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A luminescent oxygen channeling biosensor that measures small GTPase activation †

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Abstract

We established a homogeneous luminescent oxygen channeling sensor for measuring activation states of small GTPases. The assay quantifies activated GTPases in cell lysates, can be applied to different GTPases, and can be used for multiplex screening. The study will provide guidelines for determining activation states of diverse GTPases in various biological contexts.

Small GTPases are molecular switches essential for many fundamental cellular processes. They are activated when bound to GTP and switched off in their GDP-bound state.¹ Cycling between these states is promoted by guanine nucleotide exchange factors (GEFs) that catalyze GDP/GTP exchange, and GTPase activating proteins (GAPs) that accelerate GTP hydrolysis.

Due to the key role of this class of molecular switches in diverse cellular processes,² information about their activation states is crucial for understanding GTPase regulated events. However, the most common method currently available for determining the nucleotide-bound state of a given GTPase is based on pull-down assays with specific effector proteins that involve multiple steps of handling.³ This procedure is tedious and requires several time consuming separation steps. To cope with these limitations, here we report the development of a homogenous biosensor system that allows rapid, sensitive, and accurate quantification of the activation state of a given GTPase in a reliable, simple, and reagent-saving fashion. Our biosensor-system is based on luminescent oxygen channeling (LOC) and can be applied to different GTPases in highly versatile fashion. It is suitable for application in cell lysates, and in high throughput setups. These advantages make the sensor ideal for approaches in biochemistry and cell biology.

The general principle of LOC relies on the generation of singlet oxygen (¹O₂) at photosensitizer-coated donor beads and the initiation of luminescence at luminescent-derivatized acceptor beads. The distance between donor and acceptor beads can be up to 200 nm, allowing size-independent molecular interactions to be detected.⁴ LOC has been applied

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for measuring interactions between various biomolecules,⁵ but until now no LOC-based setup for measuring the activation -state of a G-protein switch has been described.

A pilot LOC-based biosensor designed for this purpose is shown in Suppl. Fig. 1A.[†] As a proof of principle, we first quantified the activation of the small GTPase Rac1. The interaction of GTP-bound Rac1 with the Rac1/Cdc42 specific effector protein Pak1 (p21 activated kinase 1)⁶ leads to complex formation that is ultimately measured in the LOC-based assay. The C-terminus of the GTPase is captured by an anti-Rac1 antibody which itself is bound on protein A acceptor beads. Based on structural data (PDB: [2QME](#)) the effector binding epitope of Rac1 (switch I) should not be recognized by the anti-Rac1 antibody that targets the C-terminus of Rac1. Pak1 (aa 70-117) was immobilized *via* its GST fusion partner⁷ on glutathione-functionalized or *via* biotin to streptavidine-functionalized donor beads (Suppl. Fig. 1A and D[†]).

To maximize signal sensitivities of the active GTPase, we determined Pak1 and anti-Rac1-mAB concentrations resulting in optimal bead-surface loading. This was achieved at 7.6 nM anti-Rac1 antibody (Suppl. Fig. 1B[†]) and 100 nM GST-Pak1 (Suppl. Fig. 1C[†]), respectively. When exceeding 7.6 nM anti-Rac1 mAB (Suppl. Fig. 1B[†]) or Pak1 (Suppl. Fig. 1C[†]) the signal became weaker, due to concentration-dependent unproductive interactions between the analyte and free ligand, as frequently observed in LOC.

We then determined the dynamic range for the quantification of GTP-bound Rac1 by titrating Rac1-GTP. Using GSH donor beads, the concentration limit of Rac1-GTP was measured to be around 25 nM (Suppl. Fig. 2A[†]). To test the effect of different immobilization strategies, we captured the effector on streptavidin donor beads after biotinylation of GST-Pak₁₇₀₋₁₁₇ with sulfo-NHS-biotin. We anticipated that streptavidin/biotin immobilization increases the surface loading of the donor beads. Indeed, the absolute signal intensity increased more than ten-fold, allowing to use the effector at considerably lower concentrations.

We therefore repeated the Rac1-GTP titration experiments using 50 nM biotinylated GST-Pak1 and streptavidin-coated donor beads. Remarkably, the lowest quantification limit (LQL) under these conditions was as low as 200 pM (Suppl. Fig. 2B[†]).

This is nearly 100-fold lower than the K_d of the Rac1/Pak1 complex.⁸ Presumably, the enhanced sensitivity is due to the high local concentration of both GTPase and effector on the bead surface. This increases their effective concentration considerably, thus allowing reliable quantification well below the K_d value of roughly 30 nM.⁸

To test how the sensor system performed under more demanding conditions we measured Rac1-GTP in cell lysates. Cell lysates obtained from NIH 3T3 cells expressing endogenous levels of Rac1 were treated with either GTP γ S or GDP to simulate activated or inactivated Rac1. As evident from Fig. 1A, a clear difference between the active and inactive GTPase was observed. In fact, active Rac1 could be detected using only 0.05 $\mu\text{g } \mu\text{L}^{-1}$ (1.0 μg in 20 μL reaction volume) lysate. We found that cell lysate somewhat reduced the signal intensity in the LOC assay. Using biotinylated IgG as a positive control we observed a concentration dependent signal decrease in the presence of cell lysate (not shown). This effect is most likely due to quenching of $^1\text{O}_2$ or unspecific protein binding blocking specific interaction and was also observed for different combinations of donor and acceptor beads. However, this effect is compensated for by the high sensitivity of the system resulting in the reliable

[†]Electronic supplementary information (ESI) available: Methods description. Comparison LOC/pulldown. Determination Rac1-GTP fraction. Assay setup. Rac1-GTP dynamic range. See DOI: 10.1039/c1cc11944c

detection of endogenous levels of activated GTPase. To compare the LOC signal with an ordinary pull-down assay, the same cell lysate (400 μ g) was treated with biotinylated GST-Pak1 and streptavidin-agarose, resulting in an easily detectable signal in the Western Blot analysis (Fig. 1B). In contrast, no signal was detected when pulldown assays were performed with 40 μ g lysate or less (Suppl. Fig. 4[†]). Similar results were obtained when using H460 lung cancer cells (data not shown). Thus, the LOC assay is at least 10-fold more sensitive than a pulldown. The robust signal of the LOC assay facilitates quantitative comparison of active GTPases in different samples. It is even possible to determine the fraction of active GTPase in cell lysates if a calibration curve using purified GTPase is generated under the same conditions (Suppl. Fig. 5[†]).

To demonstrate a broader versatility of the LOC based sensor systems, we established similar assays for Ras, Arf1, and Arf6 (Fig. 2). For Ras, we used the RalGDS-Ras binding domain (Fig. 2A),⁹ for Arf1 the GGA3 protein as effectors (Fig. 2B),^{10,11a,b} both as GST-fusion proteins, biotinylated if needed, and immobilized on either GSH or streptavidin donor beads. Without optimization, the system again clearly discriminates between activated and non-activated forms of these GTPases with sensitivities similar to those observed for Rac1-GTP. We also efficiently detected the interaction of Arf6 with the Arf effector GGA3 using a different set of donor and acceptor beads. Purified Arf6 and GGA3 were directly coupled to the beads *via* their His-tag or GST-tag, respectively, leading to clear distinction of an active from an inactive GTPase (Fig. 2C). This example nicely demonstrates the modularity of the assay and further broadens the spectrum of different applications possible with the described system. Because in this setup Arf1 and Arf6 both share a common effector no discrimination between the two is possible. However, switching to specific antibodies for both proteins will distinguish between these related GTPases (compare Suppl. Fig. 1A[†]).

Our results show that the sensor system described here is widely applicable and should considerably simplify the determination of the activation state of small GTPases from different subgroups of the Ras superfamily. The choice of antibodies for other GTPase - effector -pairs is probably the most crucial part of this sensor system, as the antibodies need to interact with the native protein with high affinity and should bind to the antigen at a site different from the switch regions of the GTPase.

To test the biosensor under multiplex conditions, we screened 86 randomly chosen small molecules and two known Rac1 inhibitors for interference with the GTPase -effector interaction (Fig. 3). The Z' -value of 0.85 shows that the assay performed reliably under these conditions. The known¹² Rac1 inhibitor EHT1864 interfered with the GTPase /effector binding, as evident from a significant drop below the 3σ -threshold in the signal. In a LOC-independent pull-down experiment the interaction of the GTPase with Pak1 was also abrogated by EHT1864 (Suppl. Fig. 7[†]). However, the second Rac1 inhibitor NSC23766 had no effect in the assay, consistent with the observation that NSC23766 does not interfere with effector binding.¹³ EHT1864 neither interfered with donor/acceptor bead conjugation, nor acted as a singlet oxygen quencher or light scatterer (Fig. 3B), indicating that the assay performs reliably under HTS-like conditions.

We have established a luminescent oxygen channeling-based biosensor that measures the GTP and GDP-bound state of small GTPases. The simplicity of design and the sensitive and robust nature of this assay should make it applicable to a wide range of GTPase activation studies. The sensor is amenable to high-throughput screening for small molecules that interfere with GTPase activation by GEFs,¹¹ GTP hydrolysis by GAPs¹⁴ or effector complexation as it can be completed within short time and shows considerably higher sensitivity than established pull down assay formats. The high background often encountered with fluorescence-based assays is circumvented by excitation at a long

wavelength and emission at a shorter wavelength. In addition, the assay does not appear to be more prone to false-positives than other screening assays; the number of singlet oxygen quenchers is manageable, even when highly diverse compound libraries are used.¹⁵ Moreover, the LOC-based assay can outcompete standard pull down-based Rac1 detection kits, as evidenced by its higher sensitivity obtained with only a tenth of cell lysate. Our data on Arf and Ras shows that this concept can be employed for GTPases other than Rac1. It is anticipated that the LOC based assay described here will become a valuable tool for quantifying the activation state of GTPases and the discovery of small molecules to be used as starting points for lead discovery or as chemical biology probes.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Quantification of activated Rac1 in NIH 3T3 cell lysates. (A) The indicated amounts of NIH 3T3 cell lysate were directly used for the LOC-based determination of the activation ($n = 4$, bars illustrate fold signal change compared to 0 µg GDP preloaded lysate). Black bars: GTPγS; white bars: GDP. (B) Pull down assay with 400 µg of the same cell lysate. All experiments used GTPγS-loaded GTPases to prevent hydrolysis. Nonetheless, when performing the experiments shown in Suppl. Fig. 2A using GTP instead of GTPγS we obtained identical results (Suppl. Fig. 3[†]).

Fig. 2.

Determination of K-Ras-GTP and ARF1-GTP. (A) GST-RalGDS-Ras binding domain coupled to GSH donor beads and panRas antibody coupled to protein A acceptor beads. Black bars: GTP γ S; white bars: GDP. (B) GST-GGA3-biotin coupled to streptavidin donor beads and anti-Arf1 antibody coupled to protein A acceptor beads. (C) 50 nM GST-GGA3 was coupled to GSH donor beads, and purified His-tagged Arf6 to Ni²⁺ chelate acceptor beads. This modified setup (Suppl. Fig. 6[†]) also yielded excellent signal-to-background rates.

Fig. 3.

Pilot screen with 88 model compounds including the known Rac1 inhibitors EHT1864 and NSC23766. (A) Rows 12 and 13 represent 8 replicates of positive (+) and negative (-) controls including 3.3% DMSO and GDP-loaded Rac1, respectively. Z' -value: 0.85. Hit threshold: $3\sigma_p$ (grey field) (B) EHT1864 does not unspecifically interfere with the assay system at 10 μ M (3.3% DMSO). Description of biotin-IgG and TrueHits assays: see Supplementary Methods.[†]