Review Article

Photoactivatable Fluorophores

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Photoactivatable fluorophores switch from a nonemissive to an emissive state upon illumination at an activating wavelength and then emit after irradiation at an exciting wavelength. The interplay of such activation and excitation events can be exploited to switch fluorescence on in a defined region of space at a given interval of time. In turn, the spatiotemporal control of fluorescence translates into the opportunity to implement imaging and spectroscopic schemes that are not possible with conventional fluorophores. Specifically, photoactivatable fluorophores permit the monitoring of dynamic processes in real time as well as the reconstruction of images with subdiffraction resolution. These promising applications can have a significant impact on the characterization of the structures and functions of biomolecular systems. As a result, strategies to implement mechanisms for fluorescence photoactivation with synthetic fluorophores are particularly valuable. In fact, a number of versatile operating principles have already been identified to activate the fluorescence of numerous members of the main families of synthetic dyes. These methods are based on either the irreversible cleavage of covalent bonds or the reversible opening and closing of rings. This paper overviews the fundamental mechanisms that govern the behavior of these photoresponsive systems, illustrates structural designs for fluorescence photoactivation, and provides representative examples of photoactivatable fluorophores in actions.

1. Introduction

Certain organic molecules emit light in the form of fluorescence upon excitation at an appropriate wavelength [1]. This behavior offers the opportunity to probe biological structures and processes with optical methods [2]. Indeed, labeling strategies to introduce fluorescent probes within biological samples, in combination with microscopic techniques to excite the labels and collect their fluorescence, permit the noninvasive visualization of a diversity of specimens in real time [3]. In particular, the basic operating principles of a fluorescence microscope (Figure 1) involve the illumination of the labeled specimen with an excitation source through an objective lens. The radiations focused on the sample excite the many fluorescent labels from their ground state (S_0 in Figure 2) to one of their excited singlet states (e.g., S_2). The excited species relax thermally to the first singlet excited state (S_1) and then radiatively to S_0 . The emitted light is collected on the detector, through the very same objective lens, to reconstruct an image of the

labeled sample with micrometer resolution on a millisecond timescale. In fact, the fluorescence microscope has become an indispensable analytical tool in the biomedical laboratory for the routinary investigation of cells and tissues.

The power of fluorescence microscopy in permitting the visual inspection of biological samples revolves around the ability of individual fluorescent probes to absorb exciting radiations and emit as a result [1]. Thus, the identification of viable strategies to manipulate the photophysical properties of organic molecules is central to the further development of this discipline. In particular, mechanisms to switch molecules from a nonemissive to an emissive state under the influence of optical stimulations translate into the opportunity to photoactivate fluorescence. Interest in such photoactivatable fluorophores was sparked initially by the idea of monitoring the course of photochemical reactions with fluorescence measurements [4] and then by the possibility of using such compounds for photographic applications [5]. It became eventually apparent, however, that photoactivatable fluorophores can be a valuable complement to conventional ones

FIGURE 1: The fluorescence microscope records images of samples labeled with fluorescent molecules by exciting the labels and collecting their emission.

FIGURE 2: The excitation of a fluorescent molecule from the ground state (S_0) to the second singlet excited state (S_2) is followed by internal conversion to the first singlet excited state (S_1). The molecule in S_1 can either decay nonradiatively or emit light in the form of fluorescence to regenerate S_0 . Alternatively, it can undergo intersystem crossing to populate the first triplet excited state (T_1) and then either decay nonradiatively or emit light in the form of phosphorescence to regenerate S_0 .

in a diversity of imaging applications [6–12]. Indeed, fluorescence photoactivation permits the monitoring of dynamic processes in real time [13–24] as well as the acquisition of images with spatial resolution at the nanometer level [25–42]. As a result, significant research efforts are presently directed to the development of strategies for fluorescence photoactivation with fluorescent proteins and synthetic dyes together with their implementation in imaging applications. In fact, the former can conveniently be encoded genetically in a diversity of biological samples and permit the visualization of living specimens [17–22]. The latter can be engineered

with superior photochemical and photophysical properties, relying on the aid of chemical synthesis [6–12], and their small dimensions, relative to their natural counterparts, ensure minimal steric perturbations to labeled samples [43]. In this paper, we overview the basic principles governing the photophysical properties of organic molecules, highlight the main mechanisms developed so far to photoactivate fluorescence with synthetic chromophores, illustrate representative examples of photoactivatable synthetic probes, and discuss their promising applications.

2. Photophysical Properties of Organic Molecules

An organic molecule can adopt a finite number of electronic configurations with distinct energies [44]. These electronic states differ in the distribution mode of the many electrons among the available molecular orbitals, and their energy separations approach the energies associated with ultraviolet and visible radiations. As a result, a molecule can gain a sufficient amount of energy to change its electronic configuration by absorbing an ultraviolet or a visible photon.

The electronic configuration with the lowest possible energy (ground state) of a neutral organic molecule distributes pairs of electrons with opposite spin in the half of available molecular orbitals with lowest energy. Therefore the sum (χ) of the individual electronic spins is equal to 0 in the ground state. This value corresponds to a multiplicity ($2\chi + 1$) of 1 and, hence, this particular electronic state is a singlet and is indicated with the notation S_0 (Figure 2).

Upon absorption of a photon with appropriate energy, an electron in one of the occupied molecular orbitals moves to one of the unoccupied molecular orbitals. During this transition, the electron retains its spin and the multiplicity of the overall system does not change. Thus, the resulting electronic state (excited state) is also a singlet, even though it has two unpaired electrons.

Distinct singlet excited states can be accessed from the very same ground electronic configuration. Indeed, a molecule in the initial state S_0 has multiple pairs of occupied and unoccupied molecular orbitals available to permit electronic transitions with different energies. Therefore, excitation from S_0 can populate any one of the accessible singlet excited states, depending on the energy of the absorbed photon. For example, the diagram in Figure 2 shows a transition from S_0 to the second singlet excited state (S_2) upon excitation.

The electronic transition that accompanies the absorption of a photon from S_0 occurs on a femtosecond timescale [1]. During this extremely fast process, the nuclear configuration of the molecule does not change, and the geometry adopted in S_0 is retained in the final electronic state (e.g., S_2 in Figure 2). After the transition, however, the nuclei respond to the change in electronic configuration by adjusting their relative positions. As a result, the excited molecule alters its geometry to release a fraction of the energy gained with excitation and, eventually, changes its electronic configuration to decay nonradiatively to S_1 . This relaxation process,

called internal conversion (Figure 2), occurs generally on a timescale of picoseconds. Once S_1 is populated, the excited molecule can return back to S_0 by either releasing thermal energy or emitting light in the form of fluorescence. The decay from S_1 to S_0 tends to occur over times ranging from hundreds of picoseconds to a few nanoseconds.

The two unpaired electrons of a molecule in S_1 have opposite spin. One of them can change its spin to produce an electronic state with a total spin (χ) of 1 and a multiplicity ($2\chi + 1$) of 3. Therefore, this particular state is a triplet and is indicated with the notation T_1 . The process responsible for the transformation of S_1 into T_1 that is called intersystem crossing (Figure 2) occurs generally over several nanoseconds and is followed by either the release of thermal energy or the emission of light in the form of phosphorescence to populate S_0 . The transformation of T_1 into S_0 , however, requires one of the two unpaired electrons to change its spin and happens over times ranging from few nanoseconds to several microseconds.

Photochemical reactions also offer the opportunity to release the excitation energy of a molecule [45]. Specifically a compound in S_1 can undergo a structural transformation to produce a different species, which can then decay to its ground electronic configuration. Thus four distinct relaxation pathways are available to deactivate S_1 , but only one of them is responsible for fluorescence. Indeed S_1 can (1) decay to S_0 thermally, (2) decay to S_0 radiatively, (3) intersystemly cross to T_1 , or (4) undergo a photochemical reaction. As a result, the quantum efficiency of fluorescence is ultimately dictated by the rates of these competitive processes [1]. In particular the fluorescence quantum yield (ϕ_F) is the ratio between the number of emitted photons (N_E) and the number of the absorbed photons (N_A) and is related to the rate constants of fluorescence (k_F), nonradiative decay (k_{NR}), intersystem crossing (k_{ISC}), and photochemical conversion (k_{PC}), according to (1). Therefore the ability of a fluorophore to emit light can, in principle, be controlled by manipulating these four rate constants with appropriate structural modifications:

$$\phi_F = \frac{N_E}{N_A} = \frac{k_F}{k_F + k_{NR} + k_{ISC} + k_{PC}}. \quad (1)$$

3. Mechanisms and Structural Designs for Irreversible Fluorescence Photoactivation

Photoactivatable fluorophores switch from a nonemissive to an emissive state upon illumination at an activating wavelength (λ_{Ac} in Figure 3). The photogenerated species is then irradiated at an exciting wavelength (λ_{Ex} in Figure 3) to emit light in the form of fluorescence. This behavior can be implemented with two general mechanisms [6–12]. One of them acts on the ability of S_0 to absorb the exciting radiations and the other controls the nonradiative deactivation of S_1 . In the first instance, the switchable system is designed to absorb at λ_{Ex} only after the transformation induced by λ_{Ac} . Under these conditions, activation enables the population of the excited state responsible for fluorescence. In the second instance,

FIGURE 3: Photoactivatable fluorophores switch from a nonemissive to an emissive state upon illumination at an activating wavelength (λ_{Ac}) and then emit upon irradiation at an exciting wavelength (λ_{Ex}).

the initial species can also absorb at λ_{Ex} , but nonradiative decay dominates its relaxation. After the transformation induced by λ_{Ac} , k_{NR} is designed to decrease significantly, relative to k_F , in order to enhance ϕ_F , according to (1).

Both mechanisms for fluorescence activation can be implemented by integrating switchable and fluorescent components within the same covalent skeleton [6–12]. The local excitation of the former can then be achieved with an activating beam operating at λ_{Ac} to induce the cleavage of one or more covalent bonds. This photolytic process separates irreversibly the two components and, in some instances, is also followed by a rearrangement or a supramolecular event. Ultimately, the photoinduced structural transformation either enables the absorption of the fluorescent fragment at λ_{Ex} or suppresses the nonradiative decay of its excited state. This general design logic is extremely versatile and can easily be adapted to most of the main families of fluorescent probes with the aid of chemical synthesis. Indeed, photoactivatable acridinone [46], anthracene [47], benzofurazan [48], borondipyrromethene [49], chromone [50], coumarin [51–56], dihydrofuran [57–59], fluorescein [60–67], naphthalene [68], pyranine [69], pyrazolines [70], resorufin [71], rhodamine [66, 72–76], thioxanthone [77] and xanthone [78] derivatives have been developed on the basis of these operating principles. Furthermore, this general strategy has been adapted to activate also the luminescence of conjugated oligomers [79] and semiconductor quantum dots [80, 81].

The behaviors of compounds **1a** [57] and **2a** [64] (Figure 4) are representative examples of the two main mechanisms for fluorescence photoactivation. The former has a photocleavable azide group attached to a dihydrofuran fluorophore. The latter has a photocleavable nitrobenzyl group attached to a fluorescein fluorophore. The absorption spectrum of **1a** in ethanol shows a band centered at 424 nm [57]. Upon illumination at a λ_{Ac} of 407 nm, the azide group cleaves to generate irreversibly **1b**. This structural transformation shifts the absorption band to 570 nm. Thus, irradiation at a λ_{Ex} (594 nm) positioned within this band can excite exclusively the photogenerated species **1b**. It follows

FIGURE 4: Illumination of **1a** at an activating wavelength (λ_{Ac}) cleaves the azide group to generate **1b** and enable absorption at an exciting wavelength (λ_{Ex}). Irradiation of **2a** at a λ_{Ac} cleaves the nitrobenzyl appendage to produce **2b** and discourage nonradiative decay upon excitation at a λ_{Ex} . Both processes translate into irreversible fluorescence activation.

that the fluorescence of this molecule is observed only after activation at λ_{Ac} and excitation at λ_{Ex} .

The absorption spectrum of **2a** in neutral buffer reveals an intense band in the visible region for the heterocyclic chromophore [64]. Upon illumination at λ_{Ex} of 490 nm, **2a** changes its electronic configuration to populate S_1 . However, the transfer of one electron from the phenoxy appendage to the heterocyclic fragment encourages the nonradiative deactivation of S_1 . As a result, the fluorescence quantum yield of **2a** is negligible. Upon irradiation at a λ_{Ac} of 350 nm, the nitrobenzyl group attached to the heterocyclic chromophore cleaves to generate **2b**. The anionic character of the photo-generated species prevents intramolecular electron transfer in S_1 and favors, instead, the radiative decay of this state. In fact, illumination of **2b** at λ_{Ex} is accompanied by significant emission. Thus, also for this particular system, fluorescence is observed only after activation at λ_{Ac} and excitation at λ_{Ex} .

The irreversible photolysis of the azide group of **1a** and nitrobenzyl appendage of **2a** releases an intact fluorescent fragment that can then emit upon excitation at an appropriate wavelength [57, 64]. In some instances, however, the released fragment must associate with a supramolecular partner in order to discourage nonradiative decay and enable emission [67, 68]. In other instances, the photochemical reaction does not release the intact fluorophore and, instead, generates an intermediate species that spontaneously rearranges into the final fluorescent fragment [50, 52, 54, 56]. Alternatively, such photolytic events can be coupled with energy transfer to activate fluorescence [65, 66]. For example, compound **3a** (Figure 5) pairs a coumarin donor to a fluorescein acceptor within its covalent skeleton [65]. The nitrobenzyl appendage attached to the donor cleaves upon illumination at a λ_{Ac} of 365 nm in neutral buffer. This structural transformation shifts bathochromically the absorption of the coumarin fragment and enables its excitation at a λ_{Ex} of 410 nm. The excited donor can then transfer energy to the

fluorescein acceptor, which then emits at 520 nm. Thus, the fluorescence of the acceptor is eventually activated through the manipulation of the absorption properties of the donor.

4. Mechanisms and Structural Designs for Reversible Fluorescence Photoactivation

The operating principles for fluorescence activation associated with **1a–3a** are centered around the photolysis of a covalent bond [57, 64, 65]. This photoinduced process is irreversible and, therefore, the fluorescence of the photochemical product cannot be switched off after activation. Reversible fluorescence activation can, instead, be achieved relying on the inherent reversibility of photochromic transformations [82–87]. Indeed, photochromic compounds switch from a colorless to a colored state under illumination at an appropriate wavelength and return to the original form either thermally or upon irradiation at another wavelength. In fact, the photoinduced coloration of a photochromic compound is, essentially, imposing a bathochromic shift on the absorption spectrum similar to that associated with the interconversion of **1a** into **1b**. It follows that the photogenerated state of the photochromic component can, eventually, be excited selectively at an appropriate λ_{Ex} . If this species is fluorescent, then its emission is observed after activation at a λ_{Ac} designed to trigger the photochromic transformation and illumination at λ_{Ex} to excite the photogenerated state [87–94]. This behavior is identical to that associated with the photoinduced conversion of **1a** into **1b** except for the fact that the photochromic process is reversible. Therefore, the original nonemissive state can be regenerated and the system can undergo multiple fluorescence activation/deactivation cycles. Nonetheless, the fluorescence quantum yields of most photochromic compounds are fairly modest, relative to those of the main families of fluorescent probes, with the exception

FIGURE 5: Illumination of **3a** at an activating wavelength (λ_{Ac}) cleaves the nitrobenzyl group to generate **3b** and enable the local excitation of the coumarin donor upon irradiation at an exciting wavelength (λ_{Ex}). The process is followed by the transfer of energy to the fluorescein acceptor and, ultimately, results in the activation of the fluorescence of the latter.

of certain diarylethenes [95–97], rhodamines [98–102], and spiropyrans [103, 104].

The behaviors of compounds **4a** [98] and **5a** [97] (Figure 6) are representative examples of reversible fluorescence activation on the basis of photochromic transformations. The former has a lactam ring connected to a xanthene heterocycle through a spirocenter. Upon illumination at a λ_{Ac} of 366 nm in a poly(vinyl alcohol) matrix, the [C–N] bond at the spirocenter cleaves to generate the rhodamine **4b**. This transformation is accompanied by a significant bathochromic shift in absorption. In fact, the photogenerated isomer can be excited selectively with a λ_{Ex} of 530 nm. The diarylethene **5b** has three adjacent [C=C] bonds at its core. Upon irradiation at a λ_{Ac} of 313 nm in dioxane, they undergo an electrocyclic rearrangement to close a six-membered ring in the form of compound of **5b**. Once again, this structural transformation imposes a significant bathochromic shift in absorption and the photogenerated isomer can be excited selectively at a λ_{Ex} of 488 nm. Thus, both systems offer the opportunity to photoactivate fluorescence with a mechanism that is essentially identical to that governing the behavior of **1a**. In these instances, however, the photogenerated species **4b** and **5b** can switch back to the original nonemissive states **4a** and **5a**, respectively, offering the opportunity to perform multiple activation/deactivation cycles. Nonetheless, **4b** is thermally unstable, while **5b** is thermally stable. The former reverts spontaneously to **4a** on a millisecond timescale and the latter converts to **5a** photochemically under visible illumination.

The photochromic transformations of **4a** and **5a** result in the formation of a fluorescent chromophore that was not originally present in the initial structure [97, 98]. Alternatively a preformed fluorophore can be connected to an appropriate photochromic component and the photoinduced interconversion of the latter can be exploited to control the emission of the former [87, 89–94]. Indeed, numerous examples of fluorophore-photochrome dyads have already been developed successfully, relying on this general design logic [105–127]. In most of these systems, however, the photogenerated state of the photochromic component is engineered to quench the emission of the fluorescent partner on the basis of either electron or energy transfer. As a

result, the behavior of these functional compounds translates into fluorescence deactivation. A remarkable exception is a family of dyads specifically designed for fluorescence activation [128–130]. In these compounds, the photochromic component regulates reversibly the ability of the fluorescent fragment to absorb at λ_{Ex} and permits the implementation of an activation mechanism similar to that governing the behavior of **1a**. For example, compound **6a** (Figure 7) pairs a coumarin fluorophore to an oxazine photochrome within its molecular skeleton. Upon illumination at a λ_{Ac} of 355 nm in acetonitrile, the oxazine ring opens to generate the zwitterionic isomer **6b**. This structural transformation brings the coumarin appendage in conjugation with the cationic fragment of the photogenerated isomer and shifts its absorption band bathochromically by *ca.* 160 nm. As a result, irradiation at a λ_{Ex} of 532 nm excites selectively **6b**. Therefore, the fluorescence of this species is observed only after activation at λ_{Ac} and excitation at λ_{Ex} . The photogenerated species, however, reverts spontaneously to the original one on a microsecond timescale and, in fact, the fluorescence of this system can be switched on and off for hundreds of cycles, relying on the reversible interconversion of the two isomers.

5. Photochemical and Photophysical Parameters Associated with Fluorescence Activation

The operating principles behind most photoactivatable fluorescent probes revolve around the coupling of a photochemical reaction (activation) with a photophysical process (fluorescence). The first of these two steps requires the nonemissive species to absorb photons at λ_{Ac} and switch to the emissive state. As a result, the molar extinction coefficient (ϵ_{Ac}) of the former at λ_{Ac} and the quantum yield (ϕ_P) for this photochemical transformation should be as large as possible. The second step demands the emissive state to absorb photons at λ_{Ex} and emit light in the form of fluorescence. Thus, the molar extinction coefficient (ϵ_{Ex}) of this species at λ_{Ex} and ϕ_F should also be as large as possible. As representative examples, the values of these parameters for **1a–6a** are listed in Table 1.

FIGURE 6: Illumination of **4a** and **5a** at an activating wavelength (λ_{Ac}) encourages the formation of **4b** and **5b**, respectively. The photogenerated isomers can be excited selectively with irradiation at an appropriate exciting wavelength (λ_{Ex}) to emit light in the form of fluorescence. Both transformations are reversible. The original nonemissive state **4a** is regenerated thermally, while **5a** is reformed photochemically.

FIGURE 7: Illumination of **6a** at an activating wavelength (λ_{Ac}) opens the oxazine ring to form **6b** and extends the conjugation of the coumarin fluorophore to enable absorption at an appropriate exciting wavelength (λ_{Ex}). The photogenerated and fluorescent isomer reverts spontaneously back to the original one in microseconds.

Compounds **2a–6a** have acceptable ϵ_{Ac} and ϕ_P values to switch to the respective emissive counterparts at moderate activation intensities [64, 65, 97, 98, 128]. However, they require a λ_{Ac} in the ultraviolet region. In fact, most of the photoactivatable fluorophores developed so far have essentially the very same limitation. Indeed, biological samples have plenty of intrinsic chromophores able to absorb in the ultraviolet region and, therefore, can coabsorb activating radiations in this wavelength range with harmful consequences. This problem can be overcome with structural modifications of the photocleavable group aimed at shifting its absorption from the ultraviolet to the visible region. For example, the introduction of two methoxy substituents on a nitrobenzyl group can be exploited to enable activation with wavelengths longer than 400 nm [8, 9]. Alternatively, two-photon absorption processes can be invoked to induce activation [46, 51–53, 69, 98, 99]. Under these conditions, the simultaneous absorption of two near-infrared photons can encourage the transformation of the nonemissive species into the emissive one. Such protocols offer also the excellent

penetration depths and spatial resolutions inherent to two-photon excitation schemes. Nonetheless, they are limited to photocleavable groups with sufficiently large two-photon absorption cross sections.

Compounds **1b–6b** can all be excited with a λ_{Ex} in the visible region and have all a relatively large ϵ_{Ex} [57, 64, 65, 97, 98, 128]. Both conditions generally apply to most of the photoactivatable fluorophores developed so far for biological applications. Instead, ϕ_F tends to vary over a broad range of values. The ϕ_F of **1b**, for example, is only 0.025 [57], while that of **5b** is up to 0.87 [97]. Nonetheless, a modest ϕ_F is often compensated by a large ϵ_{Ex} and the product of the two (brightness) for **1b** is sufficiently large to permit the detection of this fluorophore at the single-molecule level [57].

In addition to the wavelengths, molar extinction coefficients, and quantum yields listed in Table 1, two additional parameters contribute to define the performance of a photoactivatable fluorophore. One of them is the ratio (contrast) between the emission intensity of the emissive state and that

TABLE 1: Photochemical and photophysical parameters [a] associated with representative examples of photoactivatable fluorophores.

	λ_{Ac} (nm)	ϵ_{Ac} (mM ⁻¹ cm ⁻¹)	ϕ_P	λ_{Ex} (nm)	ϵ_{Ex} (mM ⁻¹ cm ⁻¹)	ϕ_F	Reference
1a/b	407	29	0.0059	594	54	0.025	[57]
2a/b	350	—	0.03	490	—	0.86	[64]
3a/b	365	28	0.3	410	80	—	[65]
4a/b	366	—	—	530	—	—	[98]
5a/b	313	17	0.42	488	46	0.87	[97]
6a/b	355	—	0.02	532	83	0.09	[128]

[a] The reported parameters were measured in EtOH for **1a/b**, neutral buffer for **2a/b** and **3a/b**, poly(vinyl alcohol) for **4a/b**, dioxane for **5a/b**, and MeCN for **6a/b**. ϵ_{Ac} and ϵ_{Ex} were determined at the wavelengths corresponding to the maxima of the corresponding absorption bands, rather than at the actual λ_{Ac} and λ_{Ex} . The missing data were not reported in the original articles.

of the nonemissive one. Ideally, the nonemissive state should not fluoresce upon illumination at λ_{Ex} . In a photoactivatable system operating on the basis of changes in the rate of nonradiative decay, however, the nonemissive state can also absorb at λ_{Ex} and the partial radiative deactivation of its excited state might contribute some fluorescence. For example, **2a** absorbs at λ_{Ex} and emits with a ϕ_F close to 0.001 [64]. After activation and the formation of **2b**, ϕ_F increases to 0.86 with a significant enhancement in detected fluorescence. Nonetheless, the minimal fluorescence contribution of the nonemissive state in such systems can have detrimental effects on contrast and might limit the use these probes in imaging applications requiring negligible background signal.

The other parameter of significance is the photobleaching resistance of the emissive state. The population of the S_1 of this species, upon excitation at λ_{Ex} , can be followed by intersystem crossing to T_1 . Both S_1 and T_1 can undergo photochemical transformations that result in the bleaching of the emissive species. These processes are generally not particularly efficient but, over the course of multiple excitation cycles, degradation can become significant. It follows that photoactivatable fluorophores with limited ability to resist photobleaching might not be particularly useful in imaging applications requiring prolonged excitation.

6. Applications of Photoactivatable Fluorophores

The unique combination of photochemical and photophysical properties of photoactivatable fluorophores translates into the opportunity to control the spatial and temporal distribution of fluorescence. As a result, these functional fluorescent probes permit the implementation of imaging and spectroscopic protocols that are otherwise inaccessible with conventional fluorophores. Indeed, photoactivatable fluorophores are routinely used as calibration standards in photolysis experiments [131–133], to probe the activity and dynamics of biomolecular systems [48, 49, 55, 61, 62, 68, 71, 134–142], to monitor the flow dynamics of fluids [143–152], and to reconstruct subdiffraction images [57–59, 74–76, 98, 99, 103, 104, 129, 153–160]. The common theme of all these strategies is the interplay of activating and exciting

beams to switch fluorescence on within a defined region of space at a given interval of time.

The ability to activate fluorescence under optical control can be exploited to monitor the diffusion of molecules in a diversity of media [13–24]. In fact, the dynamics of a photoactivatable fluorophore within a substrate of interest can be probed by irradiating at λ_{Ac} , a portion of the sample and at λ_{Ex} , the entire substrate, while measuring the intensity of the emitted light. This illumination protocol permits the monitoring of the temporal change in the spatial distribution of the activated fluorescence. For example, the photoinduced transformation of the nonemissive species **7a** (Figure 8) into the emissive one **7b** offers the opportunity to follow the diffusion of the latter in glycerol on a millisecond timescale [161]. Specifically, the tip of an optical fiber can be positioned on a glycerol drop containing **7a** to illuminate a relatively small area of the sample at a λ_{Ac} of 365 nm. This irradiation conditions cleave the two nitrobenzyl groups to generate **7b** within the illuminated area. The simultaneous irradiation of the whole substrate at a λ_{Ex} of 490 nm reveals fluorescence mostly within the activated area (Figure 8(a)). However, images recorded with the same illumination protocol after a delay of 400 and 900 ms (Figures 8(b) and 8(c)) show clearly a significant widening of the fluorescent area. Thus, the photogenerated species **7b** is diffusing away from the activated area over the course of this temporal scale.

The illumination protocol to monitor diffusion with photoactivatable fluorophores is conceptually related to fluorescence recovery after photobleaching (FRAP) [162–164]. However, FRAP demands high-irradiation intensities to bleach fluorophores and switch emission off in the illuminated area. By contrast, moderate illumination intensities are generally sufficient to switch photoactivatable fluorophores and, therefore, they can conveniently be adapted to monitor diffusion in biological specimens [13–24]. In a seminal study, for example, the dynamics of actin microfilaments could be visualized on a timescale of seconds with the aid of a photoactivatable resorufin [71]. Specifically, one of cystein residues of actin can be connected covalently to a photoactivatable label in the form of the conjugate **8a** (Figure 9). Illumination of **9a** at a λ_{Ac} of 360 nm cleaves the nitrobenzyl group to generate **8b**, which can be excited at a λ_{Ex} of 575 nm to emit light in the form of fluorescence.

FIGURE 8: Illumination of **7a** at an activating wavelength (λ_{Ac}) of 365 nm cleaves the two nitrobenzyl groups to generate **7b**, which then emits upon irradiation at an exciting wavelength (λ_{Ex}) of 490 nm. As a result, a sequence of images of a glycerol drop containing **7a** that recorded exciting at λ_{Ex} reveal fluorescence only after illumination at λ_{Ac} . Specifically, pulses at λ_{Ac} applied after 100 (a), 500 (b), and 1000 (c) ms from the onset of the frame sequence to the central region of the imaging field activate fluorescence. Interestingly, the fluorescent area widens over time indicating that the activated probes diffuse over this temporal scale. The drawing in (a) represents the tip of the optical fiber used to deliver the activating radiations. The scale bar in (b) corresponds to 10 μm (reproduced with permission from [161]).

FIGURE 9: Illumination of **8a** at an activating wavelength (λ_{Ac}) of 360 nm cleaves the nitrobenzyl group to release **8b**, which can be irradiated at an exciting wavelength (λ_{Ex}) of 575 nm to emit light in the form of fluorescence. The injection of **8a** in goldfish epithelial keratocytes results in the incorporation of this conjugate within intracellular actin microfilaments. Images of the labeled sample that recorded exciting at λ_{Ex} 4 (a), 81 (b) and 136 s (c) after illumination at λ_{Ac} of a portion of a keratocyte show a spatial redistribution of fluorescence over time. This change is indicative of microfilament dynamics. The scale bar in (a) corresponds to 10 μm (reproduced with permission from [71]).

The injection of **8a** into goldfish epithelial keratocytes results in its incorporation within the native actin microfilaments. Illumination at λ_{Ac} of a defined area within a labeled keratocyte activates fluorescence. Sequences of images (Figures 9(a)–9(c)), recorded upon irradiation at λ_{Ex} over the course of several seconds, reveal clearly a change in the spatial distribution of the activated fluorescence that is indicative of microfilament dynamics. In fact, similar imaging protocols have been extended to the investigation of the dynamics of a diversity of biomolecular systems [134, 135, 138–142].

In addition to the diffusion of individual molecules within a media of interest, similar configurations can be adapted to the investigation of fluid dynamics [143–152].

In particular, a solution of a photoactivatable fluorophore flowing through the channels of a microfluidic device can be probed, once again, on the basis of activation and excitation events. For example, a dilute solution of **7a** in basic buffer can be introduced within a rectangular channel of micrometer dimensions fabricated within a poly(dimethyl siloxane) matrix [150]. Illumination at a λ_{Ac} of 337 nm of a fixed position within the channel cleaves the two nitrobenzyl groups of **7a** to generate its emissive counterpart **7b**. The activated species travels along the channel to reach, eventually, an area that is illuminated at a λ_{Ex} of 488 nm. The fluorescence emitted from the excited area can be measured simultaneously to build a temporal profile of the number of

FIGURE 10: Illumination of a solution of **7a** flowing through a microscaled channel with an activation source generates **7b**. The emissive species travels through the channel to reach an area irradiated with an excitation source. The emitted photons are detected at this position, and their number is plotted against the delay from activation. The resulting profiles show a decrease in delay with an increase in flow rate from 11 cm s^{-1} (a) to 16 m s^{-1} (b) (reproduced with permission from [150]).

detected photons. The resulting plots show that photons are detected after a delay from activation that is directly related to the flow rate. Indeed, an increase in flow rate from 11 cm s^{-1} to 16 m s^{-1} results in a decrease in delay from milliseconds to microseconds (Figures 10(a) and 10(b)).

The phenomenon of diffraction limits the lateral resolution of the fluorescence microscope to hundreds of nanometers [2]. As a result, individual fluorophores can be resolved in space only if their distance is significantly greater than their physical dimensions. It follows that this convenient imaging technique cannot appreciate the subtle factors that govern biological processes and define biological structures at the molecular level. These stringent limitations can be overcome with the aid of photoactivatable fluorophores [26–42]. Indeed, the ability to switch fluorescence on under optical control permits the separation of distinct probes in time and the sequential reconstruction of images with subdiffraction resolution. Specifically, a biological sample can be labeled with photoactivatable probes in their nonemissive state and illuminated at λ_{AC} with low intensities. Under these conditions, only a small fraction of probes switches

to their emissive state (Figure 11), ensuring a large physical separations between them. At this point, the activated sample can be illuminated at λ_{EX} , and the few emissive species can be localized at the single-molecule level with nanoscaled precision, if their brightness and contrast are designed to be sufficiently large. The coordinates of the localized probes can be recorded, and the emissive species can be irradiated further at λ_{EX} until they bleach. Then, the sequence of activation, excitation, localization, and bleaching events can be reiterated multiple times, recording for every single sequence the coordinates of a new subset of probes. Eventually this procedure permits the accumulation of a sufficient number of coordinates to compile a single image of the sample with a lateral resolution that is no longer controlled by diffraction. For example, the tubulin structure of PtK2 cells can be immunolabeled with the photochromic rhodamine **4a** [98]. Reiterative sequences of activation, excitation, localization, and bleaching events can then be exploited to map the spatial distribution of the fluorescent probes with subdiffraction resolution. Comparison of the resulting image with its diffraction-limited counterpart (in Figures 11(a) and 11(b))

FIGURE 11: The tubulin network of PtK2 cells can be stained with the photoactivatable fluorophore **4a**. Illumination at an activating wavelength (λ_{Ac}) converts **4a** into **4b**, which can be irradiated at an exciting wavelength (λ_{Ex}) to emit light in the form of fluorescence and eventually bleach. Reiterative sequences of activation and excitation events can be exploited to localize sequentially multiple fluorescent labels and reconstruct an image (a) with a significant improvement in spatial resolution relative to a conventional diffraction-limited counterpart (b). The scale bar in (a) corresponds to $2\ \mu\text{m}$ (reproduced with permission from [98]).

clearly reveals a drastic enhancement in resolution. Individual tubulin filaments can be distinguished in the former, while they appear as blurred objects in the latter. In fact, this powerful imaging protocol has been extended already to other numerous photoactivatable fluorophores [57–59, 74–76, 98, 99, 103, 104, 129, 153–160] to permit the visualization of a diversity of biological specimens with a resolution that is otherwise impossible to achieve with conventional probes.

7. Conclusions

Synthetic fluorophores can be designed to emit light in the form of fluorescence upon excitation at one wavelength only after activation at another wavelength. Such photoactivatable fluorophores can be constructed by connecting a photocleavable group to a fluorescent chromophore. The photoinduced cleavage of the former can be exploited to control the ability of the latter to absorb at the exciting wavelength. Alternatively, this photochemical process can alter the rate of nonradiative decay of the fluorescent component. Both mechanisms translate into irreversible fluorescence activation. Similarly, photochromic transformations can be invoked to control the ability of a fluorescent component to absorb at the exciting wavelength. The inherent reversibility of photochromic processes, however, offers also the opportunity to deactivate the activated fluorophore. Indeed, multiple activation/deactivation cycles can be performed under optical control with these switchable systems.

The photochemical and photophysical behavior of photoactivatable fluorophores permits the control of the spatial and temporal distribution of fluorescence. These unique

characteristics translate into the possibility of implementing imaging and spectroscopic protocols that are otherwise impossible with conventional fluorophores. Specifically, the diffusion of chemicals labeled with photoactivatable fluorophores and the dynamics of fluids containing such switchable compounds can both be probed in a diversity of media, relying on the interplay of activation and excitation events. Furthermore, the ability to activate the emission of individual fluorophores at different intervals of time offers the opportunity to resolve temporally probes that are separated by relatively short distances. This protocol permits to overcome the limitations imposed by diffraction on the resolution of conventional fluorescence images and, hence, enables the visualization of biological specimens at the nanometer level. Thus, photoactivatable fluorophores are versatile molecular probes for the noninvasive investigation of the dynamics and structures of a diversity of samples.

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