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Posttranslational Modifications

Simultaneous Measurement of SUMOylation using SNAP/CLIP-Tag-Mediated Translation at the Single-Molecule Level**

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SUMOylation, the covalent attachment of a SUMO monomer (SUMO = small ubiquitin-like modifier) or polySUMO chains to a target protein, regulates a variety of cellular processes.[1,2] Perturbation of this modification system causes serious growth defects in yeast[3] and embryonic lethality in mice.[4] SUMOylation is also involved in the pathogenesis of several human diseases, including cancer,[5] Parkinson's diseases,[6] Huntington's disease,[7] type 1 diabetes (T1D),[8] and heart diseases.[9] The molecular mechanism underlying these various effects and functions is the diversity of SUMOylation substrates within the cell.[1,10] To date, more than 750 proteins have been identified as targets of SUMOylation.[11,12] These targets include tumor suppressors,[13] transcription factors/cofactors,[14,15] DNA repair proteins,[16] signal transduction proteins,[1] nuclear core complex,[17] chromosome-organization proteins,[2] telomere-binding proteins,[18,19] cell cycle regulators,[20] and viral proteins.[3,21] Because of the large number of SUMOylation substrates and their vital roles in cells, systematic characterization of SUMOylation is imperative for a comprehensive understanding of SUMO-mediated post-translational modification. So far, several approaches including computation-based SUMOylation predication and mass spectrometry-based proteomic assay have been developed as SUMOylation assays. Although both methods can analyze SUMOylation on a large scale, the outputs of the computation-based SUMOylation predication are theoretical outcomes and additional experiments are needed to verify the results,[22] and for the mass spectrometry-based SUMOylation assay, the large remnant of the SUMO peptide upon tryptic digestion complicates the fragment-ion spectra[23] and requires a special database searching strategy[24] or site-directed substitution[25] to facilitate data analysis. A recently developed Ubc9-fusion-directed SUMOylation (UFDS) system[13] is capable of identifying constitutive and inducible SUMOylation in vivo,[26] however, the UFDS system omits the effects of SUMO ligases completely. In addition, artificial fusion of Ubc9with the substrate proteins might restrict the flexibility of Ubc9, resulting in false negatives.[27] Therefore, a facile method for simultaneous SUMOylation measurement is highly desired.

Recently, a variety of chemical-tagging techniques have been developed to append organic fluorophores to individual proteins.[28–34] The SNAP/CLIP-tag labeling technology, which was developed by Johnsson and co-workers, is derived from the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT).[30–33] The SNAP/CLIP-tag can be covalently labeled by O^6-benzylguanine (BG) derivatives and O^2-benzylcytosine (BC) derivatives both in vivo[34] and in vitro,[32] and can be used simultaneously for multiprotein labeling. Herein, by taking advantage of SNAP/CLIP-tag-mediated translation and total internal reflection fluorescence (TIRF)-based imaging,[35] we developed a new approach for the simultaneous measurement of intracellular SUMOylations at the single-molecule level. As shown in Scheme 1, the fusion of the SNAP/CLIP-tag with SUMO gives it alkyltransferase activity.[36] In the case of SUMOylation, the SNAP/CLIP-tag can convert intracellular SUMO modifications into fluorescent signals when the SUMOylated proteins react with fluorescently labeled BG/BC derivatives. Because the SNAP-tag and the CLIP-tag react with BG derivatives and BC derivatives specifically,[32] diverse SUMOylations will be translated into distinguishable fluorescence signals. As a proof of concept, we use the tumor supressor p53[13] and GTPase-activating protein RanGAP1,[17] two well-known SUMOylation substrates, as model targets. Our results demonstrate that SUMO-1-mediated modification of p53 and RanGAP1 can be detected as different fluorescent signals and are well differentiated at the single-molecule level. The sensitivity of our method is as much as 100-fold higher than the conventional immunoblotting assay. More importantly, simultaneous measurement of SUMOylation under different physiological conditions reveals the involvement of diverse molecule mechanisms in response to extracellular stimulations.

A prerequisite for simultaneous SUMOylation detection was that the resulting fluorescent signals should be well discriminated by TIRF microscope. To this end, we measured the emission spectra of BG-Alexa Fluor 647 and BC-ATTO 488 at single-molecule level. As shown in Figure 1 A, the illumination of a single BC-ATTO 488 molecule with 488 nm laser light produced a remarkable emission spectrum with a maximum wavelength of 521 nm, while the excitation

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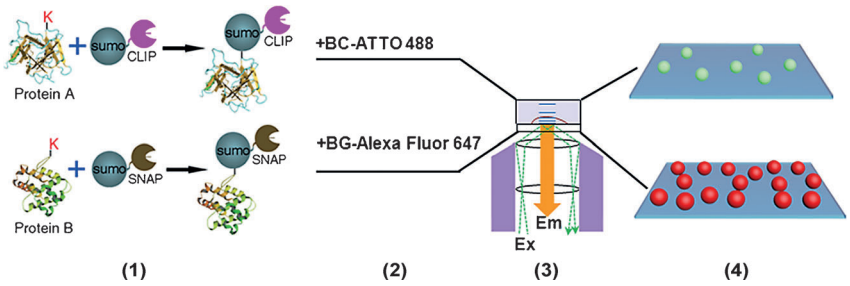

Scheme 1. Scheme for simultaneous SUMOylation assay using SNAP/CLIP-tag-mediated translation and single-molecule detection. The SNAP/CLIP-tag fused to SUMO acts as a bridge between SUMO and two distinguishable fluorescent molecules (BG/BC substrates) by way of a covalent reaction. The fluorescent signals are differentiated by dual-wavelength TIRF microscope at the single-molecule level. The whole process can be recapitulated as: 1) simultaneous SUMOylation in vivo; 2) fluorescent labeling; 3) TIRF imaging; 4) single-molecule detection. K = lysine.

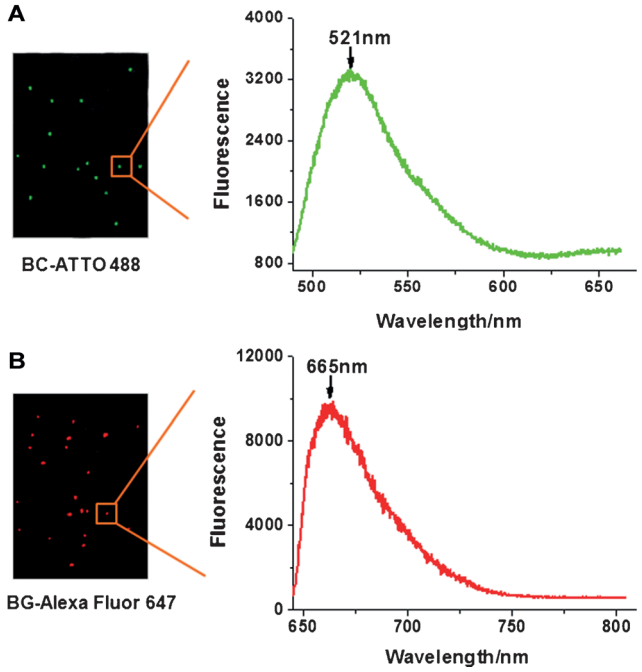

Figure 1. Single-molecule emission spectra of A) BC-ATTO 488 and B) BG-Alexa Fluor 647. The BC-ATTO 488 molecule and BG-Alexa Fluor 647 molecule were excited with lasers at 488 nm and 640 nm wavelength light, respectively. The fluorescent spots are shown as pseudocolors.

of a single BG-Alexa Fluor 647 molecule with 640 nm laser light produced an emission spectrum with a maximum wavelength of 665 nm (Figure 1B). Despite some differences in the fluorescence intensity, the emission spectra of the two fluorescent dyes were well differentiated with negligible overlapping (Supporting Information, Figure S1), suggesting that the ATTO 488/Alexa Fluor 647 fluorescence pair was suitable for the simultaneous SUMOylation assay at the single-molecule level.

To demonstrate the capability of the proposed method for simultaneous detection of two kinds of SUMOylation, the p53 and RanGAP1 proteins were SUMOylated by CLIP-SUMO-1 and SNAP-SUMO-1, respectively. After their incubation with BC-ATTO 488 and BG-Alexa Fluor 647, the SUMOylated p53 protein (p53-SUMO-1) and SUMOylated RanGAP1 protein (RanGAP1-SUMO-1) were imaged by dual-wavelength TIFR microscope. Western blotting analyses were also performed in parallel to verify the expression and modification of p53 and RanGAP1 proteins in the cells. As shown in Figure 2A–H, visible fluorescent spots were observed for SUMOylated p53 with excitation at 488 nm (Figure 2B) and for SUMOylated RanGAP1 with excitation at 640 nm (Figure 2G). In contrast, only background signal was observed for non-SUMOylated p53 (Figure 2A) and non-SUMOylated RanGAP1 (Figure 2E) proteins, indicating that the SUMOylation signals were specifically converted into fluorescent signals. Accordingly, a mixture of SUMOylated p53 and SUMOylated RanGAP1 produced fluorescent spots with dual excitation channels (Figure 2D,H). Notably, the number of fluorescent molecules in the 488 nm channel was much less than that in the 640 nm channel, agreeing well with the heterogeneity of SUMOylated p53 and SUMOylated RanGAP1 proteins inside the cells (Figure 2I, upper panel). These results clearly indicated that the proposed method could detect multiple SUMOylations efficiently and specifically.

The bottleneck in the SUMOylation assay was that only a small portion of the substrates was SUMOylated at steady state,[1,2,10,36] such a low level of modification restricted the SUMOylation identification and in vivo function characterization.[13] To assess whether the proposed method was

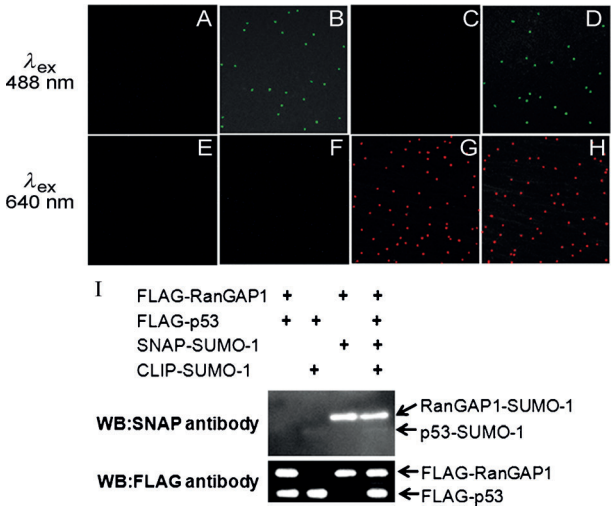

Figure 2. A–H) Simultaneous detection of p53 and RanGAP1 SUMOylation at the single-molecule level under different conditions: A,E) neither p53 nor RanGAP1 were SUMOylated; B,F) only p53 was SUMOylated; C,G) only RanGAP1 was SUMOylated; D,H) both p53 and RanGAP1 were SUMOylated. The images had the background subtracted and were pseudocolored. I) Measurement of the expression (lower panel, FLAG antibody) and SUMOylation (upper panel, SNAP antibody) of p53 and RanGAP1 in (A–H) by western-blot assay.

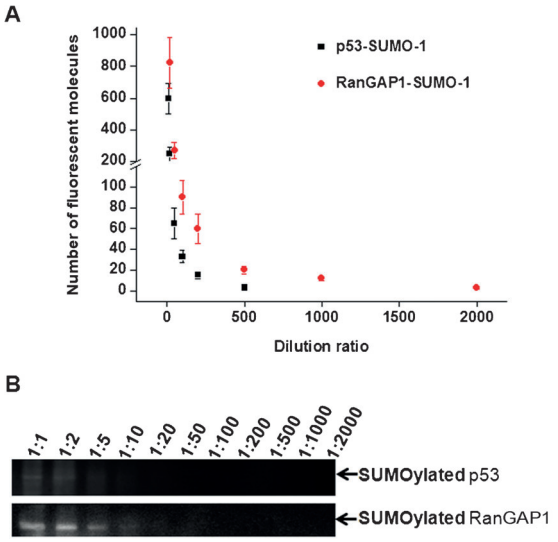

Figure 3. Variance of the number of fluorescent molecules (A) and the amount of SUMOylated target protein (B) as a function of serial dilution. SUMOylated p53 and SUMOylated RanGAP1 proteins were probed with anti-SNAP antibody. Error bars show the standard deviation from three experiments.

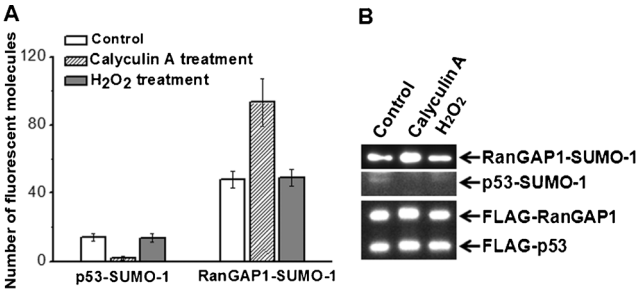

Figure 4. Simultaneous SUMOylation measurement under different physiological conditions. A) Number of fluorescent molecules measured in response to phosphatase inhibition (Calyculin A) or oxidative stress (H_2O_2). Cells without any treatment were used as a control. Error bars show the standard deviation from three experiments. B) Measurement of SUMOylated RanGAP1 (upper panel), SUMOylated p53 (middle panel), and the total protein (lower panel) in (A) by western-blot assay.

capable of overcoming this barrier, 2.1 μg of protein mixture (0.98 μg of p53 protein and 1.12 μg of RanGAP1 protein; Supporting Information, Figure S2) including the SUMOylated p53 and SUMOylated RanGAP1 proteins were diluted at various ratios for subsequent single-molecule detection. As shown in Figure 3A, the number of fluorescent molecules decreased monotonically as a function of the decrease of SUMOylated p53 and SUMOylated RanGAP1 proteins. Notably, individual fluorescent molecules were still observed even after 500-fold dilution of p53 protein and 2000-fold dilution of RanGAP1 protein using single-molecule detection (Figure 3A). In contrast, fivefold dilution of p53 protein and 20-fold dilution of RanGAP1 protein led to an undetectable SUMOylation signal with the western-blot assay (Figure 3B). Therefore, the proposed method was about 100-fold more sensitive than the conventional immunoblotting assay. This improved sensitivity could be attributed to two factors: 1) the specific and efficient reaction of the SNAP/CLIP-tag with the BG/BC derivatives[30,31] to translate the SUMO-mediated modifications to distinct fluorescence signals; 2) the high signal-to-noise ratio of single-molecule detection.[37] It should be noted that simultaneous detection of SUMOylated p53 and SUMOylated RanGAP1 could be achieved in a wide range of concentrations by single-molecule detection (Figure 3A) as compared with the western-blot assay (Figure 3B). Therefore, our method was capable of detecting multiple SUMOylated proteins effectively.

An important feature of SUMOylation is that the conjugation is reversible and dynamic, meaning intracellular SUMOylation can be dramatically affected by various stresses.[12] To determine whether our method is suitable for a comprehensive SUMOylation assay, simultaneous measurement of p53 and RanGAP1 SUMOylations was performed under various physiological conditions. As shown in Figure 4A, the addition of Calyculin A, a potent inhibitor of serine/threonine phosphatases 1 and 2A, decreased the SUMO-1 modified p53[38] as compared with the control. In contrast, Calyculin A treatment increased the SUMOylation of RanGAP1 dramatically (Figure 4A,B) in addition to a small increase in the total protein level (Figure 4B, lower panel), implying that phosphorylation might regulate p53 and RanGAP1 SUMOylations in different manners. Unlike Calyculin A, the treatment of transfected cells with 5 μM H_2O_2,[39] which produces oxidative stress within the cells, did not cause a noticeable change in either single-molecule detection (Figure 4A) or the western-blot assay (Figure 4B), indicating that oxidative stress did not alter the SUMOylation level of p53 or RanGAP1 under these conditions. These differences in SUMO modification in response to diverse extracellular stimulations and different SUMOylation substrates implied that the SUMO system adopts diverse responses to various stresses.

In conclusion, simultaneous detection of intracellular SUMOylation at the single-molecule level was demonstrated for the first time by using SNAP/CLIP-tag-mediated translation. The orthogonal substrate specificities of the SNAP/CLIP-tag[32] ensured the separation of dual SUMOylation signals, and the spectroscopically distinguishable ATTO 488/Alexa Fluor 647 fluorophore pair guaranteed signal differentiation. The efficient reaction of the SNAP/CLIP-tag with BG/BC derivatives in combination with the high signal-to-noise ratio of single-molecule detection[35] made it possible for simultaneous detection of multiple SUMOylations. It should be noted that the common translation module allows this method to be applied to a wide range of SUMO isoforms in a similar manner (Supporting Information, Figure S3). In theory, the proposed method could be expanded to any ubiquitin-like modifications[40,41] by genetic fusion of the ubiquitin-like modifiers with the SNAP/CLIP-tag. Therefore, this method could prove powerful for comprehensive understanding of the roles of post-translational protein modifications in biological processes and human diseases.

Experimental Section

To generate SNAP/CLIP-tagged SUMO, active versions of SUMO-1 and SUMO-2 cDNA,[42] were amplified and inserted into the pSNAP-tag (m) vector or the pCLIP-tag (m) vector (New England Biolabs, Inc., USA) at the BamHI and XhoI restriction sites.

HEK293T cells were either transfected with 2.5 μg of FLAG-P53 and FLAG-RanGAP1 constructs alone, or along with 2.5 μg of SNAP-SUMO-1 and CLIP-SUMO-1 expression constructs using the standard calcium phosphate precipitation method. For the drug treatment experiments, the cells were either incubated with 5 μM H_2O_2 for 20 min,[39] or treated with 0.1 μM Calyculin A for 45 min[38] before lysis.

Cells were sonicated in the lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 1 mM DTT, 1 mM N-ethylmaleimide and protease inhibitors), and the cell debris was removed by centrifugation. The supernatants were then incubated with anti-FLAG M2 affinity resin (Sigma, USA) at 4°C for 1 h. After washing three times with cold lysis buffer, the beads were incubated with 1 μM BG-Alexa Fluor 647/BC-ATTO 488 in the reaction buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20, 1 mM DTT) at 25°C for 1 h. After extensive washing, the conjugates were eluted from the beads with 0.1M glycine (pH 2.6) and the elution was neutralized with the neutralization buffer (0.5M Tris, 1M NaCl).

An inverted microscope (IX71, Olympus, Japan) was equipped with a UAPON 100X TIRF objective (1.49 NA, Olympus). A sapphire 488 nm laser (50 mW, Coherent, USA) and a cube 640 nm laser (100 mW, Coherent, USA) were used for the excitation of ATTO 488 and Alexa Fluor 647. Two long-pass edge filters (BLP01-488R-25 and BLP01-635R-25, Semrock, USA) were placed between the objective lens and the iXon X3 single-photon detector (DU-897, Andor, UK) to collect the emitted photons. The Shamrock SR-303i imaging spectrograph (Andor, UK) coupled with a Newton EM camera (DU970P-BV, Andor, UK) were used to measure the single-molecule fluorescence spectra, and the spectra were acquired by Andor solis software in FVB readout mode. Otherwise, the images were obtained by the Micro-manager 1.4 software with an exposure time of 100 ms and analyzed by Image J software (version 1.46, NIH, Bethesda, MD). The number of fluorescent molecules were determined according to a reported method.[43]

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