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Truly quantitative analysis of the firefly luciferase complementation assay☆

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ABSTRACT

Luciferase complementation assays detect protein-protein interactions within living cells using bioluminescence. Since the first report using plant cells was published in 2007, over 100 peer-reviewed articles have been published describing the detection of protein-protein interactions within plant cells by the assays. The assays have also been used to analyze networks of protein-protein interactions in plants. Although the assays have a high dynamic range, they remain qualitative with respect to determining the affinities of interactions. In this article, we first summarize the luciferase complementation assays developed in the past years. We then describe the mechanism of the firefly luciferase complementation that is most widely used in plants, and the reason it is qualitative rather than quantitative using a mathematical model. Finally, we discuss possible procedures to quantitatively determine the affinity of a protein pair using the firefly luciferase complementation assay.

1. Luciferase complementation assays and their use for the network analysis of protein–protein interactions in animal cells

Luciferase complementation assays (LCAs) detect protein–protein interactions within living cells using bioluminescence. In the assays, complementary DNA (cDNA) of luciferase is first split into the N- and C- terminal fragments and then fused to cDNAs of a protein pair of interest. A cell of interest is transformed or transfected with the resulting recombinant cDNAs so that a pair of the recombinant proteins is expressed within the cell. When the recombinant proteins interact with each other, the enzymatic activity of split luciferase is reconstituted. Compared with other assays that detect protein–protein interaction in living cells, these assays have a high dynamic range of interaction signals due to extremely low background signals in the samples [1]. Accordingly, LCAs are suitable to conduct high-throughput screening in which high degrees of differentiation between a positive and negative signal is required.

The research group of Umezawa first published the principle of the LCA using luciferase obtained from fireflies (*Photinus pyralis*) in 2001 [2]. In the publication, insulin dose-dependent interactions of phosphorylated insulin receptor substrate 1 (IRS-1) and the N-terminal SH2 domain of PI 3-kinase in living Chinese hamster ovary (CHO) cells was described. As the assay was further modified, the research group of Jacob published the network analysis of protein–protein interactions in Human Embryonic Kidney 293 (HEK 293) cells with LCA using luciferase from copepod (*Gaussia princeps*) in 2012 [3]. The analyzed proteins are composed of a total of 2167 viral and human protein pairs. To identify the interacting protein pairs, they first benchmarked the assay using 100 randomly selected protein pairs for the negative result, and 143 protein pairs known to interact for the positive [4]. The detected luminescence was normalized by dividing the luminescence of a tested protein pair by the luminescence measured in control experiments. In the control experiments, they measured the luminescence emitted by the random interactions of the N- and C- fragments of luciferase. Frequency distributions for the normalized luminescence of positive and negative protein pairs were used to determine the threshold luminescence for an interacting protein pair.

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2. Use of luciferase complementation assays for the network analysis of protein–protein interactions in plant cells

We published the application of LCA in Arabidopsis protoplasts, for the first time, to detect the interaction of a histone protein pair using luciferase from sea pansy (*Renilla reniformis*) in 2007 [5]. We further published the network analysis of protein–protein interactions in Arabidopsis protoplasts using the same LCA in 2010 [6]. The analyzed proteins in the network are composed of 38 pairs of SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein). To identify the interacting protein pairs, we benchmarked the assay by comparing the previously published results of co-immunoprecipitation assays. Eleven negative protein pairs and 8 positive protein pairs identified using co-immunoprecipitation assays were compared to the LCA results [6]. The luminescence detected in the LCA was normalized by dividing the luminescence by the luminescence emitted by luciferase activity from the click beetle (*Pyrophorus plagiophthalamus*) that depends on the transformation efficiency of the cells. Distributions for the normalized luminescence values for the positive and negative protein pairs were then used to determine the threshold luminescence for an interacting protein pair.

In 2011, the research group of Li published the result of the network analysis of protein–protein interactions, composed of 96 auxin response factors (ARFs) and auxin-modulated transcription factors (Aux/IAA) protein pairs, in Arabidopsis protoplasts using the LCA with firefly luciferase [7]. The detected luminescence was normalized by dividing the luminescence by the activity of β-glucuronidase, which depends on the transformation efficiency of the cells. They compared the results of a co-immunoprecipitation assay and the luminescence. Because the normalized luminescence was linearly and positively correlated to the amount of co-immunoprecipitated proteins, the reliability of the LCA was affirmed. They then quantitatively analyzed the interaction network of 96 ARF and IAA protein pairs [7].

Furthermore, in 2014, research groups published LCA methods to analyze protein–protein interaction networks in tomato and rice protoplasts, independently, although they have not yet analyzed the networks [8,9].

3. Six types of luciferase with different enzymatic characteristics are used to detect protein–protein interactions in living cells

As noted above, LCAs have been developed based on different types of luciferase. Because the characteristics and substrates are different in each luciferase, it is important to know which luciferase is used to determine protein–protein interactions in the cells of your interest. As of today, six different types of luciferase are used to detect protein–protein interactions *in cellulo* and *in vivo* [10]. They are from firefly (Photinus pyralis) known as FLuc [11], sea pansy (*R. reniformis*) known as RLuc [12,13], copepod (Gaussia princeps) known as GLuc [14], click beetles (*P. plagiophthalamus* and *Cratomorphus distinctus*) known as CBR and ELuc, respectively [15,16], or deep sea shrimp (Oplophorus gracilirostri) known as NanoLuc [17]. Each luciferase has a unique enzymatic character (Supplemental Table S1). For instance, the substrate for the luciferases from firefly and click beetles (FLuc, CBR, and ELuc) is D-luciferin, while that for the luciferases from sea pansy, copepod, and deep sea shrimp (RLuc, GLuc, and NanoLuc) is coelenterazine. The wavelengths of luminescence emitted from the activities of each luciferase are also different. While FLuc has its emission peak at 560 nm, RLuc and GLuc have their emission peak at 480 nm. CBR has its emission peak at 613 nm. Moreover, brightness of luminescence produced by the enzymatic activity differs. For instance, in cultured human cells, codon optimized GLuc shows 200-fold brighter signal than that from codon optimized FLuc [18]. The size of each peptide also differs. While FLuc is composed of 550 amino acids (61 KDa), NanoLuc is composed of 171 amino acids (19 KDa).

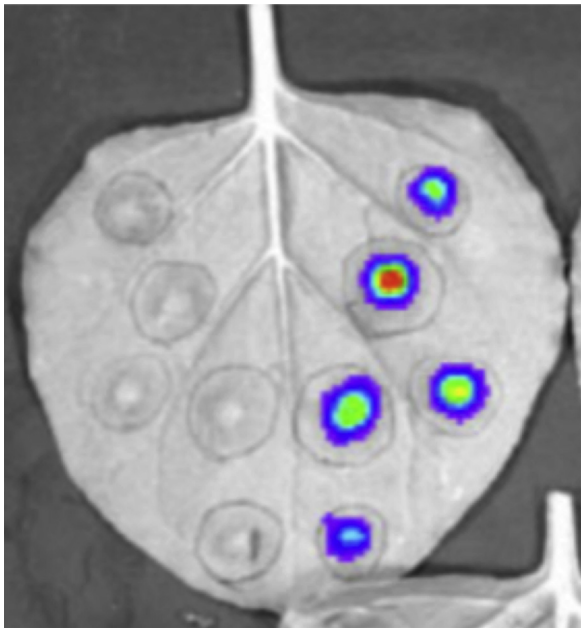

Fig. 1. Agrobacterium infiltration method. Identification of protein-protein interactions in a *Nicotiana benthamiana* leaf by the Agrobacterium infiltration method. Agrobacterium carrying a T-DNA that expresses a protein pair fused to N- and C-terminal domain of luciferase was infiltrated in different locations on the leaf. The protein pairs on the right are interacting with each other, but not the protein pairs on the left. The luminescence signals were measured using a CCD camera. An image of a luminescence heat-map is superimposed on a black-and-white image. (Kato and Popescu, *unpublished*).

4. Firefly luciferase complementation assay is most widely used for plant cells

Over 100 peer-review articles that use luciferase complementation assays to detect protein–protein interactions within plant cells have been published since 2007. We randomly selected 70 of the articles and summarized protein pairs and the type of luciferase used (Supplemental Table S2). As seen in the table, among the six types of luciferase used, firefly luciferase (FLuc) is most widely used in plants. Nearly 75% of the articles (52 of 70 articles) use the Agrobacterium infiltration method to determine protein–protein interaction by FLuc (Fig. 1). The Agrobacterium method allows expressing split FLuc in tobacco (Nicotiana benthamiana) leaves. The method was coined luciferase complementation imaging (LCI) by the research group of Zhou who published the method for the first time in 2008 [19]. In LCI, a charge-coupled device (CCD) camera detects luminescence after D-luciferin is sprayed onto the leaves. In 14 articles (20% of the 70 articles), Arabidopsis or tobacco protoplasts are transformed with plasmids expressing split FLuc using polyethylene glycol (PEG). The protoplasts are placed into 96-well plates, and D-luciferin is added directly to the wells. A photomultiplier tube (PMT) or a CCD detects luminescence. In these articles, the assay is typically conducted together with another assay that also detects protein–protein interactions such as a co-immunoprecipitation assay.

LCI has given high impact results in the field of plant biology. For instance, the research group of Zhou tested interaction of 9 effector proteins from *Pseudomonas syringae* with plant proteins