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FRET biosensor to visual Lck kinase activity during TCR Activation

A Thesis submitted in partial satisfaction of the requirements  
for the degree Master of Science

in

Bioengineering

by

Jenny Wan Fong Wu

Committee in charge:

Professor Peter Yingxiao Wang, Chair  
Professor Adam Engler  
Professor Shyni Varghese

2015

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2015

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## ABSTRACT OF THE THESIS

FRET biosensor to visualize Lck kinase activity during TCR activation

by

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Master of Science in Bioengineering

University of California, San Diego, 2015

Professor Peter Yingxiao Wang, Chair

On the molecular level, the immune response begins in T-cells where lymphocyte-specific protein tyrosine kinase p56-Lck (Lck), a Src family kinase (SFK), is one of the first molecules involved in early T-cell activation at T-cell receptors (TCRs). A biosensor utilizing fluorescence resonance energy transfer (FRET) was designed to better understand

and monitor Lck kinase activity during TCR activation by undergoing FRET change when an active Lck phosphorylates the biosensor. According to the *in vitro* data, the Lck FRET biosensor can also report proto-oncogene tyrosine-protein kinase p59-Fyn (Fyn) activity faster than to Lck activity, implying that the Fyn kinase has a higher activity level than the Lck kinase. However, mammalian HeLa cell data showed that the biosensor is more specific to Lck in a physiological setting where kinase-dead Lck(K273R) and Lck(Y394F) can induce a higher FRET change than Lck(WT). In J.CaM1.6 cells, Lck-deficit T-cells, kinase-dead Lck(K273R) did not elicit a FRET change, but kinase-dead Lck(Y394F) continued to elicit a higher biosensor response than Lck(WT). This suggests that Lck may have an adaptor function to recruit other Src family kinases (SFKs) for continuous phosphorylation on the biosensor.

## Chapter 1: Background

### T-cell Activation

Understanding T -cell development and activation, key activities during an immunological response, allows for better insight into how the body fights off diseases, how immune-related diseases arise, and ultimately provide a basis for how to fight against or even prevent such diseases. Activation begins with coupling the major histocompatibility complexes (MHCs) on antigen presenting cells (APCs) to T-cell receptors (TCRs) on T-cells creating immunological synapses (ISs), which initiates a signaling cascade that leads to T-cell activities such as proliferation, differentiation, gene expression, etc.[2] Following the formation of the ISs, lymphocyte-specific protein tyrosine kinase p56-Lck (Lck), a Src family kinase (SFK), is one of the earliest molecules to continue the signal cascade.[1, 2] Although whether Lck activation and recruitment occur before or after the MHCs dock with TCRs is still under debate, roughly 20 – 40% of Lck is constitutively active in naive T cells.[1-5] Maintained at this basal level of phosphorylation in clusters at the plasma membrane, Lck anchors to the membrane by myristoylated and palmitoylated residues on its N-terminus.[5, 6] A unique region in this domain contains a di-cysteine motif that tethers Lck to the cytoplasmic domains of CD4 and CD8 co-receptors integrated in T-cells' transmembrane.[7, 8] Following this region is a Src-homology 3 (SH3), a Src homology 2 (SH2) domain, and the catalytic tyrosine kinase domain that include the positive regulatory tyrosine residue Y394, and negative regulatory Y505 at the C-terminus.[1, 2, 9]

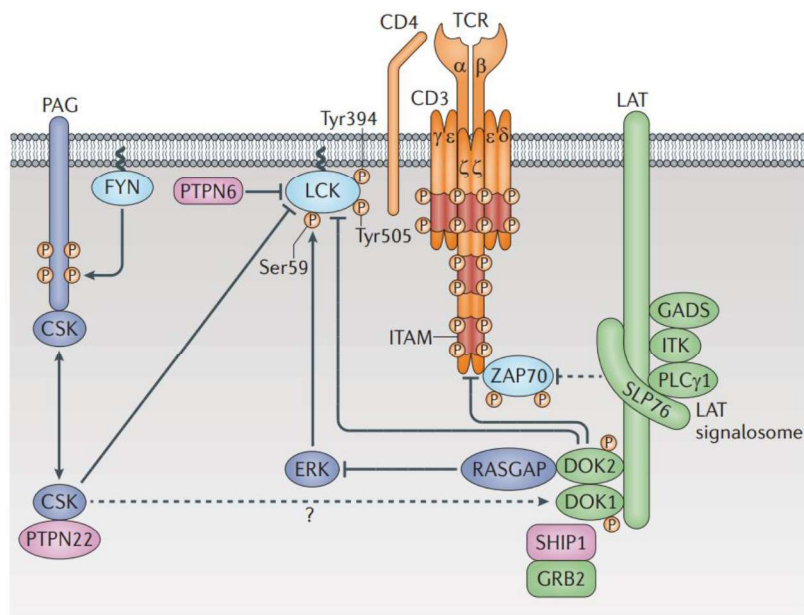


Figure 1: In T-cells, Lck can be dephosphorylated (inactive), phosphorylated on Y505 (inactive), phosphorylated on Y394 (active), or doubly phosphorylated on both Y394 and Y505 (active). Lck's negative regulating Y505 is phosphorylated by Csk and dephosphorylated by CD45. The activating Y394 is autophosphorylated or phosphorylated by Fyn while dephosphorylated by CD45, PTPN22 or PTPN6. [2]

Phosphorylation at the Y505 residue inhibits kinase function by inducing a conformational change that folds the kinase domain onto its SH2 domain, causing the protein to adopt a closed conformation further stabilized by interactions between the SH3 domain and SH2-kinase linker region.[1, 2, 10] PAG/Cbp, a transmembrane phosphoprotein associated with a glyco-sphingolipid-rich SH2 domain, recruits C-terminal Src kinase (Csk) to phosphorylate Y505, but proto-oncogene tyrosine-protein kinase p59-Fyn (Fyn) can dephosphorylate PAG, which releases Csk to bind with PTPN22 and inhibits Csk from phosphorylating Y505.[2, 5, 11] Fyn, a SFK also found in T-cells, shares overlapping common substrates with Lck, thereby allowing Fyn to compensate for many of Lck's early signaling pathways in T-cell development.[12, 13] Moreover, Fyn can also phosphorylate Lck at Y394, the activating residue, which otherwise autophosphorylates thereby activating Lck by inducing an open conformation that separates the kinase domain

from the SH2 domain. This exposes Lck's catalytic site while dephosphorylation by CD45 at Y394 will cause Lck to readopt the closed conformation.[1, 14] Figure 1 depicts a graphical representation of Lck's regulatory pathways in further details while Figure 2 shows the difference between Lck's inactive and active forms.

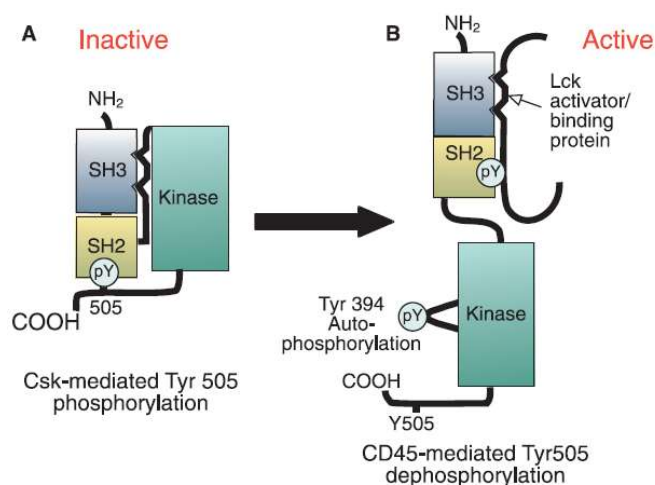


Figure 2: (A) Csk phosphorylation of the Y505 results in an intra-molecular association between the SH2 domain and C-terminal tail further stabilized by additional interactions between the SH3 domain and a polyproline sequence in the SH2-kinase linker region. (B) CD45 dephosphorylation of Y505 results in an open conformation and exposes the activation loop (A-loop) containing the activating Y394 residue, which then undergoes trans-phosphorylation to promote enzymatic activity.[1]

Upon activation and recruitment to the activated TCR, Lck phosphorylates two tyrosine residues on separate CD3 chains along the immunoreceptor tyrosine-based activation motifs (ITAMs) with a D/ExxYxxI/Lx(6-12)YxxI/L peptide sequence.[15-17] ITAMs occur once on the CD3 $\gamma$ , CD3 $\epsilon$ , and CD3 $\delta$  chains and three times on the CD3 $\zeta$  chains, where Lck phosphorylation on the  $\zeta$  chains has the highest tendency to recruit zeta-chain associated protein of 70kDa (Zap70), a Syk family kinase, to continue the signaling process.[16, 18] Zap70 adopts similar active and inactive conformations to Src kinases involving interactions between the regulatory segment and the linker to the kinase domains that reduces flexibility. A doubly phosphorylated ITAM recruits Zap70 through its tandem-

SH2 domains, bringing Zap70 closer to the plasma membrane where Lck then phosphorylates Y315 and Y319, which induces a more flexible conformation by disrupting the interaction between the regulatory segment and linker region. The new conformation opens Zap70's activation loop and allows for Y493 phosphorylation, which activates ZAP-70.[19, 20] The activated Zap70 then phosphorylates the key adapter protein linker for activation of T cells (LAT) that eventually leads to actin reorganization, cell adhesion, gene expression, etc. as summarized in Figure 3.

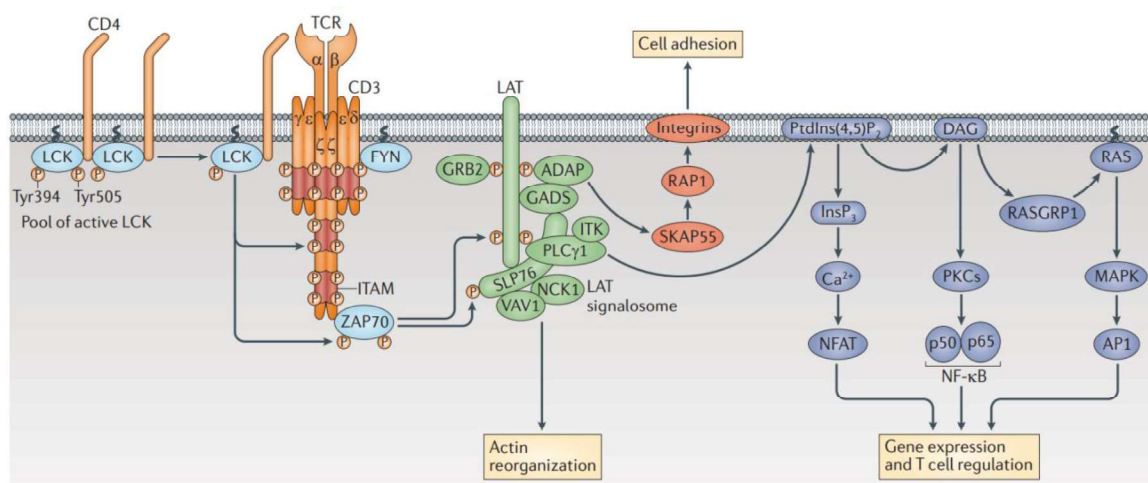


Figure 3: Lck, the first molecule recruited to the TCR–CD3 complex, can phosphorylate the ITAMs of the CD3 $\gamma$ , CD3 $\delta$  chain, CD3 $\epsilon$ , and CD3  $\zeta$  chains. Phosphorylation of the CD3  $\zeta$  chains recruits ZAP70, which is then activated by Lck phosphorylation. Activated ZAP70 recruits and phosphorylates a multi-protein complex termed the LAT signalosome that initiates other downstream signal pathways.[2]

### Fluorescence Resonance Energy Transfer Biosensor Design

Most notable in T-cell activation of the immune system is Lck, the kinase that essentially drives most of the upstream signaling process; hence, tracking that molecule will provide fundamental information into the dynamics of TCR activation. By utilizing fluorescence resonance energy transfer (FRET) technology, a Lck FRET biosensor can be designed to monitor Lck kinase activity.

Exciting fluorophores or fluorescent proteins at specific wavelengths in the electromagnetic spectrum will cause them to emit a different wavelength of light when they return to their original energy state. FRET is the phenomenon that occurs when the emission spectrum of a donor fluorophore overlaps the excitation spectrum of an acceptor fluorophore, thus allowing energy transfer that manifest as a measurable visual signal of the FRET ratio, the acceptor/donor emission ratio, which depends on the proximity of the two fluorophores. The biosensor changes orientation by adopting a different conformation when a molecule of interest binds to or interacts with its substrate region, which can bring two fluorophores closer or further in proximity. The Lck FRET biosensor's different orientations are shown in Figure 4 where the two fluorophores are in close proximity initially at its basal state and exhibit FRET. The substrate is designed to become phosphorylated by an activated Lck upon TCR activation, which then interacts with the SH2 domain by folding on itself, blocking the two fluorophores from each other and disrupting FRET. The specified SH2 domain is designed from Src's SH2 domain with a C185A mutation that has shown to have increased sensitivity to a phosphorylated substrate thereby inducing a FRET change at even low kinase activity. (data not published yet) The fluorophores used for the FRET pair are cyan fluorescent protein (ECFP) with an excitation at a peak of 433nm and emission of 475nm, and yellow fluorescent protein (YPet) with an excitation peak of 517nm and emission of 530nm.[21]



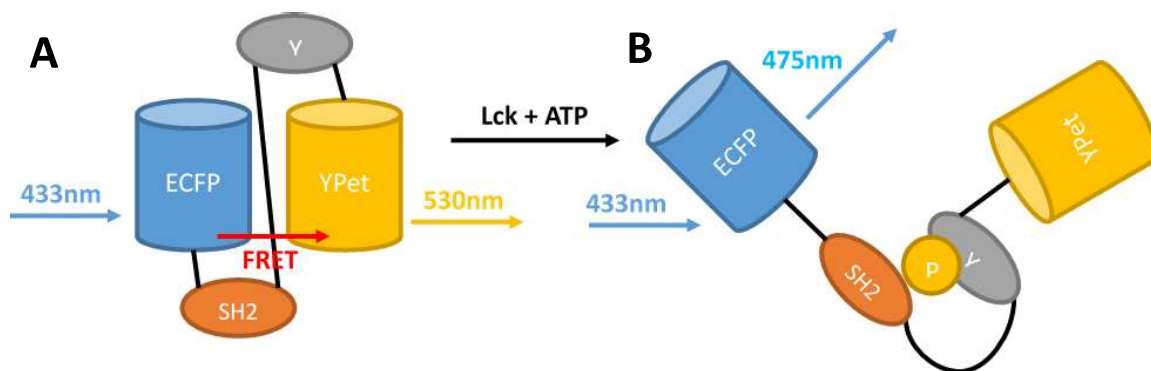


Figure 4: Generalized graphic representation of the FRET biosensor showing (A) the initial orientation of the two fluorophores in close proximity inducing FRET. (B) Upon phosphorylation on the substrate, the biosensor reorients and blocks the two fluorophores from inducing FRET.

As detailed in Chapter 1: Background, potential substrates to implement into the biosensor includes Zap70's Y315, Y319, and Y493 tyrosine sites, the tyrosine sites on the CD3 $\zeta$  chains' ITAMs, and Lck's own Y394, all of which are sites that endogenous Lck readily phosphorylates. For Zap70, since Y493 is much further away from the TCR where Lck docks, the tyrosine site is speculated to be nonspecific to Lck; moreover, Zap70 with a point mutation on Y493 can still interact with Lck and be phosphorylated.[22, 23] For the remaining up-regulation tyrosine sites on Zap70, one substrate design combines the Y315 and Y319 sites with a second alternative substrate design that mutates Y319F to prevent competitive binding problems; in addition, the mutation on the Y315 has shown to have minimal inhibitory effects on TCR activation.[22] Of the three ITAMs located on the CD3 $\zeta$  chains, the last motif has shown to be the ITAM that is mostly likely to recruit Zap70 and therefore may have the greatest affinity for Lck phosphorylation.[18, 24] Due to the length of the ITAM, two separate substrate designs encompassing the first and second tyrosine sites respectively were created. Since Lck can autophosphorylate itself on Y394 *in trans*, the last substrate design includes that region of Lck. [1, 2, 14, 25] Figure 5 summarizes the different substrate designs.

Original Polypeptide	Substrate Sequence
Zap70	MDTSV <b>Y(315)</b> ESP <b>Y(319)</b> SDPEE
Zap70	MDTSV <b>F(315)</b> ESP <b>Y(319)</b> SDPEE
CD3 $\zeta$ 3 <sup>rd</sup> ITAM (first tyrosine site)	GHDGL <b>Y</b> QGLST
CD3 $\zeta$ 3 <sup>rd</sup> ITAM (second tyrosine site)	ATKDT <b>Y</b> DALHM
Lck	IEDNE <b>Y(394)</b> TAREG

*Figure 5: The proposed substrate designs for the Lck FRET biosensors are (1) Zap70Y315-Y319, (2) Zap70Y315-Y319F, (3) CD3 $\zeta$  chain's third ITAM at the first tyrosine site, (4) CD3 $\zeta$  chain's third ITAM at the second tyrosine site, and (5) LckY394 (top to bottom).*

## Chapter 2: Results

### *in vitro* Kinase Assay

The purified biosensors were subjected to an *in vitro* kinase assay for a specificity check with different SFKs. Given the similarities between Fyn and Lck, both of which are SFKs, the initial specificity check includes the Src, Fyn, and Lck kinases. The Fyn biosensor created by Dr. Mingxing Ouyang in Dr. Peter Yingxiao Wang's lab (Wang Lab) proven to have a robust response to the Fyn kinase is also included to ensure Fyn activeness. (data not published yet) Figure 13 (Appendix) displays all the different test conditions for the *in vitro* kinase assay and their resulting normalized absorbance response at the ECFP emission wavelength (476nm) divided by the YPet emission wavelength (528nm). Figure 6 removed all other data except for the biosensors' responses to Lck and Fyn for direct comparison. The biosensors' fluorescence spectrum is depicted in Figure 14 (Appendix) where the absorbance peaks at 530nm, YPet's emission wavelength, showing that the biosensor undergoes FRET at the basal state before kinase or ATP addition to initiate phosphorylation as designed.

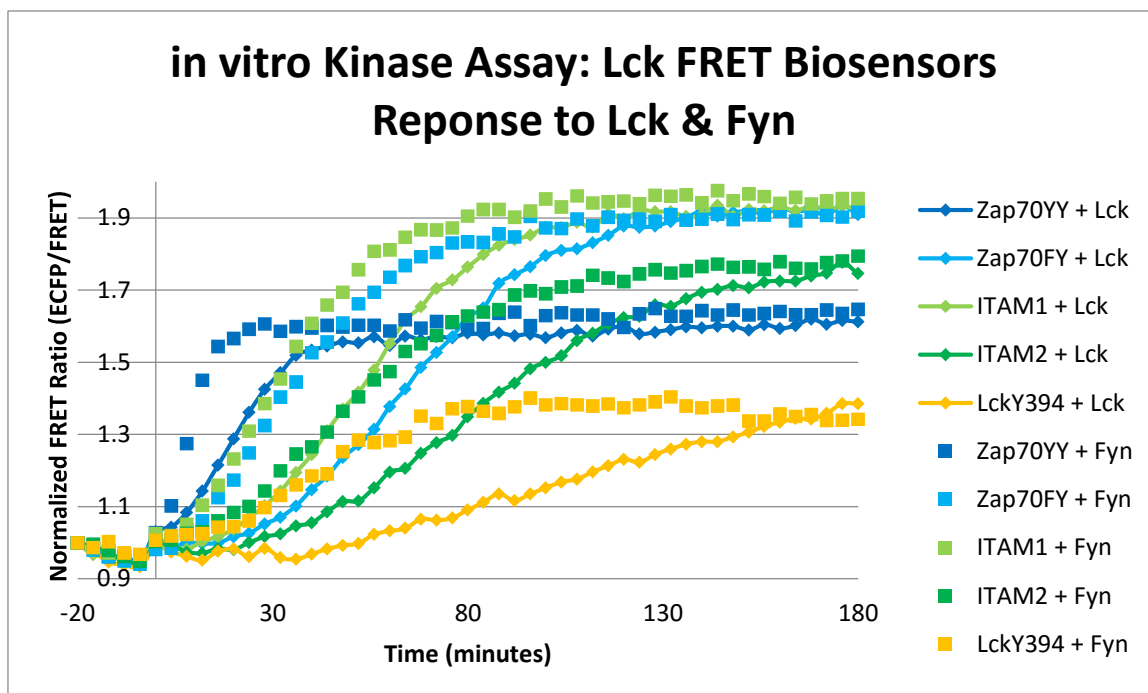


Figure 6: *in vitro* responses of all Lck FRET biosensors with the substrates: Zap70YY, Zap70FY, ITAM1, ITAM2, and LckY394 to Lck and Fyn kinase.

The *in vitro* data shows that the Lck FRET biosensors response to Src is similar to the negative control of no kinase, namely no response. For the remaining Lck and Fyn kinases, each Lck FRET biosensor, regardless of the substrate, displayed an overall tendency to react quicker to Fyn, thereby indicating a greater specificity between Fyn and the substrate. However, increasing the Lck kinase amount until the specified activity level of the Lck kinase matches the activity level of Fyn kinase according to their datasheets resulted in a near identical response as shown in Figure 15 (Appendix). The Zap70YY substrate showed the quickest response within ten minutes of adding ATP followed by the ITAM1 and Zap70FY substrates that have a very similar response strength within ten minutes of each other. The LckY394 substrate shows the slowest and weakest change in FRET response, indicating a lower specificity between the kinases to the substrate design so it will not be further pursued as a candidate for the Lck FRET biosensor.

## Mammalian Cell Characterization

Initial cellular imaging in SYF(-/-) cells, shown in Figure 16 (Appendix), displayed contradicting results where the control group, co-transfection of the biosensor with an empty mammalian vector in place of a kinase plasmid, displayed a robust biosensor response. From this data, ITAM2 displayed the lowest response consistent with the *in vitro* data, which led to the cessation of using that substrate design. However, the same imaging data also establishes that the Lck FRET biosensors are comparatively less sensitive to Fyn and Src as shown in Figure 16 (Appendix). To determine if the biosensor may be reacting to the platelet-derived growth factor (PDGF) used to stimulate the cells, PDGF receptor (PDGFR) and epidermal growth factor receptor (EGFR) was added to the purified biosensors in a second *in vitro* assay. As depicted in Figure 17 (Appendix), the biosensors response to PDGFR is quicker and stronger than the responses to Lck or Fyn kinase as compared to in Figure 6, reporting a FRET change in less than ten minutes with over a 2-fold change. This does not pose an issue since the Lck FRET biosensor shall be utilized in T-cells, which do not express PDGFR endogenously and will be stimulated by CD3 antibodies for physiological relevance. However, SYF(-/-) cells will no longer be used for mammalian characterization in favor of HeLa cells that use EGF for stimulation, which has shown to not elicit a false response from the biosensors as noted in Figure 17 (Appendix).

Imaging data for HeLa cells after EGF stimulation continued to show a robust FRET response as seen in Figure 7 across all the different co-transfection conditions. Co-transfection agents include an empty vector for control, Lck(WT), Fyn(WT), kinase-dead Lck(K273R), kinase-dead Lck(Y394F), and kinase-dead Fyn(K299M). Research has shown that the latter kinase-dead versions exhibit low phosphorylation in downstream

substrates.[26-28] In both the Zap70YY and ITAM1 substrate designs, co-transfection with Lck(WT) displayed a pre-activated biosensor that showed no further change after stimulation. In both the Zap70YY and Zap70FY substrate designs, kinase-dead Fyn(K299M) produced a delayed response ranging from 10 – 20 minutes after stimulation that ended in a FRET change similar in level to the different WT or kinase-dead kinases. Both of these designs also showed a stronger basal response between the biosensor to Lck(WT) and Fyn(WT) as opposed to the other kinases, although the Zap70FY does display a stronger FRET change for Lck(WT) than Fyn(WT). For the Zap70YY design, the control shared a similar response to the kinase-dead Lck(K273R) and Lck(Y394F) in both the basal FRET ratio and FRET change after stimulation. Zap70FY on the other hand showed a clearer distinction between the control and kinase-dead Lck in both cases. All biosensor designs that were pretreated with PP1, a SFK inhibitor, exhibited a lowered basal FRET ratio and stunted FRET change or no response at all when EGF was added. However, due to the relative lower basal FRET ratio and FRET change for the ITAM1 substrate design, the design was determined to be unsuitable for continued testing. Moreover, since the Zap70FY substrate is able to show a more distinct differentiation between the different kinases and had an overall stronger FRET change than the Zap70YY substrate, ongoing biosensor testing will be conducted on the Zap70FY substrate design.

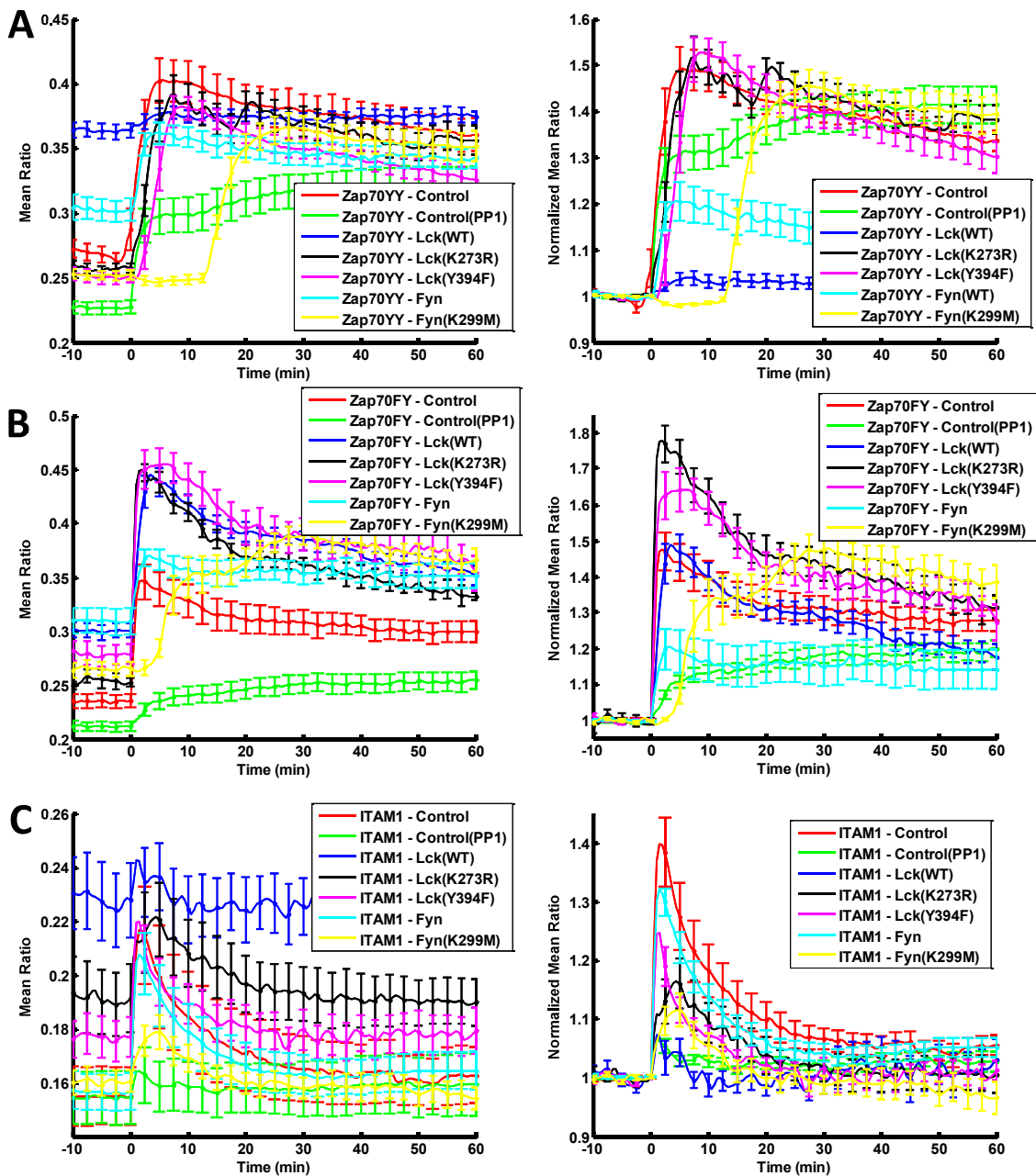


Figure 7: Real (left) and normalized (right) FRET responses in HeLa cells co-transfected with the Lck FRET biosensors of different substrate designs and specified kinases for (A) Zap70YY, (B) Zap70FY, and (C) ITAM1 upon EGF stimulation at 0 minutes for all conditions.

To confirm that the FRET response from the imaging data is strictly from phosphorylation on the biosensor, HeLa cells were co-transfected with the negative Zap70FF (Y315F,Y319F) Lck FRET biosensor or negative Zap70FF biosensor with the doubly mutated SH2 domain (R175V, C185A) in addition to the different kinases as shown in Figure 8. Due to the highly sensitive nature of the mutated SH2 domain (C185A) to kinase-like peptides, conformation change on the FRET biosensor can still be induced even without direct phosphorylation albeit only at a minimal level. Mutating the R175V residue can remove potential non-specific interactions between the SH2 domain and kinase-like peptides without interfering with the affinity between the domain and a phosphorylated tyrosine residue on the substrate. [29] Figure 18 (Appendix) depicts a representative set of FRET images with the subtracted background for the different co-transfection conditions in HeLa cells. FRET images for the doubly mutated SH2 domain are not shown.

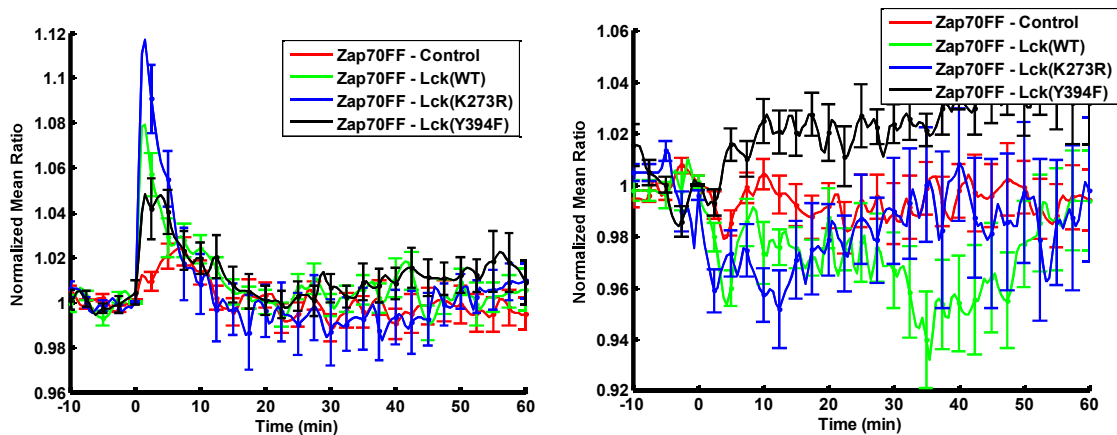


Figure 8: Negative Lck FRET biosensor, Zap70FF, (left) and Zap70FF biosensor with doubly mutated SH2 domain (R175V, C185A) (right) in response to the specified co-transfected kinases upon EGF stimulation added at 0 minutes for all conditions.



## T-Cell Response

Functionality of the Lck FRET biosensor in the T-cell model was confirmed by transfection of the normal biosensor into Jurkat cells, an immortal T-cell line, as shown in Figure 9. The FRET change from the biosensor response comes from kinase activity as seen by the decrease in FRET ratio upon PP1 addition. Another group of Jurkat cells were transfected with the negative biosensor, Zap70FF, to show that the FRET change is induced by phosphorylation as seen by the lack of response even after adding the stimulant.

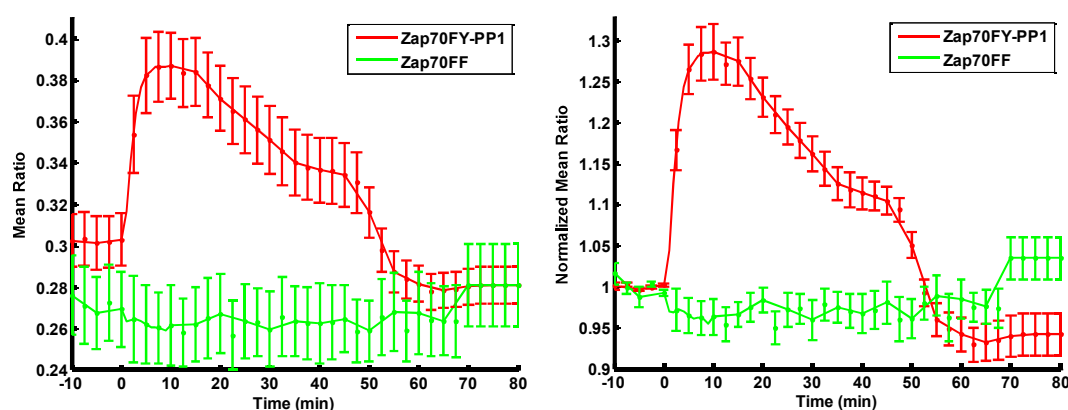


Figure 9: Real (left) and normalized (right) FRET responses in Jurkat cells transfected with the Zap70FY Lck FRET biosensor ( $n = 19$ ) or Zap70FF negative biosensor ( $n = 10$ ). Stimulant and PP1 inhibitor was added at 0 and 45 minutes respectively.

J.CaM1.6, a derivative mutant of the Jurkat cell line deficit in Lck, was co-transfected with the Lck FRET biosensor along with an empty vector, Lck(WT), kinase-dead Lck(K273R), or kinase-dead Lck(Y394). As expected from Figure 10 the empty vector serves as a control to show that the Lck FRET biosensor does not respond in a Lck-deficit environment. Lck(WT) co-transfection was able to restore some kinase activity as compared to responses in the Jurkat cells. As opposed to both kinase-dead Lck kinases causing a biosensor response as seen in HeLa cells, only Lck(Y394F) triggered a FRET

change that is not only greater than the change exhibited in the cells co-transfected with Lck(WT), but also matches closer to the response in Jurkat cells.

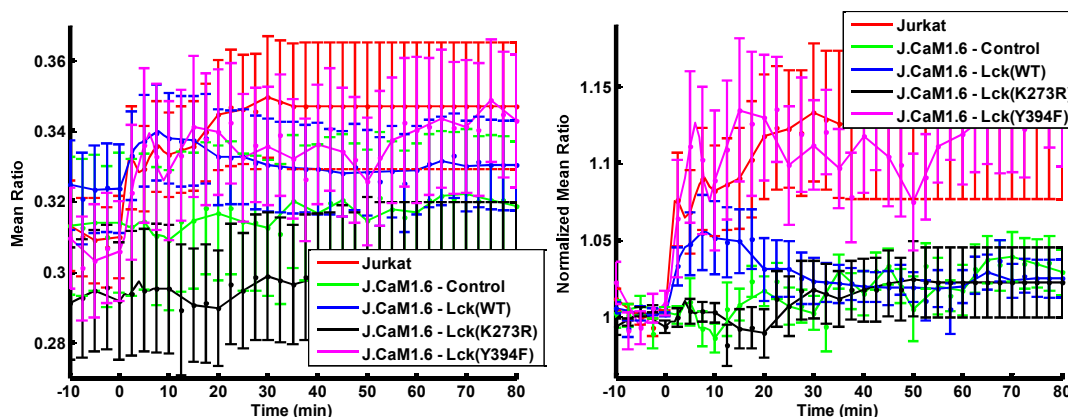


Figure 10: Real (left) and normalized (right) FRET responses in Jurkat cells transfected with the Zap70FY Lck FRET biosensor along with J.CaM1.6 cells co-transfected with the same biosensor and specified kinases. 8 out of the 12 imaged Jurkat cells, 12 out of the 26 imaged J.CaM1.6 cells co-transfected with Lck(WT), and 7 out of the 25 imaged J.CaM1.6 cells co-transfected with Lck(Y394F) exhibited the FRET change. None of the 26 J.CaM1.6 cells co-transfected with the control or 14 J.CaM1.6 cells co-transfected with Lck(K273R) exhibited any change. Stimulant was added at 0 minutes for all conditions.

The Zap70FY Lck FRET biosensor response is due to SFK activity as confirmed by the decrease in the FRET ratio after adding PP1 as seen in Figure 11. The negative Zap70FF biosensor co-transfection with Lck(Y394F) in J.CaM1.6 cells also shows no response upon stimulation further validating that the recorded response is due to a real phosphorylation event. Representative FRET images for both Jurkat and J.CaM1.6 cells in the different specified conditions are displayed in Figure 19 and Figure 20 (Appendix) respectively for reference.

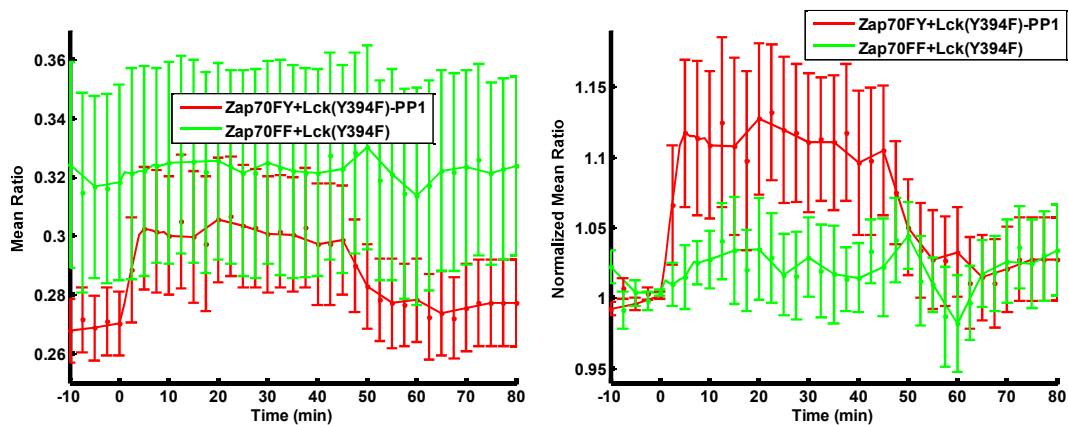


Figure 11: Real (left) and normalized (right) FRET responses in *J.CaM1.6* cells co-transfected with the Zap70FY Lck FRET biosensor and negative Zap70FF biosensor with kinase-dead Lck(Y394F). 4 out of the 12 imaged *J.CaM1.6* cells co-transfected with Zap70FY exhibited the FRET change and none of the 10 imaged *J.CaM1.6* cells co-transfected with Zap70FF showed change. Stimulant and PP1 inhibitor was added at 0 and 45 minutes respectively.

### Chapter 3: Discussion

In the *in vitro* kinase assay, the designed Lck FRET biosensor shows specificity towards both Lck and Fyn, but not Src, which follows the trend of Lck and Fyn having redundant functionality in T-cells.[1, 2, 9, 12] Research has shown that Lck(-/-) T-cells showed partially impaired T-cell differentiation, Fyn(-/-) T-cells showed no significant impairment, and Lck(-/-) + Fyn(-/-) T-cells showed a complete lack of differentiated T-cells, thus, providing evidence that Fyn can compensate for Lck during early stages of T-cell differentiation.[13, 30, 31] Regardless of the substrate design, the biosensors showed a consistent tendency of reacting quicker with the Fyn kinase than with the Lck kinase. This alludes to the fact that while Lck may play a greater role in initiating many of the signal pathways in T-cells, Fyn's greater activity level may play a greater role in amplifying the same early signaling events in downstream pathways.[12]

In the mammalian HeLa cell model, the biosensor shows a greater response towards the co-transfected Lck as opposed to the co-transfected Fyn kinase, which establishes that the biosensor has greater specificity towards Lck in a more physiological setting. A notable phenomena that occurred during the characterization was that the kinase-dead versions of Lck were able to elicit a stronger biosensor response than Lck(WT). The kinase-dead Lck Lck(K273R) has lost the ability to transfer the phosphate group from ATP to phosphorylate downstream peptides and kinase-dead Lck(Y394F) is rendered constitutively inactive due to the mutation on its activation residue.[26, 27] Control testing with PP1 treatment and the negative Zap70FF Lck FRET biosensor confirms that the FRET change is due to a real phosphorylation event that occurs from an SFK. This indicates that although the kinase-

dead Lck can no longer phosphorylate downstream peptides, the kinase is still able to recruit other SFKs to initiate phosphorylation. Since Fyn is similar to Lck with overlapping substrates, Fyn was also studied to determine if kinase-dead Fyn shares any adaptor-like function by co-transfecting HeLa with kinase-dead Fyn (K299M) whose phosphorylation function is impaired similarly to Lck(K273R).[28] Although Fyn(K299M) transfection was able to show a robust FRET change that is comparable to the kinase-dead Lck kinases in terms of magnitude, the response is noticeably delayed. This indicates that although kinase-dead Fyn(K299M) can adopt some adaptor-like function, the function is not primary and appears to encounter preliminary interference. Although the biosensor can also react to Fyn as seen in the *in vitro* data, the biosensor is not as sensitive to Fyn since the substrate design is specific to Lck. Hence, although a Fyn kinase can intermittently interact with the Lck FRET biosensor, lower affinity means the Fyn kinase is not likely to remain long on the biosensor. Within that small window of interaction, if Fyn does not quickly phosphorylate the substrate, the biosensor will not undergo any FRET change, thus explaining why Fyn(K299M) does not immediately elicit a response upon stimulation.

In the Jurkat T-cell line, the only place where Lck is endogenously found in the human body, the Lck FRET biosensor reported a FRET change and performed as expected establishing physiological significance. Additionally, in J.CaM1.6 cells, the biosensor reported a phenomena where kinase-dead Lck(Y394F) co-transfection displayed greater kinase activity than Lck(WT) co-transfection similarly to the trend displayed in HeLa cells. Although unlike the HeLa cell model, the other kinase-dead Lck(K273R) did not report a similar increased biosensor response. Since the Zap70FY Lck FRET biosensor design uses a section of the Zap70 from M310 – E324, mutated at Y315F, where Y319 is known to

undergo autophosphorylation, the FRET change may be due to Zap70 activity and not Lck.[19, 20, 22, 32] Further control testing showed that the biosensor response is solely due to phosphorylation and related to SFKs activity as noted by the lack of or decreased response in the negative biosensor transfection and PP1 inhibition in Jurkat cells and J.CaM1.6 cells co-transfected with Lck(Y394F). Since Lck(Y394F) has lost its phosphorylation ability, the proposed mechanism behind why a kinase-dead co-transfection can still induce kinase activity may be due to Lck's possible adaptor function. Lck clusters are usually found in varying size, locations, and states before, during, and after TCR activation that appears to be dependent on Lck's active/opened or inactive/closed conformations.[3, 4, 6] Given that the regulatory C-terminal tail interacts with the SH2 domain, which in turn affects how the SH2 domain interacts with the SH3 domains of other kinases, Lck's conformation appears to play a role in how Lck cluster with each other.[33, 34] In fact, a phosphorylated Y505 not only triggers Lck's closed inactive conformation, but also folds Lck in such a way that Lck can potentially dimerizes with other Lck kinases in a head-to-tail orientation that further discourages autophosphorylation between them.[35] Moreover, upon phosphorylation on Y394, the kinase domain reorients itself to interact with the SH2 domain to expose the catalytic center around K273 where ATP is anchored and the phosphate group is transferred for phosphorylation activity.[33, 36] This suggests that in addition to the regulatory C-terminal tail, Lck's other regulatory residue may also play a part in how Lck interacts with other Lck or similar SFKs during clustering. Instead of clustering only with other Lck kinases, the mutated Lck(Y394F) may potentially recruit other SFKs, which is not uncommon given that endogenous Lck can interact with Fyn upon TCR stimulation. [37, 38]

Since the kinase-dead Lck(Y394F) appears to induce a higher FRET change in the biosensor, the mutation may possibly be (1) lowering Lck's affinity to cluster with other Lck and/or (2) increasing the affinity between kinase-dead Lck with active SFKs. In scenario (1), although Lck is more likely to cluster with other Lck in the endogenous setting, the kinase-dead Lck(Y394F) may have a lower tendency to cluster with itself, thereby allowing active SFKs to cluster instead. In addition to having a lower affinity with itself, for scenario (2), kinase-dead Lck(Y394F) may also increase the affinity between kinase-dead Lck and active SFKs such that the SFKs remain near the biosensors continuously phosphorylating them. In short, even though mutation of Y394F on Lck can lead to the loss of the exposing the catalytic region for ATP anchorage, the potential recruitment of other SFKs, especially Fyn who shares similar substrates to Lck in T-cells, can still phosphorylate the biosensor. Figure 12 displays the proposed mechanism of how the Lck FRET biosensor can still report a FRET change when the kinase-dead Lck(Y394F) recruits a SFK, potentially Fyn, to phosphorylate the biosensor.

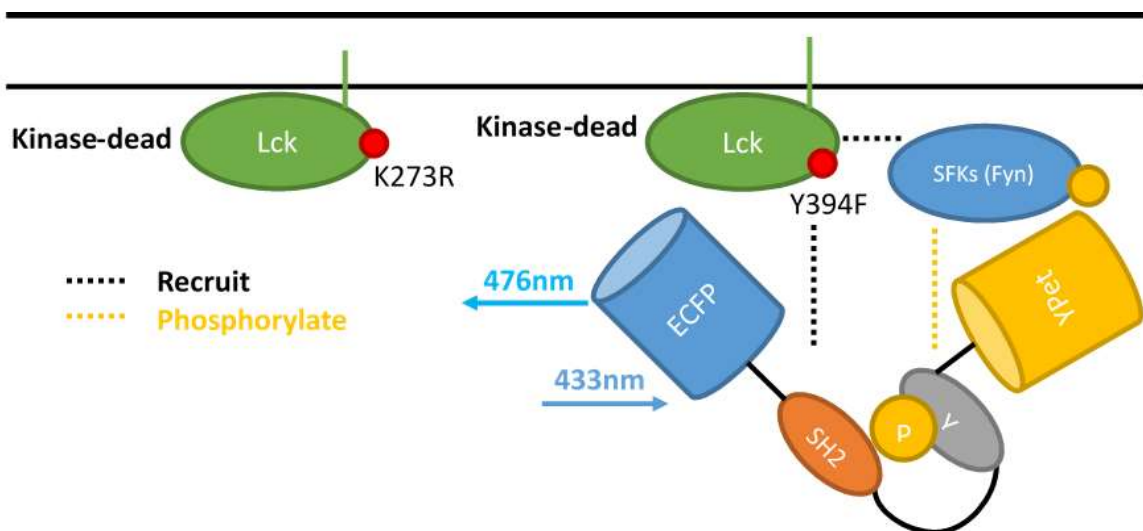


Figure 12: Proposed mechanism of how the Lck FRET biosensor can still report a FRET change in a Lck-deficient environment with the kinase-dead Lck(Y394F), which may potentially be recruiting other SFKs, most likely Fyn to phosphorylate the biosensor.

## Chapter 4: Materials & Methods

### Plasmids

pRSET-B bacterial expression constructs of the different Lck substrates were designed using a Src biosensor created in Wang Lab as the template. (data not published yet) The fluorophores and mutated SH2 domain (C185A) from Src were conserved while the Src-specific substrate is replaced with the Lck-specific substrates. The constructs were transformed into DH2 $\alpha$  competent *E. coli* cells for DNA amplification. Mammalian expression constructs were constructed in a similar manner, but using the pRSET-B bacterial constructs as the template into a modified pCAGGS mammalian vector named pCB1.[39, 40] The biosensor with the ITAM2 substrate was constructed into a pcDNA3.1 vector using Gibson Assembly (New England Labs) due to difficulties in constructing a pCB1 version. The negative Zap70FF biosensor was constructed with site-directed mutagenesis from the pRSET-B version and then constructed into a pcDNA3.1 vector with Gibson Assembly. The biosensor with the doubly mutated SH2 (R175V, C185A) domain to further discourage conformational change was constructed with site-directed mutagenesis from the negative Zap70FF biosensor.

Lck(WT)-CFP, Lck(K273R)-CFP, and Lck(Y392F)-CFP were gifts from the Centre for Vascular Research and Australian Centre for Nanomedicine, University of New South Wales, Sydney, Australia. The CFP tags were removed from Lck(WT), Lck(K273R), and Lck(Y394F) by introducing a stop codon at the end of the kinase and before the CFP sequences. Src, Fyn, and Fyn(K299M) plasmids were from the Wang Lab.



All plasmids were purified with the QIAquick Gel Extraction Kit and QIAprep Spin Miniprep Kit (Qiagen).

#### *in vitro* Assay

Biosensor plasmids were transformed into BL21 competent cells for protein amplification and purified with the Kimble-Chase protein purification kit (Fisher Scientific). Active Lck, Fyn, Src, PDGFR, and EGFR proteins (Sigma-Aldrich) were pre-mixed with the purified biosensor in a 96-well microplate in the *in vitro* assay with 2mM DTT diluted with kinase buffer and stimulated with 1mM ATP. Absorbance data was collected with Tecan infinite M1000 PRO using the i-control 1. 10. software

#### Cell Preparation

SYF (-/-) and HeLa cells were cultured in Advanced DMEM media (Gibco) added with 10%.FBS, penicillin-streptomycin, and L-glutamine, transfected in transfection media (no penicillin-streptomycin added), and starved in starvation media (no FBS or penicillin-streptomycin add). Cells were cultured at 37°C in a 5%-CO<sub>2</sub> and 95%-humidified incubator. Transfection was conducted with the Lipofectamine 3000 kit (Sigma-Aldrich) 48 hours before imaging in transfection media. Cells were then plated onto glass-bottom dishes (Cell E&G) coated overnight with fibronectin at 10µg/mL concentration in starvation media 24 hours before imaging. During imaging, cells were stimulated with PDGF or EGF at 100ng/mL and inhibited by PP1 for half an hour at 10µM before imaging for the PP1 pre-treatment.

Jurkat E6.1 and J.CaM1.6 cells (ATCC) were cultured in RPMI medium (Gibco) added with 10%.FBS, penicillin-streptomycin, and sodium pyruvate, transfected in

transfection media (no penicillin-streptomycin added), and starved in starvation media (no FBS or penicillin-streptomycin add). Cells were cultured at 37°C in a 5%-CO<sub>2</sub> and 95%-humidified incubator. Transfection was conducted by electroporation 24 hours before imaging in starvation media and plated onto glass-bottom dishes (Cell E&G) coated overnight with nonspecific IgG secondary antibodies at 10µg/mL ten minutes before imaging. During imaging, cells were stimulated with a mixture of CD3 and co-stimulatory CD28 antibodies at 10µg/mL and 5µg/mL respectively that were pre-clustered with IgG conjugated with biotin and further clustered with streptavidin. Cells were inhibited by PP1 at 10µM 45 minutes after stimulation was added.

#### Image Acquisition and Analysis

A Nikon Eclipse Ti inverted microscope installed with a 300 W Xenon lamp (Atlas Specialty Lighting), an electron multiplying (EM) CCD camera (QuantEM:512SC, Photometrics), and a 100x DIC Nikon microscope objective (NA 1.4) was used to capture all imaging data with the MetaMorph 7.8.8.0 software (Molecular Devices). The microscope is further equipped with a 420DF20 excitation filter, 455DCXRU dichroic mirror, and two emission filters controlled by a filter changer (480DF40 for ECFP and 535DF25 YPet) (Chroma Technology). Image analysis for all the acquired images was conducted on Fluocell, an image analysis software tool developed in the Wang Lab. (data not published yet)

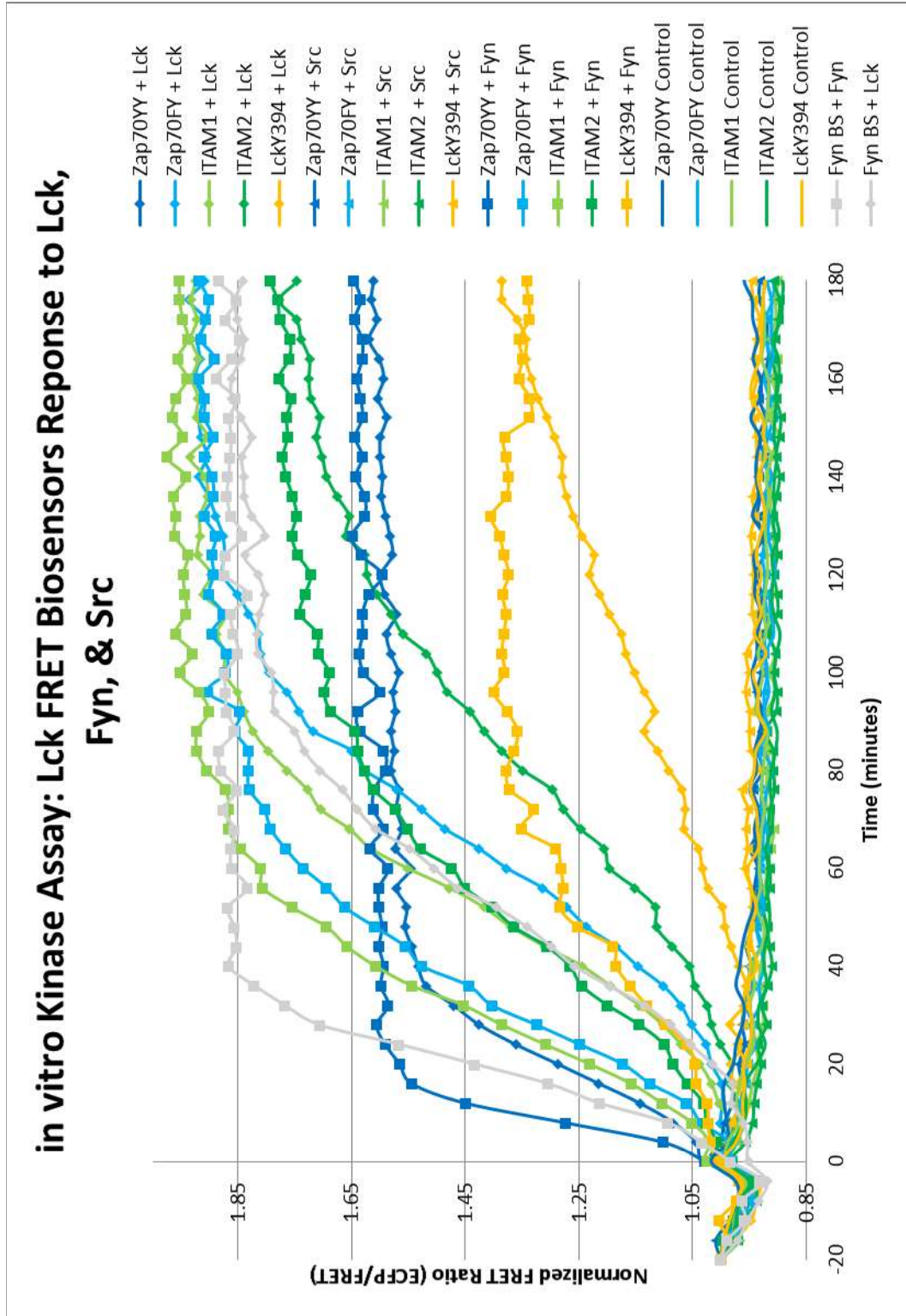


Figure 13: Normalized responses of all Lck FRET biosensors for the following substrates: Zap70YY, Zap70FY, ITAM1, ITAM2, and LckY394 to Lck, Src, Fyn, and no kinase as a control. Also shown is the Fyn FRET biosensor response to the Fyn and Lck kinase.

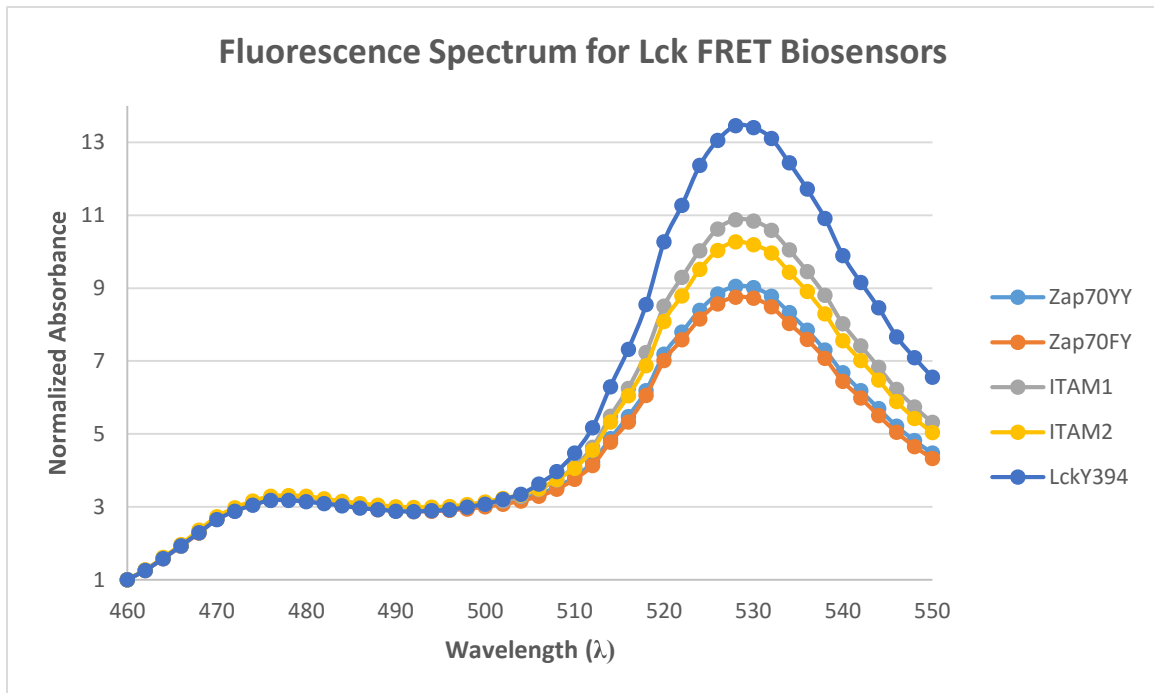


Figure 14: Fluorescence spectrum of the Lck FRET biosensors with the specified substrate designs at the basal state before activation with the kinase or ATP. The absorbance peak is at 530nm, YPet's emission wavelength, showing that the biosensor undergoes FRET at the basal state.

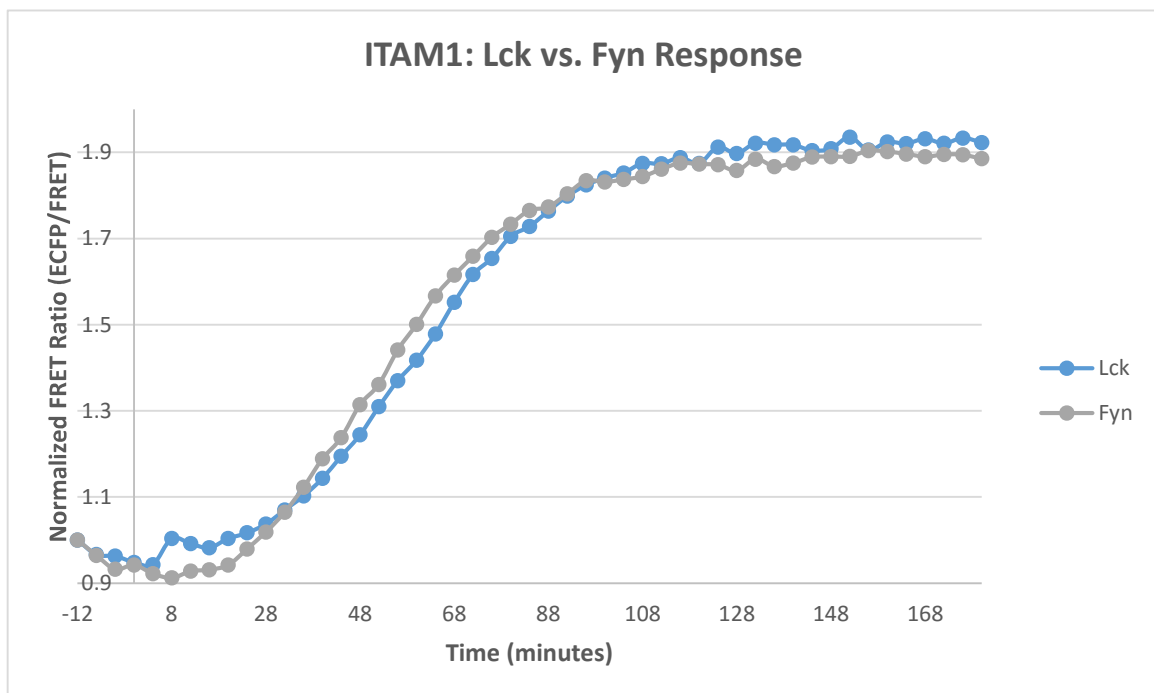


Figure 15: Fyn activity level was approximately 3x than that of Lck's activity level so increasing Lck kinase amount to 1.8x of the Fyn kinase resulted in a similar response for the Lck FRET biosensor with the ITAM1 substrate.

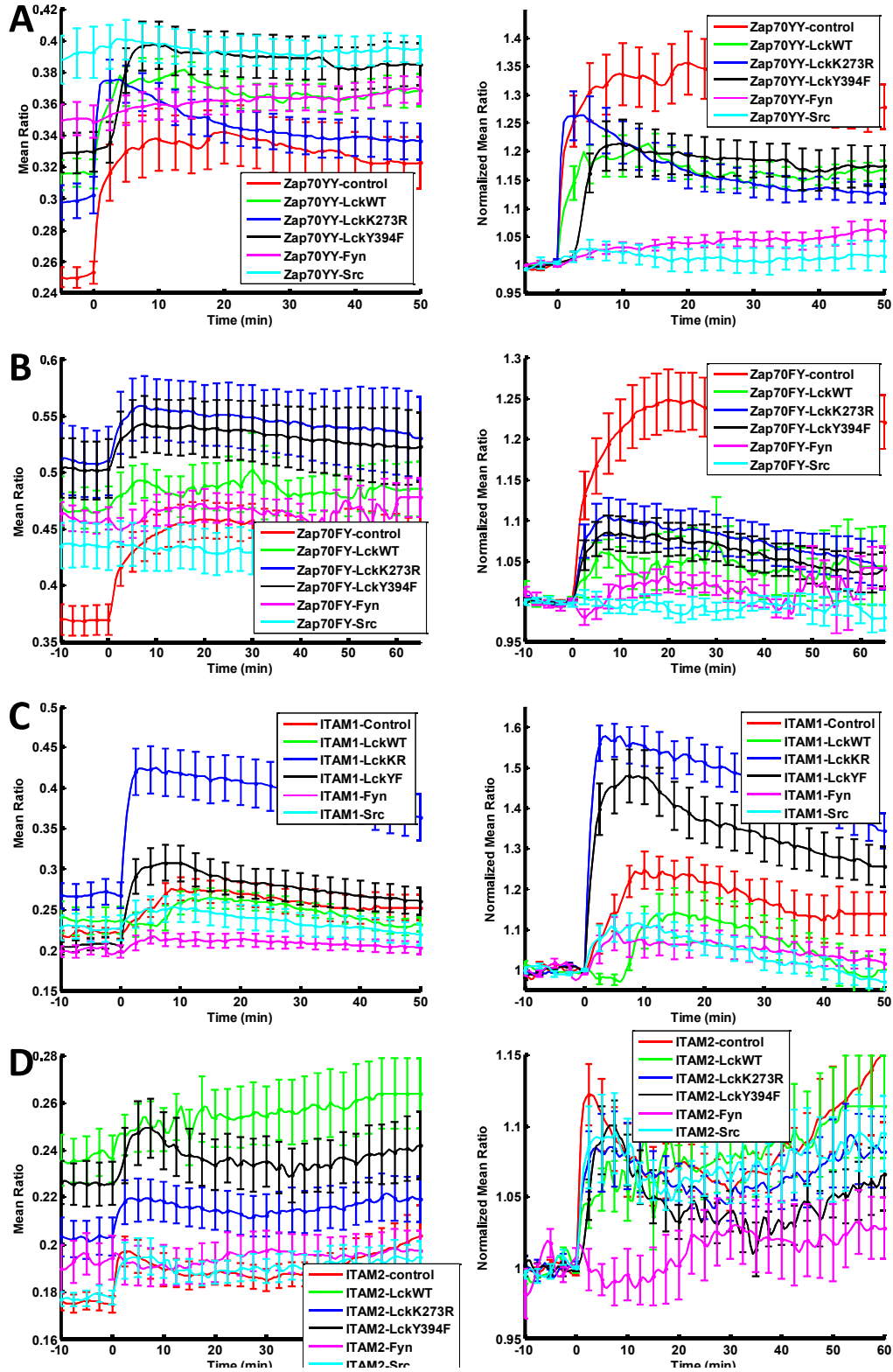


Figure 16: Real (left) and normalized (right) FRET responses in SYF(-/-) cells co-transfected with the Lck biosensors with different substrate designs and corresponding kinases for (A) Zap70YY, (B) Zap70FY, (C) ITAM1, and (D) ITAM2 upon PDGF stimulation at 0 minutes.

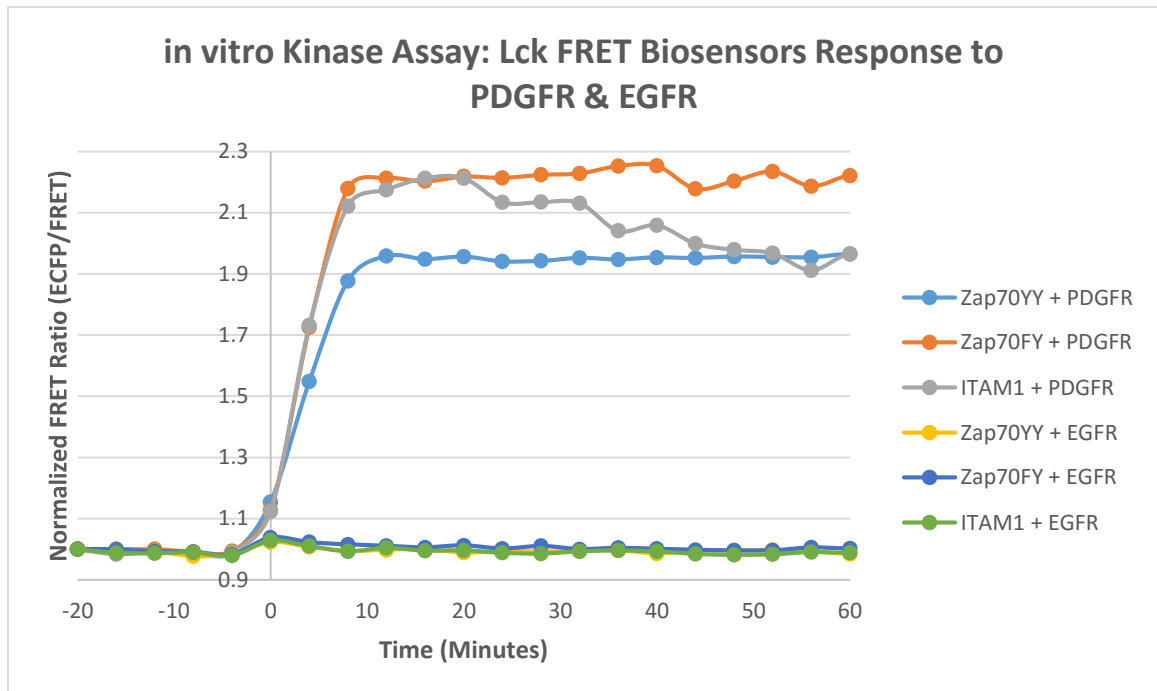


Figure 17: Normalized in vitro responses of Lck FRET biosensors with substrates: Zap70YY, Zap70FY, and ITAM1 to PDGFR and EGFR.

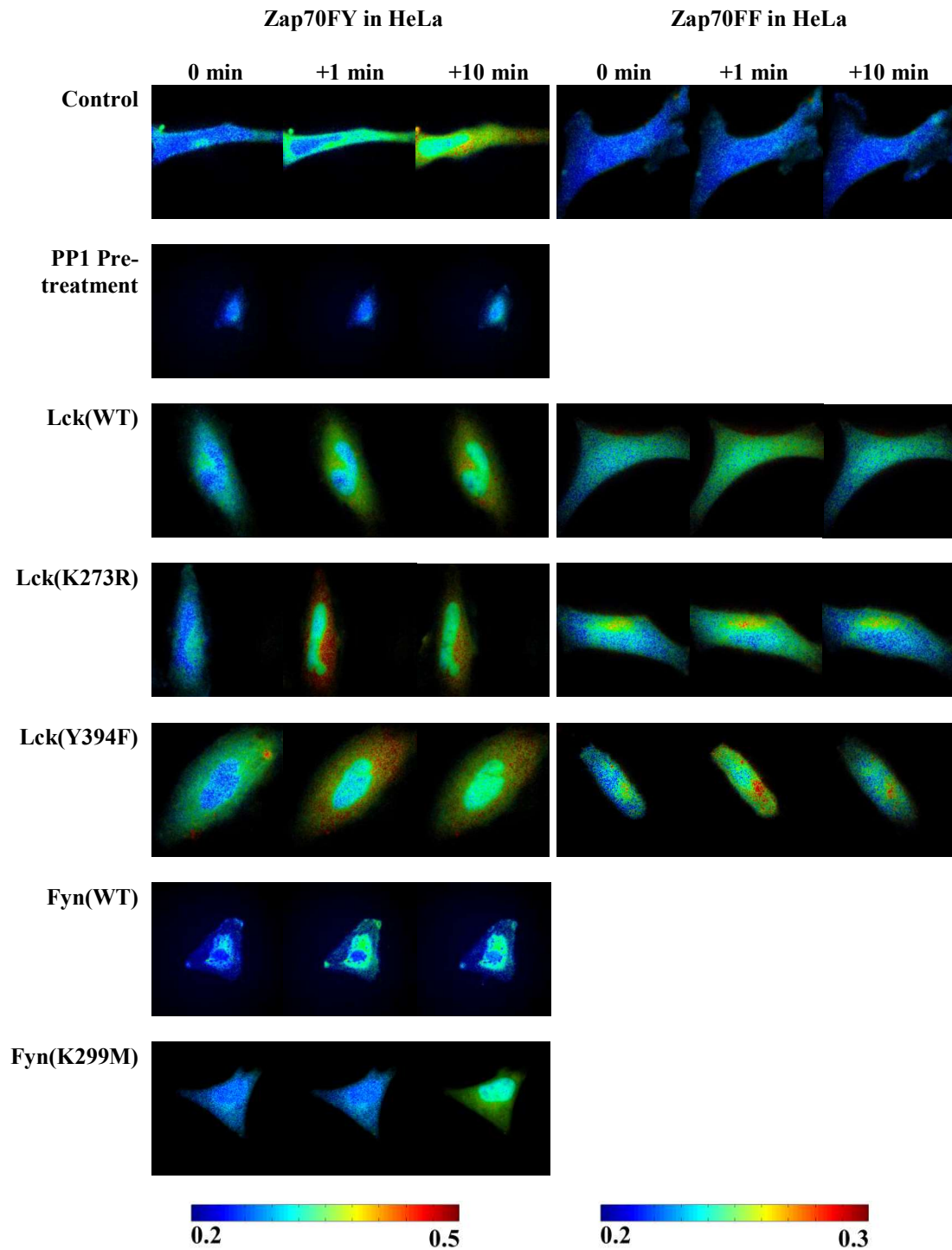


Figure 18: A representative set of FRET images with the subtracted background for HeLa cells transfected with the Zap70FY Lck FRET biosensor (left) or Zap70FF negative Lck FRET biosensor (right) in different co-transfection conditions as specified: control (empty pcDNA3.1 vector), Lck(WT), Lck(K273R), Lck(Y394F), Fyn(WT), and Fyn(K299M) (top to bottom). The FRET ratio in the images ranges from 0.2 – 0.5 (EGF stimulation) or 0.2 – 0.3 (PP1 inhibition) as shown by the respective color bar.

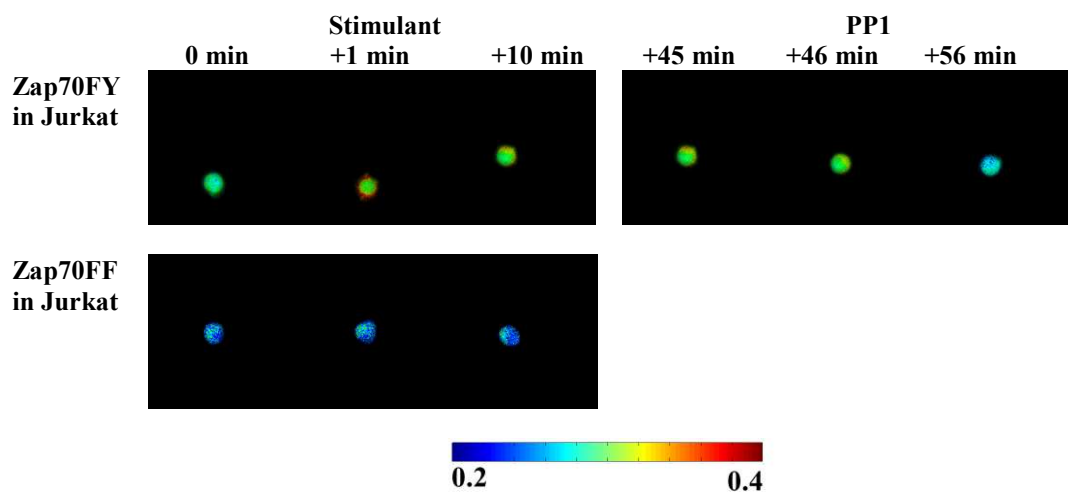
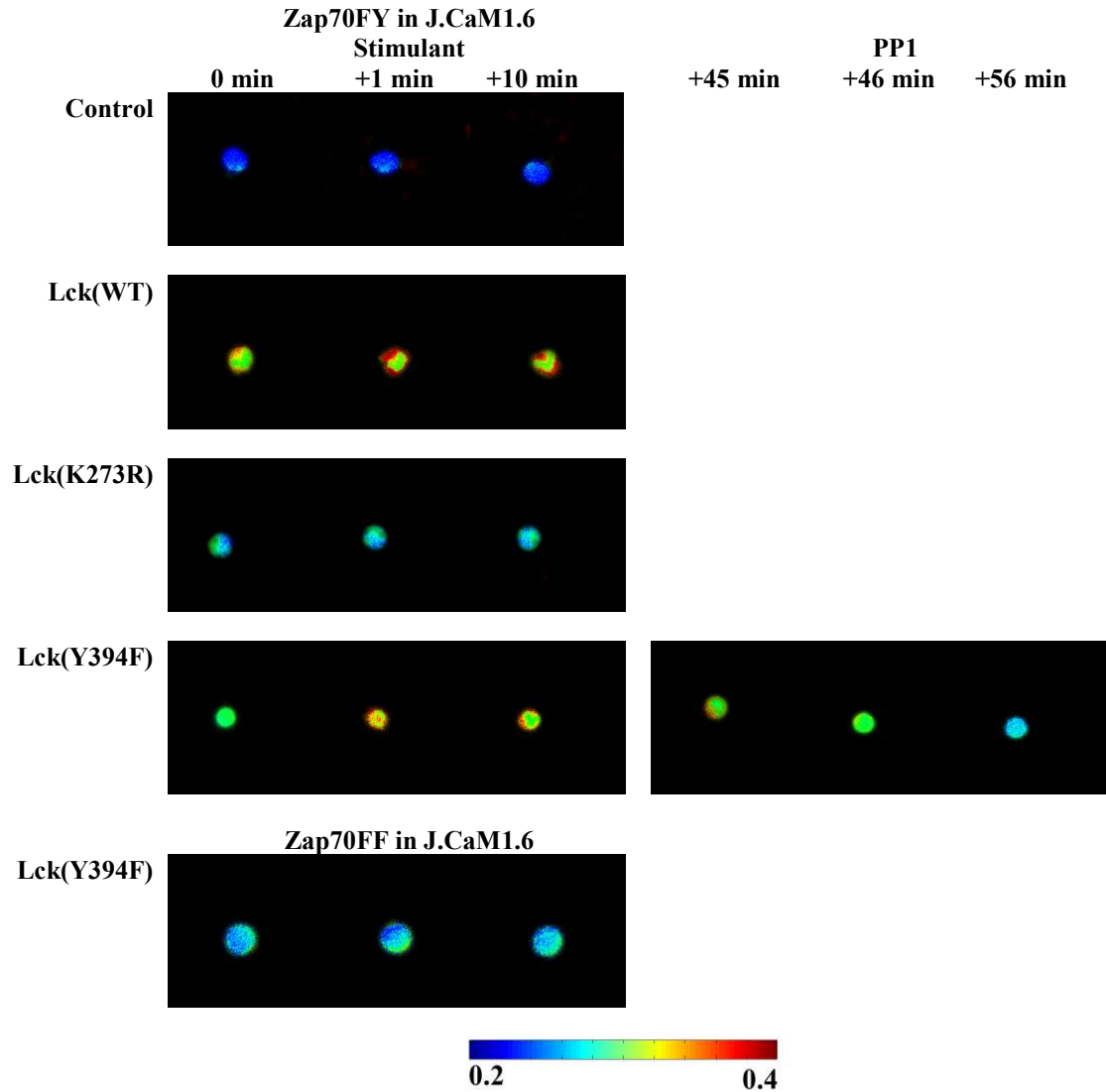


Figure 19: A representative set of FRET images with the subtracted background for Jurkat cells transfected with the Zap70FY (top) and Zap70FF (bottom) Lck FRET biosensor treated with the stimulant (left) or PP1 (right). The FRET ratio in the images ranges from 0.2 – 0.4 as shown by the color bar.





*Figure 20: A representative set of FRET images with the subtracted background for J.CaM1.6 cells co-transfected with the Zap70FY Lck FRET biosensor or Zap70FF negative Lck FRET biosensor in different co-transfection conditions as specified: control (empty pcDNA3.1 vector), Lck(WT), Lck(K273R), and Lck(Y394F) treated with the stimulant (left) or PP1 (right). The FRET ratio in the images ranges from 0.2 – 0.4 as shown by the color bar.*

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