Detection of NAD(P)H-dependent enzyme activity by time-domain ratiometry of terbium luminescence

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ABSTRACT

NAD(P)H-dependent oxidoreductases play important roles in biology. Recently, we reported that the luminescence lifetime of some Tb³⁺ complexes is sensitive to NAD(P)H, and we used this phenomenon to detect activities of these enzymes. However, conventional time-resolved luminescence assays are susceptible to static quenchers such as ATP. Herein we describe a detection methodology that overcomes this issue: the intensity of the sample is measured twice with different delay times and the intensity ratio value is used as an index of NAD(P)H concentration. The method is more robust than single-point measurement, and is compatible with high-throughput assays using conventional microplate readers.

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Oxidized and reduced nicotinamide adenine dinucleotide (phosphate), abbreviated as NAD(P)^+ and NAD(P)H , are among the most important cofactors of oxidoreductases. NAD(P)H -dependent enzymes transfer two electrons from NAD(P)H to their substrates, affording NAD(P)^+ and the products, or they may catalyze the reverse reaction. These enzymes play important roles in various metabolic pathways, such as the TCA cycle, biosynthesis of amino acids, and the respiratory chain in mitochondria,¹ and therefore they are considered as potential therapeutic targets.² Traditionally, progress of NAD(P)H -dependent enzyme reactions has been monitored by measuring absorption of NAD(P)H itself at around 340 nm.^{2d} Although the method is simple and general, it is unsuitable for application in high-throughput screening (HTS) of drug candidates, because monitoring absorption in the UV region gives poor sensitivity (especially in small volumes such as 384- or 1536-well microplates) and low reliability.³ Use of colorimetric formazan dyes,⁴ fluorescence measurement of NAD(P)H at around 460 nm upon excitation at 340 nm, or the use of fluorescent probes for NAD(P)^+ and NAD(P)H ⁵ are alternative methods, but they also have problems in HTS applications, including low sensitivity at micromolar concentration of NAD(P)H (the most

commonly used concentration range in HTS), an inner filter effect at higher NAD(P)H concentration, insufficient stability of reagents, or unsuitability for continuous assays. Although highly sensitive methods using special converting enzymes have been developed and are commercially available,⁶ they are generally expensive and not so robust.

To overcome these limitations and to establish a reliable, HTS-compatible method for detecting activities of NAD(P)H -dependent enzymes, we recently reported a novel assay platform based on luminescence quenching of Tb^{3+} complexes.⁷ Compared with organic fluorophores, luminescent lanthanide complexes, in particular those of Tb^{3+} or Eu^{3+} , have extremely long luminescence lifetimes of millisecond order,⁸ which allows for sensitive detection of target molecules by measuring only the long-lived component of luminescence. Taking advantages of this “time-resolved measurement”, a number of biochemical assays using $\text{Tb}^{3+}/\text{Eu}^{3+}$ -labelled antibodies⁹ or functional luminescent probes¹⁰ have been developed. Because time-resolved assays are particularly favored in the field of drug discovery,⁹ due to their high S/N ratios derived from the low background signals, development of Tb^{3+} -based probes for NAD(P)H -dependent enzymes should be practically significant and useful in HTS. Therefore, in our previous work,⁷ stimulated by work from Parker *et al.* showing that some lanthanide complexes are susceptible to dynamic quenching by some biological reductants,¹¹ we first confirmed that this kind of intermolecular dynamic quenching is mediated efficiently by NAD(P)H for several positively-charged Tb^{3+} complexes including A1Tb (Figure 1a, Table S1, Figure S1), whereas NAD(P)^+ , an oxidized form of NAD(P)H , is not a luminescence-quencher.¹² Then, based on these findings, we developed a general luminescence intensity- and lifetime-based assay for NAD(P)H -dependent enzyme activities using A1Tb (Figure 1b). Because the method was continuous, reversible, and sensitive, we expected that it would be suitable for real-time monitoring of $[\text{NAD(P)H}]$ and related enzyme activities *in vitro*.

Figure 1. Summary of $[\text{NAD(P)H}]$ detection.⁷ (a) Schematic illustration of the interaction of Tb^{3+} complex (A1Tb) and NADH . (b) Cartoon representation of enzyme assay using A1Tb . Luminescence intensity/lifetime of A1Tb increases upon enzymatic consumption of NAD(P)H (quencher) to form NAD(P)^+ .

However, subsequent studies revealed that, for use in a wide range of HTS applications, including coupled enzyme assays,¹³ there is still a problem with these Tb^{3+} complexes. Generally speaking, single-point luminescence or fluorescence intensity measurement is simple and fast, but it is also susceptible to many sources of error. Time-resolved luminescence of lanthanide complexes is no exception,^{10d} and the obtained signal is perturbed by variation of probe concentration, absorption of excitation/emission light by surrounding components (dye compounds, etc.), and static quenching from ATP.^{14,15} So, standard time-resolved intensity measurement of A1Tb , although it can be easily performed on microplates, does not always provide high-quality data in HTS when ATP or analogous compounds are present in the solution (as shown below). Because these compounds are quite common in biological samples,¹⁶ this problem must be solved. On the other hand, luminescence lifetime measurement, where a multipoint decay curve is acquired and fitted by least-squares methods,¹⁷ is more reliable because it does not suffer from the above-mentioned factors; however, most commercial multifunctional plate readers are not compatible with lifetime measurement. Although a few instruments are equipped with lifetime measurement, is too time-consuming (>10 sec/well) for practical real-time monitoring of many samples. To circumvent these problems and to take advantage of luminescence *lifetime* changes even in HTS, we focused on a simpler and convenient lifetime-based measurement called the rapid lifetime determination (RLD) method,¹⁸ and modified it for enzyme assays using widely available instruments. In RLD, the luminescence intensities in two (successive) time regions are measured and then the lifetime is mathematically calculated from the length of the time gates and the ratio of the gated areas under the luminescence decay curve.^{18b} So far, RLD has been applied for spatial imaging of oxygen concentration,¹⁹ protein-protein interaction,²⁰ and hydrogen peroxide.²¹ But, although efforts to improve the algorithm for accurate lifetime determination have been made,²² it is often difficult to obtain the absolute lifetime by means of RLD.^{18b} So, we decided to further simplify RLD, avoiding the need for complicated

mathematical calculation while retaining the essential advantages of RLD, by simply taking the ratio of luminescence intensities at two specific delay times (I_1/I_2 in Figure 2) and using the ratio value itself as an index.²³ In principle, this ratio value, for fixed values of t_1 , t_2 , and window (or gate) time, uniquely reflects the change of [NAD(P)H] accompanying the progress of the enzyme reaction, and is independent of [ATP] and many other factors (Figure 2). In this paper, we call this method time domain ratiometry (TDR). TDR, unlike conventional lifetime measurement, is fast (~1 sec/well) and compatible with most commercially available multifunctional plate readers.

Figure 2. Schematic illustration of the TDR method. The ratio of luminescence intensities (I_1/I_2 and I'_1/I'_2) is dependent on [NAD(P)H], but independent of ATP concentration, etc.

To experimentally examine the usefulness of the TDR technique, we first titrated A1Tb with NADH or ATP, and measured (time-resolved) luminescence intensity (Figure 3a). ATP is the main biological energy source and is present at high intracellular concentration (1 – 10 mM); it is also essential for many enzyme assays.¹⁶ However, as mentioned earlier, ATP is reported to statically quench the luminescence of some Tb³⁺ complexes.¹⁴ Indeed, as shown in Figure 3a, not only NADH but also ATP decreased the luminescence intensity of A1Tb in a concentration-dependent manner. The expected static quenching of A1Tb by ATP was confirmed by Stern-Volmer analysis up to the concentration of 100 μ M (Figure S2). However, TDR analysis successfully nullified the influence of ATP, while maintaining the sensitivity to NADH (Figure 3b). This is because only the quenching by NADH is dynamic (Figure S2), as demonstrated earlier.⁷ Although less efficient than ATP, ADP also resulted in quenching of the luminescence intensity, but not lifetime, of A1Tb (Figure S2). These data indicated that at least in assays where ATP or ADP is present, intensity-based measurements of enzyme reactions with A1Tb might not work well, and TDR analysis should be a promising alternative.

Figure 3. (a) Luminescence intensities of A1Tb measured with a delay time of 50 μ s upon titration of NADH (left) or ATP (right). (b) The ratio of luminescence intensities measured with delay times of 50 μ s and 200 μ s upon titration NADH (left) or ATP (right). Error bars represent S. D. (n = 5).

Next, we investigated whether TDR analysis is really applicable to such assays. As an example of coupled enzyme assays involving ATP, hexokinase (EC 2.7.1.1) was chosen as the target. Hexokinase is an enzyme which phosphorylates hexoses such as glucose,¹ and is related to diseases such as cancer²⁴ and diabetes.²⁵ Also, its activity can be measured in the presence of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), which couples the reaction product of hexokinase, glucose-6-phosphate (G6P), with conversion of NADP⁺ to NADPH (Figure 4a).⁴ In this coupled assay, production of NADPH would result in a decrease of probe luminescence, while simultaneous consumption of ATP would work in the opposite direction. To circumvent the resulting complexity, TDR analysis would be useful.

First, we confirmed that glucose and NADP⁺ did not greatly influence the luminescence properties of the probe (Figure S3), and we also checked the progress of the enzyme reactions in the presence of A1Tb by absorption measurement of NADPH (Figure S4). We then performed a luminescence intensity- and lifetime-based assay using A1Tb with high probe and enzyme concentrations in a quartz cuvette (Figure S5). Because NADPH is a dynamic quencher, decrease of luminescence lifetime was observed as the reaction proceeded. Under this idealized condition, intensity-based measurement also worked well. Next, we moved to detection in a more realistic and practical situation using plastic microplates (Figure 4b). In conventional time-resolved measurement of luminescence intensity, no significant difference was observed up to 0.3 U/mL hexokinase. On the other hand, with TDR, hexokinase activity as low as 0.1 U/mL could be detected, largely due to the lower coefficient of variation (CV) of the ratiometry compared with that of single time-resolved intensity measurement (Table 1). This could be attributed to elimination of the effect of static quenching by ATP and ADP (Figure S2), as well as elimination of the effect of small but non-negligible inter-well differences, e.g., in probe concentration and sample volume (see Figure S6). Overall, the results show that our TDR analysis provides higher reliability than single intensity measurements, at least when enzyme activity is low and the reaction volume is small. Because the activities of many other enzymes are currently quantified by means of assays coupled to NAD(P)H production or

consumption,¹³ we expect that this approach will be applicable to a variety of enzymes.

In conclusion, we have developed a novel assay method for NAD(P)H-dependent enzymes by utilizing the luminescent lifetime change of Tb³⁺ complexes. Specifically, we applied and validated TDR analysis, which retains the advantages of lifetime measurements while being much simpler, faster, and compatible with commercially available equipment. Compared with widely used single-point time-resolved measurements,⁸ TDR has the advantages that it eliminates errors arising from differences in probe concentration, reaction volume, etc. (Figure S6), and it is more robust with respect to interfering compounds such as ATP. TDR analysis cannot completely eliminate the effect of certain compounds, such as GTP,^{7,15} but it is markedly better than intensity-based detection using A1Tb (Figure S7). TDR should also be applicable to other luminescent probes that exhibit lifetime changes. Although TDR analysis using A1Tb would not be suitable for cell-based assays and certain biochemical assays because of the probe's poor membrane permeability and susceptibility to dynamic quenching by some reductants,^{7,14} we believe it would be a good option for a number of enzyme assays in vitro. We are currently planning to apply this method for inhibitor screening and activity profiling of NAD(P)H-dependent enzymes, as we have already reported for some other enzymes.²⁶

Figure 4. (a) Schematic illustration of the coupled assay of hexokinase. G6P: glucose-6-phosphate, G6PDH: glucose-6-phosphate dehydrogenase. (b) Monitoring hexokinase activities by detecting time-resolved luminescence intensity of 1 μM A1Tb at the delay time of 800 μs (left) or by TDR at delay times of 50 μs and 800 μs (right). Glucose was excluded from control samples. Error bars represent S. D. (n = 5). Asterisks indicate $p < 0.01$ vs control. Detailed reaction conditions are given in supplementary information.

Table 1. CV (%) of coupled assay.^a

	control	hexokinase 0.1 U/mL	hexokinase 0.3 U/mL
Intensity assay	6.9	3.5	1.7
TDR assay	1.5	1.2	0.9

^aThe values were calculated from the average and S.D. of the same 5 wells at 25 min after the reaction was started. Reaction conditions were identical with those for Figure 4.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016>.

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