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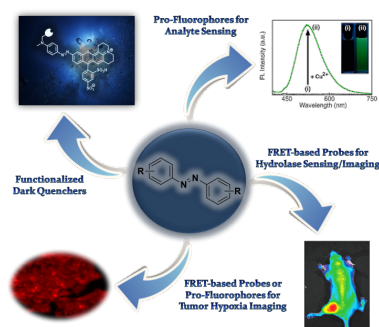
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Azo-based Fluorogenic Probes for Biosensing and Bioimaging: Recent Advances and Upcoming Challenges

Arnaud Chevalier,^[a] Pierre-Yves Renard^{*[a]} and Anthony Romieu^{*[b, c]}



Abstract: The use of non-fluorescent azo dyes as dark quenchers in activatable optical bioprobes based on Förster resonance energy transfer (FRET) mechanism and designed to target a wide range of enzymes, has been well established for over two decades. The key value of the azo moiety (-N=N-) to also act as an efficient "ON-OFF" switch of fluorescence once introduced within the core structure of conventional organic-based fluorophores (mainly fluorescent aniline derivatives) has been recently exploited in the development of alternative reaction-based small-molecule probes based on the "pro-fluorescence" concept. These unprecedented "azobenzene-caged" fluorophores are valuable tools for the detection of a wide range of reactive (bio)analytes. This review highlights the most recent and relevant advances that have been made in the design and biosensing/bioimaging applications of azo-based fluorogenic probes. Emphasis is also placed on relevant achievements in synthesis of bioconjugatable/biocompatible azo dyes used as starting building blocks in the rational and rapid construction of these fluorescent chemodosimeters. Finally, a brief glimpse of the possible future biomedical applications (theranostics) of these "smart" azobenzene-based molecular systems is presented.

Keywords: azo dyes • azoreductase • bioconjugation • dark quenchers • FRET • hypoxia • pro-fluorescence • "smart" probes

1. Introduction

Azobenzene derivatives (or azo compounds, ABs) are a well-known class of organic dye molecules that share the core azobenzene structure, simply defined as the combination of two phenyl rings attached together through a nitrogen-nitrogen double bond (-N=N-), with different substituents and/or further (hetero)cyclic rings fused to the benzene moieties. Such molecules exhibit unique large UV-vis absorbance spectra mainly due to the significant number of vibronic states in each energy levels S_n . This mostly leads to high molar absorption coefficients in solution with a wide range of colors relative to their absorbance maximum value. That is why these molecules have been widely used as pigments since the 19th century.^[1] This is all the more legitimate given their exceptional chemical stability under harsh conditions, excepted in reducing media.

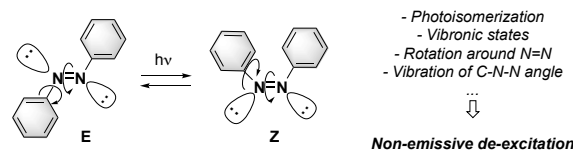


Figure 1. (E-Z) Photoisomerization of ABs leading to non-radiative deactivation of their excited states, explaining the weak or non-fluorescence of these chromophores.

The most important photophysical property of ABs is undoubtedly the prominence of a non-radiative deactivation of their electronic excited states S_1 and S_2 , related to photochemical isomerization of their azo bridge, either through a rotation mechanism around the -N=N- double bond or an inversion mechanism, in which a planar variation of one of the C-N-N angles can occur at the excited state (Figure 1).^[2] Consequently, ABs are usually weakly or non-fluorescent compounds. The preferred way to get fluorescent AB derivatives is to add a chemical functionality such as an ESIPT-active moiety^[3] or a bis(pentafluorenyl)boryl (or silyl) group to form an intramolecular N-B (or N-Si) dative bond to restrict rotation and isomerization.^[4] Thus, associated to another emitting dye (fluorescent or luminescent) in a molecular system in which all prerequisites for the occurrence of an effective FRET are fulfilled^[5], ABs can play the role of acceptor which dissipates readily the energy through non-radiative pathways. As a practical consequence, the fluorescent donor is turned "OFF", as long as the FRET process can occur. Interestingly, other photophysical mechanisms such as intramolecular photoinduced electron transfer (PeT) or internal charge transfer (ICT) have also been implemented for the rational design of non-emissive dyes (also known as quenchers) from fluorescent derivatives.^{[6],[7]} Among the different kinds of organic-based quenchers currently available, the different members of Black Hole Quencher[®] family integrate

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within their backbone structure one or several azo groups to take advantage of their non-emissive de-excitation.^[8, 9] The successful use of these azo quenchers in enzymatically activatable FRET-based probes has been extensively described in the literature, particularly for the sensitive and selective detection of biologically relevant proteases, and comprehensive reviews on this topic have already been published.^[10] Surprisingly, until five years ago, few research efforts were devoted to the synthetic chemistry of these azo-based quenchers with the aim of optimizing both their spectral and physico-chemical properties (e.g., solubility in aq. media) and introducing a bioconjugatable tether compatible with emerging and promising (bioorthogonal) reactions belonging to the repertoire of "click chemistry".^[11] We and other research groups have recently proposed innovative chemical solutions to develop non-conventional azo dyes particularly useful for: (1) a convenient access to enzyme-sensitive (mainly, proteases and reductases) FRET-based probes suitable for bioimaging experiments in living cells and/or *in vivo* and (2) the construction of unprecedented pro-fluorophores^[12] based on protection-deprotection (*i.e.*, reductive release of aniline-based fluorophores)^[13] or "covalent-assembly" probe design principle (*i.e.*, *in situ* formation of a fluorescent scaffold)^[14] for sensing (bio)analytes in various contexts. This review focuses on these recent advances (both synthetic aspects and bioanalytical applications) related to fluorescence quenching ability of ABs but their other important applications in the field of chemical biology, namely molecular photoswitches for reversible photocontrol of biological systems, related to photochromism of azo compounds will be not covered here.^[15]

Arnaud Chevalier received his Ph.D. in bioorganic chemistry in 2014 at the University of Rouen under the co-supervision of Profs. Pierre-Yves Renard and Anthony Romieu. His research works were focused on the development of new advanced chemical tools for applications in molecular imaging. As a postdoctoral fellow under the guidance of Prof. Sidney M. Hecht at the Arizona State University (United States of America), he is carrying out chemical biology research to address some issues related to biochemistry of mitochondria. He is co-author of more than 15 scientific papers and co-inventor of three patents.



Prof. Pierre-Yves Renard received his Ph.D. in organic chemistry in 1994 at the Université Paris-Sud/Ecole Polytechnique under the supervision of Prof. Jean-Yves Lallemand. After a nine year period (1994-2003) as French Department of Defense procurement agency staff (Délégation Générale pour l'Armement) at CEA Saclay, where he conducted research on biocompatible means to detoxify organophosphorus nerve agents under the supervision of Dr. Charles Mioskowski, he was appointed professor in 2003 at the University of Rouen (France), and junior member of the French University Institute (IUF) in 2006. He has, since then, led the bio-organic chemistry group, and his current research fields are the development of "smart" probes for the detection of



bioanalytes and chemical tools for bioconjugation and medicinal chemistry. He is co-author of more than 125 research papers and co-inventor of 15 international patents, two of them have given rise to the ChemForAse start-up, specialized in the development of purification methods for proteins of (bio)medical interest.

Prof. Anthony Romieu obtained his Ph.D. from the University Joseph Fourier (Grenoble, France) under the guidance of Drs Jean Cadet and Didier Gasparutto (Laboratory of Nucleic Acids Damages, CEA-Grenoble) in 1999. After serving for two years as a CNRS study engineer in the lab of Pr. B.-P. Roques at the University Paris 5, in the fields of medicinal chemistry and peptide synthesis, he joined in June 2001 a private biotech company, Manteia 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 commercialization 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 Prof. Pierre-Yves 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 co-author of more than 85 scientific papers and co-inventor of over a dozen patents, three of them being actively pursued by the Illumina Company (Genome Analyzer DNA sequencing technology).



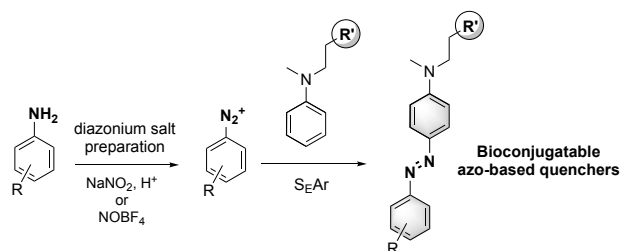
2. Design and synthesis of novel functionalized azo-based quenchers with enhanced spectral properties

The preparation of dark quenchers bearing one or two azo groups has received substantial interest among (bio)organic chemists in the early 2000s when Biosearch Technologies company (now part of LGC group) commercialized first polyaromatic azobenzene derivatives as bioconjugatable (activated *N*-hydroxysuccinimidyl (NHS) esters) quenchers named Black Hole Quenchers[®].^{[8, 9], [16], [17]} Associated to DABCYL^[18] and BlackBerry 650 (BBQ-650, recently developed and commercialized by Berry & Associates company)^[19], these molecules constitute the most popular set of azo-based quenchers currently used for designing activatable FRET-based probes and their obvious usefulness has been well proven (Figure 2). Carboxylic acid and some phosphoramidite derivatives are commercially available as reagents suitable for labeling of peptides and oligonucleotides through solution or solid-phase methodologies (*i.e.*, amidification reaction or phosphoramidite coupling followed by mild oxidation). However, these reagents are not suitable for biolabeling schemes/applications based on bioconjugation reactions developed during the past decade (*i.e.*, those belonging to the

repertoire of "click chemistry" and/or bioorthogonal reactions) and having the wind in their sails (e.g., copper-catalyzed azide-alkyne cycloaddition (CuAAC), oxime ligation, ...).^[11] Therefore, to expand the scope of these azo dyes in challenging biolabeling applications, some academic research groups have developed novel azo-based quenchers, bearing alternative bioconjugatable handles.

2.1. Synthesis of new bioconjugatable dark quenchers

Even if many synthetic methods have been reported for the synthesis of ABs^[20], the strategy currently used to prepare the azo-based quenchers always relies on a conventional azo-coupling reaction between a diazonium salt (derived from one of the two aniline coupling partners) and an electron-rich *N,N*-dialkylaniline, whose mechanism is closely related to that of a S_EAr reaction (Scheme 1). The use of an unsymmetrical mono *N*-substituted tertiary aniline with a short alkyl chain ended by a protected or free functional group is the preferred synthetic way to readily functionalized azo dyes.

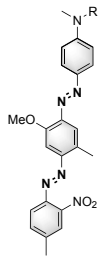
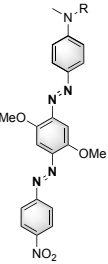
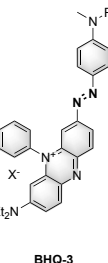


Scheme 1. General synthetic scheme for the preparation of functionalized azo-based quenchers through azo-coupling reaction.

This obvious synthetic strategy was primarily used by the Burkart and Fossey groups for the preparation of a primary alcohol derivative of **BHQ-2** and diol-appended methyl red analogs respectively.^[21, 22] The primary alcohol of **BHQ-2** was converted into activated *para*-nitrophenyl carbonate to achieve solid-phase labeling of Ybbr peptide, used in combination with a tetramethylrhodamine-CoA conjugate to develop an HTS assay for assessing inhibitors of the phosphopantetheinyl transferase Sfp. Diol-appended methyl red analogs were involved in FRET-based probes using fluorescein as reporter and suitable for monitoring boronate ligation reaction. In 2012, the Kool group developed a set of aldehyde-reactive fluorescence quenchers by introducing α -nucleophile such as oxyamine and hydrazine moieties.^[23] Their great ability to quench the emission of fluorescein was assessed in the context of duplex DNA oligonucleotides made of two complementary strands labeled with an aminoxy/hydrazine quencher through oxime/hydrazone ligation and fluorescein respectively. Interestingly, aminoxy and hydrazine quenchers were also applied to quenching of an aldehyde-containing fluorophore (i.e., 7-*N,N*-diethylamino-3-formylcoumarin) associated with living cells, demonstrating cellular fluorescence quenching within one hour. In 2013, Jing and Cornish reported the first synthesis of primary amine-modified **BHQ-1** through a conventional azo-coupling between *N*-Boc derivative of *N*-(3-aminopropyl)-*N*-methylaniline and Fast Corinth V salt, followed by Boc removal by TFA treatment.^[24] This building block was used for the construction of a FRET-based fluorogenic trimethoprim (TMP) tag (i.e., a trimeric TMP-quencher-fluorophore molecule) which can be recognized by *E. coli*

dihydrofolate reductase (e-DHFR)-tagged proteins and subsequently cleaved (i.e., quencher displacement) in a proximity-induced S_N2 reaction mediated by a cysteine residue. This enables live cell imaging of intracellular proteins with high signal-to-background. During the same period, we have revisited the chemistry of **BHQ-3**, a dark quencher commonly used for switching-off the emission of far-red or near-infrared (NIR) fluorophores belonging to the cyanine family, to expand the range of reactive and/or bioconjugatable groups available within the core structure of this chromophore (i.e., primary amine, azido, α -iodoacetyl, ketone, terminal alkyne, vicinal diol).^[25] Our synthetic strategy was based on the synthesis of functionalized unsymmetrical tertiary anilines and their use in azo-coupling reaction with methylene violet 3RAX performed under mild non-acidic conditions (i.e., nitrosonium tetrafluoroborate $NOBF_4$ as diazotization reagent^[26] in CH_3CN at 0 °C). Interestingly, further functionalization of some of these **BHQ-3** derivatives (e.g., azido derivative) can be readily achieved through post-synthetic "click" reactions (e.g., copper-catalyzed azide-alkyne cycloaddition (CuAAC)), aimed at introducing (1) a novel valuable chemical function whose stability is not necessary full-compatible with the azo-coupling reaction conditions, (2) neutral or ionic (charged) hydrophilic group(s) to impart water solubility and biocompatibility, or potentially (3) targeting moiety/balancing domain to improve *in vivo* performances including specificity, biodistribution and pharmacokinetics of NIR fluorescence imaging agents based on FRET pairs including **BHQ-3** as acceptor. Such simple and reliable synthetic methodology was also applied to shorter-wavelength dark quenchers namely **BHQ-1** and **BHQ-2**, especially by us^[27, 28] and the Wagner group.^{[29, 30, 31],[32]} The bioconjugation ability and quenching efficiency (QE) of some of these BHQ derivatives were demonstrated through the preparation and enzymatic activation (both *in vitro* and in living cells) of FRET-based fluorogenic substrates for urokinase plasminogen activator (uPA), a key protease in cancer invasion and metastasis.^[25, 27, 28] Table 1 compiles all bioconjugatable BHQ derivatives published in the last six years. Depending on the biological vector to label and/or the conjugation site/reaction chosen, researchers may select the most appropriate BHQ within this valuable synthetic "tool box".

Table 1. Bioconjugatable BHQ derivatives currently available and suitable for biolabeling.

Quencher scaffold	Reactive function	React with	Reaction type	Ref
 <p>BHQ-1 Abs λ_{max} = 535 nm QR = 480-580 nm ϵ = 34 000 M⁻¹ cm⁻¹</p>	Carboxylic Acid	Primary amine	Amidification (peptide-type coupling)	[8]
	Diol	Boronic acid	Boronate ligation	[22]
	Amine	Carboxylic acid	Amidification (peptide-type coupling)	[24]
	Oxyamine	Aldehyde / Ketone	Oxime ligation	[23]
	(Acyl)Hydrazine	Aldehyde / Ketone	Oxime ligation	[23]
	Aldehyde	Oxyamine / Hydrazine	Oxime / Hydrazone ligation	[27]
	Terminal alkyne	Organic azide / Halogeno-aromatic	CuAAC / Sonogashira	[27]
	Azido	Terminal alkyne / Phosphine	CuAAC / Staudinger ligation	[27]
	Maleimide	Thiol	Thioalkylation (Michael addition)	[27]
Phosphoramidite	Alcohol (nucleoside)	Phosphitylation followed by oxidation	[8]	
 <p>BHQ-2 Abs λ_{max} = 579 nm QR = 550-650 nm ϵ = 38 000 M⁻¹ cm⁻¹</p>	Carboxylic Acid	Primary amine	Amidification (peptide-type coupling)	[8]
	Diol	Boronic acid	Boronate ligation	[22]
	Primary amine	Carboxylic acid	Amidification (peptide-type coupling)	[31]
	Oxyamine	Aldehyde / Ketone	Oxime ligation	[23]
	(Acyl)Hydrazine	Aldehyde / Ketone	Hydrazone ligation	[23],[31]
	Aldehyde	Aldehyde / Ketone	Oxime ligation	[28]
	Activated carbonate	Primary amine	Acylation (urea bond formation)	[21]
	Terminal alkyne	Org. azide / Haloaromatic	CuAAC / Sonogashira	[28]
	Azido	Terminal alkyne / Phosphine	CuAAC / Staudinger ligation	[28]
	Maleimide	Thiol	Thioalkylation (Michael addition)	[28]
Phosphoramidite	Alcohol (nucleoside)	Phosphitylation followed by oxidation	[8]	
Thiol	Maleimide	Thioalkylation (Michael addition)	[30]	
 <p>BHQ-3 Abs λ_{max} = 672 nm QR = 620-730 nm ϵ = 42 000 M⁻¹ cm⁻¹</p>	Carboxylic Acid	Primary amine	Amidification (peptide-type coupling)	[8]
	Diol	Boronic acid	Boronate ligation	[8, 25]
	Primary amine	Carboxylic acid	Amidification (peptide-type coupling)	[33, 34]
	Terminal alkyne	Azide / Halogeno-aromatic	CuAAC / Sonogashira	[25, 35]
	Azido	Terminal alkyne / Phosphine	CuAAC / Staudinger ligation	[25]
	Ketone	Oxyamine / Hydrazine	Oxime / Hydrazone Ligation	[25]
	Aldehyde	Oxyamine / Hydrazine	Oxime / Hydrazone Ligation	[25]
	α -Iodoacetyl	Thiol	Thioalkylation (S _N 2-type reaction)	[25]
	Phosphoramidite	Alcohol (nucleoside)	Phosphitylation followed by oxidation	[8]
	5-Alkoxyoxazole	Maleimide	Kondrat'eva ligation	[34]

2.2. Synthesis of "tuned" dark quenchers for specific applications

Even though significant advances have been achieved to expand the nature of reactive groups and/or functionalities that provide

specific properties to **DABCYL**, **BHQ** or **BBQ** derivatives, synthetic efforts were exclusively focused on the side part of these dye molecules (*i.e.*, tertiary aniline moiety) without altering their core structure and spectral characteristics. However, the development of more innovative "smart" fluorescent imaging agents and/or biosensing assays requires the availability of more sophisticated azo-based dark quenchers whose molecular scaffold is dramatically different from this of the parent chromophore, especially to obtain multivalent molecular platforms allowing access to unprecedented photoactive (bio)molecular systems, or to improve both spectrum width (full-width half-maximum, $\Delta\lambda_{1/2\text{ max}}$) and QE parameters. In this context, in 2014, Kovaliov et al. reported a nucleoside-**DABCYL** analogue, namely dU^{DAB} , which could be incorporated anywhere in an oligonucleotide sequence and in any number, and used as a quencher in different hybridization sensitive probes (Figure 3a).^{[36],[37]} The key step in synthesis of phosphoramidite synthon of dU^{DAB} was a Suzuki Pd-catalyzed cross-coupling reaction between 5'-O-DMTr-5-iodo-2'-deoxyuridine and pinacol boronate ester of 4-(*N,N*-dimethylamino)azobenzene (DAB). In addition to its ability to quench fluorescein emission, photoisomerization of dU^{DAB} moiety upon irradiation at 365 nm confirms its function as a photoswitch, particularly useful for the photocontrol of DNA/RNA duplex formation. By analogy with multivalent bioconjugation platforms currently developed for applications in chemical biology and drug discovery^[38], heteromultifunctional azobenzene scaffolds that harness sequential "click" and/or bioorthogonal reactions should find significant utility, especially in the rapid construction of broad spectrum dark quenchers and emerging multicomponent fluorogenic ensemble chemosensing systems including dual-FRET-based fluorescence probes for the sequential detection of two distinct enzyme activities^[39] or probes installed with dual-reactive and dual-quenching groups for highly selective and sensitive sensing of a single analyte.^[40] The first examples of heteromultifunctional ABs have been developed by our research group and are shown in Figure 3b-c. Using two pre-functionalized primary and tertiary anilines in the synthesis of **DABCYL** core, we designed an azido-carboxylic acid azo-based quencher which was used for the preparation of a water-soluble "clickable" **DABCYL** analog through "post-synthetic" sulfonation approach.^[41] At that time, this enabled to fill a gap in the chemistry of azo-based quenchers namely a versatile synthetic method to rapidly access highly polar derivatives without using harsh conditions (*i.e.*, concentrated H_2SO_4 to perform aromatic sulfonation, see example of **BHQ-10**^[42], Figure 3d) or an hydrophilic tertiary aniline in azo-coupling (*e.g.*, *N*-alkyl-*N*-sulfopropylanilines)^[43] which may sometimes exhibit poor reactivity/solubility depending on the conditions used for this reaction. A clever extension to this preliminary work has also led to the first heterotrifunctional thiol-alkyne-carboxylic acid azo-based dark quencher derived from a nitro-**DABCYL** core.^[44] The derivatization of this trivalent AB derivative with two other non-fluorescent azo dyes (**BHQ-1** and **BHQ-3**), through effective thioalkylation and CuAAC reactions has led to an universal dark quencher (UDQ), able to turn "OFF" an array of organic-based fluorophores covering the UV/NIR (300-750 nm) spectral range (*vide infra*).

2.3. Toward universal dark quenchers

The choice of dark quenchers as the donor part in FRET pairs is often limited by the required overlap between the absorbance spectrum of the donor and the emission spectrum of the acceptor (fluorophore). Despite some examples highlighting the possibility of an effective quenching through the formation of a ground-state complex (intramolecular dimer between quencher and fluorophore)^[45] between two FRET partners not complementary from a spectral point of view, this static quenching approach, highly depending on the chemical structures of donor and acceptor units, cannot be generalized. In this context, some recent research works have been devoted to the expansion of quenching range of azo-based dark quenchers. The first achievements from our research group in this field, was based on the covalent association of three spectrally distinct and complementary azo dyes within a same molecular architecture and using the heterotrifunctional nitro-**DABCYL** (structure shown in Figure 3c) as the central core.^[45] Basically, the simultaneous quenching action of these three azo dyes provided a QE up to 95% for organic-based fluorophores whose emission is centered in the range 340-770 nm (except for a sulfocyanine dye Cy 7.0, QE = 81%). The major drawback of this unusual azo-based quencher is obviously its considerable size which could be a major limitation in biological applications through preventing its cell permeability or that of bioconjugates containing it. The issue of the extension of quenching range using smaller molecules was firstly addressed in 2011 when the Kool group had the ingenious idea to associate two donors to one acceptor within a small azo- or bis-azo-based scaffold, aimed at allowing a multiplicity of conjugation pathways of varied length, leading to a positive effect of broadening the absorption spectrum of the resulting multi-path quenchers (MPQs).^[46] **MPQ-6** (Figure 4a) exhibits the most-red-shifted absorption maxima (536 and 656 nm) and a valuable spectrum width (full-width half-maximum, $\Delta\lambda_{1/2\text{ max}}$) of 270 nm, ranging from 460 to 730 nm, when conjugated to DNA oligonucleotides (15- or 20-mer). This quencher is able to turn "OFF" the emission of a wide range of fluorescent organic dyes ranging from Alexa Fluor® 350 (7-aminocoumarin derivative) to Quasar 670 (cyanine Cy 5.0 derivative). QE was determined in the context of DNA oligonucleotide duplexes placing fluorophore and **MPQ-6** within contact distance (to assess the efficiency of contact quenching, found in the range 72-98% depending on fluorophore) or beyond this distance (to assess the efficiency of mixed contact-FRET quenching mechanism and FRET quenching, found in the range 65-95% and 50-78% respectively). Historically, this work represents the first example of structural tuning of azo-based quenchers to improve their spectral properties.

By analogy with this promising approach, we have designed another class of broad spectrum azo-based quenchers by using anilinium moiety of unsymmetrical rhodamines as acceptor group and named azo-sulforhodamine dyes (Figure 4b, **SR101-Q-X**).^[47] The use of an aniline-based fluorophore (*e.g.*, 7-aminocoumarins, bodipys bearing an amino group in 2/6 position, pyronins, rhodamines, rhodols, *N*-substituted 4-amino-1,8-naphthalimides, ...) as the primary aniline partner in azo-coupling reactions is also beneficial to easily bring valuable functionalities (*e.g.*, targeting and/or water-solubilizing moieties) onto the resulting azobenzene-caged fluorophore by taking advantage of the reactive groups already available on the starting fluorescent aniline (*e.g.*, onto the *meso*-phenyl ring of xanthene dyes). The recent synthesis of unsymmetrical sulforhodamine SR101-110

achieved by our research group^[48], has enabled the practical implementation of this approach involving azo-coupling reaction mediated by NOBF_4 ^[26] and leading to quenchers **SR101-Q-X**. By changing only the *N*-substituent of the tertiary aniline partner, it was possible to introduce a wide range of biconjugatable handles (primary amine, terminal alkyne, azido, carboxylic acid and maleimide) onto **SR101-Q** scaffold while retaining two sulfonate groups on the *meso* phenyl ring, particularly beneficial for water solubility. The bioconjugation ability and excellent QE (in the range of 93-99% depending on fluorescent donor) of these **SR101-Q-X** derivatives were demonstrated through the preparation and *in vitro* enzymatic activation of FRET-based fluorogenic substrates of uPA.

2.4. Future prospects

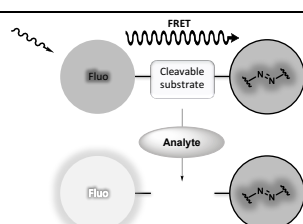
All innovations in molecular design and synthetic chemistry of azo-based quenchers accomplished during the past five years, have led to a wide array of valuable photoactive building blocks suitable to meet present and future challenges of biosensing/bioimaging involving the use of fluorescent chemodosimeters. Nevertheless, there is still an urgent need for broad spectrum dark quenchers capable of completely switching "OFF" fluorophores emitting in the Cy 7.0/Cy 7.5 spectral region (*i.e.*, > 750 nm, QE > 95%). In 2015, the Hanaoka group has described Si-rhodamine-based NIR dark quenchers (**SINQs**), which show broad absorption covering this region.^[49] They are non-fluorescent independently of solvent polarity and pH, probably due to free rotation of the bond between the *N*-atoms and the xanthene moiety (Figure 5a). A similar strategy was recently applied to a dihydroxanthene-hemicyanine fused scaffold^[50] (Figure 5b) but in both cases, the synthetic access to these sophisticated molecules involves reactions more complicated than a conventional azo-coupling. Thus, the development of effective azo-based NIR dark quenchers remains a major challenge in the field of chemistry of such azo dyes. With regard to biolabeling of peptides/proteins with an azo-based quencher, the recent development of bench-stable diazonium reagents and mild conditions to perform tyrosine (or its genetically encoded 2-naphthol analog)-selective modifications of

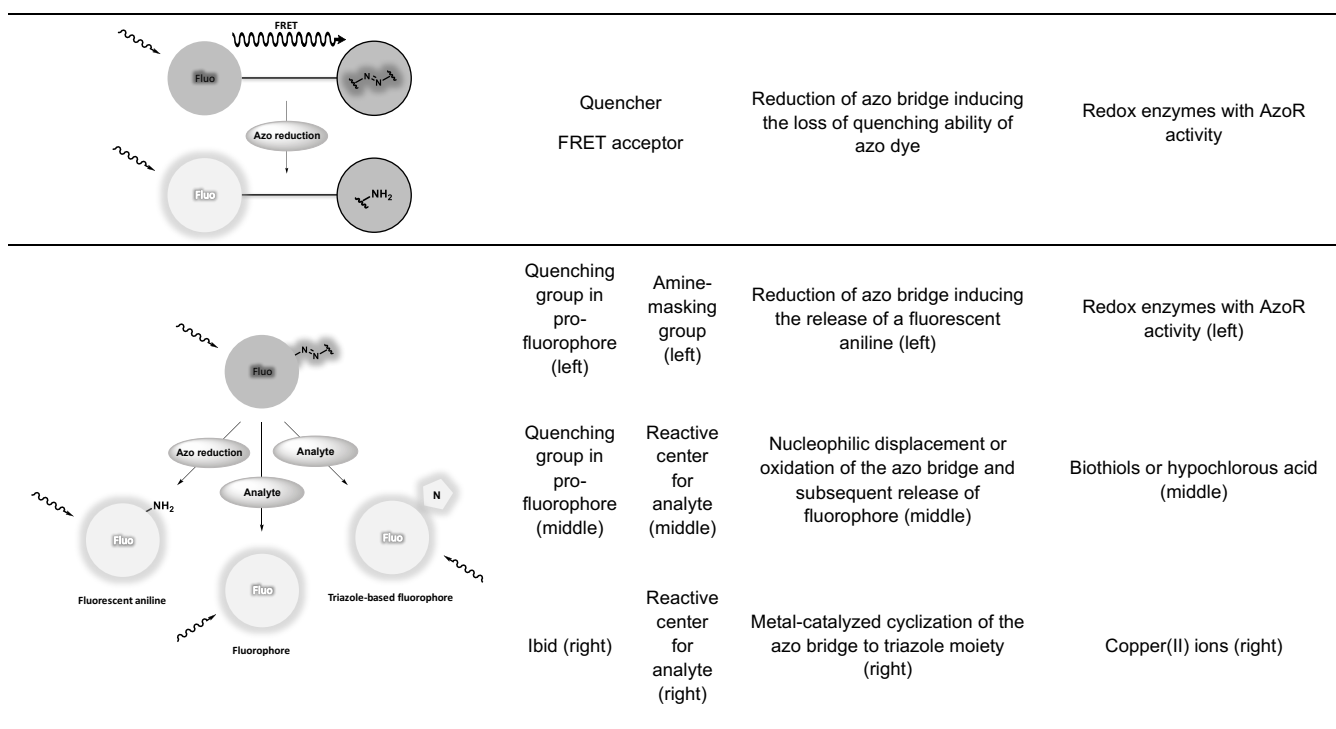
peptides/proteins through site-specific azo coupling^[51], provides a smart alternative to generate FRET pairs from a peptide-based biopolymer (Figure 6). Construction of azo-based quenchers using an amino acid residue as partner in azo-coupling reaction, may have multiple benefits such as: (1) facile access to structural diversity (and specific spectral characteristics) without resorting to total synthesis of isolated azo-based dark quenchers and (2) the non-necessity of a rational functionalization of azo-based dark quenchers for their conjugation to biological vectors.

3. Azo-based fluorogenic probes

A survey of literature on azo-based fluorogenic probes identified three main distinct probe design principles, which are summarized in Table 2. Azo dye can either be used as a dark quencher in the context of FRET-based probes (approaches 1 and 2) or as a pro-fluorophore defined as an azobenzene-caged fluorophore whose activation is based on a "mild" chemical event induced by the targeted (bio)analyte (most often, reductase or chemical reducing agent, approach 3). For the first approach, numerous FRET-based activatable probes bearing an enzyme-cleavable substrate for the detection of biologically relevant hydrolytic enzymes (mainly proteases) in various biological contexts (*i.e.*, extracellular fluids to cells, tissues and various *in vivo* animal models), have been reported in the literature and/or already compiled in some comprehensive reviews.^[10] Here, we will focus only on the detailed description of the two other approaches to turn "ON" or ratiometric fluorescence detection (entries 2-3 in Table 2) upon either reductive decomposition of FRET-based probes (approach 2) or conversion of an azo-based pro-fluorophore into a fluorescent organic dye through reductive decomposition, elimination or metal-catalyzed cyclization to triazole of its $-\text{N}=\text{N}-$ double bond (approach 3). Interestingly, alternative activation mechanisms producing optical signal enhancement have been also implemented in azo-based chromogenic/fluorogenic chemosensors for anion/cation detection but they will not be discussed here.^[54, 55]

Table 2. Overview of azo-based fluorogenic probes (FRET-based probes and pro-fluorophores) currently used for reaction-based biosensing/bioimaging: schematic representation and principle for the activation.

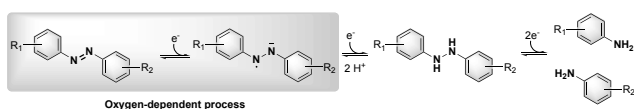
Probe design principle	Origin/function of azo moiety	Activation mechanism	Targeted analyte(s)
	Quencher FRET acceptor	Cleavage of the linkage between fluorophore and quencher inducing the loss of FRET	Hydrolytic enzymes such as esterases, phosphatases, phospholipases, proteases, ... ^[10] or nucleophilic species such as biothiols ^[52,53]



Scheme 2. Mechanism generally assumed for the AzoR-mediated reduction of azo dyes. Please note: co-factor NADH or NADPH is omitted for clarity.

3.1. Approach 2: reductive decomposition of azo-based quencher (acceptor) of FRET probes. Application to detection of azoreductase (AzoR) activity and tumor hypoxia imaging

The sensitivity of $-N=N-$ double bond towards reducing agents such as sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) is well known and has promoted the use of azobenzene derivatives as cleavable linkers in chemical biology^[56, 57]. Thus, it is obviously relevant to take advantage of this chemical feature, to design fluorogenic probes for selective and sensitive detection of a (bio)analyte acting as reductant. Among all the species known to directly cleave azo bonds, redox enzymes named azoreductases (AzoRs) and working in tandem with a co-factor (NADH or NADPH)^[58] are prominent and frequently involved in remediation processes directed to removal and treatment of azo dyes from waste waters generated by dye industry.^[59] The accepted mechanism to explain the AzoR-mediated conversion of azo dye into two aromatic amines is summarized in Scheme 2, and based on stepwise reduction process involving sequential mono-electronic transfers^[33]. The first step leading to the radical-anion intermediate is strongly dependent of oxygen concentration (*i.e.*, oxygen partial pressure $p\text{O}_2$) in the medium. Low levels of oxygen (hypoxia status, $p\text{O}_2 = 0.1\text{-}5\%$) prevents the radical anion from reverting back to the neutral AB, and the radical is stabilized and further reduced to hydrazine whose $-\text{NH}-\text{NH}-$ single bond is finally cleaved to give the two corresponding anilines.



From a biological standpoint, hypoxia is a feature of tumor tissue and a notable phenomenon in the occurrence and growth of solid tumors. In oncology, the formation of hypoxic tumors usually leads to poor response to curative treatments and poor clinical outcome due to their resistance to radiation and chemotherapy (indeed, abnormal microvessels of such tumors limit the perfusion of cytotoxic chemotherapeutic drugs). Thus, assessment of the tumor hypoxia degree is of great importance in predicting anticancer therapies efficacies. Among the different methods currently available to achieve this^[60], those based on the fluorescence detection of hypoxia biomarkers (*e.g.*, reductases and hypoxia inducible factor (HIF)) are widely explored.^[61, 62] In this, context, a particular attention is paid to fluorogenic probes selectively activated by AzoRs. Interestingly, pioneering works from Biosearch Technologies have shown that some AzoRs (*e.g.*, group II AzoR BT11 working in tandem with NADPH) are able to readily cleave azo bond of quenchers of fluorescence (*i.e.*, **BHQ-10** and Janus Green B (JGB) the non-bioconjugatable version of **BHQ-3**).^[42] As a natural extension of these findings, FRET-based probes (named QCys) involving the covalent association of a far-red fluorescent cyanine dye (Cy 5.0, Cy 5.25 or Cy 5.5 derivative) with the complementary azo-based quencher **BHQ-3** through a carboxamide linkage, have been developed by the Nagano group for *in vivo* imaging hypoxia related to ischemia (Figure 7).^[33] The reductive cleavage of the azo bond of **BHQ-3** induces a loss of its quenching ability and as a consequence the restoration of far-red emission of cyanine (at 670 nm) for high level of hypoxia ($p\text{O}_2 = 1\%$ or less). This claimed activation mechanism of AzoR-sensitive fluorogenic QCy probes was demonstrated through an *in vitro* assay using rat liver microsomes placed under normoxic and hypoxic conditions. Next, the probe **QCy5** was successfully

applied both for visualizing the hypoxic status of MCF-7 breast cancer cells, and for *in vivo* imaging ischemic organs (liver and kidney) in live mice. More recently, the Jayagopal group published a similar azo-based FRET probe, namely **HYPOX-3**, involving a commercial mono-sulfonated derivative of Cy 5.25 as fluorescent donor (named NIR-667-carboxylic acid, Abs/Em 667/689 nm) and **BHQ-3** as dark acceptor, both being linked together through a carboxamide linkage. Similar results than those obtained with **QCy5** probe were observed for fluorescence imaging of hypoxic status of retinal cells (R28 cells). Further investigations have demonstrated the potential utility of **HYPOX-3** for early detection and timely treatment of retinal diseases (*i.e.*, blinding retinal vascular diseases including age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity), through *in vivo* retinal imaging of hypoxia^[63].

Furthermore, in the field of bioreductively activated FRET probes, the Qian group has reported an alternative donor-acceptor pair: rhodamine B (RhoB) and a novel azo-based quencher derived from 8-*N*-substituted 4-amino-1,8-naphthalimide scaffold and named **HPN**, covalently associated through an ester linkage (Figure 8).^[64] This corresponding turn "ON" probe **HP** appeared to be an obviously more sensitive chemodosimeter with detection observed as from 10% of *pO*₂ proving that this novel FRET strategy is also a good way to detect low levels of hypoxia. The excellent oxygen sensitivity of **HP** in living cells (HeLa cells) is linked to reduction potential of its azo moiety, undoubtedly lower than that of the -N=N- double bond of **BHQ-3**. Nevertheless, we should express some reserves about *in cellulo* results because the ester linkage involved in the construction of this AzoR-sensitive FRET pair, may be cleaved by esterases, to give the same fluorescence response (Em. 570-600 nm upon excitation at 540-560 nm) than that obtained after bioreduction of azo bridge. As conclusion, all these published results clearly show the usefulness of azo-based quenchers as reduction-responsive unit in FRET probes for the detection of hypoxia.^[65] Furthermore, the same probe design principle was also applied to other applications including (1) the tracking of microbial degradation of azo-based organic pollutants through green fluorescence unveiling of a *N*-substituted 4-piperidinyl-1,8-naphthalimide scaffold^[43] (Figure 9a) and (2) the validation of a new concept of chemically deactivatable quencher for FRET-based probes that turn "ON" their blue fluorescence (7-*N,N*-diethylaminocoumarin-3-carboxamide derivative as donor) by either an enzymatic cleavage or a chemical reagent (sodium dithionite), applied both to quantification of caspase-3 activity in solution and visualization of unreacted probes in apoptotic cells (Figure 9b).^[57] The only noticeable drawback associated with all bioreductively activated FRET probes, is their big molecular size and large structure which may negatively influence cell penetration and the affinities of the involved redox enzymes to these fluorogenic two-component assemblies. A valuable alternative is the use of small molecule activatable agents (also known as pro-fluorophores) derived from common organic-based fluorophores (generally, fluorescent anilines) and designed both to integrate an azo-based quenching unit within their core structure and to selectively react with their target (*i.e.*, redox enzyme or reactive analyte).

3.2. Approach 3: pro-fluorophores containing an azo moiety as quenching unit.

3.2.1 Fluorescence activation through reductive cleavage of the azo bridge and subsequent release of luminescent aniline.

The growing interest in alternative chemical structures of azo dyes required for high-tech applications (*e.g.*, dye sensitized solar cells, anion sensors, ...) has recently led chemists to focus on the synthesis of hybrid structures based on the functionalization of organic-based fluorophores with one or several (di)aryl azo moieties. As partially illustrated in Figure 10, several fluorescent organic dyes belonging to different families including bodipys^[66, 67], fluoresceins^[68], 1,8-naphthalimides^[53, 55], porphyrines^[69], perylenes^[70] and pyrenes^[71] have been converted into non-fluorescent azo dyes either through diazotization of their primary amino group or through covalent conjugation to a conventional bis-aryl azo dye.

Based on this unconventional strategy for fluorophore quenching, we have considered the reversible masking of primary aniline(s) of rhodamines through the direct conjugation of the -N=N- group(s). In addition to the ultrafast E/Z photoisomerization process of -N=N- bond(s), the locking of the xanthenic platform in a non-conjugated spirolactone form^[72] may also contribute to a greater QE than that obtained with FRET mechanism. Once again, the use of mild diazotization conditions previously mentioned (see part 2) has enabled us to efficiently synthesize the first bioreductively activatable pro-fluorophore derived from green-emitting rhodamine 110 dye (**Rho110**), and suitable for the detection of AzoR activity both *in vitro* and in the context of bacterial cultures (Figure 11).^[73] A similar approach was used by Li *et al.* to rationally design a turn "ON" reactive probe for the quantification of endogenous sulfides (*i.e.*, predominant forms of gas transmitter H₂S at physiological pH) in rat tissues.^[74] A set of azobenzene-caged O-methyl rhodols were synthesized using Mills reaction (*i.e.*, condensation between substituted nitrosobenzenes and O-methyl rhodol). The pentafluorophenyl derivative was emerged as the best candidate in light of its high reactivity towards the reducing analyte, resulting in high sensitivity (148-fold fluorescent switch-on response at 517 nm, upon excitation at 468 nm), good selectivity (22-fold more selective towards sulfides than other biothiols such as cysteine, glutathione (GSH) and homocysteine) and low detection limit (500 nM) (Figure 12). Further extension of this fluorogenic reduction process to other xanthenic scaffolds belonging to the family of rosamines (2Me rhodamine green (**2Me RG**) and its red-emitting silicon analog **2Me SiR600**) was explored by the Nagano group, to detect different levels of hypoxia (Figure 13a).^[75] Promising small molecule imaging agents namely **MAR** and **MASR** were obtained and **MAR** was successfully used to visualize retinal hypoxia in a rat model of retinal artery occlusion. For the dual purpose of red-shifting fluorescence and tuning the physicochemical properties (water solubility and cell permeability) of these hypoxia imaging agents without resorting to multi-step *de novo* syntheses, azobenzene-caged sulforhodamine dyes bearing either two methyl groups or two "clickable" azido groups were designed and prepared by our group (Figure 13b).^[76] Post-synthetic derivatization with a positively-charged or zwitterionic terminal alkyne and through CuAAC reaction has led to compounds **SR101-NaphtNH₂-Hyp-X** (X = sulfobetain or ammonium). These two fluorogenic probes were found to be readily activated by reductases and NADPH under physiological conditions to give back **SR101-NaphtNH₂**, leading to 45- and 58-fold fluorescence emission enhancement at ca. 625 nm within 1 h for sulfobetain and ammonium respectively. However, their ability to image the hypoxic status in living A549 cells was a failure, possibly explained by their too large size and/or too high hydrophilic character that limits their cell penetration. Conversely and positively, fluorescence enhancement inside cells under severe hypoxia (0.1% O₂) was obtained for non post-

functionalized **SR101-NaphtNH₂-Hyp-diMe** (Figure 14). Likewise, Shin *et al.* have recently conducted a systematic study on azorhodamines as fluorogenic substrates through the synthesis of a library of 10 compounds, in order to establish a relationship between structure and reactivity towards AzoR. They found that azorhodamines bearing an electron-donating group (typically *N,N*-dimethylamino) underwent two-electron reduction of -N=N- to the hydrazine by AzoR, followed by nonenzymatic reaction to afford the fluorescent product **2-Me RG**, and appeared to be suitable fluorogenic substrates for an AzoR reporter system.^[77] With the aim of obtaining a turn "ON" response in the far-red or NIR range, the same AzoR sensing strategy was recently implemented to a dicyanomethylene-4*H*-chromene (DCMC) fluorophore^[78] whose emission is centered at 650 nm (Figure 15).^[79]

The installation of an azo bridge between two 2,2'-bipyridinyl (bpy) chelating units of a phosphorescent dinuclear ruthenium(II) or iridium(III) complex has also been considered by the Chao group and reductively activatable probes suitable for sensing biothiols and endogenous (bi)sulfites (predominant forms of sulfur dioxide) respectively were obtained (Figures 16a-c and 17a).^[80-82] The main advantages of these phosphorogenic probes compared to conventional aniline-based pro-fluorophores previously discussed are red emission (around 600 nm), large Stokes' shift (typically 165-200 nm) and the opportunity to make time-resolved fluorescence measurements even if this latter feature was not used in these studies. As a highly relevant application, azo-based iridium(III) complexes **IrX** have been used as multicolor phosphorescent probes to detect hypoxia both in adherent living cells but also in oxygen-gradient 3D multicellular spheroids at depths of over 100 μm, which have many similarities to the *in vivo* environment (Figure 16d).^[83] More recently, the Choi group has shown that an azo-based binuclear ruthenium(II) complex **Ru-azo** could be also used as a "smart" photosensitizer in two-photon photodynamic anticancer therapy (Figure 17b).^[84] Indeed, this complex showed high accumulation in the mitochondria of cancer cells and produced efficient photocytotoxicity upon bioreductive activation by GSH and two-photon excitation at 810 nm.

In addition to all these reaction-based probes where the analyte-responsive azo unit is always directly connected to aniline-based luminophore, it is worth mentioning the unusual ratiometric azo-probes reported by the Freeman group and for which the azo moiety acts as a conformational locking group promoting the formation of a fluorescent dimer/excimer (Figure 18).^[85, 86] Bacterial reduction of the azo group leads to a conformational change of the cyclohexane ring and subsequent dimer/excimer disruption revealed by a blue-shifted fluorescence emission.

3.2.2 Fluorescence activation through elimination of the (di)aryl azo moiety.

As for examples discussed above, this second class of azo-based pro-fluorophores were developed based on the quenching ability of the AB moiety either fused to a fluorescent core or covalently conjugated to an organic-based fluorophore. However, their activation mechanism does not necessarily involve the reductive cleavage of their azo bridge but also its displacement through nucleophilic substitution or oxidation reactions mediated by the target (bio)analyte. The first example illustrating these alternative reaction-based strategies was published in 2014 by Lim *et al.* and was focused on the development of a NIR fluorescent probe **MitoGP** based on an azo-heptamethine cyanine fused chromophore, for the selective detection of mitochondrial GSH over cysteine and homocysteine.^[87] A dramatic fluorescence turn

"ON" response at 810 nm was obtained upon removal of PeT-active 4'-nitro-4-hydroxyazobenzene moiety through addition-elimination process induced by GSH. Furthermore, the lipophilic cation unit available within the core structure of cyanine dye (*i.e.*, indole unit of Cy 7.0 *N*-quaternarized with propionic acid butyl ester) acted as an effective mitochondria-targeting moiety (Figure 19). An other non-emissive fluorophore-azo dye conjugate namely **Hyp-Ly** was recently reported by Luo *et al.* that relies on the covalent association between 4-(*N,N*-dimethylamino)-4'-methylazobenzene and a 3-*N*-piperazinyl-bodipy through a carbamate linkage (Figure 20).^{[88],[89]} By analogy with azo-based pro-fluorophores described in section 3.2.1, this fluorogenic compound can be readily activated by reductive enzymes (typically AzoR activity) but the formed aniline (a *para*-aminobenzyl derivative) is able to undergo a further 1,6-elimination (self-immolation process) to release the green-yellow fluorescent bodipy **BOD-Ly**. This latter piperazine-based bodipy can be protonated in acidic environment and so trapped within cellular organelles such as lysosomes (pH 4.5-5.5) that are vital machines in mediating a variety of physiological processes, including protein degradation and restoration, intracellular transport and plasma membrane repair. Live-cell fluorescence microscopy experiments with human liver cancer cell line HepG2 have shown the potential of this azo-based pro-fluorophore to visualize lysosomes in hypoxic tumor cells (Figure 21).

In addition to these works, an unprecedented activation mechanism of azo-based pro-fluorophores through oxidation of -N=N- double bond by hypochlorous acid (HClO) was recently and independently reported by two chinese academic research groups (Figure 22).^[90] The non-fluorescent probe **YDN** was readily obtained through a conventional azo-coupling between *meta*-phenylenediamine and 5-aminofluorescein. Green fluorescence of parent fluorescein (centered at 516 nm) was unveiled upon reaction with HClO under simulated physiological conditions (*i.e.*, PBS, 10 mM, pH 7.4). Relatively stable and good performances in the fluorescence turn "ON" response were obtained within the pH range 5-9. High selectivity of **YDN** for HClO was demonstrated through competitive experiments with other ROS/RNS. Finally, the potential of **YDN** for imaging HClO was evaluated both in living cells (HeLa cells) and in live mice. The same probe design principle was also applied to an ES IPT-fluorophore namely 2-(2-hydroxyphenyl)benzothiazole (**HBT**) whose fluorescence emission maximum is in the blue region (centered at 466 nm). The corresponding probe **PHC2** featured high degree of selectivity and sensitivity for HClO with a low detection limit (13.2 nM) under simulated physiological conditions (*i.e.*, PBS, 10 mM, pH 7.4 + 1% EtOH). However, contrary to **YDN**, no fluorescence imaging experiments in cells or in more complex biological systems were performed, no doubt because the blue fluorescence emitted by the released **HBT** is only really full-compatible with *in vitro* measurements.

These recent examples contribute to make AB leaving groups valuable assets in the design of fluorogenic turn "ON" probes for the detection of a wide range of reactive (bio)analytes, especially nucleophilic species. Considering the large number of nucleophilic biomolecules present in living cells (*e.g.*, proteins/peptides, thiols, gaseous signaling molecules, ROS/RNS, ...) which would be useful to detect, this way to design reaction-based fluorescent probes will probably be developed in the coming years, provided that the probe presents a high selectivity for the targeted biological nucleophile.

3.2.3 Fluorescence activation through metal-catalyzed cyclization of the azo bridge to triazole moiety

The last fluorescence activation mechanism implemented in the context of azo-based pro-fluorophores relies on the inhibition of N=N- isomerization and subsequent oxidative cyclization to triazole moiety catalyzed by the target analyte. With the aim of developing a turn "ON" reactive probe for fluorescence detection of copper(II) ions in neutral aq. solutions, Jo *et al.* have synthesized a water-soluble and non-emissive azoaniline dye **A** (*i.e.*, AB derived from *meta*-phenylenediamine).^[91] Coordination of Cu(II) ion favors a cascade of electron and proton transfer events leading to *in situ* formation of a highly fluorescent 2-phenylbenzotriazole derivative **B**. This chemistry can be readily effected by μM -level concentrations of Cu(II) ion in water (pH = 6-8) at room temperature to elicit >80-fold enhancement in the green emission at 530 nm. Remarkably, this azo-based probe showed excellent sensitivity to and selectivity for Cu(II) over other metal ions (Figure 23). More recently, the same fluorescence turn "ON" signaling mechanism was implemented to more sophisticated anilines with the dual aim of widening working pH range and improving sensitivity of this unusual class of copper(II)-sensitive probes (Figure 24a). This was achieved with coumarin-azo dye **C** that exhibited excellent sensitivity within a wide range of pH (3-10).^[92] An unprecedented detection limit of 20 nM at physiological pH was obtained (in addition, a lower value of 14.4 nM was reported very recently with azoaniline **TPI-AD**).^[93] Conversely, there has been no significant improvement in the sensitivity with azoaniline dye **D** (detection limit = 10 μM) despite a broad working pH range (5-11) for fluorescence turn "ON" detection of Cu(II) analyte.^[94] Interestingly, the ability of both probes to image intracellular Cu(II) was demonstrated through fluorescence microscopy experiments with human colon adenocarcinoma cell line HT29 and liver cancer cell line HepG2 respectively. A clever extension of this promising reaction-based strategy was recently studied by the Lee group for devising "reactive" fluorescent molecular logic gates useable in dual analyte (bio)sensing (Figure 24b). Two bifunctional fluorescent chemodosimeters based on (*E*)-4-((2,4-diaminophenyl)diazanyl)phenol scaffold were developed.^[95] The first one **D** was successfully used for the sequential as well as the individual detection of fluoride and Cu(II) ions through colorimetric change (42 nm bathochromic shift as the result of fluoride-mediated *tert*-butyldimethylsilyl (TBDMS) ether deprotection) followed by a blue emission response (at 484 nm, induced by Cu(II)). Similar red-shifted absorbance and fluorescence outputs and a tunable detection time were obtained with dual responsive molecular probe **E** rationally designed for the sensing of diethyl chlorophosphate (DCP), a nerve agent mimic, and Cu(II) ions (*i.e.*, 63 nm bathochromic shift of absorbance maximum as the result of phenol phosphorylation reaction and turn "ON" emission at 455 nm induced by Cu(II)). Some other organometallic reaction approaches leading to conversion of azo quenching unit to substituent promoting larger π -conjugation, will likely be considered to monitor other biologically relevant metals (especially those implicated in diseases) in a variety of oxidation states.

4. Summary and upcoming challenges

In this review, major advances in the design and synthesis of site-specifically functionalized azo dyes with enhanced spectral properties, over the past 15 years, were presented. The main goal of these works was to provide effective fluorescence quenchers full-compatible with challenging analytical applications in various biological systems. The practical implementation of these

advanced chemical tools in the context of analyte-sensitive fluorogenic probes constitutes the second part of this literature survey. Thus, representative examples of small molecule fluorescent probes whose activation mechanism is based on chemical/enzymatic deactivation of their (di)aryl azo unit, were discussed. Except some specific and rare examples, a turn "ON" emission increase was preferred and obtained both to dramatically improve signal-to-noise ratio and to maximize spatial resolution in bioimaging experiments. The most popular FRET-based probes where the azo dye acts as an effective dark quencher, are mainly used for the detection of hydrolytic enzymes activities. However, the recent identification of biocompatible chemical reactions triggered by relevant (bio)analytes and known to selectively affect the chemical integrity of (di)aryl azo moiety, opens new promising perspectives in this field of bioimaging. The use of azo-based quenchers as sonophores for optoacoustic imaging, recently demonstrated by a first proof-of-concept reported by the Grimm group, is certainly a complementary path that needs exploring.^[96] Indeed, **BHQ-1** was found to exhibit strong and concentration-dependent optoacoustic signal, and its conjugation to a cyclic RGD-peptide led to a targeted contrast agent, effective for detecting $\alpha_v\beta_3$ -integrin expressing glioblastoma tumors *in vivo*. In addition to these innovations related to diagnostics, some research groups had the idea to take advantage of the hypoxia-dependent bioreductive metabolism of azo compounds in the prodrug design of anticancer agents. Some self-immolative prodrugs have been designed to generate the corresponding active drug upon reductive activation by AzoR and subsequent domino process often involving cyclization reactions^[97]. Further implementation of this concept of hypoxia-activated prodrugs to nanocarriers such as polymer-drug conjugates (through a covalent strategy), micelles, nanoparticles, and polymersomes (through encapsulation strategies), has already been extensively studied for cancer therapy.^{[62, 98], [99]} The possible synthetic access to unsymmetrical azobenzene derivatives acting as both azo-based pro-fluorophore and prodrug should promote advances in small molecular theranostics (Figure 25a).^[100] Indeed, these compact and biocompatible (bio)molecular conjugates are attractive candidates for both diagnosis and therapy due to their unique features such as target delivery, imaging abilities, facile modification, reliability, real-time information on drug release and pharmacokinetics. The first practical example of this dual imaging/therapy strategy was recently published by Verwilt *et al.* and concerned the design and construction of a theranostic drug delivery system (DDS) for cancer therapy (Figure 25b).^[101] It is based on an AB scaffold acting both as a fluorescence quencher (*i.e.*, non-fluorescent azo-rhodamine B hybrid) and deactivator of an alkylating anticancer drug (*i.e.*, nitrogen mustard *N,N*-bis(2-chloroethyl)-*para*-phenylenediamine), and it also bears a triphenylphosphonium group that combines excellent targeting of the hyperpolarized mitochondria of cancer cells in solid tumors with a convenient means for drug resistance evading. As claimed by the authors and supported by preliminary *in vivo* therapeutic experiments, this bioreductively activated theranostic DDS could result in the selective eradication of hypoxic regions within solid tumors providing better treatment options for this challenging fraction of cells within solid tumors. Without a doubt, this first spectacular achievement will boost further innovations in this field and practical biomedical applications.

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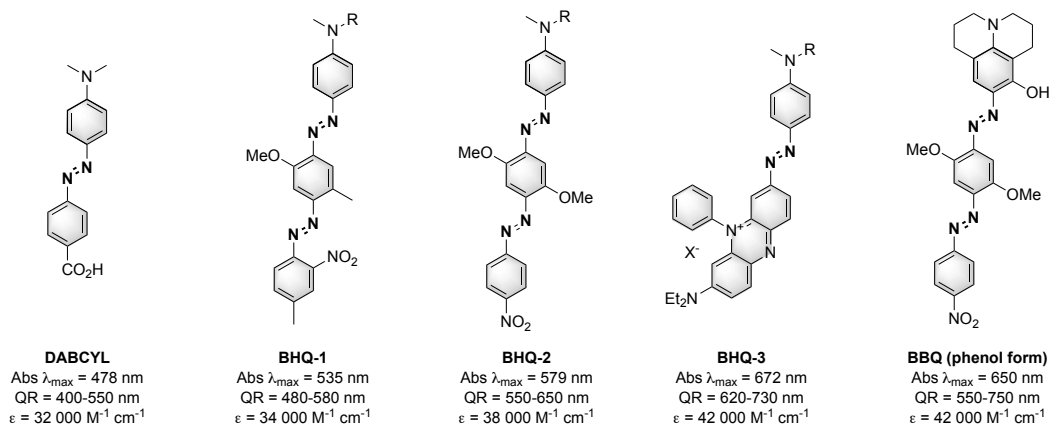


Figure 2. Structures and spectral absorption features of the most popular azo-based dark quenchers (QR = quenching range). Please note: **BBQ-650** = phosphoramidite derivative of glycerol ether form of phenolic **BBQ** (in MeOH, Abs λ_{max} = 598 nm and ϵ = 40 667 M⁻¹ cm⁻¹).

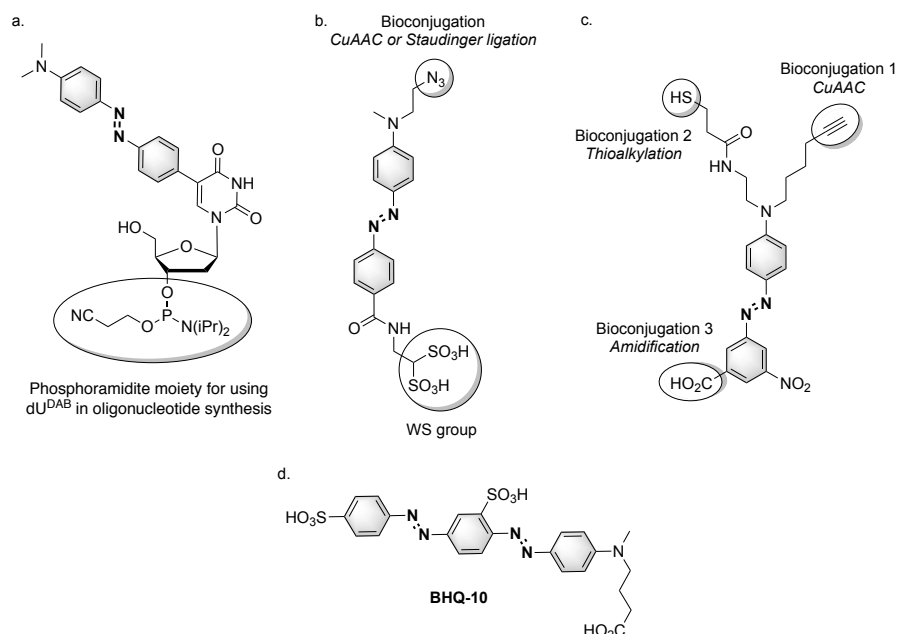


Figure 3. Structures of "tuned" azo-based dark quenchers for oligonucleotide synthesis (a)^[36] and (multiple) bioconjugation (b-c)^{[41],[44]} and **BHQ-10** (iPr = isopropyl, WS = water-solubilizing).

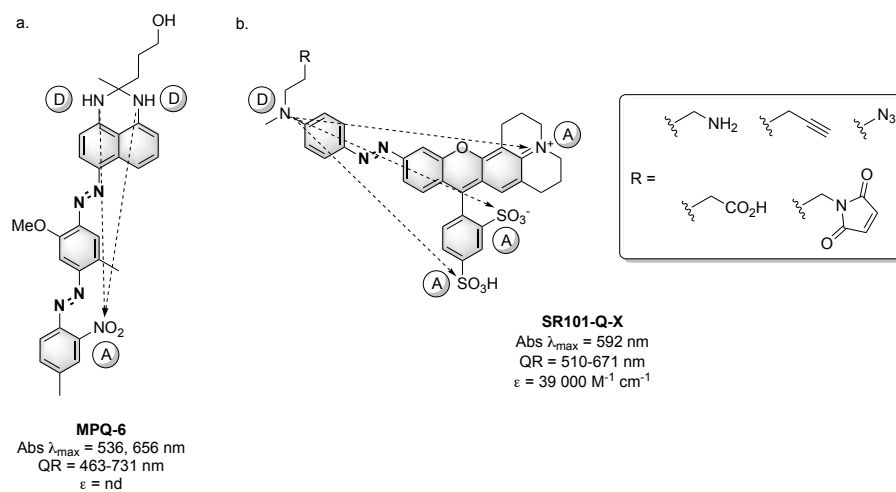


Figure 4. Structures and spectral absorption features of universal azo-based dark quenchers recently reported in the literature^{[46],[48]} (QR = quenching range, nd = not determined). Please note: for **MPQ-6**, Abs maxima and QR were determined in the context of a 20-mer quencher-oligonucleotide conjugate.

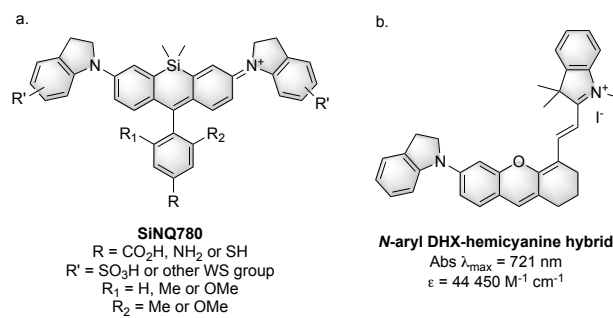


Figure 5. Structures of NIR dark quenchers recently reported in the literature^{[49],[50]} (WS = water-solubilizing, DHX = dihydroxanthene). Please note: spectral absorption features of *N*-aryl DHX-hemicyanine hybrid was determined in EtOH.

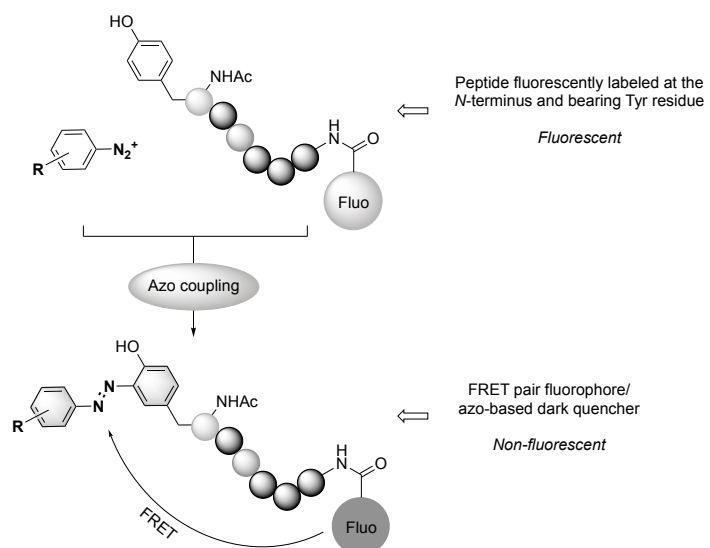


Figure 6. Tyrosine-selective modification of peptides through site-specific azo-coupling with the aim of creating a FRET pair (Ac = acetyl, Fluo = organic-based fluorophore, Tyr = L-tyrosine).

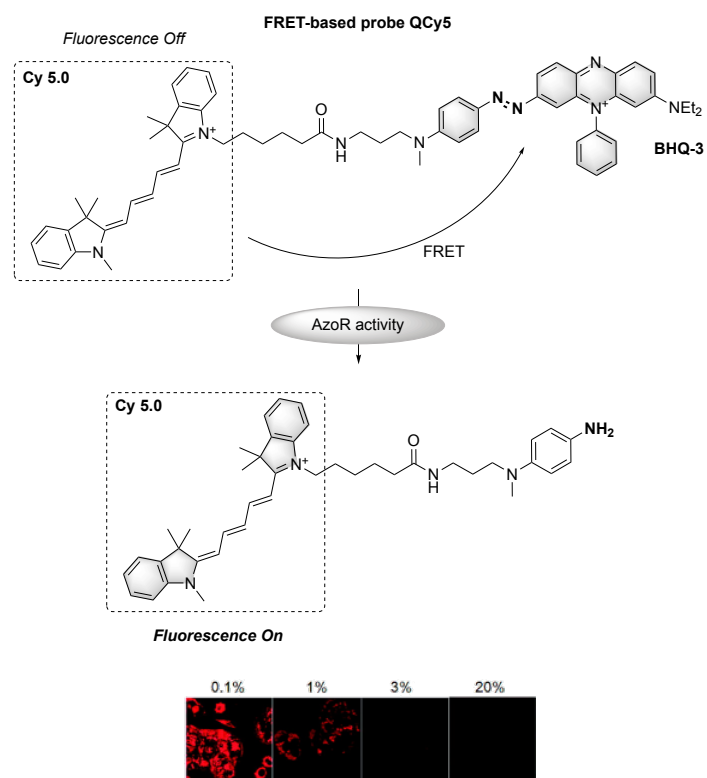


Figure 7. Structure and fluorescence revealing mechanism of FRET-based probe **QCy5** for hypoxia imaging, reported by the Nagano group^[33] (top). Oxygen dependency of **QCy5** fluorescence (bottom). MCF-7 cells loaded with 1 μM **QCy5** were incubated at various oxygen levels. Scale bar, 50 μm . Copyright 2010 American Chemical Society. Please note: the counterion (trifluoroacetate) of probe **QCy5** is omitted for clarity.

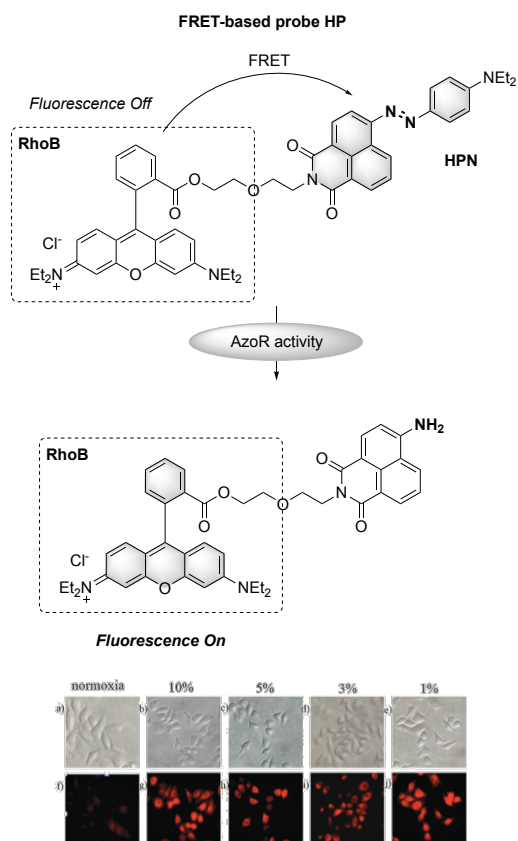


Figure 8. Structure and fluorescence revealing mechanism of FRET-based probe HP for hypoxia imaging, reported by the Qian group^[64] (top). Fluorescence and bright-field images of HeLa cells at different oxygen concentrations (bottom). The concentration of DMSO in the culture medium is 1%. Filter type is Texas-Red (Ex: 540-560 nm, Em: 570-600 nm). Copyright 2015 Royal Society of Chemistry.

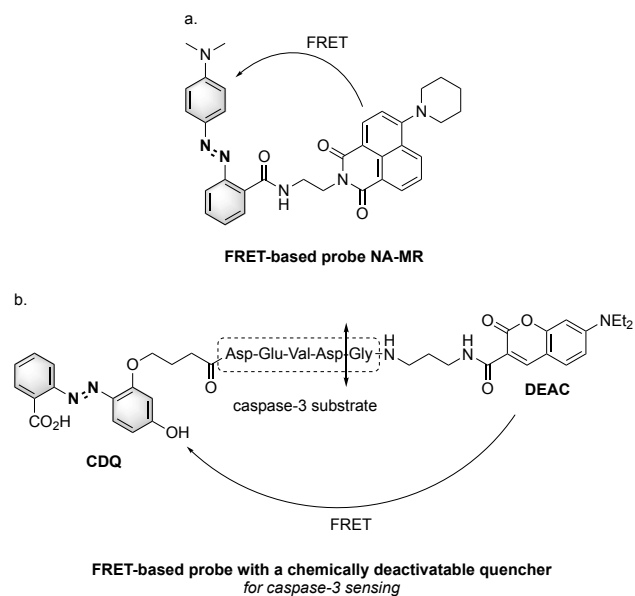


Figure 9. Structures of FRET-based probes reported by the Xu group for the tracking of microbial degradation of azo-based organic pollutants^[43] (a) and the Wagner group for the quantification of caspase-3 activity in solution and visualization of unreacted probes in apoptotic cells^[57] (b) (CDQ = chemically deactivatable quencher, DEAC, 7-*N,N*-diethylaminocoumarin, MR = methyl red, Asp = L-aspartic acid, Glu = L-glutamic acid, Gly = glycine, Val = L-valine).

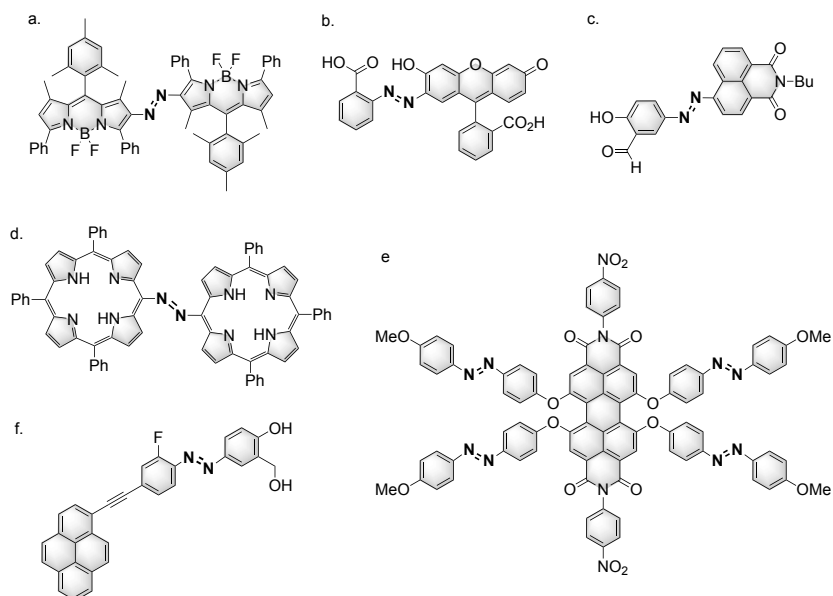


Figure 10. Structures of non-fluorescent azo dyes derived from organic-based fluorophores (bodipy^[66] (a), fluorescein^[68] (b), 1,8-naphthalimide^[55] (c), porphyrin^[69] (d), perylene^[70] (e) and pyrene^[71] (f) and already reported in the literature (Ph = phenyl, Bu = butyl).

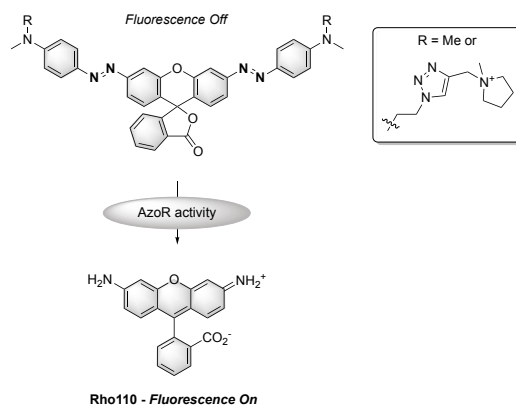


Figure 11. Structure and fluorescence revealing mechanism (reductive cleavage of the azo bridge and subsequent release of fluorescent aniline) of the first pro-fluorophores containing an azo moiety as quenching moiety, reported by us.^[73] These are azobenzene-caged rhodamine 110 derivatives suitable for the detection of AzoR activity both *in vitro* and in the context of bacterial cultures (Rho110 = rhodamine 110). Please note: the counterion (trifluoroacetate) of the "clicked" bis-cationic probe is omitted for clarity.

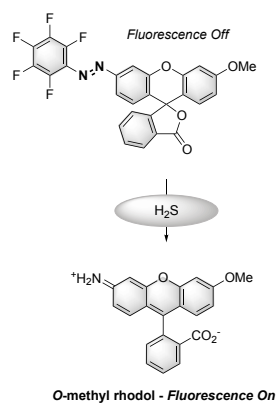


Figure 12. Structure and fluorescence revealing mechanism (reductive cleavage of the azo bridge and subsequent release of fluorescent aniline) of the pro-fluorophore developed for the biosensing of gasotransmitter H₂S.^[74]

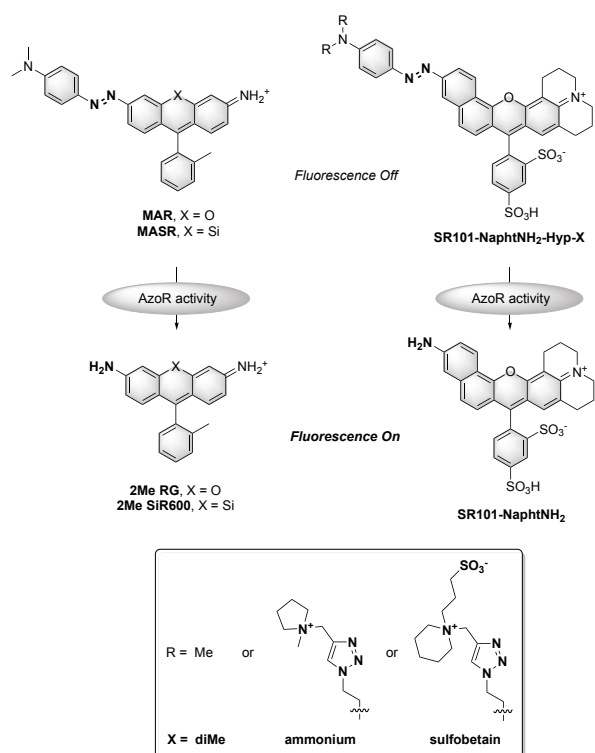


Figure 13. Structure and fluorescence revealing mechanism (reductive cleavage of the azo bridge and subsequent release of fluorescent aniline) of azo-based profluorophores developed for the intracellular detection of hypoxia in living cells.^{[75],[76]} Please note: the counterion (trifluoroacetate for **MAR**, **MASR** and **SR101-NaphtNH₂-Hyp-ammonium** and triethylammonium for **SR101-NaphtNH₂-Hyp-sulfobetain**) is omitted for clarity.

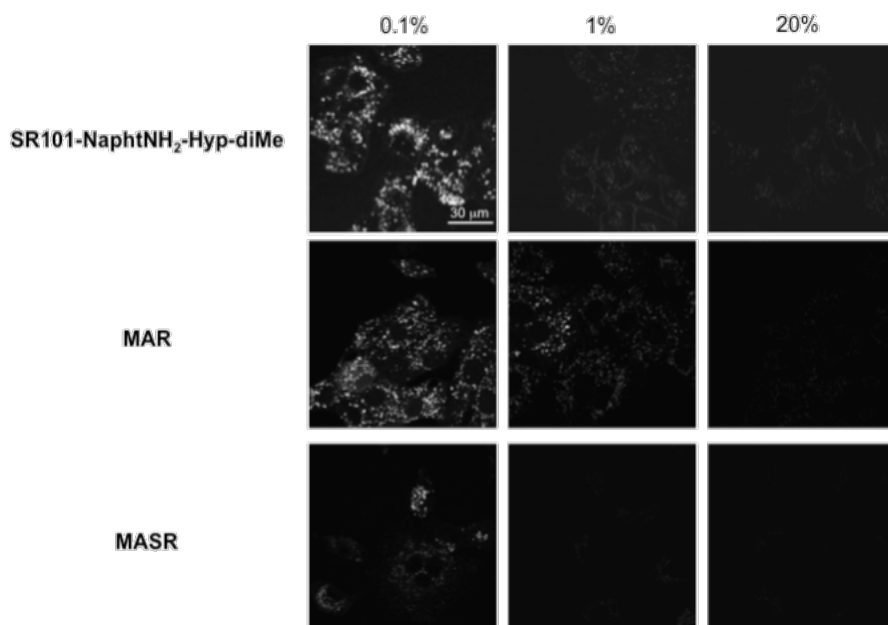


Figure 14. Fluorescence confocal microscopy images of **SR101-NaphtNH₂-Hyp-diMe**, **MAR** or **MASR** in live A549 cells.^[76] The A549 cells were incubated with **SR101-NaphtNH₂-Hyp-diMe**, **MAR** or **MASR** (1 μM) containing DMSO (0.1%) as a cosolvent at various oxygen concentrations (0.1%, 1% and 20%) for 6 h. Scale bar: 30 μm. The excitation wavelength and emission range of detection were 488 nm and 515-553 nm for **MAR**, 590 nm and 600-670 nm for **SR101-NaphtNH₂-Hyp-diMe**, and 600 and 620-700 nm for **MASR**, respectively. Copyright 2015 IOP Publishing Ltd.

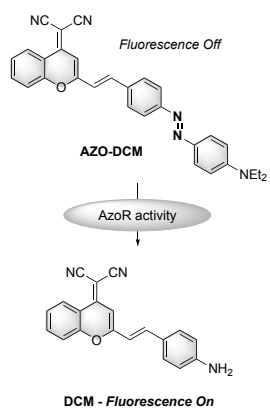


Figure 15. Structure and fluorescence revealing mechanism (reductive cleavage of the azo bridge and subsequent release of fluorescent aniline) of far-red turn "ON" fluorescent probe **AZO-DCM** ($E_m \lambda_{max} = 650 \text{ nm}$) applied in cytochrome P450 reductase (AzoR activity) detection and hypoxia imaging in tumor cells.^[79]

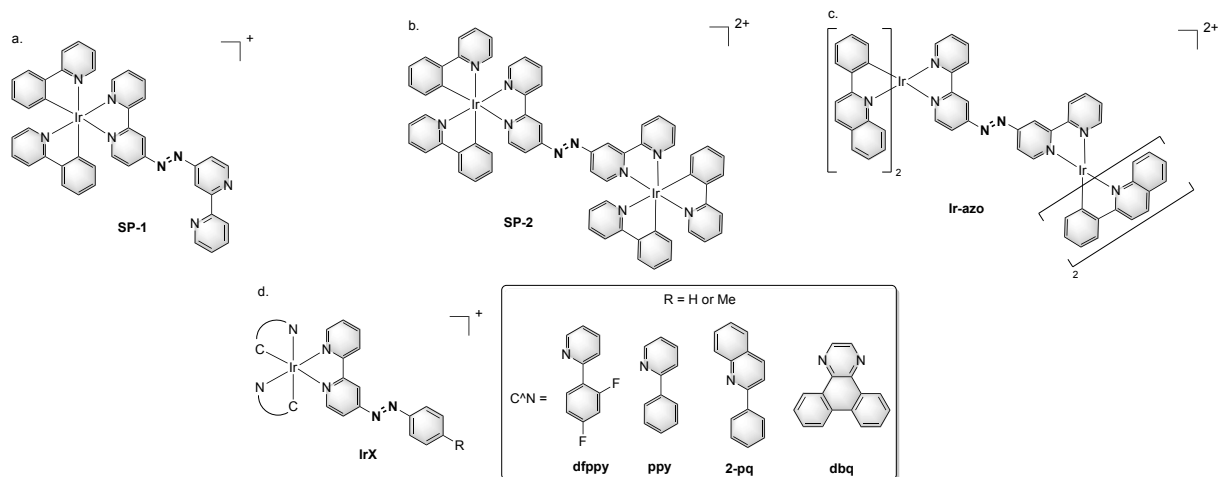


Figure 16. Structures of turn "ON" phosphorescent probes based on an Ir(III)-azo complex for imaging sulfite/bisulfite^[80] (a-b), biothiols^[82] (c) and hypoxia^[83] (d) in living cells.

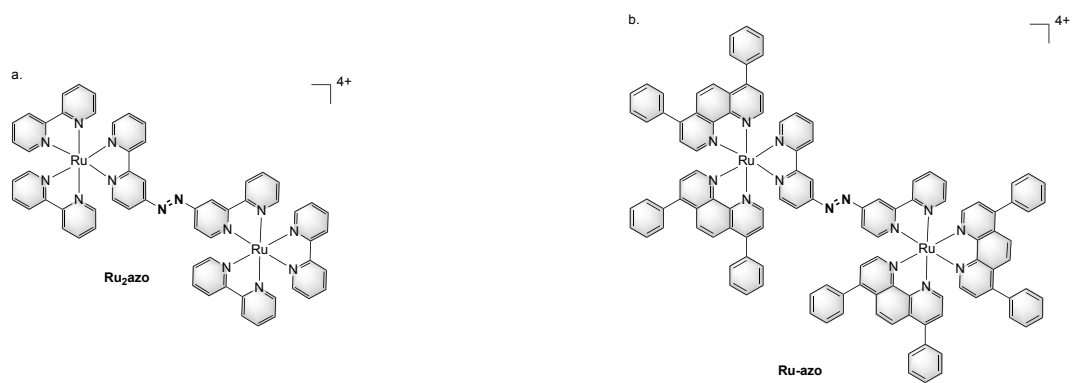


Figure 17. Structures of a turn "ON" phosphorescent probe based on an Ru(II)-azo complex for the detection of biothiols^[81] (a) and a GSH-activatable Ru(II)-azo photosensitizer for two-photon photodynamic therapy^[84] (b) (GSH = glutathione).

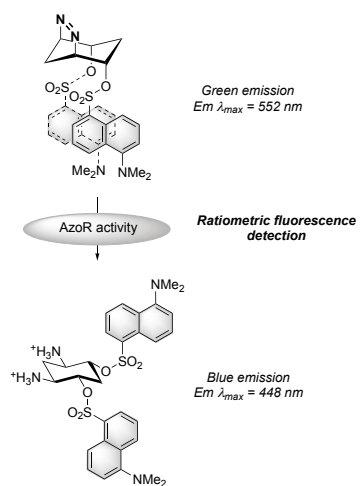


Figure 18. Structure and activation mechanism of ratiometric fluorescent probe reported by the Freeman group for detection of bacteria.^[65]

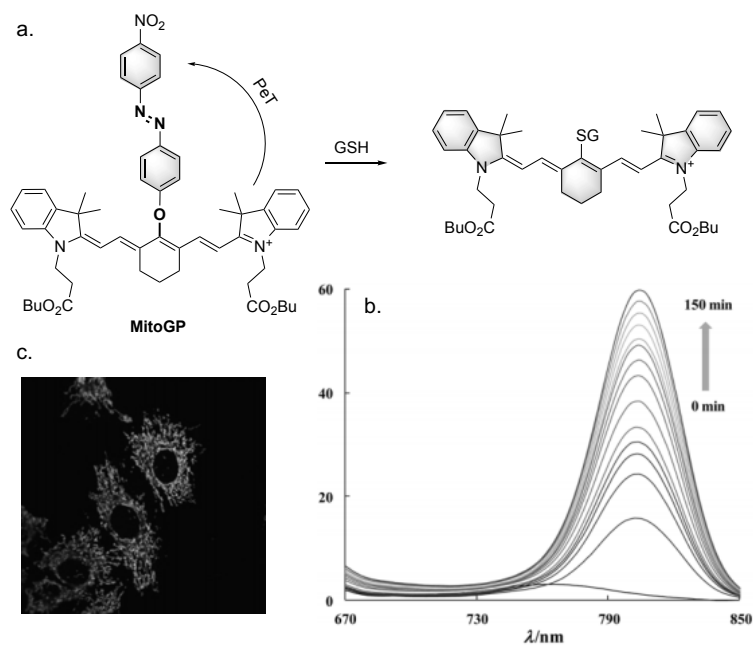


Figure 19. (a) Structure and fluorescence revealing mechanism (nucleophilic displacement leading to the release of fluorophore) of azo-based pro-fluorophore **MitoGP** for the selective detection of mitochondrial GSH over cysteine and homocysteine^[87] (Bu = butyl, GSH = glutathione, PeT = photoinduced electron transfer). (b) Time-dependent fluorescence emission spectrum of **MitoGP** (10 μ M, Ex at 600 nm) upon addition of GSH (10 mM) in HEPES buffer (0.10 M, pH 7.4). (c) Confocal laser scanning microscopic image of **MitoGP** in living HeLa cells (1.0 μ M, 30 min, Ex at 555 nm). Copyright 2014 American Chemical Society. Please note: the nature of counterion of **MitoGP** is not specified by the authors.

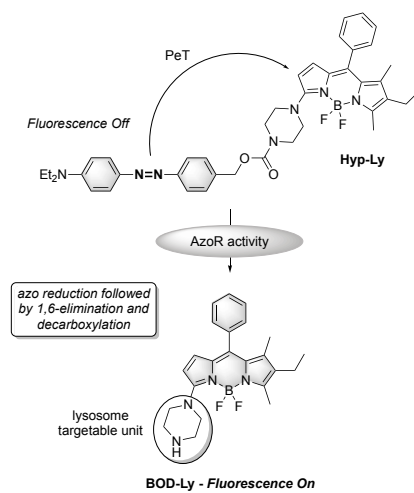


Figure 20. Structure and fluorescence revealing mechanism (reductive cleavage of the azo bridge and subsequent domino reaction leading to the release of fluorophore) of turn "ON" fluorescent probe **Hyp-Ly** for visualizing lysosomes in hypoxic tumor cells.^[88]

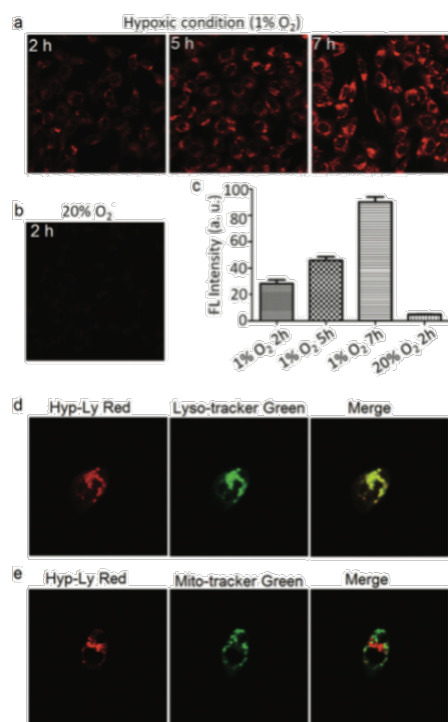


Figure 21. Fluorescence confocal microscopy images of **Hyp-Ly** for monitoring cellular hypoxia in HepG2 cells.^[88] (a) HepG2 cells were treated with **Hyp-Ly** (10 μ M) under hypoxic conditions (1% oxygen) with varying incubation times; (b) HepG2 cells were incubated with **Hyp-Ly** under 20% oxygen for 2 h; (c) Average intensity in fluorescence images. Data represent mean standard error. Ex at 514 nm and the emission was collected at 550-600 nm. (d-e) Confocal fluorescence images for intracellular localization of **Hyp-Ly** (10 μ M) in cells: HepG2 cells were incubated with **Hyp-Ly** under hypoxic conditions for 6 h and then co-stained with (d) Lyso-Tracker Green (1 μ M) and (e) Mito-Tracker Green (200 nM) for 30 min. Red channel, Ex at 514 nm, Em = 550-600 nm; green channel, Ex at 488 nm, Em = 500-530 nm. Copyright 2016 Royal Society of Chemistry.

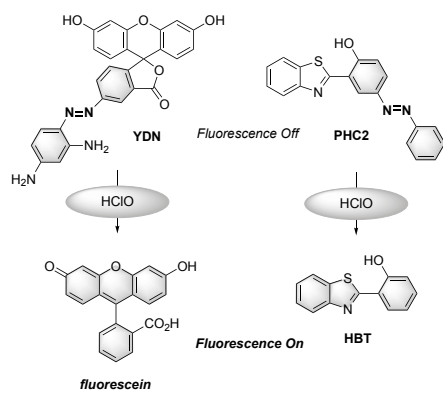


Figure 22. Structures and fluorescence revealing mechanism (oxidation of the azo bridge and subsequent release of fluorophore) of azo-based pro-fluorophores developed for the detection of hypochlorous acid (HBT = 2-(2-hydroxyphenyl)benzothiazole).^[90]

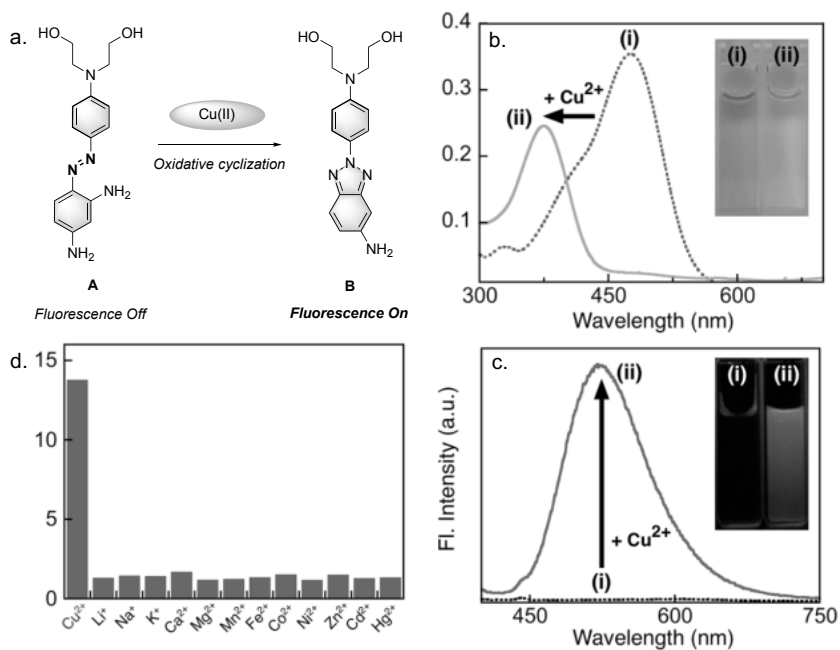


Figure 23. (a) Structure and fluorescence revealing mechanism (metal-catalyzed cyclization of the azo bridge leading to the formation of triazole-based fluorophore) of turn "ON" fluorescent probe **A** for the detection of copper(II) ions in water.^[91] (b-c) Oxidative cyclization of **A** (10 μ M) to **B**, triggered by copper(II) ions (100 equiv) in water (pH = 7.0; HEPES, 50 mM) and monitored by (b) UV-vis and (c) fluorescence spectroscopy after 10 min. Digital images in the insets were obtained (with ambient light for (b) shift from yellow to colorless, and a hand-held UV lamp (Ex at 365 nm) for (c) shift from dark to green) for the samples prepared using the same experimental conditions. (d) Fluorescence response of **A** (10 μ M) toward metal ions in water at pH = 7.0 (HEPES, 50 mM). The signal intensity at $\lambda = 530$ nm (= I) is normalized with that of the probe-only sample (= I_0). The bars represent I/I_0 of **A** in the presence of various metal ions (100 equiv) screened (HEPES = 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid). Copyright 2012 American Chemical Society.

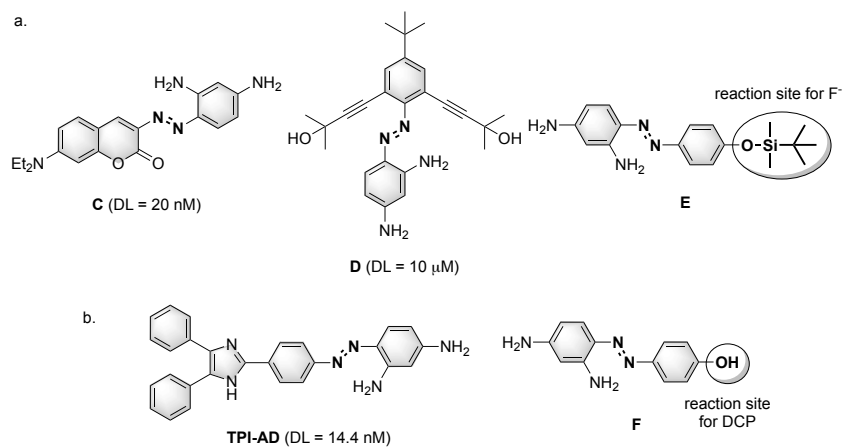


Figure 24. (a) Structures of azoaniline dyes recently developed with the dual aim of widening working pH range and improving sensitivity of this unusual class of copper(II)-sensitive probes (DL = detection limit).^[92-94] (b) Structures of bifunctional azoaniline-based chromogenic/fluorogenic probes for dual detection of fluoride and Cu(II) ions (left) or nerve agent mimic DCP and Cu(II) ions (right)^[95] (DCP = diethyl chlorophosphate).

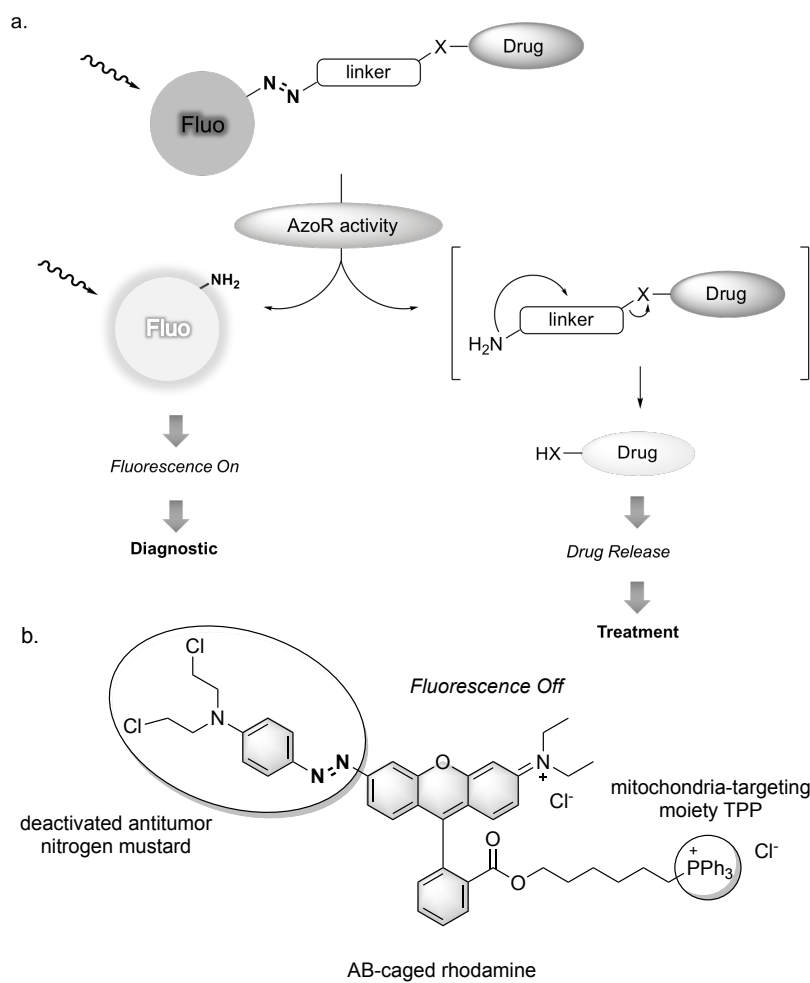


Figure 25. (a) Principle of small conjugate-based theranostic agents activated through the bioreduction of azo bridge. (b) Structure of hypoxia-activated prodrug recently reported by Verwilt *et al.*^[101]