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Article
Modulation of Dynamic Dimerization in Fluorogenic SNAP-tag Probes for Long-term Super-resolution Imaging

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Abstract
The combination of super-resolution microscopy and synthetic fluorescence probes have emerged as a universal tool to monitor dynamic biological events at the nanometer scale. However, the limited site-specificity and fluorogenicity of synthetic fluorescent probes make it still difficult to realize long-term super-resolution imaging. Herein, we introduce a dynamic dimerization mediated SNAP-tag fluorogenic probe, **BGAN-Aze**, which can specifically bind to various SNAP-tag fusion proteins with 41-fold fluorescence enhancement. The equilibrium between the non-fluorescent dimer (D) and the fluorescent monomer (M) of **BGAN-Aze** acts as an effective method to reduce the fluorescence background and
endow **BGAN-Aze** with the capability of conducting washing free super-resolution imaging of various intracellular and extracellular proteins. Using this probe, we monitored multiple dynamic biological events, such as MMC, mitophagy, the fusion of nucleolus, and the growth and contact of filopodia. We expect that **BGAN-Aze** will become a widely used SNAP-tag for super-resolution imaging of dynamic biological events and the D-M equilibrium can be a general strategy for designing fluorogenic probes.

**Introduction**

Live cells are highly complex machines driven by a series of dynamic biological events. Our deep understanding of their inner mechanism critically depends on the visualization of dynamic cellular contents with high spatial and temporal resolution.\(^1\) In recent years, the combination of fluorescence microscopy and synthetic fluorescence probes has successfully evolved as a universal tool to investigate these biological events in real-time or over a period of time.\(^2,3\) Especially, the emerging super-resolution imaging has surpassed the optical diffraction limit and allowed the imaging of cellular contents at the nanometer scale.\(^4-6\) However, a significant challenge in long-term super-resolution imaging of dynamic biological events still exists, as synthetic fluorescence probes always display limited site-specific labeling, cell permeability, and fluorogenicity in complex cellular environments.\(^7-9\)

To improve the specificity of synthetic probes, self-labeling protein tags were proposed. These tags can be flexibly combined with various synthetic probes through specific enzymatic reactions, thus effectively improving the optical properties of traditional organic fluorophores.\(^10-12\) Nowadays, various protein fusion tags have been developed and widely used in labeling proteins of interest (POI), including HaloTag\(^13\), PYP-tag\(^14, 15\), SNAP-tag\(^16\), TMP-tag\(^17-19\), and BL-tag\(^20\). One of the most prominent is SNAP-tag, an engineered variant of the human repair protein \(O^6\)-alkylguanine-DNA alkyltransferase (hAGT), which can specifically react with a broad variety of \(O^6\)-benzylguanine (BG) derivatives through the formation of a stable thioether bond.\(^16, 21\) Its diverse applications have been demonstrated in efficient and precise editing
of endogenous transcripts\textsuperscript{22}, super-resolution fluorescence imaging\textsuperscript{23} and biomolecular tracking inside living cells\textsuperscript{24}, therefore generating extensive development of synthetic SNAP-tag probes.

However, most synthetic SNAP-tag probes exhibit high background and low cell permeability, which limit their applications in the study of dynamics biological events\textsuperscript{25}, especially in long-term super-resolution imaging. These false signals from the background seriously affect the accurate judgment of the occurrence of biological events. Therefore, it is important to design fluorogenic SNAP-tag probes which show great increase in fluorescence upon the binding of SNAP-tags\textsuperscript{26}. So far, several fluorescence activation strategies have been applied in constructing fluorogenic SNAP-tag probes, such as introducing quenchers,\textsuperscript{27, 28} inhibiting molecular twisting,\textsuperscript{29, 30} conjugating environment-sensitive fluorophores\textsuperscript{31, 32, 33} and molecular switches. Furthermore, controlling the assembly of synthetic probes evolves as an optimized way of tuning optical properties of dyes and enabling new design strategies for fluorescence tools including fluorogenic SNAP-tag probes.\textsuperscript{34, 35} Rhodamine dyes have nowadays become the preferred fluorophore for designing fluorogenic SNAP-tag probes for super-resolution imaging,\textsuperscript{36, 37} due to the equilibrium between a non-fluorescent spirocycle (L) form and a fluorescent zwitterion (Z) form.\textsuperscript{38-40} The key factor for their fluorogenicity is that the aggregation of unreacted or unspecific binding of rhodamines to hydrophobic structures will stay in the non-fluorescent L-form, thereby reducing the background noise.\textsuperscript{41} However, the L-Z equilibrium of rhodamine is severely affected by the charged microenvironment of POI and the linker length between rhodamine and BG group, since only negatively charged proteins cause the L-to-Z transition of rhodamines through electrostatic attraction.\textsuperscript{42} Moreover, the benzylguanine (BG) and the positive charge of rhodamine make it difficult to penetrate cell membranes and image intracellular contents at the nanometer scale. Therefore, there is an urgent need for fluorogenic SNAP-tag probes with universal labeling ability for different types of target proteins.

In this paper, we introduce a dynamic dimerization equilibrium, which triggers fluorescence “ON” and “OFF”, to design a fluorogenic SNAP-tag probe \textbf{BGAN-Aze} used for wash-free long-term super-resolution imaging of dynamic biological events (Fig. 1). The three components in \textbf{BGAN-Aze},
Fig. 1 The proposed D-M equilibrium of fluorogenic SNAP-tag probe BGAN-Aze.

azetidine substituted naphthalimide, benzene ring, and guanine moieties, are highly flat which induces
BGAN-Aze to form a stable and quenched dimer in aqueous solution. The equilibrium between non-
fluorescent dimer (D) and fluorescent monomer (M), which always remain neutral, can enhance
fluorogenicity and cell permeability simultaneously. Once BGAN-Aze reacts with SNAP-tag, its
fluorescence will be activated and enhanced by 41-fold with the dissociation of the dimer. Therefore,
BGAN-Aze was successfully applied to long-term super-resolution imaging of various dynamic
organelles and subcellular organelles, including mitochondria, mitochondrial cristae, nucleolus, and
filopodia. We further monitored the process of nucleolar fusion and its duration via super-resolution
imaging for the first time. And the growth curve of filopodia was also successfully tracked in situ at the
nanometer scale.

Results and Discussion

Existence of Dimer and Quenching Effect

As the core regulatory trigger, D-M equilibrium was firstly demonstrated through HRMS,
computational analysis, fluorescence, and UV-vis absorption spectroscopy. Firstly, we found an obvious
dimer ion peak at 1011.3828 ([2M+H]^+, calculated 1011.3803, ESI as ionization source) in the HRMS
spectrum besides a monomer ion peak at 506.1958 ([M+H]^+, calculated 506.1941). These peaks prove the
existence and high stability of the dimer (Fig. 2a, S1). In the meanwhile, an UV-vis absorption peak of the
dimer at 525 nm was observed, in contrast to the monomer peak at 450 nm (Fig. S2). The redshift in the dimer was consistent with our TD-DFT calculations, which shows that the dimer of BGAN-Aze demonstrated a noticeable redshift in the UV-vis absorption spectrum, in comparison to that of the monomers (Fig. S3).

These results inspired us to simulate various aggregation conformations between two monomers of BGAN-Aze and explore its aggregation pattern, using molecular dynamics (MD) (Fig. 2b, S4-6). The most stable dimer conformation from the MD calculations was further optimized and analyzed with reference to a monomer, using density functional theory. Our results show that both the azetidine substituted naphthalimide (Fig. S7, crystal of a reference compound AN-Aze) and guanine moieties in BGAN-Aze are highly flat. Such a highly flat π-conjugated network affords a strong tendency for aggregation. Consequently, two monomers of BGAN-Aze could form a “folded” dimer via van der Waal (vdW) interactions (Fig. 1). The calculated intermolecular interaction energy (∆E) amounts to -2.09 eV, indicating a substantial aggregation tendency. On the other side, we found that BGAN-Aze showed obvious red-shifted absorption and emission compared with respect to the reference compound AN-Aze (BGAN-Aze: λ\text{abs} = 470 nm, λ\text{em} = 571 nm; AN-Aze: λ\text{abs} = 465 nm, λ\text{em} = 555 nm) (Fig. S8, Table S1).

Similarly, with the concentration of BGAN-Aze increasing in aqueous solution, the maximum absorption and emission wavelengths shifted from 556 nm (0.1 μM) to 572 nm (20.0 μM), which suggested that BGAN-Aze was apt to form J-aggregates (Fig. 2c). This also could be confirmed by the normalized fluorescence spectra: as the excitation wavelength (λ\text{ex}) shifts from 400 nm to 500 nm, the peak emission wavelength (λ\text{em}) also progressively shift from 545 nm to 570 nm (Fig. 2d, S9). A quite subtle excitation shoulder, which appeared at 490 nm, also became stronger when monitored at a longer emission wavelength (Fig. S10). These exceptional spectral profiles again demonstrated the J-aggregation of BGAN-Aze.
Fig. 2 Characterization and quenching effect of dimer. (a) HRMS spectra of BGAN-Aze in high purity water. (b) Plots of the reduced density gradient $s$ versus the electron density multiplied by the sign of the second Hessian eigenvalue $\text{sign}(\lambda_2)\rho$. $\text{sign}(\lambda_2)\rho > 0$ denotes strong repulsion, $\text{sign}(\lambda_2)\rho < 0$ denotes strong attraction, and $\text{sign}(\lambda_2)\rho \approx 0$ denotes vdW interactions. (c) Fluorescence spectra of BGAN-Aze at various concentrations from 0.1 μM to 20 μM. (d) Fluorescence spectra of BGAN-Aze in PBS (20 mM, pH = 7.4), excited at various wavelengths from 400 to 500 nm (with a step size of 10 nm). (e) SEM (Scanning Electron Microscopy) images aggregates of BGAN-Aze. Scale bar: 200 nm. (f) SIM imaging of aggregates of BGAN-Aze which were standing for 48 h in PBS (pH = 7.4, 20 mM). Scale bar: 5 μm. (g) Fluorescence spectra of BGAN-Aze in various solvents. [BGAN-Aze] = 10 μM. (h) Fluorescence spectra of 5 μM BGAN-Aze in the absence and presence of 10 mM SDS in PBS (20 mM, pH = 7.4). (i) Absorption spectra of 5 μM BGAN-Aze with different concentration of SDS (0.8 - 2.0 mM) in PBS (20 mM, pH = 7.4). Inset: plot of the fluorescence intensity at 539 nm as a function of the concentration of SDS (0 - 2.0 mM).

We also analyzed the nature of such strong intermolecular interactions, using the electron density and reduced density gradient (RDG) analysis developed by Yang group\textsuperscript{43}, whereby a small value of $\text{sign}(\lambda_2)\rho$...
close to 0 indicates vdW interactions. Indeed, our calculations reveal a low-density and low-gradient spike for the dispersion-bounded dimer, as highlighted in a green circle (Fig. 2b). These results implied that vdW interactions are the main driving force for molecular aggregation. These vdW interactions could further promote the dimer of **BGAN-Aze** to form nanoparticles, as confirmed by the DLS experiments (Fig. S11). After standing for 48 h in PBS, yellow aggregates were formed and directly prove the larger aggregates of **BGAN-Aze** (Fig. S12). SEM images also showed that **BGAN-Aze** aggregated to ~50 nm nanoparticles (Fig. 2e) which could also be seen as weak fluorescent dots through super-resolution imaging (Fig. 2f).

To validate the quenching effect of the dimer, we investigated the absorption and fluorescence spectra of **BGAN-Aze** and **AN-Aze** in different solvents (Fig. 2g and S8). Due to the sensitivity of naphthalimide to the microenvironment, their absorption wavelength and fluorescence wavelength all exhibited redshifts as the solvent polarity increased (Table S1). However, several differences between **BGAN-Aze** and **AN-Aze** are of note. The fluorescent brightness and molar extinction coefficient of **BGAN-Aze** decreased sharply in PBS while **AN-Aze** kept moderate. Consequently, the quantum yield of **BGAN-Aze** was below 0.016 which was much lower than that of **AN-Aze** (0.20). It was indicated that the guanine group indeed made **BGAN-Aze** form dimer in PBS and the photoinduced electron transfer from guanine to naphthalimide quenched the fluorescence.

**Fluorogenicity of binding to SNAP-tag**

The quenched dimer of **BGAN-Aze** provided a novel strategy for designing fluorogenic probes toward SNAP-tags and encouraged us to investigate whether the dimer could be disassembled and thereby recover high fluorescence intensity simultaneously. With the addition of surfactant SDS, the fluorescence of **BGAN-Aze** (5.0 μm in PBS, pH = 7.4) was greatly enhanced by more than 130 folds (Fig. 2h, S13, Table S2). On the contrary, the fluorescence intensity of **AN-Aze** only increased by less than 3 folds at various concentrations of SDS from 0.1 μM to 20.0 μM (Fig. S14). Surprisingly, we found the fluorescence enhancement mainly originates from the disassembly of dimer absorbing at 525 nm (Fig. 2i,
S15, 16). Upon the addition of SDS (0 - 0.8 mM), the nanoparticles would first disassemble with a significant decrease at the absorption wavelength between 450-510 nm but negligible fluorescence enhancement (Fig. S15). Subsequently, the dimer would gradually split into monomer as more SDS was added (0.8 - 5 mM). Importantly, with the continuous decrease of absorption wavelength at 525 nm the fluorescence was enhanced greatly by 86.9 folds from 0.8 to 2.0 mM of SDS (Fig. 2i). These results indicated that turn-on fluorescence response was driven by the disassembly of the dimer, and thus ensured its fluorogenicity to SNAP-tag.

We then used BGAN-Aze to label SNAP-tag in vitro and found the fluorescence intensity enhanced 41 folds after BGAN-Aze reacted with SNAP-tag which indicated excellent fluorogenicity for SNAP-tag (Fig. 3a, S17). DLS measurements further validated that BGAN-Aze (5.0 μM) aggregated into nanoparticles with a diameter around 600 nm in PBS (20 mM, pH = 7.4). These nanoparticles were indeed disassembled by SNAP-tag and the size decreased to ~4 nm after the conjugation to SNAP-tag (Fig. 3b). The quantum yield of BGAN-Aze increased from 0.016 to 0.39 once binding to SNAP-tag (Table 1 and Fig. 3a). It was worth noting that the emission wavelength of BGAN-Aze shifted to 540 nm from 571 nm after it binds to SNAP-tag (emission at 540 nm in MeOH). Since naphthalimide was a polarity-sensitive fluorophore (its emission experiences blueshifts with the decrease of polarity), we could speculate that the environment at the binding pocket of the SNAP-tag was highly polar. The protein labeling process was next monitored by recording the fluorescence intensity of BGAN-Aze at 535 nm to study the kinetics of reaction rate, in which the total concentration of BGAN-Aze and SNAP-tag was 5 μM. And the time required for 50% labeling of SNAP-tag was about 56 s for BGAN-Aze (Fig. 3c). The further kinetic analysis showed the second-order rate constant (K₂) of reaction between BGAN-Aze and SNAP-tag was 1998 ± 275 M⁻¹s⁻¹ (Fig. S18). It was indicated that the dimer of BGAN-Aze could be effectively disassembled by SNAP-tag along with great fluorescence enhancement.
Fig. 3. The response of BGAN-Aze to SNAP-tag. (a) Fluorescence spectra of 5 μM BGAN-Aze in the absence and presence of 5 μM SNAP-tag. (b) DLS analysis of 5 μM BGAN-Aze in the absence and presence of 5 μM SNAP-tag in PBS. (c) Time course of fluorescence intensity of 5 μM BGAN-Aze in the presence of equal purified SNAP-tag. (d)-(f) No-wash HeLa live-cell imaging of SNAP-tag proteins labeled with BGAN-Aze. HeLa cells transiently expressing SNAP-Vector, SNAP-H2B, and SNAP-Cox8A were incubated with 0.5 μM BGAN-Aze for 30 min. Graph: intensity profile of regions of interest (ROI) across cells.

No-washing long-term super-resolution imaging in living cells using BGAN-Aze.
Inspired by the above results, we next attempted to carry out wash-free fluorescence imaging of various intracellular proteins based on **BGAN-Aze**. Firstly, living HeLa cells, expressed SNAP-vector, were incubated with **BGAN-Aze** for 30 min and directly imaged by confocal microscopy without washing step. Obvious green fluorescence was captured in the cytoplasm with good SNR (13.0 folds) which indicated high cell permeability of **BGAN-Aze** that is available for intracellular proteins labeling (Fig. 3d). On the contrary, non-transfected cells displayed negligible fluorescence. To further inspect the location accuracy of **BGAN-Aze** to SNAP-tag, commercialized plasmids pSNAPf-H2B, pSNAPf-Cox8A, pSNAPf-NPM1 and pSNAPf-ADRβ2 fusing SNAP-tag to human histone H2B, cytochrome c oxidase subunit 8 (Cox8A), nucleophosmin and β-2 adrenergic receptor, respectively, were transiently transfected to HeLa cells. It's satisfactory to observe strong fluorescence appearing in specific locations with a high signal-to-background ratio. Co-staining of **BGAN-Aze** and Hoechst 33342 was next performed which demonstrated that **BGAN-Aze** could light nuclear-localized SNAP-H2B with an excellent nuclei-to-cytosol signal ratio (~22.5 folds, Fig. 3e). The intensity profiles of the linear regions across transfected cells displayed in close synchrony and further indicated colocalization between **BGAN-Aze** and Hoechst 33342. Moreover, the labeling of SNAP-Cox8A and SNAP-ADRβ2 fusion proteins with **BGAN-Aze** revealed mitochondrial and membranal outlines for its excellent fluorogenicity and location accuracy (Fig. 3f, S19). These results suggested that **BGAN-Aze** is highly suited for the visualization of various proteins at a specific location without a washing procedure.

The excellent performance of our probe in imaging different types of target proteins prompted us to investigate its application in long-term super-resolution imaging of different organelles and subcellular organelles. Mitochondria are highly dynamic organelles. They are highly sensitive to phototoxicity, as reflected by a significant change in membrane potential. This makes many mitochondrial probes incompatible in long-term super-resolution imaging. Because these probes are lipophilic cations and target mitochondria depend on the negative mitochondrial membrane potential. Once the mitochondrial membrane potential is lost, a mass of probes will dissociate into the cytoplasm and induced high background fluorescence. Therefore, we intended to inspect the performance of our probe, which could
label mitochondria inner membrane through a stable thioether bond between probes and SNAP-Cox8A fusion proteins without any lipophilic cations, in long-term SIM imaging and explore mitochondrial dynamics.

Mitochondrial cristae, formed by the inner membrane of mitochondria, are highly dynamic sub-organelle structures that are still challenging for super-resolution imaging. As they house the megadalton complexes of the electron transport chain and ATP synthase, their morphologies are recognized as an evaluation criterion for mitochondrial health. As shown in Fig. 4, due to its specific location in the inner membrane, **BGAN-Aze** could clearly image mitochondrial cristae via SIM (Fig. 4, A1-6). Most of the cristae were perpendicular to the mitochondrial macro axis and formed sheet-like mitochondrial cristae. However, there existed a large empty space, labeled by white arrows in A1-6, at the junctions of mitochondria. To monitor the dynamic network of mitochondrial cristae, we tracked single branching mitochondrion under super-resolution imaging for 10 mins. Fig.4 b shows the tanglesome mitochondrial cristae at 40 s gradually changed into lamellar ones during 40 – 120 s. It was worth noting that the three sheet-like mitochondria in dotted line frame arranged parallelly and kept close or far away from each other in real-time. While, some of them, labeled by pink arrows, fractured into smaller sheets at 200 s. As a consequence, the network of mitochondrial cristae was highly dynamic and could be imaged by our probes at the nanometer scale. Furthermore, the instantaneous contact between two isolated mitochondria was also observed in Fig. 4c. Mitochondria, labeled by a blue arrow, turned into rotund ones and stretched vimineous “tentacle” like branch towards right mitochondria at 20 s, simultaneously. Intimate contact was revealed in a blue dotted line frame during 40 s-60 s. The two mitochondria soon separated at 60 s and returned to the initial state. These results inspired us to further chase the change of mitochondrial cristae during mitochondria-mitochondria interaction. Fortunately, we monitored the fusion of two mitochondria during 100-240 s (Fig. 4d). At 100 s, the two mitochondria went close to each other and showed intimate contact. As the event went on, the left mitochondrion displayed a clear sheet and gradually turned into a rotund one from a clubbed one, along with the disappearance of many
Fig. 4 Super-resolution imaging of mitochondria using BGAN-Aze. (a)-(h) SIM imaging of mitochondria and mitochondrial cristae based on SNAP-tag-Cox8A fusion protein labeled with BGAN-Aze. (i) Intensity profile of ROI across mitochondria and lysosome in (f). (j) Intensity profile of ROI across mitochondria and lysosome in (h) at different time points. (k) Time dependence of the ratios of fluorescence intensities $I_{mito}$ and $I_{lyso}$. 
mitochondrial cristae. However, after complete fusion at 240 s, the newly formed mitochondrion again showed distinct lamellar mitochondrial cristae. Although mitochondria are considered semi-autonomous organelles, they also engaged in many physiological processes via dynamic interactions with other subcellular organelles, including fission, mitophagy, and apoptosis. As mitophagy act a crucial role in cellular stress caused by aberrant oxidative bursts in which damaged mitochondria are removed by autolysosomes, we next carried out long-term dual-color SIM imaging to reveal the dynamic interactions between mitochondria and lysosomes (Fig. 4e). The mitochondria were stained with our probe and lysosomes were labeled with Lyso-Tracker Red respectively. As shown in Fig. 4f, the lysosome, lined out by a green arrow, was stranded in the center of the annular mitochondrion and displayed intimate contact with each other. While at 20 s the annular mitochondrion was gradually cut off at the contact site and turned into a linear one. The intensity profiles of the linear regions in Fig. 4i further indicated the left section of the annular mitochondrion gradually disappeared with the decrease of fluorescence intensity. Moreover, some prolonged contacts between lysosomes and mitochondria had been captured. The lysosome in Fig. 4g moved around a linear mitochondrion (marked by a yellow arrow) and showed frequent contact during 1-100 s. While another lysosome remained motionless at an unchanged contact site. Due to the stable covalent labeling of BGAN-Aze to mitochondria, mitophagy was monitored in which damaged mitochondria would be phagocytosed by acidic lysosomes. It was shown in Fig. 4h that the lysosome kept close contact with the joint of mitochondrion during 1-300 s. But we could observe the red lysosome change to light grey step by step. The intensity profiles across the lysosome and mitochondrion in Fig. 4j which show separated signals at 1 s increasingly exhibited co-localization and indicated partial mitochondrion fused with this lysosome and the occurrence of mitophagy. We also calculated the ratio of $I_{mito}$ to $I_{lyso}$ (Fig. 4k) and inspected the whole process of mitophagy which indicated the process lasted about 120 s. These results demonstrated the ability of BGAN-Aze in long-term super-resolution imaging of mitochondrial dynamics for its excellent specificity, stability, and fluorogenicity.
As the largest membrane-less organelle, nucleolar fission and fusion act crucial roles in liquid-liquid phase separation (LLPS). However, it is still difficult to imagine the whole process of fusion at the nanometer scale because of its long duration.\textsuperscript{44, 45} Inspired by the successful application in long-term super-resolution imaging, we monitored the overall process of nucleolar fusion at the nanometer scale for the first time and found that the process lasted ~20 min (Fig. 5, S20). As shown in Fig. 5a, the two nucleoli appeared completely separated at 0 min and formed a connection bridge at 3 min which gradually became bulky (Fig. 5b). Furthermore, relative fluorescence intensity was adopted to further illustrate the formation of the connection bridge (Fig. S21). The intensity between the initial two nucleoli continuously increased until almost equal to the intensity of the two nucleoli which indicated the formation of a stable connection bridge (Fig. 5c). The increase of fluorescence intensity at the distance of 800 nm was analyzed in Fig. 5d and indicated that the process lasted about 20 min. We also observed the behavior of H2B, a major protein in chromatin, at the nanometer scale (Fig. S22).

**Fig. 5** Super-resolution imaging of nucleolar fusion using BGAN-Aze. (a) SIM imaging of nucleolus based on SNAP-tag-NPM1 fusion protein labeled with BGAN-Aze. (b) Schematic diagram for the nucleolar fusion and connection bridge formation. (c) Relative intensity profile of connection bridge at different time points. (d) Time dependence of the maximum fluorescence intensities in (c).
Besides intracellular proteins, we also tracked membranal dynamics through SNAP-ADRβ2 in which SNAP-tag was exposed to the extracellular side of the membrane (Fig. 6). As plasma-membrane protrusions, filopodia primarily work as antennae to probe the surrounding environment which is involved in many biological events such as cell migration, wound healing, and neurite outgrowth. However, filopodia are highly dynamic with a nanometer size, and their growth rate is still unclear. Fortunately, using **BGAN-Aze** we observed the whole growth process of several filopodia and calculated their growth rate (Fig. 6a-b). Initially, two bulges arose and were subtle which were marked by blue and yellow circles at 0 min. The bulge, labeled by a blue arrow, firstly stretched out the filiform bubble and extended 0.95 μm after 1 min. Differently, the bulge, which was labeled by a yellow arrow, underwent a long-term growth process during 5-24 min. Through the time-dependent growth curve, we could find that the growth rate was dynamically changing and appeared slow in the initial stage (Fig. 6c). Moreover, another growth model was captured. Bulge marked by a pink arrow was formed at 11 min and fused with lateral mature filopodia during 17-23 min. Then, the newly syncretic filopodia continued to extend 0.62 μm during 23-24.5 min. Besides the formation of fresh filopodia through original protrusions and fusion, filopodia could also be newly formed by the fission of mature filopodia (Fig. 6d). The intact filopodia, labeled by white arrows, showed obvious protrusions at 9.5 min which split into two filopodia during 9.5-19.5 min and indicated the fission of mature filopodia. In addition, dynamic contacts between different filopodia were captured: the filopodia which displayed linear at 0.5 min changed into dendritic one and showed fickle morphology. It also contacted the adjacent filopodia during 9.5-23.5 min and dissociated at 24 min. Unlike the prolonged contact, as shown above, it again contacted the same filopodia at 25.5 min and dissociated at 27 min with less than 1.5 min contact.
In conclusion, through the D-M equilibrium of BGAN-Aze, we developed a fluorogenic SNAP-tag probe and realized long-term super-resolution imaging of universal intracellular and extracellular proteins. BGAN-Aze forms quenched dimers with minimal background fluorescence. Upon binding to the SNAP-tag, the fluorescence of BGAN-Aze increased by 41-fold, as a result of the dissociation of the dimer. Finally, BGAN-Aze was successfully applied to long-term super-resolution imaging of dynamic mitochondrial cristae, nucleolus, and filopodia, and revealed various dynamic biological events, including MMC (mitochondria-mitochondria contact), mitophagy, the fusion of nucleolus, growth, and contact of filopodia. We believe the D-M equilibrium as shown in BGAN-Aze will become a promising new strategy for developing fluorogenic probes.
**Materials and instrument**

Unless otherwise specifically stated, all reagents were purchased from commercial suppliers (Sigma-Aldrich, J & K, Innochem) and used without further purification. Solvents [dimethyl sulfoxide (DMSO), methanol] were purchased from J&K and used without further treatment or distillation. Silica gel (200-300 mesh) were purchased from Innochem. pSNAPf-Cox8A, pSNAPf-Vector and pSNAPf-ADRβ2 were purchased from New England Biolabs (NEB). SNAP-H2B was a gift from Prof. Yi Xiao, Dalian University of Technology. SNAP-NPM1 was a gift from Prof. Yang Wang, Dalian Medical University.

All $^1$H-NMR and $^{13}$C-NMR spectra were recorded on a Bruker 400 spectrometer with TMS as an internal standard. Chemical shifts were given in ppm and coupling constants ($J$) in Hz. High resolution mass spectrometry data were obtained with a HP1100LC/MSD mass spectrometer and a LC/Q-TOF MS spectrometer. UV-vis absorption spectra were collected on an Agilent Cary 60 UV-Vis Spectrophotometer. The blank correction was performed using the respective pure solvent as a reference. Fluorescence measurements were performed on an Agilent CARY Eclipse fluorescence spectrophotometer.

**Cell culture and transfection**

HeLa (helacyton gartleri) cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, BI) which were cultured in a humidified atmosphere of 5% CO2/95% air at 37 °C. Before the imaging experiments, HeLa cells were seeded on glass bottom cell culture dish (Nest, polystyrene, Φ 15 mm) for 1 – 2 days to reach 40–90% confluency. The cells were then used for further experiments.

Transfection experiment was performed according Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Briefly, 2 μL Lipofectamine 2000 (Invitrogen) and appropriate plasmid were firstly diluted in 20 μL DMEM (dulbecco's modified eagle medium), respectively. 5 min later, the diluted plasmid in 20 μL
DMEM was added to the diluted Lipofectamine 2000 (Invitrogen) with homogeneous mixing. Another 10 min later, the mixture was added to the cell-culture dish in 1 mL DMEM. The final concentration of plasmid was controlled at 500-1000 ng/mL. After 4 h in 37 °C, the culture medium was changed from DMEM to DMEM with 10% FBS. The transfected cells were used for super-resolution and confocal imaging after 48 h.

### Confocal and SIM imaging

Confocal images were recorded with FV1000 (Olympus) with a100 × / NA 1.44 oil immersion objective lens. Super-resolution images were performed by Nikon N-STORM/SIM 5.0 Super-Resolution Microscope System with a motorized inverted microscope ECLIPSE Ti2-E, a 100 × / NA 1.49 oil immersion TIRF objective lens (CFI HP), LU-NV series laser unit (405 nm, 488 nm, 561 nm, 647 nm), and an ORCA-Flash 4.0 SCMOS camera (Hamamatsu Photonics K.K.).

Confocal imaging was firstly conducted to demonstrate the labelling specific and fluorogenic by Olympus FV1000. through Structured Illumination Microscopy (SIM; Nikon N-SIM Ti-2E). The cells, which were transfected with SNAP-tag fusion plasmid including pSNAPf-Cox8A, pSNAPf-Vector, pSNAPf-ADRβ2, SNAP-H2B, SNAP-NPM1, were incubated with BGAN-Aze respectively. Ex:405 nm, collected: 435-485 nm; Ex:488 nm, collected: 500-545 nm; Ex:561nm, collected: 570-640 nm. The nucleus was stained with Hoechst 33342. Mitochondria were stained with Mito Tracker Red.

### Culture of crystal

5 mg AN-Aze was firstly dissolved in 5 mL CHCl₃. Then HPLC MeOH was dropwise added to the solution until a slight turbidity appeared. Another 1 mL CHCl₃ was next added. The clear solution was further filtered through a 0.22 μm filter. The mixture in 10 mL vial was placed in darkroom with constant temperature of 20°C.

### Calculation details
The molecular aggregation effect was further investigated by molecular dynamics (MD) and DFT calculations. To determine a reliable dimer structure, 30 ps MD simulations were firstly carried out at 298 K in NVE ensemble with a time step of 1 fs using the DFTB+ 1.0.1 package. The SK-parameters of mio-1-1 for C, N, H, and O were employed. The total electronic energy evolution shows that the dimer reached thermal equilibrium within 30 ps of simulation time. After that, the most stable dimer was selected from MD simulations, and subjected to further geometry relaxation using M062X/6-31G(d,p) as implemented in Gaussian 16 program package. The solvent effect was taken into consideration using the SMD model with water as a solvent.

**Data Availability**

The source data underlying Fig. 2a,c,d,g-i, 3, 4i-k, 5c-d, 6c are recorded in a Source Data file. The total raw and processed imaging dataset can be accessed by emailing the corresponding author. The data generated in this study are provided in the supplementary information.

**References**


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Authors contributions
Z.X. lead the project. Z.X. and Q.Q. conceived and designed the project. W.L. synthesized the probe.

W.C. and X.L performed the calculations. Q.Q., W.L. and J.C. performed confocal and SIM imaging.

Q.Q., W.L., W.Z., N.X., J.L. performed fluorescence and UV experiments. Y.T., Y.Z., Y.C. and L.M. were responsible for cell culture and purification of SNAP-tag. Q.Q. and Z. X. wrote the manuscript.

Ethics declarations
Competing interests

The authors declare no competing interests.
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