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Monitoring PARKIN RBR Ubiquitin Ligase Activation States with UbFluor

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Abstract

PARKIN is a RING-Between-RING (RBR) E3 ligase, which ubiquitinates mitochondrial proteins in response to mitochondrial damage. Ser⁶⁵ of PARKIN is phosphorylated by kinase PINK1 (pPARKIN), which causes partial PARKIN activation. PINK1 also phosphorylates Ser⁶⁵ of ubiquitin (pUb), which activates pPARKIN further. Due to the lack of precise and quantitative assays to quantify the activity of PARKIN there were many conflicting reports on the role of pUb as a PARKIN activator, whether S65E PARKIN is a true phosphomimetic of pPARKIN, and the effect of substrate of PARKIN turnover was also not known. This protocol provides a step-by-step guide on the use of UbFluor probe to precisely quantitate changes in the activity of PARKIN in response to phosphorylation, allosteric activation by pUb, protein substrates, and activating structural mutations. These results pave the way forward to discover PARKIN activators and to precisely quantify the activity of other RBR E3s.

Keywords

PARKIN; Ubiquitin; UbFluor; Mitophagy

INTRODUCTION

Rapid therapeutic response to drugs targeting mutant genes in cancer suggest that similar approaches can be used to treat neurodegenerative diseases. PINK1 and PARKIN are highly mutated in patients with autosomal-recessive juvenile parkinsonism (AR-JP), suggesting that these genes are viable drug targets to pursue (Pickrell and Youle, 2015). To test this therapeutic hypothesis, there is a need to develop small-molecule probes for PINK1 and PARKIN (Hertz et al., 2013). PINK1 kinase senses mitochondrial damage and activates ubiquitin ligase PARKIN (Narendra et al., 2010). Activated PARKIN causes extensive polyubiquitination of mitochondrial outer membrane proteins and triggers mitophagy (Sarraf et al., 2013). Three major factors that contribute to PARKIN activation are: a) translocation of PARKIN to mitochondria in response to mitochondrial damage, b) phosphorylation of Ser⁶⁵ of PARKIN by PINK1 (pPARKIN), and c) phosphorylation of Ser⁶⁵ of ubiquitin by PINK1 (pUb) (Aguirre et al., 2017; Caulfield et al., 2014; Heo et al., 2015; Iguchi et al., 2013; Kane et al., 2014; Kazlauskaitė et al., 2014a; Kazlauskaitė et al., 2014b; Kondapalli et al., 2012; Koyano et al., 2014; Ordureau et al., 2015; Ordureau et al., 2014; Shiba-Fukushima et al., 2012; Wauer et al., 2015a; Wauer et al., 2015b; Yamano et al., 2015; Zheng and Hunter, 2013). Many genetic mutations of PARKIN inactivate the enzyme,

necessitating the discovery of PARKIN activators (Fiesel et al., 2015; Hampe et al., 2006; Henn et al., 2005; Kazlauskaitė et al., 2015; Kitada et al., 1998; Lucking et al., 2000; Matsuda et al., 2006; Safadi and Shaw, 2007; Spratt et al., 2013; Sriram et al., 2005; Valente et al., 2004). Typically, the activity of PARKIN is monitored using western blotting techniques, which are neither quantitative nor high-throughput. Furthermore, the typical enzymatic assay requires ATP, E1 and E2 enzymes, and a mixture of polyubiquitin chains is formed. Such complexity of the assays makes it difficult to quantify changes in biochemical activity of PARKIN and to discover small-molecule activators of PARKIN.

Fluorescence polarization (FP) can be readily used to monitor enzyme activity in a plate-based format, including studies monitoring the effects of biochemical activity mutations or of small-molecule modulators on enzyme activity (Levine et al., 1997; Liang et al., 2014). The ubiquitin thioester probe UbFluor recapitulates the native biochemical mechanisms of E3 ligases that rely on a catalytic cysteine for covalent ubiquitin transfer (Figure 1) (Krist et al., 2016; Park et al., 2017). By mimicking the E2~Ub thioester, UbFluor provides a stable yet reactive substrate for nucleophilic attack by the catalytic cysteine of a HECT or RBR E3 ligase. The release of fluorescein thiol (FluorSH) in this reaction can be readily observed by a drop in FP. Unlike traditional gel-based readouts of ubiquitination assays, UbFluor allows continuous monitoring of ubiquitination reactions and is readily adapted for low-volume, high-throughput purposes (Krist et al., 2016). Previously, we have demonstrated the utility of the ubiquitin thioester probe UbFluor for biochemical and drug discovery studies of HECT E3 ubiquitin ligases using FP as a readout (Foote et al., 2017; Krist et al., 2017; Krist et al., 2016).

More specifically, we have shown that the pseudo-first order determination of E3 ligase reaction rates and apparent rate constants using UbFluor provides a biochemically relevant and rapid means of characterizing E3 ligase variants (Krist et al., 2016). E3 ligases can be characterized in two regimes: single turnover (ST), in which ligase is in five- to twenty-fold excess over UbFluor, or multiple turnover (MT), in which UbFluor is in at least five-fold excess over ligase. ST reactions typically require 30–60 minutes for complete UbFluor consumption, while MT reactions can require several hours to exceed 50% consumption of UbFluor. While ST reactions measure only the initial transthiolation of the ubiquitin moiety onto the E3 catalytic cysteine, MT reactions report on both the transthiolation, and on the subsequent isopeptide ligation and regeneration of the free catalytic cysteine (Figure 1). Thus, combined ST and MT analysis of E3 ligases can reveal previously unknown information about the mechanistic details of point mutations, post-translational modifications, or interacting molecules such as inhibitors, activators, or ubiquitination substrates. Critically, the FP detection properties of UbFluor allow the continuous measurement of reaction progress in the initial 1–15 minutes of a reaction, which is essential for determining the initial reaction rate and making quantitative comparisons.

In the case of PARKIN, we verified previously studied activation states and discovered additional activation states of PARKIN (Park et al., 2017). By titrating PARKIN and pPARKIN with increasing concentrations of pUb, we demonstrated that UbFluor can accurately recapitulate and measure the initial reaction rates of these various PARKIN activation states. We further demonstrated that the addition of the PARKIN substrate

Mitochondrial Rho 1 (Miro1) results in further activation of PARKIN by distinct mechanisms, and verified mechanistic details of PARKIN activation under ST and MT conditions by adding lysine to reactions as a potential ubiquitin acceptor. Collectively, we showed that UbFluor effectively measures PARKIN activity due to a variety of effectors. This protocol outlines basic steps to measure the activity of PARKIN E3 ligase using UbFluor.

BASIC PROTOCOL 1. PARKIN FLUORESCENCE POLARIZATION (FP) ASSAY WITH UBFLUOR

The ubiquitin thioester probe UbFluor recapitulates the native biochemical mechanisms of E3 ligases that rely on a catalytic cysteine for covalent ubiquitin transfer, allowing continuous monitoring of ubiquitination reactions at low volumes (Krist et al., 2016; Park et al., 2017). This enables measurement of initial reaction rates for multiple PARKIN variants in combination with allosteric effector proteins and substrates. The mixture of PARKIN and any other interacting partners is prepared first. UbFluor is then added and the mixture is subsequently analyzed in a plate-based format to determine the initial reaction rate of PARKIN under the assayed conditions. By combining insights from ST and MT experiments, it is possible to quantitatively compare activation states and understand the mechanisms of the enzyme in various activation states.

Due to the considerable activation of PARKIN by point mutations, post-translational modifications and effectors, empirical determination of the optimal reaction conditions may be necessary depending on the PARKIN construct(s) used. It is essential that UbFluor reactions be run in the strict absence of free thiols such as BME and DTT, as they will react with UbFluor and alter the apparent reaction rate even in concentrations as low as 1 μ M. TCEP may be used as a reducing agent in their place. UbFluor does not react with free lysine up to at least 100 mM in the absence of E3 ligase, and it also is generally inert to lysine-rich substrate proteins in the absence of E3 ligase. However, active E3 ligases can transfer the ubiquitin moiety of UbFluor onto a substrate lysine, or onto free lysine in solution in an analogous manner. These key features allow users to study the effects of free lysine or lysine-containing substrates on the activity of PARKIN and other E3 ligases, as the presence of an acceptor lysine can alter the overall turnover rate of the enzyme.

Materials

UbFluor, from previously published procedures (Krist et al., 2017)

PARKIN E3 ligase, pPARKIN, or PARKIN with point mutations (available from Boston Biochem or LifeSensors and obtainable from *E.coli* expression) (Park et al., 2017)

S65-phospho-ubiquitin (pUb) (available from Boston Biochem and obtainable by published procedures) (Klosowiak et al., 2016)

Mitochondrial Rho 1 (Miro1), truncated Miro1 (MiroS), (obtainable by published procedures) (Klosowiak et al., 2013), or other PARKIN substrate protein(s).

240 μ M Tween-20

25 mM TCEP

UbFluor Assay Buffer (1.5 M NaCl, 500 mM HEPES, pH 7.5; see recipe)

Lysine UbFluor Assay Buffer (1.5 M NaCl, 500 mM HEPES, 0.1–1 M lysine, pH 7.5. For each final concentration; see recipe. Lysine is not reactive with UbFluor, but is reactive toward UbFluor in the presence of RBR E3 ligase.)

1.5 mL microcentrifuge tubes

Centrifuge for 1.5 mL microcentrifuge tubes (Thermo Scientific Legend Micro 21R or equivalent)

384-well plates (low binding, low volume, Corning, cat no. 3820)

Centrifuge for 384-well plates (Jouan RC1022 or comparable)

Synergy 4 fluorescence plate reader with Gen5 software (BioTek).

Procedure for FP assay with UbFluor

1. Determine the concentrations and amounts of each reaction component needed for each planned reaction, for up to 4 ST or 12 MT reactions to be run simultaneously.

Our reactions typically are 25 μ L and contain the following buffer components at the given final concentrations: 150 mM NaCl, 50 mM HEPES, pH 7.5 (use 2.5 μ L UbFluor Assay Buffer or Lysine UbFluor Assay Buffer), 6 μ M Tween-20, 0.5 mM TCEP, 0–100 mM lysine. pUb concentration typically ranges from 0–25 μ M. Miro1/MiroS substrate concentration typically ranges from 0–15 μ M. For ST reactions, PARKIN concentration ranges from 1–5 μ M with 0.05 – 1 μ M UbFluor. For MT reactions, PARKIN concentration ranges from 0.2–2 μ M with 5 – 20 μ M UbFluor.

2. Except for the UbFluor, combine all components of each reaction in a 1.5 mL microcentrifuge tube. If pUb, Miro, or lysine is added, incubate reaction at room temperature for at least 10 minutes to ensure equilibration/binding of various components.

Preparing a master mix of the buffer components that are being held constant over a set of reactions can streamline the preparation of the reactions. If possible, prepare the reactions and the UbFluor stock solution such that the volume of UbFluor stock solution to be added is between 2 and 5 μ L.

3. Prepare a 384-well plate for use in the Synergy 4 plate reader. Prepare the plate reader hardware and software to monitor the appropriate number of wells for the desired length of time. Pre-heat the plate reader to the desired reaction temperature, typically 25 $^{\circ}$ C.

ST reactions are run for at least 20 minutes, and should be analyzed every 15 seconds. MT reactions are analyzed for at least 30 minutes. MT reactions should be analyzed every 15–30 seconds depending on how many reactions are being run simultaneously; the Synergy4 plate reader requires approximately 2 seconds to read each well.

4. Add the required amount of UbFluor stock solution (determined above in Step 1) to the side of each microcentrifuge tube, making sure the ligase does not touch the UbFluor solution at the bottom of the tube.

The surface tension of the stock solution will allow the droplet of UbFluor to adhere to the side of the centrifuge tube.

5. Centrifuge all microcentrifuge tubes at room temperature at $10,000 \times g$ for seven seconds to start the reactions.
6. Transfer each reaction to a well in the 384-well plate (load 20 μ L), pipetting each solution five times while loading to ensure complete mixing.
7. Once all reactions have been loaded to the plate, centrifuge the plate at room temperature at $1,500 \times g$ for 10 seconds.
8. Place the plate in Synergy 4 plate reader and begin fluorescence polarization readings. As outlined in step 3, typical reaction time is 20 minutes for ST and 30 minutes for MT, but may be optimally determined by the user. Reactions are typically run at 25°C.

The dead time between spinning UbFluor into solution and FP measurements is typically ~2 minutes. This can be expected when measuring 4 – 8 reactions during a given reading. For slower reactions, especially MT, 12 reactions and ~3 minutes of dead time are tolerable. The typical dynamic range of the assay is ~100 mP when UbFluor is completely consumed, but full consumption is not necessary for each experiment if the goal is analysis of initial reaction rates.

As an optional orthogonal assay, the reactions can be quenched with SDS-PAGE loading buffer and analyzed on a gel.

ALTERNATE PROTOCOL. SINGLE-WELL FLUORESCENCE POLARIZATION (FP) ASSAY WITH UBFLUOR FOR RAPID REACTIONS

This alternate to Basic Protocol 1 for FP assays with PARKIN and UbFluor allows the measurement of initial rates of rapid reactions with highly activated forms of PARKIN. In particular, the addition of pUb or a substrate to reactions with pPARKIN can accelerate reactions such that they are complete in less than 5 minutes. For quantitative comparison of initial reaction rates, the standard protocol cannot capture the initial phase of such reactions because of the 2–3 minute dead time. This alternate protocol, while slightly more laborious on a per-well basis, reduces the dead time to 10–30 seconds, which we found to be tolerable for almost all conditions tested. By performing multiple repetitions of steps 5–9 below, we

were able to measure initial reaction rates in multiple replicates across a range of reaction conditions, allowing us to accurately quantify highly reactive PARKIN activation states. In particular, this protocol was used to measure initial reaction rates of pPARKIN with UbFluor in the presence of pUb, Miro, pUb + Miro, and pUb + lysine.

Materials

See Basic Protocol 1

Procedure for FP assay with UbFluor for fast reactions

1. Determine the concentrations and amounts of each reaction component needed for each planned reaction in a sequence. For example, perform triplicate analysis of pPARKIN reaction with UbFluor at fixed concentrations without pUb and with three increasing concentrations of pUb, for a total of 12 reactions.

Our reactions typically are 25 μ L and contain the following buffer components at the given final concentrations: 150 mM NaCl, 50 mM HEPES, pH 7.5 (use 2.5 μ L UbFluor Assay Buffer or Lysine UbFluor Assay Buffer), 6 μ M Tween-20, 0.5 mM TCEP, 0–100 mM lysine. pUb concentration typically ranges from 0–25 μ M. Miro1/MiroS substrate concentration typically ranges from 0–15 μ M. For ST reactions, PARKIN concentration ranges from 1–5 μ M with 0.05 – 1 μ M UbFluor. For MT reactions, PARKIN concentration ranges from 0.2–2 μ M with 5 – 20 μ M UbFluor

2. Except for UbFluor, combine all components of each planned reaction in a 1.5 mL microcentrifuge tube.
3. Transfer the contents of each 1.5 mL tube in the reaction series, i.e. all reaction components except UbFluor, into successive wells in a 384-well plate.
4. Centrifuge the plate at $1,500 \times g$ at room temperature for 10 seconds.
5. Prepare the Synergy 4 plate reader hardware and software for FP readings of the single well that is to be read. Pre-heat the plate reader to 25°C or the desired reaction temperature.

For rapid reactions, the plate reader should be set to make FP readings of the single well every 2 seconds for 5 minutes.

6. Collect and prepare the 384-well plate containing the reaction mixture, the UbFluor stock solution, pipettors, and centrifuge prior to conducting steps 7–9.
7. Add the required amount of UbFluor stock solution to the first well in the reaction series, pipetting up and down three times to ensure complete injection of the solution into the reaction mixture. This starts the reaction; timing of the reaction should begin here.

Our reactions typically contain 0.1 – 20 μ M UbFluor final concentration.

8. Using a separate pipettor set to 15 μ L, pipet the reaction mixture 5 times to fully mix the solution in the well, taking care not to introduce air bubbles into the mixture.

This step can be omitted if the UbFluor stock solution volume added in step 7 is large enough to fully mix the reaction. However, typically the UbFluor stock solution concentration is >5 times the final HECT concentration, resulting in a small addition volume which is insufficient for complete mixing of the reaction. If elimination of this step is desired, stock solutions of PARKIN and other additives used to prepare the reaction mixture in steps 1 and 2 should be of high concentration, and UbFluor stock solution can then be diluted to a concentration that results in a 10–15 μ L addition volume in step 7.

9. Place plate in Synergy 4 plate reader and begin FP readings.

Typically, the dead time between adding UbFluor to the reaction well and the start of FP measurements is 10–30 seconds.

Repeat steps 5–9 until all reactions prepared in steps 1–4 have been completed.

BASIC PROTOCOL 2. DATA ANALYSIS FOR UBFLUOR FP ASSAY

Previously, we have demonstrated a linear relationship between polarization and UbFluor cleavage, which allows us to directly convert polarization values to UbFluor concentration. Before running any reactions of UbFluor with enzyme, it is important to determine the baseline polarization values (mP) for ‘intact’ and ‘cleaved’ UbFluor. Under typical ST conditions (0.1 – 1.0 μ M UbFluor) we observe a polarization value of ~155 mP for ‘intact’ UbFluor and ~30 mP for ‘cleaved’ UbFluor. Under typical MT conditions (5 – 20 μ M UbFluor) we observe a polarization of ~135 mP for ‘intact’ UbFluor and ~30 mP for ‘cleaved’ UbFluor. These values may vary between plate readers and may also shift if the G-factor of your plate reader is recalibrated. We have observed at most a 3 mP variation between batches of UbFluor. Nonetheless, we recommend using a single batch of UbFluor for experiments involving direct comparisons whenever possible to eliminate the need to account for variation between batches.

Materials

UbFluor, from previously published procedures (Krist et al., 2017)

240 μ M Tween-20

25 mM TCEP

UbFluor Assay Buffer (1.5 M NaCl, 500 mM HEPES, pH 7.5; see recipe)

PBS, 1x, with 20 mM β -mercaptoethanol (BME)

1.5 mL microcentrifuge tubes

Centrifuge for 1.5 mL microcentrifuge tubes (Thermo Scientific Legend Micro 21R or equivalent)

384-well plates (low binding, low volume, Corning, cat no. 3820)

Centrifuge for 384-well plates (Jouan RC1022 or comparable)

Synergy 4 fluorescence plate reader with Gen5 software (BioTek).

Procedure for data analysis of UbFluor FP assay

1. To determine the baseline polarization of 'intact' UbFluor, run an FP experiment with at least 3 reactions according to the method described above in Basic Protocol 1, with no PARKIN added, or with catalytically inactive PARKIN (C431 mutated) if available. Measure the polarization values for 30 minutes. The average of these polarization values can be considered the 'intact' polarization level.

Under MT reaction conditions, we always observe a slight initial decrease in UbFluor polarization signal, even in the absence of enzyme or the presence of catalytically inactive enzyme.

2. To determine the baseline polarization of 'cleaved' UbFluor, run an FP experiment with at least 3 reactions according to the method described above in Basic Protocol 1, with 1.25 μ L of PBS with 20 mM BME (final BME concentration of 1 mM). Measure the polarization values over the course of 30 minutes. The average of these polarization values can be considered the 'cleaved' polarization level; if the polarization values are still decreasing in the first 5–10 minutes, discount these values when calculating the average.

In-gel fluorescence scanning can also be used to confirm the complete cleavage of UbFluor in the presence of 1 mM BME. This provides further assurance that the determined 'cleaved' polarization level corresponds to fully cleaved UbFluor.

3. Perform steps 1 and 2 for each concentration of UbFluor you plan to use.

Multiple concentrations can be assayed simultaneously.

4. To determine the conversion factor between mP and UbFluor concentration, use the determined 'intact' and 'cleaved' values to generate a linear transformation for each concentration of UbFluor assayed above. Using the standard equation for a straight line ($y=mx+b$), polarization is the independent variable (x) and UbFluor concentration is the dependent variable (y).

Example: For 10 μ M UbFluor, determine polarization values for 'intact' (155 mP, 10 μ M UbFluor) and 'cleaved' (30 mP, 0 μ M UbFluor). Calculate the equation of a line between these two points. For all subsequent experiments using 10 μ M UbFluor, use this equation to convert raw polarization values to units of UbFluor concentration.

5. For any subsequent experiments performed as outlined in Basic Protocol 1 or the Alternate Protocol, convert the initial linear region of the UbFluor consumption reaction by PARKIN or other E3 ligase from raw mP values to UbFluor concentration using the equations determined in step 4. Plot the converted UbFluor concentrations (μM) vs time (min). The slope of the line is the rate of UbFluor consumption in $\mu\text{M}/\text{min}$. In experiments where UbFluor and enzyme concentration are held constant, direct comparison of initial rates can be used to characterize the effects of added effectors such as pUb or substrate proteins.

The linear region of UbFluor consumption can be the first 1–15 minutes of the reaction depending on the speed of the reaction. Less than 20% of the total UbFluor present should be consumed; if this condition is not met, reduce enzyme concentration and/or switch to the Alternate Protocol.

6. For further comparison of a set of PARKIN variants, it is possible to calculate the apparent bimolecular rate constant for each variant using a set of experiments in which UbFluor concentration is varied while enzyme concentration is held constant. Under pseudo-first order reaction conditions far below K_M ($[\text{UbFluor}] \ll 800 \mu\text{M}$), the observed initial UbFluor consumption rate is linear with respect to both enzyme and UbFluor concentration. Thus, the rate = $k_{\text{obs}}[\text{enzyme}][\text{UbFluor}]$. Plotting the rate of UbFluor consumption (y) as a function of UbFluor concentration (x) should produce a linear relationship. The slope of this line is $k_{\text{obs}}[\text{enzyme}]$. By dividing the slope by $[\text{enzyme}]$, the value of k_{obs} can be determined.

Comparison of k_{obs} , the apparent bimolecular rate constant, between enzyme variants allows researchers to show that a particular mutation or modification produces a robust and consistent change in enzyme activity across a range of UbFluor concentrations.

REAGENTS AND SOLUTIONS

Use ddH₂O from Thermo Scientific Nanopure or equivalent purification system

UbFluor Assay Buffer: 1.5 M NaCl, 500 mM HEPES, pH 7.5, 10x

1.5 M NaCl

500 mM HEPES

Adjust pH to 7.5 by addition of solid NaOH pellets and/or dropwise addition of 1 M NaOH

Store at room temperature for up to 1 month in a dark place; check pH to ensure quality.

UbFluor Lysine Assay Buffer: 1.5 M NaCl, 500 mM HEPES, 0.1–1 M lysine pH 7.5, 10x

1.5 M NaCl

500 mM HEPES

0.1–1 M lysine (for each desired final concentration of lysine, make a batch of buffer with 10x the desired final concentration)

Adjust pH to 7.5 by addition of 1 M NaOH or 1 M HCl

Store at room temperature for up to 1 week in a dark place; check pH to ensure quality.

PBS, 1x, with 20 mM β -mercaptoethanol (BME)

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

1.8 mM KH₂PO₄

Adjust pH to 7.4 if necessary by addition of 1 M NaOH or 1 M HCl.

20 mM BME

Use within 1 day of BME addition; PBS solution without BME can be stored up to 1 month at room temperature in the dark, and BME can be added to a small aliquot immediately prior to use.

COMMENTARY

Background Information

HECT (~28 known) and RBR E3s (~13 known) are ubiquitin ligases that have a catalytic cysteine and form an obligatory E3~Ub thioester prior to ubiquitin transfer onto the catalytic cysteine (An et al., 2014; Spratt et al., 2014). Both families of E3 ligases have complex mechanisms of regulation and are involved in unique disease-related signal transduction pathways. There is strong interest in the development of small-molecule inhibitors or activators of HECT E3s and RBR E3s, along with proteolysis-targeting chimeras (PROTACs) and molecular glues (thalidomide-like) that promote interactions between E3 ligases and non-canonical substrates to induce substrate ubiquitination and degradation (Bondenson et al., 2015; Kronke et al., 2014; Lu et al., 2014; Lu et al., 2015; Ruegger et al., 1998; Sakamoto et al., 2001; Tan et al., 2007; Winter et al., 2015). However, the complexity of ubiquitination reactions, which require ATP, Ub, E1 enzyme, E2 enzyme, E3 ligase, and protein substrate, makes existing assays expensive and complicated. Typically, additional reagents are employed to quantify the formation of reaction products, which are complex mixtures of polyubiquitin chains. The ideal minimalistic biochemical assay for HECT/RBR E3 ligase should satisfy two major criteria: a) it should contain E3 ligase and some form of ubiquitin (elimination of ATP, E1, and E2 simplifies the assay), and b) the assay readout should monitor the consumption of the ubiquitin substrate, rather than formation of the reaction products. The second criterion is critical, because the ubiquitin substrate is homogeneous, but reaction products are heterogeneous mixtures of polyubiquitin chains

with different linkages and lengths. By quantifying the consumption of the ubiquitin substrate, the assay is further simplified.

Attempts to develop such assays have been undertaken previously with C-terminal ubiquitin electrophiles (Love et al., 2009). These probes are suicide inhibitors of HECT/RBR E3 ligases that bind the ubiquitin binding site near the catalytic cysteine, followed by covalent bond formation reaction. These probes have been used extensively to monitor conformational changes of RBR E3 ligases, and the covalent bond formation reaction is typically associated with the activation of RBR E3s. However, this approach has several drawbacks. First, the covalent labeling efficiency for both HECT and RBR E3s is low. Second, the covalent labeling reaction is stoichiometric and not catalytic as in the natural system. Finally, C-terminal ubiquitin electrophiles cannot be used to monitor the effect of mutations or small molecules on the isopeptide ligation step. To avoid these pitfalls, we have developed C-terminal ubiquitin thioesters UbMES and UbFluor that mimic Ub~E2 thioesters but with the E2 enzyme removed (Krist et al., 2017; Krist et al., 2016; Park et al., 2017). Even though they lack E2 enzymes, such ubiquitin thioesters can be directly charged to the catalytic cysteine of HECT and RBR E3s ligases, generating catalytically active HECT/RBR E3~Ub thioesters. The fluorescently labeled thioester UbFluor can robustly and simply measure the enzymatic activity of PARKIN in response to Ser⁶⁵ phosphorylation, pUb, and structural activating mutations. UbFluor is a general probe suitable for measuring the enzymatic activity of HECT E3s, RBR E3s, and perhaps bacterial E3 ligases (NEL and HECT-like) that also harbor a catalytic cysteine (Maculins et al., 2016).

Critical Parameters

Since UbFluor is a thioester, assays must be strictly run in the absence of free thiol such as DTT or BME, which will cleave the thioester between Ub and fluorescein. When there is a need to use reducing agent with UbFluor, TCEP should be the reagent of choice. UbFluor is generally stable in the presence of 100 mM of lysine. In addition, caution should be exercised when small molecules are screened against RBR E3 ligase using UbFluor: if such small molecules have thiol or amine groups they may cleave UbFluor even in the absence of RBR E3.

Some small molecules and biological reagents can also cause an increase in fluorescence polarization over time. We observed that bovine serum albumin increases polarization of UbFluor in a time-dependent manner. Fluorescent small molecules may also disrupt fluorescence polarization readings; thus, such molecules should be excluded from use in UbFluor assays as modulators or labeling agents.

Troubleshooting

See Table 1.

Anticipated Results

Basic Protocol 1 will produce the data about the activity of PARKIN as measured by UbFluor. Basic protocol 2 serves the purpose to analyze the data generated from the basic protocol 1.

Time Considerations

For typical experiments with RBR E3 ligase, assays are run in the presence of upstream E1 and E2 enzymes, ATP and ubiquitin. Assembling these components and maintaining laboratory stocks can be time consuming and expensive. UbFluor, on the other hand, allows a simple, two-component reaction that can be monitored by fluorescence polarization, obviating the need for more labor intensive SDS-PAGE gels. Preparation of UBE1 enzyme should require 3–5 days, and the subsequent preparation of Ub~MES requires one day for synthesis and FPLC size exclusion purification. Conjugation of FluorSH to Ub~MES should require 1–2 days for the reaction and subsequent purification. A skilled chemist can complete the synthesis of FluorSH in 7–10 days. 30–60 minutes should be allowed to prepare a set of UbFluor reactions for FP measurement. Data analysis can require several hours to days depending on the size of the dataset.

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Significance Statement

Human genome sequencing uncovered many disease genes, which are potential drug targets for personalized therapy. Approximately 50% of all autosomal recessive juvenile parkinsonism (AR-JP) cases are caused by mutation in PARK2 gene that encodes ubiquitin ligase PARKIN. Many identified mutations in PARKIN are inactivating mutations, and therefore small molecule activators of PARKIN are thought to be useful to treat Parkinson's disease. However, the assays to monitor the activity of PARKIN ligase upon its activation are not known. This protocol outlines a simple fluorescence polarization assay to monitor the activity of PARKIN in response to its activation by Ser⁶⁵ phosphorylation, by structural point mutations, and by Ser⁶⁵ phosphorylated ubiquitin.

Figure 1.
(A) Synthesis of UbFluor. (B) Mechanism of UbFluor reaction with RBR E3s.

Table 1

Symptom	Causes	Solutions
More than 90% of UbFluor is consumed before the FP measurement begins.	The selected enzyme and UbFluor concentrations resulted in a very rapid reaction.	Use a lower concentration of E3 ligase in the reaction. Utilize the Alternate Protocol 1 'Fast' protocol.
The blank (no enzyme) sample demonstrates a slight drop in polarization over the first 10–20 minutes of reaction.	This is a common FP phenomenon that we have observed and is typically more pronounced under MT conditions than ST conditions.	Using a higher concentration of enzyme may help if reactions are difficult to distinguish from the blank. Pre-equilibrate enzyme (in plate) and UbFluor to the temperature used by the plate reader (typically 25–30°C) if plate reader is set above room temperature.