Supplementary Information for

Efficient Bioimaging with Diaminodicyanoquinodimethanes: Selective Imaging of Epidermal and Stomatal Cells and Insight into the Molecular Level Interactions

N. Senthilnathan, Ch. G. Chandaluri and T. P. Radhakrishnan*

Details of characterization of the dyes, confocal laser scanning microscopy studies of pea stomata, different leaf stomata, mammalian and bacterial cells, autofluorescence analysis of pea epidermal cells, isothermal titration calorimetry, infra-red spectra, FESEM, EDX analysis, absorption and fluorescence spectra, photo-stability studies and cytotoxicity assay.

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Characterization of BT$_2$ and DPZDQ

**BT$_2$**

Yield = 75%, recrystallized from acetonitrile; m.p. 310°C (dec.); FTIR (KBr): $\nu$ cm$^{-1}$ = 2177.4, 2138.6, 1136.1; $^1$H NMR (d$_6$-DMSO): $\delta$/ppm = 9.0 (s, 4H), 7.49 (d, 4H), 7.38 (d, 2H), 7.12 (d, 4H), 6.88 (d, 2H), 3.8 (m, 4H), 3.46 (m, 4H), 2.2 (s, 6H); $^{13}$C NMR (d$_6$-DMSO): $\delta$/ppm = 21, 36, 41, 43, 48, 113, 118, 123, 125, 128, 132, 138, 139, 145, 150, 171; elemental analysis (calcd., found for BT$_2$H$_2$O i.e. C$_{32}$H$_{40}$N$_6$O$_7$S$_2$): %C = (56.23, 56.14), %H = (5.82, 5.85), %N = (12.15, 12.28). $\lambda_{\text{abs}}^{\text{max}}$: 415 nm, $\lambda_{\text{emi}}^{\text{max}}$: 538 nm, Stokes shift: 123 nm, quantum yield: 14.2 % (solid state), 0.05 % (solution state), lifetime: 0.63 ns, $\varepsilon$: 10,912 M$^{-1}$cm$^{-1}$, brightness: 154.942 M$^{-1}$cm$^{-1}$.

**DPZDQ**

Yield = 80%, recrystallized from DMF-ether; m.p. 300°C (dec.); FTIR (KBr): $\tilde{\nu}$ cm$^{-1}$ = 2171.6, 2130.3, 1593.1; $^1$H NMR (d$_6$-DMSO): $\delta$/ppm = 7.3 (d, 2H), 6.8 (d, 2H), 3.5 (s, 2H), 3.3-3.1 (m, 8H), 2.8 (m, 8H); $^{13}$C NMR (d$_6$-DMSO): $\delta$/ppm = 168, 149, 132, 123, 118, 114, 53, 46, 34; elemental analysis (calcd., found for C$_{18}$H$_{22}$N$_6$): %C = (67.15, 67.8), %H = (6.81, 6.83), %N = (26.15, 26.08). $\lambda_{\text{abs}}^{\text{max}}$: 391 nm, $\lambda_{\text{emi}}^{\text{max}}$: 522 nm, Stokes shift: 131 nm, quantum yield: 5.8 % (solid state), 0.07 % (solution state), lifetime: 0.75 ns, $\varepsilon$: 17,871 M$^{-1}$cm$^{-1}$, brightness: 103.652 M$^{-1}$cm$^{-1}$.
Comparison with other dyes

Table S1. Comparison of commercial dyes used for stomatal imaging with BT₂ in terms of conditions and selectivity of staining.

| Dye           | Solvent        | Concentration (mM) | Staining time (min) | Part stained          | Ref.   |
|---------------|----------------|--------------------|---------------------|-----------------------|--------|
| Aniline Blue  | Water          | 0.67               | -                   | Cell wall             | S1     |
| Aniline Blue  | Water          | 0.67               | -                   | Cell wall             | S2     |
| DAF-2DA*      | DMSO           | 0.02               | 30                  | Full cell             | S3, S4 |
| DAF-2DA**     | DMSO           | 0.01               | 30                  | Full cell             | S5     |
| BCECF-AM*     | DMSO           | 0.005              | 30                  | Full cell             | S5, S3 |
| H2DCF-DA**    | Ethanol        | 0.03               | 30                  | Full cell             | S5     |
| BT₂           | Water, DMSO    | 0.029              | 10                  | Inner cell walls      | This work |

Sample in buffer-free state

| Dye           | Solvent        | Concentration (mM) | Staining time (min) | Part stained          | Ref.   |
|---------------|----------------|--------------------|---------------------|-----------------------|--------|
| PI*           | Water          | 1.5                | 2                   | Epidermis cell wall   | S6     |
| PI*           | Water          | 0.03               | 1-5                 | Cell wall             | S7     |
| PI*           | Water          | 0.02               | 5                   | Cell wall of viable cells | S8     |
| Safranin      | Ethanol-water  | 28.5               | -                   | Cell wall             | S9     |
| Safranin      | Water          | 28.5               | -                   | Cell wall             | S10    |
| Safranin      | Ethanol-water  | 28.5               | -                   | Cell wall             | S11    |
| Safranin      | Water          | 28.5               | 4-8                 | Cell wall             | S12    |
| BT₂           | Water, DMSO    | 1.0                | 2                   | Cell walls / nucleus  | This work |

*Dark state required.

#DAF-2DA: 4,5-diaminofluorescein diacetate; BCECF-AM: 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; H2DCF-DA: 2',7'-dichlorofluorescein diacetate; PI: propidium iodide.

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Imaging of pea stomata with BT$_2$ in DMSO

Images of samples in the buffer medium stained by BT$_2$ in DMSO, without and with irradiation; staining of the inner guard cell wall and the response to light are clearly observed again.

**Figure S1.** CLSM fluorescence images (at different magnifications) of pea epidermis kept in buffer medium (500 µL) and stained with BT$_2$ solution in DMSO (15 µL of 1 mM) under different conditions: (a, b) irradiated for 3 h and stained, (c, d) kept in the dark for 3 h and stained. Scale bars: (a), (c) = 20 µm; (b), (d) = 10 µm.
Imaging of different leaf stomata

Figure S2. CLSM images of stomata in the leaves of dicotyledon plants, (a) crape jasmine (*Tabernaemontana divaricata*), (b) paper rose (*Bougainvillea glabra*), and (c) thale cress (*Arabidopsis thaliana*), as well as a monocotyledon plant, (d) onion (*Allium cepa*) maintained in 500 µL buffer and stained using 15 µL aqueous solution of BT$_2$ having concentrations, 5 mM (a-c) and 0.05 mM (d). Scale bar: 20 µm.

Imaging experiments carried out with leaves of dicotyledon plants showed that BT$_2$ is useful for a range of specimens.
Comparison of CLSM images

**Figure S3.** Comparison of CLSM images of pea epidermis in buffer-free state, stained using 15 µL of 1.75 mM solutions of different DADQ derivatives (structures shown) in water and DMSO for 2 min, with and without washing. Images recorded for control samples without the dye under identical microscope settings are also shown. Scale bar: 20 µm.

| Dye  | In water | In DMSO | Control |
|------|----------|---------|---------|
|      | No wash  | With wash | No wash | With wash |
| BT₂  | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| DPZDQ | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| BMPDQ | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| BMDQ | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| BPDQ | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
Isothermal titration calorimetry

Table S2. Binding and thermodynamic parameters from the ITC experiments on BT<sub>2</sub> and DPZDQ with PGA<sup>−</sup>Na<sup>+</sup>; errors are shown in parenthesis.

| Parameter | Value (error) | PGA<sup>−</sup>Na<sup>+</sup> : BT<sub>2</sub> | DPZDQ : PGA<sup>−</sup>Na<sup>+</sup> |
|-----------|--------------|----------------------------------|----------------------------------|
| N         | 1.74 (± 0.012) | 0.117 (± 0.15)                  |
| K (10<sup>5</sup> dm<sup>3</sup> mol<sup>−1</sup>) | 13.3 (± 2.9) | 0.116 (± 0.016) |
| ΔH<sup>o</sup> (kJ mol<sup>−1</sup>) | -5.970 (± 0.0741) | -161.5 (± 217.2) |
| ΔG<sup>o</sup> (kJ mol<sup>−1</sup>) | -34.9 | -23.2 |
| ΔS<sup>o</sup> (J mol<sup>−1</sup> K<sup>−1</sup>) | 97.1 | -464 |

Errors in the parameters determined for the DPZDQ / PGA<sup>−</sup>Na<sup>+</sup> are very large; the data and the fitting are therefore not meaningful.

Figure S4. (a) Raw and (b) integrated thermograms from the isothermal titration of DPZDQ (2 mM) into PGA<sup>−</sup>Na<sup>+</sup> (75 µM) in aqueous solution. Fitting of the integrated thermogram is shown in (b).
FESEM, EDX analysis

Figure S5. FESEM image and EDX spectra of selected regions (ECJ: epidermal cell junction; IGCW: inner guard cell wall) shown in the image, of pea epidermis stained using DPZDQ solution in water (0.5 mL of 5 mM) for 2 min.

Figure S6. Average Ca and K content in the ECJ region of the pea epidermis under different conditions.
Table S3. Atom % of Ca and K (with standard deviations) on the inner guard cell wall (IGCW) and epidermal cell junction (ECJ) regions (Figure 7a, b of main text, Figure S5) of fresh (in water) and stained epidermis layer of pea plant leaf, determined using EDX spectroscopy with FESEM; dyes used are BT$_2$, DPZDQ, propidium iodide.

| Sample treatment               | IGCW          |           | ECJ          |           |
|-------------------------------|---------------|-----------|--------------|-----------|
|                               | Ca       | K        | Ca          | K        |
| Water                         | 0.122    | 0.0537   | 0.197       | 0.039    |
|                               | (0.0568) | (0.0315) | (0.0917)    | (0.0162) |
| BT$_2$ (1 mM in water)        | 0.0549   | 0.0438   | 0.0519      | 0.0400   |
|                               | (0.0358) | (0.0220) | (0.0290)    | (0.016)  |
| BT$_2$ (5 mM in water)        | 0.0406   | 0.0544   | 0.0466      | 0.0421   |
|                               | (0.0222) | (0.032)  | (0.0230)    | (0.020)  |
| DPZDQ (5 mM in water)         | 0.121    | 0.056    | 0.172       | 0.0467   |
|                               | (0.0610) | (0.035)  | (0.0567)    | (0.0195) |
| Propidium iodide (1 mM in water) | 0.0500  | 0.0555   | 0.0744      | 0.0503   |
|                               | (0.0330) | (0.0277) | (0.0332)    | (0.0180) |

Table S4. Atom % of Ca and K (with standard deviations) on the inner guard cell wall (IGCW) and epidermal cell junction (ECJ) regions, of epidermis layer of pea plant leaf maintained in water, MES buffer and MES buffer containing BT$_2$.

| Sample treatment               | IGCW          |           | ECJ          |           |
|-------------------------------|---------------|-----------|--------------|-----------|
|                               | Ca       | K        | Ca          | K        |
| Water                         | 0.122    | 0.0537   | 0.197       | 0.0445   |
|                               | (0.0568) | (0.0409) | (0.0917)    | (0.0345) |
| MES buffer (pH=7.0)           | 0.0475   | 0.186    | 0.173       | 0.525    |
|                               | (0.0378) | (0.111)  | (0.118)     | (0.173)  |
| BT$_2$ (1 mM in water + MES buffer) | 0.0180  | 0.219    | 0.093       | 0.731    |
|                               | (0.00920)| (0.0708) | (0.0450)    | (0.162)  |

In the MES buffer, Ca$^{2+}$ ions appear to leach out of the epidermis, and K$^+$ to move in.
Absorption and fluorescence spectroscopy

Absorption and fluorescence Spectra:

Figure S7. (a) Absorption and (b) fluorescence spectra of BT$_2$ and DPZDQ.

Relative changes of the spectral properties of BT$_2$ on adding PGA$^-$Na$^+$

As the polymer: BT$_2$ mol ratio increases, the fluorescence intensity increases and begins to saturate above a ratio of ~ 200:1.

Figure S8. (a) Absorption and (b) fluorescence spectra of BT$_2$-PGA$^-$Na$^+$ from mole ratio 1:1 to 1:300.
Auto fluorescence analysis of pea epidermal cells

The auto fluorescence of the pea epidermal cells was monitored under CLSM at different wavelengths, laser power and gain. The images recorded are shown in Figure S9.

**Figure S9.** (A) CLSM images using auto fluorescence of fresh epidermis layer: (a) excitation wavelength : 514 nm, emission range: 540-600 nm, laser power: 100%, master gain : 1077 V, (b) excitation wavelength : 488 nm, emission range: 490-570 nm laser power: 100%, master gain : 940 V, (c) bright field, (d) excitation wavelength : 405 nm, emission range: 410-470, laser power: 35%, master gain : 881 V, (e) overlay of fluorescence with bright field. Scale bar: 20 µm. (B) Fluorescence intensity profiles [along the yellow lines marked on the stomata in the images in (Aa, Ab, Ad)].

The contrasting images of stomata stained by BT₂ are shown in Fig. S10. It is seen that the auto fluorescence images obtained even with significantly higher laser power and gain, are quite dull compared to those obtained with BT₂ based imaging.

**Figure S10.** (A) CLSM Images (fluorescence and overlay of fluorescence with bright field) of pea epidermis kept in buffer medium (500 µL): (a, b) control, and (c, d) stained using BT₂ solution in water (15 µL of 1 mM); excitation wavelength: 488 nm, emission range: 490-570 nm, laser power: 5% and master gain: 700 -800 V. Scale bar: 20 µm. (B) Fluorescence intensity profile [along the yellow line marked on the stomata in the image in (Ac)].
Imaging of mammalian and bacterial cells

In order to demonstrate the generality of DADQs as fluorescence probes for bio-imaging, we have carried out the fluorescence imaging experiments with live DU145 human prostate cell lines (live mammalian cells) and cyanobacteria (fixed cells) using BT₂ and BBEDQ respectively.

**DU145 cell line**
DU145 cell lines were stained by directly introducing 15 µl of the aqueous solution of the BT₂. Cell lines were immediately washed with PBS and imaged in a CLSM. The dye molecules entered the cells and stained it completely (Fig. S11).

![Figure S11. CLSM images (fluorescence and overlay of fluorescence with bright field) of the stained DU145 cell lines using BT₂. Scale bar: 20 µm.](image)

**Cyanobacteria cell**
As BT₂ was found to be less efficient in this case, imaging was carried out using another DADQ derivative, 7,7-bis(benzylamino)-8,8-dicyanoquinodimethane (BBEDQ).⁵¹³ 5 µl of 5 mM DMSO solution of BBEDQ was added on 0.2 ml of synechocystis pcc 6803 cells in buffer; the cells were imaged in a CLSM. The dyes entered into the cells and stained them completely.

![Figure S12. CLSM images (fluorescence and overlay of fluorescence with bright field, different magnification) of the stained synechocystis pcc 6803 cells by using 5 mM DMSO solution of BBEDQ. Scale bar: 5 µm.](image)

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**Photostability studies**

The dyes were excited at 488 nm and the fluorescence monitored. BT$_2$ is found to be very photo-stable; DPZDQ is slightly less stable.

**Figure S13.** Fluorescence intensity variation of (a) BT$_2$ and (b) DPZDQ with irradiation time.
Cytotoxicity assay

Cytotoxicity assay was carried out by Pondicherry Center for Biological Sciences, Pondicherry, India (http://http://pcbscience.webs.com/). 2 - 4 × 10^5 HeLa cells were seeded in a 96-well cell culture plate with DMEM medium (Himedia) containing 1% anti-mycotic antibiotic (Himedia) and 10% FBS (Himedia), and incubated for 24 h in a CO₂ incubator at 37°C. The cells were treated initially with the test sample (BT₂, DPZDQ) in different concentrations (25, 50, 100, 250, 500 µg/mL) and incubated for 24 h. The medium was aspirated from the cells at the end of the treatment period. 0.5 mg/mL MTT and 1% PBS were added and incubated at 37°C for 4 h. After incubation, the medium containing MTT was discarded from the cells. The crystals formed were dissolved in 100 µL of DMSO; the absorbance at 570 nm was measured using a micro-plate reader to estimate the cell viability. IC₅₀ values of BT₂ and DPZDQ are found to be 1468 ± 345.4 µg/ml and 790.70 ± 98.73 µg/ml respectively.

**Figure S14.** Percent cell viability and inhibition of L929 cell colony formation in presence of different concentrations of (a) BT₂ and (b) DPZDQ at 24 h.
(b) The graph shows the percent of cell viability against different concentrations in µg/ml. The concentrations tested are 25, 50, 100, 250, and 500 µg/ml. The graph indicates a trend where cell viability decreases as concentration increases. The images below correspond to the concentrations tested: Control, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml.
Figure S15. Percentage cell viability and inhibition of HeLa cell colony formation in presence of different concentrations of (a) BT₂ and (b) DPZDQ; the corresponding plots to determine the IC₅₀ values are also shown. IC₅₀ values are calculated by extra platting the curve.
(b)