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Emerging fluorescence sensing technologies: From photophysical principles to cellular applications

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Fluorescent molecular sensors are poised to solve problems in many disciplines. This report outlines their design basis and their applications in the fields of gene expression and neuronal activity.

After a long induction period, fluorescent molecular sensors are showing several signs of wide-ranging development (1, 2). The clarification of the underlying photophysics, the discovery of several biocompatible systems, and the demonstration of their usefulness in cellular environments are some of these signs. Another sign is that the beneficiaries of the field are multiplying. They run from medical diagnostics through physiological imaging, biochemical investigations, environmental monitoring, and chemical analysis to aeronautical engineering.

The design of fluorescent molecular sensors for chemical species combines a receptor and a fluorophore for a "catch-and-tell" operation. The receptor module engages exclusively in transactions of chemical species. On the other hand, the fluorophore is concerned solely with photon emission and absorption. Molecular light emission is particularly appealing for sensing purposes owing to its near-ultimate detectability, "off/on" switchability, and very high spatiotemporal resolution including video imaging. The commonest approaches to combining fluorophore and receptor modules involve integrated or spaced components (3).

The integrated or intrinsic sensor format relies on internal charge transfer within the excited state. The partial electronic charges so separated can interact with the target species when it is trapped by the receptor. The energy of the excited state is thereby disturbed and shows up as a blue- or red-shifted light absorption and/or emission. Roger Tsien's intracellular Ca^{2+} sensor fura-2 (Fig. 1) is a nice example (4).

The separation of charges within the spaced or conjugate sensor format occurs after excited state creation. This is photo-induced electron transfer (PET), which competes against fluorescence to dominate the energy dissipation of the excited state, i.e., fluorescence is switched off when the target species is absent. When it arrives, however, PET is arrested and fluorescence regains the upper hand, i.e., fluorescence is switched on. Czarnik's compound **1** (5), de Silva's compound **2** (6), and Calcium Green-1 from Molecular Probes (7) respond dramatically to Zn^{2+}, H^+, and Ca^{2+}, respectively (Fig. 1).

Sensors for cell-based applications developed in this manner reveal that intracellular ionic signals are heterogeneous at the single-cell level (4). To analyze whether this heterogeneity is preserved in downstream events, a sensitive, single-cell assay for gene expression was developed. The reporting molecule is the bacterial enzyme β-lactamase, which generates an amplified signal by changing the fluorescence of a substrate made available intracellularly (8).

Fluorogenic substrates, derivatives of cephalosporins, have been designed, synthesized, and tested (Fig. 2). In substrate *A*, the
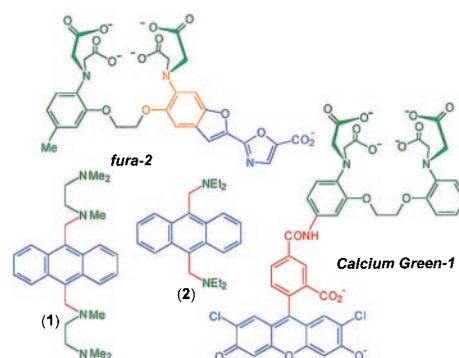

Fig. 1. Representative fluorescent sensor molecules. Color code: fluorophore = blue; receptor = green; spacer = red; mixed region = orange.

fluorophore is in conjugation with the β-lactam nitrogen that undergoes conversion from an amide to an amine on hydrolysis. This conversion leads to the desired fluorescence emission shift from 553 nm to 652 nm but is accompanied by an almost total loss of fluorescence of the product.

Substrate *B* is designed to generate an intracellularly trapped fluorescent product in a β-lactamase-initiated domino reaction. Here, a nonfluorescent rhodol derivative is attached to the cephalosporin via a carbamate linker. Hydrolysis of the β-lactam, followed by spontaneous hydrolysis of the carbamic acid and opening of the two lactones, generates the polar, green fluorescent dye. Although β-lactamase-dependent fluorescence is observed in buffers, mammalian cells become fluorescent even in the absence of reporter enzyme, presumably due to the direct hydrolysis of the linker or the opening of the lactones by intracellular nucleophiles.

In substrate *C*, β-lactam hydrolysis leads to the loss of fluorescence energy transfer between two dyes with appearance of blue donor fluorescence. An ester derivative of *C* is converted to the free substrate in living cells and detects gene expression with high fidelity and sensitivity. It reveals that receptor activation increases the probability of gene expression, with increasing stimulus levels leading to more cells expressing the reporter, as opposed to a graded increase of the response in all cells of the population (8). When messenger ion concentrations inside cells are modulated using caged reagents, it is found that the level of gene expression depends on the frequency at which the messenger is released (9).

Neurobiology has been a special beneficiary of fluorescence sensing techniques when combined with high-resolution microscopy. The reason for this is in the nature of the central nervous
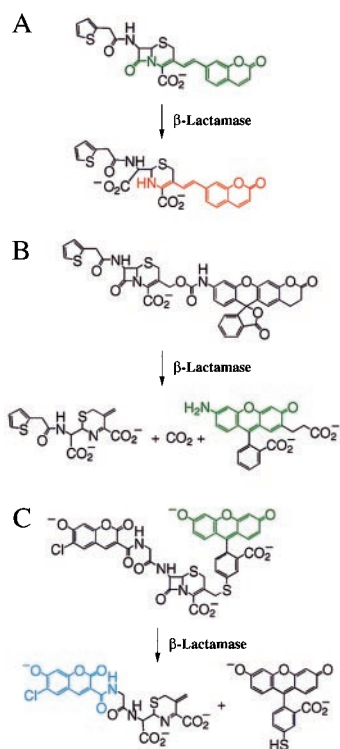

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FIG. 2. β-Lactamase activity detected by hydrolysis of a fluorescent cephalosporin (*A*), expulsion of a fluorogenic leaving group (*B*), and disruption of fluorescence resonance energy transfer (*C*).

FIG. 3. Confocal fluorescence imaging of neuronal Ca^{2+} signals during induction of LTD. (*A*) Cerebellar Purkinje neuron in a brain slice preparation. The cell was loaded with the fluorescent sensor Calcium Green-1 via a patch electrode (shown on the right). The electrical responses as well as $[Ca^{2+}]_i$ was monitored while afferent inputs were activated. *Inset* shows the localized Ca^{2+} transient that occurred during induction of LTD at these inputs. (*B*) Time course of LTD induction. The peak amplitudes of the electrical responses (EPSCs) were normalized to the mean baseline values. The horizontal bar indicates the induction period. Modified from ref. 10.

system (CNS), which represents a highly complex network of billions of cells (neurons), each of them with an elaborate morphology (Fig. 3). For an understanding of the CNS, one would like to study neuronal activity at the network level as well as with subcellular resolution.

An almost ubiquitous marker of neuronal activity is the intracellular calcium concentration ($[Ca^{2+}]_i$). Although extremely low under resting conditions (<100nM), $[Ca^{2+}]_i$ may rise to several micromolars during ongoing activity or during activity-dependent rewiring of the neuronal network (10). Although such signals can easily be studied with fluorescent molecular sensors (4), conventional microscopy in neuronal tissue is severely resolution-limited due to image blurring by out-of-focus light. Fortunately, this limitation has been overcome by confocal and, more recently, by two-photon laser-scanning microscopy (11). Thus, recordings from neuronal processes can now be performed even in intact animals, an approach that allows one to study the neuronal response to stimulation of well defined sensory inputs (12). In brain slice preparations, the optical resolution can be high enough to detect Ca^{2+} signals in femtoliter-size compartments as, for example, in single dendritic spines (13, 14). Moreover, the Ca^{2+} dynamics of distinct neuronal compartments can be monitored during the induction of long-term depression (LTD) (Fig. 3) (10), a process thought to be involved in memory formation. Such experiments assign new functional roles to neuronal calcium signals that could underlay a form of signal integration that relies on biochemical rather than on electrical computation (15).

Another application where two-photon laser-scanning microscopy has shown particularly promising results is the simultaneous fluorometric recording from up to several hundreds of dye-loaded cells in native tissue (16, 17). This approach will facilitate studies of large-scale network dynamics as well as cell migration and network formation in the developing central nervous system.

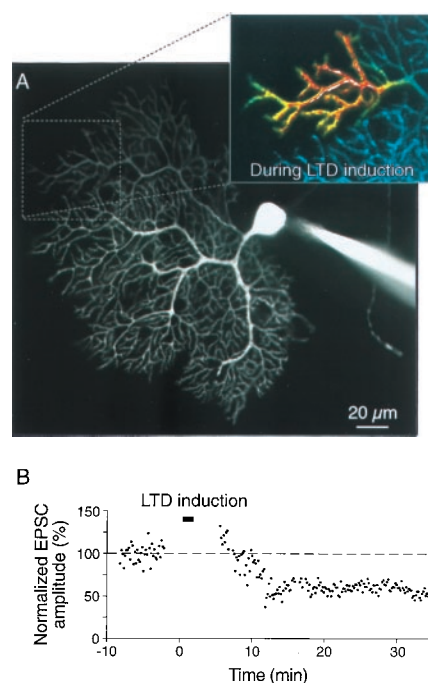Our hope is that this brief sortie will tempt the reader to explore the many possibilities that fluorescent molecular sensors are bringing to scientists at large. Yielding to that temptation can only be beneficial.

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