Running title:

A simple *in situ* cell-based SUMOylation assay

A simple *in situ* cell-based SUMOylation assay with potential application to drug-screening.

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Abbreviations: SUMO, small ubiquitin-related modifier; GFP, green fluorescent protein

Abstract

Here, we show that the SUMO conjugation reaction can be visualized by simply incubating GFP-SUMO-1 with permeabilized cells in the presence of ATP for 15 min. Neither special equipment for protein purification nor trained skills for recombinant technologies are required, making the assay potentially applicable to large-scale drug-screening strategies for the identification of drug(s) that can inhibit or enhance SUMOylation.

Keyword: post-translational modification, SUMO, cell-based assay, drug-screening

Text

SUMO-1 is a small ubiquitin-related protein modifier that is conserved from yeast to human. The attachment of SUMO-1 to a target protein, referred to as SUMOylation, is mediated by the enzymatic cascade reaction[1)]. SUMOylation begins with a SUMO-activating enzyme (also called an E1), which consists of an Aos1 and Uba2 heterodimer that carries out an ATP-dependent activation of the SUMO-1 C terminus. The activated SUMO-1 is then transferred to a SUMO-conjugating enzyme Ubc9 (E2). Finally SUMO-1 is transferred from Ubc9 to the substrate with or without the assistance of one of several SUMO-1 ligases (E3s).

Pichler et al.[2)] and Saitoh et al.[3)] demonstrated that when a yellow fluorescent (YFP) or green fluorescent (GFP) protein fused to SUMO-1 was incubated with permeabilized cells in the presence of ATP and recombinant SUMO-E1 and -E2 enzymes, YFP/GFP-SUMO-1 was efficiently conjugated to nuclear pore associated factors, including RanBP2/Nup358 and RanGAP1, thereby enabling the visualization of SUMOylation inside permeabilized cells[2, 3)]. This assay can detect the SUMOylation reaction *in situ*, and is, therefore, referred to as the *in situ* SUMOylation assay [3]. As shown in **Fig. 1**, this assay appears relatively simple. However, the preparation of the recombinant proteins required, including a heterodimer of Uba2 and Aos1 for SUMO-E1, Ubc9 for SUMO-E2 and YFP/GFP-SUMO-1, is time-consuming and the purification of these recombinant proteins

requires specialized equipment and trained skills.

To provide a SUMOylation assay that can be performed by researchers without experience of recombinant technologies and protein purification, we simplified and improved the *in situ* SUMOylation assay. Our new procedure is schematically represented in **Fig. 1**. This method requires only cultured cells and crude bacterial lysate containing GFP-SUMO-1. We briefly summarize the process as follows. The expression in *E. coli* of $(His)_6$-GFP-SUMO-1gg and $(His)_6$-GFP-SUMO-1g, a SUMOylation deficient mutant, were described previously[3, 4]. The bacterial pellet was harvested by centrifugation and dissolved in 5 ml of TRB [20 mM Hepes (pH 7.3), 110 mM KOAc, 2 mM $MgCl_2$, 1 mM EGTA]. The bacteria were then sonicated twice for 30 sec on ice. The supernatant was collected and stored at -20˚C until use. Approximately 1.0 μg of $(His)_6$-GFP-SUMO-1 was present in 10 μl of bacterial lysate. The procedure used to detect *in situ* cell-based SUMOylation was as follows. HeLa cells were grown on a coverslip in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 incubator. The coverslip was briefly rinsed with cold TRB plus 2 mM dithiothreitol (DTT), and the cells were then permeabilized for 5 min on ice with TRB plus 2 mM DTT and 500 μg/ml digitonin, a detergent that preferentially permeabilizes the plasma membrane and leaves the nuclear envelope intact (Merck-Calbiochem, Darmstadt, Germany). Cells were then rinsed twice with cold TRB. 10 μl of bacterial lysate containing ATP (final concentration: 1 mM) was added to 1 ml of TRB. This mixture was then applied to the digitonin-permeabilized cells followed by incubation for 15 min at 25˚C. The cells were then washed twice for 5 min with pre-warmed TRB and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were then rinsed with PBS for 5 min three times. A coverslip was then applied using mounting medium (2.5% DABCO in 80% glycerol). During the secondary wash, DNA was stained with 1 μg/ml 5,6-diamidino-2-phenylindole (DAPI). Samples were analyzed with a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka).

Fig. 1

When approximately 50-80% confluent (exponentially growing) or confluent (growth-arrested) Hela cells were permeabilized and incubated with the bacterial lysate containing $(His)_6$-GFP-SUMO-1gg, GFP signals at the nuclear rim were detected in cells cultured

Fig. 2

under both growth conditions (**Figs 2A and C**). However GFP signals in this assay were somehow weaker than the signals detected in previous methods (data not shown). In contrast, when the bacterial lysate containing $(His)_6$-GFP-SUMO-1g was used, no prominent signals were detected (**Fig. 2B**). Since SUMO-1g is a SUMOylation deficient mutant[3], these results imply that the GFP signals detected by $(His)_6$-GFP-SUMO-1gg might represent covalent conjugation, rather than non-covalent interaction with components at the edge of the nucleus. Unlike previous methods, we could detect the GFP-SUMO conjugation at the nuclear rim in the absence of excessive amounts of recombinant E1 and E2, suggesting the existence of endogenous E1 and E2 that might remain after digitonin-treatment. It should be noted that, when GFP-fused SUMO-3gg was used in this simple *in situ* SUMOylation assay, we found that the distribution of GFP-SUMO-3gg was similar to that of GFP-SUMO-1gg; i.e. the nuclear rim was clearly labeled with GFP-SUMO-3gg (data not shown). Thus these results indicate that the improved *in situ* SUMOylation assay is highly applicable for studying SUMOylation using cells cultured under the different growth conditions.

Using a previously developed *in situ* SUMOylation assay to screen small molecules, Fukuda et al. reported the identification of ginkolic acid and kerriamycin B, which inhibit SUMOylation[4, 5]. To demonstrate that our simple cell-based *in situ* SUMOylation assay provides a basis for the development of drugs targeted against diseases involving aberrant SUMOylation, we tested whether ginkgolic acid impairs the signals of GFP-SUMO-1 at the nuclear rim. As shown **Fig. 3A**, the GFP signals were significantly reduced in the presence of 100 μM of ginkgolic acid, supporting the previous observation by Fukuda et al.[4] In addition to the effect of this drug, we demonstrated that the signals at the nuclear rim were not observed in the absence of ATP (**Fig. 3B**), supporting not only the idea that our assay is useful for investigation of small compounds, but also the notion that accumulation of GFP-SUMO-1gg at the nuclear rim represents active SUMOylation, rather than non-covalent SUMO-protein interaction.

Fig. 3

In sum, we develop an *in situ* cell-based SUMOylation method which is simpler, cheaper and more rapid than previously described assays. The assay described here will be more easily performed by researchers and may be particularly useful in large-scale screening approaches for the identification of drug(s) that can inhibit or enhance SUMOylation, thereby contributing to the

development of therapeutic drugs.

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Figure Legends.

Fig. 1 Schematic representation of our improved *in situ* SUMOylation assay, relative to previous assays.

Typically, cells are grown on coverslips and permeabilized with detergents, such as digitonin. In the improved assay (*right panel*), the permeabilized cells are incubated with GFP-SUMO-1gg in the presence of ATP and the reaction is stopped by fixing the cells with paraformaldehyde. Compared with previous methods described by Pichler et al. [2] and Saitoh et al. [3] (*left panel*), the requirements of the improved assay are straight forward; i.e. the improved method requires only the

bacterial lysate containing GFP-SUMO-1gg, whereas the method of Pichler et al. requires four different recombinant proteins, including purified fluorescent-labeled SUMO-1gg, Aos1/Uba2 heterodimer for SUMO-E1 and Ubc9 for SUMO-E2.

Fig. 2 Application of the improved *in situ* SUMOylation assay to Hela cells cultured under different growth conditions.

(A) Asynchronously grown HeLa cells (approximately 50% confluent) were permeabilized with digitonin and mixed with bacterial lysate containing $(His)_6$-GFP-SUMO-1gg. The GFP and the DAPI (*inset*) signals were observed using fluorescence microscopy.

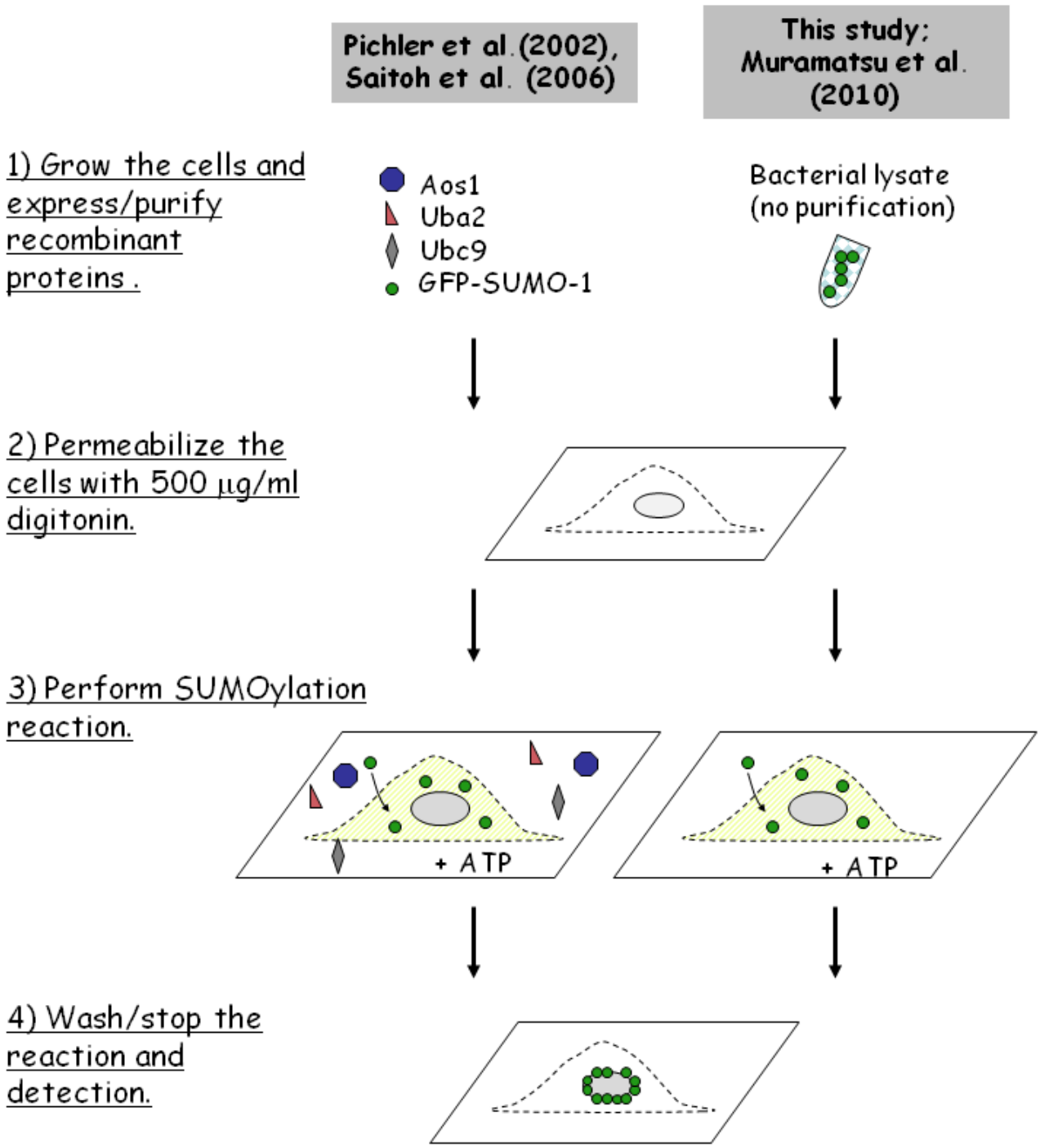
(B) Asynchronously grown HeLa cells (approximately 50% confluent) were permeabilized with digitonin and mixed with bacterial lysate containing $(His)_6$-GFP-SUMO-1g. The GFP and the DAPI (*inset*) signals were observed using fluorescence microscopy. Identical exposure conditions were used to compare SUMO-1g versus SUMO-1gg proteins.

(C) Hela cells cultured to near confluence (growth arrested state) were tested with the improved assay. $(His)_6$-GFP-SUMO-1gg was incubated with the cultured cells as indicated. The GFP and the DAPI (*inset*) signals were observed using fluorescence microscopy. *Bars* indicate 20 μm.

Fig. 3 Application of the improved *in situ* SUMOylation assay to drug-screening strategies.

(A) Asynchronously grown HeLa cells (approximately 50% confluent) were permeabilized with digitonin and mixed with bacterial lysate containing $(His)_6$-GFP-SUMO-1gg in the presence (*left panel*) or absence (*right panel*) of 100 μM ginkgolic acid. The GFP (*larger panel*) and the DAPI (*inset*) signals were observed using fluorescence microscopy. *Bars* indicate 20 μm.

(B) Asynchronously grown HeLa cells (approximately 50% confluent) were permeabilized with digitonin and mixed with bacterial lysate containing $(His)_6$-GFP-SUMO-1gg in the presence (*left panel*) or absence (*right panel*) of 1 mM ATP. The GFP (*larger panel*) and the DAPI (*inset*) signals were observed using fluorescence microscopy.



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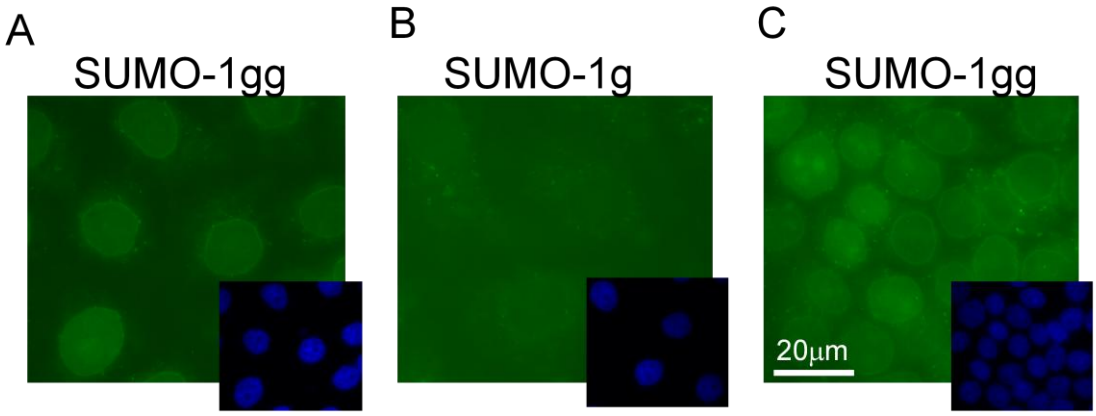

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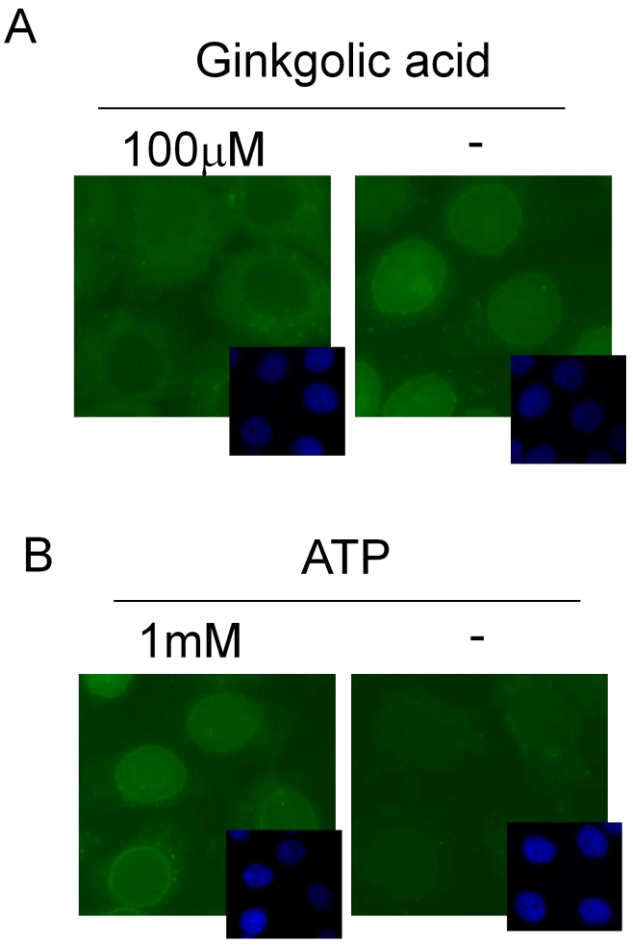

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