Supplementary Methods

M**ultiphoton TCSPC FLIM**. A scan head specifically designed for multi-photon excitation was used (Trim-scope, LaVision Biotec, Germany) to control all beam scanning and data acquisition. Detection of fluorescence was by non-descanned detectors (NDD) (Hamamatsu H6780-01-LV 1M for < 500 nm detection and H6780-20-LV 1M for > 500 nm detection) located at the back focal plane of the objective. A dichroic filter (Chroma 455 nm DCXR) was used to spectrally separate the second harmonic generated signal, when present, from the CFP emission of the Src biosensor. Band pass filters (Semrock 470/40 and Chroma 500 nm LP) were used to further filter the emission for the CFP channel. For the measurement of fluorescence excited state lifetime, a 16-anode PMT (FLIMx16, LaVision Biotec, Germany) was used in a time correlated single photon counting (TCSPC) scheme. Dectector array speeds data acquisition by enabling detection of multiple photons per excitation event. The increased photon count rate enables accurate lifetime to be measured in situations not previously accessible. The TCSPC detector was used with a gain setting of 250 (0-255 full range) and configured with 75 time bins, each of which were 0.04 ns wide, based on the 80 MHz repetition rate of the laser.

Frequency domain FLIM. A modulated 445 nm LED was used as light source to measure FLIM-FRET by frequency domain. Fluorescein (10 μM in 0.1 M Tris-Cl, pH >10) was used as reference standard with a known lifetime of 4.0 ns. Donor (D) lifetime, τ, was analyzed using the FLIM software (version 1.2.11; Lambert Instruments, Netherlands). Representative images of at least 3 independent experiments are shown (1265 cells in total were assessed for organotypic culture). Columns, mean; error bars represent ± SE, P value by unpaired Student's t-test.

Data Analysis. Raw data was analyzed using the built-in TCSPC fluorescence lifetime analysis functionality of ImSpectorPro (LaVison Biotec, Germany). A single exponential fit with offset was performed on fluorescence decay data from specific regions of each cell and the lifetime fit parameter recorded. Statistically significant differences were assessed by performing an unpaired Student's t-test on the data sets. Lifetime maps were also produced using ImspectorPro, where a single exponential fit was performed for each pixel across the time bins spanning the peak to the end of the decay. An intensity threshold was applied with the value set to the average background pixel value for the summed data set. A 3 x 3 smoothing operation was performed on the raw data while calculating the FLIM map. A standard rainbow color look up table with the limits 1 and 3.5 ns was used to display all FLIM maps for ease of comparison. Low basal Src activity is represented in the lifetime color maps as blue, while high Src activity is represented as warm red/yellow colors and areas of low signal to noise ratio in which an accurate lifetime measurement cannot be achieved are black. A 5 x 5 median filter was applied to the Tau map.

Western blotting. Src activity upon drug treatment was determined using auto-phosphorylation of Src on tyrosine 416 as described previously [25]. Src-416 and total Src (Cell Signalling Technology, USA) actin (Sigma Aldrich).