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

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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.

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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).

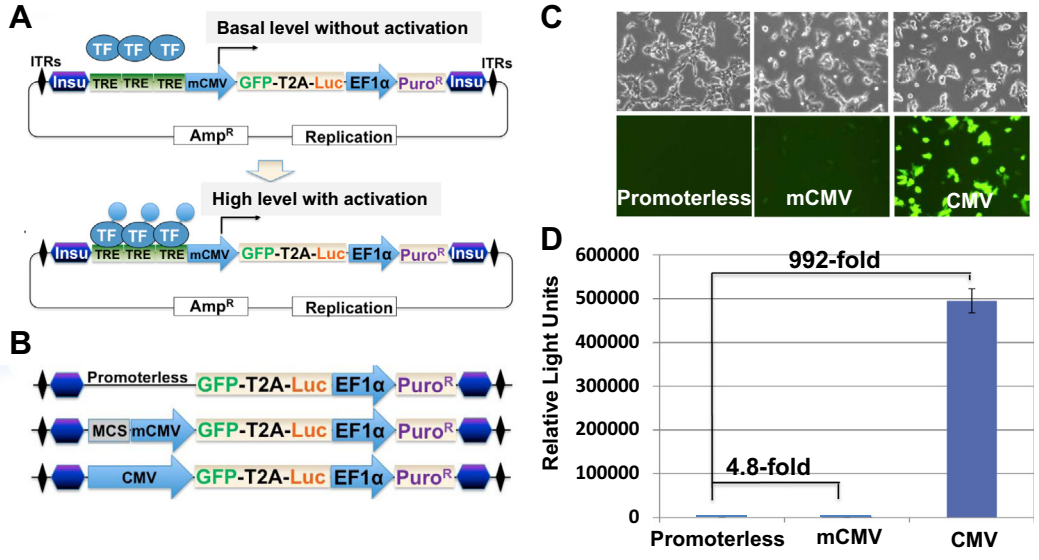

Fig. 1. A dual reporter strategy to visualize and quantify signaling pathway activation. (A) Schematic representation of main features of the dual reporter system, depicting the dual reporters GFP and firefly luciferase (Luc), separated by T2A (self-cleavage peptide), under the control of a minimal CMV 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, $n = 3$).

2.4. Drug selection scheme and establishment of stable cell lines

Three days after transfection, cells were sub-cultured in the presence of 5 μg/ml puromycin (Sigma, St. Louis, MO, USA). Cells that survived for at least one month under puromycin selection were considered stable cell lines harboring the dual-reporter integrated in the genome. Before being used for any experimentation, the stable cell lines were switched to normal culture medium without puromycin selection pressure for at least two passages to avoid potential interference of puromycin.

2.5. Microscopy and reporter gene assay

All microscopy was performed on live cells in 6-well plates. Cells were imaged using a LEICA DMI3000B fluorescent microscope. Data collection and processing were performed with LAS 3.8 software. For the reporter luciferase assay, 2×10^5 cells were seeded in each well of a 24-well plate in completed DMEM with 10% FBS, if not stated otherwise. After 24 h and at 60–80% confluency, the medium was switched to DMEM with 1.5% FBS, in the absence or presence of the specific signaling pathway inducers. 24 h later, cells were lysed on plate and harvested at indicated time points. The lysate was cleared by centrifugation, and luciferase activity was measured with a BioTeck Synergy HT plate reader (BioTek, Winsooki, VT), using a luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI). The resulting relative light units were normalized against protein input. Total protein concentration of cell lysates was determined by NanoDrop Lite (Thermo Scientific, Wilmington, DE, USA).

2.6. Live cell luciferase assay

NF-κB stable cells were seeded into a 96-well plate with a density of 5×10^4 cells/well. At 60–80% confluency after 24 h, cells were switched to low serum DMEM (1.5% FBS), containing 1× D-luciferin substrate (SBI, Mountain View, CA, USA) in the presence or absence of cytokine. The luciferase activities were measured at indicated time points using a luminometer. For the live luciferase reporter assay, all data were collected using the same detection settings for comparison. Care was taken to keep plates sterile during the assay, and plates were returned to the incubator immediately after measurements.

2.7. Data collection and presentation

For live cell monitoring, cultured cells were monitored at indicated time points under a fluorescent microscope. GFP live cell images were taken using the same exposure condition and magnification within the group for comparison.

3. Results and discussion

3.1. Design and construction of dual-reporters suitable for the establishment of stable cell lines

The ability to monitor and quantify the temporal activation of a signaling pathway in living cells, both at the individual cell level and within a population, is a desirable goal toward uncovering the functional relevance and mechanisms of signal transduction. To achieve this goal, we developed a dual reporter, based on a system for monitoring TALE (transcription factor-like effector) activation [25], making use of the unique properties of both GFP for visualization of individual cell responses and firefly luciferase (Luc) for quantification. Furthermore, we designed a general strategy to monitor cellular signaling pathways by placing GFP and luciferase genes under the control of specific transcription factors (TFs) (Fig. 1A). Activation by the specific TF will result in the expression of both GFP and luciferase. Because the TF activity is correlated to the activation of the monitored signaling pathways, dual-reporters serve as readouts of signaling activities.

To facilitate monitoring the activity of a TF, tandem repeats of transcription factor response elements (TREs) were placed immediately upstream of the mCMV promoter (Fig. 1A). Thus, transcription factor binding sites are optimally located within 300 bp upstream of the transcription start site. Without signaling activation, the pathway-responsive transcription factors are not activated and remain unbound to the cognate TREs, with expression of GFP and firefly luciferase at basal levels (Fig. 1A, upper panel). In response to specific stimuli, the corresponding TFs become activated and bind to the TREs situated at the dual reporter promoter region, resulting in high level expression of both reporters (Fig. 1A, lower panel).

To facilitate the establishment of stable cell lines, a drug selection marker gene was incorporated into the vector. We chose a constitutive promoter (EF1a) to drive the selection drug resistant gene puromycin^R, which has been demonstrated to function well in various mammalian cells. To minimize any non-specific influence of transcription, insulator sequences were implemented, flanking the reporter functional units. Additionally, short transposon-specific inverted terminal repeat sequences (ITRs) were placed on both ends of the core insulator (Fig. 1A). This enables the reporter cassettes between the two ITRs to be easily mobilized into target genomes when transposase expression is utilized. This option offers an important alternative path for the establishment of stable reporter cell lines in a controlled manner [26]. To evaluate background activities, a promoterless reporter was constructed as negative control (Fig. 1B). Similarly, a full-length CMV promoter reporter was constructed as positive control to validate experimental parameters such as transfection protocols (Fig. 1B).

We next determined the function of these reporters in cultured HEK293 cells. As expected, transfection of HEK293 cells with promoterless reporter showed no GFP-positive cells (Fig. 1C). Under the same experimental conditions, a few weakly GFP-positive cells were detected with the minimal CMV promoter reporter. In contrast, more than 80% of cells showed strong GFP expression for the full-length promoter CMV-driven reporter (Fig. 1C). In parallel, while an ~5-fold increase of luciferase activity was observed for the minimal promoter vs. promoterless control, a robust 992-fold increase was detected for the positive control reporter vs. promoterless control (Fig. 1D). These results confirm that the dual reporter functions as predicted.

3.2. The dual-reporter strategy provides a general platform for monitoring signaling pathways

To examine whether this dual-reporter strategy can serve as a general platform for monitoring various signaling pathways, we focused on several well-characterized pathways that play critical roles in cellular function and are regulated by known signaling molecules. We initially chose the inflammatory pathway, which can be activated in response to stimuli such as proinflammatory cytokines (TNFα, IL-1β), and subsequently amplifies the inflammatory cascades. A master mediator, NF-κB, mediates this important pathway. Accordingly, we created an NF-κB pathway reporter by subcloning an 84 bp sequence, including four NF-κB binding sites, upstream of the dual reporter. Similarly, other pathway reporters were built by inserting different TREs to monitor various signaling pathways. In addition to inflammatory pathway, we used this strategy for monitoring signaling pathways related to cell growth and differentiation, apoptosis, cell cycle regulation, pluripotency and cell metabolism (Table 1). Transient transfection results

Table 1
Construction and assessment of dual-reporters for monitoring various signaling pathways in mammalian cells.

Signaling pathway	Transcription factor	Response element	Activation fold-change[a] (inducer)	Cell line
Inflammation (NF-κB)	NF-κB	GGGACTTTCC	80 (TNF α) 42 (IL-1 β)	HEK293
Growth (MAPK)	AP1	GGTGACTCAGT	24 (PMA)	HEK293
Growth and differentiation (PKC)	NFAT	GGAGGAAAAACTG	1192 (PMA) 5068 (PMA + ionomycin)	HEK293
Apoptosis	P53	GGACATGCCCGGGCATGTCC	7 (Nutlin)	Jurkat
Cell cycle regulation	E2F-DP1	TTTCGCGGGAAA	626 (PMA) 1234 (PMA + serum)	HEK293
MYC/MAX Pluripotency	Myc	CACGTG	12 (h-c-Myc cotransfection)	Hep3G
PKA metabolism	CREB	GGTGACGTCA	7.5 (PMA)	HEK293
MAPK Cell division and differentiation	EGR1	GGAAGTCCATATTAGGA	8 (PMA)	HEK293

[a] Luciferase activity of inducer-treated versus untreated transiently transfected cells.

revealed that all signaling pathway reporters were significantly activated when exposed to their specific pathway inducers, as compared to uninduced controls (Table 1). These findings demonstrate the versatility of dual-reporter tools for studying signaling pathways important for cell biology and disease processes.

3.3. Monitoring the specificity of NF-κB reporter in stable cell lines

After monitoring various signaling pathways using transient transfection, we made use of the special design features of our dual reporter construct and established a number of stable cell lines. The genomes of these stable cell lines either harbor the NF-κB dual reporter or a control reporter without TREs as described earlier (Section 2). We next determined the effect of proinflammatory cytokines TNFα and IL-1β on the dual-reporter of NF-κB. As expected, treatment with TNFα resulted in a marked increase (57-fold) in GFP expression, whereas the same dosage of IL-1β resulted in less prominent (13-fold) GFP increase (Fig. 2A). In parallel, a 57- and 14-fold increase in luciferase activity was observed in NF-κB reporter cells treated with TNFα or IL-1β, correspondingly (Fig. 2B). In contrast, no GFP-positive cells were seen in the control reporter cells treated with either TNFα or IL-1β (Fig. 2C), corresponding to the low luciferase activity in the cells that both in the absence or presence of TNFα or IL-1β treatment (Fig. 2D). Because both reporter cell lines harbored the reporter constructs and only differed in the presence or absence of transcription response elements, these results demonstrate the specificity of the dual-reporter system for monitoring transcription factor-mediated pathway signaling.

3.4. Dynamic monitoring and sensitivity of NF-κB-mediated pathway activation

One important advantage of the dual-reporter system is that GFP enables real-time monitoring of pathway activation in living cells, while luciferase allows for easy quantification. To test the capacity of GFP as an effective visual tool to monitor pathway signaling, we next performed a time-course study on NF-κB reporter cells. As shown in Fig. 3A, as early as 3-h after TNFα treatment, weakly GFP positive cells started to appear; with time, the proportion of GFP-positive cells and GFP intensity steadily increased up to 48 h. As predicted, untreated controls showed only few weakly GFP-positive cells, and GFP expression did not increase over time (Fig. 3A, lower panel). In agreement with GFP expression, we found a corresponding time-dependent increase in luciferase activity upon TNFα treatment (Fig. 3B). These results confirm the specificity of the dual

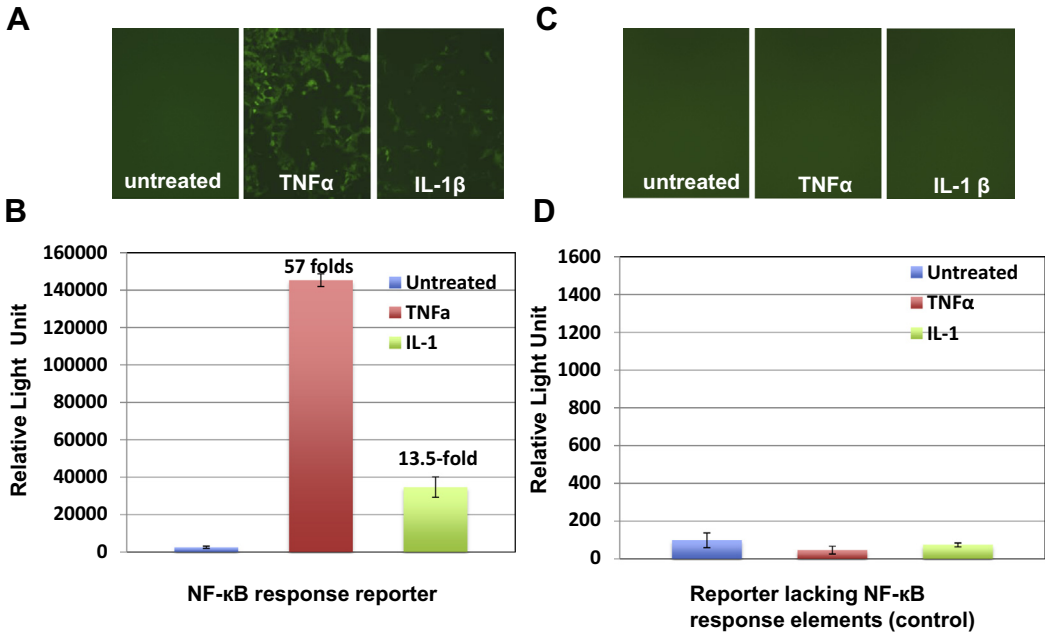

Fig. 2. The specificity of the NF-κB dual-reporter. The specificity of the NF-κB reporter was examined using stable NF-κB reporter cell lines and known NF-κB activators of pro-inflammatory cytokines, TNFα and IL-1β. Cells were treated with proinflammatory cytokine TFNα or IL-1β, or untreated for comparison. 24 h after treatment, GFP expression was recorded (A) and luciferase assays were performed (B), using cell lysates. Cells harboring the dual reporter without NF-κB response elements were treated identically and served as controls, and the GFP expression (C) and luciferase assays (D) were carried out in parallel. The luciferase activities are expressed as relative light units (RLU), normalized against protein input, and presented as fold increase over untreated control (mean ± SD, $n = 3$).

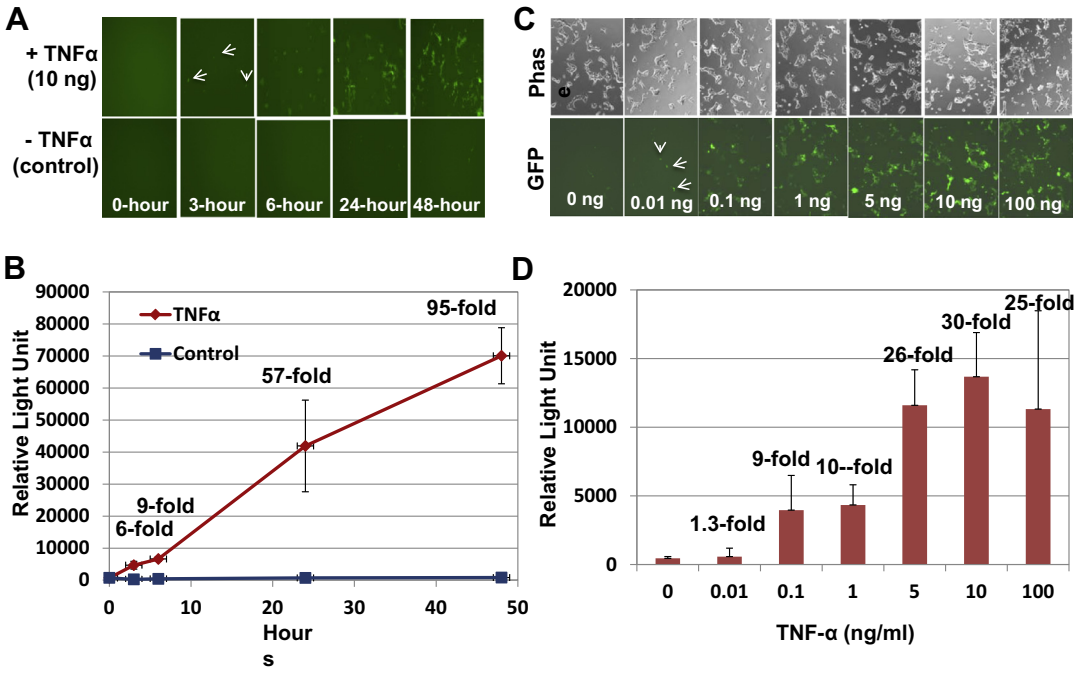

Fig. 3. Time-course and dose–response of NF-κB reporter cells responding to TNFα treatment. The time-dependent and dose-responding activation of TNFα on NF-κB pathway was examined in our NF-κB reporter cells. Cells from a stable NF-κB reporter line were treated with 10 ng/ml TNFα for a period of times (0, 3, 6, 24, 48 h), untreated cells served as negative controls. GFP expression was recorded (A) and luciferase assays were conducted (B). For the dose–response, reporter cells were treated with increasing concentration of TNFα (0, 0.01, 0.1, 1, 5, 10, 100 ng/ml) in 1.5% FBS medium for 24 h, and GFP expression was recorded (C), and luciferase assay were performed using cell lysates (D), expressed as relative light units (RLU), normalized against protein input, and presented as fold increase over controls (mean ± SD, $n = 3$).

reporter system and show its capacity for dynamic monitoring of signaling pathways. Our results demonstrate a strong correlation between the visual assessment by GFP and luciferase quantification.

To examine the sensitivity and dynamic range of the dual reporter, we performed a dose–response experiment. As shown in Fig. 3C, as low as 0.01 ng/ml, TNFα increased the GFP signal (white arrows), and the proportion of GFP-positive cells steadily went up with increasing dosages of TNFα up to 10 ng/ml. Higher dosage (such as 100 ng/ml) did not result in further increase of GFP intensity, indicating the saturation of the TNFα effect (Fig. 3C). Consistent with GFP results, we observed a dose-dependent increase in luciferase activity (Fig. 3D), with a marked 30-fold increase in luciferase activity in cells treated with 30 ng/ml, comparing to the untreated controls (Fig. 3D). The dose–response data are in line with other reporters and relevant to observations during inflammation and sepsis [27]. Together, our results strongly support the notion that the dual reporter can serve as a sensitive tool to dynamically visualize and quantify pathway signaling in mammalian cells.

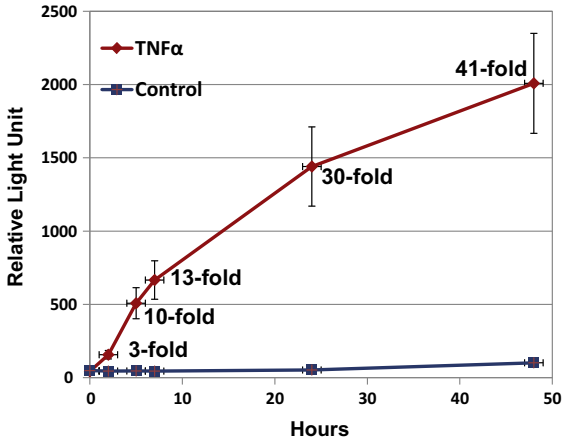

Fig. 4. Live cell luciferase monitoring for high-throughput experimentation. Cells were seeded for 24 h, then switched into low serum DMEM medium (1.5% FBS), in the absence or presence of 10 ng/ml TNFα and 1× D-luciferin substrate. Subsequently luciferase activity was assayed at 0, 2, 5, 7, 24 and 48-h and plotted against the control group without TNFα.

3.5. High-throughput monitoring of inflammatory signaling in HEK293 cells

We next examined whether the dual-reporter system is easily amenable to high-throughput applications. We cultured the reporter cells in a high-throughput format of 96-well plates. By adding the substrate D-luciferin directly into the culture medium, we were able to achieve multiple condition assessment at various time points without the need to prepare cell lysates. As shown in Fig. 4, TNFα treatment resulted in dynamic changes of luciferase activities. The changes observed in live cells in high-throughput 96-well format are similar to those using cell lysates in a low-throughput setting (Fig. 3B). These results demonstrate that the dual-reporter system is easily amenable to high-throughput applications, such as large-scale drug screening in live cells.

Our study demonstrates the potential of dual-reporter tools to characterize signaling pathways important for biology and disease processes. Together, this approach allows researchers to monitor signaling transduction in real-time and to screen drugs in a high-throughput manner, which ultimately may yield new therapeutic candidates.

Conflict of interest

BL declares financial competing interest as SBI employee. AA and CUS declare competing interest as collaborators with SBI.

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