Chapter 17

FRET-Based In Vitro Assays for the Analysis of SUMO Protease Activities

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Abstract

In humans cells three SUMO paralogues (SUMO-1, SUMO-2 and SUMO-3) and six SUMO specific proteases (SENP1-SENP3 and SENP5-SENP7) are expressed. Together the SUMO proteases perform three distinct functions. They: (1) process the immature pro-SUMO proteins into the active forms, (2) remove SUMO molecules conjugated to protein targets, and (3) depolymerise SUMO conjugated within polymeric chains. By regulating these processes the SENPs play a crucial role in regulating the sumoylation state of target proteins in cells, and therefore are academically and pharmacologically interesting enzymes. Gel-based techniques for SENP analysis are well established and can be used for many applications, but their laborious methodology makes them cumbersome tools for kinetic analysis or inhibitor screening. Therefore in vitro FRET-based assays have been developed to test the three major functions of the SENPs. These use fluorescent protein fusions of the SUMOs, and together facilitate high-throughput, real-time analysis of the three major SUMO protease activities.

Key words: SUMO, sentrin **e**protease, SENP·FRET, inhibitory peptide, sumoylation, isomerisation.

1. Introduction

The post-translational conjugation of ubiquitin-like proteins (Ulps) into specific target proteins represents an important group of regulatory mechanisms in cells. The conjugation of members of one Ulp sub-family, the three small ubiquitin-like modifiers SUMO-1, SUMO-2 and SUMO-3, onto protein targets is no exception, and is known to play a significant role in a variety of cellular functions (*1*). SUMO-1 has approximately 50% sequence identity with SUMO-2 and SUMO-3, which differ from one another

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N-terminally to the diglycine motif only by 3 amino acids, and are thought to be largely functionally redundant (*2–4*). Dependent on the substrate, sumoylation (the conjugation of SUMO onto proteins) can regulate protein-protein and protein-DNA interactions with a variety of physiological consequences, including transcriptional control, maintenance of genome integrity, protein trafficking and protein stability. Unsurprisingly, with such a diverse range of consequences sumoylation substrates are not restricted to any particular subsection of the proteome, although many are involved in chromatin organization, transcriptional regulation and RNA metabolism (*1*, *5*).

Importantly, the SUMO modification state of a particular protein is regulated not only at the level of attachment, but also removal. Thus, the extent to which proteins are conjugated to SUMO is a consequence of the balance of activities of the specific conjugation enzymes E1, E2 and E3, as well as the deconjugating proteases. In eukaryotes a single heterodimeric E1 enzyme (SAE1/SAE2 or Uba2-Aos1) and a single E2 (Ubc9), co-operate with a number of E3 ligases to control SUMO attachment. The reverse reaction is catalyzed by the SUMO-specific proteases, also known as the Sentrin proteases (or SENPs). In the yeast *S. cerevisiae* two proteases, Ulp1 and Ulp2, are known to act upon the yeast SUMO orthologue Smt3, while in humans the SENP family contains six members, Senp1-3 and Senp5-7 (*6*). The SUMO proteases perform three major functions in cells (**Fig. 17.1**). Firstly, as discussed above, they oppose the conjugation enzymes by deconjugating SUMO from target proteins. This deconjugation function is also known as isopeptidase activity, because sumoylation results in the formation of an isopeptide bond between the SUMO C-terminus and the ε-amino group of the target lysine. Secondly, due to the fact that the primary translation products of the three SUMOs are inactive, requiring removal of C-terminal residues to expose the diglycine motif essential for conjugation, the SUMO proteases are also required for the maturation of the SUMO proteins by a function known as C-terminal hydrolase activity, or processing activity. Finally, owing to the fact that SUMO can conjugate onto an internal lysine on other SUMO molecules, thus forming polymeric conjugates, the SUMO proteases are responsible for depolymerizing SUMO back to monomers.

Genetic studies in yeast have shown that Ulp1 and Ulp2 are non-redundant, with the *ulp1* deletion mutant being lethal (G2 to M progression block) (*7*, *8*). Cells lacking Ulp2, although viable, are defective in sporulation, are temperature-sensitive for growth, have decreased plasmid and chromosome stability, and display unusual cell morphology (*9*). In vivo Ulp1 and Ulp2 have distinct sets of target substrates, and it is Ulp1 that is responsible for Smt3 C-terminal hydrolase activity (*9*) although both are

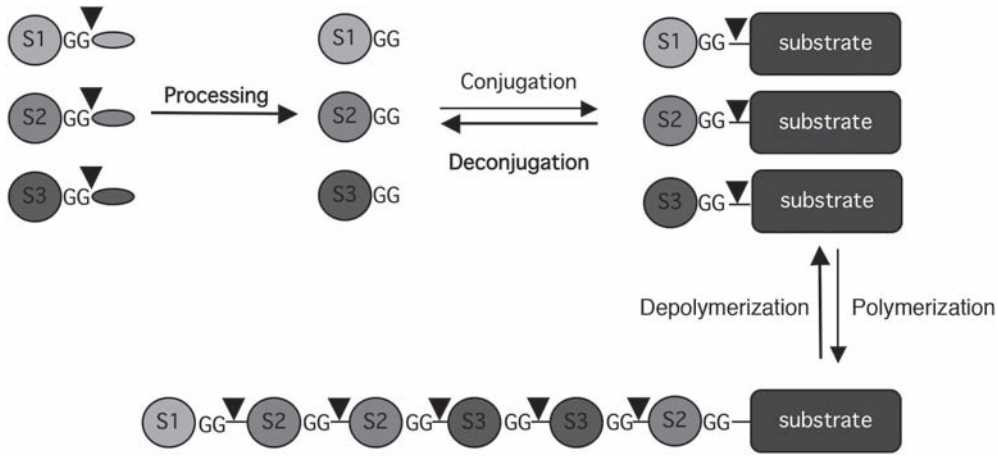

Fig. 17.1. The three modes of action of SUMO proteases. The SUMO proteases perform three major functions in vivo. Before the newly translated SUMO-1 (S1), SUMO-2 (S2), or SUMO-3 (S3) can be conjugated to protein targets, inhibitory C-terminal peptides (shaded ovals) must be removed. This is known as processing or maturation and is the result of the C-terminal hydrolase activity of the SUMO proteases. SUMO proteases, via their isopeptidase activity, also deconjugate SUMO moieties attached to protein substrates, and remove SUMO from other SUMO molecules when functioning to depolymerize SUMO polymers. Filled arrowheads indicate point of protease action, and bold arrows and text show direction of protease reaction progress (Adapted from Ref. *6*).

known to possess hydrolase and isopeptidase activities in vitro. Ulp2 appears to be responsible for depolymerizing poly-Smt3 in vivo (*10*). In humans four of the SUMO proteases, SENP1, SENP2, SENP3 and SENP5, are most closely related to Ulp1, while SENP6 and SENP7 are closer relatives of Ulp2. These similarities are also reflected in function, with SENP1, SENP2, SENP3 and SENP5 displaying varying degrees of isopeptidase and C-terminal hydrolase activities towards the three SUMOs, while SENP6 and SENP7 function to depolymerize SUMO chains. Importantly, in yeasts and humans, the different SUMO proteases are localized to specific cellular compartments, which likely play a significant part in their function in vivo. For a current summary of the substrate specificity and cellular distribution of the SUMO proteases, see Ref. (*6*).

The SENPs are cysteine proteases and all contain a conserved protease domain of about 200 amino-acids that harbors the Cys-Asp-His catalytic triad. The remaining regions of the SENPs are largely unrelated and vary in length from around 350 to 900 residues. These domains are thought to be important for directing the SENPs to particular subcellular localizations and to regulate substrate specificity (*11*).

There are a number of methods available for the in vitro analysis of SUMO proteases. The simplest gel-based approaches have the advantage of using the 'native' proteins, but are laborious, technically demanding and difficult to quantitate (*12*, *13*).

However, recently a number of high-throughput methods have been developed (*14–18*) that provide a means of real-time, quantitative analysis of the progress of a protease reaction. Of these, the FRET-based assays are the only ones that have been adapted to analyze all thee SUMO protease functions in a SUMO paralogue-specific manner.

2. Materials

2.1. Production of ECFP and YFP Fusion Proteins

1. Luria Broth (Merck): prepared as described by the manufacturer and stored in 10–500 ml volumes at 4°C after autoclaving.
2. Kanamycin: dissolved in distilled water to 50 mg/ml, 0.22 μm filter-sterilized and stored in 100–1000 μl aliquots at –20°C (single use).
3. Isopropyl-β-D-thiogalactopyranoside (IPTG): just before use dissolve in Luria broth to 400 mM and 0.22 μm filter-sterilize.
4. Complete protease inhibitor tablets (Roche): dissolve the desired number of tablets in the lysis buffer just before use.
5. Imidazole: dissolve in distilled water to 1 M and stored at –20°C in 100–1000 μl aliquots.
6. Phosphate-buffered saline (PBS).
7. Lysis buffer: PBS with 0.3 M NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol and complete protease inhibitors.
8. Wash buffer: PBS with 0.3 M NaCl, 30 mM imidazole, 1 mM PMSF, 1 mM benzamidine, 5 mM β-mercaptoethanol.
9. Elution buffer: PBS with 0.3 M NaCl, 250 mM imidazole, 1 mM PMSF, 1 mM benzamidine, 5 mM β-mercaptoethanol.
10. Dialysis buffer: 50 mM Tris-HCl, pH7.5, 10 mM NaCl, 2 mM β-mercaptoethanol.
11. *Escherichia coli* BL21DE3 transformed with the following plasmid DNAs (*16*, *19*):
    C-terminal hydrolase assays:
    - pHis-TEV-30a-YFP-SUMO-1(1-101)-ECFP
    - pHis-TEV-30a-YFP-SUMO-2(1-103)-ECFP

    Depolymerisation assays:
    - pHis-TEV-30a-ECFP-SUMO-1(1-97)
    - pHis-TEV-30a-YFP-SUMO-1(1-97)
    - pHis-TEV-30a-ECFP-SUMO-2(1-92)
    - pHis-TEV-30a-YFP-SUMO-2(1-92)

    Isopeptidase assays:

- pHis-TEV-30a-YFP-SUMO-1(1-97)
- pHis-TEV-30a-YFP-SUMO-2(1-92)
- pHis-TEV-30a-ECFP-RanGAP1(418-587)

Plasmids are available from R. T. Hay lab by request. Bacterial cultures should be stored as 15% glycerol stocks at –80°C.

12. Nickel-NTA agarose resin (Quiagen).
13. Q Sepharose resin (SIGMA).
14. UV/visible spectrophotometer.

2.2. In Vitro SUMO Conjugation Assays Using Fluorescent Proteins

1. Recombinant human SAE2/SAE1 (Uba2/Aos1) and human Ubc9 (Alexis Biochemicals or BIOMOL international).
2. Tris-HCl: 1 M aqueous solution, pH 7.5, stored at room temperature.
3. $MgCl_2$: 100 mM aqueous solution, stored at room temperature.
4. ATP (disodium salt): dissolve in distilled water, titrate to pH 7.0 with NaOH and adjusted to 100 mM. Store in 10–1000 µl aliquots at –20°C.
5. β-mercaptoethanol: Store at room temperature in supplied container.

2.3. In Vitro SUMO Protease FRET Assays

1. Multi-well fluorimeter with an excitation wavelength of 400–410 nm and capable of measuring emission at 480 and 530 nm (for example: BMG Labtech NOVOstar fluorimeter with 405-10, 530-10 and 480-10 filters and NOVOstar software (v1.20)).
2. Polystyrene multiwell plates with flat-bottom, black sides and clear base (Corning 384 well non-sterile plates or equivalent).
3. A source of SUMO protease, such as cell extract or recombinant protein. As a positive control for these assays, the recombinant SENP1 catalytic domain (available from BIOMOL international) can be used.
4. Assay buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA).

3. Methods

3.1. Protein Expression and Purification

1. Streak an L-agar plate including 50 µg/ml kanamycin with each of the bacterial stocks to be used for protein induction and incubate at 37°C overnight.
2. Pick a single colony from each plate and grow at 37°C with 200 rpm agitation in a 10 ml culture of L-broth with 50 µg/

ml kanamycin for 12–16 h or until the bacterial suspension has reached an $OD_{600} > 1.0$.

3. Inoculate a 1 l culture of L-broth with 50 µg/ml kanamycin with each 10 ml mini-culture and incubate at 37°C with 200 rpm until the OD_{600} is between 0.6 and 1.0.
4. Chill cultures in iced water for 10 min before adding IPTG to 0.4 mM.
5. Incubate at 22°C with 200 rpm for between 4 and 16 h. Cultures expressing fluorescent proteins should become colored.
6. Pellet bacteria from suspension by centrifugation at ~3,000g for 15 min.
7. Resuspend bacteria fully in ~40 ml PBS and pellet in a 50 ml Falcon tube, ~3,000g for 15 min. At this point the pellets can be stored at –80°C if required.
8. Lyse bacteria by sonication in 25 ml lysis buffer.
9. Centrifuge lysates for 30 min at 20,000g and 4°C.
10. 0.22 µm filter-sterilize the supernatants and load onto a 5 ml Nickel-NTA Sepharose column preequilibrated with lysis buffer without protease inhibitors.
11. Wash each column with 5 column volumes (CV) of wash buffer.
12. Elute bound proteins by addition of elution buffer. Either collect fractions of 0.5 CV each and analyze by SDS-PAGE (*see* **Note 1**) before pooling, or collect protein-containing eluate (colored fractions) into one single tube.
13. Dialyze fluorescent proteins with three buffer changes of at least 100-fold dilution against dialysis buffer.
14. Pass protein solutions over individual 3 ml Q Sepharose columns pre-equilibrated with dialysis buffer, and elute with dialysis buffer containing a NaCl gradient from 100 to 500 mM. Analyze colored fractions by SDS-PAGE, and pool the purest fractions.
15. Re-dialyze against dialysis buffer as described in Step 13 and freeze in 10–1000 µl aliquots at –80°C.

3.2. Calculation of Fluorescent Protein Concentration

1. Dilute a sample of each of the YFP-containing proteins 1:20 in dialysis buffer.
2. Set a spectrophotometer to 515 nm, and set the blank to the same buffer.
3. Read the absorbance value A for your diluted protein sample.
4. Use Beer's law to calculate your protein concentration.
   Beer's law: $A = \varepsilon \times c \times l$
   where: A is the absorbance at 515 nm

ε is the extinction coefficient for YFP (92 200 $M^{-1}{\cdot}cm^{-1}$ at 515 nm)

l is the length of light path (typically 1 cm)

c is the protein concentration (M)

5. Repeat Steps 1–4 using undiluted samples of your ECFP-containing proteins, measuring the absorbance at 435 nm, and use the extinction coefficient for ECFP ε (28 750 $M^{-1}{\cdot}cm^{-1}$ at 435 nm) to calculate the concentration of ECFP in the sample (*see* **Note 2**).
6. Yields are in the region of 1 µmol pure protein per liter of culture.

3.3. Conjugation of SUMO to RanGAP1 and SUMO

Of the proteins expressed directly from the plasmids (*see* **Sect. 2.1**), only the immature SUMO proteins N- and C-terminally linked to YFP and ECFP are ready to use in protease assays. The substrates used to test isopeptidase and depolymerization activities of SUMO proteases first need to be conjugated to SUMO before they can be deconjugated by the protease (**Fig. 17.2**). This is done by in vitro SUMO conjugation using recombinant SUMO specific E1 (SAE2/SAE1) and E2 (Ubc9) enzymes. Examples of conjugation assays used to generate these constructs are shown in **Table 17.1** (*see* **Notes 3** and **4**).

3.4. FRET Analysis of SUMO Protease Activities

How you perform your FRET assays very much depends on your fluorimeter. Obviously, if you have only a single sample unit, you will have to analyze one sample at a time. A multi-well fluorimeter will have the advantage of giving the user the option of running many samples simultaneously, although, unless it can read multiple wells simultaneously (most models can only read one well at a time), you will need to plan your experiment more carefully (*see* **Notes 5–14** for caveats to consider).

We use a NOVOstar (BMG labtech), which has the facility to use 384 well plates and contains an integral pipettor for in situ dispensations, and which reads one well at a time. It uses NOVOstar v1.20 software, which allows the user to write individual programs to perform each task required to run an assay. For a SUMO protease FRET assay three programs are written for each experiment:

1. Dispensation of the FRET substrate:

   The instrument's internal pipettor is used to transfer the FRET substrate (typically 20 µl of 50–1000 nM per well) into the desired position of the 'measurement' plate from either a 'reagent' plate or a microcentrifuge tube, followed by single readings at 480 and 530 nm.
2. Protease injection and initial rate monitoring:

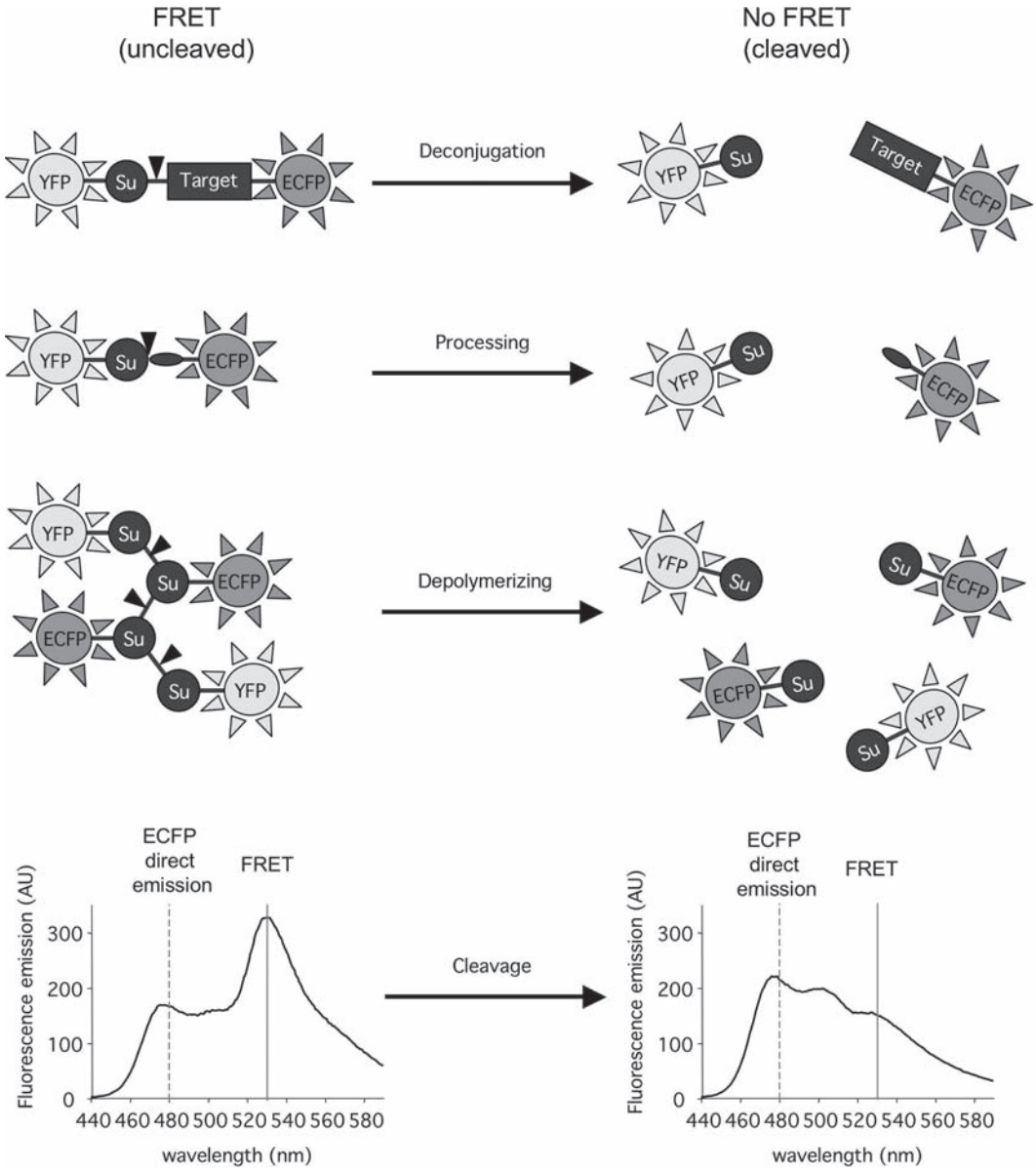

Fig. 17.2. Overview of the three FRET-based SUMO protease assays. Three FRET SUMO substrates were developed whose cleavage state is detectable by measurement of light emitted at 480 and 530 nm upon irradiation at 400–405 nm. The substrates are a (i) SUMO-target protein conjugate, (ii) the immature forms of SUMO, and (iii) polymerized SUMO, all fused to the fluorescent proteins ECFP and YFP. After cleavage by the protease, the 530 nm FRET signal decreases in intensity while the 480 nm signal (direct emission by ECFP) increases due to reduced quenching (see dashed and solid lines on lower charts). Both signals can be used to determine the amount of uncleaved substrate in a reaction at any given time. The lower charts show emission scans of YFP-SUMO-1(1-101)-ECFP before (*left*) and after (*right*) cleavage by SENP1. Similar data are obtained for all substrates described.

The instrument's internal pipettor is used to transfer the protease sample (typically 5 µl) into the desired position of the 'measurement' plate from either a 'reagent' plate or a microcentrifuge tube, followed by continuous readings at 480 and 530 nm for between 2 and 5 min.

Table 17.1
Examples of in vitro conjugation assays used to prepare YFP-SUMO-RanGAP1-ECFP conjugate and YFP/ECFP-SUMO polymeric conjugates. Incubation times and enzyme concentration are given, but it is worth monitoring the reaction by sampling at different time-points during incubation and analysing by SDS-PAGE

Assay component/feature	Isopeptidase substrate	Depolymerization substrate
Fluorescent protein 1 (0.25 µmol)	YFP-SUMO1(1-97) or YFP-SUMO2(1-92)	YFP-SUMO-2
Fluorescent protein 2 (0.25 µmol)	ECFP-RanGAP1(418-587)	ECFP-SUMO-2
Tris-HCl, pH7.5	50 mM	50 mM
NaCl	150 mM	150 mM
$MgCl_2$	5 mM	5 mM
β-mercaptoethanol	5 mM	5 mM
SAE2/SAE1	0.22 µM	0.22 µM
Ubc9	1.66 µM	13.3 µM
Reaction volume	Up to 7.0 ml with dH_2O	Up to 7.0 ml with dH_2O
Temperature	37°C	37°C
Incubation time	2 h	4 h

3. Long-term progress monitoring:

   Emission intensities at 480 and 530 nm are measured for all samples across the entire plate for up to 5 h.

Note that both the protease and FRET substrate samples are prepared in assay buffer. The settings used for each program are outlined in **Table 17.2**.

3.5. Conversion of Fluorimeter Raw Output Data into Concentration of Cleaved Substrate

Data output from NOVOstar are automatically exported into an Excel spread sheet that relates time and temperature to fluorescence emission measurements for each well of the plate monitored. This allows the user to plot charts of the fluorescence intensity at each wavelength monitored with respect to time (**Fig. 17.3A**). For quantitative comparisons, these data need to be converted into concentrations of cleaved substrate. Since the magnitude of the fluorescence intensity at 480 nm and the inverse of the magnitude of the fluorescence intensity at 530 nm is proportional to the concentration of cleaved substrate, these two figures can be used to directly calculate the unknown. The following section outlines a method for converting 480 and 530 nm fluorescence intensity to concentration of cleaved substrate, although it should be pointed out that this is not the only method to do this.

Table 17.2
Summary of the NOVOstar fluorimeter settings generally used for SUMO protease experiments

Fluorimeter setting	Program 1 (Substrate loading)	Program 2 (Protease addition & short-term read)	Program 3 (Long-term read)
Mode	Well	Well	Plate
Temperature	~25°C	~25°C	~25°C
Positioning delay (s)	0.2	0.2	0.2
Number of kinetic windows	1	1	1
Measurement start time (s)	~10	~8.5	0
Number of intervals	1	50–100	30–100
Flashes per well per interval	20–50	20–50	20–50
Duration of data collection	Single point	2–5 min	2–5 h
Measuring fluorescence intensity	Yes	Yes	Yes
Excitation wavelength	405 (±10) nm	405 (±10) nm	405 (±10) nm
Emission wavelength(s)	480 (±10) nm	480 (±10) nm	480 (±10) nm
	530 (±10) nm	530 (±10) nm	530 (±10) nm
Gain (both wavelengths)	1500–2000	1500–2000	1500–2000
Volume of solution added	15–20 µl	5–10 µl	—
Pump speed	100 µl/s	100 µl/s	—
Shaking	None	None	None
Rinse cycles	1	1	—
Air gap	Yes	Yes	Yes
Number of mix cycles	0	3	0
Mix volume	—	10 µl	—

1. Where: E_{480} is the measured emission at 480 nm (**Fig. 17.3A**)
   E_{530} is the measured emission at 530 nm (**Fig. 17.3A**)
   E_{480}^{max} is the maximum measured emission at 480 nm (see **Note 15**)
   E_{480}^{min} is the minimum measured emission at 480 nm
   E_{530}^{max} is the maximum measured emission at 530 nm

E_{530}^{min} is the minimum measured emission at 530 nm (*see* **Note 15**)

First we need to determine the full scale range (FSR) for each wavelength measured for the reaction proceeding from 0 to 100% cleavage.

So:

$$FSR_{480} = E_{480}^{max} - E_{480}^{min} \quad (17.1)$$

$$FSR_{530} = E_{530}^{max} - E_{530}^{min} \quad (17.2)$$

2. Next we need to standardize the measured E_{480} and E_{530} data to the 'zero' signal at time 'zero' and invert the 530 nm data to yield an increase in signal over time rather than a decrease (**Fig. 17.3B**). This will give us E_{480}^{stand} and E_{530}^{stand}:

$$E_{480}^{stand} = E_{480} - E_{480}^{min}$$

and

$$E_{530}^{stand} = -1 \cdot (E_{530} - E_{530}^{max})$$

From this we can calculate the percentage of the substrate cleaved according to data acquired at 480 nm ($E_{480}^{\%}$) and 530 nm ($E_{480}^{\%}$) (**Fig. 17.3C**) by the following:

$$E_{480}^{\%} = 100 \cdot (E_{480}^{stand} / FSR_{480})$$

$$E_{530}^{\%} = 100 \cdot (E_{530}^{stand} / FSR_{530})$$

3. At this point we can combine both sets of data to give a mean value $E_{mean}^{\%}$ for each time point:

$$E_{mean}^{\%} = (E_{480}^{\%} + E_{530}^{\%}) / 2$$

This can be standardized back to a scale of 0–100% (**Fig. 17.3D**):

$$E_{mean}^{\%\ stand} = 100 \cdot (E_{mean}^{\%} / E_{mean}^{\%max})$$

Where $E_{mean}^{\%\ max}$ is the average of the $E_{mean}^{\%}$ values once the reaction is complete. This is important because the average of the $E_{mean}^{\%}$ values will probably not be 100%.

4. This can then be converted to the actual amount of cleaved substrate, $E_{mean}^{substrate}$ (**Fig. 17.3E**), by:

$$E_{mean}^{substrate} = [S]_{t=0} \cdot (E_{mean}^{\%\ stand} / 100)$$

Where $[S]_{t=0}$ is the initial concentration of substrate in the assay.

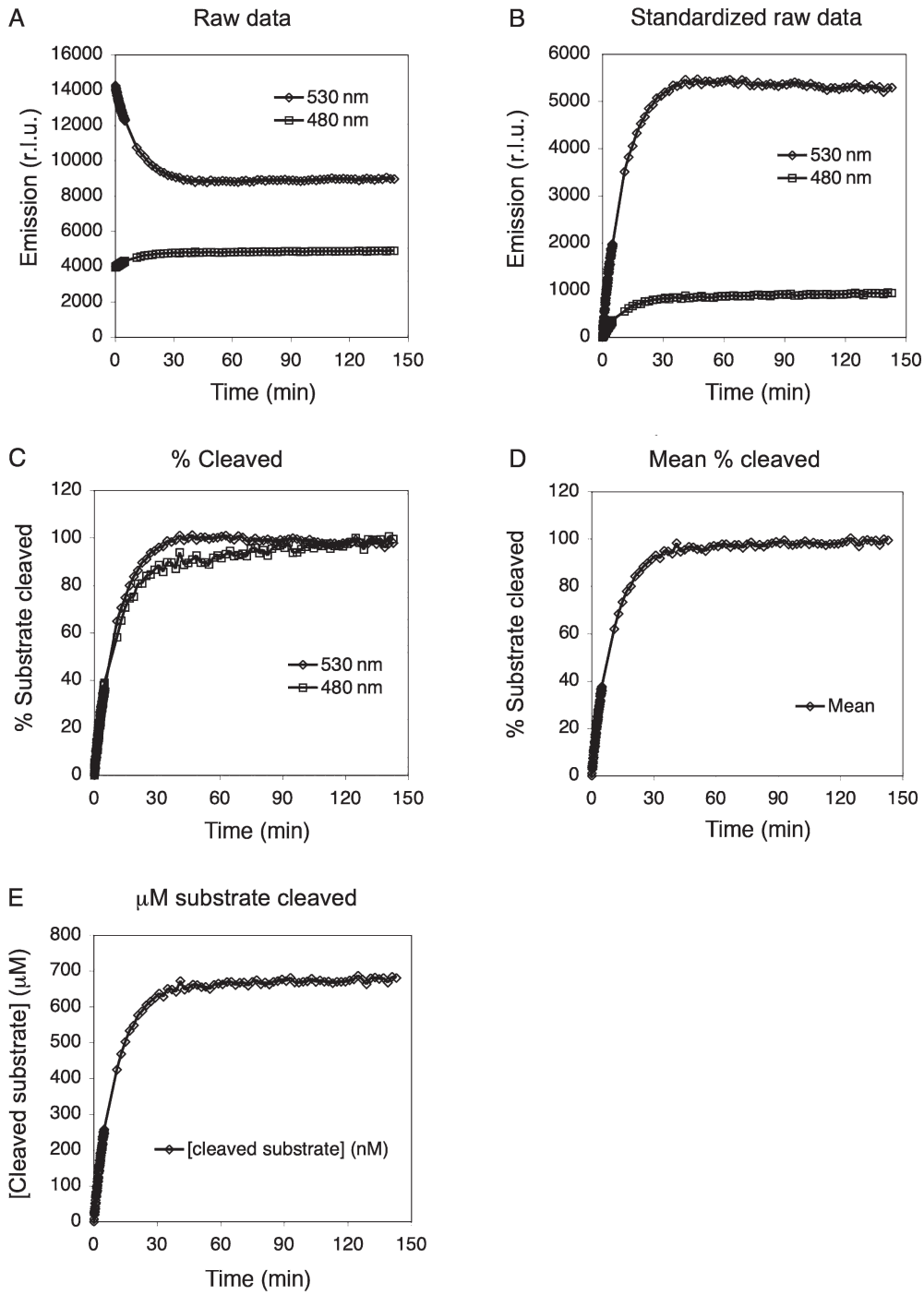

Fig. 17.3. Processing raw data output from the fluorimeter to calculate the concentration of cleaved substrate in the reaction. (**A**) Example of data output from the NOVOstar fluorimeter for an experiment using 685 nM YFP-SUMO-1(1-101)-ECFP digested by 1 nM SENP1(415–644) in a 25 μl reaction. Fluorescence intensity at 480 and 530 nm was measured using 405 nm incident light. The reaction was monitored at 3 s intervals for 5 min after the addition of protease, then at 2 min intervals for a further 130 min. Note that the gap in the data series is due to the fact that this experiment was one of over 30 assays run simultaneously, and represents the time taken for the fluorimeter to start other reactions, before reading the entire plate every 2 min (see Notes 11 and 14). (**B**) Data from panel (a) standardized to 'zero' emission at time = 0 and to give an increase in r.l.u during the reaction progress. (**C**) Percentage of cleaved substrate according to 480 and 530 nm outputs calculated from panel (b). (**D**) Mean percentage of cleaved substrate using 480 and 530 nm data shown in (c). Concentration of YFP-SUMO-1(1-101)-ECFP cleaved by SENP1 calculated from data shown in (d). See text for details of calculations.

4. Notes

1. The jellyfish fluorescent proteins and their derivatives are extremely stable. This is advantageous when it comes to their analysis by SDS-PAGE, because if protein samples are not boiled before fractionation, the fluorescent proteins remain folded in the gel. They will then fluoresce in the gel under UV irradiation (**Fig. 17.4A**), which allows the user to see exactly which species are fluorescent and also to verify the identity of each fluorescent protein due to the difference in colors between YFP and ECFP. If quantification is required, such gels can also be scanned using a gel reader with appropriate lasers fitted. Gels can be Coomassie-stained later if required.
2. If necessary for protein preps containing equal amounts of both YFP and ECFP, both methods can be used and compared. They should agree to within about 10%.
3. Monitor your in vitro conjugation assays by sampling at 30 min intervals and analyze by fractionation on SDS-PAGE. If your assays are progressing too slowly more SAE2/SAE1 and Ubc9 can be added. SUMOs, RanGAP1 and fluorescent proteins are very stable, so extended incubations (up to 24 h) at 37°C are not usually a problem.
4. After conjugation the newly-synthesized substrates should be purified away from the SUMO conjugation enzymes by Nickel-NTA Sepharose affinity chromatography as described in **Sect. 3.1**, Steps 10–15, but including an extra wash step with buffer containing 1 M NaCl.
5. Always run your reactions to completion if you want to quantify them in terms of substrate concentration. You need to know the magnitude of the signals at 480 and 530 nm both before and after complete digestion with the protease to determine the 'uncleaved' and 'cleaved' values (0 and 100% cleavage) for your particular quantity of substrate, and hence the full scale range (FSR) for your assay. If you know the concentration of the FRET substrate then this can easily be used to calibrate the signal intensities at 480 and 530 nm to a concentration value (*see* **Sect. 2.5**. for details).
6. Avoid bubbles in your FRET assays at all costs. Check your plates by eye after dispensation of the FRET substrate to make sure none are present. If they are present, bubbles can usually be removed by centrifugation of the plate (suitable centrifuge and rotor required). It is always worth checking after your assays have finished just to make sure bubbles haven't been inadvertently introduced and may have interfered with your analysis.

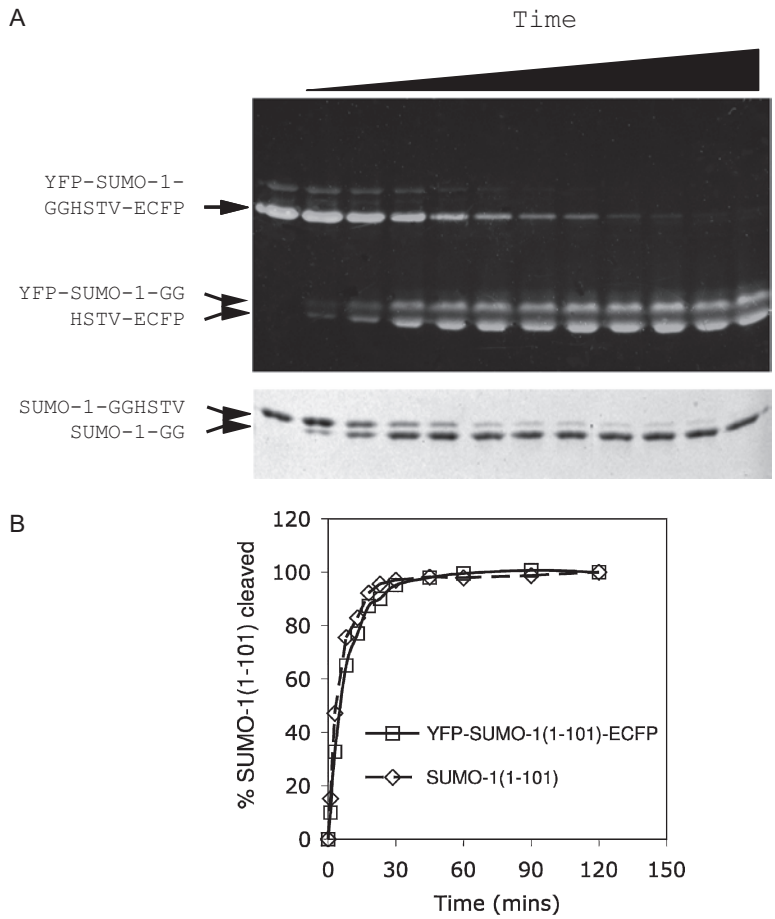

Fig. 17.4. Fluorescent protein adducts do not significantly affect SUMO processing rates. Analysis of samples of YFP-SUMO-1(1-101)-ECFP, and SUMO-1(1-101) during the progress of a SENP1 digestion show that the rate of cleavage is unaffected by the fusion with fluorescent proteins. (**A**) (*upper panel*) Photograph of a UV-irradiated SDS-PAGE gel showing a fractionation of the YFP-SUMO-1(1-101)-ECFP samples from a SENP1 protease assay (labeled YFP-SUMO-1-GGHSTV-ECFP to indicate the amino acid sequence of the linear fusion protein). The image shows the gradual disappearance of a low mobility species (uncleaved substrate) and the concomitant appearance of two higher mobility species (YFP-SUMO-1(1-97) and ECFP fused to residues 98-101 of SUMO-1 (HSTV), which can be distinguished by color (not shown). A Coomassie-stained SDS-PAGE gel of an equimolar quantity of the 'native' SUMO-1(1-101), cleaved in parallel by SENP1, shows a similar rate of cleavage. (**B**) Densitometric analysis of the image shown in (a). (Adapted from Ref. *16*).

7. Since the plates are uncovered during analysis, evaporation can cause sample concentrations to increase, which affects data collection. To avoid this we have found that it is best to adjust the concentration of protease used in the assays to allow reaction completion in less than 5 h. Where this is not possible, most plate suppliers manufacture clear plastic sealing film for their plates, which can be applied at a suitable point in the experiment. Make sure to recalibrate your data if these affect the signal strength.
8. The magnitude of fluorescence signals is affected by temperature, so it is important to run your assays at a constant temperature. The NOVOstar has an integral heating plate, and the equipment can be used in a cold room. Thus, fixed temperatures below

room temperature can be achieved if required. We usually run assays at 25°C.

9. Avoid using the wells at the edge of the plate for experiments as their spectral qualities tend to differ slightly from those of the internal wells.
10. Using the buffers described here and water as the wash solution, the NOVOstar integral pipettor appears to have a slight carry-over issue that can interfere with assays. Firstly, it usually takes two dispensations of FRET substrate before it consistently dispenses the same quantity. Secondly, after having just dispensed protease, it takes at least three dispensations (with two washes in between each) of buffer only, before absolutely no protease activity is detectable. To allow for these problems, set up your experiment with a number of wells with 'trial' assays where FRET substrate or protease are dispensed just to equilibrate the pipettor. This may be an idiosyncrasy of the NOVOstar machine under the conditions described, but it is more likely to be a feature to some degree of any fluorimeter with an internal pipettor.
11. Unless you have a multi-channel fluorimeter capable of dispensing into and reading multiple wells simultaneously you will need to plan a multiple-sample experiment carefully. For example, if you have intend to analyze 40 assays for 2 min each immediately after protease addition, it will take about 100 min for these to be analyzed before the long-term analysis can begin. In order to get as much curve information as possible, it is important to arrange your assays from slow to fast so that those predicted to finish most quickly are started latest. This limits the time between the short constant analysis and the long-term analysis.
12. If you are interested in the initial rates of your reaction, try to cover at least the first 15% of cleavage of your substrate with at least 20 data points. This should yield enough points to accurately estimate the initial rate.
13. When manually adding liquids to 384 well 'reagent' plates, aspirate the liquid as normal with a hand pipette (Gilson or similar). Subsequently, while touching the bottom corner of the well with the pipette tip, dispense the liquid, but only to the first 'click' of the pipette. By dispensing to the second 'click' you risk adding bubbles to the well. Add a twofold excess of sample to those wells from which the automatic pipettor is set to remove a sample, in order to avoid any further problems with bubbles.
14. When starting program 2 (addition of protease) in your experiment, start a timer. Note the time at which you start program 3 (long-term analysis). You will need this time to

relate the timings of the two programs when combining the data later.

15. When determining the 'maximum' and 'minimum' values for the ECFP and FRET emissions, signal variation needs to be considered. Therefore these values should be the average of the values measured once the reaction has reached completion rather than the highest or lowest single number measured.

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