ACCOUNTS
of chemical research

Photoswitching-Enabled Novel Optical Imaging: Innovative Solutions for Real-World Challenges in Fluorescence Detections

ZHIYUAN TIAN*,† AND ALEXANDER D. Q. LI*,‡

†School of Chemistry and Chemical Engineering, University of Chinese Academy of Sciences (UCAS), Beijing 100049, P. R. China, and ‡Department of Chemistry and Center for Materials Research, Washington State University, Pullman, Washington 99164, United States

RECEIVED ON APRIL 12, 2012

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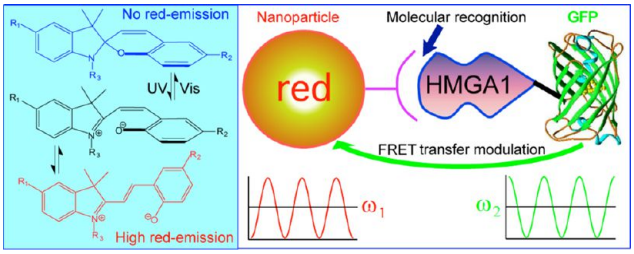

Because of its ultrasensitivity, fluorescence offers a noninvasive means to investigate biomolecular mechanisms, pathways, and regulations in living cells, tissues, and animals. However, real-world applications of fluorescence technologies encounter many practical challenges. For example, the intrinsic heterogeneity of biological samples always generates optical interferences. High background such as autofluorescence can often obscure the desired signals. Finally, the wave properties of light limit the spatial resolution of optical microscopy.

The key to solving these problems involves using chemical structures that can modulate the fluorescence output. Photoswitchable fluorescent molecules that alternate their emissions between two colors or between bright-and-dark states in response to external light stimulation form the core of these technologies. For example, molecular fluorescence modulation can switch fluorophores on and off. This feature supports super-resolution, which enhances resolution by an order of magnitude greater than the longstanding diffraction-limit barrier. The reversible modulation of such probes at a particular frequency significantly amplifies the frequency-bearing target signal while suppressing interferences and autofluorescence.

In this Account, we outline the fundamental connection between constant excitation and oscillating fluorescence. To create molecules that will convert a constant excitation into oscillating emission, we have synthesized photoswitchable probes and demonstrated them as proofs of concept in super-resolution imaging and frequency-domain imaging. First, we introduce the design of molecules that can convert constant excitation into oscillating emission, the key step in fluorescence modulation. Then we discuss various technologies that use fluorescence modulation: super-resolution imaging, dual-color imaging, phase-sensitive lock-in detection, and frequency-domain imaging. Finally, we present two biological applications to demonstrate the power of photoswitching-enabled fluorescence imaging. Because synthetic photoswitchable probes can be much smaller, more versatile, and more efficient at high-performance modulation experiments, they provide a complement to photoswitchable fluorescent proteins. Although new challenges remain, we foresee a bright future for photoswitching-enabled imaging and detection.

1. Introduction

Fluorescence imaging has become an indispensable tool for direct visualization of dynamic protein interactions, intracellular networks, and material transport in living cells. However, several real-world challenges limit fluorescence detections from being widely applied in practical settings. One such limitation is that the wave property of light prevents resolving optical features beyond the diffraction-limit barrier. The second limitation concerns high fluorescence background such as autofluorescence, frequently inundating weak signals such as single-molecule fluorescence. Third, ubiquitous fluorescent interferences render low reliability in fluorescence detections.

Published on the Web 10/24/2012 www.pubs.acs.org/accounts
10.1021/ar300108d

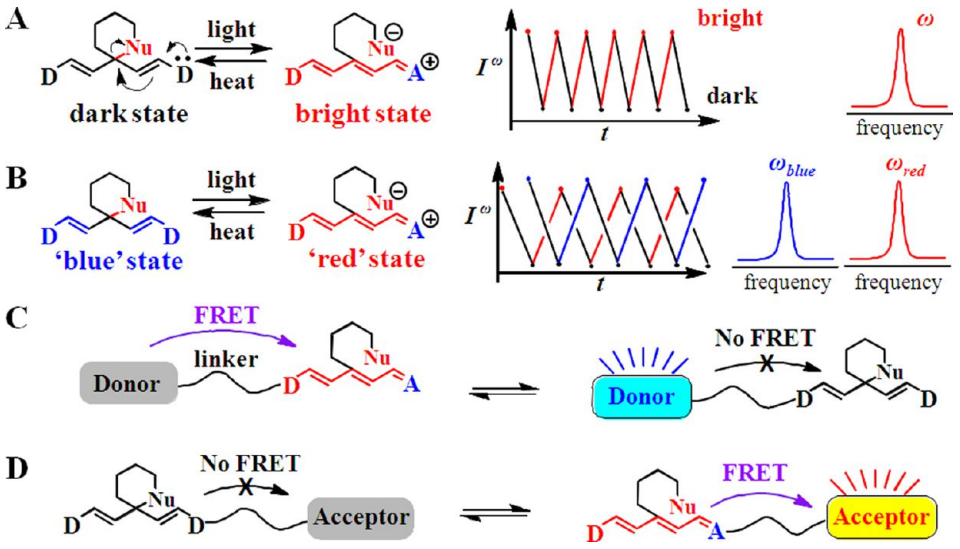

FIGURE 1. Fluorescence photoswitching enabled by molecular design. (A) An intramolecular nucleophile interrupts the π-bridge and thus switches long-wavelength absorption and fluorescence on and off. (B) When both states in (A) are fluorescent, photoswitching will induce dual-color fluorescence modulations that have an antiphase ($\phi_{blue} - \phi_{red} = \pi$) and identical frequency ($\omega_{blue} = \omega_{red}$). (C) Because of FRET, photoswitching of one molecule can induce fluorescence modulation in the nearby molecules. If the nearby molecule is a fluorescent donor and the photoswitch is a fluorescent quencher, the donor fluorescence can be modulated on and off. When the photoswitch is a fluorescent acceptor, dual color double modulations are observed. (D) If the nearby molecule is a fluorescent acceptor with respect to the photoswitch, this transfer the photoswitch fluorescence modulation such as occurred in (A) to the long-wavelength acceptor.

The diffraction limit of conventional microscopes can be circumvented using fluorescence photoswitching.[1−5] In a diffraction limited area, resolving multiple fluorophores simultaneously is impossible due to the Abbe diffraction limit. However, multiple photoswitchable fluorophores can be turned on and thus imaged within the diffraction-limited area one at a time, allowing molecules with spatially overlapping images to be separated in time and subsequently their positions determined precisely via mathematical reconstruction. Thus, fluorescence photoswitching has enabled super-resolution imaging techniques, including stochastic optical reconstruction microscopy (STORM),[6] photoactivation localization microscopy (PALM or FPALM),[7,8] photoactuated unimolecular logical switching attained reconstruction (PULSAR) nanoscopy,[9] and others,[10] which reveal previously unimagined subwavelength details. The second salient feature of photoswitching is that it encodes a modulation frequency to the fluorescence signal, thus distinguishing photoswitched signals from the others such as background noise, autofluorescence, and interferences. Obviously, it is highly desirable to eliminate the noises, suppress the interfering fluorescence below noises, and lock in detection reliability. The encoded frequency in fluorescence makes all such desirability come to fruition.

In this Account, we will first introduce strategies to modulate fluorescence signals under *constant excitation power*. Once modulated signals are generated, demodulation becomes important to process the encoded data. Therefore, we will highlight the methods of processing modulated fluorescence signals and their roles in biological fluorescence detection applications.

2. Strategies for Molecule-Initiated Fluorescence Modulation

The key enabling factor in recent breakthroughs in fluorescence imaging technologies such as the super-resolution imaging and frequency domain imaging (FDI) is that the fluorescent molecules under laser excitation can be modulated one way or the other. Although many molecular mechanisms may impart fluorescence switching, we will focus on two central strategies here: one is to interrupt the π-conjugation, and the other use energy transfer or electron transfer to nearby emitters or quenchers (Figure 1). Interrupting the π-conjugate bridge limits the delocalization extent and modulates electron communication across the bridge typically connecting a donor (D) and an acceptor (A) (Figure 1A and B). When the π-conjugate bridge is broken, the limited delocalization of π-electrons has high-energy emissions, resulting in either no visible or blue/green fluorescence. When the π-bridge is reconnected, however, extended conjugation generates low energy emissions typically near red-fluorescence. In photoswitching, the

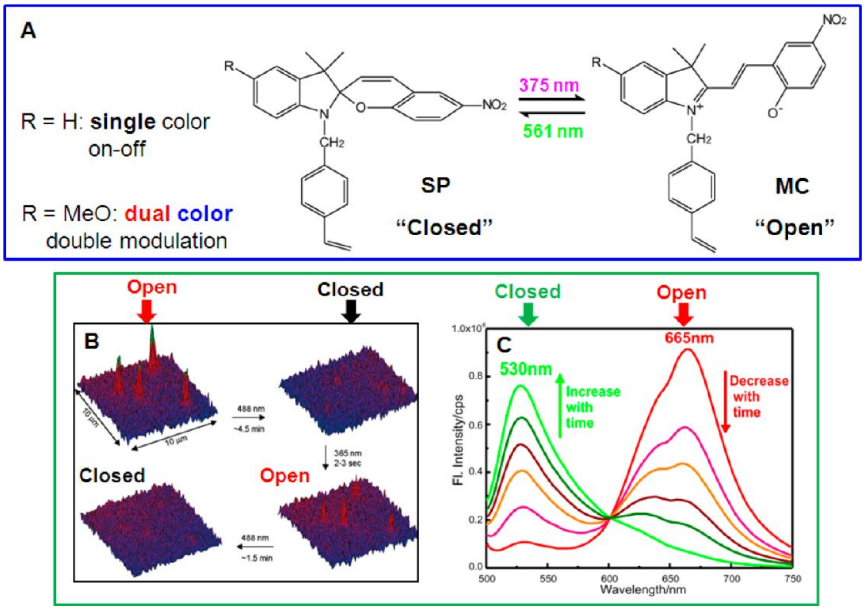

FIGURE 2. (A) Photochromic SP dyes photoswitch either between fluorescence ON and OFF states or between two fluorescent states with distinct colors, depending on the substitution. (B) Reversible on-and-off fluorescence switching of single particle induced by alternating UV (365 nm) or visible (488 nm) illumination. (C) Red-to-green dual-color fluorescence photoswitching based on photochemical reaction shown in (A). Reprinted with permission from ref 12 and ref 13. Copyright (2006) and (2009) American Chemical Society.

π-bridge is controlled by external photons, creating sudden yet highly localized changes in the energetic landscape. Such sudden energy changes in landscape influence the emission of near-by emitters via Förster (fluorescence) resonance energy transfer (FRET) or photoinduced electron transfer (PET) (Figure 1C and D). Therefore, interrupting the π-conjugate bridge is the primary means to enable molecule-initiated fluorescence modulation, whereas FRET approaches are secondary means to modulate probe-enabled fluorescence.

Interrupting the π-Bridge between the Electron Donor and Acceptor. Nucleophilic attacks to a π-bridge, especially a positively charged π-conjugation, convert an sp^2 carbon in the bridge to an sp^3 carbon. Conversely, a photoelimination removes the nucleophile, thus recovering the π-bridge and reestablishing the electron communication between D and A. These two processes represent the essential steps of photoswitching mechanisms by interrupting the π-bridge. If the nucleophilic attack comes from an intramolecular group, the photoswitching can be made reversible because the intramolecular group can attack again and again. For the photochromic spiropyran (SP) with molecular structure shown in Figure 2, two moieties, that is, the indole unit and the pyran unit, are orthogonally arranged and connected via a spiro sp^3 atom. Such a tetrahedral bonding carbon at the center connects two conjugate moieties via σ-bonds or a σ-bridge. The σ-bridge serves as a spacer and interrupts the π-conjugation, thus efficiently controlling the electron communication within the whole molecule. Because of this, SP with its spiro-ring closed has restricted π-delocalization and therefore either is nonfluorescent or emits in the blue-green region. Upon photoelimination, SP is converted to the open form, merocyanine (MC), in which two previously orthogonally arranged moieties are reconnected through a planar π-conjugation, with the π-bridge allowing electrons from both moieties to mix. The newly formed π-bridge in MC facilitates charge transfer excitations from the electron-rich (Ph-O^-) group to electron-poor group (R_4N^+), thus inducing long-wavelength absorption and potential red-fluorescence.

In common organic solvents and aqueous systems, MC absorbs intensely but fluoresces weakly in the visible region with a typical fluorescence quantum yield of $\leq$0.01.[11] Such intense absorption and weak emission features make photoswitchable MC, when exposed to solvents, as effective fluorescence quenchers. However, the fluorescence quantum yield of MC increases up to 0.24 when SP-MC dyes are incorporated into the hydrophobic core of core–shell nanoparticles.[12] Thus, MC functions as a photoswitchable fluorescent acceptor and emits red-fluorescence while protected in hydrophobic environments. Interestingly, UV- or blue-light (<420 nm) causes photoelimination, thus photochemically converting SP to MC,

whereas visible light (>420 nm) accelerates back conversion from MC to SP. Using UV-irradiation at 0.97 mW, ~90% SP was switched to MC in ~3.5 min while ~90% MC was converted back to SP within ~2.0 min upon 16–20 mW visible light illumination. Therefore, rationally designed SP-MC systems photoswitch reversibly, either as fluorescence quenchers (Figure 1A) or as red-fluorescence emitters (Figure 1B).

Attaching an electron-donating methoxy group onto the indoline 5′ position (MSP) resulted in green-fluorescence SP (centered at 530 nm) (Figure 2C), which was attributed to the charge transfer transition from the indole unit to the pyran unit even though they are out of plane. Such a proposed mechanism is supported by tuning the electron-withdrawing group on the pyran unit. Replacing the nitro group with cyano group yielded a closed SP form, which emits blue fluorescence (centered 470 nm). It is reasonable to assign such a blue-shift of fluorescence, from 530 to 470 nm, to a relatively weak charge transfer between methoxy indole and cyano pyran. Upon UV irradiation, the barrier that blocks the π-bridge is removed, and the newly formed larger π-conjugation reconnects the electron donor and the electron acceptor and results in red fluorescence emission of the MC form. These dual-alternating-color chromophores emit either blue-green fluorescence at 470–530 nm in the SP form *or* red fluorescence at 665 nm in the MC form. Again, the SP to MC conversion is totally reversible, thus emitting alternating green/blue or red photons while being photoswitched. To the best of our knowledge, this is the first paradigm where a single small molecule, undergoing light-induced reversible structural rearrangement, emits two distinct alternating colors: red and green or blue, one at a time.[13] Other systems that are thus far known to respond to stimuli-induced dual-color fluorescence conversion are photoswitchable fluorescent protein (FPs) such as Kaede and EosFP and SNARF dye as the pH probes.[14,15] However, the light-driven green-to-red conversion of FPs, hundreds of times bigger than a small molecule, is *nonreversible*, while the pH-sensitive SNARF dyes are not photoswitchable and need physical contact to stimulate the probes. Therefore, the dual-color reversibly photoswitchable SP-MC systems can be applied to innovative applications (vide infra), but the other systems cannot.

Using FRET or PET to Modulate a Nonswitchable Donor or Acceptor. Most fluorescent chromophores do not possess intrinsic photoswitching capability. When certain spectroscopic criteria are met, however, their fluorescence can be modulated via a photoswitch. Specifically, the nonphotoswitchable dye and the photoswitch can form a FRET pair,

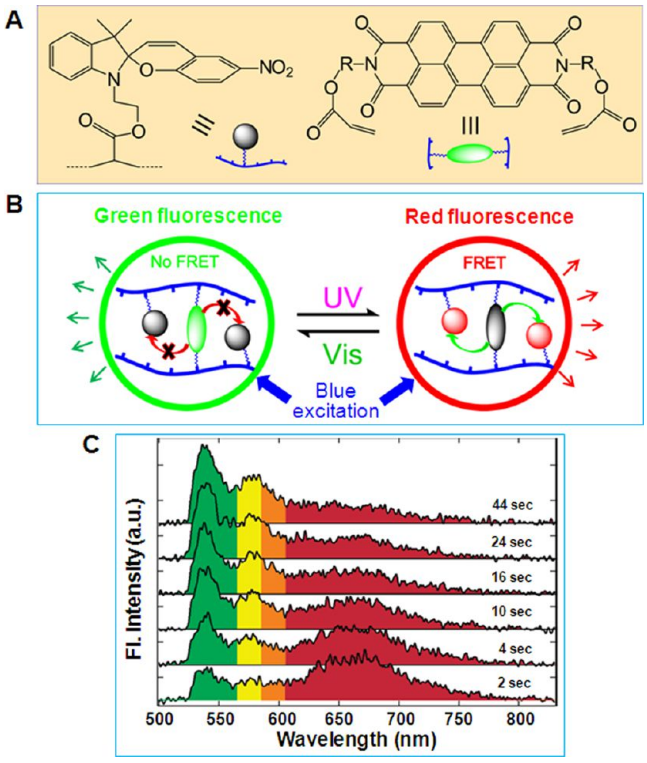

FIGURE 3. (A) Fluorescent photoswitchable acceptor and fluorescent donor were copolymerized with other monomers into photoswitchable nanoparticles. (B) Resulting photoswitchable nanoparticles show dual-color photoswitching via FRET mechanism. (C) Green-to-red fluorescence conversion of a single photoswitchable nanoparticle under 488 nm excitation after a 2 min preillumination of the sample with a UV lamp. Reprinted with permission from ref 20. Copyright (2007) American Chemical Society.

which is photoswitchable. Such a FRET pair will respond to light that triggers the photoswitch and energy transfer will allow the photoswitch to modulate its partner, thus yielding either fluorescence on–off modulation or dual-color modulation. Based on this FRET mechanism, many systems gained fluorescence photoswitching features by combining a photochromic component with various fluorescent donors, such as low-molecular-weight dyes, conjugated polymers, inorganic semiconductor quantum dots (QDs), green fluorescent proteins (GFP), and others.[4]

When the energy-accepting state of the photochromic component merely functions as a fluorescence quencher, pairing up such photochromic units with matching fluorescent donors simply creates system with fluorescence on–off properties.[16–19] A universal way to generate a dual-color system is to pair a single color photoswitch (e.g., the SP-MC dye) with a fluorescent donor. For example, green-emitting perylene diimide (PDI) dyes can be paired with red-emitting SP-MC dyes to construct a dual-dye system that photoswitch to fluoresce either green or red color. PDI dyes cannot

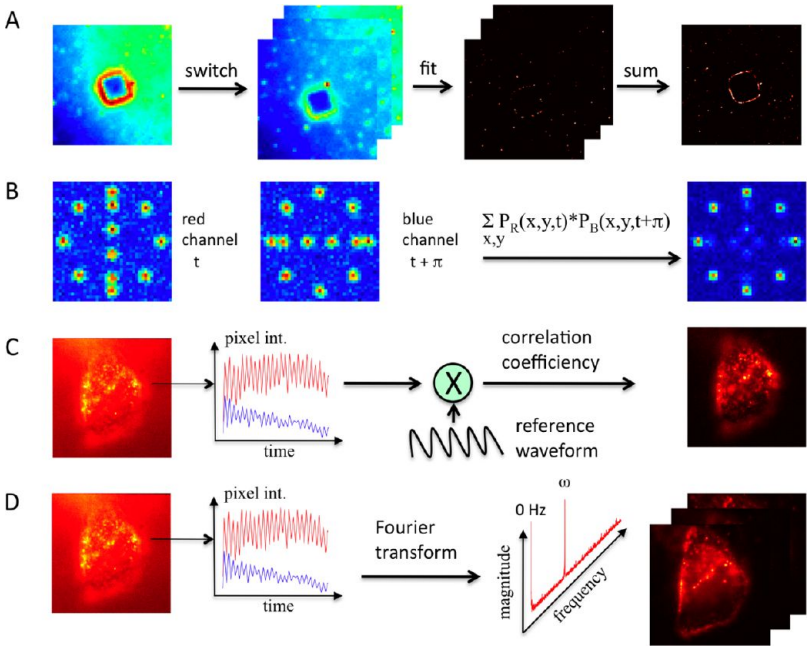

FIGURE 4. Methods to demodulate photoswitching fluorescence signals. (A) Lowering the excitation power such that approximately only one molecule or less is switched on within a diffraction limit area generates a frame with a subset of the total molecular population. Mathematically processing many such frames acquired in a related sequence reconstructs the super-resolution image. (B) Dual-color double modulation uses antiphase relationship and dual-color-correlation produce unambiguous results. (C) OLID uses an internal reference waveform to increase the intensity of pixels matching to the reference. Two nanoparticles (red and blue curves), having different sample waveforms, make it difficult to choose a single universal reference waveform. Consequently, OLID breaks down for single molecules or when nanoparticles become smaller. (D) Unlike OLID, FDI does not need reference waveform and produce amplified, sharp, and high-contrast images.

transfer energy to spiropyran because the energetic process is uphill, and therefore, the system emits PDI's green fluorescence. After SP is reversibly photoswitched to MC, excitation of PDI dyes transfers the energy downhill to the merocyanine, which give off red-fluorescence. PDI dyes and SP-MC dyes can be integrated into a single nanoparticle through emulsion polymerization, which results in dual-alternating-color fluorescence switching, as illustrated in Figure 3.[20] Complementarily, reversible PET provides an alternative mechanism for fluorescence switching.[21]

3. Processing Modulated Fluorescence Signals

Fluorescence modulation is the first step to introduce time-dependent changes in signal amplitude and phase. Aiming at a specific target, each methodology processes the time-domain modulated data in a unique way. For example, super-resolution imaging disassembles a conventional image into numerous frames while photoswitching on one molecule at a time within a diffraction-limited area (Figure 4A). Fitting each single-emitter reveals its precise location in each individual frame. Reassembling these numerous frames into a single image yields the desired super-resolution image. For molecular probes that can be photoswitched between blue-fluorescence and red-fluorescence, they can be unambiguously determined in the presence of fluorescence interference or background. Because fluorescence dual-color double modulation emits two colors alternately at antiphase, the signal ($P_R(x,y,t)$) at pixel x and y at time t in the red channel should correlate strongly to the signal ($P_B(x,y,t+\pi)$) at the same position at $t+\pi$ in the blue channel (Figure 4B). Thus statistical confidence that positive detection at x and y position is proportional to the product of $P_R(x,y,t) \times P_B(x,y,t+\pi)$. In optical lock-in detection (OLID) as shown in Figure 4C, a "representative" internal standard must be placed next to the sample. The internal standard typically must be a large bead to reproduce the ensemble properties of photoswitching and thus produces a reference waveform, against which every single spot is compared. Single-molecule detections and small nanoparticles cannot be detected reliably because the stochastic nature of their emissions does not have a fixed waveform. Finally, FDI simply applies Fourier transform (FT) to the oscillating data in the time domain (Figure 4D). FDI has many advantages because it does not need reference waveforms while amplifying signals and suppressing interferences and noise. The pros and cons of each methodology will be detailed below.

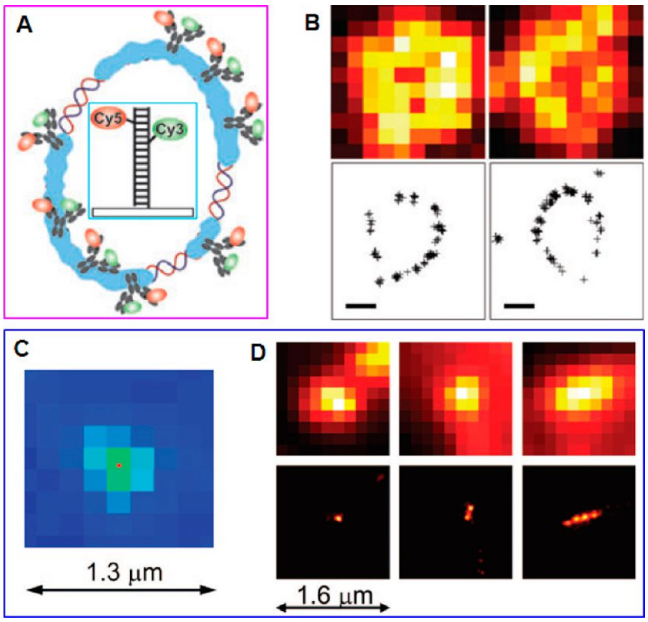

FIGURE 5. (A) Illustration of a circular DNA plasmid. (B) Indirect immunofluorescence images with switch-labeled secondary antibody imaged by a total internal reflection fluorescence (TIRF) microscopy (top); the reconstructed STORM images of the same filaments (bottom). Scale bars, 300 nm. Reprinted with permission from ref 6. Copyright (2006) Nature Publishing Group. (C) Precisely locating a single molecule at nanometer precision by locating its centroid position. (D) PULSAR nanoscopy easily resolves one, two, or four nanoparticles within a diffraction-limited area, whereas conventional microscope cannot. Reprinted with permission from ref 9. Copyright (2008) American Chemical Society.

Super-Resolution Fluorescence Imaging. Photoswitching on one molecule within a diffraction-limited area is the key to enabling super-resolution nanoscopy. This allows localization of a single fluorescent emitter by fitting the detected peak to a Gaussian mask to find its centroid position.[22] For instance, detection of 10^5 photons from a single-dye molecule will yield its position as accurate as ~1 nm. Both nonperiodic photoactivation and periodic photoswitching can enable super-resolution imaging because the dye only needs to be switched once. In PULSAR nanoscopy, a short 0.03 s UV laser pulse, ~1−100 mW/cm^2, and a 2 s 532 nm imaging pulse, ~ 600 W/cm^2, were applied in an alternating sequence. The former photoswitched on a sparse, optically resolvable subset of SP molecules into the emitting MC bright state and the latter recorded the single-molecule positions.[9,23] This photoswitching control in the time domain allows molecules forming spatially overlapping images within the diffraction limit to be separated in time, and subsequently allows their positions to be precisely determined mathematically. The 532 nm pulses also accelerate the MC to SP back conversion with a rate constant of 0.02 s^{-1}; this is favorable because PULSAR nanoscopy prefers to turn off most fluorophores or photobleach them after finishing imaging each individual frame. Repeating the photoswitching and probe imaging pattern for many cycles, thousands of frames were accumulated. Summing these individual frames reconstructed the final high-resolution fluorescence image (Figure 5).

Fluorescence photoswitching or photoactivation has enabled many subwavelength resolution technologies. As shown in Figure 5, STORM applied cyanine dye such as Alexa647 or Cy5 as the photoswitchable probe and 20 nm imaging resolution was achieved, in which the photoswitching of cyanine dye required the removal of oxygen and the fluorescence recovery depended critically on the close proximity of an additional activator fluorophore (Cy3).[6] PALM applied photoactivatable or photoconvertible fluorescent proteins, which could be genetically attached to a target protein for convenient cellular imaging.[7] However, proteins that belong to this class usually bear a disadvantage of low brightness or photon yield, which directly translates into localization accuracy and final achievable optical resolution.[22] The usage of photoactivatable proteins produces considerable random noise when those fluorescent proteins in previous frames are not photobleached. In this aspect, photoswitching reversibility such as the SP-MC dyes plays additional roles in reducing noise when compared with those irreversibly photoswitched probes. Moreover, reversibly photoswitchable probes enable the ability to sample the same structure repeatedly and therefore allow many snapshots of a time-dependent target to be recorded, facilitating imaging of dynamic processes in living cells using photoswitching-based techniques such as STORM.[24,25]

Dual-Color Correlation Imaging. Real-world fluorescence detections frequently encounter ubiquitous interferences or overwhelming background such as cell autofluorescence. Encoding intrinsic correlations among signals of different colors is an effective method to achieve unambiguous detections. The dual-alternating-color photoswitchable probes have built-in intrinsic correlations between signals at time t in one channel ($P_R(x,y,t)$) and signals at $t + \pi$ in other channel ($P_B(x,y,t + \pi)$). Using this intrinsic relationship to modulate these two channels, hence double modulation, allows unambiguous detection of the photoswitchable probes, a statistical confidence that other probes do not have.[13] For example, MSP-containing nanoparticles alternately emit red-fluorescence at 665 nm and green-fluorescence at 530 nm with an antiphase correlation. Figure 6 displays a dominating feature in the red channel, but its

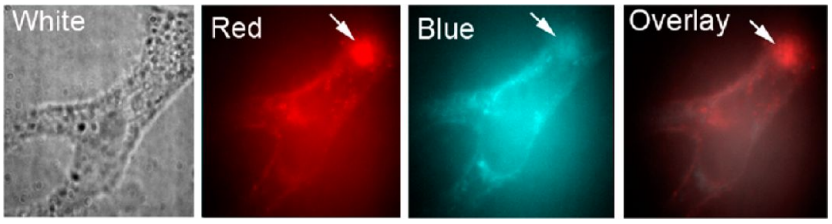

FIGURE 6. Dual-color images of the same cell on red and blue channels, which unambiguously discerns false positives in live cell imaging using demodulation strategy in Figure 4B. The intense spot indicated by an arrow in the red channel has no blue counterpart, indicating it must be from interfering cellular autofluorescence even though its intensity dominates the image. Reprinted with permission from ref 13. Copyright (2009) American Chemical Society.

correlation to the blue channel at the antiphase is rather weak. Therefore, this dominating feature in the red channel is a false positive, not the photoswitchable probe delivered into the cell. Using such a demodulation algorithm (Figure 4B), dual-color correlation imaging can automatically reject false positive signals and boost the statistical confidence of the correlated data, which cannot be accomplished in conventional imaging modality.

Phase-Sensitive Lock-in Detection. Figure 4 displays the substantial difference between OLID and FDI. OLID requires an internal standard in the samples to produce the calibrating reference waveform, whereas FDI does not. As a result, OLID still can produce false positives and "is subjected to the sophistication of the applied waveforms and deconvolution algorithms."[26] Because single-molecule emissions are stochastic,[27,28] a universal reference waveform to establish cross-correlation for OLID does not exist for single-molecule imaging. For example, fluorescent protein, Dronpa, tends to transition to a nonswitchable state and loses its ability to switch over the course of imaging.[29] Therefore, a "standard" waveform for OLID cannot be universally applied to image Dronpa. By contrast, FDI has no such limitations, because FDI scans across the entire frequency range up to the Nyquist frequency.[26] However, OLID can be applied to bulk solutions and large beads where and when time-domain ensemble profiles conform to the reference waveform. In a GFP-MC-SP system, periodically tuning the MC-to-SP ratio in the nanoparticles either strengthens or weakens the FRET efficiency between GFP and MC-SP, thus modulating both the red-fluorescence of the nanoparticles and the green-fluorescence of GFP at a specific and identically locked frequency.[30] Photoswitching of the photoswitchable probes generates periodic fluorescence oscillation; such acceptor fluorescence oscillation will in turn induce fluorescent donors within the Förster proximity to undulate in a locked frequency. Therefore, interactions or binding between the donor (labeled by GFP) and acceptor (labeled by MC-SP) create frequency-locked double modulations, as shown in Figure 7. Such

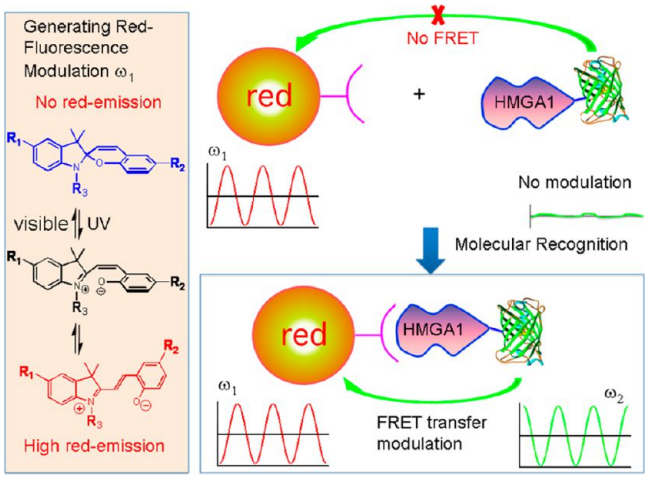

FIGURE 7. Photoswitching induces red-fluorescence modulation in SP-MC-containing nanoparticles. When a green fluorescent protein (GFP) is molecularly recognized by the nanoparticle, the modulated red-fluorescence also induces modulation in the green fluorescence in an antiphase manner by changing the FRET efficiency between nanoparticle and GFP. Consequently, the original modulation frequency (ω_1) is faithfully transferred from the nanoparticle to the analyte—GFP ($\omega_1 = \omega_2$). Reprinted with permission from ref 30. Copyright (2011) American Chemical Society.

fluorescence modulation originates from the sample due to photoswitching between two distinct molecular structures, not from modulation of the excitation source, which is fundamentally different from the secondary long-wavelength laser-driven fluorescence modulation technique that relies on the modulation of the excitation source for selective fluorescence enhancement,[31,32] although long-wavelength illumination typically yields low noise.

In the distance-sensitive FRET-based fluorescence analysis, only bound analytes are within the Förster proximity on average and interfering fluorophores are randomly distributed and typically outside the Förster proximity; thus, fluorescence noises will not oscillate with the signal. Therefore, a signal can be "extracted" from strong interfering noises using the lock-in technology. To the above GFP-MC-SP system, fluorescein as the noise surrogate was added such that the

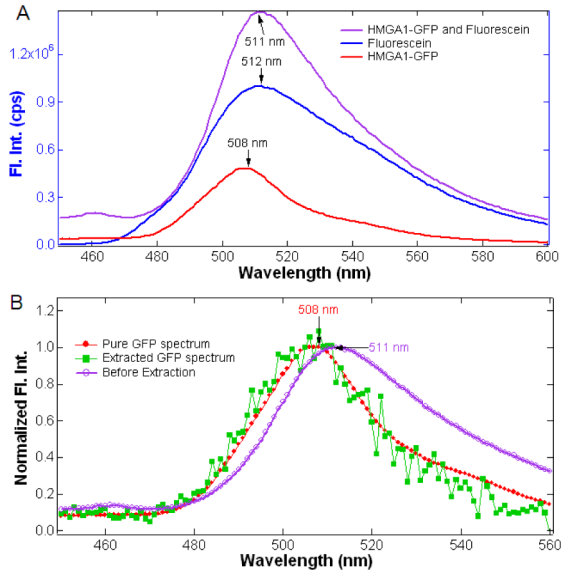

FIGURE 8. (A) Fluorescence spectra of HMGA1-GFP (red), fluorescein (blue), and their mixture (purple) at a 1:2 fluorescence ratio. (B) The inundated GFP signal can be recovered from overwhelming fluorescein using OLID. Reprinted with permission from ref 30. Copyright (2011) American Chemical Society.

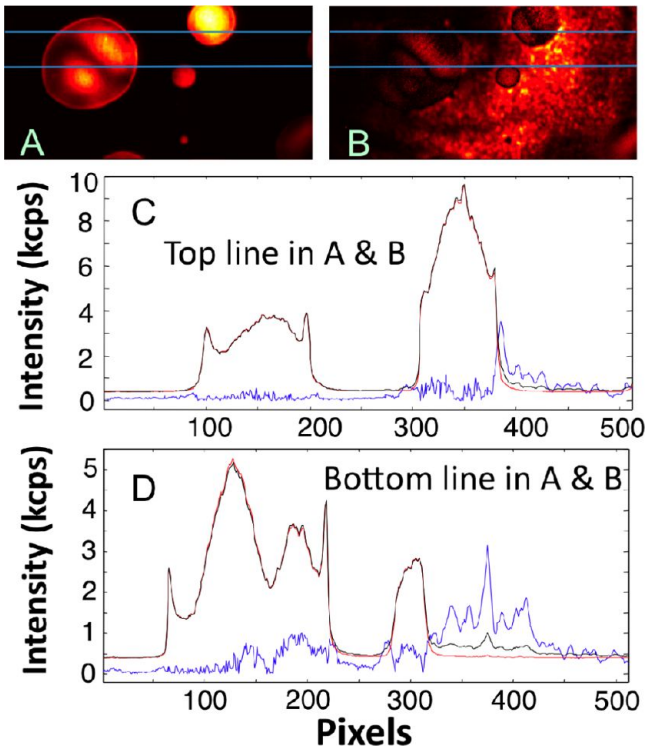

FIGURE 9. Time-domain imaging (A) and frequency-domain imaging (B) of the same area, revealing that very strong Nile Red (NR) interferences are suppressed into noise, whereas weak SP-MC periodic signals are amplified orders of magnitude higher. (C and D) Line profiles in (A) and (B) confirm the amplified periodic signals (blue) and removal of the interference (red) devoid of modulation frequency (black and red). Reprinted with permission from ref 33. Copyright (2011) American Chemical Society.

noise intensity was twice as strong as that of the GFP signal. In such a case, the fluorescence of GFP was completely obscured by the noise, as shown in Figure 8. The crude OLID data contained the superposition of both frequency encoded HMGA1-GFP signal (analyte) and the static (DC) fluorescein noise. Therefore, the "real" spectrum of HMGA1-GFP can be extracted by applying a reference waveform conformed to photoswitching profile of nanoparticles containing SP-MC. The OLID intelligently removed background noise of fluorescein, and the "unpolluted" spectrum of the HMGA1-GFP, similar to Figure 8, could be revealed. Based on such a frequency-encoded detection method, frequency-modulated signals can be easily separated from noise even if the latter are 10 times stronger that the former. As a parallel example, image extraction from large background was developed by dynamically decreasing the FRET rate and simultaneously increasing the donor fluorescence quantum yield.[32] In such a strategy, simultaneous acceptor excitation at wavelength longer than the donor emission and modulation of the secondary excitation source were employed. As a result, only signals from those species whose FRET was modulated can be recovered via demodulation, empowering selective and synchronous signal amplification from high background environments.

Frequency-Domain Imaging. The probe brightness is ultimately important for fluorescence imaging. Increasing the molecular probe brightness without increasing its molar mass has really run into a stone wall in the time domain. For photoswitchable molecular probes, however, they have super brightness in FDI.[33] The simulation results reveal that periodically oscillating fluorescence signals could be made superbright in the frequency domain, via photoswitching-enabled Fourier transform (PFT), even though their time-domain intensity was limited. In sharp contrast, other signals such as those that evolve linearly, exponentially, constantly, suddenly like a burst, serendipitously like oscillation with the locked frequency, and on-and-off like blinking are significantly suppressed. Thus, single dye activation without using photoswitches is undesirable because of lack of periodicity. Experimentally, time-domain signals of 95 cps from MC were dramatically augmented up to 3000 photon counts in the frequency domain, whereas the time domain interference of 10 000 cps from Nile Red was reduced to $\leq$150 photon counts simultaneously (Figure 9). In the time domain, the signal-to-interfering-noise ratio is $\sim$1/105 and the signal-to-shot-noise ratio is $\sim$1/1.05. Thus, signals cannot be effectively resolved. In FDI, however, the signal-to-noise

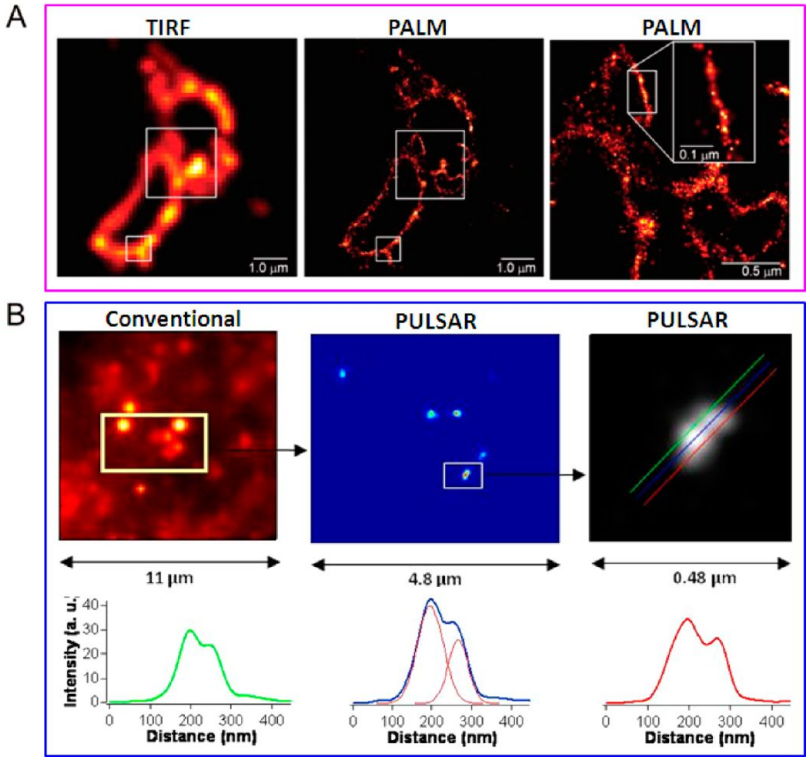

FIGURE 10. (A) TIRF (left) and PALM (middle) images of the same region within a cryoprepared thin section from a COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP Kaede. The larger boxed region in the middle panel, when viewed at higher magnification, reveals more detailed structural information of interacting lysosomes or late endosomes that are not resolvable by TIRF (right). Reprinted with permission from ref 7. Copyright (2006) AAAS. (B) PULSAR nanoscopy detects two nanoparticles tangent to each other in a lysosome of a fixed HeLa cell (upper panel). Lines drawn along the two overlapping 70 nm nanoparticles reveal the fluorescence intensity profile and yield a center-to-center distance of ~69 nm (bottom panel). Reprinted with permission from ref 9. Copyright (2008) American Chemical Society.

ratio is amplified to about 20. Because photon shot noise is random, it essentially disappears into the baseline in FDI. For most signals, the signal-to-interference ratio improved dramatically from 0.013 to 0.0067 (immeasurable) in the time domain to ~12 (clearly observed) in the frequency domain, a factor of 880−1800 amplification. Such gain in signal and loss in noise between time and frequency domains make FDI a powerful technique; it can detect single-molecule emitters against overwhelming fluorescence interferences. Compared with conventional Fourier transform based signal extraction, including the above-mentioned secondary laser modulation methods that generate periodical signals by modulating the excitation light source, PFT does not modulate the excitation source; that is, the fluorescence excitation is under a single wavelength and a constant power for all imaging frames.

4. Applying Photoswitching to Biological Imaging

Super-Resolution Fluorescence Imaging. Photoswitching has enabled visualization of biological samples with resolution approaching the molecular level. Figure 10A shows PALM imaging of lysosome membrane using photoactivatable fluorescent proteins (PA-FP) as tags.[7] In parallel, nanoparticles containing photoswitchable MC-SP dyes were used and functionalized with the HMGA1 protein for high-resolution imaging. Living cells automatically endocytosed such protein-decorated particles and transported them from endosomes to lysosomes. At this point, live cells were fixed and studied under PULSAR nanoscopy. In the as-synthesized nanoparticles, the photoswitchable dye existed in the SP form because SP was the favorable form in hydrophobic solvents. Although there were many dyes per nanoparticle, the UV laser used was so weak that on average it only photoswitched on one dye or less within a diffraction-limited area. Therefore, each single dye molecule within a nanoparticle was localized independently and their close proximity reconstituted the bright image of the nanoparticle at high resolution (Figure 5). Accordingly conventional images scale with photon counts, where PULSAR images scale with the number of photoswitching events. Confocal optical microscopy could not resolve multiple nanoparticles

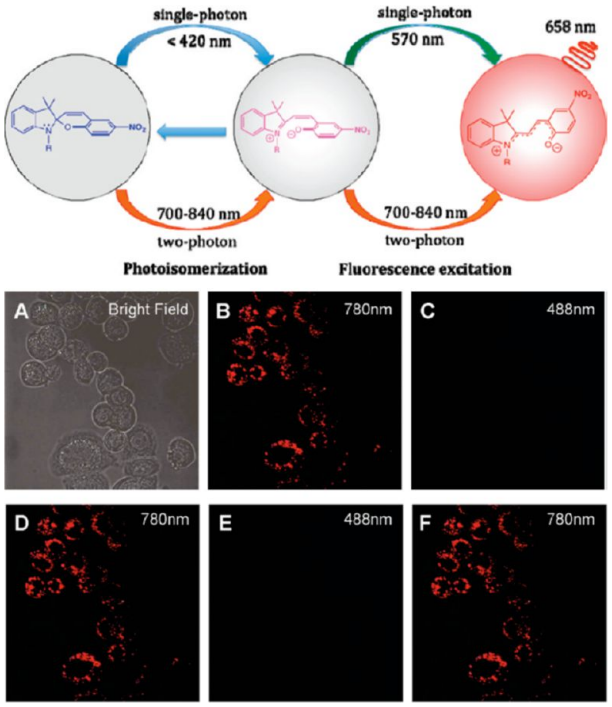

FIGURE 11. Bright-field imaging of SK-BR-3 cells labeled with anti-Her2 antibody-conjugated photoswitchable nanoparticles (A). Two-photon photoswitching and two-photon imaging at 780 nm of Her2 receptors on the cellular membranes switch on red-fluorescence (B). Single-photon excitation at 488 nm switches off red fluorescence (C). This on−off photoswitching can be repeated ≥20 times (C−F). Reprinted with permission from ref 34. Copyright 2011 American Chemical Society.

residing in a single lysosome because this organelle size was smaller than the diffraction limit. The PULSAR nanoscope, however, had resolved the individual nanoparticles inside a single organelle. Figure 10B reveals that there were at least two particles residing in this single lysosome. Fitting the intensity profile yields the projected nanoparticle center-to-center distance 69 nm between these two bright features, coincidentally matching the center-to-center distance of two tangent 70 nm nanoparticles residing in the lysosome.

Two-Photon Photoswitching and Imaging of Nanoparticles Targeted to Cancer Cells. Avoiding UV-induced physiological damage, two-photon photoswitching and imaging were recently developed.[34] Alternating 780 nm NIR two-photon and 488 nm single-photon excitation induced reversible "on−off" fluorescence photoswitching of SP-MC-containing nanoparticles. These nanoparticles were conjugated with the monoclonal anti-Her2 antibody, which specifically binds to the Her2 cancer marker overexpressed on the surface of the human breast cancer cell line SK-BR-3. Specifically, the 780 nm NIR two-photon process triggered the conversion from SP state to MC state and also excited the MC species to emit red fluorescence while the one-photon process drove MC state back to SP state, therefore deactivating the red fluorescence (Figure 11). Such on-and-off cycles modulated by NIR and visible laser illumination could be repeated for at least 20 times. Both single-photon excitation in the visible region (488−570 nm) and two-photon excitation in the NIR region (700−840 nm) can impart strong MC red fluorescence. However, single-photon excitation in the visible region (488−570 nm) also photoswitches MC back to SP, whereas two-photon excitation does not. Interestingly, the two-photon imaging process has decoupled from backward photoswitching, unlike the case of the single-photon imaging process.

5. Conclusion and Perspectives

Photoswitching revolutionized fluorescence imaging. A key determinant to these developments is the photoswitchable fluorescent molecules. As super resolution fluorescence imaging methods continue to evolve, new fluorescent probes with optimized properties will be crucial for improving imaging capabilities. The average total number of photons emitted per emitter prior to photobleaching, or the "death number",[19] which is gauged by the ratio of the fluorescence quantum yield to the photobleaching quantum yield, ultimately determines the spatial resolution. Thus, developing new photoswitchable fluorescent probes with high brightness and well-behaved photoswitching behavior remains as the key-enabling factor. For FDI, developing probes with high on-to-off switching ratio and better photostability continues to be future focal points because the FDI brightness scales linearly with both the oscillation amplitude and the number of modulated period. At molecular-scale resolution, the physical size of the label becomes important, suggesting that development of small and bright photoswitchable fluorescent probes will have significant impact on future imaging applications. The intrinsic trade-off between temporal and spatial resolutions needs to be balanced, and new probes should optimize their brightness, on−off ratio, and better photoswitching behavior.

This work was supported by National Natural Science Foundation of China (Grant No. 21173262) and the National Science Foundation (Grant Nos. CHE-0805547, CHE-1213358, and CHE-1212429).

BIOGRAPHICAL INFORMATION

Zhiyuan Tian graduated from Wuhan University, China with a B.Sc. in 1995 and received his Ph.D. degree in 2005 at the Institute of Chemistry, Chinese Academy of Sciences (CAS). He received his

postdoctoral research trainings at Queen's University in Canada, Washington State University, University of Washington, and Clemson University from 2005 to 2010. In 2011, he joined UCAS as a professor of chemistry. His research interests include nanoscaled fluorescent tags and sensors and their biological applications.

Alexander D. Q. Li received his B.Sc. at Jilin University, China in 1984, and his M.S. in 1986 and his Ph.D. from Northwestern University (Evanston, IL) in 1990. After graduation, he went to Los Alamos National Laboratory (Los Alamos, NM) as a postdoctoral director fellow and later became a Technical Staff Member. In 2000, he joined the faculty at Washington State University (Pullman, WA) as an associate professor, and currently he is a full professor of chemistry and materials science at WSU. His research interests include macromolecular design and engineering, advanced materials synthesis, biofunctionality and nanotechnology integration, self-assembly, and supramolecular systems.

FOOTNOTES

*E-mail: zytian@gucas.ac.cn (Z.T.); dequan@wsu.edu (A.D.Q.L.).
The authors declare no competing financial interest.

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