Video Article

Using *In Vitro* Fluorescence Resonance Energy Transfer to Study the Dynamics Of Protein Complexes at a Millisecond Time Scale

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Abstract

Proteins are the primary operators of biological systems, and they usually interact with other macro- or small molecules to carry out their biological functions. Such interactions can be highly dynamic, meaning the interacting subunits are constantly associated and dissociated at certain rates. While measuring the binding affinity using techniques such as quantitative pull-down reveals the strength of the interaction, studying the binding kinetics provides insights on how fast the interaction occurs and how long each complex can exist. Furthermore, measuring the kinetics of an interaction in the presence of an additional factor, such as a protein exchange factor or a drug, helps reveal the mechanism by which the interaction is regulated by the other factor, providing important knowledge for the advancement of biological and medical research. Here, we describe a protocol for measuring the binding kinetics of a protein complex that has a high intrinsic association rate and can be dissociated quickly by another protein. The method uses fluorescence resonance energy transfer to report the formation of the protein complex *in vitro*, and it enables monitoring the fast association and dissociation of the complex in real time on a stopped-flow fluorimeter. Using this assay, the association and dissociation rate constants of the protein complex are quantified.

Introduction

Biological activities are ultimately carried out by proteins, most of which interact with others for proper biological functions. Using a computational approach, the total amount of protein-protein interactions in human is estimated to be ~650,000[1], and disruption of these interactions often leads to diseases[2]. Due to their essential roles in controlling cellular and organismal processes, numerous methods have been developed to study protein-protein interactions, such as yeast-two-hybrid, bimolecular fluorescence complementation, split-luciferase complementation, and co-immunoprecipitation assay[3]. While these methods are good at discovering and confirming protein-protein interactions, they are usually non-quantitative and thus provide limited information about the affinity between the interacting protein partners. Quantitative pull-downs can be used to measure the binding affinity (e.g., the dissociation constant K_d), but it does not measure the kinetics of the binding, nor can it be applied when the K_d is very low due to an inadequate signal-to-noise ratio[4]. Surface plasmon resonance (SPR) spectroscopy quantifies the binding kinetics, but it requires a specific surface and immobilization of one reactant on the surface, which can potentially change the binding property of the reactant[5]. Moreover, it is difficult for SPR to measure fast association and dissociation rates[5], and it is not appropriate to use SPR to characterize the event of exchanging protein subunits in a protein complex. Here, we describe a method that allows measuring rates of protein complex assembly and disassembly at a millisecond time scale. This method was essential for determining the role of Cullin-associated-Nedd8-dissociated protein 1 (Cand1) as the F-box protein exchange factor[6,7].

Cand1 regulates the dynamics of Skp1•Cul1•F-box protein (SCF) E3 ligases, which belong to the large family of Cullin-RING ubiquitin ligases. SCFs consist of the cullin Cul1, which binds the RING domain protein Rbx1, and an interchangeable F-box protein, which recruits substrates and binds Cul1 through the adaptor protein Skp1[8]. As an E3 ligase, SCF catalyzes the conjugation of ubiquitin to its substrate, and it is activated when the substrate is recruited by the F-box protein, and when Cul1 is modified by the ubiquitin-like protein Nedd8[9]. Cand1 binds unmodified Cul1, and upon binding, it disrupts both the association of Skp1•F-box protein with Cul1 and the conjugation of Nedd8 to Cul1[10,11,12,13]. As a result, Cand1 appeared to be an inhibitor of SCF activity in vitro, but Cand1 deficiency in organisms caused defects that suggests a positive role of Cand1 in regulating SCF activities in vivo[14,15,16,17]. This paradox was finally explained by a quantitative study that revealed the dynamic interactions among Cul1, Cand1, and Skp1•F-box protein. Using fluorescence resonance energy transfer (FRET) assays that detect the formation of the SCF and Cul1•Cand1 complexes, the association and dissociation rate constants (k_{on} and k_{off}, respectively) were measured individually. The measurements revealed that both Cand1 and Skp1•F-box protein form extremely tight complex with Cul1, but the k_{off} of SCF is dramatically increased by Cand1 and the k_{off} of Cul1•Cand1 is dramatically increased by Skp1•F-box protein[6,7]. These results provide the initial and critical support for defining the role of Cand1 as a protein exchange factor, which catalyzes the formation of new SCF complexes through recycling Cul1 from the old SCF complexes.

Here, we present the procedure of developing and using the FRET assay to study the dynamics of the Cul1•Cand1 complex[7], and the same principle can be applied to study the dynamics of various biomolecules. FRET occurs when a donor is excited with the appropriate wavelength, and an acceptor with excitation spectrum overlapping the donor emission spectrum is present within a distance of 10-100 Å. The excited state is

transferred to the acceptor, thereby decreasing the donor intensity and increasing the acceptor intensity[18]. The efficiency of FRET (E) depends on both the Förster radius (R_0) and the distance between the donor and acceptor fluorophores (r), and is defined by: $E = R_0^6/(R_0^6 + r^6)$. The Förster radius (R_0) depends on a few factors, including the dipole angular orientation, the spectral overlap of the donor-acceptor pair, and the solution used[19]. To apply the FRET assay on a stopped-flow fluorimeter, which monitors the change of the donor emission in real-time and enables measurements of fast k_{on} and k_{off}, it is necessary to establish efficient FRET that results in a significant reduction of donor emission. Therefore, designing efficient FRET by choosing the appropriate pair of fluorescent dyes and sites on the target proteins to attach the dyes is important and will be discussed in this protocol.

Protocol

1. Design the FRET assay.

1. Download the structure file of the Cul1•Cand1 complex from the Protein Data Bank (file 1U6G).
2. View the structure of the Cul1•Cand1 complex in PyMOL.
3. Use the **Measurement** function under the **Wizard** menu of PyMOL to estimate the distance between the first amino acid of Cand1 and the last amino acid of Cul1 (**Figure 1**).
4. Load the online spectra viewer (see **Table of Materials**) and view the excitation and emission spectra of 7-amino-4-methylcoumarin (AMC) and FlAsH simultaneously (**Figure 2**). Note that AMC is the FRET donor and FlAsH is the FRET acceptor.
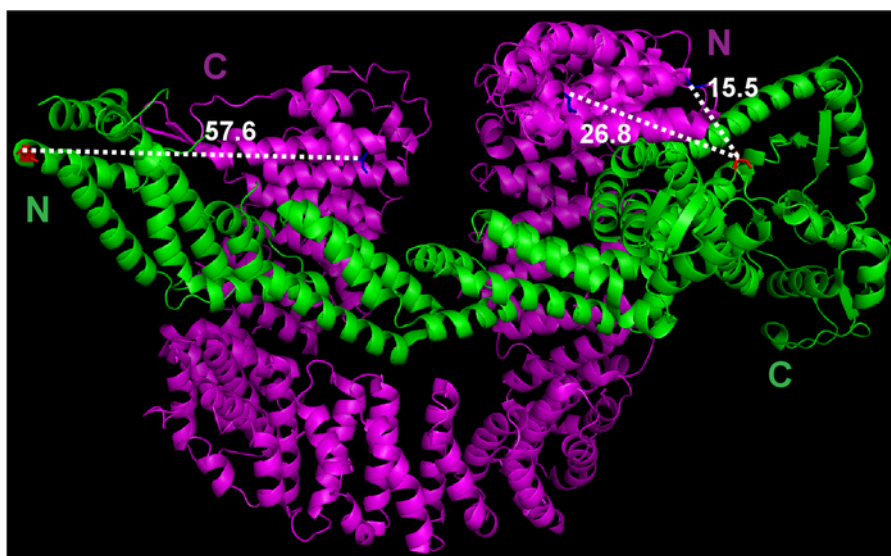
Figure 1: The crystal structure of Cul1•Cand1 and measurement of the distance between potential labeling sites. The crystal structure file was downloaded from Protein Data Bank (File 1U6G), and viewed in PyMOL. Measurements between selected atoms were done by PyMOL. Please click here to view a larger version of this figure.
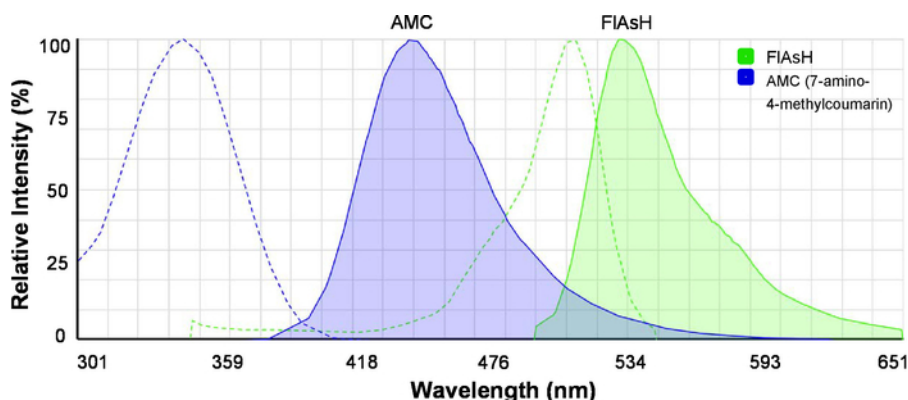
Figure 2: The excitation and emission spectra of the fluorescent dyes for FRET. Spectra of AMC (7-amino-4-methylcoumarin) and FlAsH are shown. Dashed lines indicate excitation spectra, and solid lines indicate emission spectra. The image was originally generated by the Fluorescence SpectraViewer and was modified for better clarity. Please click here to view a larger version of this figure.

2. Preparation of Cul1^{AMC}•Rbx1, the FRET donor protein

1. Construct plasmids for expressing human Cul1^{sortase}•Rbx1 in E. coli cells. Note that the two plasmids co-expressing human Cul1•Rbx1 in E. coli cells are described in detail in a previous report[20].

1. Add a DNA sequence coding "LPETGGHHHHHH" (sortase-His[6] tag) to the 3' end of Cul1 coding sequence through standard PCR and cloning methods[21,22].
2. Sequence the new plasmid to confirm the gene insert is accurate.

2. Co-express Cul1[sortase]•Rbx1 in E. coli cells. The method is derived from a previous report[20].
    1. Mix 100 ng each of the two plasmids with BL21 (DE3) chemically competent cells for co-transformation using the heat shock method[23]. Grow cells on LB agar plate containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37 °C overnight.
    2. Inoculate 50 mL of LB culture with freshly transformed colonies and grow overnight at 37 °C with 250 rpm shaking. This gives a starter culture.
    3. Inoculate 6 flasks, each with 1 L of LB medium, with 5 mL starter culture each and grow at 37 °C with 250 rpm shaking until the OD_{600} is ~1.0. Cool the culture to 16 °C and add isopropyl-β-D-thiogalactoside (IPTG) to 0.4 mM. Keep the culture at 16 °C overnight with 250 rpm shaking.
    4. Harvest the E. coli cells through centrifugation at 5,000 x *g* for 15 min and collect cell pellets in 50 mL conical tubes.
    NOTE: The cell pellets can be processed for protein purification or be frozen at -80 °C before proceeding to the protein purification steps.

3. Purification of the Cul1[sortase]•Rbx1 complex. This method is derived from a previous report[20].
    1. Add 50 mL of lysis buffer (30 mM Tris-HCl, 200 mM NaCl, 5 mM DTT, 10% glycerol, 1 tablet of protease inhibitor cocktail, pH 7.6) to the pellet of E. coli cells expressing Cul1[sortase]•Rbx1.
    2. Lyse the cells on ice with sonication at 50% amplitude. Alternate between 1-second ON and 1-second OFF and run for 3 min.
    3. Repeat step 2.3.2 2-3x.
    4. Transfer the cell lysate into a 50-mL centrifugation tube and remove the cell debris by centrifugation at 25,000 x *g* for 45 min.
    5. Incubate the clear cell lysate with 5 mL of glutathione beads at 4 °C for 2 h.
    6. Centrifuge the beads-lysate mixture at 1,500 x *g* for 2 min at 4 °C. Remove the supernatant.
    7. Wash the beads with 5 mL of lysis buffer (with no protease inhibitors) and remove the supernatant after centrifugation at 1,500 x *g* for 2 min at 4 °C.
    8. Repeat step 2.3.7 2x.
    9. Add 3 mL of lysis buffer to the washed beads and transfer the bead slurry into an empty column.
    10. Add 5 mL of elution buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM reduced glutathione, pH 8.0) into the column. Incubate for 10 min and collect the eluate.
    11. Repeat step 2.3.10 3-4x.
    12. Add 200 µL of 5 mg/mL thrombin (see **Table of Materials**) to the eluate from the glutathione beads and incubate overnight at 4 °C.
    NOTE: The protocol can be paused here.
    13. Dilute the protein sample with Buffer A (25 mM HEPES, 1 mM DTT, 5% glycerol, pH 6.5) threefold.
    14. Equilibrate a cation exchange chromatography column (see **Table of Materials**) on an FPLC system with Buffer A.
    15. Load the protein sample to the equilibrated cation exchange chromatography column at a 0.5 mL/min flow rate.
    16. Elute the protein with a gradient of NaCl in 40 mL by mixing Buffer A and 0 to 50% Buffer B (25 mM HEPES, 1 M NaCl, 1 mM DTT, 5% glycerol, pH 6.5) at a 1 mL/min flow rate.
    17. Check the eluted protein in different fractions using SDS-PAGE[24].
    NOTE: The protocol can be paused here.
    18. Pool the eluate fractions containing Cul1[sortase]•Rbx1.
    19. Concentrate the pooled Cul1[sortase]•Rbx1 sample to 2.5 mL by passing the buffer through an ultrafiltration membrane (30 kDa cutoff).

4. Add AMC to the C-terminus of Cul1 through sortase-mediated transpeptidation[21,22].
    1. Change the buffer in the Cul1[sortase]•Rbx1 sample to the sortase buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl[2], pH 7.5) using a desalting column (see **Table of Materials**).
        1. Equilibrate a desalting column with 25 mL of sortase buffer.
        2. Load 2.5 mL of Cul1[sortase]•Rbx1 sample into the column. Discard the flow-through.
        3. Elute the sample with 3.5 mL of sortase buffer. Collect the flow-through.

    2. In 900 µL of the Cul1[sortase]•Rbx1 solution in the sortase buffer, add 100 µL of 600 µM purified sortase A solution and 10 µL of 25 mM GGGG[AMC] peptide. Incubate the reaction mixture at 30 °C in the dark overnight. Note that this step will generate Cul1[AMC]•Rbx1.
    CAUTION: Fluorescent dyes are sensitive to light, so avoid exposing them to ambient light during the protein and sample preparation as much as possible.
    NOTE: The protocol can be paused here.
    3. Add 50 µL of Ni-NTA agarose beads to the reaction mixture and incubate at room temperature for 30 min.
    4. Pellet the Ni-NTA agarose beads by centrifugation at 5,000 x *g* for 2 min and collect the supernatant.
    5. Equilibrate a size exclusion chromatography column (see **Table of Materials**) with buffer (30 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 10% glycerol) on the FPLC system.
    6. Load all the Cul1[AMC]•Rbx1 sample on the size exclusion chromatography column. Elute with 1.5x column volume of buffer.
    7. Check the eluate fractions by SDS-PAGE[24].
    8. Pool the eluate fractions containing Cul1[AMC]•Rbx1.
    9. Measure the protein concentration using its absorbance at 280 nm with a spectrophotometer.
    10. Aliquot the protein solution and store at -80 °C.
    NOTE: The protocol can be paused here.

3. Preparation of ^{FlAsH}Cand1, the FRET acceptor protein

NOTE: Most of steps in this part are the same as Step 2. Conditions that differ are described in detail below.

1. Construct the plasmid for expressing human ^{TetraCys}Cand1 in E. coli cells.
    1. Add DNA sequence coding "CCPGCCGSG" (tetracysteine/TetraCys tag) before the 15th amino acid of Cand1 through regular PCR[25] (primer sequences: TGCTGTCCGGGCTGCTGCGGCAGCGGCATGACATCCAGCGACAAGGACTTTAG; CTAACTAGTGTCCATTGATTCCAAG).
    2. Insert the PCR product into a pGEX-4T-2 vector. Sequence the plasmid to confirm the gene insert is accurate and in frame.

2. Express ^{TetraCys}Cand1 in E. coli cells in the same way as step 2.2, except that the plasmid is transformed into Rosetta competent cells.
3. Purification of ^{TetraCys}Cand1 from the E. coli cells.
    1. Lyse the E. coli cell pellets and extract ^{TetraCys}Cand1 using glutathione beads. These steps are the same as steps 2.3.1–2.3.12.
    2. Dilute the protein eluate from the glutathione beads with Buffer C (50 mM Tris-HCl, 1 mM DTT, 5% glycerol, pH 7.5) by three folds. Equilibrate an anion exchange chromatography column (see **Table of Materials**) on the FPLC system with Buffer C and load the diluted protein sample at a 0.5 mL/min flow rate.
    3. Elute the protein with a gradient of NaCl in 40 mL by mixing Buffer C and 0 to 50% Buffer D (50 mM Tris-HCl, 1 M NaCl, 1 mM DTT, 5% glycerol, pH 7.5) at a 1 mL/min flow rate. Check the eluted protein in different fractions using SDS-PAGE[24], and pool the fractions containing ^{TetraCys}Cand1. Note that ^{TetraCys}Cand1 has a larger retention volume than free GST.
    4. Concentrate the pooled ^{TetraCys}Cand1 sample by passing the buffer through an ultrafiltration membrane (30 kDa cutoff).
    5. Equilibrate the size exclusion chromatography column with labeling buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM TCEP, 1 mM EDTA, 5% glycerol) on the FPLC system. Load ^{TetraCys}Cand1 sample (500 µL each time) and check the eluate fractions by SDS-PAGE[24].
    6. Pool all the fractions containing ^{TetraCys}Cand1 and concentrate the protein to ~40 µM by passing the buffer through an ultrafiltration membrane (30 kDa cutoff). Estimate the protein concentration using its absorbance at 280 nm. Store the protein as 50 µL aliquots at -80 °C.
        NOTE: The protocol can be paused here.

4. Preparation of ^{FlAsH}Cand1.
    1. Add 1 µL of FlAsH solution (see **Table of Materials**) to 50 µL ^{TetraCys}Cand1 solution.
    2. Mix well and incubate the mixture at room temperature in the dark for 1-2 h to get ^{FlAsH}Cand1.
        NOTE: The protocol can be paused here.

4. Preparation of Cand1, the FRET chase protein

NOTE: The protein preparation protocol is similar to Step 3, with the following modifications.

1. Insert the coding sequence of full-length Cand1 into the pGEX-4T-2 vector.
2. Change the buffer used in step 3.3.5 to a buffer containing 30 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 10% glycerol.
3. Eliminate steps 3.3.7 and 3.3.8.

5. Test and confirm the FRET assay

1. Prepare the FRET buffer containing 30 mM Tris-HCl, 100 mM NaCl, 0.5 mM DTT, 1 mg/mL ovalbumin, pH 7.6, and use at room temperature.
2. Test the FRET between Cul1^{AMC}•Rbx1 and ^{FlAsH}Cand1 on a fluorimeter.
    1. In 300 µL of FRET buffer, add Cul1^{AMC}•Rbx1 (FRET donor) to a final concentration of 70 nM. Transfer the solution into a cuvette.
    2. Place the cuvette in the sample holder of a fluorimeter. Excite the sample with 350 nm excitation light and scan the emission signals from 400 nm to 600 nm at 1 nm increments.
    3. Repeat step 5.2.1 but change Cul1^{AMC}•Rbx1 to ^{FlAsH}Cand1 (FRET acceptor). Scan the ^{FlAsH}Cand1 sample using the same method as in step 5.2.2.
    4. (Optional) Excite the ^{FlAsH}Cand1 with 510 nm excitation light, and scan the emission signal from 500 nm to 650 nm.
    5. In 300 µL FRET buffer, add both Cul1^{AMC}•Rbx1 and ^{FlAsH}Cand1 to a final concentration of 70 nM. Analyze the sample in the same way as in step 5.2.2.

3. Confirm the FRET between Cul1^{AMC}•Rbx1 and ^{FlAsH}Cand1 by adding the chase (unlabeled Cand1) protein (**Figure 3C**).
    1. In 300 µL of FRET buffer, add 70 nM Cul1^{AMC}•Rbx1 and 700 nM Cand1. Scan the sample emission as in step 5.2.2.
    2. In 300 µL of FRET buffer, add 70 nM ^{FlAsH}Cand1 and 700 nM Cand1. Scan the sample emission as in step 5.2.2.
    3. In 300 µL of FRET buffer, add 70 nM Cul1^{AMC}•Rbx1 and 70 nM ^{FlAsH}Cand1, and incubate the sample at room temperature for 5 min. Then add 700 nM Cand1, and immediately after the addition, scan the sample emission as in step 5.2.2). Note that this step is similar to step 5.2.5.
    4. In 300 µL FRET buffer, sequentially add 70 nM Cul1^{AMC}•Rbx1, 700 nM Cand1, and 70 nM ^{FlAsH}Cand1. Incubate the sample at room temperature for 5 min, and scan the sample emission as in step 5.2.2. Note that this is the chase sample (green line in **Figure 3C**).

6. Measure the association rate constant (*k$_{on}$*) of Cul1•Cand1

NOTE: Details of operating a stopped-flow fluorimeter has been described in a previous report[26].

1. Prepare the stopped-flow fluorimeter for measurement.
    1. Turn on the stopped-flow fluorimeter according to the manufacturer's instruction.
    2. Set the excitation light at 350 nm, and use a band-pass filter that allows 450 nm emission light to pass and blocks 500-650 nm emission light.
    3. Keep the sample valves at the **FILL** position, and connect a 3 mL syringe filled with water. Wash the two sample syringes (A and B) with water by moving the sample syringe drive up and down several times. Discard all the water used in this step.
    4. Keep the sample valves at the **FILL** position, and connect a 3 mL syringe filled with FRET buffer. Wash the two sample syringes with the FRET buffer by moving the sample syringe drive up and down several time. Discard all the FRET buffer used in this step.

2. Take a control measurement (**Figure 4C**).
    1. Connect a 3 mL syringe and load Syringe A with 100 nM Cul1^{AMC}•Rbx1 in the FRET buffer. Turn the sample valve to the **DRIVE** position.
    2. Connect a 3 mL syringe and load Syringe B with the FRET buffer. Turn the sample valve to the **DRIVE** position.
    3. Use the **Control Panel** under **Acquire** in the software to take five shots (mix equal volume of samples from Syringe A and Syringe B) on the stopped-flow fluorimeter without recording the results.
    4. Open the **Control Panel** under **Acquire** in the software, and program to record the emission of Cul1^{AMC} over 60 s. Then take a single shot.
    5. Repeat step 6.2.4 2x.
    6. Turn the sample valve to the **FILL** position. Empty Syringe B and wash with the FRET buffer.

3. Measure observed association rate constants (k_{obs}) of Cul1•Cand1 (**Figure 4B**).
    1. Keep the sample in Syringe A the same as in step 6.2.1.
    2. Connect a 3 mL syringe and load Syringe B with 100 nM ^{FlAsH}Cand1 in the FRET buffer. Turn the sample valve to the **DRIVE** position.
    3. Use the **Control Panel** under **Acquire** in the software to take five shots without recording the results.
    4. Open the **Control Panel** under **Acquire** in the software, and program to record the emission of Cul1^{AMC} over 60 s. Then take a single shot.
    5. Repeat step 6.3.4 2x.
    6. Empty Syringe B and wash with the FRET buffer.
    7. Repeat steps 6.3.1–6.3.6 several times with increasing concentrations of ^{FlAsH}Cand1 in the FRET buffer.
    8. Fit the change (decrease) in fluorescent signals measured over time from each shot to a single exponential curve. This will give k_{obs} in each measurement, and the unit is s^{-1}. Note that the basis of this calculation has been well discussed in a previous report[27].

4. Calculate the average and standard deviation of k_{obs} for each ^{FlAsH}Cand1 concentration used. Plot the average k_{obs} against Cand1 concentration (**Figure 4D**), and the slope of the line represents the k_{on} of Cul1•Cand1, with a unit of M^{-1} s^{-1}.

7. Measure the dissociation rate constant (k_{off}) of Cul1•Cand1 in the presence of Skp1•F-box protein.

NOTE: This step is similar to Step 6, with the following modifications.

1. In Syringe A, under the **FILL** position, load a solution of 100 nM Cul1^{AMC}•Rbx1 and 100 nM ^{FlAsH}Cand1 in the FRET buffer. Turn the sample valve to the "DRIVE" position.
2. In Syringe B, under the **FILL** position, load a solution of Skp1•Skp2 (prepared following a previous report[20]). Turn the sample valve to the "DRIVE" position.
3. Open the **Control Panel** under **Acquire** in the software, and program to record the emission of Cul1^{AMC} over 30 s. Then take a single shot. The fluorescent signals increase over time after mixing solutions from Syringe A and Syringe B (**Figure 5**).
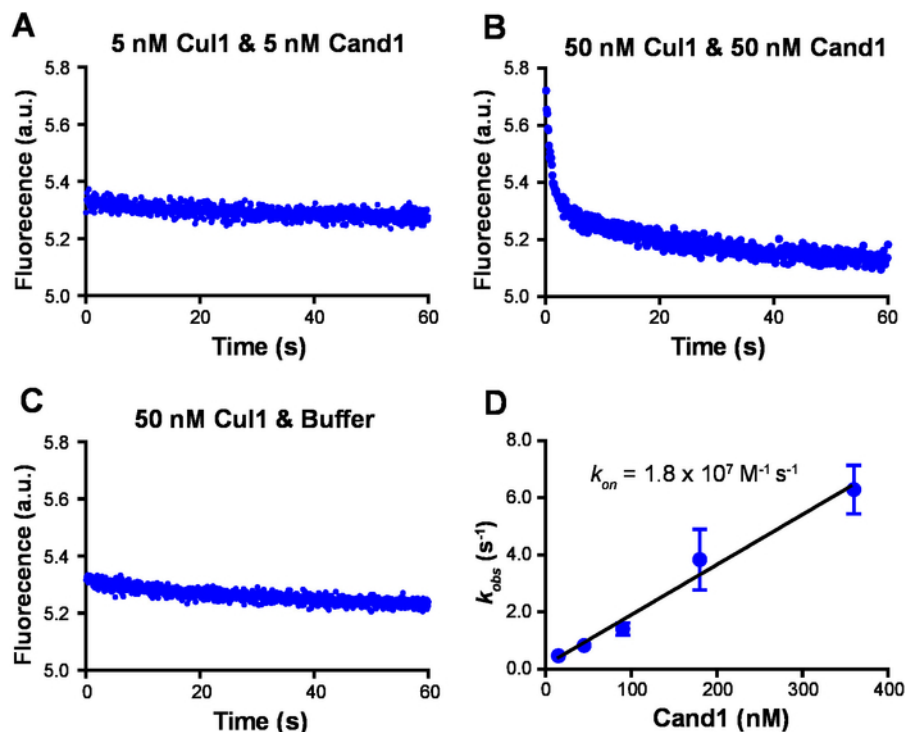
Representative Results

To test the FRET between Cul1^{AMC} and ^{FlAsH}Cand1, we first determined the emission intensity of 70 nM Cul1^{AMC} (the donor) and 70 nM ^{FlAsH}Cand1 (the acceptor), respectively (**Figure 3A-C**, blue lines). In each analysis, only one emission peak was present, and the emission of ^{FlAsH}Cand1 (the acceptor) was low. When 70 nM each of Cul1^{AMC} and ^{FlAsH}Cand1 were mixed to generate FRET, two emission peaks were present in the emission spectra, and the peak of Cul1^{AMC} became lower and the peak of ^{FlAsH}Cand1 became higher (**Figure 3A-C**, red lines). When the full-length Cand1 was used for FRET, the donor peak showed a 10% reduction in intensity (**Figure 3A**, red line), and when Cand1 with its first helix truncated was used, the reduction of donor peak intensity was increased to 30% (**Figure 3B-D**, red lines), suggesting higher FRET efficiency. To confirm that the signal changes were resulted from FRET between Cul1^{AMC} and ^{FlAsH}Cand1, 70 nM Cul1^{AMC} (the donor) was mixed with 700 nM unlabeled Cand1 (the chase) and 70 nM ^{FlAsH}Cand1 (the acceptor). As a result, the donor peak was fully restored and the acceptor peak was decreased (**Figure 3C**, green line), which confirmed that the observed FRET depends on the formation of the Cul1^{AMC}•^{FlAsH}Cand1 complex. Adding 700 nM Skp1•Skp2 to the 70 nM Cul1^{AMC}•^{FlAsH}Cand1 also fully restored the donor peak (**Figure 3D**, green line), suggesting that the Cul1•Cand1 complex was fully disrupted by Skp1•Skp2 at equilibrium.
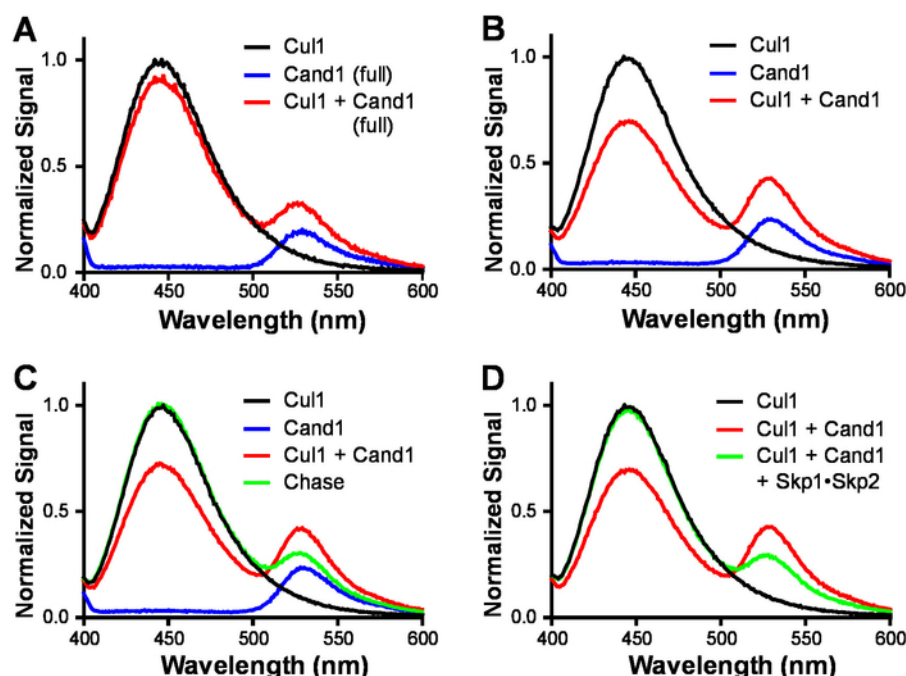
Figure 3: Representative FRET assay for Cul1•Cand1 complex formation. Samples in the FRET buffer (30 mM Tris-HCl, 100 mM NaCl, 0.5 mM DTT, 1 mg/mL ovalbumin, pH 7.6) were excited at 350 nm, and the emissions were scanned from 400 nm to 650 nm. **(A)** Emission spectra of 70 nM Cul1[AMC], 70 nM [FlAsH]Cand1 (full), and a mixture of the two (FRET). Cand1 (full) stands for full length Cand1. **(B)** Emission spectra of 70 nM Cul1[AMC], 70 nM [FlAsH]Cand1, and a mixture of the two (FRET). Cand1 with its first helix deleted was used in this experiment and thereafter. **(C)** Emission spectra of 70 nM Cul1[AMC], 70 nM [FlAsH]Cand1, a mixture of the two (FRET), and chase control for FRET (Chase). The chase sample contained 70 nM Cul1[AMC], 700 nM Cand1 and 70 nM [FlAsH]Cand1. **(D)** Emission spectra of 70 nM Cul1[AMC], a mixture of 70 nM Cul1[AMC] and 70 nM [FlAsH]Cand1 (FRET), and 700 nM Skp1•Skp2 added to the 70 nM Cul1[AMC]•[FlAsH]Cand1 complex. In each plot, the emission signals were normalized to the emission of 70 nM Cul1[AMC] at 450 nm. Please click here to view a larger version of this figure.

To measure the k_{on} of Cul1•Cand1 using the established FRET assay by monitoring the reduction of the donor signal over time on the stopped-flow fluorimeter, we first tested and determined the concentration of the protein to be used. When 5 nM each of Cul1[AMC] and [FlAsH]Cand1 were used, very little signal change was observed (**Figure 4A**), whereas, when the concentration of each protein was increased to 50 nM, the reduction of the signal over time was observed (**Figure 4B**) and this change was abolished if buffer without [FlAsH]Cand1 was added (**Figure 4C**). Therefore, 50 nM Cul1[AMC] was used for further analyses, and a series of observed association rate constants (k_{obs}) were measured by mixing 50 nM Cul1[AMC] with increasing concentrations of [FlAsH]Cand1. The k_{obs} for each experiment was calculated by fitting the points to a single exponential curve, and the k_{obs} obtained from the same [FlAsH]Cand1 concentration were averaged. By plotting the average k_{obs} with the Cand1 concentration and performing a linear regression (**Figure 4D**), the k_{on} was determined[7,27].

Figure 4: Representative measurement of association rate constant. (A) The fluorescence of 5 nM Cul1[AMC] was monitored by a stopped-flow fluorimeter over time upon addition of 5 nM [FlAsH]Cand1. **(B)** The fluorescence of 50 nM Cul1[AMC] was monitored by a stopped-flow fluorimeter over time upon addition of 50 nM [FlAsH]Cand1. **(C)** The fluorescence of 50 nM Cul1[AMC] was monitored by a stopped-flow fluorimeter over time upon addition of the FRET buffer. **(D)** k_{on} for Cand1 binding to Cul1. k_{obs} of Cul1•Cand1 at different concentrations of Cand1 are plotted. Linear slope gives k_{on} of 1.8×10^7 M^{-1} s^{-1}. Error bars represent ±SEM; n = 4. All samples were prepared in the FRET buffer and excited at 350 nm. A band-pass filter was used to collect signals from AMC and exclude signals from FlAsH. No data were normalized. Please click here to view a larger version of this figure.

Similar to the measurement of k_{on}, we measured the observed dissociation rate constant of Cul1•Cand1 by monitoring the increase (restore) of the donor signal over time on the stopped-flow fluorimeter. Cul1[AMC] and [FlAsH]Cand1 were mixed first, and then Skp1•Skp2 was added to the preassembled Cul1[AMC]•[FlAsH]Cand1 on the stopped-flow fluorimeter. The donor signal increased quickly and it revealed a k_{obs} of 0.4 s^{-1} (**Figure 5**). In contrast, when buffer was added to the preassembled Cul1[AMC]•[FlAsH]Cand1, no signal increase was observed, suggesting the fast dissociation of Cul1•Cand1 was triggered by Skp1•Skp2.
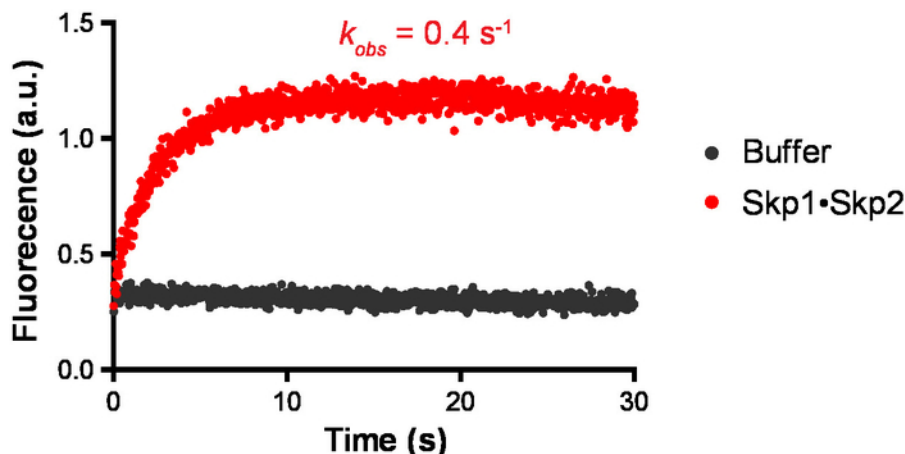
Figure 5: Representative measurement of dissociation rate constant. The change in donor fluorescence versus time was measured following addition of 150 nM Skp1•Skp2 or the FRET buffer to 50 nM Cul1[AMC]•[FlAsH]Cand1 complex. Signal changes were fit to a single exponential curve, and it gives observed dissociation rate constant of 0.4 s^{-1}. The experimental conditions were similar to that described in **Figure 4**. Please click here to view a larger version of this figure.

Discussion

FRET is a physical phenomenon that is of great interest for studying and understanding biological systems[19]. Here, we present a protocol for testing and using FRET to study the binding kinetics of two interacting proteins. When designing FRET, we considered three major factors:

the spectral overlap between donor emission and acceptor excitation, the distance between the two fluorophores, and the dipole orientation of the fluorophores[28]. To choose the fluorophores for FRET, we overlaid the excitation and emission spectra of the fluorophores, and searched for fluorophores whose emission peaks are well separated while the emission spectrum of the donor significantly overlaps with the excitation spectrum of the acceptor (**Figure 2**). To optimize the interfluorophore distance and orientation, we used the crystal structure for assistance, and we primarily considered attaching fluorophores to the protein termini, mainly due to the lower risk of disrupting the protein structure and activity. Because the distance between the N-terminus of Cand1 (Cand1[NTD]) and the C-terminus of Cul1 (Cul1[CTD]) is shorter than the distance between the C-terminus of Cand1 and the N-terminus of Cul1 (**Figure 1**), we decided to label Cand1[NTD] and Cul1[CTD]. Because we had been able to achieve 80%–90% labeling efficiency of the N-terminus of a protein using the tetracysteine tag[25], we chose to label Cand1[NTD] with FlAsH through the tetracysteine tag. We initially labeled the Cul1[CTD] with cyan fluorescent protein (CFP), which has a few advantages over AMC. First, it is a genetically encoded fluorescent tag, so it does not require additional labeling process nor steps that purify the labeled fluorescent protein from its unlabeled precursor. Second, CFP is brighter than AMC, and therefore, it offers higher assay sensitivity (see discussion below). Third, CFP has a larger spectrum overlap with FlAsH, potentially yielding more efficient FRET with FlAsH. Despite these advantages, however, CFP protein is much larger than the AMC tag, which may interfere with the protein conformation and the inter-molecular interaction. Indeed, we did not observe FRET between Cul1[CFP] and [FlAsH]Cand1 in our test, which is likely due to inadequate dipole orientation, or disruption of the Cul1-Cand1 interaction by the CFP tag. We then labeled the Cul1[CTD] with AMC, and we observed FRET between Cul1[AMC] and [FlAsH]Cand1. The initial FRET using the full-length Cand1 led to 10% reduction of the donor emission (**Figure 3A**). We further found that by deleting the first helix of Cand1, the distance between Cul1 and Cand1 is shortened from 26.8 Å to 15.5 Å (**Figure 1**), and Cand1 lacking the first helix yielded a much stronger FRET with Cul1, showing 30% reduction of the donor emission peak (**Figure 3B**). In addition, one may improve the FRET efficiency by choosing a donor fluorophore with higher quantum yield and an acceptor fluorophore with a larger extinction coefficient.

A characteristic feature of FRET is that when FRET occurs, the emission of the donor decreases and the emission of the acceptor increases (**Figure 3B**). However, fluorophores may display sensitivity to their environment, and thus, the emission intensity may change when another protein is present, even in the absence of FRET[29]. To confirm that the changed emission we observed is due to FRET between Cul1[AMC] and [FlAsH]Cand1, we added 10x excess amount of unlabeled acceptor protein (Cand1) as a chase. The chase converts the emission of the donor and the acceptor back to the normal level (**Figure 3C**), which supports that the changed emissions of Cul1[AMC] and [FlAsH]Cand1 depend on protein-protein interaction, and therefore, this result confirms that we established a FRET assay that reports the association and dissociation of Cul1•Cand1. Furthermore, we added Skp1•Skp2 to the preassembled Cul1[AMC]•[FlAsH]Cand1 complex, and Skp1•Skp2 is known to be able to disrupt the Cul1-Cand1 interaction[6]. We found that the FRET between Cul1[AMC] and [FlAsH]Cand1 disappeared (**Figure 3D**, green line) and the emission spectrum became similar to the emission spectrum of the chase sample (**Figure 3C**, green line), suggesting Cul1[AMC]•[FlAsH]Cand1 is dissociated by Skp1•Skp2 and Cul1[AMC] does not display abnormal emission when Skp1•Skp2 is present.

By monitoring the change in donor emission, we can directly observe the association and dissociation of Cul1[AMC]•[FlAsH]Cand1 on a stopped-flow fluorimeter. The stopped-flow system works by injecting reactants to a mixing chamber to rapidly mix the reactants, and stopping the flow once the mixed reactants are moved into an observation chamber[5]. The signal detection usually starts 1-2 millisecond (ms) after the mixing, which enables studying interactions that occur on the millisecond timescale. However, when the half-life of the observed reaction is shorter than the time required to mix the reactants on a particular device, this approach is not sensitive enough and is no longer appropriate[30]. Stopped-flow analyses have been used to determine rate constants ranging 10^{-6}–10^{6} s^{-1} for first-order reactions, and 1–10^{9} M^{-1} s^{-1} for second-order reactions[5]. To obtain reliable measurement of the kinetic parameters using the stopped-flow fluorimeter, a significant change of the fluorescent signal between the starting and equilibrium points is necessary. We used the [FlAsH]Cand1 lacking the first helix as the acceptor, because it yields better FRET with Cul1[AMC], and we tested the concentration of the proteins to be used for measurement. When 5 nM each of Cul1[AMC] and [FlAsH]Cand1 were mixed, no change in Cul1[AMC] signal was observed (**Figure 4A**), suggesting this concentration is insufficient for the measurement. When the protein concentrations were increased to 50 nM, the decrease in Cul1[AMC] signal over time became apparent (**Figure 4B**), and this change does not occur when the acceptor protein was absent (**Figure 4C**). Based on this result, we used Cul1[AMC] at 50 nM concertation to measure the k_{on} for Cul1•Cand1. The concentration of the donor protein used for measurement can vary when different fluorophores are used. For example, when Cul1 is labeled with CFP, 5 nM of [CFP]Cul1 is sufficient for the measurement of k_{on}[6]. Therefore, the optimal concentration of the protein should be tested for each FRET pair, and in principle, brighter fluorophores allow the use of lower protein concentrations and provide better signal-to-noise ratio[31].

To study protein-protein interactions by FRET, it requires attaching fluorophores to proteins at appropriate positions without disrupting the protein structure and activity, which potentially limits the use of FRET. In this protocol, we labeled the N-terminus of Cand1 using a tetracysteine tag, which specifically binds the FlAsH dye and the FlAsH dye becomes fluorescent only after it binds the target protein[32]. We labeled Cul1 using sortase-mediated transpeptidation, which attaches a short peptide carrying a fluorophore to the sortase tag at the C-terminus of Cul1[21,22]. The labeling efficiency is > 80% with the tetracysteine tag, and almost 100% with the sortase-His$_6$ tag after removing the unreacted protein using Ni-NTA beads (steps 2.4.3–2.4.4). Both methods add a few extra amino acids to the target protein, introducing minimal alterations to the protein structure. Similar approaches, such as the transglutaminase recognition sequence (Q-tag)[33] and the ybbR tag[34], can also be used to label the target protein in a site-specific manner. In addition, photostability of the fluorescent dyes can also limit the use of FRET. Repetitive or long exposure to excitation light can lead to photobleaching of the fluorophore, resulting in inaccurate quantification results[35]. Therefore, the donor and acceptor proteins should be protected from light during the preparation and storage steps. When using FRET to measure events that occur slowly, instead of constantly monitoring the change in donor emission over time, short readings of the donor emission should be taken at longer time intervals and the sample should be kept in the dark during the entire experiment[6].

Because FRET is sensitive and quantitative, it has become an important tool for studying the interaction between macromolecules. This protocol presents an example of using FRET to study the dynamics of a protein complex in solution. FRET has also been used together with live cell imaging to study molecular interactions in living cells[36], which is powerful in revealing the dynamics of protein complexes under physiological conditions. Furthermore, FRET can also be used at the single-molecule level to study the real-time dynamics of macromolecular complexes[37,38], providing insights into the conformational change of the complex. To improve the efficiency and detection of FRET, fluorophores and biosensors with enhanced brightness and photostability are of great interest, which are actively engineered and studied[28,39].

Disclosures

The authors have nothing to disclose.

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