Chapter 1

Design of Functional Dyes for Advanced Microscopy and Life-Sciences
1.1 Introduction

Light plays a vital role in our daily life. It is a fascinating phenomenon that ties together physics, chemistry and biology. Moreover, light is an enabler in many interdisciplinary scientific fields in the twenty-first century. Regarding the physics aspect, light is unmatched in its ability to confer information with temporal and spatial precision and has been used to map objects on the scale of tens of nanometers ($10^{-8}$ m) to light years ($10^{16}$ m). This information, gathered through super-resolution microscopes or space-based telescopes, is ultimately funneled through the human visual system, which is a miracle in itself.

From the chemistry perspective, photochemistry is a branch of chemistry concerned with the study of chemical processes induced by the absorption of light.\textsuperscript{1} Photochemical reactions can occur in natural processes such as photosynthesis of plants and in pathological processes such as photo aging of skin. Nature achieve these processes through a small set of chromophores, such as retinal, flavins and tetrapyrrols that are attached, usually covalently, to a protein envelope. These chromophores are produced through ancient biochemical pathways and have proven so successful that they appear to have remained unaltered over the course of evolution. The interaction of these chromophores and many others with electrons and photons provide the molecular basis for photochemical and photophysical switching processes. For this reason, dyes based on aromatic $\pi$-systems are well suited for use as probes for analytical and sensor chemistry and as building blocks for the fabrication of photo-controlled biomolecules.

Dyes, specifically designed for these applications, are generally called functional dyes. The design and synthesis of functional dyes bear great potential for chemistry, biology and life sciences. This field has made significant contribution to our understanding of many important natural processes as well as the scientific discovery of the man-made world. Well-established applications of functional dyes in medicine include diagnostics (biochips and bio-imaging/sensing), photopharmacology and therapeutics (biomaterials for artificial organs, medical adhesives, dental materials, drug-delivery systems, tissue engineering and photodynamic therapy).\textsuperscript{2} This chapter will focus on functional dyes including organic fluorophores and photoswitchable biomolecules that are most commonly employed in photochemistry and applications for advanced microscopy techniques and life-sciences.

1.2 Organic fluorophores for super-resolution microscopy
During the last decades, fluorescence imaging with high temporal and spatial resolution has been a milestone in life sciences research and attracted widespread interest in areas such as the observation of specific components or processes in living systems (cells, tissues and whole organisms). Great efforts have been made to overcome the physical diffraction limit of light (< 250nm), until the 1990s, a super-resolution image of a biological sample was obtained by a fundamentally new microscopy named super-resolution microscopy for the first time.

In contrast to ameliorate the spatial resolution through near-field optics and aperture filters, improvements in fluorescent probe technology play a key role to overcome the diffraction limit and therefore improve the far-field fluorescence microscopy, such as to spatially or temporally modulate the transition between two molecular states of fluorophores. For example, the super resolution of Stimulated Emission Depletion Microscopy (STED), Saturated Structured-Illumination Microscopy (SSIM) and Ground-State Depletion Microscopy (GSD) are based on narrowing the point-spread-function of an ensemble image of fluorophores; Stochastic Optical Reconstruction Microscopy (STORM), Photoactivated Localization Microscopy (PALM) and Fluorescence Photoactivation Localization Microscopy (FPALM) achieve their super resolution by fluorophore localization of photo-switchable fluorescent molecules. The Nobel Prize in Chemistry was awarded to Stefan Hell, Eric Betzig and William Moerner for the development of super-resolved fluorescence microscopy in 2014.

1.2.1 Fluorescent probes

Fluorescent probes used for super-resolution imaging are generally divided into two classes: fluorescent proteins (FPs), which are discovered in nature and transferred into science; synthetic fluorophores such as small-molecule organic dyes and quantum dots (inorganic semiconductor nanocrystal particles). These fluorescent probes are suitable for research in the life sciences because of their high fluorescence quantum yields, long-lasting photostability and a range of colors that span the visible spectrum. Such properties are not found in the pristine building blocks of proteins and nucleic acids. However, these properties of fluorescent probes are prerequisite for super-resolution fluorescent microscopy, one of the most sensitive, versatile and indispensable tools in biology related research.

Appropriate choice of fluorescent labels is a fundamental constituent to the success of the entire biological labeling and imaging experiment. The revolutionizing development of genetically encoded fluorophores such as fluorescent proteins has allowed unknown processes and dynamics in biological systems to be elucidated.
However, the enhancement of their low brightness and poor photostability are desirable. Quantum dots (QDs) are inorganic semiconductor nanocrystal particles. They exhibit exceptional photophysical characteristics such as high extinction coefficient, high photoluminescence quantum yield (QY), high signal to noise ratio, stability against photobleaching and broad excitation spectra but narrow sharply defined emission peak. All these features of quantum dots allow a wide variety of possibilities for applications including fluorescent tagging, multiple color imaging and live-cell imaging. However, cytotoxicity and poor biocompatibility of quantum dots to cells are their major drawbacks when used in biological research. Besides, their large physical size (10-100nm) is a crucial hindrance to diffusing across cellular membranes. Among all available probes, synthetic organic fluorescent dyes are a popular choice. Comparing to fluorescent proteins (2.5-4nm) and even bigger quantum dots, the appealing advantage of these organic dyes is their small size, which is only 1-2nm (Figure 1.1). Correspondingly, once properly positioned at the target biomacromolecule, the perturbation to the system of interest from these small molecules is less significant.

**Figure 1.1:** Size comparison of extrinsic fluorophores. From left to right: the organic fluorophore Cy5 (maleimide conjugate), green fluorescent protein, and a quantum dot coated with a passivating polymer layer (red) and a bioconjugating molecule layer (blue). Cyan spheres represent hydrodynamic radii.

Furthermore, another remarkable preference of small organic fluorescent probes is the capability of chemical manipulation to fulfill the desirable properties for super-resolution imaging, without genetic engineering by biological experiments. For each application, in order to extracting maximum amount of information and getting successful outcomes, it proves paramount to understand how to appropriately choose the organic fluorophores and how to design and interpret experiments and results.
Among the physical and chemical properties which are demanded in super-resolution techniques, the most important are: high photostability, low population of the dark triplet state, large quantum yield of fluorescence (greater than 0.5), good solubility in water, high signal to noise ratios (up to over 1,000) and easy to handle especially stable enough in aqueous solutions. Moreover, conjugation procedures require fluorescent molecules with different reactive groups for linking chemistry, which allow specific and efficient attachment to biological targets such as proteins (peptides), nucleic acids, lipids, carbohydrates, toxins, (modified) oligo-nucleotides and various other ‘small’ biomolecules.

However, in practice, there are no “omnipotent fluorophores” to fulfill all these requirements and it seems that minor modifications to a fluorescent molecule may result in considerable changes in their properties. Therefore, it is worthwhile to supply a wide array of organic fluorescent dyes, offering the selection of markers with the optimal combination of several chemical, biological and physical characteristics.

Recently, ingenious chemical design of small organic markers has dramatically expanded their performance and utility in almost every imaging application. A gamut of different dyes are commercially available in the form of maleimides, isothiocyanates and succinimidyl esters for specifically coupling with sulfhydryl- and amino groups in biological targets.
With a few exceptions, rhodamines (ATTO 488, ATTO 532, ATTO 565, ATTO 590, Alexa 488, Alexa 532, Alexa 546, Alexa 555), carbocyanines (Cy2, Cy3, Cy3B, Cy3.5, Cy5, Cy5.5, Cy7, Alexa 647), oxazines (ATTO 655) and carbopyronine (ATTO 647N) are four basic chemical families of single molecule fluorescent probes. Figure 1.2 shows the typical fluorescent dyes of each class often used in single molecule fluorescence research.

Here, we focus our discussion on the photochemical and photophysical processes of small organic fluorophores which are frequently used in single molecule fluorescence and super-resolution techniques.

### 1.2.1.1 Rhodamines

Along with eosin and fluorescein dyes, rhodamine dyes belong to the family of xanthenes. Figure 1.3 displays the general chemical structure of xanthene and rhodamine. They have been widely used as fluorogenic markers and laser dyes in biological imaging and single-molecule based spectroscopy, due to their desirable photophysical and photochemical properties such as excellent fluorescent quantum yield, high molar extinction coefficients, high photostabilities, low degree of triplet formation (tolerance to photobleaching) and the ability to adjust properties as platforms.

Despite their long history, the preparation and post-synthetic modifications of rhodamine dyes have not been deeply investigated.

![Molecular structures of xanthene (A) and rhodamine dyes (B).](image)

In general, rhodamine derivatives can be modified from commercially available Rhodamine dyes or prepared directly through a condensation reaction with previously functionalized reactants, such as modification of the amino groups of xanthene moiety (Figure 1.3, positions 3 and 6), modification of the carboxylic acid group (Figure 1.3B, position 2') and replacement of different atoms at the 10-position from the core skeleton of the xanthene dyes.

#### 1.2.1.1.1 Modification of the amino groups
Introduction of different substituents at the amino groups of xanthene can change the photophysical properties of Rhodamine dyes. The quantum yield of Rhodamine B and tetramethyl rhodamine with fully alkylated amino groups is sensitive to viscosity and temperature. However, quantum yield of Rhodamine 101 with the amino groups as part of aliphatic rigidized ring and rhodamine 6G with only partially alkylated amino groups are close to unity. Fluorinated rhodamine derivatives synthesized by Mitronova et al. show significant improvement in photostability through the modification of fluorinated N-alkyl groups without dramatic change in spectral properties.

Rhodamine 110 (4, Figure 1.4) is the simplest rhodamine with non-alkylated and more reactive amino groups, which is a good candidate for latent fluorophores (pro-fluorophores), with an advantage over “conventional” fluorophores whose bulk fluorescence can obscure valuable information in biological experiments, such as 5, 6, 7 and 8 shown in Figure 1.4. In recent years, several Rhodamine 110 derivatives have been synthesized as precursor dyes widely used in biomolecular imaging, monitoring of the proteolytic activity of cathepsin C in live cells, detection of thiols and characterization of enzyme kinetics of DT diaphorase.

1.2.1.1.2 Modification of the carboxylic acid group

One striking feature of rhodamine dyes is their equilibrium between a ring closed or open form. The photophysical properties of these two forms can be readily different. The ring open quinoid form is colored and fluorescent while the ring closed lactone form is colorless and nonfluorescent.

As already mentioned above, secondary amides of rhodamine are usually in a non-fluorescent spirolactam form, which prevents their use in biological experiment. Therefore, tertiary amide group were introduced, which prevent the cyclization to the lactone form as well as are starting materials for introduction of several other functional groups as a handle for bioconjugation. In 2003, Nguyen and Francis reported novel synthetic routes for the preparation of piperazine amide derivatives. The secondary amine group of these derivatives can be easily further functionalized through common organic chemistry transformations like alkylation of the amine. Cincotta and Foley introduced a method for amidation of the carboxylic acid group of Rhodamine B for the first time. Kim et al. reviewed rhodamine derivatives with modification of the carboxylic acid group used as chemosensors.

Red-emitting fluorescent dyes with absorption and emission in the far-red to near-infrared (IR) optical region (longer than 600 nm) have significant advantage for in
vivo bioimaging. Compared to ultraviolet-visible (UV-vis) light, red light is characterized by less scattering, drastically lower photo-damage to biological samples and provide good tissue penetration in depth and minimizes background interference from tissue auto-fluorescence. Thus, with great effort researchers developed a diverse array of new long wavelength fluorescent dyes in recent years.

**Figure 1.4: Variants of rhodamine dyes.**

Kolmakov and co-workers described the synthesis, properties and applications of larger bathochromic shift rhodamine derivatives used for STED microscopy. The large red shift is achieved by introducing several fluorine atoms into the phenyl ring attached to C-9 (mesoposition in the xanthene scaffold), which is quite reactive towards nucleophiles together with other modifications, such as additional double bonds and cycles, rigidized frame-works and donor substituents at nitrogen atoms.
Replacement of oxygen atom at the 10-position in the xanthene ring by other atoms, such as group 14 metalloids (silicon, germanium and tin), also can modulate their fluorescence properties. Due to the relatively low-lying LUMO energy levels of silicon, Nagano and co-workers synthesized a series of Si-Rhodamine dyes like compound 7 (Figure 1.4) that showed great bathochromic shift in excitation and emission wavelengths.49-51

1.2.1.2 Carbopyronines

Carbopyronines or Carbo-rhodamines are a class of rhodamine derivatives that are modified by introducing a germinal dimethyl group (C(CH₃)₂) to substitute the bridging oxygen atom at the 10-position of the xanthene fragment.52 This structural modification can cause a remarkably bathochromic shift in the absorption and emission bands. Since Aaron and Barker introduced a series of carbopyronine dyes, they have been widely used as laser dyes.53 Several carbopyronines with much longer emission wavelengths than the classic rhodamines including dye 6 (Figure 1.4) have been described by Kolmakov et al.54 ATTO 647N is one of the most popular ones and several carbopyronine dyes are commercially available (Figure 1.2). Especially, ATTO 647N shows good performance in STED microscopy with absorption at λ=644nm and emission at λ= 669nm.

1.2.1.3 Oxazines

Oxazine fluorescent dyes are known as planar cationic dyes with p-benzoquinone imine or diamine scaffolds as their chromophores. This family of compounds provide a sufficient red-shift in absorption. Compared to rhodamine or cyanine derivatives, oxazine dyes show high electron affinity. Due to their high oxidation potential, which minimizes the oxidation by molecular oxygen and stabilizes the reduced state, they are more resistant to photobleaching in aerated conditions.55

ATTO 655, ATTO 680 and ATTO 700 are common commercially-available oxazines56 that are widely applied in areas like optical sensors, biomacromolecular labels and dye lasers.57,58 They are also used as fluorescence probes to investigate processes like rotational relaxation,59 electron transfer60 and solvatochromism.61 Among them, ATTO 655 (Figure 1.2) exhibits especially good performance in single-molecule microscopy.62 ATTO 655 exhibits good solubility in water with a sulfonic acid residue as a polar group. It is known that highly polar dyes can bind more specifically with the target, produce less background fluorescence, possess high fluorescent quantum yield in conjugates present in aqueous solutions and yield contrast-rich images with high optical resolution.
1.2.1.4 Cyanines

Cyanine dyes are coloured organic heterocyclic chromophores containing two nitrogen atoms, which are connected by a conjugated polymethine bridge with odd number of carbon atoms. Their names rely on the number of methine groups within the chain. Figure 1.2 shows the general structure of cyanine dyes.

Cy3 and Cy5 are two popular cyanine dyes with a wide range of applications. Cy3 containing a trimethine unit as the polymethine chain exhibits absorption in the region of 500-600 nm. The addition of each double bond to the methine bridge leads to a red-shift of about 100 nm. Hence, this results in the absorption and emission of 600-700 nm for Cy5 along with 700-800 nm for Cy7. This near-infrared optical region is a vital wavelengths range for in vivo imaging as was mentioned before. It is worthwhile to note that the so-called Cy3 and Cy5 dyes are utilized to describe both the sulfonated and non-sulfonated versions rather to well-defined chemical compounds. The sulfonated cyanine dyes, which are commercially available in maleimide and succinimidyl ester form, show increased solubility of the compounds in water and reduced aggregation.

A distinct property of cyanine dyes is their capability to form a non-fluorescent isomer through cis/trans photoisomerization reaction. In the ground state, cyanine dyes exist in trans form. Upon light absorption, the rotation around a C–C bond of the polymethine chain results in a twisted intermediate, which deactivates rapidly to the ground state to yield trans or cis isomers. Due to the negligible fluorescence efficiency of the cis isomer, its formation results in a non-fluorescent state. The cis isomer is thermodynamically not stable and reverts back to the trans isomer. Therefore, cyanine dyes are of particular interest in super resolution spectroscopy, since their photophysical properties can be mainly dictated by these processes.

Cyanine dyes have several appealing properties such as high photostability, generally non-toxic, exceptional biocompatible, can be strongly modified and commercially available in the form of isothiocyanates, succinimidyl esters and maleimides for specific labeling of proteins and nucleic acids. Therefore, they have found a wide range of applications including analytical chemistry, biology, photography and laser technology.

Lately, cyanine dyes with red absorption and emission spectra were found to exhibit robust photo-switching behavior in the presence of thiols. These reversible photoactivatable molecules (also called photoswitches) are successfully applied in the super-resolution microscopy methods such as STORM.
1.2.2 Fluorophore properties

Fluorophores for in vivo applications must fulfill a set of requirements as for their optical, chemical and biological properties.\textsuperscript{20} The most desirable properties are summarized below.\textsuperscript{68}

1.2.2.1 Brightness, fluorescence intensity

Fluorophores should possess high brightness, which is the product of the molar absorption coefficient at the excitation wavelength and the fluorescence quantum yield of the fluorophore at the emission wavelength.\textsuperscript{42} Brightness is used as a measure for the intensity of the fluorescence signal obtainable upon excitation at a specific wavelength or wavelength region. Thus, preferably fluorophores should have both high absorbance and fluorescence quantum yield. However, fluorochrome is not an inert color tag but a molecule that their fluorescence properties can be affected significantly by local environmental factors, such as the interaction with adjacent fluorophores or other biomolecule species in the surrounding environment as well as solvent conditions (pH, polarity and viscosity); accurate evaluation of brightness should be performed with the fluorophore incorporated at the site of interest and in the appropriate assay buffer.

1.2.2.2 Stokes shift

Stokes shift is the difference (in wavelength or frequency units) between the spectral positions of the band maxima of the absorption and emission spectra arising from the same electronic transition.\textsuperscript{70} The ideal organic fluorescent dye should possess a stokes shift large enough to avoid detection of scattered light from the excitation beam. Therefore, for fluorescence measurements large stokes shifts are desirable.

1.2.2.3 Near-infrared dyes

Organic dyes active in the near infrared (NIR) region have been dedicated immense attention owing to their diverse applications in in vivo and live cell imaging. The wavelength range of 650−1450nm fall in the region of the spectrum with low absorption and autofluorescence from organisms and tissues, which minimize background interference, reduce light scattering and enhance tissue penetration depth.\textsuperscript{71}

1.2.2.4 Solubility
Fluorophores must retain their bright fluorescence in the biological milieu, such as in relevant buffers, cell culture media or body fluids. So, ideally, they should be soluble in water and should not aggregate in aqueous solution, or at least one should be able to formulate them in a form in which they stay fluorescent in the biological environment.

The water solubility and suppressed aggregation can be imparted by the attachment of hydrophilic groups to the skeleton of the organic dyes, such as sulfonates, carboxylates, phosphonates, quarternary ammonium salts, di(hydroxyethyl) amine, oligoethylene glycol chains, sulfonated peptides, nitrilotriacetic acid residue, nucleotides and sugars.

Recent publications on this topic describe watersoluble terrylenediimides, soluble quaterrylenediimides, bisanthenes, new hydrophilic BODIPY derivatives, squaraine dyes and dicyanomethylene dihydrofuranes.

1.2.2.5 Labelling

Small organic dyes should have functional groups for site-specific labeling. Labeling of biomolecules (such as amino acids, peptides, proteins, oligonucleotides, DNA primers, double-stranded DNA and antibodies) with a fluorophore should be highly efficient, selective and adaptable to these target molecules, preferably establishing a covalent linkage between the synthetic organic probe and a specific residue in the target molecule. Indeed, fluorophores can be attached to biomolecules via specific functional groups, such as amino groups, carboxyl groups, thiol groups, azide groups and tetrazine groups. The striking features of small organic dyes are their commercial availability of a toolbox of functionalized dyes, in conjunction with established labeling protocols, purification and characterization techniques for dye bioconjugates, as well as information on the site-specificity of the labeling procedure.

1.2.2.6 Biocompatibility and Cytotoxicity

In general, small organic fluorophores for in vivo applications should exhibit good biocompatibility and low cytotoxicity. Biocompatibility means that fluorophores should not interact (or should not change their optical characteristic upon interaction) with biomolecules and interfere normal biological processes,
particularly should have minimal interference on the folding and biological functions of the target protein.

With the exception of DNA intercalators, toxicity of organic dyes is rarely reported as a significant problem. Due to the increasing interest in in vivo imaging applications and the obvious importance of cytotoxicity data of fluorescent reporters for in vivo applications, there are reviews available on the cytotoxicity of widely used fluorophores at present.\textsuperscript{81} Moreover, it has to be kept in mind that during continuous imaging, bleached dye species are formed and/or reactive oxygen species (ROS) are generated, which can be toxic to live cells in addition to the initially used fluorophore.

\subsection{1.2.2.7 Photostability}

Photostability is a critical feature in most fluorescence applications, especially to achieve contrast in fluorescence imaging and in single molecule microscopy experiments. A fluorescent label must be stable under relevant conditions (that is, in the buffer, cell medium or support used), in the presence of typical reagents at common temperatures and under a typical excitation light flux over routinely used detection times. Compared to QDs, organic fluorophores intrinsically suffer from transient excursions to dark states (blinking) as well as irreversible destruction (photobleaching).\textsuperscript{15,82} Both processes fundamentally limit their applicability and have, for a long time, hampered the development of advanced microscopy techniques with single-molecule sensitivity or optical super-resolution <250 nm.\textsuperscript{83}

\subsection{1.2.2.7.1 Origins of fluorophore instability}

In order to improve the photostability of organic fluorophores, it is important to understand the origins of fluorophore instability.

\subsubsection{1.2.2.7.1.1 Singlet and triplet state}

Before excitation, organic fluorescent molecules are in the ground state $S_0$. Once illuminated with light, which matches the excitation wavelength, fluorophore can absorb a photon and transit into several higher electronic states. The transitions are presented as $S_0 \rightarrow S_1$ or $S_0 \rightarrow S_2$ after an absorption of a specific energy $\hbar \nu$ ($S_1$ and $S_2$ in Figure 1.5). Its instantaneous absorption takes about $10^{-15}$ s, which does not offer any displacement of the nucleus. Following rapid vibrational relaxation (picoseconds, rate $k_{\nu vib}$), the fluorophore equilibrates into the lowest vibrational level of the first singlet excited state ($S_1$) which is called the Frank-Condon principle.
A fluorophore in $S_1$ can return to the ground state $S_0$ through either radiative photon emission (fluorescence) or a non-radiative thermal deactivation (internal conversion) pathway. The timescales of fluorescent lifetimes are on the order of $10^{-10} - 10^{-9}$ seconds. An ideal fluorophore rapidly cycles between $S_1$ and $S_0$, resulting in continuous photon emission. However, the fluorophore in $S_1$ can also occupy the first triplet state $T_1$ (an excited state lasting up to several milliseconds that is characterized by a spin-forbidden transition to the ground state) through intersystem crossing. Selection rules for optical absorption states are that spins must not change during an electronic transition. Therefore, transitions between singlet and triplet states are said to be forbidden. This pathway results from an overlap of a higher vibrational singlet state and triplet state. Thus, a low rate for $S_1$ to $T_1$ and $T_1$ to $S_0$ transition results in a long triplet lifetime (typically $10^{-6} - 10^{-3}$ seconds).

**1.2.2.7.1.2 Blinking and photobleaching**

Efficient intersystem crossing leads to a large decrease in the signal intensity, which can be considered as non-fluorescent. Therefore, when one molecule is observed at a time, "on-off" fluctuations are detected as strong variations in the fluorophore’s intensity (triplet blinking). Additionally to the triplet blinking, there are other possibilities for blinking like redox blinking, photochromic blinking and photochemistry. On the other hand, excursions to the triplet state also open
multiple photochemical reaction pathways to irreversible damage (photobleaching). The time until the molecule bleaches is called bleaching time.

In a measurement in solution that contains molecular oxygen (O₂), blinking is rare because oxygen quenches the triplet state and repopulates the singlet state. However, due to the formation of singlet oxygen during the energy transfer for triplet state depopulation as well as the formation of superoxide radical during electron transfer, it happens that a positive charged radical ion of the fluorophore is generated. The oxygen species result in plenty of redox reactions forming other reactive oxygen species. It is known that reactive oxygen species lead to a decomposition of fluorophores or damage biomolecules and therefore contribute to poor fluorophore performance. Contrary, oxygen removal leads to a lack of triplet state quenching, thus, the triplet state lifetime and photobleaching lifetime increase, with negative effects for the brightness of the fluorophore and increased probability for other follow-up reactions from the triplet state. A common method to remove oxygen is an enzymatic oxygen scavenging system.

Because intersystem crossing to the triplet state is unavoidable, a variety of strategies have been proposed to minimize triplet lifetimes in order to mitigate photobleaching and blinking, hence increase photostability. Photo-induced electron transfer reactions produce non-fluorescent radical states of fluorophore (F⁺ and F⁻) originating mostly from the triplet state. These radical states can be long lasting, therefore pronounced blinking and photobleaching occur.

### 1.2.2.7.2 Triplet state quencher

Several chemical additives named as “protective agents” have been identified to decrease photobleaching and achieve significant improvement in fluorophore performance (Figure 1.6). Two main categories of anti-fading compounds have been investigated to improve the photostability of fluorophores: antioxidants/reductants (used to recover photoionized fluorophores and to remove singlet oxygen), examples are cysteamine, ascorbic acid (AA), N,N-methyl viologen (MV), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, TX), n-propyl gallate (nPG), p-phenylenediamine, 1,4 diazabicyclo[2.2.2]octane (DABCO), or 4-nitrobenzyl alcohol (NBA); the second type of additives are triplet state quenchers such as mercaptoethylamine (MEA) and 1,3,5,7-cyclooctatetraene (COT).
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Figure 1.6: Triplet state quenchers: ascorbic acid (AA), N,N-methyl viologen (MV), 1,3,5,7-cyclooctatetraene (COT), 4-nitrobenzyl alcohol (NBA), Trolox (TX) and 1,4-diazabicyclo[2.2.2]octane (DABCO).

These protective agents may operate through a wide range of mechanisms. The most effective remedy to elegantly address the triplet and radical states is the reducing and oxidizing system (ROXS). Reducing/oxidizing agents are used to convert the triplet state to radical ions of the dye (F<sup>+</sup> and F<sup>-</sup>), which are subsequently re-oxidized or re-reduced by oxidizing/reducing reagents in the buffer to recover the fluorophore back to the singlet ground state. Due to the rate of reduction and oxidation are larger than the rate of the path leading to the photobleached products, when such quenching occurs rapidly, triplet and radical states are shortened resulting in a non-blinking and long-lasting fluorescent signal (Figure 1.7).

Figure 1.7: Photo-induced electron transfer of a fluorophore. Due to an excitation of the molecule, two decay pathways are possible: 1) a more frequent radiative decay or 2) the infrequent inter system crossing to the triplet state. Caused by a collision with an oxidizing or...
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Reducing compound a reactive radical ion is produced that can either photobleach or repopulate the singlet ground state.

In particular, the combination of ascorbic acid (AA) with methyl viologen (MV), Trolox, phenylenediamine (PPD) and DABCO have been shown to function through ROXS mechanism. When using ascorbic acid/methyl viologen performed as ROXS antifades, the triplet state is rapidly depleted by electron transfer either through oxidation by methylviologen (MV) forming a radical cation F·+ or through reduction by AA yielding a radical anion F·-. The two possible radical ions are rapidly recovered to singlet ground-state fluorophores by the respective reduction (in case of a radical cation it is reduced by AA) or oxidation (in case of a radical anion it is oxidized by MV). This rapid recovery by ROXS prevents the formation of the photobleaching product P.

During the dissolving process in aerated buffer solution, Trolox can react with molecular oxygen and form oxidizing derivative Trolox quinone (TQ). The reversible redox equilibrium of Trolox leads to a reducing and oxidizing system (ROXS) (see Schema 1.1), therefore, it can be used as a single-molecule redox sensor, which influences blinking and furthermore blinking on longer time scales. However, the enhancement of the photobleaching lifetime varies with the use of different fluorophores, sometimes it declines.

![Schema 1.1: Redox reactions between TX and TQ.](image)

Another triplet state quencher, 4-nitrobenzyl alcohol (NBA), works reverse to Trolox in an oxidation followed by a further reduction. NBA shortens OFF-state lifetime $\tau_{\text{off}}$ and the summarized time of OFF-states (Total $\tau_{\text{off}}$) due to increasing the rate of photo-resurrection of Alexa and cyanine dyes.

Besides ROXS, COT is also an efficient triplet quencher, which decreases photobleaching and extends the observation time of the fluorophores through a non-vertical triplet energy transfer from a triplet donor to COT. Due to the conformational changes of COT, it functions as a flexible acceptor, which leads to a lowering of the triplet excitation energy and thus a better acceptor for non-vertical triplet energy transfer. This process of surface crossing and triplet energy transfer
can be illustrated by a triplet-triplet annihilation or Dexter energy transfer mechanism (see Figure 1.8).\textsuperscript{90}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1_8.png}
\caption{Electron exchange or Dexter interaction: stepwise (top) or concerted (bottom).}
\end{figure}

### 1.2.2.7.3 Self-healing

The most common photo-protection strategies to stabilize different organic fluorophores involve the removal of molecular oxygen from imaging buffers\textsuperscript{91,92} and the addition of ROXS or other triplet state quenchers to solution, resulting in photobleaching lifetime $\tau_{\text{bleach}}$ increase or rather blinking decreasing, which is called solution-based healing.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1_9.png}
\caption{(a) solution-based healing of a fluorophore $F$ due to an addition of a photostabilizer $P$ to the solution that depopulates the triplet state $T_1$. (b) Self-healing due to a covalent bond in between the fluorophore $F$ and the photostabilizer $P$.}
\end{figure}
The mechanism of solution-based healing is based on a collision between photostabilizer and fluorophore on the microsecond timescale (see Figure 1.9). To ensure frequent collisions, sufficient amount of additive have to be added, normally millimolar concentration are typically required. This concentration regime (at, or near, the solubility limit for many of these compounds) is particularly challenging in live-cell imaging applications, since high concentration of hydrophobic organic compounds may lead to a non-specific inhibition of biological activities. Moreover, the effect of protective agents may depend on the fluorophore type, the labeling context as well as solution conditions.

To circumvent these limitations, Blanchard and co-workers covalently conjugated photostabilizing compounds Trolox, COT and NBA to the commonly used organic fluorophore Cy5 to achieve a new class of intramolecular ‘self-healing’ reaction. Additionally, Zheng and co-workers showed that linked COT leads to unique photostabilization performance with each cyanine fluorophore spanning the visible and near-infrared spectrum (Cy2, Cy3, Cy3.5, Cy5.5, and Cy7), where the difference in photon count varied by several orders of magnitude. A covalently linked photostabilizer-dye conjugate can achieve the highest effective local concentration of the protective agent, which is ten orders of magnitude beyond their aqueous solubility limit, thereby mitigating interference with the biological system as happening for protective agents. Tinnefeld and Cordes speculated that the intramolecular triplet state quenching occurs via a ‘ping-pong’ mechanism (see Figure 1.10).

![Figure 1.10: Mechanism of self-healing of a fluorophore F with the protector molecule TX: Reduction of the triplet state and formation of a radical anion followed by a further oxidation.](image)

1.3 Photoswitches for photocontrolled biomolecules

The ability to influence key properties of chemical or biological systems by external stimuli has attracted wide-spread interest for the fields of materials and life sciences. Beyond magnetic/electro-functional materials, extensive efforts have been dedicated to develop photo-regulated biotechnology that use light as the external
trigger to regulate biological activities and processes in living systems. Light offers unparalleled opportunities as a non-invasive regulatory element for biological applications, exerting high temporal and spatial control, with a wide range of intensities and wavelengths.\textsuperscript{97,98} A variety of approaches have been taken to chemically modify a biomolecule in a manner aimed at endowing it with light-controlled function. Generally, two strategies are applied in photo-controllable biological systems: irreversible photo-activation or photo-cleavage molecules which undergo irreversible photochemistry, such that once uncaged/activated, the biomolecule remains active until it is removed in some other manner; reversible photochromic molecules bearing the feature of reversibility of photo-regulation. Photoswitches are compounds that may undergo a reversible photoisomerization between two isomeric forms upon irradiation with two different wavelength of light, and in some cases one of the isomerization processes may also be induced by thermal energy. In addition to a change in absorption spectra, these reversible transformations are accompanied by changes in the physical and chemical properties of the species involved, such as alterations in polarity, charge distribution and geometrical structure. Many classes of molecules with photoswitchable behavior have been developed (Figure 1.11),\textsuperscript{99} which are of particular interest for constructing reversible photosensitive systems. The chromophores undergo Z/E isomerization (azobenzenes,\textsuperscript{100} stilbenes\textsuperscript{101} and hemithioindigos\textsuperscript{102}) or interconvert between open and closed forms (spiropyans,\textsuperscript{103} diarylethenes\textsuperscript{104} and fulgides\textsuperscript{99}). They are commonly characterized by the absorption maxima of their isomeric forms, and the photochromic reaction usually reach a photo-equilibrium or photostationary state (PSS) after photo-induction, defined as a mixture state with both photo-isomers in a dynamic equilibrium.

\textbf{Figure 1.11:} Selected molecular structures of photoswitches and their switching characteristics.
1.3.1 Overview of the Most Commonly Used Photoswitches and Their Properties

1.3.1.1 Azobenzenes

Azobenzenes are by far one of the largest and most studied classes of photochromic molecules that are extensively used in biological applications.\(^{105}\) The reason behind this include their easy synthesis, relatively high quantum yield, fast switching and low rate of photobleaching. Azobenzene undergoes a photochemical Z/E isomerization of the central diazene double (\(-\text{N=N-}\)) bond (Figure 1.11). The E conformation is nearly planar and exhibits a dipole moment near zero.\(^{106}\) Irradiation with near-ultraviolet (UV) light stimulates the conversion to the Z form, which adopts a bent conformation with its phenyl rings twisted out of the plane from the azo group (Figure 1.11) with a dipole moment of \(~3\text{D}~\). The E conformation of azobenzene is \(~10~\text{kcal/mol}~\) more stable than the Z isomer so that, in the dark at equilibrium, E form is the dominant isomer (\(>99.99\%\)).\(^{107}\) The reverse Z to E isomerization occurs thermally by dark-adapting the solution to re-establish the equilibrium or can be achieved by irradiation with visible light (\(>460~\text{nm}\)). Irradiation produces a photostationary state with a maximum of \(~80\%~Z~\) or \(~95\%~E~\). The half-life of unmodified Z-azobenzene is on a timescale of 2 days at room temperature.\(^{108}\) In addition to the shape and polarity changes, the end-to-end distance of each isomer is also substantially different; the distance between the carbons at the para positions of the rings changes by \(~3.5\text{\text{A}}\).\(^{106}\) Substituents on the phenyl rings of the azobenzene core structure and the choice of solvent have a strong influence on these properties such as leading to changes in the absorption maximum and influencing the activation barrier for thermally induced relaxation from Z-azo to the energetically more favourable E-azo isomeric state.\(^{109}\)

Aside from stability in vivo, a key feature that will determine the success of azobenzene-based tools for biological research and photopharmacology is the wavelength required to cause photoisomerization, which must be compatible with cells and tissues. Since UV light is unable to deeply penetrate tissue and can be absorbed by other biomolecules in the cell, it causes severe damage to living systems. A straightforward solution is to use light in the red to near-infrared (near-IR) region, that is, in a wavelength range where penetration through body tissue is orders of magnitude better.\(^{110}\) The design of redshifted azobenzene derivatives has been achieved by several methods, based on tetra orthofluoroazobenzenes, introduced by Hecht and co-workers,\(^{111}\) tetra-ortho-methoxyazobenzenes and tetra-ortho-
chloroazobenzenes, reported by Woolley and co-workers\textsuperscript{112} as well as by ethylene bridges, pioneered by Siewertsen.\textsuperscript{113}

### 1.3.1.2 Stilbenes

Stilbene\textsuperscript{114} is a versatile scaffold, characterized by two aromatic rings linked by an ethylene bridge (Figure 1.11). Structurally, they are divided into Z-type and E-type, based on the configuration of their central double bond; this can undergo Z/E isomerization, changing the overall configuration and eliciting different biological activities. The trans or E form is thermodynamically more stable, however, the barrier of the thermal Z/E reisomerization is 41−46 kcal/mol, which means that the thermal Z/E isomerization at room or body temperature is negligible. Many E-stilbenes have been reported to exhibit more potent activity than the corresponding Z form across biological screens including anticancer and antioxidant activities.\textsuperscript{115} The major drawback of using stilbenes is their tendency to undergo irreversible cyclization/oxidation of the Z isomer.\textsuperscript{116}

### 1.3.1.3 Diarylethenes

Diarylethene\textsuperscript{117} (DAETs) molecules, containing two heterocyclic thiophene rings connected via a cyclopentene system, undergo a reversible photo-induced electrocyclic ring closure/ring opening reaction (Figure 1.11). The open form lacks extensive electron delocalization over the central double bond and the absorption maximum is in the UV range. Conversely, ring closure leads to a conjugated planar structure in which the absorption maximum extends into the visible range of the spectrum. Their isomerization is accompanied by a relatively small change in molecular conformation. The thermal back-reaction is symmetry-forbidden, giving good thermal stability to the closed form. The reverse transition from the closed form to the open form occurs only by irradiating the open form with visible light. The open form is colorless or weakly colored in general, whereas the closed form is colored yellow, red, green or blue depending on the substituents. By attaching different substituents to the diarylethene ring system, the switching wavelengths can be tuned to range from the UV through the whole visible spectrum to IR frequencies. Some diarylethenes show excellent reversibility of photoswitching, superb resistance to photodegradation and a favorable location of the photostationary states with near-quantitative yields of both isomerization reactions.

### 1.3.1.4 Spiropyans
Spiropyran$^{118}$ comprise an indoline and a chromene moiety bound together via a spiro junction, oriented perpendicular with respect to one another. The molecule reversibly switches between the colorless spiro (SP) form, which is nonpolar and uncharged, and a colored merocyanine (MC) form with a polar, conjugated and zwitterionic structure. Irradiation with UV light results in transformation into the MC forms, which reverse back into the SP forms both thermally and photochemically (by irradiation with visible light). The equilibrium in the PSS can be tuned both by the nature of the substituents or by the solvent.

1.3.2 Application

1.3.2.1 Photo-control of peptides

From the point of view of molecular structure, peptides that consist of short chain of amino acids linked by amide bonds exist in disordered or regularly folded structures (such as random-coil, α-helix and β-sheet), typically existing in protein.$^{119}$ They possess an important functional role in biology, serving as hormones, ligands (coenzymes and cofactors) or linkers to other bio-macromolecular assemblies (DNA, RNA, etc.).$^{120}$ When photochromic molecules, such as azobenzene or spiropyran units, are attached to these macromolecular chains, polypeptides may respond to light, resulting in photoinduced structural change. It is anticipated that this might enable general approaches to the photo-control of proteins. Both backbone and side-chain incorporation of photoswitches has been explored.$^{121}$ Three different methods used to incorporate photoresponsive switches in peptides are depicted in Figure 1.12.

![Figure 1.12](image)

Figure 1.12: Three approaches used to incorporate molecular switches in peptides. (a) Copolymers of an amino acid and molecular switch-functionalized amino acid derivative have been used to photomodulate the conformational states of poly-α-amino acids. (b) Molecular
switches have been introduced into the backbone of both cyclic and linear peptides. (c) Peptides have been cross-linked with a molecular switch between side chains.\textsuperscript{122}

Diarylethene, as mentioned before, is famous for its high thermo-stability. Thus, it was selected by Fujimoto and colleagues as a cross-linker to reversibly regulate peptide helices.\textsuperscript{123} When the dithienylethene cross-linker is in its open form, the peptides fold into stable $\alpha$-helices. Upon UV irradiation, the closed isomer of diarylethene is formed, which destabilizes the helical structures of peptides (Figure 1.13). The photo-conversion efficiency of DAET-tethered peptides is over 90\% with excellent photoreversibility. This photo-modulation motif was further introduced to peptide–DNA binding studies, which were followed by quartz crystal microbalance (QCM) analysis. Upon interaction with the DNA, the open form linked peptide, which possessed a more stable $\alpha$-helical structure, exhibited a higher binding affinity than its closed photo-isomer counterpart, which dissociated the $\alpha$-helical structure. Upon alternating irradiation with UV/Vis light, the [$\theta_{222}$] of CD spectra varies around 30–40\%, indicating a reversible photoisomerization of dithienylethene, which regulates the helical structures of the DNA-binding peptides.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Photo-regulation of $\alpha$-helix by a diarylethene derivative.}
\end{figure}

1.3.2.2 Photo-control of nucleic acids

Nucleic acids are critical for storing and encoding genetic information in all life forms; from a biological perspective, the ability to reversibly control their structure and function artificially using external stimuli offers great potential for a wide variety of applications, such as photoswitchable RNA silencing, reversible control of gene transcription, reversible aptamer recognition and the reversible photocontrol of DNAzymes. Photocontrol of nucleotide hybridization or binding properties can be achieved by introducing a photochromic group into sites on the nucleotide via two main approaches: (i) non-covalent interaction between photochromic molecules and specific base pairs in DNA strands, for example, a ligand with light-dependent affinity or intercalation ability;\textsuperscript{124,125} (ii) covalent tethering photochromic molecules
to the oligonucleotide structure, such as incorporating photoswitches into the strand backbone in place of a nucleobase or ribose moiety and also by appending a photoswitch to an existing base pair. The powerful approach of in vitro selection has been used to generate aptamers or ribozymes that interact with photoswitchable small molecules in a light dependent fashion. Hayashi et al. used a short azobenzene-containing tetrapeptide KRAzR (Lys-Arg azobenzene-Arg) on agarose beads to screen for RNA aptamers that would bind the photoswitch in the trans or E form and not in the cis or Z form. The effect of photoswitching the azobenzene on the strength of RNA binding was studied by surface plasmon resonance spectroscopy. Discrimination between the two isomeric forms was found to be roughly 10-fold. The guanidinium groups of the two arginine residues were expected to interact with RNA electrostatically as well as via hydrogen bonding. When the photoswitch was immobilized on a gold surface, UV irradiation to produce the cis form decreased aptamer binding by more than 90% and irradiation at 430 nm to regenerate the trans isomer restored binding. A proposed secondary structure and a consensus sequence were identified, which are necessary for specific binding between RNA and the trans photo-responsive peptide.

**Figure 1.14:** Schematic of azobenzene-modified DNA-controlled reversible release system. Visible irradiation at 450 nm (trans azobenzene) leads to hybridization of the linker and the complementary DNA arm. Irradiation with UV (365 nm) converts azobenzene to the cis form, leading to dehybridization and pore opening.

Tan’s group reported a photon-manipulatable mesoporous release system based on azobenzene-modified nucleic acids (Figure 1.14). To achieve hybridization with azobenzene-modified DNA (denoted as azo-DNA) to form a cage structure, a single-
stranded DNA arm (arm-DNA) was synthesized which contains a complementary sequence to azo-DNA. Once hybridization of both strands is performed, a cap over the pore mouth is formed. Upon exposure to UV light (365 nm), the pore opens as the arm-DNA is released into solution. Rhodamine 6G (Rh6G) and Doxorubicin (DOX) were used as model guest molecules to demonstrate the loading and release behavior of this system. After loading the pores with a Rh6G or DOX molecule, the system was irradiated with UV light, the guest molecules were rapidly liberated into solution because the azobenzene-incorporated DNA was dehybridized from the arm-DNA on the silica surface and unblocked the pore. The release of the entrapped guest molecules was restricted by changing to visible light irradiation at 450 nm due to pore re-blocking. The rapid capping/uncapping response to light provided by this system allows exact point-to-point drug release, enabling this release system to have potential applications in cancer therapy in the future.

1.3.2.3 Photo-control of proteins

The properties of proteins can be photo-regulated through two general approaches.\(^{120}\) One approach is to develop photoswitchable ligands to directly control protein function. The second approach involves directly connecting photoswitches with a protein to trigger an alteration in the protein conformation and activity. These strategies have provided an unprecedented temporal control of purposely designed nanodevices, as demonstrated, for example, by the light-mediates regulation of the activity of enzymes and biochannels. During the synthesis of biomolecules, proteins can be selectively modified through covalent attachment of appropriate photo-switchable moieties to a modified amino acid, which is introduced into the protein through mutation, typically cysteine, at or in the vicinity of an active site or other critical areas required for function.

1.3.2.3.1 Photoswitchable ligands

This method of photocontrol can be achieved by influencing the binding between ligand and protein. As the photoswitch toggles between its two configurations, the efficacy of the ligand changes, thereby triggering the desired biological effect in a reversible fashion. The ligand can change its efficacy upon photoswitching or could even be an agonist in one form and an antagonist in the other.

A greater degree of photo-control can be achieved by covalently tethering the photoswitchable ligand to the target protein. In a pioneering report, Erlanger and colleagues showed that photoregulation of acetylcholinesterase could be achieved by linking azobenzene to phenylcarbamoyl chloride as an inactivator of
chymotrypsin. Several groups have reported that the photocontrol of ion channels can be mediated by photochromic ligands.

In 2004, Trauner and his colleagues realized the control of K⁺ channels in nerve cells. The tethered photoswitchable ligand, maleimide azobenzene-quaternary ammonium (MAQ) containing the pore blocker, was applied to the voltage-gated potassium ion channel by selectively conjugating it to a loop on the rim of the pore, resulting in synthetic photoisomerizable azobenzene regulated K⁺ (SPARK) channels that could be blocked and unblocked by light (see Figure 1.15). The extended trans form is long enough to allow the pendant ethylammonium group to block the pore, that is, at 500 nm or in the dark. However, when it is converted to cis-isomer under irradiation with a wavelength of 380 nm, the distance of the aromatic benzene rings is shortened and the molecular length becomes smaller, which prevents the terminal quaternary ammonium salt group effectively blocking the potassium channels and therefore allows potassium ions passing through.

![Figure 1.15: Engineered potassium channels with a photoswitchable tethered ligand. (A) Chemical structures of trans (left) and cis (right) MAQ (Maleimide-Azobenzene-Quaternary ammonium). The end-to-end distance between the para positions of the azobenzene shortens by ~3 Å upon isomerization from trans to cis. (B) Scheme of a genetically-encoded, photoswitchable K⁺ channel. MAQ is covalently attached on an engineered cysteine located on the extracellular surface of a K⁺ channel. In the extended trans configuration (dark or 500 nm light), MAQ blocks ion conduction. Photoisomerization to cis with 380 nm light shortens MAQ and relieves blocking. (C) Current through a photoswitchable Kv 3.1 channel labeled with MAQ, under both wavelengths of light.](image)

Non-tethered, diffusible azobenzene ligands can also be used to control a wide variety of channels. Successful endeavors include analyzing the behavior of Purkinje
cells using a reversibly photo-switchable kainate receptor agonist, modulating insulin secretion via ATP-sensitive potassium channels with photoswitchable sulfonylurea, versions of the transient receptor potential pain receptor ion channel TRPV1 and endowment of retinal function on blind mice using photochromic AMPA receptor agonist.

The emergence of antibiotic resistance has been rising to dangerously high levels all around the world. Recently, Feringa and co-workers have reported photoswitchable antibiotic analogues with selective activation/inactivation upon light irradiation (Figure 1.16). The bactericidal activity of quinolones stems from their binding to DNA gyrase and blocking DNA replication with a pharmacophore consisting of a benzene ring fused with a carboxypyridone moiety. A photoresponsive moiety was incorporated into the structure of quinolones by replacing a piperazine moiety with an aryldiazo group. This replacement led to the selective turning “on” and “off” of the activity of this compound upon irradiation with UV light since the incorporation of the aryldiazo group made the antibiotic photoresponsive by adopting either the trans or cis isomer. The compound showed activity against both Escherichia coli and Micrococcus luteus. The authors showed that the cis form was the most active isomer. After irradiation with light at 365 nm the amount of the cis form increased, its activity was increased up to 8-fold when tested on the Gram-positive bacterium Micrococcus luteus. Moreover, the thermodynamically unstable cis form of quinolone derivative converts thermally to the trans form over time with a half-life of ca. 2 h and led to a loss in antibacterial activity, which renders the photoswitchable quinolone superior to self-destructive compounds by being temporarily activated when needed to suppress infections. These approaches provide unconventional strategies to control antimicrobial activity, prevent accumulation of the active antibiotic in the environment and hence might avoid the emergence of resistance.

\[ \text{trans} \leftrightarrow \text{cis} \]

Figure 1.16: Chemical structures of trans and cis isomers of photoswitchable quinolone.
1.3.2.3.2 Photoswitchable amino acid side-chains and cross-linkers

Many proteins can be photo-regulated by changes in their structure or conformation via photoswitch isomerization, which consequently determines their catalytic activity.\textsuperscript{139} These conformational changes may result from the binding/dissociation of different effectors or regulators.\textsuperscript{140,141}

However, in early reports, the azobenzene linker (derived from a free amino group) underwent non site-specific reactions with free amino groups or carboxy groups in the protein, two very common protein functional groups. Riklin and coworkers reported that the photo-regulation of papain involved non-specific labelling of lysine residues using 4-carboxyazobenzene.\textsuperscript{142} The modified papain contained an average of five azobenzenes per enzyme and showed a three-fold change in activity upon photoisomerization. Site-specific modification, in which several sites close to the active center of the protein are altered to achieve a better effect, has been commonly achieved by introducing a thiol-reactive group to the photoswitch. The cysteine residues of the protein react with the thiol-reactive group in a highly specific manner and the protein can be reversibly photocontrolled.\textsuperscript{143,144} Woolley and colleagues incorporated phenylazophenylalanine (PAP) at various sites in the S-peptide of RNase S using solid phase peptide synthesis.\textsuperscript{145} These peptides were then combined with S-protein to produce RNase S analogues with site-specific incorporation of azobenzene at specific side chain locations near the active site. Photoisomerization led to \(\sim\)5-fold changes in rates of RNA cleavage by the modified enzyme.

1.4 Motivation and Thesis Overview

Over the last decades, photochemistry has contributed significantly to the understanding of biological processes and to achieve functional systems in biomedicine and the life sciences. Biomedical applications such as anticancer treatment including photodynamic therapy, fueled the need for novel dyes to meet new and demanding requirements. \textbf{Chapter 1} provides an overview of the recent developments in functional dyes with special emphasis on the biological and biomedical context. Attention is paid to the recent design of dye molecules, which are useful for single-molecule fluorescence analysis, such as fluorescent probes for super-resolution microscopy, and photocontrolled biomolecules such as photochromic materials for optogenetics and photopharmacology.

Fluorescent compounds based on synthetic small molecules are powerful tools to visualize fundamental processes in the life sciences, which explains the recent
success of this type of probes. Improvement of fluorescent dyes, including an increase of photostability and the control of blinking at the single molecule level, is a prerequisite to move single-molecule and super-resolution microscopy into the mainstream of biological research. For these purposes, it has become common practice to add photostabilizing, protecting additives to the specimen in demanding fluorescence applications. To date, the use of intramolecular photostabilization in biophysical or microscopy research was still of a “proof-of-principle” nature due to experimental problems and the multi-step chemical synthesis route that was needed to conjugate an organic fluorophore to a biomolecular target. These synthetic challenges represent a fundamental hurdle for researchers not trained as chemists to use the self-healing concept. In order to extend these stability enhancements across the visual spectrum and to chemically distinct fluorophore species, in chapter 2, we introduce a general and simple design concept using unnatural amino acids (UAAs) as flexible scaffold to link many organic fluorophore types covalently to a photostabilizer on an arbitrary biomolecular target. We will introduce a synthesis method that allows the utilization of different classes of commercially available organic fluorophores. Their photostability can be improved through intramolecular photostabilization as “self-healing” fluorophores. Different experiments will be presented demonstrating the fluorophore stability and brightness as well as signal-to-noise ratios in super resolution fluorescence imaging. The overall goal for the fluorophore performance enhancement is to show the potential of these “self-healing” fluorophores in biological research. In chapter 3, due to their desirable photochemical properties, easy and cost-effective accessibility, rhodamine dyes (RhodamineB and KK114) are used as examples to pursue improved imaging in a biological context. The unnatural amino acid p-nitrophenylalanine is used to link Rhodamine B and KK114 fluorophores covalently to biomolecular targets in a single step, i.e., DNA, antibodies and proteins. RhodamineB- and KK114-photostabilizer derivatives are applied in solution-based single-molecule Förster resonance energy transfer (smFRET) microscopy and super-resolution (STED) fluorescence microscopy, where the improved characteristics of these conjugates increase the sensitivity (Chapter 3).

Besides labelling biomacromolecules with dye molecules, small bioactive compounds were functionalized with functional chromophores. Resistance to aminoglycoside antibiotics has had a profound impact on clinical practice. The most prevalent source of clinically relevant resistance against these drugs is conferred by the enzymatic modification of the antibiotic. Aside from developing novel aminoglycoside antibiotics and antimicrobial agents, molecular approaches have become another significant method to overcome the problem of pathogen drug
resistance. Pharmacologically active compounds have been rendered reversibly light-switchable to provide enhanced spatial and temporal regulation of their bioactivity. In an attempt to overcome the emerging resistance to antibiotics, in chapter 4, we present novel photoswitchable aminoglycosides as dimeric conjugates that are less susceptible to modifying enzymes while simultaneously demonstrating selective antibiotic activities upon visible light irradiation. Azobenzene as a photoresponsive element is introduced into the structure of paromomycin. The biological evaluation of this photoswitchable derivative shows clearly the potential to obtain antibiotic candidates with optical control property to overcome bacterial resistance in a straightforward manner. The synthesis of the antibiotic dye conjugates and antimicrobial tests in vitro are presented in chapter 4.

A variety of interactions between RNAs and small organic molecules in living cells play a crucial role in the modulation of biological functions. A straightforward method to prepare photoswitchable aminoglycoside dimers is reported in chapter 4. The novel type of modification provides a promising way for the development of new ligands effectively targeting ribosomal RNA. In chapter 5, we report the development of photoswitchable ligand–RNA aptamer pairs, in which the interactions could be modulated by appropriate photoirradiation. Azobenzene chromophores are incorporated into dimeric paromomycin derivatives and a well characterized aptamer is found to distinguish between the two isomeric forms. The best candidate shows a 100-fold discrimination and a micromolar binding dissociation constant. Three different photoswitchable aminoglycoside derivatives and their binding to a corresponding aptamer are evaluated especially making use of isothermal calorimetry measurements.
References

Design of Functional Dyes for Advanced Microscopy and Life-Sciences