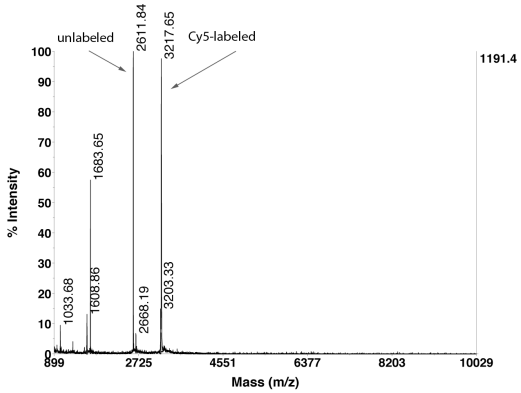
Table of Contents:

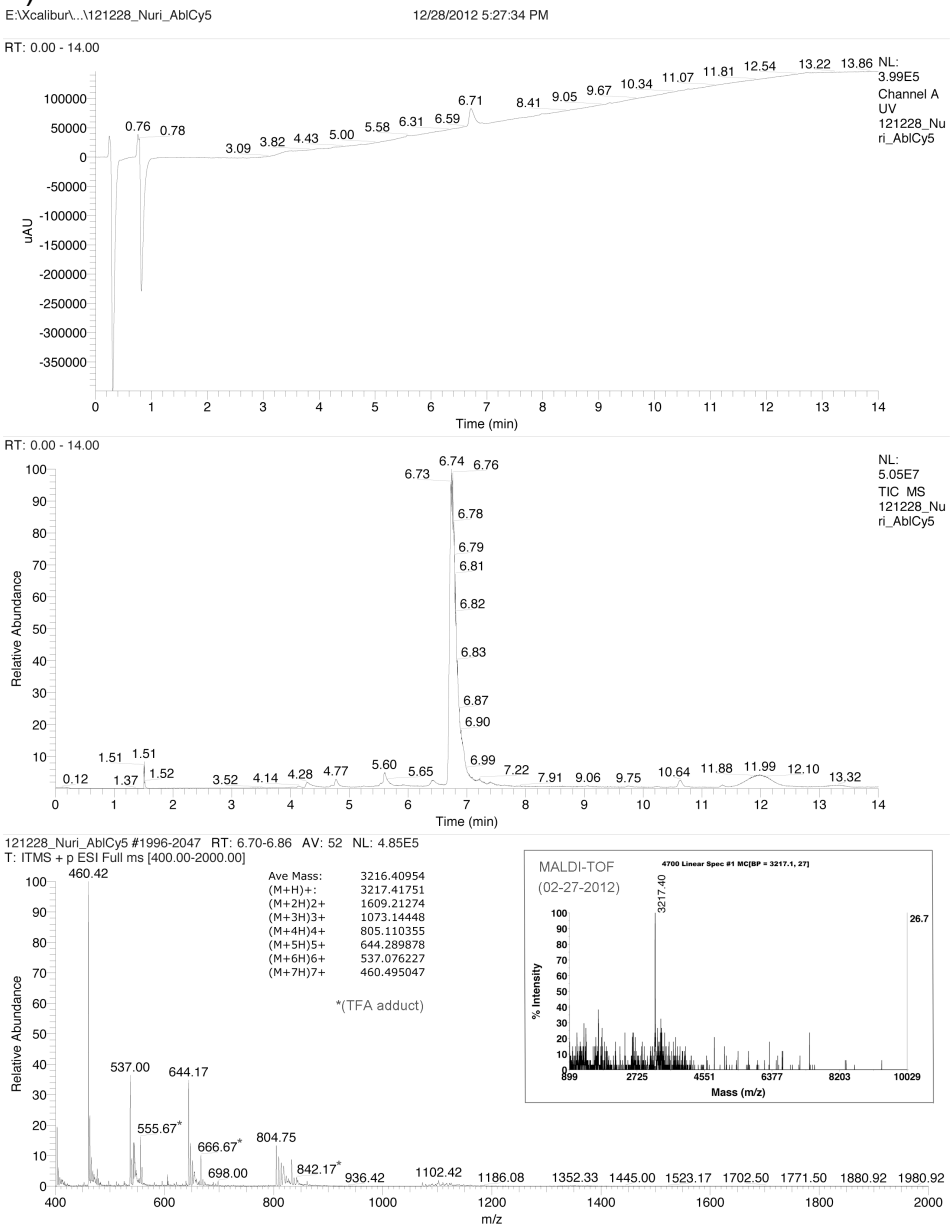
Peptide labeling with Cy5-maleimide. Peptide (7-10 mg) was dissolved to a final concentration of 1 mM in reaction buffer (2 ml, 6M guanidinium-HCl/100mM phosphate) containing triscarboxyethylphosphine (TCEP, 1 mM) and adjusted to pH 6.5 with HCl/NaOH as necessary. Cy5-maleimide (1-2 mg, Lumiprobe, Inc., USA) was dissolved in DMSO (20 μl) and added, and the solution vortexed to mix. The reaction was allowed to proceed in the dark at room temperature for 10 h. Reaction progress was checked at 2 h by analyzing an aliquot (1 μl) diluted into water/acetonitrile/trifluoroacetic acid (50/50/0.1%) by MALDI-TOF-MS (linear positive mode) using α-cyano-4-hydroxycinnamic acid as matrix. Labeled peptide was purified by preparative HPLC using a C18 column (Agilent 1200 system with Agilent Zorbax column) and fractions containing predominantly labeled material (as detected by MALDI-TOF MS) were pooled and lyophilized to give 1-2 mg final peptide. Identity and purity were initially confirmed by MALDI-TOF MS. After long-term storage (10 months) in phosphate buffered saline at -20 ^{o}C, samples were analyzed by LC/MS (ThermoFinnegan Accela-LTQ system) on a C18 column (2.1 I.D. x 50 mm L, 1.7μm particle size, Phenomenex) with a gradient of water/acetonitrile/formic acid (initially 95/5/0.1% rising to 35/65/0.1% at a rate of 6%/min).

Cy5-labeled Abl-TAT (GGEAIYAAPC$_{Cy5}$GGRKKRRQRRRPQ) A) MALDI-TOF analysis of labeling reaction, crude (2 hours) B) LC/MS of purified labeled material after 10 months storage at -20 ^{o}C in phosphate-buffered saline. Inset: MALDI-TOF analysis of purified material obtained at time of initial preparation.

A)

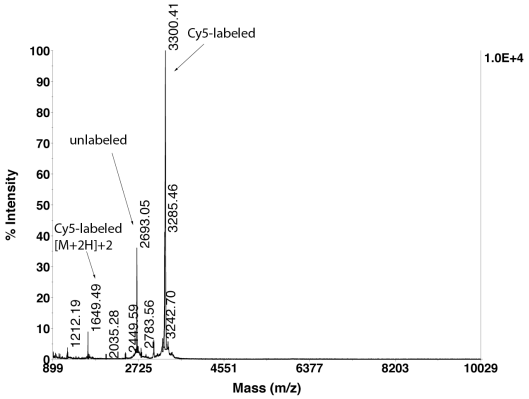


B)

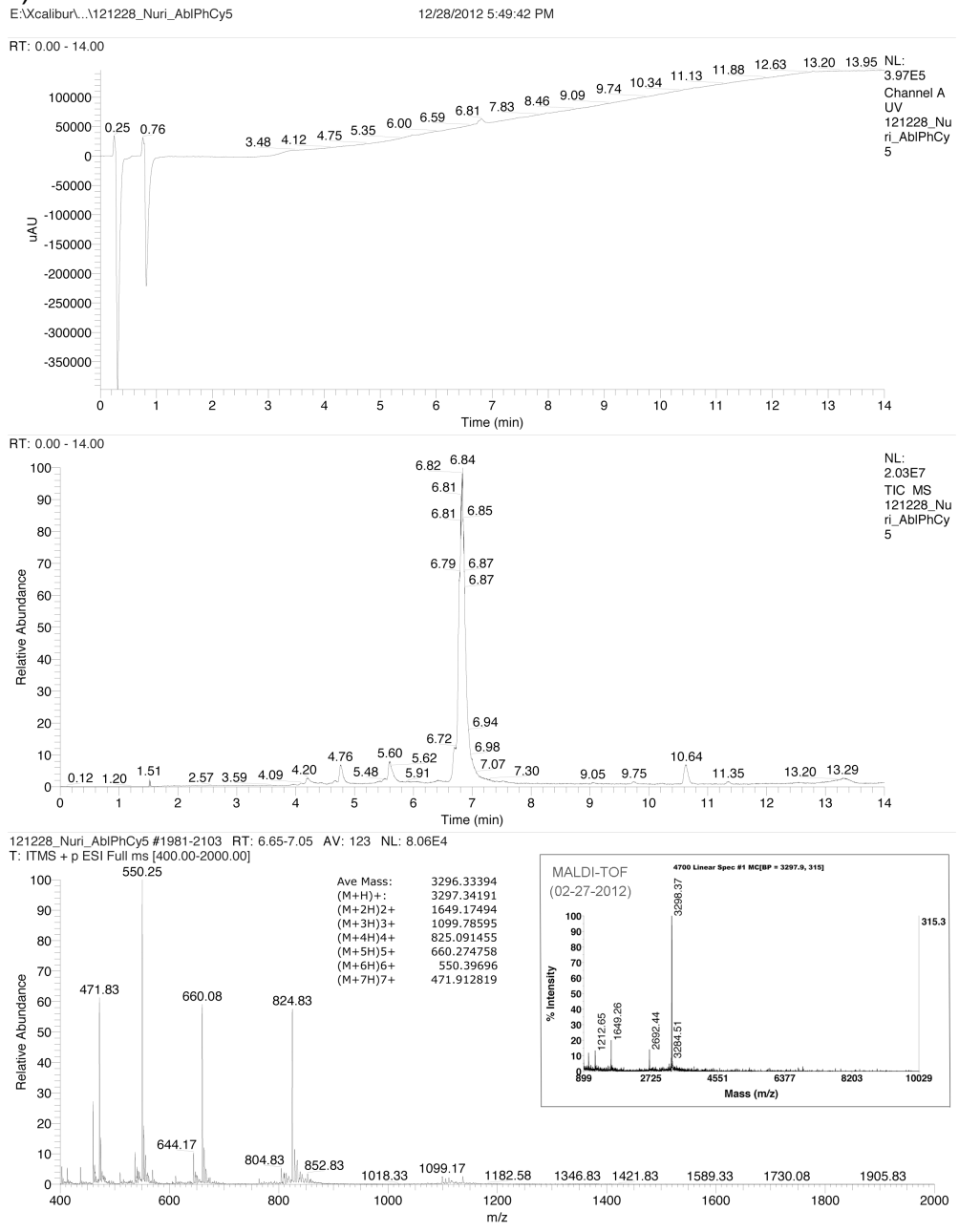


Cy5-labeled Abl-phospho (GGEAIY$_{phospho}$AAPC$_{Cy5}$GGRKKRRQRRRPQ) A) labeling reaction, crude (2 hours) B) LC/MS of purified labeled material after 10 months storage at -20 ^{o}C in phosphate-buffered saline. Inset: MALDI-TOF analysis of purified material obtained at time of initial preparation.

A)

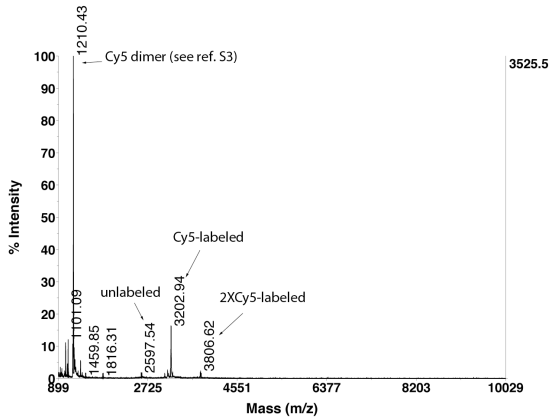


B)

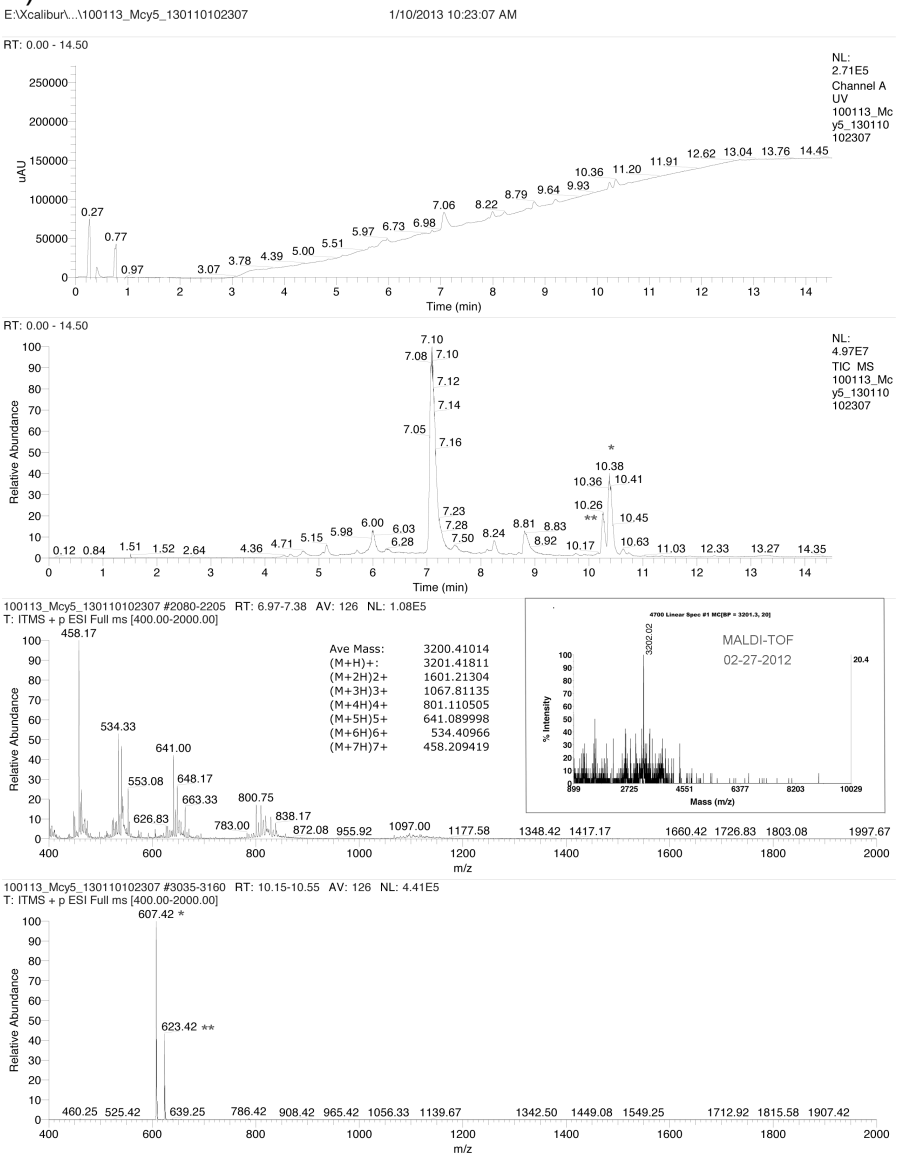


Cy5-labeled Abl-F-mutant (GGEAIFAAPC$_{Cy5}$GGRKKRRQRRRPQ). A) labeling reaction, crude (2 hours). Cy5-maleimide dimer is observed, a byproduct that has been reported in Cy5 labeling reactions in which the molar equivalents of dye exceed the equivalents of peptide[1, 2] (as was the case in this particular labeling reaction). According to the literature, this species in non-fluorescent. B) LC/MS of purified labeled material after 10 months storage at -20 ^{o}C in phosphate-buffered saline. Inset: MALDI-TOF analysis of purified material obtained at time of initial preparation.

A)



B)



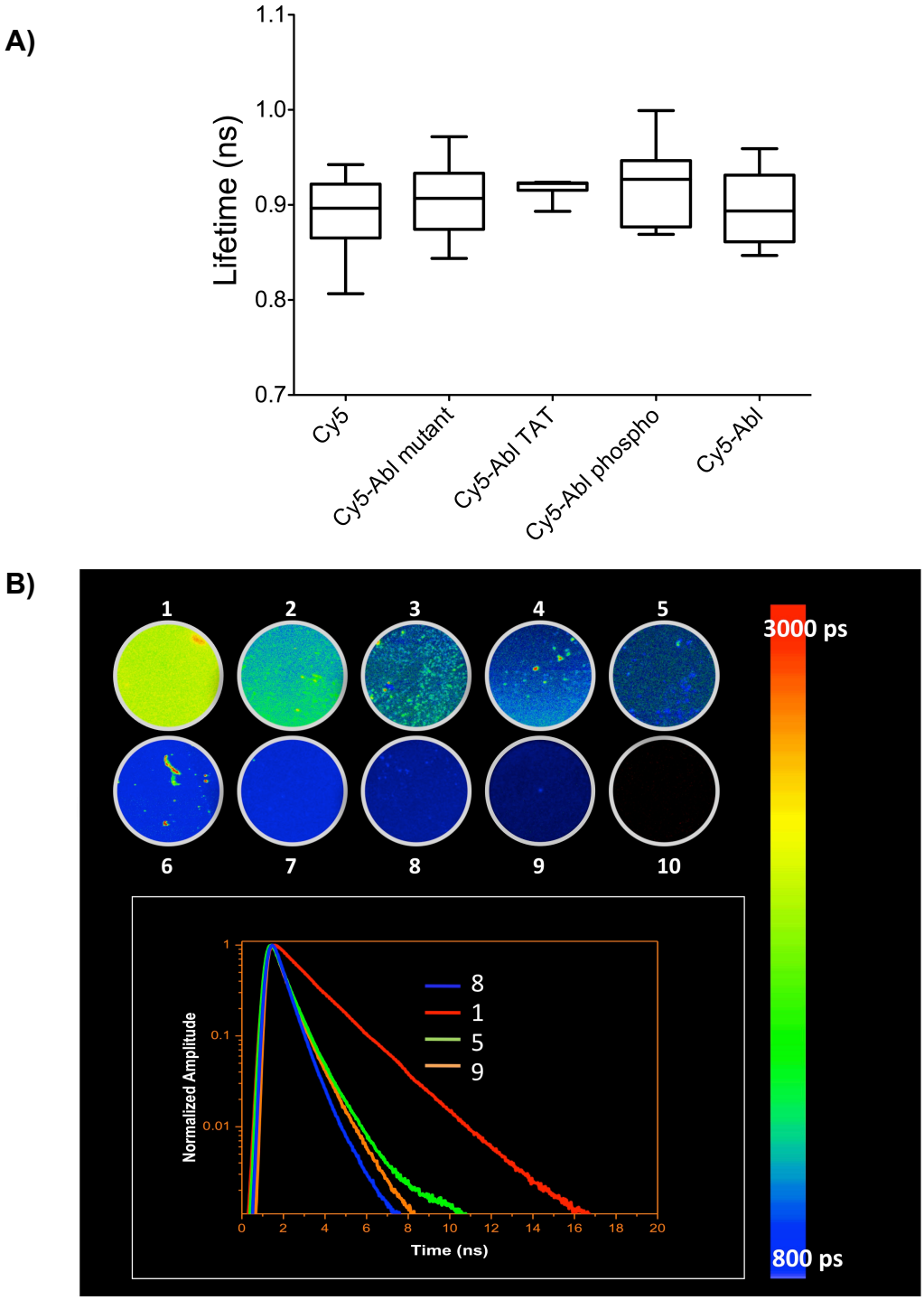
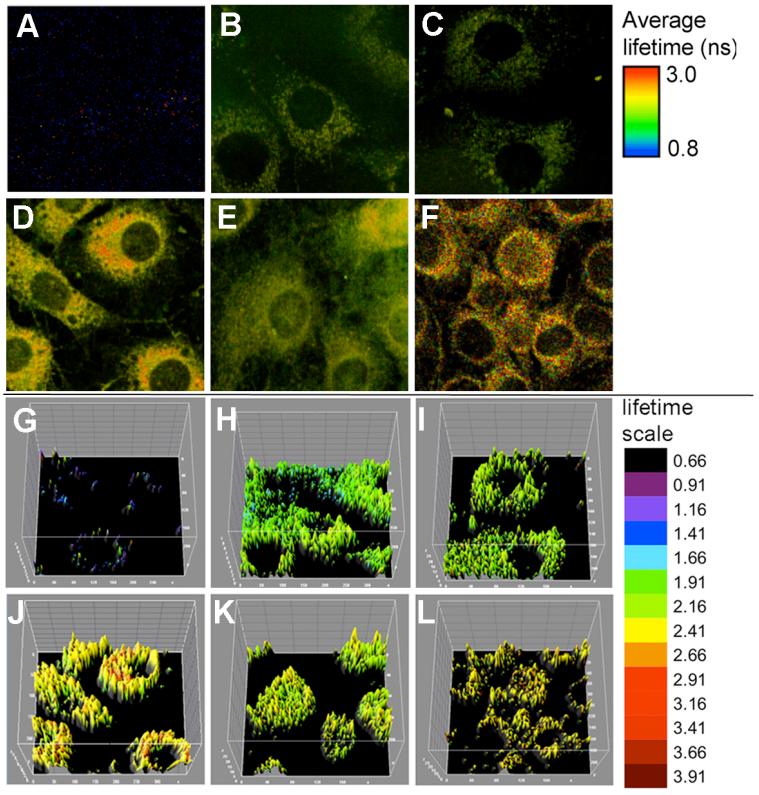


Figure S1. Fluorophore lifetime measurements in solution. A) Bulk solutions of each peptide were prepared in PBS (50 nM), dropped onto a cover slip. Measurements were taken by collecting images and lifetimes were averaged for selected ROIs (randomly chosen from the field of view). These values were plotted and the data are shown as box and whisker plots, in which the mean is shown as a horizontal line and error bars represent the minimum and maximum for each. In addition to the four species described for in-cell experiments, a version of the Abl substrate that lacked the TAT group was also included as a control for an affect of the TAT group on Cy5 lifetime. Overall the differences in mean lifetimes were statistically significant (analyzed using one-way ANOVA with Tukey's post-test for multiple comparisons). A significant difference ($P < 0.05$) was noted between the free Cy5 and each of the peptide conjugated species, as well as between the TAT-tagged biosensor and the Abl substrate without TAT, indicating that some intramolecular factors can

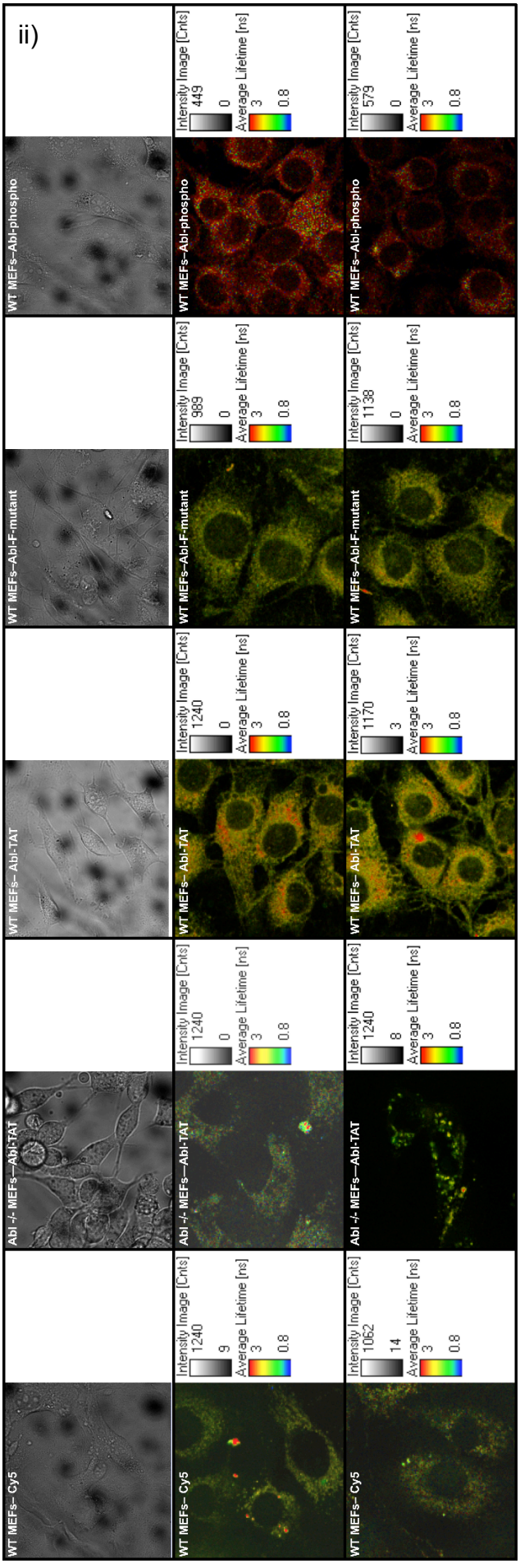
contribute to differences in Cy5 lifetime once it is conjugated to a peptide. However, no significant difference in lifetime was observed between the Abl-TAT and Abl-phospho (unphosphorylated and phosphorylated forms of the biosensor). B) The Cy5-labeled Abl-phospho peptide was incubated with recombinant c-Abl kinase at a ratio of 1:1 (1 μM final concentration for each) and dropped onto a cover slip for FLIM analysis (as described for S1A above). 1.) The mixture of the labeled phosphopeptide and c-Abl (1:1) exhibited significantly longer lifetime than observed for 9. the labeled phosphopeptide or 10. c-Abl alone. 2-6.) c-Abl was pre-incubated with increasing amounts of unlabeled phosphopeptide followed by addition of labeled phosphopeptide in the following ratios: (c-Abl:unlabeled:labeled) 2.) 1:0.25:1, 3.) 1:0.5:1, 4.) 1:0.75:1, 5.) 1:1:1, 6.) 1:2:1. Shorter lifetimes were observed for the solution as the relative amount of unlabeled phosphopeptide increased, indicating that the unlabeled peptide blocked an interaction between the labeled phosphopeptide and the c-Abl protein. 7.) the Cy5 unphosphorylatable Y→F mutant Abl-F-mutant was also incubated with c-Abl protein at 1:1 and the lifetime of this mixture was not significantly longer than for 8. the Abl-F-mutant peptide alone.

Figure S2. A) 2D lifetime maps corresponding to Fig. and B) representative bright field and raw FLIM images from replicate experiments. i) Raw FLIM images shown in A-F (raw image counts given in parentheses): (A, G) autofluorescence (3), (B, H) Cy5 only (619), (C, I) Abl-TAT in Abl (-/-) MEFs (836), (D, J) Abl-TAT in WT MEFs (1037), (E, K) Abl-F-mutant (1240), (F, L) Abl-phospho (300). Data extracted from raw FLIM images shown in G-L as lifetime/intensity maps: 2.5D plot of intensity (z-axis) color-coded by lifetime (with longer lifetimes shaded towards red), showing that even for regions with sufficient fluorescence intensity, lifetimes were different, and were consistent with the controls for phosphorylation dependence. Full color scale for the lifetime range is shown at right. ii) Bright field images (left column) showed that cell morphology was normal in the presence of biosensor peptides and Cy5. Additional fields of view are shown (right two columns) from FLIM analyses for replicate experiments of the conditions described in Fig. 3 (main manuscript).

i)



ii)



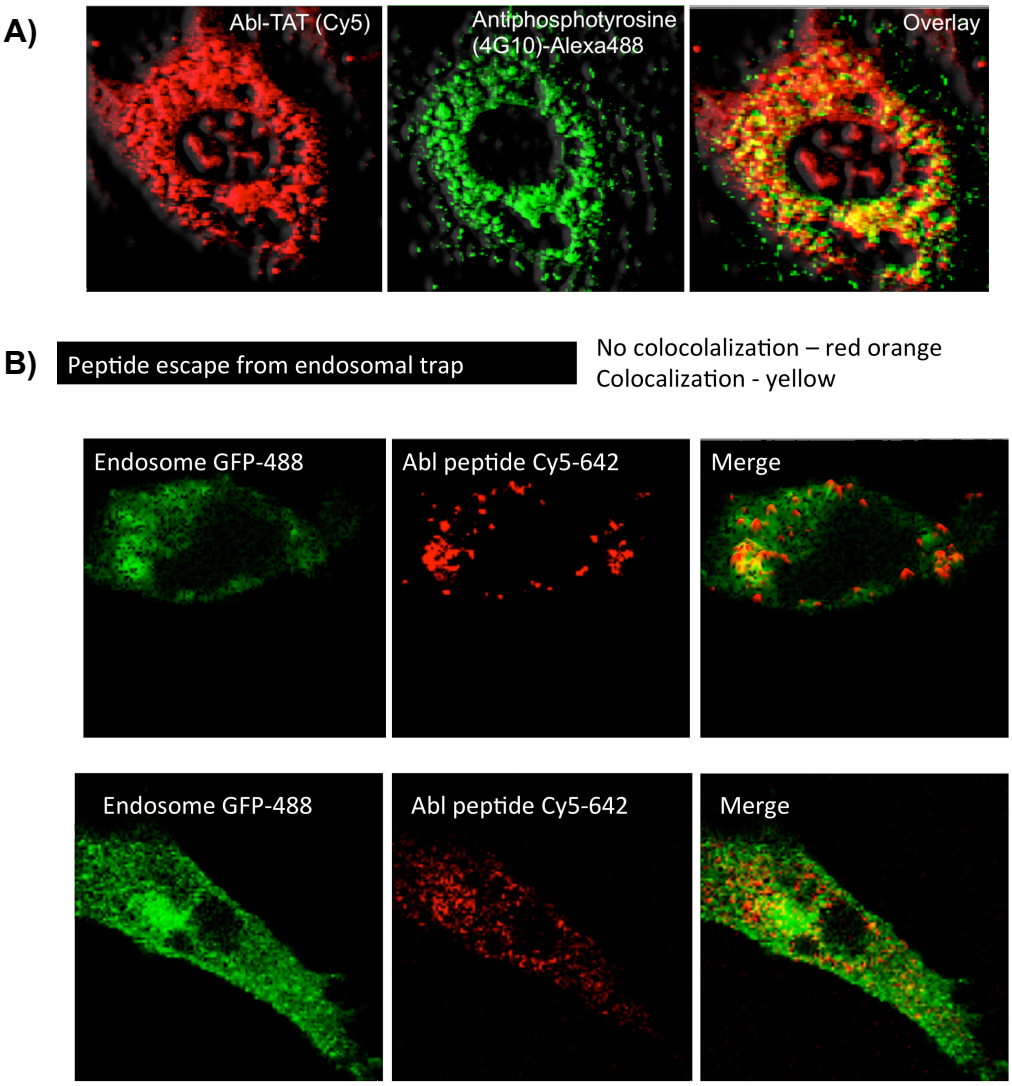


Figure S3. Immunocytochemistry controls staining for phosphotyrosine in the presence of the biosensor and colocalization between the biosensor and endosomes. Cells were plated on circular cover slips for imaging and after 75% confluency, incubated with peptide for 24 hours, washed with PBS and fixed in ice cold acetone for 5 min. After fixation, cells were washed twice (5 minutes each) with ice cold PBS and then incubated in 2% BSA in TBST for 90 minutes to prevent non-specific binding. Cells were then incubated at 4^{o}C for 20 hrs at 1:1000 dilution of antiphosphotyrosine-Alexafluor 488 (Biolegend) labeled antibody in PBST solution with 2% BSA. Cells were mounted in the imaging chamber for imaging. Colocalization analyses were performed using image J software.

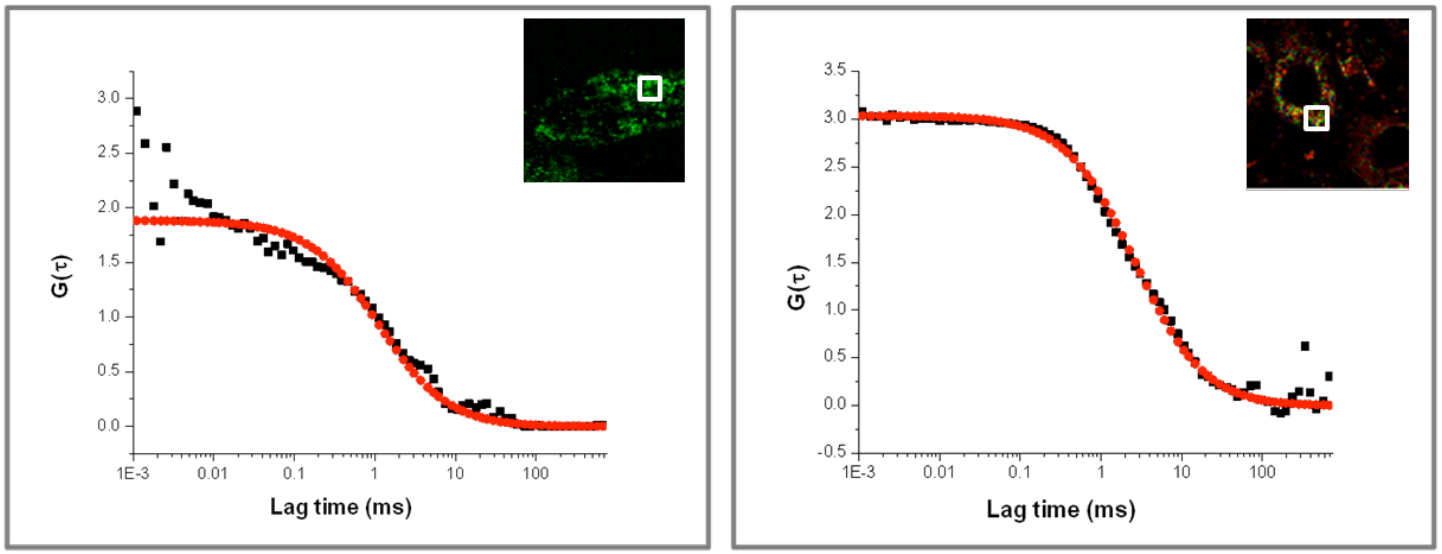


Figure S4. Fluorescence correlation spectroscopy experiments. FCS measurements were performed using cells incubated with either Cy5 alone (left) or Abl-TAT biosensor (right) using the Microtime 200 confocal system (Picoquant). 3T3 cells were incubated with Cy5 (5 nM) or Cy5-Abl TAT (5 nM) peptide for 24 hours and their diffusion characteristics were measured in the cytoplasm area (see inset). The normalized autocorrelation for both Cy5 and Cy5-Abl TAT was fitted with 3D diffusion model: $G(\tau) = \frac{1}{(N)}\left(1+\frac{\tau}{\tau D}\right)^{-1}\left(1+\frac{\tau}{\tau D \cdot k2}\right)^{-1/2}$. The diffusion time for Cy5 and Cy5-Abl TAT in the cytoplasm was measured as 0.99 and 2.66 ms respectively.

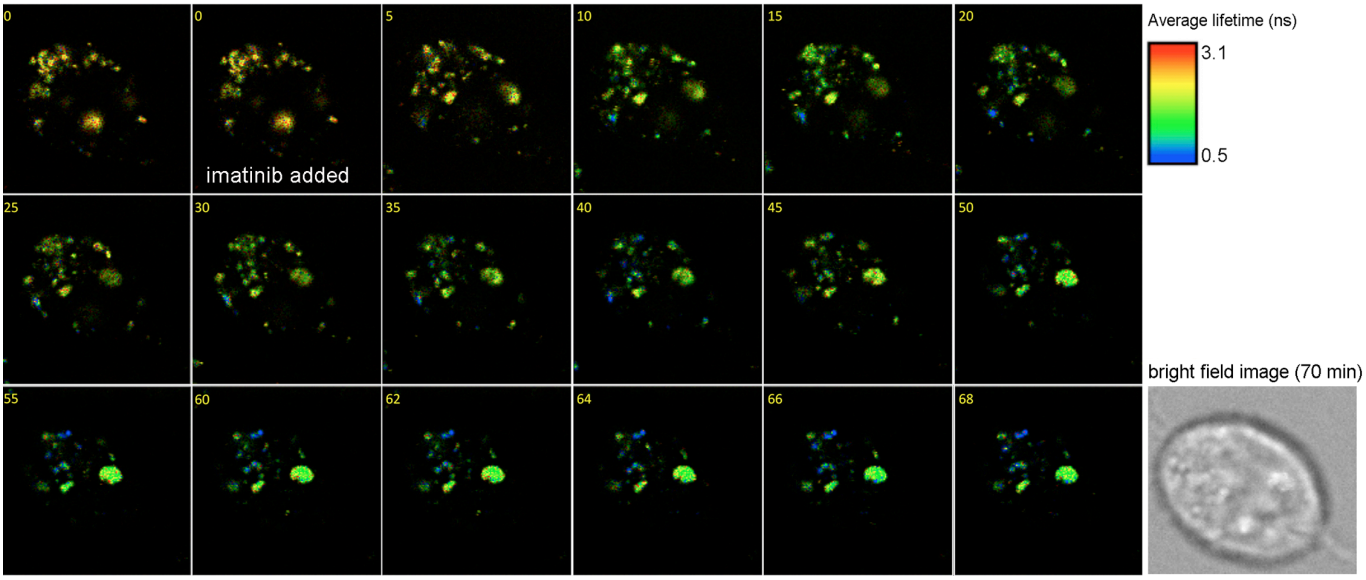


Figure S5. Real-time inhibition of biosensor phosphorylation in a live cell. The biosensor was incubated with cultured MEF cells (stably transfected with nuclear localization-enriched Abl kinase[3]) as before. Focus was established on a single cell and the cell was imaged using FLIM pre- (t=0) and post-addition of imatinib (1 μM) every 2-5 min. Timepoints are given in the upper left corner. The lifetime color code scale was standardized between images and the intensity threshold set at 200 counts; from the raw images, intensity ranged between 300-450 counts total and was maintained throughout the time course (indicating that photobleaching was not a factor). Bright field image of the cell at the end of the experiment (70 min) shows maintenance of normal morphology. See supplemental movie S5 for time lapse. Also see supplemental movie file available online at:
https://plus.google.com/photos/110476973448933646127/albums/5808140733446419969?authkey=CJet_56Hh7PfHA

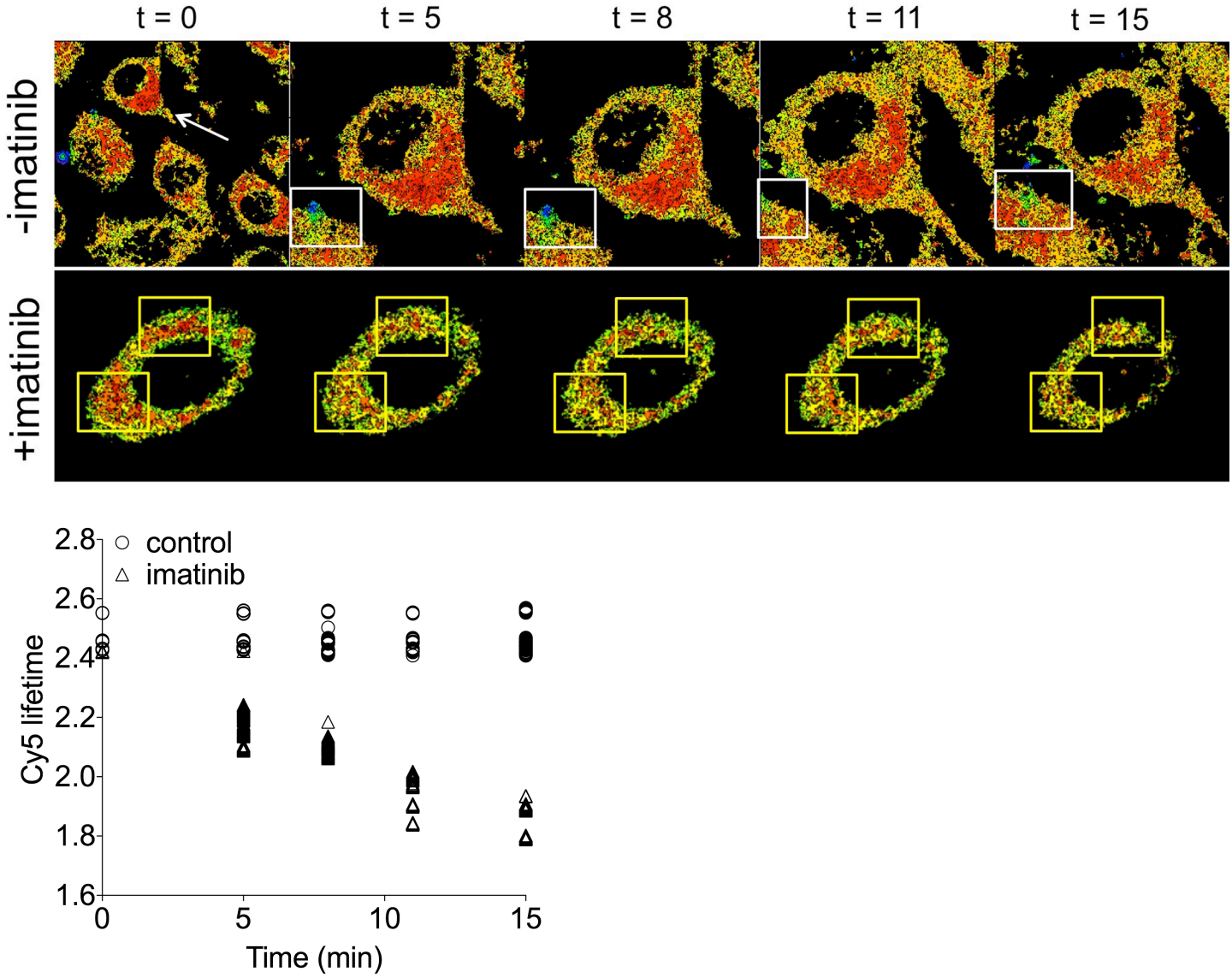


Figure S6. Quantification of lifetime changes after imatinib treatment. Additional time points are shown for the experiment described in Fig. 4 in the manuscript. In the control not treated with imatinib, lifetimes were dynamic over the course of the experiments. White boxes denote regions showing fluctuations towards longer lifetimes, while the central region of the main cell in the image shows some fluctuation towards shorter lifetime. In the cell treated with imatinib, changes tended towards shorter lifetimes (highlighted by yellow boxes). Below is a graph of ROIs from multiple cells, showing trends towards shorter lifetimes in the presence of imatinib. Open triangles denote ROIs from cells treated with imatinib, open circles (control) denote ROIs from untreated cells.

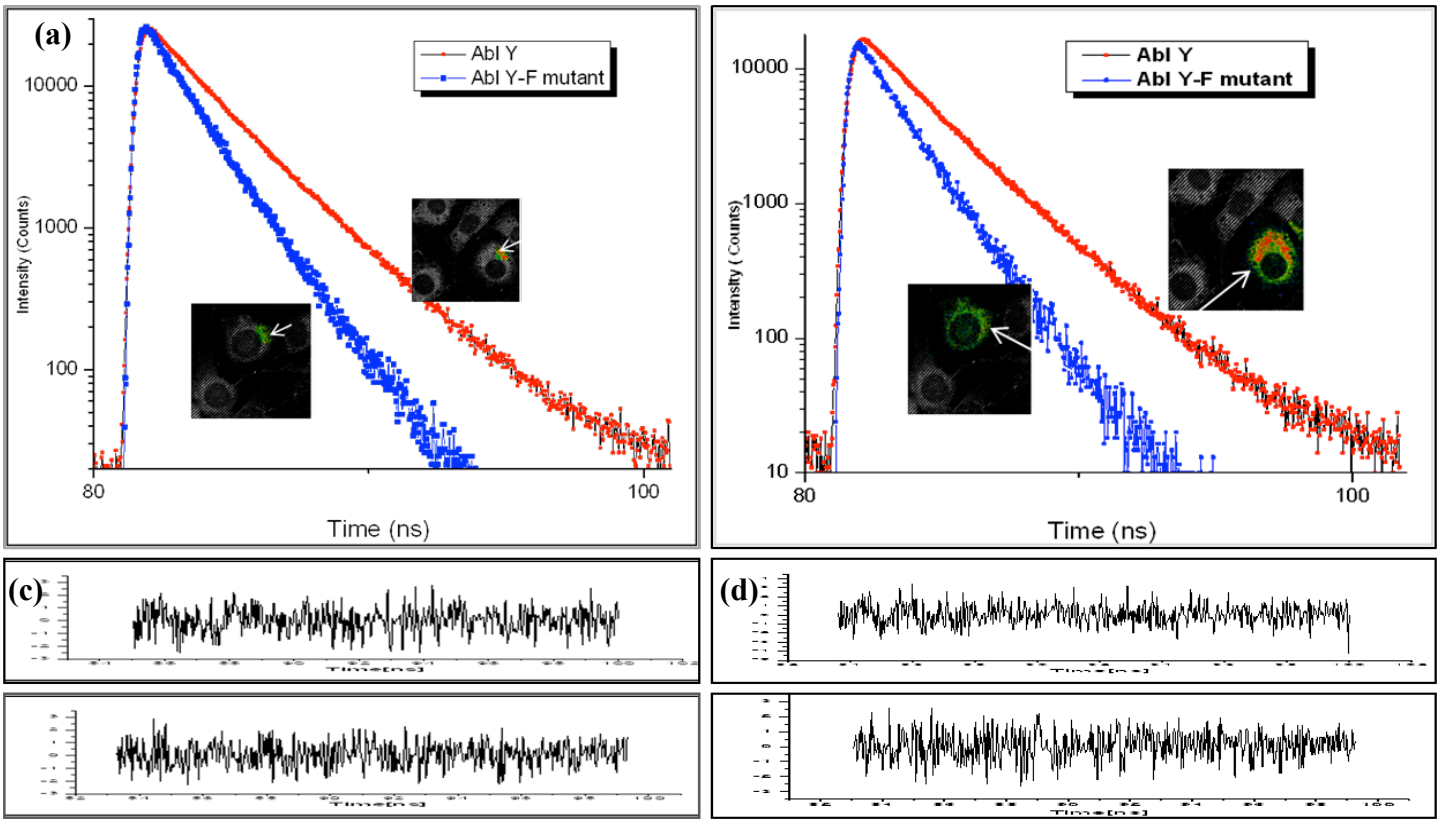


Figure S7. Representative example of multiexponential fitting. An example of a time-resolved fluorescence decay plot extracted for a single pixel in the ROI (a) and single cell (entire ROI) (b) used for fitting the lifetime of different probes (Abl peptide, red curve and Abl mutant peptide, blue curve). In this example, the decay curves for the single pixel (a) have the same trend as the whole cell average (b). The respective residuals (c, d) from fitting for the two peptides show no pattern.

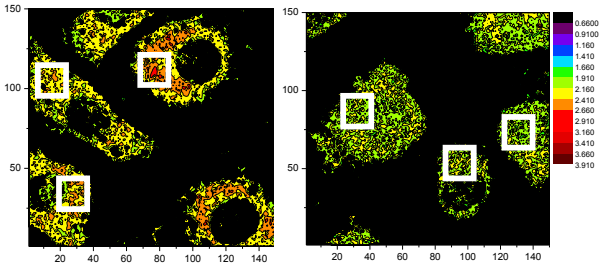


Figure S8. Representative example illustrations of ROI selection and plotted data from pixel-by-pixel lifetime quantification.

1. Schobel, U., Egelhaaf, H.J., Brecht, A., Oelkrug, D. & Gauglitz, G. New donor-acceptor pair for fluorescent immunoassays by energy transfer. *Bioconjug Chem* **10**, 1107-1114 (1999).
2. Qin, Q.P., Peltola, O. & Pettersson, K. Time-resolved fluorescence resonance energy transfer assay for point-of-care testing of urinary albumin. *Clin Chem* **49**, 1105-1113 (2003).
3. Jin, H. & Wang, J.Y. Abl tyrosine kinase promotes dorsal ruffles but restrains lamellipodia extension during cell spreading on fibronectin. *Mol Biol Cell* **18**, 4143-4154 (2007).