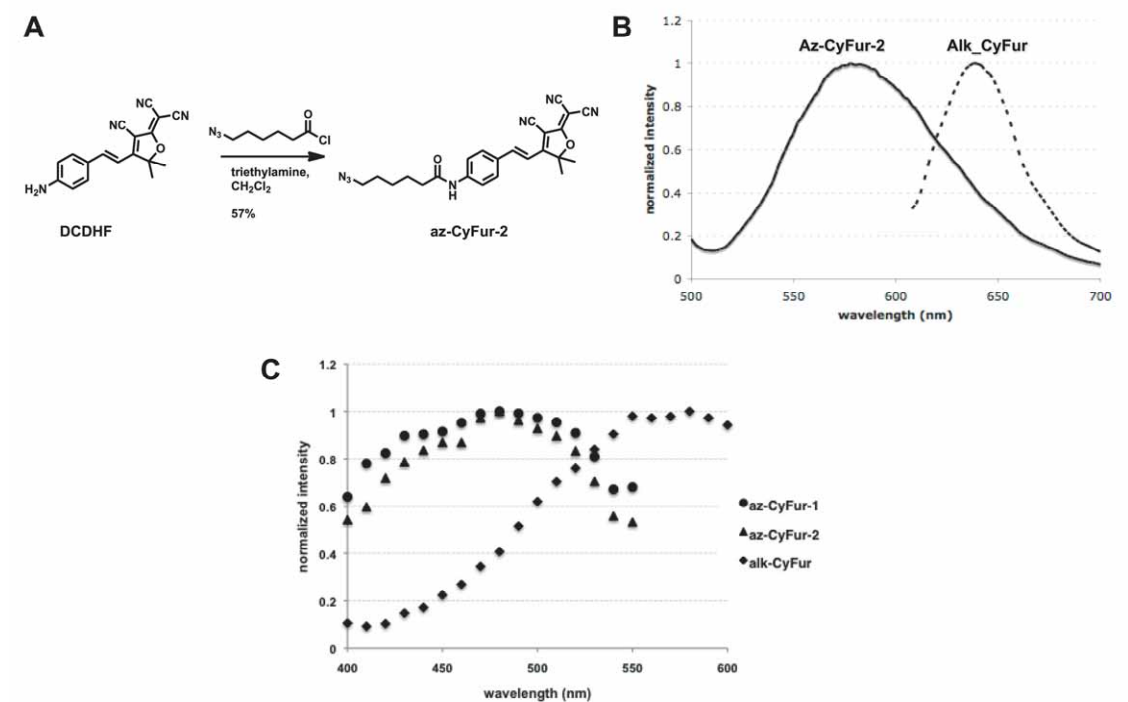


Supporting Information for:
Clickable Fluorescent Dyes for Multimodal Bioorthogonal Imaging

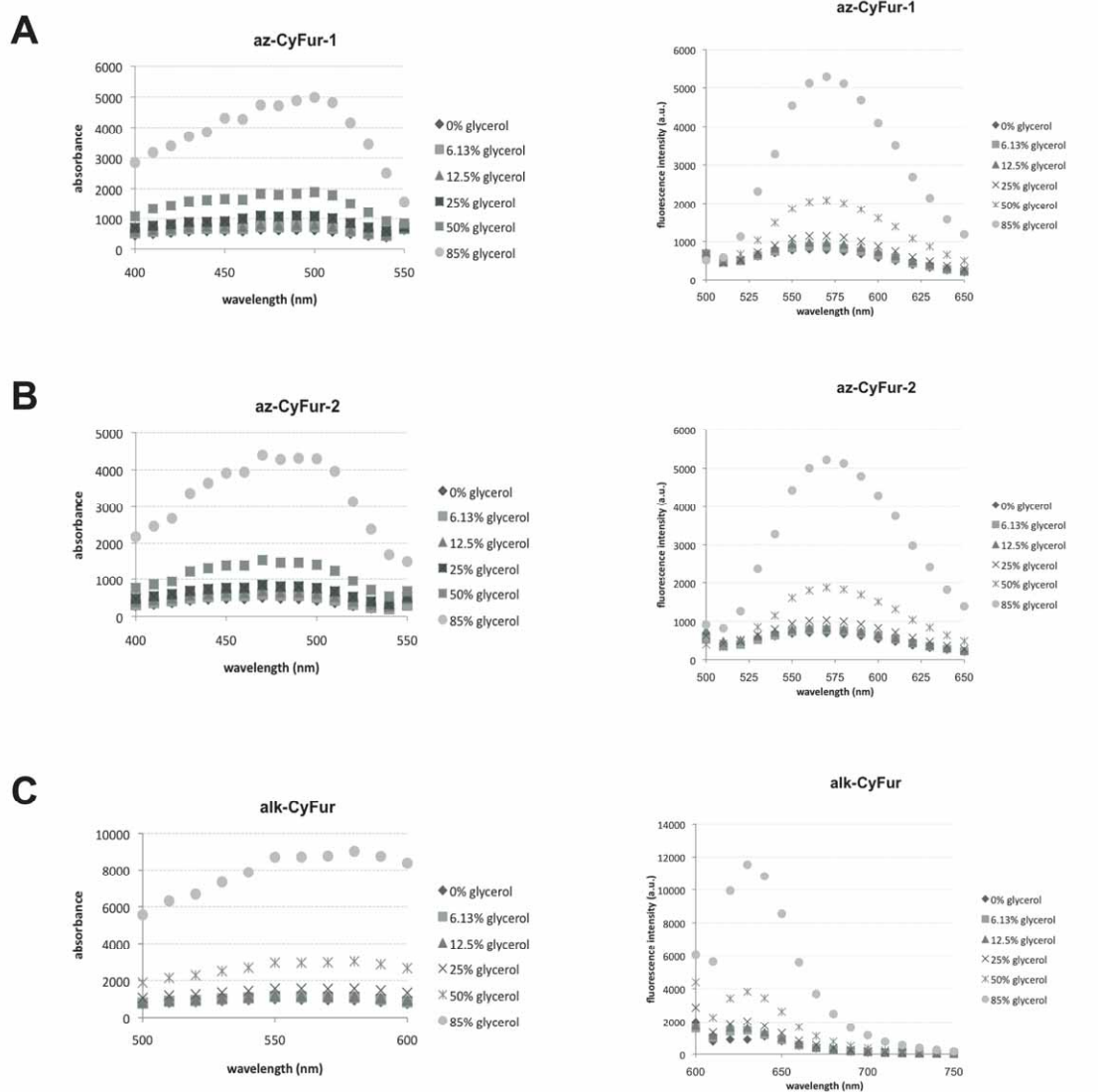
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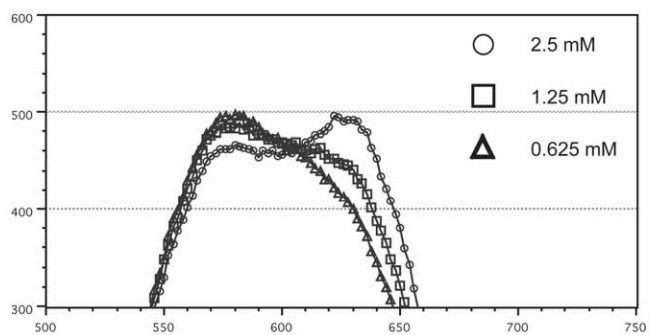
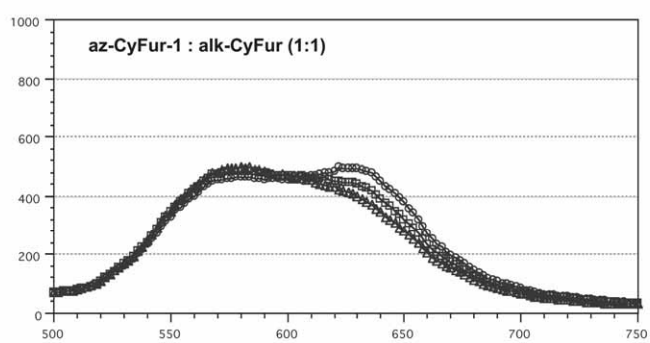
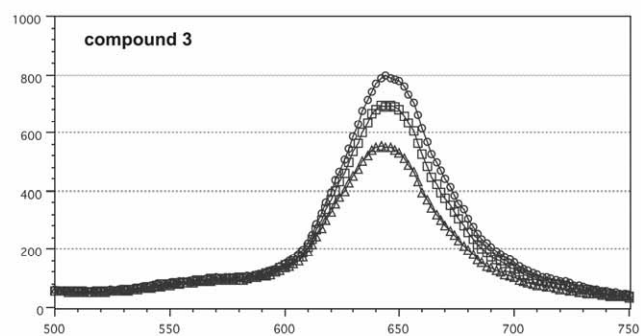
Supporting Information Figures



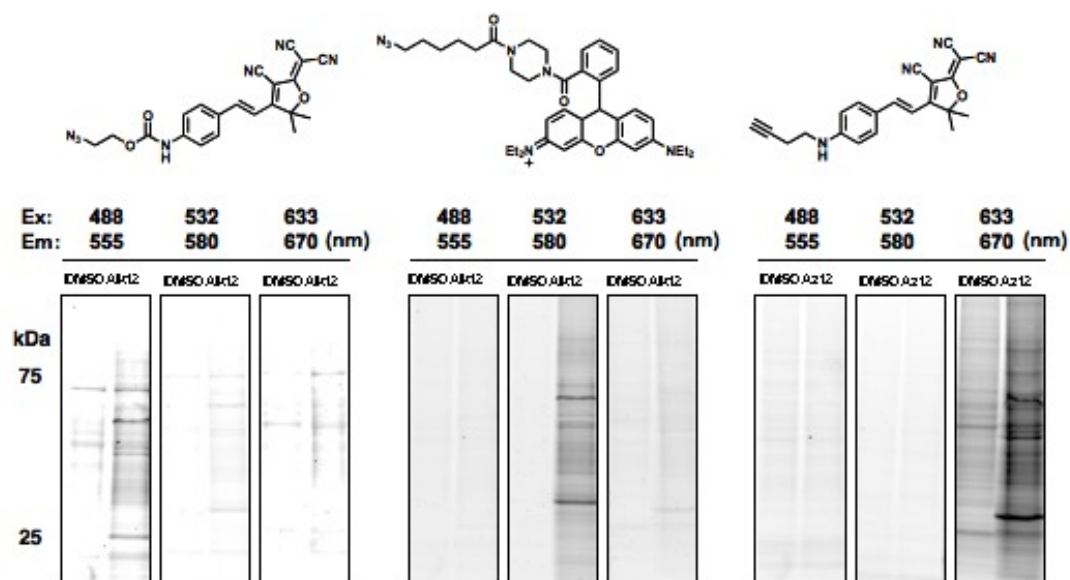
Supporting Information Fig. S1. Synthesis and spectral properties of az-CyFur-2. (A) Synthesis of az-CyFur-2. (B) Spectral properties of az-CyFur-2. (C) Absorbance spectra of az-CyFur-1, az-CyFur-2 and alk-CyFur.



Supporting Information Fig. S2. Spectra of CyFurs (2.5×10^{-4} M) in glycerol/methanol solutions with increasing viscosity. Absorption and fluorescence spectra ($\lambda_{\text{ex}} = 470$ nm) of az-CyFur-1 (A), az-CyFur-2 (B). (C) Absorption and fluorescence spectra ($\lambda_{\text{ex}} = 580$ nm) of alk-CyFur.



Supporting Information Fig. S3. Concentration-dependent fluorescent properties of clicked fluorophore **3** in comparison of az-CyFur-1:alk-CyFur (1:1) mixture.



Supporting Information Fig. S4. In-gel fluorescence imaging of alk-12-modified proteins from metabolically labeled Jurkat T cells after bioorthogonal ligation with az-CyFur-2. While detection of alk-12-modified proteins with az-CyFur labeling appeared less efficient, available settings on the 9400 Typhoon gel scanner is not optimal for visualization of acylated-DCDHF dyes (Fig. 1 and Supporting Information Fig. 1B) and would likely be better with more appropriate filter sets.

Metabolic labeling and preparation of cell lysates.

Jurkat T cells and HeLa cells were cultured and metabolically labeled with DMSO, azido-fatty acids (az-12 and az-15) or alkynyl-fatty acids (alk-12) as previously described¹. Jurkat T cell lysates used for protein labeling studies were prepared as previously described¹.

Cu^I-catalyzed Huisgen [3+2] cycloaddition/click chemistry.

Cell lysates (50 µg) in 44.5 µL of buffer (150 mM NaCl, 50 mM triethanolamine pH 7.4, 4% SDS) were reacted with freshly prepared click chemistry reaction cocktail: [azido- or alkynyl-CyFurs or az-rho (100 µM, 5 mM stock solution in DMSO), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in deionized water), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA) (100 µM, 2 mM stock solution in DMSO) and CuSO₄ ·5H₂O (1 mM, 50 mM freshly prepared stock solution in deionized water)] for a total reaction volume of 50 µL for 1 h at room temperature. Following methanol-chloroform precipitation, the protein pellet was redissolved in 18 µL of buffer (4% SDS, 50 mM triethanolamine pH 7.4, 150 mM NaCl) and separated by SDS-PAGE¹.

In-gel Fluorescence Scanning. Proteins separated by SDS-PAGE were visualized by first soaking the gel in 40% MeOH, 10% acetic acid in water with shaking for 20 mins and directly scanning the gel on an Amersham Biosciences Typhoon 9400 variable mode imager. Proteins labeled by az-CyFur-1 were visualized with excitation at 488 nm and 555 nm emission filter with 30 nm band-pass. Proteins labeled by az-Rho were

visualized with excitation at 532 nm and 580 nm emission filter with 30 nm band-pass. Proteins labeled by alk-CyFur were visualized with excitation at 633 nm and 670 nm emission filter with 30 nm band-pass.

Fluorescence imaging. Cells for fluorescence microscopy were prepared as previously reported.¹ Slides were mounted with Prolong Gold with DAPI from Invitrogen. Confocal images were collected using a Zeiss LSM 510 META laser scanning confocal microscope equipped with a C-Apochromat 40x/1.20 water objective. DAPI was excited at 405 nm with a Diode laser and emission was measured through a band-pass 420-480 nm filters. Az-CyFur-1 was excited with an argon laser at 488 nm and emission was collected at a LP560 nm filter. Az-rho was excited with a HeNe laser at 543 nm and emission was collected through a band-pass 560-615 nm filter. Alk-CyFur was excited with a HeNe laser at 633 nm, and emission was collected through a band-pass 646-753 nm filter.

Absorbance and fluorescence studies. Absorption spectra and fluorescence data were collected on SpectraMax M2 multi-detection reader (Molecular Devices). The spectra in solution were obtained at 25 °C using a quartz cuvette with a path length of 1 cm. Fluorescence quantum yields (Φ_F) of CyFur dyes were determined against cresyl violet ($\Phi_F = 0.54$ in methanol).

Chemical synthesis. All chemicals were obtained either from Sigma-Aldrich, MP Biomedicals, Alfa Aesar, TCI, Fluka or Acros and were used as received unless otherwise noted. The silica gel used in flash column chromatography was Fisher S704

(60-200 Mesh, Chromatographic Grade). Analytical thin layer chromatography (TLC) was conducted on Merck silica gel plates with fluorescent indicator on glass (5-20 μm , 60 Å) with detection by ceric ammonium molybdate, basic KMnO_4 or UV light. The ^1H and ^{13}C NMR spectra were obtained on a Bruker AVANCE-600 spectrometer equipped with a cryoprobe. Chemical shifts were reported in δ ppm values downfield from tetramethylsilane and J values were reported in Hz. MALDI-TOF mass spectra were obtained on an Applied Biosystems Voyager-DE. Literature procedures were followed for synthesis of the precursors *tert*-butyl-4-formylphenylcarbamate² and 3-cyano-2-dicyanomethylene-4,5,5-trimethyl-2,5-dihydrofuran³. *tert*-butyl-4-formylphenylcarbamate was isolated in 82% yield over 2 steps from commercially available 4-aminobenzylalcohol. 3-cyano-2-dicyanomethylene-4,5,5-trimethyl-2,5-dihydrofuran was isolated in 68% yield. $(\text{BimC}_4\text{A})_3$ was obtained following reported synthetic procedure⁴.

(E)-tert-butyl 4-(2-(4-cyano-5-(dicyanomethylene)-2,2-dimethyl-2,5-dihydrofuran-3-yl)vinyl)phenylcarbamate (1): 3-cyano-2-dicyanomethylene-4,5,5-trimethyl-2,5-dihydrofuran (500 mg, 2.5 mmol), *tert*-butyl-4-formylphenylcarbamate (555 mg, 2.5 mmol) and ammonium acetate (193 mg, 2.5 mmol) were dissolved in a mixture of THF (10 mL) and anhydrous EtOH (2.5 mL). The mixture was stirred overnight under argon in the dark at room temperature, turning from pale yellow to orange over the course of the reaction. The solution was diluted in water and extracted two times with 100 mL of ethyl acetate followed by 200 mL of brine wash and then dried over anhydrous Na_2SO_4 and filtered. Evaporation of the solvents under reduced pressure afforded crude product

that was purified by silica column chromatography using 2:1 hexanes:ethyl acetate ($R_f = 0.25$) as eluant to yield the final product as reddish-orange solid (726 mg, 72%). ^1H NMR (600 MHz, CD_2Cl_2): $\delta = 7.64$ (d, 2H, $J = 8.4$ Hz), 7.60 (d, 1H, $J = 16.3$ Hz), 7.52 (d, 2H, $J = 8.6$ Hz), 6.97 (d, 1H, $J = 16.4$ Hz), 1.78 (s, 6H), 1.51 (s, 9H); ^{13}C -NMR (125 MHz, CD_2Cl_2): $\delta = 176.3, 174.9, 152.4, 147.5, 143.6, 131.1, 128.7, 118.7, 113.6, 112.5, 111.9, 111.1, 99.1, 98.4, 81.8, 57.1, 28.4, 26.8$; MALDI-TOF: calcd. for $\text{C}_{23}\text{H}_{22}\text{N}_4\text{NaO}_3$ $[\text{M}+\text{Na}]^+$ 425.16, found 425.47.

(E)-2-(4-(4-aminostyryl)-3-cyano-5,5-dimethylfuran-2(5H)-ylidene)malononitrile (2):

To a 25 mL round bottom flask loaded with **1** (200 mg, 0.5 mmol), 10 ml of 20% TFA in dry CH_2Cl_2 was added. The mixture was stirred under argon at room temperature for 2 hrs. The solvent was removed under reduced pressure and dried on high vacuum overnight to give product as a purple solid (150 mg recovered, 99%). The product was used for subsequent reactions without further purification. ^1H NMR (600 MHz, CD_2Cl_2): $\delta = 7.60$ (d, 1H, $J = 16.0$ Hz), 7.53 (d, 2H, $J = 8.3$ Hz), 6.83 (d, 1H, $J = 16.0$ Hz), 6.72 (d, 2H, $J = 8.4$ Hz), 1.76 (s, 6H); ^{13}C -NMR (125 MHz, CD_2Cl_2): $\delta = 176.8, 175.4, 152.6, 148.9, 132.7, 124.5, 115.4, 112.9, 112.4, 111.7, 110.7, 98.0, 96.6, 55.8, 26.9$; MALDI-TOF: calcd. for $\text{C}_{18}\text{H}_{14}\text{N}_4\text{NaO}$ $[\text{M}+\text{Na}]^+$ 325.11, found 325.12.

(E)-2-(4-(4-(but-3-ynylamino)styryl)-3-cyano-5,5-dimethylfuran-2(5H)-

ylidene)malononitrile (alk-CyFur): To a 10 mL round bottom flask equipped with a condenser, **2** (30 mg, 0.099 mmol) and 98% NaH (9 mg, 0.4 mmol) were dissolved in dry DMF and stirred at 70°C under argon for 1 hr. 1-bromobutyne (0.131g, 0.99 mmol,

10 equiv.) was then added to the mixture and the temperature was increased to 100 °C for overnight stirring. Another 2 equivalence of NaH and 5 equivalence of 1-bromobutyne were added to the reaction mixture and allowed to stir for 1 hr at room temperature. The mixture was cooled to room temperature and quenched with 1 mL of MeOH. The reaction mixture was diluted with 200 mL of water and extracted twice with ethyl acetate (100 mL each time). The resulting organic layer was washed with 10% HCl, brine, dried with anhydrous Na₂SO₄ and concentrated to yield purple crude product. Silica gel chromatography was used to purify the title compound, eluting with 1:1 ethyl acetate:hexanes (R_f = 0.5) as the mobile phase to give product as purple solid (25 mg, 72%). ¹H NMR (600 MHz, CD₂Cl₂): δ = 7.62 (d, 1H, *J* = 16.0 Hz), 7.56 (d, 2H, *J* = 8.7 Hz), 6.81 (d, 1H, *J* = 16.0 Hz), 6.69 (d, 2H, *J* = 8.7 Hz), 3.44 (t, 2H, *J* = 6.6 Hz), 2.55 (dt, 2H, *J* = 2.6 Hz, *J* = 6.6 Hz), 2.12 (t, 1H, *J* = 2.6 Hz), 1.76 (s, 6H). ¹³C-NMR (125 MHz, CD₂Cl₂): δ = 176.9, 175.3, 152.9, 148.9, 132.8, 124.0, 113.6, 113.0, 112.5, 111.9, 110.2, 97.8, 81.4, 70.9, 55.4, 42.3, 30.2, 27.0, 19.5; MALDI-TOF: calcd. for C₂₂H₁₈N₄NaO [M+H]⁺ 355.15, found 355.42.

(*E*)-2-azidoethyl 4-(2-(4-cyano-5-(dicyanomethylene)-2,2-dimethyl-2,5-dihydrofuran-3-yl)vinyl)phenylcarbamate (az-CyFur-1): To a stirred solution on ice of 1:1 mixture of dry CH₂Cl₂:THF (10 ml) containing **2** (5 mg, 0.017 mmol), triphosgene (14 mg, 0.05 mmol), anhydrous pyridine (12 µL, 0.15 mmol) was added. The mixture was stirred under argon for 2 hrs and the volume was reduced under argon to half to get rid of excess phosgene (Caution: perform in the properly working hood when working with larger scale). Azidoethanol (50 µL, 0.57 mmol, 35 equiv.) with 10 equiv. of

triethylamine (20 μ L) were then added to the solution, which then turned from yellow to reddish-orange. After stirring for another 2 hr, the solvent was diluted with 100 mL of water, extracted twice with 50 mL of CH_2Cl_2 , washed with 1% HCl and then brine. The organic layer was dried over anhydrous Na_2SO_4 , filtered, and concentrated under pressure. The crude product was purified by column chromatography on silica gel using 3:2 hexane:acetone ($R_f = 0.4$) as the mobile phase to give product as orange solid (4.6 mg, 65%). ^1H NMR (600 MHz, CD_2Cl_2): $\delta = 7.67$ (d, 2H, $J = 8.7$ Hz), 7.62 (d, 1H, $J = 16.4$ Hz), 7.56 (d, 2H, $J = 8.6$ Hz), 7.08 (br, 1H), 6.99 (d, 1H, $J = 16.4$ Hz), 4.35 (t, 2H, $J = 5.0$ Hz), 3.56 (t, 2H, $J = 5.0$ Hz), 1.78 (s, 6H). ^{13}C -NMR (125 MHz, CD_2Cl_2): $\delta = 176.3, 174.9, 152.9, 147.3, 142.7, 131.1, 129.7, 119.3, 114.3, 112.4, 111.9, 111.1, 99.8, 98.5, 64.7, 50.7, 26.8, 25.8$; MALDI-TOF: calcd. for $\text{C}_{21}\text{H}_{17}\text{N}_7\text{NaO}_3$ $[\text{M}+\text{Na}]^+$ 438.13, found 438.35.

(E)-6-azido-N-(4-(2-(4-cyano-5-(dicyanomethylene)-2,2-dimethyl-2,5-dihydrofuran-3-yl)vinyl)phenyl)hexanamide (az-CyFur-2): One drop of DMF (catalytic) was added to a stirred solution at room temperature of 6-azidohexanoic acid (10 mg, 0.063 mmol) and 20 equiv. of oxalyl chloride in dry CH_2Cl_2 . Reaction mixture was then concentrated under pressure and placed on high vacuum for 30 minutes. A solution of dry CH_2Cl_2 (5 mL) containing **2** (5 mg, 0.017 mmol) and 10 equiv. of triethylamine (20 μ L) was then added to the flask containing the activated 6-azidohexanoyl chloride. After 1 hr reaction time, the solvent was diluted with 100 mL of water, extracted twice with washed with 1% HCl (50 mL) and then brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4 , filtered, and concentrated under pressure. The crude product was purified by

silica gel with 3:2 hexanes:acetone ($R_f = 0.4$) as eluant to provide product as orange solid (4.2 mg, 57%). ^1H NMR (600 MHz, CD_2Cl_2): $\delta = 7.71$ (q, 4H, $J = 9$ Hz, $J = 6.2$ Hz), 7.62 (d, 1H, $J = 16.4$ Hz), 7.44 (br, 1H), 7.03 (d, 1H, $J = 16.4$ Hz), 3.33 (t, 2H, $J = 6.8$ Hz), 2.43 (t, 2H, $J = 7.4$ Hz), 1.82 (s, 6H), 1.80-1.75 (m, 2H), 1.69-1.65 (m, 2H), 1.51-1.48 (m, 2H). ^{13}C -NMR (125 MHz, CD_2Cl_2): $\delta = 191.3, 176.2, 174.8, 147.2, 142.9, 130.9, 120.1, 114.3, 112.4, 112.0, 111.8, 111.0, 99.7, 98.4, 51.8, 37.9, 29.1, 26.8, 25.8, 25.3, 25.2$; MALDI-TOF: calcd. for $\text{C}_{24}\text{H}_{23}\text{N}_7\text{NaO}_2$ $[\text{M}+\text{Na}]^+$ 464.18, found 464.33.

2-(4-(2-(4-((*E*)-2-(4-cyano-5-(dicyanomethylene)-2,2-dimethyl-2,5-dihydrofuran-3-yl)vinyl)phenylamino)ethyl)-1*H*-1,2,3-triazol-1-yl)ethyl 4-((*E*)-2-(4-cyano-5-(dicyanomethylene)-2,2-dimethyl-2,5-dihydrofuran-3-yl)vinyl)phenylcarbamate (3):

Az-CyFur-1 (1.3 mg, 3.13 μmol) and **alk-CyFur** (1.2 mg, 3.38 μmol) were dissolved in 1 mL of MeOH and stirred at room temperature under argon in a 5 mL round bottom flask. A mixture of 1 mg of CuSO_4 (1.3 equiv.), 1 mg of sodium ascorbate (1.6 equiv.) and 3 mg of $(\text{BimC}_4\text{A})_3$ (1.4 equiv.) in 250 μL of water was added to the stirred solution. After 1 hr of reaction time, the mixture was diluted with 30 mL of water, extracted twice with ethyl acetate (15 mL each time), washed with 1% HCl, brine, dried over anhydrous Na_2SO_4 and filtered. The concentrated crude product was purified by silica gel chromatography with 20:1 ethyl acetate: MeOH ($R_f = 0.3$) as the mobile phase to give product as purple solid (2.1mg, 90%). ^1H NMR (600 MHz, CD_2Cl_2): $\delta = 7.68$ (d, 2H, $J = 8.6$ Hz), 7.64 (dd, 2H, $J = 16.4$ Hz, $J = 16.0$ Hz), 7.56 (m, 4H), 7.14 (s, 1H), 7.02 (d, 1H, $J = 16.4$ Hz), 6.81 (d, 1H, $J = 16.0$ Hz), 6.70 (d, 2H, $J = 8.6$ Hz), 4.70 (t, 1H, $J = 5.0$ Hz), 4.61 (t, 1H, $J = 4.9$ Hz), 3.81 (t, 1H, $J = 5.0$ Hz), 3.78 (t, 1H, $J = 5.4$ Hz), 3.63 (t, 1H),

3.56 (t, 1H, $J = 5.0$ Hz), 3.51 (t, 1H, $J = 5.4$ Hz), 3.10 (t, 1H), 1.82 (s, 6H), 1.78 (s, 6H).

MALDI-TOF: calcd. for $C_{43}H_{35}N_{11}O_4$ $[M+Na]^+$ 770.29, found 770.79.

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