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Peptic Fluorescent "Signal-On" and "Signal-Off" Sensors Utilized for the Detection Protein Post-Translational Modifications

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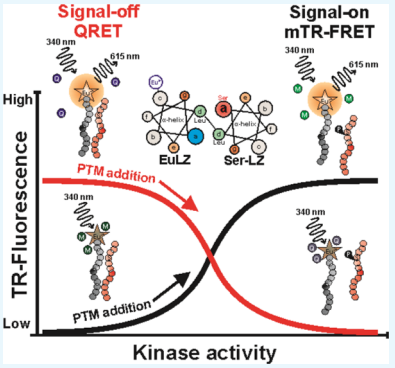
Supporting Information

ABSTRACT: Protein post-translational modifications (PTMs) are typically enzyme-catalyzed events generating functional diversification of proteome; thus, multiple PTM enzymes have been validated as potential drug targets. We have previously introduced energy-transfer-based signal-modulation method called quenching resonance energy transfer (QRET), and utilize it to monitor PTM addition or removal using the developed peptide-break technology. Now we have reinvented the QRET technology, and as a model, we introduced the tunable fluorescent "signal-on" and "signal-off" detection scheme in the peptide-break PTM detection. Taking the advantage of time-resolved fluorescence-based single-label detection technology, we were able to select the signal direction upon PTM addition or removal by simply introducing different soluble Eu^{3+}-signal-modulating molecule. This enables the selection of positive signal change upon measurable event, without any additional labeling steps, changes in assay condition or Eu^{3+}-reporter. The concept functionality was demonstrated with four Eu^{3+}-signal modulators in a high-throughput compatible kinase and phosphatase assays using signal-on and signal-off readout at 615 nm or time-resolved Förster resonance energy transfer at 665 nm. Our data suggest that the introduced signal modulation methodology provides a transitional fluorescence-based single-label detection concept not limited only to PTM detection.



1. INTRODUCTION

Post-translational modifications (PTMs) are usually enzyme-catalyzed covalent events occurring in protein side or main chains.[1,2] Wide variety of different modifications increase the size and complexity of proteome, and through that, they regulate important cellular functions. Phosphorylation is the most frequent modification and is controlled by two classes of enzymes: kinases phosphorylate and phosphatases dephosphorylate. The balance between activities of these two enzyme class determines the input−output relationship and the overall outcome.[3] Due to the crucial role of phosphorylation and other PTMs throughout the protein life cycle, deregulation of PTMs is often present in a variety of disease states.[4−6] Thus, the measurement of various PTMs and methodological development of PTM assays is constantly under the scope. Thus far, simple and sensitive universal method for PTM detection is yet to be discovered, and even today, the research partly relies on laborious heterogeneous assays like the enzyme-linked immunosorbent assay.[7,8] Nowadays, mass spectrometry (MS) is the only method that can be considered as a universal PTM detection platform, but it usually lacks throughput for large-scale screening, and still not all PTMs can be reliably measured. Recently, the introduction of self-assembled monolayers for matrix-assisted desorption/ionization has significantly improved the high-throughput screening (HTS) suitability of the MS methodology by increasing the throughput.[9,10] During the past years, homogeneous group-specific assays based on specific PTM-recognizing antibodies or secondary reaction products, e.g., nucleotide diphosphate monitoring, have also been developed.[11−13] These luminescence-based methods are homogeneous and HTS compatible, but lack similar universality for different PTMs as the MS. These assays need to be developed for all individual PTMs separately, which increases the time needed for setup and to develop the assay, thus increasing the costs. This complicated assay development is partly due to the need for specific antibodies for each PTM.

Luminescence, in its various forms, is one of the most used readout method in bioanalytical assays and especially in HTS-type applications.[14] On the basis of the signal direction of the measured reaction, biomolecular luminometric detection methods can be broadly divided into "signal-on" and "signal-off" sensors.[15] Signal-on is the desired direction of the signal change when following biomolecular interactions, as the increase in signal is directly proportional to molecular interactions. However, the signal-off-type readout is also highly applicable in cases like inhibition of enzymatic activity where it prevents molecular interaction and, therefore, provides high signal. Often, the signal-on readout referred as Förster resonance energy transfer (FRET) between two fluorophores

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and the signal-off is readout is referred as FRET between fluorophore and quencher.[11,15,16] All FRET assays rely on two label-conjugated molecules, providing measurable energy transfer from donor to acceptor only when labels are in close proximity. Depending on fluorophore or quencher used, this energy transfer can be monitored from increased or decreased signal. However, signal direction cannot be changed without new label conjugation, which might also affect the optimal distance between fluorophores. Single-label fluorescence polarization assays can be also counted as signal-on techniques.[17,18] Signal-on architecture is usually the preferred option, as the signal-off sensors are limited by the maximal signal suppression. Signal-on architecture has a high potential to improve the assay sensitivity because these assays are in theory not limited to signal increase at the upper end of the signal range.[15] With the signal-on readout, also the optimal measurement window with sufficient signal-to-background (S/B) ratio is easier to obtain. To enable and expand signal selection options for the end user, tunable detection methodologies easily suitable for both signal-on and signal-off sensing are thus of high interest. These methods could easily enable the selection of increasing signal upon the monitored reaction, without any additional changes in assay conditions.

We have previously developed a signaling technique, named as quenching resonance energy transfer (QRET) technology, which is so far mainly performed in the signal-off format.[19,20] The single-label QRET technique is based on energy transfer between lanthanide (Eu^{3+} or Tb^{3+}) chelate conjugated small-molecular-weight molecule and soluble quencher molecule.[19−21] Upon binding, the Ln^{3+}-conjugated molecule is protected from the soluble quencher, and the binding is monitored for high time-resolved fluorescence (TRF) signal. Previously, we have utilized QRET for multiple targets and also to monitor enzymatic PTM reactions.[20,22−24] Now, we have edited the QRET principle to enable signal-on and signal-off sensing by simply introducing different soluble Eu^{3+}-signal-modulating molecule but without any further changes in the assay conditions or Eu^{3+}-reporter peptide. The method was named as modulated time-resolved fluorescence resonance energy transfer (mTR-FRET), whose principle is now demonstrated using peptide-break platform for enzymatic phosphorylation/dephosphorylation monitoring. In these assays, we demonstrate the simplicity of the selection between signal-on and signal-off scheme, by changing no assay parameter other than the soluble modulator. Both assays with all four Eu^{3+}-signal-modulating molecules showed equal nanomolar sensitivity in both signal-on and signal-off modes measured at 615 nm and also the ability to monitor the reaction using TR-FRET at 665 nm.

2. RESULTS AND DISCUSSION

Here, we report a tunable signal-on and signal-off signaling method for enzymatic PTM assays as a monitored model reaction using the peptide-break technology (Figure 1). This technique distinguishes the nonmodified peptide complex from dissociated peptides after PTM addition.[25] Using the QRET detection technology (signal-off) in peptide-break concept, high TRF signal is monitored upon peptide complex formation. Enzymatic addition of PTM disrupts the binding between peptides and disposes the Eu^{3+}-label to soluble quencher to produce low TRF-signal.[23,24] The previously introduced QRET detection technology has proved its functionality in different HTS-compatible assay formats.[19−25]

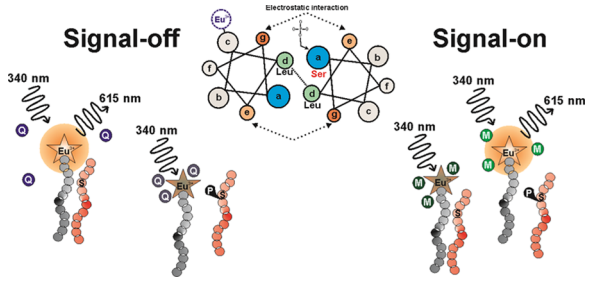

Figure 1. Principle of the leucine-zipper-based peptide-break technology for PTM detection. Leucine-zippers are coiled structures with repeated leucine residues at every seventh position. Leucines are located at the d position of the heptad repeat, and the structure is further stabilized with the β-branched amino acids at a position and amino acids creating electrostatic interactions between g and e positions. In the conventional signal-off-type QRET assay, high Eu^{3+}-signal is detected upon the formation of peptide complex, and the PTM enzyme activity is monitored from the reduced signal when Eu^{3+}-label is exposed to soluble quencher due to the zipper-complex dissociation. In the signal-on signaling mode (mTR-FRET), high Eu^{3+}-signal is detected with dissociated peptides containing the PTM, and the enzymatic PTM removal reduces the monitored TRF-signal upon the zipper-complex formation. Both signaling modes also allow TR-FRET signal detection using soluble Eu^{3+}-signal modulator molecule as a monitored energy transfer acceptor.

In the current study, the introduced signal-on method, called modulated time-resolved fluorescence resonance energy transfer (mTR-FRET), was constructed by modifying the QRET detection to provide a selectable positive signal change upon choice, also with dissociated peptides and not only with peptide complex. In the mTR-FRET approach, high TRF-signal is obtained, as the peptide carrying a PTM is incapable to form a peptide complex with the Eu^{3+}-chelate conjugated reporter peptide (EuLZ) detection peptide. This is based on the positive Förster-type energy transfer between a nonbound Eu^{3+}-labeled peptide and soluble modulator. The introduced signal-on modulation technique (mTR-FRET) along with the signal-off technique (QRET) provides the option to select the signal readout direction supporting the given modification and assay preferences. For example the signal-on method is a method of choice for phosphorylation, whereas the signal-off is preferred for dephosphorylation to obtain a signal increase upon modification. The selection between signaling modes is simply based on the selection of the soluble Eu^{3+}-signal modulator molecule, without additional changes in the other used assay parameters. The functionality of the positive and negative signal modulations and energy transfer between EuLZ and modulators (time-resolved Förster resonance energy transfer, TR-FRET) were demonstrated with protein kinase A (PKA) serine kinase and protein tyrosine phosphatase 1B (PTP1B).

The conventional QRET is based on the signal protection of the Ln^{3+}-chelate-labeled small-molecular-weight ligand upon binding to a target receptor. We have proven the functionality of the QRET detection by utilizing peptide−peptide interaction for enzymatic PTM activity monitoring with leucine zipper and charged peptides.[23,24] However, inhibition of drug candidates for PTM removal, e.g., screening for phosphatase inhibitors blocking the phosphate removal, reducing the peptide−peptide interaction and thus the TRF-signal (signal-off). Typically, the signal-on readout is always preferred and obviously not always enabled by the conven-

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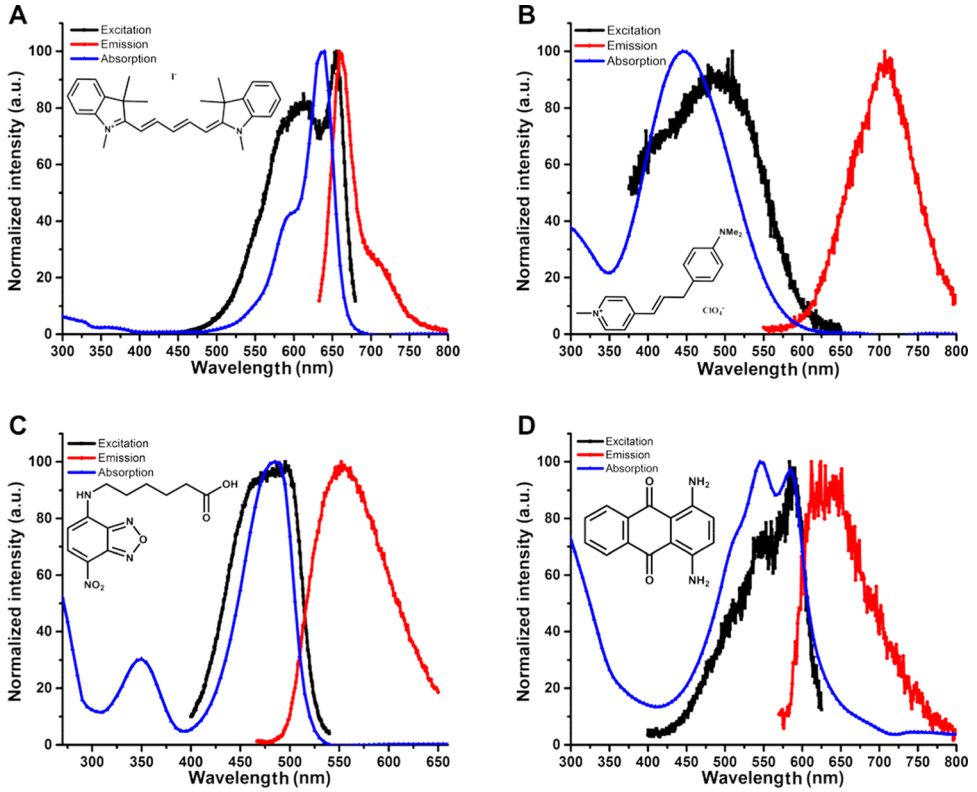

Figure 2. Normalized excitation, emission, and absorption spectra for quenchers and modulators used in this study. Excitation (black), emission (red), and absorption (blue) spectra for quenchers (A) Dy1 (1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide) and (B) Dy2 (4-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl-pyridinium perchlorate; pyridine 2) and modulators (C) Dy3 (6-*t*-7-nitrobenzofurazan-4-ylaminohexanoic acid) and (D) Dy4 (1,4-diamine anthoaquine). Signals are individually normalized to the maximum.

tional QRET. As the typical QRET provides either positive (binding) or negative (no binding) signal change without the possibility for the selection of the desired readout direction, we have now studied the effect of varying modulator molecules to address this issue. The technique introduced here provides tunable signal-on and signal-off readout by simply selecting the desired Eu^{3+}-signal-modulating molecule supporting the direction of the PTM monitored (Figure 1).

First, we selected two positive and two negative signal modulators from the dye library. The selected positive modulators were used to provide signal-on readout and quenchers for traditional signal-off assays. The Eu^{3+}-chelate spectra for EuLZ detection peptide and the spectral properties of the modulators and quenchers are presented in Figures 2 and S1 and are listed in Table S1. As for reference quencher, we selected 1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide (Dy1), which we have previously introduced in a QRET application and whose functionality was already known (Figure 2a).[25] In case of Dy1, excitation and absorption spectra overlapped with the Eu^{3+}-chelate main emission peak, as the EuLZ excitation and emission maxima were monitored at 332 and 619 nm, respectively (Figure S1 and Table S1). The excitation spectra of the second quencher, 4-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl-pyridinium perchlorate, pyridine 2 (Dy2), showed only a minor overlap with the Eu^{3+}-emission spectra (Figures 2b and S1). For signal-on modulators, 6-*t*-7-nitrobenzofurazan-4-ylaminohexanoic acid (Dy3) and 1,4-diamine anthoaquine (Dy4) were selected (Figure 2c,d). Dy3 excitation and absorption spectra have no overlap with the Eu^{3+}-emission, whereas the Dy4 excitation and absorption spectra are clearly overlapping with the Eu^{3+}-chelate emission peak (Figures 2c,d and S1). All four selected Eu^{3+}-signal-modulating molecules were selected primarily to enable different signal readout modes for signal-on and signal-off sensor development. However, the spectral studies showed that Eu^{3+}-signal-modulating molecules may also enable TR-FRET detection with the single-label strategy with EuLZ. The Eu^{3+}-chelate main emission peak overlaps especially with the Dy1 and Dy4 excitation, and also their emission is close to 665 nm, which is highly suitable for TR-FRET detection (Table S1). After the spectral property studies, the applicability of these quenchers and modulators were investigated in kinase and phosphatase reactions, using the peptide-break leucine-zipper platform.[23]

Peptide break was originally demonstrated to enable detection of different PTMs.[23] However, to keep the current assay simple and to study different detection modes, we selected only serine phosphorylation and tyrosine dephosphorylation for this study. Unlike previously, the EuLZ reporter was now conjugated with isothiocyanate (ITC)-activated heptadentate Eu^{3+}-chelate.[23,26] This chelate is less stable than the previously used nonadentate Eu^{3+}-chelate,[23,24] and thus better suited for energy-transfer applications.[26] First, to demonstrate the peptide-break technique for the selective signal readout, we performed enzymatic PKA (5 nM) assay using two well-characterized inhibitor compounds, staurosporine and H-89. Assays with different detection modes were performed simultaneously using the same protocol: PKA peptide (LZ-S) (100 nM) was used as a substrate and EuLZ (5 nM) was used for detection. For all assays, 340 nm excitation was used and signal readout was performed at 615 nm (signal-on and signal-off) or 665 nm (TR-FRET).

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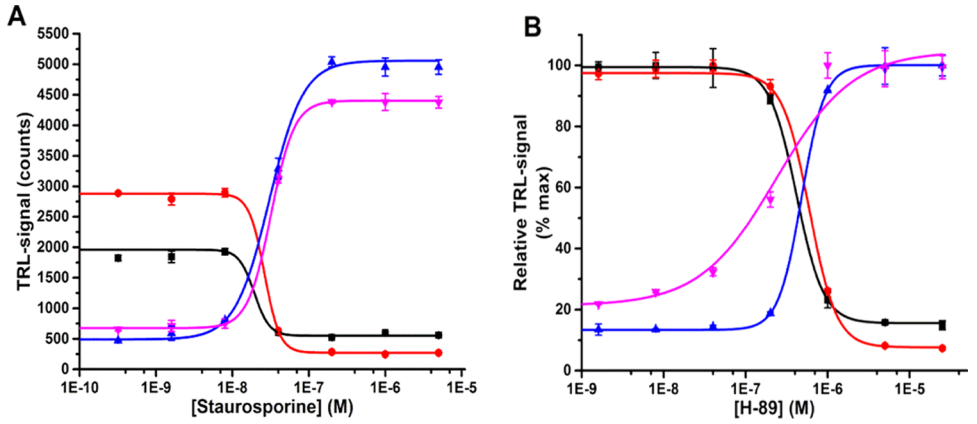

Figure 3. Dose response measurements for the two known PKA inhibitors. PKA (5 nM) inhibitor titrations were performed using (A) staurosporine (0−25 μM) and (B) H-89 (0−25 μM) with quenchers, Dy1 (magenta) and Dy2 (blue), and modulators, Dy3 (red) and Dy4 (black), in a assay using leucine-zipper peptides EuLZ (5 nM) and LZ-S (100 nM). Data represent mean ± standard deviation (SD) (n = 3).

Using the signal-off QRET readout with Dy1 and Dy2, PKA enzyme activity inhibition increased the monitored Eu^{3+}-signals, as phosphorylation was inhibited and LZ-S interacted with EuLZ reporter (Figure 3). Signal-on modulators gave high Eu^{3+}-signals when PKA was active, as LZ−peptide duplex formation was blocked due to the PTM. In this case, the enzyme inhibition was monitored from decreasing signal (Figure 3). Next, we monitored the TR-FRET signals at 665 nm for PKA reaction. Only Dy3 did not show any TR-FRET signal over background level, which was expected based on its nonoverlapping spectra with EuLZ (Figure 2c). Due to these nonoverlapping spectra of Dy3 and EuLZ, it is also clear that the modulation function with Dy3 occurs with different mechanisms than with the other dyes used.[27] Dy1 showed high signal at 665 nm but no reliable response to inhibition under the studied conditions. With Dy2 and Dy4, the PKA activity inhibition was detected also in the TR-FRET channel, but only the assay with Dy4 responded expectedly to peptide complex separation (Figure S2). In the case of Dy2, the change of the TR-FRET signal was due to the phosphopeptide separation and subsequent Eu^{3+}-signal quenching, which was also detected at 665 nm. Even the signal with Dy2 followed the PKA inhibition reaction expectedly, this detection was not considered as a true TR-FRET but "negative TR-FRET". Independent of which quencher/modulator or signal readout mode, either at 615 or 665 nm, was applied, the IC_{50} values for staurosporine and H-89 were in the same range: 16−32 nM and 0.24−0.60 μM, respectively (Table 1). These values are similar to the ones previously reported by us and others.[23,24,28,29]

Next, the signaling concept was demonstrated with the same EuLZ reporter using phosphorylated protein tyrosine phosphatase 1B substrate peptide (LZ-pY) as a substrate in a PTP1B assay. In the phosphatase inhibition, the signal-off assay (QRET) provided decreasing signal, and positive signal change was observed with the signal-on (mTR-FRET) technique upon PTP1B inhibition (Figures 1 and 4a). The used substrate peptide was not optimized for PTP1B, but only the two serine residues from the LZ-S were replaced with phosphotyrosines to allow side-by-side comparison between detection modes in both directions. The calculated IC_{50} values for Na_3VO_4, PTP1B inhibitor, were 14−40 nM when signal from the Eu^{3+}-channel (615 nm) was monitored (Figure 4a and Table 1). Again, the values were comparable to the ones reported previously.[23,30] The TR-FRET readout at 665 nm tends to produce somewhat higher IC_{50} values for PTP1B inhibition with Na_3VO_4 (53−120 nM) compared to the ones monitored using Eu^{3+}-signal readout (Figure 4b and Table 1). As in phosphorylation assay, Dy2 and Dy4 gave inhibitor-dependent TR-FRET signal change at 665 nm but in different directions. In case of PTP1B also Dy1-enabled TR-FRET-based enzyme activity was measured at 665 nm (Figure 4b). This was due to improved S/B ratio monitored with PTP1B compared to the PKA assay (1.2 vs 1.9). However, the S/B ratio monitored with Dy1 is still low due to nonoptimal detection conditions for the TR-FRET. This is mainly because the quencher and the modulator concentrations were optimized for the TRF-signal readout at 615 nm. The Dy1 concentration in PTP1B assay was lower as in PKA assay, which directly increased the detected S/B ratio at 665 nm. However, we did not further optimize the concentrations of the quenchers and modulators for TR-FRET detection.

Based on the (de)phosphorylation assays, tunable signal readout can be developed solely by selecting different soluble Eu^{3+}-signal modulator molecules. All studied modulators function as efficient energy-transfer acceptors for Eu^{3+}-chelate, and the distance between Eu^{3+}-chelate and soluble modulator provides the basis for the signal change during peptide−duplex association and dissociation. As the assay conditions were solely selected for Eu^{3+}-channel readout (signal-on and signal-off), it provided higher S/B ratios (3.8−12.1) compared to TR-FRET (1.9−3.9) detection (Table 1). However, the data suggest that the TR-FRET detection functionality can be improved by modulator concentration adjustment. As showed with two phosphorylation cycle enzymes, selection between signal-on and signal-off readout has no effect on the monitored S/B ratio or IC_{50} values. Only the IC_{50} values monitored in the PTP1B reaction using the TR-FRET readout (665 nm) were constantly slightly higher as the ones monitored at 615 nm. As introduced here with peptide-break PTM assay, different signaling options enable the selection of the signal direction. We already have indications that signal-on platform is applicable to other peptide-based assays than the one introduced here (data not shown). It is yet to be discovered if the previously developed QRET detection methods can benefit widely from the positive signal readout.

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Table 1. Summary of the PKA and PTP1B Inhibitor Titrations Using Different Signal Readouts[a]

enzyme	Eu^{3+}-signal modulator	inhibitor	mode 1 (615 nm)	IC_{50} (nM)	S/B	mode 2 (665 nm)	IC_{50} (nM)	S/B
PKA	Dy1 (1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide)	staurosporine	signal-off	32.0 ± 1.1	6.7			
		H-89		237 ± 108	4.6			
PKA	Dy2 pyridine 2	staurosporine	signal-off	30.4 ± 3.5	10.7	neg. TR-FRET[b]	21.0 ± 2.7	3.2
		H-89		481 ± 62	9.4		308 ± 46	3.9
PKA	Dy3 6-*t*-7-nitrobenzofurazan-4-ylaminohexanoic acid	staurosporine	signal-on	26 ± 14	11.8			
		H-89		599 ± 37	12.1			
PKA	Dy4 1,4-diamine anthoaquine	staurosporine	signal-on	19 ± 11	3.8	TR-FRET	16.0 ± 1.6	3.7
		H-89		418 ± 8	6.3		278 ± 29	3.6
PTP1B	Dy1 (1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide)	Na_3VO_4	signal-off	14.3 ± 3.5	7.1	TR-FRET	53.6 ± 7.1	1.9
PTP1B	Dy2 pyridine 2	Na_3VO_4	signal-off	39.6 ± 4.5	4.9	neg. TR-FRET	73.0 ± 7.7	2.1
PTP1B	Dy3 6-*t*-7-nitrobenzofurazan-4-ylaminohexanoic acid	Na_3VO_4	signal-off	26.7 ± 1.9	4.3			
PTP1B	Dy4 1,4-diamine anthoaquine	Na_3VO_4	signal-off	40 ± 11	4.9	TR-FRET	120 ± 12	2.9

[a]IC_{50} values and obtained S/B values using the peptide-break technology with signal readouts at 615 nm (QRET and mTR-FRET) or 665 nm (TR-FRET and negative TR-FRET). [b]In the negative TR-FRET, the TR-FRET signal occurs through donor quenching detected also at 665 nm.

3. CONCLUSIONS

We have successfully introduced a signal modulation method that allows the development of single-label signal-on or signal-off peptide sensors to follow enzymatic PTM modifications. The novel signal modulation technique was demonstrated with the universal peptide-break technology to monitor the enzymatic phosphorylation/dephosphorylating cycle. The key benefit of the signal modulation methods was its ability to always select the increasing signal upon monitored reaction. For the end-user, the assay operation is simple as the assay conditions, substrate peptide containing the target sequence, and the Eu^{3+}-conjugated reporter peptide are kept constant. To change the detection mode, only the soluble quencher/modulator is selected to define the direction of the signal. The introduced mTR-FRET method provides a range of possibilities to monitor interactions with increasing signal readout, currently not always possible with the conventional signal-off QRET technique. In the peptide-break system, the developed signal-on scheme allows the use of nanomolar enzyme concentration in a 384-well HTS compatible format with standard plate reader enabling time-resolved Eu^{3+}-signal detection. mTR-FRET can be monitored similarly to the QRET technique and without any loss of assay functionality. Our data suggest that the signal-on methodology provides a transitional fluorescence-based detection concept for different HTS applications, also outside PTM detection.

4. EXPERIMENTAL SECTION

4.1. Reagents. Substrate peptides for PKA, LZ-S (REELRKRRAELRRR**S**AQLRQRREQLRRR**S**ANLRKE), and PTP1B, LZ-pY (REELRKRRAELRRR**pY**AQLRQRREQLRQR**pY**ANLRKE), were purchased from Pepmic Co., Ltd (Suzhou, China). Heptadentate isothiocyanate (ITC)-TEKES-Eu(III) chelate (4-[2-(4-isothiocyanatophenyl)-ethynyl]-2,6,-bis{[*N*,*N*-bis(carboxymethyl)-amino]methyl}-pyridine) conjugated EuLZ detection peptide was from QRET Technologies (Turku, Finland). Used enzymes, catalytic domain of PKA and recombinant human PTP1B, were from New England Biolabs (Ipswich, MA) and Cayman Chemicals (Ann Arbor, MI). The soluble modulator molecules, 1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide (Dy1), 4-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl-pyridinium perchlorate; pyridine 2 (Dy2), 6-*t*-7-nitrobenzofurazan-4-ylaminohexanoic acid (Dy3), and 1,4-diamine anthoaquine (Dy4) were from Radiant Dyes Laser & Accessoires GmbH (Wermelskirchen, Germany), Thermo Fisher Scientific (Waltham, CA), and Sigma-Aldrich (St. Louis, MO). H-89 and Na_3VO_4 inhibitors were from Santa Cruz Biotechnology (Dallas, TX) and MP Biomedicals (Santa Ana, CA). Black Corning 384 well low-volume assay plates were used in all assays (Corning, Tewksbury, MA). All other reagents, including analytical-grade solvents, adenosine triphosphate (ATP), and staurosporine were acquired from Sigma-Aldrich. All assays were made in assay buffer containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (pH 7), 1 mM $MgCl_2$, 5 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, and 0.01% Triton X-100.

4.2. Instrumentation. Emission and excitation spectra for EuLZ and used Eu^{3+}-signal-modulating molecules (Dy1−4) were recorded using Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Mulgrave, Australia). TRF excitation spectra (200−500 nm) for EuLZ were

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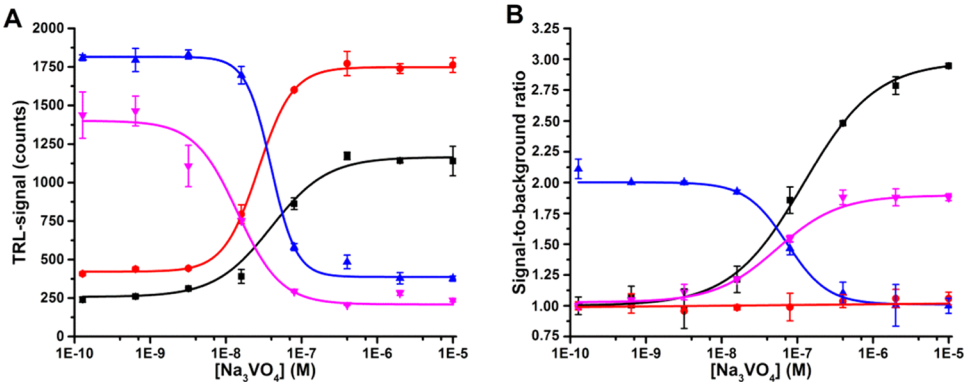

Figure 4. Dose response measurements for the Na_3VO_4 PTP1B inhibitor. PTP1B (5 nM) inhibitor titration with Na_3VO_4 (0−10 μM) was performed using quenchers, Dy1 (magenta) and Dy2 (blue), and modulators, Dy3 (red) and Dy4 (black). (A) Signal-off (QRET) and signal-on (mTR-FRET) assays monitored at 615 nm gave characteristic sigmoidal inhibitor curve with Na_3VO_4, producing IC_{50} values from 14 to 40 nM. (B) Dy1, Dy2, and Dy4, but not Dy3, enabled TR-FRET detection at 665 nm. IC_{50} values for PTP1B inhibition were between 53 and 120 nM. Data represent mean ± SD (n = 3).

recorded with 20 nm slit, 615 nm emission with 5 nm slit, 100 μs delay, 20 ms decay, and 600 μs gate time. The TRF emission spectra (550−750 nm) for EuLZ were recorded with 20 nm slit, 340 nm excitation with 5 nm slit, 100 μs delay, 20 ms decay, and 600 μs gate time. Fluorescence emission spectra for Dy1−4 were recorded with 1.5 nm slit, 618 nm (Dy1), 460 nm (Dy2), 447 nm (Dy3), and 550 nm (Dy4) excitation wavelengths. Emission scans for Dy1, Dy2, Dy3, and Dy4 were performed at 630−850, 475−850, 430−650, and 545−800 nm with 5 nm slit, respectively. Fluorescence excitation spectra for Dy1−4 were recorded with 5 nm slit and using 700 nm (Dy1), 740 nm (Dy2), 555 nm (Dy3), and 670 nm (Dy4) emission wavelengths, and scanning was performed at 450−680 nm (Dy1), 400−725 nm (Dy2), 400−545 nm (Dy3), and 400−650 nm (Dy4) with 1.5 nm slit, respectively. The absorption spectra for Dy1−4 were recorded using Cary 60 UV−vis spectrometer (Agilent Technologies). The TRF-signals and TR-FRET signals were obtained with standard Labrox plate reader from Labrox Ltd (Turku, Finland) or Victor 1420 multilabel counter from PerkinElmer Life and Analytical Sciences, Wallac (Turku, Finland). The TRF-signal (QRET and mTR-FRET) was monitored using 340 nm excitation and 615 nm emission wavelengths, 400 μs window time, and varying delay time (400−1500 μs). TR-FRET signals were monitored using 340 nm excitation and 665 nm emission wavelengths, 400 μs window time, and constant 50 μs delay time.

4.3. EuLZ and Dy1−4 Spectral Characterization. The EuLZ (10 nM) spectrum was recorded in quartz cuvette at 20 μL volume. The Dy1−4 emission/excitation/absorption spectra were recorded in quartz cuvette at 1 mL volume of assay buffer. The used concentrations for Dy1, Dy2, Dy3, and Dy4 were 4, 100, 250, and 4 μM, respectively.

4.4. PKA and PTP1B Activity Monitoring. All enzymatic PKA (5 nM) and PTP1B (5 nM) assays were performed using LZ-S and LZ-pY substrate peptides (100 nM), respectively. The PKA reactions were studied with two inhibitors, H-89 (0.32−25 000 nM) and staurosporine (0.32−25 000 nM), which were added in the plate in 2.5 μL volume. Similarly, Na_3VO_4 (1−10 000 nM) was used in case of PTP1B. The PKA reaction was initiated using 50 μM ATP, and both enzyme reactions were performed in 7.5 μL final volume and using 60 min incubation time (room temperature). Detection components were added to reach the final 20 μL volume, using EuLZ (5 nM) and selected soluble Eu^{3+}-signal-modulating molecule, Dy1 (3−4 μM), Dy2 (25−100 μM), Dy3 (200−250 μM), and Dy4 (3−4 μM). Eu^{3+}-signals (615 nm) and TR-FRET (665 nm) signals were monitored for the first time after 15 min and thereafter at multiple time points during the 90 min incubation.

4.5. Data Analysis. In all assays, the S/B ratio was calculated as μ_{max}/μ_{min} and coefficient of variation (CV%) as $(\sigma/\mu) \times 100$. In all formulae, μ is the mean value and σ is the standard deviation (SD). The data were analyzed using Origin 8 software (OriginLab, Northampton, MA), and the half maximal inhibitory concentration (IC_{50}) values were obtained using standard sigmoidal fitting functions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03672.

EuLZ spectra (Figure S1), EuLZ and Dy1−4 spectral properties (Table S1), PKA inhibition using TR-FRET readout (Figure S2) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): K.K. and H.H. are involved in the QRET Technologies Ltd. Company.

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ABBREVIATIONS

ATP, adenosine triphosphate
CV%, coefficient of variation
Dy1, 1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide
Dy2, 4-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl-pyridinium perchlorate
Dy3, 6-*t*-7-nitrobenzofurazan-4-ylaminohexanoic acid
Dy4, 1,4-diamine anthoaquine
EuLZ, Eu^{3+}-chelate conjugated reporter peptide
FRET, Förster resonance energy transfer
HTS, high-throughput screening
IC_{50}, half maximal inhibitory concentration
ITC, isothiocyanate
LZ-pY, protein tyrosine phosphatase 1B substrate peptide
LZ-S, protein kinase A substrate peptide
MS, mass spectrometry
mTR-FRET, modulated time-resolved fluorescence resonance energy transfer
PKA, protein kinase A
PTM, post-translational modification
PTP1B, protein tyrosine phosphatase 1B
QRET, quenching resonance energy transfer
S/B, signal-to-background ratio
SD, standard deviation
TRF, time-resolved fluorescence
TR-FRET, time-resolved Förster resonance energy transfer

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