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A Multi-Enzyme Bioluminescent Time-Resolved Pyrophosphate Assay

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Abstract

We have developed a high-sensitivity assay for measurement of inorganic pyrophosphate (PPi) in adenosine 5'-triphosphate (ATP) contaminated samples. The assay is based on time-resolved measurements of the luminescence kinetics and implements multiple enzymes to convert PPi to ATP that is, in turn, utilized to produce light; and to hydrolyze PPi for measurement of the steady-state background luminescence. A theoretical model for describing luminescence kinetics and optimizing composition of the assay detection mixture is presented. We found the model is in excellent agreement with the experimental results. We have developed and evaluated two algorithms for PPi measurement from luminescence kinetics acquired from ATP-contaminated samples. The first algorithm is considered to be the method of choice for analysis of long, i.e., 3-5 min, kinetics. The activity of enzymes is controlled during the experiment; the sensitivity of PPi detection is about 7 pg/ml or 15 pM of PPi in ATP-contaminated samples. The second algorithm is designed for analysis of short, i.e., less than 1-min luminescence kinetics. It has about 20 pM PPi detection sensitivity and may be the better choice for assays in microplate format, where a short measurement time is required. The PPi assay is primarily developed for RNA expression analysis, but it also can be used in various applications, which require high sensitivity PPi detection in ATP-contaminated samples.

Keywords

Bioluminescent assay; Enzyme kinetics; Inorganic pyrophosphate; Time-resolved kinetics; Gene expression; Nucleic acid detection

I. Introduction

Bioluminescence assays for detecting inorganic pyrophosphate at sub-nanomolar concentrations can become a promising new tool for RNA expression analysis for both messenger (mRNA) and micro-RNA [1,2]. The bioluminescence RNA quantification method is based on detection of released inorganic pyrophosphate (PPi) during RNA reverse transcription or replication [3,4]. During the polymerization reaction the PPi is released as the result of nucleotide incorporation by polymerase. The released PPi can be converted into adenosine 5'-triphosphate (ATP) by ATP-sulfurylase, and subsequently, the ATP provides the energy for luciferase to oxidize luciferin and generate light [5]. The bioluminescence PPi assay can be extremely sensitive and potentially can detect a single target RNA molecule. This is possible because the synthesis of a single large cDNA can require incorporation of thousands

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of deoxynucleotides, consequently producing thousands of ATP molecules and resulting in the emission of hundreds of photons per each cDNA copy synthesized. Yet, to achieve such a level of sensitivity the development of a new generation of bioluminescence PPi assay is required. One promising approach has been reported recently, which employs a bioluminescence regenerative cycle system (BRC) [1,3]. The regenerative cycle uses the ATP-sulfurylase enzyme to convert PPi to ATP. In the presence of luciferin and luciferase the consumption of the ATP molecule results in light emission and formation of a PPi molecule as by-product. This by-product PPi molecule can be re-used in another cycle of ATP production and subsequent light emission. The advantage of the regenerative bioluminescence system is that each PPi molecule can initiate potentially hundreds of light emission cycles, producing steady-state emission with the intensity proportional to the number of PPi molecules in sample. Yet, the regenerative system also has serious drawbacks. The real biological samples unavoidably carry some residual amount of ATP molecules (ATP contamination), that alone with target PPi molecules, can be involved as a substrate in the regenerative cycle. In addition, the biological samples exhibit steady-state luminescence (luminescence background), which is originated by non-ATP substrate(s) in samples [6]. The ATP contamination and steady-state luminescence background reduces the sensitivity and distorts the accuracy of PPi measurement by the regenerative cycle system.

To address the drawbacks of the regenerative cycle system we have developed an alternative high-sensitivity PPi assay, which allows, in a single experiment, measurement of the three components of the luminescence: the PPi component, the ATP component, and the steady-state background luminescence. This is achieved by formulating a multi-enzyme assay mixture, which produces luminescence kinetics with easily distinguishable fast and slow decay components. The fast component is due to conversion of PPi to ATP, and the slow component is driven by two enzymatic reactions: one involves the consumption of ATP by luciferase and the other the hydrolysis of PPi to inorganic phosphate (Pi) by inorganic pyrophosphatase (PPase) [7]. The use of PPase allows assessment of the intensity of steady-state luminescence in biological samples for increasing sensitivity and accuracy of PPi measurements.

The principle difference of our approach vs. the regenerative cycle system is the use of time-resolved measurement (i.e., kinetics assay) instead of the measurement of the steady-state luminescence intensity by regenerative cycle system. In this report we present the theoretical analysis of the multi-enzyme assay for kinetics measurements, describe the experimental procedures and algorithms for analysis of experimental data, and present results of PPi measurement at a sensitivity of about 7 pg/ml or 15 pM of PPi in ATP-contaminated samples. The assay is about three orders of magnitude more sensitive than the previously reported methods of PPi quantification (i.e., 15 pM-20 pM vs. ~20 nM sensitivity of the commercial assay) [8-12]. The kinetics assay was primarily developed for application in mRNA and microRNA expression analysis [2,4], but it also can be used for PPi measurement in other applications. It was used, for instance, to measure PPi and ATP in patient's breath condensate, where the PPi was detected at ~0.8 ng/ml and ATP at less than 0.1 ng/ml levels in 5 μ l fluid samples (unpublished results). The assay is designed for application with commercially available photon-counting luminometers and can be implemented in single-tube or microplate format to match the specific requirements of various applications.

II. Theoretical Consideration of Time-Resolved PPi Assay

In the simplified model of PPi kinetics assay, three linked reactions determine the kinetics of luminescence: (1) the reaction of conversion of PPi to ATP by ATP-sulfurylase, $E_{\text{ATP-Sulf}}$; (2) the reaction of consumption of PPi by inorganic pyrophosphatase, E_{PPase} ; and (3) the reaction of consumption of ATP by luciferin-luciferase $E_{\text{L-L}}$, that results in photon emission [13]:

Here γ is the rate of corresponding enzymatic reaction. In the simplified model (1)-(3) only products that are essential for further discussion are shown. Under the excess of enzymes $\text{E}_{\text{ATP-Sulf}}$, E_{PPase} , and $\text{E}_{\text{L-L}}$ the concentrations of ATP and PPi in solution are given by equations for a first-order biochemical reaction:

$$d[\text{PPi}]/dt = -(\gamma_{\text{sulf}} + \gamma_{\text{PPase}})[\text{PPi}] + \gamma_{\text{L-L}}[\text{ATP}] \quad (4)$$

$$d[\text{ATP}]/dt = \gamma_{\text{sulf}}[\text{PPi}] - \gamma_{\text{L-L}}[\text{ATP}] \quad (5)$$

$$I_{h\nu} = \gamma_{\text{L-L}}[\text{ATP}] \quad (6)$$

where $I_{h\nu}$ is the intensity of luminescence measured in photons/sec. Eqs.(4-6) assume the co-substrates, i.e., luciferin and molecular oxygen are at constant concentration, which is valid at typical assay conditions. Figure 1 shows examples of the luminescence vs. time detected at typical experimental conditions by a mixture of ATP-sulfurylase/PPase/Luciferin-Luciferase in solution containing different amount of PPi and ATP at $t = 0$. If there is a little or no ATP in solution at $t = 0$, the luminescence increases rapidly until it reaches the maximum intensity followed by a slower exponential decay to a steady-state luminescence level, I_{Backgr} , as illustrated in Figure 2. The luminescence kinetics in Figure 2 can be considered as a sum of three components described by five parameters: the fast and slow exponential component with amplitude A_{Fast} , B_{Slow} and rates γ_{Fast} and γ_{Slow} for the fast and slow components respectively and the steady-state luminescence level I_{Backgr} . The background luminescence I_{Backgr} is due to slowly reacting impurities, that often are present in samples. The I_{Backgr} is not described by the simplified reaction model given by Eqs. (1-3) and can be introduced as an additional phenomenological parameter of the model when interpreting experimental results. The fast and slow components of the luminescence kinetics can be expressed through the parameters of the model given by Eqs.(4-6) by substituting $I(t)$ for $I_{h\nu}$:

$$I(t) = A_{\text{Fast}} \exp(-\gamma_{\text{Fast}} t) + B_{\text{Slow}} \exp(-\gamma_{\text{Slow}} t) \quad (7)$$

where kinetics observed experimentally can be approximated by $I_{\text{Exper}}(t)$:

$$I_{\text{Exper}}(t) = I(t) + I_{\text{Backgr}} \quad (8)$$

From Eq.(8) and Eq.(4-6) and assumption of $\gamma_{L-L} \ll (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}})$ to simplify the following expressions for γ_{Fast} and γ_{Slow} , we find for concentrations of ATP and PPi:

$$[\text{ATP}] = \gamma_{L-L}^{-1} A_{\text{Fast}} \exp(-\gamma_{\text{Fast}} t) + \gamma_{L-L}^{-1} B_{\text{Slow}} \exp(-\gamma_{\text{Slow}} t) \quad (9)$$

$$[\text{PPi}] = (\gamma_{L-L} - \gamma_{\text{Fast}})(\gamma_{L-L} \gamma_{\text{Sulf}})^{-1} A_{\text{Fast}} \exp(-\gamma_{\text{Fast}} t) + (\gamma_{L-L} - \gamma_{\text{Slow}})(\gamma_{L-L} \gamma_{\text{Sulf}})^{-1} B_{\text{Slow}} \exp(-\gamma_{\text{Slow}} t) \quad (10)$$

$$\gamma_{\text{Fast}} \approx \gamma_{L-L} + \gamma_{\text{PPase}} + \gamma_{\text{Sulf}} \quad (11)$$

$$\gamma_{\text{Slow}} \approx \gamma_{L-L} \gamma_{\text{PPase}} / (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}}) \quad (12)$$

where A_{Fast} and B_{Slow} can be found from the concentrations $[\text{ATP}]_0$ and $[\text{PPi}]_0$ of the ATP and PPi at $t = 0$:

$$[\text{ATP}]_0 = \gamma_{L-L}^{-1} A_{\text{Fast}} + \gamma_{L-L}^{-1} B_{\text{Slow}} \quad (13)$$

$$[\text{PPi}]_0 = (\gamma_{L-L} - \gamma_{\text{Fast}})(\gamma_{\text{Sulf}} \gamma_{L-L})^{-1} A_{\text{Fast}} + (\gamma_{L-L} - \gamma_{\text{Slow}})(\gamma_{\text{Sulf}} \gamma_{L-L})^{-1} B_{\text{Slow}} \quad (14)$$

Efficiency of detection of PPi and ATP molecules

Assay efficiency to detect PPi and ATP molecules can be characterized by the total number of photons, N_{Phot} , emitted per each PPi and ATP molecule by the assay solution. The number is given by the integral over the luminescence intensity $I(t)$:

$$N_{\text{Phot}} = \int (A_{\text{Fast}} \exp(-\gamma_{\text{Fast}} t) + B_{\text{Slow}} \exp(-\gamma_{\text{Slow}} t)) dt = A_{\text{Fast}} / \gamma_{\text{Fast}} + B_{\text{Slow}} / \gamma_{\text{Slow}} \quad (15)$$

For sample solutions having only ATP molecules at $t = 0$, we have $[\text{PPi}]_0 = 0$ and from Eqs. (11)-(15) the number of photons, n_{ATP} , emitted by a unit volume of the assay solution:

$$n_{\text{ATP}} = \{\gamma_{L-L} / (\gamma_{\text{Fast}} - \gamma_{\text{Slow}})\} \{(\gamma_{L-L} - \gamma_{\text{Slow}}) / \gamma_{\text{Fast}} + (\gamma_{\text{Fast}} - \gamma_{L-L}) / \gamma_{\text{Slow}}\} [\text{ATP}]_0 \quad (16)$$

The number of photons emitted per each ATP molecule in solution:

$$n_{\text{ATP}} / [\text{ATP}]_0 = \{\gamma_{L-L} / (\gamma_{\text{Fast}} - \gamma_{\text{Slow}})\} \{(\gamma_{L-L} - \gamma_{\text{Slow}}) / \gamma_{\text{Fast}} + (\gamma_{\text{Fast}} - \gamma_{L-L}) / \gamma_{\text{Slow}}\} \approx 1 + \gamma_{\text{Sulf}} / \gamma_{\text{PPase}} \quad (17)$$

Now, for sample solutions having only PPi molecules and no ATP molecules at $t = 0$, the number of photons, n_{PPi} , emitted by a unit volume of the assay solution is given by:

$$n_{\text{PPi}} = \{(\gamma_{\text{Sulf}} \gamma_{\text{L-L}}) / (\gamma_{\text{Fast}} \gamma_{\text{Slow}})\} [\text{PPi}]_0 \quad (18)$$

and for the number of photons per each PPi molecule we have:

$$n_{\text{PPi}} / [\text{PPi}]_0 = (\gamma_{\text{Sulf}} \gamma_{\text{L-L}}) / (\gamma_{\text{Fast}} \gamma_{\text{Slow}}) \approx \gamma_{\text{Sulf}} / \gamma_{\text{PPase}} \quad (19)$$

The efficiency of photon generation by PPi vs. the efficiency of ATP luminescence in the same assay solution can be found from Eqs.(17) and (19) at $[\text{PPi}]_0 = [\text{ATP}]_0$:

$$n_{\text{PPi}} / n_{\text{ATP}} = \gamma_{\text{Sulf}} (\gamma_{\text{Fast}} - \gamma_{\text{Slow}}) / (\gamma_{\text{Slow}} (\gamma_{\text{L-L}} - \gamma_{\text{Slow}}) + \gamma_{\text{Fast}} (\gamma_{\text{Fast}} - \gamma_{\text{L-L}})) \approx \gamma_{\text{Sulf}} / (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}}) \quad (20)$$

The ratio $n_{\text{PPi}}/n_{\text{ATP}}$ also can be measured from luminescence kinetics by calculating the ratio of the areas under the corresponding $I(t)$ curves. For assay mixture used to carry out the measurements shown in Figure 1(a), (b) we found $n_{\text{PPi}}/n_{\text{ATP}} = 0.38$.

The ratio $n_{\text{PPi}}/n_{\text{ATP}}$ increases as the rate of conversion of PPi to ATP increases and decreases as the concentration of inorganic pyrophosphatase (PPase) in the assay mixture increases. Although the inclusion of PPase in the detection mixture reduces the efficiency of emitting photons by PPi molecules, it plays an overall positive role by allowing measurement of the steady-state luminescence background for accurate determination of luminescence from ATP and PPi molecules in sample.

Design of PPi detection assay

Two main requirements determine the design of the assay mixture for PPi detection: (1) requirement for high detection sensitivity, which, to large extent, is determined by $\gamma_{\text{Sulf}}/\gamma_{\text{PPase}}$; (2) requirement for sufficient time to complete the assay, which determines the concentrations of enzymes for achieving the desirable rates of the fast and slow components of the luminescence kinetics. In this study the objective is to adjust the PPi assay mixture for optimal performance with a photon counting luminometer (Sirius and Orion models, Berthold Detection Systems, GmbH, Pforzheim/Germany). The Sirius single-tube luminometer acquires data at a rate of 5 measurements per second, and when used with a luciferin-luciferase mixture having $\gamma_{\text{L-L}} = 0.0120 \text{ s}^{-1}$ (Sigma, Cat. No. FL-AA, diluted :2 according to the manufacturer protocol) it is able to detect less than 10 fg or about 20 attomole of ATP molecules [3]. Considering 1-5 minutes as a desirable time to complete the PPi assay, the required rates of the assay reactions can be set to have $\gamma_{\text{Fast}}^{-1} \approx 20 \text{ sec}$ and $\gamma_{\text{Slow}}^{-1} \approx 100 \text{ sec}$. At these reaction rates 300-1500 measurement points can be acquired over 1-5 min assay time. Subsequently, the five parameters of the model given by Eqs. (4-6) can be measured from the luminescence kinetics. For the PPi assay mix we have $\gamma_{\text{Fast}} \approx (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}}) = 0.05 \text{ s}^{-1}$ and $\gamma_{\text{Slow}} \approx \gamma_{\text{L-L}} \gamma_{\text{PPase}} / (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}}) = 0.01 \text{ s}^{-1}$. From the reaction rate of the luciferin-luciferase mixture (Sigma, Cat. No. FL-AA, :2 fold diluted) which has $\gamma_{\text{L-L}} = 0.012 \text{ s}^{-1}$ and from $\gamma_{\text{L-L}}$, γ_{Fast} and γ_{Slow} we find the other reaction rates of the PPi assay: $\gamma_{\text{PPase}} \approx 0.042 \text{ s}^{-1}$ and $\gamma_{\text{Sulf}} \approx 0.008 \text{ s}^{-1}$. With our luminometer the detection limit of PPi molecules, n_{PPi} , for the selected set of reaction parameters can be estimated from the efficiency of PPi vs. ATP detection given by Eq. (19) and ATP detection sensitivity of $n_{\text{ATP}} = 20 \text{ attomole}$. Due to lower efficiency of PPi vs. ATP detection we have: $n_{\text{PPi}} \approx (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}}) / \gamma_{\text{Sulf}} n_{\text{ATP}} \approx 125 \text{ attomole}$. This estimation represents

the limit for detection sensitivity of the PPI assay due to the sensitivity of the luciferin/luciferase mixture when the background luminescence of the sample solution, I_{Backgr} , can be neglected. Figure 3 shows the calculation of the assay sensitivity vs. the assay time $\gamma_{\text{Slow}}^{-1}$ at three different concentrations of luciferin-luciferase in the detection mixture. In Figure 3 the concentrations of ATP-Sulfurylase and PPase are calculated from Eqs. (11-12) to have the overall assay time $\gamma_{\text{Slow}}^{-1}$ shown in the figure for luciferin-luciferase concentrations corresponding to $\gamma_{\text{L-L}}$ value of 0.012 s^{-1} ; 0.024 s^{-1} ; and 0.048 s^{-1} and the requirement of $\gamma_{\text{Fast}} = 0.05 \text{ s}^{-1}$. From data in Figure 3 the lower PPI amount can be detected by increasing the assay time or by increasing the concentration of assay reagents. Obviously this latter option will increase the assay cost. The various factors, which influence assay performance have to be taken into consideration when designing the assay for use in single-probe or microplate format. The single-probe format often does not require the shorter assay time. The higher detection sensitivity can be achieved during 3-5 min measurement by optimizing ATP-sulfurylase and PPase for performing the assay more economically. The microplate format assay may require shorter, i.e., ~1 min assay time, which can be achieved by performing the assay with higher concentration of reagents or by accepting somewhat lower detection sensitivity.

Measurement of PPI and ATP from luminescence kinetics

The Eqs.(13)-(14) can be used to measure the amount of $[\text{ATP}]_0$ and $[\text{PPI}]_0$ in a sample by first measuring the five parameters A_{Fast} , B_{Slow} , γ_{Fast} , γ_{Slow} , and I_{Backgr} from the luminescence kinetics and then calculating $[\text{ATP}]_0$ and $[\text{PPI}]_0$ according to Eqs. (13)-(14). The amplitudes of the fast and slow components of luminescence kinetics A_{Fast} and B_{Slow} can be used to calculate $[\text{ATP}]_0$ and $[\text{PPI}]_0$ according to:

$$[\text{ATP}]_0 = \eta(A_{\text{Fast}} + B_{\text{Slow}}) \quad (21)$$

$$[\text{PPI}]_0 = \kappa(B_{\text{Slow}} - \mu A_{\text{Fast}}) \quad (22)$$

where η , κ , and μ are the parameters which are determined by calibration, i.e., by measuring known amount of ATP and PPI in two reference solutions: i.e., with known amount of PPI and no ATP, and known amount ATP and no PPI.

An additional advantage of measuring the kinetics parameters by the proposed assay is the option for monitoring the enzymes' activities and making corrections to the measured value during a prolonged, i.e., a few hours long, experiment. The assay's enzymes, and primarily the luciferase, are known to lose their activity when stored at room temperature and often show some decline of activity when stored on ice over a few hours time span [14]. The measurement of γ_{Fast} and γ_{Slow} in each experiment can be used to detect possible changes of enzymes activity:

$$\gamma_{\text{Fast}} + \gamma_{\text{Slow}} = \gamma_{\text{L-L}} + \gamma_{\text{PPase}} + \gamma_{\text{Sulf}} \approx (\gamma_{\text{PPase}} + \gamma_{\text{Sulf}}) \quad (23)$$

$$\gamma_{\text{Fast}} \gamma_{\text{Slow}} = \gamma_{\text{L-L}} \gamma_{\text{PPase}} \quad (24)$$

here, the measurements of the fast and slow rates of luminescence kinetics can be used to assess the possible change of $\gamma_{\text{L-L}}$, γ_{PPase} , and γ_{Sulf} . Often the dominant factor for poor cross-experiment reproducibility is the decline of the luciferase activity, which can be described by

the ratio of $(\gamma_{L-L} / \gamma_{L-L}^{(0)}) \approx (\gamma_{Slow}^{(0)} / \gamma_{Slow})$, where γ_{L-L} and $\gamma_{L-L}^{(0)}$ are the reaction rates of the luciferin-luciferase in the current, γ_{L-L} , and the calibration experiment, $\gamma_{L-L}^{(0)}$, respectively. To reflect the possible change of the luciferin-luciferase activity, the measured values $[ATP]_M$ and $[PPi]_M$ have to be corrected to give the true $[ATP]_O$ and $[PPi]_O$ values:

$$[ATP]_O = [ATP]_M (\gamma_{Slow}^{(0)} / \gamma_{Slow}) \quad (25)$$

$$[PPi]_O = [PPi]_M (\gamma_{Slow}^{(0)} / \gamma_{Slow}) \quad (26)$$

Application of the approach given by Eqs. (21-22) will be further discussed in the following Experimental Section.

III. Experimental Section

Material and Methods

The ATP-detection solution has been prepared from luciferin-luciferase assay mix (Sigma, Cat. No. FL-AAM) containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine buffer salts (pH 7.8). The stock solution has been prepared by dissolving the lyophilized mix in 1.5 ml of sterile water. Adenosine 5'-phosphosulfate sodium salt (APS) was purchased from Sigma, Cat. No. A5508. The inorganic pyrophosphatase (PPase) was purchased from USB, Cat. No. 70953Y (USB, Cleveland, OH). The adenosine 5'-triphosphate sulfurylase (ATP-sulfurylase) was purchased from Sigma, Cat. No. A8957. Inorganic pyrophosphate (PPi) and ATP are from Sigma, Cat. No. 221368 and FL-AAS respectively. ATP and PPi test solutions have been prepared by dissolving the reagents in ATP-free water, Promega Cat. No. F201A.

The luminescence was measured by the Sirius single-tube photon-counting luminometer (Berthold Detection Systems, GmbH, Pforzheim/Germany) interfaced with a personal computer. PPi detection mixture, prepared as described below, was kept on ice before using for measurements. The sample was prepared by adding 40 μ l of the PPi detection mixture to the disposable 5 ml measurement tube (Cat. No. 55.526, Sarstedt, Newton, NC) and kept in dark for 5 min at room temperature to allow the background luminescence to reach a steady-state level. Then 5 μ l of sample solution was added to the PPi detection solution and mixed by pipetting for 2 sec while avoiding air bubbles. The tube was inserted into the luminometer chamber and measurement of luminescence was started 8 sec $\pm$ 1 sec after addition of the sample to the PPi detection solution. Luminescence data were acquired over 5 min period at the rate of 5 measurements per second. The acquired data were saved on an Excel worksheet (Microsoft Co.) for further analysis by an in-house developed macros and stand-alone software package.

Preparation of assay mix for PPi detection—The main components of the PPi assay solution include the luciferin-luciferase mixture for ATP detection; APS/ATP-sulfurylase for PPi to ATP conversion; and inorganic pyrophosphatase (PPase) for producing the slow component of the kinetics for measurement of the luminescence background. The composition of the assay solution has to satisfy certain requirements: The amount of APS substrate in solution is one of the dominant factors that determine the dynamic range of the assay. To ensure there is no decline of the assay performance due to depletion of APS substrate, the number of APS molecules has to outnumber the PPi molecules in sample. For an assay designed for

analysis of sample containing 100 attomole – 100 femtomole PPi molecules about 400 femtomole of APS in assay solution may be needed.

The fast rate of the luminescence should be in the range in which the kinetics can be accurately captured by the instrument (luminometer). For an instrument with the data capture rate of 5 measurements per second, the fast component of the kinetics having $\gamma_{\text{Fast}}^{-1} \sim 20$ sec rise time yields ~ 50 measurement points, and, in this study, was considered to be adequate for accurate measurement of the kinetics parameters. The $\gamma_{\text{Fast}}^{-1} \sim 20$ sec leads to the requirement of $\gamma_{\text{PPase}} + \gamma_{\text{Sulf}} \approx 0.05 \text{ sec}^{-1}$ and was used in this study to determine the amount of ATP-sulfurylase and PPase in the assay solution.

The slow component of the kinetics allows measurement of the luminescence background. To complete an assay in 1-5 minutes, the slow component of the kinetics was adjusted to $\gamma_{\text{Slow}}^{-1} \sim 100$ sec by selecting the concentrations of the luciferin/luciferase and PPase in the assay solution.

The PPi detection solution was prepared by adding 400 femtomole of APS, ~ 100 mU of ATP-sulfurylase and ~ 10 mU of PPase per each 40 μl ATP-detection solution. The exact amount of ATP-sulfurylase and PPase was adjusted to achieve the desirable rates of γ_{PPase} and γ_{Sulf} by measuring the luminescence kinetics of test samples prepared from solutions of 0.4 nM of ATP and 2 nM of PPi in assay dilution buffer (Cat. No. FL-AAB, Sigma). The prepared PPi detection solution was kept on ice for at least 40 min before the use in experiments. During the 40-min “cool-down” time the initial luminescence background from the presence of residual PPi and ATP in enzyme solutions and reagents decreased by more than an order of magnitude from its initial level to the steady-state level of about 15,000 RLU/sec (Relative Light Units per second), as measured by the Sirius luminometer. Figure 4 shows examples of luminescence kinetics from samples prepared in aqueous solution by mixing the known amount of ATP and PPi and by adding 5 μl of sample to 40 μl of PPi assay solution. Figure 4 shows a small set of luminescence kinetics from a larger set of data used to investigate different algorithms for measuring ATP and PPi content of the sample and to determine the assay sensitivity when measuring PPi in ATP-contaminated samples.

PPi measurement—Different algorithms can be used to measure A_{Fast} , B_{Slow} , γ_{Fast} , γ_{Slow} , and I_{Backgr} from the experimental kinetics. Nonlinear fitting using Eqs.(7-8) may be too computationally intensive for processing large amount of experimental data [15]. We have developed an alternative algorithm that is based on the measurement of the total number of emitted photons, N_i , over a time intervals T_i during the rise and decline of the luminescence kinetics, as illustrated in Figure 5. Five intervals are selected during the luminescence kinetics: two adjacent intervals at the beginning of the kinetics and three adjacent intervals at the kinetics tail. The total number of emitted photons is calculated for each time interval, N_i :

$$N_i = \int \{A_{\text{Fast}} \exp(-\gamma_{\text{Fast}} t) + B_{\text{Slow}} \exp(-\gamma_{\text{Slow}} t) + I_{\text{Backgr}}\} dt \quad (27)$$

and

$$N_i = \sum I_{\text{Experi}}(t_k) \quad (28)$$

where the sum is taken over the corresponding time interval T of the luminescence kinetics observed in experiment. At the tail of the luminescence kinetics $A_{\text{Fast}} \exp(-\gamma_{\text{Fast}} t) \ll B_{\text{Slow}}$

$\exp(-\gamma_{\text{Slow}}t)$ and for the difference in the number of emitted photons for adjacent time intervals N_3 , N_4 , and N_5 :

$$N_4 - N_5 = (B_{\text{Slow}}/\gamma_{\text{Slow}})(1 - \exp(-\gamma_{\text{Slow}} T))\exp(-\gamma_{\text{Slow}} T)\exp(-\gamma_{\text{Slow}} t_3) \quad (29)$$

$$N_3 - N_4 = (B_{\text{Slow}}/\gamma_{\text{Slow}})(1 - \exp(-\gamma_{\text{Slow}} T))\exp(-\gamma_{\text{Slow}} t_3)\exp(-\gamma_{\text{Slow}} t_3) \quad (30)$$

From Eqs.(29-30) we find:

$$\gamma_{\text{Slow}} = (1/T) \ln\{(N_4 - N_5)/(N_3 - N_4)\} \quad (31)$$

$$B_{\text{Slow}} = (1/(t_4)) \{(N_3 - N_4)^2/(N_3 - 2N_4 + N_5)\} \ln\{(N_4 - N_5)/(N_3 - N_4)\} \quad (32)$$

$$I_{\text{Backgr}} = (1/T)\{N_3 - (t_3/T)(N_4 - N_5)\} \quad (33)$$

where γ_{Slow} , B_{Slow} , and I_{Backgr} are expressed through the parameters measured from the experimental data; t_4 is the delay of N_4 interval as shown in Figure 5. After subtracting the slow component and background from the kinetics, the N_1' and N_2' are calculated from the remaining fast component of the kinetics. Next, γ_{Fast} and A_{Fast} can be calculated as follows:

$$\gamma_{\text{Fast}} = (1/T) \ln(N_1'/N_2') \quad (34)$$

$$A_{\text{Fast}} = (N_1' - N_2')(N_2'/N_1')^2 \ln(N_1'/N_2') \quad (35)$$

The smoothed line in Figure 5 shows an approximation of the experimental data by Eq.(7) with the parameters determined by the above described algorithm. The R^2 value for the fit in Figure 5 is 0.998, thus indicating an excellent consistency between the model of Eq.(7) and the experimental data and also illustrating a robust algorithm for measurement the kinetics parameters [15,16].

The algorithm given by Eq.(31)-(35) was used to process experimental kinetics from samples carrying known amounts of PPi and ATP. The amount of PPi was determined according to Eq. (21)-(22) by calibrating the response using two reference samples carrying 50 pg PPi and 5 pg ATP. Figure 6 shows examples of measurement of the amount of PPi in samples, in which ATP contamination was simulated by adding 0, 1 pg, and 2 pg of ATP. In samples with ~40 pg of PPi the precision of PPi measurement is 6%, where the precision is defined as the ratio $D(X)/X$ of the value's standard deviation $D(X)$ to the value's average X . The assay with the described data processing algorithm is able to detect less than 0.3 pg or 0.6 femtomole of PPi in 45 μ l samples with amounts of ATP exceeding the amount of PPi.

Algorithm for fast PPi measurement—The algorithm given by Eqs.(31-35) requires capture of the slow component of the kinetics by acquiring data over a relatively long, i.e., ~ 300 sec, period of time. We have investigated an alternative approach for analyzing data and making measurements from kinetics acquired over less than 1 min. This alternative approach is based on the linearity of Eqs.(4-6). The linearity allows the luminescence kinetics of a composite sample to be presented by a sum of two luminescent kinetics for PPi and ATP components of the mixture. If at $t = 0$ Sample 1 has a known amount, $Q_{ATP}^{(0)}$, of ATP and no PPi and Sample 2 has known amount, $Q_{PPi}^{(0)}$, of PPi and no ATP, and the luminescence kinetics from these samples are $I_{ATP}(t)$ and $I_{PPi}(t)$ respectively, then the kinetics, $I_{Sample}(t)$, of a sample with unknown amount of ATP and PPi can be approximated by a linear combination of kinetics $I_{ATP}(t)$ and $I_{PPi}(t)$:

$$I_{Sample}(t) = a I_{ATP}(t) + b I_{PPi}(t) + c \quad (36)$$

where c is the steady-state luminescence intensity. The amount of ATP and PPi in the sample can be calculated using the values a and b from Eq.(36):

$$Q_{ATP} = a Q_{ATP}^{(0)} \quad (37)$$

$$Q_{PPi} = b Q_{PPi}^{(0)} \quad (38)$$

The parameters a , b and c can be calculated from the experimental data by using linear least-square fitting [15]:

$$a = (N \langle I_{ATP} I_{Sample} \rangle - \langle I_{ATP} \rangle \langle I_{Sample} \rangle) / (N \langle I_{ATP}^2 \rangle - \langle I_{ATP} \rangle^2) \quad (39)$$

$$b = (N \langle I_{PPi} I_{Sample} \rangle - \langle I_{PPi} \rangle \langle I_{Sample} \rangle) / (N \langle I_{PPi}^2 \rangle - \langle I_{PPi} \rangle^2) \quad (40)$$

$$c = (N \langle I_{Sample} \rangle - \langle I_{ATP} \rangle \langle I_{Sample} \rangle - \langle I_{PPi} \rangle \langle I_{Sample} \rangle) / (N - \langle I_{ATP} \rangle - \langle I_{PPi} \rangle) \quad (41)$$

$$D = (N \langle I_{ATP}^2 \rangle - \langle I_{ATP} \rangle^2) + 2 \langle I_{ATP} I_{PPi} \rangle - \langle I_{ATP} \rangle \langle I_{PPi} \rangle - \langle I_{ATP} \rangle^2 - \langle I_{PPi} \rangle^2 - \langle I_{ATP} \rangle \langle I_{PPi} \rangle \quad (42)$$

where

$$\langle I_{ATP}^2 \rangle = \sum I_{ATP}^{(i)} I_{ATP}^{(i)} \quad (43)$$

$$\langle I_{PPi}^2 \rangle = \sum I_{PPi}^{(i)} I_{PPi}^{(i)} \quad (44)$$

$$\langle I_{Sample}^2 \rangle = \sum I_{Sample}^{(i)} I_{Sample}^{(i)} \quad (45)$$

$$\langle I_{ATP} I_{PPi} \rangle = \sum I_{ATP}^{(i)} I_{PPi}^{(i)} \quad (46)$$

$$\langle I_{ATP} I_{Sample} \rangle = \sum I_{ATP}^{(i)} I_{Sample}^{(i)} \quad (47)$$

$$\langle I_{PPi} I_{Sample} \rangle = \sum I_{PPi}^{(i)} I_{Sample}^{(i)} \quad (48)$$

$$\langle I_{ATP} \rangle = \sum I_{ATP}^{(i)} \quad (49)$$

$$\langle I_{PPi} \rangle = \sum I_{PPi}^{(i)} \quad (50)$$

$$\langle I_{Sample} \rangle = \sum I_{Sample}^{(i)} \quad (51)$$

here $I^{(i)}$ is the respective luminescence intensity $I_{ATP}(t)$, $I_{PPi}(t)$, and $I_{Sample}(t)$ measured at $t = t_i$; N is the number of experimental points t_i ; and the sum is taken over the all experimental points of the corresponding kinetics. Figure 7 shows an example of fitting the experimental kinetics by a linear combination of two kinetics acquired from reference samples A and B with known amount of PPi and ATP. In Figure 7 the sample kinetics is C and the fitted curve is shown by D. The R^2 value for the experimental kinetics C and fit curve D is $R^2 > 0.96$, thus indicating the accuracy of the model to describe the experimental data.

To compare two algorithms for PPi measurement given by Eqs.(31-35) and by Eq.(36) the same set of kinetics has been analyzed by each algorithm. With the algorithm given by Eq.(36) the analysis was performed using only the first 45-sec of the entire 300-sec data set. Figure 8 shows results of PPi measurements by 45-sec assay using the algorithm given by Eq.(36) vs. the 300-sec assay and the algorithm of Eqs.(31-35). The results show high consistency with $R^2 = 0.99$. The sensitivity of PPi detection, DX/X , by short- kinetics assay was of 0.4 pg in 45 μ l sample and is slightly lower than the sensitivity of 0.3 pg in 45 μ l sample achieved by

analyzing the longer 300-sec kinetics as described above. Yet, the shorter 45-sec measurement procedure may have a significant advantage for assay designed in the microplate format for high-throughput analysis of large number of samples.

Measurements of PPi released during RNA reverse transcription—The assay has been used for quantitation of RNA target in complex RNA samples by carrying out reverse transcription reaction. During the RNA reverse transcription incorporation of each nucleotide base to cDNA sequence release one PPi molecule into solution. We have previously demonstrated method for programmable termination of the reverse transcription reaction by incorporating ddNTP into cDNA sequence [2,4]. The method allows producing the same number of PPi molecules per each cDNA copy independently of the size of RNA target. The total number of PPi molecules released by terminated reverse transcription reaction is proportional to the number of the corresponding RNA target molecules in sample. Yet, luminometric quantitation of PPi molecules in the reverse transcription mixture is challenging. At the typical experimental conditions a picogram quantity of PPi have to be detected in presence of a substantial background luminescence due to contaminants and reaction reagents known to be non-specific substrates for luciferase (i.e., 2',3'-Dideoxyadenosine-5'-O-(1-Thiotriphosphate) and adenosine 5'-phosphosulfate of the PPi assay). Here we present the use of time-resolved PPi assay for detection the quantity of RNA spike in complex RNA sample for gene expression analysis. Known quantities of RNA spike, a transcript of phage lambda DNA (Cat. No. K1611, Fermentas), has been added to 1 µg of human total RNA (Cat. No. 540031, Stratagene). The reverse transcription reaction has been carried out using reagents and by following the protocol of the first strand cDNA synthesis kit (Cat. No. K1611, Fermentas). For reducing background luminescence dATP in reverse transcription mixture was replaced by 2',3'-Dideoxyadenosine-5'-O-(1-Thiotriphosphate) [1,2]. After performing reverse transcription (RT) reaction at 37°C for 1 hour the amount of PPi in sample was measured by adding 10 µl of the RT solution to 30 µl of detection solution of the PPi assay. Time-resolved luminescence kinetics have been captured and processed as illustrated and described in Figure 3,5, and 7. Figure 9 shows PPi luminescence signal vs. the amount of RNA spike in sample. The sensitivity of RNA detection in complex RNA mixture achieved in this experiment is estimated to be 0.06 fmol of RNA target. Here the use of time-resolved PPi assay allows achieving higher sensitivity of RNA detection by subtracting background luminescence, which obscure analytical signal at low PPi content.

IV. Conclusion

In this report we presented a new high-sensitivity assay for measurement PPi in ATP-contaminated samples. The assay is based on time-resolved measurements of the luminescence kinetics and can be carried out using the common commercially available photon-counting luminometers. The assay implements multiple enzymes detection solution to convert PPi to ATP; to produce light by consuming ATP; and to hydrolyze PPi for measurement of the steady-state background luminescence. We have developed a theoretical model for describing luminescence kinetics from multi-enzyme PPi assay. The model provides accurate description of experimental results at the reagents concentration used in this study. The theoretical model has been used to formulate the multi-enzyme assay with luminescence kinetics having easily distinguishable fast and slow decay components. The kinetics have been used to measure five main parameters of the model and allow assessment of the PPi luminescence signal in presence of ATP contamination and steady-state background luminescence. We have developed and evaluated two algorithms for PPi measurement from luminescence kinetics acquired from ATP-contaminated samples. The first algorithm is designed for analysis of long, i.e., 3-5 min, kinetics and was able to provide the sensitivity of PPi detection at 7 pg/ml or 15 pM of PPi in ATP-contaminates samples. The second algorithm is designed for analysis of short, i.e., less than 1-min luminescence kinetics and it has ~30% lower PPi detection sensitivity of 10 pg/ml

or 20 pM. However, the second algorithm may have significant advantage for implementation with assays in a microplate format, where the short time of analysis is advantageous.

The time-resolved assay has detection sensitivity about three orders of magnitude better than the previously reported methods and commercial PPi assay [8-12]. Although no data available for PPi detection sensitivity of the bioluminescence regenerative cycle system [1,3], from the data available for ATP measurement by BRC system the time-resolved assay is expected to have from 10 to 50 times better ATP and PPi detection sensitivity than the regenerative system. An additional advantage of the time-resolved assay is its ability to measure the enzymes activity in each experiment. This can be used to reduce the experimental variability due to the change of assay conditions and enzyme(s) failure during prolonged experiments. The overall advantage of the time-resolved assay is its high sensitivity for PPi measurement in samples with endogenous ATP contamination.

The assay was developed primarily for applications in RNA expression analysis, but it also can be used in various life science and clinical applications [17-22]. In some clinical applications, such as analysis of PPi in body fluids, the assay can detect sub-femtomolar quantities of PPi in microliter-scale samples often without pre-treatment to reduce the endogenous ATP.

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Figure 1. Luminescence kinetics detected from three samples, containing: (a) 1.8 fmol ATP; (b) 11.2 fmol PPi; and (c) a mixture of ATP and PPi.

Figure 2. Example of fitting the luminescence kinetics, $I_{\text{Exper}}(t)$, by sum of the fast, $A(t)$, the slow, $B(t)$, and the steady-state background, I_{Backgr} , components.

Figure 3. Detection sensitivity of PPi assay vs. the assay time and concentration of the luciferin-luciferase in the assay solution: (a) 2-diluted ATP-detection solution, $\gamma_{L-L} = 0.012 \text{ s}^{-1}$; (b) x1 ATP-detection solution, $\gamma_{L-L} = 0.024 \text{ s}^{-1}$; and (c) x2 ATP-detection solution, $\gamma_{L-L} = 0.048 \text{ s}^{-1}$.

Figure 4.

Luminescence kinetics from samples prepared by mixing known amount of ATP and PPI. Shown are kinetics replicates acquired from samples with amount of PPI in the range of 0.6 pg – 60 pg and ATP amount of 0, 1 pg, and 2 pg in 5- μ l sample : (a) 60 pg PPI; (b) 20 pg PPI and 2 pg ATP; (c) 20 pg PPI and 1 pg ATP; (d) 20 pg PPI, etc. The set of kinetics have been used to evaluate different algorithms for measuring PPI in ATP-contaminated samples.

Figure 5.

Selection of time intervals for application of the algorithm given by Eqs. (38-42). The number of photons emitted during corresponding time interval is N_i . The smooth solid line is the fit of the experimental data by Eq. (7) with kinetic parameters determined by algorithm described in the text.

Figure 6. Measurement of PPI in three samples with simulated contamination by ATP. The amount of ATP is varied between samples and is 0, 1 pg and 2 pg of ATP.

Figure 7.

Two reference kinetics A and B are used to calculate the fit curve for sample kinetics C. The fit curve D is given by a linear combination of the kinetics A and B (see the Eq.(36). The coefficients in the Eq.(36) are calculated using data acquired during the first 45 sec, as indicated by dashed line.

Figure 8.

Measurement of PPI by 45-sec assay vs. the measurements by 300-sec assay. The dashed line shows the least square linear fit.

Figure 9.
Detection of RNA spike in complex RNA sample by time-resolved bioluminescence assay.