Journal of
Materials Chemistry B

HIGHLIGHT

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Cite this: *J. Mater. Chem. B*, 2016, **4**, 7761

AIE opens new applications in super-resolution imaging†

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With the rapid development of image processing for scene recognition and visual communication, there is an intense demand to provide the observer with a high-resolution image not only for offering better visualization but also for extracting additional details. In recent years, the invention of super-resolution imaging techniques has overcome the diffraction barrier and has provided clear insights into biological processes at the cellular and molecular scale. In general, the commonly used probes for super-resolution imaging are focused on fluorescent proteins, quantum dots and organic small-molecule fluorophores. Their photostability, biocompatibility and specificity, however, leave much to be desired. Aggregation-induced emission (AIE), a fascinating photoluminescence phenomenon, has found a wide range of applications in fluorescent sensors, biological probes and smart nanomaterials. Herein, we introduce a new class of AIE-based bioprobes for super-resolution imaging, which has recently been reported by Tang and co-workers. The results will inspire the design of AIE luminogens for specific super-resolution imaging in more fields.

Received 14th October 2016,
Accepted 14th November 2016

DOI: 10.1039/c6tb02681h

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Fluorescence imaging has become an important tool in biological and biomedical sciences for three-dimensional imaging of living cells, tissues and animals.[1,2] In contrast to other imaging techniques such as electron microscopy, fluorescence microscopy offers an advantage because it allows dynamic and minimally invasive imaging in living cells. The drawback of fluorescence microscopy, however, has been the fact that the spatial resolution is limited to about 200 nm in the imaging plane due to optical diffraction.[3] This diffraction limit is comparable to or larger than the sizes of many subcellular structures, leaving them too small to be discerned in detail.

It was not until the 1990s that several novel fluorescence microscopy techniques fundamentally revolutionized the imaging field, for the first time overcoming the lateral resolution diffraction limit.[4,5] These techniques are collectively called super-resolution imaging techniques. The key to overcoming the diffraction limit is to spatially and/or temporally manipulate the switch between two molecular states of a fluorophore (for example, a bright state and a dark state). Some techniques achieve super-resolution by sharpening the point-spread function of an ensemble image of fluorophores, such as stimulated emission depletion (STED),[6] ground-state depletion (GSD),[7] reversible saturable optically linear fluorescence transitions (RESOLFTs)[8]

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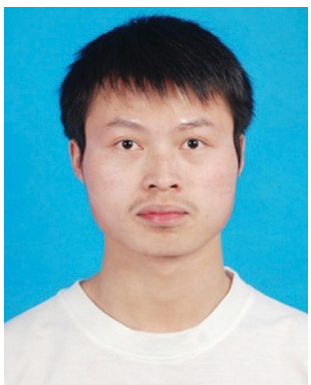
† Dedicated to Professor Ben Zhong Tang in celebration of his 60th birthday.



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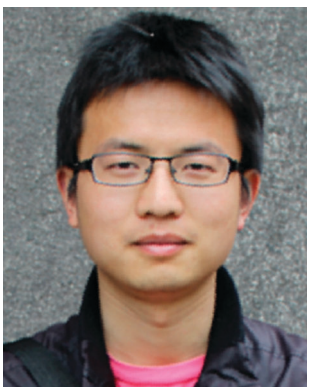

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and saturated structured-illumination microscopy (SSIM).[9] Other super-resolution imaging techniques are based on the localization of single fluorescent molecules, such as photoactivated localization microscopy (PALM),[10] fluorescence photoactivation localization microscopy (FPALM),[11] stochastic optical reconstruction microscopy (STORM)[12] and direct STORM (dSTORM).[13] These techniques have yielded an order of magnitude improvement in spatial resolution in all three dimensions over conventional light microscopy. They can be applied to biological processes and provide new and exciting views on the structural organization of cells and the dynamics of biomolecular assemblies in a wide range of timescales.[14,15]

However, in building a practical super-resolution system, many important challenges lay ahead. The commonly used probes for super-resolution imaging are mainly focused on fluorescent proteins, quantum dots and organic small-molecule fluorophores.[14] To be specific, fluorescent proteins are generally bigger, dimmer and less photostable than small-molecule fluorophores.[16] Quantum dots are traditionally coated with a passivating layer to improve solubility, and are conjugated to targeting biomolecules, such as antibodies.[14] The commercially available quantum dots (*e.g.*, ZnSe, CdS and PdTe) contain heavy metal constituents and therefore are highly toxic or carcinogenic in oxidative environments.[17] Small organelle-specific probes easily cause unexpected background auto-fluorescence and more phototoxicity.[18] Adding features such as robustness, computation efficiency, image registration, and a performance limit in super-resolution methods will be the ultimate goal for super-resolution researchers and practitioners in the future.[19]

Traditional organic fluorophores induce bright emission in dilute solution but provide weak or quenched emission in the aggregated state, which is notoriously known as aggregation-caused quenching (ACQ).[20] Fluorescein is a representative ACQ fluorophore (upper panel in Fig. 1). The molecular mechanism causing the ACQ phenomena is always the π–π stacking of their conjugate planes. This ACQ effect has significantly limited the scope of fluorophores for practical applications. Aggregation-induced emission (AIE), a unique photophysical phenomenon

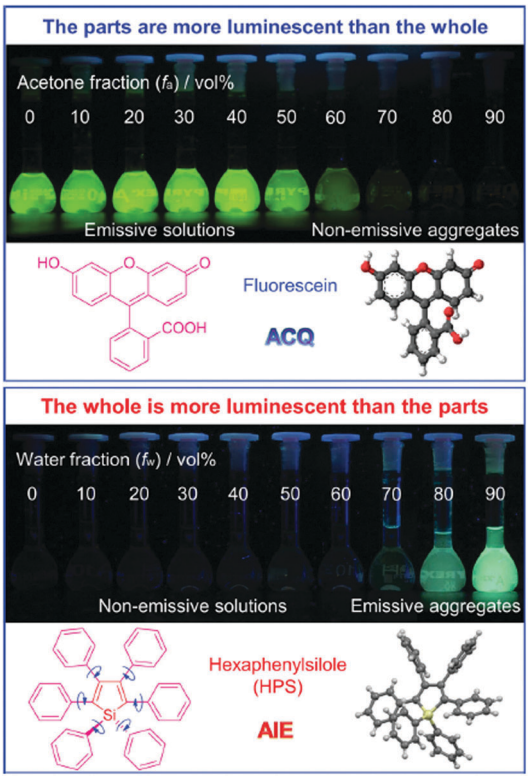

Fig. 1 Fluorescence photographs of the solutions and suspensions of (upper panel) fluorescein (15 μM) in water/acetone mixtures with different fractions of acetone (f_a) and (lower panel) hexaphenylsilole (HPS; 20 μM) in THF/water mixtures with different fractions of water (f_w). (Reprinted with permission from John Wiley & Sons, Inc.)[23]

that shows exactly the opposite behavior to the ACQ effect, was first reported by Tang and co-workers in 2001.[21] The new luminogen is non-emissive when dissolved in good solvents but becomes highly emissive when aggregated. A classic example of an AIE luminogen (AIEgen) is revealed in the lower panel of Fig. 1. The widely accepted mechanism of the AIE effect occurs through restriction of intramolecular motions (RIM), which inhibit rotations and vibrations of the aromatic rings (Fig. 2).[22,23] The free motions are restricted due to the physical constraint in the aggregated states, which diminish the nonradiative decay of the excited state energy, resulting in strong emission. The novel AIE effect provides an excellent platform for developing new molecular luminogens that can be utilized in a series of high-tech applications, including optoelectronic devices, luminescent sensors, cell imaging and other advanced functional materials.[24–32]

In this paper, we introduce a new breakthrough in the field of AIE by Tang and co-workers utilizing a new class of fluorescence turn-on photoactivatable AIE-based bioprobes for super-resolution imaging.[33] They synthesized a novel photoactivatable fluorophore (*o*-TPE-ON+) with positive charges that showed specificity to mitochondria *via* the membrane potential gradient (Fig. 3). Upon light irradiation, *o*-TPE-ON+ underwent an unconventional regioselective photocyclodehydrogenation, transforming it from a faint fluorophore to a strongly emissive *c*-TPE-ON+. The strong photostability of the AIEgens helped to produce a

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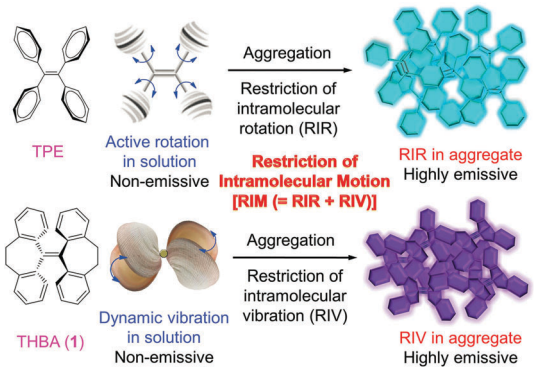

Fig. 2 Propeller-shaped tetraphenylethene (TPE) molecule is non-luminescent in a dilute solution but becomes emissive upon aggregation, due to the restriction of intramolecular rotation (RIR) of its phenyl rotors against its ethylene stator in the aggregate state. A shell-like 10,10-lik,11′-tetrahydro-5,5′-bidibenzo[*a*,*d*][7]annulenylidene (THBA; **1**) molecule behaves similarly, owing to the restriction of intramolecular vibration (RIV) of its bendable vibrators in the aggregate state. (Reprinted with permission from John Wiley & Sons, Inc.)[23]

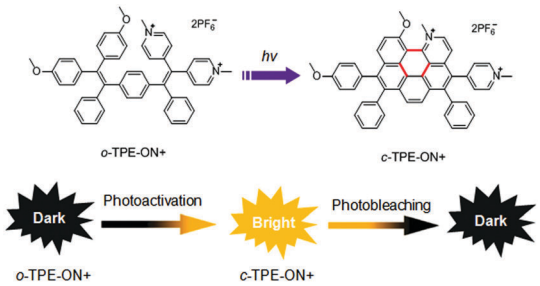

Fig. 3 Photocyclodehydrogenation process of *o*-TPE-ON+ and the general strategy of applying *o*-TPE-ON+ for super-resolution imaging. (Reprinted with permission from John Wiley & Sons, Inc.)[33]

cyclized product with a high quantum yield, a long excitation wavelength of above 500 nm and pH/environment-insensitive fluorescence. Then, the emissive *c*-TPE-ON+ could be photobleached to the dark state using an appropriate light. Subsequently, super-resolution imaging of mitochondria using *o*-TPE-ON+ was achieved without additives (like thiols or oxygen-scavenging agents that are generally required in STORM applications).[34] Besides, *o*-TPE-ON+ was applied to the super-resolved observation of dynamic multiple fission and fusion behaviors of mitochondria, proving its practicability for live-cell super-resolution imaging. The results demonstrated that it was a win–win strategy to utilize the strong photostability of the AIEgens for super-resolution imaging, which not only facilitated the development of the related fields but also provided new insights into fluorophores.

By using photoluminescence (PL) spectroscopy, the photophysical properties of *o*-TPE-ON+ and *c*-TPE-ON+ in aqueous medium were investigated. *o*-TPE-ON+ was almost non-emissive in aqueous solution corresponding to the non-radiative decay caused by the active intramolecular rotations of the phenyl rings. It should be emphasized that the fluorescence emission in the aggregates was extremely weak with an undetectable fluorescence quantum yield (Φ_F), which was dramatically different from normal AIEgens. The twisted intramolecular charge-transfer (TICT) effect was responsible for this unconventional phenomenon, because *o*-TPE-ON+ bore two electron-donating (D) methoxy groups and two electron-accepting (A) 1-methylpyridinium groups. Upon UV irradiation, *o*-TPE-ON+ can be efficiently converted to a strong emitter *c*-TPE-ON+ by photocyclodehydrogenation with a high Φ_F value of 18%. The mechanism was attributed to the activation of the restriction of the intramolecular rotation (RIR) process by chemically locking the phenyl rings. Interestingly, this probe exhibited high mitochondrial specificity, arising from its pyridinium salt functionalities.

On the contrary, the fluorescence of highly emissive *c*-TPE-ON+ can be further turned to the off-state by using appropriate light (514 nm) to photobleach its light emission. The sequential photoswitching between dark-to-bright photoactivation of *o*-TPE-ON+ and bright-to-dark photobleaching of *c*-TPE-ON+ using light of different wavelengths ensured the construction of the accurate localization-based super-resolution images.[14] Ultimately through stochastically switching it on/off and imaging, repeating this process for many cycles allowed the reconstruction of super-resolution images.

A customized STORM microscope was utilized to study the photoswitching dynamics of *o*-TPE-ON+ (Fig. 4). By tracking the intensity trace of an individual fluorophore, important blinking parameters including photon counts on time and localization precision of *o*-TPE-ON+ were measured. The resultant high photon counts and low fluorescence-on time of *o*-TPE-ON+ guaranteed its high localization precision in live-cell super-resolution imaging. And more importantly, no external additives (such as thiols or oxygen-scavenging agents that are normally used in STORM imaging) were required to control the ON/OFF photoswitching of the probe, thus rendering live-cell super-resolution imaging with *o*-TPE-ON+ better than other systems. The STORM image exhibits a clear structure of mitochondria with more structural details (Fig. 4e) because of the small full-width at half-maximum. Multiple fission and fusion events can be clearly observed in Fig. 4g–i, demonstrating the unique photoactivation characteristics of *o*-TPE-ON+, which make it suitable for super-resolution imaging under physiological conditions.

The results reported by Tang and co-workers were the first attempt to use a new class of fluorescence turn-on photoactivatable AIE-based bioprobes for super-resolution imaging. Compared with the normal probes for super-resolution imaging, this AIEgen-based probe possesses several unparalleled advantages that have been discussed above: (1) the strong photostability of the AIEgens helped in producing the cyclized product with a high quantum yield, a long excitation wavelength of above 500 nm and pH/environment-insensitive fluorescence; (2) no external additives (such as thiols or oxygen-scavenging agents that are normally used in STORM imaging) were required to control the ON/OFF photoswitching of the probe; (3) the photoactivation behavior of *o*-TPE-ON+ is oxygen-promoted owing to the involvement of oxygen in photocyclodehydrogenation; (4) *o*-TPE-ON+ can spontaneously blink under physiological conditions with advantages of high photon counts, low fluorescence-on time, and high localization precision. This work can be considered as a significant step forward in the

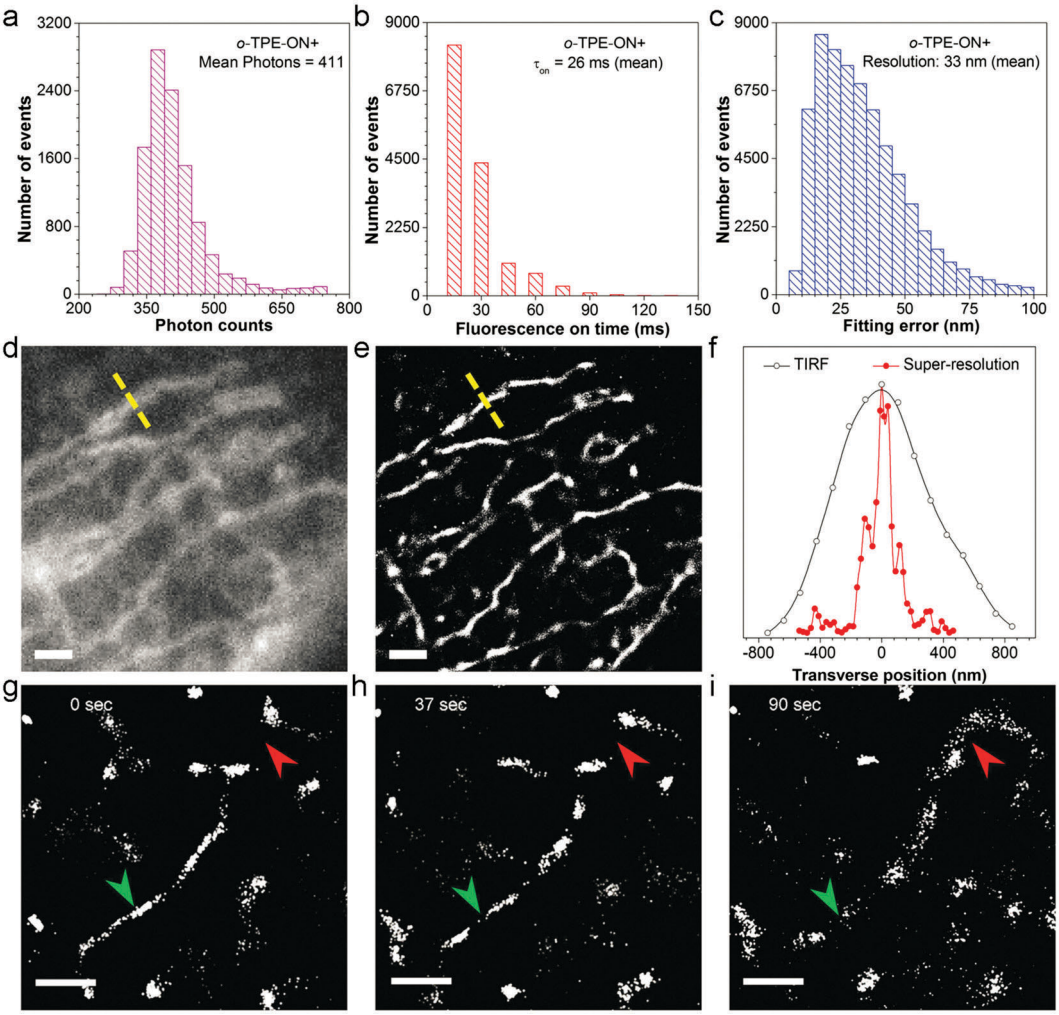

Fig. 4 (a) Photon counts, (b) fluorescence-on time, and (c) localized precision of *o*-TPE-ON+ in a single-molecule state. Super-resolution imaging of mitochondria in a fixed HeLa cell: (d) diffraction-limited TIRF image with a totally blurred structure. (e) Super-resolution image. (f) Transverse profiles of the single mitochondrion along the yellow dotted line marked in the images (d) and (e). Scale bar: 2 μm. Mitochondrial dynamics in a live HeLa cell stained with *o*-TPE-ON+ (10×10^{-6} M): fission (green arrowheads) and fusion (red arrowheads) events captured by a time series of 2.5 s STORM. The images were acquired at (g) 0, (h) 37, and (i) 90 s. Scale bars: 500 nm. (Reprinted with permission from John Wiley & Sons, Inc.)[33]

field of super-resolution imaging for developing a novel AIE-based bioprobe with a high on–off contrast and a fast photoswitching rate. We are convinced that this exploratory work will inspire more image processing researchers to develop other AIE-based fluorescent probes that are brighter, more photostable and used as switchable fluorophores to gain new biological insights into super resolution microscopy.

On the other hand, since the discovery of the AIE effect by Tang and co-workers in 2001, luminescent materials with AIE attributes have experienced exponential growth in research interest and enjoyed a wide range of high-technological applications, especially in optics, electronics and biological sciences. The AIE phenomenon opens a new door for the study of solid-state luminescence processes. Because it is of great academic value and practical implication, a large amount of excellent work with tremendous prosperous results has been generated in this exciting area of research. With such prospects, it is anticipated that this research may serve as a ''catalyst'' to stimulate new enthusiasm of scientists to be involved in this emerging and promising field, and to trigger new ideas and accelerate the advancement in the design and synthesis of novel AIE-based fluorescent probes with versatile properties for high-tech innovative applications.

At the same time, given the remarkable photophysical properties of AIE molecules, the development of novel luminescent materials for detecting multiple analytes and *in vivo* imaging-guided therapy will be the major focus of future research. Combining the AIE characteristics with the desired traits of compounds, a wide variety of advanced materials with the joint advantageous properties will be obtained. Meanwhile, the development of new AIEgens with diverse structures and emission colors covering the full visible spectrum seems more significant. In particular, the AIEgens with the emission locating in the near-infrared (NIR) or far-red regions are highly desired, because they show very small photo-damage to biological samples, minimum interference from background auto-fluorescence, and high sensitivity in the

living systems. Last but not least, the ability of targeted navigation inside a physiological environment will be another interesting exploration for sensing the focus regions or diseases with great specificity.

Acknowledgements

This work was supported by the National Basic Research Program (2013CB834502), the National Natural Science Foundation of China (21434005, 91527301), the Fundamental Research Funds for the Central Universities, the Key Science Technology Innovation Team of Zhejiang Province (2013TD02), and the Open Project of State Key Laboratory of Supramolecular Structure and Materials (sklssm201611).

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