# **Supplementary Information**

## Fluorogenic sydnonimine probes for orthogonal labeling

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#### Abbreviations

The following abbreviations are used throughout the text of the SI file: BHQ, black hole quencher; Boc, tert-butoxycarbonyl; calcd., calculated; CDCl<sub>3</sub>, chloroform-d; DMSO, dimethylsulfoxide; DIPEA, N,N-diisopropylethylamine; DMF, N,Ndimethylformamide; DCM, dichloromethane; equiv., equivalent; ESI, electrospray ionization; Et<sub>2</sub>O, diethyl ether; EtOAc, ethyl acetate; M, mol/L; MeCN, acetonitrile; MeOH, methanol; MS, Mass Spectrometry; NaOAc, sodium acetate; PBS, phosphate buffered saline; PE, petroleum ether; RP-HPLC, reversed-phase high performance liquid chromatography; r.t., room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; HATU, 2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TSTU, N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate; h, hour; HRMS, high resolution mass spectrum; 3RAX, 3-amino-7-(diethylamino)-5phenylphenazinium chloride; TAMRA, 2-(3,6-bis(dimethylamino)xanthylium-9-yl)-5carboxybenzoate; Cy5, 1-(5-carboxy-pentyl)-3,3-dimethyl-2-((1E,3E)-5-((E)-1,3,3trimethyl-5-sulfoindolin-2-ylidene) penta-1,3-dien-1-yl)-3H-indol-1-ium-5-sulfonate.

#### **Computational details**

All calculations were performed with Gaussian  $09^{[1]}$ . Geometry optimizations of all the minima and transition structures were carried out at the M06-2X level of theory<sup>[2,3]</sup> with the 6-31G(d) basis set. Vibrational frequencies were evaluated at the same level to verify that optimized structure is an energy minimum or a transition state and to compute zero-point vibrational energies (ZPVE) and thermal corrections at 298 K. Solvent effects in water were calculated at the M06-2X/6-311+G(d,p) level using the gas-phase optimized structures with the CPCM model<sup>[4-6]</sup>, where UFF radii were used. The predicted second-order rate constants shown in Figure S2 were calculated by using the corrected activation free energies  $[\Delta G^{\dagger}_{-} \operatorname{corr} = (\Delta G^{\dagger}_{-} \operatorname{compt} + 8.4)/1.6]^{[7]}$ , according to Eyring equation at 298 K.



Figure S1. The optimized structure of sydnonimine 4'.



**Figure S2.** DFT-computed activation free energies for cycloadditions of **Ph-SIN** with  $2\pi$  cycloaddends at the CPCM(water)-M06-2X/6-311+G(d,p)//M06-2X/6-31G(d) level of theory and the predicted rate constants in water at 25 °C.

4'	C -9.900140 2.795531 -0.790937
<i>G</i> (Water) = -1882.027398 Hartree	C -8.781162 2.081810 -0.364117
	O 8.170762 -1.462029 -1.303154
N 1.938298 -0.881444 -0.092767	N 8.267066 0.384624 0.010479
N 1.432042 -1.954363 0.428846	C 9.721218 0.435263 -0.013142
O 0.098723 -1.737066 0.411301	C 10.215808 1.807951 0.420041
C -0.211788 -0.502241 -0.119786	C 11.737550 1.893756 0.429148
C 1.041639 0.052303 -0.451323	H 1.300036 0.970575 -0.946505
C 3.365353 -0.797659 -0.224517	Н 3.555713 -2.878584 -0.724865
N -1.404611 -0.029111 -0.258450	Н 6.055722 -2.707677 -0.965136
C 4.079141 -1.943463 -0.560802	H 5.854489 1.467207 -0.017117
C 5.457666 -1.847035 -0.685446	H 3.398825 1.301317 0.255404
C 6.107230 -0.630585 -0.468333	H -3.786651 1.075456 0.531142
C 5.365209 0.506881 -0.146998	H -3.961670 0.431023 -1.121808
C 3.983303 0.429169 -0.017935	H -5.533264 -1.320090 -0.310265
C 7.606004 -0.619045 -0.628171	H -5.248210 -0.821984 1.364080
S -2.671710 -0.968002 0.257201	H -6.304231 1.369275 0.942176
C -4.005649 0.180361 -0.058532	H -6.423991 1.009514 -0.791505
O -2.846901 -2.123839 -0.617294	Н -7.602922 -0.172029 2.475556
O -2.590995 -1.186025 1.699515	H -8.961088 -1.063543 1.764584
C -5.332342 -0.458374 0.334712	H -7.320818 -1.697802 1.599873
C -6.465555 0.557514 0.205833	H -10.231034 -0.956016 0.038770
N -7.773385 -0.053583 0.358872	H -12.208612 0.323938 -0.662806
C -8.882406 0.722372 -0.041029	H -9.792188 3.849075 -1.032650
C -7.919300 -0.783639 1.613860	Н -7.833208 2.599258 -0.270593
C -10.140404 0.107121 -0.162614	H 7.798613 0.870402 0.763176
C -11.250727 0.828190 -0.573526	H 10.140942 -0.348475 0.633233
C -11.141464 2.181911 -0.892802	H 10.034858 0.204664 -1.034950

H 9.805882 2.567945 -0.258144 H 9.856122 2.048596 1.428397 H 12.133068 1.152895 1.133461 H 12.121729 1.620418 -0.568614 H -12.010356 2.743362 -1.220229 N 12.153061 3.215473 0.890622 H 13.165037 3.249179 0.983098 H 11.909465 3.912093 0.189349

#### Ph-SIN

G(Water) = -1136.105866 Hartree

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N -1.080131 -0.615939 0.143371 N -0.931911 -1.872886 0.417504 O 0.405910 -2.053238 0.444881 C 1.083323 -0.884634 0.196308 C 0.064511 0.072739 -0.007642 N 2.377886 -0.935556 0.190529 S 3.158194 0.499805 -0.022014 O 2.362111 1.415382 -0.848632 O 3.655968 0.993372 1.254885 H 0.124642 1.104622 -0.303001 C 4.549095 -0.047485 -0.985845 H 5.078015 -0.814061 -0.419713 H 4.184726 -0.441375 -1.934464 H 5.185145 0.824777 -1.145457 C -2.413496 -0.092452 0.032274 C -3.413187 -0.907157 -0.486863 C -2.655917 1.210164 0.450637

C -4.699911 -0.390379 -0.586148 H -3.176740 -1.916745 -0.803410 C -3.947707 1.712569 0.335746 H -1.857252 1.809163 0.875772 C -4.966711 0.915475 -0.180068 H -5.493602 -1.010150 -0.989412 H -4.156424 2.726884 0.658459 H -5.972447 1.313516 -0.265848

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#### 3,3-Ср

G(Water) = -195.1257316 Hartree

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C 1.262172 0.000000 -0.647569 C 1.262611 -0.000000 0.647379 C -0.094464 0.000000 0.000147 H 1.809548 -0.000000 1.578518 C -0.937167 -1.267178 0.000026 H -1.586180 -1.305243 0.883394 H -1.586035 -1.305322 -0.883478 H -0.305240 -2.159455 0.000062 C -0.937167 1.267178 0.000026 H -1.586180 1.305244 0.883394 H -1.586180 1.305244 0.883394 H -0.305240 2.159455 0.000062 H 1.809449 0.000000 -1.578528

#### TS1

G(Water) = -1331.184636 Hartree

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C 0.061224 1.378754 -0.858453 C 0.804561 2.000871 0.087044 C -0.492768 2.659619 -0.287192 H 0.183626 1.010641 -1.868851 H 1.825462 2.331826 0.229154 C -0.398375 3.889218 -1.187790 H -0.297361 4.800471 -0.586715 H 0.458658 3.827466 -1.864942 H -1.307337 3.991167 -1.792167 C -1.704639 2.759937 0.628464 H -1.660766 3.708654 1.175730 H -2.619850 2.773725 0.024507 H -1.792296 1.959515 1.357802 N 1.183486 -0.469047 0.649970 N 0.968312 0.458005 1.582205 O -0.371929 0.308338 1.876852 C -1.001717 -0.368978 0.883951 C 0.047487 -0.739256 -0.026168 N -2.279243 -0.513076 0.957725 S -2.994588 -1.381472 -0.258964 O -2.234892 -1.251103 -1.507484 O -3.322289 -2.719032 0.213334 H -0.020033 -1.460734 -0.823323 C -4.502105 -0.454433 -0.432654 H -5.023576 -0.464247 0.524335 H -4.250684 0.562727 -0.733502 H -5.091995 -0.954223 -1.202749 C 2.520535 -0.644332 0.193877 C 3.556696 -0.539409 1.119728

C 2.768697 -0.915675 -1.149047 C 4.865035 -0.709612 0.683758 H 3.325875 -0.330750 2.158287 C 4.083402 -1.097496 -1.565161 H 1.953034 -0.970811 -1.862101 C 5.131316 -0.993435 -0.654316 H 5.678530 -0.629211 1.397198 H 4.286404 -1.311344 -2.609274 H 6.154603 -1.132755 -0.987132

#### norbornene

G(Water) = -272.5457539 Hartree

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C -1.275344 -0.668267 -0.500391 C -0.084771 -1.124602 0.325215 C 1.179937 -0.777633 -0.521120 C 1.179960 0.777613 -0.521143 C -1.275321 0.668278 -0.500390 C -0.084742 1.124606 0.325239 C -0.030779 0.000005 1.376394 H -1.915633 -1.325220 -1.079425 H -0.114261 -2.151779 0.692510 H 2.080314 -1.172541 -0.039936 H 1.123410 -1.201886 -1.526509 H 1.123255 1.201826 -1.526539 H 2.080396 1.172565 -0.040118 H -1.915564 1.325267 -1.079429 H -0.114276 2.151779 0.692547 H -0.897291 0.000013 2.042551

H 0.896013 -0.000019 1.961528

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#### TS2

G(Water) = -1408.604663 Hartree

C 0.781512 1.771943 0.266431 C -0.067371 2.910775 0.802239 C -1.296020 1.906000 -0.804003 C 0.028698 1.162279 -0.742586 H 1.863323 1.867920 0.226054 H 0.467451 0.775550 -1.658569 C -1.496749 2.361624 0.653679 H -2.242730 3.160727 0.725219 H -1.762405 1.576300 1.357596 C -0.067172 3.962520 -0.350865 H -0.533723 4.891920 -0.009380 H 0.945114 4.202992 -0.687908 C -0.924399 3.274052 -1.454721 H -1.831515 3.848714 -1.667057 H -0.382748 3.147955 -2.396268 H 0.221874 3.303632 1.778358 H -2.111588 1.367321 -1.289845 N 1.251889 -0.751136 0.664695 N 1.036170 0.142563 1.640320 O -0.294695 -0.062275 1.960363 C -0.928544 -0.691129 0.941934 C 0.100860 -0.979166 -0.014639 N -2.208098 -0.845984 1.017461 S -2.909256 -1.658781 -0.242553

O -2.269336 -1.292680 -1.512166
O -3.042678 -3.075016 0.073286
H 0.020892 -1.661107 -0.845338
C -4.523060 -0.912619 -0.203292
H -4.966500 -1.101931 0.774105
H -4.415716 0.157380 -0.383687
H -5.107407 -1.385411 -0.994331
C 2.576003 -0.869482 0.167619
C 3.639884 -0.690762 1.052089
C 2.796107 -1.159451 -1.177507
C 4.939266 -0.803760 0.573853
H 3.435439 -0.471023 2.093741
C 4.103941 -1.282770 -1.635755
H 1.964365 -1.281712 -1.862928
C 5.175883 -1.103685 -0.766480
Н 5.770629 -0.665617 1.257376
H 4.281208 -1.511624 -2.681437
H 6.193039 -1.198031 -1.132046

#### 1,3-Ср

G(Water) = -403.09152 Hartree

C 2.075599 -1.340850 0.012149 C 1.179573 -0.220732 0.465535 C 2.636057 -0.176795 0.099311 H 2.147467 -2.397607 -0.195902 H 0.897003 -0.165379 1.522917 C 3.803917 0.725252 -0.003608 H 4.699318 0.184751 -0.318783 H 3.602976 1.526128 -0.722534 H 3.997732 1.200296 0.963537 C 0.120322 0.364743 -0.450773 H 0.488809 0.355860 -1.485625 H -0.117259 1.397517 -0.182545 N -1.132117 -0.364483 -0.357339 H -1.099803 -1.369377 -0.449124 C -2.311529 0.229449 -0.018235 O -2.425244 1.428718 0.166352 C -3.488557 -0.716488 0.138989 H -4.363135 -0.258682 -0.325626 H -3.316392 -1.702051 -0.300716 H -3.702233 -0.837288 1.204745

#### TS3

G(Water) = -1539.157085 Hartree

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C 0.063075 -0.822882 0.319228 C -0.804351 -1.316670 -0.799087 C 0.659644 -1.564361 -0.641228 H -0.031990 -0.774858 1.396066 H -1.132734 -0.599031 -1.557386 C 1.613182 -2.691189 -0.835175 H 2.504350 -2.571165 -0.211674 H 1.119243 -3.629101 -0.553816 H 1.921855 -2.772016 -1.880049 C -1.860372 -2.358324 -0.490637 H -1.466751 -3.129917 0.185321 H -2.200311 -2.870325 -1.398391

N -2.980533 -1.675748 0.128895 H -2.891429 -0.685096 0.334386 C -4.123576 -2.344076 0.417824 O -4.259035 -3.536009 0.184632 C -5.219375 -1.507458 1.051554 H -5.473780 -1.942024 2.021369 H -4.933924 -0.461202 1.184735 H -6.110255 -1.564999 0.421439 N 2.102133 0.659997 -0.516487 N 1.752418 0.088063 -1.669958 O 0.614678 0.780587 -2.038716 C 0.080644 1.406143 -0.965534 C 1.023167 1.193405 0.094984 N -1.080578 1.958631 -1.102993 S -1.837340 2.401052 0.288550 O -2.757679 1.330345 0.682357 O -0.897621 2.885089 1.302889 H 1.061967 1.730473 1.029013 C -2.808529 3.770620 -0.288086 H -2.133210 4.561337 -0.614982 H -3.435660 3.424050 -1.109403 H -3.417367 4.102108 0.554469 C 3.290128 0.188876 0.108209 C 4.404570 -0.062446 -0.689691 C 3.311215 -0.033253 1.482185 C 5.559406 -0.550173 -0.091466 H 4.349339 0.120558 -1.757017 C 4.481477 -0.507461 2.067427 H 2.424276 0.142368 2.081954

C 5.602464 -0.769215 1.284742 H 6.432316 -0.754008 -0.702795 H 4.508329 -0.684011 3.137517 H 6.509726 -1.145893 1.745453

#### cyclooctyne

G(Water) = -311.7869537 Hartree

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C 0.681129 1.341424 0.376746 C -0.682343 1.341190 -0.376880 C 0.603634 -1.453056 0.028187 C -1.857400 0.581825 0.279100 C -0.602258 -1.452377 -0.028549 C -1.957951 -0.910097 -0.119865 C 1.856814 0.582765 -0.278986 C 1.958707 -0.909006 0.120049 H -0.539204 0.960586 -1.396591 H -1.762279 0.646840 1.369540 H -0.998167 2.383992 -0.489899 H -2.803074 1.070506 0.016803 H -2.672084 -1.434049 0.523759 H -2.326611 -0.999077 -1.149093 H 2.802035 1.072273 -0.016545 H 1.761937 0.647589 -1.369447 H 2.673865 -1.432067 -0.523167 H 2.326957 -0.997634 1.149457 H 0.996525 2.384355 0.489920 H 0.538104 0.960678 1.396452

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#### TS4

G(Water) = -1447.855654 Hartree

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C -0.034920 1.024580 0.325790 C -0.764613 1.316672 -0.639356 C 0.783942 1.438389 1.486242 H 0.164910 1.386058 2.393058 H 1.617906 0.746527 1.643629 C -1.498781 2.267996 -1.487823 H -2.501859 1.884639 -1.707269 H -0.979814 2.341288 -2.453281 C 1.303275 2.871419 1.302399 H 1.882509 3.126892 2.197792 H 2.006269 2.886411 0.459522 C 0.221905 3.944871 1.082667 H -0.656672 3.702986 1.696915 H 0.616627 4.881640 1.490519 C -1.580923 3.647761 -0.821139 H -2.053818 4.332813 -1.534334 H -2.253212 3.585756 0.043366 C -0.226857 4.230223 -0.373362 H -0.290642 5.317612 -0.487132 H 0.556411 3.904187 -1.071192 N -1.022683 -1.267662 -0.603129 N -0.971273 -0.505365 -1.697552 O 0.367432 -0.554549 -2.058400 C 1.114289 -0.896688 -0.982651 C 0.155743 -1.204883 0.052952 N 2.398540 -0.858560 -1.097003

S 3.299026 -1.462024 0.149250
O 2.584930 -1.351645 1.429603
O 3.848290 -2.758721 -0.216950
H 0.357062 -1.732266 0.971345
C 4.621283 -0.271217 0.163364
H 5.095915 -0.273774 -0.817828
H 4.206021 0.710374 0.394894
H 5.323574 -0.588991 0.935888
C -2.302502 -1.452271 -0.011922
C -3.368441 -1.812024 -0.833132
C -2.467427 -1.262540 1.356846
C -4.622807 -1.991630 -0.262957
H -3.199333 -1.949603 -1.895621
C -3.726379 -1.462260 1.914858
H -1.629869 -0.945238 1.968973
C -4.802476 -1.824238 1.109294
H -5.460776 -2.274221 -0.891588
H -3.866094 -1.321116 2.981604
H -5.782692 -1.974124 1.550091

#### DIBAC

G(Water) = -785.236177 Hartree

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C 1.792874 -1.272296 0.126711
C 1.745302 0.101539 -0.228318
C 2.934150 0.759350 -0.538324
C 4.154740 0.094635 -0.457710
C 4.199516 -1.249526 -0.094739
C 3.022046 -1.932408 0.185954

H 2.893238 1.799067 -0.849368 H 5.070937 0.626362 -0.693658 H 5.151013 -1.768365 -0.039702 H 3.040084 -2.982878 0.456690 C 0.511670 -1.854456 0.359067 C -0.700455 -1.811226 0.393815 C -1.973158 -1.207373 0.158655 C -3.202887 -1.651505 0.647399 C -1.881186 -0.014442 -0.597546 C -4.349338 -0.905905 0.399387 H -3.247866 -2.569310 1.224231 C -3.038413 0.726491 -0.809560 C -4.265882 0.284912 -0.319439 H -5.307407 -1.247608 0.778171 H -2.967716 1.665686 -1.350525 H -5.159573 0.875285 -0.496177 C -0.559765 0.402912 -1.220638 N 0.502604 0.794068 -0.268845 H -0.159080 -0.422280 -1.814735 H -0.741841 1.253657 -1.883558 C 0.261942 1.962841 0.427708 O -0.731419 2.626376 0.188166 C 1.239576 2.360256 1.516372 H 1.700126 1.498114 2.002256 H 2.035686 2.988962 1.107396 H 0.681126 2.951026 2.242812 \_\_\_\_\_

#### TS5

G(Water) = -1921.308232 Hartree

C -0.233042 -1.504886 0.742980 C 0.890835 -2.332146 0.982926 C 0.764332 -3.460456 1.791961 C -0.449529 -3.772702 2.393931 C -1.558950 -2.964122 2.163983 C -1.450375 -1.845004 1.346774 H 1.631852 -4.098688 1.932966 H -0.528156 -4.651223 3.026389 H -2.514894 -3.198511 2.621534 H -2.325177 -1.224751 1.179579 C -0.052750 -0.353498 -0.110431 C 0.711399 0.280998 -0.878874 C 1.924460 0.354802 -1.663413 C 2.340718 1.509683 -2.339804 C 2.723728 -0.807641 -1.722677 C 3.542417 1.521691 -3.037350 H 1.710542 2.390715 -2.324968 C 3.932631 -0.769439 -2.409036 C 4.345841 0.385319 -3.066361 H 3.850573 2.422792 -3.558339 H 4.558445 -1.656867 -2.409979 H 5.291402 0.397058 -3.599261 C 2.250017 -2.093799 -1.090763 N 2.135271 -2.031901 0.375101 H 1.258566 -2.351061 -1.473464 H 2.944383 -2.899133 -1.349993 C 3.322950 -1.911978 1.068801 O 4.385952 -1.910829 0.474134

C 3.247798 -1.735154 2.572751 H 3.294629 -2.705676 3.075221 H 4.124505 -1.158880 2.869810 H 2.334393 -1.228548 2.890036 N -0.896078 2.147807 -0.043811 N -0.339238 2.059391 -1.245237 O -1.307841 1.431784 -2.010398 C -2.202123 0.804044 -1.208353 C -1.775929 1.140215 0.134788 N -3.123545 0.107654 -1.773313 S -4.385661 -0.460117 -0.869964 O -4.165273 -0.246021 0.567875 O -5.624989 0.019848 -1.452831 H -2.335249 1.001894 1.044945 C -4.249152 -2.206896 -1.184455 H -5.091443 -2.685427 -0.681438 H -4.301871 -2.364219 -2.261722 H -3.301072 -2.565401 -0.779127 C -0.188582 2.810408 0.999886 C 1.181803 3.026422 0.886665 C -0.903294 3.219323 2.123279 C 1.842536 3.664854 1.932015 H 1.721118 2.690979 0.007746 C -0.224958 3.846863 3.160861 H -1.976821 3.066328 2.173705 C 1.146968 4.071535 3.067125 H 2.911359 3.835473 1.856340 H -0.774444 4.169103 4.039112 H 1.671761 4.566414 3.877662

#### **General information**

All chemical reagents were obtained from commercial sources and used without further purification. TLC analysis was performed with silica gel-coated plates with 0.2 mm silica gel-coated HSGF 254 plates. Compounds were purified by column chromatography on silica gel (200-300 mesh) or neutrality Al<sub>2</sub>O<sub>3</sub> (200-300 mesh).

<sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker Avance III HD 500 spectrometer. Chemical shifts for <sup>1</sup>H NMR spectra are reported as  $\delta$  in units of parts per million (ppm) downfield from SiMe<sub>4</sub> ( $\delta$  0.0) and relative to the signal of chloroform-d ( $\delta$  7.26, singlet) or dimethyl sulfoxide- $d_6$  (DMSO- $d_6$ ) ( $\delta$  2.50, quintet). Multiplicities were given as: s (singlet); d (doublet); t (triplet); q (quartet); dd (doublets of doublet) or m (multiplets). The number of protons (n) for a given resonance is indicated by n H. Coupling constants are reported as a J value in Hertz. Chemical shifts for  ${}^{13}C$  NMR spectra are reported as  $\delta$  in units of parts per million (ppm) downfield from SiMe<sub>4</sub> ( $\delta$ 0.0) and relative to the signal of chloroform-d ( $\delta$  77.16, triplet), or dimethyl sulfoxide $d_6$  (DMSO- $d_6$ ) ( $\delta$  39.52, heptet). High-resolution mass spectra (HRMS) analyses were performed on an Agilent quadrupole time flight high resolution mass spectrometer mass spectrometer (6540 Q-TOF LC/MS). Liquid chromatogram was detected by Shimadzu HPLC (LC-20AD, SPD-M20A detector). Analyses were performed using an ACE Excel 5 AQ column ( $4.6 \times 250$  mm, 5 µm). Samples were prepared by semi-preparative RP-HPLC (LC-20A, SIL-10AP, SPD-20A detector, Shimadzu) with ACE Excel 5 AQ column ( $10 \times 250$  mm, 5 µm). Fluorescence spectra were recorded at room temperature by LS55 (PE, America) spectrophotometer from molecular devices using a cuvette with 1 nm path length. Fluorescence microscopy images were taken on a: Zeiss LSM 710 laser scanning confocal microscope.

#### **Organic synthesis**

Ethyl 4-((cyanomethyl)amino)benzoate (6). To a solution of ethyl 4-aminobenzoate (5) (2.0 g, 12.1 mmol, 1 equiv.) in toluene (25 mL) was added KI (200 mg, 1.21 mmol, 0.1 equiv.), TEA (4.9 g, 48.4 mmol, 4.0 equiv.) and chloroacetonitrile (1.37 g, 18.2 mmol, 1.5 equiv.) successively. The mixture was refluxed for 48 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate and water. The organic layer was separated and washed with brine solution, dried over sodium sulfate, concentrated under reduced pressure and recrystallization to give the desired product 6 (2.27 g, 92%).

<sup>1</sup>**H** NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  7.96 (d, J = 8.8 Hz, 2H), 6.69 (d, J = 8.8 Hz, 2H), 4.33 (q, J = 7.1 Hz, 2H), 4.15 (s, 2H), 1.37 (t, J = 7.1 Hz, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  166.7, 149.3, 131.4, 120.8, 116.7, 112.2, 60.5, 31.7, 14.2; **HRMS** (ESI<sup>+</sup>): calcd. for C<sub>11</sub>H<sub>12</sub>N<sub>2</sub>NaO<sub>2</sub><sup>+</sup> [M+Na]<sup>+</sup>: 227.0791, found: 227.0789.



**5-amino-3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium chloride (7).** To a solution of ethyl 4-((cyanomethyl)amino)benzoate (6) (2.27 g, 11.1 mmol, 1.0 equiv.) in dry THF (30 mL) was added isoamyl nitrite (1.95 g, 30 mmol, 1.5 equiv.). The mixture was stirred for 2 hours at room temperature. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure. Then a solution of 4 M HCl in dioxane (5 mL) was added to the mixture and the reaction was stirred 24 hours at room temperature. The precipitate was collected by filtration and washed with dry diethyl ether. The desired product **7** is obtained (808 mg, 27%).

<sup>1</sup>**H NMR** (500 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  9.93 (s, 2H), 8.75 (s, 1H), 8.29 (d, *J* = 8.7 Hz, 2H), 8.20 (d, *J* = 8.7 Hz, 2H), 4.39 (q, *J* = 7.1 Hz, 2H), 1.36 (t, *J* = 7.1 Hz, 3H); <sup>13</sup>**C NMR** (125 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  169.6, 164.3, 135.8, 134.1, 131.0, 123.3, 102.8, 61.7, 14.1; **HRMS** (ESI<sup>+</sup>): calcd. for C<sub>11</sub>H<sub>12</sub>N<sub>3</sub>O<sub>3</sub><sup>+</sup> [M–Cl<sup>-</sup>]<sup>+</sup>: 234.0873, found: 234.0877.



((3-chloropropyl)sulfonyl)(3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)amide (8). To a solution of 5-amino-3-(4-(ethoxycarbonyl)phenyl)-1,2,3oxadiazol-3-ium chloride (7) (500 mg, 1.85 mmol, 1.0 equiv.) in dry DCM (10 mL) was added 3-Chloropropanesulfonyl chloride (393 mg, 2.22 mmol, 1.2 equiv.) and cooled to -30 °C. The mixture was stirred and added a solution of TEA (468 mg, 4.63 mmol, 2.5 equiv.) in dry DCM (5 mL) slowly. Then kept the temperature for 5 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure. The crude product was purified by flash chromatography on neutrality  $Al_2O_3$  (PE:EtOAc = 1:1) to give the desired product **8** (622 mg, 90%).

<sup>1</sup>**H NMR** (500 MHz, CDCl<sub>3</sub>)  $\delta$  8.33 (d, J = 8.5 Hz, 2H), 7.90 (d, J = 8.6 Hz, 2H), 7.86 (s, 1H), 4.45 (q, J = 7.1 Hz, 2H), 3.72 (t, J = 6.3 Hz, 2H), 3.33 – 3.26 (m, 2H), 2.42 – 2.31 (m, 2H), 1.44 (t, J = 7.1 Hz, 3H); <sup>13</sup>**C NMR** (125 MHz, CDCl<sub>3</sub>)  $\delta$  170.5, 164.4, 136.4, 135.3, 132.0, 121.8, 100.8, 62.3, 51.7, 43.2, 27.3, 14.4; **HRMS** (ESI<sup>+</sup>): calcd. for C<sub>14</sub>H<sub>17</sub>ClN<sub>3</sub>O<sub>5</sub>S<sup>+</sup> [M+H]<sup>+</sup>: 374.0572, found: 374.0567.



(3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-(methyl(phenyl) amino)propyl)sulfonyl)amide (9) To a mixture of ((3-chloropropyl)sulfonyl)(3-(4- (ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)amide (8) (530 mg, 1.41 mmol, 1.0 equiv.) in toluene (20 mL) was added successively KI (23.2 mg, 0.14 mmol, 0.1 equiv.), TEA (712 mg, 7.05 mmol, 5.0 equiv.) and *N*-methylaniline (302 mg, 2.82 mmol,

2.0 equiv.). The mixture was stirred at 120 °C for 24 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was filtrated and concentrated under reduced pressure. The crude product was purified by flash chromatography on neutrality  $Al_2O_3$  (MeOH:DCM = 1:100) to give the desired product **9** (250 mg, 40%).

<sup>1</sup>**H NMR** (500 MHz, CDCl<sub>3</sub>)  $\delta$  8.33 (d, *J* = 8.8 Hz, 2H), 7.86 (d, *J* = 8.8 Hz, 2H), 7.80 (s, 1H), 7.21 (dd, *J* = 8.8, 7.3 Hz, 2H), 6.72 (d, *J* = 8.1 Hz, 2H), 6.69 (t, *J* = 7.3 Hz, 1H), 4.46 (q, *J* = 7.1 Hz, 2H), 3.49 (t, *J* = 7.2 Hz, 2H), 3.24 – 3.14 (m, 2H), 2.94 (s, 3H), 2.23 – 2.13 (m, 2H), 1.44 (t, *J* = 7.1 Hz, 3H); <sup>13</sup>**C NMR** (125 MHz, CDCl<sub>3</sub>)  $\delta$  170.4, 164.4, 149.2, 136.4, 135.2, 131.9, 129.4, 121.8, 116.7, 112.5, 100.7, 62.3, 52.1, 51.2, 38.4, 21.7, 14.4; **HRMS** (ESI<sup>+</sup>): calcd. for C<sub>21</sub>H<sub>25</sub>N<sub>4</sub>O<sub>5</sub>S<sup>+</sup> [M+H]<sup>+</sup>: 445.1540, found: 445.1541.



(3-(4-((3-((tert-butoxycarbonyl)amino)propyl)carbamoyl)phenyl)-1,2,3-oxadiazol -3-ium-5-yl)((3-(methyl(phenyl)amino)propyl)sulfonyl)amide (11). To a solution of (3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-(methyl(phenyl)amino) propyl)sulfonyl)amide (9) (200 mg, 0.45 mmol, 1.0 equiv.) in THF (10 mL)was added a solution of LiOH (58.9 mg, 2.25 mmol, 5.0 equiv.) in water (2 mL) at room temperature and stirred for 3 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure and poured into water (5 mL), adjusted the pH to 1-2 with 1 M HCl aqueous solution, then extracted with dichloromethane. The organic layer was separated and washed with brine solution, dried over sodium sulfate and concentrated under reduced pressure to give desired product 10. The crude product 10 was used directly for the next step without any purification process.

A solution of **10** (100 mg, 0.24 mmol, 1 equiv.) in DMF (5 mL) was added HATU (137 mg, 0.36 mmol, 1.5 equiv.) and DIPEA (124 mg, 0.96 mmol, 4.0 equiv.) and stirred for

15 minutes at room temperature. Then *N*-Boc-1,3-diaminopropane (84 mg, 0.48 mmol, 2.0 equiv.) was added and stirred for 5 hours. After the starting material was consumed completely which was detected by TLC, water was added to quench the reaction and extracted with EtOAc. The organic layer was separated and washed with brine solution, dried over sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash chromatography on neutrality  $Al_2O_3$  (MeOH:DCM = 1:50) to give the desired product **11** (120 mg, 87%).

<sup>1</sup>**H NMR** (500 MHz, CDCl<sub>3</sub>)  $\delta$  8.19 (d, J = 8.4 Hz, 2H), 8.02 (s, 1H), 7.85 (d, J = 8.6 Hz, 2H), 7.79 (s, 1H), 7.21 (dd, J = 8.5, 7.4 Hz, 2H), 6.72 (d, J = 8.2 Hz, 2H), 6.68 (t, J = 7.3 Hz, 1H), 4.87 (s, 1H), 3.53 (dd, J = 11.9, 6.1 Hz, 2H), 3.49 (t, J = 7.2 Hz, 2H), 3.29 (d, J = 5.6 Hz, 2H), 3.22 – 3.14 (m, 2H), 2.93 (s, 3H), 2.22 – 2.12 (m, 2H), 1.77 – 1.71 (m, 2H), 1.47 (s, 9H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  170.5, 164.6, 157.7, 149.2, 139.5, 135.2, 129.6, 129.4, 121.8, 116.7, 112.5, 100.6, 80.3, 52.1, 51.2, 38.4, 37.0, 36.1, 30.0, 28.5, 21.8; HRMS (ESI<sup>+</sup>): calcd. for C<sub>27</sub>H<sub>37</sub>N<sub>6</sub>O<sub>6</sub>S<sup>+</sup> [M+H]<sup>+</sup>: 573.2490, found: 573.2491.



(3-(4-((3-ammoniopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-

(methyl(phenyl)amino)propyl)sulfonyl)amide 2,2,2-trifluoroacetate (4) A solution of 11 in DCM was added CF<sub>3</sub>COOH, the mixture was stirred for 3 hours at room temperature. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure to give desired product 4. The crude product 4 was used directly for the next step without any purification process.

**HRMS** (ESI<sup>+</sup>): calcd. for  $C_{22}H_{29}N_6O_4S^+$  [M–CF<sub>3</sub>COO<sup>-</sup>]<sup>+</sup>: 473.1966, found: 473.1981.



(3-(4-((3-aminopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-((4-((*E*)-(2,5-dimethoxy-4-((*E*)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl) amino)propyl)sulfonyl)amide (13) Fast black K salt hemi (zinc chloride) salt (dye content ~30%) 12 (24 mg, 28.6 µmol, 1.2 equiv.) was dissolved in a mixture of 130 µL NaOAc buffer (0.1 M, pH 4.0) and 130 µL MeCN. The resulting mixture was stirred at r.t. for 10 min, and then sonicated to get near complete solubilization. In another roundbottom flask, the compound 4 (14 mg, 23.9 µmol, 1.0 equiv.) was dissolved in MeCN (60 µL) and cooled to 0 °C. The diazonium salt solution was directly filtrated and added to the solution of compound 4 and the resulting mixture was stirred at r.t. for 2 h. The fine precipitate newly formed was recovered by filtration and washed twice with a mixture of deionized water and MeCN (1:1, v/v). The resulting solid was then dried by lyophilization to afford the desired product 13 (11.7 mg, 52%).

<sup>1</sup>**H NMR** (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.95 (t, *J* = 5.5 Hz, 1H), 8.44 (dd, *J* = 10.8, 5.7 Hz, 2H), 8.17 (q, *J* = 9.2 Hz, 4H), 8.07 (d, *J* = 9.0 Hz, 1H), 7.85 – 7.73 (m, 4H), 7.45 (s, 1H), 7.37 (s, 1H), 6.91 (d, *J* = 9.1 Hz, 1H), 4.00 (s, 3H), 3.95 (s, 3H), 3.64 (dd, *J* = 16.6, 9.0 Hz, 4H), 3.18 (dd, *J* = 14.8, 6.8 Hz, 2H), 3.09 (s, 3H), 2.88 (d, *J* = 11.1 Hz, 2H), 2.08 – 1.96 (m, 2H), 1.89 – 1.80 (m, 2H). **HRMS** (ESI<sup>+</sup>): calcd. for C<sub>36</sub>H<sub>40</sub>N<sub>11</sub>O<sub>8</sub>S<sup>+</sup> [M+H]<sup>+</sup>: 786.2777, found: 786.2774.



(3-(4-((3-(4-(3,6-bis(dimethylamino)xanthylium-9-yl)-3-carboxylatobenzamido) propyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-((4-((*E*)-(2,5-dimethoxy -4-((*E*)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)propyl) sulfonyl)amide (1a) A solution of TAMRA (7.8 mg, 17.4 µmol, 1.1 equiv.) in DMF (4 mL) was added DIPEA (20.7 mg, 0.16 mmol, 10.0 equiv.) and TSTU (5.2 mg, 17.4 µmol, 1.1 equiv.) in sequence and stirred at room temperature for an hour. After TAMRA was consumed completely which was detected by MS, 13 (12.4 mg, 15.8 µmol, 1.0 equiv.) was added into the reaction mixture and stirred for another 5 hours in the dark. DMF was removed under reduced pressure and the dye-conjugate was purified by semi-preparative RP- HPLC with 80% to 100% MeOH in water over 20 minutes at a flow rate of 5 mL/min. Visible detection was achieved at  $\lambda = 254$  nm. The product-containing fractions were lyophilized to give 1a (6.6 mg, 35%).

**HRMS** (ESI<sup>+</sup>): calcd. for  $C_{61}H_{60}N_{13}O_{12}S^+$  [M+H]<sup>+</sup>: 1198.4200, found: 1198.4196.



Figure S3. HRMS of 1a.



Figure S4. HPLC trace of purified 1a using a gradient of 70% to 100% MeOH in water over 30 min (548 nm).



(*E*)-(3-(4-((3-aminopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-((4-((7-(diethylamino)-5-phenylphenazin-5-ium-3-yl)diazenyl)phenyl)(methyl)amino) propyl)sulfonyl)amide chloride (15) Methylene Violet 3RAX 14 (24.3 mg, 0.064 mmol, 1.0 equiv.) was dissolved in H<sub>2</sub>O (1 mL), then added 200µL concentrated hydrochloric acid and cooled by ice-salt bath. The solution was added a solution of NaNO<sub>2</sub> (4.8 mg, 0.07 mmol, 1.1 equiv.) in H<sub>2</sub>O (1 mL), and stirred for 30 min until the reaction mixture turned dark blue. Solid NaOAc (280 mg) was added and adjusted the pH to 7-8 to preform *N*-nitrosamine/diazonium salt intermediate. A solution of 4 (37.5 mg, 0.064 mmol, 1.0 equiv.) in THF (5 mL) was slowly added into the preformed *N*nitrosamine/diazonium salt intermediate and stirred for a further 5 hours. The reaction mixture was concentrated under reduced pressure and the crude product was purified by semi-preparative RP-HPLC with 70% MeOH in 0.1% TFA distilled water as eluents at a flow rate of 5 mL/min. Visible detection was achieved at  $\lambda = 254$  nm. The productcontaining fractions were lyophilized to give **15** (17 mg, 50%).

<sup>1</sup>**H NMR** (500 MHz, MeOD)  $\delta$  8.28 (d, J = 9.0 Hz, 1H), 8.22 (s, 1H), 8.15 – 8.03 (m, 6H), 7.96 – 7.92 (m, 3H), 7.87 (dd, J = 9.9, 2.6 Hz, 1H), 7.70 – 7.64 (m, 4H), 7.24 (d, J = 1.9 Hz, 1H), 6.82 (d, J = 9.3 Hz, 2H), 5.83 (d, J = 2.5 Hz, 1H), 3.81 (s, 2H), 3.73 – 3.66 (m, 2H), 3.50 (t, J = 6.7 Hz, 2H), 3.42 (s, 2H), 3.23 (t, J = 7.2 Hz, 2H), 3.13 (s, 3H), 3.02 (t, J = 7.4 Hz, 2H), 2.22 – 2.13 (m, 2H), 2.02 – 1.94 (m, 2H), 1.34 (s, 3H), 1.08 (s, 3H). **HRMS** (ESI<sup>+</sup>): calcd. for C<sub>44</sub>H<sub>48</sub>N<sub>11</sub>O<sub>4</sub>S<sup>+</sup> [M–Cl<sup>-</sup>]<sup>+</sup>: 826.3606, found: 826.3624.



((3-((4-((*E*)-(7-(diethylamino)-5-phenylphenazin-5-ium-3-yl)diazenyl)phenyl) (methyl)amino)propyl)sulfonyl)(3-(4-((3-(6-(3,3-dimethyl-5-sulfonato-2-((1*E*,3*E*)-5-((*E*)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1ium-1-yl)hexanamido)propyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl) amide chloride (1b) A solution of Cy5 (5 mg, 7.5 µmol, 1.0 equiv.) in DMF (2 mL) was added DIPEA (9.7 mg, 75 µmol, 10.0 equiv.) and TSTU (2.5 mg, 8.3 µmol, 1.1 equiv.) in sequence and stirred at room temperature for an hour. After Cy5 was consumed completely which was detected by MS, 15 (7.78 mg, 8.3 µmol, 1.1 equiv.) was added into the reaction mixture and stirred for another 5 hours in the dark. DMF was removed under reduced pressure and the dye-conjugate was purified by semi-preparative RP- HPLC with 45% MeCN in 0.1% TFA distilled water as eluents at a flow rate of 5 mL/min. Visible detection was achieved at  $\lambda = 254$  nm. The product-containing fractions were lyophilized to give 1b (9.8 mg, 84%).

**HRMS** (ESI<sup>+</sup>): calcd. for  $C_{76}H_{84}N_{13}O_{11}S_3^+$  [M–Cl<sup>-</sup>]<sup>+</sup>: 1450.5570, found: 1450.5561.







Figure S6. HPLC trace of purified 1b in 70% CH<sub>3</sub>OH/H<sub>2</sub>O over 20 min (254 nm).



2,5-dioxopyrrolidin-1-yl 5-(((2-methylcycloprop-2-en-1-yl)methyl)amino)-5oxopentanoate (1,3-Cp-NHS) (16) was prepared from cyclopropene alcohol according to the literature procedure<sup>[8]</sup>.



**Tetrazine-BODIPY 504 (Tz-BDP) (17)** was prepared in two steps from 4(aminomethyl)benzonitrile and formamidine acetate salt according to the literature procedure<sup>[9]</sup>.



**2,5-dioxopyrrolidin-1-yl4-(didehydrodibenzo[b,f]azocin-5(6H)-yl)-4-oxobutanoate (DIBAC-NHS) (19)** was prepared from DIBAC **2** according to theliterature procedure<sup>[10]</sup>.

Preparation of DIBAC/1,3-Cp-TG beads<sup>[11-13]</sup>



Scheme S1. Synthesis of DIBAC/1,3-Cp-TG beads.

TentaGel S NH<sub>2</sub> resin (29 mg, 7.25  $\mu$ mol, 130  $\mu$ m beads) was swollen in double distilled water for 24 hours. Water was removed by centrifuged and the beads were rapidly washed with DCM/Et<sub>2</sub>O (55:45) (2 × 2 mL).

#### **DIBAC/1,3-Cp-TG beads**

A solution of DIBAC-NHS 19 (2.8 mg, 7.25 µmol) in 2 mL DCM/Et<sub>2</sub>O (55:45) was

added to the beads, followed by DIPEA (2.5  $\mu$ L, 15  $\mu$ mol) and rotated in a plastic tube for 30 min to modify the outer layer of the beads. The beads were then centrifuged and washed with DCM/Et<sub>2</sub>O (55:45) (1 × 30 s), DCM (3 × 30 s) and DMF (5 × 30 s). Then swollen in 2 mL DMF then added 1,3-Cp-NHS **16** (2.1 mg, 7.25  $\mu$ mol) and DIPEA (2.5  $\mu$ L, 15  $\mu$ mol) successively. The beads were rotated for 1.5 h then centrifuged and washed with DMF (5 × 30 s). The beads were dried under vacuum to give **DIBAC/1,3-Cp-TG beads**.

#### **Kinetics measurement**

The kinetics of cycloadditions of sydnonimine **11** with DIBAC **2** were measured by HPLC in 1:1 DMSO/H<sub>2</sub>O at 25 °C. Stock solutions of sydnonimine **11**, DIBAC **2** and internal standard (2, 6-dichloropyridine) in DMSO were prepared. Prepared respective concentration solutions in 1:1 DMSO/H<sub>2</sub>O, leading to the final concentration of sydnonimine **11** was 50  $\mu$ M, DIBAC **2** and internal standard was 500  $\mu$ M. Reactions were monitored by the absorption peak area of sydnonimine **11** compared with the internal standard. Consumption of materials followed a second-order equation and the second-order rate constants were obtained by least squares fitting of the data to a linear equation.



Figure S7. Kinetic measurement of cycloaddition of sydnonimine 11 with DIBAC 2, measured second-order rate constant in DMSO/H<sub>2</sub>O is  $0.71 \pm 0.007 \text{ M}^{-1} \cdot \text{s}^{-1}$ . [A]—concentration of sydnonimine 11 (M).

## Mutually orthogonal labeling of two fluorogenic probes

Sydnonimine 11 (2 µmol) and 1,3-Cp-NHS 16 (2 µmol) were dissolved in 500 µL DMSO- $d_6/D_2O$  (9:1, V/V). The mixture was thoroughly mixed, incubated at room temperature and monitored by <sup>1</sup>H NMR several time points. After 24 hours, the components signals stayed the same.



8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6 6.5 6.4 6.3 6.2 6.1 6.0 5.9 5.8 fl(ppm)

Figure S8. <sup>1</sup>H NMR analysis of sydnonimine 11 and 1,3-Cp-NHS 16 in DMSO-d<sub>6</sub>/D<sub>2</sub>O (9:1, V/V).

## **Fluorescence spectra**



Figure S9. Fluorescence emission spectra of 1a. Measurement conditions: 20  $\mu$ M in 1% DMSO/PBS, 25 °C and  $\lambda_{ex} = 559$  nm.



Figure S10. Fluorescence emission spectra of 1b. Measurement conditions: 20  $\mu$ M in 1% DMSO/PBS, 25 °C and  $\lambda_{ex} = 622$  nm.



Figure S11. Fluorescence emission spectra of **3a**. Measurement conditions: 20  $\mu$ M in 1% DMSO/PBS, 25 °C and  $\lambda_{ex} = 559$  nm.



Figure S12. Fluorescence emission spectra of 3b. Measurement conditions: 20  $\mu$ M in 1% DMSO/PBS, 25 °C and  $\lambda_{ex} = 622$  nm.

## Confocal fluorescent microscope imaging

A small portion of the beads (2 mg) was added in 25  $\mu$ L CH<sub>3</sub>CN/H<sub>2</sub>O (1:1) in a plastic tube, followed by 10  $\mu$ L **1b** (1 mM) and 10  $\mu$ L **17** (1 mM) in DMSO. After 20 min, the beads were imaged under a Leica confocal microscope with corresponding filters without washing.

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# NMR spectra











