SUPPORTING INFORMATION

A Fluorogenic Green-Inside Red-Outside (GIRO) Labeling Approach Reveals Adenylyl Cyclase-Dependent Control of BKα Surface Expression

Christopher P. Pratt,[†§#] Jianjun He[‡§], Yi Wang[†§] Alison L. Barth[†#], Marcel P. Bruchez*[†‡§]

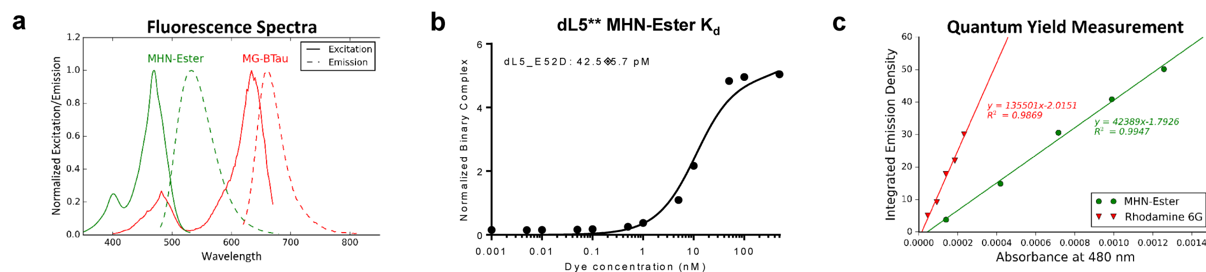
†Department of Biological Sciences, Carnegie Mellon University, Pittsburgh PA 15213
‡Department of Chemistry, Carnegie Mellon University, Pittsburgh PA 15213
§Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh PA 15213
#Center for the Neural Basis of Cognition, Carnegie Mellon University, Pittsburgh PA 15213

Table of Contents:

Supplemental Figure S1	3
Supplemental Figure S2	4
Supplemental Figure S3	5
Supplemental Figure S4	6
Supplemental Figure S5	7
Supplemental Figure S6	8
Supplemental Figure S7	9
dL5**-BKα plasmid map and sequence	10-11
Supplemental Experimental Procedures	12-14
References	15

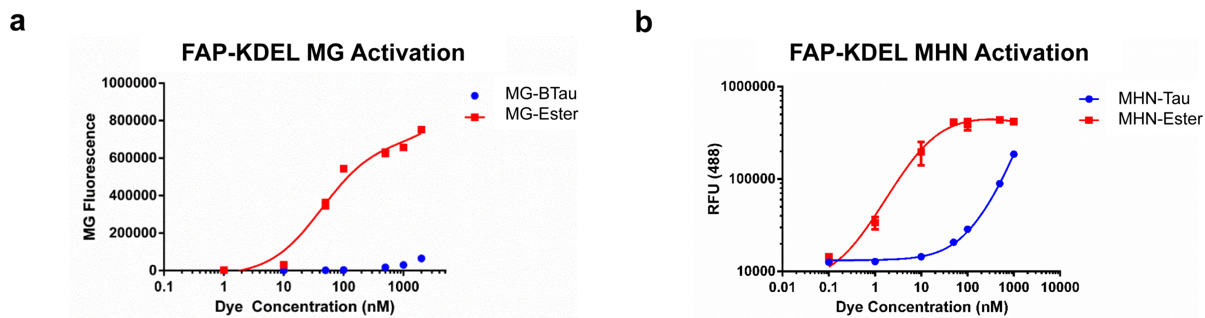
Supplemental Data

Figure S1



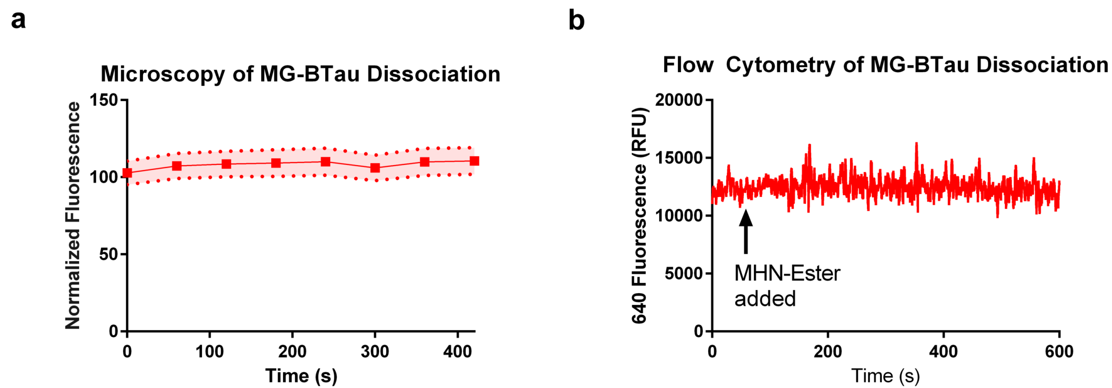
Supplementary Figure S1: Determination of dL5+MHN-ester fluorescence properties** (a) MHN-ester and MG-BTau excitation (solid lines) and emission (dashed lines) spectra bound to excess dL5** protein. Refer to Table 1 for peak values. (b) Kd determination of purified dL5**+MHN-ester complex by serial dilution and fluorescence measurement yielded a Kd of 42.5 (±5.7) pM. (c) Quantum yield measurement of dL5**+MHN-ester complex was determined using Rhodamine 6G properties to yield $\Phi x = 0.30$.

Figure S2



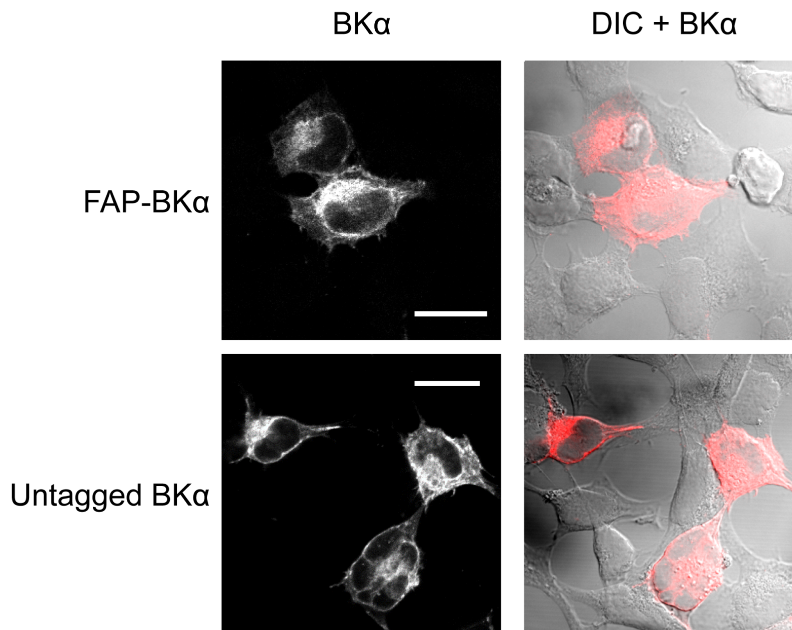
Supplementary Figure S2: Endoplasmic reticulum localized FAP-KDEL demonstrates cell exclusion and permeability of modified dyes (a) Fluorescence labeling of ER-targeted FAP with sulfonated cell impermeable MG-BTau compared to cell permeable MG-ester shows a lack of fluorogen activation due to cell exclusion. Curves were fit using GraphPad Prism 6 using a total (including nonspecific) binding model. (b) A sulfonated form of MHN (MHN-Tau) shows cell exclusion at low concentrations compared to the readily cell-permeable MHN-ester. Curves were fit using GraphPad Prism 6 using a total (including nonspecific) binding model.

Figure S3



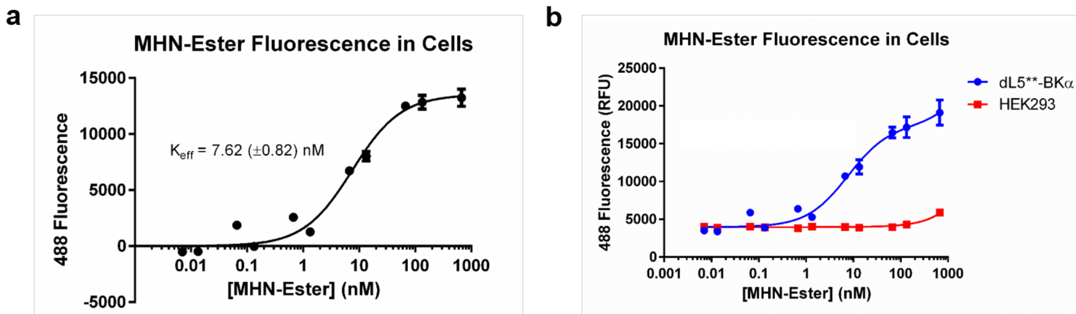
Supplementary Figure S3: No measurable MG-BTau dissociation is observed with addition of MHN dyes by microscopy or flow cytometry (a) Microscopy of cells labeled with 300 nM MG-BTau show no decline of signal over time following the addition of 300 nM MHN-Tau dye (*n* = 5 fields, approx. 25 cells, shaded area indicates S.E.M.) (b) Flow cytometry of cells complexed with 300 nM MG-BTau show no decline in median signal following addition of 300 nM MHN-ester. Events were aggregated as described in methods using a custom python script.

Figure S4



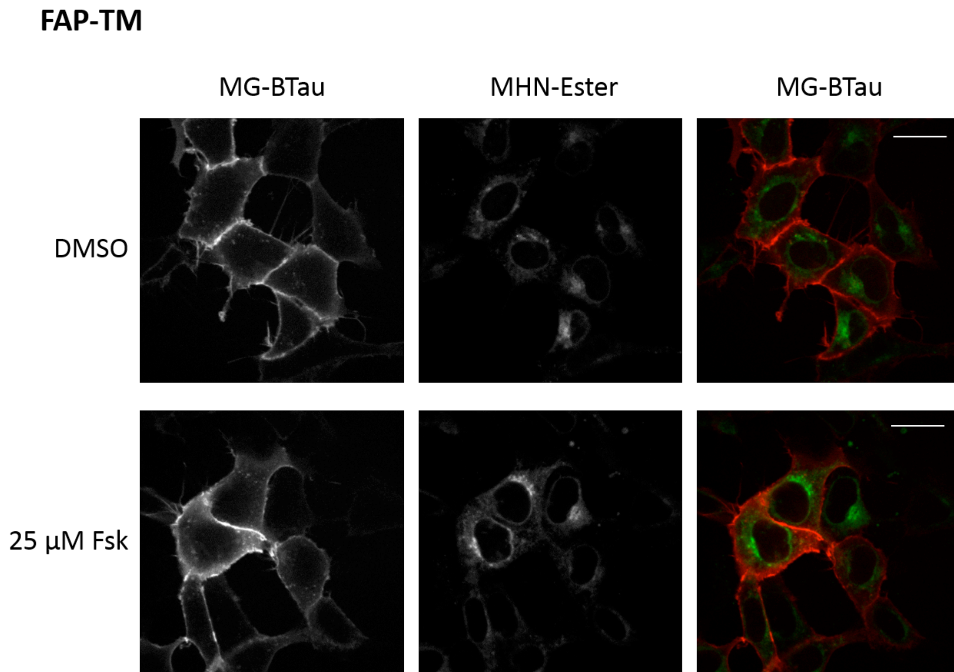
Supplementary Figure S4: Immunofluorescence of untagged BKα and dL5-BKα show similar localization to a predominantly internal compartment.** HEK293 cells were transiently transfected with FAP-BKα or untagged BKα and fixed 24 hours later. Antibodies against mouse BKα showed a similar localization to an internal compartment in both FAP-BKα and untagged BKα. Adjacent, untransfected cells act as an internal control. Scale bars are 20 μm.

Figure S5



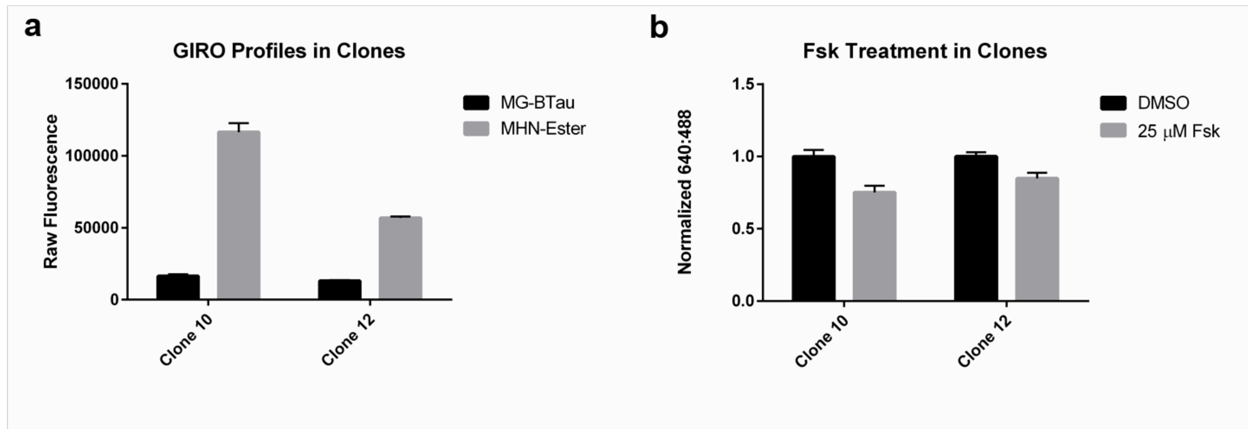
Supplementary Figure S5: Determination of Effective Dissociation Constant K_{eff} of dL5+MHN-Ester on Cells** (a) Titration of MHN-ester dye added to FAP-BKα cells shows an effective dissociation constant K_{eff} of 7.62 (±0.82). (b) Raw fluorescence data from dL5**-BKα expressing stable cells (blue) and non-expressing HEK293 cells (red) treated with MHN-Ester shows an increase in nonspecific fluorescence at high concentrations, but no observable nonspecific fluorescence at dye concentrations below 500 nM. >5000 cells were quantified per replicate, 3 replicates.

Figure S6



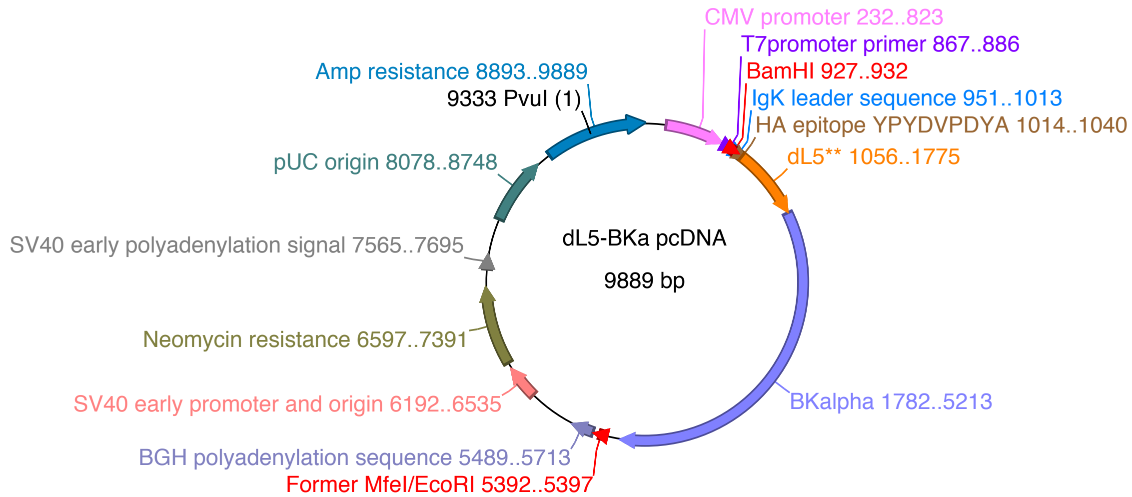
Supplementary Figure S6: No changes are seen in FAP-TM cells by microscopy when treated overnight with Fsk. Representative images of FAP-TM expressing cells imaged at 40x with 18 hour treatment with 25 µM fsk compared to DMSO control. Scale bars are 40 µm. The FAP-TM cells are a heterogenous stable population, quantitation of fluorescence and RSE was done using flow cytometry.

Figure S7



Supplementary Figure S7: Clones used for experiments exhibit unique FAP-BKα expression levels, analogous response to forskolin. Two stably expressing clones, numbered 10 and 12 were derived from transfected FAP-BKα HEK293 cells and used in all experiments. (a) Running flow cytometry in parallel on both clones using identical conditions gives a higher expressing clone (10) and a lower expressing clone (12). Raw fluorescence is plotted in arbitrary units. Clone 10 shows an approximately twofold higher expression level than clone 12. (b) Treatment of clones 10 and 12 overnight with Fsk shows a similar response in normalized RSE, despite intrinsic differences in raw surface expression ratios. Error bars are ±SEM.

Graphical map of dL5**-BKα in pcDNA3.1:



dL5**-BKα sequence:

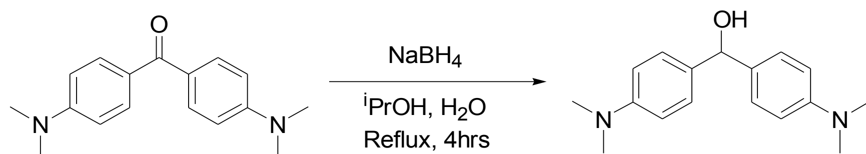
Igκ signal sequence
HA (YPYDVPDYA)
dL5**
BKα

atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggtgactatccat
atgatgttccagattatgctggggcccagccggccCAGGCCGTCGTTACCCAAGAACCTAGTGTTACCGT
TAGCCCAGGTGGTACTGTTATACTTACTTGTGGAAGTGGTACGGGTGCCGTCACATCTGGTCATTATGCA
AATTGGTTTCAACAAAAACCAGGACAAGCTCCAAGAGCTTTGATTTTTGATACTGATAAGAAGTATTCTT
GGACCCCAGGTAGATTTTCTGGATCTTTGCTGGGAGCAAAGGCAGCTTTGACAATATCAGATGCTCAGCC
TGAGGACGAAGCCGAGTATTACTGTTCTCTTAGCGACGTGGATGGCTACTTGTTTGGCGGTGGAACACAA
CTGACGGTTCTGTCCGGTGGTGGCGGCTCTGGTGGCGGTGGCAGCGGCGGTGGTGGTTCCGGAGGCGGCG
GTTCTCAGGCTGTGGTGACTCAGGAGCCGTCAGTGACTGTGTCCCCAGGAGGGACAGTCATTCTCACTTG
TGGCTCCGGCACTGGAGCTGTCACCAGTGGTCATTATGCCAACTGGTTCCAGCAGAAGCCTGGCCAAGCC
CCCAGGGCACTTATATTTGACACCGACAAGAAGTATTCCTGGACCCCTGGCCGATTCTCAGGCTCCCTCC
TTGggGCCAAGGCTGCCCTGACCATCTCGGATGCGCAGCCTGAAGATGAGGCTGAGTATTACTGTTCGCT
CTCCGACGTTGACGGTTATCTGTTCGGAGGAGGCacccagctgaccgtcctctccACTAGTATGGATGCG
CTCATCATACCGGTGACCATGGAGGTGCCGTGCGACAGCCGGGGCCAACGCATGTGGTGGGCTTTCTTGG
CCTCCTCCATGGTGACTTTCTTCGGGGGCCTCTTCATCATCTTGCTCTGGCGGACGCTCAAGTACCTGTG
GACCGTTTGCTGCCACTGCGGGGGCAAGACGAAGGAGGCCCAGAAGATAAACAATGGCTCCAGCCAGGCA
GATGGTACTCTCAAGCCAGTGGACGAAAAAGAGGAGGTGGTGGCAGCCGAGGTCGGCTGGATGACATCTG
TGAAGGACTGGGCAGGGGTGATGATATCCGCCCAGACACTGACTGGCAGAGTCCTGGTTGTGTTAGTCTT
TGCTCTCAGCATTGGTGCCCTCGTAATATACTTCATAGACTCGTCAAACCCAATAGAATCCTGCCAGAAT
TTCTACAAAGATTTCACATTACAGATCGACATGGCTTTCAACGTGTTCTTCCTCCTCTACTTTGGCTTGC
GGTTTATTGCAGCCAACGATAAGCTGTGGTTCTGGCTGGAAGTGAATTCAGTAGTAGATTTCTTCACAGT
CCCTCCTGTGTTTGTGTCTGTGTACTTAAACAGAAGTTGGCTTGGCTTGAGATTTTTAAGAGCTCTCAGA
CTGATACAGTTTTCAGAGATTTTGCAGTTTCTGAATATCCTTAAAACAAGTAACTCCATCAAGCTGGTGA
ATCTGCTCTCCATATTTATCAGCACGTGGCTGACTGCAGCTGGATTCATCCACTTGGTGGAGAATTCAGG
GGACCCATGGGAAAATTTCCAAAACAACCAGGCACTTACGTACTGGGAATGTGTCTACTTACTCATGGTC
ACAATGTCTACAGTGGGTTATGGGGACGTTTATGCAAAAACCACACTTGGACGCCTCTTCATGGTCTTCT
TCATCCTCGGGGGACTGGCCATGTTTGCCAGCTACGTCCCTGAAATCATAGAGTTAATAGGAAACCGCAA

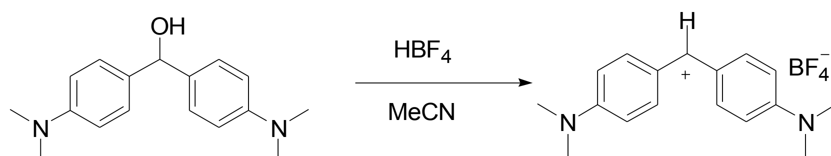
GAAATACGGGGGCTCCTATAGCGCGGTTAGTGGAAGAAAGCACATTGTAGTCTGTGGACACATTACTCTG
GAGAGTGTCTCTAACTTCCTGAAGGACTTTCTGCACAAGGACCGGGATGATGTCAACGTGGAGATTGTCT
TTCTTCACAACATCTCCCCTAACCTTGAGCTTGAGGCTCTGTTCAAACGGCATTTCACTCAGGTGGAGTT
TTATCAGGGCTCTGTCCTCAATCCACATGATCTTGCCAGAGTCAAGATAGAGTCAGCAGATGCATGCCTG
ATCCTTGCCAATAAGTATTGCGCTGACCCGGATGCAGAAGATGCCTCCAACATCATGAGAGTGATCTCCA
TCAAAAACTACCACCCAAAGATCAGGATCATCACTCAGATGCTGCAGTATCACAACAAGGCCCATCTGCT
CAACATCCCCAGCTGGAACTGGAAAGAGGGTGATGACGCAATATGCCTTGCAGAGCTCAAGTTGGGTTTC
ATAGCCCAGAGCTGTCTGGCTCAAGGCCTCTCCACAATGCTTGCCAATCTCTTCTCTATGAGGTCATTCA
TAAAGATTGAGGAAGACACATGGCAGAAATACTACTTGGAAGGAGTCTCCAATGAAATGTACACAGAATA
TCTCTCCAGTGCCTTCGTGGGTCTGTCCTTCCCTACTGTTTGTGAGCTGTGTTTTGTGAAGCTTAAGCTC
CTGATGATAGCCATTGAGTACAAGTCTGCCAACAGAGAGAGCCGAAGCCGAAAGCGAATATTAATTAACC
CTGGGAACCACCTTAAGATCCAAGAAGGTACTTTAGGATTTTTCATCGCAAGTGATGCCAAAGAAGTTAA
AAGGGCATTTTTTTACTGCAAGGCCTGTCATGATGACGTCACAGATCCCAAAAGAATTAAAAAATGTGGC
TGCAGGCGGCTTGAAGATGAGCAGCCGCCAACCCTGTCACCAAAAAAAAAACAACGTAATGGGGGCATGA
GGAACTCGCCCAACACCTCCCCGAAGCTGATGAGGCATGACCCCTTGTTAATTCCTGGCAATGATCAGAT
TGACAACATGGACTCCAATGTGAAAAAGTACGACTCCACTGGAATGTTTCACTGGTGTGCACCCAAGGAG
ATTGAGAAAGTCATCTTGACTCGAAGTGAAGCTGCCATGACTGTCCTGAGTGGCCATGTCGTAGTCTGCA
TCTTTGGGGATGTCAGCTCAGCCCTGATTGGCCTCCGGAACCTGGTGATGCCACTCTGTGCTAGCAACTT
TCACTATCATGAGCTCAAACACATTGTGTTTGTGGGCTCCATTGAGTACCTCAAGAGGGAGTGGAAACA
CTGCACAACTTCCCGAAAGTGTCCATATTGCCTGGTACACCATTAAGTCGGGCTGATTTAAGGGCTGTCA
ACATCAACCTCTGTGACATGTGCGTTATCCTGTCAGCCAATCAGAATAATATTGATGATACTTCGCTTCA
GGACAAGGAATGCATCTTGGCGTCACTCAACATCAAATCTATGCAGTTTGATGACAGCATCGGGGTCTTG
CAGGCTAATTCCCAAGGATTCACACCTCCTGGAATGGACAGATCATCACCCGACAACAGCCCAGTGCACG
GGATGTTACGCCAGCCGTCCATCACAACTGGGGTCAACATTCCCATCATCACGGAACTCGCTAAGCCGGG
CAAGTTGCCTTTGGTATCAGTCAATCAGGAAAAAAACAGTGGGACGCACATTCTAATGATAACGGAGTTG
GTGAATGATACCAATGTTCAGTTTTTGGACCAAGACGATGACGATGACCCTGACACAGAGCTGTACCTCA
CACAGCCCTTTGCTTGTGGGACAGCATTTGCCGTCAGCGTCCTGGACTCACTCATGAGCGCGACATACTT
CAATGACAATATCCTCACCCTAATACGGACCCTGGTGACAGGAGGAGCCACACCAGAGCTCGAGGCTCTA
ATAGCTGAGGAGAATGCACTTCGAGGAGGCTACAGCACTCCGCAGACATTGGCCAACAGGGACCGTTGCC
GAGTGGCCCAGTTAGCCCTGTTAGATGGTCCCTTTGCAGACTTAGGGGATGGTGGTTGTTATGGTGATCT
GTTCTGCAAAGCTCTGAAAACATATAATATGCTTTGTTTTGGAATTTACCGGCTGAGAGATGCCCACCTC
AGCACCCCCAGCCAGTGTACAAAAAGGTACGTCATCACCAATCCTCCCTACGAGTTTGAGCTCGTACCAA
CAGACCTGATCTTCTGCCTGATGCAGTTTGACCACAACGCTGGCCAATCCCGGGCCAGTCTGTCTCATTC
CTCCCACTCCTCACAGTCGTCCAGTAAGAAGAGCTCCTCCGTCCACTCCATCCCGTCCACAGCAAATCGG
CCGAACCGGCCCAAGTCCAGGGAGTCCCGCGACAAACAGAACAGAAAAGAAATGGTTTACAGATAG

Supplemental Experimental Procedures:

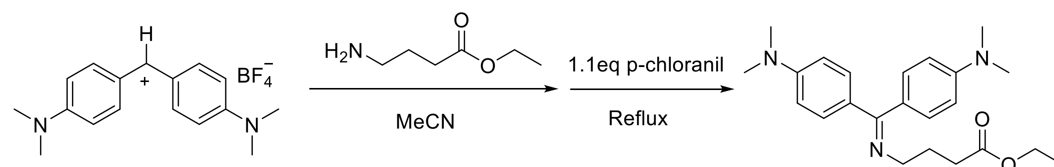
Synthesis of MHN-Ester:



MHN-Ester was prepared as follows: 2.68g bis[4-(dimethylamino)phenyl]-Benzophenone (Michler's ketone) was dissolved in 12mL/0.7mL of iPrOH/H_2O, added 0.3g $NaBH_4$. Then, the reaction mixture was heated to reflux for 4 hours. Thin layer chromatography (TLC) was used to monitor reaction progress. After completion, the reaction was cooled and filtered, washed and recrystallized in ethanol (EtOH) to give the desired product without further purification (yield: 97%).



270 mg bis[4-(dimethylamino)phenyl]-Benzophenol (Michler's hydrol) was dissolved in 20 mL MeCN, 1 equivalent HBF_4 (50% solution in H_2O) was added to the solution and allowed to stir at room temperature for 30 minutes. The reaction mixture was then dried for later use.[1]



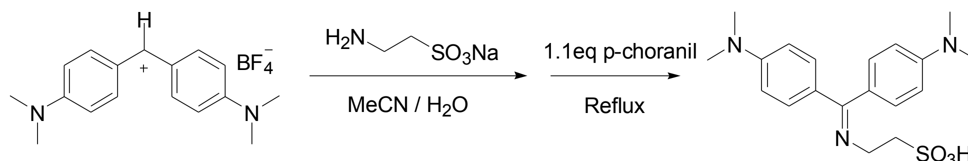
2-5 fold of amine was added to the 4,4-bis(dimethylamino)benzhydrylium tetrafluoroborate MeCN (50%) solution. Following a loss of deep violet color to a colorless solution, 1.1 eq of p-chloranil was added and then heated to 50°C for 2 hours. The reaction mixture was poured directly onto silica gel, ethyl acetate (EtOAc)/EtOH (60% / 40%) was used as eluent to separate the final product: 4-{[Bis-(4-dimethylamino-phenyl)-methylene]-amino}-butyric acid ethyl ester (MHN-ester) (yield: 24%).

1H NMR (300 MHz, CDCl3): δ 7.48(d, 2H), 7.36(d, 2H), 6.86(m, 4H), 4.07(q, 2H), 3.82(t, 2H), 2.41(t, 2H), 2.09(m, 2H), 1.19(t, 3H),

13C NMR (100 MHz, CDCl3): δ 176.3, 172.9, 154, 133.1, 128, 111.3, 60.5, 39, 30.8, 24.6, 13.1, MS (EI): m/z (%): 382.3. (Theoretical 381.24)

Synthesis of MHN-Tau:

4,4-bis(dimethylamino)benzhydrylium tetrafluoroborate MeCN (50%) solution was prepared as described above.



5-fold excess of sodium taurate was added to the 4,4-bis(dimethylamino)benzhydrylium tetrafluoroborate MeCN (50%) solution. Following a loss of deep violet color to a colorless solution, 1.1 eq of p-chloranil was added and heated to 50°C for 2 hours. The reaction mixture was poured directly onto silica gel, EtOAc/EtOH (60% / 40%) was used as eluent to separate the final product: 2-{[Bis-(4-dimethylamino-phenyl)-methylene]-amino}-ethanesulfonic acid (MHN-tau) (yield: 44%).

1H NMR (300 MHz, MeOD): δ 7.57(d, 2H), 7.47(d, 2H), 6.90(m, 4H), 4.22(t, 2H), 3.33(m, 1H), 3.21(t, 2H), 3.16(s, 12H)

13C NMR (100 MHz, MeOD): δ 176.5, 155.3, 154.3, 134.1, 118.9, 115.2, 111.3, 110.8, 49.0, 45.0, 38.8, MS (EI): m/z (%): 375.2 (Theoretical 375.16)

Determination of dL5+MHN-Ester quantum yield and Kd**

Quantum yields were determined by comparing integrated emission spectra of FAP/fluorogen complexes to reference dyes. Corrected emission spectra were taken on a Quantamaster monochromator fluorimeter (Photon Technology International). Rhodamine 6G in ethanol was used as standard for MHN-ester/dL5** complex. The emission spectra (500 - 700 nm) of a set of five concentrations were integrated and plotted against absorbance at 480 nm and the Φ_F was then calculated from the slopes using equation 1

$$\Phi_X = \Phi_{ST}\left(\frac{\mathrm{Grad}_X}{\mathrm{Grad}_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right)$$

eq 1.

Determination of dissociation constant: 5nM of purified dL5** was pre-complexed with a series of dye dilutions and the fluorescence intensity was measured. The fluorescence of dye without protein at each

concentration was also obtained and subtracted as background. Based on the plateau intensity in the binding curve calculated from a hyperbolic binding model, the results were normalized to the concentration of dye/protein complex. Using this scaled data, the dissociation constant was calculated from a one-site binding ligand depletion model in GraphPad Prism 6.

Transient Transfection and Generation of Stable FAP-TM and FAP-KDEL Cell Lines

Cells were seeded in 6-well plates and transfected the following day using lipofectamine 2000 (Life Technologies, Carlsbad CA) according to the manufacturer's instructions. 2 μg DNA were used per well of transfection. Transfected cells were re-seeded onto 25 mm coverslips in 6-well plates or 18 mm coverslips in 12-well plates for fixation and analysis. dL5**-TM was transfected with pBabe-dL5**-TM and selected by growth in 2 μg/ml puromycin following sorting by FACS. dL5**-KDEL, a construct localized to endoplasmic reticulum,[2] was subcloned into pcDNA3.1, linearized, and transfected using lipofectamine 2000. Cells were selected for 10 days using G418 to produce stably expressing polyclonal cells.

Measurement of MG-BTau displacement by MHN dyes

MG-BTau displacement was measured in cell suspension by dissociation of HEK293 cells stably expressing FAP-BKα using PBS containing 4 mM EDTA. 300 nM MG-BTau was added for 5 minutes to label all surface-exposed FAP. Flow cytometry was initiated and 300 nM MHN-Ester was added after one minute. Surface fluorescence was binned in 0.1s intervals and smoothed to generate traces over time. No measurable change was observed (n = 3 experiments). MG-BTau displacement was measured in adherent cells by addition of 300 nM MHN-Tau to cells pre-labeled with MG-BTau; changes in far-red (640 nm excitation / 685/70 nm emission) fluorescence was measured using a Nikon spinning disk confocal microscope (Andor). In the experiments shown, MG-BTau was not washed out prior to MHN-dye addition.

Determination of K_{eff}

Dye activation was measured using flow cytometry as described in methods. Dyes were serially diluted from 500 nM to 5 pM. Untransfected HEK293 cells were treated identically to identify nonspecific dye background. 4 replicates were acquired for each cell type and dye concentration. Median fluorescence values were averaged among replicates to get the total fluorescence for dL5-BKα and HEK293. HEK fluorescence signal was subtracted from FAP-BKα in order to determine specific binding. Background fluorescence from unbound dyes in HEK293 cells is negligible for both dyes and indiscernible from cellular background autofluorescence at concentrations below 1 μM.

References:

(1) Kanzian, T., Nigst, T. A., Maier, A., Pichl, S., and Mayr, H. (2009) Nucleophilic Reactivities of Primary and Secondary Amines in Acetonitrile. *Eur. J. Org. Chem. 2009*, 6379–6385.

(2) Telmer, C. A., Verma, R., Teng, H., Andreko, S., Law, L., and Bruchez, M. P. (2015) Rapid, Specific, No-wash, Far-red Fluorogen Activation in Subcellular Compartments by Targeted Fluorogen Activating Proteins. *ACS Chem. Biol.* 150216130031004.