SUPPORTING INFORMATION:

Rapid amplitude-modulation of a diarylethene photoswitch: En route to contrast-enhanced fluorescence imaging.

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Materials and methods

Sample preparation for spectroscopic measurements. Experimental procedures for the preparation of Dasy have been reported previously.[1] All spectroscopic measurements were performed in aqueous solution (mQ water). Samples were recorded in a macro fluorescence quartz cuvette (light path = 10 × 10 mm) or in a reduced volume cuvette (v = 45 µL, light path = 3 × 3 mm). The reduced cuvette was used in all modulation experiments, whereas the typical non-modulated experiment was performed in the macro cuvette.

Spectroscopic measurements. Ground state absorption spectra were recorded on a Cary 50 UV/vis spectrometer. In the non-modulated experiments, corrected fluorescence spectra were recorded on a SPEX Fluorolog-3 spectrofluorometer. Fluorescence lifetimes were determined on a time correlated single photon counting (TCSPC) setup using PicoQuant laser diodes (377 nm) and a PMT detector (10000 counts, 1024 channels). The emitted photons were collected at the magic angle (54.7°) at 500 nm. The measured fluorescence decays were fitted using the program FluoFit Pro v.4 (PicoQuant GmbH, Germany) after deconvolution of the data with the instrument response function (IRF). In the fluorescence modulation experiments, the light sources were a 365 nm LED (Engin LZ1 10UV00, FWHM = 11 nm, ~30 mW at the sample) and a red laser diode at 660 nm (Coherent OBIS 1185057, ~40 mW at the sample). Corrected emission was detected with a photomultiplier tube (PMT) connected to an oscilloscope (Tektronix TDS 3052) and a lock-in amplifier (LIA; EG&G Model 124A) equipped with a differential preamplifier (EG&G Princeton Applied Research Model 116). Note that this experimental setup was used for also a subset of the non-modulated experiments (steady-state experiments shown in Fig. 5). The LIA received a reference signal from the red laser diode driver. The LIA detects only the ac component of the PMT response in phase with the signal from the power supply. Frequencies at multiples of 50 Hz were excluded to avoid interference from the 50 Hz grid frequency.

Fluorescence imaging. The fluorescence properties of Dasy within cells were analyzed using an inverted Leica SP5 HyD Confocal Microscope hosted at the Centro Andaluz de Nanomedicina y Biotecnología

(Bionand). Imaging was performed using a 63x PLAN APO NA 1.4 oil immersion objective. Fluorescence was recorded between 450 and 550 nm using a HyD detector with excitation at 405 nm. Brightfield transmitted light images were captured simultaneously using the 405 nm light.

Cell culture. L929 cells (mouse fibroblast cell line, derived in 1948 from a C3H/An male mouse), obtained from the European Collection of Authenticated Cell Culture (ECACC), were cultured in complete medium (DMEM + 10% FBS + 1% Penicillin-Streptomycin + 2 mM L-Glutamine) at 37°C in a humidified environment with 5% CO_2. For microscopy experiments, L929 cells were grown on 12 mm diameter 0.170 mm thick glass coverslips in multiwell plates to approximately 50% confluency prior to starting the experiment.

Cell treatment. L929 cells were treated overnight with a complete medium solution containing Dasy at 100 µM, and fluorescence images were then recorded to corroborate its internalization. Cells were fixed with PFA 4%, washed three times with PBS 10x and then mounted in Glycerol/PBS 10x (9:1) before the modulation experiment.

Microscopy fluorescence modulation experiment. Images were taken with resonant scanner mode (scan frequency: 8000 Hz) and using a 256 x 128 pixel resolution. A sequential scanning was then configured, containing four scans or images per frame. The first two scans were acquired with simultaneous irradiation at 405 nm (115 µW) and 633 nm (20 µW), whereas in the last two scans only 405 nm irradiation was employed. Fluorescence emission was recorded during 120 s in the green spectral region, with a four-scan frame acquisition time of 135 ms. Amplitude modulated fluorescence was observed throughout the whole measurement, with an apparent modulation frequency of 7.4 Hz.

Isomerization quantum yield determination. The isomerization quantum yields for the closing and opening isomerization reactions were determined by using UV light at 365 nm from a hand-held UV-lamp (UVGL-25, 1.5 mW/cm^2) and visible light at 523 nm from an LED (Engin LZ1-10G100, FWHM = 37 nm, ~1 mW/cm^2) with a longpass filter at 470 nm, respectively. The absorbance changes as a function

of irradiation time were monitored and compared to those of the reference compound Furylfulgide 2-[1-(2,5-dimethyl-3-furyl)ethylidene]-3-isopropylidenesuccinic anhydride[2] under identical irradiation power/geometries and corrected for the molar absorption coefficients at the irradiation wavelengths.

Fluorescence quantum yield determination. For fluorescence quantum yield determination, relative measurements on a SPEX Fluorolog-3 spectrofluorometer together with reference compound (9,10-Diphenylanthracene in cyclohexane, Φ_F=0.96 were used.[3]

Thermal stability

Dasy(o) was converted to Dasy(c) using UV light at 365 nm and left for 43 hours in the dark. Ca 15% decrease in the absorption of Dasy(c) was observed. The absorbance in the wavelength region 330-380 nm where Dasy(o) displays its characteristic strong absorption band also decreased, strongly signalling that the spectral changes are due to the irreversible formation of a by-product.

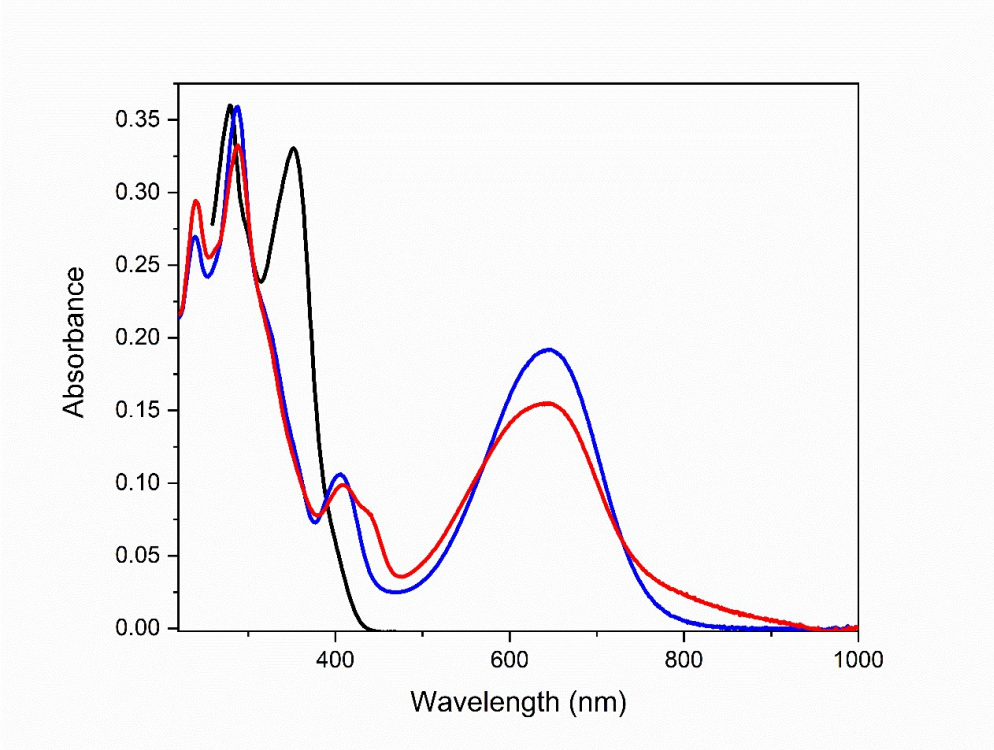

Figure S1. Thermal stability. Absorption spectra of Dasy(o) in aqueous solution before UV irradiation (black line), after UV irradiation at 365 nm to yield the photostationary state (virtually 100% Dasy(c), blue line) and after leaving the UV irradiated sample for 43 hours in the dark (red

line).

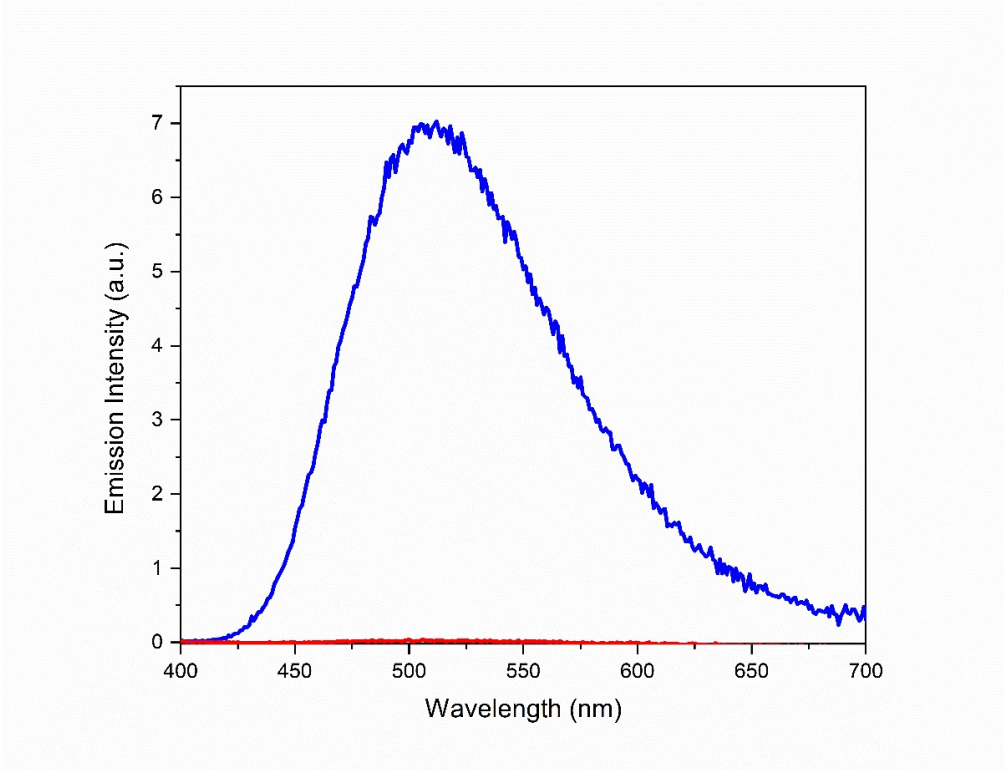

Figure S2. Emission spectra of Dasy in aqueous solution before irradiation at 365 nm (blue line, 100% Dasy(o)) and after irradiation at 365 nm to reach the photostationary state (PSS), virtually corresponding to 100% Dasy(c) (red line). Excitation wavelength = 355 nm

Modulation amplitude A_m vs. modulation frequency f_m

The experimentally obtained values of A_m versus f_m were fitted to the following equation

$$A_m = \frac{A_m(0)}{\sqrt{1 + 2\pi {f_m}^2 \tau^2}} \qquad (1)$$

where $A_m(0)$ is the maximal modulation amplitude (observed when $f_m < k_{obs}$, that is when the modulation frequency is slower than the isomerization kinetics and Dasy is being switched all the way between the two photostationary states (PSS)), and τ is the inverse rate constant ($1/k_{obs}$) for the isomerization reactions. Note that in our case, there are two isomerization reactions (Dasy(o) $\rightarrow$

Dasy(c) triggered by UV light at 365 nm only, and Dasy (c) → Dasy(o) triggered by simultaneous exposure to UV light at 365 nm and red light at 660 nm). The resulting fit shown in Fig. S4 yields a τ-value of 30 ms which represents the overall isomerization kinetics well (see Fig. S3).

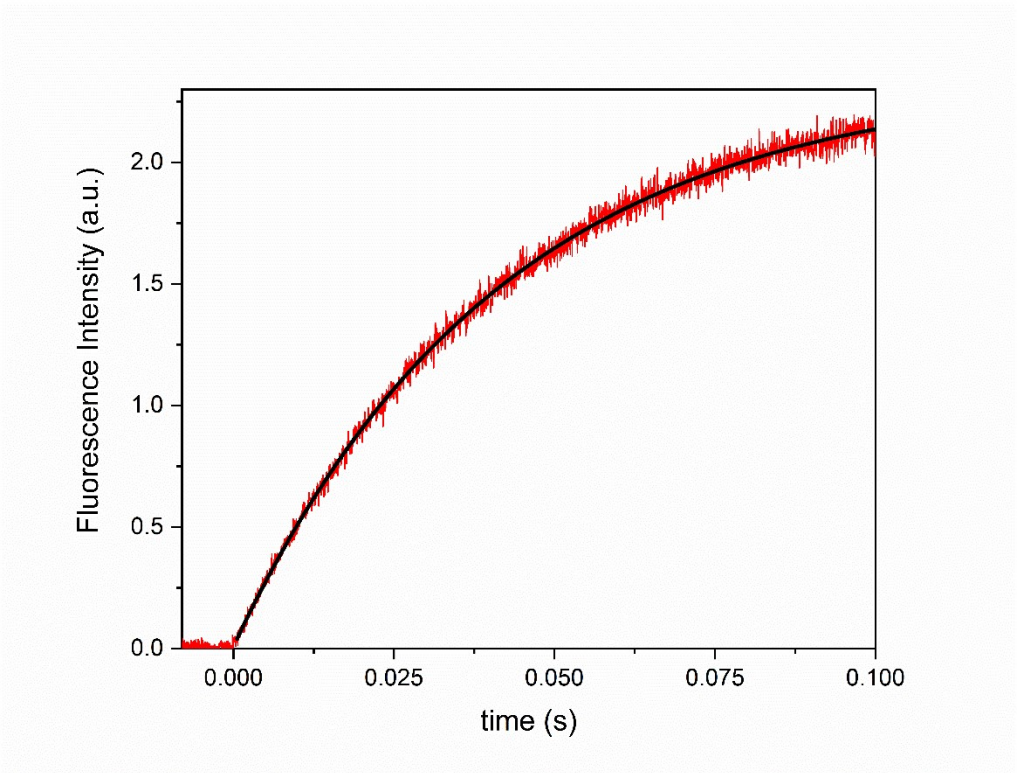

Figure S3. Changes in the fluorescence intensity of Dasy with time, corresponding to the isomerization kinetics induced by exposure to both 365 nm and 660 nm light (red line) as described above. The onset of light at 660 nm appeared at t = 0. A monoexponential fit yields a τ-value (lifetime) of 41 ms (black line).

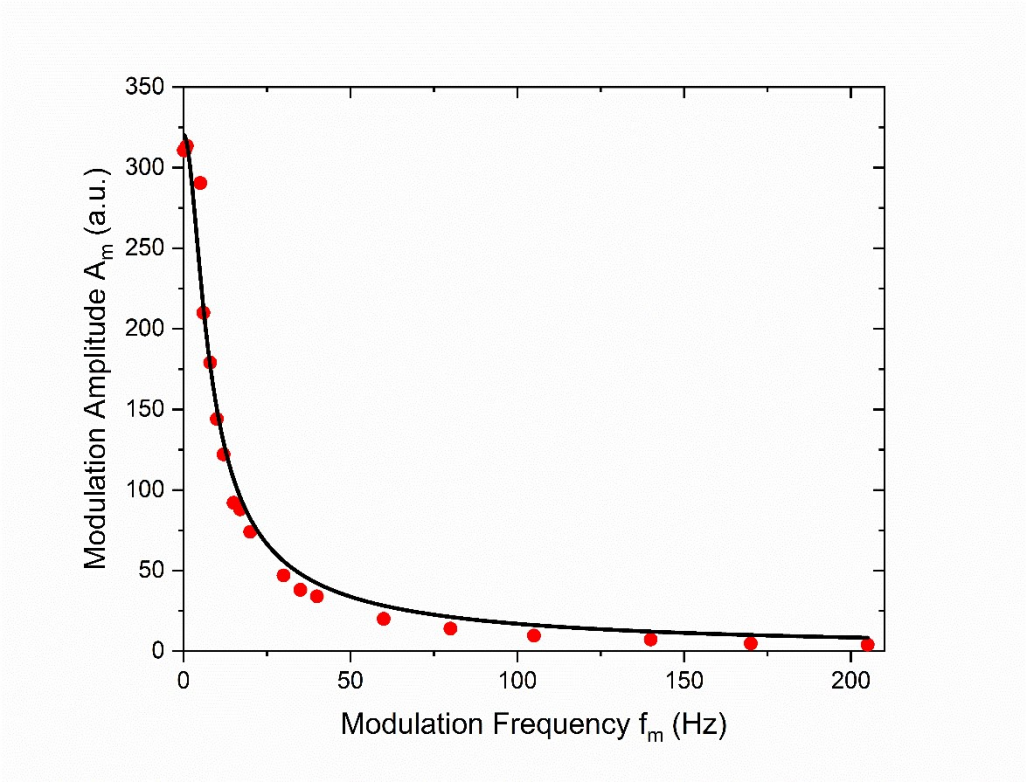

Figure S4. Modulation amplitude A_m vs. modulation frequency f_m. Experimental data (red dots) fitted to equation 1 above (black line).

Amplitude modulation of Dasy in fixed cells using fluorescence microscopy

The overall variations of Dasy fluorescence from inside the cell are displayed in Fig. S5, top panel. During the first 120 s, the cell was exposed to both 405 nm and modulated 633 nm light. This results in the establishment of a new PSS, that contains less Dasy(o), implying that the fluorescence intensity will decrease with time. The decrease in fluorescence intensity is thus not a result of photodegradation. This is proven by exposing the cell to only light at 633 nm for 180 s (axis break in the figure between 120 s and 300 s) resetting Dasy to the initial Dasy(o) form, as manifested by the total recovery of the initially observed fluorescence intensity (around 1375 a.u.). This clearly excludes photodegradation. 120 s of both 405 nm and 633 nm light exposure follows, and the sample was subsequentially fully reset to the initial state using 633 nm light only at t = 420 s. As indicated in the manuscript, the extensive applied light exposure scheme (over 700 s) is not required to yield sufficiently good data, but

is chosen to prove the excellent photostability of Dasy. The lower panel shows zoom-ins of the amplitude modulated part of the overall fluorescence intensity in time-windows spaced by 300 s (as presented also in Fig. 6b in the manuscript).

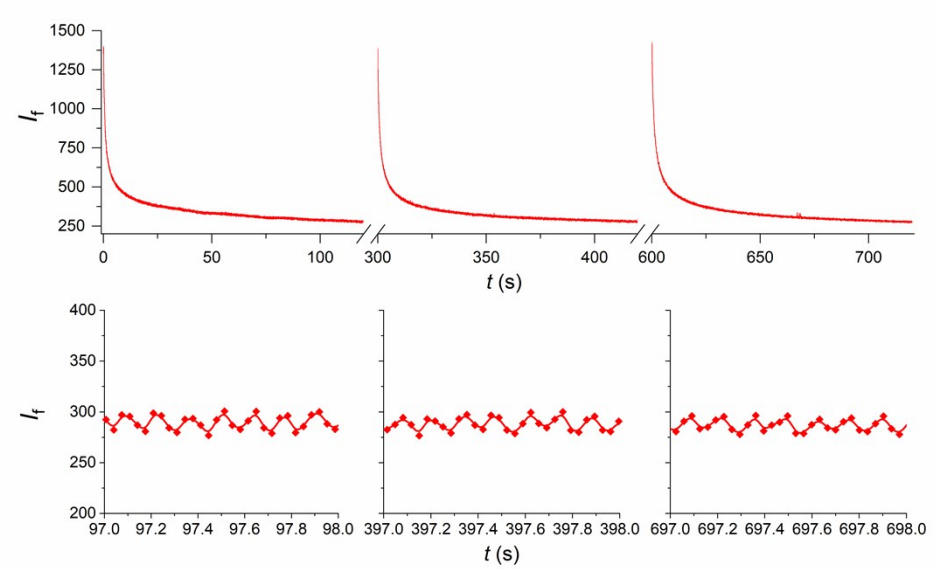

Figure S5. Amplitude modulation of Dasy in fixed cells using fluorescence microscopy. See text above for details.

Supplementary fluorescence microscopy images

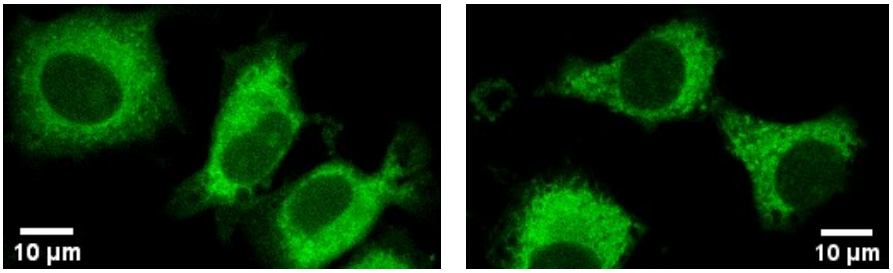

Figure S6. Fluorescence images of Dasy within L929s. No accumulation of Dasy within the cell nucleus is observed.

References

1. Naren, G. W.; Li, S. M.; Andréasson, J., A simplicity-guided cocktail approach toward multicolor fluorescent systems. *Chemical Communications* **2020,** *56* (23), 3377-3380.
2. Heller, H. G.; Langan, J. R., Photochromic heterocyclic fulgides 3. The use of (E)-alpha-(2,5-dimethyl-3-furylethylidene) (isopropylidene)succinic anhydride as a simple convenient chemical actinometer. *Journal of the Chemical Society-Perkin Transactions 2* **1981,** (2), 341-343.
3. Brouwer, A. M., Standards for photoluminescence quantum yield measurements in solution (IUPAC Technical Report). *Pure and Applied Chemistry* **2011,** *83* (12), 2213-2228.