Supplementary Material (ESI) for Chemical Communications

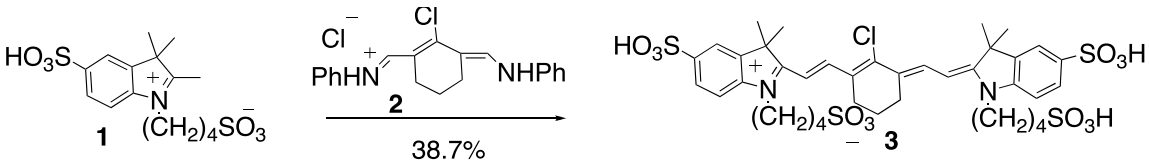
A NEAR-INFRARED DYE FOR MULTICHANNEL IMAGING

Wellington Pham, Lauren Cassell, Amelie Gillman, Dmitry Koktysh and John C. Gore*

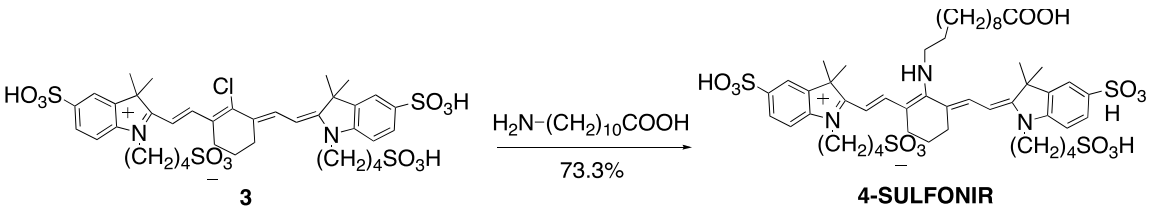
Institute of Imaging Science, Vanderbilt University

Materials. Unless otherwise stated, reagents and solvents were obtained from commercial sources without further purification. All moisture or air-sensitive reactions were carried out under a positive argon atmosphere. All spectra were obtained from distilled and deionized ultra-purified water. Nuclear magnetic resonance (NMR) were obtained using a Bruker DPX-400 in deuterated water. Chemical shifts are reported in parts per million relative to a (or the) water peak at 4.80 ppm, and coupling constants are reported in hertz. Reverse phase HPLC purification was performed on a Hitachi Lachrome Elite incorporated with a Diode Array detector L-2455 using a Vydac 218TP1010 C_{18} column (Hesperia, CA). The elution gradient was set from 0 to 20% of acetonitrile in protonated, deionized water. Detection was monitored in the 200-900 nm range. Absorbance spectra were recorded on an Agilent UV-Vis spectrophotometer. Steady-state fluorescence spectra were performed on and a PTI QM-4SE spectrofluorometer. Low resolution MALDI-TOF and high-resolution exact mass measurements (ESI) were determined from the Vanderbilt Mass Spectrometry Core Facility. All of the counter ions were removed by passing the compounds through the Dowex-50W (Sigma Aldrich) column before analysis.

*Synthesis of dye intermediate **3***



Synthesis of 4-Sulfonir



3 **4-SULFONIR**

Quantification procedure. To demonstrate this future application, we decided to quantify the fluorescence signal of each dye in their respective ranges. Briefly, the dye samples were imaged twice using the Maestro™ in vivo imaging system (Cambridge Research and Instrumentations Inc.). Both data sets were acquired using the Maestro™ "flat-field" correction functionality to compensate for possible illumination field inhomogeneities. The data sets were also single-binned to maximize resolution. Exposure times were selected via the Maestro™ "AutoExpose" data collection option. The first image data set was excited in the NIR range (Fig. 3A) and emission photons were collected from 780-950 nm. The second image data set was excited in the visible range (500-620 nm) and emission photons were collected in the 550-800 nm range. Emission data was collected in 10 nm increments over the corresponding emission ranges. The two image data sets were saved as two collections of 1392x1040.TIF files.

The TIF. File data sets were imported into Matlab for analysis. To assemble the contributions from each wavelength's .TIF file to the total image signal intensity over the entire emission range, each data set was integrated over all emission wavelengths on a pixel by pixel basis. A built-in Matlab trapezoidal integration function was used to calculate the total signal intensity of each pixel over the emission range to yield an associated matrix of values corresponding to the area under the curve of each pixels' spectra.

Subsequent to the generation of each data set's intensity matrix, pixels in the Maestro field of view (FOV) in which no dye signal was displayed were isolated as background (BG), identified by manual drawing of a region of interest outside of the dye samples. The mean and standard deviation of the BG signal were then calculated. A normal

distribution was assumed for the BG. Thus, it was considered that greater than 99% of the BG signals fall within the range of the mean BG ± three standard deviations of BG. Based on this assumption, a threshold value for determination of significant signal was established as BG + three standard deviations of BG. All pixels displaying intensity signals less than this threshold were assigned a value of zero. All pixels displaying intensity values greater than this threshold were considered significant. Significant intensity values were assigned one of the dye samples via manual drawing of regions of interest. Contributions from BG outside of the portion of the FOV covered by the dye samples were thus separated from the total signal intensity from each dye sample in each data set. The total signal intensity from each dye sample was then calculated as the sum of all non-zero values in the corresponding ROI and expressed as a fraction of the total image signal intensity.

From this analysis, we found that if NIR820 and 4-Sulfonir are used to image two events in one environment, excitation in the NIR window (>750 nm) causes the former to emit approximately 90% of the total fluorescent signal. In contrast, excitation in the visible range results in 4-Sulfonir's emission constituting around 80% of the total signal..

Quantum yield measurement of fluorescent dye.

We follow the procedure that has been reported previously [2, 3]. All of the solvents and reagents are analytical graded except where noted. The stock solutions of the dyes (4-Sulfonir and standard ICG) were prepared in volumetric flash and then further carefully diluted to provide standard solution with an absorbance value of 0.5.

After the absorbance measurement, these diluted solutions were further diluted to a factor of 10 and used for fluorescence measurements. After obtaining the emission spectra of the standard and 4-Sulfonir, these were corrected using the built-in function in the PTI modular fluorescence system using Felix software. The area under the curve were determined and the relative quantum yield was calculated based on the equation:

$$\phi_{F(x)} = \left(\frac{A_{ICG}}{A_{4-SUL}}\right)\left(\frac{F_{4-SUL}}{F_{ICG}}\right)\left(\frac{n_{4-SUL}}{n_{ICG}}\right)^2 \Phi_{F(ICG)}$$

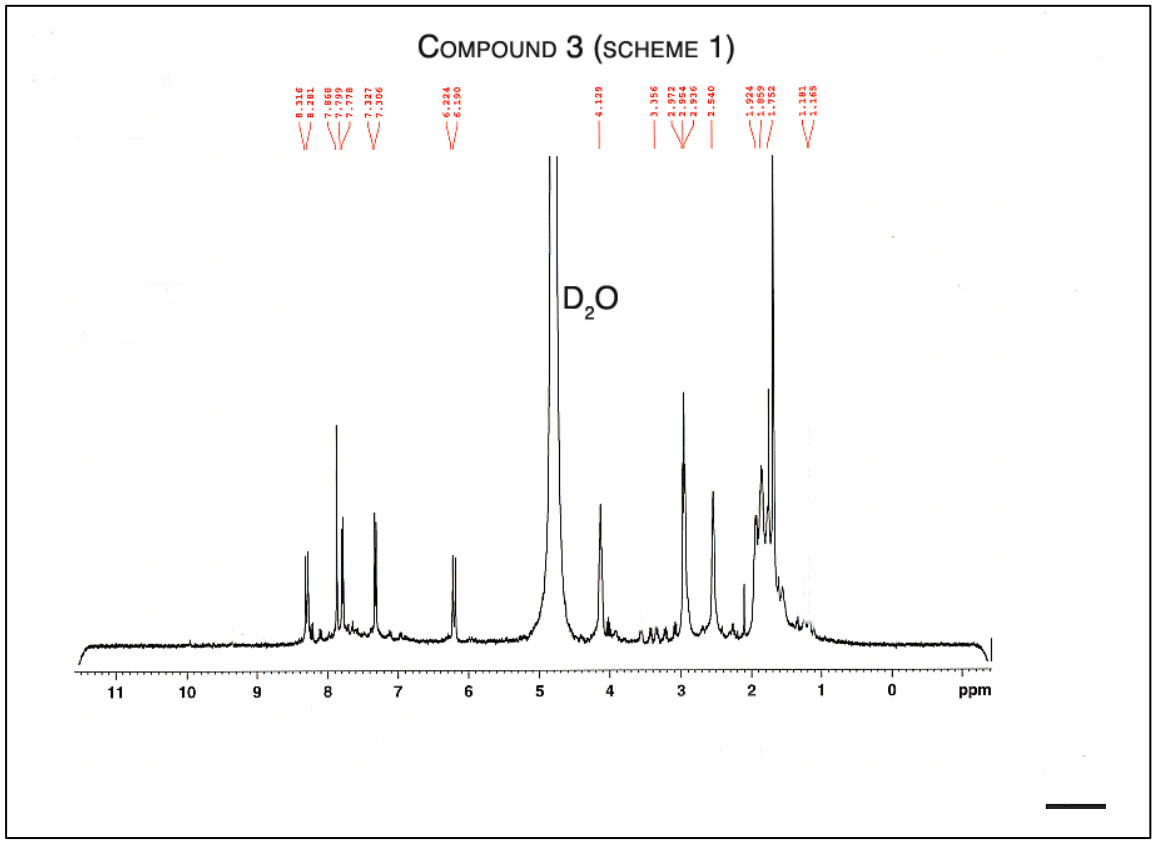
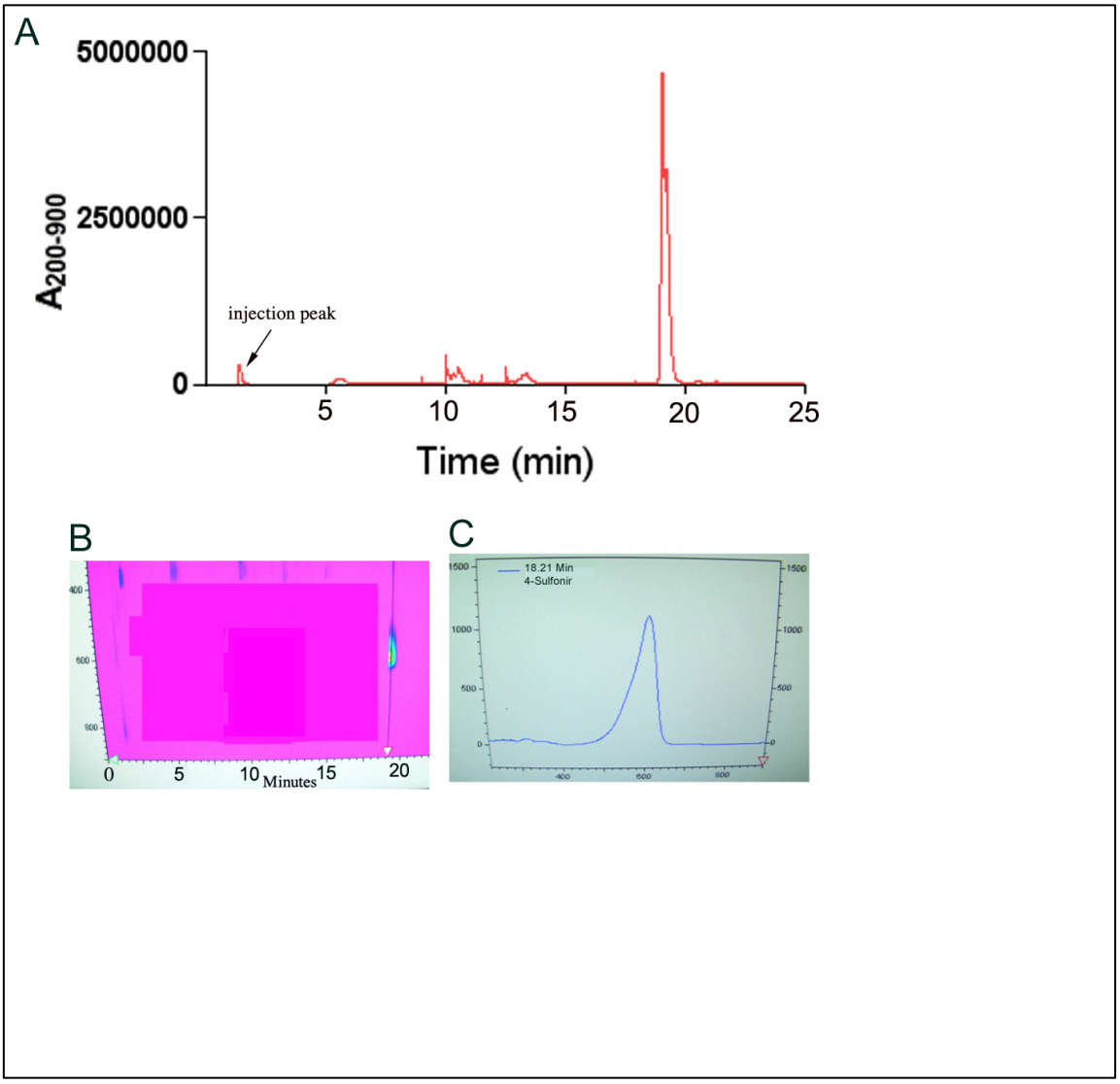


Figure 1. ^{1}H-NMR of compound 3.



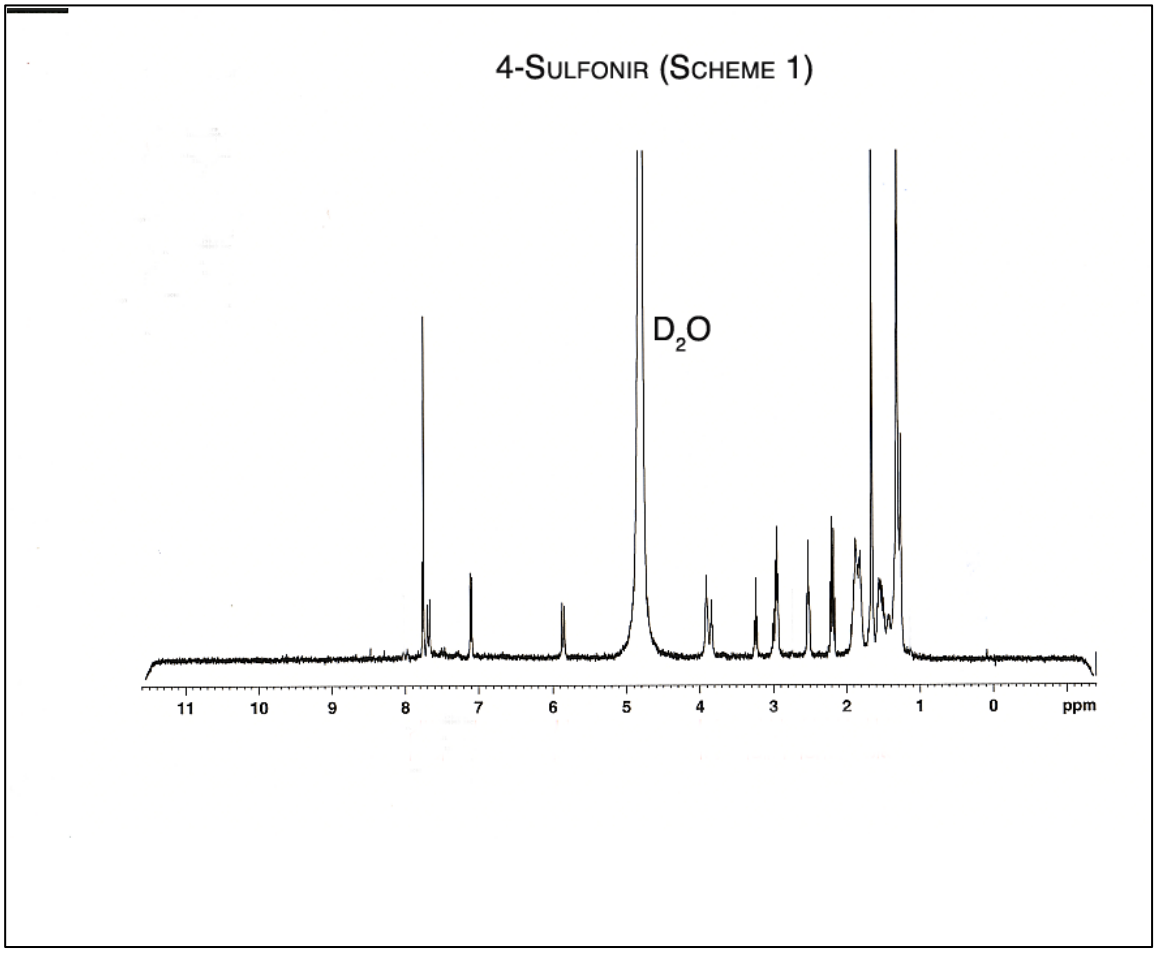


Figure 3. ^{1}H-NMR of 4-Sulfonir.