Supplementary Online Materials for:

Fluorogen Activating Protein - Affibody Probes: Modular, No-wash Measurement of Epidermal Growth Factor Receptors

Yi Wang[1,2], Cheryl Telmer[2], Brigitte F. Schmidt[2], Josef D. Franke[2a], Stephan Ort[3,4b], Donna Arndt-Jovin[3], Marcel P. Bruchez[1,2,5*]

[1]The Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, 15213, USA [2]The Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh, PA, 15213 USA, [3]Laboratory for Cellular Dynamics, The Max Planck Institute for Biophysical Chemistry, Goettingen Germany, [4]Enzyme Chemistry Research Group, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, [5]The Department of Chemistry, Carnegie Mellon University, Pittsburgh, PA, 15213, USA

[a] Present address: Creighton University, Department of Biology, Omaha, NE
[b] Present address: Edwards Lifesciences Services GmbH, München, Germany

*Corresponding author
Marcel P. Bruchez
Mellon Institute
4400 Fifth Avenue
Pittsburgh, PA 15213, USA

bruchez@cmu.edu
1-412-268-9661 (work phone)
1-412-268-6571 (fax)

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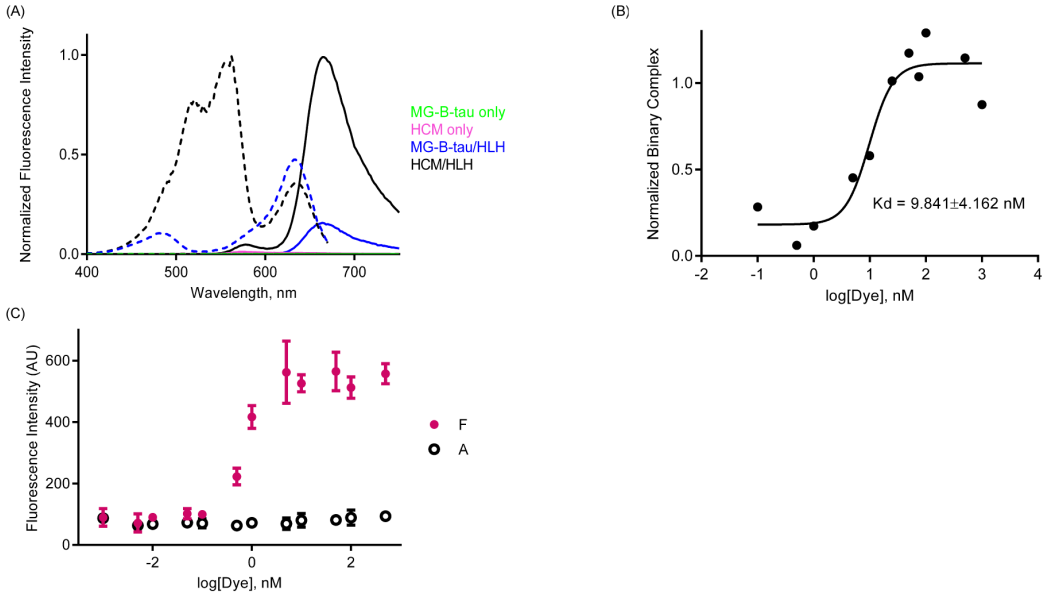
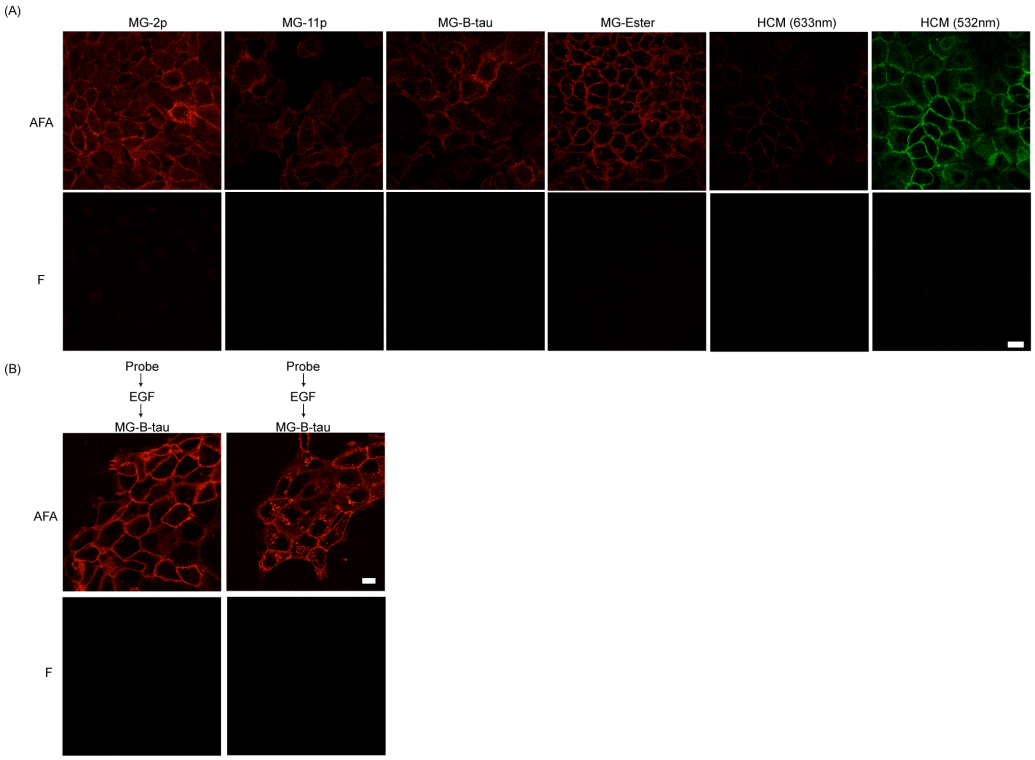
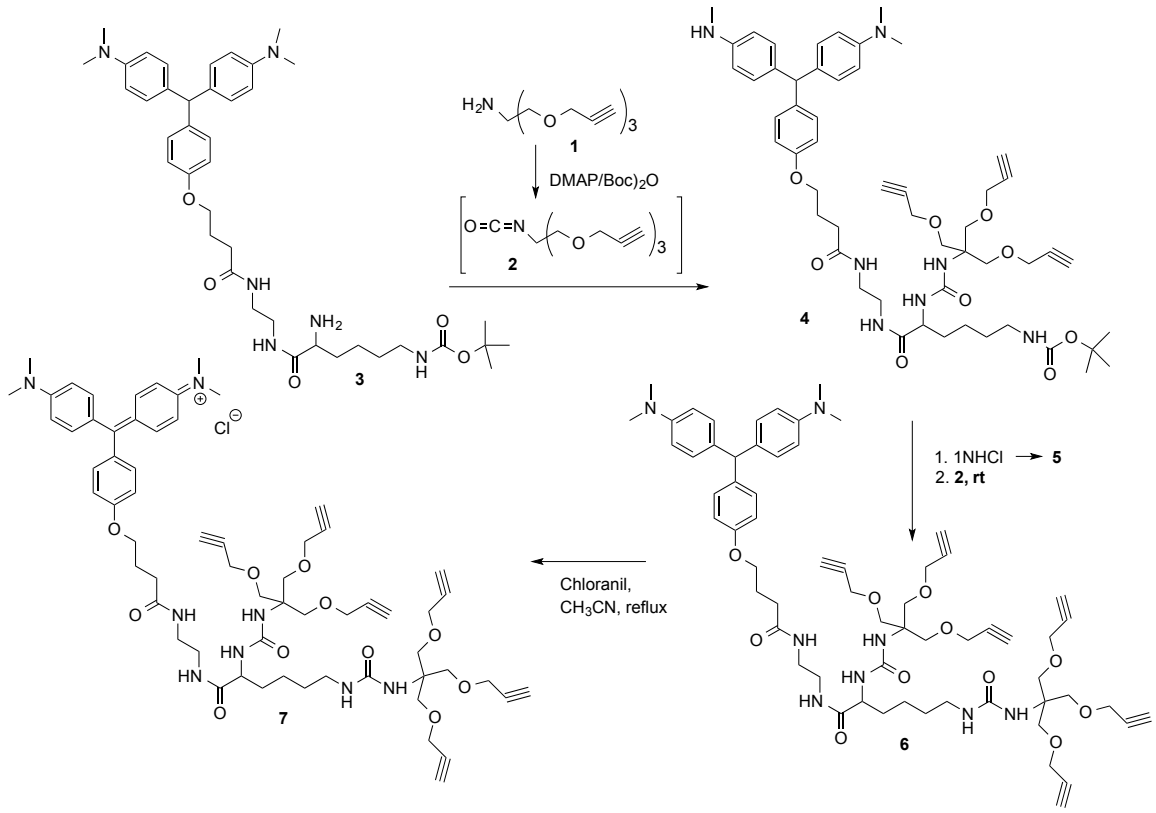


S1: Characterization of probes binding to malachite green and its derivatives. (A) Comparisons for excitation scans (dotted lines) and emission scans (solid lines) of AFA/HCM and AFA/MG-B-tau complexes. 1μM of fluorogen were pre-incubated with 10μM of probes. For excitation scans, the excitation wavelength was 400nm to 670nm with an emission at 700nm. For emission scans, the emission wavelength was 530nm to 750nm with an excitation at 500nm. The excitation and emission scans were normalized to the maximum fluorescence of HCM, respectively. Comparing the excitation intensity of MG bound to MG free produced an activation ratio of ~3300-fold, excited at 634 nm, while HCM bound relative to HCM free produced an activation ratio of ~330-fold, excited at 561 nm. (B) The binding equilibrium of AFA and HCM. 5nM of probes were assembled into fluorescence complexes as a function of HCM concentrations. The fluorescence was measured at the excitation of 530 nm and the emission of 664 nm. (C) Comparison of fluorescent activation by affibody $Z_{EGFR:1907}$ (A) and FAP_{dL5**} (F). 5nM of probes were titrated with various MG-B-tau concentrations. The fluorescence was measured at the excitation of 636 nm and the emission of 664 nm.

S2: Movie of EGF-mediated endocytosis. Cells were starved overnight and labeled with 250nM AFA for an hour followed by 100nM MG-B-tau. Then cells were then stimulated with 10ng/mL EGF and imaged by confocal microscopy every 30 seconds for 30 minutes.



S3: Fluorescence microscopy of A431 cells. Cell were with 250nM of AFA or F for 1 hour at 37°C. Then 100nM of fluorogen was added to cells 5 minutes before imaging. (A) Comparison of cell labeling with various malachite green derivatives; (B) EGFR endocytosis tracking and quantification using cell impermeable fluorogen (MG-B-tau). Scale bar 20µm.



S4: Synthetic route to HCM; preparation of the MG-scaffold

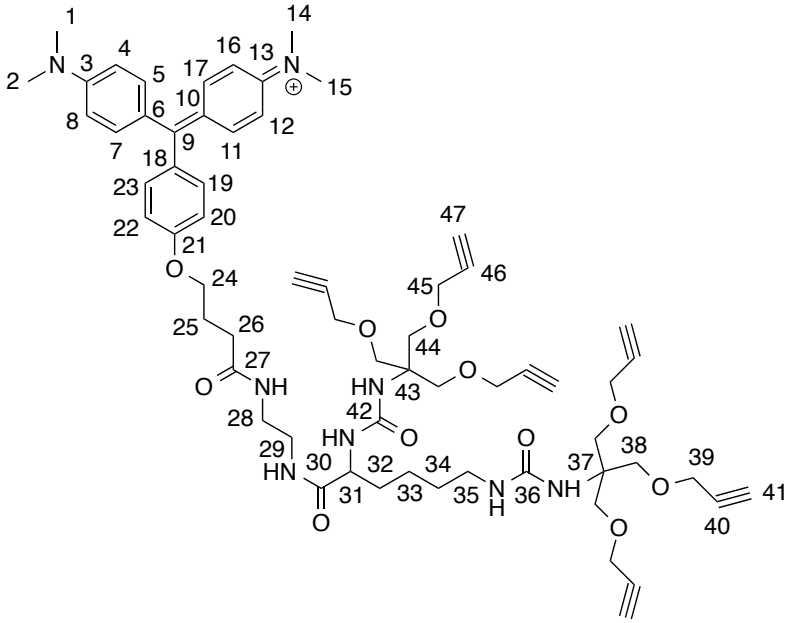
Tert-butyl (6-((2-(4-(4-(bis(4-(dimethylamino)phenyl)methyl)phenoxy) butanamido)ethyl)amino)-5-(3-(1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-yl)ureido)-6-oxohexyl)carbamate **MG[H]EDA-Lys(BOC)[1]alkyne 4**. Boc anhydride (218 mg, 0.1 mmol) was dissolved in dry methylene chloride (5 mL). Dimethylaminopyridine (122 mg, 1mmol) dissolved in dry methylene chloride (5 mL) was added at rt under argon. 1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-amine **1** [2] (235 mg/1 mmol) was added and the reaction mixture was stirred for 10 minutes. The in-situ generated "tripod-isocyanate **2**" was added to a solution of *tert*-butyl (5-amino-6-((2-(4-(4-(bis(4-(dimethylamino)phenyl) methyl)phenoxy) butanamido)ethyl)amino)-6-oxohexyl)carbamate [MG[H]-EDA-Lys(Boc)] **3** (703 mg, 1mmol) in anhydrous methylene chloride. The reaction mixture was stirred at rt overnight. The reaction mixture was concentrated and separated by column chromatography on silica gel (eluent: chloroform/10%methanol).[1]H-NMR ($CDCl_3$) 6.99 (2H,d), 6.94 (4H,d), 6.87 (1H, *NH*), 6.76 (2H,d), 6.72 (1H, *NH*), 6.63 (4H,d), 5.77 (1H,*NH*), 5.28 (1H,s), 5.15 (1H,*NH*), 4.78 (1H,*NH*), 4.11 (6H,d), 4.05 (1H,m), 3.94 (2H,t), 3.76 (6H,s), 3.37 (2H,m), 3.34 (2H,m), 3.04 (2H,m), 2.88 (12H,s), 2.44 (3H,t), 2.38 (2H,t), 2.06 (2H,m), 1.72 (1H,m), 1.55 (1H,m), 1.44 (2H,m), 1.41 (9H,s), 1.32 (2H,m).

6-Amino-*N*-(2-(4-(4-(bis(4-(dimethylamino)phenyl)methyl)phenoxy) butanamido)ethyl)-2-(3-(1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-yl)ureido)hexanamide **MG[H]EDA-Lys[1]alkyne 5**. MG[H]EDA-Lys(BOC)[1]alkyne **4** was

dissolved in ethanol and 2 equiv of 1N HCl was added. The reaction mixture was refluxed until the evolution of CO_2 ceased. The reaction mixture was cooled to rt and adjusted to pH 9 by the addition of ammonium hydroxide. The reaction mixture was concentrated to dryness. The residue was taken up in acetonitrile, filtered and dried to give a light green resin.^{1}H-NMR (MeOD) 6.97 (2H,d), 6.92 (4H,d), 6.80 (2H,d), 6.70 (4H,d), 5.27 (1H,s), 4.13 (6H,d), 4.03 (1H,m), 3.98 (2H,t), 3.77 (6H,s), 3.34 (2H,m), 3.28 (2H,m), 2.87 (12H,s), 2.82 (3H,t), 2.67 (2H, t, 2.40 (2H, t), 2.06 (2H,m), 1.72 (1H,m), 1.60 (1H,m), 1.48 (2H,m), 1.38 (2H,m). $C_{49}H_{65}N_7O_7$ MW 864.1 g/mol

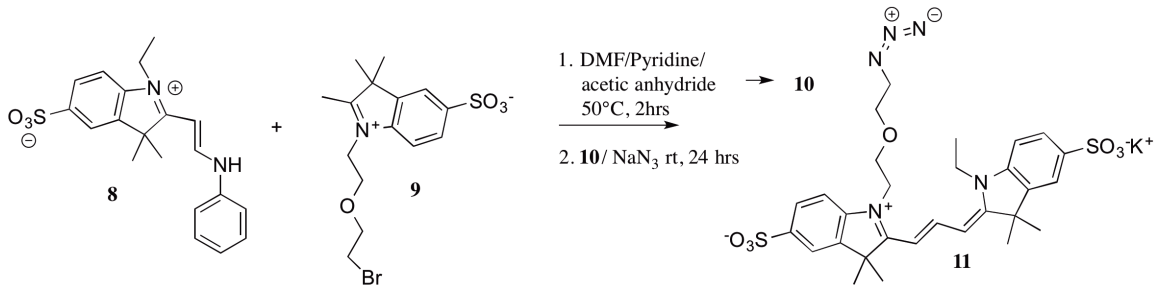
N-(2-(4-(4-(bis(4-(dimethylamino)phenyl)methyl)phenoxy)butanamido)ethyl)-2,6-bis(3-(1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-yl)ureido)hexanamide **MG[H]EDALys(6)alkyne 6.** Boc anhydride (114 mg, 0.5 mmol) was dissolved in dry methylene chloride (3 mL). Dimethylaminopyridine (61 mg, 0.5 mmol) dissolved in dry methylene chloride (3 mL) was added at rt under argon. 1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-amine **1** (116 mg/ 0.5 mmol) was added and the reaction mixture was stirred for 10 minutes. The in-situ generated "tripod-isocyanate **2**" was added to a solution of **5** (432 mg, 0.5 mmol) in anhydrous acetonitrile. The reaction mixture was stirred at rt overnight. The reaction mixture was concentrated and separated by column chromatography on silica gel (eluent: chloroform/10%methanol).^{1}H-NMR ($CDCl_3$) 7.28 (1H, *NH*), 7.05 (2H,d), 6.98 (4H,d), 6.86 (1H,*NH*), 6.79 (2H,d), 6.67 (4H,d), 6.02 (1H,d, *NH*), 5.34 (1H, *NH*), 5.48 (1H,s, *NH*), 5.32 (1H,s), 5.14 (1H,s,*NH*), 4.17 (3H,d), 4.16 (3H,d), 4.09 (1H,m), 3.97 (2H,t), 3.81 (12H,s), 3.42 (2H,m), 3.14 (2H,m), 2.92 (12H,s), 2.49 (3H,t, *alkyne*), 2.48 (3H,t, *alkyne*), 2.40 (2H,t), 2.11 (2H,m), 1.75 (1H,m), 1.59 (1H,m), 1.44 (2H,m), 1.37 (2H,m). $C_{63}H_{76}N_8O_{11}$ MW 1121.36 g/mol

N-(4-((4-((14-(3-(1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-yl)ureido)-8,15,20-trioxo-6,6-bis((prop-2-yn-1-yloxy)methyl)-4-oxa-7,9,16,19-tetraazatricos-1-yn-23-yl)oxy)phenyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)-*N*-methylmethanaminium chloride (**MG-EDA-Lys(6)alkyne**) **7.** MG[H]EDALys(6)alkyne **6** (112 mg, 0.1mmol) was dissolved in boiling acetonitrile 5 mL). Chloroanil (36 mg, 1.5 equiv) was added and the reaction mixture was refluxed for 1 hr. A silica gel column in chloroform was prepared. The reaction mixture was poured onto the column. The product adhered to the column, while excess chloroanil passed through. The column was washed with one volume of chloroform and the product was eluted with a gradient of chloroform/methanol 0-20 to yield 92 mg, 80% of **7**.



S5: Numbering of **MG-EDA-Lys(6)alkyne 7** for NMR signal assignments.

^{1}H-NMR(500 MHz, MeOD) 8.03 (1H, *NH*), 7.96 (1H, *NH*), 7.45 (4H,d, J=9Hz, *5,7,11,17*), 7.39 (2H,d,J=8.6 Hz, *19,23*), 7.21 (2H,d, J=8.6Hz, *20,22*), 7.06 (4H,d, J=9Hz, *4,8,12,16*), 4.23 (2H,t, J=7.2 Hz, *24*), 4.16 (12H,m, *39,45*), 4.02 (1H,m, *31),* 3.41 (2H,m, *28*), 3.35(12H,s, *1,2,14,15*), 3.30 (2H,m, *29*), 3.06 (2H,m, *35*), 2.84 (6H,m, *41,47*), 2.48 (2H,t, J=7.2Hz, *26*), 2.19 (2H,q, J=7.2Hz, *25*), 1.75 (1H,m, *32*), 1.59 (1H,m, *32*), 1.47 (2H,m, *34*), 1.40 (2H,m, *33*).**^{13}C-NMR (500 MHz, MeOD)** 178.26 (*C9*), 174.82/174.74 (*C30*), 174.14 (*C27*), 164.30 (C*21*), 158.92 (*C37*), 158.28 (*C42*), 156.98 (*C3,13*), 140.51/140.32 (*C5,7,11,17*), 137.56 (*C19,23*), 131.83 (*C18*), 126.91 (*C6,10*), 114.77 (*C20,22*), 113.08 (*C4,8,12,16*), 79.31/79.26 (*C40,46*), 74.73/74.62 (*C41,47*), 69.07/68.95 (*C38,44*), 67.73 (*C24*), 58.40/58.27 (*C37,43*), 58.16/58.11 (*C39*), 54.14/54.10 (*C31*), 39.52 (*C1,2,14,15*), 39.05 (*C35*), 38.75 (*C28*), 38.64 (*C29*), 32.09 (*C26*), 31.90 (*C32*), 29.52 (*C34*), 24.92 (*C25*), 22.83 (*C33*).

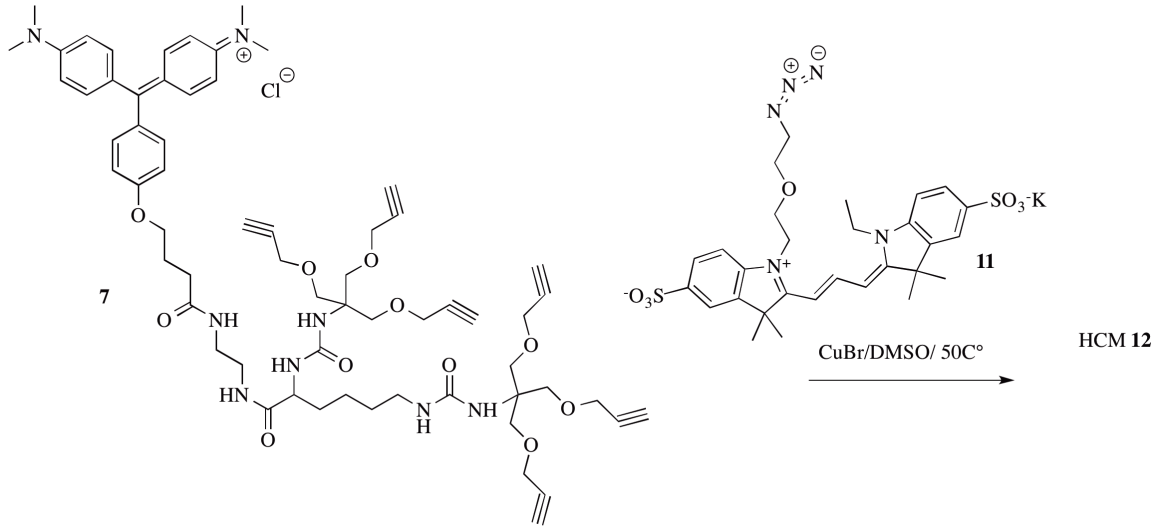


S6: Synthetic route to HCM; preparation of Cy3-P1-azide

Pyridin-1-ium 1-(2-(2-bromoethoxy)ethyl)-2-((*E*)-3-((*Z*)-1-ethyl-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3*H*-indol-1-ium-5-sulfonate

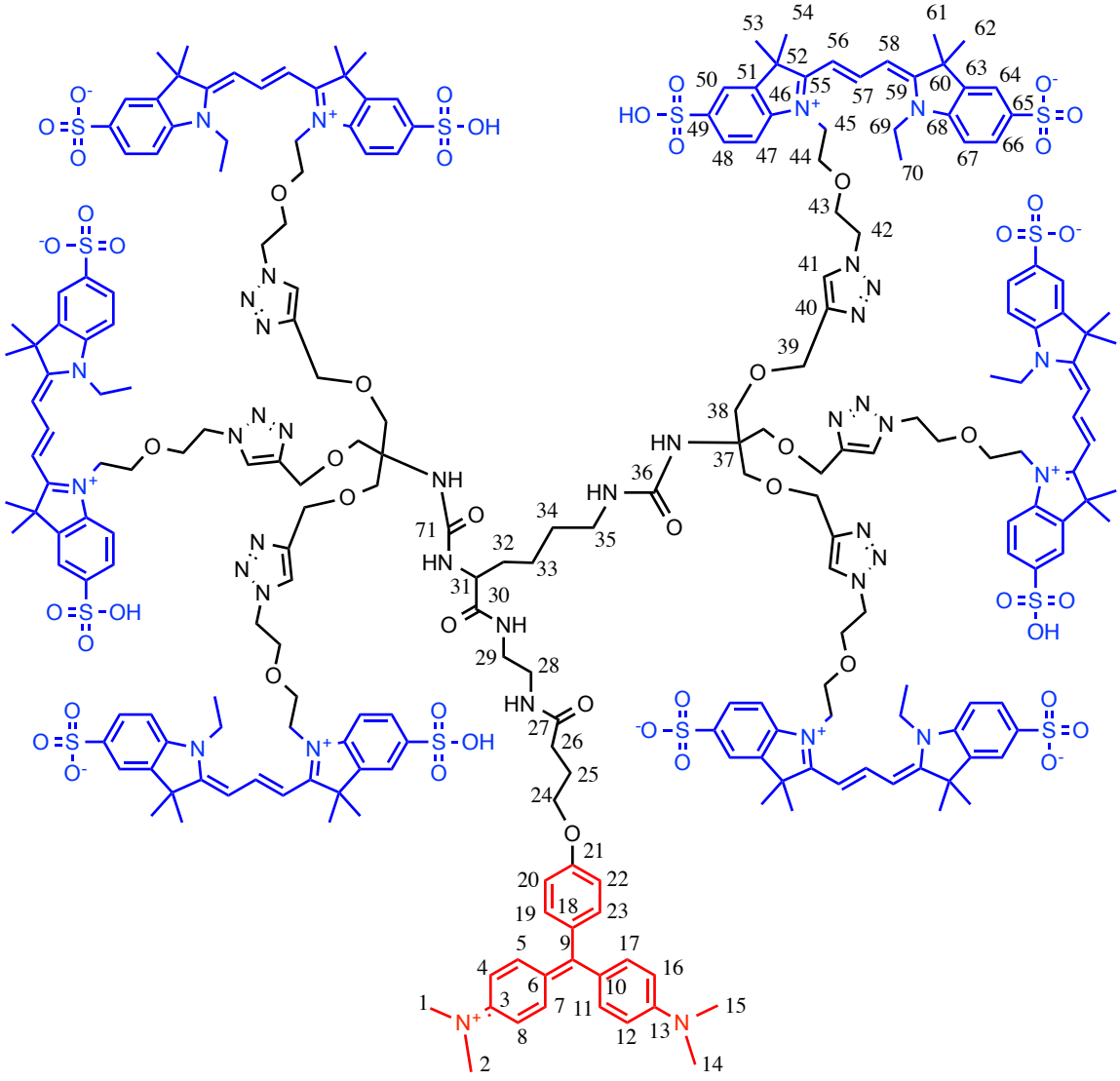
Cy3-P1-Br 10. 1-Ethyl-3,3-dimethyl-2-(2-(phenylamino)vinyl)-3*H*-indol-1-ium-5-sulfonate (338 mg / 1mmol) **8** [3] and 1-(2-(2-bromoethoxy)ethyl)-2,3,3-trimethyl-3*H*-indol-1-ium-5-sulfonate (390 mg, 1mmol) **9** were dissolved in dry DMF (3 mL), pyridine (1 mL) and acetic anhydride (1 mL). The reaction mixture was stirred at 50°C for 2 hrs. The reaction mixture was added drop wise to 50 mL of acetone under stirring precipitating the product. The organic layer was decanted. The residue was dissolve in water/10%ethanol and separated by medium pressure chromatography (Buchi Sepacore System) on a RP-18 column, eluent: water/10-30% ethanol. The product fractions were collected and concentrated to yield 517 mg (70 %) of red crystals.[1]H-NMR(MeOD) 8.89 (2H,d,*Pyr*), 8.67 (1H,m,*Pyr*), 8.54 (1H,t, J=13.4 Hz), 8.12 (2H,t,*Pyr*), 7.92 (1H,d), 7.91(1H,d), 7.86 (1H,dt), 7.40 (2H,t), 6.63 (1H,d), 6.52 (1H,d), 4.41 (2H,m), 4.20 (2H,m), 3.92 (2H,t), 3.71 (2H,t), 3.38 (2H,t), 1.76 (6H,s), 1.74 (6H,s), 1.38 (3H,t).

Potassium 1-(2-(2-azidoethoxy)ethyl)1-ethyl-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3*H*-indol-1-ium-5-sulfonate **Cy3-P1-azide 11.**Cy3-P1-Br **10** (790 mg, 1mmol) was dissolved in dry DMF (5mL). Sodium azide (130 mg, 2 equiv) were added and the reaction mixture was stirred overnight. The reaction mixture was precipitated by adding drop wise to 50 mL of acetone. The precipitated dye was taken up in water/10 % ethanol. 1M sulfuric acid (2 mL) was added and the reaction mixture was purified by medium pressure chromatography (Buchi Sepacore System) on a RP-18 column, eluent: water/10-30% ethanol. The product fractions were collected and concentrated. The residue was taken up in a minimum amount of methanol drop wise added to a solution of 1M potassium acetate in isopropanol. The potassium salt of Cy3-P1-azide precipitated from the reaction mixture and was collected and dried to yield 500mg (75%) of red crystals.
[1]H-NMR(MeOD) 8.61 (1H,t), 7.98 (1H,d), 7.97 (1H,d), 7.92 (1H,t), 7.90 1H,t), 7.44 (1H,d), 7.41 (1H,d), 6.61 (1H,d), 6.49 (1H,d), 4.44 (2H,t), 4.24 (1H,m), 3.96 (2H,t), 3.64 (2H,t), 1.83 (6H,s), 1.81 (6H,s), 1.44 (3H,t).



S7: Synthetic route to HCM: final step

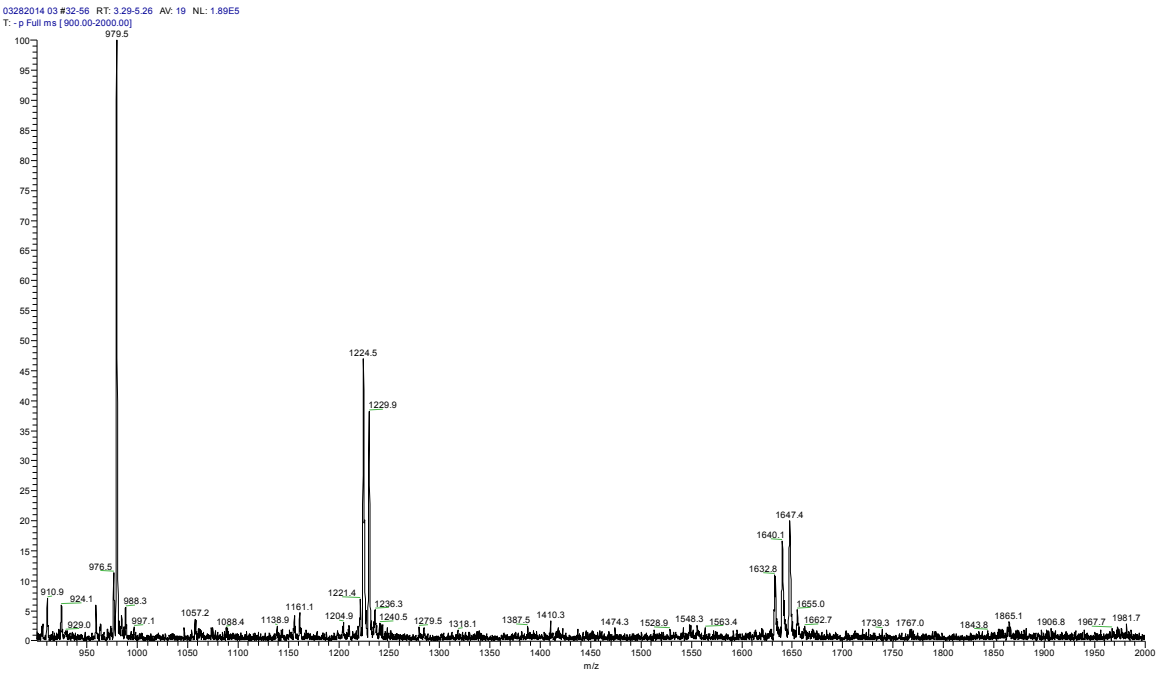
MG-EDA-Lys(6)Cy3-P1 **HCM 12.** MG-EDA-Lys(6)alkyne **7** (12 mg/0.01 mmol) and Cy311-P1-azide **11** (100 mg/ 0.025 mmol) were dissolved in anhydrous DMSO (20 mL) at 50 C°. Copper(I)bromide (45 mg/0.03 mmol) and PMDTE (1ml) added. The reaction mixture was stirred for 1 hr. After cooling to rt the reaction mixture was precipitated into 200 ml of ethyl acetate. The organic phase was decanted and the residue dissolved in diluted sodium bicarbonate (2 ml). The reaction mixture was separated by medium pressure chromatography (Buchi Sepacore System) on a RP-18 column, eluent: water/0-15% ethanol. The product fractions were further purified by size exclusion chromatography on a P4-Biogel (eluent: water) to separate small amounts of Cy3-P1-azide starting material from the product. Yield: 20 mg (40%) of HCM **12**



S8: Numbering of **HCM 12** for NMR assignments.

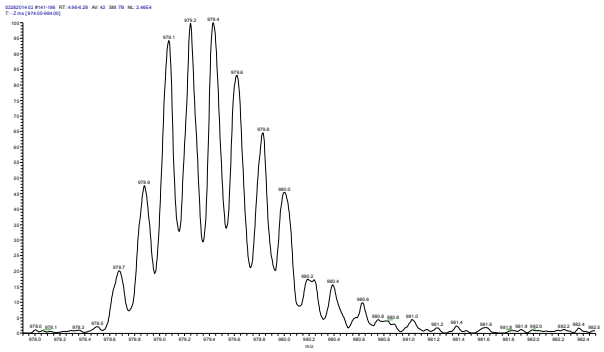
^{1}H-NMR(500 MHz, MeOD) 8.47 (t, 6H, *57*), 7.92 (6H,s, *52*), 7.9 (6H,d, *48*), 7.89 (6H,s *50*), 7.82 (6H,d, *66*), 7.74 (3H,s, *41*)/7.71 (3H,s *41*), 7.39 (6H,d, *67*), 7.31 (4H,d, *5*, *7*, *11*, *17*), 7.28 (6H,d, *47*), 7.26 (2H,d, *19,23*), 7.11 (2H,d, *20,22*), 6.96 (4H,d, *4,8,12, 16*), 6.57 (6H,dd, *58*), 6.51 (6H,dd, *56*), 4.43/4.41 (12H,s, *39*), 4.38 (12H, *42*), 4.30 (12H, *45*), 4.22

(12H, *69*,) 4.12 (2H,m, *24*), 3.89 (1H, m, *31*), 3.80 (12H, *44*,), 3.77 (12H, *43*), 3.71/3.70 (12H,s, *38*), 3.25 (12H,s, *1*,*2*,*14*,*15*), 3.19 (4H,m, *28*,*29*), 2.81 (2H,m, *35*), 2.35 (2H,m, *26*), 2.02 (2H,m, *25*), 1.71 (36H,s, *53*,*54*), 1.67 (36H,s, *61*,*62*), 1.54 (2H,m, *32*), 1.35 (18H,s,*70*), 1.22 (4H,m, *33*,*34*).**^{13}C-NMR (500 MHz, MeOD)** 177.6 (*C9*), 175.7 (*C55*), 175.2 (*C59*), 174.7 (*C30*), 174.0 (*C27*), 164.2 (*C21*), 158.76 (*C71*), 158.19 (*C36*), 156.9 (*C3*, *C13*), 151.47 (*C57*), 144.5/144.4 (*C40*), 143.9/143.8 (*C46*), 142.9 (*C49*), 142.8 (*C65*), 142.7 (*C68*), 141.1 (*C63*), 140.5 (*C5*, *C7*, *C11*, *C17*), 137.5 (*C19*, *C23*), 131.7 (*C18*), 126.9 (*C66*), 126.7 (*C6*, *C10*), 126.7 (*C48*), 124.2/124.1 (*C41*), 120.1 (*C50*), 119.8 (*C64*), 114.9 (*C20*, *C22*), 113.2 (*C4*, *C8*, *C12*, *C16*), 111.5/111.4 (*C47*), 110.8 (*C67*), 103.9 (*C56*), 103.7 (C*58*), 69.6/69.5 (*C38*), 69.1 (*C43*), 67.7 (*C24*), 67.6 (*C44*), 64.2 (*C39*), 59.1/59.0 (*C37*), 54.3 (*C31*), 49.8 (*C60*), 49.8 (*C42*), 49.2 (*C52*), 44.6 (*C45*), 39.6(*C1*, *C2*, *C14*, *C15*), 39.5 (*C14*, *C15*), 38.9 (*C35*), C38.7 (*C29*), 38.5 (*C28*), 32.1(*C26*), 31.8 (*C32*), 29.5 (*C33*), 27.08 (*C53*, *C54*), 26.83 (*C61*,*62*), 25.0 (*C25*), 23.3/22.9 (*C34*), 11.45 (*C70*).

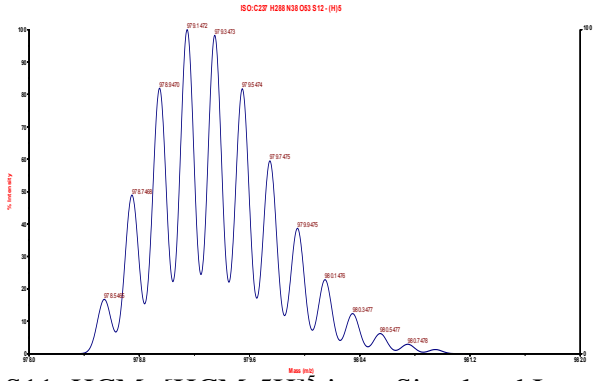


[HCM-5H]$^{5-}$ [HCM-4H]$^{4-}$ [HCM-3H]$^{3-}$

S9: HCM –Mass Spectrum 900-2000m/z range



S10: HCM -[HCM-5H]$^{5-}$ ion signal (7 pt. Boxcar Smooth)



S11: HCM -[HCM-5H]$^{5-}$ ion - Simulated Isotopic Distribution

Reference:

(1) Ozhalici-Unal, H., Pow, C. L., Marks, S. A., Jesper, L. D., Silva, G. L., Shank, N. I., Jones, E. W., Burnette, J. M., 3rd, Berget, P. B., and Armitage, B. A. (2008) A rainbow of fluoromodules: a promiscuous scFv protein binds to and activates a diverse set of fluorogenic cyanine dyes. *J Am Chem Soc 130*, 12620-12621.

(2) Chabre, Y. M., Contino-Pepin, C., Placide, V., Shiao, T. C., and Roy, R. (2008) Expeditive synthesis of glycodendrimer scaffolds based on versatile TRIS and mannoside derivatives. *J Org Chem 73*, 5602-5605.

(3) Mujumdar, R. B., Ernst, L. A., Mujumdar, S. R., Lewis, C. J., and Waggoner, A. S. (1993) Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjug Chem 4*, 105-111.