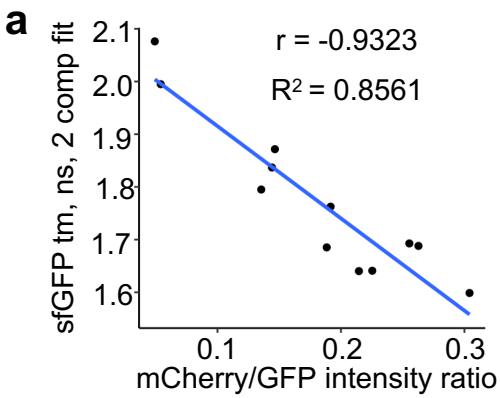
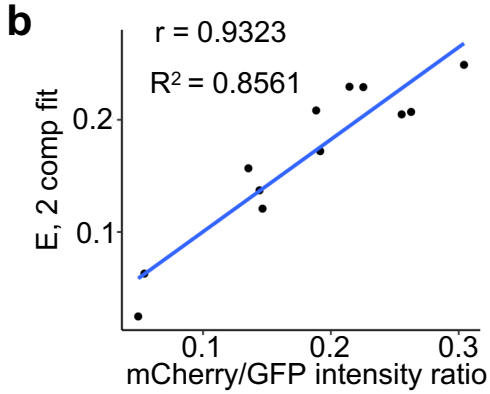
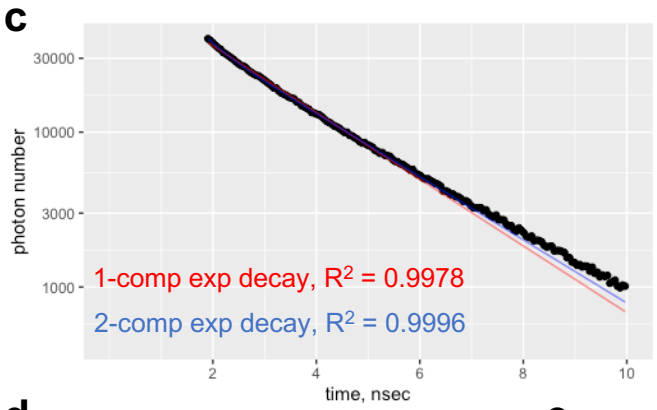
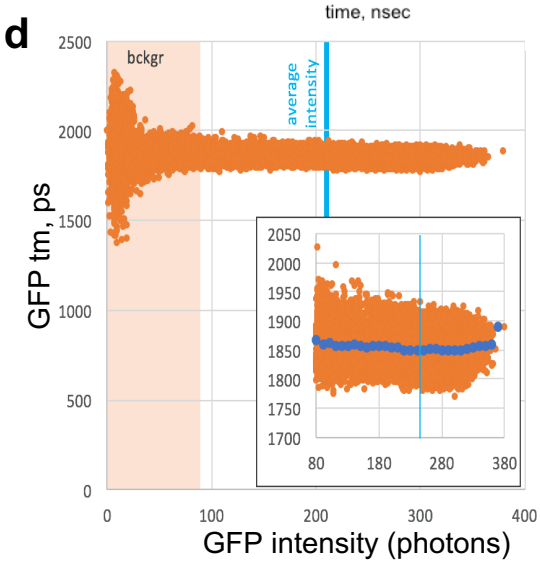


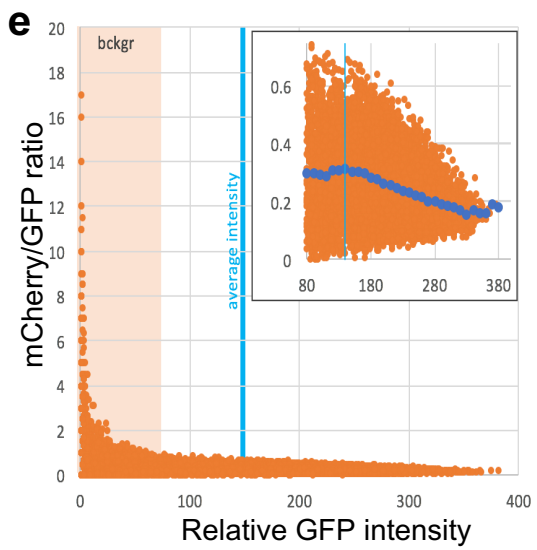




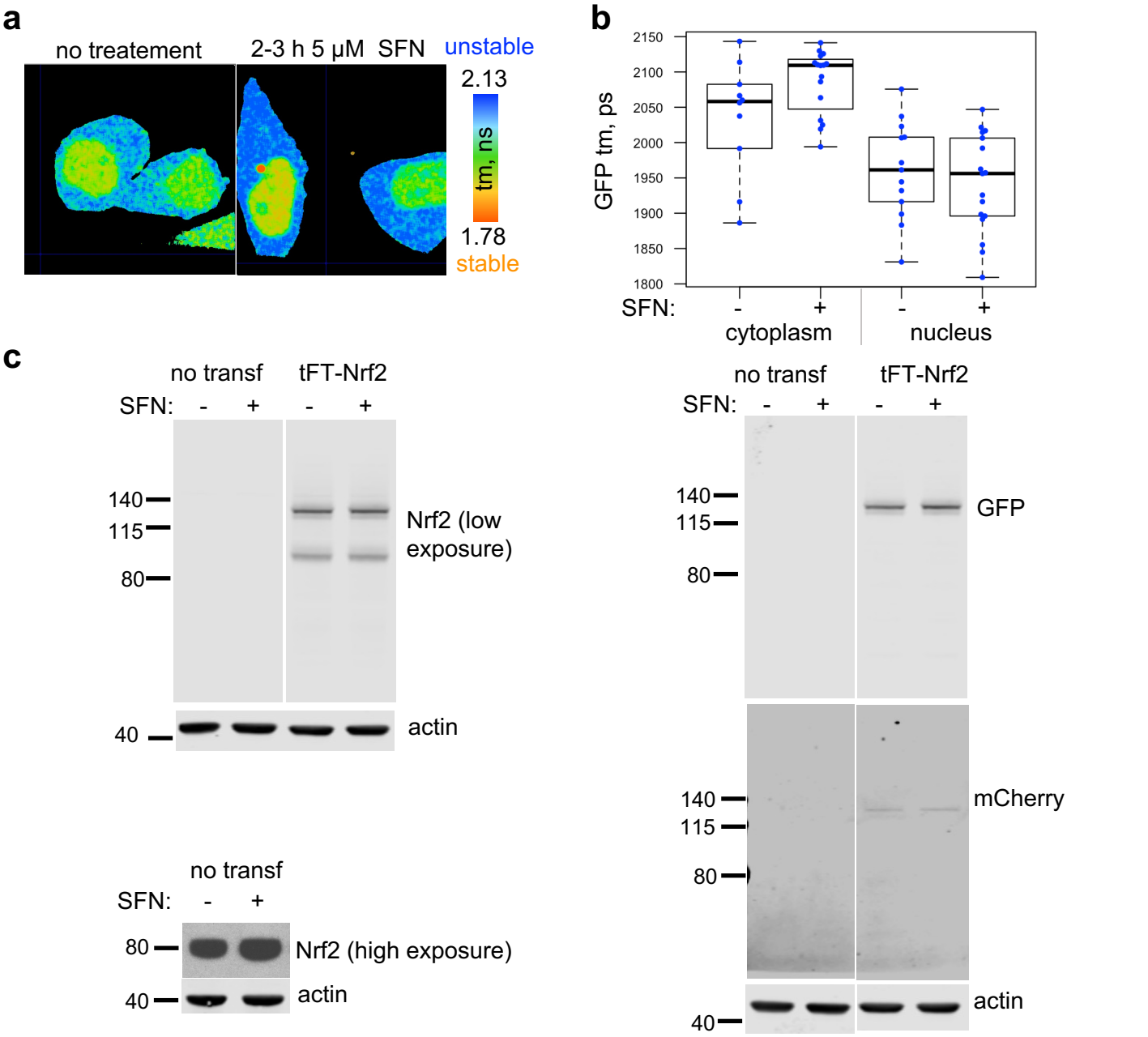
model	τ	$\tau1$	$\tau2$ (fixed)	$a1$	$a2$
1-comp	2.015				
2-comp		0.3883	2.1287	0.1148	0.8852

model	R^2	Pr(>F)
1-comp	0.9978	
2-comp	0.9996	<0.001

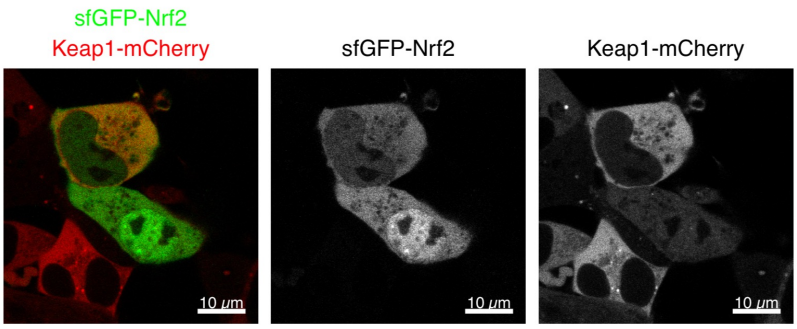




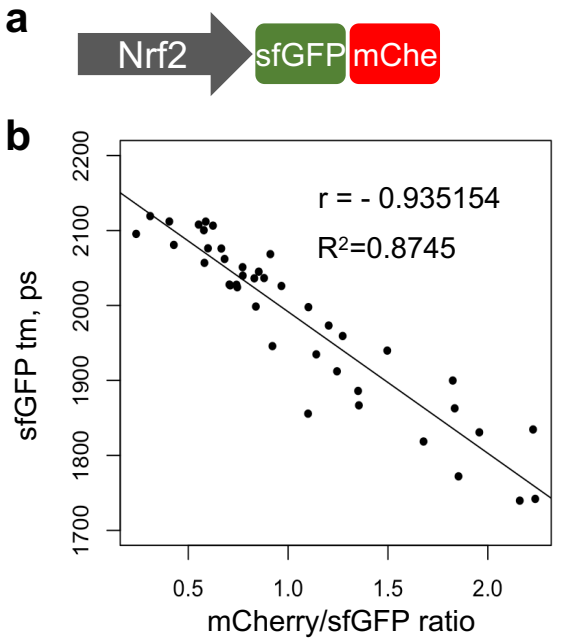
Supplementary Figure 1. Ratiometric and FLIM-based measurements of FLIM-timer. Related to **Figure 1. a-b**, Strong correlation with high goodness of fit between fluorescence lifetime determined by a 2-component exponential decay analysis (**a**) or FRET efficiency (E) (**b**) and the mCherry/GFP intensity ratios in HeLa cells transfected with sfGFP-mCherry-Nrf2 shown in **Figure 1c**. Larger component in 2-complonent decay model was fixed at 2.1287, the value of unquenched FRET donor measured in cells transfected with sfGFP-Nrf2. **c,** Analysis of the pooled photon trace data (black) from a representative measurement in **a**, performed by fitting either 1-component exponential decay model ($F(t) = ae^{-t/\tau}$, red) or 2-component exponential decay model ($F(t) = a(a1e^{-\frac{t}{\tau1}} + a2e^{-\frac{t}{\tau2}})$ with $a1 + a2 = 1$ and fixed $\tau2$ value, blue). The plot shows cumulative number of photons excited by repeated ultra-short pulses of laser illumination plotted against photon arrival time relative to the start of the corresponding pulse. The first 1.9 nsec of trace that include signal generated by the equipment (so-called Instrument Response Function) was removed from the analysis. The calculated goodness of fit (R^2) for each model is shown. Table shows fitted parameters and goodness of fit for each model as well as statistical significance (Pr(>F) value) of the difference between the two models calculated by ANOVA. **d-e,** Fluorescence lifetime (tm) values (**d**), or mCherry/GFP intensity ratios (**e**) plotted against photon number/fluorescence intensity in the same pixels of cells shown in **Figure 1d**. Background fluorescence intensities are shaded, and average cellular intensity shown as light-blue vertical lines. The inserts zoom into average values above background. Dark blue plots in insets are average values of tm (**d**) or mCherry/GFP ratios (**e**) calculated for the 10-unit bins across the range of intensities. Note the absence of correlation between average tm or its variability (thickness of the "cloud") and pixel intensity, and the increase in variability and average mCherry/GFP values at the lower range of GFP intensity.



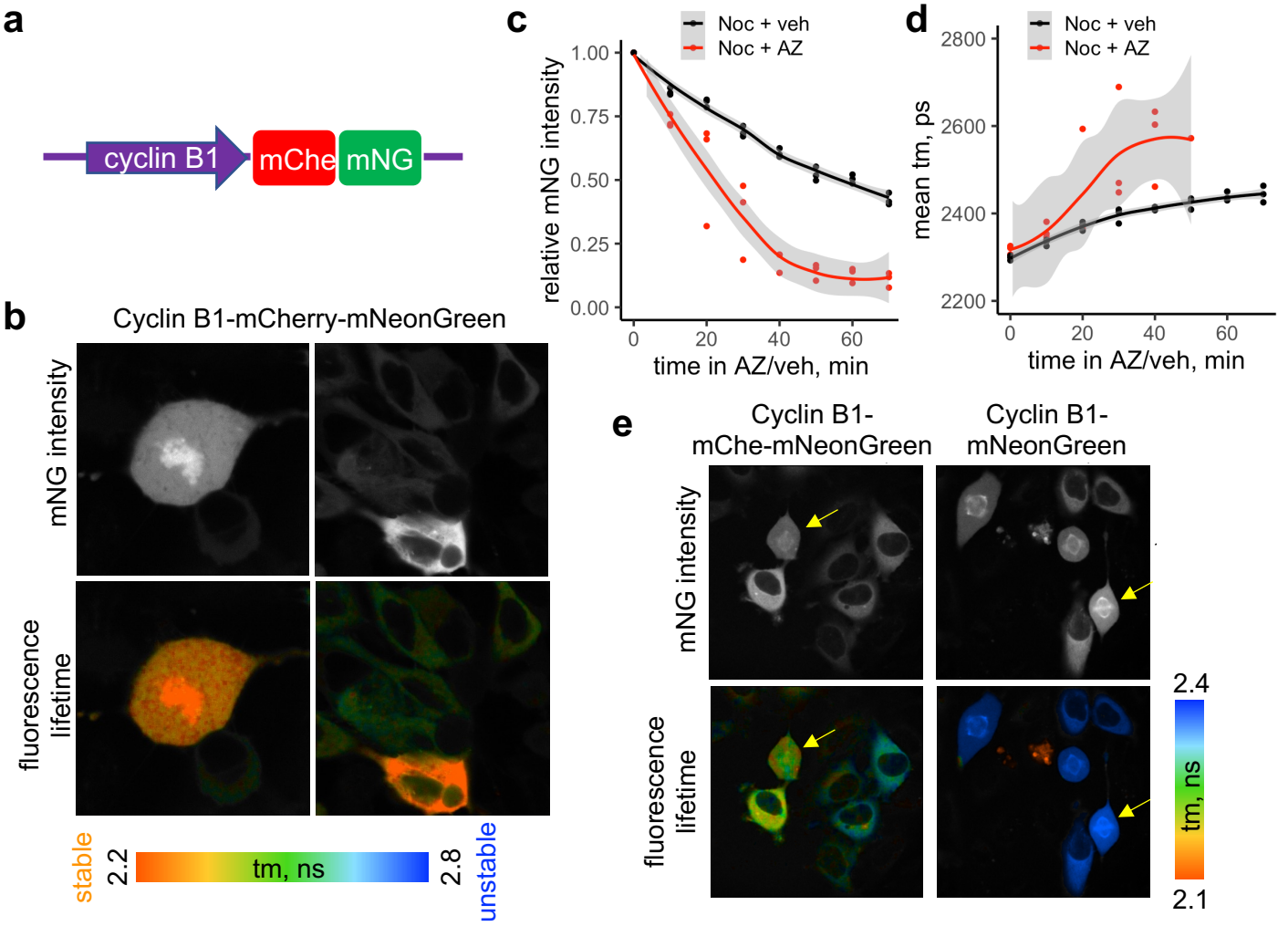
Supplementary Figure 2. Sulforaphane does not decrease the turnover of overexpressed FLIM-timer tagged Nrf2. Related to **Figure 2. a,** Examples of fluorescence lifetime heat maps in HeLa cells transfected with sfGFP-mCherry-Nrf2, before (left) and after (right) 2-3 h treatment with 5 μM sulforaphane (SFN). The colour coding for the fluorescence lifetime is shown on the right. **b**, Quantification of the fluorescence lifetime in the sfGFP-mCherry-Nrf2 expressing HeLa cells before and after SFN treatment exemplified in **a**. Each dot represents mean fluorescence lifetime within either nucleus or cytoplasm as indicated, and the box plots summarise data distribution as in **Fig. 1b. c,** Untransfected or sfGFP-mCherry-Nrf2 (tFT-Nrf2)-transfected HeLa cells were treated for 1 h with 5 μM Nrf2 inducer sulforaphane (SFN) or left untreated. 10 μg of lysates were immunoblotted with anti-Nrf2 (top left), anti-GFP (top right) or anti-mCherry (bottom right) antibodies and detected with fluorescent secondary ab using LiCor. 20 μg of lysates from un-transfected cells were re-blotted for Nrf2 followed by ECL development on film (bottom left). Actin is a loading control. Note that SFN stabilises endogenous but not endogenously overexpressed Nrf2.



Supplementary Figure 3. Variability in Keap1 and Nrf2 expression in transient co-transfections. Related to **Figure 3.** Variability among HEK293 cells co-transfected with sfGFP-Nrf2 (middle grey panel and green on overlay) and Keap1-mCherry (right grey panel and red on overlay) for 1 day, imaged using confocal microscopy.



Supplementary Figure 4. C-terminal FLIM-timer tagged Nrf2 as a readout of Nrf2 stability. Related to **Figure 4**. **a**, Schematics of Nrf2 labelled with sfGFP-based FLIM-timer at C-terminus. **b,** Negative correlation between ratiometric and FLIM-based measurements in HeLa cells transiently transfected with Nrf2-sfGFP-mCherry with or without Keap1 within either cytoplasm or nucleus, pooled. The ratio between mean mCherry and sfGFP fluorescence intensities with brightness above the threshold of 20 are plotted against globally binned fluorescence lifetime of sfGFP in the same region. Pearson Correlation Coefficient r and a goodness of fit R^2 are shown. FLIM acquisition and imaging as in **Fig.1d**.



Supplementary Figure 5. Use of mNeonGreen based FLIM-timer for endogenously tagged cyclin B1 is limited by its low photostability. Related to **Figure 5. a,** Endogenous cyclin B1 in HeLa cells was tagged using CRISPR-CAS9 with mCherry-mNeonGreen, separated by the 7 amino-acid linker described in **Fig.1a. b,** Example of fluorescence observed in HeLa cells with endogenous-level cyclin B1-mCherry-mNeonGreen. Left: mitotic cell shows bright mNeonGreen (mNG) fluorescence (top) with fluorescence lifetime (tm, bottom) close to the minimal for this FRET pair (see **Fig. 1b**), indicative of stabilised cyclin B1. Right: low fluorescence intensity (top) with longer fluorescence lifetime (bottom) in the majority of interphase cells. Fluorescence lifetimes are colour-coded for fluorescence lifetime value as shown underneath. **c-d,** HeLa cells expressing cyclin B1-mCherry-mNeonGreen were treated for 1 h with Nocodazole prior addition of 1.25 μM AZ 3146 or equivalent volume of vehicle (DMSO) for up to 70 min. Upon AZ 3146/vehicle addition, mitotic cells were followed by a time-lapse FLIM, with data acquired every 10 min and mNeonGreen fluorescence intensity normalised to that prior treatment (**c**) or the mean fluorescence lifetime (**d**) in each cell were plotted against time of treatment. Dots represent individual cell measurements, and the lines depict fitted local polynomial regression curve, with the 95% confidence interval shown as grey shading. **e,** Reduced fluorescence lifetime is not observed in mitotic cells overexpressing donor-only control, Cyclin B1-mNeonGreen. FLIM was acquired in unsynchronised culture of HeLa cells expressing cyclin B1 endogenously tagged with either mCherry-mNeonGreen (left) or mNeonGreen (right). Top panels show mNeonGreen intensity (photon numbers), and the bottom panels show fluorescence lifetime maps with color-coding shown on the right. Note that mitotic cells, identified by their morphology (yellow arrows) display low fluorescence lifetime only in the Cyclin B1-mCherry-mNeonGreen-expressing and not in Cyclin B1-mNeonGreen-expressing sample.