ADVANCING MOLECULAR TOOLS FOR SIGNAL TRANSDUCTION STUDIES IN MAMMALIAN CELLS

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By

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Dr. Maria Gallegos	6/9/2014

ABSTRACT

Despite their fundamental importance, the dynamics of signaling pathways in living cells remain challenging to study, due to the lack of non-invasive tools for temporal assessment of signal transduction in desired cell models. Here we present a dual-reporter strategy that enables researchers to monitor signal transductions in mammalian cells in real-time, both temporally and quantitatively. This is achieved by co-expressing green fluorescent protein (GFP) and firefly luciferase (FLuc) in response to signaling stimuli. To display the versatility of this approach, we constructed and assessed eight unique pathway reporters. To further validate and demonstrate the usefulness of this system, we established stable NFκB pathway reporter cell lines. Using these stable cell lines, we monitored the activity of NFκB-mediated inflammatory pathway in real-time, both visually and quantitatively. In addition, we provide evidence that the dual reporter system is readily amenable to a high-throughput format and compatible with single cell analysis. Luciferase assay has become an increasingly important technique to monitor a wide range of biological processes *in vivo* and *in vitro*. However, the mainstay protocols require an expensive luminometer to acquire and process data, therefore limiting luciferase applications in biological research. To overcome this limitation, we have developed an alternative protocol that utilizes a commonly available cooled charge-coupled device (CCCD) instead of a luminometer for data acquisition and processing. Using a CCCD-based approach, we characterized the substrate specificity, assay linearity, signal to noise levels, and detection of fold-changes of different luciferases (Firefly, Renillar, and

Gaussia). We also defined the assay parameters that are critical for the appropriate use of CCCD for the different luciferases. To demonstrate the usefulness of the proposed method in cultured mammalian cells, we conducted a case study to examine NFκB gene activation during inflammatory signaling pathway in human embryonic cells (HEK293 cells). Data collected via CCCD camera are found to be equivalent to those acquired by a luminometer, thus validating the assay protocol. By comparison, the CCCD-based protocol is more readily amenable to high-throughput applications and offers fast simultaneous data acquisition and alternative image presentation.

The findings presented here demonstrate the power of the dual reporter system, significantly improving the capacity to study signal transduction pathways in mammalian cells. In addition, the CCCD-based protocol provides an important alternative for monitoring luciferase reporters. Because CCCD-based imaging technology is widely available, this approach will enable more researchers to use luciferase for monitoring biological processes.

KEYWORDS

Dual-reporter, signal transduction, GFP, luciferase, transcription factor, NFκB, Firefly luciferase (FLuc), Renillar luciferase (RLuc), Gaussia luciferase (GLuc), luciferase assay, cooled charge coupled device (CCCD), high-throughput (HTP) screening

ABBREVIATIONS

CMV	Cytomegalovirus promoter
mCMV	Minimal CMV promoter
EF1α	Elongation Factor 1-alpha promoter
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HEK 293	Human Embryonic Kidney cell line 293
T2A	Self-cleaving 2A peptide sequence
TF	Transcription factor
ITR	Inverted terminal repeats
Insu	Core insulator sequences
Luc	Luciferase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
TNFα	Tumor necrosis factor alpha
TRE	Transcription factor response element

IL-1β	Interleukin 1 beta
Puro	Puromycin
RLU	Relative light unit

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INTRODUCTION

Signaling pathways regulate cell-specific behaviors that are important for normal development and disease processes (Clevers, 2006; Karin, 2006). 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 (Faris, Gaskin, Parsons, & Fu, 1994; He, Zong, Bernier, & Wang, 2009; Hussain et al., 2012). However, immune-based protocols rely on highly specific antibodies, tend to be laborious to perform (Dutta, Wang, Wu, Mukherjee, & Fishman, 2011), and are not suitable for pathway activations that do not involve phosphorylation (Marino, Galluzzo, & Ascenzi, 2006). Similarly, biochemical analysis of metabolites in a signaling cascade can provide clues to pathway activation (Lewis et al., 2000). However, quantification of the metabolites usually requires rigorous validation, special reagents and equipment (Pandey, Andersen, & Mann, 2000). Both immuno-based and biochemical assays typically use cell lysates, thus limiting their capability to study the dynamics of signal transduction in living cells (Antoon et al., 2011).

High-throughput, image-based cell assays have emerged as alternative approach for monitoring molecular events (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994; Hoffman, 2002; Johnson & Straight, 2013). For example, signaling molecules such as NFκB can be fused with imageable reporters such as GFP or RFP. Migration of GFP from the cytosol to the nucleus reveals NFκB pathway activation (Zhou & Kuo, 1997).

Alternatively, the fluorescent reporter proteins can be placed under the control of appropriate transcription factors to monitor pathway activation (Ferrer-Vaquer et al., 2010). In fact, numerous reporter cell lines have been established for the use of signal transduction monitoring, regulation studies, and compound screening (Apfel, Reischmann, & Müller, 2013; Collins, Torrero, & Franzblau, 1998; Kain et al., 1995; Vilas-Boas, Fior, Swedlow, Storey, & Henrique, 2011). 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 (Hadjantonakis & Nagy, 2000). To overcome this 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 (Connelly, Thomas, & Deiters, 2012; Kato et al., 2009). 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 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. With this approach, we established a number of stable cell lines harboring inflammatory NFκB reporters. We demonstrate the capability of both live visualization and quantification of NFκB pathway activation using cultured human

HEK293 cell models. As signal transduction pathways occur on the cellular level, live visualization has the capacity to reveal individual cell responses, and is compatible with single cell analysis. Furthermore, we provide evidence that the dual reporter system is readily amenable to high-throughput quantification of pathway signaling in living cells.

Bioluminescent proteins have revolutionized our molecular tools and offered us new methods to study and understand biological phenomena (Kremers, Gilbert, Cranfill, Davidson, & Piston, 2011; Sadikot & Blackwell, 2005). In particular, Firefly luciferase (FLuc), Renilla luciferase (RLuc) and Gaussia luciferase (GLuc) have been useful in a variety of fields including immunology (Bartok et al., 2013; Hiramatsu et al., 2006; Sadikot et al., 2001), drug screening (Brock et al., 2008; Galiger et al., 2013), oncology (Chung et al., 2009; Gross & Piwnica-Worms, 2005; Shah, Tang, Breakefield, & Weissleder, 2003), functional genomics (Contag et al., 1997; Hong, Yang, & Cai, 2011; Jiwaji et al., 2010), virology (Burgos, Guzman-Sanchez, Sastre, Fillat, & Valdivieso, 2006; G. D. Luker et al., 2002; K. E. Luker & Luker, 2010), mycology (d'Enfert, Vecchiarelli, & Brown, 2010; Enjalbert et al., 2009; Mosci et al., 2013), and neuroscience (Luo, Lin, Masliah, & Wyss-Coray, 2006; Tang et al., 2003).

Although luminometers are the most common instruments used to measure luciferases activity *in vitro*, they are complicated, relatively expensive and not widely available. Cooled charged-couple device (CCCD) has become a powerful photon detector device with a wide variety of applications from gel documentation to supersensitive biological imaging, including chemiluminescence (*e.g.* luciferase) and fluorescence (*e.g.* GFP) detection (Contag & Bachmann, 2002). In fact, many researchers in life sciences

are already using a CCCD apparatus, such as ChemiDoc™ from Bio-Rad and IVIS from Caliper for *in vivo* bioluminescence imaging (BLI) in model animals (Kim et al., 2010; McLatchie et al., 2013; Wang et al., 2005). The same photon counting functionality of CCCD similar to luminometer provides a promising alternative to monitor and quantify various luciferases *in vitro* (Bhaumik, Lewis, & Gambhir, 2004; Maguire et al., 2013; Sun et al., 2010). In addition to the photon counting function, CCCD may offer some advantages over a luminometer, because of its mode of simultaneous data collection and alternative image presentation. Here, we show that a CCCD-based protocol can be used to quantify activities of the three most popular luciferases (FLuc, RLuc, GLuc) in mammalian cell culture. We establish and validate the critical parameters and procedures for a CCCD-based protocol. In a case study, we demonstrate that the CCCD-based protocol produces results similar to those produced by a luminometer. Taken together, our findings demonstrate the power of dual-reporters for the study of signaling pathways in mammalian cells. In addition we show that CCCD offers a sensitive and convenient alternative to detect, quantify, and visualize luciferase expression.

MATERIALS AND METHODS

Materials and reagents

Human recombinant TNFα, IL-1β, and IL-6 were purchased from R&D Systems (Minneapolis, MN USA). 5X D-luciferin substrate for live cell luciferase activity assay was obtained from System Biosciences (Mountain View, CA, USA). Fetal bovine serum (FBS) was obtained from Atlas Biologicals (Fort Collins, CO, USA).

Cell culture

Human embryonic kidney cells (HEK293) were maintained in high glucose Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FBS (Atlas Biologicals, CO, USA), 2 mM GlutaMax (Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were incubated at 37°C with 95% air and 5% CO_2. At ~80% confluency, cells were washed with 1X PBS (Cellgro, VA, USA) and passaged with 1X trypsin-EDTA (Cellgro, VA, USA).

Design and construction of dual-reporters

The dual reporter system is configured from the 5' to 3' as follows: multiple cloning sites (MCS) to insert different transcription factor response elements (TREs), followed by a minimal CMV promoter, GFP-T2A-firefly-luciferase and a poly A signal as previously reported (Uhde-Stone, Huang, & Lu, 2012). To make the dual-reporters suitable for establishing stable reporter cell lines, a constitutive promoter EF1α-driven

puromycin resistance gene cassette was implemented (Figure 1a). Reporter and selection cassette were flanked by insulator sequences to minimize potential interference of neighboring sequences. In addition, integrase-recognizable insertion sequences were introduced and situated outside the insulator to allow for controlled integration by co-expression of integrase (Mossine, Waters, Hannink, & Mawhinney, 2013). The above dual reporter cassette was subsequently cloned into a regular plasmid with an ampicillin selection marker. In addition, 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 specific TREs were cloned into the MCS. All final constructs were subject to confirmation of double-stranded DNA sequencing.

Generation of stable cell lines

All transfections were performed in 6-well plates seeded with $2x10^5$ 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/without 0.2 μg of transpose expression vector DNA (SBI).

Transient transfection

$1x10^5$ of HEK293 cells/well were plated in 96-well plate in DMEM supplemented with 10% FBS. At ~80% confluency, HEK293 cells were co-transfected with

NFkB+Fluc+GFP (125 ng) and Gaussia+RFP (12.5 ng) constructs at 10:1 ratio using the Purefection transfection reagent according to the user manual (SBI, Mountain View, CA, USA). The amount of plasmid used, were changed accordingly to match the 6-well plate format. Transfection efficiency then was observed using inverted fluorescent microscope, 24 hours after transfection.

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 more passages to avoid potential interference of puromycin.

Drug treatment

$4x10^5$ of HEK293 cells/well were plated in 24-well plate in DMEM supplemented with 10% FBS. At ~80% confluency, cells were treated with DMEM (1.5% FBS) plus TNFα (100 ng/ml), IL-1 (100 ng/ml), LPS1 (100 ng/ml) and LPS6 (100 ng/ml). Drug-mediated luciferase activity was measured 24 hours after treatment by cooled CCD camera and luminometer.

Firefly luciferase activity assay

For the live luciferase reporter assay, NFκB stable cells were seeded in each well of a 96-well plate with a density of $5x10^4$ cells/well. After 24 hour, at ~60~80% confluence, cells were switched to low serum DMEM (1.5% FBS), containing 1X D-luciferin substrate (SBI) in the presence or absence of cytokine. The luciferase activities were recorded at indicated time points using a BioTeck Synergy HT plate reader (BioTek, Winsooki, VT) using a luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI). All data were collected using the same detection setting to allow comparison. Care was taken to keep the plates sterile during the assay, and return them to the incubator immediately after measurements. In cell lysis method, $2x10^5$ cells were seeded in each well of a 24-well plate in completed DMEM with 10% FBS. After 24 hours and at ~60-80% confluency, the medium was switched to DMEM with 1.5% FBS, in the absence or presence of TFNα or IL-1β. 24 hours later, cells were lysed with Passive Lysis Buffer (PLB)(Promega, USA), cell lysates were cleared by 2 minutes centrifugation at highest speed, and then activity was recorded by adding 100 µl of substrate to 10 µl of supernatant using Luciferase assay system kit (Promega, USA). The resulting relative light units (RLU) were normalized against total protein concentration determined by NanoDrop Lite (Thermo Scientific, Wilmington, DE, USA). In linearity experiments, serially diluted samples were prepared at 1:2 ratio from cell lysate with total protein concentration of 2 µg/µl (20 µl). Passive Lysis Buffer (PLB) was added to make the dilutions.

Gaussia luciferase activity assay

20 µl of cell-free conditioned media were collected as a source for secretory Gaussia luciferase (Wurdinger et al., 2008). 100 µl Coelenterazine (CTZ)(SBI, Mountain View, USA) substrate was added to 5 µl of conditoned media and chemiluminescence was recorded immediately after adding the substrate. In linearity experiments, serially diluted Gaussia samples were prepared from 10 µl of conditioned media at 1:2 ratio. DMEM (10% FBS) were added to make the dilutions Firefly luciferase activity was normalized by GLuc activity data.

Data collection & presentation

For live cell monitoring, cultured cells were monitored at indicated time points under a LEICA DMI3000B fluorescent microscope, and processed with LAS 3.8 software. GFP live cell images were taken using the same exposure condition and magnification within the group of comparison. For the luciferase reporter assay, all data are presented as mean ± S.D. (n = 3), unless stated otherwise. Luciferase activity was measured with either ChemiDoc XRS^{+} cooled CCD camera (BioRAD, USA) or TR 717 Microplate Luminometer (Applied Biosystems, USA). Pictures taken by cooled CCD camera were further analyzed by Image Lab software (version 3.0). Different concentrations of 95% pure luciferases (NanoLight Technologies, AZ, USA) were prepared by serial dilution in PBS. In CCD-based GLuc activity assay, CTZ substrate was added by multichannel pipette in less then 10 seconds. In live-cell luciferase activity assay, substrate was added manually along with changing the media to DMEM (1.5%

FBS). In luminometer-based cell lysate luciferase assay, substrate was injected to each and every well right before light detection by the instrument. Data were expressed as the mean ± SD (n=6), unless stated otherwise.

RESULTS

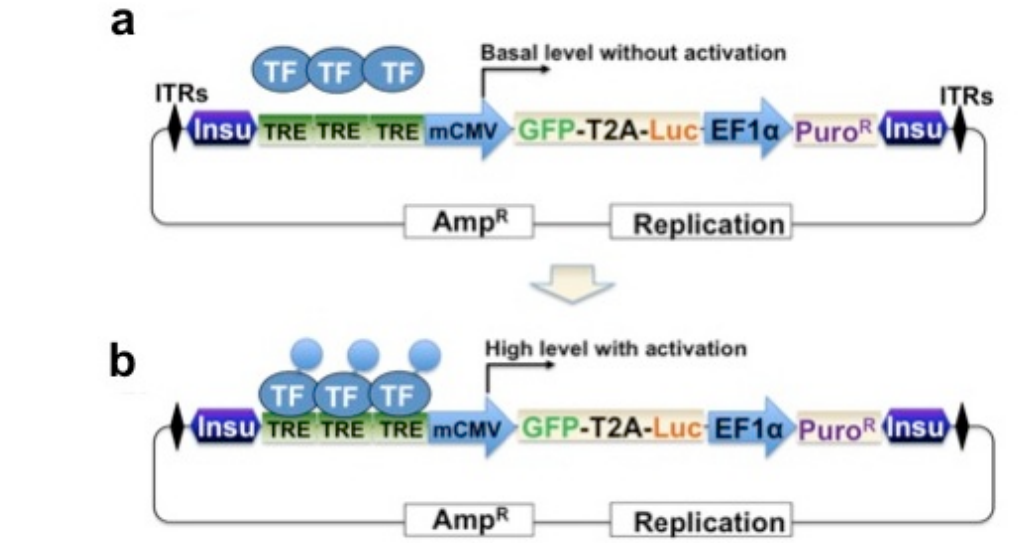
Design and construction of dual-reporters suitable for the establishment of stable cell lines

We designed a general strategy to monitor cellular signaling pathways by placing GFP and luciferase genes under the control of specific transcription factors. To facilitate monitoring the activity of a transcription factor, a tandem of transcription factor response elements (TREs) was placed immediately upstream of the mCMV promoter. Thus, transcription factor binding sites are optimally located within 300 bp upstream of the transcription start site. Activation by the specific transcription factor will result in the expression of both GFP and luciferase. 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). In response to environmental stimuli, the corresponding transcription factors become activated and bind to the TREs situated at the dual reporter promoter region, resulting in high-level expression of both reporters (Fig. 1b).

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. In addition, 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 (Li et al., 2013). To evaluate background activities, a promoterless reporter was constructed as negative control. Similarly, a full-length CMV promoter reporter was constructed as positive control to validate experimental parameters such as transfection protocols (Fig. 1c).

We next determined the function of these reporters in cultured human embryonic kidney cells (HEK293). As expected, transfection of HEK293 cells with promoterless reporter showed no GFP-positive cells. 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. 1d). In parallel, ~5-fold increase of luciferase activity was observed for the minimal promoter vs. promoterless control, whereas a robust 992-fold increase was detected for the positive control reporter vs. promoterless control (Fig. 1e). These results confirm that the dual reporter functions as intended.


a
TF TF TF
Basal level without activation
ITRs
Insu TRE TRE TRE mCMV GFP-T2A-Luc EF1α Puro^R Insu
ITRs
Amp^R
Replication
b
High level with activation
TF TF TF
Insu TRE TRE TRE mCMV GFP-T2A-Luc EF1α Puro^R Insu
Amp^R
Replication


c
Promoterless GFP-T2A-Luc EF1α Puro^R
MCS mCMV GFP-T2A-Luc EF1α Puro^R
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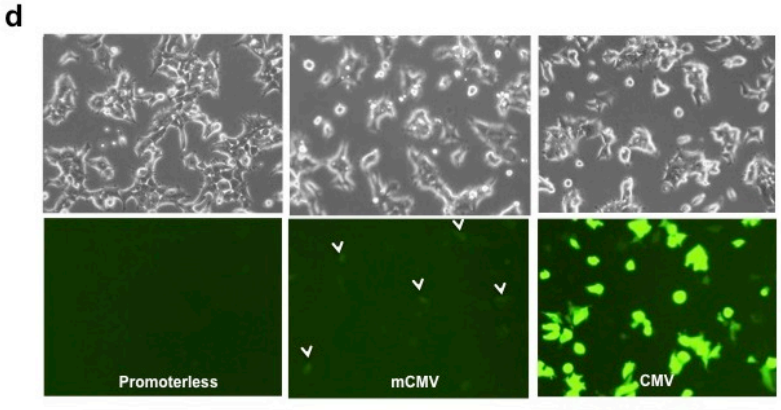
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Promoterless
mCMV
CMV

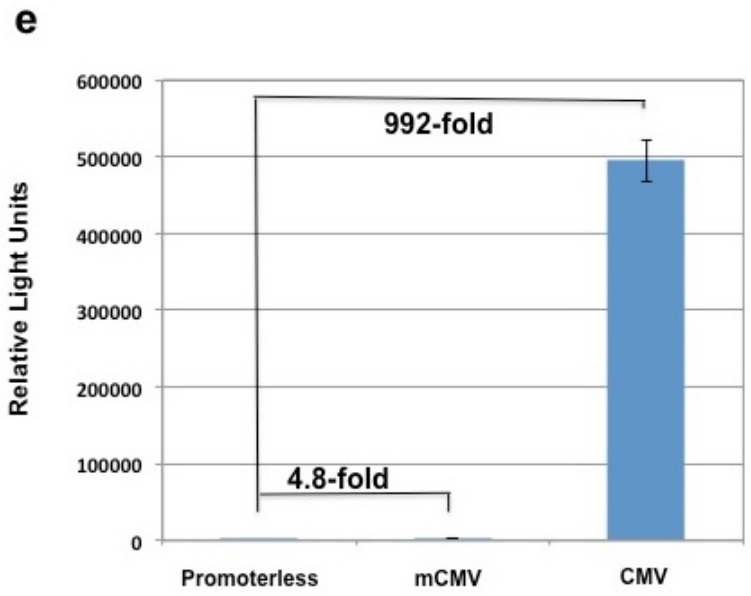


Figure 1. A dual reporter strategy to visualize and quantify signaling pathway activation. (**a**) Schematic representation of main features of the dual reporter system. Without stimuli, the transcription factor (TF) is inactivated and does not bind to TREs, therefore basal levels of expression of both GFP and Luc are expected. (**b**) Upon stimuli, the TF becomes activated and binds to TREs, resulting in high levels of expression. (**c**) Configuration and construction of the promoterless negative control reporter, mCMV-driven basal reporter, and full-length CMV promoter-driven positive control reporter. (**d**) HEK293 cells transfected with the promoterless negative control reporter showed no GFP expression, while the mCMV-driven reporter displayed few weakly GFP-positive cells. The positive control reporter showed strong GFP expression in most cells. (**e**) Relative luciferase activities of each reporter were displayed in relative light units. Error bars indicate ± SD, n = 3.

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

To assess if this dual-reporter strategy can serve as a general platform for monitoring various signaling pathways, we focused on several pathways that play critical roles in cellular function and are regulated by known signaling molecules. We created an NFκB pathway reporter by subcloning an 84-base sequence including four NFκB binding

sites. Similarly, other pathway reporters were built by inserting different TREs to monitor various signaling pathway involved in cell growth and differentiation, programmed cell death, cell cycle regulation, pluripotency status of stem cells, metabolism, and cell division and differentiation (Table 1). Transient transfection experiments confirmed that all pathway reporters were significantly activated when exposed to their specific pathway inducers, compared to uninduced cells (Table 1). These findings demonstrate the versatility of dual-reporter tools for the study of signaling pathways important for cell biology and disease processes.

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

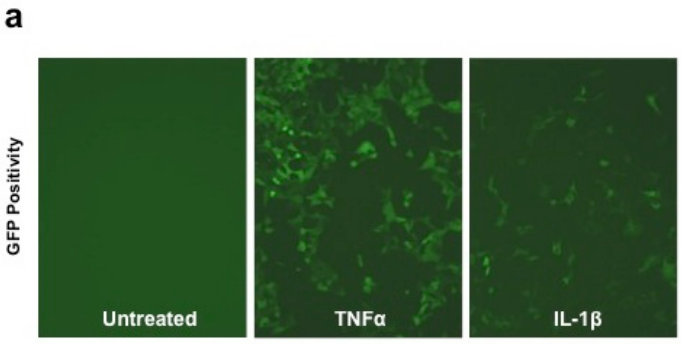
Signaling Pathway	**Transcription factor**	**Response element**	**Activation fold-change* (Inducer)**	**Cell line**
Inflammation (NFκB)	NFκB	GGGACTTTCC	80 (TNF α) 42 (IL-1 β)	HEK293
Growth and differentiation (MAPK)	AP1	GGTGACTCAGT	24 (PMA)	HEK293
Growth and differentiation (PKC)	NFAT	GGAGGAAAAAC TG	1192 (PMA) 5068 (PMA + Ionomycin)	HEK293
Apoptosis	P53	GGACATGCCCGG GCATGTCC	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	GGAAGTCCATAT TAGGA	8 (PMA)	HEK293

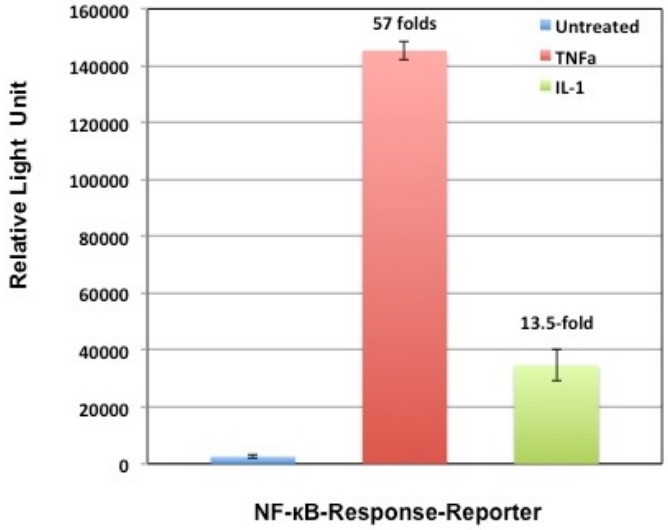
*Luciferase activity of inducer-treated versus uninduced transiently transfected cells

Monitoring of NFκB reporter in stable cell lines demonstrates specificity

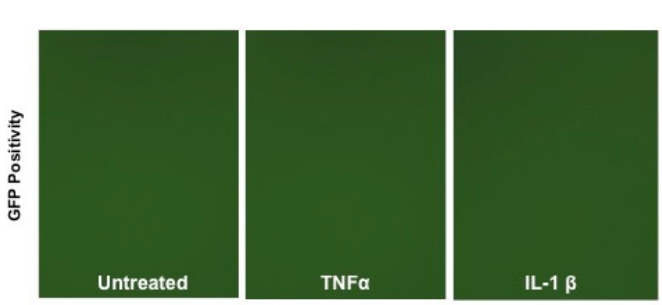
We next determined the effect of proinflammatory cytokines TNFα and IL-1β on NFκB stable reporter cell lines. As expected, treatment with TNFα resulted in a marked increase in GFP expression, whereas the same dosage of IL-1β resulted in less prominent GFP increase (Fig. 2a). Correspondingly, a 57- or 14-fold increase in luciferase activity was observed in NFκB reporter cells treated with TNFα or IL-1β (Fig. 2b). In contrast, no GFP-positive cells were seen in the control reporter cells treated with either TNFα or IL-1β (Fig. 2c) and luciferase activity remained low, both in absence and presence of TNFα or IL-1β (Fig. 2d). Because both reporter cell lines harbored the reporter constructs and only differed in the presence or absence of transcription response elements, these data demonstrate the specificity of the dual-reporter system for monitoring transcription factor-mediated pathway signaling.



b


160000
140000
120000
100000
80000
60000
40000
20000
0
Relative Light Unit
57 folds
13.5-fold
Untreated
TNFa
IL-1
NF-κB-Response-Reporter

c


GFP Positivity
Untreated
TNFα
IL-1 β

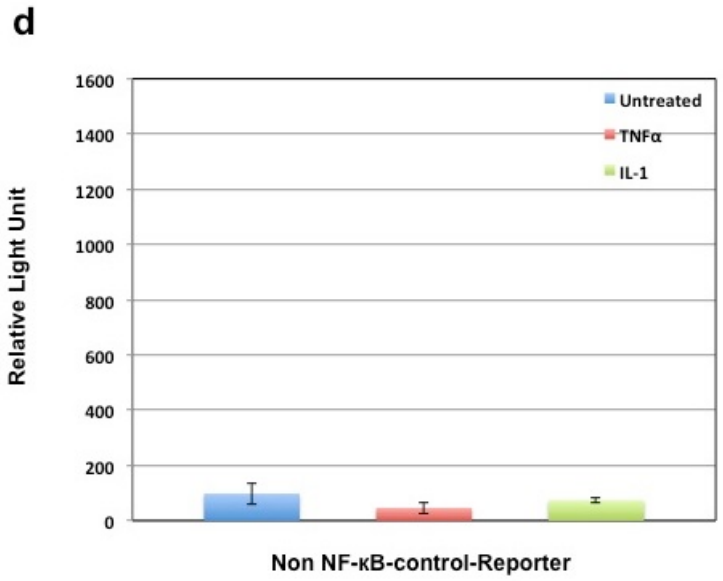


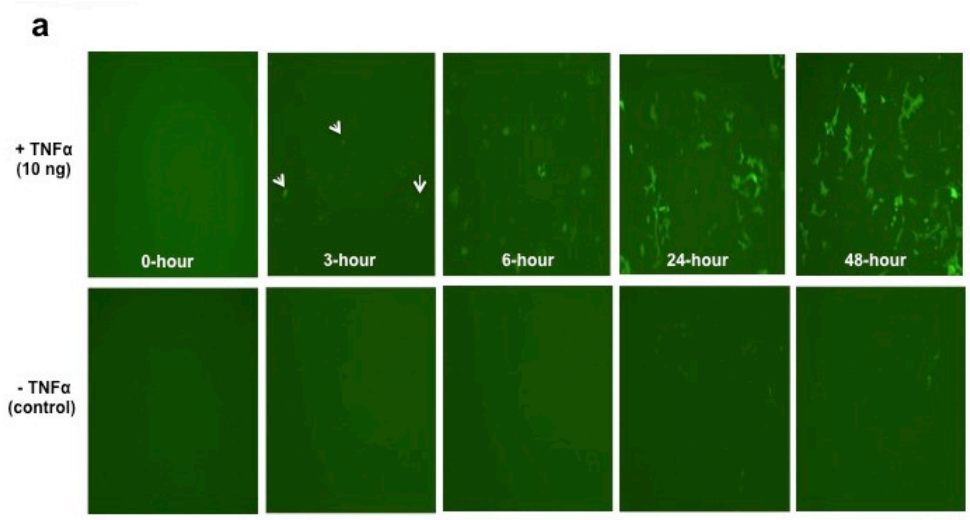
Figure 2. Specificity of the NFκB dual-reporter. The specificity of the NFκB reporter was tested using stable NFκB reporter cell lines and known NFκB activators of pro-inflammatory cytokines, TNFα and IL-1β. 24 hours after treatment, GFP expression was recorded (**a**) and luciferase assays were conducted using cell lysates (**b**). Cells harboring the dual reporter without NFκB response elements were treated identically and served as controls; the GFP expression analysis (**c**) and luciferase assays (**d**) were carried out in parallel. The luciferase activities are presented as relative light units (RLU), normalized against protein input. Data were shown as mean ± SD, n=3.

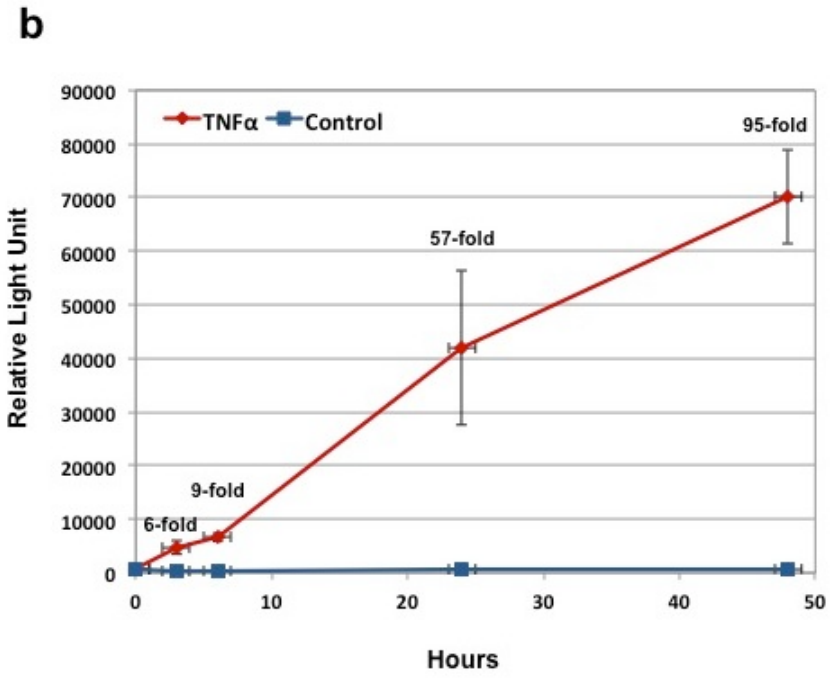
Dynamic monitoring and sensitivity of NFκB-mediated pathway activation

To test the capacity of GFP as an effective visual tool to monitor pathway signaling, we conducted a time-course study on NFκB reporter cells. As shown in Figure 3a, at the 3-hour time point after TNFα treatment, weakly GFP positive cells started to appear and both the proportion of GFP-positive cells and GFP intensity increased with time up to 48 hours. Under the same experimental conditions, untreated controls showed only few weakly GFP-positive cells that remained at low levels similar to the 0-hour time point (Fig. 3a). In agreement with GFP expression, we found a corresponding increase in

luciferase activities with time of TNFα treatment (Fig. 3b). These results confirm the specificity of the dual reporter system and show its capacity for dynamic monitoring of signaling pathways. The data demonstrates a strong correlation between the visual assessment by GFP and luciferase quantification as well.

To further examine the sensitivity and dynamic range of this dual reporter, we performed a dose-response experiment. TNFα dosage as low as 0.01ng/ml marginally increased the GFP signal (white arrows), and the proportion of GFP-positive cells increased with increasing dosages of TNFα up to 10 ng/ml. At this concentration, the activation effects appeared to be saturated, because a maximum dosage of 100 ng/ml did not result in further increase of GFP intensity (Fig. 3c). Consistent with GFP results, we observed a dose-dependent increase in luciferase activity (Fig. 3d). The lowest dose of 0.01 ng/ml TNFα resulted in a marginal 1.3-fold increase compared to the untreated controls, while the 10 ng/ml dosage resulted in a marked 30-fold increase in luciferase activity. The dose-response data are in line with other reporters and relevant to observations during inflammation and sepsis (Lu, Moser, Shigenaga, Grunfeld, & Feingold, 2010). Together, these data strongly support the notion that the dual reporter can serve as a sensitive tool to dynamically visualize and quantify pathway signaling in mammalian cells.


a
+ TNFα
(10 ng)
- TNFα
(control)
0-hour
3-hour
6-hour
24-hour
48-hour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TNFα
Control
95-fold
57-fold
9-fold
6-fold
Relative Light Unit
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Hours

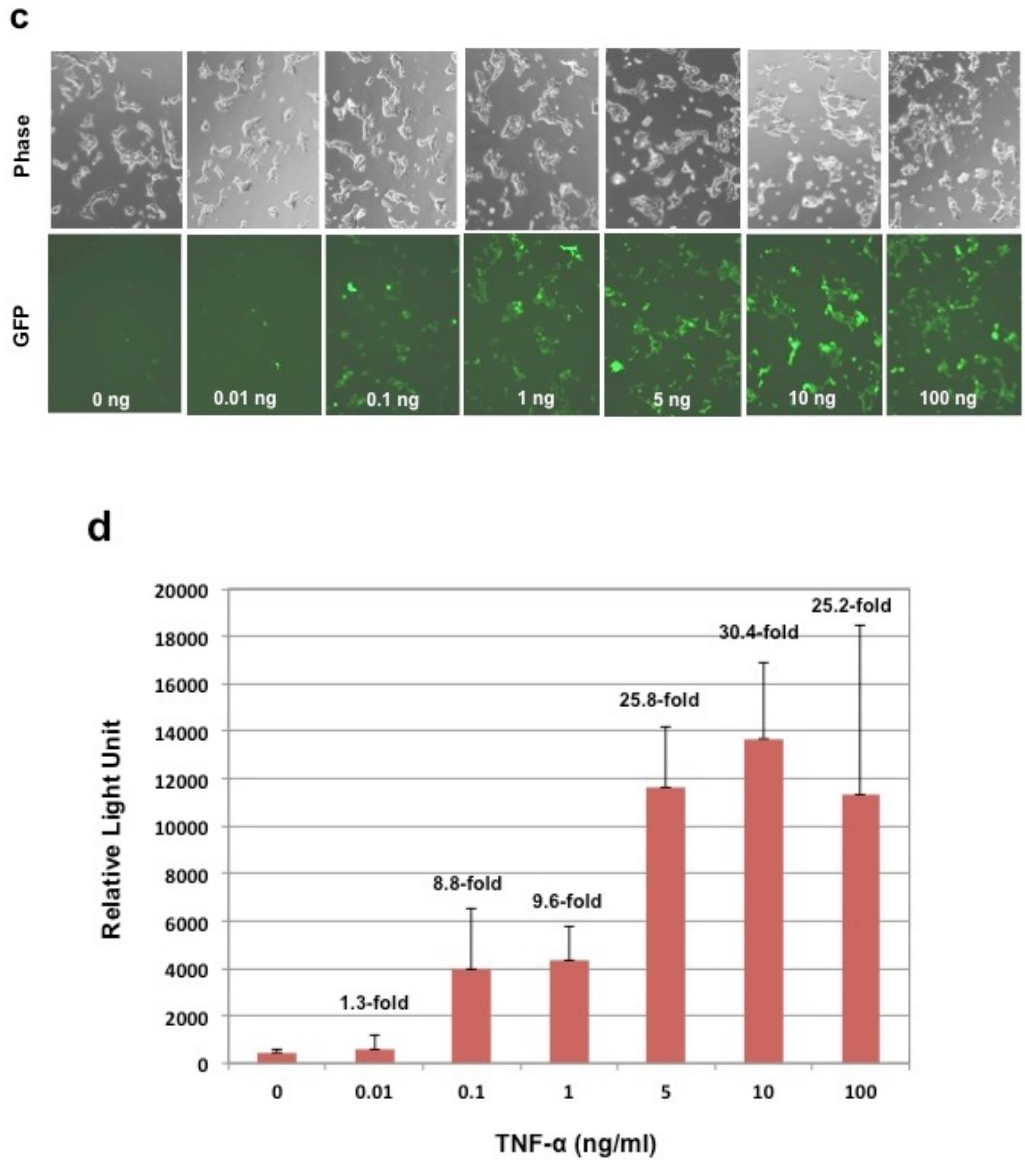


Figure 3. Time-course and dose-response studies on NFκB reporter cells. The activation effect of TNFα on NFκB pathway was analyzed over time or against increased dosage of TNFα. Reporter cells were treated with 10 ng/ml of TNFα over a period of 48 hours. Cells incubated in 1.5% FBS-supplemented DMEM without TFNα were used as negative controls. GFP expression was recorded at the indicated time-points (**a**) and luciferase assay were conducted at the same time-points (**b**). For the study of dose-response, reporter cells were treated with increasing concentration of TNFα (0, 0.01, 0.1, 1, 5, 10 and 100 ng/ml) in 1.5% FBS-supplemented culture medium. After 24-hours, the GFP intensity was recorded (**c**), and luciferase assay were performed using cell lysates (**d**). Data were shown as mean ± SD, n=3.

High-throughput monitoring of inflammatory signaling in HEK293 cells

We next considered whether the dual-reporter system is easily amenable to high-throughput applications. To this end, we cultured the reporter cells in a high-throughput format of 96-wells. By adding the substrate D-luciferin directly into the culture medium, we were able to achieve multiple condition assessment at multiple time points without the need to prepare cell lysates. As shown in Figure 4, TNFα treatment resulted in dynamic changes of luciferase activities. The changes observed in live cells in high-throughput 96-well 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.

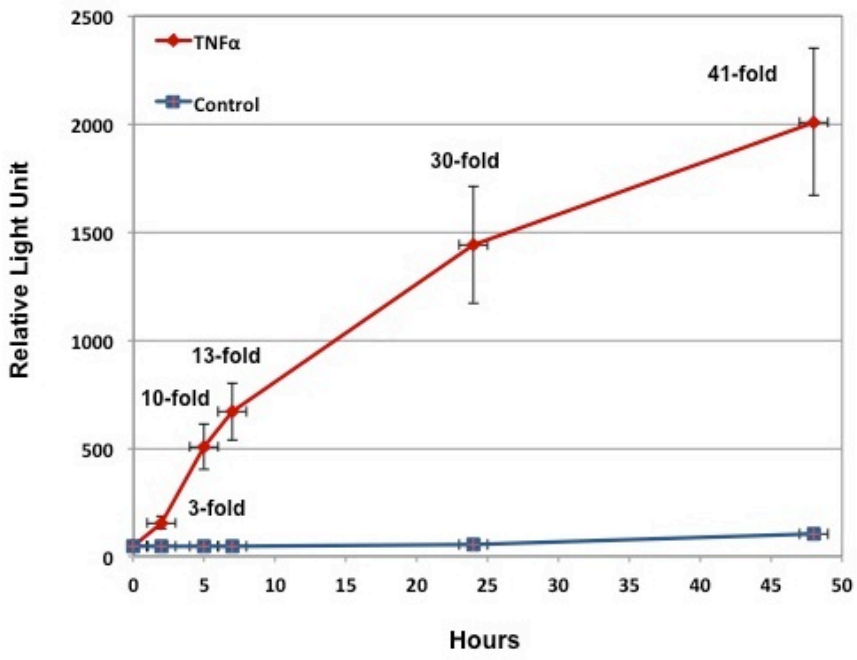


Figure 4. Live cell luciferase monitoring allows for high-throughput experimentations. At ~60~80% confluency, complete DMEM was switched into low

serum DMEM medium supplemented with 1.5% FBS in the absence or presence of 10 ng/ml TNFα and 1X D-luciferin substrate. Luciferase activity of live cells was assayed at 0, 2, 5, 7, 24 and 48-hour time-points and plotted against the control group lacking TNFα.

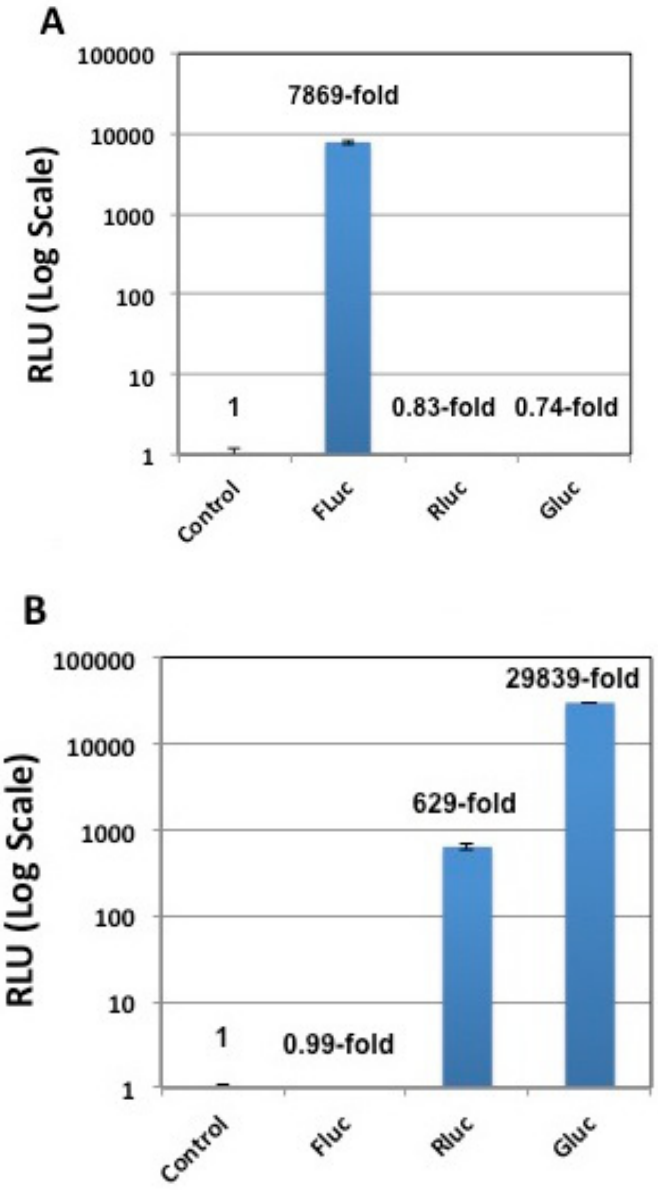
Substrate specificity of Firefly, Renilla, and Gaussia luciferases

We were interested in testing the feasibility of using more than one luciferase reporter simultaneously, *e.g.* one for gene expression monitoring, and the other for normalization. In order to assess the specificity of different luciferases, we investigated the activity of three different commercially available recombinant luciferases, FLuc, RLuc and GLuc (NanoLight Technologies, AZ, USA), on D-luciferin and CTZ substrates one at a time. 100 µl of two different substrates were added to 10 µl of pure FLuc, RLuc and GLuc (5 ng/µl in PBS) and the activity was measured by luminometer. We did not detect any activity of Renilla/Gaussia luciferases on FLuc substrate (Fig. 5a) and vice versa (Fig. 5b). This lack of cross-reaction between the substrates for FLuc and RLuc/GLuc shows that activity of both groups can be monitored individually.

Stability of FLuc, RLuc and GLuc activity

Next, we assessed the chemiluminescent stability of FLuc, RLuc and GLuc over the particular time frame. We added 100 µl of substrate to 10 µl of pure enzymes (50 ng/µl in PBS) and recorded luciferase activity for a total period of 1800 seconds (0, 30, 60, 120, 300, 600, 900 and 1800 seconds). We found that FLuc and RLuc signals remained strong for about two minutes after adding substrate, then dropped gradually by 95% over the course of 30 minutes. GLuc signals rapidly declined about 50% within two

minutes after adding substrate and by 95% over the course of 15 minutes (Fig. 5c). The graphical representation of CCCD pictures showed the same trend (Fig. 5d).



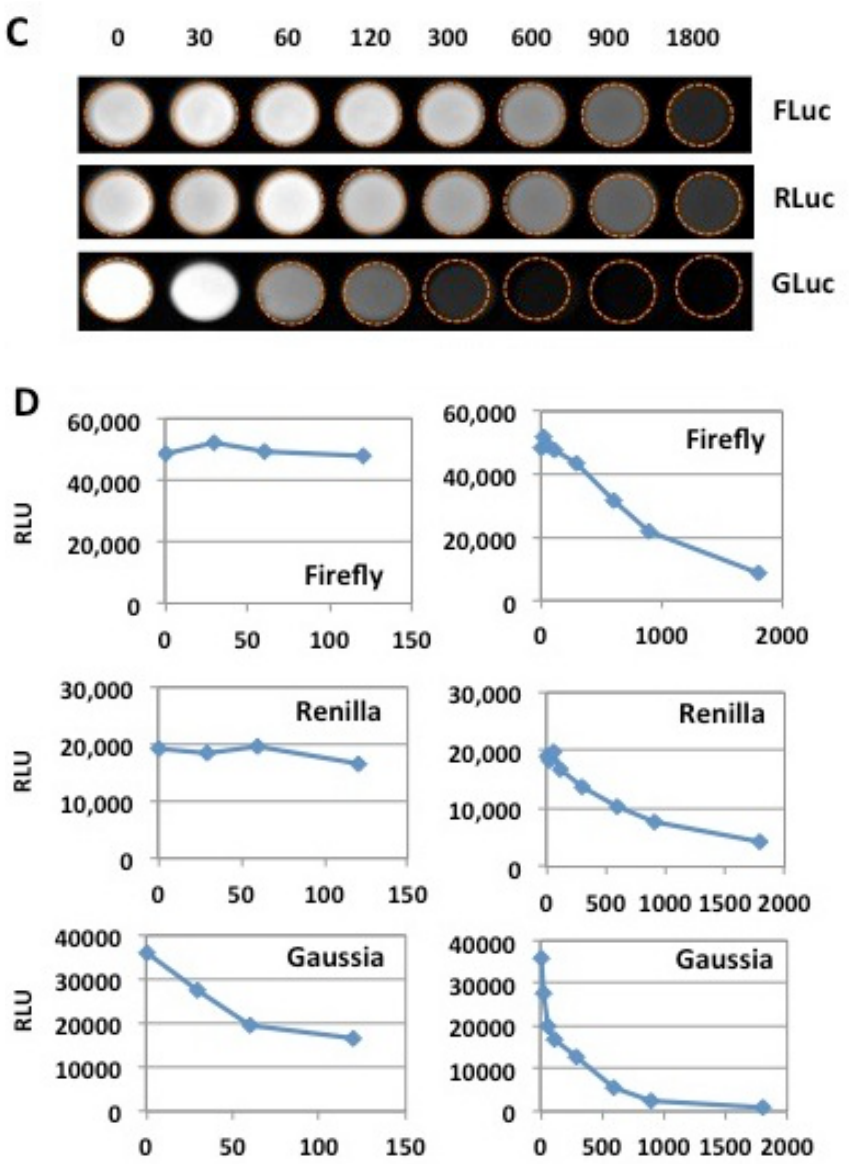
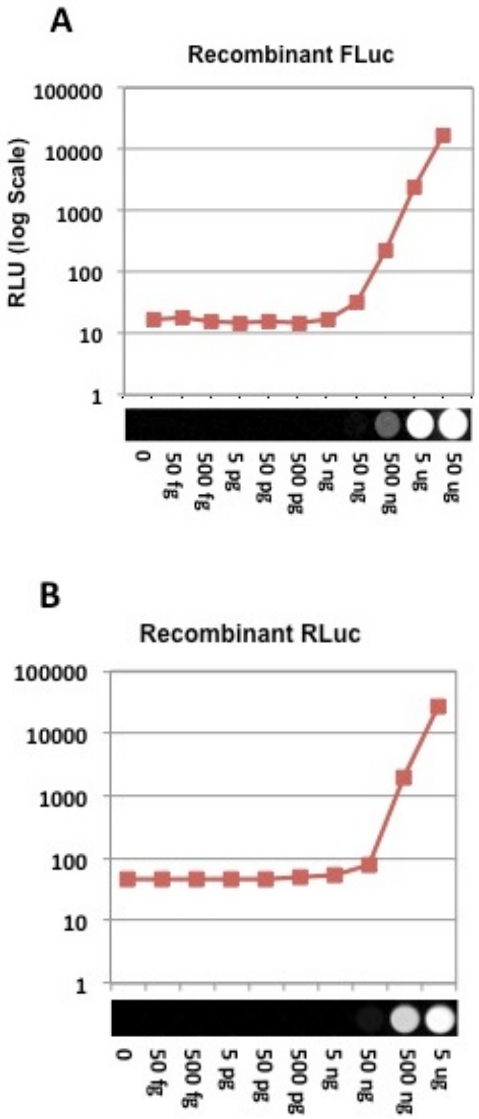


Fig. 5. Luciferases specificity and compatibility. (**a**) Firefly substrate (D-luciferin), and (**b**) Renilla/Gaussia substrate (Coelenterazine, CTZ), were added to pure recombinant luciferase proteins and luciferase activities were quantified by luminometer. Proteins were dissolved in PBS at the same concentration in all experiments; PBS was used as control. Decay rate for three different luciferases were monitored by CCCD camera for 30 minutes (1800 seconds). Substrates were added at different time points, starting from the last one. Luciferase activity was quantified by (**c**) CCCD camera and (**d**) by luminometer at two different time frames (150 and 1800 seconds). Data are expressed as mean ± SD of 2 independent experiments (n=6).

CCCD camera sensitivity in photon detection

We next determined the sensitivity of CCCD camera in detecting different ranges of signals. We assayed luciferase activity of three recombinant luciferases, FLuc, RLuc and GLuc, ranging in concentration from 0-50 µg protein (Fig. 6a, b and c). The CCCD limit of detection was 500 ng for FLuc and RLuc, and 50 ng for GLuc.



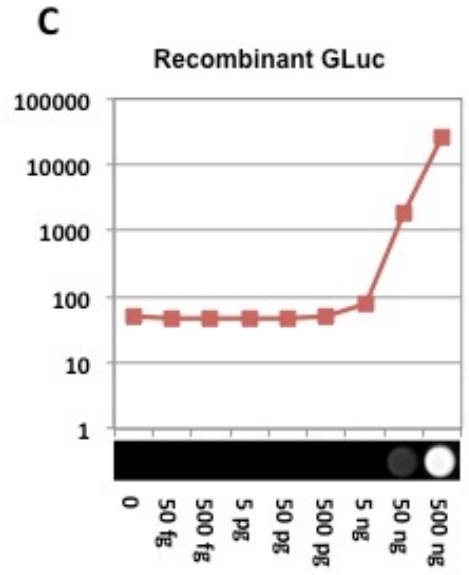


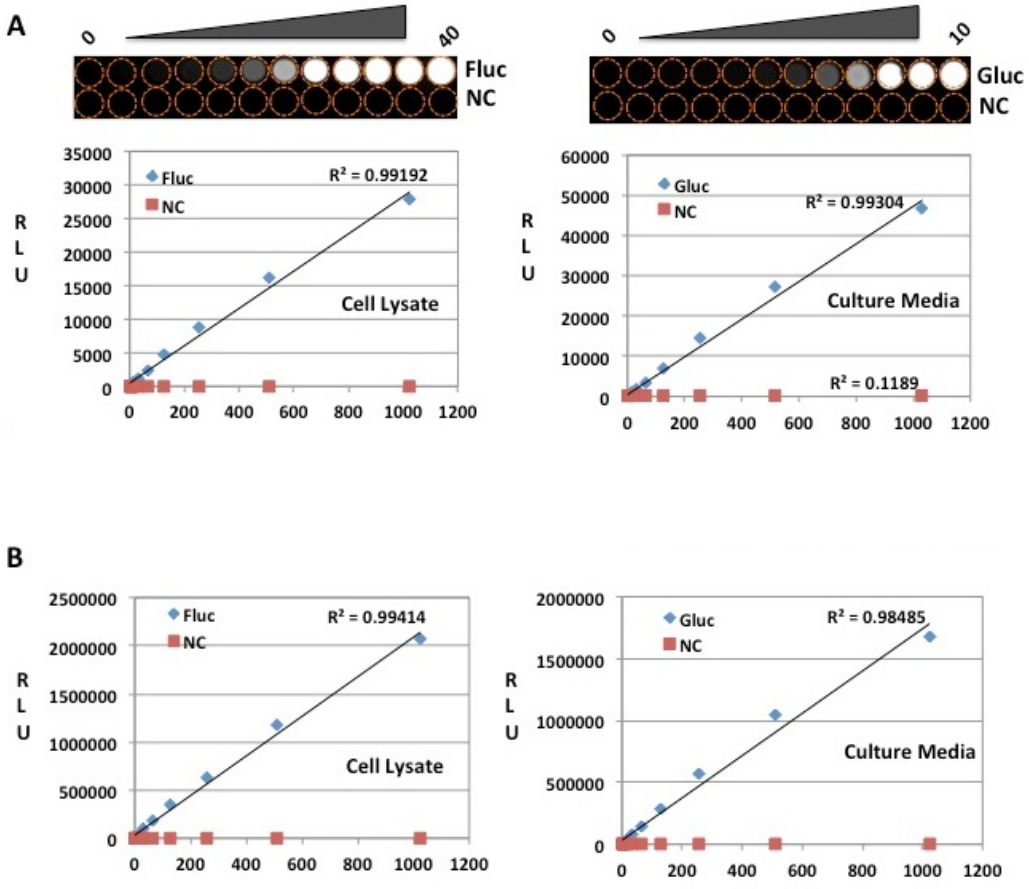
Fig 6. Cooled CCD-based assay sensitivity. Relative luciferase activity of (**a**) Firefly, (**b**) Renilla and (**c**) Gaussia were captured by cooled CCD camera and plotted against recombinant enzyme concentrations. Protein samples were prepared by serial dilution at 1:10 ratio.

CCCD reading shows linear relationship between enzyme concentration and image acquisition time

To further evaluate the reliability of CCCD camera, we examined linearity of acquired data based on two critical parameters: enzyme concentration, and exposure time. In both experiments, we compared the ability to quantify luciferase activity of CCCD camera and luminometer.

To test if luciferase activity correlates with luciferase protein concentration, we transiently transfected HEK293 cells with FLuc-GFP and GLuc-RFP constructs. 48 hours after transfection, conditioned media was taken, then cells were lysed and total protein concentration was measured. Serially diluted samples at 1:2 ratio were prepared with 20 µl of cell lysate and 10 µl of conditioned media, and were assayed for FLuc and GLuc

activity respectively by CCCD camera (Fig. 7a) and luminometer (Fig. 7b). We next tested the effects of various image acquisition times on luciferase activity measurement in CCCD-based assay. We used different concentration of FLuc as described above, but captured the signal with 2, 5, 15 and 20 seconds exposure times (Fig. 7c), and plotted the results after analyzing them with Image Lab software. We found a linear relationship between signals detected by CCCD and both, luciferase concentration and exposure time. Higher luciferase concentration or longer image acquisition time results in higher RLU readings.



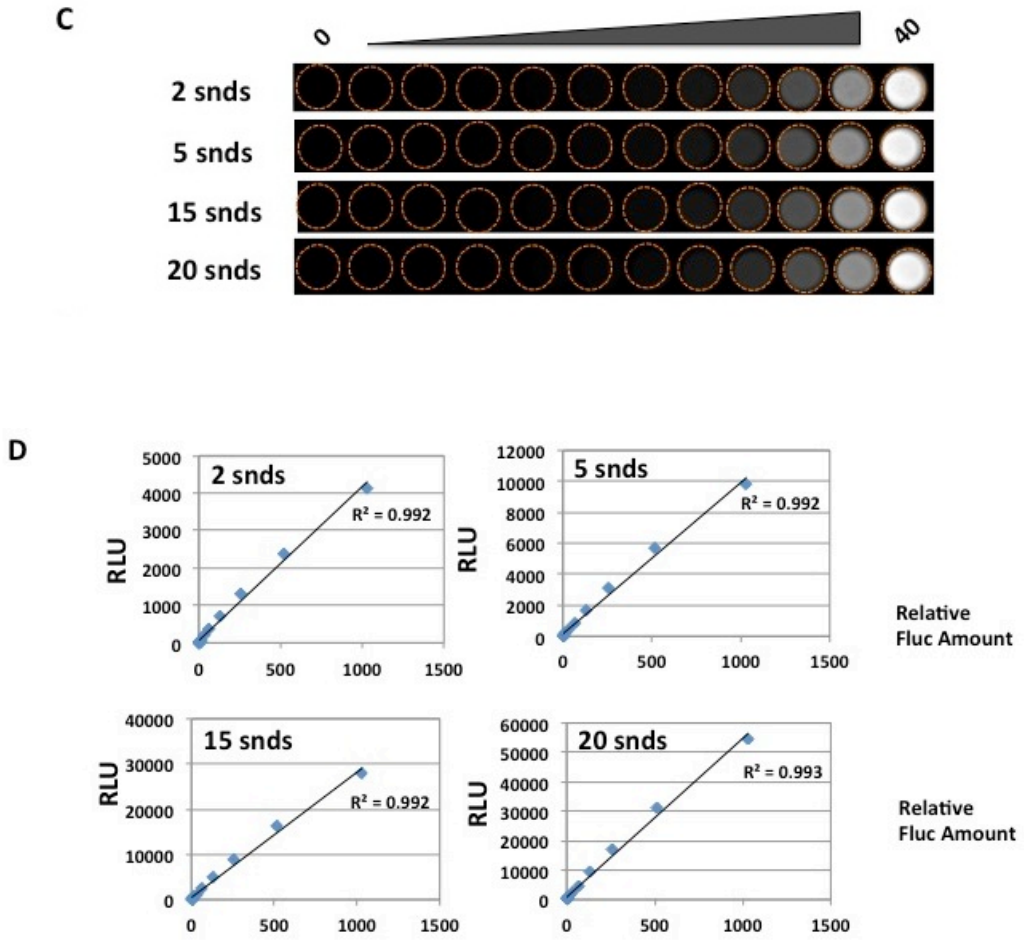


Fig. 7. Assay linearity. FLuc activity in cell lysate and GLuc activity in cell free conditioned media with different enzyme concentration were measured by (**a**) CCCD camera and (**b**) luminometer and plotted against negative control (NC). Firefly luciferase activity in samples with different enzyme concentration was measured with four different acquisition times. (**c**) CCCD camera pictures taken with 2, 5, 15 and 20 second exposure times. (**d**) Plotted results based on analysis of the acquired pictures with Image Lab software (version 3.0).

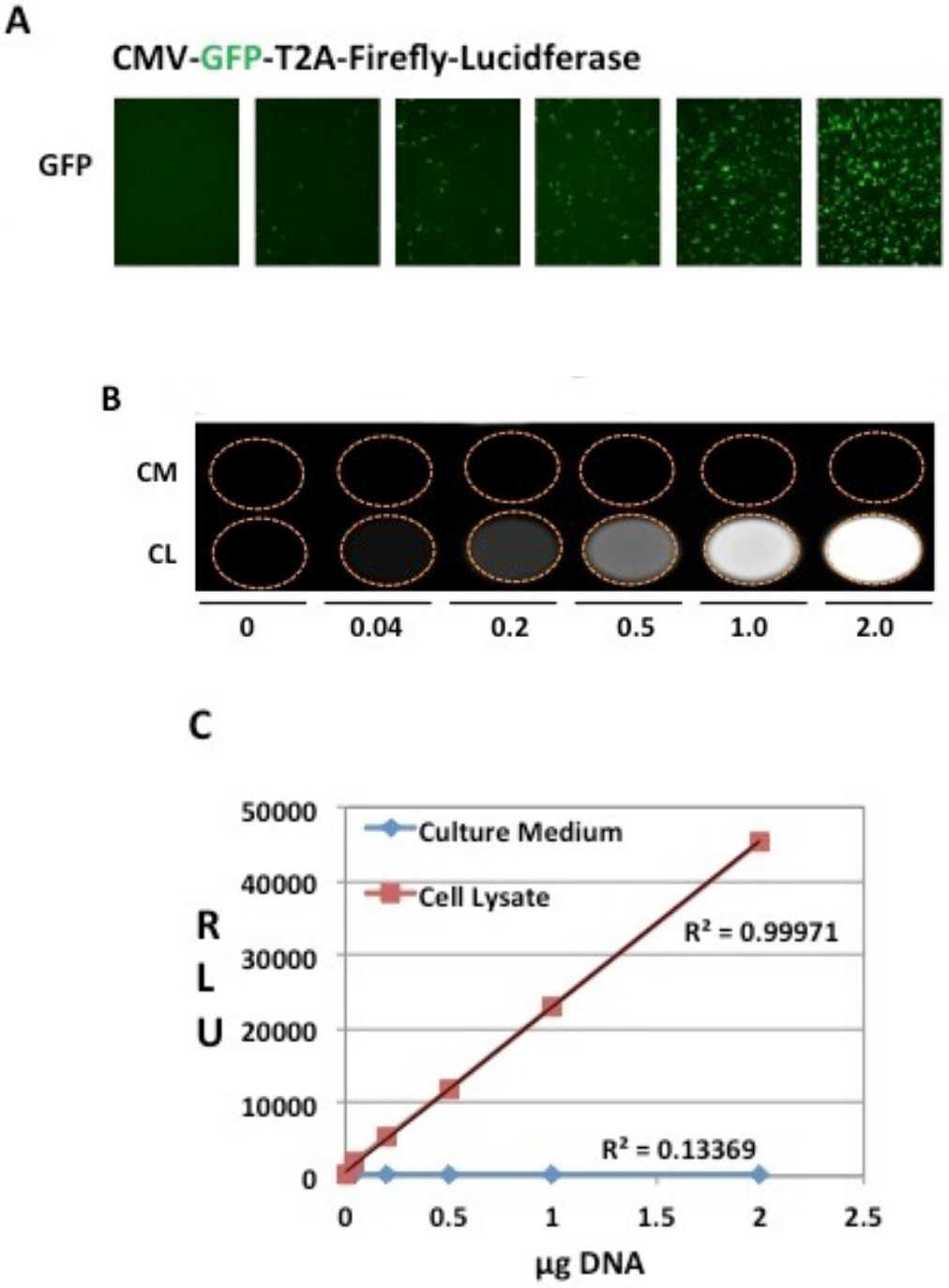
CCCD-based luciferase assay is ideal for gene regulation studies

Next, to evaluate the direct relation between plasmid expression level and luciferase activity, we transfected HEK293 cells with various amounts of two different

plasmids, CMV-GFP-FLuc and CMV-GLuc-RFP. In order to have optimal expression of reporters, we chose CMV promoter, which is highly functional in different mammalian cells. In cells transfected with FLuc-GFP, (Fig. 8a) GFP expression (Fig 8b) and CCCD-based luciferase activity assay (Fig 8c), confirmed visually and quantitatively the differences in gene expression level due to increasing amount of plasmid. Cells transfected with increasing concentrations of RFP-GLuc, showed the same trend of increased RFP expression (Fig 8d), and visual and quantitative luciferase activity (Fig 8e and f). Our results demonstrated a linear relationship between plasmid DNA input, luciferase activity and fluorescent reporter gene expression.

CCCD camera can produce quantitative data

We next investigated the potential of CCCD camera in quantifying the levels of luciferase activity and compared the results to the data acquired by luminometer. We co-transfected HEK293 cells with NFκB and Gaussia luciferase reporters. 24 hours post-transfection, chemiluminescence produced by FLuc and GLuc was assessed by both CCCD camera (Fig. 8g) and luminometer (Fig. 8h). To graph the pictures obtained by CCCD camera, we analyzed them with Image Lab software (version 3.0). Both CCCD and luminometer showed similar results in capturing the signals.


A
CMV-GFP-T2A-Firefly-Lucidferase
GFP
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40000
30000
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10000
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Culture Medium
Cell Lysate
R² = 0.99971
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1.5
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D
CMV-Gaussia-Luciferase-EF1-RFP-T2A-Puromycin
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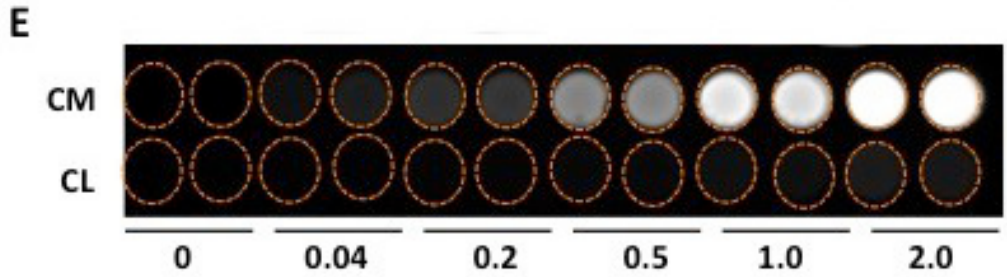
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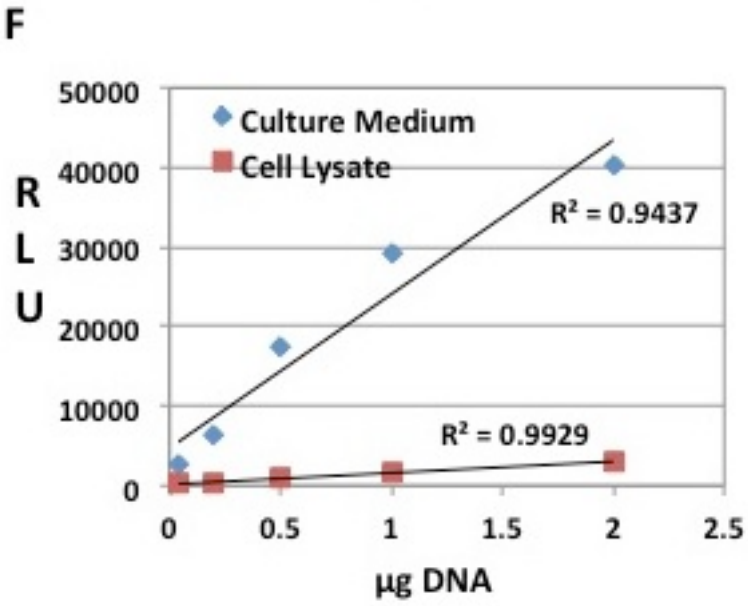
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RLU
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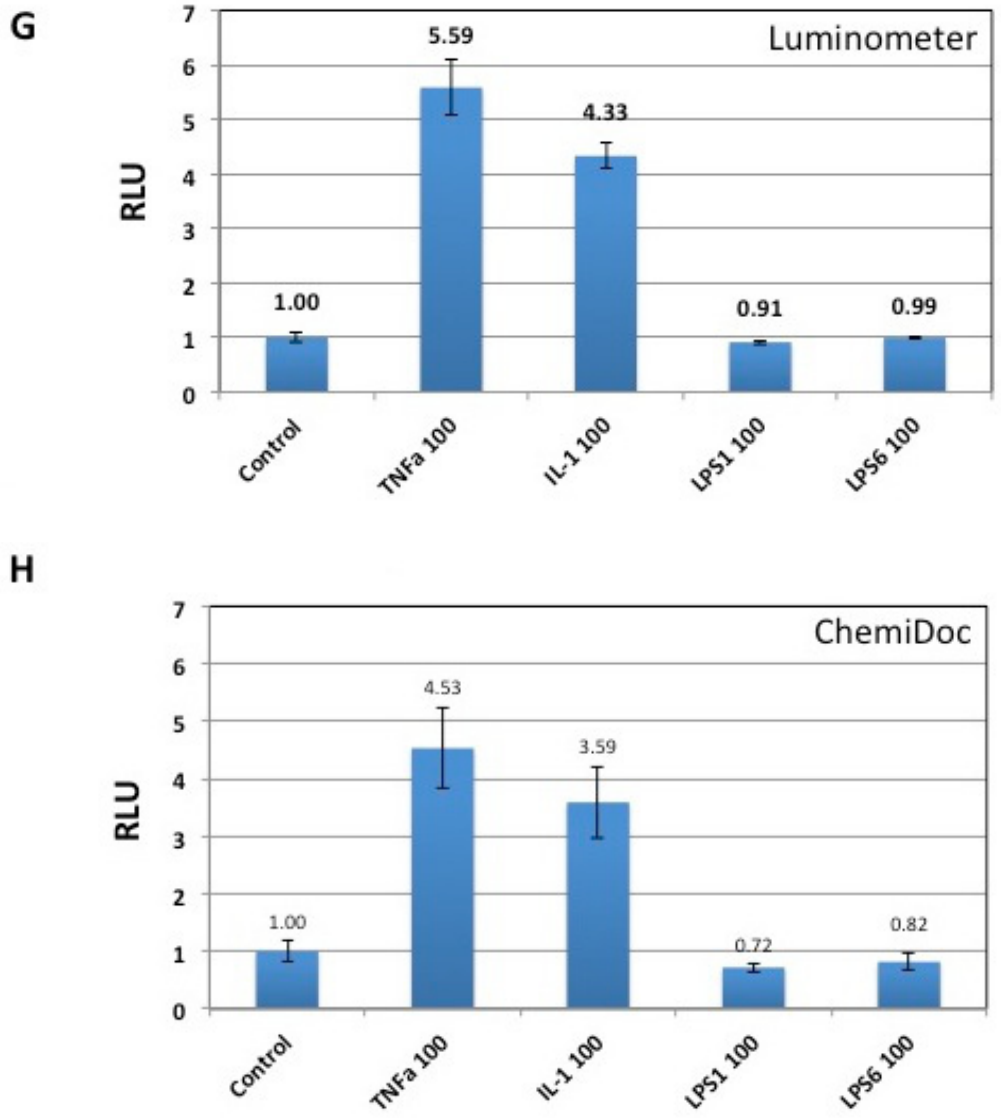
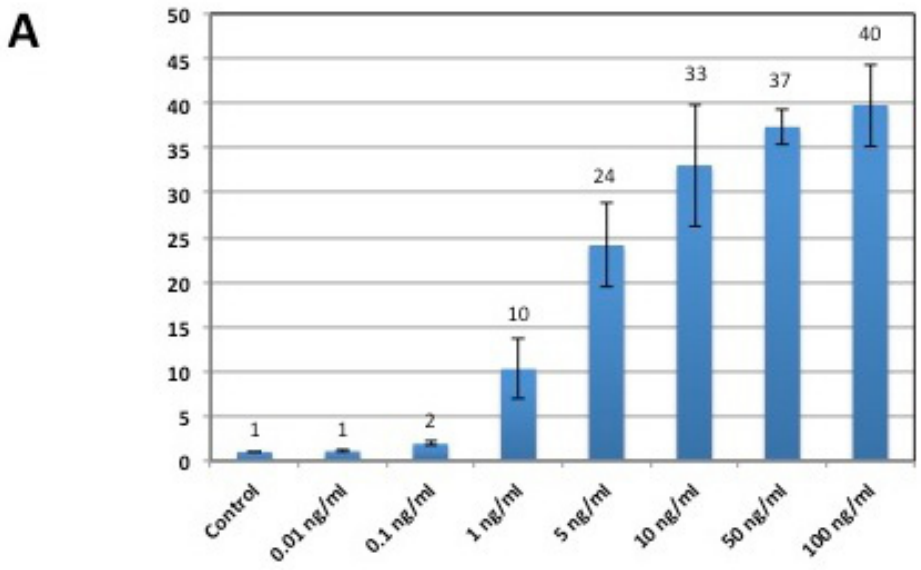


Fig. 8. Cooled CCD-based gene regulation studies. HEK293 cells were transfected with increasing concentrations of FLuc-GFP and GLuc-RFP constructs (0 to 2 µg) and gene expression level was observed and quantified. (**a**) GFP expression pattern and (**b**) FLuc activity level captured by CCCD camera is proportional to the amount of plasmid DNA and shows complete linearity on (**c**) graphical presentation. (**d**) RFP expression level, (**e**) GLuc signal intensity in cell free conditioned media and (**f**) its graphical presentation also show the same result. No FLuc activity in conditioned media (CM) and no GLuc activity in cell lysate (CL) were measured. Cells were transiently co-transfected with NFκB-FLuc-GFP and GLuc-RFP constructs. 48 hours post transfection and 24 hours after drug treatment FLuc activity was measured with (**g**) CCCD camera and (**h**) luminometer respectively. All CCCD camera results were plotted after analysis with

Image Lab software (version 3.0). FLuc activity was normalized against GLuc signal. Data are expressed as mean ± SD of 3 independent experiments with 6 repeats.

CCCD-based luciferase assay is easily amenable to high-throughput applications

To generate a platform for high-throughput applications (HTP) of CCCD camera, we monitored live NFκB stable reporter cells luciferase activity, in response to TNFα. At ~90% confluency, cells were treated with DMEM (1.5% FBS) with D-luciferin (1X) and various concentration of TNFα (0.01, 0.1, 1, 5, 10, 50, 100 ng/ml) and signals were recorded at four time points (zero, 3, 6 and 24 hours). Results were analyzed and graphed as dose-response (Fig. 9a) to show the effect of different concentration of TNFα on NFκB expression. On the same plate, a time course experiment was performed (Fig. 9b), focusing on one drug concentration (50 ng/ml) and showing its effect over the course of 24 hours. Here we achieved a platform to quantify two separate experiments with a single CCCD image capture, demonstrating its potential for high-throughput applications.



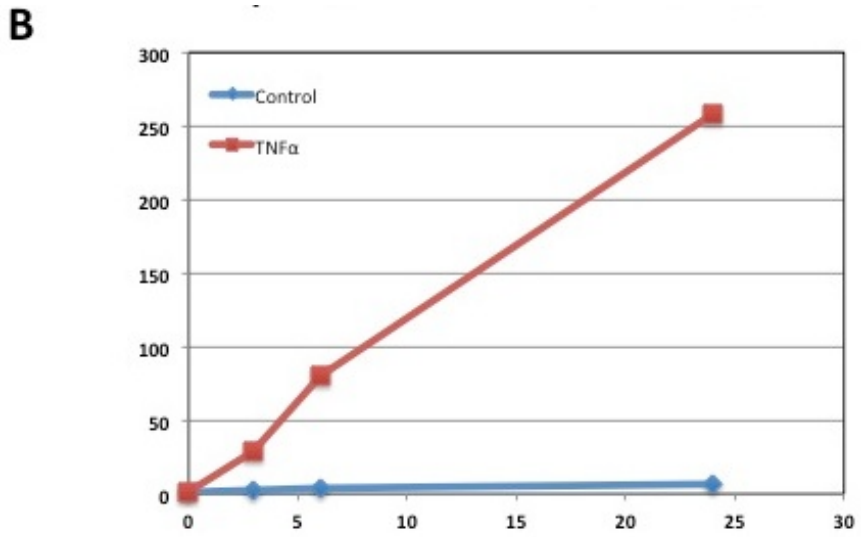


Fig. 9. NFκB stable reporter cell line luciferase activity pattern. HEK293 cells were stably transfected with NFκB reporter (inflammatory pathway) and selected for one month by puromycin. High throughput application, drug treatment and luciferase assay were performed simultaneously on live cells by adding DMEM (1.5% FBS) with different concentration of TNFα, and 1X D-luciferin substrate. (**a**) NFκB stable cell line dose-response to TNFα was plotted based on results obtained at 24 hours time point. (**b**) Time-dependent response of the cells to 50 ng/ml TNFα over the course of 24 hours.

DISCUSSION

Signal transduction is part of a complicated network of cellular communication that controls and coordinates basic activities and actions. Cell response to its environment is the basis of development, repair, and immunity as well as enables us to understand drug and disease processes in the body.

The ability to monitor and quantify the temporal activation of a signaling pathway in living cells, both on the individual cell level and within a population, is a desirable goal toward uncovering the functional relevance and mechanisms of signal transduction. Toward this goal, we reasoned that NFκB transcription factor could provide a versatile means for monitoring inflammation pathway signaling (Lawrence, 2009), in response to signals initiated by proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) (Osborn, Kunkel, & Nabel, 1989). In addition to inflammatory pathway, we tested other proposed reporters to monitor several other signaling pathways (Table I). We adapted a dual reporter for monitoring transcription factor activation (Uhde-Stone, Cheung, & Lu, 2014), making use of the unique properties of both green fluorescent protein (GFP) for visualization of individual cell responses and firefly luciferase (Luc) for quantification. Since the transcription factor activity is correlated to the activation of the monitored signaling pathway (Prell et al., 2014), dual-reporter serves as readouts of signaling activities.

We made use of the special design features of our dual reporter construct, and established NFκB stable cell lines. Absolute response of stably transfected cells to TNFα and IL-β confirms the high specificity of our designed reporter to monitor inflammatory

pathway. Later, we continued the validation of our stable reporter cell lines by conducting dose- and time-dependent experiments, which showed the sensitivity of the reporter. Results indicated the good correlation between GFP intensity and luciferase signals. We elucidated that different concentrations of stimuli over the course of 48 hours can affect the level of signaling pathway activation (Uhde-Stone, et al., 2012).

In order to expand and facilitate the application of this reporter system, we also monitored the NFκB pathway activity by widely available cooled CCD camera (CCCD). First, we conducted series of experiments and validated the ability of CCCD camera in detecting photons of different luciferases-substrate reactions (Table 2) – Firefly luciferase (Chiu & Christopoulos, 1999; de Wet, Wood, DeLuca, Helinski, & Subramani, 1987), Renilla luciferase (Lorenz, McCann, Longiaru, & Cormier, 1991), Gaussia luciferase (Tannous, Kim, Fernandez, Weissleder, & Breakefield, 2005) – their compatibility, stability and linearity over the time of experiments. The results showed that CCCD is sensitive enough to detect the signal produced by low amount of luciferase in samples. We also showed that GLuc, RLuc and FLuc have higher activity and lower stability, respectively. We planed the experiments accordingly, to be accurate, and to avoid losing the luciferase signals over the time. Since different luciferase classes oxidize different substrates, their combination enable us to monitor various biological processes such as gene expression and normalization simultaneously (Bhaumik & Gambhir, 2002). As we expected, CCCD-based assays demonstrate the linear correlation between luciferase activity and enzyme concentration, and is in agreement with GFP expression level. Together these results support the CCCD camera reliability, reproducibility in photon

counting *in vitro* when protein concentration and image acquiring time vary, and its potential in *in vitro* gene regulation studies.

Next we provided evidences and confirmed that monitoring NFκB signaling pathway activity in both transiently or stably transfected cells by CCCD camera generates outcomes equivalent to the results acquired by luminometer. We also showed that high-throughput experimentation in 96-well format is easily manageable with this transient and stable reporter cell lines. Although CCCD camera shows less sensitivity than a luminometer, for most applications the CCCD camera is sufficiently sensitive to track gene expression changes under different conditions (*e.g.* drug treatment) and can be used as a great alternative for less available luminometers in gene regulation studies.

Taken together, we demonstrated the potential of our dual-reporter system for monitoring signaling pathways activity and gene regulation studies. GFP offers real-time visualization of gene activation, while luciferase serves as quantitative tool (Afshari, Uhde-Stone, & Lu, 2014).

Table 2. Different luciferases and their specifications

	Origin (species)	Amino Acid	Weight (kDa)	Substrate	Cofactor	Light peak (nm)
FLuc	*P. pyralis*	550	62	D-luciferin	ATP/Mg^{2+}	562
RLuc	*R. reniformis*	311	34	coelentrazine	N/A	480
GLuc	*G. princeps*	185	19.9	coelentrazine	N/A	480

CONCLUSION

We have developed a dual reporter strategy for live visualization and quantification of pathway signaling in mammalian cells. We have demonstrated the versatility of this approach by monitoring various signaling pathways. In a case study, we have further validated the system by monitoring NFκB pathway signaling in a stable human HEK293 cell line by luminometer and cooled CCD alternatively. We demonstrate that CCCD is a feasible alternative to a luminometer for gene expression and regulation studies. Taken together, our study demonstrates the potential of dual-reporter tools to characterize signaling pathways important for biology and disease processes. Moreover, 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.

CONTRIBUTIONS

Dr. Biao Lu generated the dual-reporter constructs and most of the transient transfection results (Table 1). All other data were generated by Amirali Afshari.

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