Supplementary Information for:

Cyanine Dyes as Ratiometric Fluorescence Standards for the Far-Red Spectral Region

Joshua K. G. Karlsson,[‡] Owen J. Woodford,[‡] Heinz Mustroph[†] and Anthony Harriman[‡*]

[‡]Molecular Photonics Laboratory, School of Chemistry, Bedson Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom

and

[†]FEW Chemicals GmbH, Ortsteil Wolfen, Technikumstr. 1, 06766 Bitterfeld-Wolfen, Germany

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1. Steady-State Spectroscopic Characterisation of Dyes

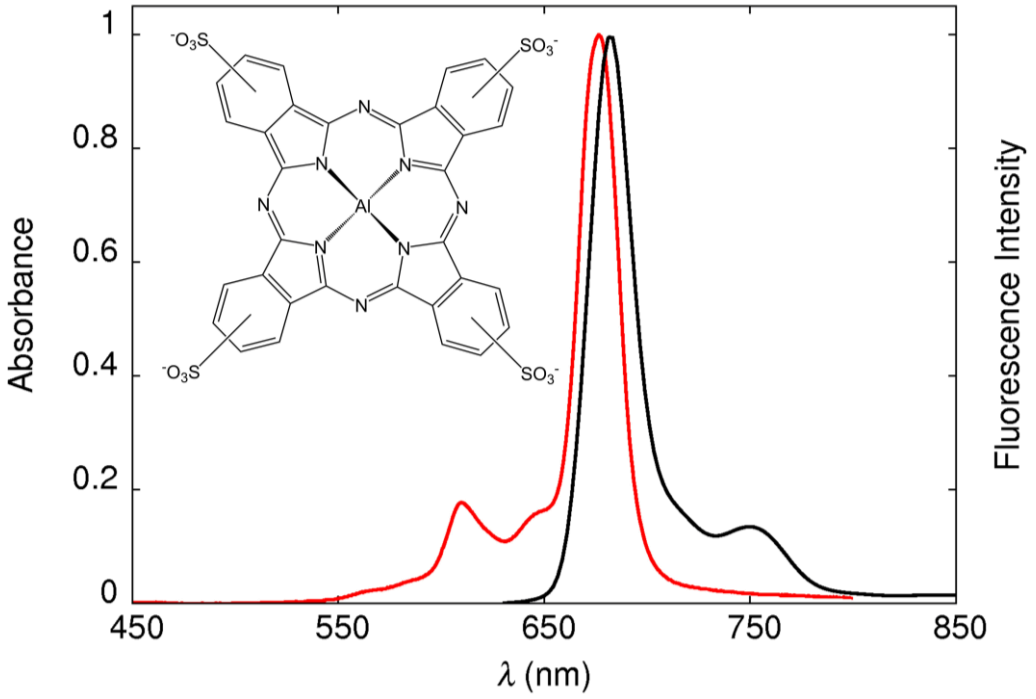

Figure S1. Normalized steady-state absorption and fluorescence spectra recorded for aluminium phthalocyanine tetrasulfonate in methanol.

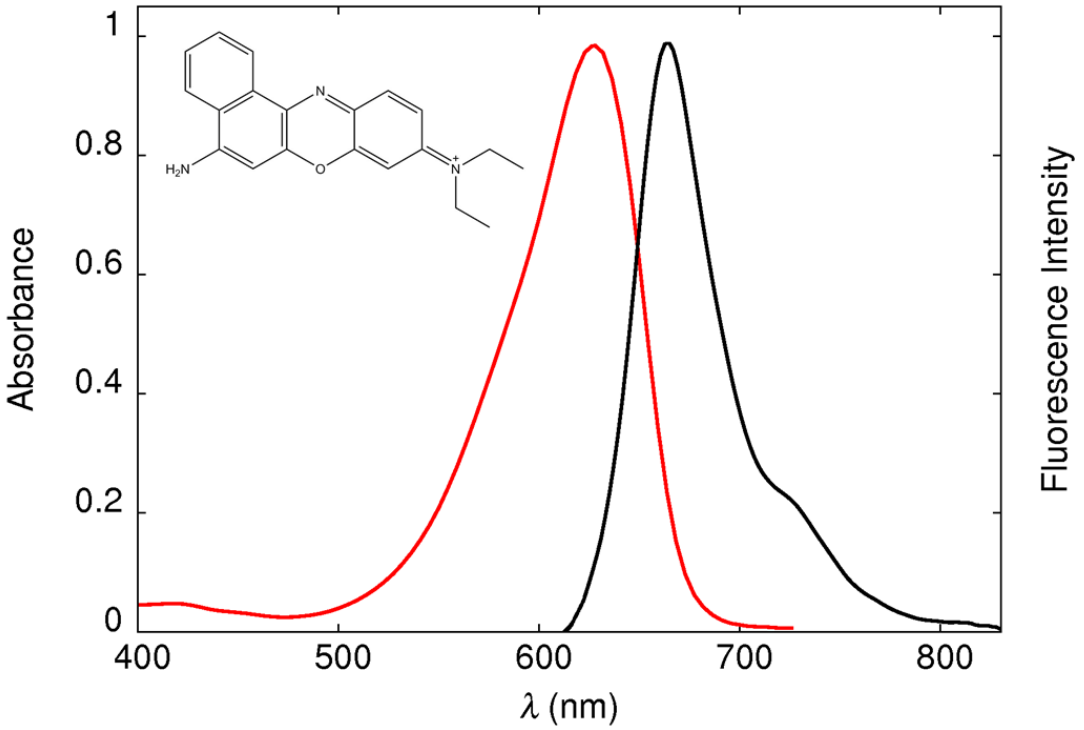

Figure S2. Normalized steady-state absorption and fluorescence spectra of Nile Blue in ethanol.

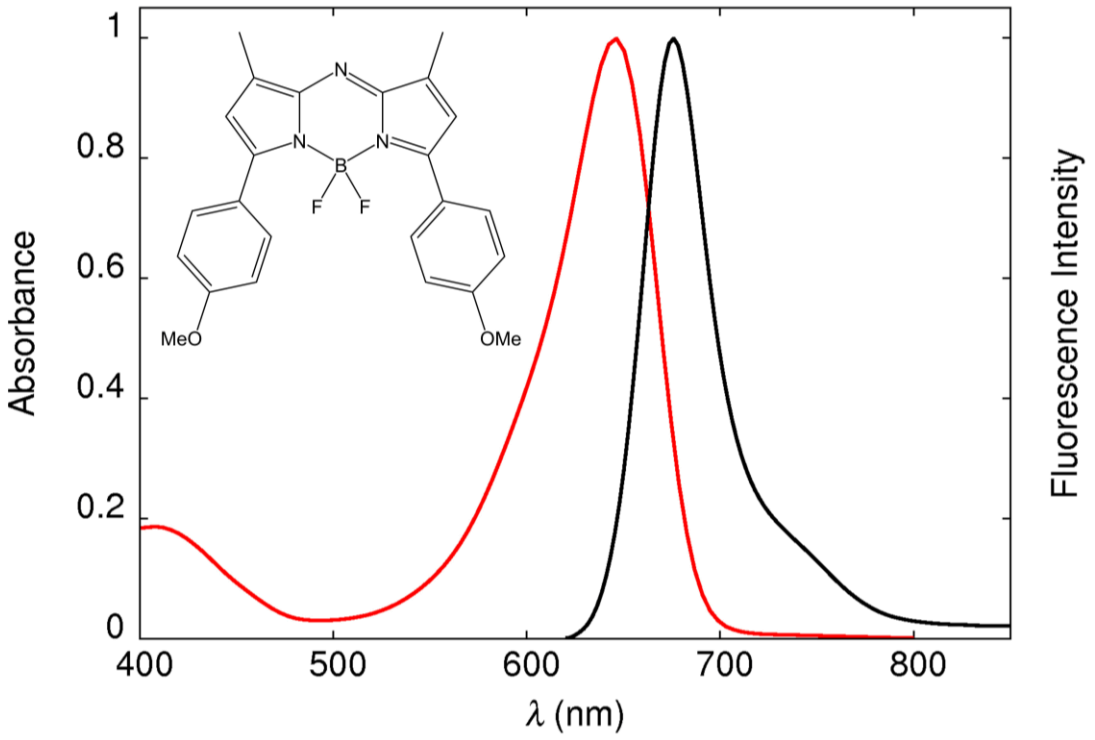

Figure S3. Normalized steady-state absorption and fluorescence spectra recorded for aza-BOD-1 in methanol.

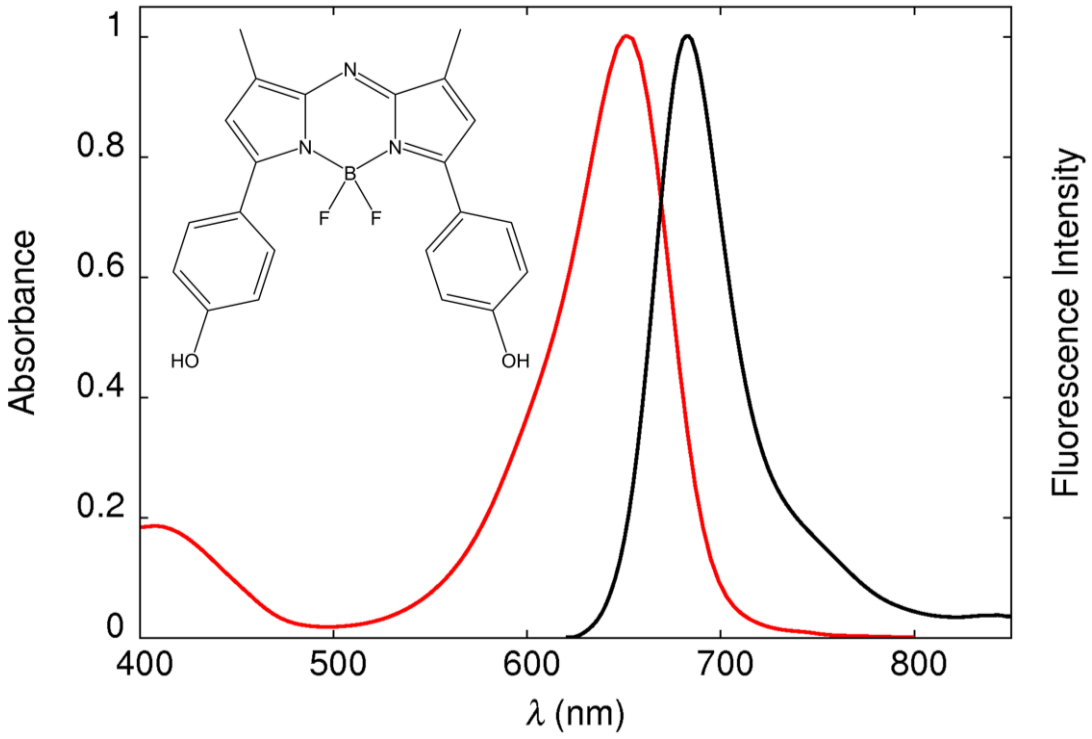

Figure S4. Normalized steady-state absorption and fluorescence spectra recorded for aza-BOD-2 in methanol.

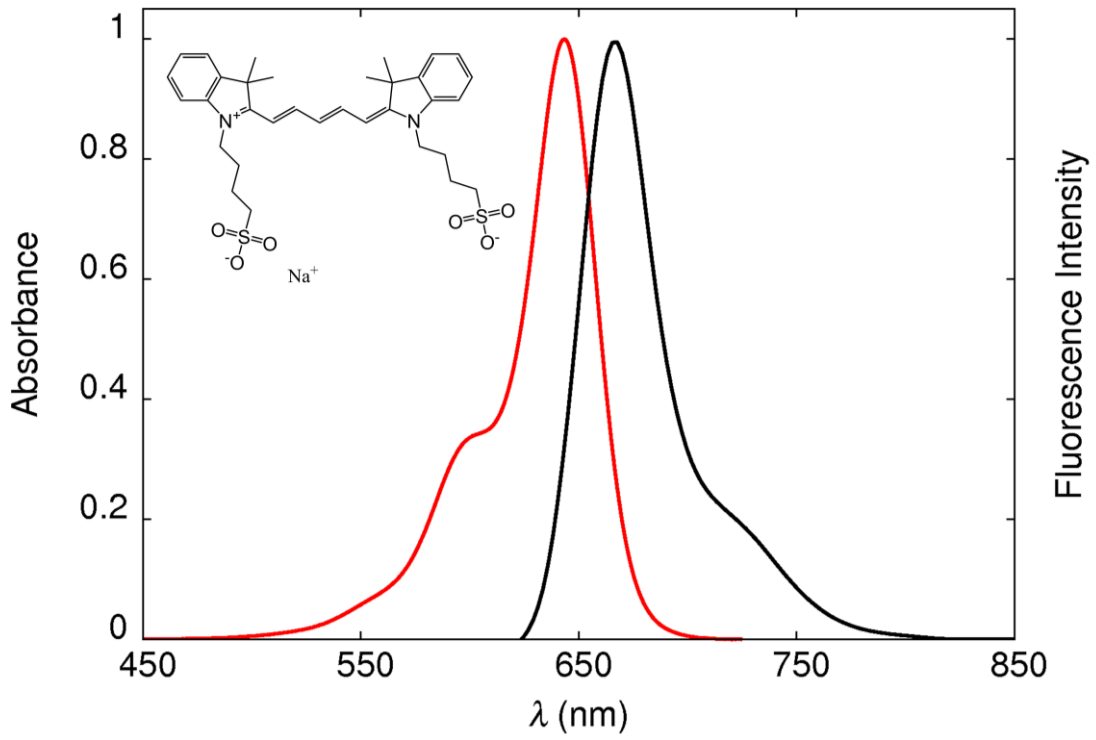

Figure S5. Normalized steady-state absorption and fluorescence spectra recorded for cy643 in methanol.

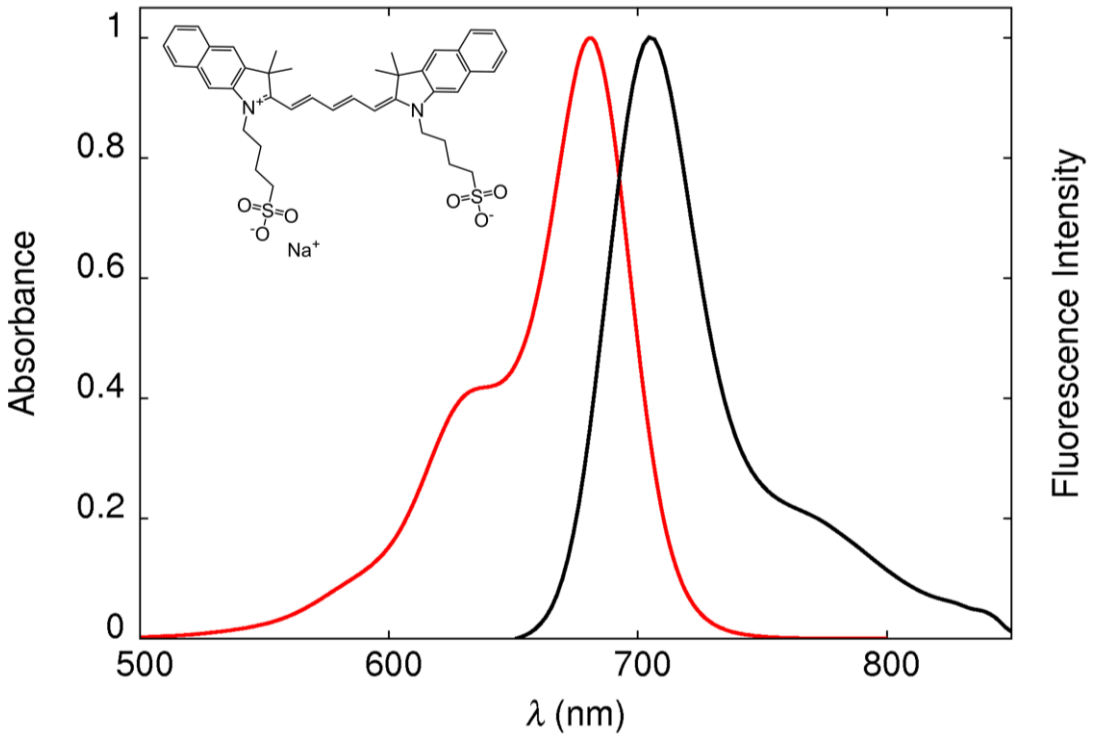

Figure S6. Normalized steady-state absorption and fluorescence spectra recorded for cy681 in methanol.

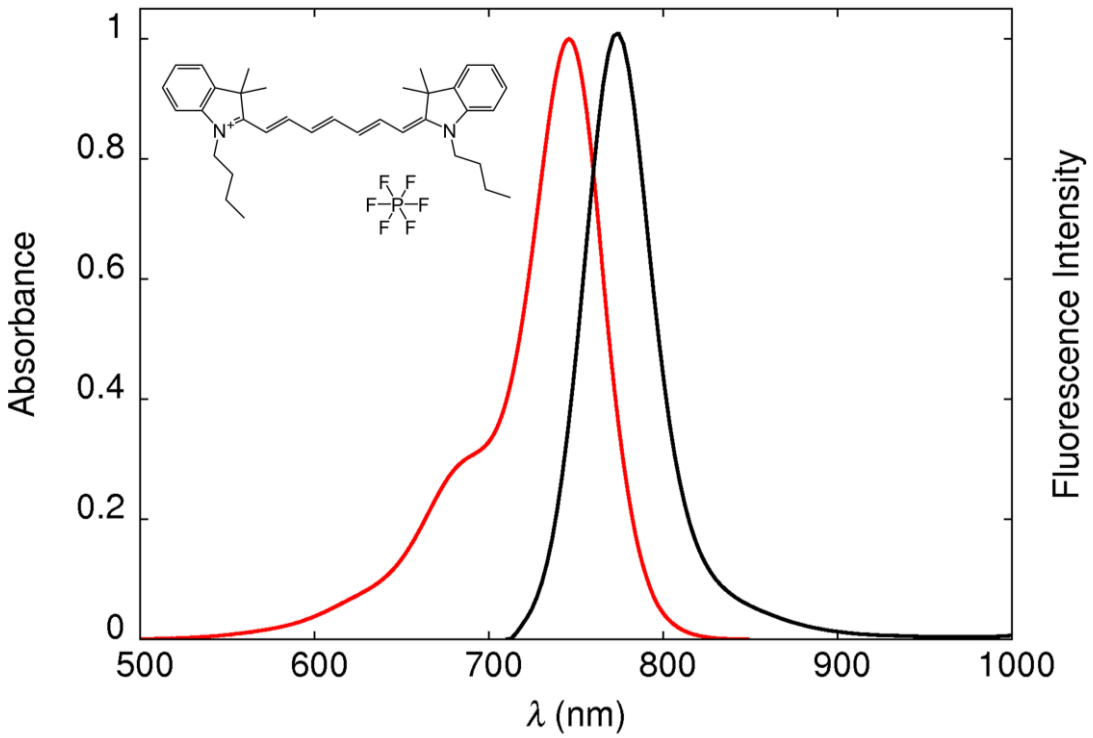

Figure S7. Normalized steady-state absorption and fluorescence spectra recorded for cy746 in methanol.

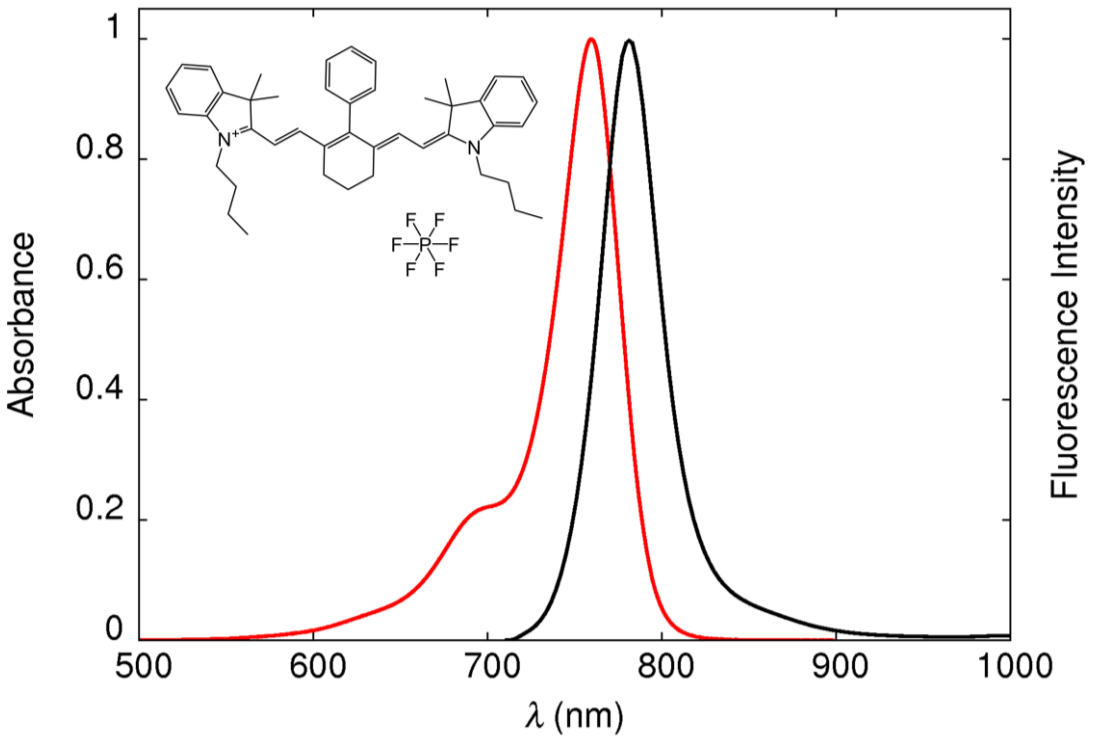

Figure S8. Normalized steady-state absorption and fluorescence spectra recorded for cy761 in methanol.

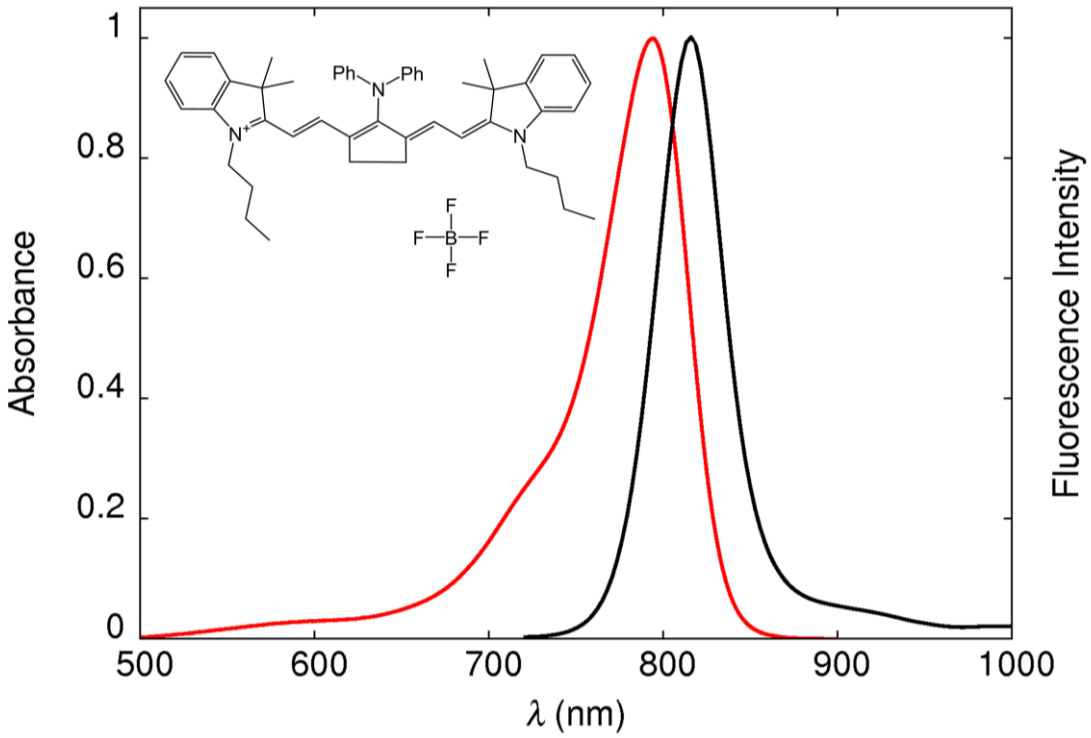

Figure S9. Normalized steady-state absorption and fluorescence spectra recorded for cy794 in methanol.

2. Beer-Lambert Plots

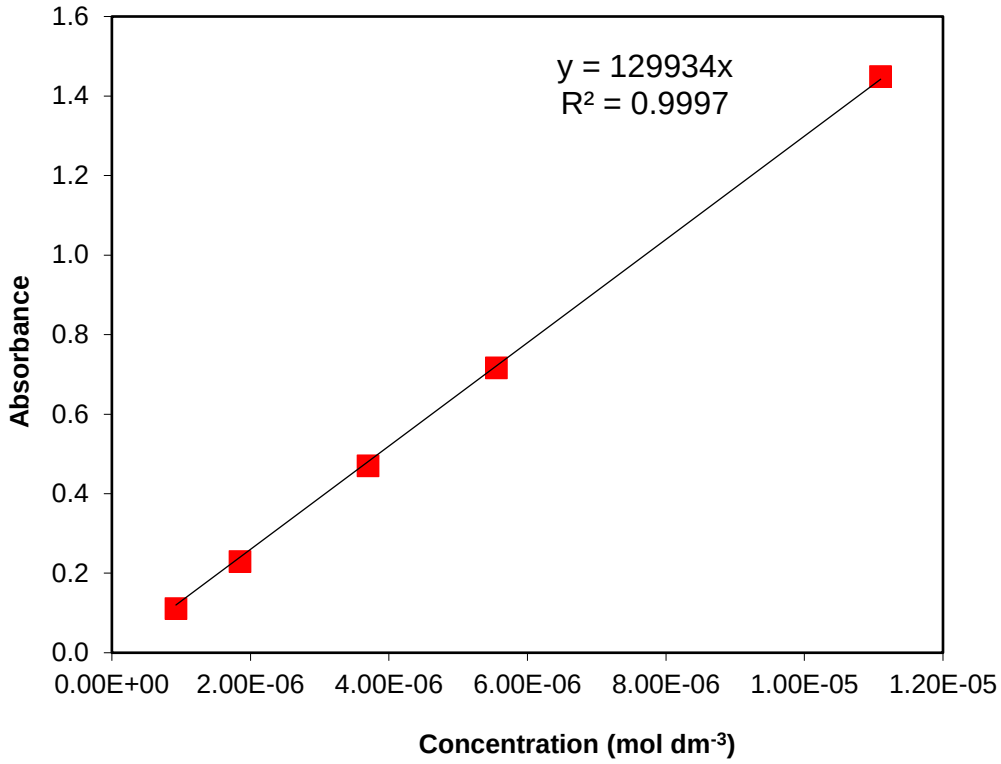

Figure S10. Beer-Lambert plot for aluminium phthalocyanine tetrasulfonate in methanol at the absorption maximum of 675 nm.

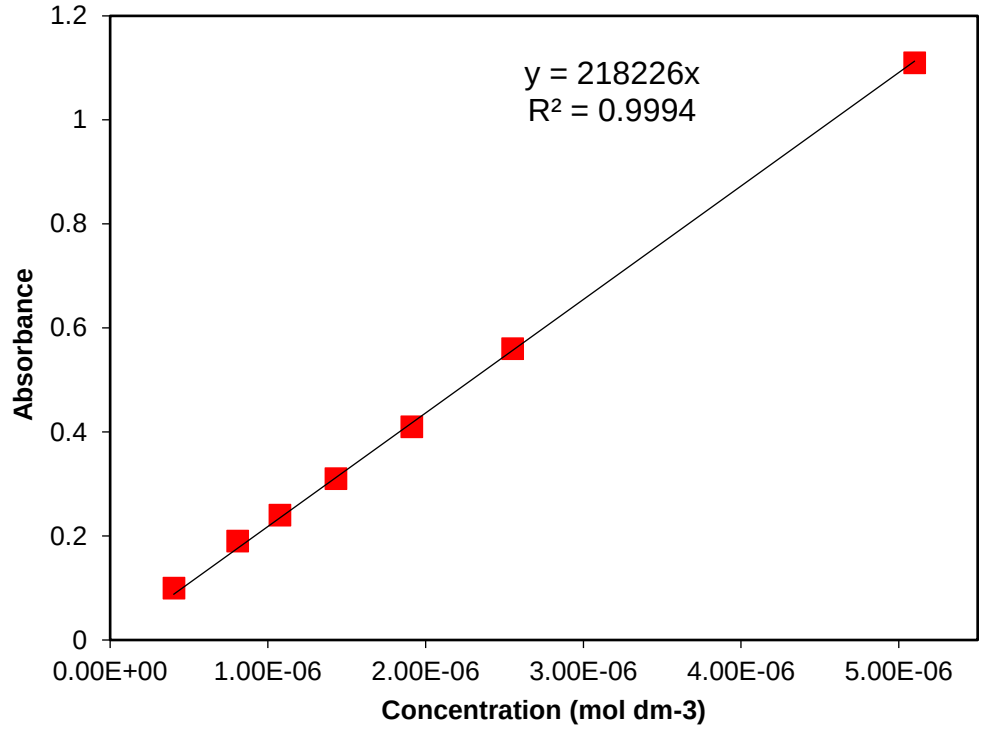

Figure S11. Beer-Lambert plot for cy643 in methanol at 643 nm.

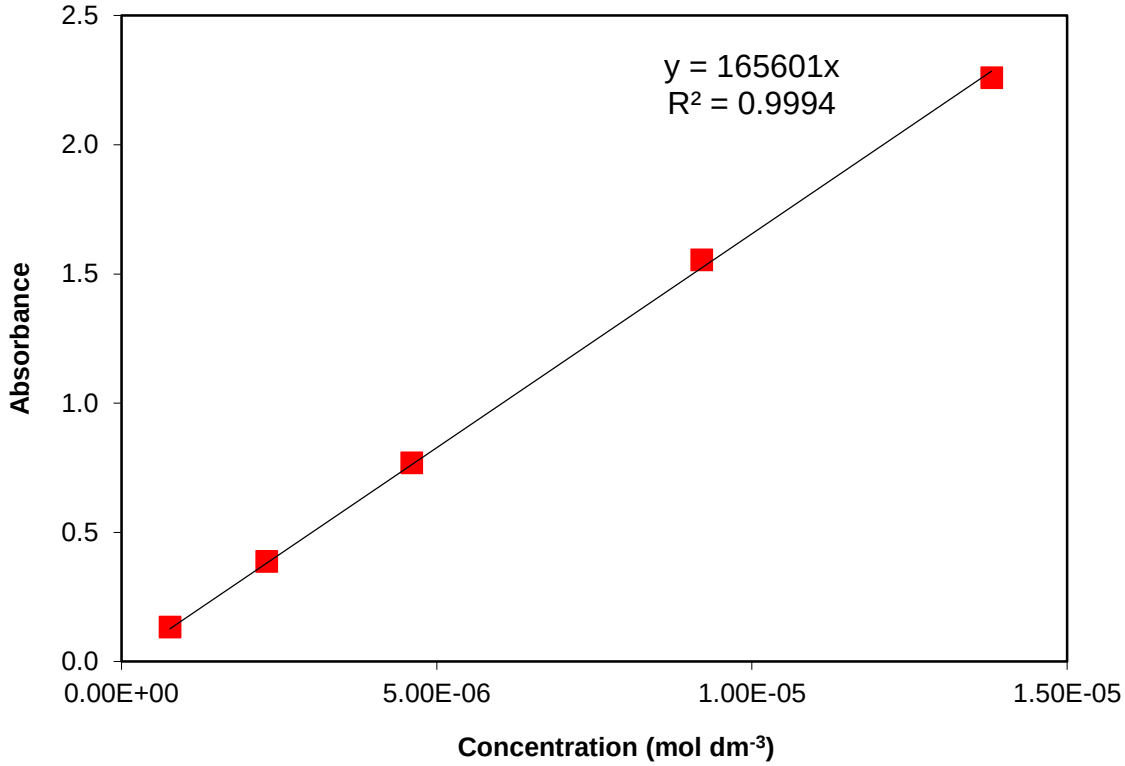

Figure S12. Beer-Lambert plot for cy681 in methanol at 681 nm.

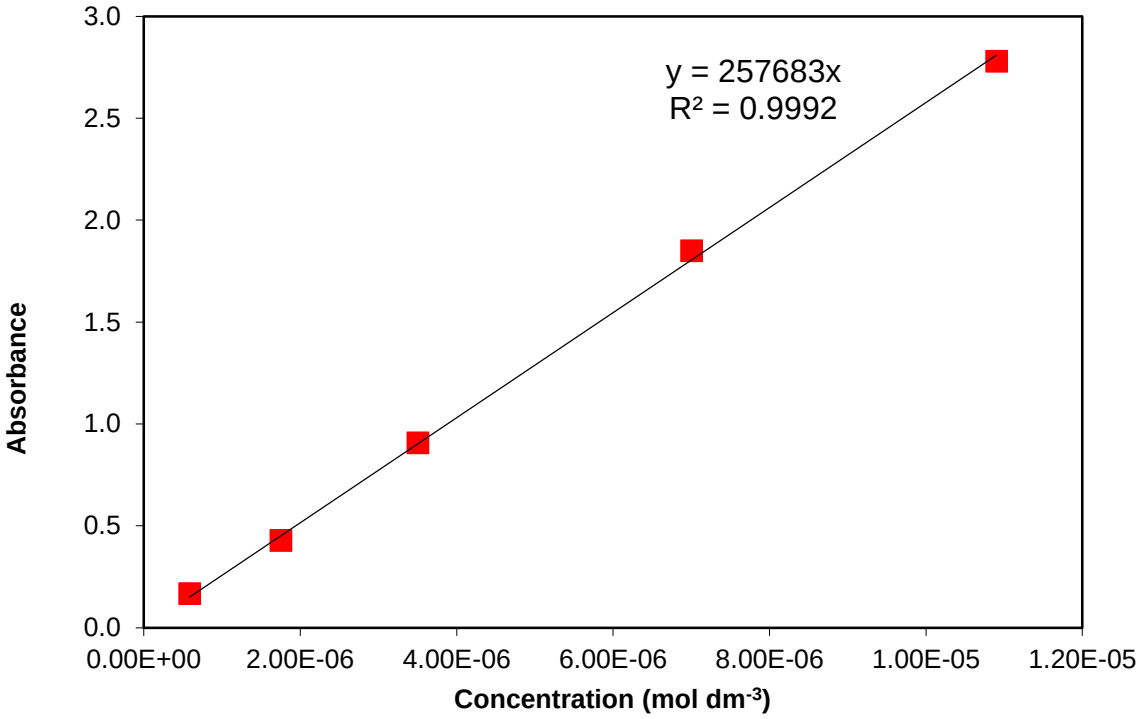

Figure S13. Beer-Lambert plot for cy746 in methanol at 746 nm.

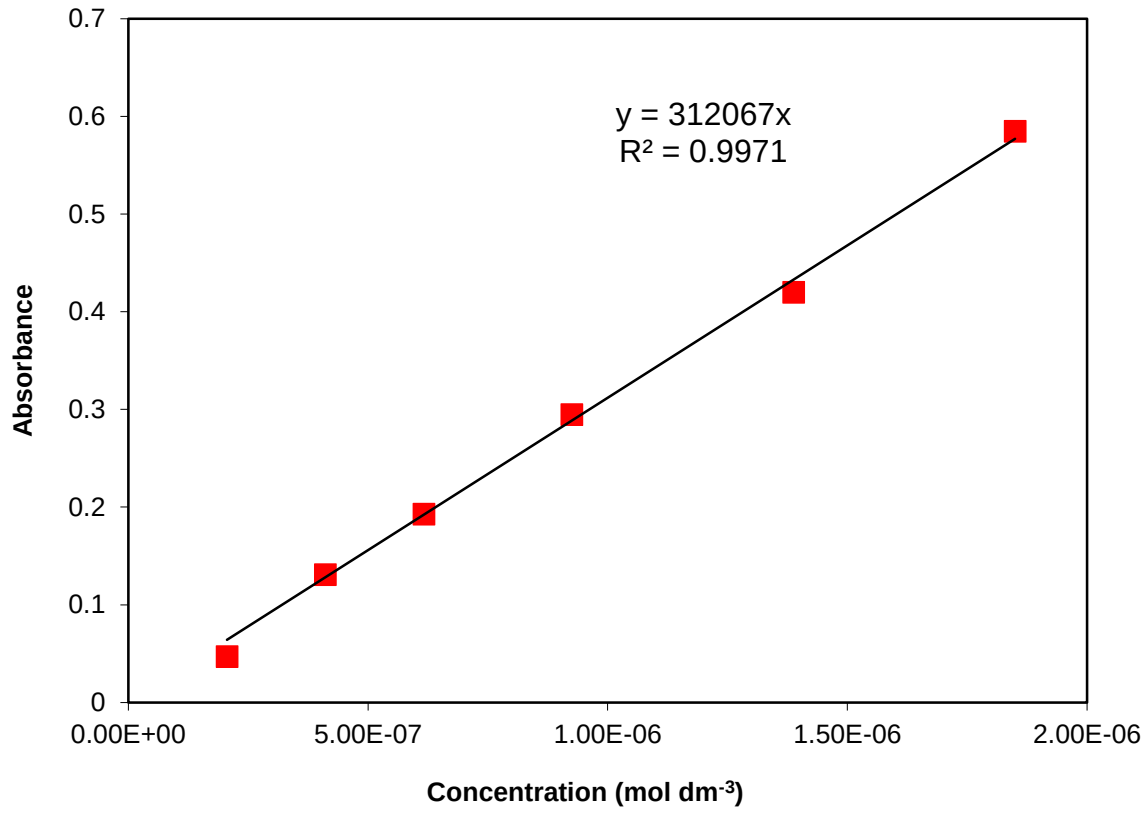

Figure S14. Beer-Lambert plot for cy761 in methanol at 760 nm.

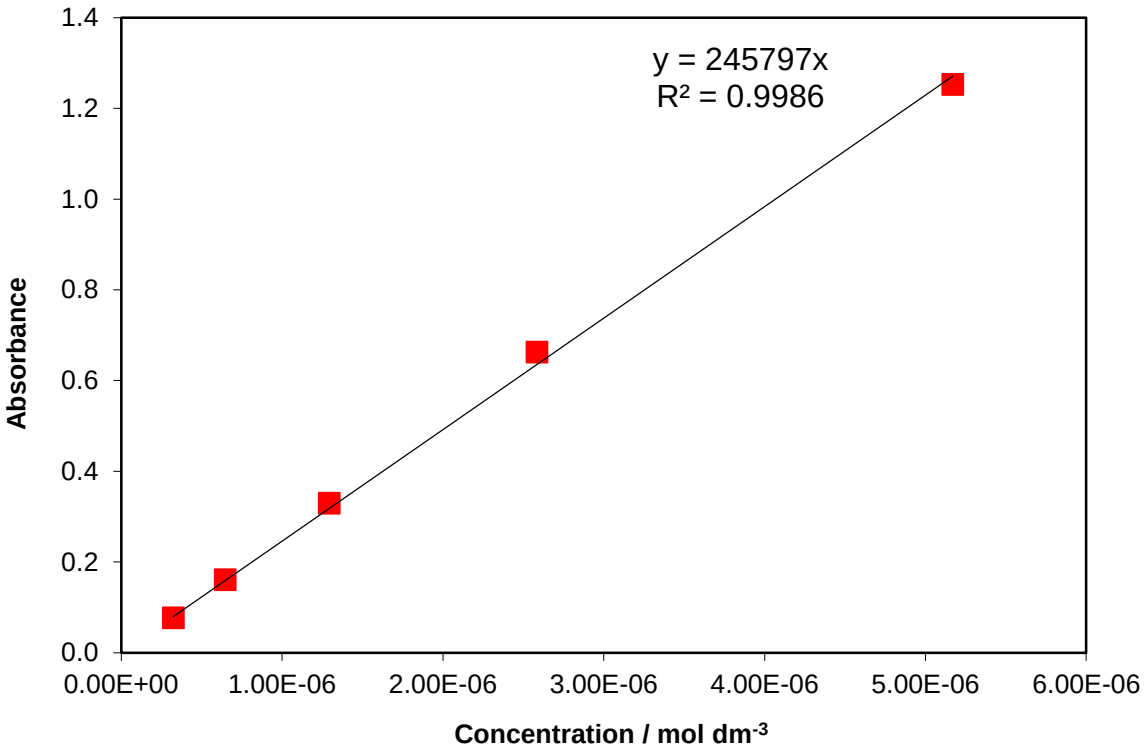

Figure S15. Beer-Lambert plot for cy794 in methanol at 794 nm.

3. Manufacturer Reference Codes for Standard Cyanine Dyes

In the paper each of the cyanine dyes examined was assigned a name based on its absorption maximum in methanol. Small differences might exist in absorption and fluorescence maxima due to “rounding-up” of the wavelengths and because of disparate resolution of the instruments. These changes are within 1 nm. The manufacturer (FEW Chemicals GmbH) refers to these dyes with the following product codes:

Dye	**Manufacturer Product Code**	**CAS-Number**
cy643	S0523	120768-44-7
cy681	S0524	64285-36-5
cy746	S2137	134339-08-5
cy761	S2138	1472004-00-4
cy794	S2025	1888324-74-0

4. Photophysics of the cyanine dyes

The fluorescence spectrophotometer was calibrated for imperfections in the spectral response using two complimentary methods. Firstly, a calibrated white light source (tungsten halogen LS-1-CAL from Ocean Optics) was used across the wavelength range covering 550 to 900 nm. At wavelengths less than 550 nm, the instrument manufacturers' calibration was applied. The validity of the calibrated source was confirmed using a low pressure Xe discharge lamp (Newport 6033), which gives a series of well-defined lines. The instrument was calibrated at regular intervals by recording the Xe discharge spectrum. Solutions were optically dilute, having absorbance values less than 0.08 at the excitation wavelength. Several solutions were used for each sample. Non-emissive glass cut-off filters were used to isolate fluorescence from any stray excitation light. Spectra were recorded at show scan rates (typically 60 nm/min) and at the narrowest slit-widths capable of giving a good signal-to-noise ratio. The effect of dissolved oxygen was negligible as regards spectral position, band-shape or intensity. Several samples of methanol were used, differing in the possible contamination by trace amounts of water, but no significant differences were observed. However, the quantum yields determined for the cyanine dyes are sensitive to changes in temperature due to the competing isomerisation step being an activated process. Temperatures were maintained at 20 ^{0}C.

The data portrayed in Figure 4 suggests a linear relationship between the fluorescence quantum yield, Φ_F, and the mean emission maximum recorded for the emitting state, ν_F. We can use this approximation to estimate the threshold wavelength at which the photo-initiated thermal blooming technique will fail to provide a meaningful Φ_F for cyanine dyes. This cut-off point corresponds to $\Phi_F = 0.03$ and equates to $\nu_F = 11{,}000$ cm^{-1} (i.e., 910 nm). On the higher energy side, Φ_F must reach a maximum value of unity. Extrapolation of the data indicates that this will not happen for dyes absorbing in the visible region. It should be stressed that the linear relationship is accidental and without theoretical basis. There is no reason to suppose that the linear relationship will hold for other emissive dyes.

5. ^{1}H-NMR Spectra for Brilliant Green

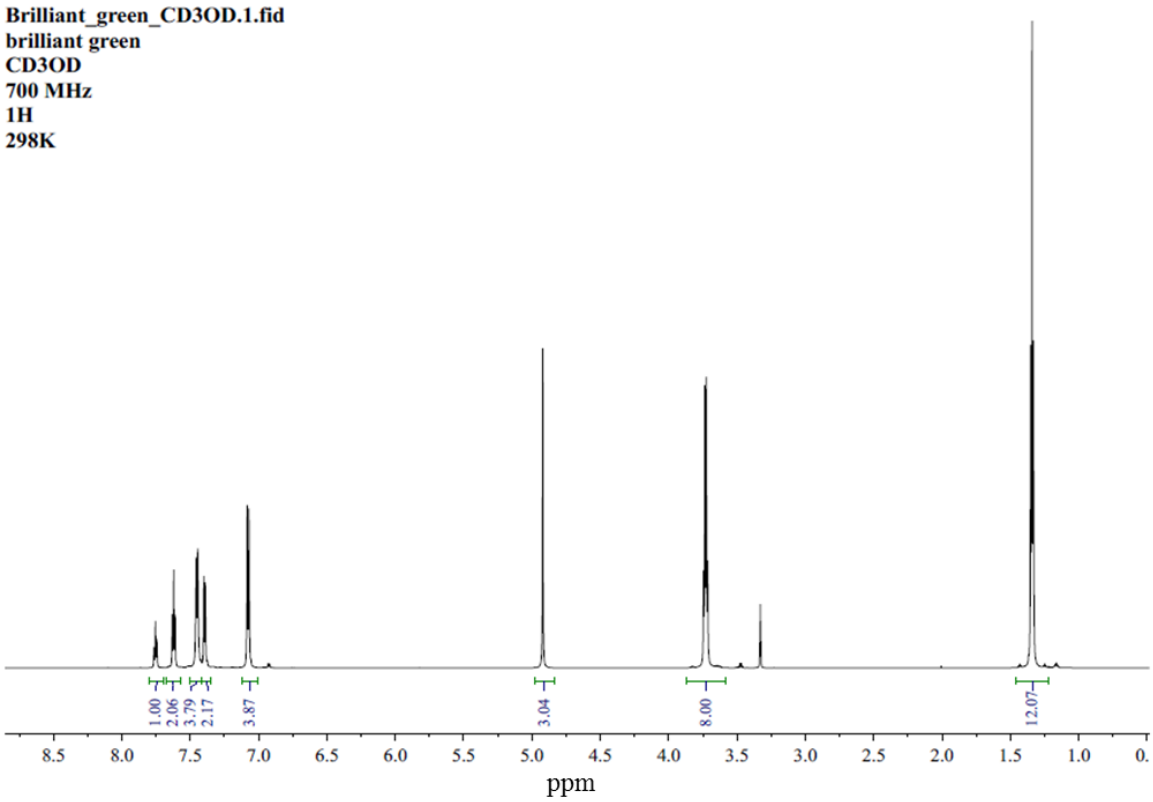

Figure S16. ^{1}H-NMR spectrum recorded for Brilliant Green in d_4-methanol.

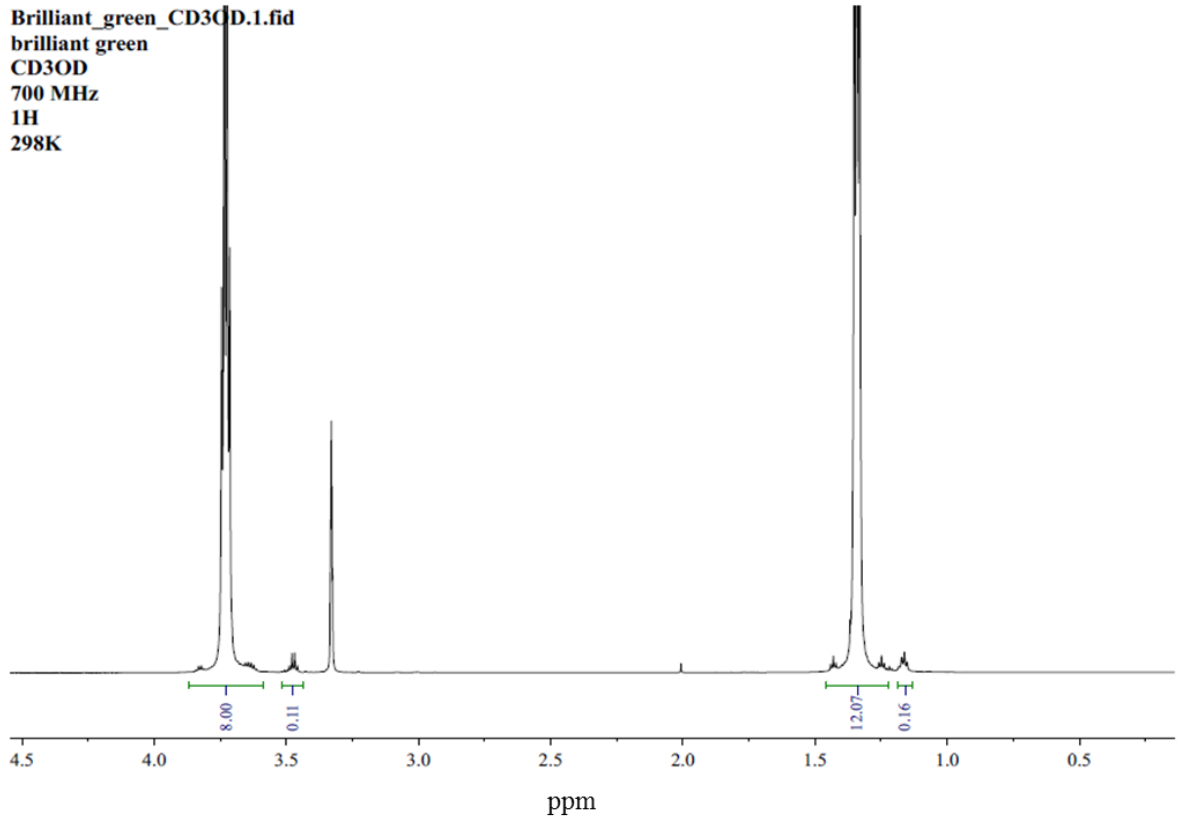

Figure S17. Expanded view of the partial ^{1}H-NMR spectrum recorded for Brilliant Green in d_4-methanol to highlight the presence of possible impurities. These impurities appear as weak peaks on the edge of the main peaks. Integration of the various signals leads to the conclusion that the sample of Brilliant Green has an estimated purity of around 99%. We thank Dr. Corinne Wills and Prof. William McFarlane for undertaking this analysis on our behalf.

6. ^{1}H-NMR Spectra for the Cyanine Dyes

The qualitative purity of the cyanine compounds was tested with high resolution ^{1}H NMR spectroscopy. The water-soluble dyes cy643 and cy681 were dissolved in D_2O and produced sharp spectra. For both cy643 cy681, there is no evidence of impurities in the NMR spectrum, the dyes can therefore be considered to be of very high purity. Cy681 showed some signal broadening at room temperature, but peaks were sharpened and resolved at 343K and assignment was obtained with the aid of homonuclear correlation spectroscopy (COSY). The signal broadening was not readily explained, but may have been the result of limited solubility, restricted molecular motion or diamagnetic anisotropy.

The remaining three dyes were examined in deuterated chloroform. Solubility data were not available and could not be thoroughly tested due to the small amounts of sample available. Although all the dyes are readily soluble in methanol up to the 10 μmol dm^{-3} region, the likelihood of them being soluble in the millimolar range was considered unlikely. It is hard to be certain of the dyes being fully dissolved at such high concentration due to solutions being very strongly coloured. Therefore, dyes cy747, cy761 and cy794 were examined in deuterated chloroform.

Cy747 yielded a sharp, high resolution spectrum. Only minor impurities in the aromatic region could be detected. Cy761 displayed broad peaks at room temperature, which sharpened upon heating to 343K. Again, only a small amount of signal was not accounted for by assignment of the dye. Cy794 also required heating in order to resolve the spectrum. Assignment of cy794 was aided by COSY at 323K, displaying only a trace impurity by way of an unexplained peak at 1.7 ppm. We once again thank Dr. Corinne Wills and Prof. William McFarlane for undertaking this analysis.

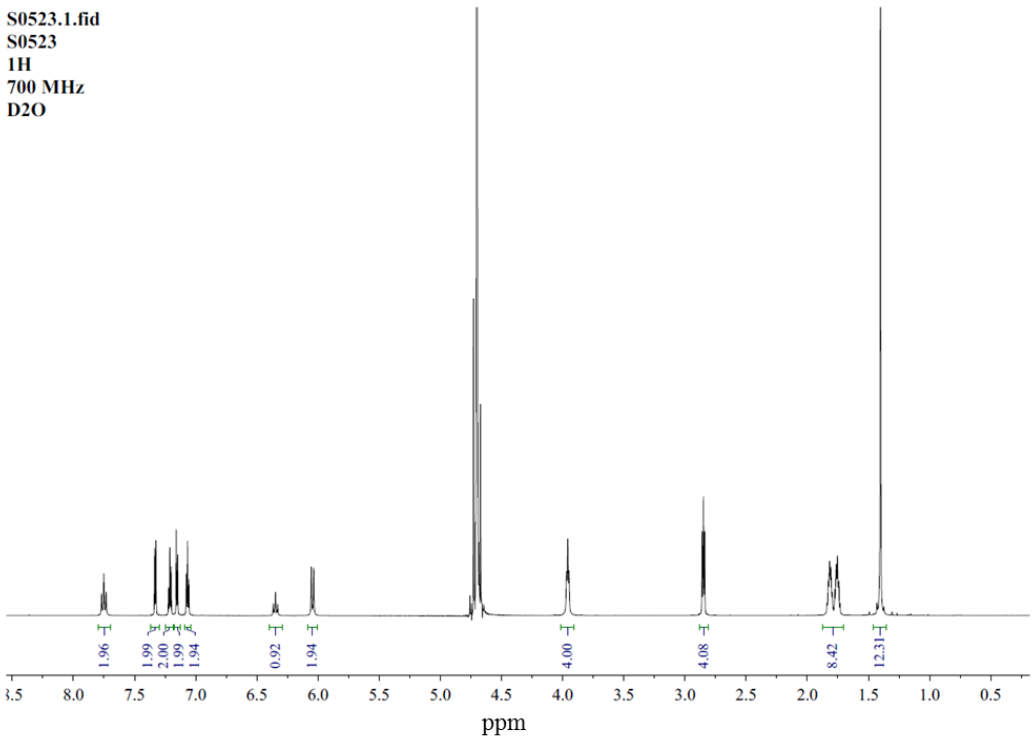

Figure S18. ^{1}H-NMR spectrum recorded for cy643 in D_2O.

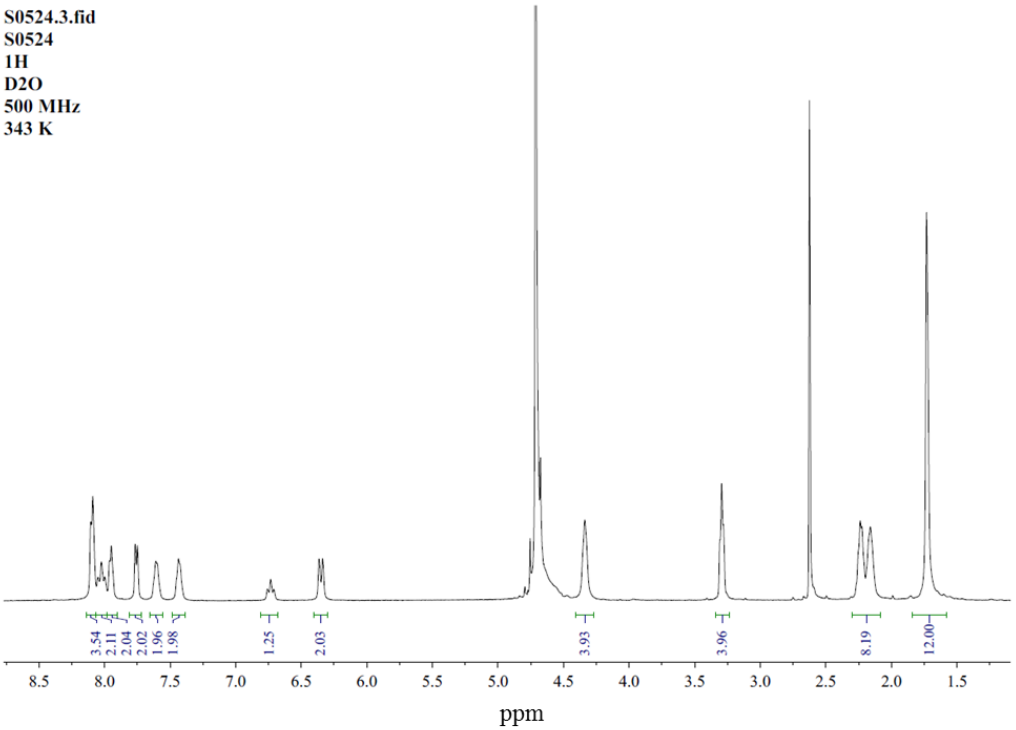

Figure S19. ^{1}H-NMR spectrum recorded for cy681 in D_2O.

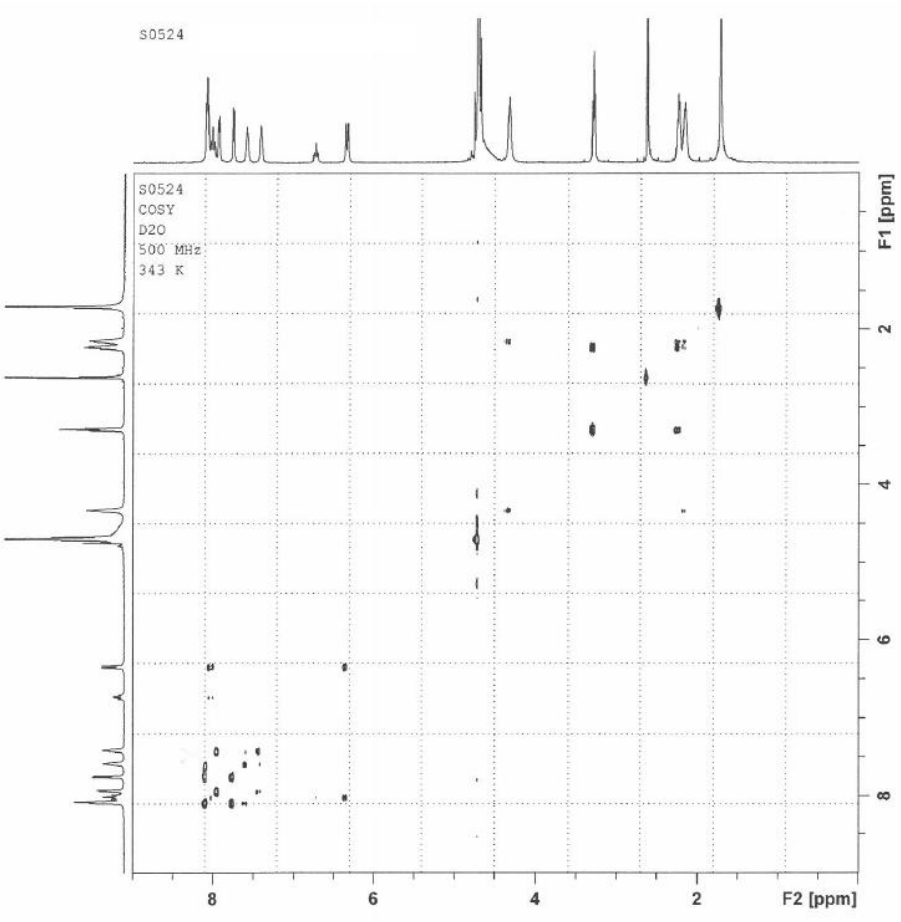

Figure S20. COSY ^{1}H-NMR spectrum recorded for cy681 in D_2O.

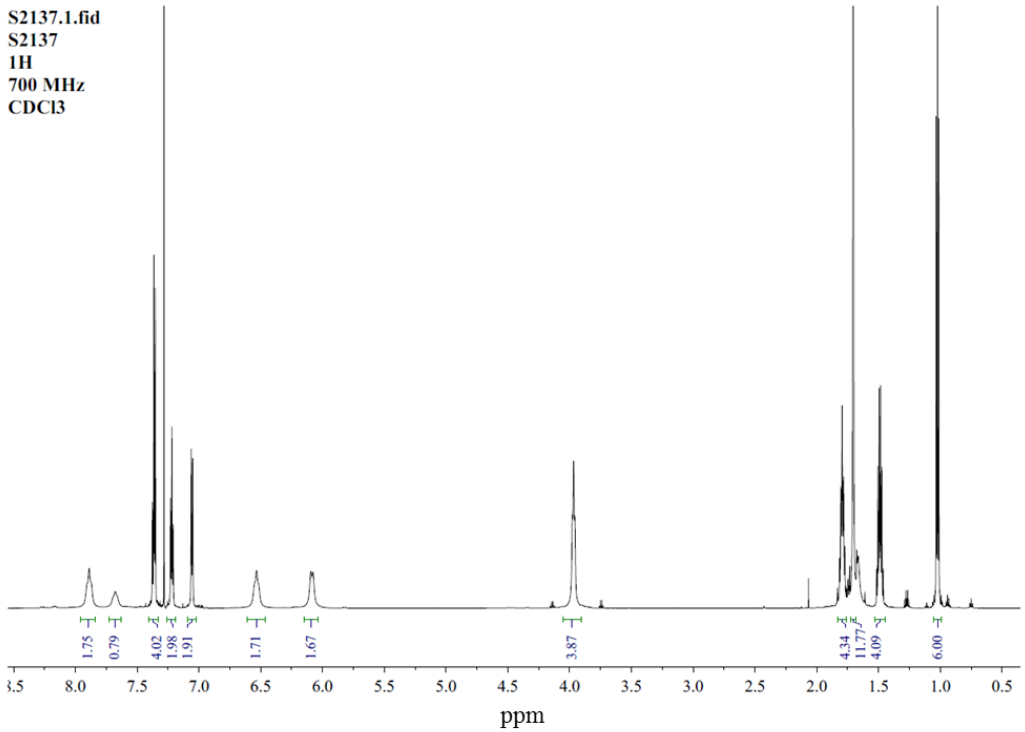

Figure S21. ^{1}H-NMR spectrum recorded for cy746 in deuterated chloroform.

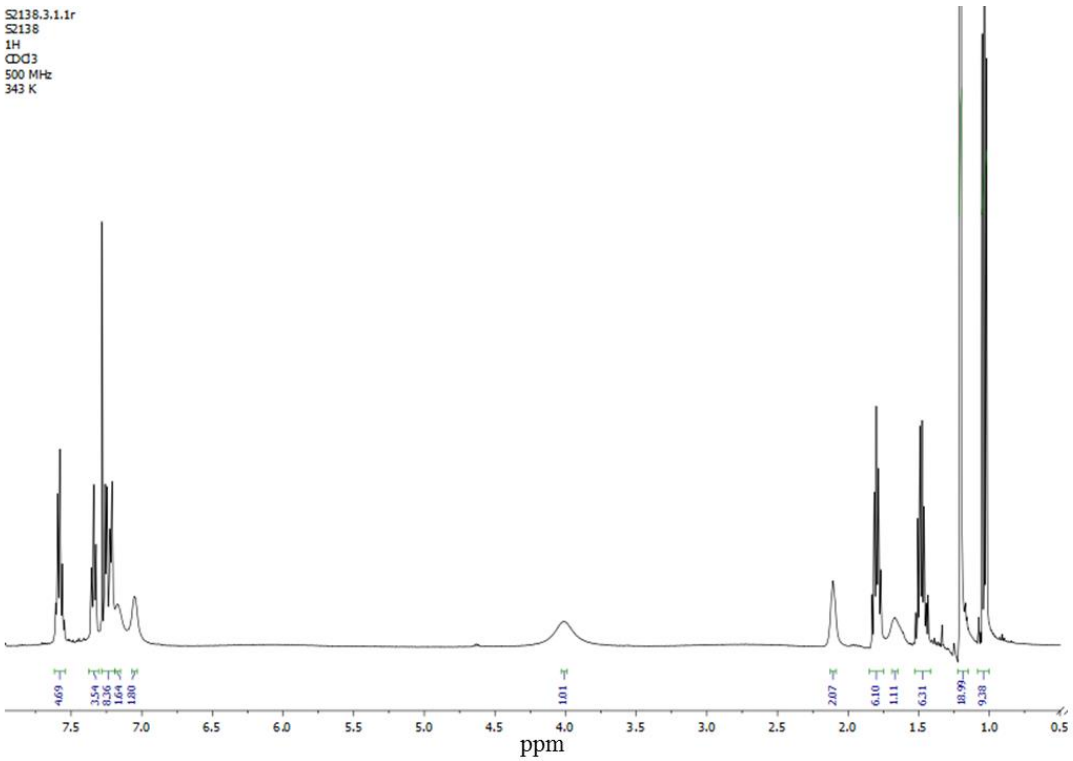

Figure S22. ^{1}H-NMR spectrum recorded for cy761 in deuterated chloroform.

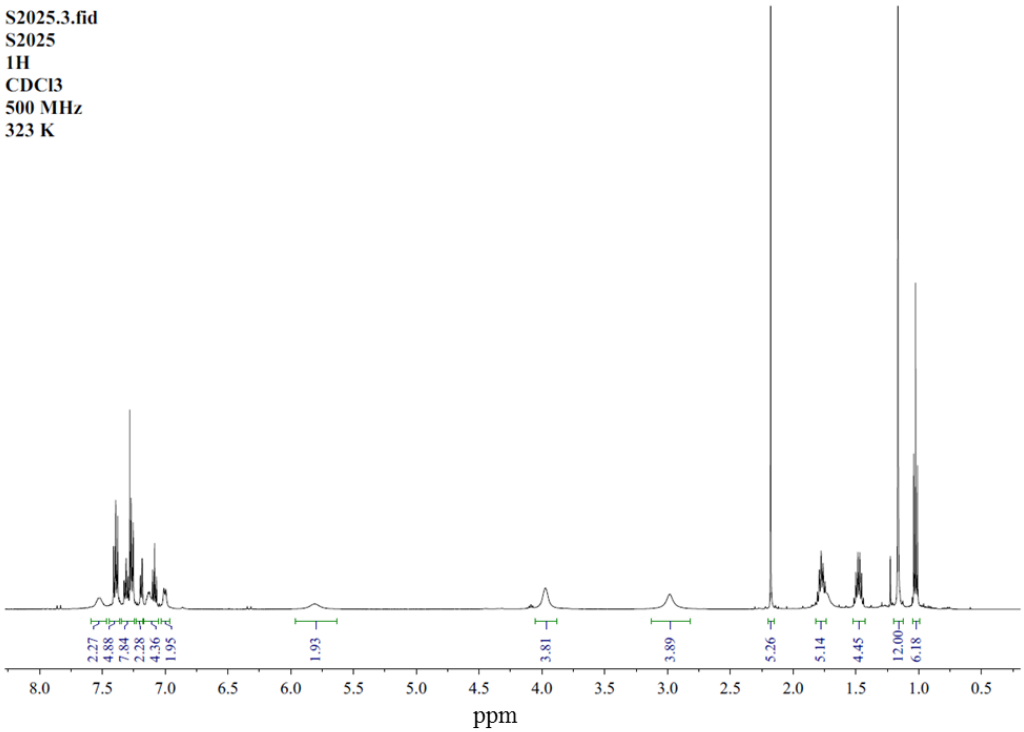

Figure S23. ^{1}H-NMR spectrum recorded for cy794 in deuterated chloroform.

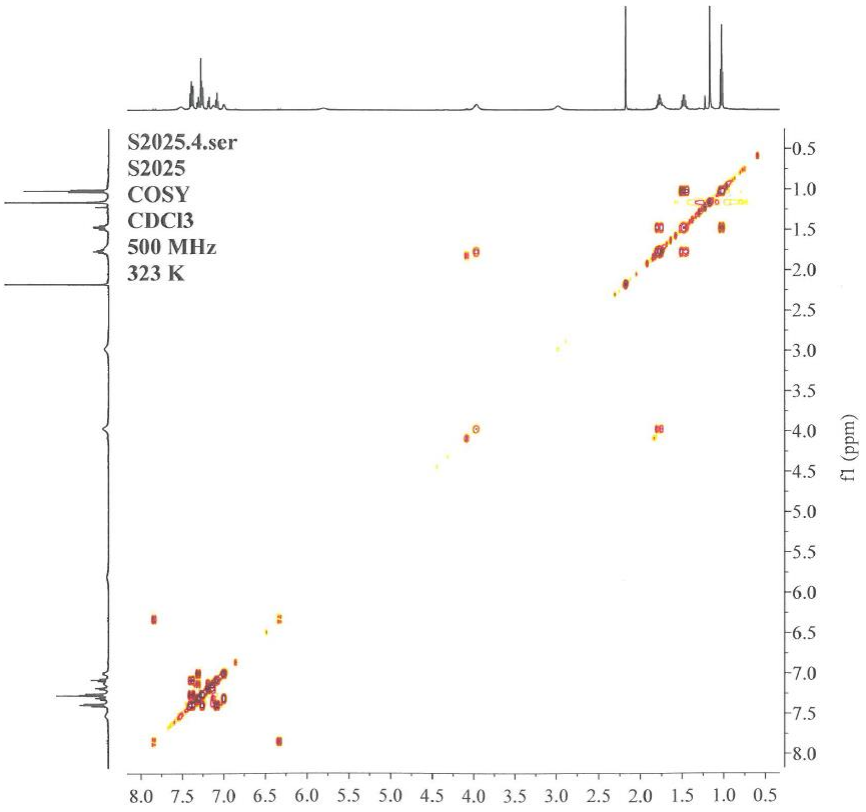

Figure S24. COSY ^{1}H-NMR spectrum recorded for cy794 in deuterated chloroform.