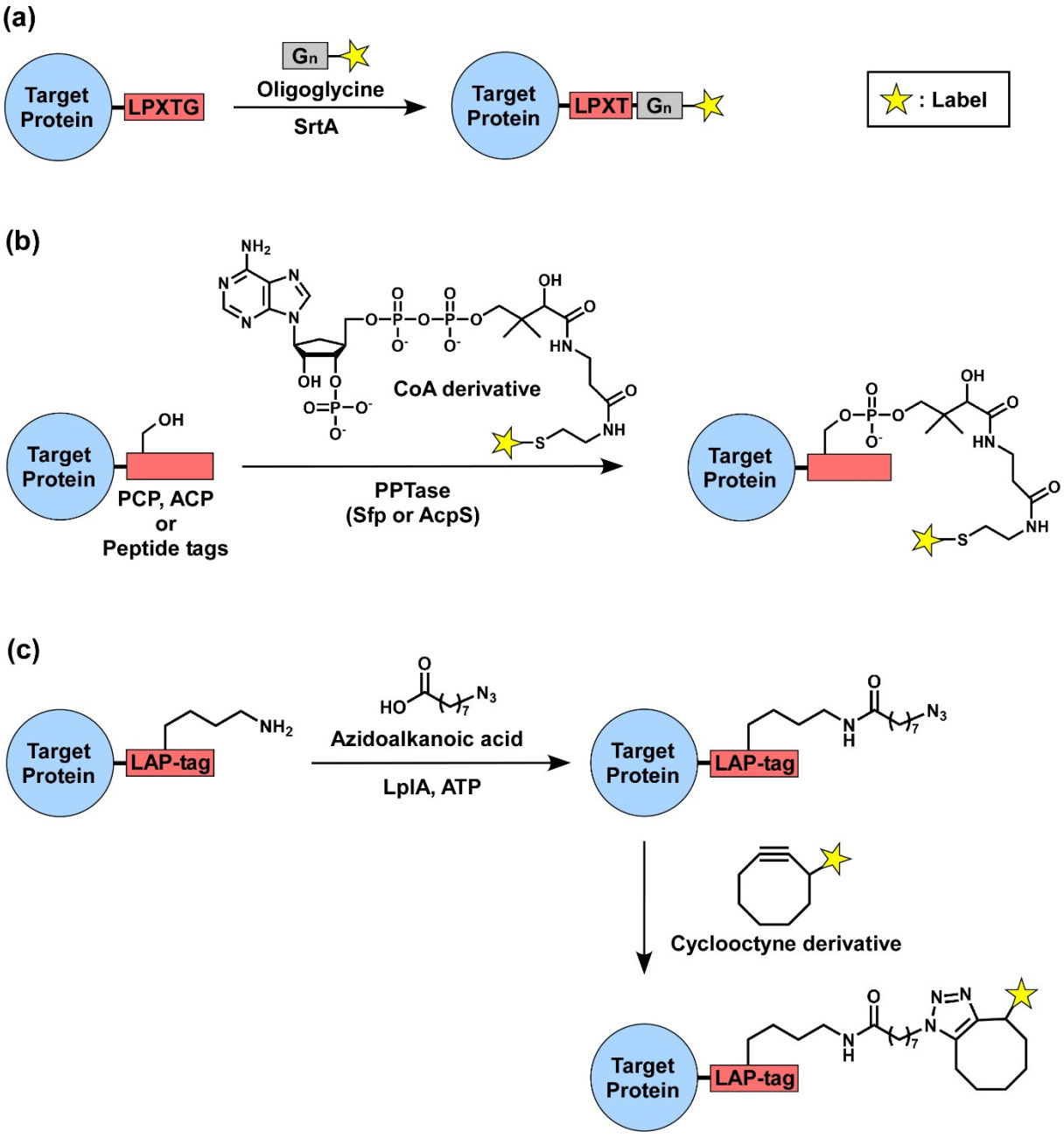
Peptide
Substrate
Enzyme
Target Protein
(b)
Target Protein
Enzyme
Substrate
Target Protein
: Label
(c)
Target Protein
Substrate protein
Enzyme
Substrate
Target Protein

Fig. 2. S. Sueda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Oligoglycine
SrtA
Target Protein
LPXT
Gn
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(b)
CoA derivative
Target Protein
PCP, ACP or Peptide tags
PPTase (Sfp or AcpS)
Target Protein
(c)
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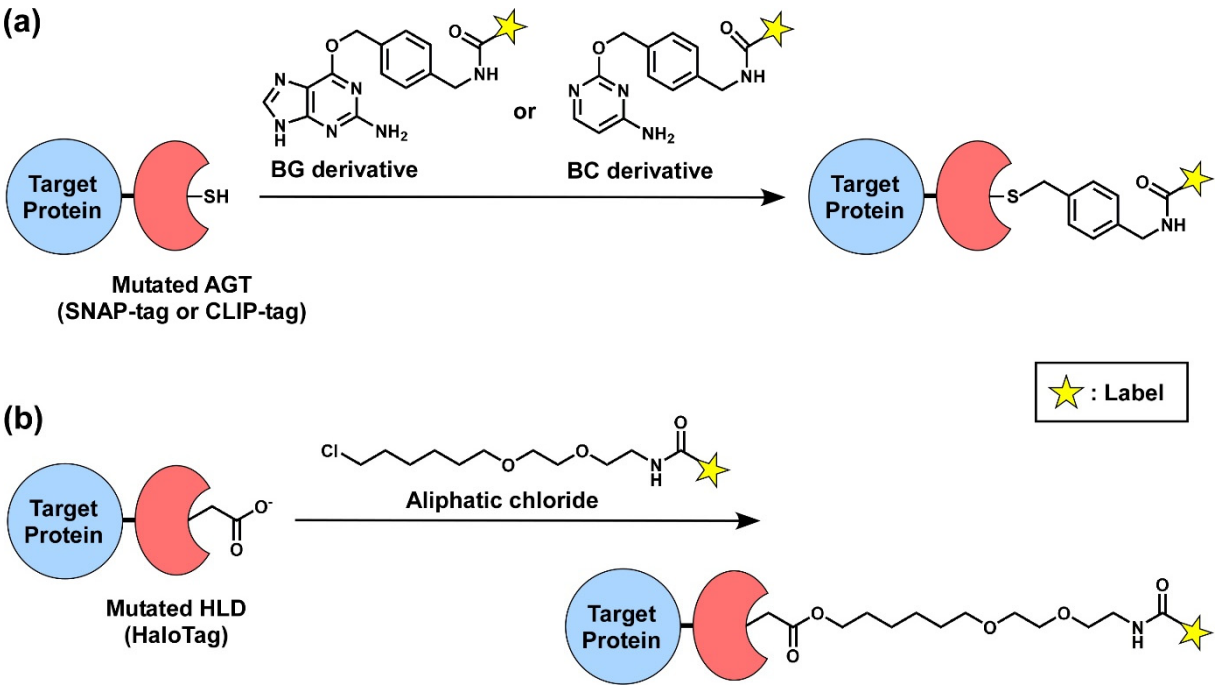
Cyclooctyne derivative
Target Protein
LAP-tag

Fig. 3. S. Sueda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Mutated AGT
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BG derivative
or
BC derivative
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(HaloTag)
Cl
Aliphatic chloride
Target Protein

Fig. 4. S. Sueda

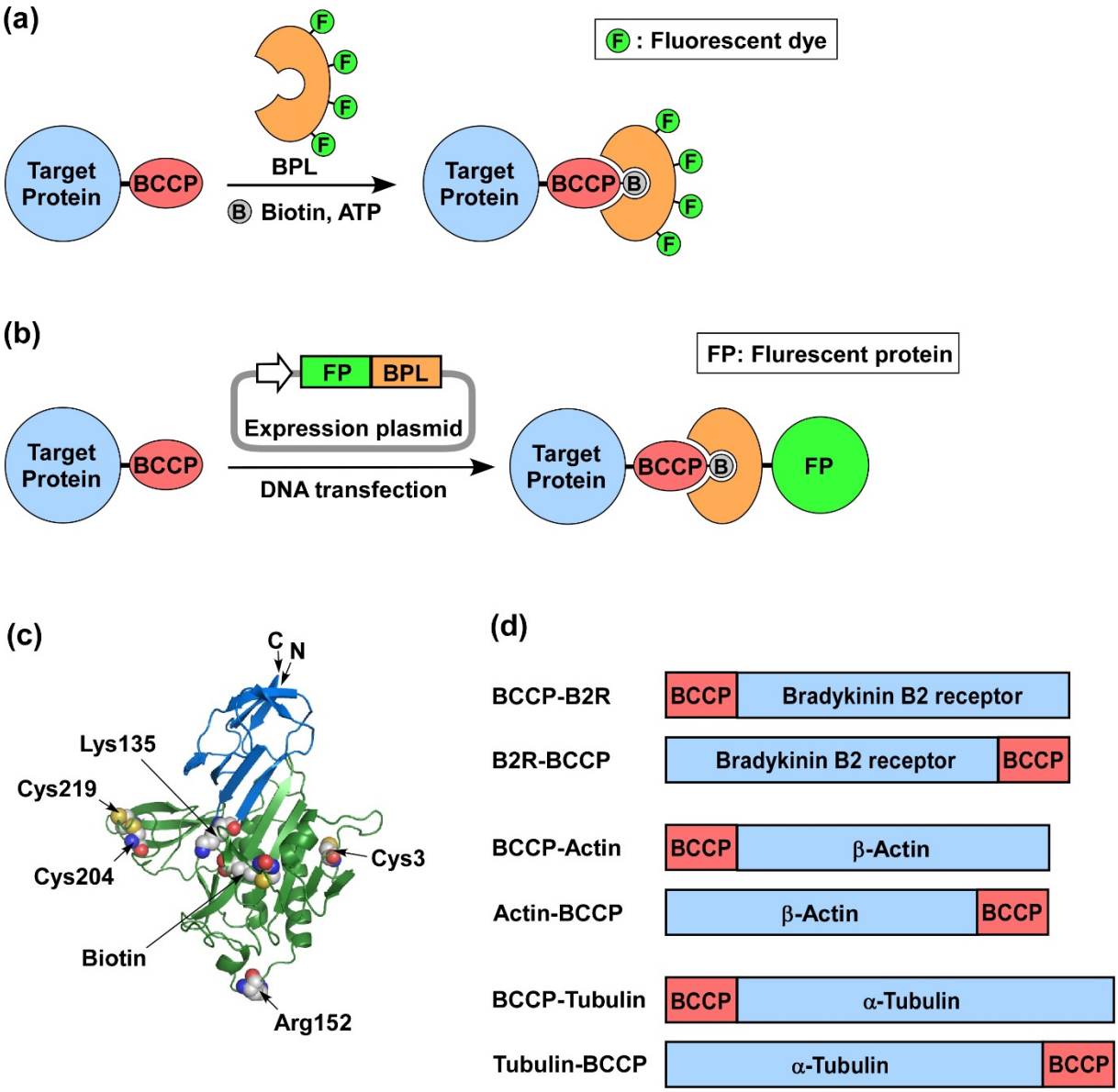
(a)
F : Fluorescent dye
Target Protein
BCCP
BPL
B Biotin, ATP
(b)
FP: Flurescent protein
FP
BPL
Expression plasmid
DNA transfection
FP
(c)
C
N
Lys135
Cys219
Cys204
Biotin
Cys3
Arg152
(d)
BCCP-B2R
BCCP
Bradykinin B2 receptor
B2R-BCCP
Bradykinin B2 receptor
BCCP
BCCP-Actin
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Fig. 5. S. Sueda

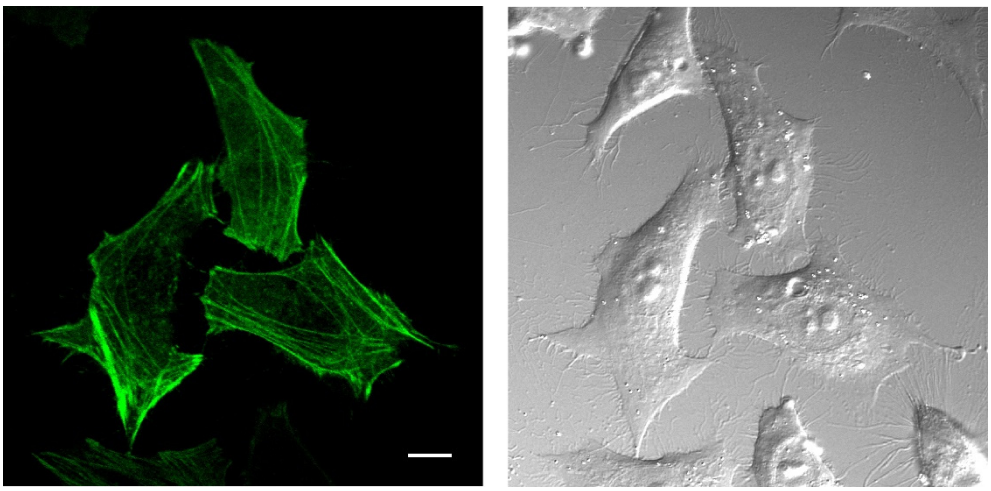
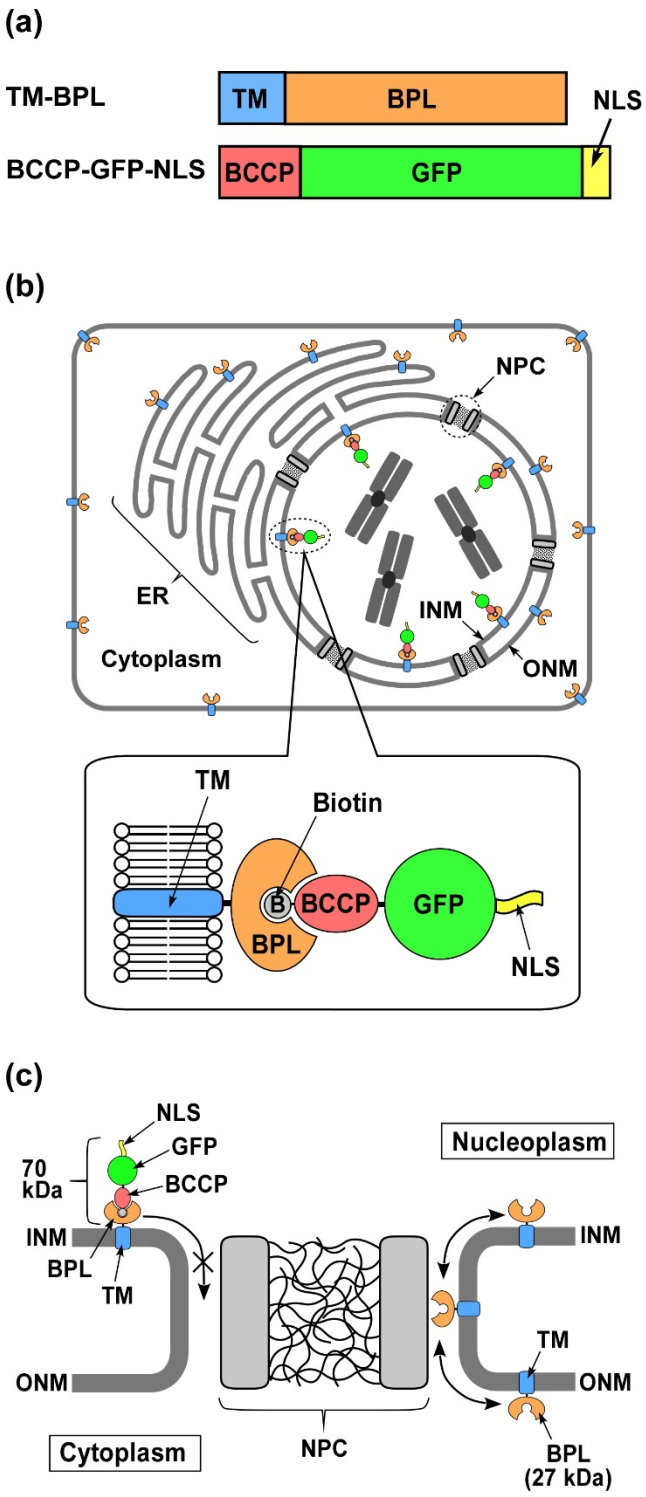


Fig. 6. S. Sueda


(a)
TM-BPL
TM
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BCCP
GFP
(b)
NPC
ER
INM
Cytoplasm
ONM
TM
Biotin
B
BCCP
GFP
BPL
NLS
(c)
NLS
GFP
70 kDa
BCCP
Nucleoplasm
INM
BPL
TM
INM
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ONM
ONM
Cytoplasm
NPC
BPL (27 kDa)