Published in final edited form as:
Anal Chem. 2013 August 6; 85(15): 7542–7549. doi:10.1021/ac401569j.

Reporter Protein Complementation Imaging Assay to Screen and Study Nrf2 Activators in Cells and Living Animals

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Abstract

NF-E2-related factor-2 (Nrf2) activators promote cellular defense mechanism and facilitate disease prevention associated with oxidative stress. In the present study, Nrf2 activators were identified using cell-based luciferase enzyme fragment complementation (EFC) assay, and the mechanism of Nrf2 activation was studied by molecular imaging. Among the various Nrf2 activators tested, pterostilbene (PTS) showed effective Nrf2 activation, as seen by luminometric screening, and validation in a high throughput-intact cell-imaging platform. Further, PTS increased the expression of Nrf2 downstream target genes, which was confirmed using luciferase reporter driven by ARE-NQO1 and ARE-GST1 promoters. Daily administration of PTS disturbed Nrf2/Keap1 interaction and reduced complemented luciferase signals in HEK293TNKS mouse tumor xenografts. This study reveals the potentials of Nrf2 activators as chemosensitizing agents' for therapeutic intervention in cancer treatment. Hence, the validated assay can be used to evaluate the identified activators pre-clinically in small animal models by non-invasive molecular imaging approach.

INTRODUCTION

NF-E2-related factor-2 (Nrf2) is a transcription factor ubiquitously expressed at low levels in cells of all human tissues [1]. As Nrf2 regulates a major cellular defense mechanism, tight regulation of its expression is crucial for maintaining cellular homeostasis. Rapid activation of Nrf2 pathway is important in preventing human diseases such as cancer, neurodegenerative, cardiovascular, ischemia, diabetes, pulmonary fibrosis, and inflammatory diseases [2]. Nrf2 is maintained in the cytoplasm as an inactive complex bound to a repressor molecule: Keap1 (Kelch-like ECH-associated protein-1) that facilitates its ubiquitination. Keap1 contains several reactive cysteine residues that serve as sensors of intracellular redox state. During redox stress, cytosolic Nrf2 is phosphorylated and translocated into the nucleus in response to activation of protein kinase C and MAP kinase pathways [3]. In the nucleus, it combines (forms a heterodimer) with a small Maf protein and binds to the Antioxidant Response Element (ARE) in the upstream promoter region of many antioxidative genes, and initiates their transcription [4]. The diverse nature of downstream target genes of Nrf2 including Heme Oxygenase 1 (HO1), NADPH: Quinone

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The authors declare no competing financial interest.

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Oxidoreductase 1 (NQO1), Glutathione S-transferase (GST), demonstrates its vital importance for cell survival and protection [5]. Conversely, high constitutive activation of Nrf2 and its downstream gene expression have been reported in many tumors, and in cancer cell lines which has been reported to protect them from the cytotoxic effects of anticancer therapies, resulting in chemo- and/or radiotherapy resistance [3,6]. Therefore, understanding Nrf2 regulation and identifying Nrf2 activators would have significant impact for study of disease prevention and/or for understanding their roles as sensitizing agents during cancer therapy.

Recently, a large number of Nrf2 activators have been under investigation for their activity in cellular protection and improvement of biological activity. The key activators include sulforaphane [7], resveratrol [8], curcuminoids [9], and green tea leaves extract [10]. These compounds play key roles in a wide variety of Nrf2-mediated functions, such as (i) cellular redox homeostasis, (ii) cell growth and apoptosis, (iii) DNA repair, (iv) the inflammatory response, and (v) the ubiquitin-mediated degradation pathway[11].

Perturbation in cellular function and overproduction of ROS may amplify the pro-inflammatory state of diseased areas, culminating in oxidative stress and loss of cellular functions [12]. These potential strategies have made Nrf2 activation a recent thrust area to overcome variety of diseased states. Even though few *in vitro* assay systems available to study Nrf2 activation, there is no sensing systems that have been constructed yet to evaluate Nrf2 activation in intact cells and living animals, which currently holds its promising application in medical research.

Sensitive and robust high throughput assays with the potential to screen and pre-clinically evaluate Nrf2 activators in living animals, are required for full diversity screening of a large compound collections and their translation to the clinic. Recently, Xie et al [13] developed a cell-based high throughput luciferase assay system based on the interaction of Nrf2 with its nuclear partner MafK or runt-related transcription factor 2 (RunX2). Recently Wu et al [14] developed a high throughput Screen for Identifying Small Molecules to Activate the Keap1-Nrf2-ARE Pathway based on the activation of ARE promoters. Since the Keap1-Nrf2 complex is believed to serve as a cytoplasmic sensor of oxidative stress, the development of screening system of Nrf2 activators to identify the breakage of cytoplasmic Nrf2-Keap1 complex is more beneficial and sensitive. We here describe an enzyme fragment complementation (EFC) system, an assay principle amenable to high throughput screening (HTS), to measure the dissociation of complex, thereby studying the nuclear entry of Nrf2. Furthermore, the assay can be used to evaluate the identified activators pre-clinically in small animal models by non-invasive molecular imaging approach.

EFC assay systems are based on the ability to express an enzyme as two separate fragments, N- and C-terminal segments that do not spontaneously assemble and become functionally active [15]. In this study, we used N- and C- terminal domains of firefly luciferase fragments which are inactive when co-expressed in cells. Luciferase activity occurs only when the two fragments are fused to respective interacting proteins, resulting in reconstituted active reporter enzyme that can be detected by luminometry or a low-light imaging device [16]. Hence, the activity of the interacting proteins is directly correlated to the signal generated and a highly sensitive assay can be established. This allows for use of small amounts of enzyme and a scalable HTS assay format [16,17]. The EFC assay has been successfully developed for commonly used reporter genes which include β-galactosidase [18], α-lactamase [19], green fluorescent protein, and luciferases [16] that are routinely used in mammalian cells, and non-invasive imaging of living animals. Taking advantage of the high sensitivity of the EFC technology, we have developed a highly specific EFC assay to measure the activity of Nrf-Keap1.

In this study, we used firefly luciferase complementation system due to its high sensitivity, accuracy, low background signal, and potential red shifted light emission for sensitive imaging in animals with minimal light attenuation and tissue scattering, which serves as a good model in sensor-based studies. In the present study, human embryonic kidney cells, HEK293T stably co-expressing fusion Nrf2 and Keap1 proteins flanked with carboxyl- and amino- terminal luciferase fragments (NLuc-Keap1 and CLuc-Nrf2), were generated. Upon Nrf2 activation by activators, CLuc-Nrf2 and NLuc-Keap1 complex dissociates leading to reduction of complemented luciferase enzymes. This reduction leads to decrease in luciferase activity which is directly proportional to the effectiveness of the complex dissociation. We optimized various aspects of developed constructs for optimal orientations. Experimental protocol was optimized in order to maximize sensitivity and efficacy of the assay system. In addition, we used several known and previously reported Nrf2 activators to evaluate Nrf2 activation, thereby providing the suitability of the sensor system for high throughput screening. Among the various Nrf2 activators tested, pterostilbene showed highest level of Nrf2 activation and hence subjected for *in vivo* studies. We demonstrated that pterostilbene (50 -200 mg/kg, i.p daily) significantly reduced the luciferase signals in tumor xenografts of HEK293T cells stably co-expressing CLuc-Nrf2 and NLuc-Keap1 fusion proteins up to 10 days from the time of tumor implantation. The results of our study not only validated our hypothesis, but also revealed several Nrf2 activators as promising candidates for therapeutic intervention targeted at oxidative stress-associated disorders and as a chemosensitizing agents in cancer treatment.

MATERIALS AND METHODS

Construction of plasmids

The vector constructs and stable cells expressing the complementation system were developed as detailed in supplemental material [16].

Evaluation of Nrf2/Keap1 complementation system with Nrf2 activators

To validate the Nrf2/Keap1 sensor system, HEK293T cells stably co-expressing complementation system were exposed to several known Nrf2 activators (RES: Reveratrol, PTS: Pterostilbene, $CoCl_2$, TTFA, EGCG, $NaAsO_3$, DEM, TUN, THA, and H_2O_2). These compounds were tested at different concentrations for either 8 or 24 h. Cells were harvested and assayed for luciferase activity using luminometer (Promega, Madison, WI, USA).

High throughput screening - 96 well assay format

HEK293T cells stably co-expressing Nrf2-Keap1 (HEK293TNKS) complementation system were seeded in 96-well plates (10,000 cells/well) and incubated for 24 h prior to treatment with different Nrf2 activators. Stocks of different Nrf2 activators were prepared in DMSO and diluted 1:100 in serum free medium. Then, 10 μl of the intermediate stock solution was added to each assay plate to achieve required final concentrations. Cells were incubated with test compounds at 37°C, 5% CO_2 for 24 h. Final DMSO concentration was <0.05%. Plate was imaged using IVIS spectrum system as described previously [20]. Media without D-luciferin served as negative control.

ARE-Luciferase Reporter Gene Assay

hNQO1-ARE-luc and GST-ARE-luc reporter gene constructs used for cell-based reporter gene assay were provided by Donna D. Zhang (College of Pharmacy, University of Arizona, Tucson, AZ). ARE-luc construct (500 ng/well) was transiently transfected into HEK293T cells in 12-well plates using Lipofectamine 2000. After 24 h, the media was changed and optimal concentrations of Nrf2 activators were added, with subsequent 8 h incubation, and

the luminometry assay was performed as previously described. Luciferase activities were expressed as fold induction relative to values obtained from control cells. Results represent mean values of at least three independent transfection experiments, each carried out in triplicates.

In vivo studies

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Stanford University (Assurance Number: A3213-01; Protocol Id: 26302). HEK293TNKS stable cells (106) were implanted into flanks of nu/nu CD-1 nude mice (Charles River Laboratories, Wilmington, MA). When tumors reached approximately 3mm in diameter, mice were randomized into 4 groups, and treatment was initiated. It involved intraperitoneal (i.p.) administration of different PTS concentrations (50, 100 and 200 mg/kg BW) dissolved in PBS/ Tween 20 (0.5%). PBS/ Tween 20 mixture without PTS was used in control group injections. The first group received single dose of PTS at above-mentioned concentrations, whereas the second group received injection of the same doses every day for a week. The experiment was continued further on third group of animals where a single dose 200 mg/kg BW of PTS everyday was used, but treatment was initiated from the day of tumor implantation.

Bioluminescence imaging

For *in vivo* bioluminescence imaging, mice were anesthetized by 2% isoflurane/air mixture (1L/min) and injected with a single dose of 150 mg/kg D-luciferin in PBS, i.p., Image acquisition was initiated immediately following injection of luciferin. Consecutive bioluminescence images were acquired every minute for 30 min. Image acquisition was carried out daily throughout the study for 15 days. Living Image 3.0 software was used for data analysis (Caliper Life Sciences).

Statistical analysis

All data were expressed as mean ± S.D and analyzed by ANOVA using SPSS version 7.5 (SPSS Inc., Cary, NC, USA). Group means were compared by t-test. Values were considered statistically significant at $p<0.05$.

RESULTS

Optimal constructs for imaging Nrf2/Keap1 interaction

The EFC assay can measure the interactions between proteins, such as two proteins involved in a signaling pathway, by fusing target proteins of interest to separate N- and C- terminal fragments of a reporter protein. Activity of the Luciferase is reconstituted when individual fragments are brought in close proximity by interactions between the fused proteins of interest **(Figure 1A)**. We used Firefly Luciferase (FL) complementation system for studying Nrf2 signaling, based on optimized FL enzyme fragments (NLuc416 and CLuc394). This EFC method builds on the established sensitivity of FL in imaging studies in intact cells and living animals. To find the optimal pair of vectors expressing fusion proteins of Nrf2 and Keap1 with reporter protein fragments, we constructed eukaryotic plasmid vectors expressing fusion proteins of full-length Nrf2 and Keap1 linked at either their N- and C-terminal domains of split-firefly luciferase in various arrangements **(Figure 1B)**. Pairs of these fusion proteins, each bearing complementing fragments of the split-firefly luciferase, were transiently co-transfected in human embryonic kidney HEK293T cells and assayed for complemented firefly luciferase activity after 24 h. As shown in **Figure 1C**, significant

difference in levels of complemented luciferase signals through heterodimerization (Nrf2-Keap1 interaction) was observed with various combinations. The combination co-expressing NLuc-Keap1 and CLuc-Nrf2 showed significantly higher level of signal than that of other combinations. This combination was used for generating stable cell line using puromycin antibiotic selection marker. A single colony isolated from stable cell was expanded and used for all cell culture and *in vivo* imaging studies.

HTS optimization and validation

HEK293TNKS bioluminescence activity and background response was compared at seeding densities from 0 to 10,000 cells per well in 96-well plates. Bioluminescence intensity was found increasing proportionally to cell numbers, indicating that signal intensity correlated directly with cell number per well (**Figure 2**). To compare the signal-to-noise ratio at each density we tested HEK293T without sensor in 0 to 10,000 cells per well in 96-well plates were imaged for bioluminescence and identified no increase in background bioluminescence proportional to the cell number, which indicates low background emission from the Firefly Luciferase (FLuc) substrate D-Luciferin. These data show that signal-to-noise ratio is constant across cell densities of 0-10,000 cells/well and is minimally affected by cell number variation. Cell-based assays often have a narrow range of cell densities required to achieve an optimal response. Our data demonstrate that variability in seeding densities did not affect signal-to-noise ratio in HEK293T at 10,000 cells/well, thereby fixing this cell density for further studies.

Based on earlier reports demonstrating the role of P-glycoprotein (PGP) in the extrusion of luciferin from intracellular compartments [21], the effect of luciferin concentration on the bioluminescent response was evaluated. HEK293T cells (10,000 cells per well) were treated with increasing doses of D-luciferin to obtain saturating substrate conditions. The obtained results demonstrate measurable bioluminescence at 1.5 μg/ml luciferin, with saturating concentrations reached at 50 μg/ml (**Figure 3**).

Testing HTS potential of the Nrf2-Keap1 sensor system by known Nrf2 activators

To test the hypothesis that luciferase complementation system developed in this study could be potentially used for measuring Nrf2 activation in a high throughput assay screening platform, we used HEK293TNKS cells in a 96 well format and imaged the complemented luciferase signal after exposure to various known Nrf2 activators such as RES, PTS, $CoCl_2$, TTFA, EGCG, $NaAsO_3$, DEM, TUN, THA, and H_2O_2 at optimal doses by optical charge coupled device camera (CCD) imaging system after the addition of substrate D-luciferin (50μg/ml). As shown in **Figure 4A**, luciferase activity decreased upon exposure of cells to various Nrf2 activators. An inverse relationship was observed between luciferase activity and the concentration of Nrf2 activators used for the study **(Figures S-1 to S-4)**. Among various activators tested, the percentage change in the level of complementation signal observed was maximum with RES, which is a known Nrf2 activator, considered as a positive control **(Figure 4B)**. Further, it showed that PTS was found as the best Nrf2 activators among various tested chemical activators tested. From the results, after carefully considering the activation time, any ligand which induces a drop in luciferase complementation signal >10% compared to solvent control, after normalizing the results for cell viability, can potentially be considered as Nrf2 activator.

To test whether the viability of the engineered HEK293TNKS cell line is modulated by the Nrf2 activators, cells treated with various Nrf2 activators were assessed by MTT assay. The results show no considerable change in the levels of cell death upon treatment with various activators, which is an encouraging evidence for a potential *in vivo* use of the Nrf2-Keap1 sensor system **(Figure 4C)**.

Evaluation of Nrf2/Keap1 complementation system in response to various Nrf2 activators

To validate the Nrf2/Keap1 complementation sensor system, several known Nrf2 activators such as RES, PTS, $CoCl_2$, TTFA, EGCG, $NaAsO_3$, DEM, TUN, THA, and H_2O_2 were used to treat stable cells co-expressing CLuc-Nrf2 and NLuc-Keap1 (HEK293TNKS) fusion proteins. Whole cell lysates were used to measure the complemented luciferase activity by luminometer assay. Since Nrf2 activation is reported to be both concentration and time dependent, the assays were performed for various activators at different dose, and by assaying at various time points after treatment. The developed sensor system showed drop in luciferase signal which is directly associated with Nrf2 activation (Nrf2-Keap1 complex breakage). PTS showed profound Nrf2 activation from 4 h to 24 h which is similar to the reference positive control, RES **(Figure S-1, S-2)**. In addition, the optimal time treatments for Nrf2 activation by EGCG, H_2O_2 and TUN were found to be 4 h, 1 h and 30 min respectively **(Figure S-3, S-4, S-5)**. Further our results clearly indicated that RES and PTS showed pronounced effects on Nrf2 activation even doses as low as 10μM concentration. After 8 h of treatment with resveratrol and PTS, luciferase signals dropped to 46% and 62.9% respectively compared to solvent control treated cells (**Figure 2A)**, and further decreased to 62.8% and 77.8%, respectively, after 24 h **(Figure 2B)**. The results demonstrated that RES and PTS showed better activity when compared to other Nrf2 activators. Change in luciferase activity indicates Nrf2 activation by various chemical inducers **(Table S-1)** in breaking complement system. The percentage change in signal decrease is directly proportional to the effective disruption of the Nrf2/Keap1 complex, i.e. lower signal intensity corresponds to the higher propensity for dissociation of the complement system that gets activated. The results indicate that RES and PTS are the best Nrf2 activators among various tested chemical activators of Nrf2.

The activation of ARE-Luciferase reporter construct by pterostilbene

ARE-dependent transcriptional activation of Nrf2 was studied using hNQO1-ARE-Luc and GSTARE-Luc reporter gene constructs by cell-based reporter gene assay. Optimized concentrations of RES and PTS were further tested in ARE-dependent reporter gene system using ARE sequence taken from either NQO1 gene promoter or GST1 gene promoter. PTS exposure to HEK293T cells resulted in two-fold induction when compared to control (untreated) cells **(Figure 5)**. These results indicate that the effect of PTS on ARE- driven Nrf2 downstream gene expression was relatively similar and promising when compared with the effect of positive control, RES.

Nrf2 activation by pterostilbene studied in living animals

As the objective of this investigation was to develop imaging sensor which can be used for screening and pre-clinically evaluating Nrf2 activators, we further tested PTS mediated Nrf2 activation in living animals by optical imaging in tumor xenograft of HEK293T cells stably expressing Nrf2/Keap1 system in mouse. Cells were implanted subcutaneously into the flanks of hind limb in 5-week old nude mice. When tumors reached approximately 3mm in diameter, mice were randomized and PTS treatment was initiated as described in “Materials and Methods”. We found a significant reduction in the bioluminescence signal in PTS-treated animals compared to vehicle control treated animals. The set of animals which received respective PTS doses (50, 100 and 200 mg/kg BW) every day showed significant Nrf2 activation as measured by bioluminescence signal, especially at the doses of 100 and 200 mg/kg BW ($p<0.05$). We continuously imaged the set of animals for up to 9 days from the start of treatment, after two weeks of tumor establishment. When the tumor size exceeded beyond 0.8 mm in diameter (close to 4 weeks from the initial tumor implantation) significant reduction in bioluminescence signal with dose-dependent PTS treatment was

observed **(Figure 6A and B)**. The results showed significant Nrf2 activation ($p<0.05$) of this treatment group as assessed by imaging.

DISCUSSION

Keap1-Nrf2 pathway plays a crucial role in determining the sensitivity of mammalian cells to chemical and oxidative insults [22]. There are strong opinions that further development of drugs that activate Nrf2 should be pursued for preventing many diseases such as neurodegenerative disorders [23], COPD [24], chronic inflammatory diseases [25] and, diabetes and associated complications [26].

Nrf2 activation is a promising approach for the enhancement of cytoprotective defense mechanism to prevent cellular diseases. It is not surprising that attention has been focused on identifying small molecule activators of the Nrf2/Keap1 pathway. A few studies have been reported for screening of Nrf2 activators from small molecule libraries using high throughput assay protocols [14]. Smirnova et al., [27] developed a Neh2-luciferase reporter system for high throughput screening as well as real-time monitoring of Nrf2 activators in vitro. The interacting surface of Nrf2 and Keap1 has been reported as ETGE motif in Neh2 domain of Nrf2 and the Kelch domain of Keap1 [28]. But it is not clear, whether Nrf2 activators can interact only with Neh2 domain and cause dissociation of the complex. Hence, the developed reporter system, using full-length Nrf2 construct allows direct monitoring of Nrf2 activators with different molecular mechanism towards dissociation of the complex.

Recently, Xie et al., [13] developed a high throughput system to identify Nrf2 activators based on the interaction of Nrf2 with its nuclear partner MafK or runt-related transcription factor 2 (RunX2) that is dependent on the reconstitution of a “split” luciferase. Under basal conditions, Nrf2 is found in association with its repressor, Keap1 in the cytoplasm. The dissociation of Nrf2 from Keap1, which is the initial event in Nrf2 activation that maintains molecular ON/OFF switching mechanism of Nrf2-ARE pathway, has not been explored. In addition, OKD48 transgenic mouse model has been developed for monitoring oxidative stress with Keap1-Nrf2 dependent manner with some limitations [29].

Upon investigation, we described here a cell-based luciferase enzyme fragment complementation assay that reports Nrf2 activation. Different reporter systems have been used to build EFC assays to monitor protein dimerization [30], protein-degradation [31], protein-folding [32], and nuclear-cytoplasmic translocation of proteins [33]. In our study, we successfully developed cell-based luciferase EFC assay for Nrf2/Keap1 system, which is sensitive and precise in detecting Nrf2 activation. We developed eukaryotic plasmid vectors carrying Nrf2 and Keap1, along with split-firefly luciferase in different orientations. CLuc-Nrf2 and NLuc-Keap1 combinations displayed high signal levels, and have been selected for making stable HEK293T cells that were studied in response to different activators. Under normal cellular conditions, both fusion proteins stay in the cytosol, interact, and complement luciferase fragments that lead to active luciferase enzyme. Upon treatment with Nrf2 activators, CLuc-Nrf2 and NLuc-Keap1 complex dissociates and CLuc-Nrf2 translocates into the nucleus, which results in reduction of complemented luciferase enzyme level.

Nevertheless, as a proof of principle, to validate the Nrf2/Keap1 complementation system, several known Nrf2 activators with different mode of action, such as RES, PTS, $CoCl_2$, TTFA, EGCG, $NaAsO_3$, DEM, TUN, THA, and H_2O_2 were studied at different concentration [29,34]. The percentage signal drop was directly proportional to the disruption in the Nrf2/Keap1 complex, i.e. lower signal intensity reflected higher propensity of dissociation of the complement system for activation. Moreover, a direct relationship

between luminescence signals and cell number was observed using two approaches, luminometry (in cell lysates) and bioluminescence imaging (in intact cells).

The first line of evidence deducted from luminescence intensity showed that resveratrol (RES) and PTS had a pronounced effect on Nrf2 activation as compared to other commercially available activators of Nrf2. Interestingly, we also identified no change in cell viability upon treatment of Nrf2 activators proving their non-toxic property in the cells. As of now, molecular mechanism of Nrf2 activation by PTS is not well documented. Given the fact that resveratrol suffers from low bioavailability and problem with metabolic structural instability [35], we continued our studies with PTS alone. Earlier reports suggest that Nrf2 activation by nuclear translocation, increased ARE-Nrf2 binding, and transactivation of its downstream genes. Optimized concentrations of RES and PTS were tested in ARE-dependent reporter gene system using ARE sequence taken from either the NQO1 gene promoter or the GST1 gene promoter and identified similar effect of PTS on ARE-driven Nrf2 downstream gene expression compared to positive control, RES.

In addition to observing all major advantages with the high throughput screening capability of this assay, we found some minor variations between the luminometer results with the intact cell imaging signals in luciferase complementation for very few ligands. This may be possibly due to the substrate utilization conditions by these two assays. In the intact cell-imaging assay, D-luciferin is used as a substrate, which normally uses ATP, Mg^{2+}, and CoA from the cells to react with luciferase enzyme to produce light. Whereas in the luminometer assay, LARII assay reagent, which possess D-luciferin substrate with sufficient amount of ATP, Mg^{2+}, and CoA, is used and hence doesn't require any additional cellular counterparts from the cell lysates to produce light. Moreover, ATP depletion and decrease in cellular ATP synthesis have been reported to be associated with some of the chemical exposures [36]. Hence, the minor change in results obtained from intact cell imaging assay with the luminometry assay may possibly be associated with these conditional variations. This could be overcome by the use of various co-factors as supplements with D-luciferin substrate while imaging luciferase enzyme levels in intact cells.

Activation of the target pathway that leads to the dissociation of the complex can be directly measured and correlated with its effects on tumor growth in vivo. In continuation of the in vitro evaluation of Nrf2 activators, we proposed to determine the feasibility and sensitivity of the sensor system for the evaluation of response to PTS in mouse tumor xenograft models. Live imaging was performed to determine the onset and duration of the Nrf2 activation in each animal. Initial imaging performed 24 h after cell implantation allowed us to verify the localization of cells in the implanted area and the homogeneity of experimental groups in terms of luminescence. To validate versatility of the system in vivo, PTS treatment (50, 100, 200 mg/Kg BW) was carried out after tumor development for 9 days. In this approach, before PTS treatment, tumors were allowed to grow for 10 days and reach 3mm in diameter. We detected significant differences between control and treated groups by bioluminescence quantification as early as 3 days after treatment initiation by observing a decrease in luminescence signals. We also found that in mouse xenograft model PTS at the dosage of 200 mg/kg BW effectively disrupted Keap1/Nrf2 interaction with concomitant suppression of tumor growth.

CONCLUSION

In conclusion, we demonstrate that luciferase complementation assay allows for non-invasive quantification of response to Nrf2 activators both *in vitro* and *in vivo*. Our studies have taken advantage of cell-based assay that allows to rapidly measuring Nrf2 activation with excellent sensitivity on a high throughput scale. Despite the fact that it requires

luciferase-expressing cell lines, it is a powerful method for estimation of Nrf2 activation with great flexibility that could be used for primary screening of compounds with Nrf2 activating potential. Use of live animal bioluminescence imaging provides more physiologically relevant information and allows for non-invasive, longitudinal monitoring of animals in preclinical setting. Since high level of Nrf2 activation leads to chemoresistance, the developed sensor system is applicable to high throughput screening of chemotherapeutic drugs and/or effects of drug candidates on Nrf2 pathway. Compared with conventional methods, high throughput model requires less time for data evaluation, and provides a rapid, real-time, non-invasive way to study the activation of Nrf2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Sanjiv Sam Gambhir, Chairman, Department of Radiology, and Canary Center at Stanford, for the facility. We thank the Department of Radiology, Stanford University for the funding supports (Dr. Ramasamy Paulmurguan). This study in part is supported by the NIH National Cancer Institute Grant 1R01CA161091 (to RP). We also thank Dr. Tim Doyle for providing technical support in imaging system at Stanford University. We thank Prof. Donna D. Zhang (University of Arizona, Tucson, AZ) for the generous gift of hNQO1-ARE-Luc and mGST-ARE-Luc reporter gene constructs. One of the authors' Dr. K.M. Ramkumar acknowledges SRM University for providing his overseas fellowship under 'Faculty Abroad Program'.

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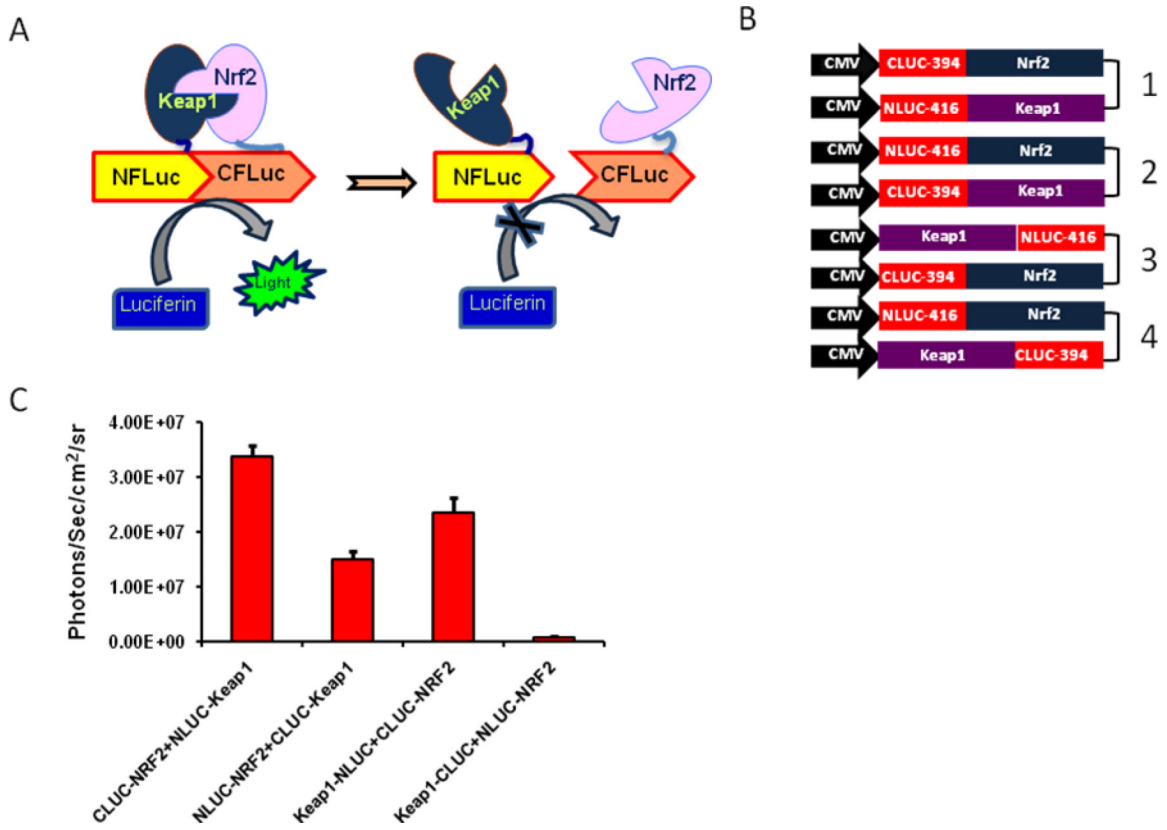

Figure 1. Split luciferase complementation assay to identify Nrf2 activators

(A) Schematic drawing of a split luciferase complementation assay to detect Nrf2 activation. The Spontaneous association of Nrf2 and its inhibitor Keap1 bring inactive fragments of luciferase into close proximity to reconstitute bioluminescence activity. **(B)** Schematic diagram of FLuc complementation constructs for imaging Nrf2 and Keap1 interaction. **(C)** Quantification of FLuc bioluminescence for various orientations and combinations of complementation systems Nrf2 and Keap1.

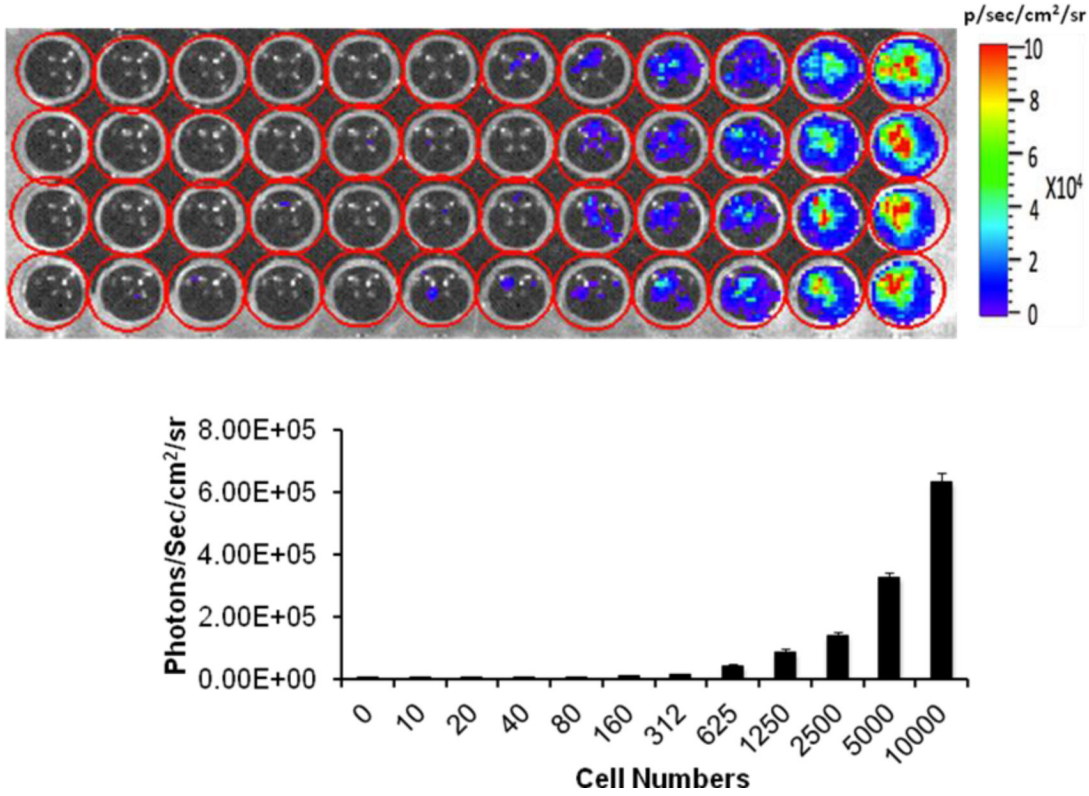

Figure 2. Effect of seeding density on signal and background in 96-well plates

Cells were seeded at 0 to 10,000 cells/well. Values represent the mean ± SEM of 2 separate experiments, each with 4 replicates.

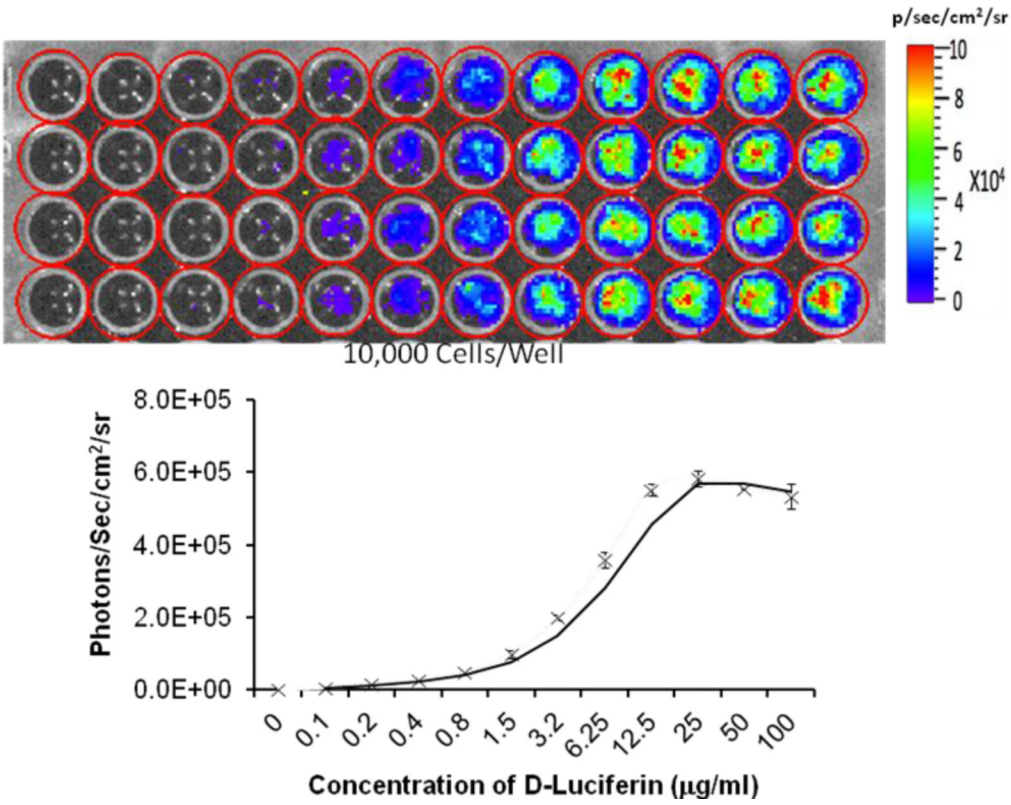

Figure 3. Effect of luciferin concentration on cellular response

Cells were treated with multiple doses of luciferin and imaged 5 to 10 min following substrate addition. Values represent the mean ± SEM of 2 separate experiments, each with 4 replicates.

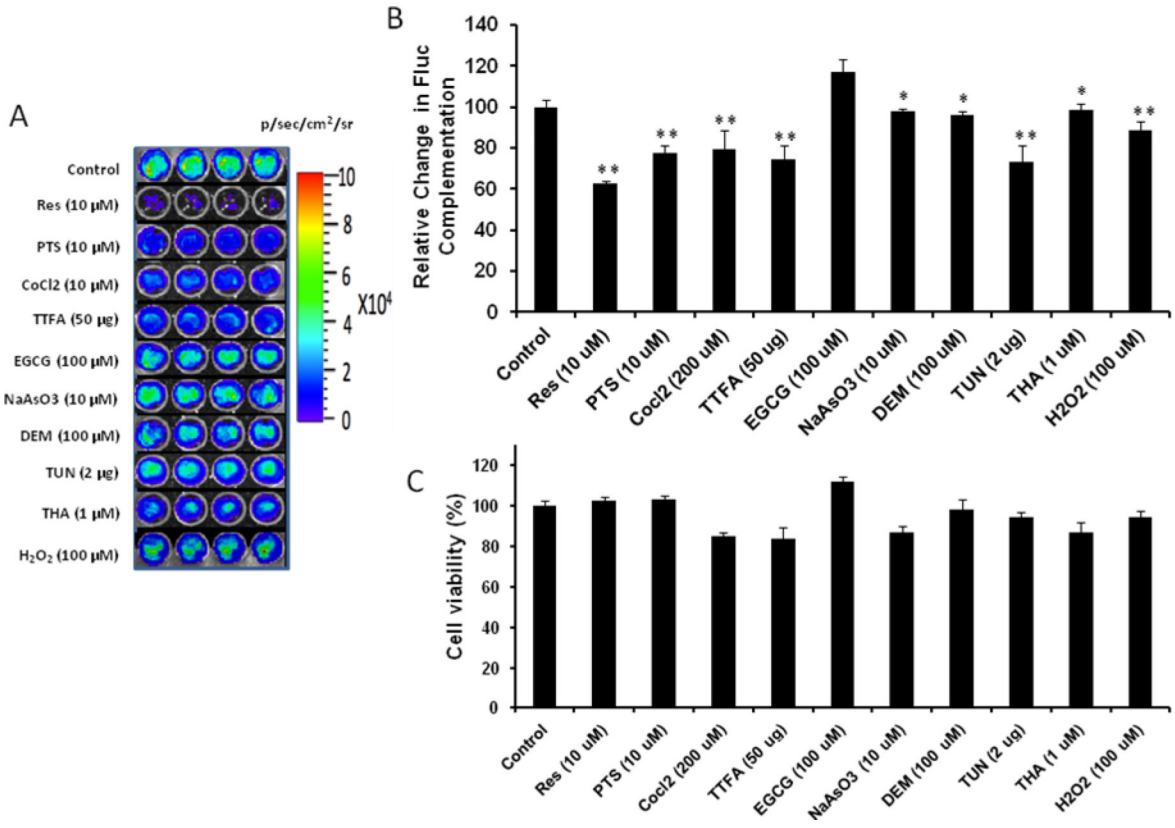

Figure 4. Nrf2 activators screening by high-throughput imaging

(A) High-throughput screening of Nrf2-Keap1 sensor system by different Nrf2 activators, **(B)** Quantified data for FLuc bioluminescence of Nrf2-Keap1 sensor system by various Nrf2 activators exposure at optimal concentrations, Photon flux data is normalized to the total protein per well and represented as percentage change. Data are shown as means ± SD (n=4: *p<0.05, **p<0.001 vs control). **(C)** Effect of Nrf2 activators on cell viability in HEK293TNKS cells. Data are shown as means ± SD (n=4: *p<0.05, **p<0.001 vs control).

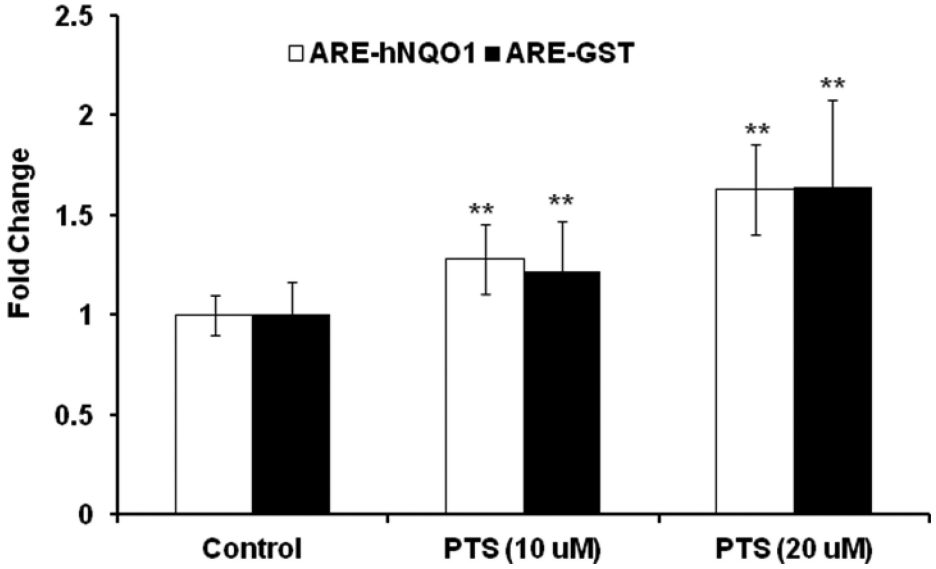

Figure 5. Effect of RES and PTS on ARE-hNQO1 and ARE-GST luciferase activation systems

HEK293T cells were transfected with either ARE-hNQO1 or ARE-GST luciferase transgene. Transfected cells were treated with RES (10μM) and PTS (10μM) for 8 h. Luciferase assay was performed as described in methods. Luciferase activities were expressed as fold induction relative to values obtained from control cells. Photon flux data was normalized to total protein and Rluc expression per well.

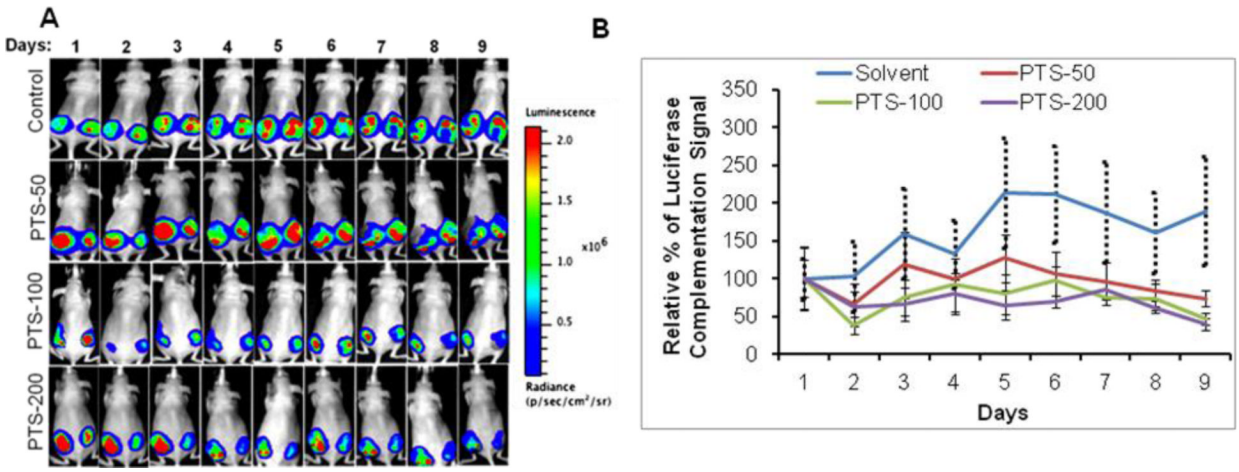

Figure 6. Nrf2 activation by pterostilbene studied in nu/nu CD-1 nude mice

HEK293TNKS stable cells (10^6) were implanted into flanks of *nu/nu* CD-1 nude mice. When tumors reached approximately 2mm in diameter, pterostilbene was administered as described in Methods. For bioluminescence imaging, mice were anesthetized using a 2% isofluorane/air mixture (1L/min) and injected with a single dose of 150 mg/kg D-luciferin in PBS intraperitoneally. Image acquisition was initiated immediately following injection. Consecutive bioluminescence images were acquired every minute for 30 min. Image acquisition was carried out daily throughout the study. Living Image 3.0 software was used for data analysis. The second set of animals which received respective doses pterostilbene (50, 100 and 200 mg/kg BW) every day and continuously imaged for up to 9 days from the start of treatment, after initial 2 weeks of tumor establishment. **B.** Quantitative analysis photons measured from animals received various treatments. Error bars indicate SEM from 10 independent determinations.