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"AND" luminescent "reactive" molecular logic gates: a gateway to multi-analyte bioimaging and biosensing

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This review outlines examples that illustrate a recent and highly innovative concept in the field of (bio)-molecular sensing, namely the simultaneous multi-analyte detection using "reactive" luminescent probes that are able to produce an optical signal only in response to multiple (bio)chemical inputs and through covalent chemical reactions with target (bio)analytes. Unlike conventional "AND" molecular logic gates based on supramolecular photochemical mechanisms, these unusual "smart" optical (bio)probes are suitable tools to track the rise and fall of a wider range of biologically relevant analytes, in complex media and with higher selectivity. The potential utility of this concept for *in vivo* molecular imaging and possible solutions to adapt the described luminogenic processes to far-red or NIR emitters are also discussed.

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Introduction

In the past decade, the scope of luminescence biosensing and bioimaging research has dramatically increased, thus finding numerous applications in diagnostics (*in vitro* or *in vivo*),¹ enzyme biotechnology,² environmental monitoring,³ food safety⁴ and forensic sciences.⁵ This tremendous development was promoted by the recent and rapid emergence of advanced chemical tools known as activatable or "smart" optical (bio)-probes.⁶ If properly designed, these synthetic probes should exhibit no signal until they interact (and/or react) with their target (bio)analyte, thus reducing the level of background luminescence (fluorescence or bio- or chemiluminescence) and potentially endowing them with greater sensitivity. Their powerful ability to offer greater temporal and spatial sampling capability is another attractive and valuable feature. Various photophysical processes such as photo-induced electron transfer (PeT),⁷ photo-induced proton transfer⁸ and resonance energy transfer (BRET, CRET, FRET or through-bond energy transfer, TBET),^{9,10} or the direct change in π -conjugated systems (CCS) induced by (bio)chemical reactions¹¹ are routinely used to design luminogenic probes suitable for the selective detection of a single (bio)analyte in a more or less complex (biological) matrix. Among the wide range of fluorogenic probes currently available, those based on an irreversible

chemical reaction in which the (bio)analyte acts either as a reactant or a catalyst are being increasingly put forward because of their rapid response, high sensitivity, and excellent selectivity.¹² They are named reaction-based fluorescent probes, fluorescent chemodosimeters or pro-fluorophores, and have been the subject of several comprehensive reviews.^{11,13} In order to develop multi-analyte detection technologies, some academic research groups are now exploring alternative "reactive" luminogenic probes which are able to produce a detectable signal only in response to multiple (bio)chemical inputs (*vide infra*).¹⁴ This means that two or three targeted (bio)analytes must work in tandem or in a sequential manner to convert the probe into a luminescent product ("turn-on" emission response) or to induce a dramatic shift in its excitation/emission profiles (ratiometric response). Ideally, these "AND" molecular logic gates should make it possible to provide an alternative to serial measurements of single (bio)analytes in either the same (biological) sample at different times or two (or more) different (biological) samples in tandem. It should also facilitate the monitoring of multiple biomolecular events leading to a common disease pathology, to obtain an optimal predictive accuracy for illness diagnosis and prognostication. The field of "AND" logic gates based on small molecules with chemical inputs and luminescence output has been the subject of numerous studies, but it still remains limited to fluorescent chemosensors that rely on non-covalent and reversible interactions with the target analytes that perturb photophysical processes within the system (known as supramolecular photochemical mechanisms).¹⁵ Therefore, these molecular devices can only be used to detect a limited number of analytes including H⁺, metal cations and anions. To over-

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come these limitations, the development of logic gates based on biomacromolecules (proteins and nucleic acids) and enzymes was also considered. In particular, nucleic acid-based logic gates utilising functional oligonucleotides such as DNAzymes which are DNA molecules possessing catalytic activity, and nucleic acid aptamers, which selectively bind to target molecules with affinity rivalling that of protein antibodies, have been successfully constructed to emulate Boolean operations such as "AND", "OR", "XOR" and "NOR".¹⁶

Herein, I outline the recent development of reaction-based molecular probes used for the simultaneous detection of two or three distinct (bio)analytes. Two main strategies for constructing this kind of luminogenic probe, namely (1) the conversion of conventional fluorogenic dyes into pro-fluorophores having several distinct reaction sites and (2) the internal construction of a bioluminophore/fluorophore scaffold controlled by the target (bio)analytes, are summarised. Also, only luminogenic probes leading to the release/



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predictive Medicine (a spin-off of the Serono group, Switzerland), as a senior scientist in bioorganic chemistry. The core business of this company was devoted to the development and commercialisation of an original high-throughput DNA sequencing technology. Following a restructuring leading to the acquisition of Manteia by Solexa Ltd (this latter company became a wholly owned subsidiary of Illumina, Inc. in January 2007), he was appointed as a lecturer in bioorganic chemistry at the University of Rouen. From February 2004 to August 2013, he co-facilitated with Pr. P.-Y. Renard the bioorganic chemistry research team from the COBRA lab (UMR CNRS 6014). In September 2013, he was appointed as a full professor at the University of Burgundy and a junior member of the French University Institute (IUF). He also joined the ICMUB lab (UMR CNRS 6302) and his current research interests mainly focus on the development of advanced chemical tools ("smart" optical (bio)probes, novel fluorogenic reactions, and cross-linking reagents for multiple bioconjugation) for biosensing and bioimaging applications. He is a co-author of 70 scientific papers and a co-inventor of over a dozen patents, three of them being actively pursued by the Illumina Company (Genome Analyzer DNA sequencing technology).

formation of a single optical reporter are focused on, excluding FRET donor-acceptor systems that are able to respond to several distinct analytes with different sets of fluorescence signals.¹⁷ The merits and limitations of the probes and possible improvements to be made in their rational design are also discussed.

Pro-fluorophores combining several distinct reaction sites

Among the numerous chemical reactions implemented in the context of single analyte-sensitive fluorogenic probes, a few of them have already been used to develop "turn-on" fluorescent sensing platforms for the simultaneous detection of multiple analytes in a biological environment. Indeed, the main difficulty associated with this multi-analyte fluorogenic approach relates to the selection of a combination of two (or more) biocompatible reactions which are fully orthogonal among themselves and easily applied to a single fluorescent scaffold. All the examples of such integrated molecular "AND" logic gates currently available in the literature have been published during the last two years. In this section, we describe them focusing on both the reaction types jointly used for the chemoselective (bio)sensing of two or three different analytes simultaneously and the proposed mechanisms to interpret the "turn-on" or ratiometric fluorescence response of these probes. Possible improvements of these promising organic reaction-based strategies, especially through their extension to more sophisticated fluorophores (*i.e.*, those exhibiting valuable spectral features in the far-red or NIR region)¹⁸ other than conventional BODIPY, coumarin and xanthene dyes, are also briefly described.

Nucleophilic addition and protonation-deprotonation equilibrium

Glass and co-workers have recently devised tunable fluorescent molecular logic gates with potential applications to neuronal imaging.¹⁹ At first, they reported a dual-analyte fluorescent chemosensor (ExoSensor 517, **ES517**) for the direct visualization of neurotransmitters released upon exocytosis. This reaction-based fluorescent probe is able to selectively label primary amine neurotransmitters (especially glutamate, Glu) through the formation of an iminium ion (carbonyl addition followed by dehydration) and allows for direct visualization of only active neurotransmitters released in the synaptic cleft by taking advantage of the pH gradient between the inside and the outside of the synaptic vesicle (pH change from 5.0 to 7.4). The implementation of such a sensing mechanism was achieved using 7-amino-4-phenylcoumarin as a fluorophore. Introduction of a formyl group in position 3 of the coumarin scaffold and conversion of its 7-NH₂ group into a sulfamide derivative easily deprotonable at physiological pH (pK_a value close to 6) have led to a fluorogenic probe reactive toward both glutamate and hydroxide anions. In its native molecular form (Fig. 1), **ES517** fluoresces *via* an intramolecular charge transfer

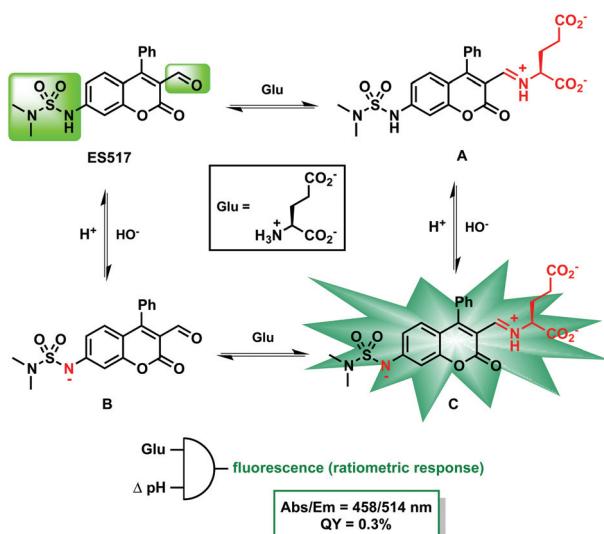


Fig. 1 “AND” molecular logic gate for neuronal imaging based on the fluorogenic reaction of ES517 with two distinct analytes, namely glutamate and hydroxide anion (ratiometric response).

(ICT) process from the moderate electron-donating sulfamide moiety to the electron-withdrawing aldehyde group. Both glutamate binding and sulfamide deprotonation enhance the charge transfer across the π -system of the fluorophore, resulting in large bathochromic shifts in both the absorption and emission profiles (Abs/Em maxima 458/514 nm for **C** compared to the blue-shifted absorption maxima of other forms clearly displayed in spectral curves. However, their emission maxima are not accurately reported within this publication). Thus, the excitation of the sensor (form **C**) at the highest wavelength of absorption (typically at 488 nm) should enable the visualization of only the “active” neurotransmitters in the synapse. Conversely, unbound sensor molecules (form **B**) would not be visualized as they absorb at a shorter wavelength (Abs maximum 428 nm for **B**). The sensing mechanism of ES517 may be summarised as follows (Fig. 2): in the cytosol, a weakly fluorescent form is predominant due to the neutral pH and relatively low concentration of amines. When ES517 enters the vesicle, it binds to the neurotransmitter whose concentration is particularly high, to produce iminium ion **A**. Form **A** would have marginal electron transfer and weak fluorescence since its sulfamide moiety is a weak donor. Upon exocytosis, the bound complex enters the synaptic cleft, becomes deprotonated (form **C**), and produces a marked fluorescence increase due to the enhanced ICT. At present, validation of this unusual ICT-based ratiometric probe was only achieved through *in vitro* fluorescence assays and under conditions mimicking exocytosis. A reaction of 20 μ M of ES517 with a saturating amount of primary amine (300 mM for glutamate, GABA and glycine and 100 mM for aromatic neurotransmitters, namely norepinephrine, dopamine and serotonin) and pH adjustment from 5.0 to 7.4 produces a fluorescence enhancement at 517 nm by factors of 10–12 for non-aromatic neurotransmitters and 1.5–5.5 for those bearing an aromatic

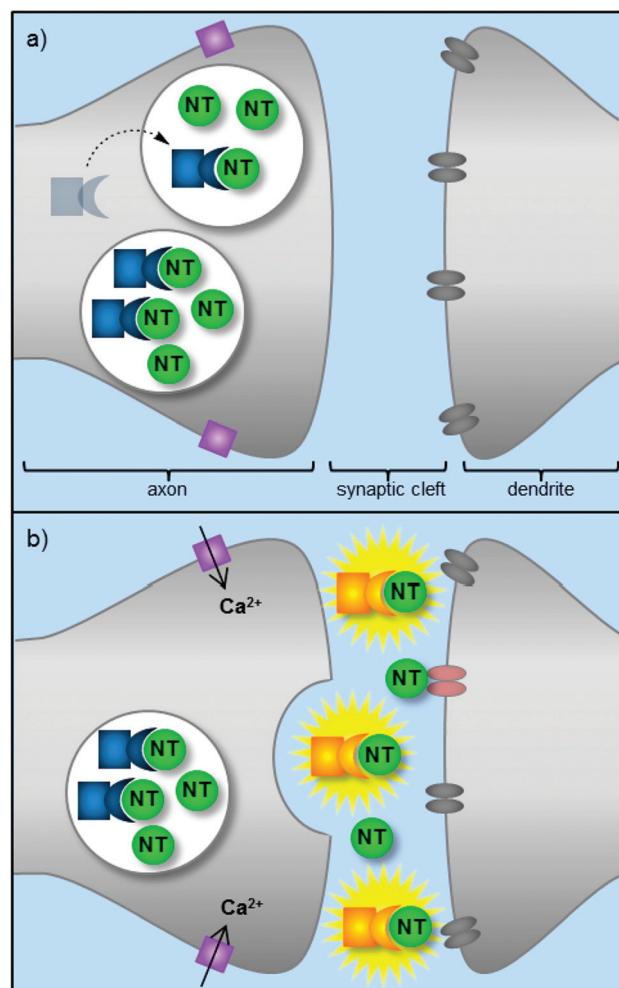


Fig. 2 Sensing mechanism of ES517. (a) The sensor enters the vesicle and selectively labels the neurotransmitter (NT). The bound complex accumulates and green fluorescence at 514 nm is “off” due to low pH (5). (b) Influx of Ca(II) triggers exocytosis. The increase in environmental pH in the synaptic cleft (7.4) switches the green fluorescence “on” for only the bound complex. Reproduced with permission from Klockow et al.¹⁹ Copyright 2013 American Chemical Society.

core. For these latter neuroanalytes, it is assumed that their electron-rich aromatic group (catechol or 5-hydroxyindole) promotes fluorescence quenching through a reductive PeT process. Despite these promising results, some improvements have to be made to this reaction-based fluorescent probe, in particular, by increasing the brightness of the fluorescent sensor–glutamate complex and possibly by red-shifting its excitation/emission profiles, aimed at meeting the challenge of high resolution imaging of neurotransmitter secretion in the brain. In continuation of this first study, the same group has recently reported a series of three-input “AND” fluorescent molecular logic gates for directly imaging the co-release of glutamate and the zinc(II) cation from glutamatergic secretory vesicles.²⁰ Concomitant detection of these two analytes is particularly relevant to gain insights into the mechanisms underlying neurotransmitter dysregulation and the progression of neurodegenerative diseases without altering the synaptic

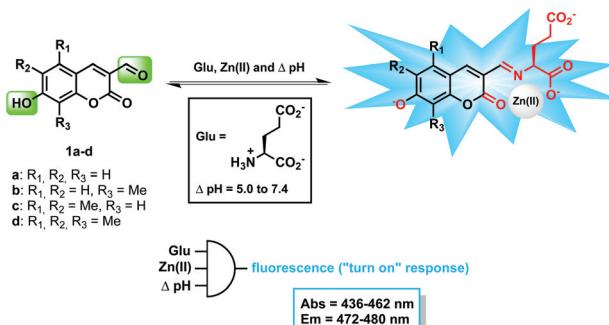


Fig. 3 "AND" molecular logic gate for neuronal imaging based on the fluorogenic reaction of **1a–d** with three distinct analytes, namely glutamate, the Zn(II) cation and the hydroxide anion ("turn-on" response).

activity. As for the previous example, these "reactive" probes are based on a 3-formylcoumarin scaffold which is able to form an imine with glutamate. This condensation reaction leads to the formation of a multidentate ligand fused to the coumarin ring and able to readily coordinate the Zn(II) cation. The 7-OH group (pK_a value close to 6) imparts pH-sensitivity which acts as the final switch for a "turn-on" fluorescence response (Fig. 3). Finally, methyl groups are appended to the coumarin scaffold to red-shift and optimize the absorption/emission maxima of the probe (red-shift of ~ 10 nm with each additional methyl group). Even though the sensors **1a–d** can be regarded as structural analogues of **ES517**, the change of the pH-sensitive group and the removal of the phenyl substituent in position 4 dramatically affect the fluorescence sensing mechanism. Indeed, at physiological pH, 3-formyl-7-hydroxycoumarin derivatives **1a–d** are blue fluorescent emitters (emission maximum in the range 460–480 nm) upon excitation at 416–440 nm (however, their quantum yields were not determined and are not reported within this publication). When the coumarin-based probe penetrates the zinc-containing secretory vesicles, a reaction with glutamate, subsequent binding of the Zn(II) cation and the acidic environment lead to a sensor-glutamate-zinc bound complex, which exhibits a moderate blue fluorescence upon excitation at a shorter wavelength (380–395 nm depending on the methyl-substitution pattern of coumarin). Upon exocytosis, this Zn(II) complex is released within the synaptic cleft, deprotonation of its phenol group occurs, and a remarkable red-shift of the excitation maximum (~ 50 nm) and a fluorescence enhancement by a factor of 6–11 (depending on the methyl-substitution pattern of coumarin) are observed. Validation of these three-input sensors was again achieved through *in vitro* fluorescence assays, using high concentrations of glutamate and zinc(II) salt (typically found in glutamatergic vesicles) and under pH conditions mimicking exocytosis. The major drawback of this sensing approach is that the unreacted probe (e.g., compound **1c**) acts as a single-input "YES" molecular logic gate, because a substantial fluorescence signal is obtained when the pH is increased from 5.0 to 7.4 in the absence of glutamate and/or the Zn(II) cation (Fig. 4). However, the authors claim that this unbound sensor is char-

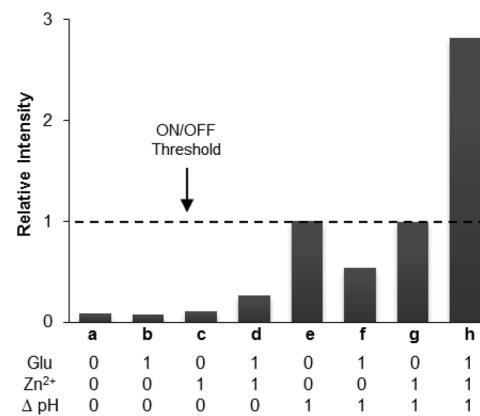


Fig. 4 Relative fluorescence intensities and truth table for **1c**, a three-input "AND" fluorescent molecular logic gate. Samples contained **1c** (1 μM) in buffer (50 mM HEPES, 120 mM NaCl, 1% DMSO) in the presence or absence of Glu (500 mM) and Zn(OAc)₂ (40 mM). ΔpH = increase in pH from 5.0 to 7.4. Reproduced with permission from Hettie et al.²⁰ Copyright 2014 American Chemical Society.

geless and would not accumulate within acidic secretory vesicles, but would instead be washed away during the preimaging cell preparation. To abolish native fluorescence of **1a–d**, a further functionalisation of its position 8 with the di-(2-picoly)amine (DPA) Zn(II)-chelating moiety and the acetylation of its phenol group could be considered (Fig. 5).²¹ Indeed, as recently demonstrated by Lippard and co-workers with a fluorescein-based Zn(II) sensor of the Zinpyr (ZP) family (**DA-ZP1-TPP**), the presence of an acetyl group provides complete fluorescence quenching that is rapidly reversed on exposure to Zn(II), the Lewis acidity of which mediated hydrolysis of the ester group to afford a large, rapid, zinc-induced fluorescence response.²² Furthermore, the use of an analogue of Fisher's base aldehyde derived from 2,4-diformylphenol as a latent fluorescent platform may be an effective way to implement this promising multi-analyte sensing approach to a spectral range more suitable for neuronal imaging applications (Fig. 6).

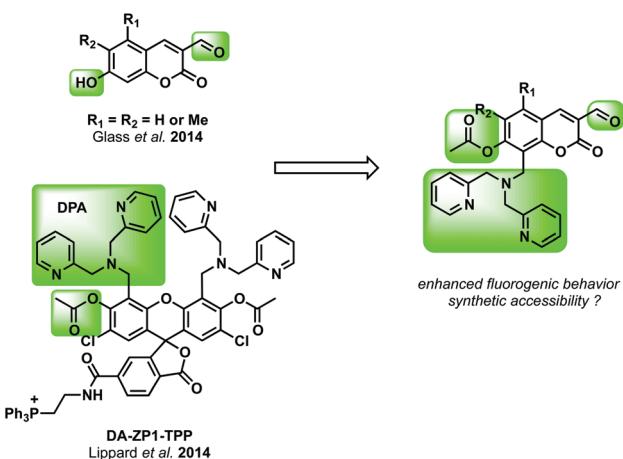


Fig. 5 A possible way to improve the fluorogenic behavior of the 7-hydroxycoumarin-based "AND" molecular logic gate, suitable for neuronal imaging.



Fig. 6 A suggested structure of the latent fluorescent platform (derived from the core structure of NIR fluorogenic dyes recently developed by Shabat and co-workers⁵⁹), to red-shift the absorption/emission maxima of the three-input "AND" fluorescent molecular logic gate suitable for neuronal imaging.

Metal-catalysed reaction and nucleophilic addition-elimination

Simultaneous detection of two reactive analytes, namely the mercury(II) cation and hydrogen peroxide (H_2O_2), known to play vital roles in the environment and in health, was recently achieved by Churchill and co-workers.²³ They designed the "turn-on" fluorogenic probe **2** based on a bis(dimethylthiocarbamate)-caged fluorescein whose deprotection involves two distinct reactions occurring sequentially: (1) a desulfurization reaction induced by the thiophilic Hg(II) cation and leading to the conversion of the thiocarbonyl group to the carbonyl one and (2) deprotection by H_2O_2 through an addition-elimination process (Fig. 7).²⁴ This fluorescein-based probe was readily prepared by a reaction of fluorescein with *N,N*-dimethylthiocarbamoyl chloride. Validation of the claimed tandem sensing mechanism was done through *in vitro* fluorescence assays performed under simulated physiological conditions (20 mM HEPES-DMSO 8 : 2, pH 7.4). First, metal ion screening involving **2** was assayed with a large excess (660 equiv.) of Ca(II), Cd(II), Co(II), Cu(II), Fe(II), Mg(II), Mn(II), Pb(II), Zn(II), Ni(II), Ag(I) and Hg(II) respectively: a dramatic 50-fold increase in the fluorescence emission intensity at 520 nm with Hg(II) was found over other metals except for thiophilic Ag(I) (a 10-fold increase in fluorescence emission intensity was obtained with this monovalent cation metal). This premature fluorescence unveiling is explained by the fact that an Hg(II)-induced desulfurization process is combined with the deprotection of one thio-

carbonyl group leading to the emissive mono-carbamate intermediate **3'**. Fortunately, further addition of the same excess amount of a second targeted analyte, namely H_2O_2 , leads to a higher increase in fluorescence (a 100-fold increase relative to that for the starting probe **2**). Thus, this fluorogenic system can be interpreted as an "AND" logic gate for Hg(II) and H_2O_2 . However, upon its "pre-activation" with Hg(II), probe **2** exhibits a very moderate selectivity for H_2O_2 over other reactive oxygen species (ROS) such as *meta*-chloroperoxobenzoic acid (*m*-CPBA), KO_2 and $NaOCl$. Finally, the biocompatibility of this reaction-based approach was demonstrated through an assay using living neuronal cells. SH-SY5Y neuroblastoma cells treated with a Hg(II) salt and H_2O_2 were incubated with probe **2**. Fluorescence confocal microscopy reveals the same pattern of responses as that observed *in vitro* (Fig. 8). Interestingly, this fluorogenic dual-reagent-mediated phenol deprotection strategy can also be applied to red-emitting naphthofluorescein derivatives, thereby facilitating dual bioimaging of Hg(II) and H_2O_2 in complex biological media (Fig. 9).²⁵ The best way to improve the selectivity of bis-caged-fluorescein probes (such as **2**) toward two distinct analytes such as Hg(II) and H_2O_2 is no doubt based on the implementation of two different reactions each one triggered by a single analyte but both leading to the phenol release. Building on previous studies on luminescent chemodosimeters for the Hg(II) cation or H_2O_2 ,²⁶ it is fairly easy to design a fluorescein-based probe whose phenolic hydroxyl groups would, for instance, be replaced by alkynyl ether and pinacol boronate moieties. Indeed, alkyne ethers readily react with the Hg(II) cation according to an oxymercuration-elimination process whereas H_2O_2 is known to promote transformation of monoboronates to phenols through an oxidative cleavage reaction. Interestingly, this fluorogenic sensing strategy based on two distinct sequential reactions affecting the same quenching moiety has been recently applied to a mitochondria-targetable fluorescent probe for dual-channel nitric oxide (NO) imaging assisted by intracellular biothiols (cysteine or glutathione (GSH), Fig. 10).²⁷ Introduction of the *ortho*-phenylene diamine (OPD) moiety in position 9 of pyronin B has led to probe **4** whose fluorescence is completely abolished by the PeT process. NO is known to react specifically with OPD to form a triazole ring, and therefore leads to the quantitative conversion of **4** into the fluorescent benzotriazole-pyronin dye **5** (Abs/Em maxima 591/616 nm). Since the benzotriazole unit is a good leaving group, this latter fluorophore subsequently reacts with GSH or cysteine through an S_NAr -type reaction to give a red-emitting thiopyronin dye **6**. In the case of cysteine, a further rearrangement reaction takes place to provide the green-emitting aminopyronin dye **7**. Therefore, pro-fluorophore **4** is able to sequentially react with two distinct bioanalytes but does not behave as an "AND" molecular logic gate.

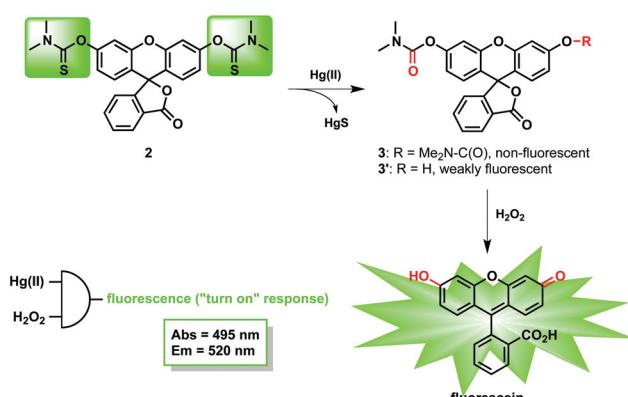


Fig. 7 "AND" molecular logic gate based on the fluorogenic deprotection of bis(dimethylthiocarbamate)-caged fluorescein **2** mediated sequentially by the Hg(II) cation and H_2O_2 ("turn-on" response).

Metal complexation and oxidation reaction

Concomitant detection of the mercury(II) cation and a ROS, namely the superoxide anion radical $O_2^{\bullet-}$, was also carried out through a dramatically different chemosensing approach based on the fluorescence exaltation of a *meso*-thienyl BODIPY

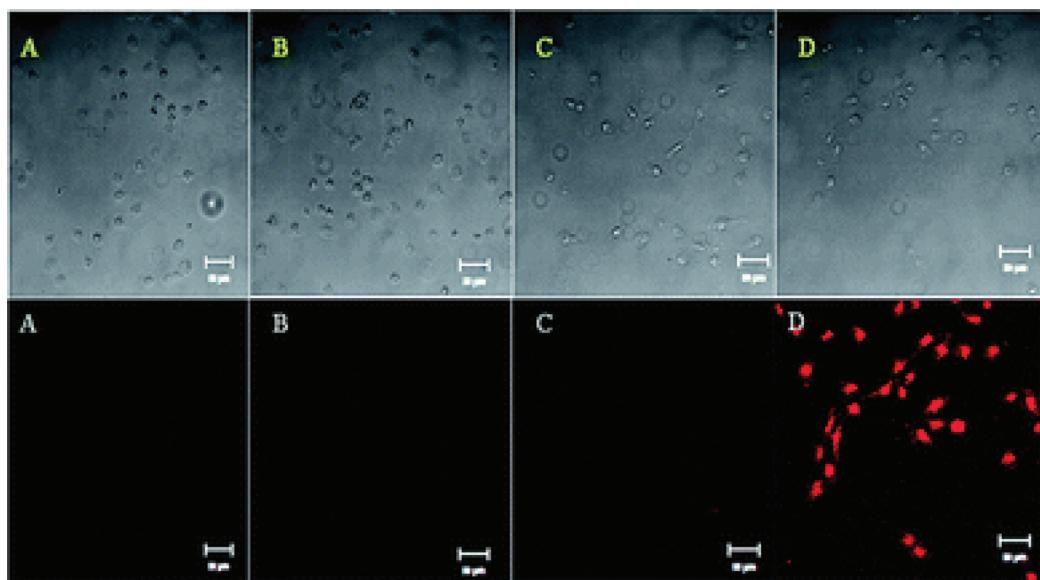


Fig. 8 Fluorescence microscopy data: (upper) bright field images, (lower) fluorescence images, (A) control, (B) SH-SY5Y neuroblastoma cells with pro-fluorophore 2, (C) cells with 2 and Hg(II), and (D) cells after incubation with 2 + Hg(II) + H₂O₂ for 1 h. Ex at 546 nm (scale bar = 50 μm). Reproduced with permission from Murale et al.²³ Copyright 2013 Royal Society of Chemistry.

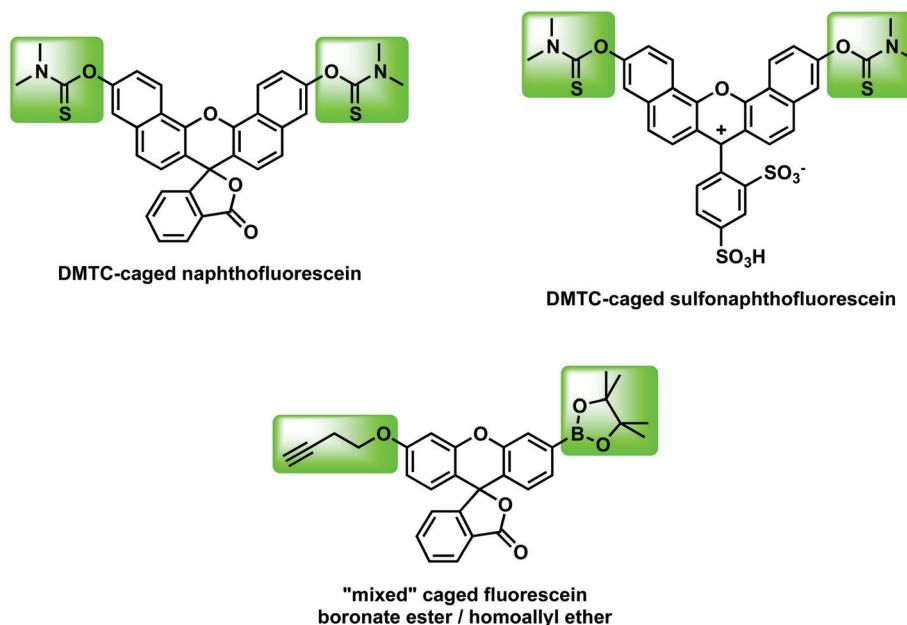


Fig. 9 Possible ways to red-shift the absorption/emission maxima (top) and to improve the selectivity (bottom) of the two-input "AND" fluorescent molecular logic gate suitable for tandem Hg(II) and H₂O₂ chemosensing (DMTC = dimethylthiocarbamate).

dye whose thia-heterocycle is substituted with one or two 2-pyridyl-sulfide arms.²⁸ The binding receptor [S_{thi},N_{py}] or [S_{thi},N_{py},N_{py}] of such PeT-based probes acts as a quencher due to its high electron-donating ability, until the coordination of the Hg(II) cation. In addition to suppressing the PeT process, metal chelation leads to enhanced steric rigidity of the *meso*-aryl group which may further help fluorescence recovery. Sulfide moieties are reactive toward the O₂[−] radical anion and their oxidation is an effective and complementary way to mini-

mise the reductive PeT effect of the Hg(II)-chelating *meso*-aryl substituent (Fig. 11). The BODIPY-based probes **10** and **11** were prepared from 2,4-dimethylpyrrole and 3-thiophene-carboxaldehyde **8** or **9** using a standard one-pot protocol. Interestingly, the two aryl-aldehydes functionalised with either one or two 2-pyridyl-sulfide arms were obtained through a single copper-catalysed cross-coupling reaction between 2,5-dibromo-3-thiophenecarboxaldehyde and 2-mercaptopuridine, in 45% and 13.5% yields, respectively. *In vitro* validation of **10** and **11**

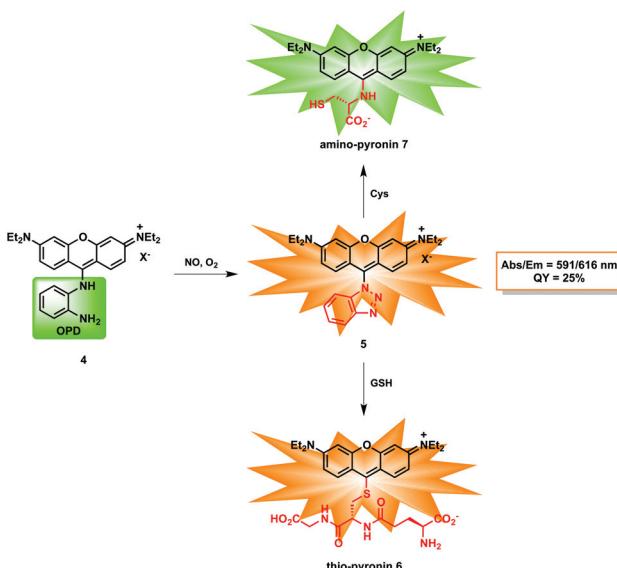


Fig. 10 Fluorescent probe OPD-pyronin B 4 for dual-channel nitric oxide (NO) imaging assisted by intracellular biothiols (Cys or GSH). The fluorogenic reaction mechanism proposed by Guo and co-workers.²⁷

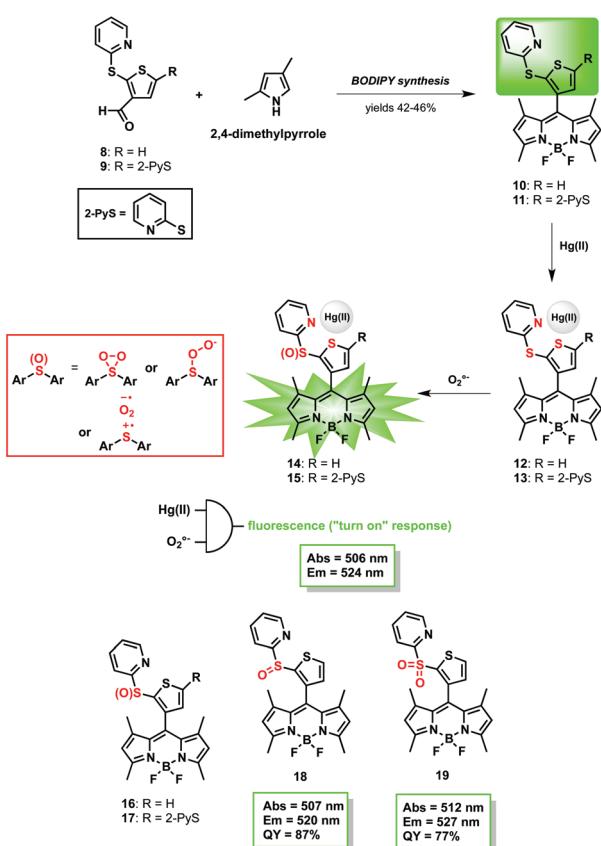


Fig. 11 "AND" molecular logic gate based on the fluorogenic metal-chelation/oxidation process of BODIPY-based probes 10 and 11, mediated by the $\text{Hg}(\text{II})$ cation and the superoxide anion radical O_2^- ("turn-on" response).

was performed in a $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ mixture ($7:3$, v/v), according to a methodology identical to that used for the bis-(dimethylthiocarbamate)-caged fluorescein 2 (*vide supra*). Selective coordination of the $\text{Hg}(\text{II})$ cation through the formation of 1:1 stoichiometry complexes was clearly demonstrated through UV-vis absorption measurements. Only the addition of this divalent cation (in its perchlorate salt form) causes the absorbance band to split into two components and the second local maximum centered at 532 nm is $\sim 25 \text{ nm}$ red-shifted compared to the absorption maximum of unchelated probes. The residual fluorescence emission of probes 12 and 13 at 524 nm ($\text{QY} \sim 2\%$ in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$, $7:3$, v/v) slightly decreases, but since no molar extinction coefficients for the $\text{Hg}(\text{II})$ probe complexes are reported, it is difficult to conclude that quenching occurs especially through an enhanced spin-orbit coupling. Further addition of KO_2 leads to a dramatic green fluorescence enhancement of the mercury(II) chelate-probes 12 and 13 (an ~ 25 -fold increase in emission intensity at 524 nm was obtained). Dual addition of analytes in reverse order: probe + O_2^- + $\text{Hg}(\text{II})$, leads to a similar strong "turn-on" fluorescence response. Such a fluorogenic reactivity toward O_2^- is also observed with unchelated probes 10 and 11 but a (lower) ~ 7 -fold increase in emission intensity is obtained. This set of results supports that pro-fluorescent BODIPY dyes 10 and 11 act as two-input "AND" logic gates for tandem $\text{Hg}(\text{II})$ and O_2^- detection (Fig. 12). Moreover, a satisfying selectivity for O_2^- over other ROS was obtained because only *m*-CPBA leads to a significant fluorescence increase of probes 10 and 11 but nevertheless six times lower than that observed with the superoxide anion radical. To elucidate the reaction pathway giving this excellent "turn-on" signal in the presence of both analytes, authentic samples of mono- and di-oxidized forms of probe 10 (*i.e.*, sulfoxide 18 and sulfone 19) were prepared by

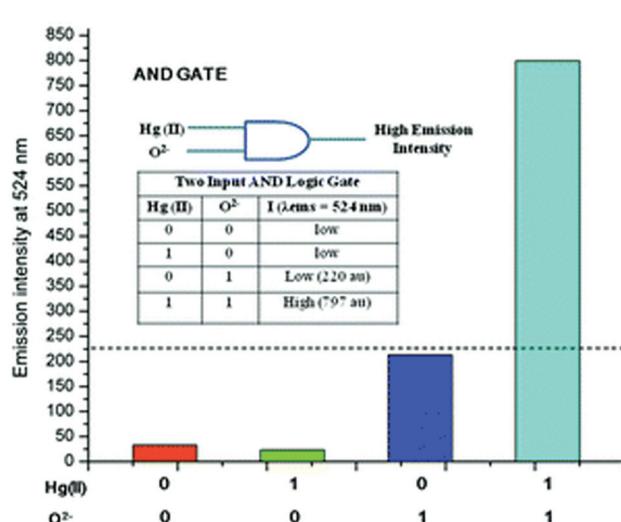


Fig. 12 Relative fluorescence intensities and truth table for 11, a two-input "AND" fluorescent molecular logic gate. Samples contained 11 ($1 \mu\text{M}$) in the buffer $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ ($7:3$, v/v) in the presence or absence of $\text{Hg}(\text{II})$ (18 equiv.) and O_2^- (18 equiv.). Reproduced with permission from Singh *et al.*²⁸ Copyright 2013 Royal Society of Chemistry.

treatment with *m*-CPBA and isolated by silica gel column chromatography in 49% and 18.5% yields respectively. These two compounds are strongly fluorescent ($QY = 87\%$ and 77% respectively), but exhibit no optical response to the Hg(II) cation. This means that for probes **10** and **11**, a long-lasting (but not isolable) oxidised species giving a signal from the superoxide is neither the sulfoxide nor the sulfone (see compounds **14** and **15** or **16** and **17** in Fig. 11). Among the numerous intermediates potentially generated in the oxidation of organic sulfides, dioxathirane, persulfoxide or sulfide radical ion superoxide pair may be involved in this fluorogenic process.²⁹ Both biocompatibility and efficacy of the superoxide-mediated sulfide oxidation reaction and the lack of cytotoxicity for this novel class of BODIPY-based probes were finally demonstrated in the context of neuroblastoma cells and a moderate fluorescence response was obtained with the probe **11** + ROS only (not Hg(II)). However, a further extension to Hg(II) imaging in live cells seems to be problematic due to the competitive chelation action of certain amino acids such as cysteine. Thus, it may be irrelevant to apply this unusual tandem chelation/oxidation fluorogenic reaction to red/NIR region BODIPY dyes³⁰ more appropriate for bioimaging applications.

In situ formation of the bioluminophore/fluorophore scaffold mediated by two distinct (bio)analytes

The multi-analyte detection strategy shown in the examples above is currently limited by the need to use fluorophores that exhibit intrinsic fluorogenic behavior, typically obtained through the reversible (bio)chemical modification of an aniline or a phenol moiety present in their core structure. Thus, such an approach may be applicable for a limited number of fluorophores whose spectral features in the visible region are often not compatible with biosensing/bioimaging in complex biological media: 7-amino-7-hydroxycoumarins, conventional xanthene dyes (*e.g.*, (naphtho)fluorescein, rhodamine 110, rhodols, *etc.*) or precursors that are able to generate a large π -conjugated system upon reaction with a single analyte (typically an intramolecular cyclisation reaction, Fig. 13, top). To overcome this limitation, a sophisticated alternative would be to use two non-fluorescent precursors that are sensitive/reactive toward targeted (bio)analytes and will then be able to readily react among themselves (typically a bimolecular process) and under mild conditions, to *in situ* generate a luminescent compound (Fig. 13, bottom). To the best of our knowledge, self-assembling fluorescent molecular sensors based on multistep reaction cascades, initiated by two (or more) distinct analytes, have not yet been reported in the literature. However, recent achievements in the field of molecular (bio)sensing focused on the development of novel rapid, efficient (and possibly biocompatible) fluorogenic reactions triggered by a single (bio)analyte and leading to *in situ* assem-

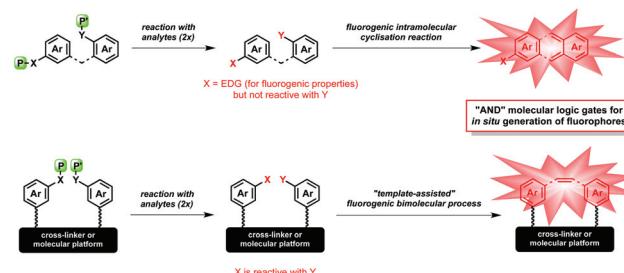


Fig. 13 "AND" fluorescent molecular logic gate based on cascades of covalent bond-modifying reactions triggered by two distinct (bio)analytes: (top) *in situ* generation of a fluorophore through an intramolecular cyclisation reaction triggered by one of the two (bio)analytes (the second one acts a deprotection reagent for the fluorogenic reactive site X, EDG = electron-donating group); (bottom) *in situ* assembly of a fluorophore from two caged precursors whose functional reactive groups X and Y are released through specific reactions with the target (bio)analytes.

bly of a fluorophore will undoubtedly allow to fill this gap.³¹ The majority of these studies display a fluorimetric assay based on the conversion of a non-fluorescent phenolic derivative to a highly fluorescent (imino)coumarin product, usually through a specific cyclisation reaction triggered by the reactive analyte (Fig. 14).³² Since 7-amino-7-hydroxycoumarins are known to be fluorogenic dyes (*vide supra*), one would assume that further functionalization of the (imino)coumarin-precursor with an aniline or a phenol moiety masked by a trigger-recognition unit reactive toward a second distinct (bio)analyte could easily lead to reaction-based probes acting as "AND" fluorogenic logic gates (Fig. 15). Interestingly, alternative reactions such as aldolisation-elimination³³ (or related reactions),³⁴ Mannich cyclisation³⁵ (or related intramolecular addition-elimination reactions),^{36,37} phenylogous Vilsmeier-Haack,³⁸ $\text{SeAr}^{39,40}$ or tandem phenol oxidation-Michael addition⁴¹ has been also implemented to detect various (bio) analytes including G-quadruplex DNA structures, penicillin G acylase (PGA), monoamine oxidases (MAO A and B), sarin mimics, NO and peroxy nitrite (OONO^-) through the formation of cyanine, pyronin B, pyrazino-benz[e]indole, pyrrocoumarin, diazachrysene and resorufin fluorescent scaffolds respectively (Fig. 16). One can also point out other fluorogenic reactions where the mechanism underlying *in situ* formation of the fluorescent scaffold remains uncertain. One good example is the conversion of 6-nitroquinoline into a fluorescent helicene named pyrido[3,2-f]quinolino[6,5-c]cinnoline 3-oxide, under hypoxic conditions (Fig. 17).⁴² For most of these examples, two distinct reactive sites involved in the fluorogenic "covalent-assembly" process were clearly identified within the core structure of the fluorophore precursor. Thus, it is reasonable to assume that the masking of these two reactive functionalities with bio-labile or analyte-sensitive protecting groups will lead to effective dual-analyte responsive fluorescent chemodosimeters. For instance, the design of a dual-analyte fluorogenic probe based on a mixed diaminoaryl ether scaffold with the following structural features: (1) a primary aniline

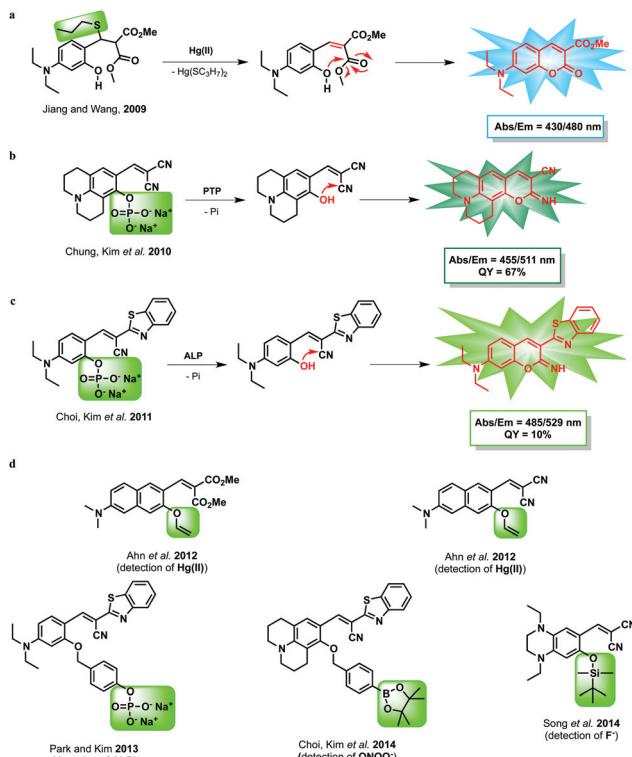


Fig. 14 “Turn-on” fluorescent (bio)analyte sensing based on *in situ* formation of (imino)coumarin scaffolds. PTP = protein tyrosine phosphatase, ALP = alkaline phosphatase, Pi = phosphate anion.

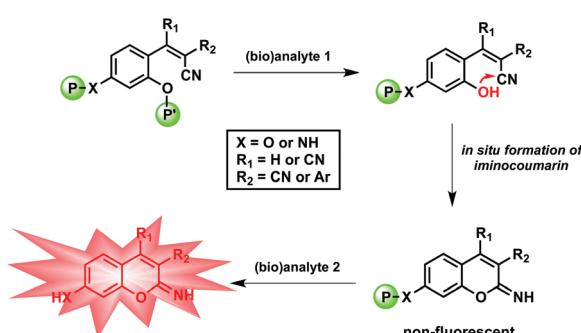


Fig. 15 “AND” fluorescent molecular logic gate based on *in situ* formation of a free 7-amino/7-hydroxycoumarin scaffold mediated by two distinct (bio)analytes (by analogy with the formalism used in Fig. 13, O = Y): an intramolecular cyclisation reaction triggered by one of the two (bio)analytes and subsequent aniline/phenol deprotection induced by the second one.

substituted with a self-immolative carbamate spacer or protected as a carboxamide and (2) a tertiary aniline *para*-functionalized with a formyl group masked as a hemithioaminal or α -alkoxy carbamate responsive to one of the two targeted analytes,⁴³ should enable the simultaneous detection of two (bio)-chemical species through the *in situ* formation of an unsymmetrical pyronin fluorophore (Fig. 18). Further extension of the “covalent assembly” approach to longer wavelength fluorescent dyes (typically far-red or NIR cyanine dyes)⁴⁴ will also

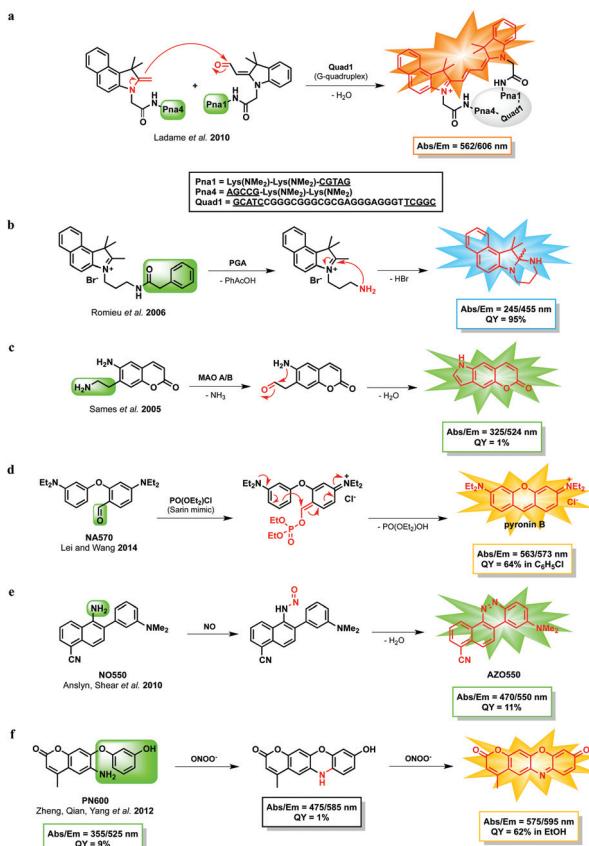


Fig. 16 Fluorogenic reactions triggered by a single (bio)analyte and leading to *in situ* formation of a fluorophore scaffold. (a) Aldolisation–elimination reaction;³³ (b) Mannich cyclisation;³⁵ (c) intramolecular addition–elimination reaction;³⁶ (d) phenylous Vilsmeier–Haack reaction;³⁸ (e) cascade reaction: nitrosation, S_EAr and dehydration;³⁹ (f) tandem phenol oxidation–Michael addition.⁴¹ The reported quantum yield values (QY) were determined in water (or related aq. buffers), unless stated otherwise.

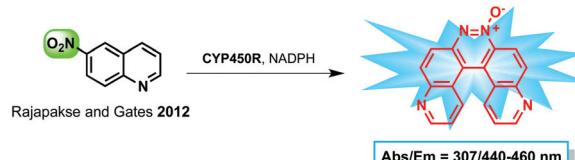


Fig. 17 An unusual example of fluorogenic reactions triggered by a reductase (CYP450R = cytochrome P450 reductase) and leading to the conversion of 6-nitroquinoline into a fluorescent helicene named pyrido[3,2-f]quinolino[6,5-c]cinoline 3-oxide. For a possible mechanism for the formation of this azoxyhelicene, see ref. 42.

be studied to allow *in vivo* applications. However, this would imply using either a heterobifunctional cross-linker (with optimal length and geometry) or a multivalent molecular platform for grafting the two complementary fluorophore precursors in close proximity to each other and through a well-defined and controlled spatial orientation,⁴⁵ thus favoring the reaction rate of the fluorogenic bimolecular process. An attractive alternative could be the use of erythrocytes (red blood

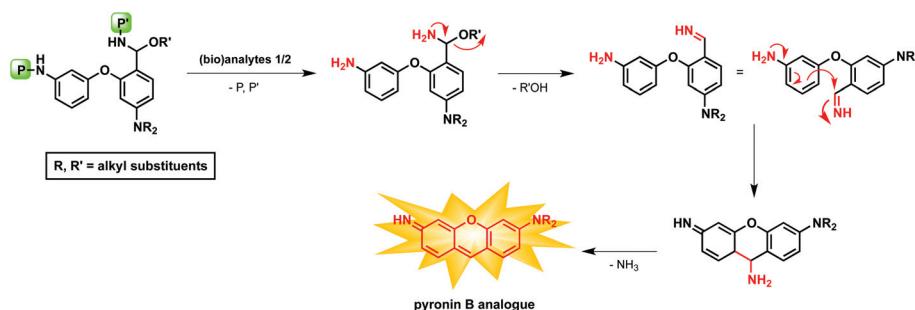


Fig. 18 One possible strategy for the simultaneous detection of two distinct (bio)analytes through the “covalent assembly” approach: *in situ* formation of an unsymmetrical pyronin fluorophore (P and P' = carboxamide or carbamate protecting groups). Please note that the intermediate imine can be hydrolysed to aldehyde but a similar intramolecular cyclisation reaction can take place to provide an unsymmetrical pyronin fluorophore (elimination of a water molecule).

cells) as the capture agent for both lipidated caged precursors (*i.e.*, pre-functionalized with a long alkyl chain) through strong interaction with the lipophilic membrane of these carriers. Such a cell-based assembly strategy has been recently applied successfully to a pair of a lipidated photo-labile prodrug (an anti-inflammatory drug covalently appended to an alkylcobalamin) and a far-red emitting fluorophore (cyanine dye Cy 5.0 acting as an antenna and an energy donor) for applications in photo-induced drug delivery (Fig. 19).⁴⁶

An interesting recent development in the field of “click” chemistry made it possible to perform concurrent *in vivo* monitoring of two distinct (bio)analytes using luciferin-based bioluminescence imaging technologies. Indeed, inspired by the Nature and after a comprehensive study of the regeneration pathway of D-luciferin (a common bioluminescent substrate for firefly luciferase) in fireflies, Rao and co-workers discovered a new, biocompatible reaction between 6-substituted 2-cyano-benzothiazole (CBT) derivatives and D-cysteine, which can be controlled by pH, redox status and hydrolytic enzyme activities to synthesize large molecules and form different nano-

structures (Fig. 20).⁴⁷ In the space of just five years, this thiol-based “click” reaction has been extensively studied and successfully applied to *in vivo* molecular imaging through the clever design of nano-aggregation fluorescent or magnetic resonance (MR) probes for the non-invasive detection of protease activities (furin and caspases) or for sensing the reducing environment.⁴⁸ In 2013, Bertozzi, Chang and co-workers implemented this “click” condensation reaction to the simultaneous *in vivo* imaging of two distinct analytes, namely H₂O₂ and caspase-8 (an apoptosis-related cysteine protease).⁴⁹ Their strategy employs *in situ* formation of firefly luciferin from two complementary caged precursors, namely “peroxy caged luciferin 2 (PCL-2)” and Z-Ile-Glu-Thr-Asp-D-Cys (IETDC) that can be unmasked through a selective reaction with the targeted ROS and protease respectively. Indeed, PCL-2 is a H₂O₂-responsive boronic acid probe that releases 6-hydroxy-2-cyano-benzothiazole (HCBT) through boronate oxidation to phenol followed by 1,6-elimination reaction whereas pentapeptide IETDC is a potent substrate of caspase-8 which hydrolyses the peptide bond after the aspartic acid residue to release D-cysteine. Once released, HCBT and D-Cys subsequently react together to yield luciferin *in situ*, resulting in a bioluminescence signal under the action of luciferase (Fig. 21). Moreover, concomitant use of PCL-2 and IETDC *in vivo* establishes a concurrent increase in both H₂O₂ and caspase-8 activity during acute inflammation in living mice. This “self-assembly” bioluminogenic approach is therefore a powerful tool for studying simultaneous oxidative stress and inflammation processes in living animals during injury, aging and disease, and may be extended to concurrent monitoring of multiple (bio)analytes. This should be made easier by the

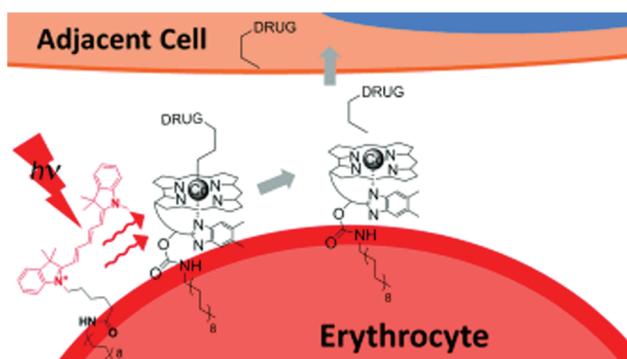


Fig. 19 A wavelength-encoded drug-release strategy. Anti-inflammatory drugs are covalently appended to cobalamin (Cbl) by means of a photolabile Co–C bond. Lipidated-Cbl and fluorophore constructs assemble on the plasma membrane of human erythrocytes. The fluorophore serves as an antenna, capturing long-wavelength light and transmitting the energy to the Cbl–drug conjugate, resulting in drug release from the erythrocyte carrier. Reproduced with permission from ref. 46. Copyright 2014 John Wiley & Sons, Inc.

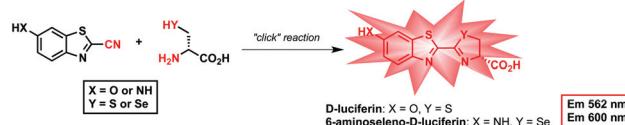


Fig. 20 The “click” reaction between free D-(seleno)cysteine and 6-amino/6-hydroxy CBT derivative for the synthesis of D-luciferin (or 6-aminoseleno-D-luciferin).

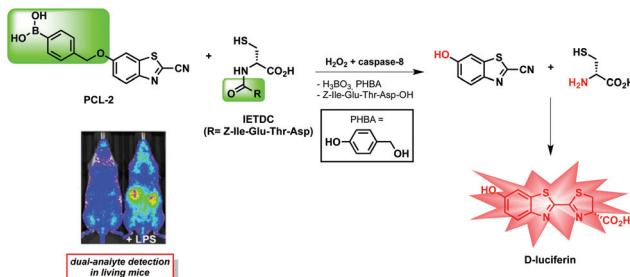


Fig. 21 "Self-assembly" bioluminogenic approach for dual-analyte luciferin imaging: *in vivo* bioluminescence detection of H_2O_2 and caspase-8 in a murine model of acute inflammation (LPS = lipopolysaccharides used to induce an acute inflammatory response). Bioluminescent images reproduced with permission from ref. 49. Copyright 2013 American Chemical Society.

current availability of a wide range of "activatable" luciferin-based bioluminescent probes (also known as caged luciferins) suitable to image a variety of enzymatic activities (*e.g.*, glycosylase, MAO, protease, and sulfatase) and physiological states^{10,50} that can serve as a relevant source of inspiration for the design and synthesis of other caged luciferin precursors. Furthermore, an extension of this bioluminogenic "click" reaction to D-selenocysteine (D-Sec) will lead to the *in situ* formation of a luciferin analogue having a luminescence maximum close to 600 nm, more suited for *in vivo* imaging (Fig. 20).^{51,52}

Conclusions and outlook

In this feature article, I hope that I have been able to demonstrate that reaction-based luminescent probes are tools well suited for devising new sensing systems for the concomitant detection of several (bio)analytes at the nano-scale and within the same sample or biological medium. Contrary to more conventional "AND" molecular logic gates based on supramolecular photochemical mechanisms (commonly employed for multi-cation and/or multi-anion detection), such "lab-on-a-molecule" prototypes can be applied to a wider range of reactive (bio)analytes including biomolecules and disease-related enzymes, by judicious selection of suitable reaction sites. This could be particularly interesting to develop innovative new diagnostic tests for diseases where the combined detection/quantification of two distinct biomarkers is preferred to avoid "false positive" results (*e.g.*, prostate cancer⁵³).⁵⁴ Dual-controlled activatable optical (bio)probes could also be used to achieve a high target-to-background signal ratio (TBR) and thus improve the sensitivity of *in vivo* molecular imaging.⁵⁵ In this context, the concept of double enzymatic activation of pre-pro-fluorophores recently published by Prost and Hasserodt and applied to the simultaneous detection of β -D-galactosidase and leucine amino-peptidase through the release of a precipitating phenol-based fluorophore is very relevant and promising.⁵⁶ Furthermore, "dual-lock" structures combining two distinct reactive/quenching groups sensitive to the same analyte have recently emerged as valuable tools for designing

reaction-based fluorescent probes with unprecedented performances (*i.e.*, selectivity and sensitivity) for single analyte sensing.⁵⁷ The discovery of novel biocompatible fluorogenic reactions will facilitate the design of "reactive" probes leading to *in situ* formation of fluorescent scaffolds with valuable spectral features in the NIR wavelength range, known as the "therapeutic" window,⁵⁸ will also contribute to this challenging goal.

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