



Cite this: *Chem. Commun.*, 2015, 51, 15382

Received 9th July 2015,  
Accepted 21st August 2015

DOI: 10.1039/c5cc05700k

www.rsc.org/chemcomm

## A two-photon activatable amino acid linker for the induction of fluorescence†

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**A new one- and two-photon activatable fluorophore based on ATTO565 was developed using a photolabile linker that simultaneously acts as a quencher. It is especially interesting for protein and peptide applications because it can be incorporated by standard peptide chemistry. The application of the new fluorogenic construct in super-resolution microscopy of antibody conjugates is shown.**

New fluorescence imaging probes are of high interest for bioimaging at the subcellular level. For the control of fluorescence within biological or artificial systems in time and space, many different photoactivatable fluorophores were developed in the last few years.<sup>1</sup> These caged fluorophores are weakly or non-fluorescent molecules. Irradiation with the appropriate wavelength releases the fluorescent dye. The use of light as an external trigger has the great advantage of being a non-invasive approach to control the time and area of the fluorescence if the wavelength is carefully chosen.

Photoactivatable fluorophores are also the key to single-molecule super-resolution techniques.<sup>2</sup> In these techniques, the resolution limit of light microscopy is bypassed by a temporally confined detection of single fluorophores, the determination of their position and the reconstruction of an image from single-molecule coordinates.<sup>3,4</sup> Organic fluorophores exhibit a high photon yield and typically show a better performance than fluorescent proteins; however, photoswitching often requires the addition of redox chemicals to the image buffer which are cytotoxic. In order to achieve the best results in single-molecule super-resolution imaging, it is thus desirable to develop caged

organic fluorophores which exhibit a high contrast ratio between the fluorescent and the quenched state.

There are different strategies for the development of caged organic fluorophores. A simple but very effective strategy is to attach a photolabile group to the fluorophore at a position that interrupts the conjugated  $\pi$ -system and suppresses fluorescence. Since the first reports in this field (using fluorescein derivatives),<sup>5</sup> the strategy was also applied to other chromophore classes like rhodamines,<sup>6</sup> acridinone,<sup>7</sup> rosamine,<sup>8</sup> coumarins,<sup>9</sup> carborhodamines<sup>10</sup> and boron-dipyrromethane (BODIPY).<sup>11</sup> Also, the efficiency and the properties of the photolabile groups were enhanced in different studies.<sup>12</sup> In recent publications the use of 2-diazoketone as a very small caging group for fluorophores was presented. Irradiation of these molecules releases nitrogen and causes a rearrangement reaction resulting in the recovery of fluorescence. This strategy was applied to a variety of different fluorophores and their application in super-resolution microscopy was shown.<sup>13</sup>

Other approaches that generate light activatable fluorophores without the use of caging groups trigger, for instance, the photoinitiated oxidation of a ZnSalen from a quenched thioether to a fluorescent sulfoxide.<sup>14</sup> Another example of a quenched cyanine dye used the photocleavage of a C–Br bond to restore the fluorescence. The fluorescence is quenched by bromides by the induction of intersystem crossing.<sup>15</sup> A completely different strategy uses photoactivated click chemistry of a tetrazole with different dipolarophiles to form fluorescent cycloadducts. Irradiation of tetrazole induces the elimination of nitrogen. The resulting highly reactive nitrile imine can form fluorescent products with appropriate alkenes.<sup>16</sup>

The release of nitrogen was also used in the design of photoactivatable push–pull fluorophores.<sup>17</sup> The electron donating amino group is masked as an azide moiety. Upon illumination, the aryl azide releases nitrogen and is transformed into an aryl amine, thus restoring fluorescence. The compatibility of these systems with super-resolution imaging was also demonstrated.<sup>18</sup>

An alternative way to trigger fluorescence with light is to attach a fluorophore and a quencher in close proximity. The photolabile

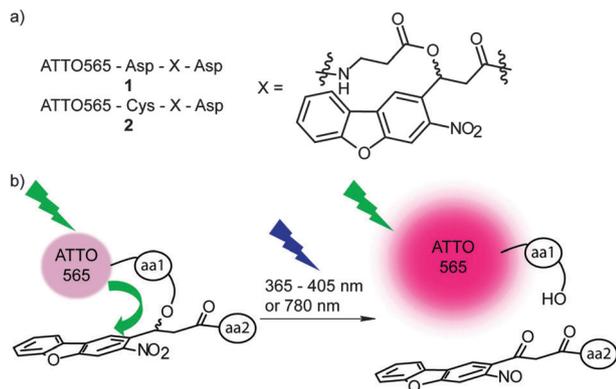
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† Electronic supplementary information (ESI) available: Synthetic procedures and characterization of the new compounds, one- and two-photon illumination procedures, widefield and super-resolution microscopy details. See DOI: 10.1039/c5cc05700k





**Fig. 1** (a) Small peptides **1** and **2** with the new linker in the middle and the fluorophore on the N-terminus were synthesized. (b) Photocleavable activation of ATTO565 can be performed with various wavelengths. The quenching effect is indicated with the green arrow.

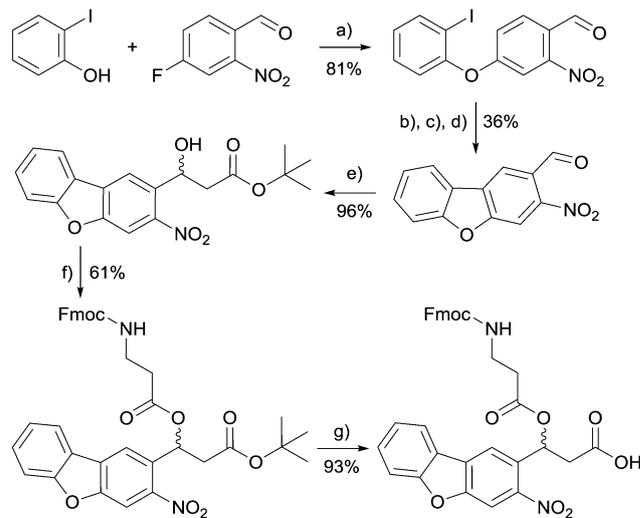
groups act either as a linker between the fluorophore and the quencher<sup>19</sup> or the cages force a structure in a special conformation. Upon irradiation the separation of the fluorophore and quencher takes place and fluorescence is restored. For instance, both of these techniques were used to follow mRNA distribution in cells with molecular beacons.<sup>20</sup>

In this work we present a new strategy where the photolabile group is not directly attached to the fluorophore. Our newly synthesized linker molecule combines the properties of a quencher and a photolabile group (Fig. 1). This linker is especially interesting for protein and peptide applications because it can be incorporated into standard peptide coupling chemistry. We prepared small peptides containing the new linker and investigated the photochemical properties of the new constructs using one-photon excitation (shown here for 365–568 nm) and two-photon excitation (shown here for 780 nm). To the best of our knowledge the new linker is the first example of a photolabile quencher.

Furthermore, the new construct offers the possibility to be used as a strand break that can be activated with IR light. Strand breaks are prominent tools to control biomolecules such as oligonucleotides<sup>21</sup> and peptides.<sup>22</sup> Future applications in which the linker could trigger self-assembly processes<sup>23</sup> or control the activity of enzymes and proteins with high spatial resolution<sup>24</sup> is possible.

We observed in a previous study that the nitrodibenzofuran (NDBF) caging group possesses the ability to quench the fluorescence of the rhodamine fluorophore ATTO565 presumably by contact quenching.<sup>25</sup> The NDBF group is known to be cleaved by UV light and also above 400 nm.<sup>26</sup> NDBF possesses a good two-photon absorption cross-section, which allows the triggering of the photoreaction with IR light.<sup>27</sup>

The first step of the synthesis was adapted from an already published procedure.<sup>28</sup> 4-Fluoro-2-nitro-benzaldehyde was coupled with 2-iodophenol in an Ullmann reaction. The resulting aldehyde was protected as an acetal and the dibenzofuran core was generated by palladium-catalysed cross-coupling. After acidic deprotection, the aldehyde was transferred to an alcohol by the



**Scheme 1** Synthesis scheme of the new linker molecule. (a) CuBr, K<sub>2</sub>CO<sub>3</sub>. (b) Ethyleneglycol, *p*-toluenesulfonic acid. (c) Cs<sub>2</sub>CO<sub>3</sub>, Pd(OAc)<sub>2</sub>. (d) HCl. (e) Riecke-Zn, *tert*-butyl chloroacetate. (f) Fmoc- $\beta$ -alanine, EDC, DMAP. (g) TFA. For detailed procedures and analytical data see the ESI.†

Reformatsky reaction and further coupled to Fmoc- $\beta$ -alanine. Final deprotection of carboxylic acid resulted in the new linker, which can then be used in standard Fmoc peptide coupling chemistry (Scheme 1).

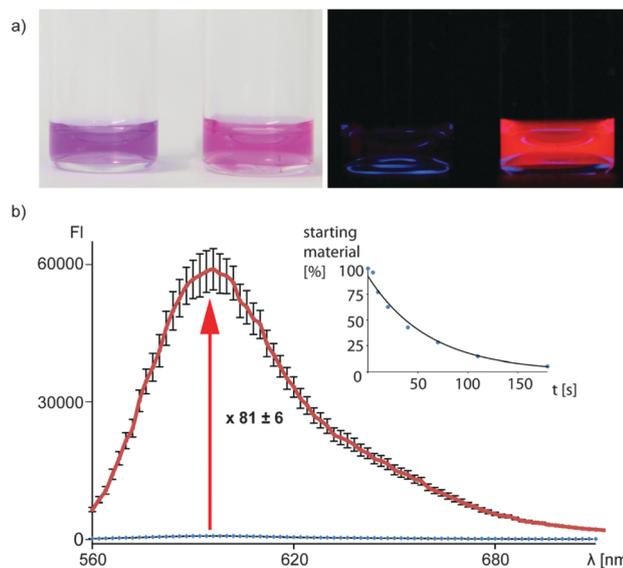
First, we checked if base-induced elimination is a problem in further applications of the new linker and confirmed that it is sufficiently stable in 20% (v/v) piperidin in DMF – a typical cleavage condition for Fmoc groups in the solid-phase peptide synthesis (ESI†).

To study our new linker it was embedded into two short peptides **1** and **2** (Fig. 1). Both contained aspartic acid residues for solubility purposes and an optional cysteine (peptide **2**) for maleimide coupling. The N-terminus was labelled with an ATTO565-*N*-hydroxysuccinimide (NHS) active ester. Peptide **1** was soluble in small volumes of aqueous Tris and HEPES buffered saline (HBS) buffers (pH 7.5) to at least 2 mM. We observed less than 5% degradation of the linker over 170 h in the dark and at room temperature in HBS buffer.

The uncaging quantum yield of peptide **1** was determined to be 0.13 at 365 nm using ferrioxalate actinometry.<sup>29</sup> A significant fluorescence increase of this compound was observed upon uncaging (81  $\pm$  6-fold increase in the absolute fluorescence, Fig. 2, 22-fold increase in fluorescence quantum yield, ESI†). This makes the new construct very interesting for bioimaging applications. The dye could also be photoactivated using a widefield microscope at 405 nm illumination and even at 568 nm (ESI†).

To prove that the NDBF group incorporated into the peptides is still two-photon active, molecule **2** was immobilized in a maleimide-containing hydrogel. The irradiation was performed using a laser-scanning microscope at 780 nm in a defined square region with increasing laser power (Fig. 3). Fig. 3b (middle) shows that indeed an excellent three-dimensional activation could be obtained by two-photon excitation. At high

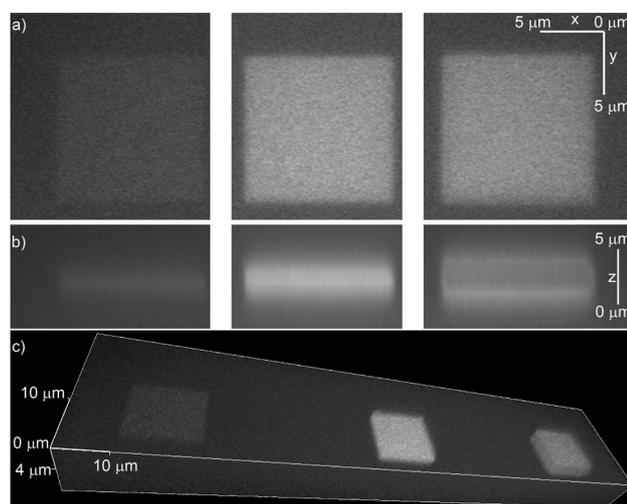




**Fig. 2** Fluorescence increases after irradiation of peptide **1** at 365 nm using a 250 mW LED. (a) Photographs of 44 nmol peptide in 200  $\mu$ L HBS buffer before and after irradiation for 3 min under white light (left picture) and using a TLC lamp at 366 nm (right picture). (b) Fluorescence spectra of the new construct before (blue line) and after UV illumination (red line). The inset shows the time course of the uncaging reaction determined by HPLC.

laser intensities bleaching of ATTO565 in the middle of the focal plane was observed (Fig. 3b, right).

At the correct settings two-photon irradiation offers the possibility to activate and bleach the fluorophore consecutively. Due to the activation with IR light, this procedure allows high tissue penetration depths with little phototoxicity.

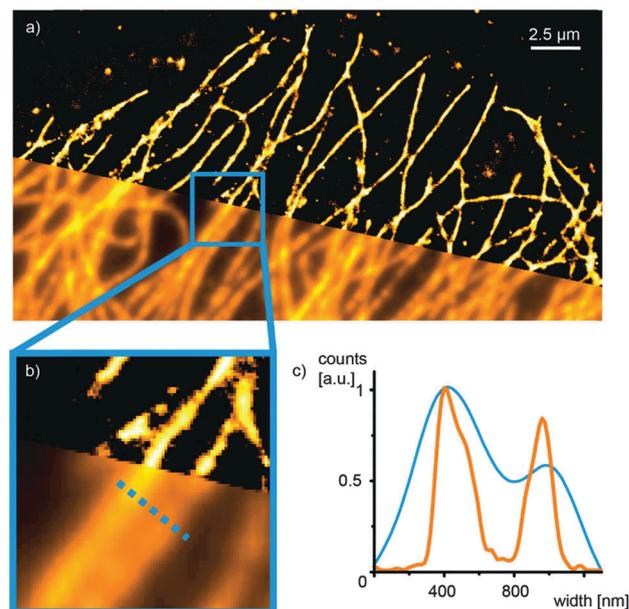


**Fig. 3** Two-photon activation of the fluorescence of **2** in a hydrogel. Panels (a)–(c) show different projections of the same sample. Irradiation was performed at 780 nm using a laser-scanning microscope. The fluorescence was recorded continuously at 561 nm. The left square was irradiated with 6 mW, the middle with 14 mW, and the right ones with 20 mW, each time 20 scans. The fluorescence increases (left squares to the middle ones) until bleaching starts (right squares).

To make the new construct a valuable marker for biomolecules, peptide **2** was reacted with 6-maleimido-hexanoic acid *N*-hydroxy-succinimide ester, a bifunctional linker. The resulting NHS ester was coupled to an antibody and used for staining of microtubules of U2OS cells *via* immunofluorescence. These filamentary structures are an ideal target to evaluate the suitability of a fluorophore for super-resolution microscopy following the concept of single-molecule localization.<sup>3</sup>

We demonstrated one-photon activation of the antibody conjugate with a short 405 nm laser pulse and continuous illumination at 568 nm (ESI,† Fig. S5). Furthermore, we illuminated the sample at 568 nm only and found a consecutive activation and bleaching or blinking of single peptide **2** molecule at appropriate laser intensities (ESI,† Fig. S6). We used standard localization algorithms to fit a 2D Gaussian function to the diffraction-limited intensity profile of each single emitter in order to determine their center of mass. Reconstruction of these localizations in a 2D histogram yielded an image with a significantly improved spatial resolution (Fig. 4) and an experimental localization uncertainty of 20.4 nm determined by nearest neighbor analysis.<sup>30</sup> Notably, we thereby demonstrate single-molecule super-resolution imaging with synthetic fluorophores without the need for chemical additives in the imaging buffer.<sup>4</sup>

In summary, we developed the first photolabile quencher for ATTO565 with a light/dark contrast of 81 : 1. It can be incorporated into peptides and acts both as a quencher and a strand break. The easy way to the functionalization of the linker with



**Fig. 4** Conventional and super-resolved images of the microtubulin network of an U2OS cell stained *via* immunofluorescence with the photoactivatable peptide **2** antibody conjugate. (a) Reconstructed super-resolved image (upper right) and diffraction-limited widefield (lower left) images. (b) Magnified view of the boxed region highlighted in (a). (c) Cross-sectional intensity profile through neighboring microtubulin filaments marked in (b) (blue dotted line). The intensity profile demonstrates the resolution improvement from the widefield (blue line) to the super-resolved image (orange line).



other molecules like hydrophilic amino acids results in a good solubility in aqueous buffers (pH 7.5). The linker shows very little degradation under these conditions. Irradiation can be performed with UV light, also above 400 nm, which is more favourable for living cells. Additionally, the construct is two-photon accessible that allows very high spatio-temporal resolution and high tissue penetration depths in the possible applications. The linker provides a new tool in the field of bioimaging. It reduces the number of modifications needed to generate a photoactivatable fluorophore quencher system from three (fluorophore, quencher and photolabile groups) to two (fluorophore and new linker molecule). In addition, the high contrast between the fluorescent and the dark state makes this probe ideally suited for single-molecule super-resolution microscopy. In the future, we will investigate the mechanism of the quenching effect and develop an application of the new linker to other fluorophores.

FF and AH gratefully acknowledge the Konrad-Adenauer-Stiftung for a fellowship for FF. The authors thank Erin Schuman for generous access to the microscopes at the MPI for brain research. We are grateful to Sebastian Malkusch for providing the LAMA software. This work was funded by the Deutsche Forschungsgemeinschaft (EXC 115).

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