Biochemical and Biophysical Research Communications 458 (2015) 543–548

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

# A cooled CCD camera-based protocol provides an effective solution for *in vitro* monitoring of luciferase

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ARTICLE INFO

*Article history:*
Received 21 January 2015
Available online 10 February 2015

*Keywords:*
Firefly luciferase
Renilla luciferase
Gaussia luciferase
Cooled charge coupled device (CCCD)
High-throughput screening

ABSTRACT

Luciferase assay has become an increasingly important technique to monitor a wide range of biological processes. However, the mainstay protocols require a luminometer to acquire and process the data, therefore limiting its application to specialized research labs.

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 acquiring and processing. By measuring activities of different luciferases, we characterized their substrate specificity, assay linearity, signal-to-noise levels, and fold-changes via CCCD. Next, we defined the assay parameters that are critical for appropriate use of CCCD for different luciferases. To demonstrate the usefulness in cultured mammalian cells, we conducted a case study to examine NFκB gene activation in response to inflammatory signals in human embryonic kidney cells (HEK293 cells). We found that data collected by CCCD camera was equivalent to those acquired by luminometer, thus validating the assay protocol. In comparison, The CCCD-based protocol is readily amenable to live-cell and high-throughput applications, offering fast simultaneous data acquisition and visual and quantitative data presentation.

In conclusion, the CCCD-based protocol provides a useful alternative for monitoring luciferase reporters. The wide availability of CCCD will enable more researchers to use luciferases to monitor and quantify biological processes.



## 1. Introduction

Bioluminescent proteins have revolutionized molecular tools to study and understand biological processes [1,2]. In particular, Firefly luciferase (FLuc), Renilla luciferase (RLuc) and Gaussia luciferase (GLuc) have been useful in a variety of fields including immunology [3–5], drug screening [6,7], oncology [8–10], functional genomics [11–13], virology [14–16], mycology [17–19], and neuroscience [20,21].

Luminometers are the most common instruments used to measure luciferases activity *in vitro*, but they are complicated, relatively expensive and not widely available. Cooled charge-coupled device (CCCD) has become a powerful photon detector device with a wide variety of applications, ranging from gel documentation to supersensitive biological imaging, including chemiluminescence (e.g. luciferase) and fluorescence (e.g. GFP) detection [22]. In fact, for *in vivo* bioluminescence imaging (BLI) in animal models, many researchers in life sciences are already using a CCCD apparatus, such as ChemiDoc™ from Bio-Rad or IVIS from Caliper [23–25]. The photon counting functionality of CCCD provides a promising alternative to luminometers for monitoring and quantifying luciferases *in vitro* [26–28]. In addition, CCCD offers advantages over luminometer, such as simultaneous data collection and alternative image presentation for quantitative and qualitative data analysis.

Here, we show that a CCCD-based protocol can be used to quantify activities of 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

*Abbreviations:* CCCD, cooled charge-coupled device; CMV, cytomegalovirus promoter; CTZ, coelenterazine; FLuc, Firefly luciferase; GLuc, Gaussia luciferase; RLuc, Renilla luciferase; HEK293, human embryonic kidney 293 cell line; HTP, high-throughput screening; IL-1, interleukin 1; IL-6, interleukin 6; LPS, lipopolysaccharide; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; TNFα, tumor necrosis factor alpha; RFP, red fluorescent protein; GFP, green fluorescent protein.

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http://dx.doi.org/10.1016/j.bbrc.2015.01.150
0006-291X/

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 CCCD to detect, visualize, and quantify luciferase expression level in mammalian cells as a feasible alternative to luminometer technology.

## 2. Materials & methods

### 2.1. Cell culture

Human Embryonic Kidney 293 cells (HEK293) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlas Biologicals, CO, USA), 2 mM GlutaMAX (Life Technologies, Grand Island, NY, USA), and 1% penicillin-streptomycin 100 U/ml (Life Technologies, USA). Cells were incubated at 37 °C with 95% air and 5% $CO_2$. At ~80% confluency, cells were washed with 1× PBS (Cellgro, VA, USA) and passaged with 1× trypsin-EDTA (Cellgro, VA, USA).

### 2.2. Reporter construction

For FLuc-based monitoring of gene expression, a previously described dual reporter [29,30] was used, configured as follows: CMV promoter::GFP-T2A-FLuc. As internal control (GLuc), a dual reporter was designed as follows: CMV promoter::RFP-T2A-GLuc, followed by a secretory signal peptide. To generate the NFκB::Fluc-GFP reporter, three repeats of an NFκB-specific transcription response element (TRE) were inserted upstream of a mCMV (minimal) promoter.

The components of the core sequence were PCR-amplified and fused together using gene fusion technology (SBI, Mountain View, CA, USA). The above reporter sequences were subsequently cloned into PiggyBac plasmid with a puromycin selection marker (SBI, Mountain View, CA, USA).

### 2.3. HEK293 cells transfection

HEK293 cells were transfected using Purefection transfection reagent according to the user manual (SBI, Mountain View, CA, USA). Typically, $1 \times 10^5$ of HEK293 cells/well were plated in 96-well plate in DMEM supplemented with 10% FBS. At ~80% confluency, HEK293 cells were transiently co-transfected with NFκB-Fluc-GFP (125 ng) and Gaussia-RFP (12.5 ng) constructs at 10:1 ratio using the Purefection transfection reagent. Transfection efficiency was monitored using Leica DMI3000 B inverted fluorescent microscope, 24 h after transfection with appearance of red florescent protein (RFP) positive cells.

For stable integration, cells were transfected with NFκB::GFP-FLuc vectors, and selected with 5 μg/ml puromycin (Sigma, USA) for at least one month.

### 2.4. Recombinant luciferase protein assays (FLuc, RLuc, GLuc)

Different concentrations of 95% pure recombinant luciferase proteins, FLuc, RLuc and GLuc (NanoLight Technologies, AZ, USA) were prepared by serial dilution in PBS; 5 μl were pipetted into microtiter wells. 100 μl D-luciferin and CTZ, respectively, were added as substrate, and luciferase activity was assessed immediately.

### 2.5. Firefly luciferase activity assay

For cell-lysis method, cells were lysed with Passive Lysis Buffer (PLB; Promega, USA). Cell lysates were cleared by centrifugation at highest speed for 2 min and supernatant were saved for luciferase assay. For luciferase quantification, 100 μl of substrate was added to 10 μl of supernatant, according to luciferase assay kit instructions (Promega, USA). If not stated otherwise, luciferase activity was recorded immediately after substrate addition.

In live-cell luciferase activity assay, cells were switched to low serum DMEM (1.5% FBS), containing D-luciferin (Sigma, USA) at 1× final concentration. Luciferase activity was measured after 5 min.

### 2.6. Gaussia luciferase activity assay

20 μl of cell-free conditioned medium were collected to test for secretory Gaussia luciferase. 100 μl Coelenterazine (CTZ; SBI, Mountain View, USA) substrate was added to 5 μl of conditioned medium, and chemiluminescence was recorded immediately after adding the substrate.

### 2.7. Drug treatment

$4 \times 10^5$ of HEK293 cells/well were plated in 24-well plate in DMEM supplemented with 10% FBS. At ~80% confluency, cells were treated with low serum DMEM (1.5% FBS) plus 100 ng/ml of TNFα, IL-1, LPS1, Lipopolysaccharides from *Escherichia coli* 0127:B8 (St. Louis, Sigma), and LPS6, Lipopolysaccharides from *E. coli* 026:B6 (St. Louis, Sigma), respectively. Luciferase activity was measured 24 h after drug-treatment.

### 2.8. Data collection with luminometer and CCCD camera

Luciferase activity was measured by TR 717 Microplate Luminometer (Applied Biosystems, USA) or ChemiDoc XRS$^+$ CCCD camera (BioRAD, USA). For ChemiDoc-based measurements, the "Chemi Blots" application of Image Lab software (version 3.0) was used with a signal accumulation mode of 1–30 s, 4 frames. Chemiluminescence was measured immediately after substrate addition by multichannel pipette. Note: Since chemiluminescence has the ability to travel across wells, it is necessary to use white or black 96-well plates. Pictures taken by CCCD camera were further analyzed by Image Lab software (version 3.0). Data were expressed as the mean ± SD (n = 6), unless stated otherwise.

## 3. Results and discussion

### 3.1. Substrate specificity of the luciferases FLuc, RLuc and GLuc

To establish a practical protocol, we first tested the feasibility of using more than one luciferase reporter simultaneously. We achieved this goal by analyzing substrate specificity of commonly used luciferases. In order to assess the specificity of different luciferases, we investigated the activity of three recombinant luciferases, FLuc, RLuc and GLuc, on D-luciferin and CTZ substrates by luminometer. We did not detect any activity of Renilla or Gaussia luciferases on FLuc substrate (Fig. 1A) or vice versa (Fig. 1B). The results suggest that one may use a combination of Fluc with either RLuc or GLuc for simultaneously monitoring of different signals. The absence of cross-reaction between the substrates for FLuc and RLuc/GLuc shows that activity of both groups can be observed individually, which is particularly useful for simultaneous monitoring of gene expression and normalization.

### 3.2. Stability and sensitivity of FLuc, RLuc and GLuc activity

To establish critical parameters for assay conditions, we first assessed the stability of FLuc, RLuc and GLuc chemiluminescence over time. After adding pure enzymes, we recorded luciferase activity at various time points up to 30 min (0, 30, 60, 120, 300, 900,

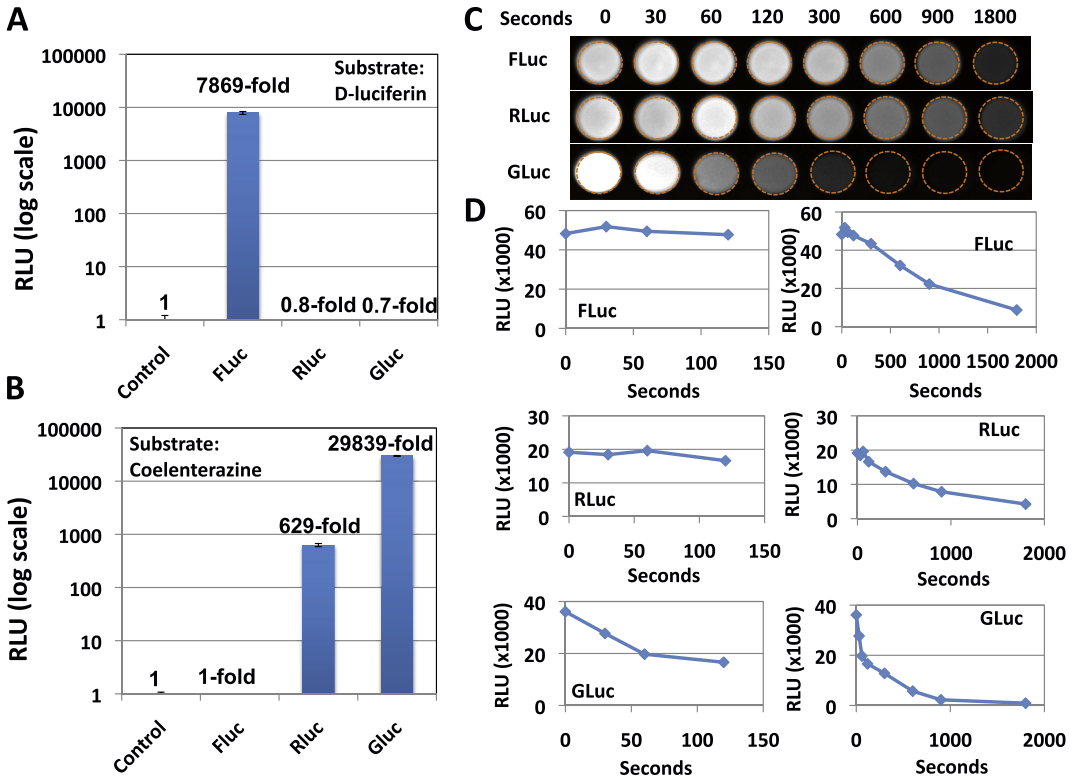


**Fig. 1.** Substrate specificity and compatibility of three different luciferases. (A) Firefly substrate (D-luciferin) and (B) Renilla/Gaussia substrate (Coelenterazine, CTZ) were added to recombinant luciferase proteins (FLuc, RLuc, and GLuc, respectively), and luciferase activities were quantified by luminometer. (C) Luciferase activity for the three different luciferase proteins was quantified by CCCD camera and (D) by luminometer, for two different time frames, 0–150 s and 0–1800 s (30 min) after substrate addition. Data is expressed as mean ± SD of 2 independent experiments (n = 6).

and 1800 s). The measurements were conducted in parallel with luminometer and CCCD camera. 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 min (1800 s). In contrast, GLuc signals rapidly declined by about 50% within two minutes after adding substrate, and by 95% over the course of 15 min (Fig. 1C). Luminometer results at two different time frames (0–150 s and 0–1800 s) showed the same trend (Fig. 1D). The results show that FLuc and RLuc display similar stability, and are stable over a longer period of time than GLuc.

We next determined the sensitivity of CCCD in detecting different ranges of signals. We assayed luciferase activity of recombinant FLuc, RLuc and GLuc, ranging in concentration from 0 to 50 μg protein (Fig. S1A–C). The CCCD lowest limit of detection was 500 ng for FLuc and RLuc, and 50 ng for GLuc. The data is consistent with previous reports that Gluc is more sensitive than that of FLuc or RLuc, but its signal degenerates more quickly. Similar stability, sensitivity, and the absence of cross-specificity, make FLuc and RLuc ideal choices for simultaneous monitoring of luciferase activity.

### 3.3. CCCD readings show linear correlation with enzyme concentration and exposure time

To further evaluate the linearity of a CCCD-based approach, we examined the correlation of acquired data based on two critical parameters: enzyme concentration, and exposure time. To test if luciferase activity correlates with luciferase protein concentration, we transiently transfected HEK293 cells with FLuc-GFP and GLuc-RFP constructs. 48 h after transfection, cells were lysed, serially diluted, and assayed for FLuc and GLuc activity by both CCCD camera (Fig. 2A) and luminometer (Fig. 2B). In a separate set of experiments, we tested the effects of various image acquisition times on luciferase activity measurement by CCCD. We used different concentration of FLuc as described above, but captured the signal at different times, namely 2, 5, 15 and 20 s (Fig. S2A). For quantification, we analyzed the data using Image lab and plotted the results separately for the 4 different exposure times (Fig. S2B).

Taken together, we found a linear relationship between signals detected by CCCD camera and both luciferase concentration and exposure time. Higher luciferase concentration or longer image acquisition time result in higher RLU readings, however, the relative luciferase activities remain linear.

### 3.4. CCCD-based luciferase assay is ideal for gene regulation studies

To evaluate the relation between plasmid expression and luciferase activity, we transfected HEK293 cells with increasing amounts of two different plasmids, CMV::GFP-FLuc and CMV::GLuc-RFP. Fluorescence microscopy (Fig. 3A) and CCCD-based luciferase activity assay (Fig. 3A, B) revealed a trend of increased reporter gene expression and luciferase activity in response to increasing amount of FLuc-GFP plasmid. Cells transfected with RFP-GLuc plasmid confirmed this trend (Fig. 3C, E). Our results demonstrate a linear correlation between plasmid DNA input, luciferase activity and fluorescent reporter gene expression.

### 3.5. CCCD camera produces quantitative data

We next compared the ability to quantify luciferase activity by CCCD camera with that of a luminometer. We co-transfected HEK293 cells with an NFκB-responsive promoter::FLuc reporter and a CMV::GLuc construct (for normalization), and assessed

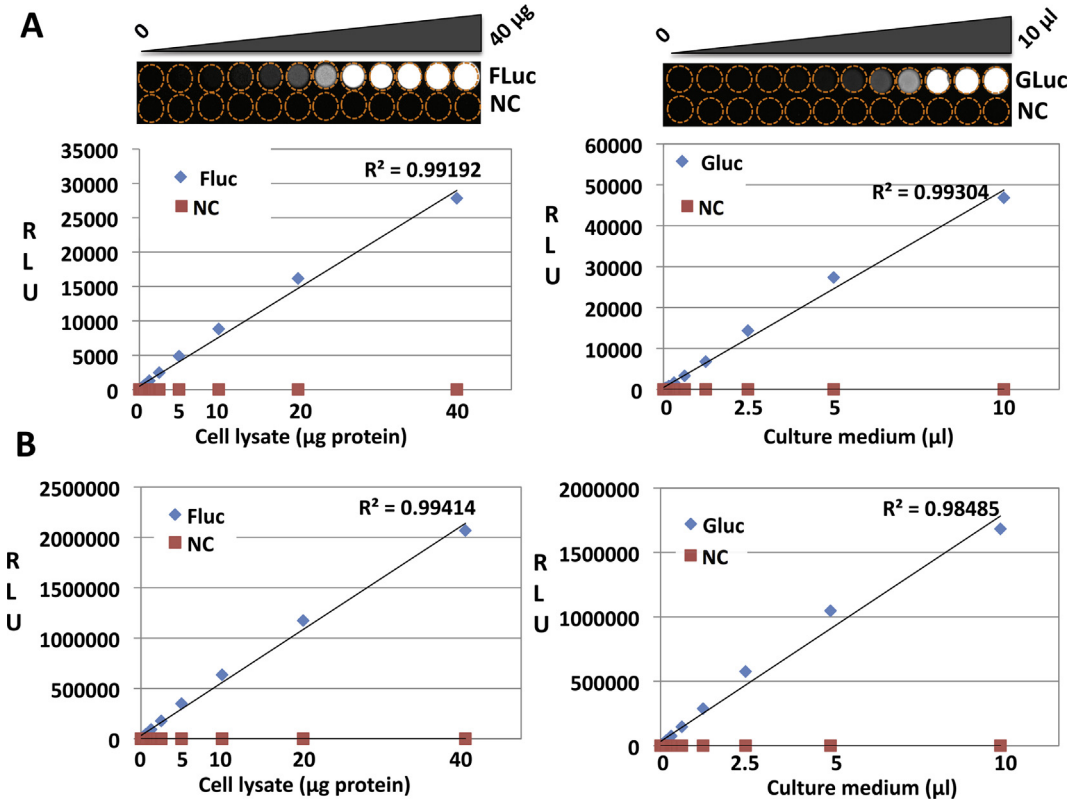


**Fig. 2.** Assay linearity. HEK293 cells were transiently transfected with FLuc and GLuc expression vectors, respectively, or with empty vector as negative control. Transfected cells were serially diluted, and FLuc activity of cell lysate and secreted GLuc activity in cell-free conditioned medium was measured by (A) CCCD camera (qualitative representation, upper panel, and quantitative representation, lower panel), and (B) luminometer. Results are shown in comparison to negative controls (NC).

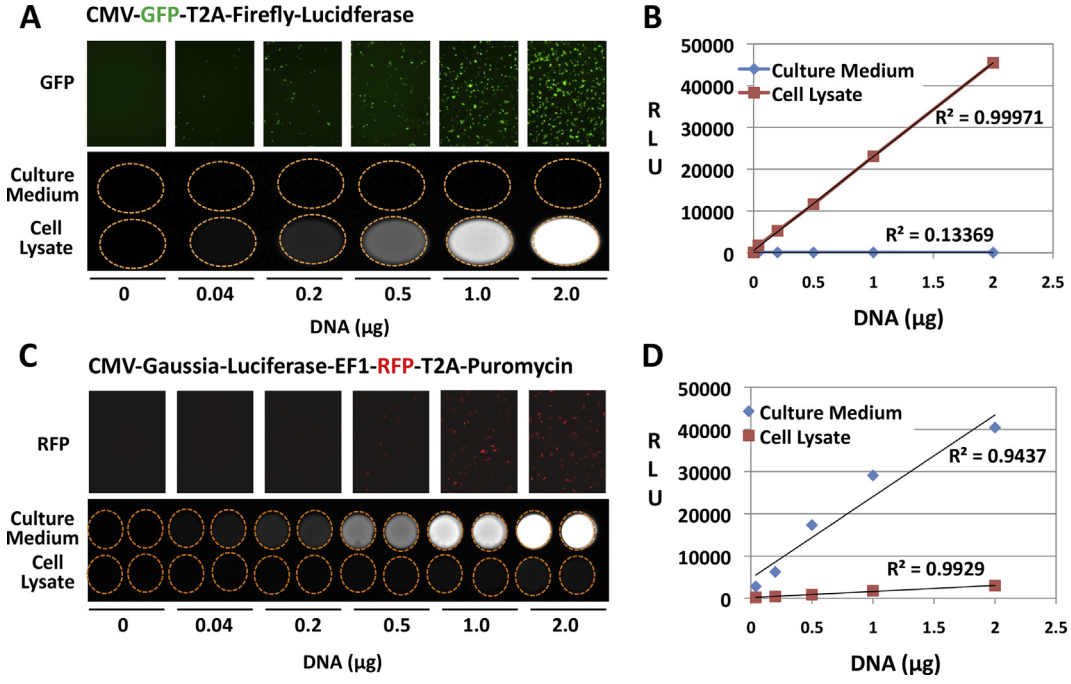


**Fig. 3.** CCCD-based gene regulation studies. HEK293 cells were transfected with increasing concentrations of FLuc-GFP and GLuc-RFP constructs (0–2 µg), and gene expression was observed and quantified. (A) GFP expression captured by fluorescence microscopy (upper panel), and FLuc activity captured by CCCD camera (lower panel). (B) Quantified CCCD results for FLuc activity, plotted against plasmid DNA concentration. (C) RFP expression captured by fluorescence microscopy (upper panel), and GLuc activity in cell-free conditioned medium, captured by CCCD camera (lower panel). (D) Quantified CCCD results for GLuc activity, plotted against plasmid DNA concentration.

luciferase activity in response to potential inducers of NFκB, namely TNFα, IL-1, LPS1 and LPS6. 24 h after transfection, chemiluminescence was assessed by both luminometer (Fig. 4A) and CCCD camera (Fig. 4B). As Fig. 4(A & B) shows, quantification of luciferase activity by CCCD and luminometer resulted in comparable data. TNFα was the most powerful inducer of NFκB expression, followed by IL-1. None of the lipopolysaccharides tested (LPS1, LPS6) resulted in induced NFκB::FLuc expression in the tested cell line (HEK293). The results confirm that luciferase activity obtained by CCCD can be quantified reliably, and results are comparable to those obtained by luminometer.

### 3.6. CCCD-based luciferase assay is amenable to high-throughput applications

To test if CCCD can serve as platform for high-throughput applications (HTP), we monitored luciferase activity in living cells. To this end, we treated stable NFκB::FLuc reporter cells with DMEM and assessed luciferase activity in response to various concentration of TNFα. Signals obtained by CCCD camera were recorded at four time points (0, 3, 6 and 24 h). Results were quantified by Image Lab software, and graphed as dose–response (Fig. 4C) to show the effect of different concentration of TNFα on NFκB expression. On the same plate, a time course experiment was performed (Fig. 4D), focusing on one drug concentration (50 ng/ml) and showing its effect over the course of 24 h. The ability to monitor luciferase in live cells, and the quantification of separate experiments with a single image, demonstrate the potential of CCCD for high-throughput applications.

**Table 1**
Key parameters of luciferases FLuc, RLuc and GLuc.

| | FLuc | RLuc | GLuc |
|---|---|---|---|
| Species | *Photinus pyralis* (firefly) | *Renilla reniformis* (sea pansy) | *Gaussia princeps* (mesopelagic copepod) |
| Substrate | D-Luciferin | Coelentrazine (CTZ) | Coelentrazine (CTZ) |
| Cofactor | ATP/$Mg^{2+}$ | N/A | N/A |
| Light peak | 562 nm | 480 nm | 480 nm |
| Specify | Very high | Very high | Very high |
| Sensitivity | High (~50 pg) | High (~50 pg) | Very high (~5 pg) |
| Range | Broader ($10^{3-5}$) | Medium ($10^{2-4}$) | Medium ($10^{2-4}$) |
| Assay stability | High (~5 min) | High (~5 min) | Low (<2 min) |
| Live cell monitoring | Excellent | Suitable | Suitable |
| HTP applications | Amenable | Amenable | Amenable |

### 3.7. CCCD provides a feasible alternative for monitoring luciferase

We previously constructed and tested a dual reporter system (GFP and FLuc) to visualize and quantify signal transduction in mammalian cells [29]. Here, in order to expand and facilitate luciferase-based applications, we tested if the widely available CCCD technology can serve as an alternative to a luminometer for detection and quantification of chemiluminescence. First, we validated the ability of CCCD camera in detecting photons of different luciferases-substrate reactions (Table 1), namely Firefly luciferase [31,32], Renilla luciferase [33], and Gaussia luciferase [34]. We assessed their compatibility, stability, sensitivity, and linearity over the time of experiments. We demonstrated that FLuc and RLuc are stable over a longer period of time than GLuc. We planned our

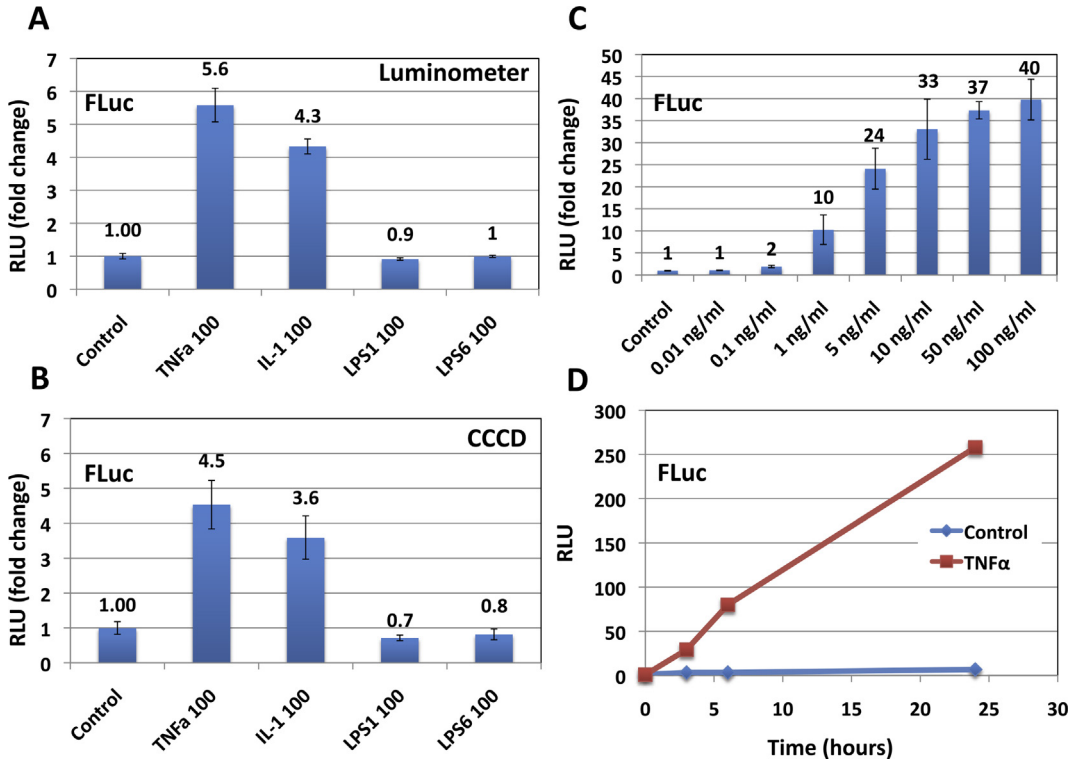


**Fig. 4.** CCCD-based monitoring of luciferase activity in live cells. To assess quantifiability of CCCD-derived data, cells were transiently co-transfected with NFκB::FLuc-GFP and, for normalization, with CMV::GLuc-RFP constructs. 48 h post transfection and 24 h after drug treatment, FLuc activity was measured with (A) luminometer and (B) CCCD camera, respectively. Stable NFκB reporter cells were treated with DMEM, and luciferase activity was assessed in response to various concentration of TNFα. Signals were recorded at four time points (zero, 3, 6 and 24 h). (C) NFκB stable cell line dose–response to TNFα was plotted based on results obtained at the 24-h time point. (D) Time-dependent response of the cells to 50 ng/ml TNFα are shown over the course of 24 h. All CCCD camera results were plotted after analysis with Image Lab software (version 3.0). FLuc activity was normalized against GLuc activity. Data are expressed as mean ± SD of 3 independent experiments, each consisting of 6 technical replicates.

experiments accordingly, minimizing loss of luciferase signals over the time. Since FLuc oxidizes a different substrate than GLuc and RLuc, their combination enables simultaneous monitoring of gene expression and normalization [35]. CCCD-based assays demonstrated a linear correlation between luciferase activity and enzyme concentration, and was in agreement with GFP and RFP expression level. Together these results show the reliability of CCCD camera-based detection of luciferase activity *in vitro* when protein concentration and image acquiring time vary, and its suitability for use in *in vitro* gene regulation studies.

We further showed that monitoring NFκB signaling pathway activity in both transiently transfected and stable cells by CCCD camera generates outcomes equivalent to the results acquired by luminometer. We further demonstrated that high-throughput experimentation in 96-well format is easily manageable with both transient and stable reporter cell lines. We conclude that the CCCD-based protocol is a feasible alternative for monitoring luciferase reporters, bringing the power of luciferase reporters to a wider range of researchers.

## Conflict of interest

BL declares financial competing interest as SBI (System Biosciences) employee. AA and CUS declare competing interest as collaborators with SBI.

## Acknowledgments

We would like to thank members of the System Biosciences Inc. for their support, technical assistance and discussions.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.150.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.150.

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