Analytical Biochemistry 489 (2015) 1–8

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A luminescent assay for real-time measurements of receptor endocytosis in living cells

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ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form
28 July 2015
Accepted 5 August 2015
Available online 13 August 2015

Keywords:
G-protein-coupled receptor
Receptor tyrosine kinase
Endocytosis
Internalization
NanoLuc

ABSTRACT

Ligand-mediated endocytosis is a key autoregulatory mechanism governing the duration and intensity of signals emanating from cell surface receptors. Due to the mechanistic complexity of endocytosis and its emerging relevance in disease, simple methods capable of tracking this dynamic process in cells have become increasingly desirable. We have developed a bioluminescent reporter technology for real-time analysis of ligand-mediated receptor endocytosis using genetic fusions of NanoLuc luciferase with various G-protein-coupled receptors (GPCRs). This method is compatible with standard microplate formats, which should decrease work flows for high-throughput screens. This article also describes the application of this technology to endocytosis of epidermal growth factor receptor (EGFR), demonstrating potential applicability of the method beyond GPCRs.

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Endocytosis is a common mechanism regulating the function of ligand-activated G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) [1–7]. These transmembrane receptors transmit signals from extracellular ligands to intracellular signaling pathways, and activation is often followed by receptor desensitization and rapid endocytosis [1]. Endocytosis generally results in attenuation of this transmitted signal, but the fate of internalized receptors is complex and can involve receptor recycling, lysosomal trafficking, degradation, or sustained signaling of the receptor to downstream effectors from endosomes [1]. Due to this integral role in regulating signal transduction, it is not surprising that perturbations in receptor endocytosis have been linked to human disease [7]. Therefore, endocytosis represents a potentially broad but largely unexplored opportunity for pharmacological intervention [3,8,9]. Despite increased interest in targeting endocytosis in chemical library screens, few quantitative methods exist for interrogating this dynamic process as it occurs in intact cell populations. Simple analytical methods to track endocytosis in cells, therefore, should enable compound screening efforts at GPCRs and other key transmembrane receptor classes.

The method described in this article enables a facile, microplate-based measurement of receptor endocytosis in living cells. In contrast to other described methods to monitor receptor internalization, our approach enables measurements of endocytosis in real time in a simple microplate format. NanoLuc (Nluc) was selected as an ideal reporter due to its extraordinary brightness and relatively small size (~19 kDa) as well as its demonstrated compatibility as a protein fusion partner [10]. We demonstrate that various integral membrane receptors tolerate Nluc fusion protein on the receptor N terminus, resulting in Nluc being localized in the extracellular milieu of unstimulated cells, and translocate into the lumen of the endosomes on receptor stimulation. To couple luminescent reporter signal with receptor internalization, we have exploited a decrease in bioluminescence of Nluc on trafficking into endosomes. The reporter strategy applied here uses a simple homogeneous format, is compatible with transiently transfected cells, and uses a nondestructive endpoint for real-time analysis of receptor endocytosis.

Abbreviations: GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; Nluc, NanoLuc; EGFR, epidermal growth factor receptor; PCR, polymerase chain reaction; ISO, isoproterenol; RP-HPLC, reverse phase high-performance liquid chromatography; DMF, dimethylformamide; DCM, dichloromethane; MFI, mean fluorescent intensity; V2R, vasopressin-2 receptor; AT1R, angiotensin II type 1a receptor.

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http://dx.doi.org/10.1016/j.ab.2015.08.005
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To demonstrate the broad utility of this assay, we have successfully applied the method to multiple GPCRs ectopically expressed in HEK293 cells. Each of these Nluc-receptor fusions responds predictably to functionally selective or nonselective endocytosis agonists in a dose- and time-dependent manner. We also successfully expanded the application of this technology to endocytosis of RTKs using epidermal growth factor receptor (EGFR) as the example. The spatiotemporal behavior of this luminescence sensor was validated with image-based analysis of receptor trafficking on a confocal microscope using HaloTag as an orthogonal fluorescent reporter. Image analysis was enabled with a novel fluorogenic, pH-sensitive probe for HaloTag that allowed time-dependent measurements of endosomal trafficking via confocal microscopy. Our results support the use of Nluc for kinetic measurements of endocytosis in live cell populations in a simplified microplate-based format.

Materials and methods

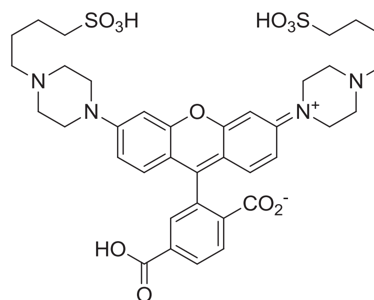
Molecular biology

N-terminal fusions of Nluc with each receptor were generated in which Nluc was placed after an IL6 secretion tag (MNSFSTSAFGPVAFSLGLLLVLPAAFPAP) to facilitate proper cell surface expression of the Nluc-receptor fusion. The Nluc luciferase was flanked on each side by flexible Gly/Ser linkers (GSSG). The Nluc–B2AR reporter vector was created by amplifying Nluc by polymerase chain reaction (PCR) and cloning it into the XhoI/SacI sites of the plasmid pGPCRINT11 to generate the arrangement described above. For AGTR1 and OPRD1, the full-length genes were amplified by PCR and inserted into the SacI/EcoRI sites of the Nluc–B2AR construct to replace the receptor. Nluc fusions with full-length EDG1 or with EGFR lacking its putative signal peptide (residues 1–24) were generated through overlap extension PCR. A PCR fragment containing the receptor was combined with a PCR fragment containing the IL6 peptide and Nluc by overlap extension. This PCR product was then cloned into the pF9a vector using the Flexi Vector System, and an XhoI/XbaI fragment containing Nluc and the receptor was transferred into the pGPCRINT11 backbone. The Nluc–Halo Tag–B2AR reporter was built by amplifying Nluc and HaloTag separately via PCR and then joining them using an overlapping PCR method. The Nluc–HaloTag PCR product was cloned into the XhoI–SacI sites of plasmid pGPCRINT11.

Endocytosis assay using Nluc

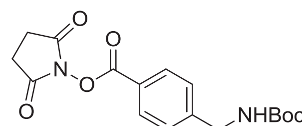
At the time of seeding into assay plates, HEK293 cells were transfected with Nluc/receptor fusions using Fugene HD. Lipid:DNA complexes were formed by first diluting the expression construct into promoterless pGEM3Z(−) DNA (Promega) to achieve lower intracellular expression levels. A dilution factor of 1:1000 (for Nluc–V2R, Nluc–DOR, Nluc–EDG1, Nluc–AT1R, and Nluc–EGFR) or 1:10,000 (for Nluc–B2AR constructs) was used prior to the addition of 3 μl of Fugene HD per microgram (μg) of DNA. For experiments using dynamin K44A to inhibit endocytosis, pGEM3Z(−) carrier DNA was replaced with plasmid DNA encoding dynamin K44A. A 1/20 volume of transfection complex was added to HEK293 cells suspended at 2×10^5 cells per milliliter (ml) in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, Gibco). Then, 100 μl/well of cells with transfection complexes was seeded into white 96-well plates (Corning), followed by 20 h of incubation in a humidified tissue culture incubator. After incubation, growth medium was replaced with Opti-MEM (Gibco) and cells were starved of serum for 2 h in a humidified tissue culture incubator set to 37 °C. After starvation, NanoGlo Live Cell Substrate was added, and cells were stimulated with serially diluted agonist. For antagonist experiments at B2AR, samples were treated with a 10× concentration of antagonist for 30 min prior to the addition of a 10× solution of isoproterenol (ISO) as described above for agonist profiling. The final ISO concentration was 1 μM. Luminescence was measured over the time indicated in a GloMax luminometer prewarmed to 37 °C. For normalization purposes, the luminescence value at each timepoint was divided by the luminescence value prior to stimulation.

Chemical synthesis of HaloTag pH sensor (PBI-4959)



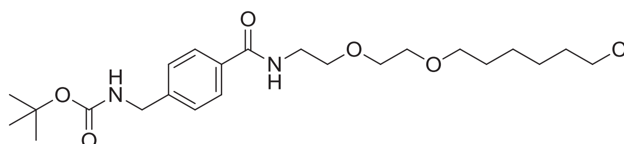
3′,6′-Bis(4-(4-sulfobutyl)piperazin-1-yl) rhodamine (5′,6′) carboxylic acid (PBI-4474)

3′,6′-Diiodorhodamine(5′,6′) carboxylic acid (50.0 mg, 0.084 mmol), 4-(piperazin-1-yl)butane-1-sulfonic acid–HCl (43.3 mg, 0.168 mmol), potassium-*tert*-butoxide (46.9 mg, 0.418 mmol), and CuI (31.8 mg, 0.168) were charged into a pressure tube, and 2,2,2-trifluoroethanol (3 ml) was added. The tube was capped and heated to 110 °C in an oil bath for 10 days. Solvent was removed under reduced pressure, and the crude was suspended in 30 ml of water, stirred for 10 min, and centrifuged. The mother liquor was decanted, filtered, concentrated to 15 ml, and subjected to reverse phase high-performance liquid chromatography (RP-HPLC), giving the product (6.5 mg, 9.5%) as a red solid. MS (ESI) *m/z* calcd for $C_{37}H_{45}N_4O_{11}S_2$ $(M + H^+)$ 785.3, found 785.5.



2,5-Dioxopyrrolidin-1-yl 4-(((tert-butoxycarbonyl)amino)methyl) benzoate

To a solution of 4-(((*tert*-butoxycarbonyl)amino)methyl)benzoic acid (0.5 g, 1.99 mmol) and *O*-(*N*-succinimidyl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (0.72 g, 2.39 mmol) in 5 ml of CH_2Cl_2, *N,N*-diisopropylethylamine (1.0 ml, 5.97 mmol) was added. The mixture was stirred for 2 h and concentrated under reduced pressure, giving a red solid that was dissolved in EtOAc and washed with 2 N HCl (aq.) solution. The organic layer was retained, dried over Na_2SO_4, and concentrated to give a white solid.



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