**Multi-excitation fluorogenic labeling of surface, intracellular and total protein pools in living cells**

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S1: Synthesis and characterization of BluR dyes

General remarks

Unless otherwise noted, all chemicals were purchased from Acros Organics, Ark Pharm Inc. Aapptec, Alfa Aesar, Sigma-Aldrich and used without further purification. When necessary, anhydrous solvents were used as purchased. Column chromatography was performed on a silica gel (particle size 20-45 μm) or reverse-phase C18 Buchi International MPLC or a Waters 600 HPLC on a μ-Bondapak 10 μm 7.8x300 mm RP-C18 column was used. ¹H, ¹³C, ¹⁹F, COSY, HSQC and HMBC Nuclear Magnetic Resonance (NMR) spectra of the compounds were recorded on either 300 MHz or 500 MHz Bruker Advance spectrometer using CDCl₃, MeOD or DMSO-d₆ as the deuterated solvent and chemical shifts are expressed in parts per million (ppm). All spectra were recorded at 300 K. Analysis was performed using Bruker TopSpin 3.2 software. Mass of the compounds was recorded using Electron Spray Ionization (ESI-MS) technique using MeOH either in the positive or negative mode on a Finnigan LCQ quadrupole ion trap mass spectrometer using Xcalibur Version 1.2. Mass analysis of the products was performed on a ThermoFisher Scientific LCQ classic with Xcalibur version 1.3 software. The sample was desalted and concentrated with a homemade 3 cm C-18 capillary liquid chromatography column and electrosprayed directly into the mass spectrometer. The purity of the samples were also determined by eluting the dye on a reverse phase C18 column on Acquity UPLC instrument using a varying gradient of water(0.1% TFA)-acetonitrile mixture with a flow rate of 0.5 mL/min over 5 mins. Analysis was carried out using Empower software that displays a UV absorption chromatogram 280 nm, 400 nm and 610 nm channels.

1. **7-hydroxy coumarin-4-acetic acid**: The chemical was obtained from Acros Organics and used without further purification. The NMR of the sample was recorded to confirm the purity.

![7-hydroxy coumarin-4-acetic acid](image)

**1H NMR**: CD₃OD, 300 MHz: 7.59 (d, 1H, J= 8.7 Hz), 6.83 (dd, 1H, J = 2.4 Hz, 8.8 Hz), 6.75 (d, 1H, J = 2.3 Hz), 6.22 (s, 1H), 3.84 (s, 2H)

**13C NMR**: CD₃OD, 300 MHz: 171.31, 162.13, 161.65, 155.43, 155.43, 150.59, 126.15, 112.99, 111.74, 102.25, 37.11

2. **MG[H]EDA**: Was synthesized and characterized according to previously reported procedure.¹
3. Synthesis of Pacific blue (6,8-difluoro-7-hydroxycoumarin-3-carboxylic acid)

**Synthesis of Pacific Blue**

![Chemical structure of Pacific blue]

**3a) 2,4-difluorobenzene-1,3-diol**

A solution of 1,3-dimethoxy-2,4-difluorobenzene (4.1 g, 23.5 mmol, 1.0 eq.) was prepared in anhydrous DCM (80 mL) was cooled to 20 °C in Argon atmosphere and stirred for 5 minutes before the addition of BBr₃ (1M in DCM, 62 mmol, 62 mL, 1.3 eq.) via an addition funnel over 25 mins. The reaction was stirred at ambient temperature for 28 hours before quenching it with water (dropwise). The mixture was subsequently stirred until all precipitate dissolved completely. DCM layer was isolated and the water layer was extracted using ethyl ether thrice. The organic layer was washed with brine and dried over sodium sulfate and the organic solvent was removed under vacuum. The solid thus obtained was subjected to a flash chromatography (silica gel) in 30% ethyl acetate in hexane, to obtain a pale orange solid. **Yield:** 3.2 g, 21.9 mmol, 93 %

**1H NMR (DMSO-d₆, 500 MHz):** 6.34 (ddd, J= 9.15, 4.95, 1 H); 6.76 (ddd, J = 2.3, 3.8, 9.1, 1H); 9.56 (br-s, 1H, Ar-OH), 9.92 (br-s, 1H, Ar-OH)
Supplementary Information

13C NMR (DMSO-d$_6$, 500 MHz): 106.04 (dd, J = 9.1, 31.85) 110.21 (dd, J = 14.5,77.3), 134.91(dd,J=53.8, 68.6 ), 141.40 (dd, J= 23.2, 945.02), 142.58(dd, J = 9.2, 41), 146.03 (dd, J = 16.4, 921)

ESI-MS (negative): obtained: 145.2 expected: 146.02

3b) 3,5-difluoro-2,4-dihydroxybenzaldehyde$^3$: 1,3-dihydroxy-2,4-difluorobenzene (3.2 g, 21.9 mmol) and hexamethylenetetramine(3.2 g, 23 mmol) were dissolved in 40 mL of trifluoroacetic acid and refluxed for 24 hours. Upon completion, the solvent was removed under vacuum. The slimy residue was then dissolved in chloroform and sodium bicarbonate solution was added to make it alkaline. The aqueous layer was then acidified with con. HCl and re-extracted multiple times with dichloromethane. The organic extract was dried using sodium sulfate, concentrated under vacuum and subject to column chromatography. A flash column chromatography using 20% EtOAc: Hexanes was carried out. The product started crystallizing as a white solid while eluting from the column. However, upon concentration, it became a yellow mass. Yield: 1.29 g, 7.4 mmol, 34%

1H NMR (DMSO-d$_6$, 500 MHz): 7.29 (dd, J = 2.0, 11, 1H), 10.04 (d, J = 2.3, 1H), 10.6-12 (br, 2H)

13C NMR (DMSO-d$_6$, 500 MHz): 109.98 (dd, J = 10.4, 76.5), 114.31 (dd, J = 6.5, 22.1), 141.51 (dd, J = 22.9, 950.8), 141.97 (dd, J = 53.8, 71.5), 146.07 (dd, J = 17.8, 941.7), 147.20 (d, J = 46.6), 189.74

ESI-MS: (negative) obtained: 173.3 expected: 174.01

3c) 6,8-difluoro-7-hydroxycoumarin-3-carboxylic acid (Pacific blue)$^{3,4}$:

3,5-difluoro-2,4-dihydroxybenzaldehyde (2.1 g, 12.2 mmol) and malonic acid (2.54 g, 24.42 mmol) were dissolved 11 mL of anhydrous pyridine in a 250 mL round flask. 1 mL of aniline was added dropwise. The mixture was stirred overnight under argon at room temperature. The obtained precipitate was washed by 17.5 mL ethanol for 1 h, filtered and washed again with hydrochloric acid water and diethyl ether. Yield: 2.18 g, 9.6 mmol, 74%

1H NMR (DMSO-d$_6$, 500 MHz): 7.39 (dd, J = 1.9, 10.5, 1H), 8.69 (s, 1H)

13C NMR (DMSO-d$_6$, 500 MHz): 164.15, 158.40, 149.52 (d, J = 26.7 Hz), 149.41 (dd, J = 965.1, 17.9 Hz), 141.44 (m), 139.08 (dd, J = 981.5, 24.4 Hz), 128.26, 113.90, 110.04 (dd, J = 84.1, 11.9 Hz), 109.24 (d, J = 39.5 Hz)

ESI-MS: (negative) obtained: 241.2 expected: 242.00

4. Synthesis of BluR dyes:
General procedure:\n
Coumarin-carboxylic acid (0.1 mmol, 1 eq.) and HOBt (32 mg, 0.2 mmol, 2 eq.) was dissolved in anhydrous DMF (600 uL). DCC (40 mg, 0.2 mmol, 2 eq.) was added to the mixture and stirred for 4 hours at room temperature. A premixed solution of MG[H]EDA (100 mg, 0.2 mmol, 2 eq.) and DIEA (23 uL, 0.2 mmol, 2 eq.) in anhydrous DMF (400 uL) was added dropwise to the coumarin-carboxylic acid mixture and left to stir at room temperature overnight. The next day, the mixture was filtered; the filtrate was dried and taken up in acetonitrile. To this chloranil (50 mg, 0.2 mmol) was added and boiled for 15 mins at 60 °C to obtain the crude BluR dye.

The crude BluR dye was cooled to room temperature, filtered to remove any undissolved particles. The filtrate was dried down, taken up in a mixture of 60:30:10 water (0.1% TFA): acetonitrile: methanol and purified using a gradient of 0-40% acetonitrile (linear gradient 1.5 hours) using a reverse-phase C18 column using MPLC. The fractions were analyzed using UPLC, appropriate fractions were combined and dried to obtain a pure BluR dye – confirmed by NMR spectroscopy recorded using MeOD and electrospray ionization – mass spectroscopy (ESI-MS).
Supplementary Information

Spectroscopic characterization of BluR dyes

BluR1 – 7-hydroxycoumarin-4-acetic acid and MGEDA conjugate

![BluR1](image)

$^1$H NMR of BluR1 (CD$_3$OD)
Supplementary Information

### $^{13}$C NMR of BluR1 (CD$_3$OD)

| Position | $^1$H (500 MHz) | $^{13}$C (500 MHz) |
|----------|-----------------|--------------------|
| 1        | 3.32, s, 12H    | 39.41              |
| 2        |                 | 156.96             |
| 3        | 7.04, d, 2H, J = 9.3 | 112.98          |
| 4        | 7.42, d, 4H, J = 14.05 | 140.46        |
| 5        |                 | 126.88             |
| 6        |                 | 178.28             |
| 7        |                 | 131.82             |
| 8        | 7.34, d, 2H, J = 8.75 | 137.40        |
| 9        | 7.15, d, 2H, J = 8.8  | 114.64            |
| 10       |                 | 164.23             |
| 11       | 4.16, t, 2H, J = 6.25 | 67.50            |
| 12       | 2.12, m, 2H     | 24.76              |
| 13       | 2.39, t, 2H, J = 7.25 | 31.93            |
| 14       |                 | 174.27             |
| 15       | 3.33, br-s, 2H  | 38.75              |
| 16       | 3.33, br-s, 2H  | 39.08              |
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|   |            |      |
|---|------------|------|
| 17 | ---------- | 169.77 |
| 18 | 3.71, s, 2H | 39.41 |
| 19 | ---------- | 155.41 |
| 20 | ---------- | 112.98 |
| 21 | 7.60, d, 1H, J = 8.75 Hz | 126.18 |
| 22 | 6.80, dd, 1H, J = 8.75, 2.3 | 111.75 |
| 23 | ---------- | 161.65 |
| 24 | 6.68, d, 1H, J = 2.3 | 102.27 |
| 25 | ---------- | 151.04 |
| 26 | ---------- | 161.97 |
| 27 | 6.1811 | 111.75 |

ESI-MS (positive mode): obtained: 675.3     expected: 675.32

BluR2 – 6,8-difluoro-7hydroxycoumarin-3-carboxylic acid (Pacific blue, PB) and MGEDA conjugate

![BluR2 Structure](image)
Supplementary Information

$^1$H NMR of BluR1 (CD$_3$OD)
### $^{13}$C NMR of BluR1 (CD$_3$OD)

| Position | $^1$H (500 MHz) | $^{13}$C (500 MHz) | $^{19}$F (300 MHz) |
|----------|-----------------|-------------------|-------------------|
| 1        | 3.32, s, 12H    | 39.41             |                   |
| 2        | -----           | 156.92            |                   |
| 3        | 7.20, d, 4H, J = 9.2 Hz | 112.94 |                   |
| 4        | 7.37, d, 4H, J = 8.9 Hz | 140.40 |                   |
| 5        | -----           | 126.75            |                   |
| 6        | -----           | 178.05            |                   |
| 7        | -----           | 131.64            |                   |
| 8        | 7.28, d, 2H, J = 8.7 Hz | 137.40 |                   |
| 9        | 7.14, d, 2H, J = 8.7 Hz | 114.63 |                   |
| 10       | -----           | 164.20            |                   |
| 11       | 4.19, t, 2H, J = 6.3 Hz | 67.50  |                   |
| 12       | 2.18, m, 2H     | 24.75             |                   |
| 13       | 2.47, t, 2H, J = 6.9 Hz | 31.84  |                   |
| 14       | -----           | 174.34            |                   |
| 15       | 3.47, t, 2H, J = 6.2 Hz | 38.52  |                   |
| 16       | 3.57, t, 2H, J = 5.2 Hz | 39.07  |                   |
ESI-MS (positive mode): obtained: 697.3  expected: 697.28

5. HCM

HCM was synthesized using the previously reported procedure\(^6\). TLC, ESI-MS was used to confirm the purity of the sample, before cell imaging.
Supplementary Information

S2: Photophysical properties of BluR dyes:

**General remarks:** All spectroscopic measurements were carried out using Beckman Coulter DU730 Life Science UV/Vis Spectrophotometer, Agilent Technologies Cary 60 UV-Vis, TECAN Infinite M1000 96-well plate reader or Quantamaster monochromator fluorimeter (Photon Technology International). A phosphate buffered saline (PBS) – 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄.7H₂O, 2mM KH₂PO₄, pH 7.4 was used for all measurements. The ratio of dye:dL5** was always 1:5. MG2p was used as a reference dye in all cases.

1. **Structure of MG2p**

![MG2p structure](image)

**Figure S2.1:** Structure of MG2p – standard dye that binds to dL5**

2. **Absorbance spectra of donor coumarins**

![Absorbance spectra](image)

**Figure S2.2a:** Absorbance spectra of donor coumarins, 7HC and PB, recorded in phosphate buffered saline (PBS) pH 7.4 and each dye was normalized to the wavelength corresponding to the highest optical density. Pacific blue (PB) shows significant absorbance cross-section at 405 nm.
3. Spectral overlap between 7HC, PB and MG2p-dL5**

Critical distance for energy transfer between the coumarins and MG2p-dL5** were calculated using the formula

$$R_0^6 = 8.785 \times 10^{-5} \kappa^2 Q_D^0 J/n^4$$

where $R_0$ is the critical distance for energy transfer or the Forster distance, $\kappa^2$ is the orientation factor (assumed as 2/3), $n$ is the refractive index of the medium (assumed as 1.4), $Q_D^0$ is the quantum yield of the donor (7HC – 0.68, PB – 0.77)$^4$, $J$ is the overlap integral between the fluorescence spectrum of the donor and the molar absorption spectrum of the acceptor

$$J = \int FD(\lambda)\varepsilon A(\lambda)\lambda^4 d\lambda$$

Where $FD$ is the peak-normalized fluorescence spectrum of the donor, $\varepsilon A$ is the absorption spectrum of the acceptor.$^7$

The normalized donor emission fluorescence data and the acceptor (MG2p-dL5**) absorbance spectra were plugged into the spreadsheet obtained from http://photobiology.info/Experiments/Biolum-Expt.html

![Figure S2.3a: Spectral overlap between (normalized) emission spectra of 7-hydroxycoumarin-4-acetic acid (7HC) and absorbance spectra of MG2p-dL5**.](image-url)
**Figure S2.3b:** Spectral overlap between (normalized) emission spectra of Pacific blue (PB) and absorbance spectra of MG2p-dL5**

4. **Absorbance of BluR dyes only**

**Figure S2.4a:** Absorbance of 5 μM BluR1, BluR2 and MG2p in phosphate buffered saline (PBS) pH 7.4. Each dye was normalized to the wavelength corresponding to the highest optical density. Absorbance features of BluR1 and MG2p are very similar, while BluR2 shows pronounced coumarin absorbance in addition to the characteristic features of MG. The extinction co-efficient of MG at 607 nm is 74,250 M⁻¹cm⁻¹ in PBS 7.4.¹
Figure S2.4b: Absorbance of 5 uM BluR1 and MG2p in phosphate buffered saline (PBS) pH 7.4. Each dye was normalized to the wavelength corresponding to the highest optical density. Absorbance features of BluR1 and MG2p are very similar, while BluR1 shows pronounced coumarin absorbance in addition to the characteristic features of MG between 300 to 400 nm. The extinction co-efficient of MG at 607 nm is 74,250 M$^{-1}$cm$^{-1}$ in PBS 7.4.$^1$

Figure S2.4c: Absorbance of BluR1 measured using phosphate buffered saline (PBS) at pH 7, pH 8, and pH 9. The plots have been offset by 0.05 using Origin 8.0 software. The red-shifting of coumarin band due to increase in pH is very pronounced indicating the formation of phenolate structures at a higher pH.
5. Absorbance spectra of BluR-dL5** and MG2p-dL5**

**Figure S2.5a:** Absorbance of BluR1, BluR2 and MG2p (3 uM) complexed with dL5** (15 uM) in phosphate buffered saline (PBS) pH 7.4 each dye-dL5** was normalized to the wavelength corresponding to the highest optical density. When bound to dL5**, BluR1 and BluR2 show red-shift similar to MG2p. The extinction co-efficient of MG – dL5** at 607 nm is 91,700 M⁻¹ cm⁻¹ in PBS 7.4.⁸

**Figure S2.5b:** Absorbance of BluR1 and MG2p (3 uM) complexed with dL5** (15 uM) in phosphate buffered saline (PBS) pH 7.4. Each dye-dL5** was normalized to the wavelength corresponding to the highest optical density. When bound to dL5**, BluR1 shows red-shift similar to MG2p. The extinction co-efficient of MG – dL5** at 607 nm is 91,700 M⁻¹ cm⁻¹ in PBS 7.4.⁸
6. Excitation spectra of BluR, MG2p and dye-dL5** complexes (em: 700 nm)

![Excitation Spectra](image)

Figure S2.6: Excitation spectra was recorded using 1 uM dye and 5 uM dL5**, \( \lambda_{em} \) was set to 700 nm. The dyes were compared to MG2p-dL5**, BluR1, BluR2 and MG2p only in phosphate buffered saline (PBS) pH 7.4. Normalization for the dye, dye-dL5** was done by setting the \( \lambda \) of dye-dL5** with highest fluorescence intensity to 1.0. BluR2 shows significant excitation cross-section at 405 nm in addition to MG-like excitation profile. The free dyes show no significant excitation.

![Excitation Spectra](image)

Figure S2.6b: Excitation spectra from 300-680 nm using emission wavelength of 700 nm, resulted in the appearance of the \( \lambda/2 \) peak at 350 nm, as anticipated. This rendered impossible any significant analysis of the very subdued 7HC peak in BluR1-dL5**

7. FRET emission spectra of BluR, MG2p and dye-dL5** complexes (ex: 405 nm)
Figure S2.7: Emission spectra was recorded using 1 uM dye and 5 uM dL5**. λex was set to 405 nm to visualize energy transfer between coumarin to MG-dL5**. The dyes were compared to MG2p-dL5**, BluR1, BluR2 and MG2p only in phosphate buffered saline (PBS) pH 7.4. Normalization was done by setting the λmax of emission of BluR2-dL5** to 1.0. When excited at 405 nm, BluR2-dL5** shows about 10-fold far-red emission compared to BluR1-dL5** and MG2p-dL5**. The free dyes show no significant emission.

8. Direct emission spectra of BluR, MG2p and dye-dL5** complexes (ex: 600 nm)

Figure S2.8: Emission spectra was recorded using 1 uM dye and 5 uM dL5**. λex was set to 600 nm to visualize direct excitation of MG-dL5** in BluR dyes and compared to MG2p-dL5** and free dyes.
BluR1, BluR2, MG2p in phosphate buffered saline (PBS) pH 7.4. Normalization for the dye, dye-dL5** was done by setting the emission maximum of dye-dL5** to 1.0. Notice that BluR2, MG2p behave similarly when bound to dL5** and BluR1-dL5** shows a noticeable red-shift in MG emission. The free (unbound) dyes show no significant emission.

9. Energy transfer efficiency

The energy transfer efficiency of the donor coumarin dyes to the acceptor MG-dL5** in the BluR-dL5** complex was calculated by determining the fluorescence of the BluR-dL5** and the respective coumarin complexes using the formula

$$E = 1 - \frac{F_{BluR-dL5**}}{F_{coumarin}}$$

Where E – energy transfer efficiency and F – fluorescence intensity

Figure S2.9a: Evaluation of FRET efficiency based on energy transfer from 7-hydroxycoumarin-4-acetic acid (7HC) (1 uM) to Malachite Green (MG) in BluR1 – dL5** (1 uM – 5 uM) was measured by the decrease in the 7HC emission when both 7HC and BluR1 – dL5** were excited at 405 nm.
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Figure S2.9b: Evaluation of FRET efficiency based on energy transfer from Pacific blue (PB) (1 uM) to Malachite Green (MG) in BluR1 – dL5** (1 uM – 5 uM) was measured by the decrease in the PB emission when both PB and BluR2 – dL5** were excited at 405 nm.

10. Quantum yield

Quantum yield of the dyes were determined using MG2p-dL5** as a standard with a quantum yield of 20% in PBS 7.4. The fluorescence emission of dye-dL5** solutions containing the same O.D at 630 nm (λex) in PBS 7.4 were obtained. The fluorescence spectra of the solutions were measured and the ratio of quantum yield of standard and unknown sample is:

\[ \frac{F_x}{F_s} = \frac{Q_x}{Q_s} \]

Where \( F \) – fluorescence intensity, \( Q \) – quantum yield; \( x \) – sample, \( s \) – standard

S3: Binding affinity of BluR dyes

Binding affinity of BluR1, BluR2 dyes was determined by titrating it against a known concentration of dL5**. Triplicate fluorescence response was determined using a 96-well plate on a TECAN Infinite M1000 96-well plate reader fluorimeter using ex/em of 636 nm/664 nm. Analysis of fluorescence response was determined using a non-linear regression using One site – Total, accounting for ligand depletion. The model, originally used for radioactivity measurements was tweaked to fit fluorescence data by fixing the volume at 0.2 mL and SpecAct was set to 1.00 on GraphPad Prism5.0 software. The ligand depletion model assumes that changes in
complex formation are associated with complementary changes in free ligand and free receptor, and are a typical model for ligand-receptor interactions when one has to work at protein concentrations that are near the Kd value. The original formula and fitting can be found at

http://www.graphpad.com/guides/prism/6/cure-fit/index.htm?reg_one_site_total_depletion.htm

**Figure S3:** Kd measurement of BluR1, BluR2 and MG2p using purified dL5**. 5 nM dL5** was incubated with a serial dilution of 500 nM to 10 pM of the respective dye dissolved in phosphate buffered saline (PBS) pH 7.4. The dye fluorescence was subtracted from the dye-dL5** fluorescence. Notice that the binding properties of BluR1, BluR2 and MG2p are similar.

**S4: Cell imaging of BluR dyes**

**Biological Materials and Methods**

**Cell culture**

HEK-293 cells were cultured in Dulbecco’s Modified Eagle Median (DMEM) with 10% fetal bovine serum (FBS) in a 5% CO₂ 37°C incubator. Cells were split at 80% confluence.

**Mammalian cell line preparation**

dL5** BKα DNA constructs and stable cell line generation was described previously ⁹

**Fluorescence microscopy**

HEK-293 cells expressing dL5** BKα were plated in glass-bottom Mattek dishes and imaged at 80% confluence. For the sequential dye Inside/Outside labeling approach, the media was aspirated and replaced with Hanks Balanced Salt Solution containing 50 nM HCM. HCM was incubated for 5 min and 1 µM BluR2 dye was added dropwise to dish and allowed to incubate for 10 min in an incubator. After the final 10 min incubation, imaging was performed on a Zeiss LSM 880 (Figure 3 and S4) using a PlanApo 63x, 1.40 NA objective
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respectively. Excitation utilized a 405 nm laser, 514 nm laser, and a 640 nm laser. All excitation wavelengths used an emission filter of 641-695 on the Zeiss microscope.

Figure S4.

**Figure S4.** Quantitative comparison of 514 nm excitation of BluR2 Only in respect to HCM Only. For both dye labeling conditions, the 514-680 mean fluorescence of the image is divided by its 640-680 mean fluorescence, resulting in a normalized, quantitative measurement of 514 nm fluorescence produced by either HCM or BluR2. When the 514/640 mean fluorescence ratios are normalized to HCM Only, BluR2 is less than 10% of HCM fluorescence. These results demonstrate that 514 nm excitation does not produce admissible BluR2 fluorescence, making it possible to differentiate HCM labeling from BluR2. Scale bar: 20 µm.
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