Supporting Information

New ICT-based ratiometric two-photon near infrared probe for imaging tyrosinase in living cells, tissues and whole organisms.

Javier Valverde-Pozo[1], Jose Manuel Paredes[1,*], M. Eugenia Garcia-Rubiño[1], Thomas J. Widmann[2], Carmen Griñan-Lison[2, 3, 4], Silvia Lobon-Moles[1], Juan A. Marchal[3, 5, 6], Jose M. Alvarez-Pez[1] and Eva M. Talavera[1,*]

[1] Nanoscopy-UGR Laboratory, Department of Physical Chemistry, Faculty of Pharmacy, Unidad de Excelencia en Quimica Aplicada a Biomedicina y Medioambiente (UEQ), University of Granada, C. U. Cartuja, 18071 Granada, Spain.
[2] GENYO, Centre for Genomics and Oncological Research, Pfizer/University of Granada/Andalusian Regional Government, 18016 Granada, Spain.
[3] Instituto de Investigación Biosanitaria (ibs.GRANADA), 18012 Granada, Spain.
[4] UGC de Oncología Médica, Complejo Hospitalario de Jaen, 23007 Jaen, Spain.
[5] Centre for Biomedical Research (CIBM), Biopathology and Regenerative Medicine Institute (IBIMER), University of Granada, 18100 Granada, Spain.
[6] Department of Human Anatomy and Embryology, Faculty of Medicine, University of Granada, 18016 Granada, Spain.
* Correspondence: etalaver@ugr.es; Tel.: +34 958243828 (optional; include country code; if there are multiple corresponding authors, add author initials); jmparedes@ugr.es; Tel.: +34 958243829

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Synthesis of DCM-HBU

To a solution of compound 1 (80 mg, 0.26 mmol) in dichloromethane (12.82 mL) under a N_2 atmosphere, triphosgene (153 μg, 5.15 x 10^{-4} mmol) was added. The mixture was then cooled to 0-5 °C to add DIPEA (88.9 μL) and then was stirred for 3 h. Then, 4-hydroxybenzylamine (127 μg) was added and the reaction was kept at room temperature for 24 h. 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 DCM-HBU (compound 2) as a pale-yellow solid with a 5 % yield. ^{1}H NMR (500 MHz, DMSO-d_4) δ 8.73 (dd, J = 8.4, 1.3 Hz, 1H), 7.92 (ddd, J = 8.5, 7.2, 1.4 Hz, 1H), 7.80 (dd, J = 8.4, 1.1 Hz, 1H), 7.76 – 7.58 (m, 8H), 7.56 (d, J = 8.8 Hz, 2H), 7.32 (d, J = 15.9 Hz, 1H), 7.09 (dd, J = 8.6, 2.3 Hz, 3H), 6.98 (s, 1H), 6.72 – 6.67 (m, 3H). TOF MS ES -, calculated for $C_{28}H_{20}N_4O_3Cl$: 495.1224 ($M+Cl^-$); found: 495.1241.

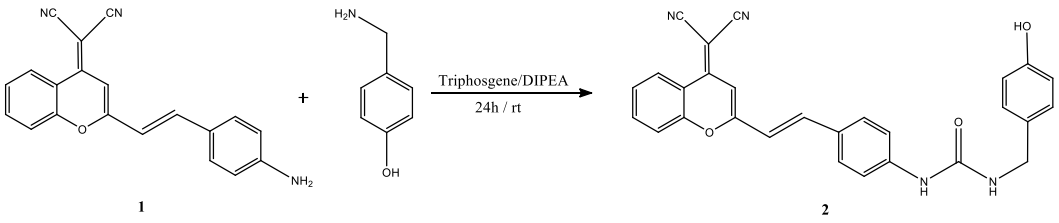


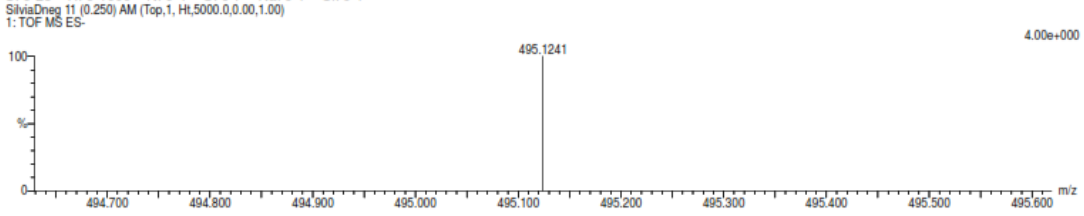
Figure S1. Synthesis of compound DCM-HBU.

Mass spectrum of compound DCM-HBU

Elemental Composition Report **Page 1**

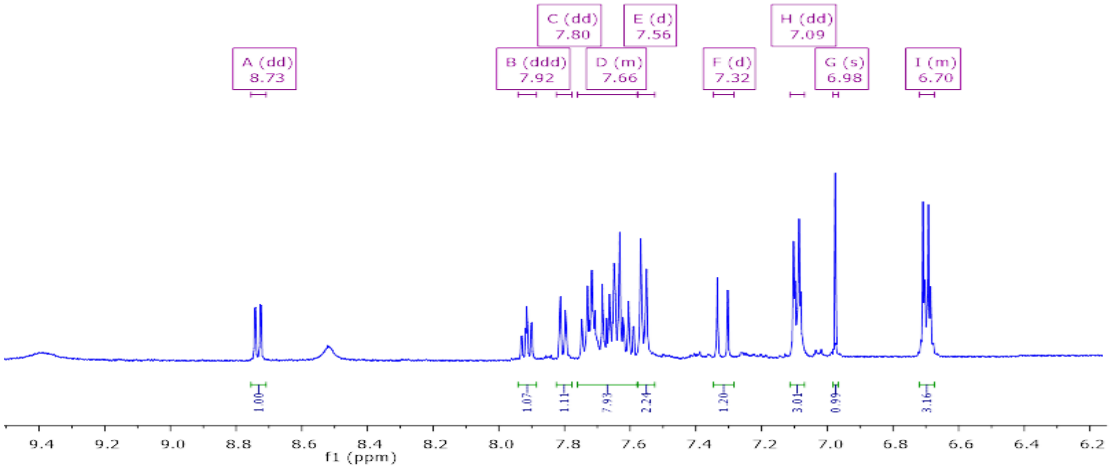
Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

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



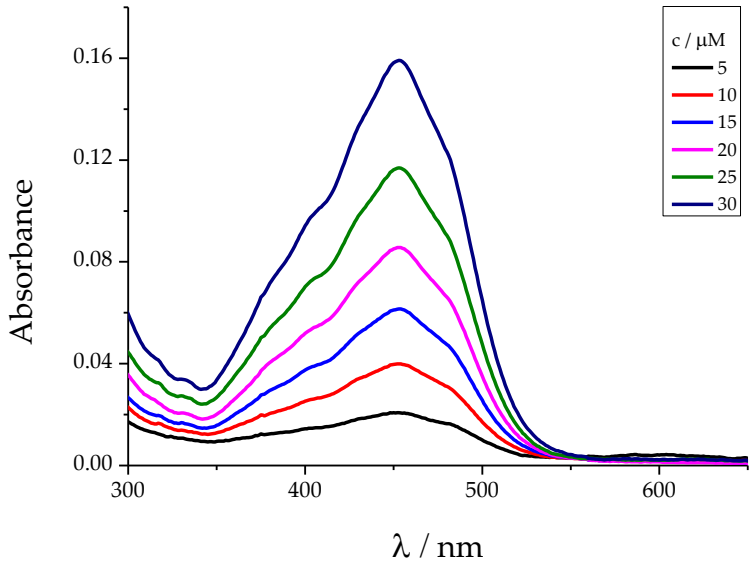


Figure S2. Absorption spectra of the DCM-HBU at different concentrations in PBS/DMSO (7/3, v/v).

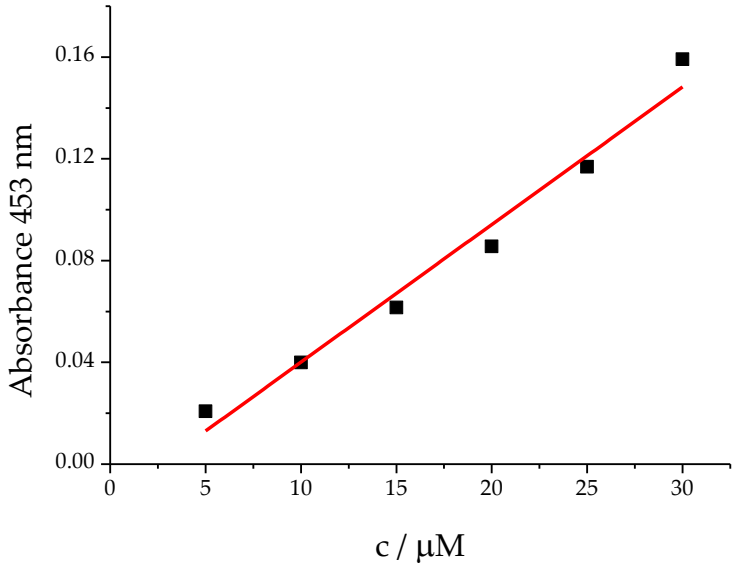


Figure S3. Determination of the molar absorptivity coefficient of the compound DCM-HBU in PBS /DMSO (7/3, v/v) at λ = 453 nm. Fit equation: y = -0.01402 + 5 409x, R^2 = 0.971.

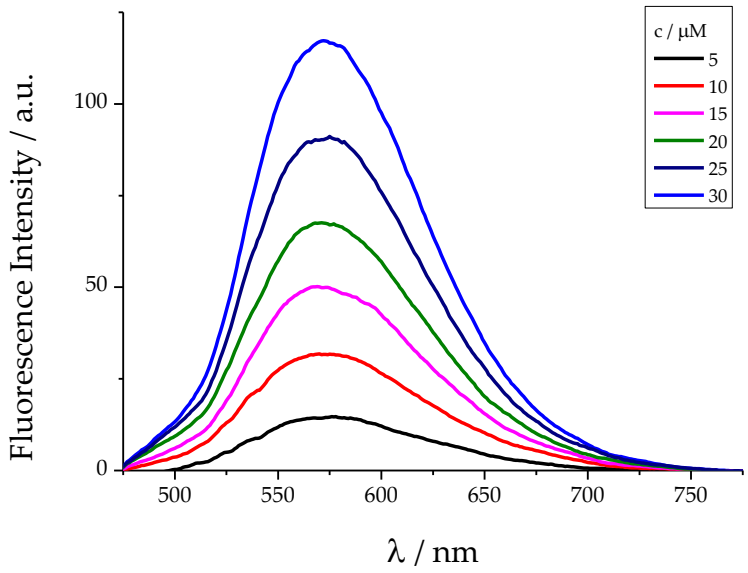


Figure S4. Emission spectra of the DCM-HBU at different concentrations in PBS/DMSO (7/3, v/v) by excitation at 453 nm.

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 when 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.

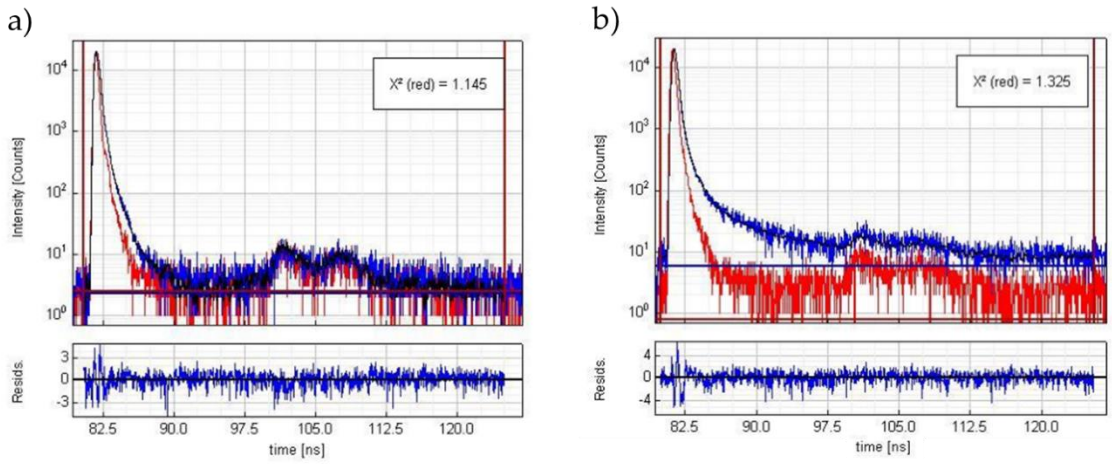


Figure S5. a) Fluorescence decay of DCM-NH_2 at λ_{ex} = 485 nm and λ_{em} = 664 nm. b) Fluorescence decay of DCM-HBU at λ_{ex} = 440 nm y λ_{em} = 570 nm.

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)).

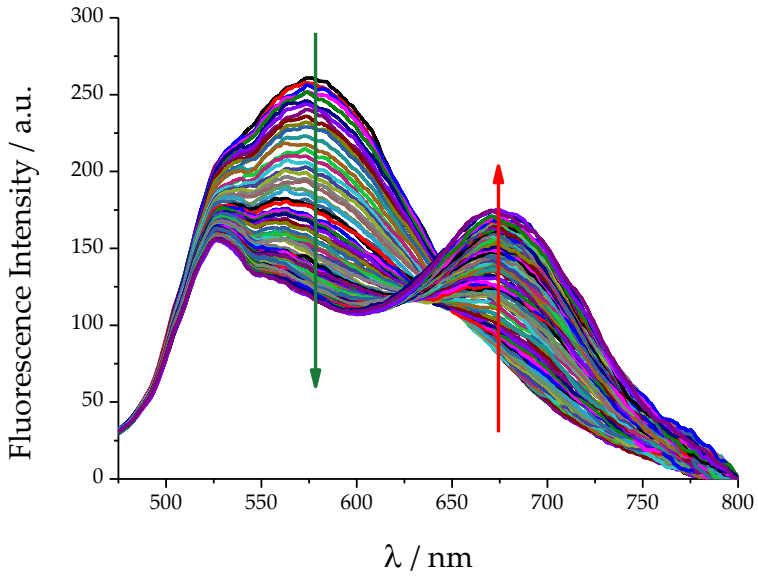


Figure S6. Evolution of the emission spectra of DCM–HBU (25 μM) with TYR (0.13 mg mL^{-1}) observed every 1 min for 2 h by excitation at 450 nm at 37 °C.

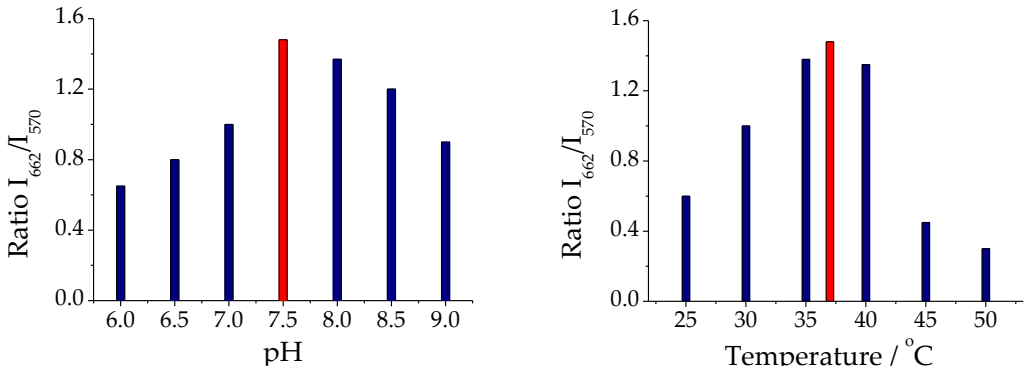


Figure S7. (**a**) Ratiometric measurements of fluorescence signals of I_{662}/I_{570} of DCM-HBU (25 μM) with DPP IV (0.13 mg mL^{-1}) after 2 h of incubation at 37 °C and different pHs by excitation at 450 nm. (**b**) Ratiometric measurements of fluorescence signals of I_{662}/I_{570} of DCM-HBU (25 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.

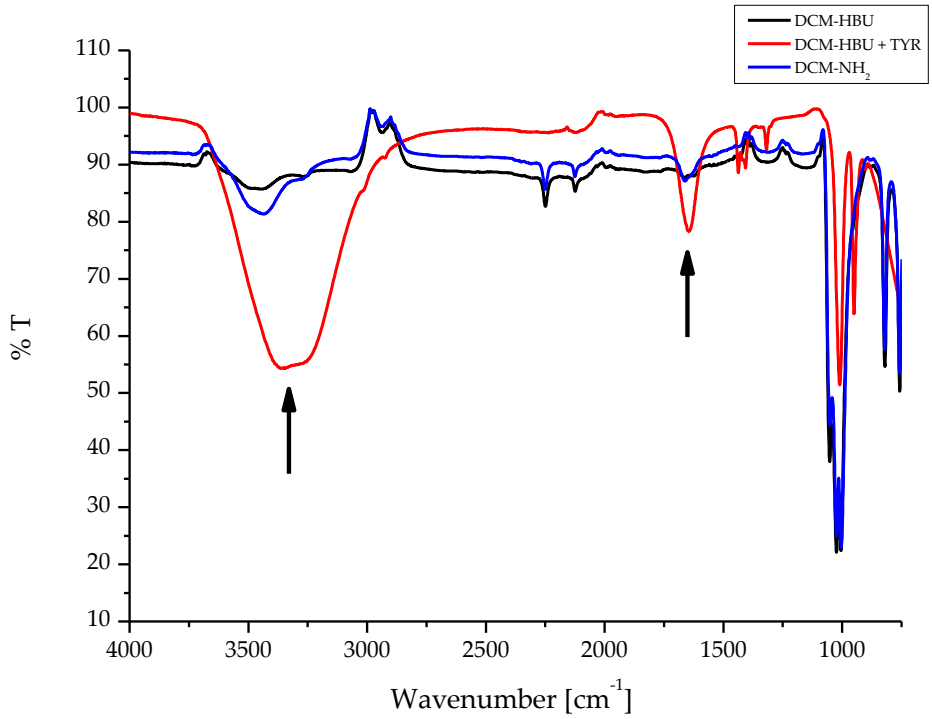


Figure S8. IR spectra of DCM-NH_2 (in DMSO), DCM-HBU (in DMSO) and DCM-HBU with TYR (in PBS/DMSO 7,3 v/v) after 2 h of reaction.

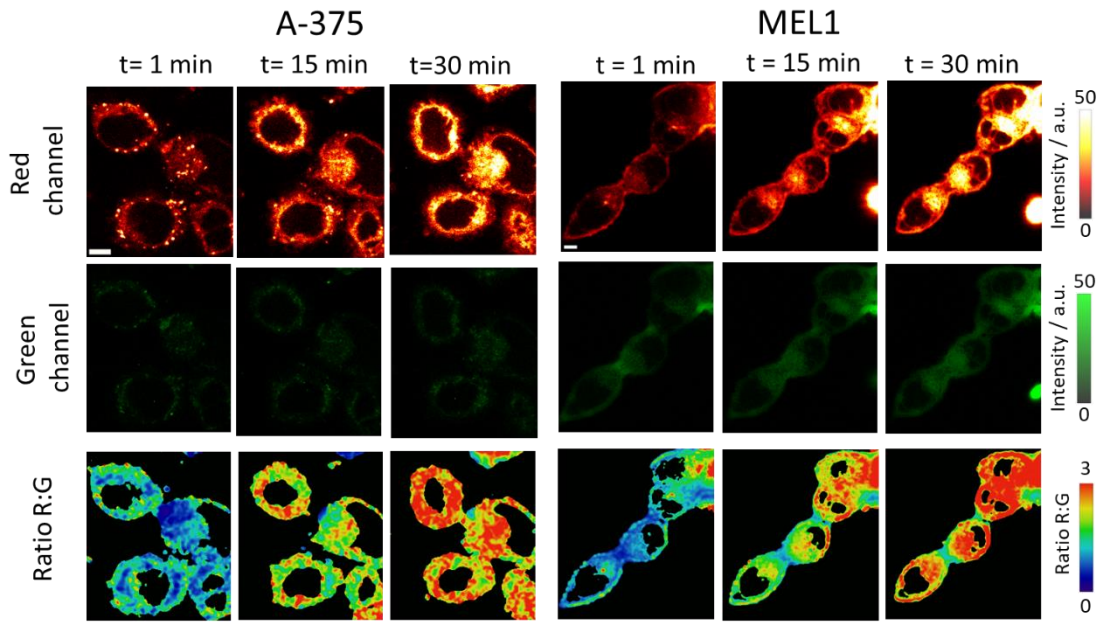


Figure S9. Representative images of the red (λ_{em} = 650-720 nm) and green (λ_{em} = 502-538 nm) intensity channels and the ratio R:G images of A-375 and MEL1 cells after adding DCM-HBU (5 μM) over time by excitation at 453 nm. Scale bars are 5 μm.

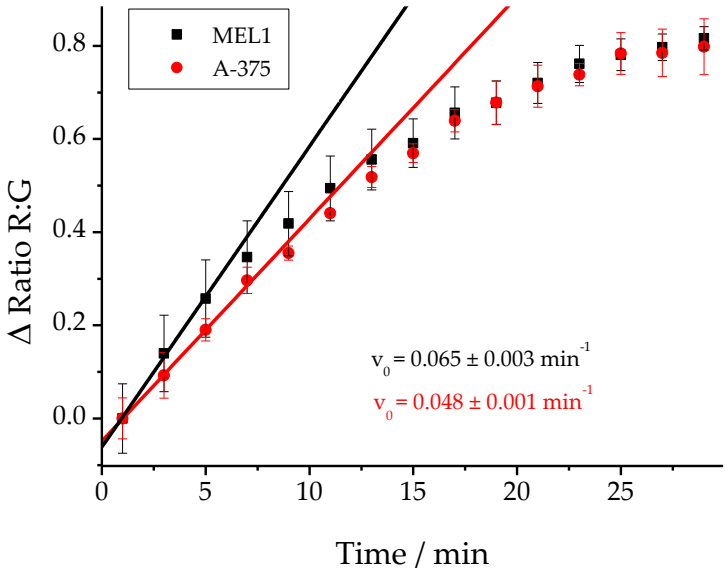


Figure S10. Increase in the value of the ratio versus time of the images of MEL1 and A-375 cells.

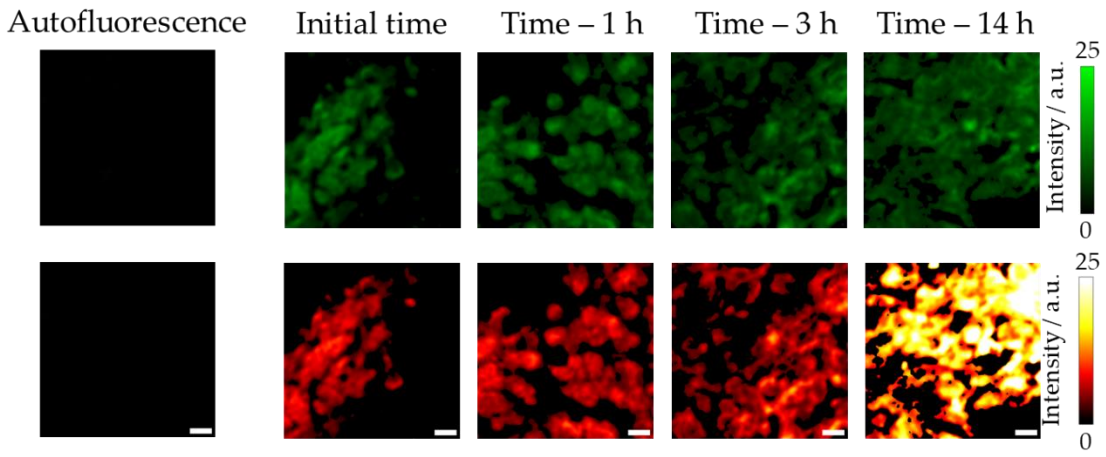


Figure S11. Representative images of the intensity green (λ_{em} = 502-538 nm) and red (λ_{em} = 650-720 nm) channels (first and second lines, respectively) of A-375 tumours after adding DCM-HBU (5 μM) using two-photon microscopy with excitation at 800 nm. Scale bars are 10 μm.

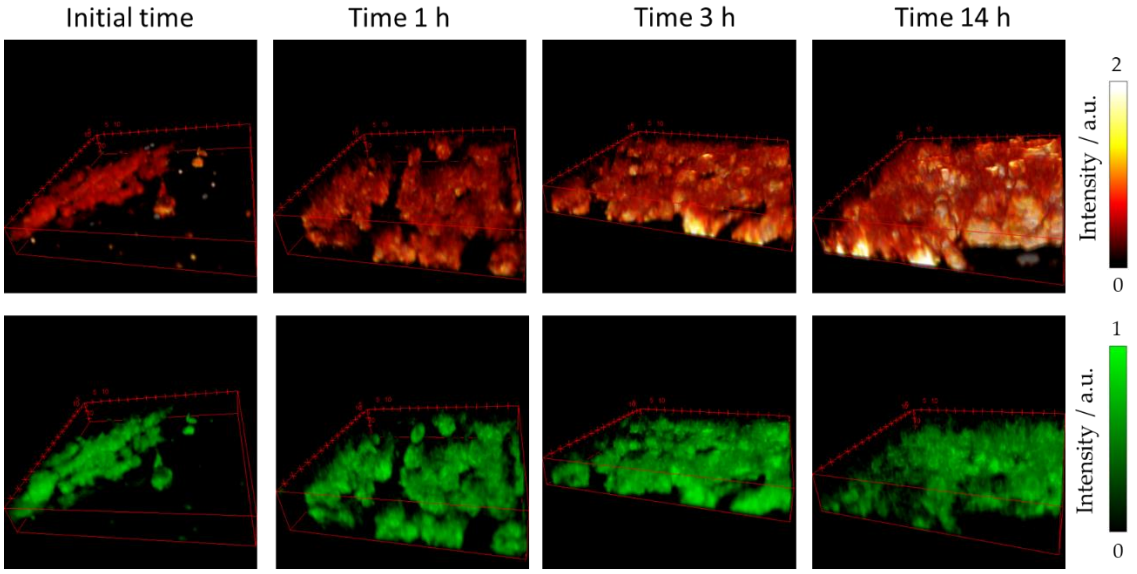


Figure S12. 3D red (first line) and green (second line) intensity images of A-375 tumours using two-photon microscopy with excitation at 800 nm

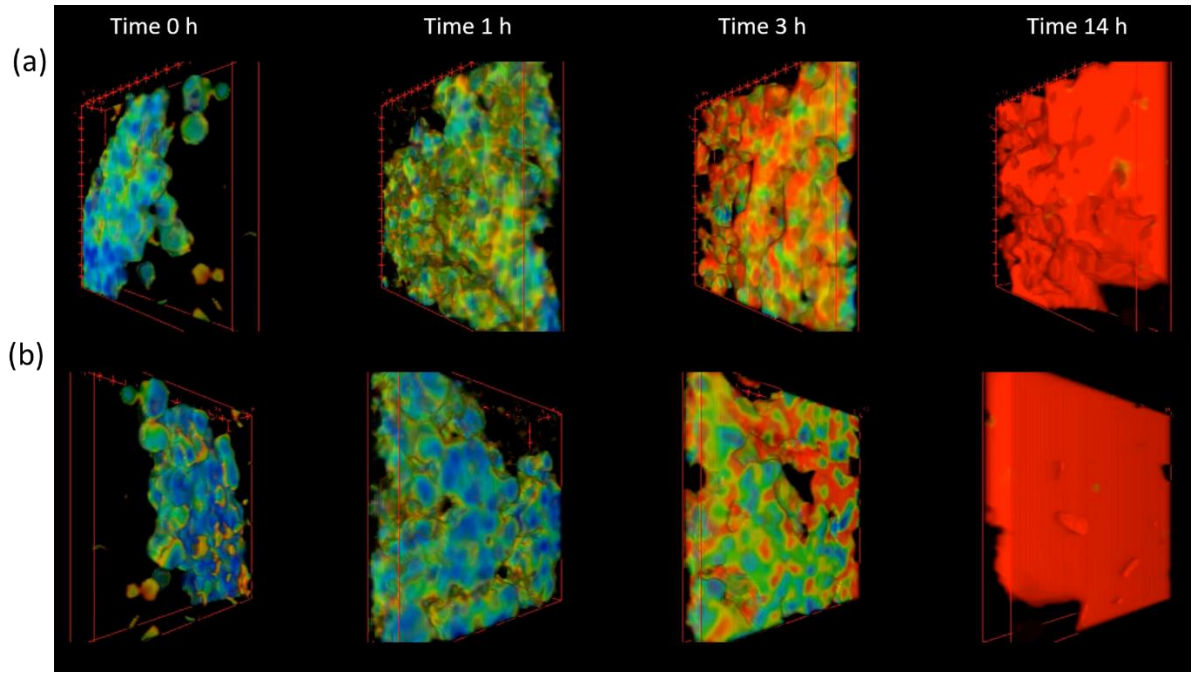


Figure S13. Top (a) and bottom (b) plane of 3D ratiometric images of A-375 tumours.

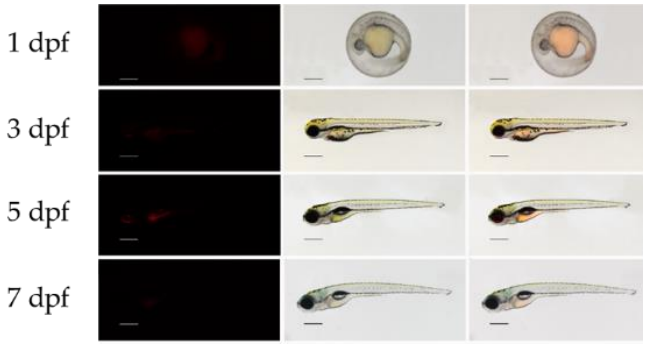


Figure S14. Living zebrafish embryos and larvae incubated with 10 μM DMSO for 3 h at different days post fertilization (dpf); red fluorescent (left), brightfield (centre), and merge (right) images are taken with a stereo microscope (λ_{exc} = 458 nm; λ_{em} = 680 nm). Scale bars: 1 dpf: 250 μm, 3-7 dpf: 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.