

Real-time monitoring of ubiquitination in living cells by BRET

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Ubiquitin has emerged as an important regulator of protein stability and function in organisms ranging from yeast to mammals. The ability to detect *in situ* changes in protein ubiquitination without perturbing the physiological environment of cells would be a major step forward in understanding the ubiquitination process and its consequences. Here, we describe a new method to study this dynamic post-translational modification in intact human embryonic kidney cells. Using bioluminescence resonance energy transfer (BRET), we measured the ubiquitination of β -arrestin 2, a regulatory protein implicated in the modulation of G protein-coupled receptors. In addition to allowing the detection of basal and GPCR-regulated ubiquitination of β -arrestin 2 in living cells, real-time BRET measurements permitted the recording of distinct ubiquitination kinetics that are dictated by the identity of the activated receptor. The ubiquitination BRET assay should prove to be a useful tool for studying the dynamic ubiquitination of proteins and for understanding which cellular functions are regulated by this post-translational event.

Ubiquitination is a rapid and reversible post-translational modification that is involved in numerous aspects of biology. The 76-residue polypeptide ubiquitin fulfils essential functions in eukaryotes through its covalent attachment to other intracellular proteins^{1,2}. Historically, the best-characterized role for this modification is the targeting of proteins for degradation by the 26S proteasome after the transfer of an ubiquitin chain of at least four units, referred to as polyubiquitination³. More recently, the addition of a single ubiquitin to one (monoubiquitination) or multiple (multiubiquitination) protein sites has been described⁴. In these cases, the post-translational modification serves as a reversible signal involved in a variety of key cellular functions including intracellular signaling, subcellular localization and protein-protein interactions.

Despite the growing interest in ubiquitination, inspired by the diversity of cellular functions that appear to be influenced by this process, the lack of tools for monitoring its dynamic regulation limits studies in this field. Western blot analysis using antibodies to ubiquitin allows the detection of protein ubiquitination. Although

this assay can be used to determine whether a protein is ubiquitinated, it cannot be easily adapted to study the dynamic nature of the ubiquitination and deubiquitination cycles. To monitor the ubiquitination process in living cells, we took advantage of a bioluminescence resonance energy transfer (BRET) method, which allows real-time detection of protein-protein interactions *in vivo*. This technique is based on the nonradiative transfer of energy between a luminescent energy donor (here, *Renilla* luciferase, *Rluc*) and a fluorescent energy acceptor (here, green fluorescent protein, GFP). This is a system of choice for monitoring both constitutive and regulated intermolecular interactions because of the strict dependence on molecular proximity ($<100 \text{ \AA}$) between the donor and acceptor molecules for energy transfer⁵.

It was recently proposed that β -arrestin 2 (β -arrestin), a versatile protein involved in the regulation of G protein-coupled receptor (GPCR) signaling⁶, is ubiquitinated in response to receptor activation⁷. Based on the stability of their interaction with β -arrestin, two broad classes of GPCRs are distinguishable^{8,9}: GPCRs known as class A receptors (for example, the β_2 -adrenergic receptor, $\beta_2\text{AR}$) interact only transiently with β -arrestin after their activation, whereas activated class B receptors (for example, the V_2 -vasopressin receptor, $V_2\text{R}$) form stable complexes with β -arrestin. Recent findings¹⁰ are suggestive of a link between the dynamics of β -arrestin ubiquitination and the nature of its interaction with the activated receptor. A stable interaction correlates with sustained β -arrestin ubiquitination, whereas a transient interaction results in short-lived ubiquitination. Using β -arrestin as a model ubiquitinated protein, we report here that BRET allows monitoring of the changes in the ubiquitination state of a protein in real time.

RESULTS

Ubiquitination monitored by BRET in living cells

As ubiquitination results from the covalent attachment of ubiquitin to specific protein substrates, BRET should be adaptable to monitoring this post-translational modification in living cells. The covalent attachment of a GFP-ubiquitin fusion protein to a substrate fused to *Renilla* luciferase (*Rluc*) should bring the *Rluc* and GFP moieties within a BRET-permissive distance, resulting in a detectable BRET signal. To determine whether we could use BRET

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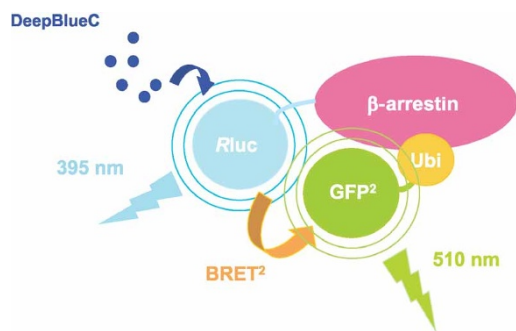


Figure 1 | Schematic representation of the β -arrestin ubiquitination BRET² assay. Upon degradation of its catalytic substrate (DeepBlueC coelenterazine), the Rluc fused to the N terminus of β -arrestin emits blue light with an emission peak at 395 nm. If the β -arrestin is ubiquitinated, a nonradiative transfer of energy will occur between Rluc and the GFP² fused to the N terminus of ubiquitin, resulting in re-emission of light with an emission peak at 510 nm.

to study ubiquitination of β -arrestin, we designed N-terminally tagged Rluc- β -arrestin and GFP-ubiquitin (GFP²-Ubi) fusion protein constructs. The close proximity of several GFP² moieties could cause quenching or interference phenomena that could lead to a decrease in the BRET signal, and indeed, we observed this (Supplementary Fig. 1 online). To limit the formation of poly(GFP²-Ubi) chains¹¹, we created a mutant GFP²-Ubi protein by changing Lys48 and Lys63, the two residues through which linkages occur, to alanine residues.

To initiate the energy transfer between Rluc and GFP² in cells coexpressing Rluc- β -arrestin and GFP²-Ubi, we used the luciferase substrate DeepBlueC coelenterazine, which readily crosses the plasma membrane. Hydrolysis of DeepBlueC coelenterazine leads to the emission of light with a spectrum overlapping the excitation spectrum of GFP², thus allowing BRET² (Fig. 1). Coexpression of a constant level of Rluc- β -arrestin with increasing concentrations of GFP²-Ubi led to an increase in the BRET² signal (Fig. 2a). The

energy transfer increased hyperbolically as a function of the GFP²-Ubi expression level, indicative of a specific interaction between the two proteins¹². To confirm that the signal specifically reflects the ubiquitination process, we used either a GFP²-tagged ubiquitin mutant that is unable to take part in the ubiquitination process as a result of the replacement of its last two glycines by alanine residues (GFP²-Ubi_{AA}) or GFP² alone as negative controls. Coexpression of Rluc- β -arrestin with GFP²-Ubi_{AA} or GFP² led to weaker signals that increased linearly rather than hyperbolically with the increase in the fluorescence/luminescence ratio, most likely reflecting random collision (bystander BRET) between Rluc- β -arrestin and these two GFP² constructs. Western blot analysis of lysates from cells expressing GFP², GFP²-Ubi_{AA} or GFP²-Ubi corroborated the lack of integration of GFP²-Ubi_{AA} into proteins (Fig. 2b). The smeared band detected above 63 kDa in lysates from cells expressing GFP²-Ubi most likely reflects ubiquitination of multiple proteins, and the sharp bands observed in lysates from cells expressing GFP²-Ubi_{AA} probably correspond to monomeric and multimeric forms of the GFP²-tagged ubiquitin. The BRET² signal detected between Rluc- β -arrestin and GFP²-Ubi did not result from the ubiquitination of the Rluc moiety itself, as no signal was detected when only GFP²-Ubi and Rluc were coexpressed (Fig. 2a). To determine whether the GFP²-Ubi protein can covalently modify β -arrestin, we immunoprecipitated Myc-tagged β -arrestin and detected GFP²-Ubi-immunoreactive species, which probably correspond to a ubiquitinated β -arrestin population (Fig. 2c). Taken together, these results demonstrated that energy transfer between GFP²-Ubi and Rluc- β -arrestin reflects a nonrandom clustering of these two proteins and the covalent ubiquitination of β -arrestin *in vivo*.

Detection of agonist-induced ubiquitination

In a recent study, *ex vivo* western blot analysis suggested that GPCR activation modulates β -arrestin ubiquitination^{7,10}. To assess whether BRET can detect receptor-regulated ubiquitination in living cells, we monitored the transfer of energy between Rluc- β -arrestin and GFP²-Ubi in the presence of coexpressed β_2 AR and

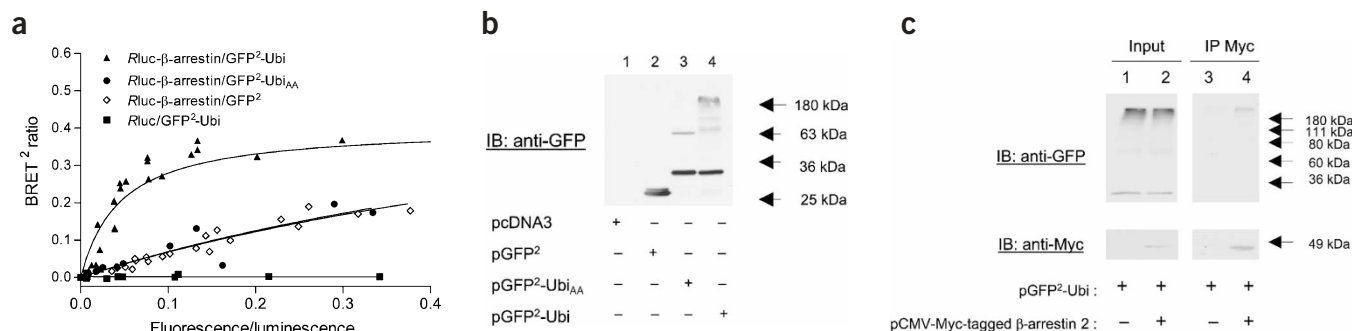


Figure 2 | Specific BRET² signal between Rluc- β -arrestin and GFP²-Ubi. (a) HEK 293 cells were cotransfected either with constant concentrations of Rluc- β -arrestin, Myc-tagged β_2 AR and HA-tagged β_2 AR DNA constructs and increasing concentrations of plasmids encoding GFP²-Ubi (\blacktriangle), GFP²-Ubi_{AA} (\bullet) or GFP² (\diamond), or with a constant concentration of Rluc and increasing concentrations of GFP²-Ubi DNAs (\blacksquare). The data shown represent pooled individual readings obtained from five to eight independent experiments. The curves were fitted using a nonlinear regression equation, assuming a single binding site (GraphPad Prism). (b) Lysates obtained from mock-transfected HEK 293 cells or cells transfected with pGFP², pGFP²-Ubi_{AA} or pGFP²-Ubi were resolved by SDS-PAGE, and the attachment of ubiquitin to proteins was assessed by western blot analysis using an antibody to GFP. Numbers on the right indicate molecular weight of protein markers. The blot shown is representative of two independent experiments. (c) Immunoprecipitation with an antibody to Myc was performed on lysates prepared from cells expressing pGFP²-Ubi or pGFP²-Ubi and pCMV-Myc-tagged β -arrestin. GFP²-Ubi protein associated with the immunoprecipitated β -arrestin was revealed as in b with an antibody to GFP, whereas the amount of β -arrestin immunoprecipitated was controlled with an antibody to Myc.

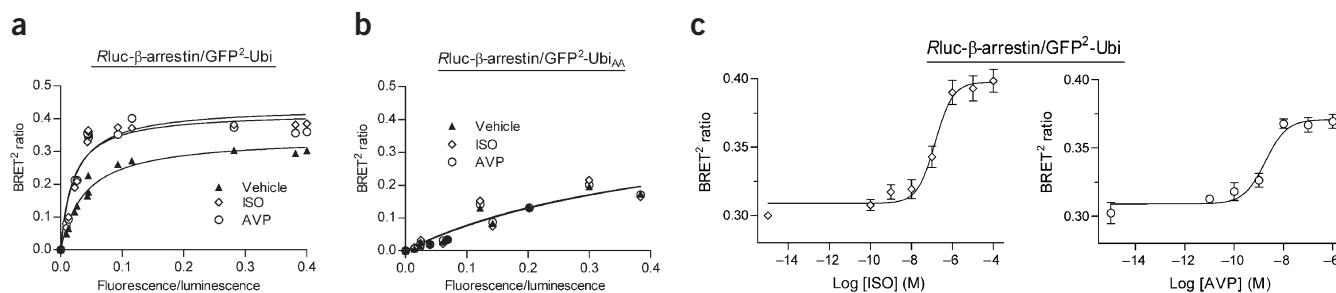


Figure 3 | Detection of GPCR-induced β -arrestin ubiquitination. (**a,b**) Cells cotransfected with a constant concentration of Rluc- β -arrestin, Myc-tagged V₂R and HA-tagged β_2 AR plasmids and increasing concentrations of GFP²-Ubi (**a**) or GFP²-Ubi_{AA} (**b**) constructs were unstimulated (\blacktriangle) or stimulated with 10⁻⁵ M ISO (\diamond) or 10⁻⁶ M AVP (\circ) for 120 s before BRET measurement. DeepBlueC coelenterazine was added in the continuous presence of the agonists and the BRET measurements were taken 20 s after the addition of luciferase substrate. The data shown represent pooled individual readings obtained from three independent experiments. (**c**) Cells expressing the same construct as in **a** and displaying a GFP²-Ubi/Rluc- β -arrestin expression ratio of 0.1 \pm 0.005 (assessed by measuring total fluorescence and luminescence signals) were stimulated with increasing concentration of ISO (left) or AVP (right) for 120 s; BRET measurements were then collected as in **a**. The curves shown represent the mean \pm s.e.m. of three independent experiments.

V₂R. Activation of each receptor with its selective agonist, isoproterenol (ISO) for the β_2 AR and arginine-vasopressin (AVP) for the V₂R, substantially increased the BRET² signal (Fig. 3a). In contrast, ligands did not have an effect on the bystander BRET² observed between GFP²-Ubi_{AA} and Rluc- β -arrestin (Fig. 3b), confirming the selectivity of the agonist-promoted ubiquitination observed with GFP²-Ubi. This increase in BRET² signal was dose dependent, and the agonist concentrations yielding a half-maximal response (EC₅₀) were 140 and 1.8 nM for ISO and AVP, respectively, consistent with the affinity of these ligands for their receptors (Fig. 3c).

Concomitant detection of ubiquitination and recruitment

It has been suggested that receptor activation may regulate the ubiquitination of β -arrestin and its recruitment to GPCRs^{10,13} in a coordinated fashion. To monitor these two events simultaneously in the same cells, we took advantage of the distinct spectral properties of two luciferase substrates that allow energy transfer with two different fluorescent proteins. Whereas the Rluc-catalyzed oxidation of DeepBlueC coelenterazine leads to BRET² between Rluc and GFP², the use of coelenterazine h leads to a distinct emission spectrum that promotes BRET¹ between Rluc and a variant of GFP, the yellow fluorescent protein (YFP)^{14–16}. We divided a culture of cells coexpressing Rluc- β -arrestin, GFP²-Ubi and V₂R-YFP into two samples. In one sample we monitored the β -arrestin ubiquitination by assessing the BRET² between

Rluc- β -arrestin and GFP²-Ubi, and in the second sample we measured BRET¹ between Rluc- β -arrestin and V₂R-YFP to evaluate the recruitment of β -arrestin to the receptor (Fig. 4). Stimulation with AVP resulted in an increase in both BRET² and BRET¹ signals, reflecting concomitant ubiquitination (Fig. 4a) and recruitment of β -arrestin (Fig. 4b). Each of the BRET signals detected was specific to the interaction monitored, as no substantial transfer of energy occurred between Rluc and GFP² in the presence of coelenterazine h or between Rluc and YFP in the presence of DeepBlueC coelenterazine.

Real time monitoring of distinct ubiquitination kinetics

As described above, one can distinguish two GPCR classes based on their profile of interaction with β -arrestin. Because the ubiquitination state of β -arrestin seems intimately linked to its interaction profile with receptors¹⁰, we followed the kinetics of agonist-promoted β -arrestin ubiquitination in real time in cells coexpressing β_2 AR and V₂R. Both ISO and AVP induced rapid and similar increases in the BRET-detected ubiquitination of β -arrestin that reached a peak at 2 minutes after activation (Fig. 5). However, although it remained stable for at least 10 min after V₂R activation (Fig. 5b,c), the BRET signal returned progressively toward basal values after activation of the β_2 AR (Fig. 5a,c). This reduction in BRET signal most likely reflects a deubiquitination process and not a degradation of β -arrestin. As BRET is a ratiometric measurement (GFP² emission divided by Rluc emission), degradation of

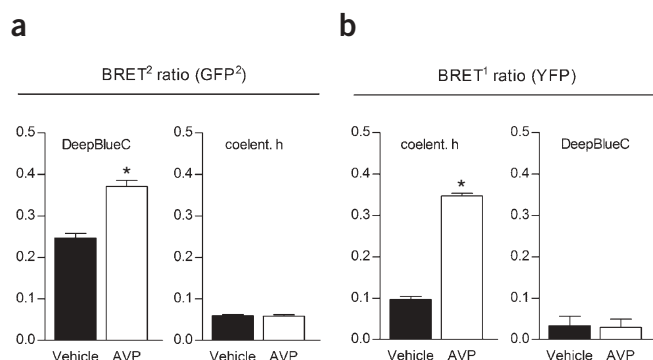


Figure 4 | Concomitant detection of β -arrestin ubiquitination and its recruitment to the activated receptor. (**a,b**) Cells cotransfected with Rluc- β -arrestin, V₂R-YFP and GFP²-Ubi were stimulated with 1 μ M AVP or vehicle alone for 10 min. DeepBlueC coelenterazine (BRET², **a**) or coelenterazine h (BRET¹, **b**) were then added in the continuous presence of the agonist and BRET measurements collected 20 s after the addition of the luciferase substrate to monitor Rluc- β -arrestin ubiquitination or its recruitment to V₂R-YFP, respectively. Bar graphs shown represent the mean \pm s.e.m. of three independent experiments. The lack of cross-talk between BRET¹ and BRET² in the same samples was verified by measuring the BRET signal between Rluc- β -arrestin and GFP²-Ubi in the presence of coelenterazine h for BRET² and between Rluc- β -arrestin and V₂R-YFP in the presence of DeepBlueC for BRET¹. Virtually no cross-talk signal was observed (right side of each panel). Statistical significance of the difference was assessed using paired Student's *t*-test. **P* < 0.05.

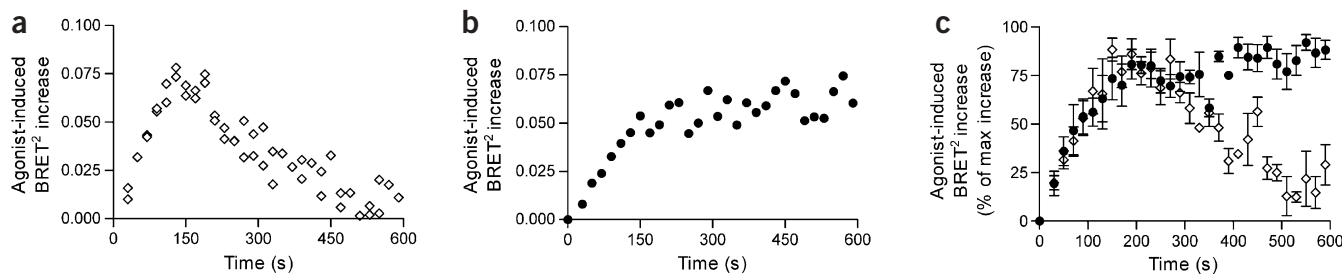


Figure 5 | Real-time assessment of receptor stimulated β -arrestin ubiquitination. Cells cotransfected with *Rluc*- β -arrestin, Myc-tagged V_2R , HA-tagged β_2AR and GFP²-Ubi (GFP²-Ubi to *Rluc*- β -arrestin ratio of 0.1 ± 0.005) constructs, were incubated in the presence of DeepBlueC coelenterazine immediately before stimulation with 10^{-5} M ISO (\diamond ; **a,c**) or 10^{-6} M AVP (\bullet ; **b,c**). BRET² measurements were then collected immediately every 10 s in the continued presence of the agonists for 10 min. (**a,b**) The real-time agonist-promoted increases in BRET are represented as the difference in the absolute BRET values obtained in the presence and in the absence of ISO or AVP. (**c**) Compilation of three independent experiments carried out as in **a** and **b**. The data shown represent the mean \pm s.e.m. and are expressed in percentage of the maximal BRET² signal obtained for each curve.

Rluc- β -arrestin should induce a decrease in luminescence and lead to an equivalent reduction in the transfer of energy to the acceptor without affecting the signal. These results, therefore, confirm that in living cells, the nature of the activated receptor determines the dynamics of β -arrestin ubiquitination.

DISCUSSION

The aim of this study was to adapt an energy transfer assay to the detection of protein ubiquitination in living cells. Using β -arrestin as a model, we demonstrated that BRET can specifically detect both basal and dynamically regulated ubiquitination processes. As compared to western blot analysis, the use of BRET presents several advantages: (i) because the BRET assay is carried out in living cells, it avoids the possible signal alterations that could result from cell lysis, protein solubilization or any purification steps before western blot analysis; (ii) the ability to follow the evolution of the BRET signal in real time allows one to capture the dynamic nature of the ubiquitination process; (iii) one can monitor changes in ubiquitination levels resulting from specific treatments in one population of cells using BRET, whereas western blot experiments require distinct cellular pools; and (iv) because of the ratiometric nature of the BRET technique, a reduction in signal truly represents a decrease in ubiquitination and does not result from the degradation of the ubiquitinated protein target.

As the BRET method is a proximity-based assay that was previously used to detect noncovalent protein-protein interactions, it was important to validate that BRET detected between *Rluc*- β -arrestin and GFP²-Ubi truly reflected the covalent attachment of the ubiquitin moiety. This was achieved by comparing the BRET signals obtained between *Rluc*- β -arrestin and either GFP²-Ubi, GFP²-Ubi_{AA} or the GFP² moiety alone. The BRET signal obtained between *Rluc*- β -arrestin and GFP²-Ubi increased hyperbolically with increasing concentration of GFP²-Ubi before reaching a plateau once the covalent attachment of ubiquitin to *Rluc*- β -arrestin attained the maximal steady state. In contrast, the smaller, linear BRET signal obtained between *Rluc*- β -arrestin and GFP²-Ubi_{AA} or GFP² most likely resulted from bystander BRET¹². Because it cannot be incorporated into proteins, the concentration of free GFP²-Ubi_{AA} available for random collision should be equivalent to that reached when the GFP² moiety alone is expressed, with both being much higher than that attained with GFP²-Ubi for equivalent GFP² constructs. It follows that the

bystander BRET component of the hyperbolic signal observed between *Rluc*- β -arrestin and GFP²-Ubi should then be much smaller than that observed with GFP²-Ubi_{AA} or GFP². The lower signal detected between the *Rluc* moiety alone and GFP²-Ubi compared to that monitored between *Rluc*- β -arrestin and GFP²-Ubi_{AA} or GFP² confirms this prediction. This makes GFP²-Ubi_{AA} a stringent control that allows one to distinguish between protein-protein interactions that are due to random collision and those due to covalent ubiquitination of a specific substrate.

Both BRET and immunoprecipitation approaches indicated that β -arrestin undergoes basal ubiquitination to a significant extent. These results are somewhat different from those of a published report^{7,10} in which ubiquitination of β -arrestin was observed almost exclusively as a result of receptor activation. The specific reasons for this difference are not known, but it probably results from differences in the experimental conditions used, such as receptor expression level or the overexpression of exogenous ubiquitin protein.

An important aspect of the BRET-based method is its ability to detect dynamic changes in the ubiquitination state of a protein and to monitor changes over time. The ubiquitination BRET assay allowed us to monitor distinct kinetics of β -arrestin ubiquitination resulting from the activation of different GPCRs. Activation of the β_2AR induced a fleeting β -arrestin ubiquitination, whereas a long-lasting ubiquitination of the regulatory protein was observed after activation of the V_2R . This is consistent with a previous study examining the ubiquitination status of β -arrestin at 1 and 15 min after receptor stimulation¹⁰. However, because the activated receptor recruits β -arrestin, the increase in ubiquitination BRET signal (**Fig. 2a**) could result from the ubiquitination of the receptor itself. This was not the case, as no BRET modulation occurred between β_2AR -*Rluc* or V_2R -*Rluc* and GFP²-Ubi after brief agonist stimulation (**Supplementary Fig. 2** online). The data show that the ubiquitination BRET assay can faithfully reflect rapid and differential changes in the ubiquitination state of a specific substrate after specific stimuli.

The GFP²-Ubi construct used for the BRET experiments was designed to lack the two major sites involved in polyubiquitination in mammals (Lys48 and Lys63) to prevent the formation of GFP chains that could interfere with the energy transfer process. Despite the constraint that such a construct could impose on the system, ubiquitination of β -arrestin, which is believed to be

polyubiquitinated^{7,10}, could be readily observed. This is not surprising given that the process of polyubiquitination permits the attachment of the GFP²-Ubi through its terminal glycine either to β -arrestin targeted lysine(s) or to growing chains of ubiquitin. As the use of the GFP²-Ubi construct, lacking the polyubiquitination sites, could interfere with the properties of ubiquitinated proteins, we compared the ubiquitination BRET signal obtained with this construct to the signal obtained in the presence of a wild-type GFP²-Ubi protein (GFP²-Ubi_{WT}). Although a specific hyperbolic BRET signal was detected between Rluc- β -arrestin and GFP²-Ubi_{WT}, it was lower than the one obtained with GFP²-Ubi (Supplementary Fig. 1 online). Despite the lower absolute signal, similar BRET modulations were observed with both constructs after agonist stimulation (Supplementary Fig. 1 online). This indicates that GFP²-Ubi expression, *per se*, does not influence the nature of the results obtained but facilitates the detection of BRET signal changes. The fact that GFP²-Ubi_{WT} could also be used in the ubiquitination BRET assay makes the method more broadly applicable.

Besides its usefulness in directly monitoring protein ubiquitination, the BRET assay offers the possibility of concomitant detection of additional protein interactions involving the ubiquitinated protein. Taking advantage of two spectrally distinct coelenterazines, we demonstrated the feasibility of such dual detection by monitoring both ubiquitination of β -arrestin and its recruitment to the V₂R in the same cells.

In addition to shedding new light on the dynamics of β -arrestin ubiquitination in living cells, our study represents proof of the principle that resonance energy transfer approaches allow the monitoring of ubiquitin attachment in real time. BRET and possibly fluorescence resonance energy transfer (FRET) could be used to study the ubiquitination of various proteins whose activity and fate are regulated by this post-translational modification. Studies of the growing number of ubiquitin-like proteins that regulate diverse cellular functions through their covalent attachment to specific protein substrates could also benefit from advances in ubiquitination BRET assays.

METHODS

Plasmids. To produce a wild type GFP²-Ubi construct (GFP²-Ubi_{WT}), we amplified the gene encoding human ubiquitin from a pQE32-Ubi plasmid and subcloned it into pGFP²-C1 (Perkin Elmer BioSignal). We then mutated by PCR lysines 48 and 63 in the ubiquitin moiety of the GFP²-Ubi_{WT} construct into alanines (GFP²-Ubi). Finally, we constructed the GFP²-Ubi_{AA}^{17,18} by mutating by PCR the two C-terminal glycines 75 and 76 into alanines. We previously described pcDNA3.1-Rluc- β -arrestin, pcDNA3.1-hemagglutinin (HA)-tagged β_2 AR, pcDNA3.1-Myc-tagged V₂R and pRK5-V₂R-YFP^{13,19–21}. pCMV-Myc-tagged β -arrestin was a kind gift from S. Marullo's laboratory.

Cell culture and transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin, 2 mM L-glutamine (Wisent) and cultured at 37 °C in 5 % CO₂. We transfected the cells 24 h after seeding using the calcium phosphate precipitation method²².

BRET measurement. Transfected cells were washed twice with PBS, detached in PBS with EDTA and resuspended in PBS with

0.1% glucose. We determined the cell number by measuring protein concentration using the Dc protein assay kit (Bio-Rad). We obtained BRET measurements using two different sets of energy transfer partners. Energy transfer known as BRET², between Rluc and GFP², was determined after the addition of the Rluc substrate, DeepBlueC coelenterazine¹⁴ (Perkin Elmer), whereas BRET¹, between Rluc and YFP, was assessed in the presence of another Rluc substrate, coelenterazine h^{15,16}.

BRET² monitoring of β -arrestin ubiquitination. We distributed cells coexpressing Myc-tagged V₂R, HA-tagged β_2 AR, Rluc- β -arrestin and GFP²-Ubi constructs in white 96-well Microplates (Corning) at a density of 100,000 cells per well. We collected BRET² measurements 20 s after the addition of the luciferase substrate, DeepBlueC coelenterazine, at a final concentration of 5 μ M. Readings were obtained using a modified TopCount NXT instrument (Packard Bioscience) that allows the sequential integration of the signals detected in the 370–450 and 500–530 nm ranges. The BRET² signal corresponds to the ratio of the light emitted by the GFP² (500–530 nm) over the light emitted by the Rluc (370–450 nm). We corrected the values by subtracting the background ratio value detected when the Rluc constructs were expressed alone. For titration experiments, the BRET signal was plotted as a function of the total fluorescence signal over the total luminescence signal (see below), a ratio used as an index of the concentration of GFP²-Ubi (or GFP²-Ubi_{AA}) expressed.

BRET¹ monitoring of β -arrestin recruitment to the V₂R receptor. After dividing the cells coexpressing Rluc- β -arrestin, GFP²-Ubi and V₂R-YFP into two samples, we initiated BRET¹ between Rluc- β -arrestin and V₂R-YFP by the addition of 5 μ M coelenterazine h (the recruitment of β -arrestin to the receptor). We collected the readings at the appropriate emission wavelengths after the addition of 1 μ M AVP using a Mithras LB 940 instrument (Berthold Technologies). We analyzed results as described above by calculating the ratio of the light emitted by the YFP (510–550 nm) to the light emitted by the Rluc (460–500 nm). We corrected the values by subtracting the background ratio value detected when the Rluc constructs were expressed alone.

Total fluorescence and luminescence measurements. We dispersed cells in white 96 well plates with clear bottoms (Costar) at a density of 100,000 cells per well¹². We measured total fluorescence of cells in a Fluorocount (Packard Bioscience) using an excitation filter at 400 or 485 nm and an emission filter at 510 or 530 nm in the case of GFP² or YFP, respectively. We then incubated cells for 10 min with coelenterazine h to measure the total luminescence using a LumiCount instrument (Packard Bioscience). In the case of fluorescence, we subtracted the background value detected in untransfected cells to give a specific fluorescence value. In the case of luminescence, where the background was negligible, we used absolute values.

Immunoblotting and immunoprecipitation. We solubilized cells expressing pcDNA3, GFP²-Ubi_{AA} or GFP²-Ubi in lysis buffer A containing 50 mM HEPES (pH7.5), 0.5 % Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 5 mM *N*-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptin, 5 μ g/ml

aprotinin and 100 μ M benzamidine. After separation of protein on SDS-PAGE and transfer to nitrocellulose, we revealed the immunoreactive bands using an antibody to GFP (Clontech), a horseradish peroxidase-conjugated antibody (Amersham) and the Western Lightning chemiluminescence Reagent Plus (Perkin Elmer). For immunoprecipitation experiments, we used cells expressing or not expressing pCMV-Myc-tagged β -arrestin 2 and pGFP²-Ubi and solubilized them in buffer A for 30 min with rocking at 4 °C. We then incubated soluble extracts with an antibody to Myc (9E10) and protein G-agarose beads overnight at 4 °C. Bound extracts were resolved by SDS-PAGE and transferred to nitrocellulose. We performed western blot analysis with an antibody to GFP, as described above, to reveal basal β -arrestin ubiquitination, and used A14 antibody to Myc (Santa Cruz) to detect the presence of Myc-tagged β -arrestin after its immunoprecipitation.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Ciechanover, A., Orián, A. & Schwartz, A.L. The ubiquitin-mediated proteolytic pathway: mode of action and clinical implications. *J. Cell Biochem. Suppl.* **34**, 40–51 (2000).
- Passmore, L.A. & Barford, D. Getting into position: The catalytic mechanisms of protein ubiquitylation. *Biochem. J.* **379** (Part 3), 513–525 (2004).
- Hochstrasser, M. Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* **30**, 405–439 (1996).
- Schnell, J.D. & Hicke, L. Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *J. Biol. Chem.* **278**, 35857–35860 (2003).
- Eidne, K.A., Kroeger, K.M. & Hanyaloglu, A.C. Applications of novel resonance energy transfer techniques to study dynamic hormone receptor interactions in living cells. *Trends Endocrinol. Metab.* **13**, 415–421 (2002).
- Wojcikiewicz, R.J. Regulated ubiquitination of proteins in GPCR-initiated signaling pathways. *Trends Pharmacol. Sci.* **25**, 35–41 (2004).
- Shenoy, S.K., McDonald, P.H., Kohout, T.A. & Lefkowitz, R.J. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* **294**, 1307–1313 (2001).
- Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S. & Caron, M.G. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J. Biol. Chem.* **274**, 32248–32257 (1999).
- Oakley, R.H., Laporte, S.A., Holt, J.A., Caron, M.G. & Barak, L.S. Differential affinities of visual arrestin, beta-arrestin 1, and beta-arrestin 2 for G protein-coupled receptors delineate two major classes of receptors. *J. Biol. Chem.* **275**, 17201–17210 (2000).
- Shenoy, S.K. & Lefkowitz, R.J. Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *J. Biol. Chem.* **278**, 14498–14506 (2003).
- Patrick, G.N., Bingol, B., Weld, H.A. & Schuman, E.M. Ubiquitin-mediated proteasome activity is required for agonist-induced endocytosis of GluRs. *Curr. Biol.* **13**, 2073–2081 (2003).
- Mercier, J.F., Salahpour, A., Angers, S., Breit, A. & Bouvier, M. Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J. Biol. Chem.* **277**, 44925–44931 (2002).
- Lavoie, C. *et al.* Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy. *J. Biol. Chem.* **277**, 35402–35410 (2002).
- Jensen, A.A., Hansen, J.L., Sheikh, S.P. & Brauner-Osborne, H. Probing intermolecular protein-protein interactions in the calcium-sensing receptor homodimer using bioluminescence resonance energy transfer (BRET). *Eur. J. Biochem.* **269**, 5076–5087 (2002).
- Angers, S. *et al.* Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. USA* **97**, 3684–3689 (2000).
- Xu, Y., Piston, D.W. & Johnson, C.H. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proc. Natl. Acad. Sci. USA* **96**, 151–156 (1999).
- Haas, A.L., Murphy, K.E. & Bright, P.M. The inactivation of ubiquitin accounts for the inability to demonstrate ATP, ubiquitin-dependent proteolysis in liver extracts. *J. Biol. Chem.* **260**, 4694–4703 (1985).
- Wilkinson, K.D. & Audhya, T.K. Stimulation of ATP-dependent proteolysis requires ubiquitin with the COOH-terminal sequence Arg-Gly-Gly. *J. Biol. Chem.* **256**, 9235–9241 (1981).
- Perroy, J., Adam, L., Qanbar, R., Chenier, S. & Bouvier, M. Phosphorylation-independent desensitization of GABA(B) receptor by GRK4. *EMBO J.* **22**, 3816–3824 (2003).
- Morello, J.P. *et al.* Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J. Clin. Invest.* **105**, 887–895 (2000).
- Terrillon, S. *et al.* Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and hetero-dimers during biosynthesis. *Mol. Endocrinol.* **17**, 677–691 (2003).
- Mellon, P., Parker, V., Gluzman, Y. & Maniatis, T. Identification of DNA sequences required for transcription of the human alpha 1-globin gene in a new SV40 host-vector system. *Cell* **27**, 279–288 (1981).