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

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1 Experimental details

1.1 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.

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.).

1.2 Spectroscopic studies

BGAN-Aze and **AN-Aze** were firstly prepared as 2 mM stock solution in HPLC DMSO. Samples for spectroscopic analysis were prepared through diluting the stock solution in respective solvents. The concentration of samples was usually prepared as required.

In order to record UV-vis and fluorescence spectra of **BGAN-Aze** and **AN-Aze**

1.3 Cell culture and transfection

1.3.1 Cell culture

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% CO_2/95% 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.

1.3.2 Cell transfection

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

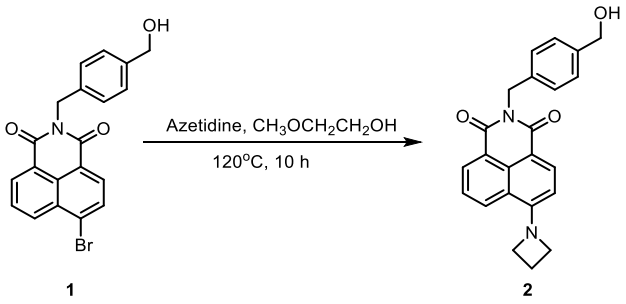
Briefly, 2 μL Lipofectamine 2000 (Invitrogen) and appropriate plasmid were firstly diluted in 20 μL DMEM (dulbecco's modified eagle medium), respectively. 5 min later, the diluted plasmid in 20 μL DMEM was added to the diluted Lipofectamine 2000 (Invitrogen) with homogeneous mixing. Another 10 min later, 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.

1.4 Imaging

Confocal imaging was firstly conducted to demonstrated 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.

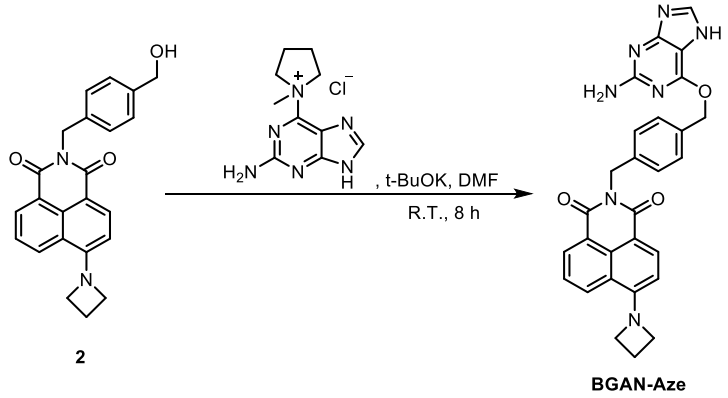
2 Synthesis

1 and **AN-Aze** were synthesized according literature.[1]



1 **2**

Compound **1** (500 mg, 1.26 mmol) in 5 mL 2-methoxyethanol was added azetidine (216 mg, 3.78 mmol) under N_2. Then, the mixture was slowly heated to 120°C and stirred at this temperature for 10 h. After cooling to room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (DCM:MeOH=50:1). The product was obtained as yellow solid 340 mg, yield 75%.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (d, J = 7.2 Hz, 1H), 8.32 (d, J = 8.4 Hz, 1H), 8.18 (d, J = 8.5 Hz, 1H), 7.56 (t, J = 7.8 Hz, 1H), 7.29 (d, J = 7.8 Hz, 2H), 7.23 (d, J = 7.8 Hz, 2H), 6.40 (d, J = 8.5 Hz, 1H), 5.19 (s, 2H), 5.12 (s, 1H), 4.58 – 4.25 (m, 6H), 2.46 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.16, 163.25, 152.74, 141.71, 136.74, 133.45, 131.45, 131.35, 130.43, 127.91, 126.89, 124.32, 121.94, 120.54, 108.34, 106.50, 63.13, 55.59, 42.78, 16.91. HRMS (ESI) m/z found 373.1542 $[M+H]^+$, calculated 373.1552 for $C_{23}H_{21}N_2O_3$.



2 **BGAN-Aze**

The solution of compound **2** (50 mg, 0.13 mmol), 1-(2-amino-9H-purin-6-yl)-1-methylpyrrolidinium (103 mg, 0.40 mmol), and *t*-BuOK (90 mg, 0.80 mmol) in 5 mL dry DMF was stirred at room temperature for 8 h. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (DCM:MeOH=15:1). **BGAN-Aze** was obtained as yellow

solid 28 mg, yield 41%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.82 – 11.88 (m, 1H), 8.47 (d, J = 7.2 Hz, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.26 (d, J = 8.5 Hz, 1H), 7.84 (s, 1H), 7.63 (s, 1H), 7.48 (d, J = 7.6 Hz, 2H), 7.40 (d, J = 7.8 Hz, 2H), 6.48 (d, J = 8.6 Hz, 1H), 6.32 (s, 2H), 5.47 (s, 2H), 5.27 (s, 2H), 4.53 (t, J = 7.3 Hz, 4H), 2.52 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.21, 163.29, 160.30, 160.08, 155.66, 152.87, 138.22, 135.94, 133.55, 131.53, 131.44, 130.51, 128.97, 128.09, 124.38, 121.96, 120.61, 113.97, 108.36, 106.57, 66.93, 55.63, 42.81, 16.92. HRMS (ESI) m/z found 506.1958 $[M+H]^+$, 1011.3828 $[2M+H]^+$, calculated 506.1941 for $C_{28}H_{24}N_7O_3$, 1011.3803 for $C_{56}H_{47}N_{14}O_6$.

3 Spectra

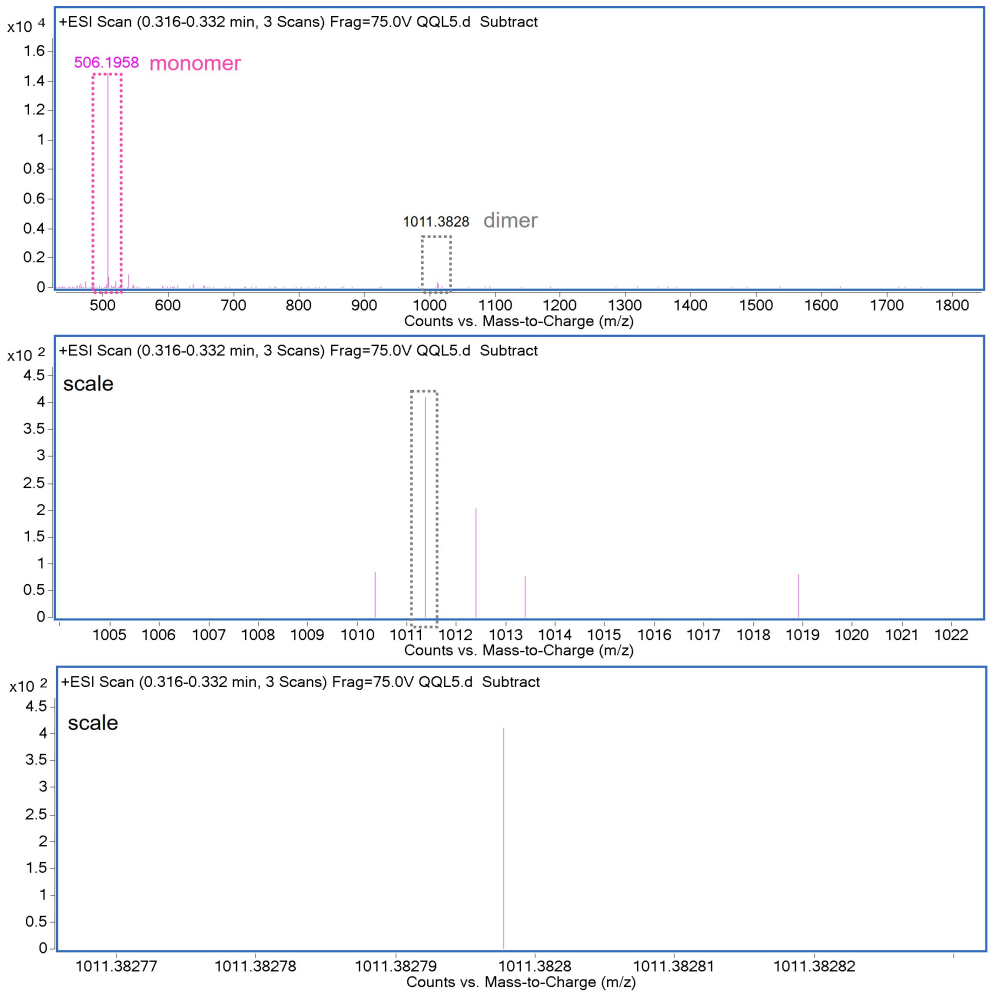


Figure S1 HRMS spectrum of **BGAN-Aze**.

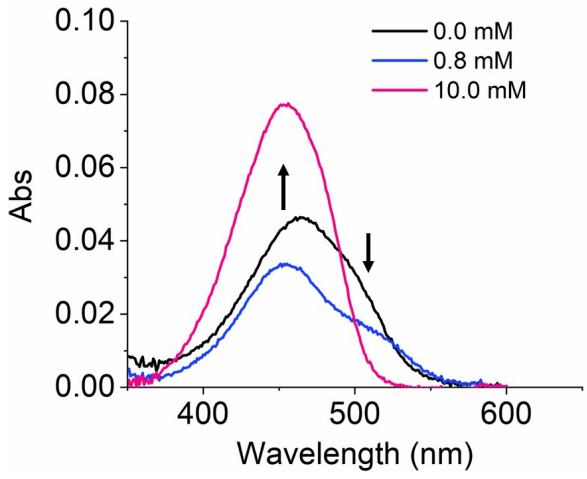


Figure S2 UV-vis spectra and (b) fluorescence spectra of 5 μM **BGAN-Aze** at different concentration of SDS. An absorption peak of dimer at 525 nm was observed.

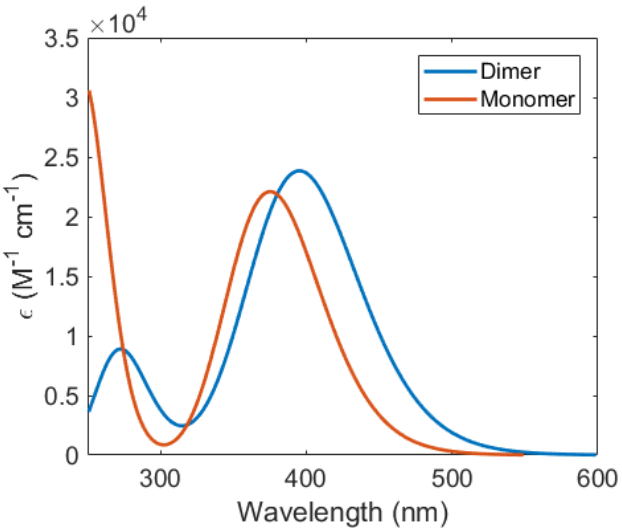


Figure S3. Calculated UV-vis absorption spectra of the monomer and the dimer of **BGAN-Aze** at M062X/6-31G(d,p) level in water. Our time-dependent density functional theory (TD-DFT) calculations further show that the dimer of **BGAN-Aze** demonstrate a noticeable redshift in the UV-vis absorption spectrum, in comparison to that of the monomers. For example, the calculated UV-vis absorption spectra peaked at 375.2 nm for monomers and 404.4 nm for dimers, respectively.

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[2]. 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 (Figure S4). 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. The optimized dimer exhibited a “folded” structure (Figure S5).

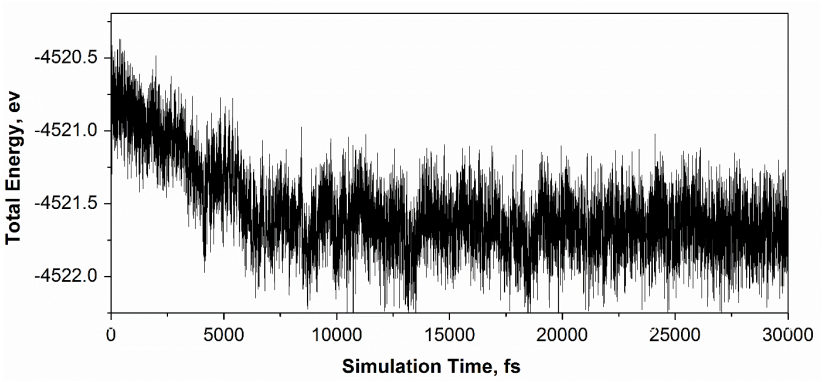


Figure S4. Evolution of the total electronic energy of a **BGAN-Aze** dimer, as a function of the simulation time.

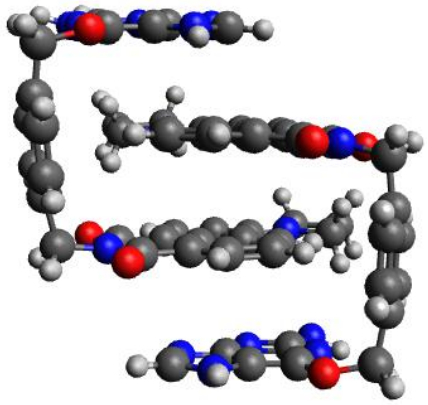


Figure S5. Optimized molecular structure of a **BGAN-Aze** dimer at M062X/6-31G(d,p) level in water.

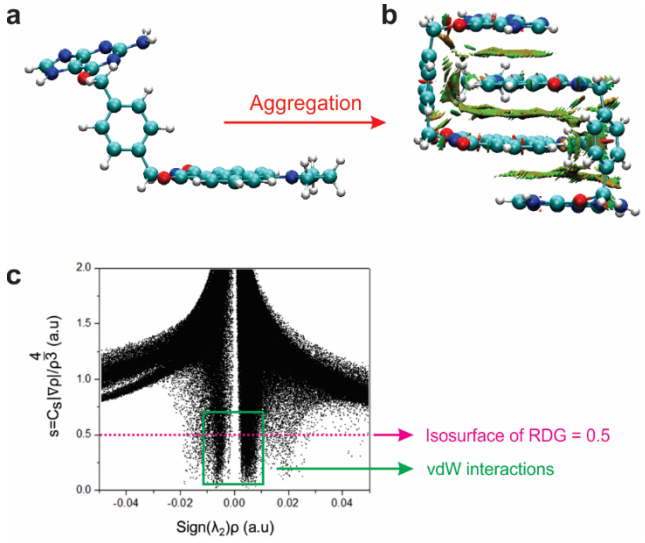


Figure S6. a. Molecular conformation of a **BGAN-Aze** monomer in water; b. Molecular conformation of a **BGAN-Aze** dimer in water, with a folded structure. The surfaces between two **BGAN-Aze** monomers are colored on a blue-green-red scale according to the values of sign(λ_2)ρ, ranging from -0.04 to +0.04 au, at the RDG isosurface *s* = 0.5. Blue indicates strong attraction, red indicates strong repulsion, and green indicates very weak interactions; c. Plots of the reduced density gradient *s* versus the electron density multiplied by the sign of the second Hessian eigenvalue [sign(λ_2)ρ]. sign(λ_2)ρ>0 denotes strong repulson, sign(λ_2)ρ<0 denotes strong attraction, and sign(λ_2)ρ ≈ 0 denotes vdW interactions.

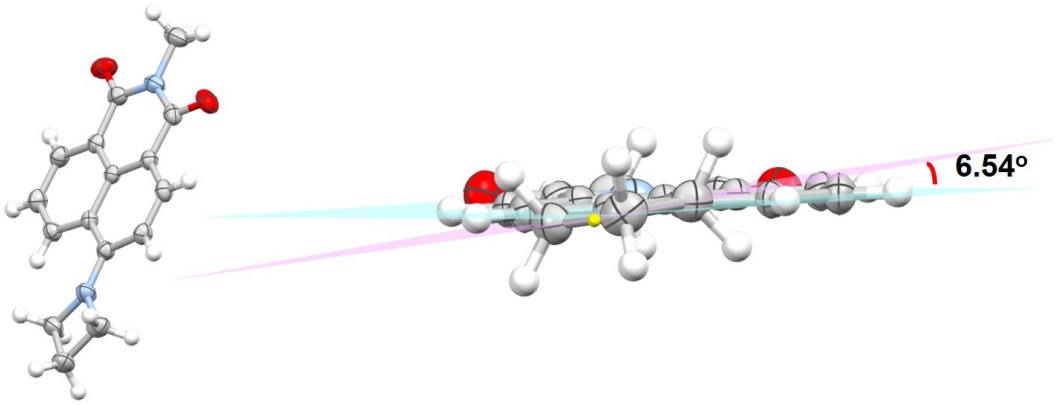


Figure S7 The single crystal of **AN-Aze** from different angles. There only exists 6.54° between the plane of naphthalimide and the plane of azetidine.

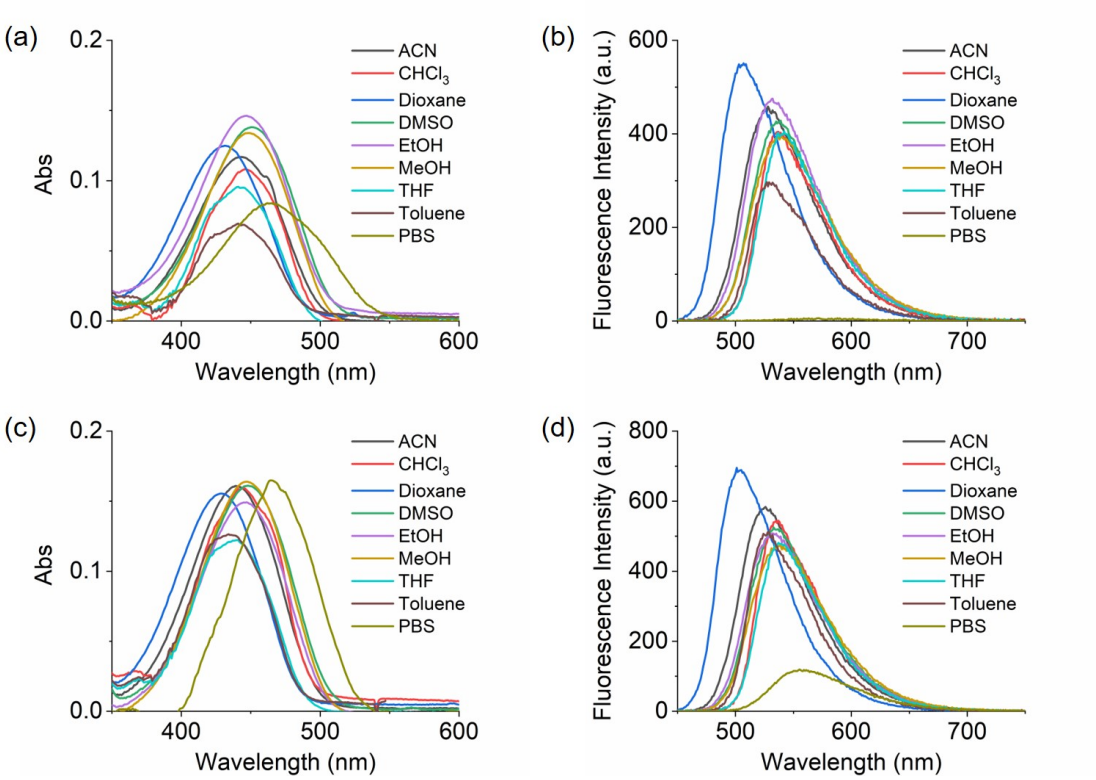


Figure S8. (a) UV-vis spectra and (b) fluorescence spectra of **BGAN-Aze** in different solvents. (c) UV-vis spectra and (d) fluorescence spectra of **AN-Aze** in different solvents. [**dyes**] = 10 μM.

Table S1. Spectroscopic data of **BGAN-Aze** and **AN-Aze** in different solvents.

solvent		Toluene	CHCl3	THF	Dioxane	ACN	EtOH	MeOH	DMSO	PBS
$_{abs}$ (nm)	**AN-Aze**	434	445	440	429	439	447	447	448	465
	BGAN-Aze	441	446	441	432	443	447	447	450	470
$_{em}$ (nm)	**AN-Aze**	528	537	537	501	526	532	541	533	555
	BGAN-Aze	527	537	542	507	528	532	540	539	571
($M^{-1}cm^{-1}$)	**AN-Aze**	11989	15226	12341	15066	15911	15076	16740	15960	15992
	BGAN-Aze	6662	11012	9505	15182	11691	14086	13918	13661	8399
(nm)	**AN-Aze**	94	92	97	72	87	85	94	85	90
	BGAN-Aze	86	91	101	75	85	85	93	89	101
	AN-Aze	0.87	0.91	0.85	0.78	0.62	0.64	0.55	0.73	0.25
	BGAN-Aze	0.76	0.85	0.75	0.61	0.67	0.64	0.57	0.73	0.016
BGAN-Aze + SNAP-tag		-	-	-	-	-	-	-	-	0.39

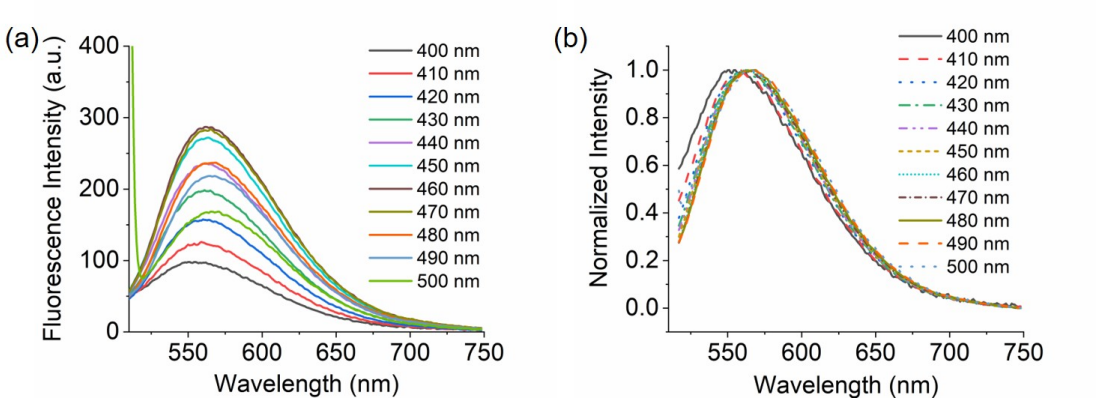


Figure S9. (a) Fluorescence spectra and (b) normalized fluorescence spectra of **BGAN-Aze** in PBS (10 mM, pH = 7.4), excited at various wavelengths from 400-500 nm. [**dyes**] = 10 μM.

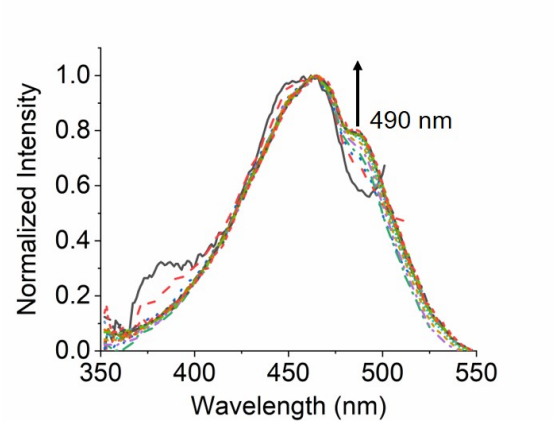


Figure S10. Normalized fluorescence excitation spectra of **BGAN-Aze** in PBS (10 mM, pH = 7.4), monitored at various emission wavelengths from 520 to 650 nm. [**dyes**] = 10 μM.

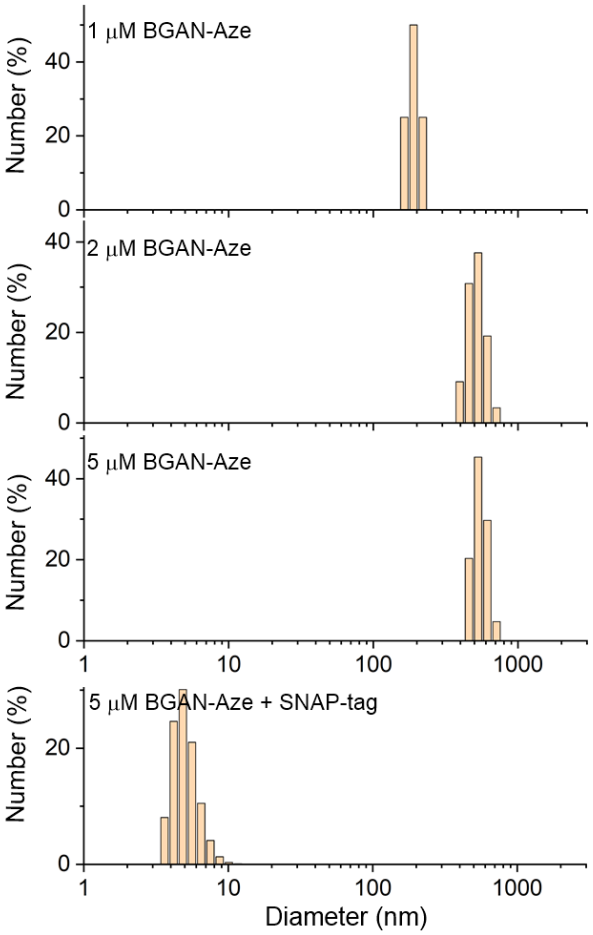


Figure S11. DLS analysis of **BGAN-Aze** at different concentration and in presence of 5 μM SNAP-tag in PBS buffer (1% DMSO). [**BGAN-Aze**] = 5 μM.

Figure S12. Image of 4 mL **BGAN-Aze** solution which stood for 48 h in PBS buffer. [**BGAN-Aze**] = 10 μM.

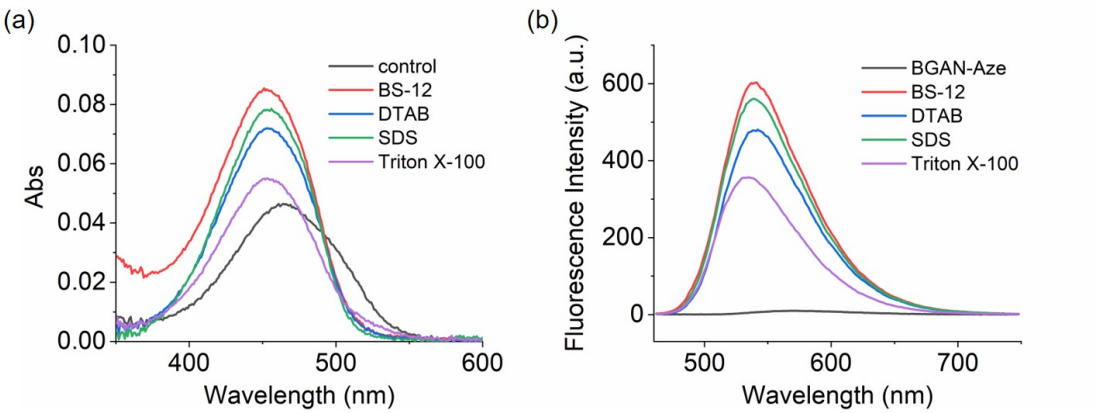


Figure S13. (a) UV-vis spectra and (b) fluorescence spectra of **BGAN-Aze** in the absence and presence of 10 mM different surfactants in PBS buffer (1% DMSO). [**BGAN-Aze**] = 10 μM.

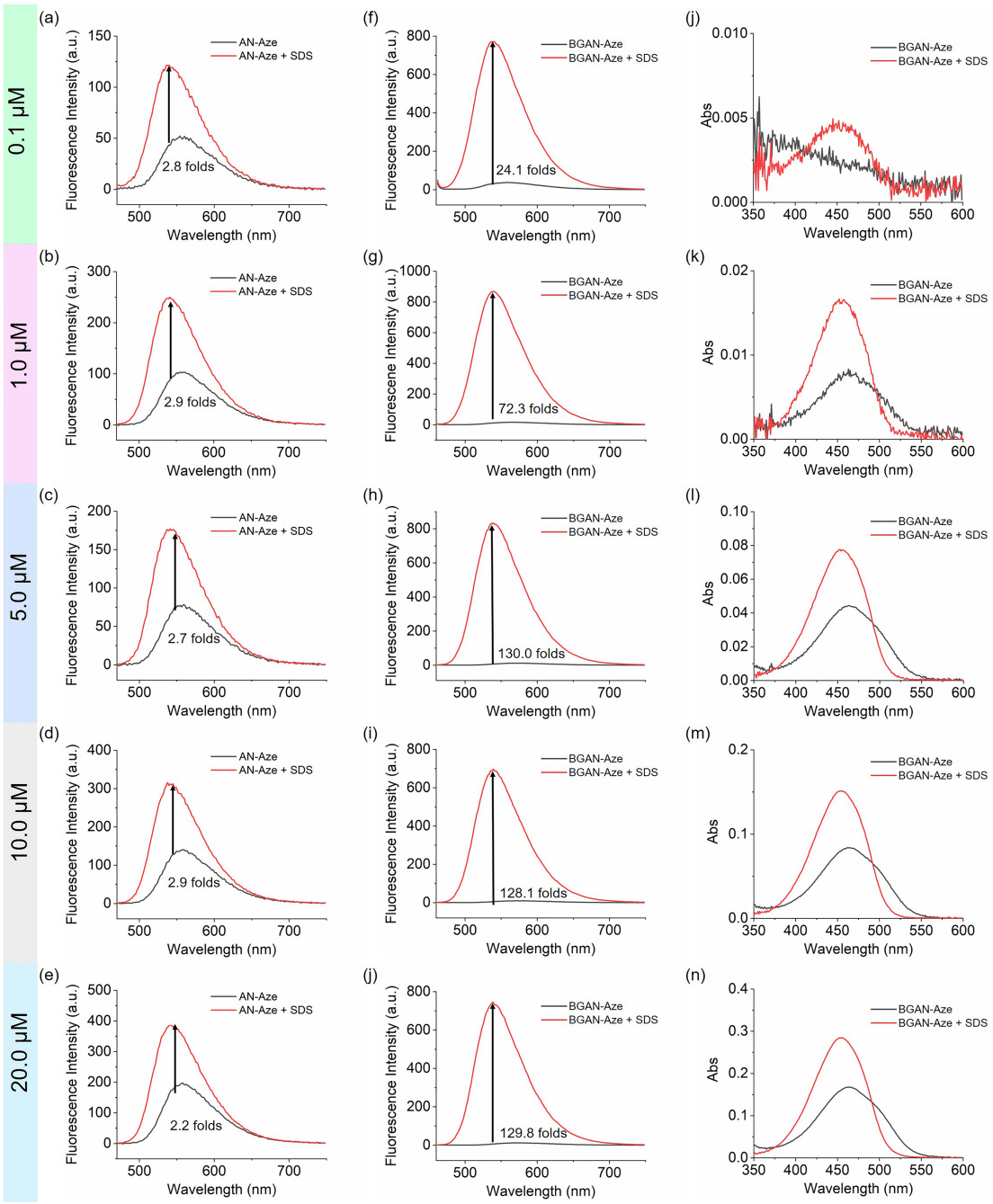


Figure S14. (a)-(e) Fluorescence spectra of **AN-Aze** in the absence and presence of 10 mM SDS in PBS buffer. (f)-(j) Fluorescence spectra and (k)-(n) UV-vis spectra of **BGAN-Aze** in the absence and presence of 10 mM SDS in PBS buffer. The concentration of dyes was selected to be 0.1, 1.0, 5.0, 10.0, 20.0 μM.

Table S2 Spectroscopic data of **BGAN-Aze** at different concentration in the absence and presence of 10 mM SDS

concentration		0.1 μM	1.0 μM	5.0 μM	10.0 μM	20.0 μM
$_{abs}$ (nm)	**BGAN-Aze**	-	463	463	465	463
	with SDS	445	433	453	453	453
$_{em}$ (nm)	**BGAN-Aze**	564	567	577	571	570
	with SDS	540	540	539	539	538
(nm)	**BGAN-Aze**	-	104	114	106	107
	with SDS	95	107	86	86	85
	BGAN-Aze	-	0.025	0.017	0.016	0.014
	with SDS	-	0.475	0.519	0.477	0.391

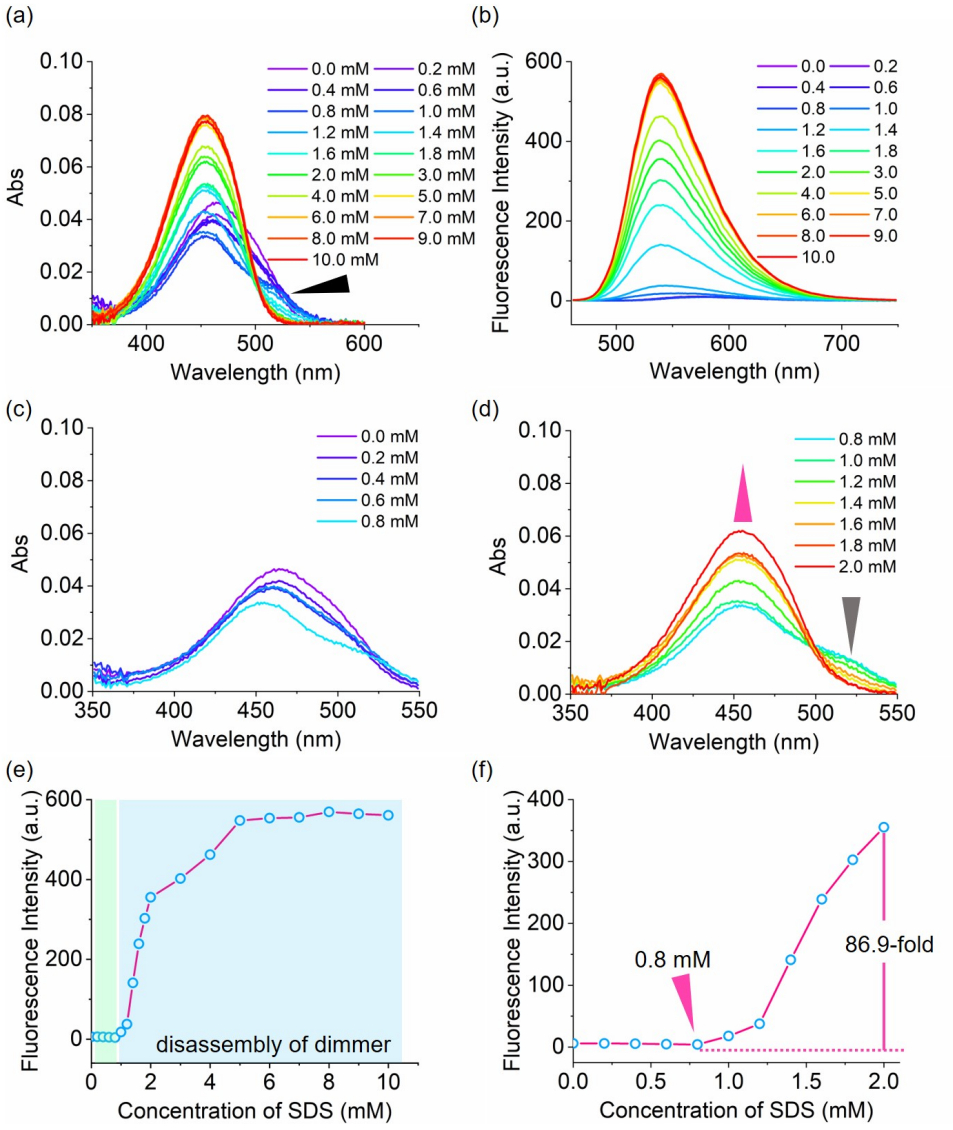


Figure S15. (a), (c), (d) UV-vis spectra of **BGAN-Aze** in the absence and presence of different concentration of SDS. We could inspected the obvious absorption peak at

525 nm indicating the absorption of dimer. (b) Fluorescence spectra of **BGAN-Aze** in the absence and presence of different concentration of SDS. (e), (f) Fluorescence responses at 539 nm to different concentration of SDS. (e) 0-10 mM, (f) 0-2 mM. The fluorescence of **BGAN-Aze** greatly increased once the dimer was disassembled. [**BGAN-Aze**] = 10 μM.

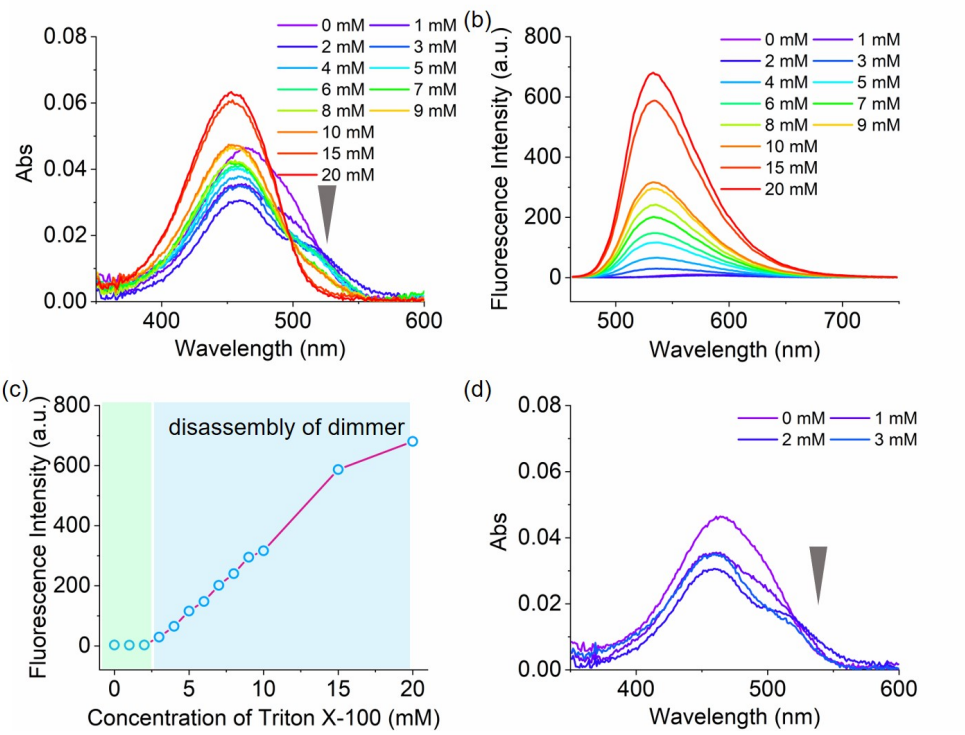


Figure S16. (a), (d) UV-vis spectra of **BGAN-Aze** in the absence and presence of different concentration of Triton-X100. We also could inspected the obvious absorption peak of dimer at ~525 nm. (b) Fluorescence spectra of **BGAN-Aze** in the absence and presence of different concentration of Triton-X100. (e) Fluorescence responses at 539 nm to different concentration of Triton-X100. The fluorescence of **BGAN-Aze** slowly increased and indicated the dimer was not easy to be disassembled by Triton-X100. [**BGAN-Aze**] = 10 μM.

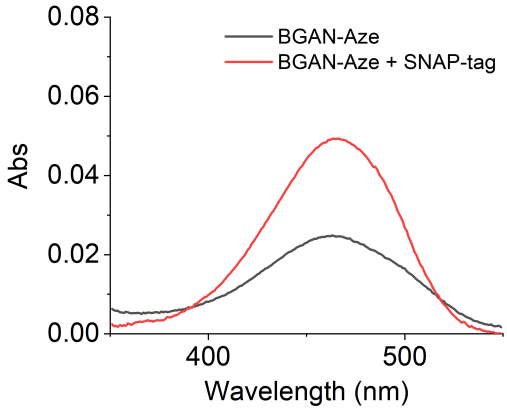


Figure S17. (a) UV-vis spectra of **BGAN-Aze** in the absence and presence of 5 μM SNAP-tag in PBS buffer (1% DMSO). [**BGAN-Aze**] = 5 μM.

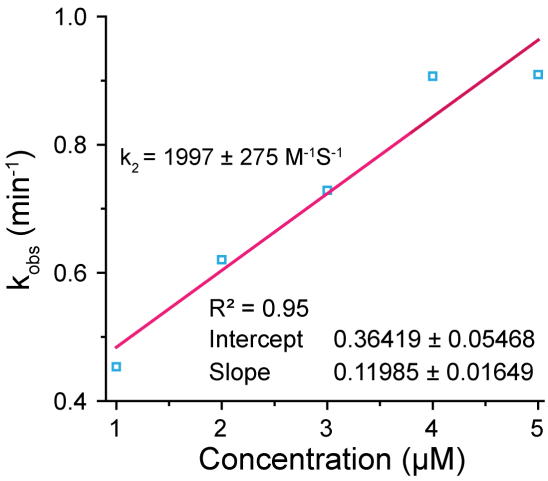


Figure S18. Plots of the pseudo-first-order rate constant (kobs) versus **BGAN-Aze** concentrations. k_2 was the slope of the fitting line.

4 Imaging details

4.1 Confocal imaging

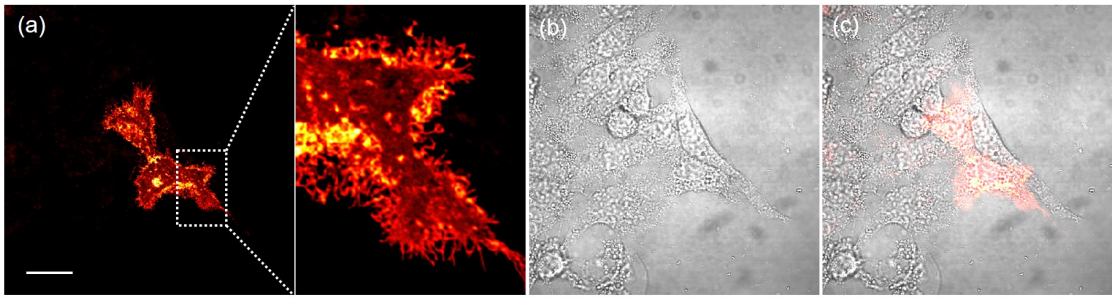


Figure S19. No-wash live HeLa cell confocal imaging of SNAP-tagged ADRβ2 labeled with **BGAN-Aze**. Cells were incubated with 200 nM **BGAN-Aze** at 37°C for 5 min (a) Confocal images, (b) bright field, (c) merger of (a) and (b). Ex: 488 nm; Slit: 500-600 nm, scale bar 20 μm.

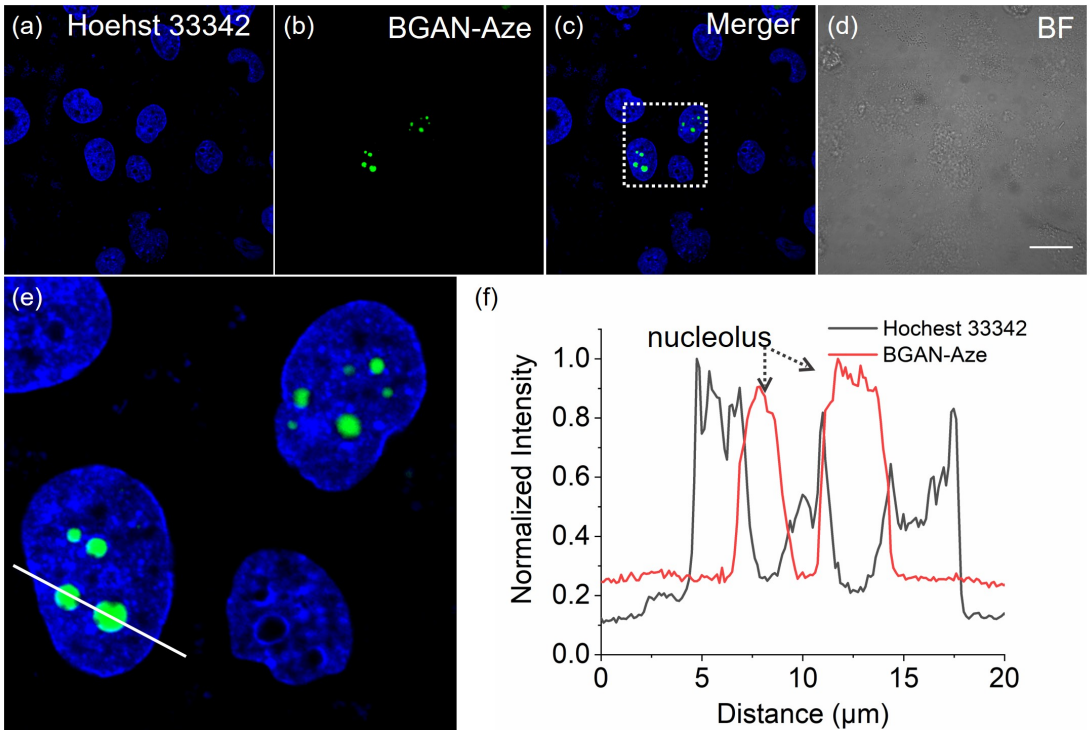


Figure S20. No-wash live HeLa cell confocal imaging of SNAP-tagged NPM1 labeled with **BGAN-Aze**. Cells were incubated with 500 nM **BGAN-Aze** and Hoechst 33342 at 37°C for 30 min. (a) Hoechst 33342. (b) **BGAN-Aze**. (c) merger of (a) and (b). (d) bright field. (e) scale of ROI in (c). (f) Intensity profile of ROI across nucleus in (e) which indicated **BGAN-Aze** could specifically stain nucleolus and shown dividual

fluorescence with Hoechst. Ex: 488 nm; Slit: 500-600 nm for **BGAN-Aze**, Ex: 405 nm; Slit: 425-475 nm for Hoechst 33342. Scale bar 20 μm.

4.2 Super-resolution imaging

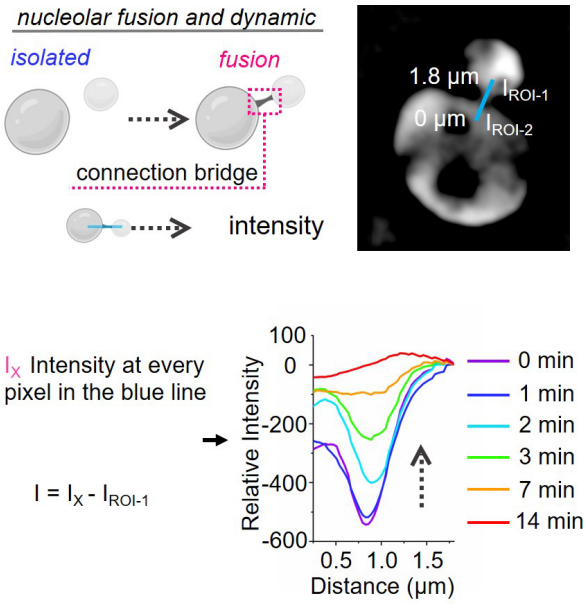


Figure S21. Analysis method for monitoring the fusion of nucleolus. The relative intensity on the line, across the connection bridge, was adopted to inspect the formation and mature of connection bridge.

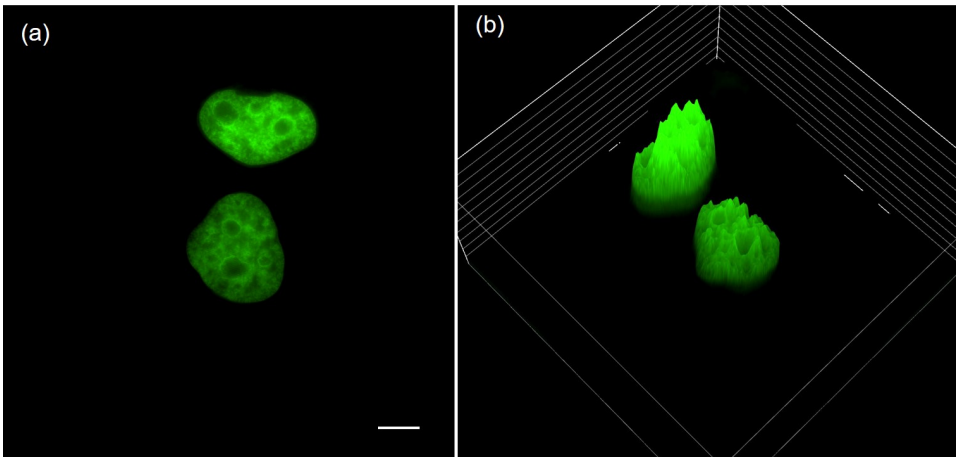


Figure S22. (a) No-wash SIM imaging of **BGAN-Aze** in live HeLa cells. Intensity surface of (a) which indicated the high nuclei-to-cytosol signal ratio. Scale bar 5 μm.

5 Crystallography experiment

5.1 Culture of crystal

5 mg **AN-Aze** was firstly dissolved in 5 mL $CHCl_3$. Then HPLC MeOH was dropwise added to the solution until a slight turbidity appeared. Another 1 mL $CHCl_3$ 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.

5.2 Single-crystal structures

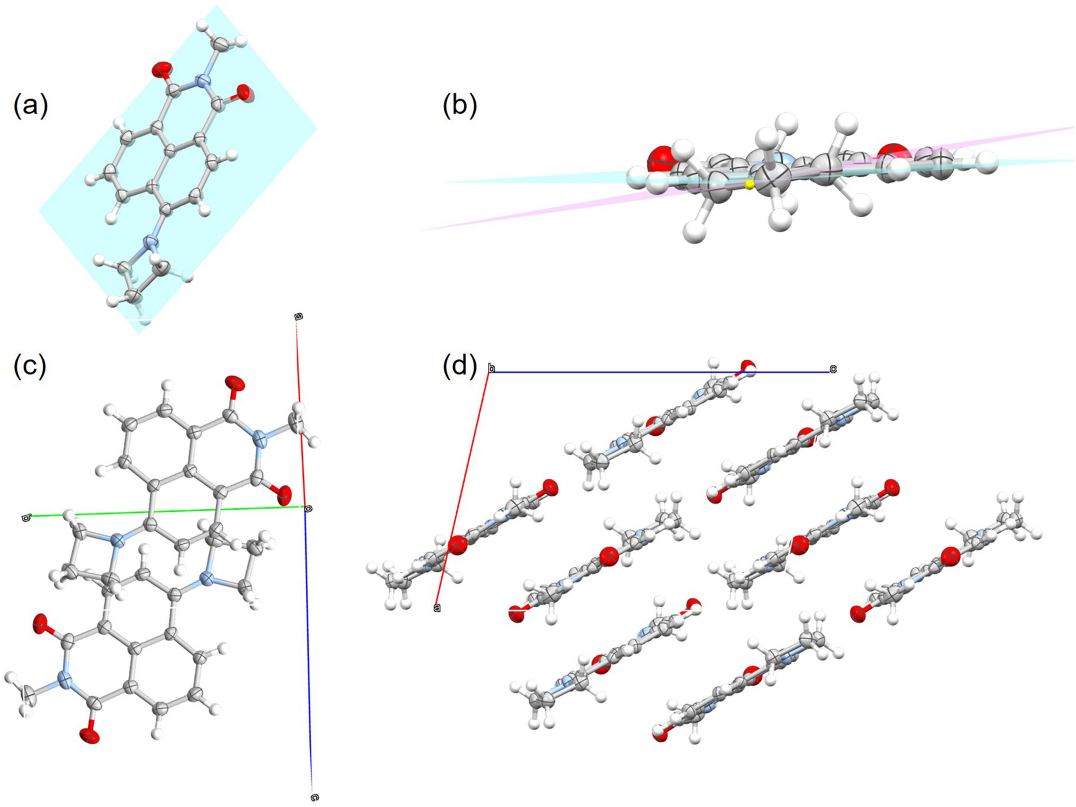


Figure S23 The single crystal of **BGAN-Aze** from different angles (a) – (e).

Table S3 Crystal data and structure refinement for **BGAN-Aze**

Identification code	**BGAN-Aze**

Empirical formula	$C_{16}H_{14}N_2O_2$
Formula weight	266.29
Temperature/K	293
Crystal system	rectangle
Space group	P 21/n
a/Å	9.4602(12)
b/Å	10.3865(12)
c/Å	13.1660(14)
α/°	90
β/°	102.807(14)
γ/°	90
Volume/$Å^3$	1261.5(3)
Z	4
F(000)	560.0
Crystal size/mm^3	0.13 × 0.14 × 0.18
Radiation	Mu

6 Spectra characterization

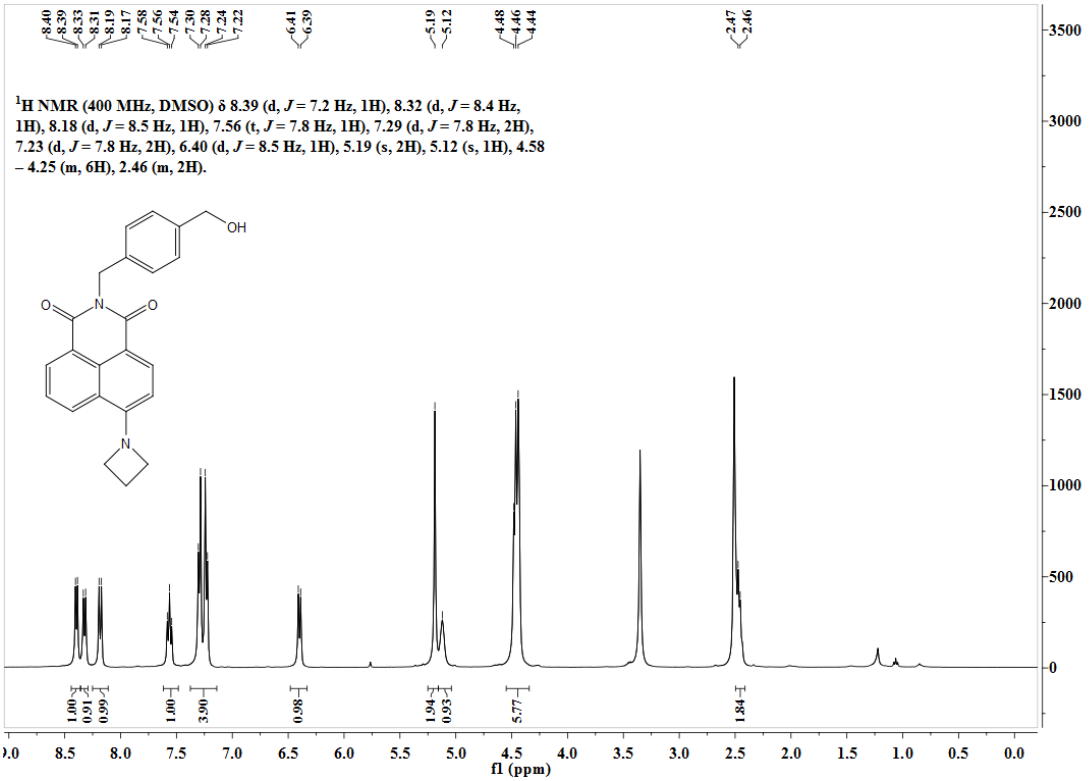


Figure S1 ^{1}H NMR spectrum of **2** in DMSO-d_6.

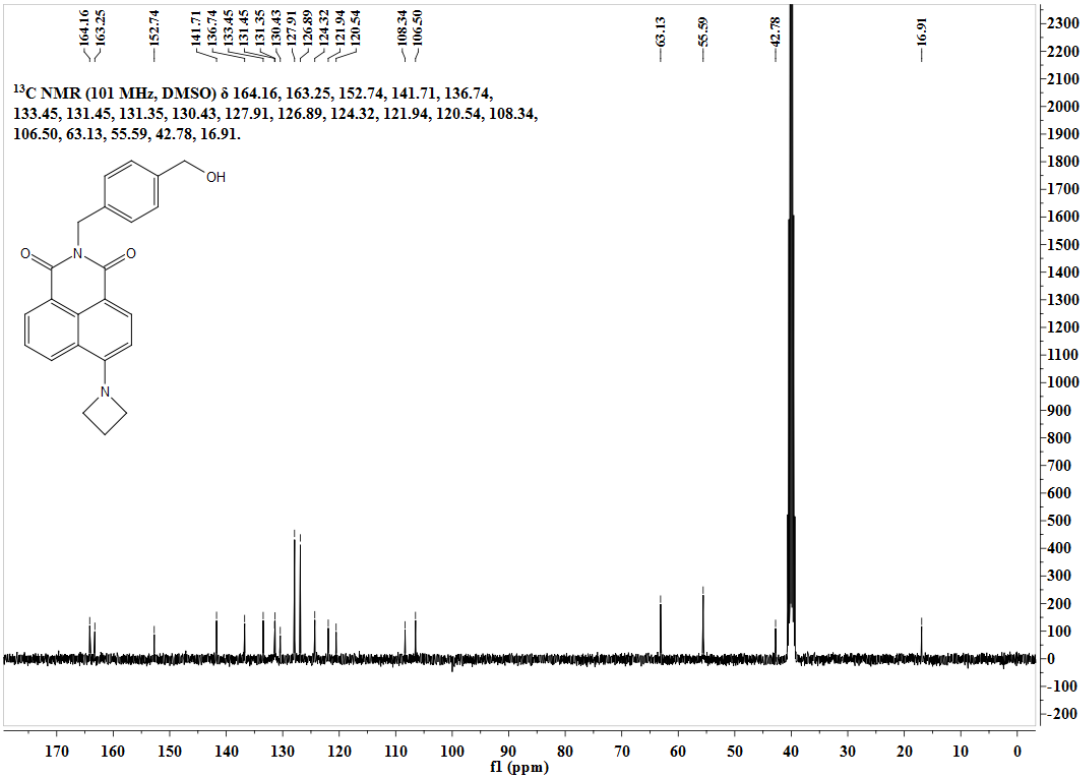


Figure S2 ^{13}C NMR spectrum of **2** in DMSO-d_6.

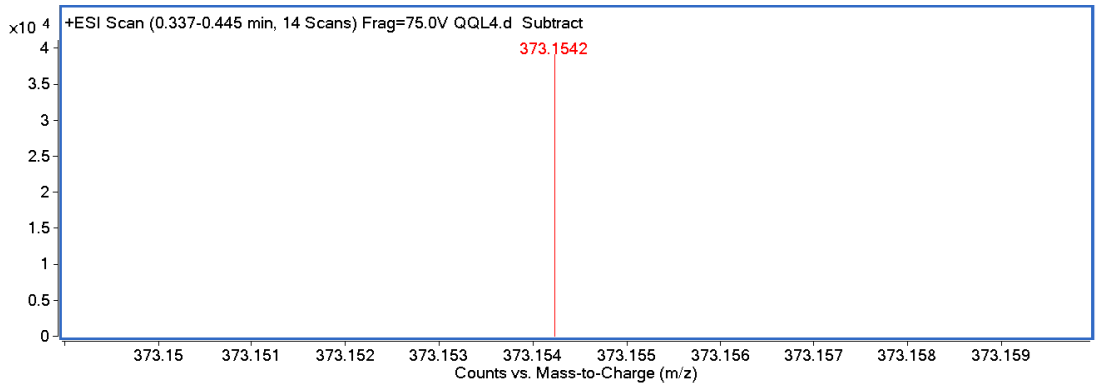


Figure S3 HRMS spectrum of **2**.

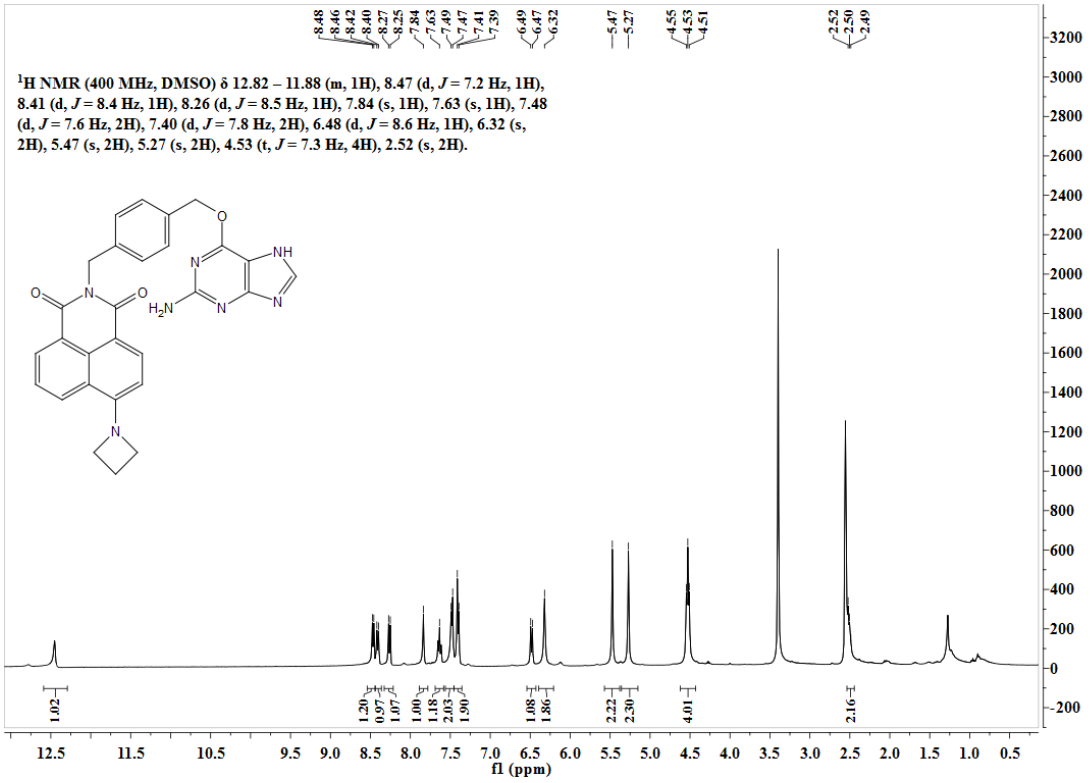


Figure S2 ^{1}H NMR spectrum of **BGAN-Aze** in DMSO-d_6.

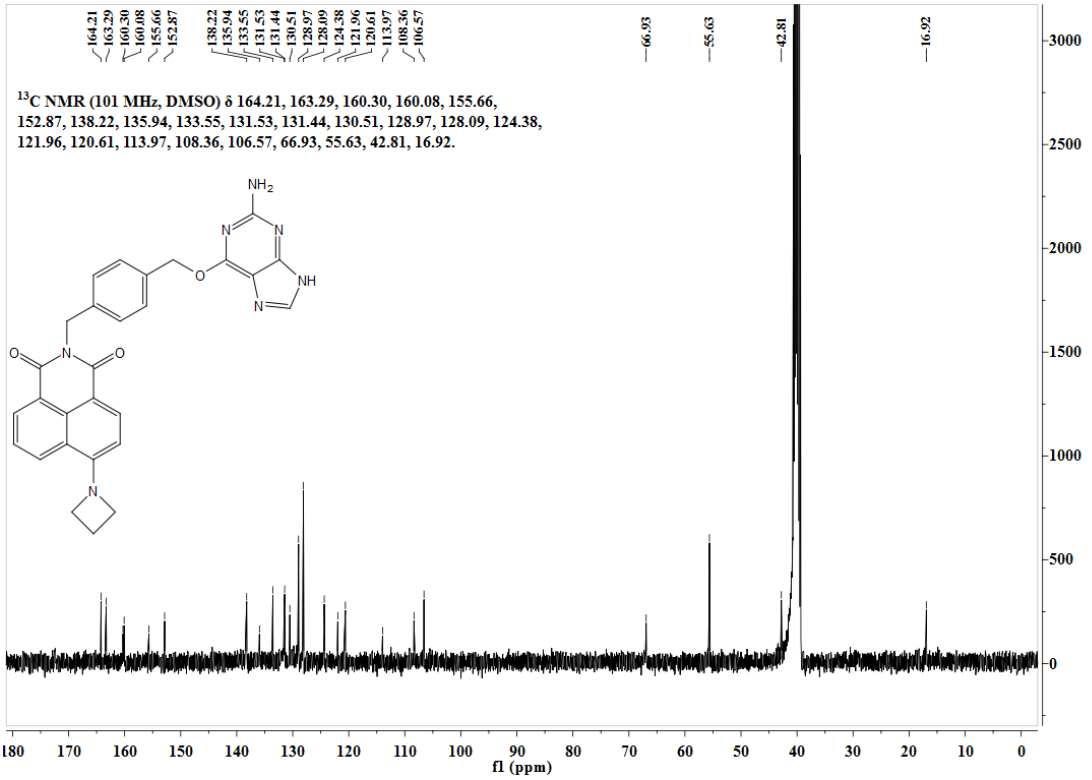


Figure S2 ^{13}C NMR spectrum of **BGAN-Aze** in DMSO-d_6.

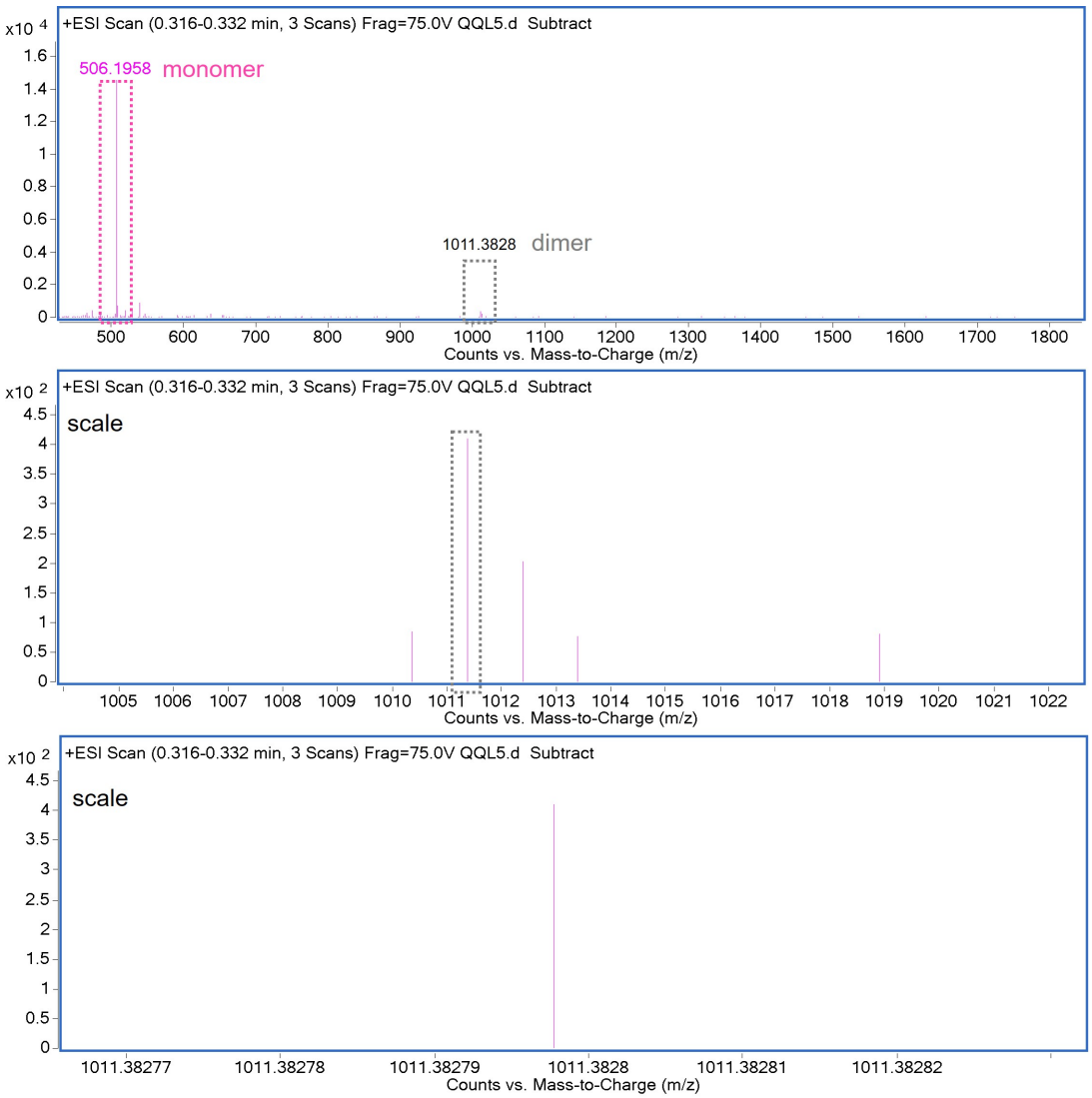


Figure S36 HRMS spectrum of **BGAN-Aze**.

7 References

[1] X. Liu, Q. Qiao, W. Tian, W. Liu, J. Chen, M. J. Lang, Z. Xu, *J. Am. Chem. Soc.* **2016**, *138*, 6960-6963.

[2] B. Aradi, B. Hourahine, T. Frauenheim, *J. Phys. Chem. A.* **2007**, *111*, 5678-5684.