An *In Vitro* Förster Resonance Energy Transfer-Based High-Throughput Screening Assay for Inhibitors of Protein–Protein Interactions in SUMOylation Pathway

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ABSTRACT

Förster resonance energy transfer (FRET) is a powerful tool in biological research and has been widely used in the study of biomolecular interactions. SUMOylation is an important post-translational modification that is involved in many key biological processes. As a multi-step cascade reaction, SUMOylation involves multiple enzymes and protein–protein interactions. Here, we report the development of an *in vitro* FRET-based high-throughput screening (HTS) assay in SUMOylation. This assay is based on steady state and high efficiency of the fluorescent energy transfer between CyPet and YPet fused to SUMO1 and Ubc9, respectively. We optimized the assay and performed a small-scale pilot study to validate the screening platform. Carried out in 384-well plate format, our FRET-based HTS provides a powerful tool for large-scale and high-throughput applications.

INTRODUCTION

Protein post-translational modifications alter protein functions in most cells, especially in eukaryotic cells. Common modifications involve the attachment of small-chemical moieties, such as phosphate, acetyl, or methyl groups, that mediate many cellular events, including signaling transduction, DNA repair, and transcriptional regulation.^{1–3} Besides those small-chemical moieties, small proteins also function as protein modifiers. For example, in recent years, SUMO, known as small ubiquitin-related modifier, has emerged as an important protein modifier. Composed of ~100 amino acids, SUMO undergoes reversible conjugation to the lysine residues of target proteins by the actions of various enzymes (SUMOylation).^{4–6} Although structurally related to ubiquitin, SUMO shares only 18% sequence identity with ubiquitin and has distinct effects on target proteins.⁷ In a target-specific manner, SU-

MOylation affects a target protein's intracellular localization, ability to interact with other proteins, or transcriptional activity.⁸ SUMOylation may also compete with ubiquitination for the same lysine residues to increase the stability of target proteins.⁹ Given its important role in many biological processes, SUMO is required in most eukaryotic organisms. Although not yet completely understood, misregulated SUMOylation has been linked to human diseases, including cancer, neurodegenerative diseases, and viral infections.^{10–12}

Similar to the case of ubiquitination, conjugation and deconjugation of SUMO require the action of multiple enzymes. SUMO is translated from mRNA as a precursor protein. Pre-SUMO is then cleaved by SUMO-specific proteases (SENPs) to active SUMO with a C-terminal Gly-Gly motif.¹³ The heterodimer Aos1/Uba2, which is the SUMO E1 activating enzyme, forms a thioester bond to SUMO with energy from the degradation of ATP.^{14–16} SUMO is further transferred from the E1 enzyme to the active site cysteine of the SUMO E2 conjugating enzyme Ubc9.¹⁷ Catalyzed by SUMO E3 ligases, SUMO is finally transferred from Ubc9 to the lysine residues of target proteins.⁸ SUMOylated proteins are then recognized by SENPs, and free SUMO is cleaved off to be used for conjugation to other proteins.¹³

Protein–protein interactions are crucial for the SUMOylation cascade. Using X-ray crystallography and biochemical methods, such as yeast two-hybrid assay, interactions between proteins involved in the SUMOylation pathway have been investigated.^{16,18–21} The interaction of SUMO and its E2 enzyme, Ubc9, is one of the best-studied interactions in the SUMOylation pathway. Studies on the crystal structure of the protein complex identified the residues in the interaction interface of SUMO1-Ubc9, which are conserved among SUMO and Ubc9 family members.²² Biochemical studies showed that the noncovalent interaction of SUMO-Ubc9 is not essential to the SUMOylation of substrate proteins but is for forming polySUMO chains.²³ Although SUMO forms a polymeric chain *in vitro* and *in vivo*,^{24,25} its *in vivo* function still needs to be elucidated. Chemical tools that disrupt the SUMO-Ubc9 interaction will be useful for studying its biological function. However, although there has been much effort looking for small-chemical inhibitors of the

ABBREVIATIONS: DTT, dithiothreitol; FRET, Förster resonance energy transfer; HTS, high-throughput screening; IPTG, isopropyl β -D-L-thiogalactopyranoside; SENP, SUMO-specific protease; SUMO, small ubiquitin-related modifier.

SUMOylation pathway,^{26–28} not much success has been done to identify compounds that disrupt the SUMO-Ubc9 interaction.

Förster resonance energy transfer (FRET) is an energy-transfer phenomenon between two adjacent fluorophores with significant overlap in donor emission spectrum and acceptor excitation spectrum. The efficiency of energy transfer is highly sensitive to the distance between the donor and acceptor fluorophores, which makes FRET very appealing for bioanalysis. FRET-based techniques have been extensively used in biological research for identifying protein interactions, real-time monitoring of intracellular signaling activities, and high-throughput screening (HTS) of bioactive molecules.^{29–31}

Our group previously reported a cell-based FRET HTS assay to identify small-chemical compounds that disrupt the interaction of SUMO1 and Ubc9.³² In this assay, CyPet and YPet, a fluorescent protein pair developed in 2005 with enhanced FRET efficiency,³³ were tagged to SUMO1 and Ubc9, respectively. Plasmids were transfected into mammalian cells to generate cell lines that stably express these two fusion proteins, and compounds were applied to the cells to determine their effects on the FRET in the cells. However, this cell-based screening had several issues, including the signal variation between different batches of cells and the lack of flexibility to control the amount of protein expression in the cells.

Here, we report the development of an alternative FRET-based *in vitro* HTS assay for molecules that disrupt the SUMO1-Ubc9 interaction. The same FRET pair, CyPet and YPet, was used to modify SUMO1 and Ubc9. After purified recombinant CyPet-SUMO1 and YPet-Ubc9 proteins had been mixed *in vitro*, compounds were tested for their ability to inhibit FRET between two proteins. The conditions of the screening can be easily optimized by changing the amount of proteins used in the assay, and it was performed in a 384-well plate format, which readily allows repeated study and large-scale application, such as genome-wide and industrial applications.

MATERIALS AND METHODS

DNA Constructs, Protein Expression, and Purification

The protocols of DNA construct generation and protein expression were as previously described.³² Briefly, the open-reading frames of CyPet and YPet were amplified by PCR with primers containing *NheI*/*Sall* sites (CyPet/YPet) or *Sall*/*NotI* sites (SUMO1/Ubc9), and the PCR products were cloned into pCRII-TOPO vector (Invitrogen). The fragments encoding SUMO1 and Ubc9 were extracted by *Sall*/*NotI* digestion and inserted into the *Sall*/*NotI* sites of pCRII-CyPet or pCRII-YPet, respectively. After the sequences had been confirmed, the cDNAs encoding CyPet-SUMO1 and YPet-Ubc9 were cloned into the *NheI*/*NotI* sites of pET28(b) vector (Novagen). The open-reading frames of CyPet were also amplified by PCR with primers containing *NheI*/*NotI* sites and inserted into pET28(b) vector.

BL21(DE3) *Escherichia coli* cells were transformed with pET28 vectors encoding CyPet, CyPet-SUMO1, or YPet-Ubc9. After the transformed bacteria had been plated on LB agar plates containing 50 µg/mL kanamycin, a single colony was picked and inoculated in 2xYT medium. The expression of polyhistidine-tagged recombinant proteins was induced with 0.1 mM isopropyl β-D-L-thiogalactopyranoside

(IPTG) at 25°C overnight. Bacterium cells were centrifuged at 5400 *g* for 15 min, resuspended in buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 5 mM imidazole, and sonicated with an ultrasonic liquid processor (Misonix). The cell lysate containing recombinant proteins was cleared by centrifugation at 25,000 *g* for 30 min. The recombinant proteins were then bound to Ni²⁺-NTA agarose beads (QIAGEN) and eluted in buffer containing 20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 150 mM imidazole. After the proteins had been dialyzed in buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol (DTT), they were concentrated and purified by gel filtration high-performance liquid chromatography (HPLC) with a Superdex75 10/300 GL column on an ÄKTA purifier system (GE Healthcare). The purity of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining, and their concentrations were determined by Coomassie Plus Protein Assay (Thermo) with known quantities of bovine serum albumin as standards. Aliquots of final products were stored at –80°C.

Fluorescence Measurement and Analysis

The fluorescent emissions of CyPet- or YPet-tagged proteins were determined by the fluorescent plate reader Flexstation II³⁸⁴ (Molecular Devices) in 384-well plates at the excitation wavelength of 414 nm with a cutoff filter of 455 nm. The readings were later corrected by the background fluorescence of the plate to get the emission intensities of the fluorescent proteins. The emission ratio of each well was then calculated by dividing the emission intensity at 530 nm by that at 475 nm.

Optimization of HTS

To determine the urea concentration that completely disrupts the interaction of CyPet-SUMO1 and YPet-Ubc9, 15 pmole of YPet-Ubc9 protein was mixed with 15 pmole of CyPet-SUMO1 or CyPet in a total volume of 60 µL of buffer containing 20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl in the presence of 0, 0.7, 1.3, 2.7, 4, or 5.3 M urea. At each urea concentration, the emission ratio of the mixture of CyPet-SUMO1 and YPet-Ubc9 was measured and compared with the emission ratio of CyPet and YPet-Ubc9. In the SUMO1 peptide competition assay, 0.5 µM of CyPet-SUMO1 and 0.5 µM of YPet-Ubc9 were mixed with increasing concentrations of SUMO1 peptide without fluorescence protein.

To determine the best molar ratio of CyPet-SUMO1 and YPet-Ubc9 in the screening assay, the two proteins were mixed in a total of 60 µL buffer containing 20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl in the presence or absence of 2.7 M urea. The amount of CyPet-SUMO1 was fixed to 15 pmole, and the molar ratio of CyPet-SUMO1 to YPet-Ubc9 changed from 1:0.6 to 1:2.6. After the emission ratio of each condition had been determined, *Z'* factor was calculated by

$$Z' \text{ factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|},$$

where μ_p , μ_n , σ_p , and σ_n refer to the mean values and standard deviations (SDs) of the emission ratio of CyPet-SUMO1 and YPet-Ubc9 mixture in the presence and absence of urea, respectively.

To determine the best molar concentration of CyPet-SUMO1 and YPet-Ubc9 in the screening assay, two proteins were mixed in a total of 60 μ L of buffer containing 20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl in the presence or absence of 2.7 M urea. The molar ratio of CyPet-SUMO1 to YPet-Uba9 was fixed to 1:1.8, and the amount of CyPet-SUMO1 changed from 9 to 30 pmole. After the emission ratio of each condition had been determined, a Z' factor was calculated by using the equation just given.

Screening of Small-Chemical Compounds That Disrupt the SUMO1-Ubc9 Interaction

Compounds were acquired from Maybridge and dissolved in DMSO to 500 μ M. In the screening, CyPet-SUMO1 and YPet-Ubc9 proteins were mixed and aliquoted into a 384-well plate by Aquamax4 (Molecular Devices). Each well contained 12 pmole of CyPet-SUMO1 and 21.6 pmole of YPet-Ubc9 in a total of 60 μ L of buffer. The compound (1.2 μ L) was then transferred to each well and mixed with the proteins by gently shaking the plates. After the fluorescent emission intensities at 475 nm and 530 nm at the excitation wavelength of 414 nm had been determined by FlexstationII³⁸⁴ (Molecular Devices), the emission ratios of all the wells were calculated and compared to look for compounds that decrease the emission ratio.

Data Analysis

All data were processed by Prism 5 (GraphPad) and reported in the format of mean $\pm$ SD.

RESULTS

Although the noncovalent interaction of SUMO and its E2 enzyme, Ubc9, is not essential for conjugating SUMO to its substrate, the SUMO-Ubc9 interaction is required to form the polySUMO chain.²³ To develop a FRET-based HTS assay to discover chemical compounds that disrupt the SUMO1-Ubc9 interaction, we used a molecular cloning method to tag SUMO1 and Ubc9 with fluorescent proteins and to determine the feasibility of using FRET to monitor their interaction.

Our assay is based on CyPet and YPet, fluorescent proteins developed from CFP and YFP with a greater FRET efficiency than previous compounds. The excitation and emission peak wavelengths of CyPet and YPet are 414 nm and 475 nm, 515 and 530 nm, respectively. In principle, when CyPet and YPet are tagged to SUMO1 and Ubc9, respectively, the interaction of SUMO1 and Ubc9 will bring CyPet and YPet close to each other, thus resulting in energy transfer from excited CyPet to YPet and increased FRET sensitized emission from YPet. When the SUMO1-Ubc9 interaction is disrupted by small-chemical compounds, the amount of FRET-sensitized emission from YPet will be lost, and CyPet will have a higher level of emission due to less quenching (Fig. 1).

To get the fusion proteins of CyPet-SUMO1 and YPet-Ubc9, the open-reading frames of CyPet and YPet were ligated into pCRII-TOPO vector first and then fused to SUMO1 and Ubc9, respectively. The fusion genes of CyPet-SUMO1 and YPet-Ubc9 were then transferred to the bacterial expression vector, pET28(b) (Fig. 2A). The fusion proteins with poly-histidine tag were expressed in *E. coli* strain, BL₂₁(DE₃), and purified by Ni-NTA affinity column and HPLC size-exclusion chromatography.

When CyPet-SUMO1 protein was excited at 414 nm, an emission peak at 475 nm was observed (Fig. 2B, blue line). After YPet-Ubc9 protein had been added, the emission level at 475 nm was decreased (Fig. 2B, green line), and the increase in emission levels at 530 nm were greater than that from YPet alone (Fig. 2B, yellow line). This observation showed that FRET was occurring between CyPet-SUMO1 and YPet-Ubc9.

A high-quality HTS assay should have good reproducibility, a high signal-to-noise ratio, and low cost. To determine the quality of our FRET-based assay and optimize the screening conditions, we will need a compound that disrupts SUMO1-Ubc9 interaction to serve as control, which is not yet available. We instead used urea, which disrupts protein interactions non-specifically at high concentrations, to mimic the effect of positive control in our screening assay. When urea was added to the mixture of CyPet-SUMO1 and YPet-Ubc9 proteins, the disruption of SUMO1-Ubc9 interaction resulted in an increase of emission at 475 nm and a decrease of emission at 530 nm at the excitation wavelength of 414 nm (Fig. 2C).

We then determined whether disrupting the SUMO1-Ubc9 interaction would lead to decreased FRET. To estimate the relative level of FRET, we calculated the ratio of 530 nm emission to 475 nm emission when the proteins were excited at 414 nm. The emission ratio of the mixture of CyPet-SUMO1 and YPet-Ubc9 proteins gradually decreased on the addition of increasing amounts of SUMO1 without fluorescent tag (Fig. 3A) or dilution (data not shown).

We then determined the dose-dependent disruption effects by urea. Since very high concentrations of urea might disrupt the structures of fluorescent proteins, we wanted to find the minimal concentration of urea that completely disrupted the SUMO1-Ubc interactions. We cloned the open-reading frame of CyPet to pET28(b), expressed CyPet protein in *E. coli*, and purified the proteins by Ni-NTA affinity chromatography and HPLC. YPet-Ubc9 protein was then mixed with CyPet-SUMO1 or CyPet, and the emission ratios of the mixtures were measured under different concentrations of urea to determine the condition in which the emission ratio of the interacting protein pair (CyPet-SUMO1 + YPet-Ubc9) equals to that of the noninteracting control (CyPet + YPet-Ubc9). When urea concentration was increased from 0 to 5.3 M, the emission ratio of CyPet-SUMO1 and YPet-Ubc9 decreased from 0.27 (difference of emission ratios from two groups when urea = 0) to 0 (difference of emission ratios from two groups when urea is highest) after subtracting the control group fluorescent signals (CyPet + YPet-Ubc9; Fig. 3B). The IC₅₀ of urea disruption is 0.63 M. Overall, these experiment results showed that we could use FRET to detect the non-covalent interaction of SUMO1 and Ubc9, as well as the disruption of this interaction.

The concept of Z' factor was introduced in 1999 to estimate the quality of a HTS assay.³⁴ Typically, when a HTS assay has a Z factor between 0.5 and 1, its quality can be considered excellent. Using urea as the positive control, we determined the minimal amount of CyPet-SUMO1 and YPet-Ubc9 proteins used in the screening assay to get a satisfactory value of Z' factor. We first fixed the amount of CyPet-SUMO1 transferred into each well to 15 pmole and then varied the

Fig. 1. Principle of high-throughput screening of small-chemical compounds that disrupt SUMO1-Ubc9 interaction. Disrupting the interaction between CyPet-SUMO1 and YPet-Ubc9 by small-chemical inhibitors results in a decrease of the Em_{530}/Em_{475} ratio at an excitation wavelength of 414 nm.

amount of YPet-Ubc9 from 9 to 39 pmole in a final volume of 60 μ L. After the emission ratios of all mixtures had been determined, the mean values and SDs of each group were calculated, and the Z' factor at each [CyPet-SUMO1]:[YPet-Ubc9] ratio was calculated (Fig. 3C). When we increased the ratio to 1:1.4, the Z' factor was barely above 0.50. After the [CyPet-SUMO1]:[YPet-Ubc9] ratio was increased to 1:1.8 or higher, the Z' factors were all above 0.7, mainly due to the larger change of emission ratio on addition of urea. We next fixed the

molar ratio of CyPet-SUMO1 to YPet-Ubc9 to 1:1.8, and repeated this assay by transferring different amounts of proteins into each well. When we changed the amount of CyPet-SUMO1 from 9 to 30 pmole with a fixed [CyPet-SUMO1]:[YPet-Ubc9] ratio of 1:1.8, the Z' factor increased from 0.39 to 0.90 (Fig. 3D) and went above 0.5 at 12 pmole of CyPet-SUMO1. Based on the results of the experiments just mentioned, we decided that the final conditions for our screening assay would be 12 pmole of CyPet-SUMO1 and 21.6 pmole of YPet-

Fig. 2. The emission ratio of Em_{530}/Em_{475} can be used to monitor the interaction between CyPet-SUMO1 and YPet-Ubc9. **(A)** Maps of bacterial expression plasmids encoding CyPet-SUMO1 and YPet-Ubc9. **(B)** Protein interaction results in quenching of CyPet-SUMO1 emission and increased emission from YPet-Ubc9. The emission spectrum of 20 pmole of CyPet-SUMO1 or 40 pmole of YPet-Ubc9 or their mixture was determined at an excitation wavelength of 414 nm. **(C)** Fluorescence spectra show that the interaction between CyPet-SUMO1 and YPet-Ubc9 can be disrupted by urea. About 15 pmole CyPet-SUMO1 and 27 pmole YPet-Ubc9 were mixed in a total volume of 60 μ L in the presence (○) or absence (●) of 2.7 M urea. The mixture was excited at 414 nm, and emission intensities from 455 to 555 nm were collected.

Fig. 3. Optimization of the high-throughput screening assay. **(A)** Competitive inhibition of protein interaction decreases Em_{530}/Em_{475} ratio. Mixtures of 5 mM of CyPet-SUMO1 and 0.5 mM of YPet-Ubc9 were incubated in a total volume of 20 μ L in an increasing concentration of nonlabeled SUMO1 from 0 to 40 mM. Em_{530}/Em_{475} ratio at each condition was determined in triplicate. IC_{50} is 16.2 mM. **(B)** Quantification of Em_{530}/Em_{475} ratio at different concentrations of urea to find the best urea concentration to optimize the process. About 15 pmole YPet-Ubc9 were mixed with 15 pmole CyPet-SUMO1 (open circle) or CyPet (closed circle) in a total volume of 60 μ L in the presence of different concentrations of urea from 0 to 5.3 M. The Em_{530}/Em_{475} ratio at each condition was determined in triplicate. **(C)** Optimization of [CyPet-SUMO1]:[YPet-Ubc9] molar ratio. About 15 pmole CyPet-SUMO1 was mixed with different amounts of YPet-Ubc9 in a total of 60 μ L in the absence or presence of 2.7 M urea. *Left:* The Em_{530}/Em_{475} ratios determined from 16 wells at each condition. *Right:* The Z' factor at each [CyPet-SUMO1]:[YPet-Ubc9] molar ratio. **(D)** Optimization of the amount of proteins per well. Different amounts of CyPet-SUMO1 and YPet-Ubc9 proteins were mixed with a fixed molar ratio of 1:1.8 in a total of 60 μ L in the absence or presence of 2.7 M urea. *Left:* The Em_{530}/Em_{475} ratios determined from 16 wells at each condition. *Right:* The Z' factor at each amount of CyPet-SUMO1 protein.

Ubc9 in a total volume of 60 μ L of buffer in each well of 384-well plates, which represent a 0.2 μ M of CyPet-SUMO1 and a 0.36 μ M of YPet-Ubc9.

To validate our FRET-based HTS platform, we conducted a small-scale pilot screening with a compound collection purchased from Maybridge, Inc. A screening assay protocol was developed (Table 1). To eliminate interference from the fluorescent emissions from compounds during the screening, the compound fluorescence was determined before adding the proteins, and this was subtracted from the spectrum of the final mixtures to calculate the emission ratios of the

fluorescent proteins. After we had screened 300 compounds, the mean value of the emission ratio was 0.86, and the SD was 0.06 (Fig. 4B). FRET signals for most compounds were in the range of mean $\pm$ 3 SD. Only one compound had a much lower emission ratio of 0.18, which was due to the high-fluorescence background from the compound. Although we did not find any inhibitors disrupting SUMO1-Ubc9 interaction from this small collection of compounds, our data showed that this FRET-based assay has a high signal-to-noise ratio and good reproducibility, and it is suitable for screening of a broader range of compounds in the future.

Table 1. Protocol Table for the Förster Resonance Energy Transfer–Based SUMO1–Ubc9 Interaction Screening Assay

Step	Parameter	Values	Description
1	Distribute compounds	10 μ M final concentration in 60 μ L final volume	Distribute compounds in a 384-well black plate containing buffer
2	Measure Em_{530} and Em_{475} ($Ex=414$ nm)		Determine fluorescence emission of compounds as background readings by using Flexstation II384
3	Distribute CyPet-SUMO1 and YPet-Ubc9 to each well	12 pmole of CyPet-SUMO1 and 21.6 pmole of YPet-Ubc9	Distribute fluorescent proteins into assay plates
4	Measure Em_{530} and Em_{475} ($Ex=414$ nm)		Determine Förster resonance energy transfer signals as assay readings by using Flexstation II384
5	Subtract compound fluorescence to get the emission from fluorescent proteins		Substrate compound background by using Excel
6	Data analysis: calculate Em_{530}/Em_{475}		Calculate ratio metric data for each well

DISCUSSION

Using purified recombinant proteins of CyPet-SUMO1 and YPet-Ubc9, we developed an *in vitro* HTS assay for SUMO1-Ubc9 disruptors. Although FRET assay has been widely used in protein–protein interaction in *in vitro* and spectroscopic methods, it has not been developed into a robust HTS technology.^{29,35} The highly efficient FRET pair, CyPet and YPet, provided suitable tools for the high-sensitivity HTS assay.³³ The reversibility of the SUMO1 and Ubc9 interaction is well documented by the competition of free SUMO1 and the urea disruption (Figs. 2D and 3A, B). These results suggest that the interaction of SUMO1 and Ubc9 can be disrupted by other small-molecular inhibitors. Ratio metric measurement and the screening Z' have been determined from absolute amounts of CyPet-SUMO1 and ratio of CyPet-SUMO1 to YPet-Ubc9, and optimized

concentrations (12 pmole of CyPet-SUMO1 and 21.6 pmole of YPet-Ubc9) were chosen for the HTS campaign. Since the absolute amount and the relative ratio of two proteins used in the screening can be precisely controlled, we determined the optimal assay condition in which the lowest concentrations of proteins were used to get a satisfactory assay quality, and carried out the screening with high reproducibility.

Hits were selected based on their decrease of the Em_{530}/Em_{475} ratio (*i.e.*, three SD below the mean signal). In FRET assays, the acceptor emission/donor emission ratio is generally used as a criterion to determine FRET signal.^{29,30,33} However, several factors may affect the acceptor emission/donor emission ratio, cause antifactual change of the ratio, and, therefore, generate false-positive hits. First, the uneven distributions of protein FRET pair in each well may result in a decrease of FRET signal. Second, the fluorescent properties of the assay plate may also cause variations in fluorescent readings. The plastic bottom of assay plates often generates low-level fluorescent signals, and the noise may change the acceptor emission/donor emission ratio. Third, the fluorescent emission of small-molecular compounds at donor emission wavelength may cause a decrease of acceptor emission/donor emission ratio.

We previously reported a cell-based screening assay to identify disruptors of SUMO1-Ubc9 interaction, in which SUMO1 and Ubc9 were tagged with CyPet and YPet, respectively, and coexpressed in selected stable cell line before compounds were applied to the cells.³² Although the cell-based screening assay has the advantage of ruling out compounds that are degraded in the cells or cannot penetrate the cell membrane, the flexibility of controlling the amount of two proteins in the stable cell line is difficult to achieve. The *in vitro* FRET assay and cell-based FRET assay have different pros and cons. The *in vitro* FRET assay has well-controlled concentrations of components and has much less variability in HTS. However, the hits from the *in vitro* FRET assay may not be active in cells because of the lack of cell permeability and other physical chemical properties. On the

Fig. 4. Representative data from a 384-well plate. Each closed circle represents the Em_{530}/Em_{475} ratio of an individual well.

other hand, the concentrations and half life of fluorescent fusion proteins are hard to control in cells, and expression levels could be different in individual cells even from stable cell lines. Therefore, the cell-based FRET assay may not generate sufficient FRET signal, which is a major challenge in current cell-based FRET assay, and also produces much larger variations in HTS. However, hits from cell-based FRET assay should work directly in cells. The hit rate in cell-based FRET assay is smaller than the biochemical FRET assay. When the ratio of [CyPet-SUMO1]:[YPet-Ubc9] is above 1:1.4, the Z' is above 0.5, whereas as in the cell-based FRET assay, the Z' is only 0.17. The CV in *in vitro* FRET assay is 0.07, whereas CV varies from 0.06 to 0.23, depending on cell numbers. In combination with the current *in vitro* FRET platform, both screening quantitative measurements and cell permeability of compounds can be achieved.

In summary, we demonstrated a robust genetic-encoded FRET-based HTS platform for protein–protein interaction inhibitor screening. The strategy using fluorescent proteins as FRET pair for protein interaction inhibitor screening can be used as a general approach for other protein interaction inhibitor discovery. Since recombinant proteins can be obtained by multiple methods, such as insect cells or mammalian cells, they can be easily adapted to other protein–protein interaction pairs in signal transduction pathways. The assay volume, protein amount, and ratio can be controlled; this approach provides an accurate and inexpensive strategy for protein interaction inhibitor screenings. Since our approach does not require radioactive labeling, it is also environmentally friendly. The discovery of protein–protein interaction inhibitors is a very challenging task in current scientific and industrial communities due to several reasons,^{36,37} one of which we believe is the lack of robust screening technologies that can provide starting points for subsequent medicinal chemistry. Our sensitive FRET-based approach can provide a powerful tool and can be expanded to genome-wide protein–protein interaction inhibitor screening.

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DISCLOSURE STATEMENT

The authors are the inventors of this technology and have financial interests.

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