Point of VIEW

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New Approaches to In-Cell Detection of Protein Activity: Genetically Encoded Chemiluminescence Probes Pave the Way to Robust HTS Assays

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ABSTRACT New genetically encoded biosensors utilizing the modified firefly luciferase promise a great improvement in the signal-to-noise ratio and the dynamic range of response in living cells. These biosensors are particularly suitable for high-throughput screening assays that use large-well-capacity formats because of their excellent response characteristics. The biosensor design strategies are highly generalizable and will be extremely valuable for expanding the repertoire of screenable targets in living cell systems.

High-throughput screening (HTS) assays are used increasingly often to target highly specific signaling pathways isolating a handful of proteins in specific disease contexts (*1, 2*). In these screens, the availability of HTS assay reagents amenable to live-cell applications that do not compromise the dynamic range and signal-to-noise ratio (S/N) has been the primary obstacle. On page 346 of this journal, Fan and co-workers (*3*) show three generalizable approaches to producing excellent live-cell HTS assay reagents that can be multiplexed into large-screen formats because of extremely high dynamic range and S/N, all using the genetically encoded approach.

Screening strategies involving *in vitro* reactions of purified components or cell lysates can be optimized by standard approaches, including modifications to fluorescent dyes and reaction or assay conditions to yield greater dynamic range of response or better S/N. Standard approaches such as these are not easily implemented in assays that use living cells. The major barrier to implementing robust HTS assays in living cells is the transport of biosensors through the cell membrane. Approaches that use cell-permeable compounds can be successful in cases where the biosensor is a small dye molecule (such as calcium indicator dyes using acetoxymethylester modification, *etc.*) (*4*). Macromolecules and their derivatives are becoming increasingly popular in designs of biosensors, but they are simply too large for efficient transport into the cell. Strategies such as electroporation, transport reagents, and viral transduction have seen limited applicability in large-scale HTS applications (*5–9*).

Genetically encoded biosensors are a class of biosensor molecules that can be used to bypass the cell membrane transport problem altogether. Cells can be made to carry the DNA codes for the biosensor molecule in their genome, either constitutively expressing these molecules or in an inducible form where it can be turned on to produce the biosensors at carefully titrated intracellular concentrations. Although advantageous in this regard, the fluorescent proteins that are most commonly used for the detection of protein "activities" in these genetically encoded biosensors are often not optimal for producing highly robust HTS assays because of low dynamic range of response, low S/N, or both. The main problem lies in the fact that useful information gathered from using these biosensors are primarily about the state of post-translational modification of the protein targets rather than bulk localization or accumulation of fluorescently labeled material. This type of

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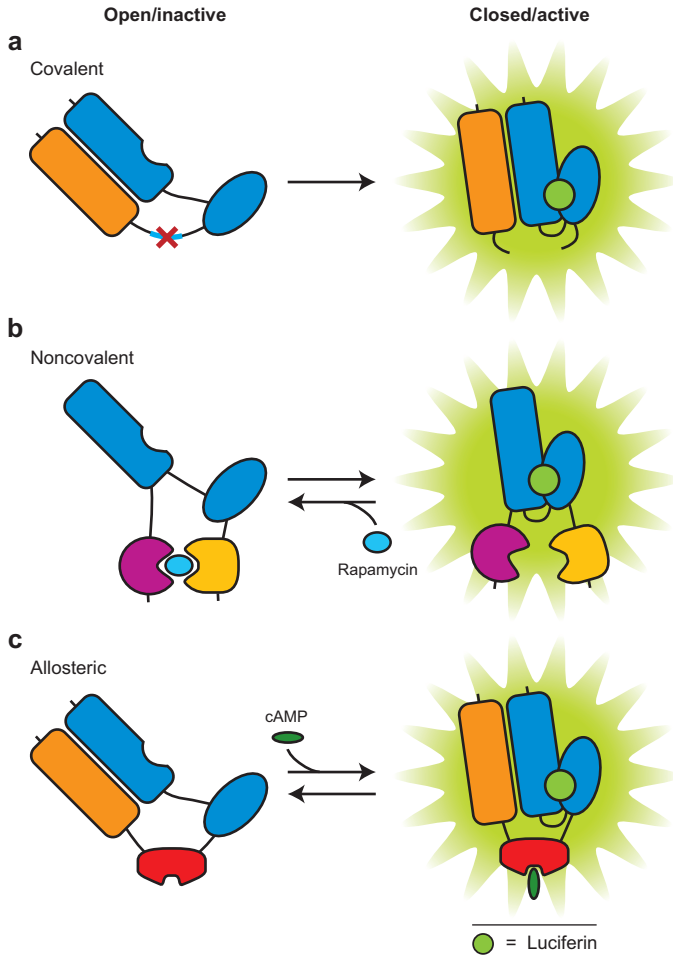

Figure 1. Three strategies for the selective activation of firefly luciferase reporters: covalent, noncovalent, and allosteric. a) Covalent: proteolytic cleavage allows formation of the closed/active conformation. b) Noncovalent: the presence of rapamycin holds the enzyme in the open/inactive conformation and depletion of the small molecule leads to activation of the reporter. c) Allosteric: binding of cAMP to an engineered binding site leads to a conformational change resulting in the closed/active form of the reporter.

detection requires some form of sensing of either conformational change to the molecule due to phosphorylation or binding of downstream targets due to protein activation. The most frequently used technique to measure protein−protein interactions or conformational changes within the protein is Fluorescence Resonance Energy Transfer (FRET) between the FRET donor and acceptor fluorescent protein pairs. In FRET, a donor fluorophore of shorter excitation and emission wavelengths in its excited state can transfer energy by a nonradiative, long-range, dipole−dipole coupling mechanism to an acceptor fluorophore of longer excitation and emission wavelengths in close proximity (<10 nm) (*10*). FRET biosensors respond to changes in molecular conformation (*via* post-translational modifications) by altering the fluorescence emission ratio between the direct donor excitation/emission and the transfer of energy to acceptor *via* the FRET, resulting in increased acceptor emission (*11*).

A typical shortfall of FRET-based biosensors is the low dynamic range of response. This is primarily caused by two factors: (i) nondirect excitation of the acceptor fluorescent protein by a nonradiative mechanism tending to produce relatively weak signals; and (ii) often, conformational changes between "on" versus "off" states are not large enough or the attachments of fluorescent proteins are suboptimal to confer great enough change in FRET efficiency. These effects together result in a typical FRET ratio change ranging anywhere from 30% up to a 2-fold change for most genetically encoded biosensors using the FRET approach (*11, 12*). These issues are not particularly amenable for a direct transfer of FRET-based genetically encoded biosensors for HTS assay development. Further compounding the problem is the relative difficulty of optimizing the FRET efficiency change as a function of biosensor states. Most biosensor optimizations involve approaches to change the dipole−dipole coupling efficiency by lengthening or shortening the linker between the FRET fluorescent protein pair or conferring 3D changes to the molecular conformation of the FRET pair by methods such as the circular permutation of fluorescent proteins (*13*). Other methods, including bimolecular fluorescent complementation, have also been used to detect protein−protein interaction, but they suffer from lack of reversibility (*14*).

Fundamentally, the best approach to attaining the greatest signal dynamic range difference as a function of protein on/off states would be to achieve a totally dark state while the biosensor is "off" and maximally bright in the "on" state. Inclusion of the reversibility of on/off states would add further specificity in target state detection. Fan and colleagues achieve this by designing a genetically encoded biosensor, based

not on FRET between two fluorescent proteins but on chemiluminescence produced by modified-luciferase enzyme acting upon its substrate luciferin only in the activated state of the biosensor. The three example approaches the authors present here, protease cleavages (Figure 1, panel a), rapamycin binding (Figure 1, panel b), and allosteric activation by binding of a signaling molecule cyclic adenosine monophosphate (cAMP) (Figure 1, panel c), all involve modulation of the hinge structure of luciferase enzyme to prevent the interaction of luciferase with the substrate luciferin while the biosensor is maintained in the "off" state. Upon activation by the protease cleavage, competitive removal of rapamycin, or binding of cAMP, the structural impingement to the closure of the hinge structure is relieved, allowing binding and oxidation of luciferin to result in luminescence.

Making these genetically encoded chemiluminescence sensors is no easy task. The naturally occurring luciferin enzyme could not have been used directly because the critical hinge domain did not allow for any type of direct modification. The authors utilized the circular permutation strategy to translocate the amino- and carboxyl-terminals of the protein to within the hinge domain so that they can be further functionalized. Circular permutation (*13*) is a technique to translocate the amino- and carboxyl terminals of a protein to completely new locations by first closing off the native amino- and carboxyl-terminals by using a short flexible linker and then creating new termini at desired locations. This is not a straightforward technique, because introducing new termini at the wrong locations could result in misfolding of the protein or loss of activity. The authors overcome these pitfalls by producing a library of luciferase, randomly incorporating a short insert fragment (to simulate functionalization) and assessing the resulting luciferase activity. The clone identified thus was tolerant to incorporation of the new termini at an ideal location for the manipulation of the hinge domain and retained ~75% of the parental wild-type enzymatic activity. Using this modified luciferase, the authors were able to produce example biosensors described in the Letter.

It is exciting that these example approaches are highly generalizable to a wide range of designer biosensors by simply exchanging the protease recognition sequence, cofactor binding domains, or allosteric elements to target a wide array of intracellular proteins of interest in living cells; these can then be translated into HTS assay platforms producing excellent S/N. These approaches produce several-fold changes in luminescence upon "activation", and the authors show example studies of live-cell applications using a HEK293T model cell system. Here, the luminescence change was on the order of ~20-fold. This is a highly significant result because the ability to obtain signals going from near-zero basal to multifold changes upon protein activation has never been available in the genetically encoded format. This will open up the HTS to the use of living cells to screen against functional targets under realistic *in vivo* conditions. Taken together, these characteristics will likely offer high specificity for the activation states of many protein targets, improving the overall HTS assay quality and expanding the HTS multiplexing possibilities to include living cell systems that were previously inaccessible.

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