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Multiplexed Bioluminescence Reporter Assay: Empowering Screening Strategy for Future Medicine

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ABSTRACT

Multiplexed cellular screens offer a powerful solution to the limitations of traditional, single-target assessment-based experiments by allowing scientists to measure multiple biological functions simultaneously. For past several decades, dual luciferase reporter assay using non-overlapping firefly and *Renilla* luciferase formed the backbone of biomedical and biotechnology research applications. Although it was highly reliable, the application could essentially allow measurement of only one gene function assessment at one go. Our recent report of 'triple reporter assay' development by integrating the mushroom luciferase (*nn*Luz) isolated from *Neonothopanus nambi* has renewed the scope of developing a non-overlapping, multiplexed reporter assay for simultaneous measurement of multiple gene functions from live cells. This development adds up strength to biomedical research applications with requirement of large volume screens for modulators against the function of multigene complexes, which are relevant in the context of normal or disease biology.

Keywords: Luciferase, Reporter gene, Multiplexed screening

Introduction

In the post-genomic era, technologies capable of identifying cross-talks between signaling pathways in the context of normal or disease conditions are of high demand. Multiplexed assay systems with distinguishable signal strengths are being developed to mitigate this requirement of enhanced screening of molecules capable of inhibiting signaling cross-talks. In this context, the luciferase-based reporter assay systems are especially attractive, as the output photonic signal is resulted from a chemical reaction, the photons emitted are literally background noise-free and thus provides high sensitivity and specificity. Relied on enzyme-substrate specificity, accurate signal distinction ability, reduced variability across biological replicates, reliable read-outs of real-time target modulatory effect by the candidate drug molecule, and extension to high-throughput screening and *in vivo* imaging are some of the major features where luciferase-based assessment satisfies the drug screening requirements. Further, a live mammalian cell-based functional screening format has shown better predicting ability of gene functions *in vivo*. However, luciferase-based screening applications could essentially allow measurement of only one gene function assessment at one time, limiting insight into broader cellular contexts. In this context, our recent work demonstrating the development of a 'triple reporter assay' involving a novel mushroom luciferase (*nn*Luz) identified from *Neonothopanus nambi* is a unique step forward [1]. The potential of this multiplexed assay developed holds immense importance for future biotechnology and biomedicine research fields.

Diversity of luciferases and their applications

A luciferase enzyme catabolizes its specific substrate, thereby yielding a photonic signal with distinctive spectral signature, which is measured using advanced photomultiplier or cooled CCD camera-based devices. Luciferases are a class of photoproteins, when obtained from natural biological entities are popularly called bioluminescent (BL) reporter and currently there are many such luciferase enzymes identified from terrestrial and deep-sea organisms [2]. In recent years several new BL cell-based screening methods utilizing genetic biosensors have been designed for measurement of a variety of dynamic cellular functions [3]. These cell-based assays often used luciferase genes that were isolated from natural resources (e.g. luciferase from Firefly, *Renilla*, *Gaussia, Vergula* and *Oplophorous*) and optimized for expression in mammalian cells as powerful tools. Interestingly, in spite of the diverse source, the commonly used luciferases fall into two broad categories, one that uniquely catabolizes substrate derivatives called luciferin and the other that catabolizes coelenterazine derivatives [4]. Since 1990s, dual luciferase reporter assay has been used as a gold-standard assay system by the research community [5]. This assay relied on using two non-overlapping luciferases with distinctive spectral signatures resourced from firefly and marine copepod *Renilla sp*. for various gene function tests. The initial designs involved placing the firefly luciferase (FLuc) as the experimental reporter, while using *Renilla* luciferase (RLuc) as an internal control for normalization. The method was adapted to various systems including yeast and mammalian cells to study gene expression, transcriptional regulation etc. Expanding its scope, a combination of firefly and *Renilla* luciferases worked well for live cell-based screening and *in vivo* imaging. Compared to live cell assays using fluorescence dyes, dual luciferase reporter assay had limited scope of integration where longitudinal monitoring and high-throughput screen are primary requirements. Requirement of cell lysis, flash signal kinetics of RLuc and ATP-dependant FLuc activity affected by the cellular energy metabolism conditions majorly hindered scope of expansion as a screening technology. Towards overcoming the bottleneck, a variant like Dual-Glo assays was also introduced that offer more sustained signals for better adaptability to serve screening requirements.

As the commonly used marine luciferase resources, i.e. RLuc, *Gaussia* Luc (GLuc) and NanoLuc (NLuc), are reactive to the coelenterazine substrate or its derivatives, technically all of these luciferase reporters can be utilized to measure one biological event at a time. Towards circumventing this limitation, researchers have applied mutagenesis approach to generate luciferase variants. There are also synthetic substrate variants produced which are spectrally distinguishable by using advanced spectral unmixing strategy [6,7]. For such multiplexed assay system development, luciferase-luciferin pairs with a 30-40 nm gap in peak emission wavelength are used for measurement of multiple linked gene activities by quantifying photonic emission peaks from luciferase [8,9]. As these multiplex assays offer higher sensitivity than fluorescent methods, broad dynamic range for signal detection, and lower intrinsic background due to lack of endogenous luminescence makes them attractive assay formats. However, unlike the fluorescence emission, the BL emissions are generally much broader and thus significant signal overlap demand background correction during output measurements. Therefore, a truly multiplexed assay by combining non-overlapping luciferases remained an unsolved puzzle to mitigate the high demand in the field.

New entity: Cracking the code of mushroom luciferase

So far, many bioluminescent mushroom species have been reported from different parts of the world, which are

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evolutionarily divergent from other known terrestrial and marine species with luciferase. One such mushroom luciferase gene, i.e. *nn*Luz was recently identified along with establishment of its bioluminescence principle. Further, elucidating its chemical biosynthetic mechanism, it was shown that the caffeic acid is metabolically converted into the unique 3OH-hispidin substrate, which is catabolized by the *nn*Luz protein to produce BL light [10], as well as suitable conversion of the gene for use in human cells fuelled the field for further breakthrough applications in the biomedical fields [1]. We found this luciferase-luciferin pair as a unique candidate with no cross-reaction with other common luciferase-luciferin pairs in use. Additionally, the photonic emission maximum (EM_{max}) of wild-type or humanized *nn*Luz (hLuz) is also spectrally distinguishable from emissions of FLuc2, and NLuc or hRLuc. Considering the utility of bringing together three independent luciferase reporters in one assay, we chose to study regulatory role of NF-κβ and p53 macromolecules on PIK3CA gene expression, a complex biology of high relevance in the context of ovarian cancer. Previous knowledge states that both p53 and NF-κβ has binding sites on the PIK3CA promoter and thereby exerts their regulatory role on the later [11,12]. In the context of epithelial ovarian cancer cells, p53 directly bind to PIK3CA promoter sequence and acts as a transcriptional repressor. Whereas, in cisplatin resistant ovarian cancer cells, enhanced NF-κβ activity causes development of acquired chemoresistance. However, it was unknown till date, whether or not NF-κβ activation may exert indirect control on p53 activation, and thereby modulate PIK3CA gene regulation in chemo-resistant cancer. For understanding such molecular cross-talk mediated gene regulation in cancer conditions, simultaneous measurement of multiple protein function using a combination of independent luciferase reporters would be ideal. We aimed to capture such information by making reporter sensors to measure NF-κβ response via hLuz, p53 transcription factor activity via NLuc and PIK3CA promoter activity via FLuc reporter. Engineered ovarian cancer cell lines expressing pNF-κβ-hLuz, p53-NLuc fusion and PIK3CA-FLuc2-tdT alone or in combination used for the multiplexed assessment. A new level of understanding came out from this study indicating the complexity of multiple drug action that overlaps with each other. In this case with triggered NF-κβ activation, repressive effect of p53 activation prevails, which in turn drives a less repressive regulatory action of the PIK3CA gene expression.

Outlook

Accurate understanding of complex biological functions in a live cell context is important. But the current methods for studying complex cell functions are slow and limited because they mostly allow tests only one function at a time, failing to capture the full picture of the intermolecular interactions and their impact in the whole cell context. Additionally, for the requirement of modulator compound screening, it is also difficult to make any comparative assessment on target molecules based on the assay read outs from different screening experiments performed in sequence. For these reasons, the assessments which can fetch the information through successive experiments need to be replaced, allowing to fetch more accurate information. Multiplexed cellular screens seek to overcome these limitations by extracting multiple readouts from a single screen, thereby adds up strength to the future screening requirements. The discovery of newer luciferase entities, such as humanized *nn*Luz, with non-overlapping reactivities and distinct emission spectra has renewed the hope of digging deeper into complexities that arise due to molecular cross-talks. The *nn*Luz reporter in combination with other existing luciferases is expected to expand the scope of multiplexed cellular screens, allowing scientists to reveal complex biological functions in future. Simultaneously, research applications aimed at *in vivo* imaging using model organisms, can also grow with enhanced scope of multiplexed luciferase imaging. These developments will eventually provide a robust, high-throughput assay framework to help in intricate understanding of real-time signalling cross-talks under the influence of various modulators.

Conflict of Interest

All authors declare no conflict of interest.

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