

Materials and Methods

Peptide Biosensors

Peptide sensors were designed based on FAK kinase domain sequences¹ containing the autophosphorylation site Tyr397, with the sequence SETDDYAEK(5FAM)IIDEEDRKKRRQRRRPQ. A delivery sequence was then added to the main kinase-recognition sequence. All peptides were prepared by Alpha Diagnostic with >90 % purity. Characterization of labeled materials are provided in the Supporting Information.

In-vitro 2D cell culture

Human endothelial colony forming cells (ECFC) were obtained as a gift from Dr. Mervin Yoder laboratory (Indiana University School of Medicine, Indianapolis, IN). Briefly, ECFC were grown in EGM-2 (Lonza, Walkersville, MD) supplemented with 10% FBS. ECFC were grown in collagen-coated culture flasks and passaged at 80% confluence; passages less than six were used. Human Mesenchymal Stem Cell (HMSC) cultured in DMEM low glucose (Invitrogen) supplemented with 5% FBS, were passaged to 70% confluence. HMSC were grown on two different substrates; on cover slips no 1.5 (neuroVitro) and on a Poly lactide-co-glycolic acid (PLGA) scaffold matrix prior to experiment with FAKSOR

Poly lactide-co-glycolic acid (PLGA) preparation

(PLGA) was dissolved in a solvent of tetrahydrofuran: N,N-Dimethylformamide (THF:DMF) to obtain a final solution of 30% w/v PLGA solution. The solution was loaded in 10 mL syringe fitted with an 18G blunt needle for electrospinning. Aligned polymer fibers were electrospun using a flow rate of 2 mL/h, electric potential of 2kV/cm and a rotation speed of 1000 rpm. Fiber mats were collected and used for further studies.

Aligned PLGA fiber mats were cross linked using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemistry. Fiber mats were treated with 1N NaOH for 1 hour to expose the surface functional groups and rinsed with water and treated with EDC at pH 7. After 2 hours, cross-linker (Cadaverin) at two different concentrations (3 mM and 6 mM) was added to cross-link the polymer structures.

Sensor delivery

For sensor delivery in 2D cultures, cells were incubated with a peptide sensor Faksor in the corresponding appropriate culture medium, washed three times with 1× PBS, and mounted in the imaging chamber.

2D imaging

Since imaging was performed in live cells, we employed a temperature controlled chamber (37°C) (INU) adapted onto the stage of either TCSPC-FLIM (Company?) or A1R-MP Confocal System (Nikon). Prior to imaging 2D cultures, cells were cultured on glass coverslips (#1.5) or on the scaffold on top of the coverslip and were transferred to live-cell chamber disk (courtesy of Dr. Paul Robinson) with fresh medium. Prior to imaging vascular networks in 3D multi tissue constructs, tissues were transferred to glass bottom dishes (#1.5; MatTek Corporation) and filled with fresh medium.

Confocal Microscopy

Nikon A1R-MP was used to capture conventional confocal images of 2D cell cultures (<https://www.nikoninstruments.com/Products/Multiphoton/A1RMP-Multiphoton>). For fluorophore excitation, a 488 nm laser was used for FAKsor. Signal from FAKsor was collected via photomultiplier tubes (PMTs). PMTs correspond to a unique detection channels with emission filter bands of 515/30 nm. Image scanning was conducted in the galvano mode using 512 × 512 pixels @ 4 fps. For all images, the pinhole was set at 1 Airy Unit (a.u.). For 2D Images, a 60× oil objective was used.

TCSPC-FLIM

To perform FLIM of the peptide biosensors we utilized a Time Correlated Single Photon Counting Fluorescence Lifetime Imaging (TCSPC-FLIM) system (MicroTime 200, Picoquant, Germany). Details of the instrumentation is reported in our previous works^{2,3}. The schematic of instrumentation is represented in Supplementary Figure 3. Briefly, pulsed picosecond diode laser (460 nm 40 MHz) was used to achieve photon excitation. A dichroic mirror (1P-dichroic, Chroma) was used to reflect a laser beam through a long

working distance objective (50x, 1.45 NA, Olympus) and into the sample. Images were acquired via raster scanning with a XY-Piezo nanopositioning stage (Physik Instrumente, Germany). Fluorescence was collected by the same objective and the emitted photons were transmitted by a dichroic mirror (1-P-dichroic, Chroma), through a 50- μm pinhole, and directed into two single photon avalanche diode detectors (SPAD, SPCM-AQR, PerkinElmer Inc.). To collect emitted photons from the FAM-5 fluorescent reporter, a 520 \pm 60 nm filter was placed in front of SPAD 1. The captured photons were then processed by TimeHarp 260 PC-board (Picoquant, Germany) using TCSPC and the data was stored in the Time Tagged Time Resolved (TTTR) mode.

Data analysis step is represented in the Supplementary Figure 4. SymPho Time (A Symphony of Photons in Time) software (Picoquant, Germany) was used to perform data analysis. Pixel-by-pixel average fluorescence lifetime was obtained by fitting experimental fluorescence decay curve with a multi-exponential model.

A non-linear least squares analysis employing iterative Levenberg–Marquardt routine was used for a pixel-by-pixel fitting of the experimentally observed fluorescence decay. Levenberg-Marquadt was chosen for its computational efficiency (Supplementary Figure 4). The equation used for this model is:

$$I(t) = \sum_i^n a_i e^{-\frac{t}{\tau_i}}$$

where $I(t)$ is the fluorescence intensity at time, t , after excitation pulse, and n is the total number of decay components in the exponential sum. The variables τ_i and a_i are the fluorescence lifetime and fractional contribution of the i -th emitting species, respectively.

In this work, a bi-exponential model was chosen to fit the experimental decay to obtain the average fluorescence lifetime per pixel. Assessment of fitting was accomplished by minimizing the Chi-square value. To ensure unbiased fitting, we excluded Chi-square values greater than 1.3. The average fluorescence lifetime was determined in each cell, with a minimum of 100 cells in each of the groups.

Statistic

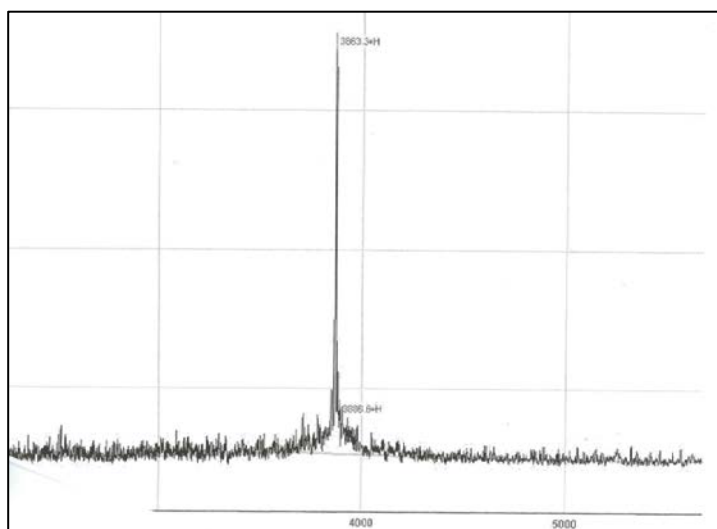
For fluorescence lifetime analysis at least 100 cells from 3 different biological replicates were used for analysis. To compare means among different groups, an ANOVA

was completed ($\alpha=0.05$) with a Fisher least significant difference (LSD) post-hoc test to determine groups that were statistically different from each other.

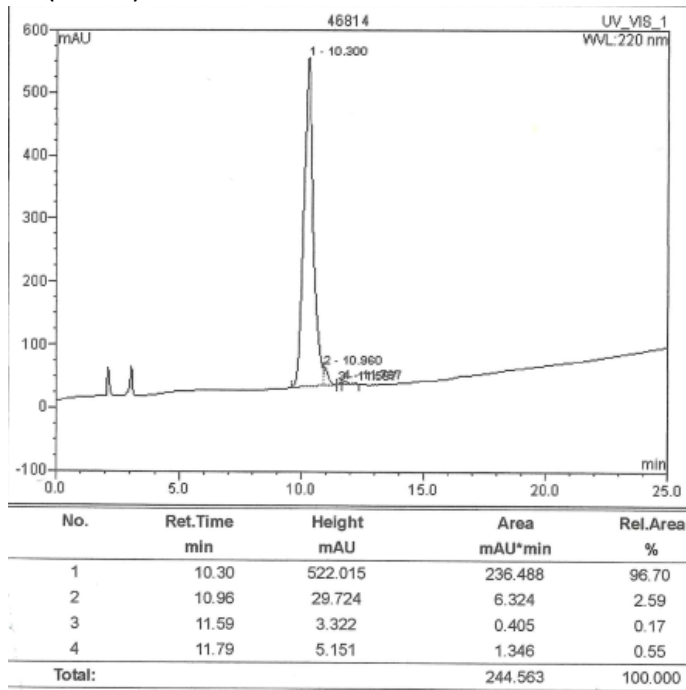
ESI References

1. D. L. Wheeler, T. Barrett, D. A. Benson, S. H. Bryant, K. Canese, V. Chetvernin, D. M. Church, M. DiCuccio, R. Edgar, S. Federhen, L. Y. Geer, Y. Kapustin, O. Khovayko, D. Landsman, D. J. Lipman, T. L. Madden, D. R. Maglott, J. Ostell, V. Miller, K. D. Pruitt, G. D. Schuler, E. Sequeira, S. T. Sherry, K. Sirotkin, A. Souvorov, G. Starchenko, R. L. Tatusov, T. A. Tatusova, L. Wagner and E. Yaschenko, *Nucleic Acids Research*, 2007, **35**, D5-D12.
2. J. Chen and J. Irudayaraj, *Analytical Chemistry*, 2010, **82**, 6415-6421.
3. N. Damayanti, A. Craig and J. Irudayaraj, *Analyst*, 2013, **138**, 7127-7134.

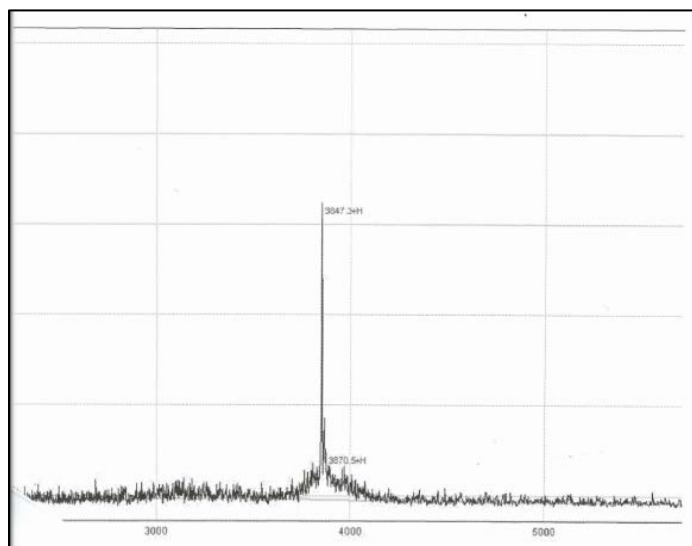
Electronic Supplementary Information



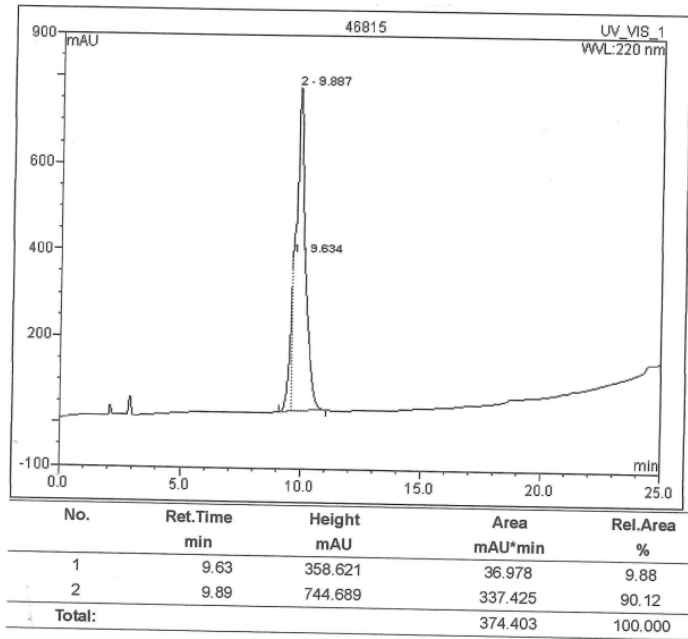
Supplementary Figure 1a | MALDI –TOF characterization of purified FAKSOR with sequence: SETDDYAEK(5FAM)IIDEEDRKKRRQRRRPQ



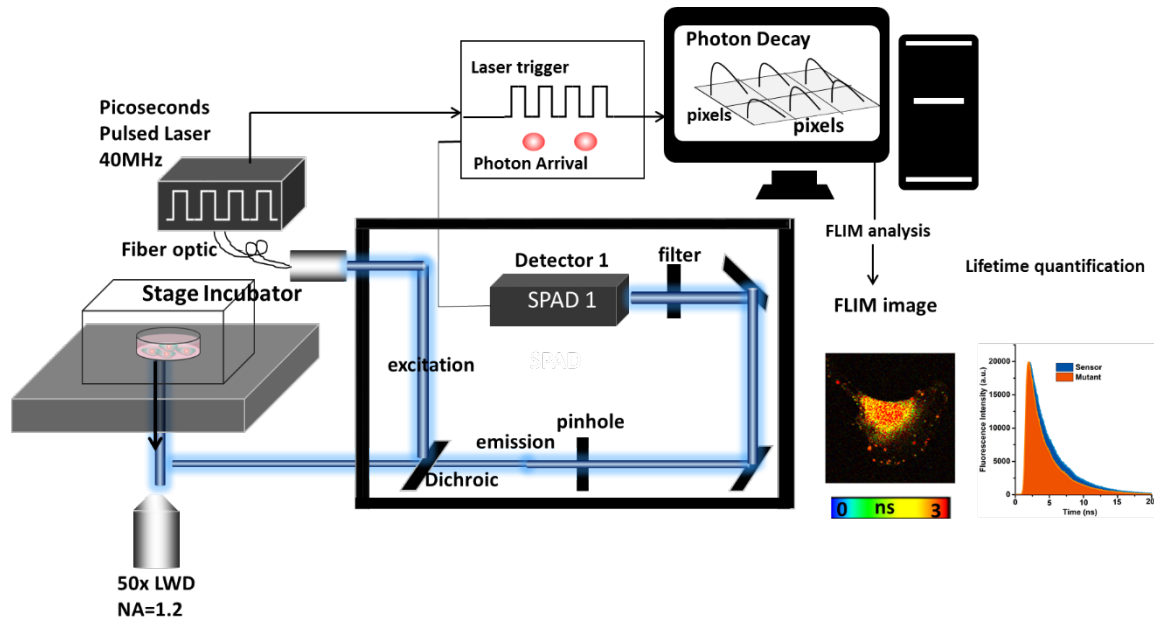
Supplementary Figure 1b | HPLC characterization of purified FAKSOR



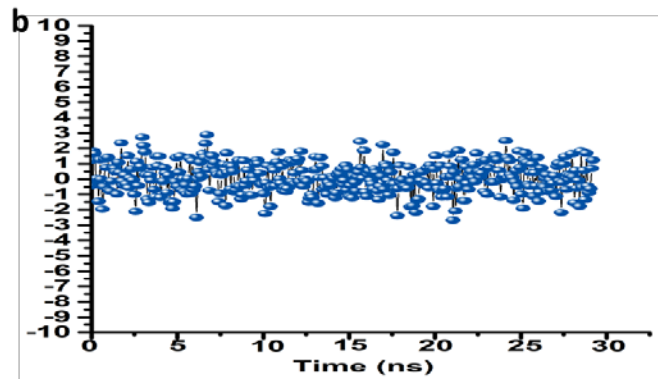
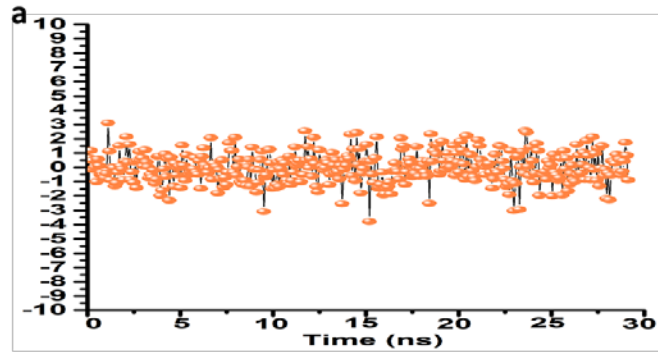
Supplementary Figure 2a | MALDI –TOF characterization of purified FAKSOR mutant Y-F SETDDFAEK(5FAM)IIDEEDRKKRRQRRRPQ



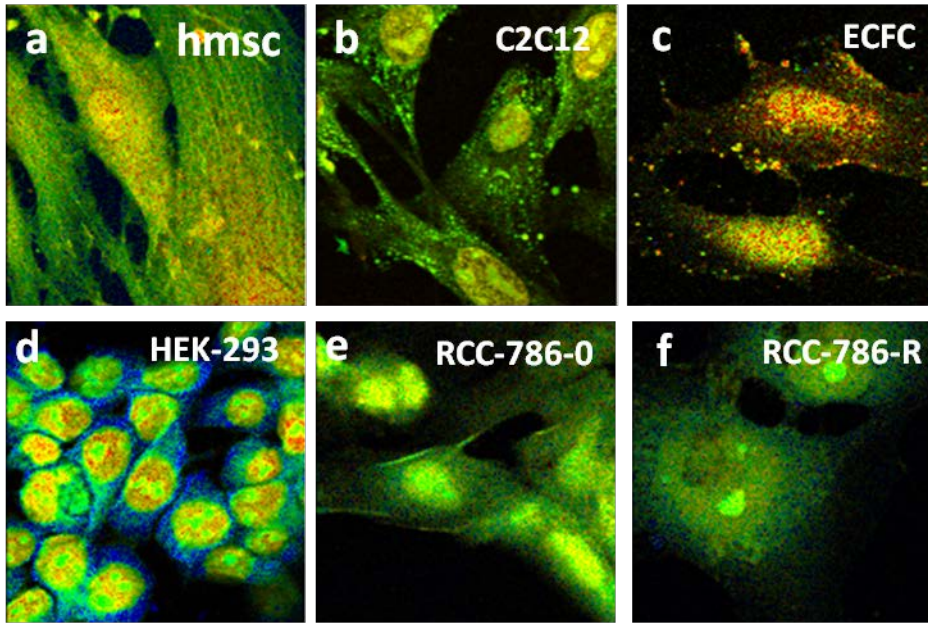
Supplementary Figure 2b | HPLC characterization of purified FAKSOR mutant Y-F



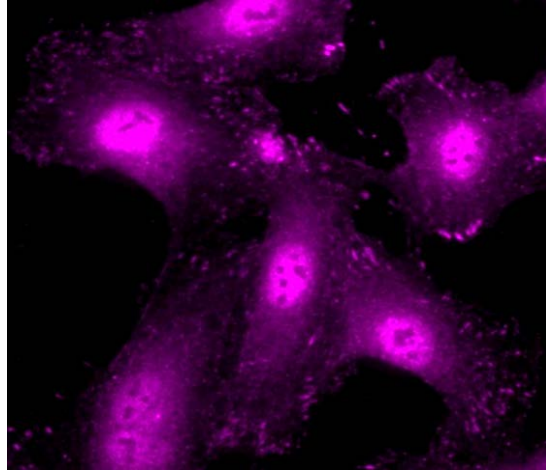
Supplementary Figure 3 | Schematic of Time Correlated Single Photon Counting Fluorescence Lifetime Imaging (TCSPC-FLIM) system (Picoquant, Germany). Photons were excited by pulsed picosecond diode laser 460 nm 0 MHz). The laser was coupled to the main MicroTime 200 Unit. Images were acquired by raster scanning with an XY-Piezo nanopositioning stage (Physik Instrumente, Germany), which allows precise X-, Y-, and Z-movement with 80 μm range and an X-, Y-, Z-resolution less than 10 nm. The laser beam was reflected towards the objective using a dichroic mirror (1P-dichroic, Chroma) and then focused into the sample through a long working distance objective (50x, 1.2 NA, Olympus). The emitted photons were collected by the same objective and transmitted by a dichroic mirror (1-P-dichroic, Chroma), through a 50 μm pinhole and directed into two single photon avalanche diode detectors (SPAD,SPCM-AQR, PerkinElmer Inc.). A 520 ± 60 nm emission filter was placed in front of SPAD 1 to collect signal from FAM-5 The emitted photons were then processed by TimeHarp 260 PC-board (Picoquant, Germany) using a time correlated single photon counting method. The data was then stored in Time Tagged Time Resolved (TTTR) mode and was finally fitted using SymphoTime software.



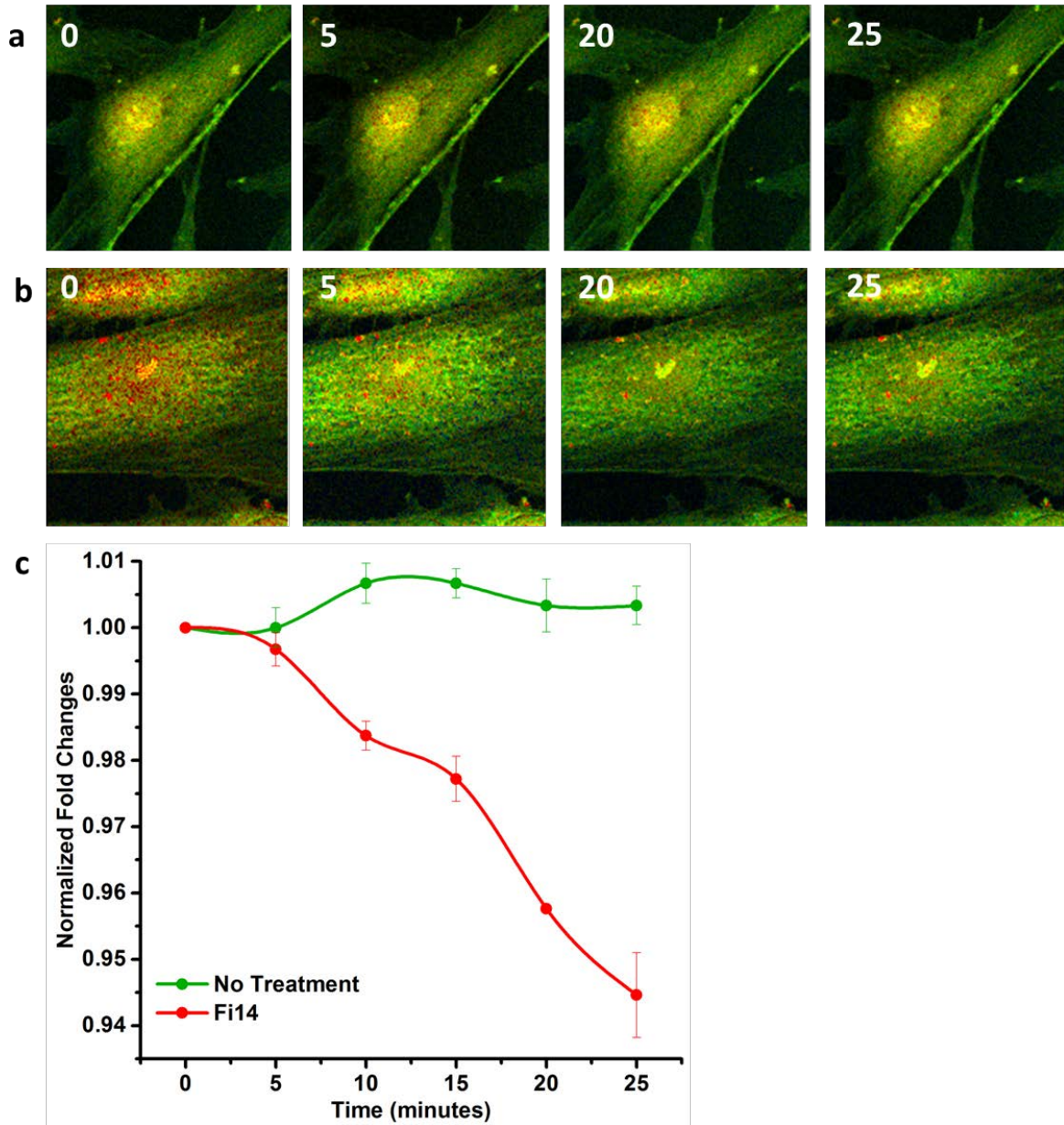
Supplementary Figure 5 | No pattern was observed in the residuals plot of decay fitting for (a) FAKSOR and (b) FAKSOR mutant Y-F



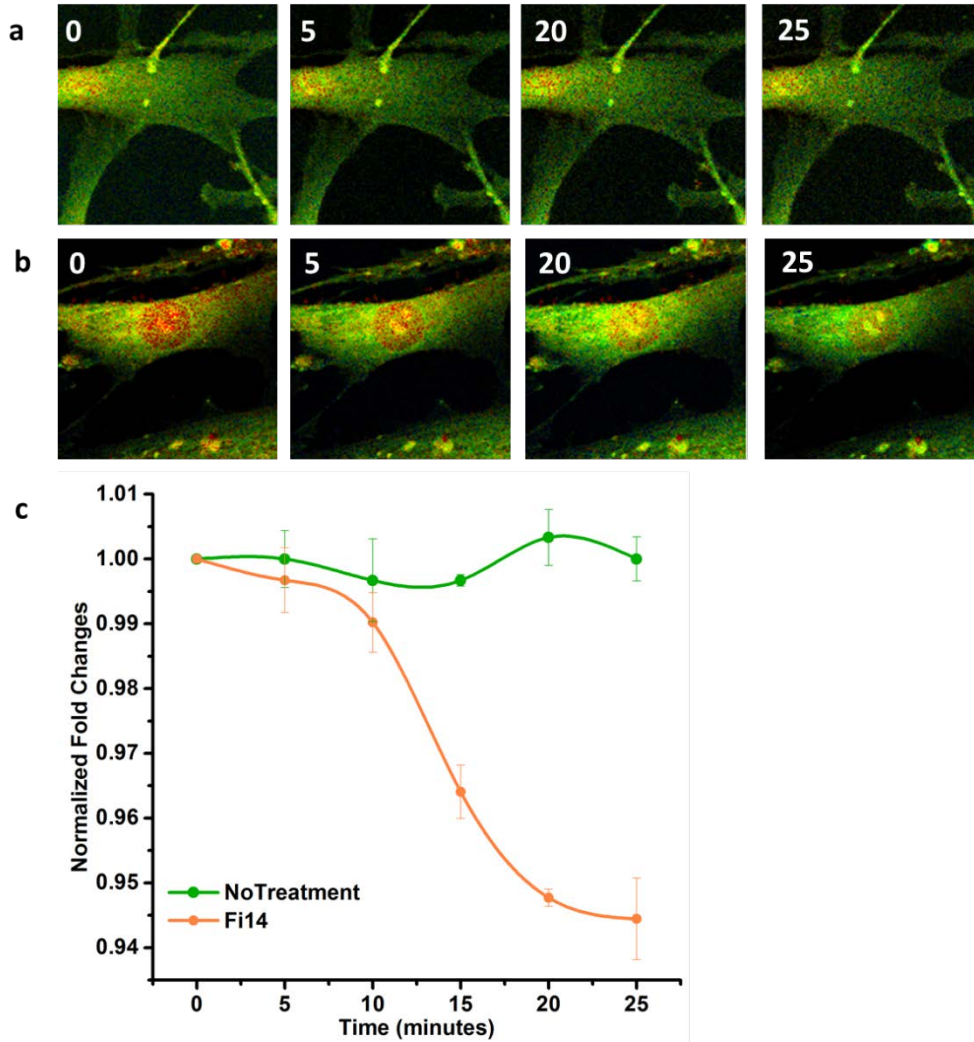
Supplementary Figure 6 FAKSOR is rapidly internalized (20 min) by different cell types. (a-f) Fluorescence lifetime images after 20 minutes of incubation with FAKSOR(20 μ M) at 37°C in different cell types cultured on 2D glass coverslips at 37°C.



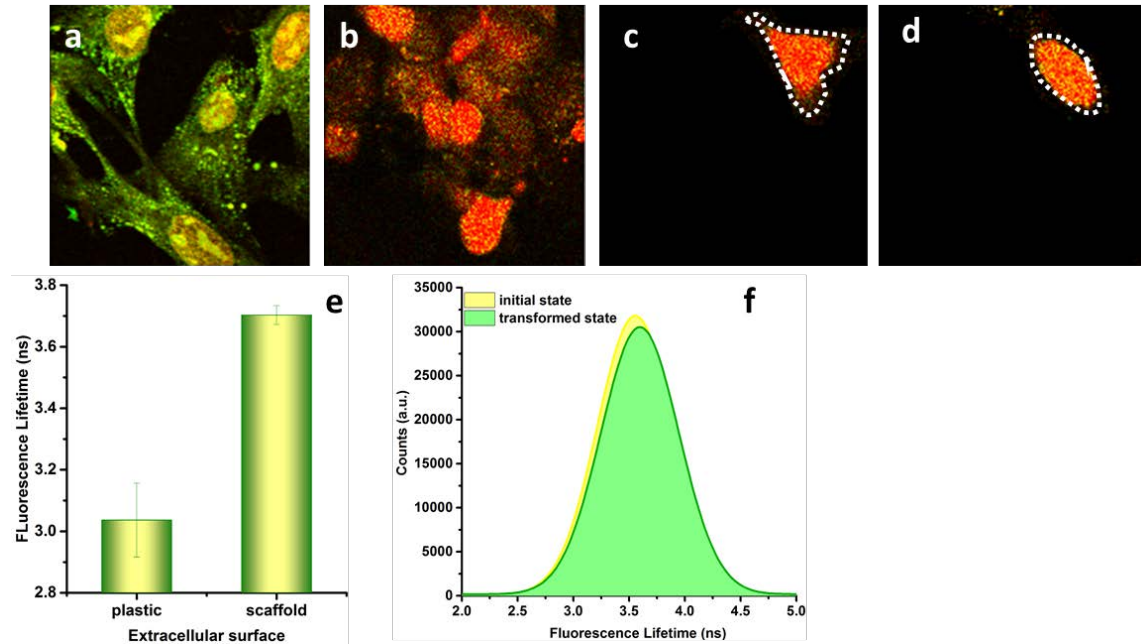
Supplementary Figure 7| Subcellular localization of FAK in ECFC visualized by Immunofluorescence. FAK is localized in the nucleus and cytoplasm, consistent with the subcellular localization of FAKSOR. The observation was consistent throughout all samples (n=100).



Supplementary Figure 8| Real time monitoring (25 minutes) of FAKSOR in C2C12 upon treatment with FAK inhibitor, F-14. Fluorescence lifetime time-course images of FAKSOR treated with FI-14 (b) show an overall trend of decreasing fluorescence lifetime for FAKSOR compared to non-treated control group (a). Over a 25-minute observation period, quantitative analysis showed that FI-14 treatment results in a decrease in fluorescence lifetime of FAKSOR compared to control. Data representative of three independent biological replicate and three technical replicates.



Supplementary Figure 9 | Real time monitoring (25 minutes) of FAKSOR in HMSC upon treatment with FAK inhibitor, F-14. Fluorescence lifetime time-course images of FAKSOR treated with FI-14 (b) show an overall trend of decreasing fluorescence lifetime for FAKSOR compared to the non-treated control group (a). Over a 25-minute observation period, quantitative analysis showed that FI-14 treatment results in a decrease in the fluorescence lifetime of FAKSOR compared to control. Data representative of three independent biological replicate and three technical replicates.



Supplementary Figure 10 | FAKSOR fluorescence lifetime depends on FAK phosphorylation, which depends on extracellular matrix stiffness. Fluorescence lifetime images of FAKSOR in C2C12 cells cultured on different substrates; (a) plastic (b) PLGA scaffold. (c), (d) The dynamics of FAK phosphorylation activity depends on the cell morphology and adherence of cells to the PLGA Scaffold (Supplementary Video 1).