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

Nur P. Damayanti,[a,d] Sampreeti Jena,[c] Joseph M. K. Irudayaraj*[a,e] and Laurie 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*

Abstract:

Most commonly employed strategies to map kinase activities in live cells require expression of genetically-encoded proteins (e.g. FRET sensors). In this work, we describe development and preliminary application of a set of cell-penetrating, fluorophore labelled peptide substrates for fluorescence lifetime imaging (FLIM) of Syk and Src-family kinase activities. These probes do not rely on FRET pairs or genetically-encoded protein expression. We also demonstrate image-by-image and pixel-by-pixel quantification of probe phosphorylation ratio, suggesting that this strategy will be useful for detailed mapping of single cell and subcellular kinase activation in live cells.

Introduction:

Protein kinases control many fundamental aspects of cell function, including the cell cycle, DNA repair, and response to exogenous stimuli. Despite the biological importance of their dynamics and subcellular localization, few tools exist for monitoring kinase activities in cells in real time. Genetically-encoded Förster resonance energy transfer (FRET) protein sensors can accomplish single cell, dynamic imaging, however these are difficult to multiplex since two fluorophores (often with relatively broad excitation and emission spectra) are required per sensor, so spectral overlap/bleed through between sensors becomes a significant problem. We previously reported that a fluorophore labelled, cell-penetrating peptide substrate for Abl kinase exhibited longer fluorescence lifetime upon phosphorylation and apparent interaction of the

phosphopeptide product with SH2 domains.[1] This novel strategy was capable of detecting Abl kinase activity and inhibition in live cells, however it was not clear if the approach would be applicable to other kinases. We recently extended this approach to monitor apparent Akt, VEGFR2 and FAK activation in a range of cell models using 5-FAM, a fluorescein-based fluorophore label.[2, 3] In this report, we demonstrate that this strategy can be applied to two other kinase families, the Syk and Src families, using three different fluorophores (DyLight 488, DyLight 550, and Cy5). We also show for the first time that the lifetime data in each cells or even each pixel can be used to extract fractional contributions from "unphosphorylated" and "phosphorylated" probe populations, paving the way to more detailed mapping of kinase activities and phosphorylation dynamics within cell populations and throughout the interior of a live cell.

Results and Discussion:

We started by exploring the generality of the fluorescence lifetime shift upon phosphorylation of the peptide probe, using Cy5, the same fluorophore as we previously observed to exhibit this behaviour. Two substrates developed by the Parker laboratory were chosen: SAStide (a Syk substrate)[4] and SFAStide-A (a Src-family substrate)[5] and combined with the TAT sequence to generate cell-penetrating probes. Peptides were synthesized with the sequences shown in Table 1, labelled on the central cysteine residue with Cy5-maleimide, and purified by preparative HPLC (as previously described[1]). The corresponding

phosphorylated and Y→F mutant probes were also synthesized as positive and negative controls, respectively.

Table 1. Peptide probe sequences

Probe	**Sequence**
SAStide-TAT	GG**DEEDYEEPDEP**GGCGG*RKKRRQRRRPQ*
SFAStide-A-TAT	GG**DEDIYEELD**CGG*RKKRRQRRRPQ*
pSFAStide-A-TAT	GG**DEDIpYEELD**CGG*RKKRRQRRRPQ*
SFAStide-A-Y→F-TAT	GG**DEDIFEELD**CGG*RKKRRQRRRPQ*
Abl-TAT (from ref. 1)	GG**EAIYAAP**CGG*RKKRRQRRRPQ*

Sequences in **bold** are the substrate portion; in *italic* is the TAT cell-penetrating module; C = the cysteine used for fluorophore labelling; pY = phosphorylated tyrosine.

The SAStide-TAT (defined as **SAStide-Cy5**) and SFAStide-A-TAT (defined as **SrcF-Cy5**) probes and controls were tested in cells for phosphorylation- and kinase activity-dependence of a fluorescence lifetime shift. Imaging was performed as described in our previous publication.[1] For the SAStide-TAT probe, MDA-MB-231 cells lacking Syk (WT, which are Syk -/-) or engineered to express Syk-EGFP under the Tet-ON promotor (Syk +)[6‡] were incubated with the peptide for 1 hour. Cells were imaged before and after stimulation with pervanadate (100 μM, 15 min), a phosphatase inhibitor that induces Syk activation.[7] In Syk-EGFP-expressing cells, regions with longer lifetime probe species were observed only after stimulation of Syk activity, whereas no increased lifetime was seen in Syk (-

/-) cells even with stimulation, consistent with dependence on Syk kinase activity (Fig. 1 A-D). For the SFAStide-A-TAT probe, it was not possible to use a Src-family kinase knockout system, since this substrate is phosphorylated by many Src family members, and knocking out all Src family kinases is not feasible (cells are no longer viable). Instead, we used a well-characterized model for Src family kinase activation downstream of epidermal growth factor receptor (EGFR) stimulation by EGF, and the Abl/Src-family inhibitor dasatinib.[8, 9] Longer lifetimes were observed upon EGF stimulation, and reverted upon treatment with dasatinib. The Y→F mutant showed no longer lifetimes even with EGF stimulation, whereas the pY positive control showed longer lifetime throughout the cell even with no EGF stimulation.

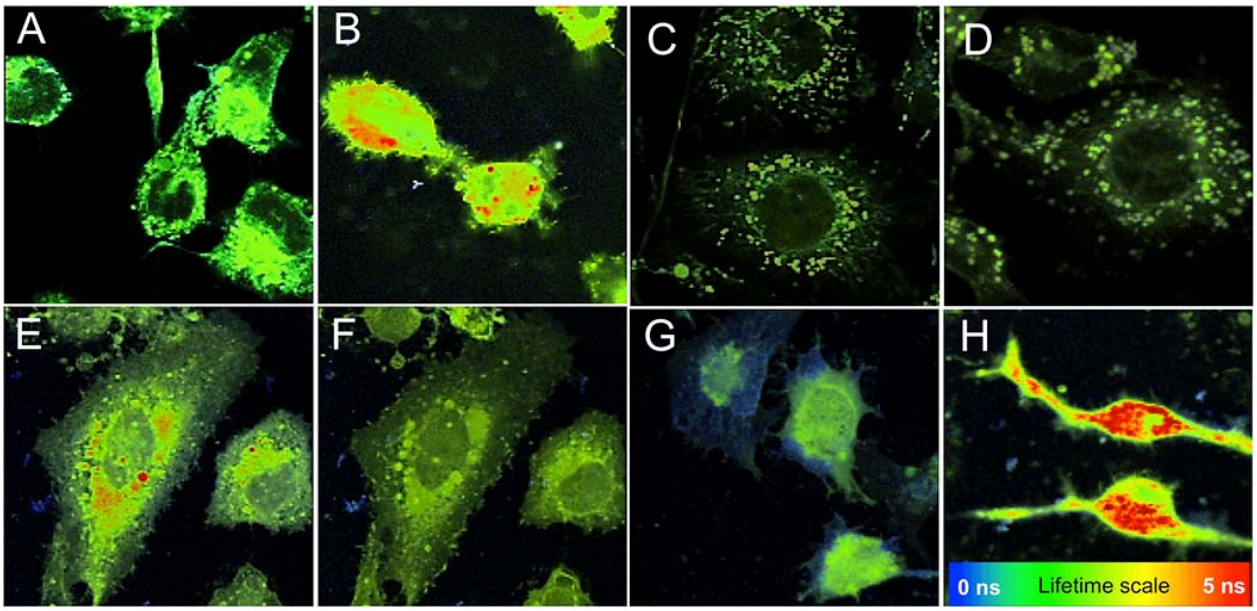
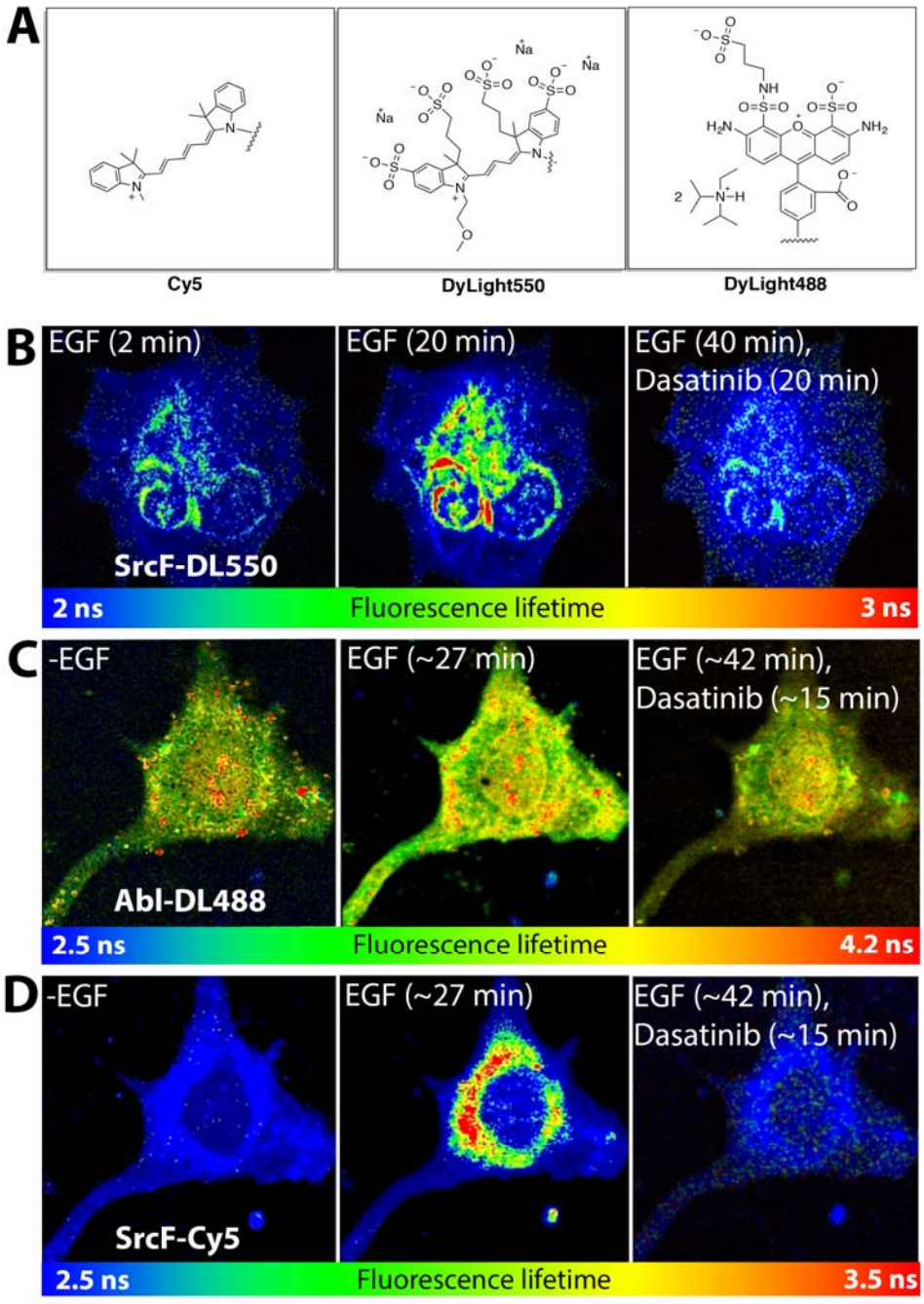


Figure 1: SAStide-TAT and SFAStide-A-TAT probe characterization in cells. (A-D) MDA-MB-231 breast cancer cells expressing Syk-EGFP (A-B) or Syk (-/-) WT (C-D) were incubated with SAStide-Cy5 (10 μM) for 1h. Cy5 lifetime was

measured before (A, C) and after (B, D) treating the cells with pervanadate (100 μM, 15 min) to activate Syk kinase. Longer lifetimes were only seen in stimulated Syk+ cells. (E-H) WT MDA-MB-231 cells were serum starved overnight, then treated with SrcF-Cy5 (10 μM) for 1h followed by EGF (10 ng) to stimulate Src-family kinase activity (E). After 15 min EGF treatment, the Abl/SrcF kinase inhibitor dasatinib was added (1 μM), decreasing longer lifetime species (F). Negative Y->F mutant (10 μM) (G) and positive pSFAStide (10 μM) (H) controls (in EGF-treated, or untreated cells, respectively) showed longer lifetime species were consistent with peptide phosphorylation. Images normalized to the lifetime scale shown.

Next, we examined the fluorescence lifetime behaviour of the SFAStide-A-TAT and Abl-TAT probes tagged with two different fluorophore labels (Fig. 2A). The Abl-TAT probe (described previously)[1] was labelled with DyLight488 maleimide (defined as **Abl-DL488**) or DyLight550 maleimide (defined as **Abl-DL550**). The SFAStide-A-TAT probe was labelled with DyLight550 (defined as **SrcF-DL550**). The SFAStide-DL550 probe was incubated with serum starved MDA-MB-231 cells, followed by kinase activation via EGF stimulation, and treatment with dasatinib. As shown in Fig. 2B, SrcF-DL550 showed longer lifetime after EGF stimulation that was subsequently decreased by dasatinib (as was seen for SrcF-Cy5), indicating that the DyLight550 fluorophore behaves similarly to Cy5 and exhibits longer lifetimes upon phosphorylation under these conditions. Similar experiments were also performed with the Abl-DL550 probe (shown in the

supporting information, Figure S2). In another experiment, the Abl-DL488 probe and SrcF-Cy5 probe were combined and incubated with serum starved MDA-MB-231 cells for two-color FLIM imaging of response to EGF. After EGF stimulation, lifetime increased for each sensor, and decreased again after adding dasatinib (Fig. 2C-E). These experiments demonstrate that the DyLight 550 and 488 dyes also produce the lifetime effects seen for Cy5 when conjugated to phosphopeptides and presumably interacting with phosphopeptide-binding protein domains (as we demonstrated previously[1]). This is also consistent with recently reported data on the 5-FAM fluorophore,[2, 3] and supports the interpretation that a range of fluorophores can be used effectively in FLIM probe peptide design.


A
Cy5
DyLight550
DyLight488
B
EGF (2 min)
EGF (20 min)
EGF (40 min), Dasatinib (20 min)
SrcF-DL550
2 ns
Fluorescence lifetime
3 ns
C
-EGF
EGF (~27 min)
EGF (~42 min), Dasatinib (~15 min)
Abl-DL488
2.5 ns
Fluorescence lifetime
4.2 ns
D
-EGF
EGF (~27 min)
EGF (~42 min), Dasatinib (~15 min)
SrcF-Cy5
2.5 ns
Fluorescence lifetime
3.5 ns

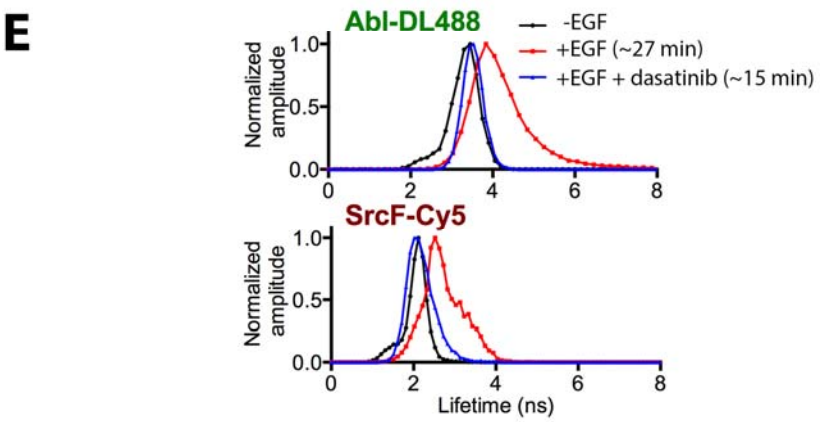

E
Abl-DL488
-EGF
+EGF (~27 min)
+EGF + dasatinib (~15 min)
Normalized amplitude
1.0
0.5
0.0
0
2
4
6
8
SrcF-Cy5
Normalized amplitude
1.0
0.5
0.0
0
2
4
6
8
Lifetime (ns)

Figure 2: Fluorophores other than Cy5 exhibit fluorescence lifetime shifts on kinase activity probes. (A) Structures of the Cy5, DyLight-488 and DyLight-550 fluorophores. (B) SrcF-DL550 (10 μM) was incubated with serum starved MDA-MB-231 cells for 1h, followed by stimulation with EGF and subsequent treatment with dasatinib. (C-D) Abl-DL488 (10 μM) and SrcF-Cy5 (10 μM) were incubated with serum-starved MDA-MB-231 cells for 1h, followed by EGF (10 ng) stimulation (total of ~45 min, ~27 min timepoint shown) and subsequent treatment with dasatinib (1 μM, 15 min). Images normalized to the respective lifetime scales shown. Average lifetime histograms for this experiment are shown in (E). Timepoints given are approximate based on the variability of exact measurement times due to alternating between detection channels.

As a preliminary application of the Abl-DL488 and SrcF-Cy5 probes to multiplexed analysis of kinase activation and inhibition, we performed experiments to image the difference between the effects of dasatinib vs. imatinib on Abl and Src-family kinase activation in EGF-stimulated MDA-MB-231 cells. As before cells were serum starved and incubated with the Abl-DL488 and SrcF-Cy5 probes followed by addition of EGF, and fluorescence lifetime was imaged for each probe over a timecourse after stimulation followed by addition of kinase inhibitor—either imatinib, which inhibits Abl but not Src-family kinases, or dasatinib, which inhibits both. Representative FLIM images in Fig. 3 show that the amount of longer lifetime species detected for the Abl-DL488 probe decreased after adding imatinib (Fig. 3A, C), whereas the SrcF-Cy5 probe

maintained a greater amount of longer lifetime species (Fig. 3B, D). In contrast, both probes showed a decrease in longer lifetime species after adding dasatinib (Abl-DL488: Fig. 3E, G; SrcF-Cy5: Fig. 3F, H). These experiments showed that kinase inhibitor selectivity could be monitored using these FLIM probes.

To quantify the relative amount of the longer lifetime species, 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 multiplexed Abl-DL488/SrcF-Cy5 EGF stimulation/dasatinib inhibition timecourse experiments. This was done by fitting the averaged decay curve for each image to a bi-exponential model function by employing the Levenberg–Marquardt routine for non-linear least squares fitting,[10] as further described in the Supporting Information. This non-biased fitting resolved two different lifetimes, as well as their fractional amplitudes, to identify the average lifetime and its proportional representation for each species. The relative intensity represented by the longer vs. the shorter lifetime species was then calculated and expressed as I_{long}/I_{total}. Results shown in Fig. 4A illustrate that cell-to-cell behaviour is fairly variable, but trends in proportion of longer lifetime (i.e. phosphorylated) species can be observed that are consistent with stimulation and inhibition of Abl and Src-family kinase activities.

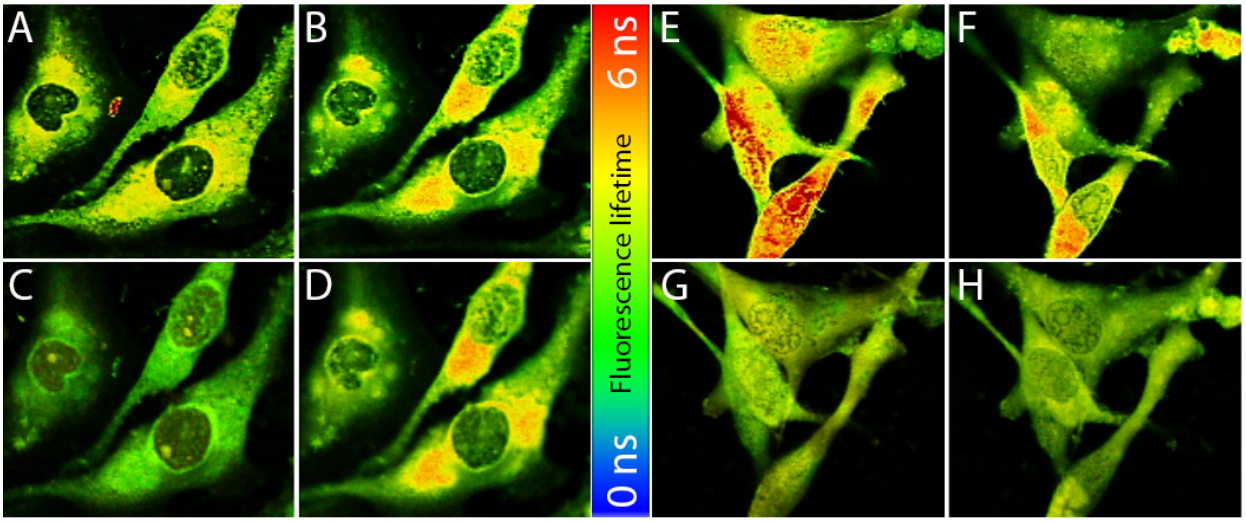


Figure 3: Imatinib inhibits phosphorylation of Abl-DL488 probe but not SrcF-Cy5 probe. Serum-starved MDA-MB-231 cells were treated with the Abl-DL488 (10 μM) (A, C, E, G) and SrcF-Cy5 (10 μM) (B, D, F, H) probes for 1 h. Kinase activity was stimulated with EGF (10 ng) for 25 min, followed by addition of imatinib (5 μM) (A-D) or dasatinib (1 μM) (E-H) for an additional 20 min. Images normalized to the lifetime scale shown.

Finally, to further extend quantification to the subcellular level, we performed proof-of-concept analyses to break the images down further and measure the proportion of longer and shorter lifetime species pixel-by-pixel. Fluorescence decay curves for each pixel were fitted using the bi-exponential model fitting routine described for the average image lifetime (and in the Supporting Information), from 150 x 150 pixel images of the Abl biosensor from –EGF and two timepoints after EGF stimulation. Fitting results were exported to MATLAB to separate matrices of each component: longer lifetime, shorter lifetime, fraction of

longer lifetime and fraction of shorter lifetime. The relative fractions of each lifetime component were then calculated in MATLAB and plotted as a heatmap in Origin (Fig. 4B). These analyses illustrate the potential to obtain subcellular maps of FLIM probe phosphorylation that may eventually be able to be used for more refined quantification of kinase activities in specific areas of the cell, paving the way towards incorporation of FLIM probe data into complex models of signalling behaviour both between and within individual cells.

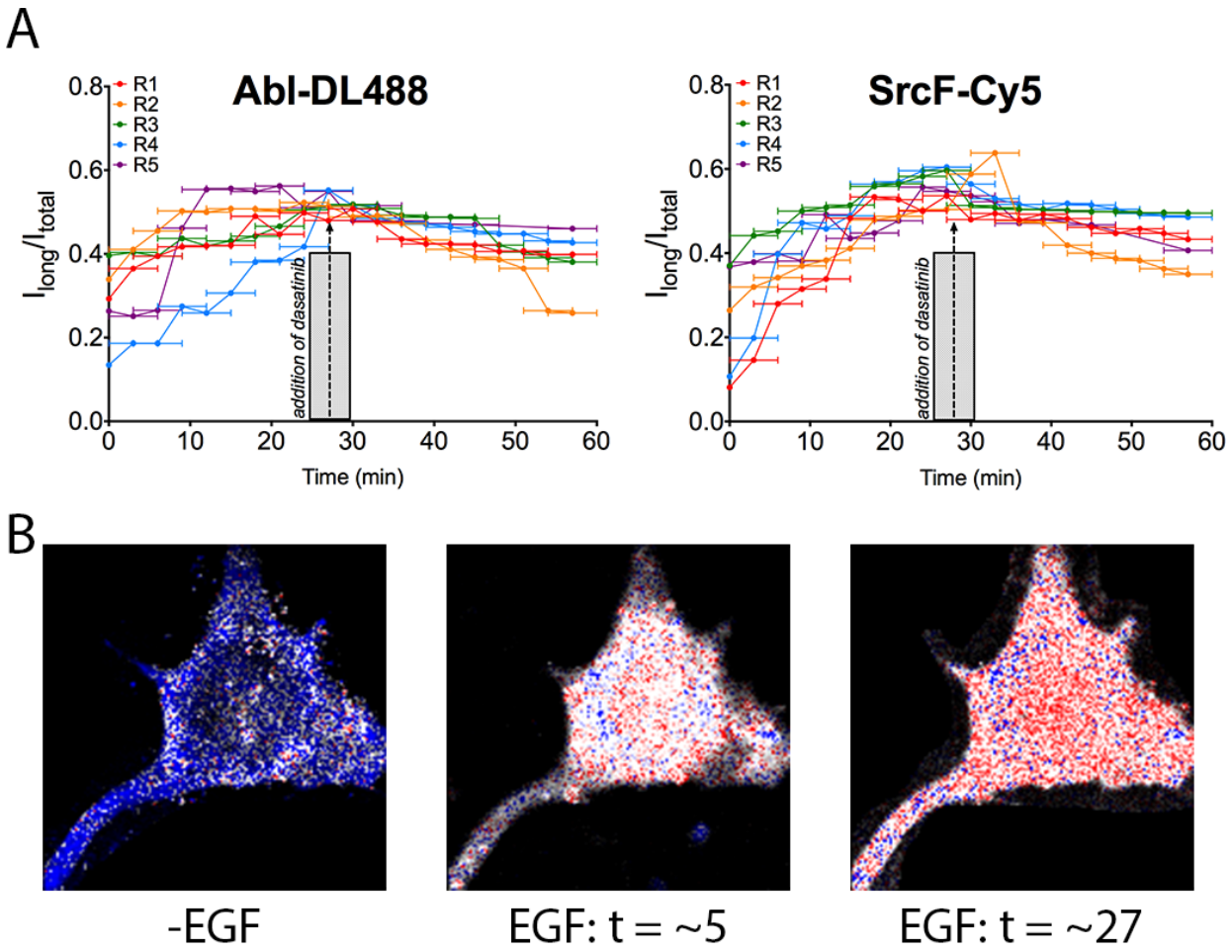


Figure 4: Quantitation of relative probe phosphorylation. *(A) Average FLIM probe phosphorylation per image.* Data from EGF stimulation/dasatinib inhibition experiments (five replicates, R1-R5) were analysed via bi-exponential fitting as described in the supporting information to quantify relative amount of phosphorylated probe per image. Relative amount of phosphorylated probe over time was plotted as the ratio of intensity for the longer lifetime species (I_{long}) to the intensity for both the longer and shorter species (I_{total}). Left: Abl-DL488 probe, Right: SrcF-Cy5 probe. Horizontal error bars (+/- 3 min each) represent the lack of precision in measurement timepoints resulting from limitations in the instrumentation and variable time required to change channels from one color to the other. (B) *Quantitative subcellular maps of relative FLIM probe phosphorylation.* Abl-DL488 FLIM data from one of the cell images from the EGF stimulation experiment were analysed to quantify the relative amount of phosphorylated probe per pixel. Data were analysed as described in the supporting information. Relative amount of phosphorylated probe over time was plotted on a per-pixel basis as the ratio of intensity for the longer lifetime species (I_{long}) to the intensity for both the longer and shorter species (I_{total}). Timepoints are given as approximations due to the instrumentation limitations described above.

Conclusions:

In this work, we showed that combining different fluorophores and peptides into Abl, Src-family and Syk kinase FLIM probes exhibited similar phosphorylation-dependent lifetime change behaviour as previously characterized for an Abl

kinase FLIM probe, implying that the effects of phosphorylation via kinase activity on fluorescence lifetime of these probes is relatively universal. We also demonstrated two-color imaging of Abl and Src-family kinase activities downstream of EGFR stimulation by EGF, and subsequent inhibition with the Abl/Src inhibitor dasatinib. We showed that using a more specific inhibitor, imatinib, produced Abl kinase-specific inhibition results. Finally, we showed for the first time that apparent probe phosphorylation can be quantified on a pixel-by-pixel basis. These results provide further evidence for the generality and multiplexability of this approach, demonstrate the potential to quantify proportions of phosphorylated and unphosphorylated species via multicomponent fitting, and provide a specific strategy for more advanced quantitative study of subcellular kinase activity using these and similar probes.

Acknowledgements: Cells used in this work were a kind gift from Prof. Robert Geahlen (Purdue University Department of Medicinal Chemistry and Molecular Pharmacology). Analyses of cells with the Abl-550 probe were performed on the Nikon AR1 FLIM system with training and support from Dr. Guillermo Marques at the University of Minnesota – University Imaging Centers, http://uic.umn.edu. This work was supported by the Purdue Center for Cancer Research (NPD and JI) and the NCI/NIH (R01CA182543, R33CA183671 and R33217780 to LLP).

References

1. N. P. Damayanti, L. L. Parker and J. M. Irudayaraj, *Angew Chem Int Ed Engl*, 2013, **52**, 3931-3934.
2. N. P. Damayanti, K. Buno, Y. Cui, S. L. Voytik-Harbin, R. Pili, J. Freeman and J. M. K. Irudayaraj, *ACS Sens*, 2017, **2**, 1225-1230.
3. N. P. Damayanti, K. Buno, N. Narayanan, S. L. Voytik Harbin, M. Deng and J. M. K. Irudayaraj, *Analyst*, 2017, **142**, 2713-2716.
4. A. M. Lipchik, R. L. Killins, R. L. Geahlen and L. L. Parker, *Biochemistry*, 2012, **51**, 7515-7524.
5. A. M. Lipchik, M. Perez, S. Bolton, V. Dumrongprechachan, S. B. Ouellette, W. Cui and L. L. Parker, *J Am Chem Soc*, 2015, **137**, 2484-2494.
6. X. Zhang, U. Shrikhande, B. M. Alicie, Q. Zhou and R. L. Geahlen, *Molecular cancer research : MCR*, 2009, **7**, 634-644.
7. B. Fei, S. Yu and R. L. Geahlen, *Biochim Biophys Acta*, 2013, **1833**, 2153-2164.
8. J. S. Biscardi, A. P. Belsches and S. J. Parsons, *Molecular carcinogenesis*, 1998, **21**, 261-272.
9. J. C. Montero, S. Seoane, A. Ocana and A. Pandiella, *Clin Cancer Res*, 2011, **17**, 5546-5552.
10. K. Levenberg, *SIAM J Appl Math*, 1944, **11**, 431-441.