Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information for:

Multiplexable fluorescence lifetime imaging (FLIM) probes for Abl and Src-family kinases

Sampreeti Jena[±,c] Nur P. Damayanti[±,a,d] Jackie Tan,[c] Erica Pratt,[c] Joseph M. K. Irudayaraj*[a,e] and L. L. Parker*[b,c]

[a]*Previous affiliation:* Department of Agricultural and Biological Engineering, Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN, 47907.

[b]*Previous affiliation:* Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue Center for Cancer Research, Purdue University, West Lafayette, IN, 47907.

[c]*Current affiliation:* Department of Biochemistry, Molecular Biology and Biophysics, Masonic Cancer Center, University of Minnesota, Minneapolis, 55455.

[d]*Current affiliation:* Indiana University School of Medicine, Indianapolis, IN, 46202.

[e]*Current affiliation:* Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, 61801.

*Co-corresponding authors: llparker@umn.edu, jirudaya@illinois.edu

Experimental procedures

Peptide Biosensors. Peptide sensors were previously designed and developed by the Parker lab[1, 2]. Complete sequences are available in Table 1. Briefly, Fmoc solid phase peptide chemistry was used to synthesize each sensor, which consists of recognition sequence modified with cysteine as reporter anchoring site, fluorescence reporter and Tat penetrating sequence to aid cellular delivery. The fluorophore maleimides (DyLight 550 or 488 from ThermoFisher Scientific) were conjugated to the cysteines for each respective sensor in buffer containing 6M guanidinium-HCl, 100 mM phosphate, and 10 mM TCEP, at pH 6.5, checking desalted aliquots with MALDI-TOF MS until reaction had gone to completion (typically ~1-2 h). Sensor peptides were purified using HPLC (Agilent 1200 preparative system, Zorbax C18 column, 20 mm inner diameter, 250 mm length). Final labeled peptides were characterized with LC/MS (see pp. 14-18).

Cell Culture. Human MDA-MB-231 were purchased from ATCC. Cells were cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin/Streptomycin at 37^{o}C, 5% CO_2.

Sensor Delivery and Imaging. Cells were serum starved for 24 h prior to EGF stimulation experiments. Live cells were then incubated with the respective biosensors (10 μM) in serum free medium for 2 hours prior to FLIM imaging experiments. Cells were washed with PBS and maintained in the low background

fluorescence medium, FluoroBrite DMEM (Gibco, USA) in a top incubator live cell imaging chamber (TOKAI HIT INUBG2ASFP). The live cell chamber was equipped with temperature feedback control to maintain stable temperature at 37^{o}C.

TCSPC-FLIM. FLIM experiments utilized a Nikon A1R laser scanning confocal microscope equipped with a FLIM, FRET, and FCS enabled system (LSM Upgrade Kit, PicoQuant, Germany)[3-9] that allows precise recording of the arrival time of photons at the detector. The Instrument Response Function (IRF)[3] was taken into consideration during measurement and data analysis. Two picosecond pulsed laser lines 488 and 561 nm were used as excitation sources and the emitted light was guided through a 50 µm pinhole. Two single photon avalanche diode detectors (PDM SPAD, PMA Hybrid PMT.) were used to collect emitted photons. A 520±30 nm emission filter was placed in front of the SPAD to ensure collection from DL488 while a 580±40 nm filter was placed in front of the Hybrid PMT to collect emission from the Dylight 550 fluorophore. Each photon was tagged with a time stamp that signified its arrival time in the detector after a laser pulse, using the time correlated single photon counting (TCSPC) in the Time Tagged Time Resolved Single Photon Mode (TTTR) (Picoharp 300, Picoquant). Average fluorescence lifetimes were measured and displayed as color contrast unless otherwise specified, using the color scales indicated for each image. For quantitative analysis, lifetime decay per image was fitted per pixel with bi-exponential fitting employing an iterative Levenberg-Marquadt algorithm (Equation 1).

$I(t) = \sum_{i=1}^{n} \alpha i exp(-t/\tau i) + C$ .....(Equation 1)

where I(t) is the fluorescence intensity at time t after the excitation pulse, n is the total number of decay components in the exponential sum, and C is a constant pertaining to the level of background light noise. The variables τi and αi are the fluorescence lifetime and fractional contribution of the emitting species, respectively.

Bi-exponential fitting

I(t) = $\alpha 1 \exp\left(-\frac{t}{\tau 1}\right) + \alpha 2 \exp\left(-\frac{t}{\tau 2}\right) + C$ ..... (Equation 2)

To ensure unbiased fitting, fluorescence decay curves collected in each pixel were fitted with (Equation 2) until two criteria were met: 1. sufficient Chi value (<1.3) and 2. no residual pattern was observed. Fitting resulted in two fluorescence lifetime values (τ 1 and $\tau 2$) as well as their population ratio in each pixel ($\alpha 1$ *and* $\alpha 2$) . Fitting was performed in SymphoTime (Picoquant) software. The results were exported to MATLAB to separate matrices of lifetime and matrices of intensity of each component. The total ratio of matrices describing the fractional intensity of longer lifetime over total intensity was calculated using the MATLAB code shown below:

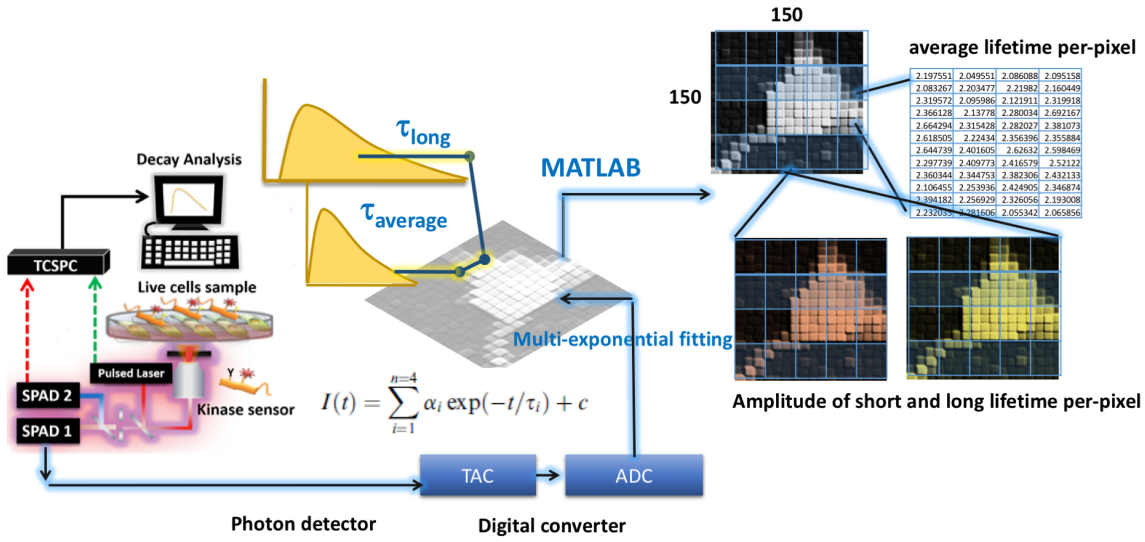
```
%% Data File
data1=Imatinib3;
data2=Imatinib1;

%% Data Process
[row,col]=size(data1); %size of file
```

```
for i=1:row
    for j=1:col
        dataSum(i,j)=data1(i,j)+data2(i,j);%sum of
twofiles
        if dataSum(i,j)>0
            dataAns(i,j)=data1(i,j)./dataSum(i,j);
%divison
        elseif dataSum(i,j)==0
            dataAns(i,j)=0;
        else
            dataAns(i,j)=dataSum(i,j);
        end
    end
end

%% Save to ascii file
save('Imatinib0.txt','dataAns','-ascii')
```

The matrices were then exported to ImageJ for mapping. A schematic of the instrumentation and analysis steps are provided below.



Analysis schematic. Cells are imaged using a TCSPC FLIM microscope, and files are analyzed by deconvoluting multiple lifetimes via multi-exponential fitting. Proportional amplitudes for different lifetime components can be extracted and re-plotted as fractional intensity of longer lifetime species.

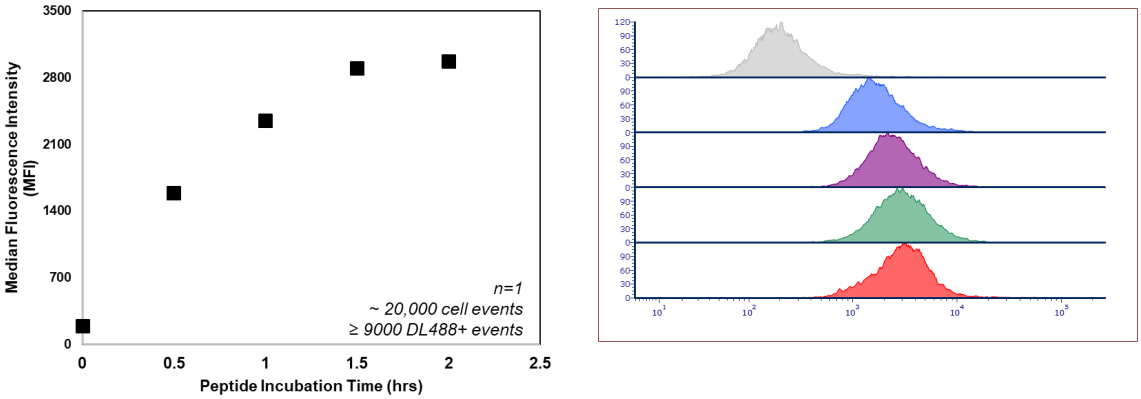


Figure S1A. Peptide uptake quantification with flow cytometry. MDA MB 231 cells serum starved overnight were incubated with FmABL-DL488 probes (10 uM) with incubation periods ranging from 0 to 2 hours. Cells were trypsinized, washed 3x with PBS and resuspended in FACS buffer (1% BSA + 2mM EDTA) at a density of 1e7 cells/mL. DAPI was added immediately prior to analysis. 405 nm and 488 laser lines of the FACSCantoA analyzer were used for exciting DAPI and DL488, respectively.

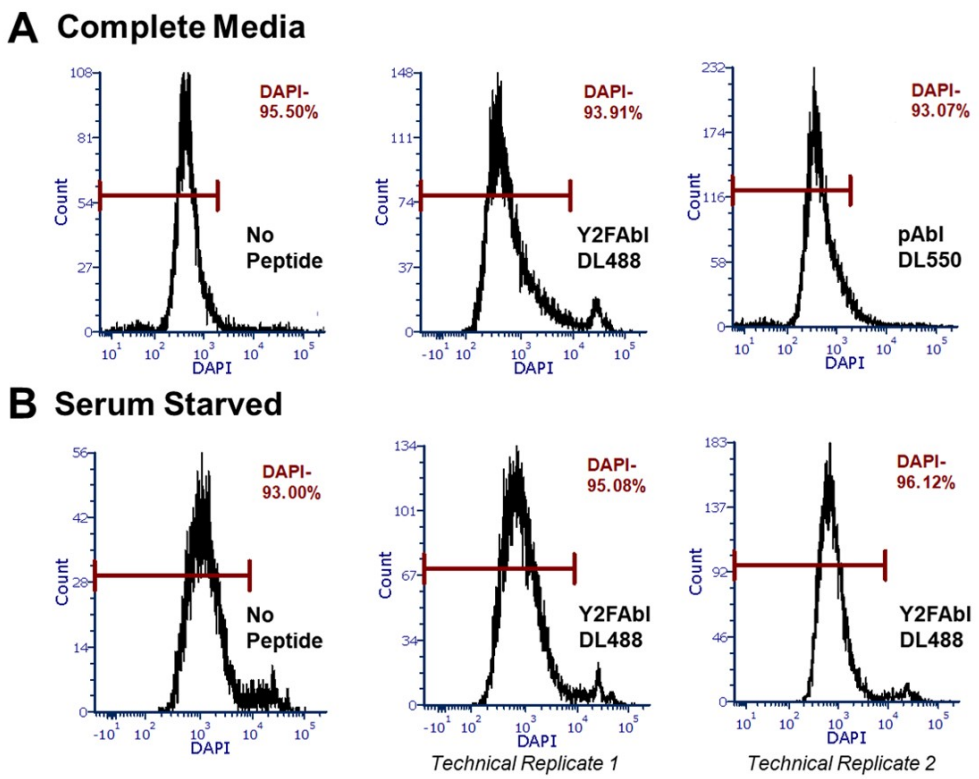


Figure S1B. Evaluation of peptide probe cytotoxicity in MDA-MB-231 cells via flow cytometry using DAPI. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain which strongly binds to DNA after penetrating the cell membrane. An intact live cell membrane is much less permissible to DAPI internalization and results in low fluorescent signal which can be used to infer cell viability. MDA-MB-231 cells were seeded in 12-well plates at least two days prior to analysis at a density of 4-6e5 cells/well. Serum-free media was introduced at least 12 hrs prior to analysis. Cells were incubated with 6-10 μM Abltide-TAT peptide probes (pY,Y2F) for two hours at 37°C and 5% CO_2. Cells were then trypsinized to cleave surface-bound probe and washed three times with PBS prior to resuspension at 1e7cells/mL in FACS buffer (PBS, 1% BSA, 2mM EDTA). DAPI was added immediately prior to analysis on the BD FACSCanto A. Approximately 10,000-20,000 cell events were captured

for all tested conditions. A) Representative flow cytometry data showing the distribution of cellular DAPI fluorescent signal and gating to define live (DAPI-) vs dead cells. The proportion of live cells when no peptide is present (95.5%) is indistinguishable from cells incubated with Y→F mutant probe (Y2FAbl, 93.91%), and cells incubated with phosphorylated Abl probe (pAbl, 93.1%). B) Representative flow data showing cell viability is similarly unaffected under serum-starved conditions. Peptide probe data are from two independent technical replicates. The detection fluorophores used (DL488, DL550) are listed below the peptide probe name.

Additional microscopy images

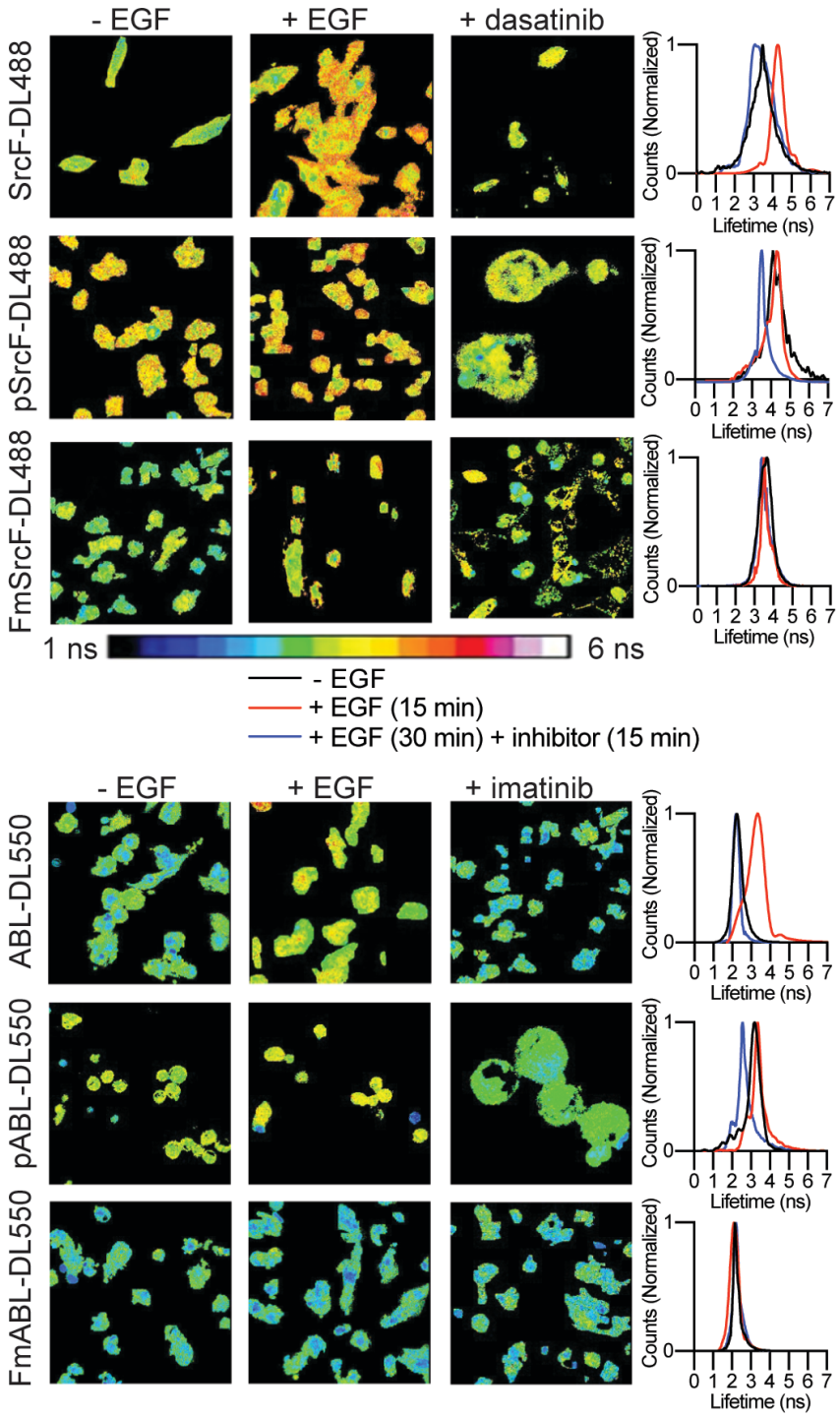


Figure S2. Probe characterization in MDA-MB-231 breast cancer cells for SrcF and Abl probes, labelled with DyLight488 or 550, respectively: SrcF-DL488,

positive mutant (pSrcF-DL488) and negative Y->F mutant (FmSrcF-DL488) (top); ABL-DL550, positive mutant (pABL-DL550) and negative Y->F mutant (FmABL-DL550) (bottom). Cells were serum starved overnight and incubated with probe (10 μM) for 2h. DL488 or DL550 lifetime was measured before (-EGF) and after (+ EGF) treating the cells with epidermal growth factor (EGF, 10 ng, 15 min) to activate Src and Abl kinases. Longer lifetimes were observed in the wild type probes following stimulation as well as both before and after stimulation in case of the positive mutant probes, with no increase in the negative mutant, indicating that the longer lifetime species were consistent with peptide phosphorylation. After 15 min of EGF treatment, the inhibitor dasatinib (for Src probes) or imatinib (for Abl probes) was added (1 μM), decreasing the longer lifetime species. Images normalized to the respective lifetime scales and average lifetime histograms per image corresponding to the wild type, positive mutant and negative mutant probes for this experiment are shown. Scale bars = 20 μm.

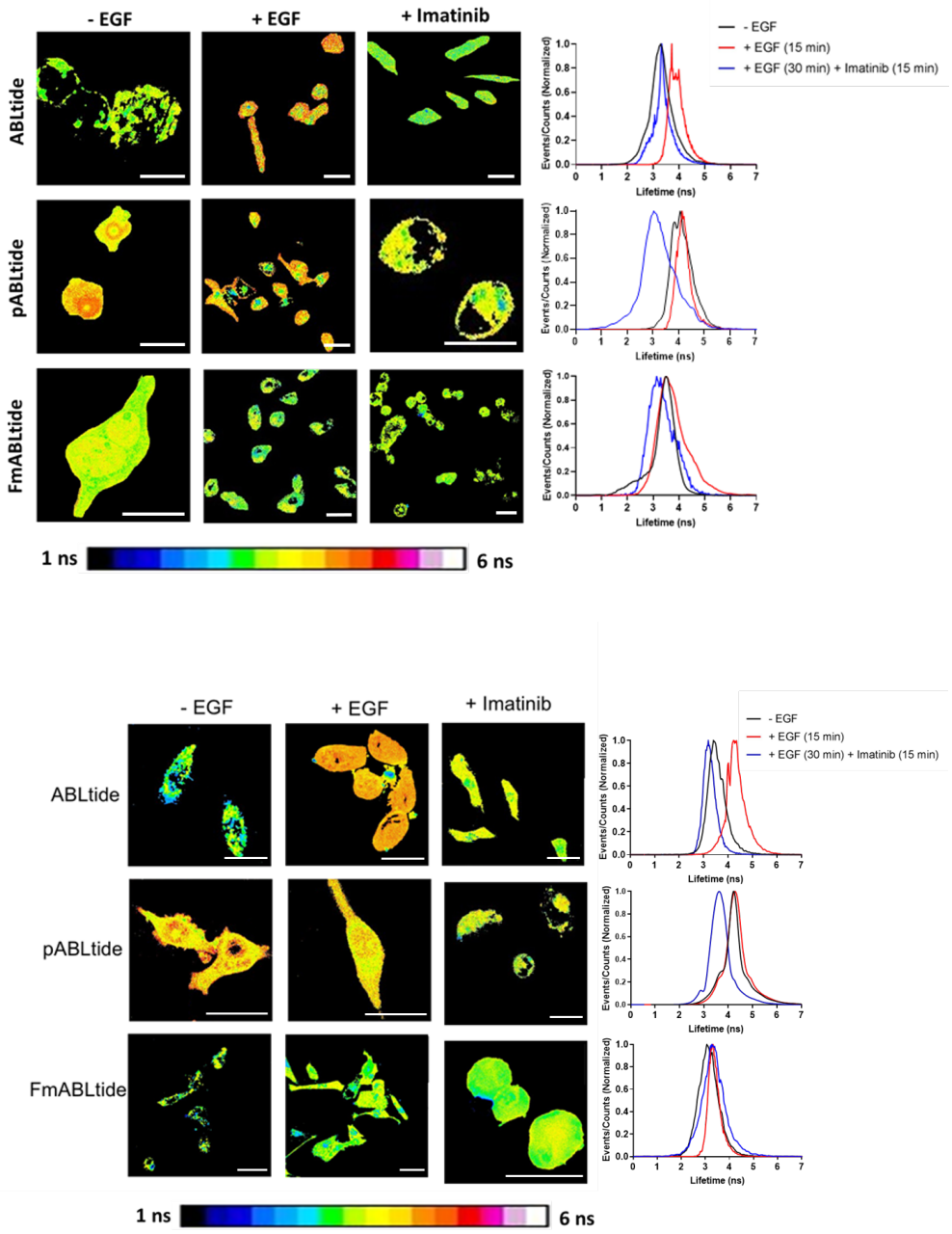


Figure S3. Abl-DL488 probes analyzed in MDA-MB-231 cells. The probes were synthesized and labeled with DyLight488 maleimide. MDA-MB-231 cells were incubated with probes and imaged before treatment, following EGF treatment and following inhibitor treatment. Representative lifetime mapped images and lifetime histogram plots are shown. Scale bars are 20 μm.

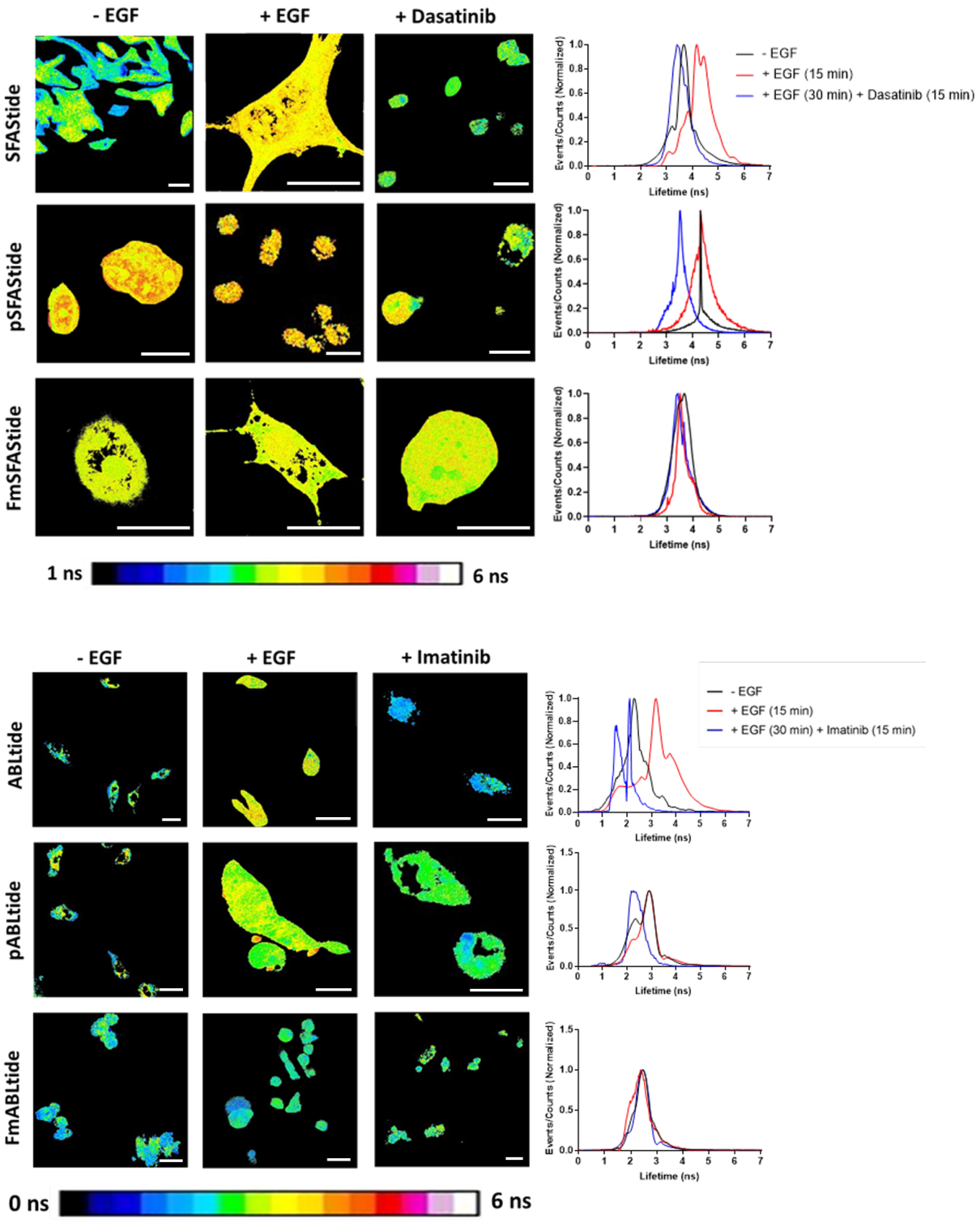


Figure S4. SrcF-DL488 and Abl-DL550 probes analyzed in MDA-MB-231 cells (duplicates of experiments shown in Fig. S2). MDA-MB-231 cells were

incubated with probes and imaged before treatment, following EGF treatment, and following inhibitor treatment. Representative lifetime mapped images and lifetime histogram plots are shown. Scale bars are 20 μm.

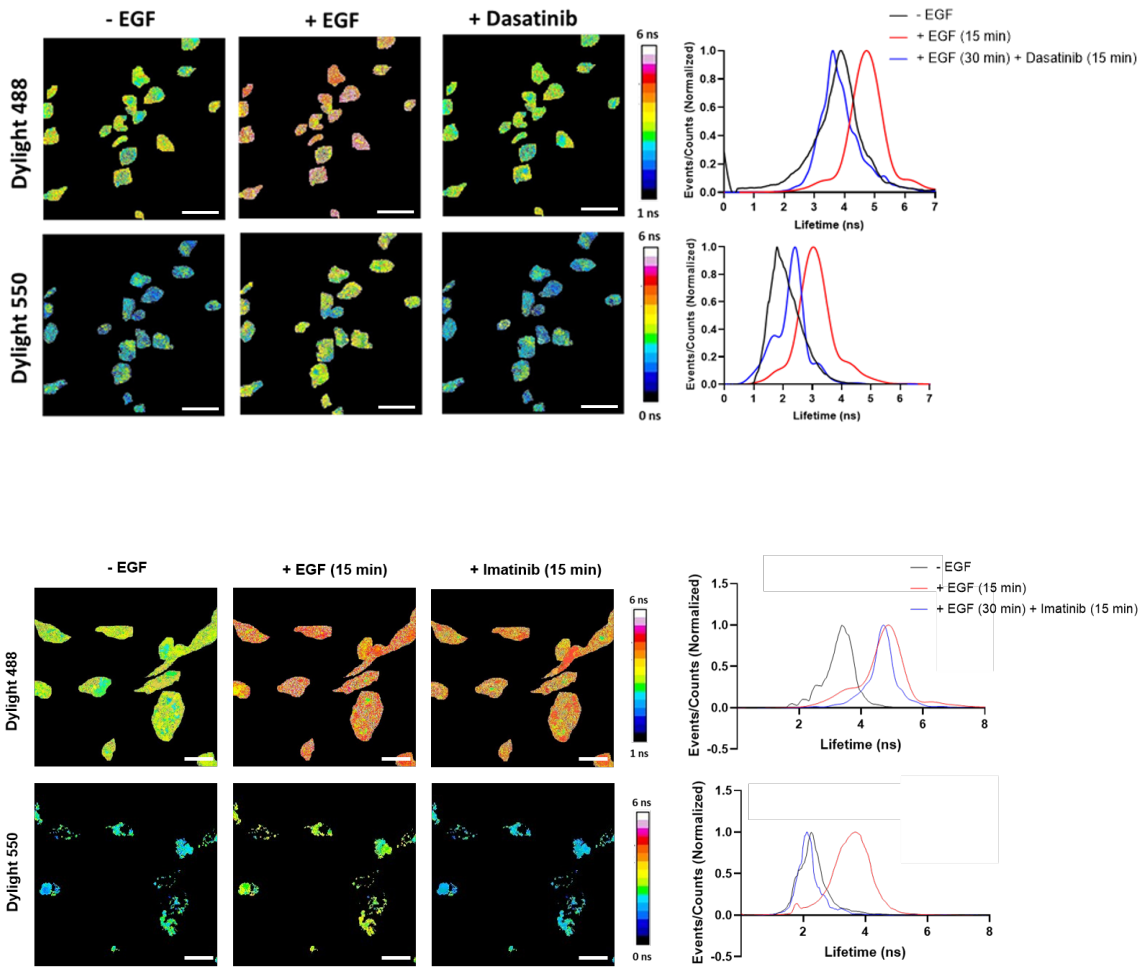


Figure S5. Multiplexing of SFAS-DL488 and ABL-DL550 probes in MDA-MB-231 cells. Representative FLIM images and lifetime histograms from duplicate experiments performed for multiplexed analysis in Figure 1 of the main manuscript. Scale bars are 20 μm.

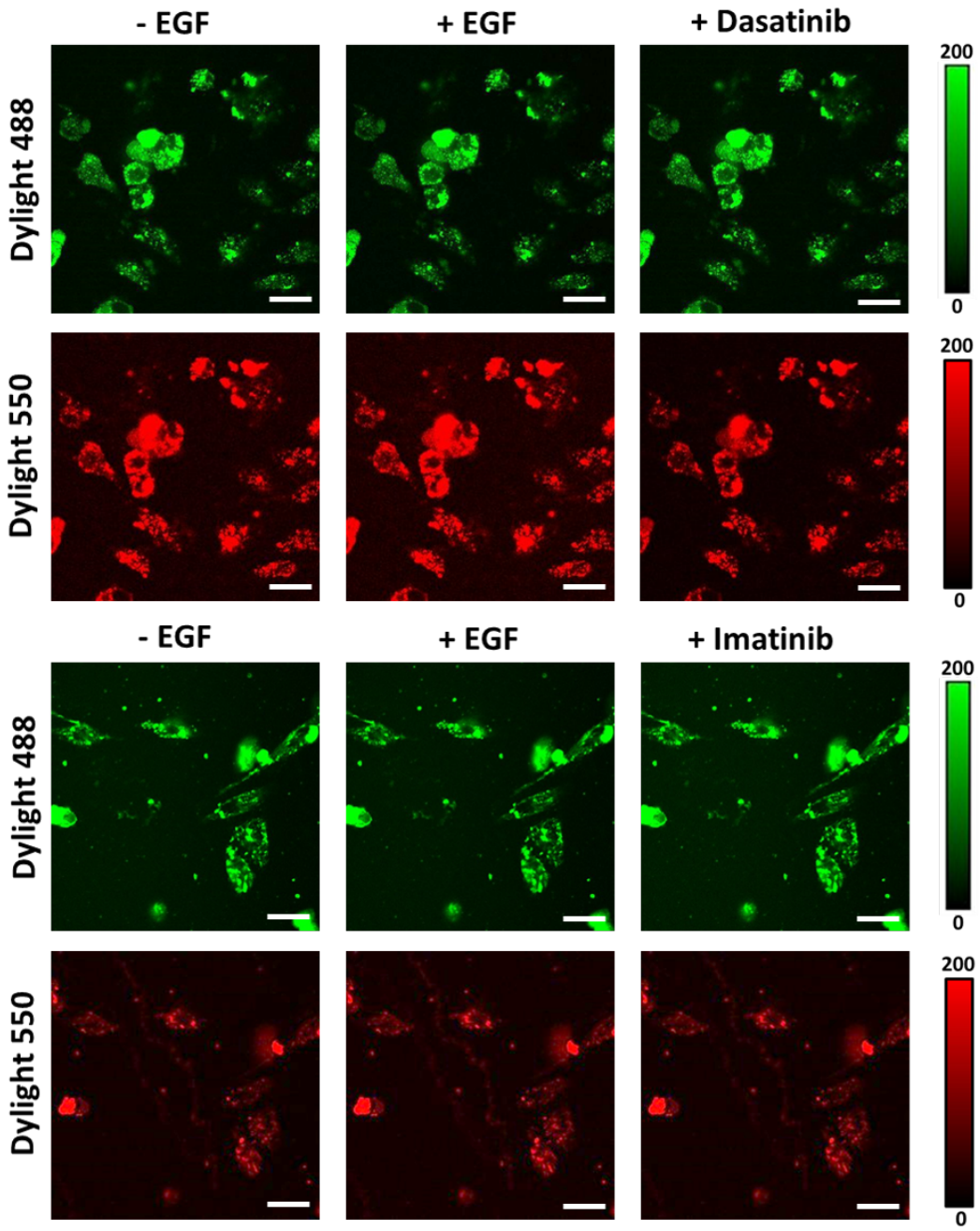


Figure S6. Representative fluorescence Intensity images of SFAS-DL488 (green channel) and Abl-DL550 probes (red channel) from multiplexed analysis. Top: Dasatinib treatment from Fig. 1 of the main manuscript. Bottom: Imatinib treatment from duplicate experiment shown in Fig. S5. Scale bars are 20 μm.

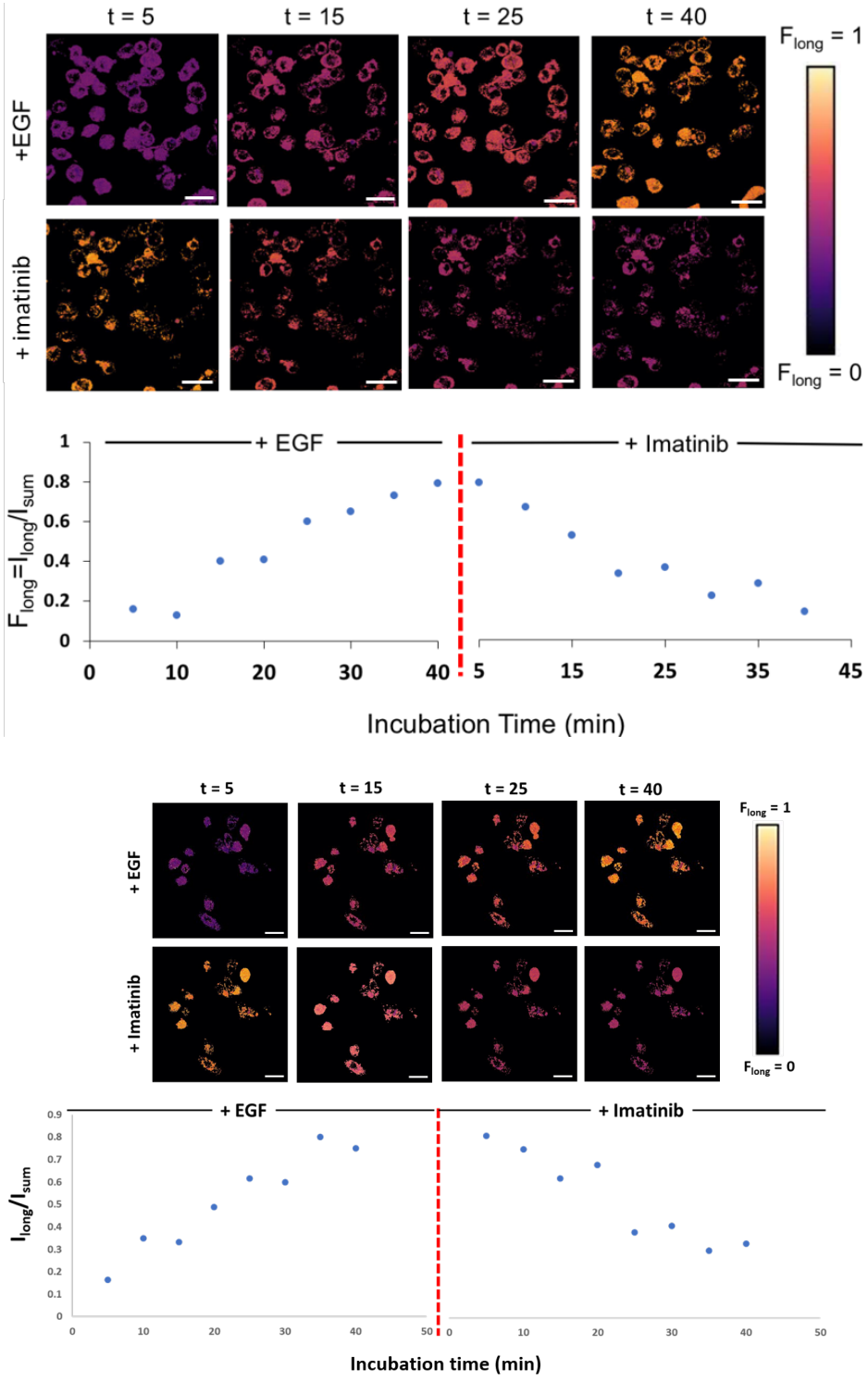


Figure S7. Relative fraction of phosphorylated ABL-DL488 probes spatially mapped in a pixel-pixel basis and average value plotted as a function of time (top set and bottom set represent duplicate experiments). We used a multi-

exponential fitting model to extract the proportion of the longer vs. shorter lifetime species on a per-image basis from the time course experiments. This was done by fitting the averaged decay curve for each image to a bi-exponential model function, employing the Levenberg–Marquardt routine for non-linear least squares fitting, as further described in the ESI methods above. This fitting algorithm resolved two different lifetimes, as well as their fraction, to identify the average lifetime for each species. The relative intensity represented by the longer vs. the shorter lifetime species was then calculated as F_{long}. To extract the fraction of the longer lifetime component for individual pixels, a similar fitting analysis was performed on all pixels, iteratively. The fraction of the longer lifetime species was mapped on a pixel by pixel basis in the images. Scale bars are 20 μm.

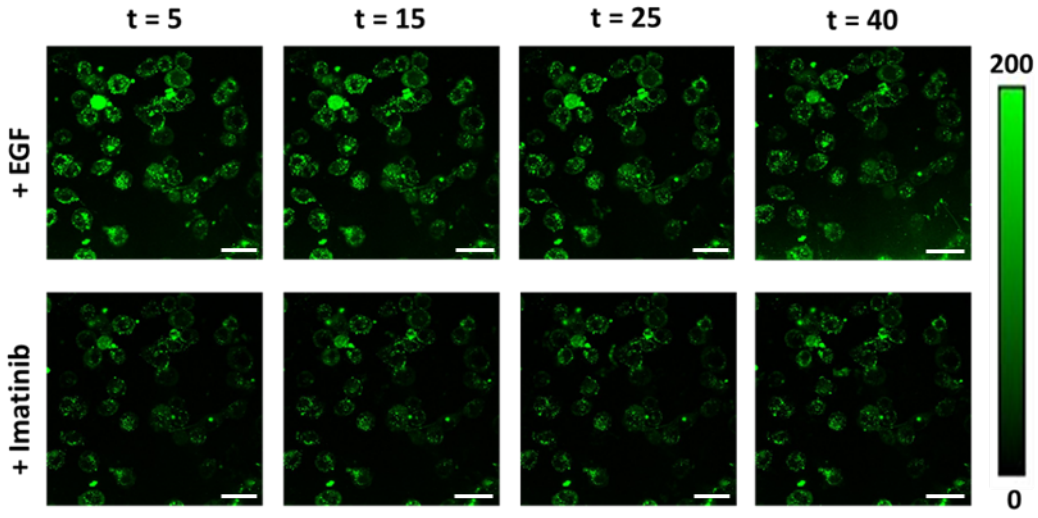


Figure S8. Fluorescence Intensity images of ABL-DL488 probes from time course analysis in Figure 3 of the main manuscript. Scale bars are 20 μm.

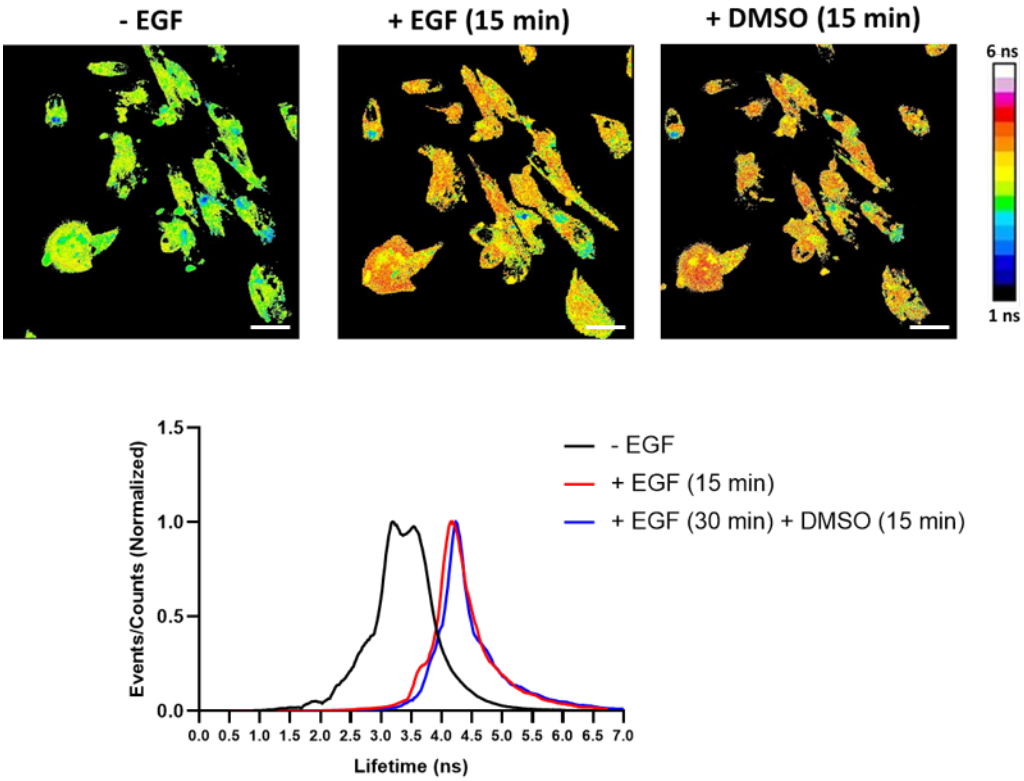


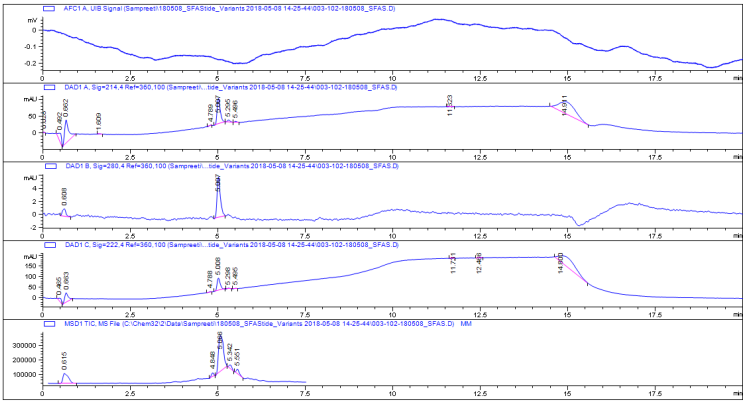
Figure S9. DMSO Control. MDA-MB-231 cells were incubated with Abl-DL488 probes and imaged before treatment, following EGF treatment and DMSO treatment. Representative lifetime mapped images and lifetime histogram plots are shown. Scale bars are 20 μm.

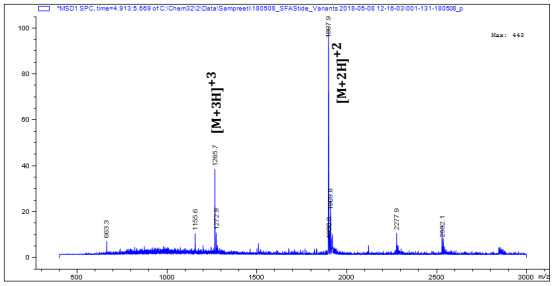
Peptide characterization data

Table S1. Peptides used in this work

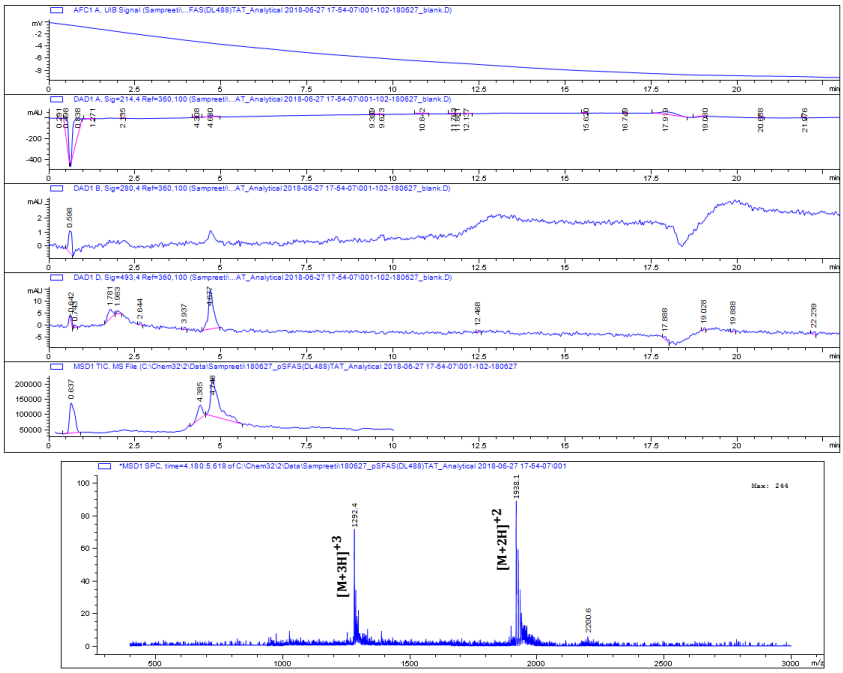
Peptide	Sequence	Characterization data available?	Fluorophore label
SFAS-DL488	GG**DEDIYEELDC**$_{DyLight488}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 488 maleimide
pSFAS-DL488	GG**DEDIYEELDC**$_{DyLight488}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 488 maleimide
FmSFAS-DL488	GG**DEDIFEELDC**$_{DyLight488}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 488 maleimide
Abl-DL488	GG**EAIYAAPC**$_{DyLight488}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 488 maleimide e
pAbl-DL488	GG**EAIpYAAPC**$_{DyLight488}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 488 maleimide
FmAbl-DL488	GG**EAIFAAPC**$_{DyLight488}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 488 maleimide
Abl-DL550	GG**EAIYAAPC**$_{DyLight550}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 550 maleimide
Abl-DL550	GG**EAIYAAPC**$_{DyLight550}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 550 maleimide
Abl-DL550	GG**EAIYAAPC**$_{DyLight550}$GG*RKKRRQRRRPQ*	Yes (HPLC)	DyLight 550 maleimide

SFAS-DL488 GGDEDIYEELDC$_{DyLight488}$GGRKKRRQRRRPQ MW 3796.3

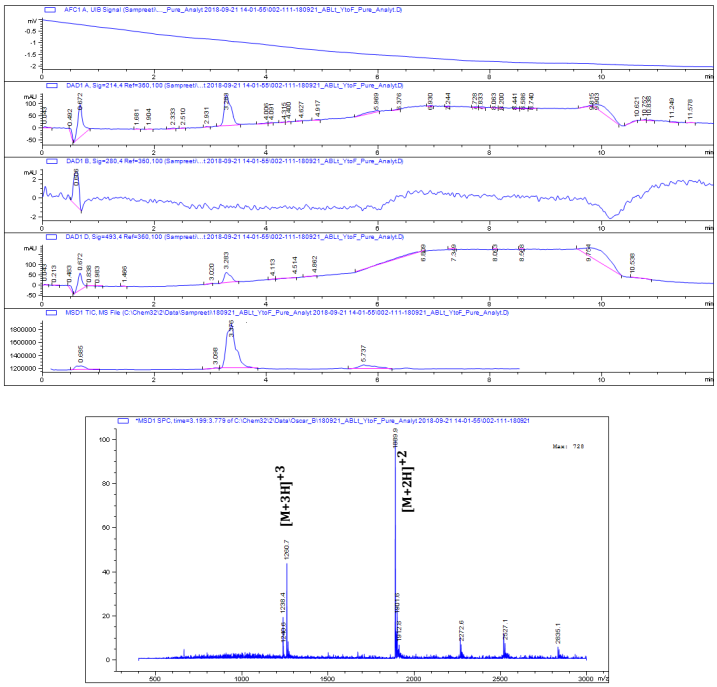




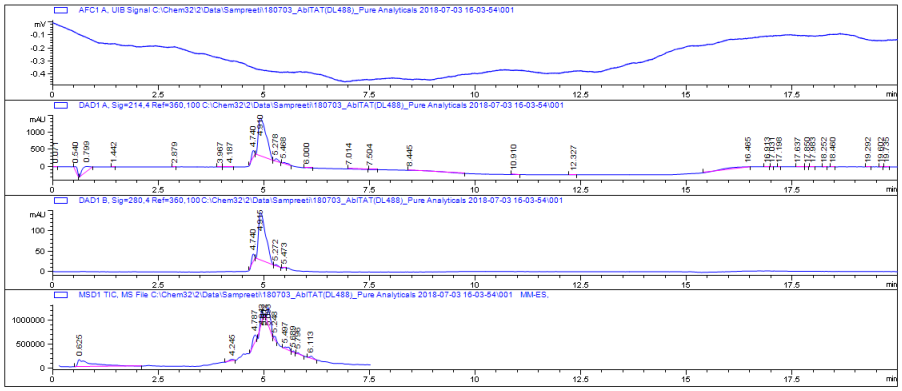
pSFAS-DL488 GGDEDIYEELDC$_{DyLight488}$GGRKKRRQRRRPQ MW 3874.3

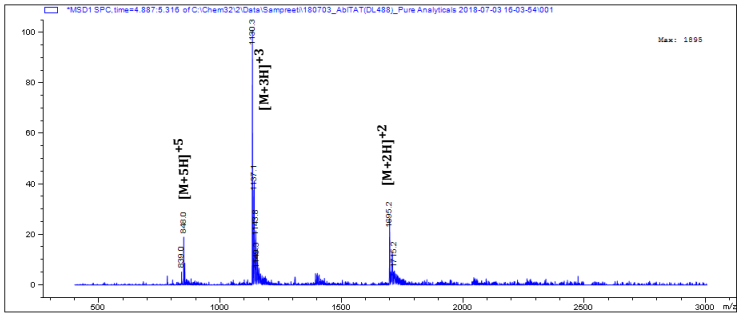


FmSFAS-DL488 GGDEDIFEELDC$_{DyLight488}$GGRKKRRQRRRPQ MW 3780.3

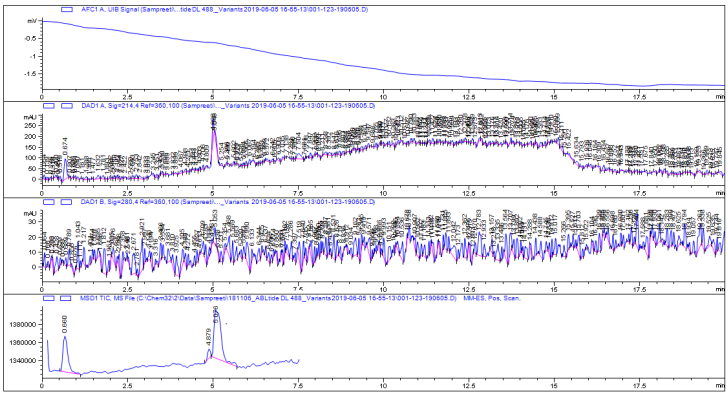


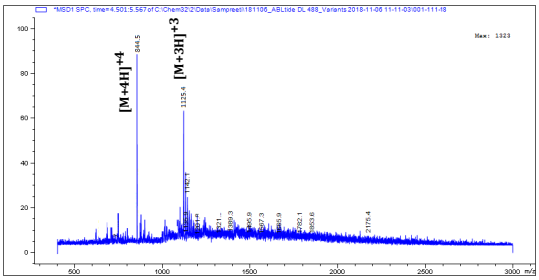
Abl-DL488 GG**EAIYAAP**C$_{\text{DyLight-488}}$GG*RKKRRQRRRPQ* MW = 3390.0



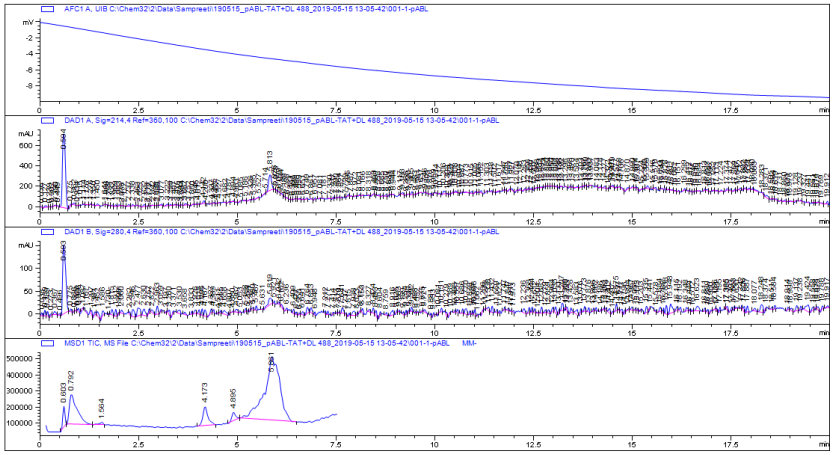


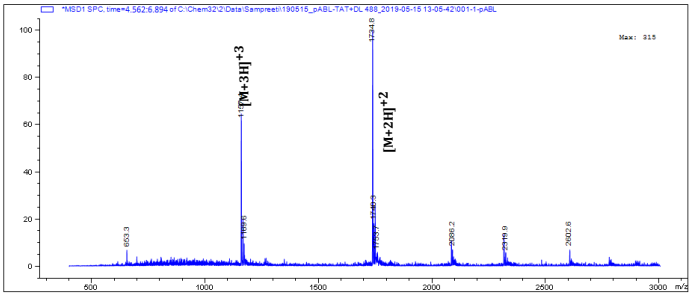
FmAbl-DL488 GG**EAIFAAP**C$_{\text{DyLight-488}}$GG*RKKRRQRRRPQ* MW = 3374.0



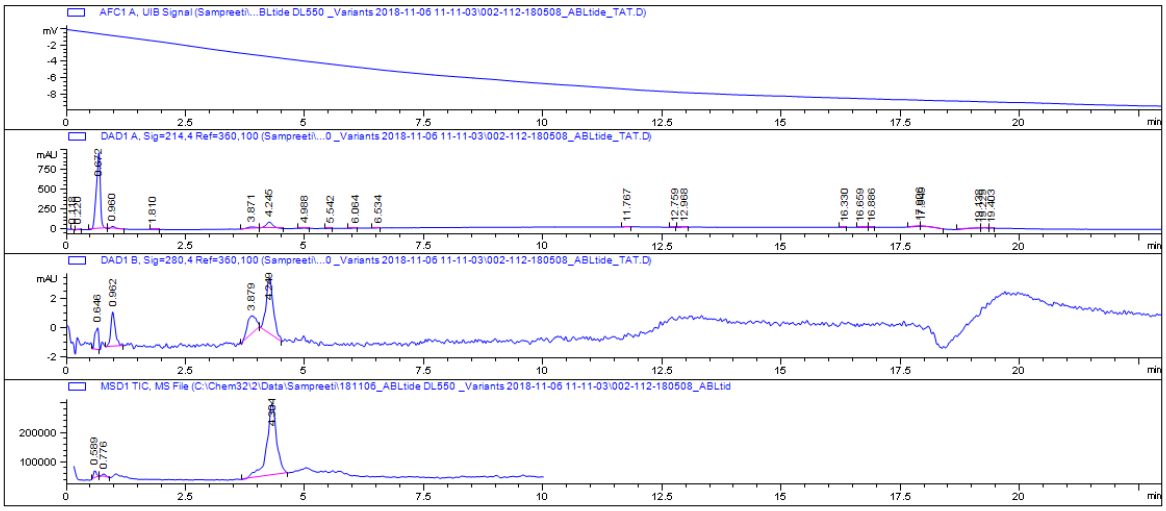


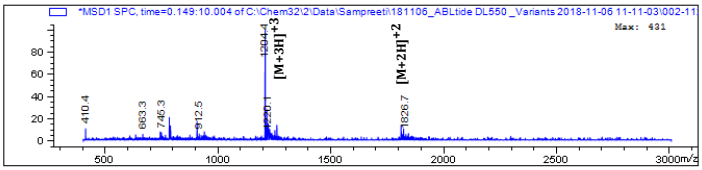
pAbl-DL488 GG**EAIYAAP**C$_{\text{DyLight-488}}$GG*RKKRRQRRRPQ* MW = 3468.0



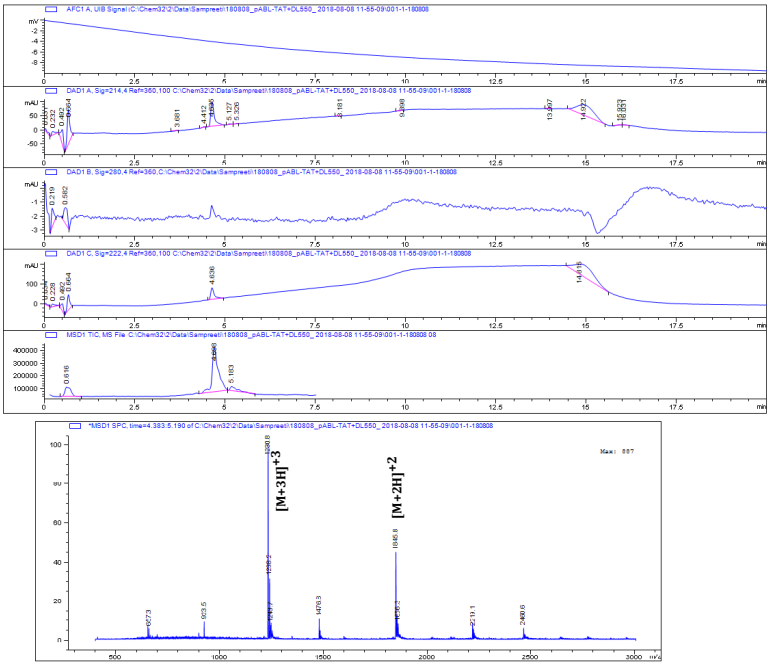


Abl-DL550 GG**EAIYAAP**C$_{\text{DyLight-550}}$GG*RKKRRQRRRPQ* MW = 3612.0

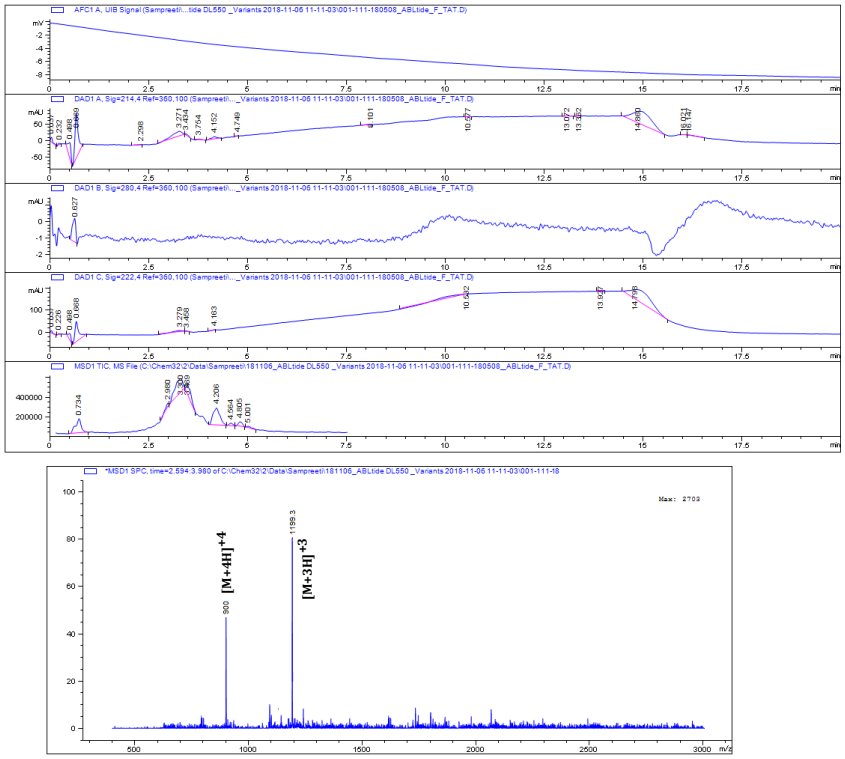




pAbl-DL550 GG**EAIpYAAP**C$_{\text{DyLight-550}}$GG*RKKRRQRRRPQ* MW = 3690.0



FmAbl-DL550 GG**EAIFAAP**C$_{\text{DyLight-550}}$GG*RKKRRQRRRPQ* MW = 3596



References

1. Lipchik, A.M., Killins, R.L., Geahlen, R.L. & Parker, L.L. A peptide-based biosensor assay to detect intracellular Syk kinase activation and inhibition. *Biochemistry* **51**, 7515-7524 (2012).
2. Lipchik, A.M. et al. KINATEST-ID: a pipeline to develop phosphorylation-dependent terbium sensitizing kinase assays. *Journal of the American Chemical Society* **137**, 2484-2494 (2015).
3. Damayanti, N., Craig, A. & Irudayaraj, J. A hybrid FLIM-elastic net platform for label free profiling of breast cancer. *Analyst* **138**, 7127-7134 (2013).
4. Chen, J. & Irudayaraj, J. Quantitative Investigation of Compartmentalized Dynamics of ErbB2 Targeting Gold Nanorods in Live Cells by Single Molecule Spectroscopy. *Acs Nano* **3**, 4071-4079 (2009).
5. Chen, J. & Irudayaraj, J. Fluorescence Lifetime Cross Correlation Spectroscopy Resolves EGFR and Antagonist Interaction in Live Cells. *Analytical Chemistry* **82**, 6415-6421 (2010).
6. Chen, J., Miller, A., Kirchmaier, A.L. & Irudayaraj, J.M. Single-molecule tools elucidate H2A.Z nucleosome composition. *J Cell Sci* **125**, 2954-2964 (2012).
7. Chen, J., Nag, S., Vidi, P. & Irudayaraj, J. Single Molecule In Vivo Analysis of Toll-Like Receptor 9 and CpG DNA Interaction. *Plos One* **6** (2011).
8. Vidi, P.-A., Chen, J., Irudayaraj, J.M.K. & Watts, V.J. Adenosine A(2A) receptors assemble into higher-order oligomers at the plasma membrane. *FEBS Letters* **582**, 3985-3990 (2008).

9. Damayanti, N.P., Parker, L.L. & Irudayaraj, J.M.K. Fluorescence Lifetime Imaging of Biosensor Peptide Phosphorylation in Single Live Cells. *Angewandte Chemie-International Edition* **52**, 3931-3934 (2013).