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ROZA-XL, an improved FRET based biosensor with an increased dynamic range for visualizing Zeta Associated Protein 70 kD (ZAP-70) tyrosine kinase activity in live T cells

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ABSTRACT

Genetically encoded FRET based biosensors allow one to visualize the spatial and temporal evolution of specific enzyme activities in live cells. We have previously reported the creation of a FRET based biosensor specific for Zeta-Associated Protein −70 kD (ZAP-70) (Randriamampita et al., 2008), a Syk family protein tyrosine kinase. ZAP-70 is essential for early T cell receptor (TCR) signaling events, T lymphocyte development and has also been implicated in integrin mediated T lymphocyte migration. In order to facilitate the study of ZAP-70 kinase activity during dynamic phenomena such as immunological synapse formation or cell migration, we have designed and prepared a second generation of ZAP-70 specific biosensors. Here we describe a novel biosensor named ROZA-XL, that displays a 3–4 times greater dynamic range than its predecessor and possesses a robust baseline FRET value when expressed in the Jurkat human T cell line. We demonstrate that the robust behavior of this biosensor allows for rapid analysis of TCR mediated of ZAP-70 kinase activity at a single cell level, as shown in a simple end point assay in which ROZA-XL expressing cells are allowed to interact with stimulatory anti-CD3epsilon coated coverslips.

1. Introduction

An important challenge to furthering our understanding of mechanisms underlying receptor mediated signal transduction is the ability to follow key signaling phenomena, notably enzymatic events, in real time at a single cell level. One of the more promising approaches available today involves genetically encoded FRET based biosensors that have been designed to report the spatial and temporal evolution of specific enzymatic activities, such as small GTPase activity, or protein kinase activity, in live cells [1]. Single chain FRET based biosensors have been applied to a number of signaling events important to the biology of T lymphocytes, notably migration, adhesion and T cell receptor (TCR) signaling ([2–4] recently reviewed in Ref. [5]). We have previously developed a biosensor for the Syk family tyrosine kinase ZAP-70 [6]. ZAP-70, expressed exclusively in T cells and NK cells, is best known for its critical role in early TCR signaling. TCR engagement leads rapidly to ZAP-70 activation and subsequent ZAP-70 mediated phosphorylation of signaling adaptors such as Linker for Activation of T cells (LAT) and SH2 domain–containing leukocyte phosphoprotein of 76 kDa (SLP-76) [7,8], both important signaling branch points leading to changes in internal [Ca2+], MAP kinase pathway activation and cytoskeletal modifications [9]. ZAP-70 has also been shown to be required for integrin mediated T lymphocyte

Abbreviations: ZAP-70, Zeta Associated Protein 70 kD; SH2, Src homology 2; LAT, Linker for Activation of T cells; PLCγ1, Phospholipase Cγ1; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa.

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migration [10]. Like other protein kinase biosensors, the ZAP-70 biosensor ROZA (Reporter of Zap-70 Activity) was designed to be a surrogate substrate for ZAP-70, and when expressed in T cells, its FRET status reflects the local balance of activated kinases and phosphatases acting on the biosensor [11].

In the majority of biochemical or cellular imaging experiments described in the literature, ZAP-70 tyrosine kinase activity is examined indirectly using methods (i.e. FACS, western blotting or immunofluorescence staining) that rely on immune-detection of the phosphorylation state of ZAP-70 residues known to be involved in regulating the kinase activity, notably the interdomain B tyrosine residue 319. These methods require cell fixation or cell lysis, thus precluding detailed study of the spatial or temporal evolution of the kinase activity. The tyrosine 319 residue and others in the interdomain B are also binding sites for SH2 domain containing signaling partners, and therefore, the immunolabeling is also an indicator of the adaptor function of ZAP-70. In addition, recent reports have demonstrated that tyrosine 319 can be phosphorylated even if the catalytic site is inactivated, emphasizing the continuing need for alternative methods for the study of this important enzyme that are both compatible with live cell imaging and provide a direct read-out of the tyrosine kinase activity [12,13].

We previously employed the ROZA biosensor to examine the spatial-temporal distribution of ZAP-70 kinase activity in primary human T cells during the formation of a super-antigen mediated immunological synapse with Raji B cells [6]. This first generation ROZA gave a ~25% change in normalized ratiometric FRET signal in a ZAP-70-dependent manner, a dynamic range similar to other FRET-based kinase biosensors [6,14]. Further study of the differential ZAP-70 response to more physiological antigenic stimuli, or the subcellular redistribution of activated ZAP-70 during synapse formation, would be facilitated by a biosensor with an increased dynamic range. We report here the elaboration of a second generation of ZAP-70 reporters based on elements of either LAT or SLP-76, from which we identified one, named ROZA-XL, who possesses a dynamic range 3–4 times greater than the first generation ROZA, and who displays a robust background FRET condition when stably expressed in the Jurkat E6 human T cell line. In a simple assay employing coverslips coated with a standard anti-CD3e antibody and epifluorescent imaging, we show that this improved biosensor can be used to rapidly assess ZAP-70 kinase activity upon TCR engagement at a single cell level opening the way to more sophisticated experiments.

2. Materials and methods

2.1. Cells and reagents

Jurkat clone E6 was from ATCC; ZAP-70-deficient clone P116 was a generous gift of Hai-Tao He, CIML, Marseille, France. YFP variant YPET was a kind gift of P Daugherty, UC Davis, USA. The following mAbs were used: anti-CD3 epsilon clone OKT3 for stimulations and FACs analysis was from BD Bioscience (Franklin Lakes NJ, USA), anti-phospho-ZAP-70 Y319 was purchased from Cell Signaling Technology (NEB (UK) Ltd); anti-ZAP-70 (2F3.2) was from Upstate (Lake Placid NY,USA); anti-actin (MAB1501R) was from Millipore (Billerica, MA USA); anti-phospho-LAT 191 from Biosource/Life Technologies (Saint Aubin France) and secondary HRP-conjugated goat anti-mouse antibodies were from BioRad (Hercules, CA, USA). DNA sequences coding for the human VAV1-SH2 domain (residues 671–769) coupled to SLP76 peptide sequences (residues 110–120 or 124–135) were custom synthesized by Eurofins MWG Biotech (Ebersberg, Germany). Linker sequences were chosen as per the published ROZA biosensor. Other ROZA variants were prepared and cloned into pCDNA3.1 as described previously [6]. Cells were cultured at the Platforme de Culture Cellulaire, INSERM, Marseille, France. The complete nucleotide sequence of ROZA-XL has been deposited in Genebank (Accession number KM979358). Expression vectors coding for ROZA-XL and ROZA-XL YF will be available from Addgene.

2.2. Cell transfections

Wild type E6 and ZAP-70-deficient Jurkat cell lines were nucleofected with the Amaxa electroporation system using Solution V, program S-018 (Lonza, Basel, Switzerland). Cells were used for stimulation and imaging experiments 24–48 h after transfection. Stable transfectants were created by culturing the cells in the presence of 1.2 μg/mL G-418 (Gibco, Carlsbad CA, USA), sorting for YPET positive cells, and cloning by limiting dilution. All Jurkat clones were evaluated for TCR expression by flow cytometry before use and clones with similar biosensor and TCR CD3ε expression were selected for study.

2.3. FRET image acquisition

Biosensor-expressing Jurkat cells were imaged in custom-made microwells cut from polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI USA) and sealed to glass coverslips via O_2-plasma oxidation. Transfected cells were rinsed into HBSS (Gibco) supplemented with 2% FCS (Lonza), 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 mM Hepes pH 7.4 and allowed to rest for 15 min at 37 °C before imaging. For coverslip stimulation experiments, the wells were treated with either 0.01% poly-L-lysine in H_20 (Sigma) for 10 min at room temp or 10 ug/mL anti-CD3ε (OKT3) in PBS, 4 °C overnight, after rinsing, wells were filled with 150 uls warm imaging buffer and placed on the pre-warmed microscopy stage. At time 0, cells were added in an equal volume of imaging buffer and FRET sequence was acquired at 5 min.

2.4. Fluorescence microscopy

All FRET experiments were performed at 37 °C using a Zeiss Z1 (Carl Zeiss, Oberkachen, Germany), automated microscope equipped with a CoolSnap HQ CCD camera (Photometrics, Tucson, AZ), a Sutter Lambda 3 emission filter wheel and piloted by μManager [15]. Brightfield and fluorescent images were collected at either 40x/1.3 or 63x/1.3 magnification (Zeiss Plan-Neofluar). For ratiometric FRET imaging, three images were collected consecutively at each time point, brightfield (10 ms), CFP (excitation 436/20 nm, dclp 455, emission 480/40 nm, 100 ms) and FRET (excitation at 436/20 nm, dclp 455, emission 535/30 nm, 100 ms). For time lapse imaging Jurkat cells expressing biosensors, all three channels were collected every 15 s for no more than 20 min to minimize photobleaching.

2.5. Image analysis

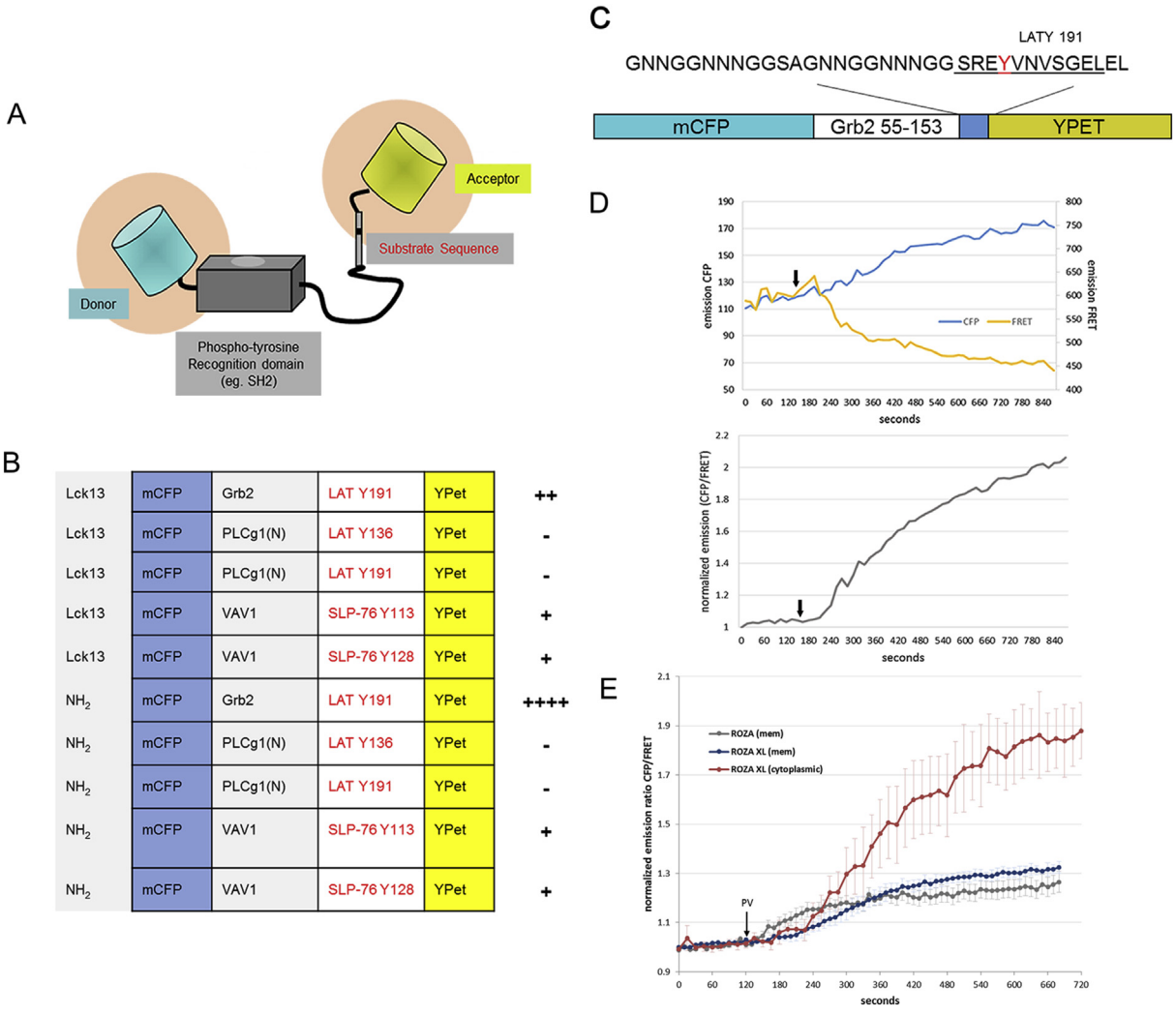
An image processing program was developed in house using MATLAB software in order to automatically detect and evaluate the average cell fluorescence intensity of each channel (CFP and FRET) in an image sequence (Mathworks, Natick, MA, USA). The principal steps of the program include background subtraction, thresholding and binarisation of the images to distinguish cells from the background and to create a mask for fluorescence intensity calculations. These steps are followed by identification and numbering of cells in the first image and following them in the next image. In subsequent steps, the fluorescence intensity for each cell identified in each image of the series is calculated and written to output. The FRET ratio was calculated as CFP/FRET intensity for each cell. In time

lapse series of Jurkat cells with pervanadate stimulation, the raw ratios were normalized to the average of the ratios calculated for the first 10 time points before stimulation. Pixel maps of Jurkat cells expressing ROZA XL deposited on anti-CD3ε coated surfaces were generated using FIJI IMAGEJ as follows. For each image in the series, the background fluorescence was first subtracted. After manually setting a threshold, a binary mask was generated in order to set all values outside of the cell localized fluorescence to zero. For each time point, the image corresponding to the CFP acquisition was divided by the image corresponding to the FRET image, the resulting ratiometric image was multiplied by a factor of 1000 in order to facilitate further analysis.

3. Results

3.1. Designing an improved FRET sensor for the ZAP-70 protein tyrosine kinase

The original ROZA biosensor follows a canonical tyrosine kinase reporter configuration and consists of a substrate sequence derived from the Linker for Activation of T cells tyrosine 171 fused to the SH2 domain of the adaptor GRB2, and flanked by CFP and YFP (Fig. 1A for general biosensor scheme) [11]. In order to generate an improved biosensor possessing a greater dynamic range, we kept mCFP as the fluorescent donor, but exchanged the FRET acceptor mYFP for YPET as the CFP-YPET pair has been reported to provide increased FRET efficiency [16,17]. We then altered the substrate sequence focusing on LAT phosphorylation sites other than that used in ROZA, and also phosphorylation sites on SLP-76 as SLP-76 has been reported to be phosphorylated by ZAP-70, for example during integrin mediated signaling [18]. A series of novel compositions was constructed in which either of the two LAT phosphorylation sites (Y191 and Y132) were assembled in fusion with their respective cognate Src Homology 2 (SH2) domains (GRB2 SH2 and N-terminal Phospholipase Cγ1 (PLCγ1) SH2) and two phosphorylation sites of SLP76 (Y113 and Y128) were fused to their cognate SH2 domain from the guanidine exchange factor VAV1. Care was taken to choose phosphorylation sites for whom anti-phospho epitope antibodies were commercially available. A non-cognate pair i.e. LAT Y191 with N-terminal PLCγ1 SH2 was also prepared. As the N terminal PLCγ1 SH2 domain can bind phosphorylated LAT Y191 with a 10 fold lower affinity than phosphorylated LAT 132, we thought this combination may lend itself to a dynamic intramolecular association (Fig. 1B) [19]. Long glycine–serine rich linker



Lck13	mCFP	Grb2	LAT Y191	YPet	++
Lck13	mCFP	PLCg1(N)	LAT Y136	YPet	-
Lck13	mCFP	PLCg1(N)	LAT Y191	YPet	-
Lck13	mCFP	VAV1	SLP-76 Y113	YPet	+
Lck13	mCFP	VAV1	SLP-76 Y128	YPet	+
NH_2	mCFP	Grb2	LAT Y191	YPet	++++
NH_2	mCFP	PLCg1(N)	LAT Y136	YPet	-
NH_2	mCFP	PLCg1(N)	LAT Y191	YPet	-
NH_2	mCFP	VAV1	SLP-76 Y113	YPet	+
NH_2	mCFP	VAV1	SLP-76 Y128	YPet	+

Fig. 1. ROZA XL, an improved ZAP-70 biosenser. A) Canonical architecture for tyrosine kinase biosensor with kinase substrate sequence and cognate phosphopeptide binding domain flanked with fluorescent donor and acceptor proteins. B) Table of new biosensor structures tested. C) Schematic representation of ROZA-XL composition. D) Upper panel: Fluorescent emission intensity of CFP (ex436/20 nm, em480/40 nm) and FRET (ex436/20 nm, em535/30 nm) channels of a single Jurkat E.6 cell expressing ROZA-XL and stimulated with 0.5 mM pervanadate at 160 s. Lower panel: Normalized emission ratio CFP/FRET of same cell as in upper panel. E) Comparison of normalized emission ratio of original ROZA and membrane (mem) and cytoplasmic variants of ROZA-XL during stimulation with 0.5 mM pervandate (arrow).

regions in all constructs were adopted as previously assessed on ROZA [6]. Each construct was generated both with and without the N-terminal 13 amino acids of Lck that directs the biosensor to the cytoplasmic membrane via a palmitoylation motif. Each construct was transiently expressed in Jurkat E6 cells and the FRET capacity was evaluated by imaging cells before and after addition of 0.5 mM freshly prepared pervanadate, which reveals tyrosine kinase activity by inhibiting constitutive phosphatase activity. Of the biosensor compositions tested, the construct that resulted in the most significant FRET change upon stimulation was that composed of the LAT 191 tyrosine phosphorylation sequence coupled to the SH2 domain of GRB2 and lacking the membrane anchor (Fig. 1C). The reciprocal intensity change of the CFP and FRET channel and the normalized emission ratio calculated CFP/FRET of a single cell, indicates that like the original ROZA and other kinase biosensors described in the literature, ROZA XL undergoes a net decrease in FRET with phosphorylation (Fig. 1D) [14]. The membrane bound version of this construct gave a similar FRET response to the original ROZA construct, albeit with slightly different early kinetics. However the cytoplasmic version was nearly 4 times more efficient, giving a 100% change in FRET as compared to the 25% change in signal for the membrane bound form (Fig. 1E) Clones were established of Jurkat E6 cells and Jurkat ZAP-70-deficient P116 cell expressing ROZA XL (ROZA XL-YF), as were clones expressing a mutated ROZA XL in which the LAT Tyr 191 residue was substituted by Phe.

3.2. ROZA XL undergoes a robust change in FRET ratio in a ZAP-70 and phosphorylation dependent fashion

Time-lapse imaging of the Jurkat cells (either wild type E6 or ZAP-70 deficient clone P116) expressing ROZA XL or ROZA XL YF and stimulated with 0.5 mM pervanadate revealed that both the presence of ZAP-70 and the biosensor with the LAT 191 tyrosine were necessary for significant FRET change (Fig. 2A). Unfortunately, the only commercially available inhibitor for ZAP-70, piceatannol, is fluorescent and therefore cannot be used in these imaging experiments. To further validate the behavior of the biosensor, samples of the Jurkat clones expressing ROZA XL or ROZA XL YF were stimulated with the anti-CD3ε mAb OKT3, lyzed and analyzed in a western blot. Probing with an anti-phospho LAT 191 mAb revealed that ROZA XL was phosphorylated in the Jurkat E.6 cells as was endogenous LAT, and this phosphorylation was also dependent upon the expression of ZAP-70 and the presence of Tyrosine 191 in the biosensor construct (Fig. 2B). Of particular interest, we noted that the raw baseline FRET value before normalization was remarkably robust from one cell to another and from one experiment to another (Fig. 2C). With pervanadate stimulation we observe stable FRET signal indicative of ZAP-70 activity throughout the time scale measured. To assess whether this observation may represent continuing ZAP-70 activity and not simply accumulation of the probe, we found that using the same stimulating agent, phosphorylated ZAP-70 can be detected in its phosphorylated state at 15 min post stimulation (Fig. 2D).

3.3. ROZA-XL allows simple and direct evaluation of ZAP-70 activity upon TCR stimulation in Jurkat T cell line

To assess the capacity of ROZA XL to respond via the TCR, we chose a very simple set up in which the Jurkat E6 cells stably expressing ROZA XL were allowed to settle on glass coverslips coated with either poly-L-lysine (PLL) or the anti-CD3ε mAb OKT3. The FRET image sequence was collected at 5 min after adding the cells to the coverslip. The population of cells interacting with anti-

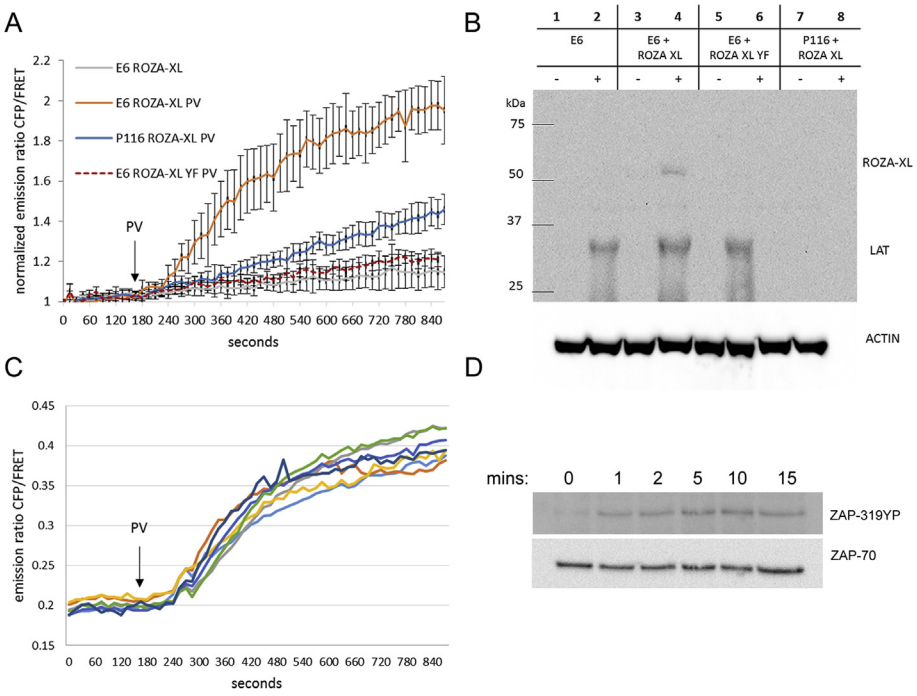


Fig. 2. ROZA XL undergoes a change in FRET status in a ZAP-70 and tyrosine phosphorylation-dependent manner. A) Clonal populations of Jurkat E6 (wt) and P116 (ZAP-70-deficient cells) stably expressing either ROZA-XL or ROZA-XL YF were imaged at 15 s intervals for 2.5 min (160 s) before addition of freshly prepared pervanadate at 0.5 mM final concentration. Figure shows average evolution of normalized emission ratio CFP/FRET (N = 25–30 cells from 3 separate experiments per condition). B) Western blot of Jurkat E6 or P116 cells stably expressing ROZA XL or ROZA XL YF. Cells were lysed with hot SDS buffer 5 min after stimulation with anti-CD3ε antibody OKT3. Western blot was probed with anti-LAT pY 191. Blot was stripped and reprobed for actin as a loading control. C) Raw CFP/FRET ratio from time lapse imaging of individual Jurkat E6 cells expressing ROZA-XL as per A. D) Jurkat E6 cells were lysed with hot SDS buffer at indicated time points after stimulation with 0.5 mM pervanadate. Western blot of lysates was probed with anti-ZAP-70 pY319, stripped and reprobed for total ZAP-70.

CD3-coated coverslips showed marked spreading as compared to cells on PLL (not shown) and increased FRET signals (Fig. 3A). After image analysis, the distribution of individual cell responses on the control (poly-L-lysine) versus activating (anti-CD3) coverslips was plotted as a histogram and revealed that strong FRET responses above the basal FRET level observed in the pervanadate experiments (>0.23) were only observed in those cells on the activating surface, consistent with the well-documented ZAP-70 activation upon T cell receptor engagement (Fig. 3B). Furthermore, cell lysates were prepared from Jurkat E.6 cells after 5 min of contact with PLL and anti-CD3ε coated surfaces and probed for pan-phosphotyrosine in a western blot. As expected, increased phosphotyrosine staining was only observed in the lysates derived from cells exposed to the anti-CD3ε antibody (Fig. 3C).

4. Discussion

Increasing numbers of FRET based biosensors have been reported in the literature, as well as novel strategies for generating new analogs, however the creation of these engineered proteins remains a largely empirical endeavor [20]. Of the novel ROZA analogs we constructed and tested, only the sequences containing the Grb2 SH2 domain gave a significant FRET change upon pharmacological stimulation, despite the relative structural similarities, i.e. substrate sequence length, nature of phosphotyrosine binding domain, or linker sequences, among the various constructs prepared. One explanation for this finding is that the Grb2 SH2 domain is unique among SH2 domains in that it binds its phosphorylated ligand with a β-hairpin structure rather than in an extended configuration more common to other SH2 domains [21]. This structural feature may facilitate the change in distance or orientation of the donor and acceptor required to alter the FRET signal during substrate phosphorylation and binding. Furthermore, the cytoplasmic form of the novel biosensor was also significantly more responsive than the membrane bound form. Several factors potentially contribute to this difference; the proximity to the cytoplasmic membrane may negatively influence the fluorescent properties of the donor CFP, while anchoring the biosensor into the membrane may restrict or alter the range of conformations that the biosensor may assume thus limiting the intrinsic FRET capacity. Furthermore the nature of the membrane anchor may lead to segregation of the probe away from active kinase (e.g. into lipid rafts), in contrast, a greater population of the cytoplasmic substrate may be accessible to the active kinase or inaccessible to relevant phosphatases. The lack of significant FRET signal in the SLP-76 based or LAT 132 based variants may be the results of structural issues that preclude FRET, or alternatively the net change in phosphorylation of the substrate sequences upon activation is not large enough to be detected in the biosensor concentration ranges expressed in the cells [22].

Another important feature of ROZA XL is that the raw non-normalized FRET ratio in the resting state of the Jurkat T cell line is very robust. Preliminary results suggest that similar baseline FRET values are also obtained in primary human lymphocytes (data not shown). When evaluating the cellular response to temporally controlled stimuli, the change in FRET ratio obtained for a given cell or subcellular region is most often reported as a value normalized to the corresponding values obtained before stimulation. However in some experimental contexts it may not always be feasible to have a "before and after" image of the same cell. In this case, a robust raw FRET value and dynamic range simplifies experimental design, and should therefore greatly facilitate future live single cell imaging experiments exploring the ZAP-70 function in T lymphocytes. The simple single time point assay using the anti-CD3ε coated coverslips present here demonstrates this point albeit with low spatial resolution fluorescent imaging. Future experiments combining ROZA-XL expressing cells with more sophisticated functionalized surfaces (e.g. lipid bilayers or nano-patterned substrates), and

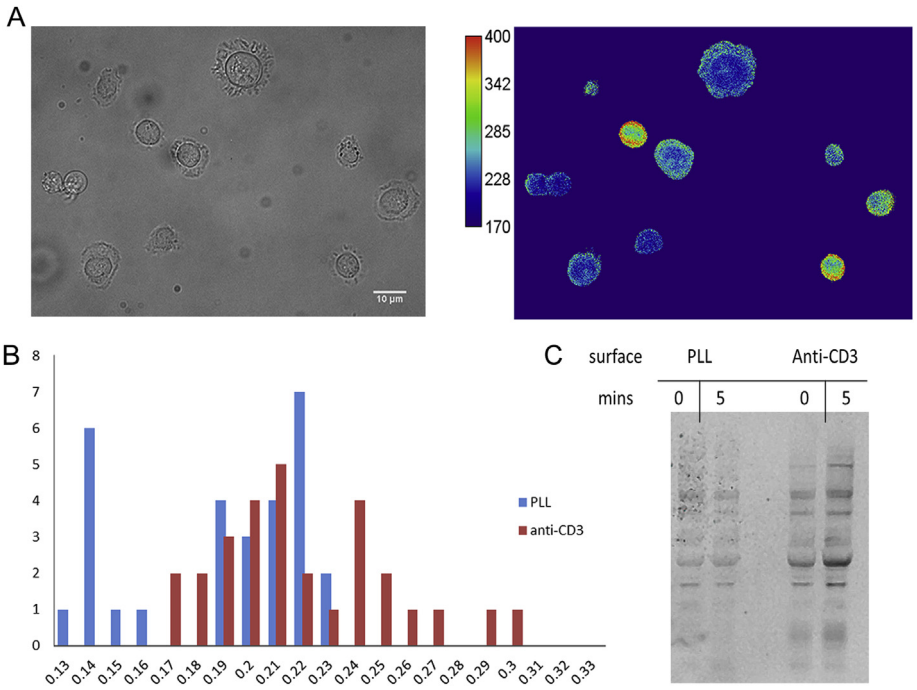


Fig. 3. ROZA XL is stimulated with surface bound anti-CD3ε A) Left Panel: Brightfield image of Jurkat E6 cells stably expressing ROZA-XL after 5 min contact with cell surface coated with 10 µg/ml anti-CD3ε (OKT3). Right Panel: FRET ratio of same cells in left panel. Magnification 40× B) Histogram of average single cell raw FRET emission ratio in Jurkat E6 cells stably expressing ROZA XL after 5 min of contact with either poly-L-lysine or anti-CD3ε (OKT3) N = 29 cells for each condition from 3 different experiments. C) Jurkat E6 cells were allowed to settle on glass surfaces pretreated with either Poly-L-Lysine or anti-CD3ε. At the times indicated, the cells were lysed with hot SDS buffer. Western blots of lysates were probed with pan-phosphotyrosine antibody 4G10.

higher resolution imaging techniques (e.g. confocal or TIRF microscopy) more adapted for examining the cell surface contact zone, should provide information highly complimentary to the various reports in the literature concerning dynamic changes in the dynamic spatial distribution of ZAP-70 as well as changes in cellular morphology during TCR engagement [23–25].

Conflict of interest

None.

Acknowledgments

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