Supporting Information

New ICT-Based Ratiometric Two-Photon Near Infrared Probe for Imaging Tyrosinase in Living Cells, Tissues, and Whole Organisms

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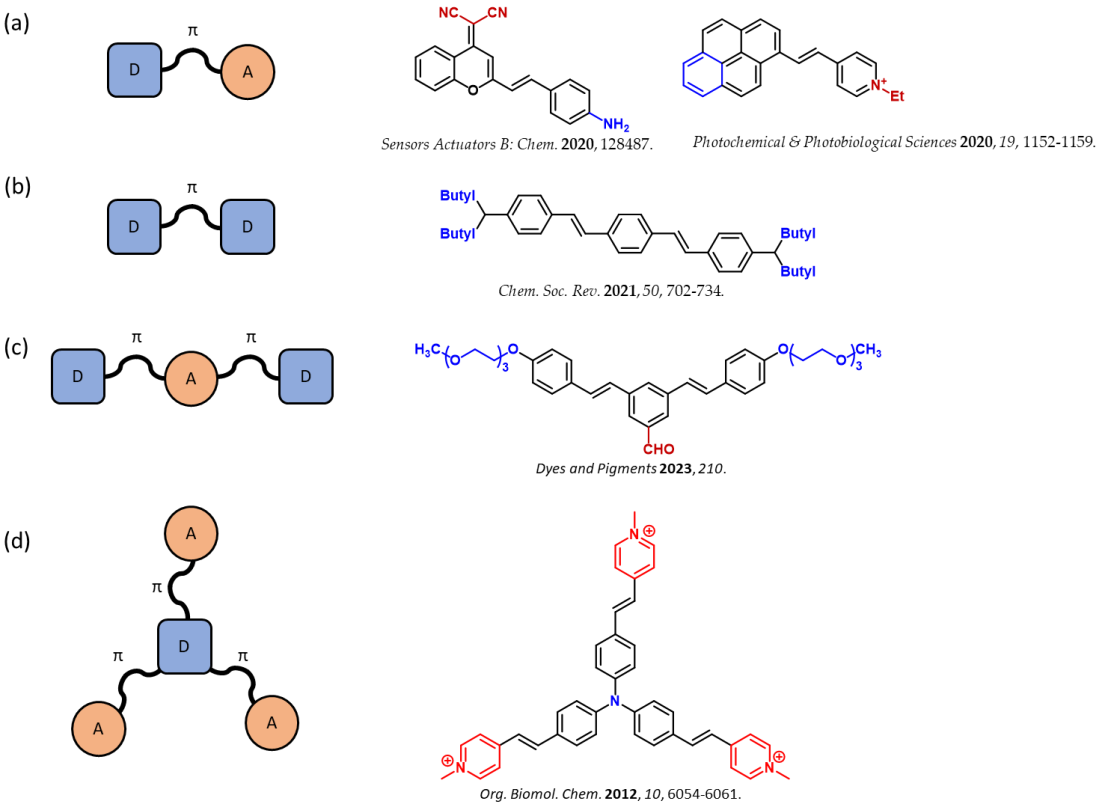
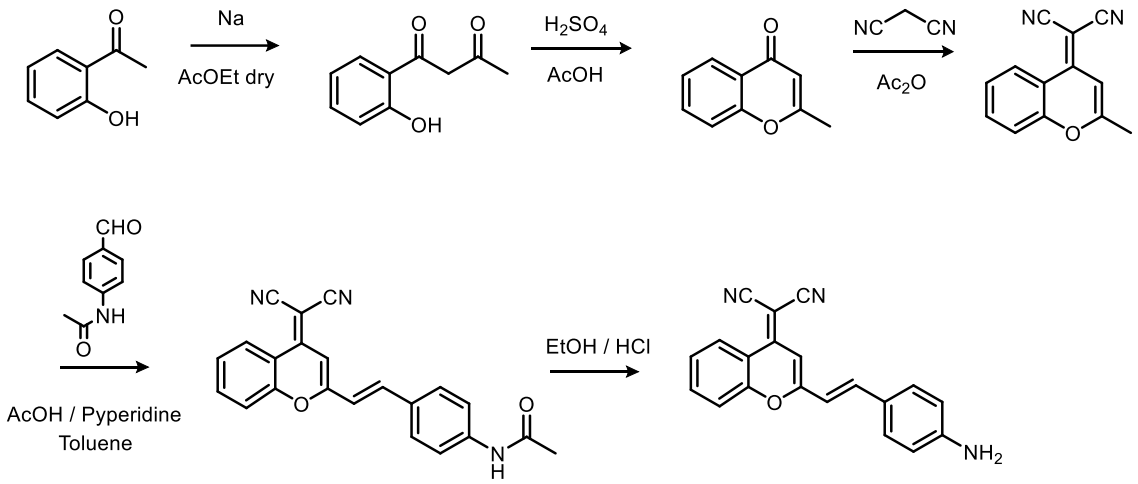


Figure S1. Representative ICT-based two photon excitable molecules of (a) D–π–A dipoles, (b) D–π–D and (c) D–π–A–π–D quadrupoles and (d) two-dimensional octupoles with some structures reported.

Synthesis of DCM-HBU

To a solution of compound DCM-NH_2 (80 mg, 0.257 mmol, 1 eq) in anhydrous dichloromethane (12.82 mL) under a N_2 atmosphere, triphosgene (153 mg, 0.513 mmol, 2 eq) was added. The mixture was then cooled to 0°C to add DIPEA (88.9 µL, 0.513 mmol, 2 eq) and was stirred for 3 h; during this time, the temperature was carefully maintained within the range of 0-5 °C by use of an ice bath. Then, 4-hydroxybenzylamine (127 mg, 1.03 mmol, 4 eq) was added and the reaction was kept at room temperature for 24 h, under a N_2 atmosphere. Then, the solvent was removed under low pressure, and the residue was submitted to flash chromatography in CH_2Cl_2:CH_3OH mixtures to produce of DCM-HBU as a pale-yellow solid (6 mg), yield 5 %, mp 172-173 °C ^{1}H NMR (500 MHz, DMSO) δ 9.88 (s, 1H), 9.29 (s, 1H), 9.16 (s, 1H), 8.74 (dd, *J* = 8.4 Hz, 1H), 7.93 (ddd, *J* = 8.5, 7.2, 1.4 Hz, 1H), 7.82 (dd, *J* = 8.4, 1.1 Hz, 1H), 7.72 (dd, *J* = 15.5, 12.4 Hz, 3H), 7.62 (ddd, *J* = 8.4, 7.2, 1.3 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 2H), 7.33 (d, *J* = 15.9 Hz, 1H), 7.10 (d, *J* = 8.9 Hz, 3H), 7.00 (s, 1H), 6.71 (d, *J* = 8.8 Hz, 3H). TOF MS ES -, calculated for $C_{28}H_{20}N_4O_3Cl$: 495.1224 ($M+Cl^-$); found: 495.1241.



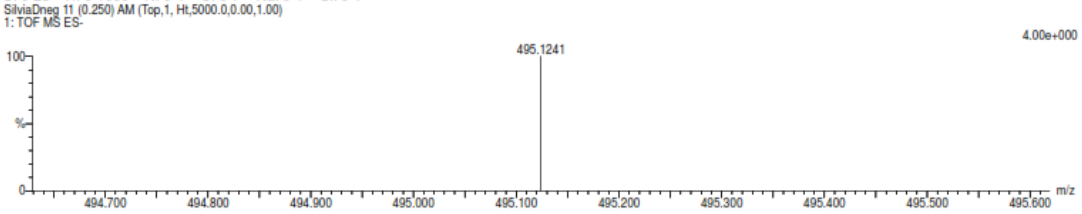
Scheme S1. Synthesis of compound DCM-NH_2.

Mass spectrum of compound DCM-HBU

Elemental Composition Report **Page 1**

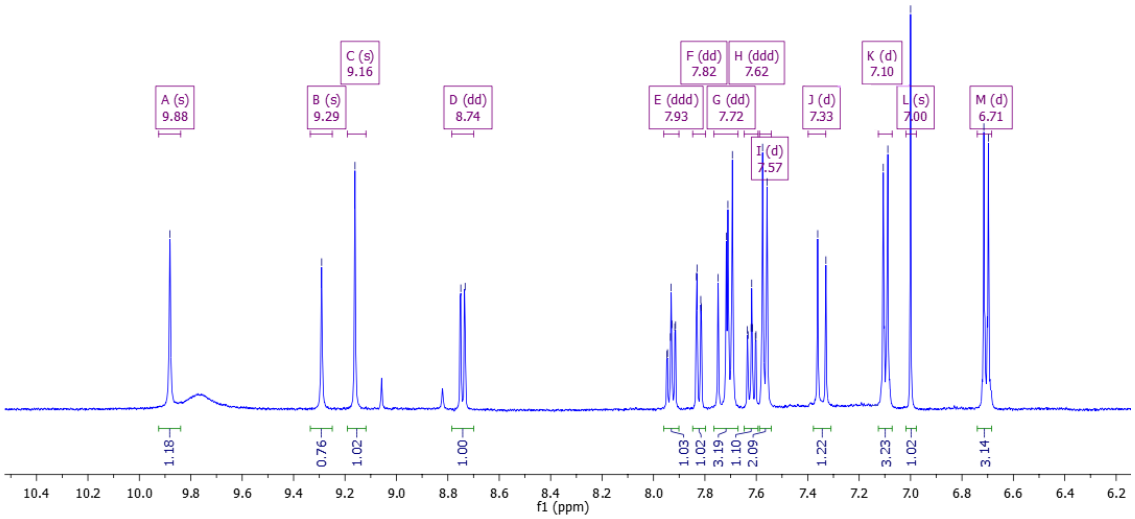
Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
418 formula(e) evaluated with 5 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-28 H: 0-1000 N: 0-4 O: 0-7 Na: 0-1 Cl: 0-1
SilviaDneg 11 (0.250) AM (Top,1, Ht,5000.0,0.00,1.00)
1: TOF MS ES-

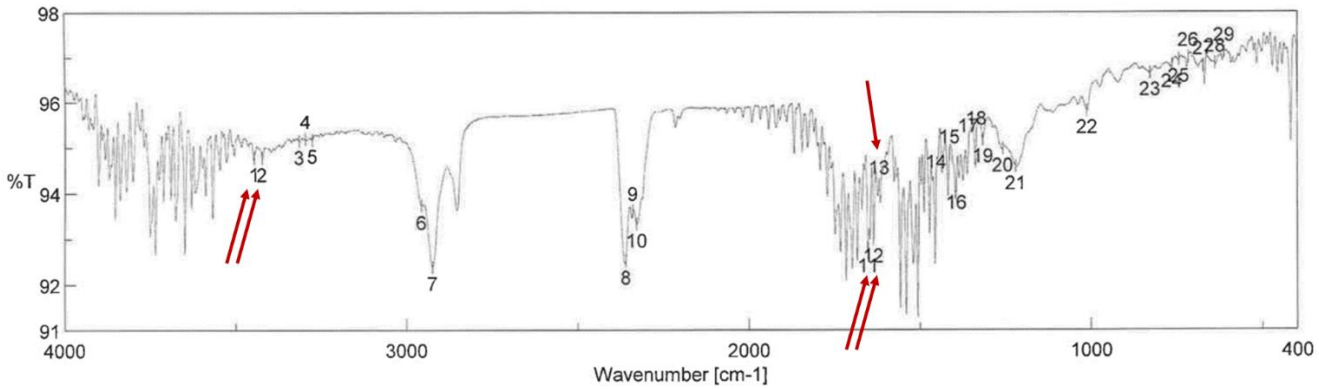


Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
495.1241	495.1224	1.7	3.4	20.5	12.6	1.5	C28 H20 N4 O3 Cl
	495.1211	3.0	6.1	15.5	12.6	1.5	C27 H24 O7 Cl
	495.1281	-4.0	-8.1	17.5	12.8	1.7	C25 H20 N4 O6 Na
	495.1200	4.1	8.3	17.5	12.6	1.5	C26 H21 N4 O3 Na Cl
	495.1192	4.9	9.9	20.5	12.8	1.8	C28 H19 N2 O7

^{1}H-NMR spectrum of compound DCM-HBU



IR spectrum of compound DCM-HBU



IR spectrum of DCM-HBU shows the bands from amide groups indicated by the arrows:

1 and 2, stretching N-H

11 and 12, stretching C=O

13, bending N-H

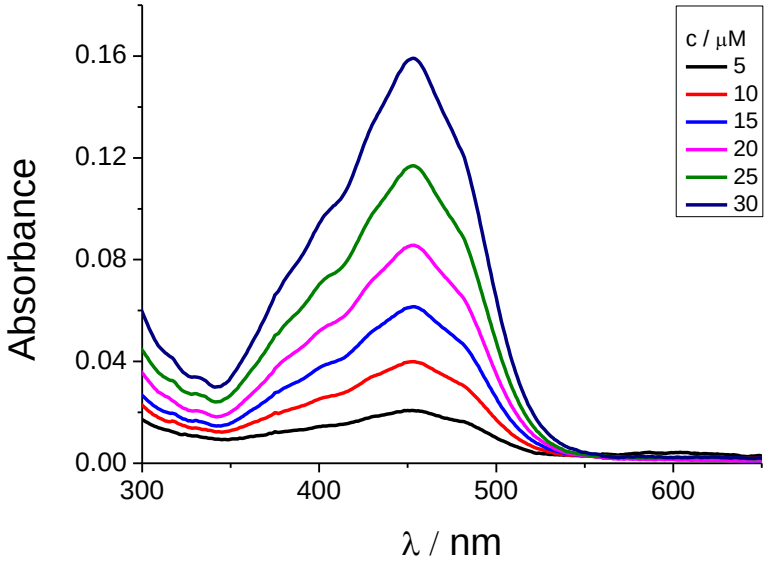


Figure S2. Absorption spectra of DCM-HBU at different concentrations in PBS/DMSO (7/3, v/v).

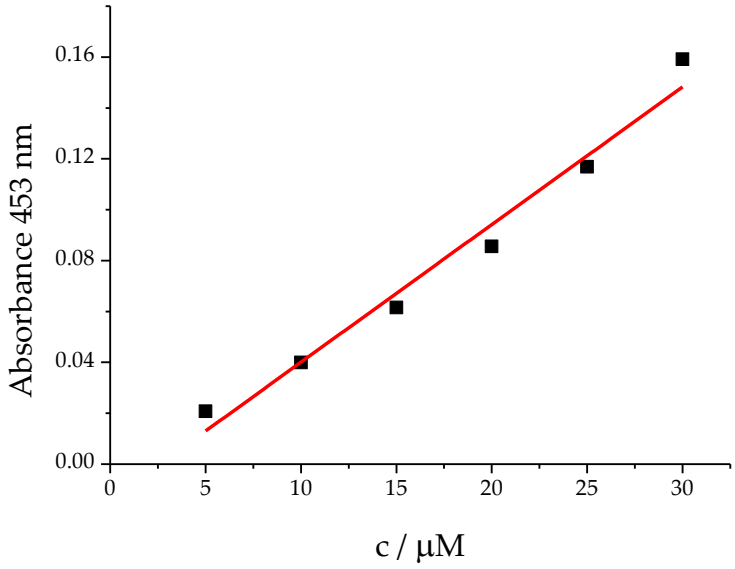


Figure S3. Determination of the molar absorptivity coefficient of the compound DCM-HBU in PBS /DMSO (7/3, v/v) at λ = 453 nm. Fit equation: y = -0.01402 + 5 409x, R^2 = 0.971.

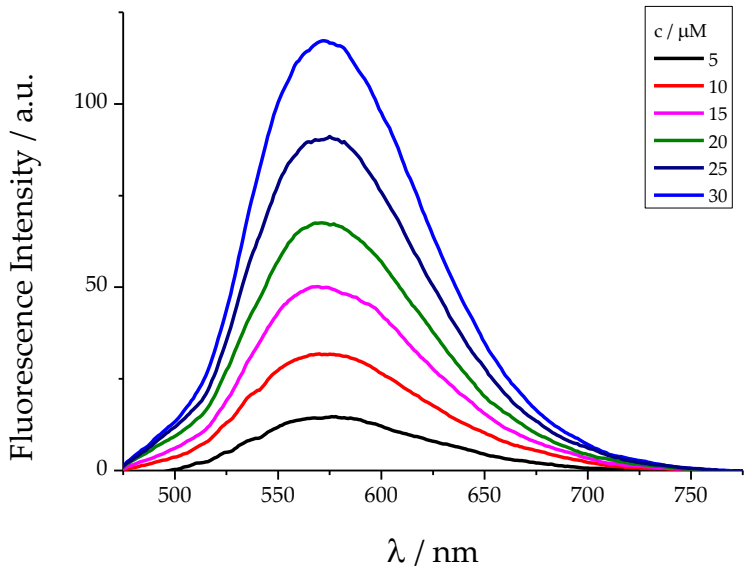


Figure S4. Emission spectra of DCM-HBU at different concentrations in PBS/DMSO (7/3, v/v) by excitation at 453 nm.

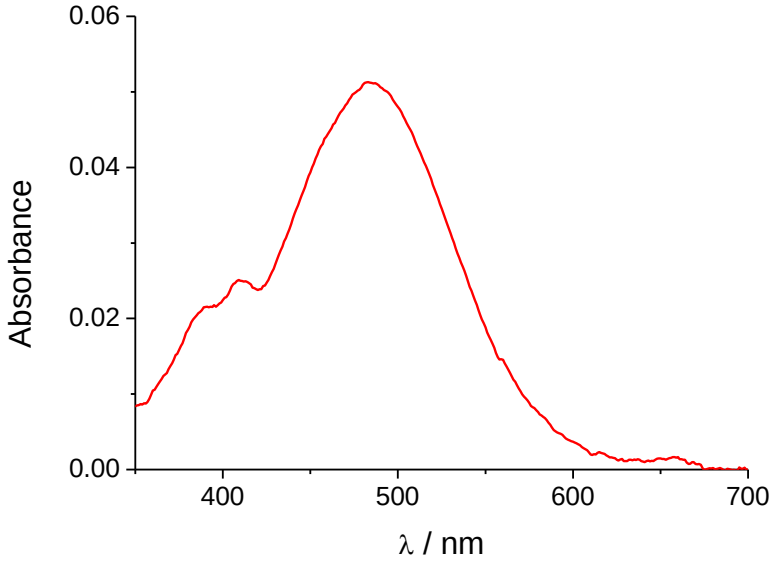


Figure S5. Absorption spectrum of DCM-NH_2 in PBS/DMSO (7/3, v/v).

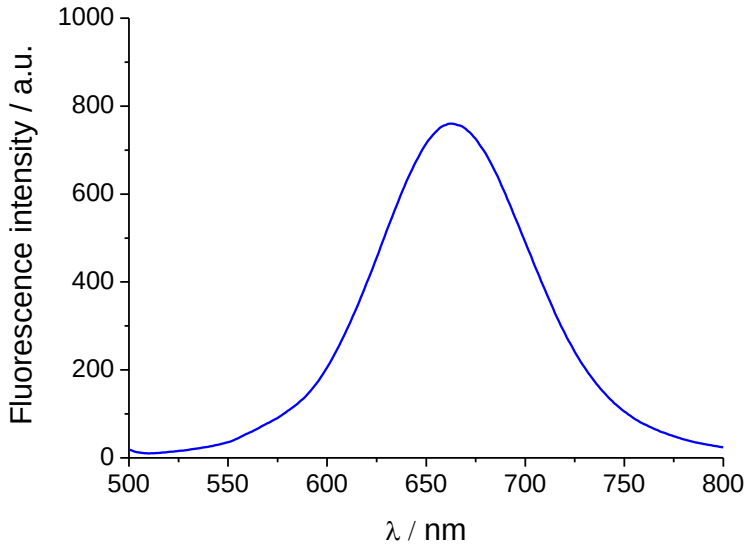


Figure S6. Emission spectrum of DCM-NH_2 in PBS/DMSO (7/3, v/v).

Quantum yield calculation

The relative fluorescence quantum yield data were obtained by integrating the areas under the fluorescence curves using the expression:

$$\Phi = \Phi_R \cdot \frac{I}{I_R} \cdot \frac{OD_R}{OD} \cdot \frac{n^2}{n_R^2}$$

where Φ and Φ_R indicate the fluorescence quantum yields of the sample and reference, respectively; I and I_R the integrated fluorescence spectra of the sample and reference, respectively; OD and OD_R are the optical density at the excitation wavelength of the sample and reference, respectively; and n and n_R are the refractive indexes of the solvents where the sample and reference are dissolved, respectively. As references, we have used Fluorescein in NaOH 0.1M (Φ = 0.91) for DCM-HBU, and Rhodamine 101 in MeOH (Φ = 1) for DCM-NH_2.

The procedure followed to calculate the QY was, firstly, prepare the samples in the solvents in a concentration with an absorption less than 0.1. Later, we selected an excitation wavelength that allows to register the complete emission spectrum. The integrated areas under the fluorescence spectra were calculated using Origin 8.5 software. The spectra used to calculate the QY are represented in the Figure 1a and 1b, S2, S4, S5 and S6.

Table S1. Fluorescence quantum yields, absorptivity coefficients and Stokes shift of DCM-NH_2

Solvent	**Φ**	λ_{abs}^{max} **/ nm**	λ_{em}^{max} **/ nm**	**Stokes shift / nm**	**ε* / M^{-1} cm^{-1}**
Acetone	7.2 %	486	639	153	54 380
Acetonitrile	10.7 %	478	640	162	47 660
Chlorobenzene	5.9 %	489	630	141	45 080
DMSO	14.6 %	520	666	146	45 510
Toluene	2.8 %	490	616	126	42 140
Water	0.4%	469	657	188	31 050
DMSO/water, 7/3, v/v	0.7 %	480	662	182	37 685

* at maxima absorption wavelength

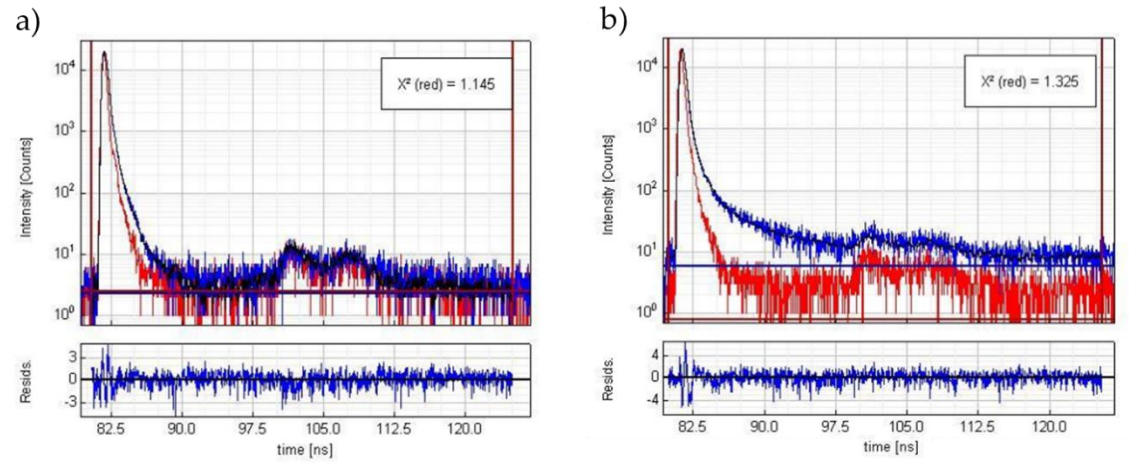


Figure S7. a) Fluorescence decay of DCM-NH_2 at λ_{ex} = 485 nm and λ_{em} = 664 nm. b) Fluorescence decay of DCM-HBU at λ_{ex} = 440 nm y λ_{em} = 570 nm.

We used the TCSPC method to register the fluorescence decay by a FluoTime 200 fluorometer (PicoQuant, Inc.). Excitation was achieved by a 485 nm or 440 nm laser-pulsed (PicoQuant, Inc.). The pulse repetition rate was 40 MHz.

Fluorescence traces were obtained in 1320 channels. The channel resolution was 36 ps. Histograms of the instrument response functions (using LUDOX scatterer) and sample decays were recorded until they reached 20 000 counts in the peak channel. Fluorescence decays were recorded at three emission wavelengths, 659, 664 and 669 nm for the sample DCM-NH_2 (7/3 PBS/DMSO (v/v)) and 565, 570 y 575 nm for the DCM-HBU(7/3 PBS/DMSO (v/v)).

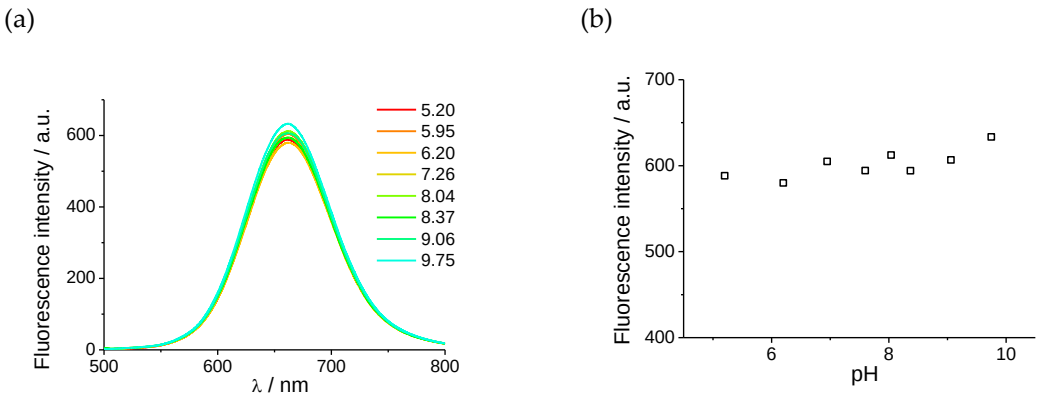


Figure S8. (a) Emission spectra of DCM-NH_2 in in PBS/DMSO (7/3, v/v) at different pH values. (b) Representation of the maximum fluorescence intensity in function of pH.

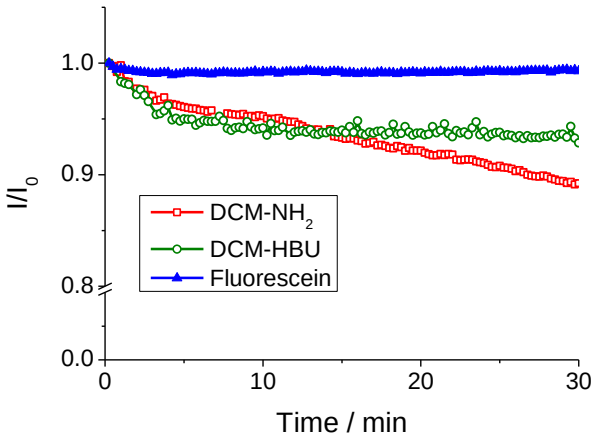


Figure S9. Normalized steady-state fluorescence intensity at its maximum value of fluorescein, DCM-HBU and DCM-NH_2 continuously irradiated with a Xe lamp.

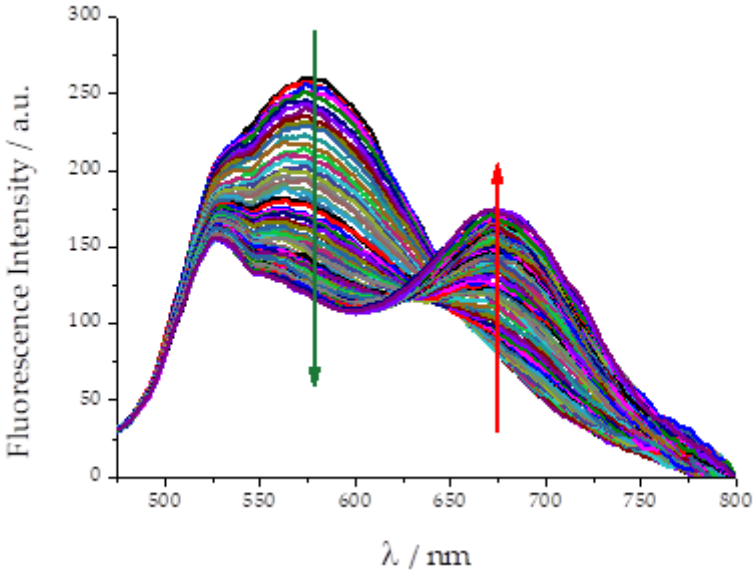


Figure S10. Evolution of the emission spectra of DCM−HBU (25 μM) with TYR (0.13 mg mL^{-1}) observed every 1 min for 2 h by excitation at 450 nm at 37 °C.

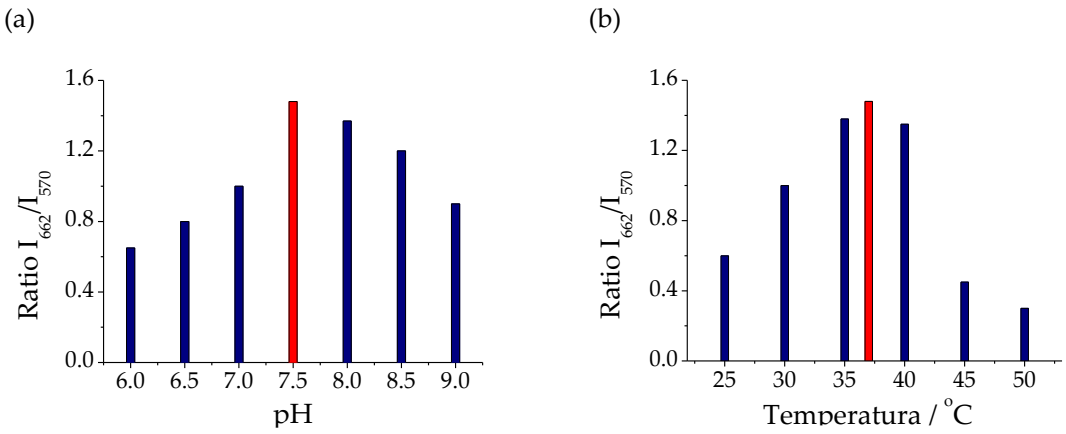


Figure S11. (**a**) Ratiometric measurements of fluorescence signals of I_{662}/I_{570} of DCM-HBU (25 μM) with DPP IV (0.13 mg mL^{-1}) after 2 h of incubation at 37 °C and different pHs by excitation at 450 nm. (**b**) Ratiometric measurements of fluorescence signals of I_{662}/I_{570} of DCM-HBU (25 μM) with DPP IV (0.13 mg mL^{-1}) after 2 h of incubation at different temperatures and pH 7.5 °C by excitation at 450 nm.

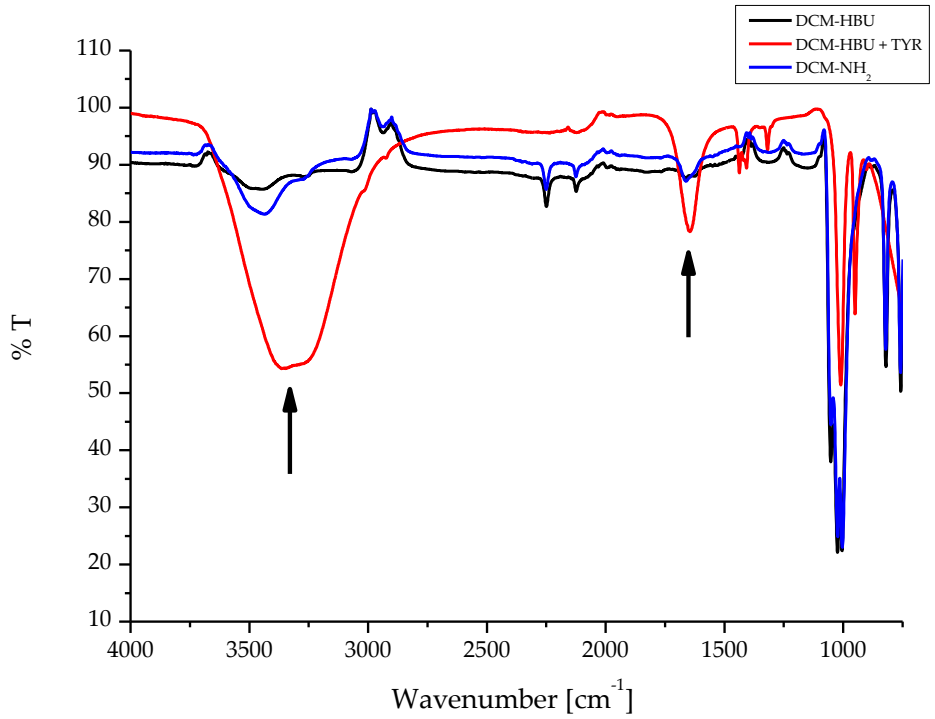


Figure S12. IR spectra of DCM-NH_2 (in DMSO), DCM-HBU (in DMSO) and DCM-HBU with TYR (in PBS/DMSO 7,3 v/v) after 2 h of reaction.

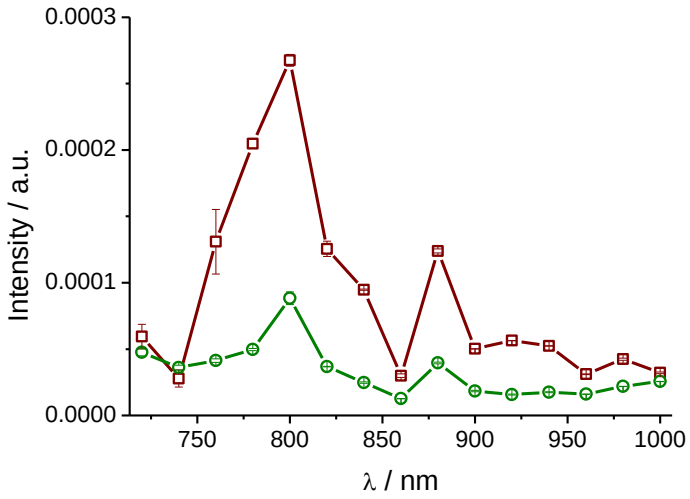


Figure S13. Two-photon excitation spectra of DCM-NH_2 (square in brown) and DCM-HBU (circles in green) measured in PBS/DMSO 7/3 v/v. Emission collected at 650–720 nm and 502-538 nm, and excitation ranging from 720 to 1000 nm, measured every 20 nm. The intensities were calculated by normalization to the power of the excitation source measured on the excitation pathway. Bars represent the standard error from three replicates.

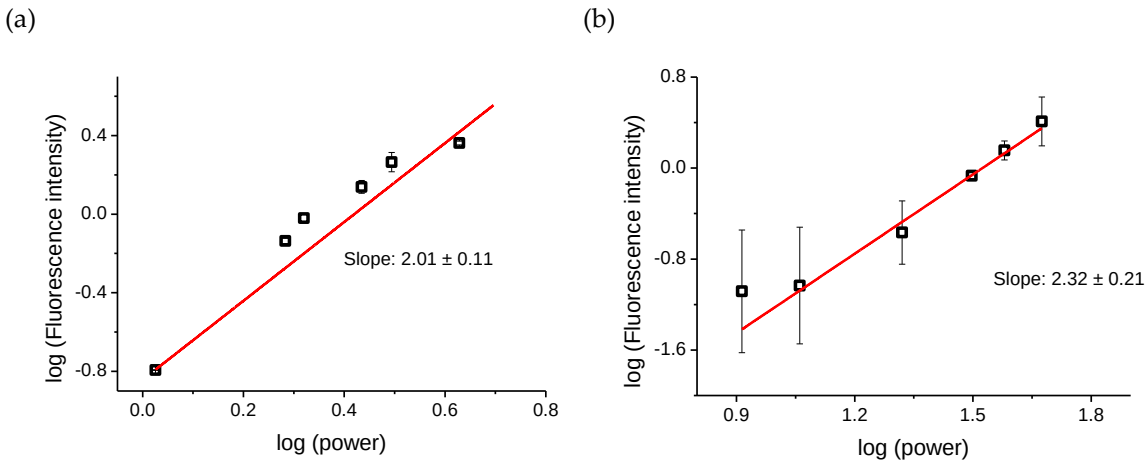


Figure S14. Logarithmic plot of the power dependence of the relative two-photon induced fluorescence intensity of (a) DCM-NH_2 and (b) DCM-HBU. Error bars represent SE.

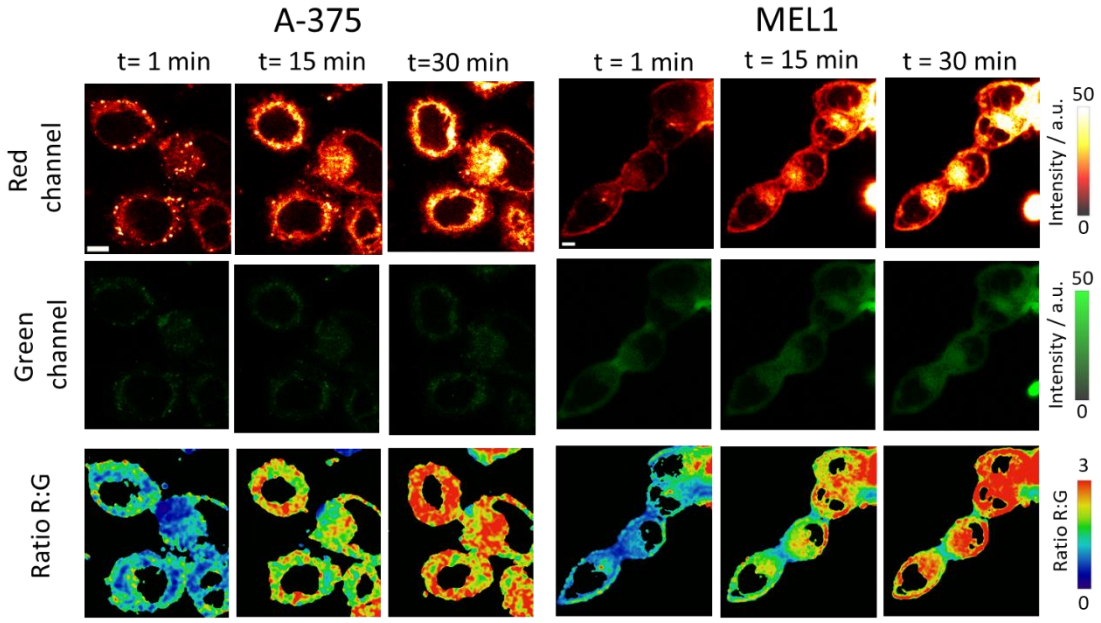


Figure S15. Representative images of the red (λ_{em} = 650-720 nm) and green (λ_{em} = 502-538 nm) intensity channels and the ratio R:G images of A-375 and MEL1 cells after adding DCM-HBU (5 μM) over time by excitation at 453 nm. Scale bars are 5 μm.

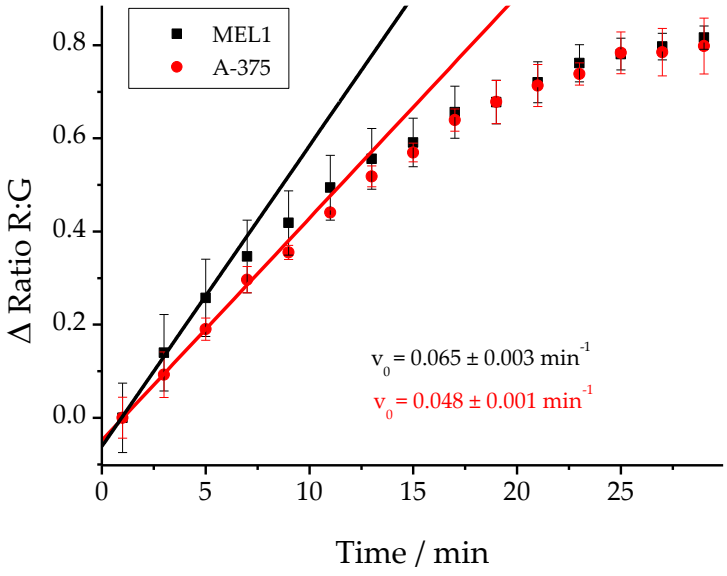


Figure S16. Increase in the value of the ratio versus time of the images of MEL1 and A-375 cells. Error bars represent SE.

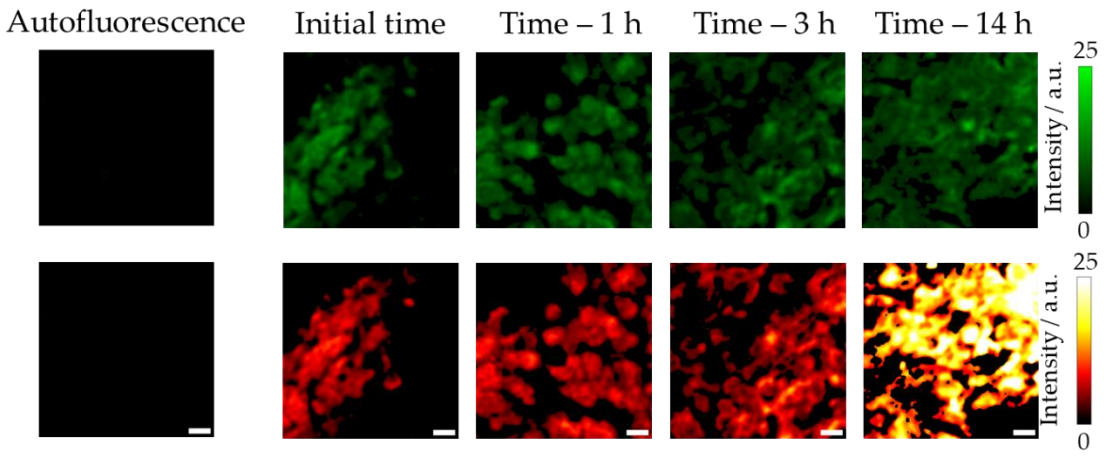


Figure S17. Representative images of the intensity green (λ_{em} = 502-538 nm) and red (λ_{em} = 650-720 nm) channels (first and second lines, respectively) of A-375 tumours after adding DCM-HBU (5 μM) using two-photon microscopy with excitation at 800 nm. Scale bars are 10 μm.

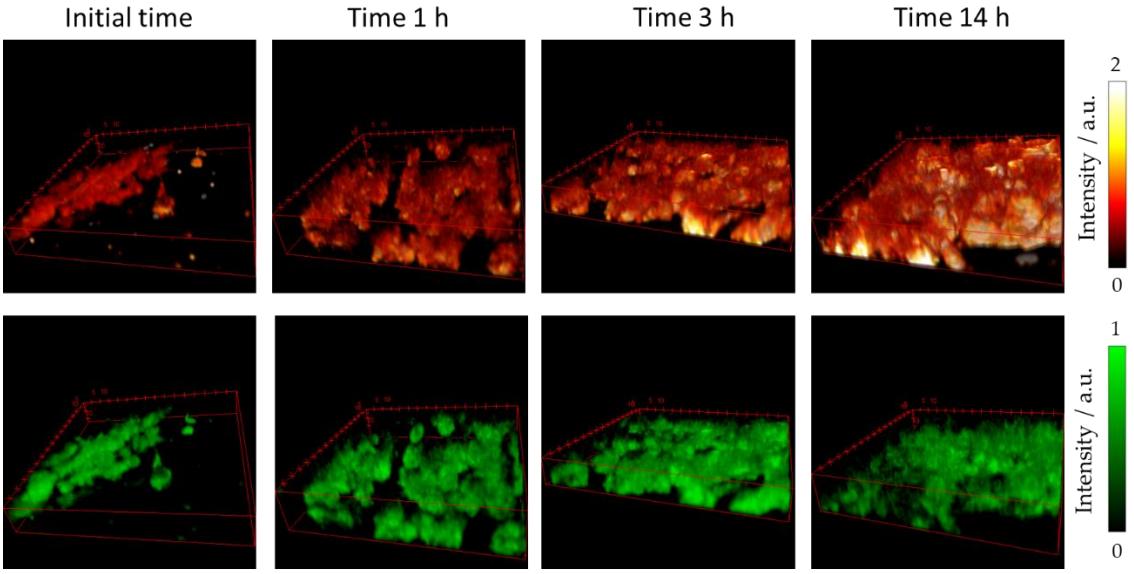


Figure S18. 3D red (first line) and green (second line) intensity images of A-375 tumours using two-photon microscopy with excitation at 800 nm

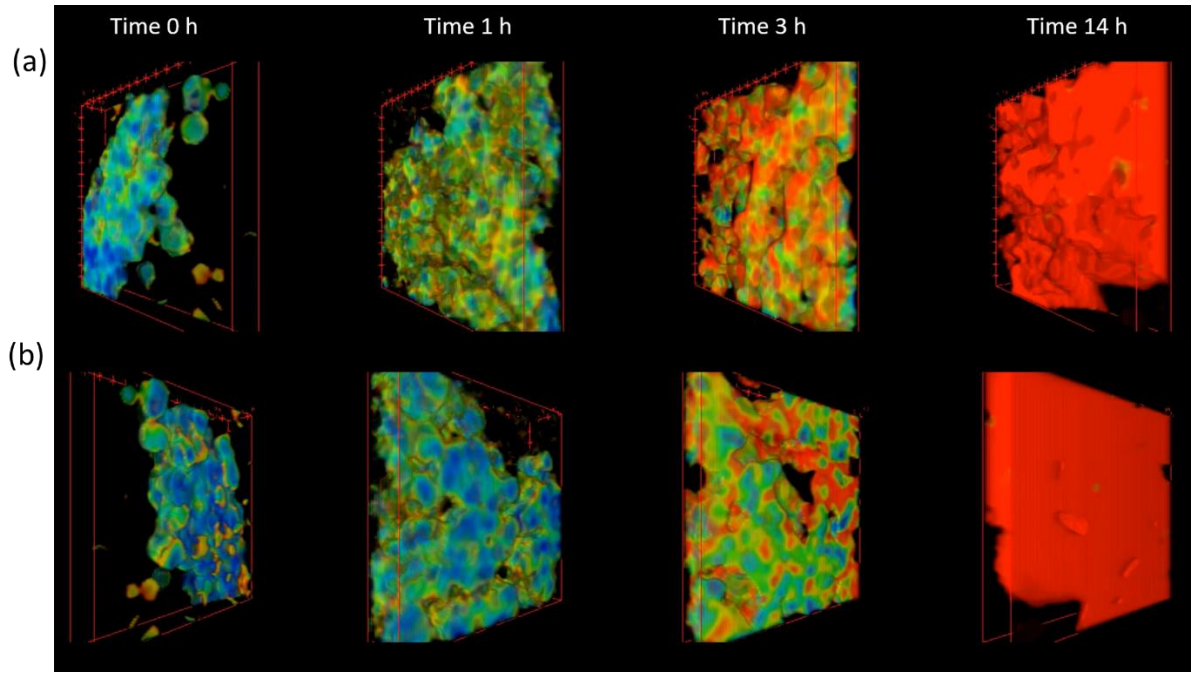


Figure S19. Top (a) and bottom (b) plane of 3D ratiometric images of A-375 tumours.

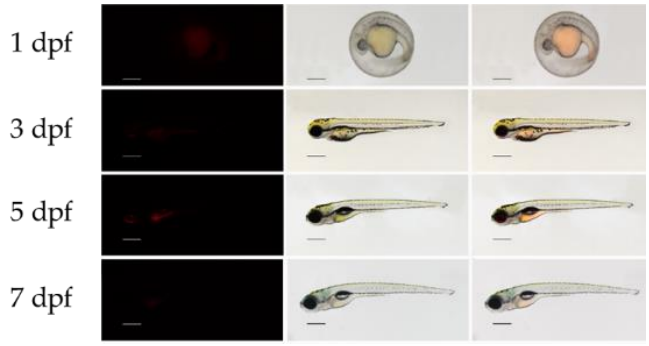


Figure S20. Living zebrafish embryos and larvae incubated with 10 μM DMSO for 3 h at different days post fertilization (dpf); red fluorescent (left), brightfield (centre), and merge (right) images are taken with a stereo microscope (λ_{exc} = 458 nm; λ_{em} = 680 nm). Scale bars: 1 dpf: 250 μm, 3-7 dpf: 500 μm.

Video S1. Fluorescence microscopy R:G ratio maps of live A-375 cell line after adding DCM-HBU and representation of the R:G ratio values over time, by two-photon excitation.

Video S2. 3D ratiometric images of A-375 tumours.