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More than a Light Switch: Engineering Unconventional Fluorescent Configurations for Biological Sensing

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Abstract: Fluorescence is a powerful and sensitive tool in biological detection, used widely for cellular imaging and \textit{in vitro} molecular diagnostics. Over time, three prominent conventions have emerged in the design of fluorescent biosensors: a sensor is ideally specific for its target, only one fluorescence signal turns on/off in response to the target, and each target requires its own sensor and signal combination. These are conventions but not requirements, and sensors that break with one or more of these conventions can offer new capabilities and advantages. Here, we review “unconventional” fluorescent sensor configurations based on fluorescent dyes, proteins, and nanomaterials such as quantum dots and metal nanoclusters. These configurations include multi-fluorophore FRET networks, temporal multiplexing, photonic logic, and cross-reactive arrays or “noses.” The more complex but carefully engineered biorecognition and fluorescence signaling modalities in unconventional designs are richer in information, afford greater multiplexing capacity, and are potentially better suited to the analysis of complex biological samples, interactions, processes, and diseases. We conclude with a short perspective on the future of unconventional fluorescent sensors, and encourage researchers to imagine sensing beyond the metaphorical light bulb and light switch combination.

Keywords: biosensor, FRET, quantum dot, gold nanoparticle, multiplexing, molecular logic, chemometrics, chemical nose.
Introduction

Fluorescence is a hugely powerful tool for bioanalysis. It has provided indispensable insight into biological structures and mechanisms, as well as molecular detection, expression, and interactions. Fluorescence methods and their many advantages have displaced alternative colorimetric and radiometric methods in many applications. Advantages include sensitivity that can reach the level of single molecules; flexibility in retrofitting various experimental setups (e.g. microfluidics, electrochemistry\(^2\)) with components for fluorescence spectroscopy and imaging; spatial and temporal resolution across many orders of magnitude (e.g. super-resolution imaging,\(^3,4\) dynamic tracking); multiparametric encoding of information in the form of intensity, wavelength, anisotropy, and lifetime; and potential to respond to physicochemical attributes of the local environment (e.g. temperature,\(^5\) viscosity,\(^6\) pH\(^7\)) and molecular interactions. The latter responses are typically via Förster resonance energy transfer (FRET),\(^8\) photoinduced electron transfer (PET),\(^9\) aggregation,\(^10\) frustration of molecular motions (e.g. rotation) or photochemical processes (e.g. proton transfer).\(^11\) Given the above, it is no surprise that fluorescence methods have an irreplaceable role in the present and future of chemical biology.

There are three general types of fluorescence methods relevant to chemical biology and other fields of bioanalysis: assays, staining and labeling, and sensing. By typical definition, sensing differs from assays, staining, and labeling in its active rather than passive fluorescence signaling. The probes used in assays and staining or labeling protocols tend to have their fluorescence turned “on” permanently. Washing steps are therefore required to remove excess and unbound materials, and measurements are typically at a single time point. In contrast, fluorescent sensors turn “on” or “off,” change color, or otherwise modulate their fluorescence in response to their chemical or biomolecular target. Washing steps are not required and real-time tracking of signal changes is possible. Fluorescent sensors have been designed to detect a vast number of biologically important analytes, including but not limited to proteins, nucleic acids, carbohydrates, and small molecules and ions, whether in solution, on the membrane or within the interior of cells, and even \textit{in vivo}.\(^1,12\)
There is widespread interest in using fluorescent sensors to address challenges in chemical biology and bioanalysis. For \textit{ex vivo} measurements, a particularly important challenge is the detection of disease through the levels of certain biomarkers in blood, urine, and other bodily fluids. For example, the proteins troponin, myoglobin and creatine kinase are markers of myocardial infarction.\textsuperscript{13} Carbohydrates such as hyaluronic acid are markers of fibrosis when free in the blood,\textsuperscript{14} or implicated in tumor progression.\textsuperscript{15} Nucleic acids are widely used in pathogen detection,\textsuperscript{16,17} and small molecules such as glucose, urea, and creatine report on pancreatic, kidney and liver function.\textsuperscript{18} For \textit{in vitro} measurements, important challenges are understanding both healthy cell functions and disease pathologies. Examples include, but are not limited to, detection of intracellular mRNA\textsuperscript{19} and single nucleotide mutations,\textsuperscript{20} as well as cell surface receptor dynamics.\textsuperscript{21} The least developed context for fluorescent sensors is \textit{in vivo}; however, there is great potential for imaging the molecular hallmarks of diseased tissue, whether for diagnostics or for guided surgery. These techniques have been particularly studied for the detection of cancers and their treatment.\textsuperscript{22}

A survey of the literature on fluorescent sensors reveals two conventions that are followed in almost all designs. The first convention is that a sensor is designed to be as selective as possible (\textit{i.e.} specific) for its chemical or biomolecular target. For example, an antibody or aptamer ideally binds only its target protein without cross-reactivity with other proteins, an oligonucleotide ideally hybridizes only with its perfect complement, and a substrate is ideally only turned over by a target enzyme. Although real sensors do not have such perfect specificity, this idealized concept is still the basis of their design and optimization. The second convention is that these highly-selective sensors respond to their targets through modulation of only one fluorescent signal. The outcome of these two conventions is a third convention: detection of $N$ targets is through deployment of $N$ probes, each with a unique chemical or biomolecular event that modulates the $N^{th}$ fluorescent signal. These signals are designed to be as orthogonal to one another as possible, typically as different colors of fluorescence with corrections for signal crosstalk or cross-reactivity between sensors as necessary. In other words, $N$ independently actuated light switches and light bulbs.
Here, we review recent developments in fluorescent sensor design that break with one or more of the conventions noted above. Some well-known conventional fluorescent sensor designs are first noted as a baseline for comparison, followed by a short overview of the materials used in the unconventional designs of fluorescent sensors that we highlight next. These designs include sensors with FRET networks that have multiple co-dependent signals but still offer orthogonal detection of multiple targets, sets of sensors that separate signals as a function of time rather than wavelength, logic sensors that that require multiple chemical or biomolecular events to output only one fluorescent signal, and cross-reactive array-based sensors that utilize a small number of fluorescent signals to report on a much larger number of targets. None of these designs can be described as a switch for single light bulb. We discuss how the capabilities of each unconventional fluorescent sensor design will develop and potentially impact chemical biology and bioanalysis in the future, including a more global perspective to conclude the review.

**Examples of Conventional Fluorescent Sensors**

Many hundreds of conventional fluorescent sensor designs have been reported. We present here a small selection of illustrative examples for comparison to unconventional designs. Readers can find many more examples of conventional fluorescent sensors in other review articles.\(^{23-25}\)

One common class of fluorescent sensors combines biomolecular probes with environmentally-responsive dyes that increase in quantum yield when target is bound. For example, Spinach probe binds and activates 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) dye,\(^{26}\) fluorescent nucleobases in an oligonucleotide probe activate upon hybridization,\(^{27}\) and both enable sequence-specific DNA and RNA sensing. These designs are examples of ‘turn on’ sensors.
Figure 1. Conceptual illustrations of some conventional (A-B) and unconventional (C-F) fluorescent sensors. FRET is shown as the most common process for modulating emission signals, where D represents the donor and A the acceptor. (A) A beacon-like sensor that changes conformation and turns on FRET upon binding target. (B) A sensor where FRET is turned off by the hydrolytic activity of an enzyme. (C) A QD-based concentric FRET sensor as an example of a FRET network. Its spectral emission pattern reports on the activity of two (or more) targets. (D) Temporal multiplexing with a Tb LLC donor: (i) time-gated FRET; and (ii) different acceptor emission lifetimes as two signals for two targets, obtained by control over the Tb LLC-acceptor separation distance. (E) Hypothetical AND logic sensor design with FRET to obtain Boolean-like output (T = true, F = false) from binding of two targets. (F) Semi-selective array sensor for detection of multiple targets with chemometric analysis of emission intensity patterns. Binding between target and sensor turns emission either on or off.

Other common classes of fluorescent sensors are based on energy and electron transfer processes (e.g. FRET, PET). These processes have a strong dependence on the distance between donor and acceptor, operating over length scales (1–10 nm) that are commensurate with the size of many biological molecules. Sensing is achieved by increasing or decreasing the donor-acceptor distance, typically through association, dissociation, or conformational changes in response to target, thereby changing the efficiency of the process, as depicted in Figure 1A-B. These designs can be ‘turn-on,’ ‘turn-off,’ or ‘color-changing’ depending on the direction of distance.
modulation and whether or not the acceptor is fluorescent. One of the original sensor designs based on FRET was a molecular beacon, which is a hairpin oligonucleotide labeled at opposite termini with a fluorophore and a quencher. When a target nucleic acid strand or other analyte binds to the loop of the hairpin, the distance between the fluorophore and quencher is increased, and so the fluorescent output is increased.\(^{28}\) Similar mechanisms can be utilized for other types of target; for example, peptide sequences labeled with donor and acceptor dyes at opposite termini to monitor hydrolytic enzyme activity,\(^{29,30}\) physical displacement of an acceptor-labeled ligand from a donor-labeled receptor by a competitive-binding analyte, or a sandwich assay where the analyte itself mediates proximity between donor and acceptor.\(^{31}\) Sensor systems can also be constructed to actuate a conformational change in a protein linker upon binding metal ions, as exemplified by the Ca\(^{2+}\) sensors that modulate the distance and thus FRET between two fluorescent proteins, altering the emission colors.\(^{32}\)

The foregoing sensor designs, which follow the conventions noted in the introduction, have served biological and analytical chemists well for two decades; however, new fluorophores and new ways of engineering fluorophore systems have emerged to enable more information-rich outputs than simple on/off switching. Several of these unconventional sensor designs are illustrated in Figure 1C-F, and are discussed throughout the remainder of this review.

**Materials to Enable Unconventional Fluorescent Sensors**

Evolving and enhancing the properties of fluorescent materials is key to the advancement of both conventional and unconventional fluorescent sensor designs. This section addresses the basic properties of selected ‘old’ and ‘new’ materials, shown in Figure 2. Repurposing of old materials and the emergence of new materials has enabled several new concepts in fluorescent sensing. A detailed discussion of each material is beyond the scope of this review; however, interested readers can find comprehensive reviews of each the following materials in refs.\(^{33,34}\) and via the citations after each subheading.
Figure 2. Common materials for engineering fluorescent sensors, drawn roughly to scale, alongside an AuNP, which is often used as a fluorescence quencher. Green fluorescent protein (FP) represents fluorescent proteins more generally, and a Tb cryptate is an example of an LLC. The grey volume around the metal nanocluster, QD, and AuNP represent an approximate space occupied by surface ligands.

Molecular fluorophores.\textsuperscript{23,35,36} The most common fluorophores for conventional sensors are small conjugated dye molecules.\textsuperscript{35} Quantum yields vary but can exceed 50%, and many popular dyes from the xanthene, cyanine, coumarin, and BODIPY families are widely available with reliable procedures for bioconjugation.\textsuperscript{36,37} Fluorescent proteins (FPs), which have a small molecule fluorophore center inside a barrel-shaped protein, are also common. FPs are inherently biocompatible, can be expressed and fused to other proteins \textit{in situ} through standard molecular biology techniques,\textsuperscript{38} and, like dyes, are available in the full rainbow of colors.\textsuperscript{39,40} Frequent disadvantages of dyes and FPs versus other materials include more narrow absorption bands, smaller spectral separation between excitation and emission, broader emission bands, sensitivity to the surrounding environment (\textit{e.g.} pH), and lower resistance to photobleaching.\textsuperscript{36} FPs in particular suffer from long maturation times, that can hinder dynamic \textit{in-situ} measurements. The lack of functionalizable surface area also limits the ways in which fluorescent dyes can be integrated with other materials. Nonetheless, fluorescent dyes and FPs remain popular—even in unconventional sensor designs—because their combination of small size, brightness, availability and convenience of use remain unmatched.

Lanthanide complexes.\textsuperscript{41-43} Luminescent lanthanide complexes (LLCs) are of interest because of their unusual photophysical properties. The occurrence of typically forbidden $f$-$f$ electron transitions in lanthanide ions ($\text{Tb}^{3+}$, $\text{Eu}^{3+}$, $\text{Dy}^{3+}$, $\text{Sm}^{3+}$) yields multiple sharp emission lines in the visible spectrum and emission lifetimes in the microsecond to millisecond regime. Multidentate
ligands or cryptands bind the lanthanide ion, enhance its photosensitization, provide large spectral separation between excitation and emission wavelengths, and offer chemical handles to enable bioconjugation.\textsuperscript{41,42} As will be seen for some unconventional sensors, the narrow emission lines and long lifetime of LLCs are more advantageous for some applications than their low brightness is disadvantageous.

**Quantum dots.**\textsuperscript{44-46} Colloidal semiconductor nanocrystals or “quantum dots” (QDs) are one of the best and most widely used fluorescent nanoparticles, with CdSe/ZnS core/shell and related materials at the forefront.\textsuperscript{44,45} Their advantageous properties include spectrally broad absorption across the UV and visible regions; spectrally narrow emission that can be precisely tuned across the visible region and into the IR through control of nanocrystal size and material composition; and emission that is much brighter and far more resistant to photobleaching than that of most dyes.\textsuperscript{46-48} Like other nanoparticles,\textsuperscript{22,49} QDs have surface area upon which various chemistries and tools can be engineered. Surface chemistry is largely controlled by appending ligands or polymer coatings, and a huge variety and large number of biomolecules can be conjugated to a single QD.\textsuperscript{50,51} This capability has also been widely exploited to array multiple copies of other chromophores and fluorophores around a central QD, frequently for the development of FRET sensors.\textsuperscript{9,52,53} The value of QDs in the design of unconventional sensors thus arises from both their fluorescence properties and surface engineering.

**Metal nanoclusters.**\textsuperscript{54,55} Noble metal nanoclusters are an emerging nanoparticle fluorophore with long-lived emission ($10^{-8}$–$10^{-5}$ s) tunable across the visible and NIR spectrum. The clusters comprise \textit{ca.} 18–100 gold and/or silver atoms, and the color of emission and brightness is modified through changes to composition (number of atoms, dopants) and surface ligands.\textsuperscript{55-57} Metal nanocluster emission tends to be broad and the brightness low. The energy and charge transfer mechanisms for these systems are non-typical and currently an area of great interest.\textsuperscript{58-60}

**Quenchers.**\textsuperscript{61} It is often useful to turn fluorescence on/off, for example, by FRET, without introduction of a second FRET-sensitized fluorescence signal. The two most common quencher materials are molecular ‘dark quenchers,’ which are dyes that relax energetically without fluorescence (zero quantum yield),\textsuperscript{62} and gold nanoparticles (AuNPs), which are generally
thought quench fluorescence through a non-FRET mechanism of energy transfer.\textsuperscript{34,63} AuNPs can be synthesized in a range of sizes (2–200 nm) and can be surface functionalized and bioconjugated through a wide variety of chemistries.\textsuperscript{64} Like QDs, many sizes of AuNP support attachment of a huge variety and large number of biomolecules and fluorophores.\textsuperscript{65} The range of molecular dark quenchers is typically limited to less than 10 nm, whereas many AuNPs can still efficiently quench fluorescence at distances in excess of 10 nm.\textsuperscript{66,67} Both materials have been widely used in conventional fluorescent sensor designs,\textsuperscript{68} and remain useful components for unconventional sensor designs.

**FRET Networks**

Conventional FRET multiplexing relies on $N$ orthogonal probes and fluorescent signals for $N$ targets. Networked FRET seeks to achieve multiplexing without true signal orthogonality, with interdependent FRET signals reporting on one or more targets. In this manner, more targets can be detected with fewer sensors and fewer fluorescence signals.

As noted earlier, FRET is a powerful sensing tool. While the use of QDs as donors or acceptors has advanced multiplexed FRET sensing beyond what is practical with sensors based on dyes and FPs, most sensor designs with QDs have still followed convention. Selected examples of conventional multiplexed FRET sensing with QDs include work by the groups of Hildebrandt,\textsuperscript{69} Stevens,\textsuperscript{70} and Willner\textsuperscript{71} for detection of three cancer-related miRNA sequences, two mRNA markers of tuberculosis in human serum, and three DNA sequences, respectively. The multiple targets were detected by an equal number of sensors, each with its own color of QD. However, the utility of $N$ different colors of fluorophore for $N$ probes in conventional multiplexing is limited by the number of emissions (considering their non-zero bandwidth) that will fit within the visible spectrum without prohibitive overlap between the $N$ signals. Although the cited examples somewhat mitigate this limitation through the use of QDs (vs. dyes), dark quenchers, or a common donor (e.g. LLC) across multiple FRET pairs, there are also the non-trivial matters of synthesizing and delivering multiple FRET probes into a system at appropriate concentration and location, and compensating for intrinsic differences in brightness between probes.
Figure 3. Networked FRET sensors. (A) cFRET imaging of the activity of chymotrypsin (yellow) and trypsin (red), as model proteases. (i) The sensor has two fluorescent dye-labeled peptide substrates, colored to match the target protease. FRET pathways are shown (1a, 1c, 2b) alongside the microscope configuration for three-color imaging. (ii) Representative time series of emission ratio images for tracking the activity of the proteases as they diffuse inward from opposite sides of a capillary containing the QD sensor. These images can be analyzed to calculate progress curves for hydrolysis at any point in the image (not shown). Adapted with permission from ref. 72. Copyright 2015 American Chemical Society.

(B) A concentric FRET system for measurement of the activity of three proteases: (i) model of the sensor, showing the central QD and three peptide substrates with three fluorescent dye labels; (ii) absorption and emission spectra for the QD and dyes; (iii) the FRET network. Four colors of emission are analyzed as three emission ratios, each dependent on the balance of competitive (1a-c, 2a-b) and sequential (1-3) FRET pathways. The sensor in panel B uses a subset of this network. Reprinted with permission from ref. 73 under a CC BY license.

(C) DNA is useful for constructing FRET network sensors. In this example, a FRET relay between four dyes (D1-D4) is disrupted when various strands are displaced from sensor by its targets (T1-T3). The targets are detected from the change in the spectral patterns of fluorescence excitation and emission. Reproduced from ref. 74 with permission under a CC BY license.
Networked FRET for the detection of multiple analytes with a single fluorescent sensor entity has been implemented by incorporating multiple FRET donors and acceptors within the same sensor architecture. For example, our group demonstrated a system that used a single QD as a scaffold and an initial FRET donor for multiple copies of two different fluorescent dyes, 1 and 2, which were themselves a donor-acceptor pair, as diagrammed in Figure 3A. In this system, which was christened concentric FRET (cFRET), the QD transfers energy to both Dye 1 and Dye 2, and Dye 1 subsequently transfers energy to Dye 2, creating a network of multiple energy transfer pathways. The combined fluorescence signal output from the QD and two dyes depends on how many of each FRET pathway (QD→Dye 1, QD→Dye 2, Dye 1→Dye 2) are available. If Dye 1 is removed from the QD, then the pathways are perturbed in a different way than if Dye 2 is removed. The size of perturbation depends on the number of each dye removed. All FRET pathways and fluorescence signals are probed simultaneously as Dye 1/QD and Dye 2/QD emission ratios. The combination of emission ratios, not the individual ratios, is unique to the precise number ratio of each dye per QD and can thereby report orthogonally on two analytes simultaneously.

The first cFRET system reported on the activity of two different proteases with Dye 1 and Dye 2 attached to the QD by two peptide substrates, one selectively targeting each protease. cFRET sensing of protease activity has been implemented in spectrofluorimetric and imaging formats, the latter depicted in Figure 3A, and extended to the detection of nucleic acid targets via toehold mediated strand displacement. Alternate colors of materials have also been used in a cFRET system to obtain longer-wavelength fluorescence for improved signal-to-background ratios in biological media. Most recently, cFRET has been extended to a one QD-three dye system, expanding the network of energy pathways to enable the simultaneous detection of three proteases with one sensor. This configuration and its FRET network are depicted in Figure 3B. A variation on the cFRET concept was also developed with a quenching network instead of a FRET network. Here, a combination of charge transfer and FRET acted on the central QD, again with the capability to detect two targets as one sensor.
Currently, cFRET systems are the most sophisticated FRET networks for multiplexed sensing; however, FRET networks based on DNA nanostructures have great potential. Two recent examples are linear multi-strand assemblies of oligonucleotides. Liang et al. were able to demonstrate *in vitro* and intracellular detection of two DNA or RNA targets, albeit qualitative, with one FRET sensor.\(^7\) A common donor dye was used for two different dye acceptors within the same sensor assembly. As shown in Figure 3C, Zhang *et al.* created a FRET cascade with a series of four dyes for the detection of three different DNA targets.\(^7\) Although the pattern of fluorescence emission encoded which targets were present in the sample, catastrophic disruption of the FRET cascade by any one target required interrogation of the sensor with multiple excitation wavelengths. This limitation and similar ones are likely to be overcome by expanding DNA FRET networks from one-dimensional to multi-dimensional structures in order to host multiple copies of multiple fluorophores. In a preliminary example, Brown *et al.* explored the potential for sensing with DNA dendrimers that had a four-dye FRET cascade from the 3\(^{rd}\)-generation periphery to the 0\(^{th}\)-generation core.\(^7\) Multi-dye, three-dimensional DNA nanostructures will readily support FRET networks that are only partially disrupted by binding of nucleic acid targets, and thereby enable single-excitation-wavelength sensing of multiple targets through changes in the spectral pattern of emitted fluorescence, analogous to cFRET.

FRET networks and their ability to detect multiple targets as a single sensor are conceptually ideal for investigating cellular signaling cascades and networks.\(^8\) To firmly understand the interplay between multiple components (*e.g.* first and second messengers, effectors) in these complex stepwise processes, single cells must be imaged in different scenarios, and multiple targets and activities simultaneously monitored in real time. In a similar vein, FRET network sensors also stand to be useful for identifying and tracking one of two or more competing pathways without *a priori* knowledge of which pathway will be followed; for example, the intrinsic or extrinsic pathways of apoptosis,\(^8\) or enzyme activity in DNA repair pathways,\(^8\) as a function of external stimuli. These prospective applications are further enhanced by the anticipated ability of networked FRET sensors to extend multiplexed detection to the level of a single sensor entity, offering the potential to interface with single-molecule fluorescence, localization, and super-resolution imaging to achieve new levels of spatial and temporal detail.
**Temporal Multiplexing**

With almost all conventional fluorescent sensors, multiplexing is achieved by separating signals by wavelength. Moving beyond this paradigm, there is the possibility of using time as an additional dimension for separating signals.
Fluorescence decay times are typically on the order of nanoseconds, but some materials have much longer-lived emission, on the order of microseconds or longer. Examples are the LLCs and noble metal nanoclusters discussed previously.\textsuperscript{85} LLCs are particularly useful in this respect because their emission decay over $10^{-4}$–$10^{-3}$ s enables relatively facile time-gated measurements, which are a built-in feature of many modern fluorescence plate readers. These measurements follow a pattern of pulsed excitation, a waiting period of microseconds, and then a period of signal acquisition. It is thereby possible to separate longer-lived emission from shorter-lived emission, even with similar emission wavelengths. With more sophisticated instrumentation, more detailed temporal information can be obtained by measuring full emission decay curves. Both formats enable multiplexing in the time domain as well as the color/wavelength domain.

Sensing based on emission decay curves takes advantage of the sensitivity of the rate of FRET to the distance between donor and acceptor. Different distances and rates of FRET result in different lifetimes of emission for the donor. Importantly, when the donor has a much longer lifetime than the acceptor (as is the case with LLCs), the acceptor emission decay will mirror that of the donor. Multiple FRET sensors for multiple targets can thus be designed with the same donor and acceptor pair by varying their separation between sensors.

Qiu et al. demonstrated the above concept for the multiplexed detection miRNA, as shown in Figure 4A.\textsuperscript{83} To detect two targets, sensors were designed with a sandwich binding format and utilized a QD as the acceptor for a Tb LLC donor. Two sensors with different distances between the QD and Tb LLC were achieved by using two methods to conjugate an oligonucleotide probe to the QD: direct conjugation, and indirect conjugation through a streptavidin spacer that yielded a distance 2.4 nm longer on average. The rate of FRET from the Tb LLC to the dye thus varied.
between the two sensors and each had a characteristic acceptor decay curve. To resolve detection of the two miRNA targets, the decay curves for the QD were divided into two time windows and the emission intensity within each window was recorded as a signal. It was possible to unmix the individual sensor signals to orthogonally quantitate each target because each sensor made a different relative contribution to the total signal in each time window. Proof of concept was also demonstrated for the detection of three targets via measurements in three time windows. Analogous to \( N \) colors for \( N \) targets, \( N \) time windows are needed for \( N \) targets. Given that the emission properties of Tb LLCs enable their concurrent use as donors for many different dye acceptors, the combination of multiple colors of acceptor and multiple time windows has potential for double-digit multiplexing capacity.

In another pair of examples, we utilized a clean separation in time between two signals for multiplexed sensing. The basis of these sensors was a FRET relay system where a Tb LLC was a donor for a QD acceptor and, in turn, the QD was a donor for a dye acceptor. The QD and Tb LLC were both excited by a pulse of excitation light and emission was measured immediately after excitation and after a 55 µs time gate. The mismatch in lifetime was such that the QD acted as a donor for the dye in the immediate or “prompt” time window, then switched its role to an acceptor for the Tb LLC in the time-gated window, followed by donor (again) for the dye. As proof of concept, two DNA sequences, one bearing a Tb LLC label and the other a dye label, were quantitatively detected upon binding to complementary oligonucleotides conjugated to the QD. The signals were the immediate Dye/QD emission ratio and the combined time-gated intensity of the QD and dye. A sensor based on this configuration was developed for the parallel detection of the activity of two proteases by conjugating the QD with two peptide substrates, one bearing a Tb LLC label and the other a dye label, as depicted in Figure 4B. The signals were the prompt Dye/QD emission ratio and the time-gated QD/Tb LLC emission ratio, both of which were altered by proteolytic removal of the Tb LLC and dye labels from the QD with loss of FRET.

Temporal multiplexing via time-gating has the potential to overcome scattering and autofluorescence backgrounds in tissues, enabling sensing experiments that would otherwise only be possible with cultured cells. Temporal rather than spectral multiplexing also leaves more
of the spectrum available and may permit sensing of multiple targets in conjunction with traditional cellular labelling and staining. This concept builds on the success of conventional colocalization fluorescence imaging in elucidating cellular function and transport,\textsuperscript{88} with the added power of active sensing. Alternatively, the high density of multiplexing promised by detection channels derived from combinations of multiple colors and time windows could, for example, enable real-time RNA imaging experiments of live cell systems with a number of a target genes that would otherwise require non-imaging methods (\textit{e.g.} PCR) or fixation (\textit{e.g.} \textit{in situ} sequencing, FISH, mass spectrometric imaging).\textsuperscript{89-91}
Photonic Logic Sensors

In the paradigm of $N$ sensors and $N$ signals for $N$ targets, detecting two targets requires a readout of two signals, and the interdependence of the two targets is inferred from the correlation (or not) between those two signals. It is possible, however to design systems that give a single output from the presence or absence of multiple targets. That is, one sensor and one signal for $N$ targets.

Figure 5. Fluorescent logic sensing. (A) Truth table for two-input Boolean functions for common logic gates. Each input represents the presence (1) or absence (0) of a target. Output is a bright (1) or dark (0) fluorescence signal. (B) Examples of two DNA oligonucleotide-based logic sensors implemented in parallel with two colors of fluorescent dye acceptor (A488, A546) and a dark quencher (labFQ) with a Tb LLC FRET donor: (i) combinations of the sensors and target inputs, and (ii) corresponding truth tables; (iii) schematics of the sensor designs, highlighting toeholds (circled) for strand displacement by the target inputs (I1, I2, I3); (iv) fluorescence outputs for each input combination. Adapted with permission from ref. 92. Copyright 2017 American Chemical Society. (C) Designs and mechanisms of (i) OR and AND logic sensors, and (ii) fluorescent output data from implementation in mammalian cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Nanotechnology (ref. 93), copyright 2015.
In the case of photonic logic, the relationship between one measured fluorescence signal and \( N \) targets is determined by a Boolean operation.\(^9^4\) Examples of turn-on sensors include OR gates, where the output signal is produced if any of the input analytes are present, and AND gates, where the output signal is produced only if all the input analytes are present. Analogous turn-off sensors include NOR and NAND gates. There also exist other less intuitive logic gates, such as XOR and XNOR, as summarized in Figure 5A.\(^9^5\) Various biomolecular probe architectures have been designed to link chemical and biological inputs with Boolean fluorescence output, as reviewed by de Silva, one of the progenitors of the field.\(^9^6\)

Many photonic logic sensor designs are based on DNA hybridization, both in terms of the targets and the assembly of the sensor itself.\(^9^7\)\(^-\)\(^9^9\) The programmable and predictable nature of DNA structures, and the utility of toehold-mediated strand displacement reactions, provides the control necessary to address the primary challenge of these sensor designs: coupling at least two biomolecular recognition events and a corresponding number of structural changes to only one fluorescent output that ideally has binary signaling behavior. The latter is easier said than done, and many real designs instead strive for a difference between high-low output states that is much larger than the variation between outputs nominally of the same binary state.

DNA logic gates can be designed to require engineered targets, whether in terms of a fluorescent label or specific nucleotide sequences, and to function with unlabeled targets of any selected nucleotide sequence. For the purpose of this review on sensing, we exclude examples of the former,\(^1^0^0\) but highlight examples of the latter below.

One design motif for two-target logic sensors is a linear assembly of multiple oligonucleotides that builds in toeholds or similar features to facilitate target binding. We recently developed a series of OR, AND, NOR, and NAND logic sensors in this format.\(^9^2\) The initial sensor assemblies set up different permutations of competitive and sequential FRET between a Tb LLC donor, a fluorescent dye acceptor, and dark quencher acceptor labels on the oligonucleotides. In each sensor, binding of nucleic acid target disrupted one or more of these FRET pathways in the assembly with a Boolean-like effect on the output signal, which was time-gated dye emission sensitized by energy transfer from the Tb LLC. These configurations had some of the highest
Boolean contrast (the signal difference between nominal output states of 0 and 1) and lowest Boolean variability (differences in signal between input configurations that yield the same nominal output state) to date. The systems also functioned in serum, and, as summarized in Figure 5B, the use of a Tb LLC donor permitted multiple colors of logic gate to be used in parallel.\textsuperscript{92} Earlier work by Yoshida and Yokobayahsi used similar design motifs to create AND and OR sensors for protein targets.\textsuperscript{101} In this case, a fluorescent dye and dark quencher were used as the FRET pair and the sensor assembly incorporated aptamer sequences. Another example, for which we make an exception to our criterion of non-engineered targets, are the AND and OR gates that Groves \textit{et al.} developed.\textsuperscript{93} As shown in Figure 5C, these sensor assemblies initially placed a fluorescent dye and quencher in close-proximity with Boolean output actuated by four-way strand exchange reactions and, importantly, functioned within living mammalian cells.\textsuperscript{93}

Oligonucleotide-based logic sensors have also been designed as complex structures with a simple donor-acceptor FRET pair. As one example, He \textit{et al.} utilized a triangular prism DNA nanostructure as a basis for an OR sensor.\textsuperscript{102} Initially, a fluorescent dye and dark quencher were in close proximity to one another; structural changes upon binding a target resulted in a reduction in FRET efficiency and a Boolean-like increase in dye emission intensity. A ternary INHIBIT sensor was also demonstrated, as were AND and XOR gates, although the latter required engineered targets. As another example, Li \textit{et al.} designed an oligonucleotide-based, four-input OR gate that responded to restriction enzymes.\textsuperscript{103} Four different enzymes acting at four different sites on the ‘tweezer’ structure of this sensor were each able to disrupt FRET between a fluorescent dye and dark quencher.

Multi-dye FRET networks with a discrete structure can also be utilized for logic sensing, both with linear and more complex DNA structures. For example, Buckhout-White \textit{et al.} demonstrated that DNA triads with linkers removable by toehold-mediated strand displacement, and with three colors of fluorescent dye at their center, had potential as OR and AND gates.\textsuperscript{104} An emission ratio between two of the dyes was the output signal. More complex logic functions (\textit{e.g.} INHIBIT, XOR) were also suggested, but with much less ideal contrast. In another example, He \textit{et al.} developed logic sensors based on changes in FRET between blue, green, and red QDs mediated by either assembly or disassembly of linear structure of oligonucleotides.\textsuperscript{105} OR, NOR,
NAND, INHIBIT, XOR, and XNOR sensors were demonstrated with the emission intensity of the blue QD as the output, and an AND sensor used the emission intensity of the green QD as the output. Only the OR and AND sensors were discrete assemblies; the remainder were multi-component sensors.\textsuperscript{105} The multi-component design is not unique to the use of QDs, as a multi-component AND sensor with a FRET pair of two fluorescent dyes was reported by Zhang and coauthors.\textsuperscript{106}

The scope of molecular logic is much broader than the fluorescent sensors discussed here. For example, beyond DNA, the groups of Katz and Willner have developed enzymatic logic systems with combinations of glucose oxidase, dehydrogenases and a peroxidase, to measure inputs of enzyme substrates such as glucose or peroxide.\textsuperscript{107,108} In general, further development of molecular logic in the context of bioanalysis needs to address a broader scope of biomolecular targets, higher contrast leading to less variability between Boolean output states, and practical analytical figures of merit.

Like FRET networks, logic sensors have the potential to directly address the interplay between two or more components in a biochemical network, particularly by deducing relationships between targets and components of pathways with fewer signals; for example, one sensor and one signal to detect both activation of an oncogene and inactivation of a tumor suppressor gene, or that both of the two main tumor-suppressor pathways (RB and p53) are inactivated.\textsuperscript{109} Logic gates also support screening for multiple targets or pathways without \textit{a priori} expectations for which will be expressed or occur. Among other prospective applications, this capability has diagnostic value for rapid screening of a panel of infectious disease or pathogen biomarkers. In addition, logic probes have the potential to help address the challenges of sensing in real biological systems, one of which is degradation of probes leading to false positive/negative signals. To this end, a hypothetical sensor can be designed with the logical requirement for signal output that the target was detected and the sensor itself remained intact (\textit{e.g.} detecting a target hydrolase while guarding against non-specific hydrolysis), or that the target was detected and a known interference or inhibitor was not. The principal benefit of logic sensors is that value-added capability is built-in at the molecular level without increasing the complexity of signal measurements.
**Fluorescent Sensor Arrays**

Chemical nose arrays operate so that the convention of $N$ sensors for $N$ analytes no longer applies—tens of different targets can be detected with an array of only a few sensor elements.\textsuperscript{110,111} Instead, these sensor systems work on the principle that an array of cross-reactive chemical sensors can give a fingerprint for each sample to which it is exposed. These fingerprints can then be statistically analyzed (via chemometric methods) to build a library of known patterns corresponding to particular analytes. It is then possible to perform recognition of samples against which the array has already been trained, or identify if an unknown sample falls outside the training of the array.\textsuperscript{112}

Sensing arrays are created in either single channel mode, where each sensor element of the array is exposed to the sample in isolation, or a multiplexed mode, where the array ensemble is exposed to the sample in a single volume.\textsuperscript{113} The latter format has advantages of reduction in the volume of sample needed, and improved cross-reactive target-sensor interactions. Multicolored fluorescent outputs lend themselves well to non-specific array based sensing through multiplexing, just as in conventional sensing.
Fluorescent nanoparticle arrays have been recently reviewed by Bigdeli et al., but we highlight some important examples here. One approach to array-based signaling has been the displacement of non-covalently bound FPs or fluorescent polymers from the surface of AuNPs by proteins in serum. Rotello and coworkers have tailored the surface of AuNPs with quaternary ammonium ions of different shape and polarity to bind proteins non-specifically on the basis of size, charge, polarity, or chemical functionality, as illustrated in Figure 6A. The FP or fluorescent polymer in proximity to the gold is quenched, but lights up when displaced by the incoming proteins, which compete for the same binding sites on the AuNP. Each AuNP sensor element with pre-bound GFP or a green fluorescent polymer was measured before and after the addition of each target protein. The change in output fluorescence in each case (single channel) was read and statistically linked to the analytes displacing the fluorophores. In these studies, linear discriminant analysis (LDA) was the chemometric method used, but principal component analysis (PCA) and hierarchical clustering analysis (HCA) are also popular methods.

Other fluorophores used for protein sensor arrays have included gold nanoclusters that vary in color and intensity dependent on the proteins present. For example, Yeung et al. used an array of eight green-emitting gold nanoclusters, each functionalized with a different small thiol, such as glutathione or (2-mercaptoethyl)amine, to introduce different surface polarity and discriminate between eight common proteins in buffer by the relative degree of fluorescence quenching. A more efficient example was shown by Ouyang et al., who created a color-changing array, where...
two different blue-emitting gold nanoclusters were synthesized and used to discriminate eight proteins on the basis of color changes, rather than simply intensity changes, as summarized in Figure 6C.\textsuperscript{115} Fluorescent metal complexes have also been demonstrated for sensing and discrimination of several clinically relevant proteins. A Ru(II) complex was synthesized and functionalized with various biomimetic groups. These metal complex sensors were able to discriminate between six proteins based on changes in their fluorescence intensity induced by protein binding.\textsuperscript{121}

The above examples with proteins are not the full extent of applications for fluorescent array-based sensing. Array-based systems have been reviewed for the sensing of biological hazards such as bacteria and bacterial metabolites,\textsuperscript{122} and Anslyn and coworkers have formulated an array of molecular fluorophores or fluorescent transition metal complexes to measure kinase activity on an array of peptides.\textsuperscript{123,124} There is also a move to sense not only proteins, but to address more complex multi-analyte problems such as fingerprinting disease states.\textsuperscript{125} It is here that array sensors can really come into their own, correlating the array output with patient health rather than specific analytes. Recent work in this area by Rotello and coworkers exploited a sensor array similar to their aforementioned protein sensing array, that was instead trained to monitor complex cell surface states, enabling the differentiation of cancerous and metastatic cells from healthy counterparts. In this mode, it was cross-reactivity between cell surface glyco-proteins and other moieties that the sensor array measured.\textsuperscript{126} Anslyn and coworkers have also discriminated between cancer cell lines with a DNA-fluorophore sensor and introduced the use of a support-vector machine analysis for going beyond simple classification and detecting samples that do not fall into the training data set.\textsuperscript{127} Rotello and coworkers have also probed the method of action of drugs on various cancers, with successful prediction of unknown reaction mechanisms through advanced HCA and LDA analysis, as shown in Figure 6B.\textsuperscript{116} Looking forward, new arrays are starting to emerge that can detect disease states more broadly (e.g. late-stage cancer) from serum samples or other body fluids, such as the one summarized in Figure 6D.\textsuperscript{117}

A key challenge with real biological systems is selectivity. For example, exogenous sensing probes introduced to a cell or tissue are often acted upon by multiple enzymes, rather than one
specific target enzyme. Analogous cross-reactivity challenges are common with sensing based on ligand-receptor or antibody-antigen motifs. Successful detection of one biomarker is rarely a sufficient diagnostic for a disease, necessitating panels of multiple biomarkers, increasing the risks of off-target interactions. Array-based sensing with chemometric analysis can obviate the need for a well-defined set of biomarkers and high selectivity. Potential applications include identifying, staging, or assessing other pathophysiology of cancers through patterns of non-specific protein expression,\textsuperscript{128} protease activity,\textsuperscript{129} and/or glycosylation.\textsuperscript{130} Assessment of other molecularly complex processes, such as stem cell differentiation,\textsuperscript{131} may also be more tractable with non-specific sensor arrays than with sensors targeted to select biomarkers.

**Conclusions and Perspective**

Specificity, modulation of only one fluorescence signal, and one sensor per analyte are common conventions of fluorescent biosensor design, but these motifs are not requirements. New capabilities are gained by engineering unconventional fluorescent biosensors that break with one or more of these conventions and move beyond simple turn-on/off behavior. To illustrate, we have summarized how FRET networks enable multiplexing with one sensor, how multiplexing in time is an alternative to multiplexing in color, how molecular logic simplifies the analysis of multiple targets, and how patterns of fluorescent output from semi-selective arrays of sensor elements are potentially more informative than specific sensors.

Unsurprisingly, many unconventional fluorescent sensor designs take advantage of materials other than dyes and FPs. Further development will continue to leverage the properties of many nanomaterials. QDs stand as versatile and enabling materials, although a future shift from CdX-based QD materials to InP,\textsuperscript{132} Si,\textsuperscript{133} and other currently less-developed alternatives is anticipated. Lanthanide-based upconversion nanoparticles (UCNPs),\textsuperscript{134} which combine many of the optical properties of LLCs with the advantageous physicochemical properties of a nanoparticle, are a promising next material for unconventional sensor designs. UCNP materials have long lifetimes, can be photoexcited in the NIR biological window, permit barcoding with multiple different colored lanthanide emitters, and offer some material-enabled methods of bioconjugation.\textsuperscript{135,136} These properties have great potential in the construction of time-gated FRET and array-based
sensors. Semiconducting polymer dots (Pdots),\textsuperscript{137} carbon dots,\textsuperscript{132} and other emerging nanomaterials will also graduate from components of conventional sensors to components of unconventional sensors; however, a better understanding of the photophysics of these materials and greater control over their surface functionalization and bioconjugation are first needed. The use of metal nanoclusters is also likely to expand, especially in array-based sensing, as these materials become better understood and controlled. New unconventional sensors based exclusively on dyes and FPs may be enabled through the use of lipid,\textsuperscript{138} polymer,\textsuperscript{139} and virus-like nanoparticles as scaffolds,\textsuperscript{140} an via the ongoing use of DNA nanostructures, which are rapidly growing in scope and sophistication.\textsuperscript{99} Innovation in fluorescent materials will continue to help drive innovation in fluorescent sensing.

The technical requirements of unconventional fluorescent sensor designs are not much different than those for conventional sensors. The possible exception is temporal multiplexing, which is more technically demanding than spectral multiplexing; however, the barriers to accessing and acquiring instrumentation for these measurements are continually decreasing. Open questions are if and how unconventional sensor designs will integrate with single-molecule detection and super-resolution imaging, but the answers to these question will not be much different than those for conventional sensors. Where unconventional sensor designs are frequently more demanding than their conventional counterparts are their requirements for data analysis, with photonic logic as the exception. FRET networks, temporal multiplexing, and sensor arrays require multi-dimensional mathematics or chemometrics for analysis.\textsuperscript{141} Far from a disadvantage, these approaches are best positioned to take advantage of advanced and emerging computational technologies, such as machine learning and data mining,\textsuperscript{142} that will further enhance capabilities.

Despite significant progress in recent years, it must be remembered that unconventional sensors are now a proven idea but not yet a proven method. The unconventional sensing of DNA, RNA, proteins, and enzymatic activity is well documented; however, much of this research and development has been with buffered or otherwise clean and controlled samples. Sensing with more complex media needs to be addressed, such as in blood or other bodily fluids, within the cell cytosol, and in tissue. In parallel, there is a need in many cases to translate unconventional sensors from detection of proof-of-concept targets to detection of targets with \textit{bona fide} clinical
relevance or for genuine biological discovery, including realistically useful limits of detection and other figures of merit.

With many of the concepts in this review in their infancy and pursued by a relatively small number of research groups, there may be a temptation to overlook unconventional sensor designs as novelties in favor of tried-and-true conventional sensor designs. It must be remembered that conventional sensor designs have had decades of research and development to become conventional. With time and effort, the unconventional sensors in this review or their progeny will one day become conventional and powerful additions to the routine arsenal of tools for chemical biology and bioanalysis. The concepts and designs represent the next evolution in fluorescence-based biological sensing and imaging.
Notes
The authors declare no competing interests.

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