Desperately seeking sensors

The list of biologically active small molecules for which fluorescent sensors would be desirable is enormous. The list of sensors actually available is surprisingly small. The reason derives in part from a lack of communication between chemistry inventors and biology end-users. A new World Wide Web board has been created to address this need.

Chemistry & Biology July 1995, **2**:423–428

If there is a culture gap between chemistry and biology, it results, at least in part, from a methods credibility gap. At the recent Bioorganic Chemistry Gordon conference, one eminent chemist was heard to remark that his goal in life is 'to design an unambiguous experiment regarding enzyme mechanism'. A respectable chemistry experiment is one in which the conclusion does not contain the '..either...or...' modifier. Understanding the reaction muck that is a cell, while of some considerable significance, remains nonetheless a daunting target for a chemistry community accustomed to the luxury of physically isolating variables. Nevertheless, life is chemistry (plus a little electricity), and biological functions result from the ebb and flow of molecule concentrations within a leaky membrane beaker. By using analytical methods to determine cell chemistry, we will come to understand the dynamic mechanisms of cell biology.

While admittedly biased, I see no avenue of approach to this challenge superior to the use of fluorosensors. A dramatic example of the type of information that can be obtained using such technology is shown in Figure 1. Fluorosensors are not fluorescent stains or dyes. Sensors in general are devices that signal the presence of matter or energy reversibly and in real-time. Like catalysts, sensors are not themselves consumed by the act of sensing. In what follows, I will review the need for sensors, the desirable characteristics of a biological fluorosensor, and list the sensors that are currently available. I will also comment on the attitudes in both the biological and chemical communities that have contributed to making the list so short.

The need for sensors, and the importance of being fluorescent

In principle, some cell components can be observed directly, but in practice the spectral fingerprints of most cell components are insufficiently resolved to observe singly. Thus, most analytes can only be sensed if they bind physically to a receptor site on a properly designed sensor. Thus, the availability of receptors (either biotic or abiotic) with appropriate binding affinity and sufficient discrimination is prerequisite for chemical sensor construction.

Beyond this, chemical sensors require a means to transduce a binding event into an easily quantitated signal event. Although it is possible to use absorption or electrical changes as the signal, fluorescent signaling offers real advantages. Because fluorescence emission occurs at a different wavelength than excitation, the background signal can be made very low and the sensitivity thus very high. Electrochemical signaling does offer the

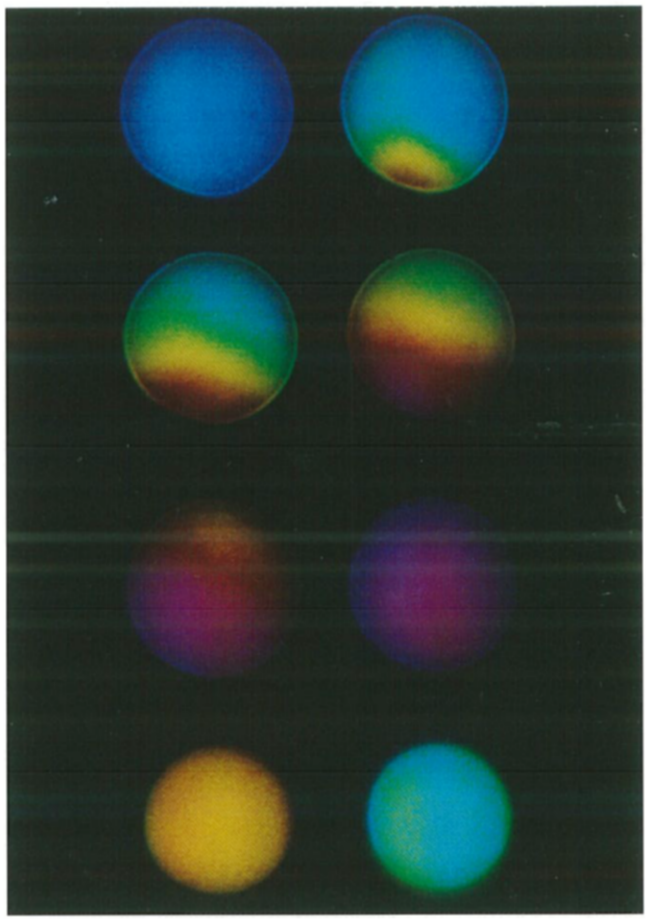


Fig. 1. Images of cytosolic free calcium in sea urchin egg (*Lytechinus pictus*) undergoing fertilization. Calcium concentrations, measured by the fluorescent indicator fura-2, are displayed as colors ranging from blue (very low Ca^{2+}) through green, yellow, orange, red, to purple (very high Ca^{2+}). From left to right, sequential views at 15, 36, 44, 52, 60, 68, 212 and 550 seconds after sperm is added to the surrounding sea water. A wave of high calcium spreads from the sperm entry point at the egg's lower left quadrant, triggering the exocytosis of the fertilization membrane and starting the program of development. Images courtesy of R. Tsien and M. Poenie.

 ISSN 1074-5521

potential of very large signal range, but at the cost of having to stick wires into a cell. Not only does light not require a wire for ingress and egress, but spatial information requires that sensors be present in every region of the cell simultaneously. Clearly, molecule-sized sensors fit the bill.

Desiderata

To detect changes in the presence and concentration of ions or other biologically active small molecules, there are some fairly straightforward criteria for a sensor that will be useful in a cellular context. These include:

Selectivity

The sensor will ideally generate a signal only as a result of the presence of one analyte. This amounts to an engineering specification, as many potentially interfering species (for example, Pb^{2+}) are not present in cells. Complete selectivity is an unachievable ideal, and we shouldn't get hung up on the fact that all sensors will signal interferences at high enough concentration.

Affinity

Just as ideal enzymes evolve to achieve K_m near the concentration of their substrate, ideal fluorosensors will be engineered to achieve K_d near the median concentrations of their analytes. By designing the sensor so that a normal concentration gives a 50 % signal, one can detect both concentration increases and concentration decreases.

Spectral properties

The desirable spectral properties of a fluorosensor include: (1) as intense fluorescence as possible in a compound retaining photostability, (2) excitation wavelengths exceeding 340 nm (to pass through glass microscope objectives and minimize UV-induced cell damage), and (3) emission wavelengths exceeding 500 nm to avoid autofluorescence (especially from NADH). Best of all would be a sensor that can be excited at a wavelength corresponding to readily available laser diode sources.

Variable detection sites

It is frequently interesting to know how concentrations are changing both inside and outside the cell. The localization of a fluorosensor will be determined by its structure.

Ease of delivery

Both drugs and sensors intended to measure intracellular concentrations of an analyte must cross cell membranes. Fortunately, delivering polar sensors to single cells is generally easier than delivering polar drugs to whole organisms, but the requirement that the sensor crosses the membrane and then becomes trapped inside is not always easy to fulfill. Naturally, toxicity remains undesirable.

Availability

Intracellular sensing is far from the only potential application of fluorosensors, but it is a very important one. Each application will bring its own criteria for what is meant by 'useful', but in every case it must be possible to buy the fluorosensor, and the sensor must have been previously shown to work, with minimal development required by the end-user. Fluorosensors that do not achieve this state will be ignored by potential end-users until they do.

The state of the art

Ask yourself: For what analytes do fluorosensors that meet this demanding list of characteristics exist? The end-user will list Ca^{2+} early. Professor Roger Tsien, currently of the University of California, San Diego, identified the need for intracellular fluorosensors in the late 1970s and has been inventing solutions ever since. The existence of fluorosensors like fura-2 and indo-1 has made the measurement of Ca^{2+} concentrations routine, and in large measure has made possible the subdiscipline called Cell Calcium. With these tools in hand, intracellular calcium concentrations can be visualized in real time (see Fig. 1).

Fluorosensors for pH, pO_2 and pCO_2 (indirect from pH) are also in common use (Table 1), and fluorosensors for Na^+, K^+, Zn^{2+}, Mg^{2+} and Cl^- exist (although continued improvement would be desirable). All of the above are examples of fluorescent chemosensors, meaning that the analyte receptor is of abiotic origin with fluorescence signaling [1]. A fluorescent biosensor for cAMP [2] has just become available commercially (from Atto Instruments, Rockville, MD).

That's pretty much the list. Now, ask yourself: For what analytes would fluorosensors be desirable? The list is long and profound. Near the top would be neurotransmitters — glutamate and acetylcholine, followed closely by glycine, aspartate and dopamine. NO and ATP sensors would be very helpful. In the long term one must aim to be able to sense all of the naturally-occurring amino acids, nucleosides, nucleotides, carbohydrates, coenzymes, inorganic phosphate and catecholamines. While we're dreaming, how about fluorosensors indicating phosphotyrosine levels of individual signal transduction proteins? You get the picture. A working fluorosensor for any one of the above analytes would create a whole new field of study.

Why so few?

The need for useful fluorosensors is acute. The chemical problems are all addressable, even if not all of them are immediately soluble. Why, then, are there still so few fluorosensors available to the scientific community?

The first step in the production of a fluorosensor is to identify or make a receptor that can be adapted to produce useful signals. Fortunately, there is an entire Molecular Recognition subfield in chemistry that exists with an eye towards understanding noncovalent bonding and receptor design. Unfortunately, most of the work done to date has not been carried out in water. However intellectually satisfying it is to invent abiotic receptors for ligands that function in organic solvents,

Table 1. Probes of dynamic biochemical signals inside living cells.

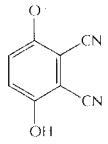
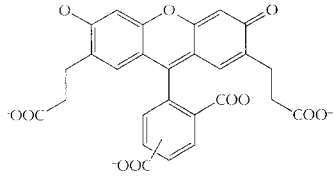
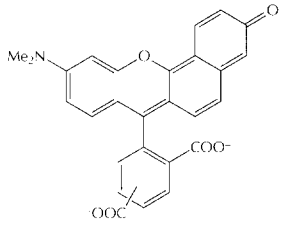
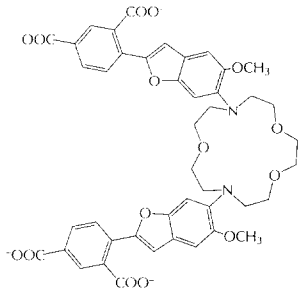
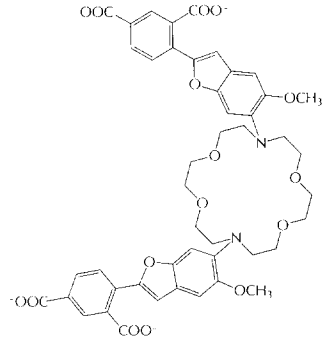
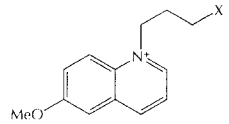
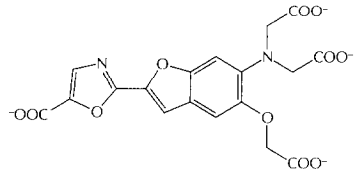
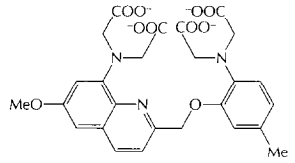
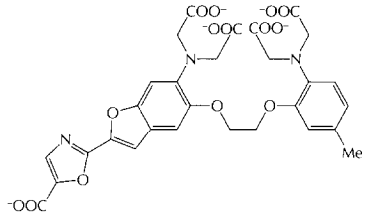
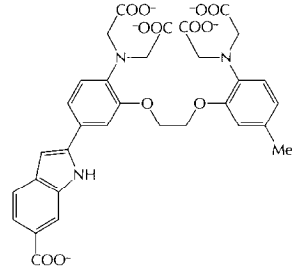
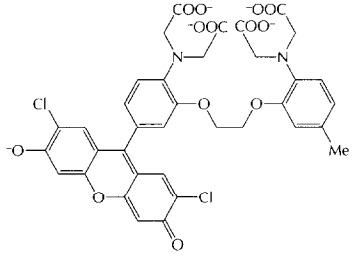
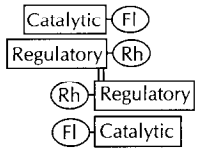
Probe	Analyte	Typical concentration in resting cells[a]	Intracellular physiological range[b]	Effective dissociation constant[c]	Year of first biological reference
 DHPN	H^+	100 nM	10–1000 nM	10 nM	1981
 BCECF	H^+	100 nM	10–1000 nM	107 nM	1982
 SNARF-1	H^+	100 nM	10–1000 nM	32 nM	1990
 SBFI	Na^+	4–10 mM	0–100 mM	18 mM	1989
 PBFI	K^+	100–140 mM	20–160 mM	100 mM	1989
 SPQ: X = SO_3^-	Cl^-	5–100 mM	0–100 mM	83 mM	1987
TMAPQ: X = NMe_3^+	Cl^-	5–100 mM	0–100 mM	50 mM	1992

Table 1 (continued).

Probe	Analyte	Typical concentration in resting cells[a]	Intracellular physiological range[b]	Effective dissociation constant[c]	Year of first biological reference
 FURAPTRA (Mag-fura-2)	Mg^{2+}	0.5–2 mM	0.2–5 mM	1.5 mM	1989
 quin-2	Ca^{2+}	50–200 nM	10 nM–10 μM	115 nM	1980
 fura-2	Ca^{2+}	50–200 nM	10 nM–10 μM	224 nM	1985
 indo-1	Ca^{2+}	50–200 nM	10 nM–10 μM	250 nM	1985
 fluo-3	Ca^{2+}	50–200 nM	10 nM–10 μM	400 nM	1989
 FlCRhR	cAMP	<50 nM	0–10 μM	100 nM	1991

Information taken from [1].
[a]Crude estimates for the cytosol of typical mammalian cells not being stimulated or knowingly perturbed.
[b]Approximate range encountered in stimulated or reasonably perturbed cells.
[c]Effective dissociation constant for analyte from the chemosensor in mammalian cytosol or ionic media mimicking cytosol.

what we learn about binding in organic solvent often does not teach us usefully about binding such ligands in water. The community needs to turn its prodigious intellect and creativity to the issues of aqueous binding. Only then will the tremendous potential of molecular recognition studies for understanding biological systems be realized. The problem is not simply one of focus; funding agencies must recognize that these problems are hard and will not be solved overnight. Calcium sensors have taken a decade to bring to practical fruition [3], despite being addressed by a brilliant individual who had the advantage of starting with a good calcium ion receptor (EGTA).

An equally fundamental issue is the need for long-wavelength fluorophores. Ideally, excitation and emission should be well resolved and should occur above about 500 nm to avoid endogenous cellular absorbance and autofluorescence. Most of the currently available sensors follow this rule (Table 2). Longer wavelength is better, and near-IR would provide the added benefit of later application to whole-organism systems. (Place a flashlight behind your hand; the red light you see tells you which band is not being absorbed). Many existing fluorophores with these spectral properties are insoluble and unstable in oxygenated aqueous solution. Many yield broad emission bands that will ultimately inhibit the simultaneous sensing of multiple analytes (imagine it!). Ideally, the fluorophore should use wavelength shift (ratiometric) signaling rather than relying on intensity changes, but even signaling with intensity changes is far from useless.

Once we have a selective receptor, and an appropriate fluorophore, we still need to build in fluorescent signal transduction. It is almost certainly not enough simply to conjugate a receptor site with a fluorophore. The precise type of conjugation will determine whether a large signal or a small signal is transduced upon binding, and what type of signal it is. Fortunately, there are multiple mechanisms by which to modulate the fluorescence output (such as photoinduced electron transfer, fluorescence resonance energy transfer, ligand-induced deconjugation and fluorescence lifetime modulation). Unfortunately, our ability to predict the structural requirements for fluorescence is still as immature as our ability to predict inhibitor activities. The best calcium fluorosensors evolved from a largely empirical search for properties. Figure 2 shows how indo-1 signals the binding of Ca^{2+}. The experience that trial and error are still important to fluorosensor design suggests that combinatorial chemistry approaches to the discovery of both binding and fluorescence properties would be powerful, if effective library generation schemes can be invented [4].

Table 2. Properties of fluorosensors from Table 1.

Probe	Typical excitation wavelengths[a] (nm)	Typical emission wavelengths[a] (nm)	Best detection mode[b]
DHPN	360/420	455/512	Em. ratio
BCECF	440/490	530	Exc. ratio
SNARF-1	517/576	587/640	Em. ratio
SBFI	340/385	530	Exc. ratio
PBFI	340/350	530	Exc. ratio or intensity
SPQ	350	442	Intensity
TMAPQ	355	450	Intensity
FURAPTRA (Mag-fura-2)	335/370	510	Exc. ratio
quin-2	340	505	Intensity
fura-2	340/380	505	Exc. ratio
indo-1	331/349	405/485	Em. ratio
fluo-3	500	530	Intensity
FlCRhR	490	520/590	Em. ratio

Information taken from [1].
[a]Typical excitation and emission wavelengths used for measuring analyte. These do not necessarily correspond to spectral maxima, because observing wavelengths are often displaced to improve discrimination between spectra of complexed and free forms or separation between excitation and emission wavelengths. When a pair of wavelengths is given, the first emphasizes the fluorescence of the complexed form while the second is preferential for the free chemosensor. Excitation and emission bandwidths are often considerably widened in order to maximize detection sensitivity.
[b]Intensity = measurement of fluorescence intensity at fixed wavelengths; Exc. ratio = measurement of fluorescence at two separate excitation wavelengths and a common emission band and ratioing of the two excitation amplitudes; Em. ratio = measurement of fluorescence at a common excitation wavelength and two separate emission bands and ratioing of the two emission amplitudes.

A call for crosstalk

Finally, a bit of editorializing. Science is a human endeavor, and some of the energetic barriers to new fluorosensor discovery are not scientific. The first is entropic. The average chemist with the background to discover new fluorescent chemosensors neither knows which analytes are of greatest interest to cell biologists, nor knows how to test new sensors in cells. For this reason, the staff of Current Biology has created a bulletin board on the World Wide Web (http://www.cursci.co.uk/BioMedNet/cmb/cmbinf.html) on which biologists are encouraged to post information on the analytes that are of interest to them (together with information on specific requirements of their system), and to sign up as beta testers of new sensors. Chemists are encouraged to monitor this board. If successful, this forum will truly advance the goals of *Chemistry & Biology* to stimulate cross-fertilization between the two communities.

The other barrier is enthalpic in nature (in other words, it can generate heat). There is concern in some chemistry circles that chemistry is becoming little more than a service function for biological research. My friends in the biological sciences assure me that this is not a perception pervasive in their community. Even so, a chemist investing years in the creation of a useful new sensor must

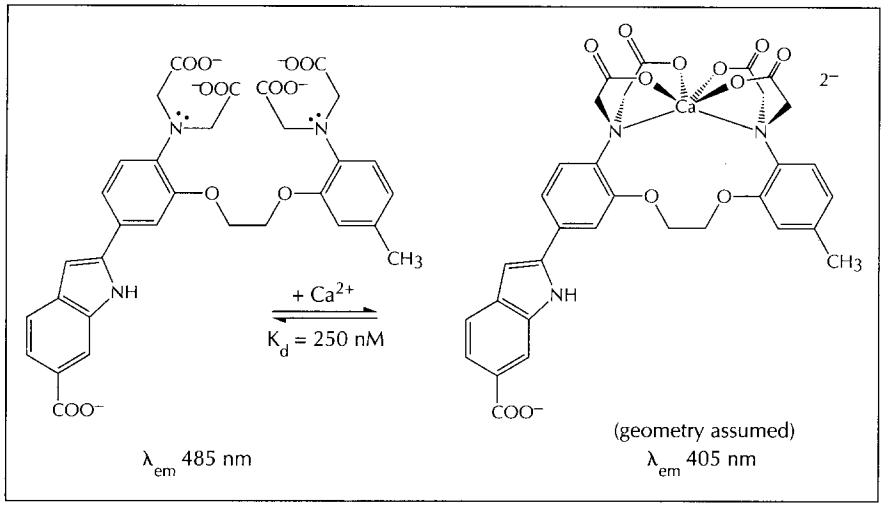


Fig. 2. Fluorescent signaling by indo-1. Coordination of calcium ion to the ligand results in the deconjugation of two aromatic amine groups. This change in geometry changes the conjugated π-system of the fluorophore, and in this case changes the emission wavelength from 485 nm to 405 nm.

receive recognition for the effort. It is the chemistry community that needs to recognize the accomplishment and the intellectual challenge. Non-synthetic end-users cannot be expected to appreciate the difficulties involved in providing a new sensor, however much they may revere the person who provides one. If we do not want the accomplishments of chemists who take on these challenges to be undervalued, we must start by valuing them ourselves.

Any agency with an interest in promoting discovery in cell biology and medicine should be interested in promoting the solving of these scientific problems. The communities that are best equipped to address these issues do not even know of the specific needs that exist. The best way forward would be to identify the most acute needs and advertise the opportunities to the Molecular Recognition chemistry community. There is plenty of work to go around. And it is pure pleasure to offer a rationale as compelling as this one for investing public funds in chemistry research.

References:

1. Tsien, R.Y. (1992). Probes of dynamic biochemical signals inside living cells. In *Fluorescent Chemosensors for Ion and Molecule Recognition*. (Czarnik, A.W., ed.), pp 130–146, ACS Books, Washington, D.C.
2. Adams, S.R., Harootunian, A.T., Buechler, Y.J., Taylor, S.S. & Tsien, R.Y. (1991). Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* **349**, 694–697.
3. Tsien, R.Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404.
4. Ellman, Jonathan A. (1995). Combinatorial organic libraries. ChemTracts-Organic Chemistry **8**, 1–4.

Anthony W Czarnik is Director of Bioorganic Chemistry at Parke–Davis Pharmaceutical Research and adjunct Professor of Chemistry at the University of Michigan, Ann Arbor, MI 48105, USA.