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Localization of SUMO-Modified Proteins using fluorescent SUMO-Trapping Proteins

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December 18, 2018

Dear Stephanie Ray Weldon:

Please find enclosed our original manuscript with the title:

Localization of SUMO-Modified Proteins using fluorescent SUMO-Trapping Proteins

The enclosed protocol and representative data describe in detail the use of a fluorescent SUMO-trapping proteins developed by us for the detection of SUMO localization in fixed mammalian and nematode cells. We believe, that this antibody-independent technique for SUMO localization will receive wide-wide-spread interest in the field.

Please do not hesitate to contact me if you have any questions regarding this manuscript.

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Sincerely,

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TITLE:

Localization of SUMO-Modified Proteins Using Fluorescent Sumo-Trapping Proteins

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KEYWORDS:
SUMO, Smt3, Smo-1, sumoylation, *C. elegans*, PNT2, budding yeast, UTAG, SUMO-trapping, UD

SUMMARY:
SUMO is an essential and highly conserved, small ubiquitin-like modifier protein. In this protocol we are describing the use of a stress-tolerant recombinant SUMO-trapping protein (kmUTAG) to visualize native, untagged SUMO conjugates and their localization in a variety of cell types.

ABSTRACT:
Here we are presenting a novel method to study the sumoylation of proteins and their sub-cellular localization in mammalian cells and nematode oocytes. This method utilizes a recombinant modified SUMO-trapping protein fragment, kmUTAG, derived from the Ulp1 SUMO protease of the stress-tolerant budding yeast *Kluyveromyces marxianus*. We have adapted the properties of the kmUTAG for the purpose of studying sumoylation in a variety of model systems without the use of antibodies. For the study of SUMO, KmUTAG has several advantages when compared to antibody-based approaches. This stress-tolerant SUMO-trapping reagent is produced recombinantly, it recognizes native SUMO isoforms from many species, and unlike commercially available antibodies it shows reduced affinity for free, unconjugated SUMO. Representative results shown here include the localization of SUMO conjugates in mammalian tissue culture cells and nematode oocytes.

INTRODUCTION:
The purpose of this method is to facilitate the study and analysis of SUMO-conjugated proteins using the recombinant SUMO-trapping UTAG (**U**lp domain **Tag**) protein. As detailed below, UTAG can be used in lieu of other reagents and approaches to purify, detect, and visualize SUMO-

modified proteins. Depending on growth conditions, cells may contain hundreds or thousands of proteins that are modified with SUMO or SUMO chains (for review see Kerscher et al. 2006[1] and Kerscher 2016[2]). This represents a considerable difficulty for the functional analyses of specific SUMO-modified proteins, especially since only a fraction of a particular sumoylation target is actually modified[3]. In addition to their roles in essential cellular processes such as transcriptional regulation, protein homeostasis, the response to cellular stress, and chromatin remodeling during mitosis and meiosis; it has now become sufficiently clear that SUMO, SUMO-modified proteins, and SUMO pathway components also have potential as biomarkers for pathologies such as cancer and neurodegenerative disorders[4-7]. This underscores the need for robust, reliable, and readily available tools and innovative approaches for the detection and functional analysis of SUMO-modified proteins in a variety of cells and samples.

In many systems, SUMO-specific antibodies are the reagents of choice for the detection, isolation and functional analyses of SUMO-modified proteins[8,9]. However, some commercially available SUMO-specific antibodies are expensive, limited in quantity or availability, prone to exhibit wildly variable affinities and cross-reactivity, and in some instances lack reproducibility[10]. One alternative approach is the expression of epitope-tagged SUMO in transformed cells and organisms, but linking epitope tags to SUMO may artificially lower its conjugation to protein targets[11]. Additionally, epitopes are not useful when untransformed cells or tissues are evaluated.

Ulp1 is a conserved SUMO protease from *S. cerevisiae* that both processes the SUMO precursor and desumoylates SUMO-conjugated proteins[12]. We developed the UTAG reagent based on the serendipitous observation that a mutation of the catalytic Cysteine (C580S) in Ulp1's SUMO processing **U**lp **d**omain (UD) not only prevents SUMO cleavage but also traps SUMO-conjugated proteins with high avidity[12]. For simplicity, we referred to this carboxy-terminal SUMO-trapping Ulp1(C580S) fragment as UTAG (short for UD TAG). UTAG is a recombinant pan-SUMO trapping protein that represents a useful alternative to anti-SUMO antibodies used for the isolation and detection of SUMO-modified proteins. Importantly, it specifically recognizes natively-folded, conjugated SUMO and not just one or several epitopes on SUMO. To improve both the protein stability and SUMO binding strength of UTAG, we generated a variant of UTAG from the stress-tolerant yeast *Kluyveromyces marxianus* (Km). KmUTAG tightly binds SUMO-conjugates with nanomolar affinity[13]. Additionally, kmUTAG is resistant to elevated temperatures (42 °C), reducing agents (5 mM TCEP - Tris(2-carboxyethyl)phosphine hydrochloride), denaturants (up to 2 M Urea), oxidizing agents (0.6% hydrogen peroxide), and non-ionic detergents. This stress tolerance is beneficial during harsh purification condition and prolonged incubation times, ensuring its stability and SUMO-trapping activity. Not surprisingly, however, KmUTAG's SUMO-trapping activity is incompatible with cysteine-modifying reagents, ionic detergents and fully denatured protein extracts. The remarkable affinity and properties of KmUTAG indicate that this reagent may become part of the standard repertoire for the study of sumoylated proteins in multiple species.

Here we provide a simple method to detect SUMO-modified proteins in mammalian cells and nematodes using a recombinant fluorescent mCherry-KmUTAG fusion protein (kmUTAG-fl).

PROTOCOL:

1. SUMO detection in fixed tissue culture cells using recombinant KmUTAG-FL SUMO-trapping protein

1.1. Grow tissue culture cells of choice on 22 mm round cover slips in 6-well TC plates until 70%–80% confluent. Perform steps 1.2–1.8 in the 6-well plate.

1.2. Wash cells briefly with 1 mL of DPBS (Dulbecco's phosphate-buffered saline)

1.3. For fixation, prepare a fresh solution of 4% Paraformaldehyde (PFA) solution (diluted in DPBS). To fix cells, add 2 mL 4% PFA in DPBS to each well. Incubate the cells for 20 min at room temperature. NOTE: All steps using PFA should be performed in a laboratory safety hood and PFA must be disposed properly.

1.4. Wash the fixed cells 3x in 1 mL DPBS while nutating the plate, 5 min each wash.

1.5. To permeabilize cells, incubate for 15 min with 0.1% Triton X-100 in DPBS

1.6. Wash the cells 3x in 1 mL DPBS while nutating the plate, 5 min each wash

1.7. Incubate the cells with 500 µL of 0.1 M Glycine-HCL (pH 2.0) for 10 s, then neutralize pH immediately with 500 µL of 10x SUMO Protease Buffer (SPB).

1.8. Wash the cells 3x in 1 mL DPBS while nutating plate, 5 min each wash

1.9. Remove the coverslips from the well and place them in a humidity chamber. Then proceed with incubations on the coverslip as follows:

1.9.1. For UTAG-fl staining only: mix 1 µg UTAG-fl with 100 µL of 1x SPB containing 5 mM TCEP in a tube, pipette the mix onto the cells on the coverslip, and incubate at room temperature for 1 h in the humidity chamber.

1.9.2. Optionally, for UTAG-FL and anti SUMO1 antibody co-staining, proceed with the following:

1.9.2.1. Mix in a tube 1 µg UTAG-FL and 0.5 µL SUMO2/3 8A2 (obtained for Developmental Studies Hybridoma Bank[9]) with 100 µL of blocking buffer, pipette the mix onto the coverslip, and incubate in room temperature for 1 h.

1.9.2.2. Wash cells on the coverslip 3 times with 200 µL DPBS, 5 min each wash.

1.9.2.3. Mix in a tube 0.5 µL anti-mouse Alexa Fluor 488 conjugated antibody with 100 µL Blocking buffer, pipette the mix onto the coverslip, and incubate in room temperature for 1 h.

1.10. To wash coverslips, pipette 200 µL DPBS on each coverslip and leave in place for 10 min. Repeat the wash 2 more times.

1.11. Remove coverslip from the last wash and invert it onto a pre-cleaned microscopy slide with a drop of mounting medium (see **Table of Materials**).

1.12. Store overnight in a -20°C freezer before viewing under the microscope. Visualize using the appropriate filter sets for DAPI (DNA), Texas Red (kmUTAG-fl), and Alexa Fluor 488 (if optional SUMO2/3 co-staining is performed).

2. SUMO detection in fixed nematode gonads using UTAG-fl

2.1. Transfer adult hermaphrodites to an 8 µL droplet of egg buffer[14] on a plus-charged slide that has been coated with poly-L-lysine. Release gonads from the worms using 27.5 G needles. Proceed with either antibody labeling or UTAG-fl labeling.

2.2. For antibody labeling samples, proceed as follows:

2.2.1. Freeze-crack samples in liquid nitrogen and then fix overnight in -20 °C methanol in a Coplin jar.

2.2.2. Also in Coplin jars, wash slides for 3 times in 1x PBS, then block for 20 min in PBS containing 0.5% BSA and 0.1% Tween 20.

2.2.3. Add 30 µL of anti-SUMO 6F2 antibody (1:10) to each slide covering the specimens. Incubate overnight in a humidity chamber at 4 °C.

NOTE: SUMO 6F2 was obtained from the Developmental Studies Hybridoma Bank[15].

2.2.4. In Coplin jars, wash slides for 2 min in 1x PBS, then cover and incubate specimens with 30 µL of DyLight 488 goat-anti mouse secondary antibody (1:200) for 1.5 hours in a humidity chamber at room temperature.

2.2.5. In Coplin jars, wash slides for 2 min in 1x PBS, perform a quick dip in dH_20, and then mount the slides with mounting medium (see **Table of Materials**).

2.3. For KmUTAG-fl labeling, proceed with the following:

2.3.1. To fix cells, add 1 volume of 8% PF to samples for a final concentration of 4% PF. Fix for 10 min in a humidity chamber and then quench the reaction by transferring slides to a Coplin jar containing 1x PBS with 0.1 M glycine for at least 5 min.

2.3.2. In Coplin jars, wash cells for 5 min in 1x PBS, then permeabilize the samples in 1x PBS containing 0.1% Triton-X for 10 min.

2.3.3. Wash in a Coplin jar for at least 5 min in 1x PBS, then add 200 µL of 0.1 M Glycine-HCl (pH = 2.0) to the samples on the slide for 10 seconds. Immediately add 200 µL of 10x SPB to neutralize the pH.

2.3.4. Wash the slide in 1x PBS in a Coplin jar for 5 min.

2.3.5. Remove slide from wash and pipette 100 µL of 1x SPB + 5mM TCEP containing 2 µg of UTAG-fl to the nematodes on the slides, and incubate in humidity chamber for 1 h without rocking.

2.3.6. Return the slide to the Coplin jar and wash for 15 min in 1x PBS

2.3.7. Remove the slide from the wash, use a laboratory wipe to dry around the sample, and then mount the slides with 5 µL of mounting medium (see **Table of Materials**). Store the slides at 4 °C. Visualize using the appropriate filter sets for DAPI (DNA), Texas Red (kmUTAG-fl), and DyLight 488 (SUMO2/3).

REPRESENTATIVE RESULTS:

KmUTAG-fl is a recombinant, mCherry-tagged SUMO-trapping protein. To produce kmUTAG-fl, we cloned a codon-optimized mCherry-kmUTAG into the pSPOT1 bacterial overexpression plasmid (**Figure 1**). After induction, the kmUTAG-fl protein was purified on Spot-TRAP, eluted, and frozen until further use. To ensure the SUMO-trapping activity of KmUTAG-fl, we confirmed binding to SUMO1-conjugated beads and precipitation of a SUMO-CAT fusion protein (data not shown, but see previous work[13]).

KmUTAG-fl incubated with fixed PNT2 cells showed a distinct nuclear staining when observed using the appropriate filter set (Chroma) and a 100x oil-immersion objective on a Epifluorescent Zeiss Axioplan Microscope (**Figure 2** – Top right panel). Both diffuse nuclear staining and distinct nuclear foci were visible. Nuclear localization was confirmed using co-staining with DAPI (**Figure 2** – top left panel). Consistent with the SUMO-trapping activity of kmUTAG-fl the nuclear localization pattern was reminiscent of SUMO2/3 staining. Co-staining with anti SUMO2/3 8A2 antibody (**Figure 2** - bottom left panel) confirmed the co-localization of kmUTAG-fl with the SUMO2/3 signal (**Figure 2** – merge, bottom right). This validates the efficacy of KmUTAG-fl to detect SUMO2/3 in mammalian cells.

Since kmUTAG-fl exhibits pan-SUMO specificity, we also tested it on *C. elegans* nematodes to determine if the localization pattern of kmUTAG-fl would match the previously reported patterns of anti-SUMO antibodies and mCherry::SUMO fusion proteins [8,15]. Isolated gonads from oocyte-producing adult hermaphrodites were processed for labeling with either anti-SUMO antibody or kmUTAG-fl. Consistent with previous reports [8], anti-SUMO antibody initially localized to the nucleoplasm of late meiotic prophase oocytes (**Figure 3A,C**). It then redistributed to the central ring complex (RC) of the paired homologs (bivalents) as the nuclear envelope broke down and the chromosomes congress towards the metaphase plate (**Figure 3B,D**). Components of the ring

complex, which is situated between the DAPI-stained homologs, include both GEI-17(a E3 SUMO ligase) and SUMO-conjugated chromokinesin [8]. In parallel preparations, gonads labeled with KmUTAG-fl revealed similar patterns (**Figure 3E,F**). KmUTAG-fl shifted from labeling the nucleoplasm to concentrating on the ring complex, as oocytes began (-1 oocyte in **Figure 3E**) and completed (-1 oocyte in **Figure 3F**) the process of nuclear envelope breakdown. These results validate KmUTAG-fl as a useful tool for the analysis of meiosis and SUMO-related processes in *C. elegans* and possibly other nematodes.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of the kmUTAG-fl SUMO-trapping fusion protein used in this method. The Spot-tag vector pSPOT1 is used for the expression of KmUTAG-fl.

Figure 2. kmUTAG-fl colocalization with SUMO2/3 in mammalian cells. PNT2 cells were grown on coverslips, fixed, and stained with both KmUTAG-fl and anti-SUMO2 8A2 antibody, before applying mounting media containing DAPI. Slides were visualized using a confocal microscope and appropriate filters for DAPI (DNA), mCherry (kmUTAG-fl), and GFP (anti SUMO2/3). kmUTAG-fl colocalized with SUMO2/3 in PNT2 cells. Scale bar = 20 μm.

Figure 3. Comparison of SUMO labeling by anti-SUMO antibodies and KmUTAG-fl during *C. elegans* oocyte maturation. (**A**) Schematic of the proximal *C. elegans* gonad. Developing oocytes enter the spermatheca (sprmth) one-by-one where they are fertilized before entering the uterus. Oocytes in the -1 position, immediately adjacent to the spermatheca undergo nuclear envelope breakdown prior to fertilization. After fertilization, the sperm chromatin remains in a condensed state until the oocyte chromosomes complete their meiotic divisions. (**B**) Schematic showing the structure of a chromosomal bivalent that forms after homologous chromosomes have recombined, disassembled their synaptomenal complex, and have condensed in preparation for metaphase. A central ring complex (RC) between the homologs is enriched not only in aurora kinase and the checkpoint protein BUB-1 but also the E3 SUMO ligase GEI-17 and chromokinesin. (**C-D**) A line of developing oocytes within the oviduct (**C**) and a newly fertilized oocyte in metaphase of meiosis I (**D**) labeled with anti-SUMO antibody. (**C-D**) Full-sized images at the right show the merged (m) and single channel DAPI (D) and anti-SUMO antibody (S Ab) images of the -2 and -1 nuclei and the metaphase I spindle region. o = metaphase I chromosomes of oocyte, s = sperm chromatin mass. (**E-F**) Developing oocytes within the oviduct labeled with KmUTAG-fl. Concentration of KmUTAG-fl at the ring complex begins shortly before nuclear envelope breakdown (-1 oocyte in **E**) and becomes largely restricted to the ring complex after nuclear envelope breakdown (-1 oocyte in **F**). Blue = DAPI. Scale bar = 5 μm.

DISCUSSION:

Here we introduce the use of kmUTAG-fl, a recombinant protein, for functional studies of SUMO in fixed mammalian cells and dissected nematode gonads. KmUTAG-fl is a stress-tolerant pan-SUMO specific reagent that recognizes and traps native SUMO-conjugated proteins and SUMO chains. Since SUMO's tertiary structure is highly conserved it is very likely that SUMO variants from additional model and non-model systems can be analyzed with the kmUTAG-fl reagent. As such, KmUTAG-fl may represent a useful alternative or secondary reagent for traditional antibody

staining protocols.

There is ample precedent for the use of non-antibody alternatives, including lectins for carbohydrate detection as well as aptamer nucleotides and affimer peptides for *in vivo* labeling of proteins [16-19]. Additionally, affimers and monobodies have been generated that bind SUMO and prevent the interaction with SUMO-interacting motifs (SIMs) on other protein [18,20,21]. However, to our knowledge, KmUTAG-fl is the first recombinant fluorescent pan-SUMO-trapping protein for the direct visualization of SUMO conjugates in fixed cells. KmUTAG-fl is derived from Ulp1 of a stress-tolerant yeast, *K. marxianus,* and this may explain its remarkable stability [13]. Unlike most antibodies, KmUTAG does not require a secondary antibody for visualization, and stained cells are readily visible under the fluorescence microscope. Both our analyses of SUMO conjugates in mammalian cells and nematode gonads suggest that KmUTAG-based imaging compares favorably to SUMO antibody-staining and shows little noise (e.g. compare **Figure 3A,B** versus **Figure 3D,E**).

Critical steps in the protocol include the use of fresh paraformaldehyde and a short fixation time to keep SUMO natively-folded so it can be recognized by kmUTAG. Also, the brief treatment of fixed cells with the low pH glycine solution increases its sensitivity for SUMO. However, not all samples may lend themselves to analysis with KmUTAG-fl. As with antibody-based protocols, when attempting to localize SUMO in new cell types or tissues, detergent choice and detergent concentrations may be important variables. Ultimately, we plan to generate additional KmUTAG-fl variants including a cell-penetrating kmUTAG protein that can be used to detect SUMO dynamics in living cells, organoids, and tissue biopsies.

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DISCLOSURES:
Recombinant kmUTAG reagents are provided to the research community via Kerafast.com.

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Figure 1

Figure 2

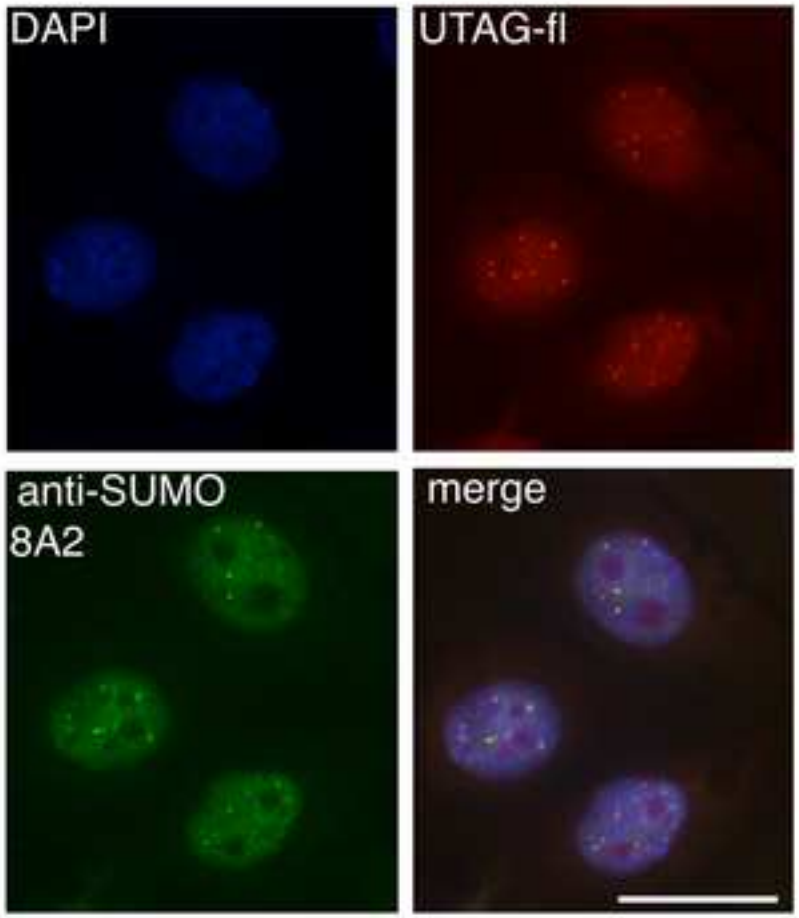
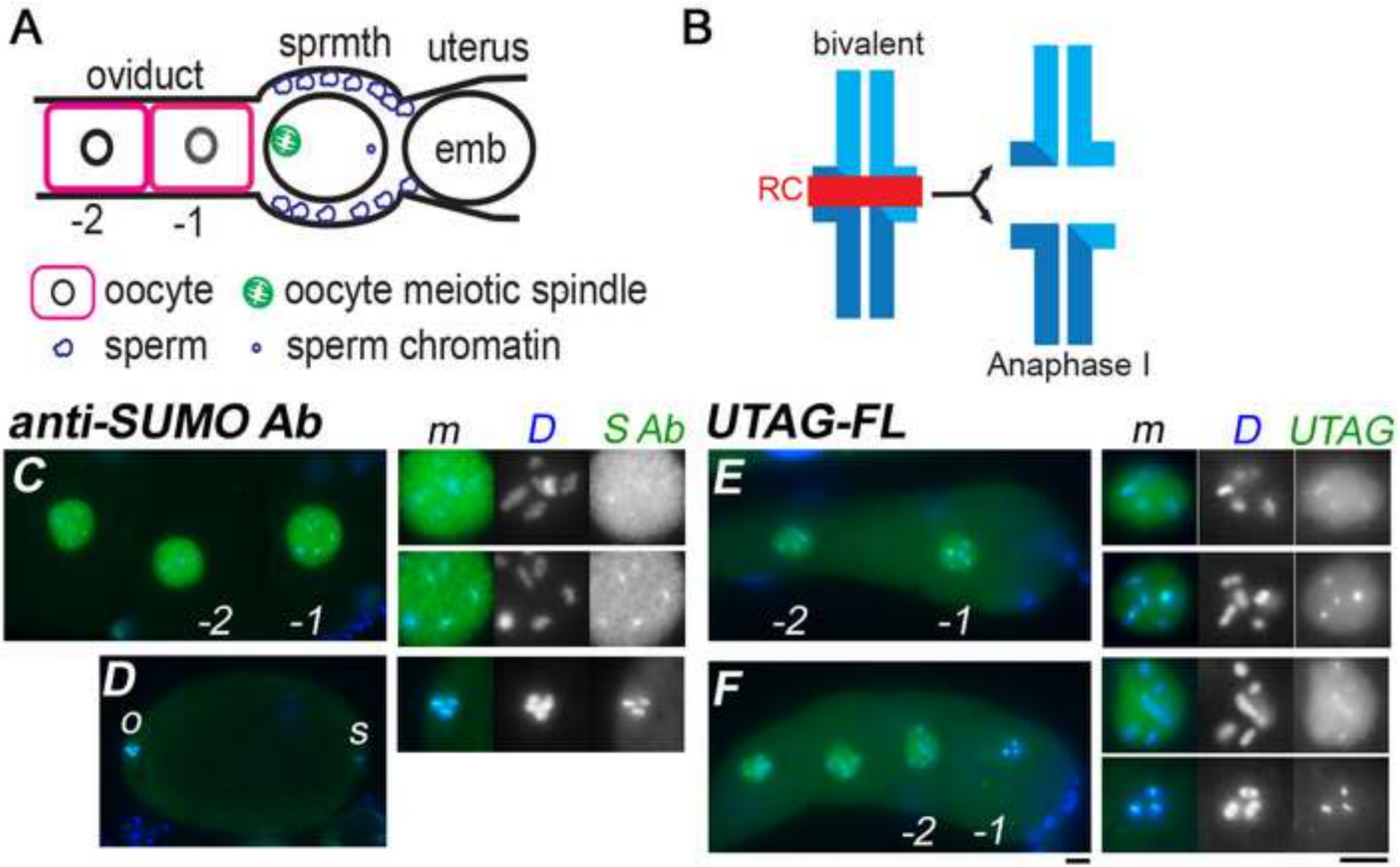


Figure 3



Name of Material/ Equipment	Company	Catalog Number
16% Paraformaldehyde (formaldehyde) aqueous solution	Electron Microscopy Sciences	30525-89-4
6-Well Cell Culture Plates	Genesee Scientific/Olympus P	25-105
Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	115-545-003
DPBS, no calcium, no magnesium	Fisher Scientific	Gibco 14190144
Dylight 488 conjugated AffiniPure Goat Anti-Moue IgG (H+L)	Jackson ImmunoResearch	115-485-146
Fisherbrand Coverglass for Growth Cover Glasses	Fisherbrand	12545101
FLUORO-GEL II with DAPI	Electron Microscopy Sciences	50-246-93)
FLUORO-GEL with DABCO	Electron Microscopy Sciences	17985-02
Glycine-HCl	Fisher BioReagents	BP3815
Glycine-HCl	ACROS Organics	6000-43-7
KmUTAG-fl	Kerafast	
Oneblock Western-CL blocking buffer	Prometheus	20-313
PBS, Phosphate Buffered Saline, 10X Solution	Fisher BioReagents	BP3994
PNT2 cell line	Sigma-Aldrich	95012613
pSPOT1	(ChromoTek GmbH)	ev-1
SUMO 6F2	DSHB	SUMO 6F2
SUMO protease buffer [10x]		
SUMO-2 Antibody 8A2	DSHB	SUMO-2 8A2
TCEP-HCL	GoldBio	51805-45-9
Triton X-100	Fisher BioReagents	9002-93-1

Comments/Description

Used as a secondary antibody for mouse monoclonal antibody

Used as a secondary antibody for mouse monoclonal antibody

Mounting media in step 1.11
With DAPI added to 1 µg/mL; mounting media in step 2.2.5

KmUTAG reagents are available on Kerafast.com

Normal prostate epithelium immortalized with SV40.
https://www.chromotek.com/fileadmin/user_upload/pdfs/Datasheets/pSpot1_v1.pdf
SUMO 6F2 was deposited to the DSHB by Pelisch, F. / Hay, R.T. (DSHB Hybridoma Product SUMO 6F2)
500 mM Tris-HCl, pH 8.0, 2% NP-40, 1.5 M NaCl
SUMO-2 8A2 was deposited to the DSHB by Matunis, M. (DSHB Hybridoma Product SUMO-2 8A2)
Used as a reducing agent at a concentration of 5mM
Used for permeablization at 0.1% in DPBS/PBS(for worms)

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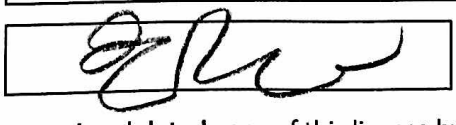
Signature:	
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January 30, 2019

Dear Dr. Philip Steindel:

Please find enclosed our revised manuscript submission (JoVE59576) with the title:

Localization of SUMO-Modified Proteins using fluorescent SUMO-Trapping Proteins

We would like to thank you and the reviewers for very helpful comments that have greatly improved the manuscript. The edited manuscript, figures, table and rebuttal document have been uploaded together with this letter.
We hope that you will now find this protocol suitable for publication in JoVE.

Please do not hesitate to contact me if you have any questions regarding this manuscript.

.
Sincerely,

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Associate Professor, Biology
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Revisions for JoVE submission JoVE59576

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. We inspected the manuscript for spelling mistakes and grammar to the best of our ability.
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For example: Dylight, Kimwipe, Chromotek, Zeiss

Protocol:
1. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps. Protocol was edited as suggested.

Specific Protocol steps:
1. 1.9.2: Which of the following steps are part of this one (co-staining)? They should be substeps of this one, as it’s unclear now. 1.9.2 was removed as it is an optional step (now labeled OPTIONAL)

Figures, Tables, and Figure Legends:
1. Please Remove ‘Fig.1’, ‘Fig.2’, and ‘Fig.3’ from the Figures themselves. Removed Fig.#
2. Figures 2: Please include a scale bar here. Bar added
3. Figure 3: What is ‘m’ and ‘D’? **m**erge and **D**api, respectively. Now added in the new Figure legend.

References:
1. Please ensure references have a consistent format. References have been edited

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol. Checked for completeness.
2. Please remove trademark (™) and registered (®) symbols from the Table and Materials. Removed as indicated above.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Kerscher and colleagues describe the use of a novel fluorescent recombinant SUMO-trapping protein to localize SUMO in mammalian and C. elegans cells. The trapping protein is an mCherry-tagged catalytically inactive fragment of the SUMO protease Ulp1 from the stress-tolerant K. marxianus. This reagent is simpler to use than anti-SUMO antibodies because it does not require a secondary antibody. The representative results demonstrate that this reagent yields similar results to conventional anti-SUMO antibodies in two systems. Given their simplicity and broad species specificity, the tool and technique are likely to be of great interest to the broader SUMO research community. After the authors address relatively minor concerns, this work is worthy of publication in JoVE. We would like to thank Reviewer #1 for insightful comments and suggestions. Please find our edits below!

Major Concerns:

* The following is an issue of clarity and data presentation and should be readily addressable. The main result in Figure 3 is clear: anti-SUMO antibody and kmUTAG-fl yield very similar data, and therefore kmUTAG-fl represents a viable alternative to antibodies for visualization in cells. However, the authors should clarify the Results paragraph (206-214) and Figure legend for Figure 3. These are difficult to follow. My questions and comments include:
o The sentence on lines 210-211 is difficult to interpret.
o Are there supposed to be visually obvious differences between the -1 and -2 oocytes? The authors write in the figure legend "In the -2 oocyte, SUMO begins to localize to the chromosomes (-2 oocyte) before the pattern becomes specifically restricted to the meiotic ring complex between the paired homologs".
o What specifically is changing from Fig 3a to Fig 3b? Is the rightmost diffuse mass in 3b a fertilized oocyte that had once been a highly compact occyte as pictured in 3a?
o How does Fig3c relate to Fig 3b?
o What are the right panels (m, D, 6F2/UTAG) illustrating? Is D DAPI? Is m merge? What is each different row of the righthand panels? I think these cells match up with cells from the images to their left, but clear labeling would help.

To improve clarity and data presentation we have replaced the indicated part of the results section with the following paragraph and a new figure 3 and figure legend:

Since kmUTAG-fl exhibits pan-SUMO specificity, we also tested it on *C. elegans* nematodes to determine if the localization pattern of kmUTAG-fl would match the previously reported patterns of anti-SUMO antibodies and mCherry::SUMO fusion proteins (Pelisch et al., 2014; Pelisch et al., 2017). Isolated gonads from oocyte-producing adult hermaphrodites were processed for labeling with either anti-SUMO antibody or kmUTAG-fl. Consistent with previous reports (Pelisch et al., 2017), anti-SUMO antibody initially localized to the nucleoplasm of late meiotic prophase oocytes (Fig. 3A,C). It then redistributed to the central ring complex (RC) of the paired homologs (bivalents) as the nuclear envelope breaks down and the chromosomes congress towards the metaphase plate (Pelisch et al. 2017; Fig. 3B,D). Components of the ring complex, which is situated between the DAPI-stained homologs, include both GEI-17(a E3 SUMO ligase) and SUMO-conjugated chromokinesin (Pelisch et al., 2017). In parallel preparations, gonads labeled with KmUTAG-fl revealed similar patterns (Fig. 3E-F). KmUTAG-fl shifted from labeling the nucleoplasm to concentrating on the ring complex, as oocytes began (-1 oocyte in Fig. 3E) and completed (-1 oocyte in Fig. 3F) the process of nuclear envelope breakdown. These results validate KmUTAG-fl as a useful tool for the analysis of meiosis and SUMO-related processes in *C. elegans* and possibly other nematodes.

Fig 3. **Comparison of SUMO labelling by anti-SUMO antibodies and KmUTAG-fl during *C. elegans* oocyte maturation** (A) Schematic of the proximal *C. elegans* gonad. Developing oocytes enter the spermatheca (sprmth) one-by-one where they are fertilized before entering the uterus. Oocytes in the -1 position, immediately adjacent to the spermatheca undergo nuclear envelope breakdown prior to fertilization. After fertilization, the sperm chromatin remains in a condensed state until the oocyte chromosomes complete their meiotic divisions. (B) Schematic showing the structure of a chromosomal bivalent that forms after homologous chromosomes have recombined, disassembled their synaptomenal complex, and have condensed in preparation for metaphase. A central ring complex (RC) between the homologs is enriched not only in aurora kinase and the checkpoint protein BUB-1 but also the E3 SUMO ligase GEI-17 and chromokinesin. (C-D) A line of developing oocytes within the oviduct (C) and a newly fertilized oocyte in metaphase of meiosis I (D) labeled with anti-SUMO antibody. (C-D) Full-sized images at the right show the merged (m) and single channel DAPI (D) and anti-SUMO antibody (S Ab) images of the -2 and -1 nuclei and the metaphase I spindle region. Metaphase I chromosomes of oocyte (o). Sperm chromatin mass (s). (E-F) Developing oocytes within the oviduct labelled with KmUTAG-fl. Concentration of KmUTAG-fl at the ring complex begins shortly before nuclear envelope breakdown (-1 oocyte in E) and becomes largely restricted to the ring complex after nuclear envelope breakdown (-1 oocyte in F). Blue (DAPI). Scale bar = 5 microns.

Minor Concerns:

* Authors should be careful to spell out abbreviations on first appearance (e.g. UTAG on line 45, UD domain on line 73, dPBS on line 98, etc. spelled out abbreviations as indicated
* Some additional information about the function of Ulp1 should be provided in the introduction Additional information and a reference has been added in the 3rd paragraph of the introduction.
* The sentence beginning on Line 76 is confusing. It indicates that authors generated "generated a variant of the UTAG from the stress-tolerant yeast Kluyveromyces marxianus". What species does the UTAG described in the previous sentences come from? *S. cerevisiae* – this has now been clarified in the introduction.
* There is a reference in line 80. The fact in line 78 and the fact in lines 82-84 need references. If the reference for line 80 (Peek et al 2018) apply to the others, that reference should either be placed after the first fact in the sequence of three (line 78), or at the end of these facts (line 84). The reference was moved
* Line 88: "simply" should be "simple" corrected
* Line 89: this is the first mention of the term "kmUTAG-fl". I assume fl is fluorescent, but the authors should spell this out. Fluorescent was spelled out
* Line 102: "off" should be "of"
* Line 118: What volume of SPB? 100µl this was added
* Line 122 indicates authors will be staining with anti-SUMO1 antibody, but line 124 (and following) indicate anti-SUMO2/3 antibody is used. Authors should clarify or correct. anti-SUMO2/3 antibody this was corrected (Thanks for catching this)
* Line 124: Replace comma with "and" corrected
* Authors are using Alexa-Fluor 488 secondary antibody for SUMO2/3 antibody. This is green. They're also using DAPI (blue) for DNA and mCherry (red) for kmUTAG-fl. However, in lines 139-140, they only recommend imaging using red and blue channels. What about the green filter for SUMO2/3 secondary? This oversight was corrected
* Line 145: "coated" would be better than "subbed" (more formal) – edited!

* Line 158: L should be capitalized in DyLight corrected
* Line 186: Authors don't indicate what filters are recommended for use here, as they did in first protocol. Also, recommend replacing "view" with "visualize" edited and information was added
* Line 226: title of figure caption should include organism title was updated
* Line 248: "precedence" should probably be "precedent" edited
* Line 259: Authors indicate that "a short fixation time" is critical "to keep SUMO intact". The authors should expand upon this. Are the authors talking about preventing SUMO chain disassembly, deconjugation from substrate, denaturation, or something else? We added the following sentence: … short fixation time to keep SUMO natively-folded so it can be recognized by kmUTAG
* Line 263: suggest replacing "useful" with "important" edited as suggested

Reviewer #2:

Manuscript Summary:
Yin et al describe a method for immunofluorescent localisation of SUMO proteins using a fragment derived from yeast Ulp1. The efficacy of this procedure has been demonstrated thoroughly in their previous work (Elmore et al, and Peek et al). The manuscript is generally well written, the protocol is clear to follow, and the data appear convincing. This protocol will be a useful addition to the field. In my opinion, the manuscript would be acceptable in its current form, but I have a couple of minor points that the authors may consider addressing before publication. We would like to thank Reviewer #2 for insightful comments and suggestions. Please find our edits below!

Major Concerns:
I have no major concerns regarding this manuscript.

Minor Concerns:

1. Being a SUMO biologist who does not work on C.elegans oocytes, I found figure 3 a little hard to follow. I wasn't entirely clear on what was represented in each panel. The authors may consider adding some clearer labels to the figure, and slightly more detail in the legend as to what each panel represents, in order to help the reader. In addition, if images have been spliced together, the authors should probably indicate this clearly with a dotted line. As detailed above, to improve clarity and data presentation we have replaced the indicated part of the results section with the following paragraph and a new figure 3 and figure legend.

2. The authors state in the discussion that to their knowledge 'this is the first method for the specific in vitro detection of SUMO conjugates in fixed cells, using a fluorescent SUMO-trapping recombinant protein.' While their technique appears to work very well, the approach is somewhat similar to that of a previous group who used a recombinant anti-SUMO monobody to either stain fixed cells for SUMO, or to isolate SUMOylated proteins from cell/tissue lysates (Berndt et al, Biochemical Journal 2013). If they feel inclined, they may wish to mention this. We appreciate the reviewer sharing this information and we have added three references. Additional we amended our statement in the discussion.

3. While the primary protocol described here relates to the immunofluorescent localisation of SUMO in fixed cells, the authors discuss the utility of this approach in isolating SUMOylated species from cell lysates. However, they note that their recombinant SUMO-binding protein is sensitive to the addition of NEM in the pulldown. Since NEM is commonly used to preserve SUMOylated species in cell lysates, have the authors experienced any problems resulting from the loss of SUMOylated species in their lysates as a result of the necessity to omit NEM? If so, does the benefit of the high affinity of their SUMO-binding protein outweigh this cost? No protease inhibitors are needed for this protocol due to fixation with paraformaldehyde. For applications using cell lysates (not described here) we recommend the Promega 50x protease inhibitor cocktail G6521. In our hands this protease inhibitor cocktail does a great job to maintain SUMO conjugates and does not interfere with kmUTAG/SUMO binding.

4. The pSPOT-mCherry-KmUTAG construct outlined in figure 1 is very elegant, and would allow interested researchers to perform the protocol described here in full. It would be very useful to the field if the authors would consider making this plasmid available on Addgene or a similar repository. We will soon make the kmUTAG-fl protein at cost available to the community on Kerafast.com.

5. While generally well written, the manuscript may benefit from close reading by a native English speaker in a few places. Done!