Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Highly Selective Staining and Quantification of Intracellular Lipid Droplets with a Compact Push-Pull Fluorophore based on Benzothiadiazole

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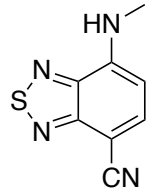
Table S1. Summary of photophysical data for CBD-Fluor.[1]



solvent	Polarity value[a]	Abs λ_{max} (nm)	Em λ_{max} (nm)	log ε	Φ_F
Hexane	0.009	427	515	3.60	0.39[b]
Toluene	0.099	435	532	3.64	0.30[b]
Dioxane	0.164	437	546	3.65	0.28[b]
Dichloromethane	0.309	436	545	3.65	0.26[b]
Acetonitrile	0.460	437	569	3.64	0.17[b]
Ethanol	0.654	441	578	3.71	0.14[c]
Water	1	441	612	3.48	0.005[c]

[a] Relative solvent polarity scale.[2] [b] Quantum yields determined relative to coumarin 153 in ethanol (ϕ=0.54) [b] Quantum Yields determined relative to $Ru(bpy)_3$ in 0.5M H_2SO_4 (ϕ=0.028)

Determination of the Limit of Detection for CBD-Fluor

Limit of Detection was calculated following the procedure outlined by Wasatch Photonics. https://wasatchphotonics.com/applications/fluorescence-limit-detection/

A series of dilutions from 2.1 μmol to 0.21 nmol of CBD-Fluor were prepared in dioxane and the fluorescence spectra were measured in a cuvette with a 10 mm pathlength. Each sample was excited at 437 nm and the total emission intensity between 447 nm to 700 nm was measured.

Each sample concentration was measured three times along with three measurements of pure dioxane as the blank. Each set of spectra were then averaged and the signal for each concentration was calculated as a function of wavelength

(signal = average sample intensity - average blank intensity).

The standard deviation (σ) for the blank measurements was calculated as a function of wavelength.

The signal-to-noise ratio (SNR) was calculated with the following equation:

$$SNR = \frac{Max\ Signal_{\lambda}}{\sigma_{\lambda}}$$

Where σ_{λ} is the standard deviation of the blank at the same wavelength as the max signal.

The natural logarithm of the SNR (**ln(SNR)**) was plotted against the **ln(concentration)**. A trendline was applied and the concentration at which SNR = 3 was considered to be the limit of detection.

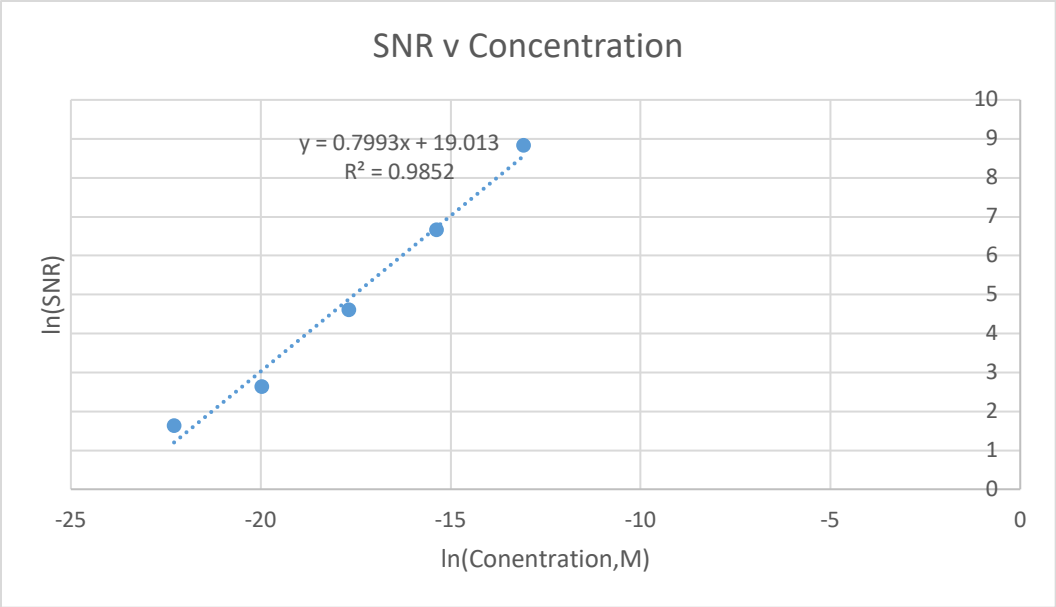


Figure S1: Limit of detection determined at the concentration that produced a ratio of signal to noise (SNR) of 3. Limit of detection determined to be 184 pM.

Procedure for the SDS experiment for Figure 3B.

2 µL of CBD-Fluor in dioxane (0.01 M) was added to 2 mL of water or 2 mL of 8 mM SDS. Each sample was excited at 437 nm and the emission was measured from 447 nm to 700 nm. This procedure was repeated 3 times and the data in Figure 3B is the average of all 3 trials.

General

All cell imaging was acquired using a Carl Zeiss Laser Scanning Confocal Microscope 880 with Airyscan (Oberkochen, Germany) using 40x/1.1NA Water Objective. Stock solutions were prepared in biological grade DMSO and diluted in 35 mm dishes for imaging to total no more than 0.1% (v/v) in media. Imaging: CBD-Fluor: ex: 488 nm, em: 539 nm; Nile Red: ex: 488 nm, em: 565 nm; and Hoechst ex: 405 nm, em: 453 nm.

Cell culturing

HeLa (human cervical cancer) cells were cultured and maintained appropriately according to the American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Media was supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and

1% L-glutamine (ThermoFisher). Cells were split at 70–80% confluency into T-75 culture flasks every 2–3 days and were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air.

Fluorescent Assays

At 70–80% confluency, cells (2 mL) were seeded onto 35 mm glass-bottom round plates (MatTek Corporation, Ashland, MA) at a density of 200K cells/mL. After adherence of cells (24 h), media was aspirated and cells were washed with phosphate buffered saline (PBS 1X, pH 7.4). Cells were then incubated for 25 min with either CBD-Fluor or Nile Red (NR) with concentrations of 20 μM and 90 ng/mL, respectively.[3] Cells were then washed with PBS twice more and were kept hydrated in phenol red-free media for imaging. Both CBD-Fluor and Nile Red were imaged at 488 nm (1% laser power), and their resulting emission spectra were saved (Figure S4). Cells were then co-incubated with CBD-Fluor and Nile Red at the above concentrations for 25 minutes. To confirm colocalization, cells were excited at 488 nm (1% laser power) and ZEN Blue software was used for un-mixing of the resulting emissions. The LSCM was set to a pinhole yielding 1 Airy Scan unit and spectrally separated CBD-Fluor from Nile Red using lambda scanning settings.

Cell Fixation

Cells were maintained and grown as described above. After cell adhesion overnight, the MatTek plate was washed with PBS to remove non-adherent cells. 1 mL of paraformaldehyde (4% in PBS) was added and allowed to incubate at room temperature for 20 min. Cells were then washed with PBS once more, rehydrated with PBS, and stained similarly as with live cells.

Co-incubation with nuclear counter stain

Live cells were co-incubated with Hoechst 33258 and CBD-Fluor for 25 minutes. After washing with PBS three times, cells were re-hydrated in phenol red-free media for imaging. CBD-Fluor: ex: 488 nm, em 539 nm; Hoecsht: ex: 405 nm, em: 453 nm (Figure S5).

Generation of fluorescence intensity histogram

For CBD-Fluor and Nile Red (ex: 488 nm), regions of interest (ROI) were generated by plotting the relative fluorescence intensity of both dyes versus the distance across a cell. At any given ROI, Nile Red displayed significantly higher levels of background noise than did CBD-Fluor. Three different histograms were generated at random using Zen Blue software and the resulting S/N ratios were reported as the average +/– STD (Figure S2).

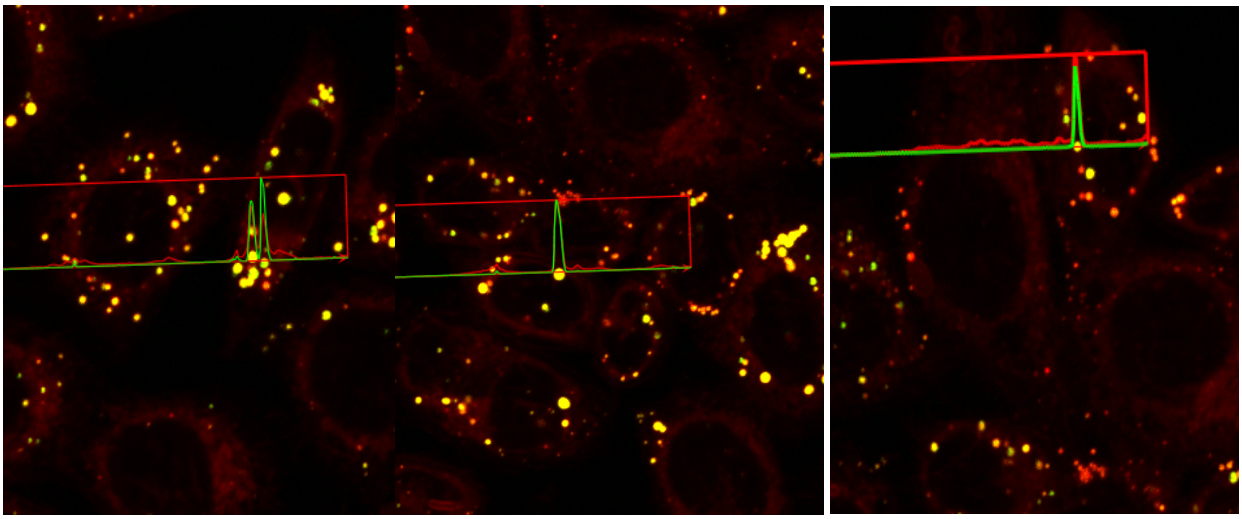


Figure S2: Representative ROI for assessing signal-to-noise for both Nile Red and CBD-Fluor. The highest fluorescence intensity for each channel was divided by the average noise across the entire cell. Signal-to-noise: Nile Red: 28.2 +/− 10.7 and CBD-Fluor: 331 +/− 106.

Orthogonal Analysis

To ensure that CBD-Fluor was localizing in spherical structures within the cell, a Z-stack analysis was performed (Figure S3). A total of 11 slices (10 μm) across the Z-axis were taken and the cross hairs were placed over a spherical structure at random. Each axis confirms the presence of staining within the cell.

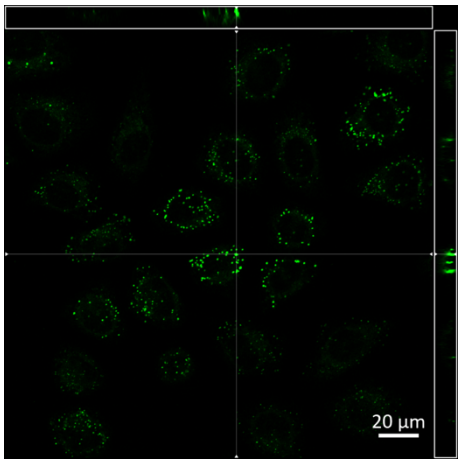


Figure S3: Orthogonal analysis displays the presence of CBD-Fluor within the lipid structures as opposed to aggregating on the surface.

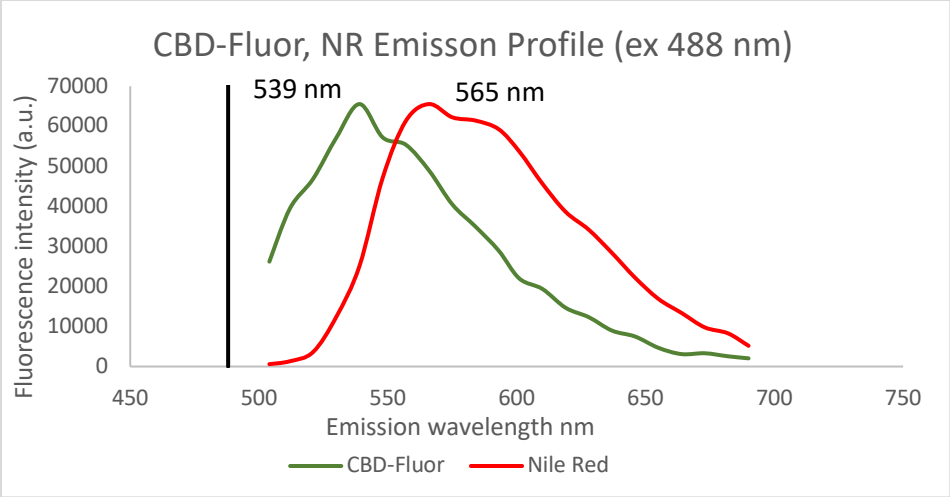


Figure S4: Emission profiles for CBD-Fluor and Nile Red (ex: 488 nm) obtained from C. Zeiss LSM 880.

Lipid Structure Quantification

Lipid count was investigated using CellProfiler. For experimental and control signals, a background threshold was applied using Adaptive Otsu three-class thresholding. When CBD-Fluor was excited at 488 nm, cells were limited to a 2–20 pixel size with a minimum lower-bound threshold of .06 for all groups for consistency. Clumped objects were distinguished by their shape while method for dividing those shapes was determined by intensity.

Cytotoxicity

HeLa cells were seeded onto a 96-well plate at a density of 5,000 cells per well and were allowed to adhere overnight at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air. After 24 h, CBD-Fluor was added at differing concentrations and cells were incubated for 2 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air. Media was then aspirated and replaced with fresh media containing 10% (v/v) Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies) and cells were further incubated 3 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air. The resulting absorbances were then recorded at 450 nm using a 96-well plate reader (BioTek SYNERGY-HTX multimode reader, Winooski VT). 10% CCK-8 (v/v) in media and in the absence of cells was used as a negative control (0% viability). DMSO was used as the vehicle for delivery of CBD-Fluor and as a positive control (100% viability) with a final concentration of less than 0.1% (v/v). Results are reported as the average +/− standard deviation from three independent experiments (Figure S5).

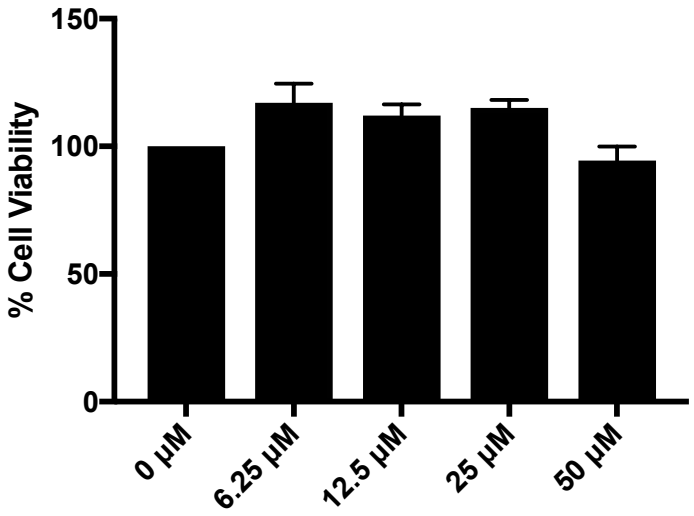


Figure S5: Cell viability assay conducted with HeLa cells and CBD-Fluor over a 2 h incubation period.

References

1. Thooft, A. M.; Cassaidy, K.; VanVeller, B. J. Org. Chem. **2017**, *82*, 8842–8847.

2. Solvent Effects in Organic Chemistry, 4th ed.; Reichardt, C., Welton, T., Eds.; Wiley-VCH: Weinheim, **2011**, pp 425–508.

3. Greenspan P.; Mayer E. P.; Fowler S. D. *J. Cell Biol.* **1985**, *100*, 965–973. 10.1083/jcb.100.3.965.