Biochemical and Biophysical Research Communications 448 (2014) 281–286

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Live visualization and quantification of pathway signaling with dual fluorescent and bioluminescent reporters

Amirali Afshari [a], Claudia Uhde-Stone [a], Biao Lu [b,*]

[a] Department of Biological Sciences, California State University, East Bay, 25800 Carlos Bee Blvd, Hayward, CA 94542, United States
[b] System Biosciences (SBI), 265 North Whisman Rd., Mountain View, CA 94043, United States

ARTICLE INFO

Article history:
Received 15 April 2014
Available online 1 May 2014

Keywords:
Dual-reporter
Signal transduction
GFP
Luciferase
Transcription factor
NF-κB

ABSTRACT

Despite their fundamental importance, the dynamics of signaling pathways in living cells remain challenging to study, due to a lack of non-invasive tools for temporal assessment of signal transduction in desired cell models. Here we report a dual-reporter strategy that enables researchers to monitor signal transduction in mammalian cells in real-time, both temporally and quantitatively. This is achieved by co-expressing green fluorescent protein and firefly luciferase in response to signaling stimuli. To display the versatility of this approach, we constructed and assessed eight unique signaling pathway reporters. We further validated the system by establishing stable NF-κB pathway reporter cell lines. Using these stable cell lines, we monitored the activity of NF-κB-mediated inflammatory pathway in real-time, both visually and quantitatively. Live visualization has the power to reveal individual cell responses and is compatible with single cell analysis, In addition, we provide evidence that this system is readily amenable to a high-throughput format. Together, our findings demonstrate the potential of the dual reporter system, which significantly improves the capacity to study signal transduction pathways in mammalian cells.

1. Introduction

Signaling pathways regulate cell-specific behaviors that are important for normal development and disease processes [1,2]. Despite their importance, real-time monitoring of pathway signaling has remained a challenge, mainly due to a lack of tools to visualize and quantify the dynamics of signal transduction in living cells. For decades, immune-based analysis of protein phosphorylation has been a mainstay of signaling pathway analysis [3–5]. However, immune-based protocols rely heavily on specific antibodies, are cumbersome [6] and not suitable for pathway activations without phosphorylation [7]. Similarly, biochemical analysis of metabolites in a signaling cascade can provide clues to pathway activation [8]. However, quantification of the metabolites usually requires rigorous validation, special reagents and equipment [9]. Moreover, both immune-based and biochemical assays typically use cell lysates, thus limiting their capability to study the dynamics of signal transduction in living cells [10].

High-throughput, image-based cell assays have emerged as alternative approach for monitoring molecular events [11–13]. For example, signaling molecule such as NF-κB can be fused with imageable reporters such as green fluorescent protein (GFP) or red fluorescent protein (RFP). Migration of GFP from the cytosol to the nucleus reveals NF-κB pathway activation [14]. Alternatively, the fluorescent reporter proteins can be placed under the control of appropriate transcription factors to monitor pathway activation [15]. In fact, numerous reporter cell lines have been established for the use of signal transduction monitoring, regulation studies and compound screening [16–19]. Although fluorescent proteins allow real-time monitoring of individual cell responses, quantification tends to be tedious, time-consuming and costly, due to the requirement of specialized imaging systems or fluorescent-activated cell sorters [20]. To overcome this

Abbreviations: CMV, cytomegalovirus promoter; EF1α, elongation factor 1-alpha promoter; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK 293, human embryonic kidney cell line 293; IL-1β, interleukin 1 beta; Insu, core insulator sequences; ITR, inverted terminal repeats; Luc, luciferase; MAPK, mitogen activated protein kinase; mCMV, minimal CMV promoter; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-myristate-acetate; Puro, puromycin; RFP, red fluorescent protein; T2A, self-cleaving 2A peptide sequence; TF, transcription factor; TNFα, tumor necrosis factor alpha; TRE, transcription factor response element.

* Corresponding author.
E-mail addresses: afshari.amirali@gmail.com (A. Afshari), Claudia.stone@csueastbay.edu (C. Uhde-Stone), BLu@systembio.com (B. Lu).

http://dx.doi.org/10.1016/j.bbrc.2014.04.108
0006-291X/

drawback, bioluminescent proteins are used in place of GFP/RFP. Actually, the firefly luciferase-based reporter system has greatly simplified quantification procedures and enabled the integration of plate-handling and data-collection amenable for high-throughput applications [21,22]. However, luciferase lacks a robust visualization capability, and thus is not suitable for monitoring signaling dynamics within single cells.

Combining the advantages of fluorescent and bioluminescent reporters, we developed a dual-reporter system to both visualize and quantify pathway signaling in living mammalian cells. We incorporated features to allow easy and reliable establishment of stable reporter cell lines with reduced background noise, and minimized potential genomic influences. To test this system, we constructed and assessed eight unique signaling pathway reporters, and established stable cell lines harboring inflammatory NF-κB reporters. We demonstrate the capability of both live visualization and quantification of NF-κB pathway activation using cultured human HEK293 cell models. Live visualization reveals individual cell responses and is compatible with single cell analysis. We further provide evidence that the dual reporter system is readily amenable to high-throughput quantification of pathway signaling in living cells. Our findings demonstrate the power of dual-reporters for studying signaling pathways in mammalian cells.

2. Materials and methods

2.1. Materials and reagents

Human recombinant TNFα, IL-1β, and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). Nutlin-3, PMA (phorbol-12-myristate 13-acetate) and ionomycin were purchased from Sigma, St. Lois, MO, USA). 5× D-luciferin substrate for live cell luciferase activity assay was obtained from System Biosciences (SBI, Mountain View, CA, USA). Fetal bovine serum (FBS) was obtained from Atlas Biologicals (Fort Collins, CO, USA).

2.2. Design and construction of dual-reporters

The dual reporter system is configured from the 5′ to 3′ as follows (Fig. 1A): multiple cloning sites (MCS) to insert different transcription factor response elements (TREs), followed by a minimal CMV promoter (mCMV), GFP-T2A-firefly-luciferase and a poly A signal as previously reported [23]. To make the dual-reporters suitable for establishing stable reporter cell lines, a constitutive promoter EF1α-driven puromycin resistance gene cassette was implemented. Reporter and selection cassette were flanked by insulator sequences to minimize potential interference of neighboring sequences. To obtain a controlled integration by co-expression of integrase [24], integrase-recognizable insertion sequences were introduced and situated outside the insulator. The above dual reporter cassette was subsequently cloned into a regular plasmid with an ampicillin selection marker. Additionally, reporters lacking the mCMV or harboring a full-length CMV promoter were similarly constructed for system validation. For specific signaling pathway reporters, four to eight repeats of the corresponding TREs were cloned into the MCS. All final constructs were confirmed by double-stranded DNA sequencing.

2.3. Cell culture and transfection

Human embryonic kidney cells (HEK293) were maintained in high glucose Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS, 2 mM GlutaMax (Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, and 100 U/ml streptomycin. All transfections were performed in 6-well plates seeded with 2×10^5 cells per well the day before transfection. At 30–50% confluency, cells were transfected with the dual reporter plasmid with or without transposase-expression vector, using Purefection transfection reagent according to the user manual (SBI, Mountain View, CA, USA). For all transfection experiments, 2 μg of dual-reporter DNA was used, with or without 0.2 μg of transpose expression vector DNA (SBI, Mountain View, CA, USA).

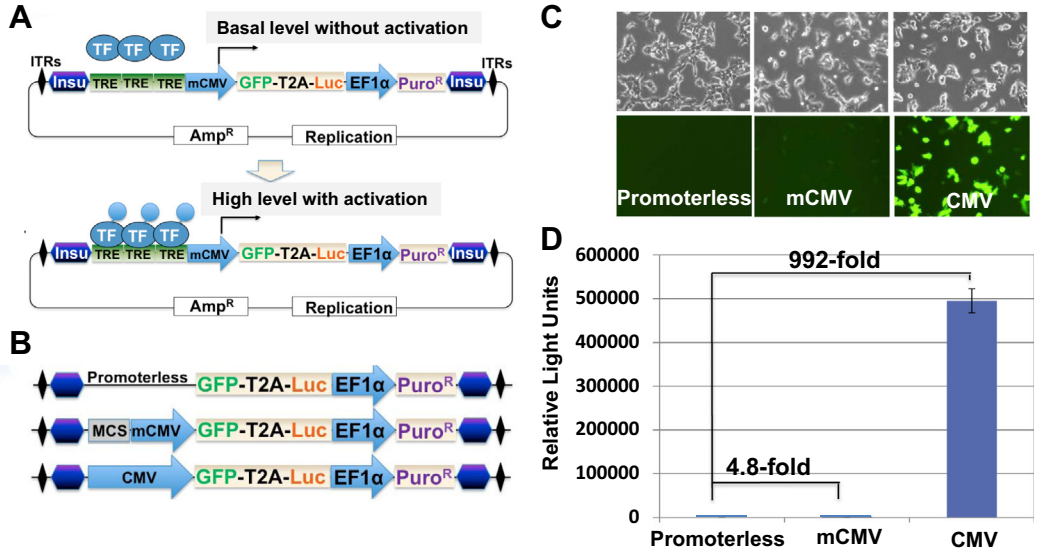

Fig. 1. A dual reporter strategy to visualize and quantify signaling pathway activation. (A) Schematic representation of main features of the dual reporter system, depicting the dual reporters GFP and firefly luciferase (Luc), separated by T2A (self-cleavage peptide), under the control of a minimal CMV promoter. The multiple cloning sites were incorporated to facilitate the insertion of transcription factor response elements (TREs). Without stimuli, the transcription factor (TF) is inactivated and does not bind to TREs, therefore basal levels of expression of both GFP and Luc are expected (upper panel). Upon stimulation, the TF becomes activated and binds to TREs, resulting in high levels of expression (lower panel). (B) Configuration and construction of a promoterless negative control reporter, a mCMV-driven basal reporter, and a full-length CMV promoter-driven positive control reporter. (C) HEK293 cells were transfected with the promoterless negative control reporter, the mCMV-driven reporter, and the positive control, and GFP expression was recorded. (D) Relative luciferase activities of each reporter were presented as fold increase over promoterless control (mean ± SD, $n = 3$).