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Fabrication of molecular tension probes

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ABSTRACT

A unique bioluminescent imaging probe is introduced for illuminating molecular tension appended by protein–protein interactions (PPIs) of interest. A full-length luciferase is sandwiched between two proteins of interest via minimal flexible linkers. The ligand-activated PPIs append intramolecular tension to the sandwiched luciferase, boosting or dropping the enzymatic activity in a quantitative manner. This method guides construction of a new lineage of bioassays for determining molecular tension appended by ligand-activated PPIs.

The summary of the method is:

- Molecular tension appended by protein–protein interactions (PPI) is visualized with a luciferase.
- Estrogen activities are quantitatively illuminated with the molecular tension probes.
- Full-length *Renilla* luciferase enhances the optical intensities after bending by PPI.

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Method details

To date, several potential techniques have been established for determining protein–protein interactions (PPIs), including (i) Bioluminescence resonance energy transfer (BRET) based on energy transfer between bioluminescent donor and fluorescent acceptor proteins [1–3]; (ii) Mammalian/

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yeast two-hybrid assay reflecting interactions between “Prey” and “Bait” proteins [4]; (iii) Protein-fragment complementation assay (PCA) making use of split-reporter protein and its conditional reconstitution [5,6].

We previously developed a unique bioluminescent probe called “strain probe” for illuminating PPIs [7]. We initially hypothesized that any luciferase has talent to change its enzymatic activity according to the molecular tension artificially appended by PPIs. This molecular tension may cause distortion of the active site, which modulates the enzymatic activity.

In this method, we introduce how to fabricate *molecular tension probe*s emitting bioluminescence in response to molecular tension appended by PPIs in detail.

Materials

- pcDNA 3.1(+) (Invitrogen)
- a mammalian expression vector
- Restriction enzymes (*Hind*III, *BamH*I, *Kpn*I, *Xho*I)
- African green monkey kidney fibroblast-derived COS-7 cells
- A 96-well clear bottom microplate (Nunc)
- Dulbecco’s modified Eagle’s medium (DMEM)
- Fetal bovine serum (FBS; Gibco)
- Penicillin/streptomycin (P/S; Gibco)
- TransIT-LT1 (Mirus), a lipofection reagent
- 17β-Estradiol (E_2; native estrogen)
- 4-Hydroxytamoxifen (OHT; synthetic antiestrogen)
- Phosphate-buffered saline (PBS)
- A lysis buffer (E291A, Promega)
- An assay buffer (E290B, Promega)
- Native coelenterazine (nCTZ)
- A Bradford reagent for determining total protein amounts
- Hank’s buffered salt solution (HBSS) buffer (Gibco)

Basic concept for designing molecular tension probes

The basic design of *molecular tension probes* consists of four different ingredients, i.e., a full-length luciferase, a pair of proteins of interest (called proteins “A” and “B”), and a flexible linker, where the luciferase is sandwiched between the two proteins of interest via a minimal length of flexible linkers (Fig. 1). The luciferase is tensed by the ligand-activated PPIs. The minimal flexible linkers as possible connecting the ingredients are advantageous to efficiently convert the molecular tension to practical distortion of the sandwiched luciferase.

Any luciferases basically have talent to vary their enzymatic activity more or less according to the molecular tension appended by an intra-molecular PPI. A globular marine luciferase may be advantageous over beetle luciferases, which consist of N- and C-terminal domains connected by a flexible hinge region [8] (Fig. 1A). A globular marine luciferase like *Renilla reniformis* luciferase (RLuc) easily receives tension from PPIs, whereas the flexible region in beetle luciferases relaxes the intra-molecular tension.[1] The active site of RLuc8 is close from the C-terminal end [10], thus is prone to be influenced by protein-tagging and molecular tension appended by adjacent proteins.

In this protocol, we exemplify a *molecular tension probe* that is made of RLuc8 as a model luciferase sandwiched between the ligand-binding domain of the human estrogen receptor (ER LBD) as an intracellular receptor member of the nuclear receptor superfamily and Src homology domain 2 of *v*-Src (SH2), based on our previous papers [7,11]. Upon ligand activation, Tyr537 of ER LBD is

[1] The C-terminal domain of firefly luciferase (FLuc) is rotated to release luciferin in the light-emitting process [9], which is hampered by the molecular tension in the probe.

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