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ORIGINAL PAPER

High Sensitivity, Quantitative Measurements of Polyphosphate Using a New DAPI-Based Approach

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Abstract Polyphosphate (poly-P) is an important metabolite and signaling molecule in prokaryotes and eukaryotes. DAPI (4′,6-diamidino-2-phenylindole), a widely used fluorescent label for DNA, also interacts with polyphosphate. Binding of poly-P to DAPI, shifts its peak emission wavelength from 475 to 525 nm (excitation at 360 nm), allowing use of DAPI for detection of poly-P in vitro, and in live poly-P accumulating organisms. This approach, which relies on detection of a shift in fluorescence emission, allows use of DAPI only for qualitative detection of relatively high concentrations of poly-P, in the μg/ml range. Here, we report that long-wavelength excitation (≥400 nm) of the DAPI-poly-P complex provides a dramatic increase in the sensitivity of poly-P detection. Using excitation at 415 nm, fluorescence of the DAPI-poly-P complex can be detected at a higher wavelength (550 nm) for as little as 25 ng/ml of poly-P. Fluorescence emission from free DAPI and DAPI-DNA are minimal at this wavelength, making the DAPI-poly-P signal highly specific and essentially independent of the presence of DNA. In addition, we demonstrate the use of this protocol to measure the activity of poly-P hydrolyzing enzyme, polyphosphatase and demonstrate a similar signal from the mitochondrial region of cultured neurons.

Keywords Fluorescence · DAPI · Polyphosphate · Inorganic phosphate · Polyphosphatase

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Introduction

Polyphosphate (poly-P) is a long-chain polymer composed of many orthophosphates linked together by high-energy ATP-like bonds. Poly-P has been found in all living organisms tested, ranging from bacteria to higher eukaryotes. Metabolism and biological functions of poly-P have been extensively studied in bacteria and yeast. It plays an important role as an energy source, as a regulator of gene expression, as a channel-forming component, as a store of inorganic phosphate, and as a chelator of heavy metals [1, 2]. Although amounts of poly-P in higher eukaryotes are significantly lower than in bacteria and yeast [3], recent studies indicate that poly-P plays important physiological roles in higher eukaryotes. Examples of these roles include: regulation of enzyme activity [4] in mammary cancer cells; participation in mitochondrial function of mammalian tissue culture cells [5, 6] and modulation of TRP channels in sensory neurons [7].

Two experimental approaches are widely used for quantitative estimation of the amounts of poly-P in vitro. The first is based on hydrolysis of poly-P by hydrochloric acid with subsequent measurements of released inorganic phosphate, P_i [8]. The second, a more specific and sensitive enzymatic method, uses exopolyphosphatase (PPX) and

polyphosphate kinase (PPK) enzymes to detect ng amounts of poly-P [9, 10].

The fluorescent probe 4′,6-diamidino-2-phenylindole (DAPI) has also been used to detect poly-P. In this approach, the DAPI-poly-P complex has been detected by a shift in the DAPI fluorescence emission spectrum; when excited at 360 nm, the peak emission wavelength of free DAPI is 475 nm, while the peak emission wavelength of the DAPI-poly-P complex is 525 nm[11]. However, protocols based on this principle allow detection of poly-P in vitro only for relatively high concentrations of poly-P (in the μg/ml range). DAPI has also been used in fluorescence microscopy for qualitative staining of poly-P in cells. In these experiments, DAPI-poly-P, when excited at 364 nm, emits a typical green-yellow fluorescence signal in organisms which are able to accumulate high levels of poly-P [12-14], or in poly-P rich cellular organelles [15, 16]. Although DAPI staining readily revealed the presence of the polymer in poly-P accumulating bacteria, poly-P was not detected in species maintaining significantly lower levels [17].

A significant limitation in the use of DAPI for detection of poly-P occurs, when the 360 nm is used to excite fluorescence, due to spectral overlap in the emission spectra of DAPI and DAPI-DNA with the DAPI-poly-P emission spectrum. Use of high concentrations of DAPI (~15 μM), coupled with fluorescence measurements made using 360 nm excitation, has allowed separation of DAPI-DNA and DAPI-poly-P signals and monitoring of temporal changes of poly-P using fluorescent microscopy [18]. However, this has been achieved only for high concentrations of poly-P (0.5–10 mM). Thus, to date, DAPI fluorescence measurements of poly-P have been successfully used only for relatively high poly-P concentrations, or for qualitative detection.

Here, we describe an improved protocol under which DAPI can be used to detect ng/ml concentrations of poly-P in vitro. With this approach, neither the fluorescence from free DAPI nor from the DAPI-DNA complex makes a significant contribution to the signal, providing a new assay, which is essentially independent of the presence of DNA in the sample. We further demonstrate that the sensitivity of the protocol is sufficient to measure activity of a polyphosphatase enzyme and a mitochondrial signal in cultured neurons.

Methods

Materials

Poly-P-75 was purchased from Sigma-Aldrich (St. Louis, MO, USA), while both poly-P-15 (with an average chain length of 15 orthophosphates) and poly-P-130 (with an average chain length of 130 orthophosphates) were kind gifts of Dr. T. Shiba (Matsumoto University, Japan), DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) was from Fluka (Buchs, Switzerland), purified exopolyphosphatase (scPPX1) was a gift from Dr. A. Kornberg (Stanford University, CA). All other chemicals were purchased from Sigma-Aldrich.

Measurements of fluorescence

DAPI fluorescence was measured using a fluorometer (DeltaRAM V, Photon Technology International, Lawrenceville, N.J., USA). All recordings were made using polymethylmethacrylate cuvettes (VWR International, PA) with 2 ml of sample buffer solution containing 150 mM KCl, 20 mM HEPES–KOH, pH=7.0. A Teflon stir bar was used to continuously mix the sample during fluorescence measurements. A 20 mM DAPI stock solution was prepared by adding DAPI to water. Stock solutions of Poly-P standards were prepared in sample buffer at final concentrations of 0.5 or 50 μg/ml. DAPI and poly-P additions were made directly to the cuvette containing the sample buffer. Unless otherwise stated, [DAPI]=10 μM in the sample cuvette. We have found that DAPI fluorescence is linear up to DAPI concentration of 40 μM when fluorescence is excited at 415 nm and emission fluorescence is measured at 550 nm. Data points for fluorescence spectra were collected every 0.5 nm. For measurement of fluorescence as a function of time, data points were collected at 0.2-s intervals. Fluorescence intensity is presented as counts per second (cps). Data was analyzed and plotted using Sigma Plot (Jandel Scientific, CA). In the results presented here, poly-P amounts are expressed as the weight of poly-P added to the cuvette containing 2 ml of sample buffer. Under these conditions, 1 μg of poly-P corresponds to ~ 6 μM of orthophosphate, calculated as $–PO_3$ monomeric units.

Cells used for in-vivo experiments

Competent *E. coli* cells were prepared by incubation in transformation buffer contained 100 mM KCl, 10 mM $CaCl_2$, 45 mM $MnCl_2$ and 10 mM MES-KOH pH=6.3 as describe previously [19]. Primary co-cultures of hippocampal neurons and astrocytes were prepared from Sprague Dawley rat pups 2–4 days postpartum (University College London breeding colony) as previously described [20].

Confocal experiments

Confocal images were obtained using a Zeiss 510 CLSM equipped with a META detection system and a 40× oil immersion objective. DAPI emission spectra were measured

using 364 or 405 nm laser for excitation. Measurements were done in the presence of 10 μM DAPI.

Results

Measurement of synthetic poly-P in vitro

In order to improve the detection sensitivity of the DAPI assay for poly-P, we first analyzed how binding of poly-P to DAPI alters the DAPI excitation spectrum. In the experiments described in this section, commercial poly-P (from Sigma) with an average chain length of 75 orthophosphates was used. Figure 1a shows the excitation spectrum of free DAPI (10 μM, solid trace) and DAPI after three sequential additions of 250 ng/ml of poly-P. The fluorescence emission intensity was measured at 550 nm. In the presence of poly-P the DAPI fluorescence intensity was increased, especially for excitation wavelengths > 400 nm. At the wavelength of maximal DAPI fluorescence excitation (360 nm) the signal intensity of DAPI emission at 550 nm increases by only ~15% with addition of 750 ng/ml of poly-P. However, at ~415 nm excitation, fluorescence emission from free DAPI is very close to the background level (~2,000 cps), whereas the fluorescence signal from DAPI-poly-P complex is significantly greater (~400% increase; Fig. 1a).

Next, we compared the emission spectra of DAPI and DAPI plus poly-P when excited at either 360 or 415 nm. When excited at 360 nm, the typical [11] red shift of the DAPI emission spectrum is seen in the presence of high concentration (1.2 μg/ml) of poly-P (Fig. 1b). However, at this excitation wavelength the fluorescence of DAPI-poly-P significantly overlaps the fluorescence contributed by free DAPI. In contrast, when excited at 415 nm, the fluorescence of free DAPI is minimal, and a significant fluorescence increase can be detected in the presence of poly-P (Fig. 1c). These results suggest that excitation at 415 nm can be used for detection of a specific increase in DAPI-poly-P fluorescence using a single emission wavelength, rather than relying on measurement of a shift in the emission maximum.

The advantage of our novel approach becomes especially evident when low concentrations of poly-P are assayed. As can be seen from Fig. 2a, when we used 360 nm excitation, the presence of 25 to 100 ng/ml concentrations of poly-P were too low to induce a detectable shift in fluorescence at 525 nm, which was only observed in the presence of high amounts of poly-P (Fig. 1b). Interestingly, at these low concentrations of poly-P the overall fluorescence of DAPI in the presence of poly-P was slightly decreased. Thus, high fluorescence signal from free DAPI and the absence of a detectable emission wavelength shift hinders the use of this

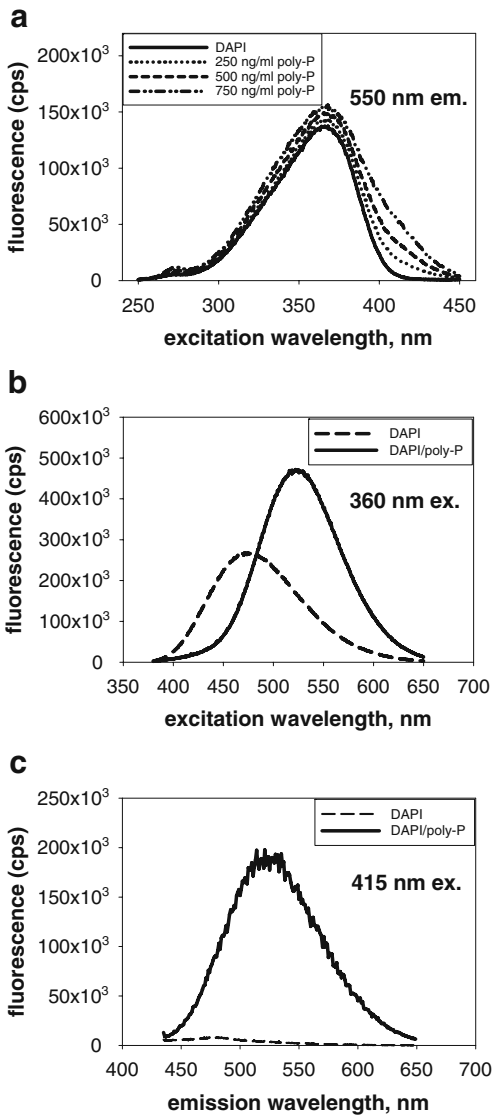

Fig. 1 Excitation and emission spectra of DAPI in the absence and presence of poly-P. **a** 10 μM DAPI was added to a 4 ml cuvette containing 2 ml of sample buffer. The excitation wavelength was scanned from 250 to 450 nm while fluorescence emission was monitored at 550 nm (*solid line*) and repeated again after three sequential additions of 250 ng/ml of poly-P-75. **b** Emission spectra of free DAPI (*dashed line*) and DAPI in the presence of 1.2 μg/ml of poly-P-75 (*solid line*). Fluorescence was excited at 360 nm. **c** Same as **b** except excitation wavelength was 415 nm

protocol for quantitative measurement of poly-P in the ng/ml concentration range.

In contrast, Fig. 2b shows changes in the emission spectrum for free DAPI and DAPI in the presence of 25, 50 and 100 ng/ml of poly-P when fluorescence was excited at 415 nm. Clearly, under these conditions even 25 ng/ml of poly-P induces a significant increase in the fluorescent signal with an emission peak at 525 nm. Figure 2c shows fluorescence changes at 550 nm, when fluorescence was excited at 415 nm, in response to multiple additions of 25 ng/ml of poly-P. As can be seen from the figure, in the

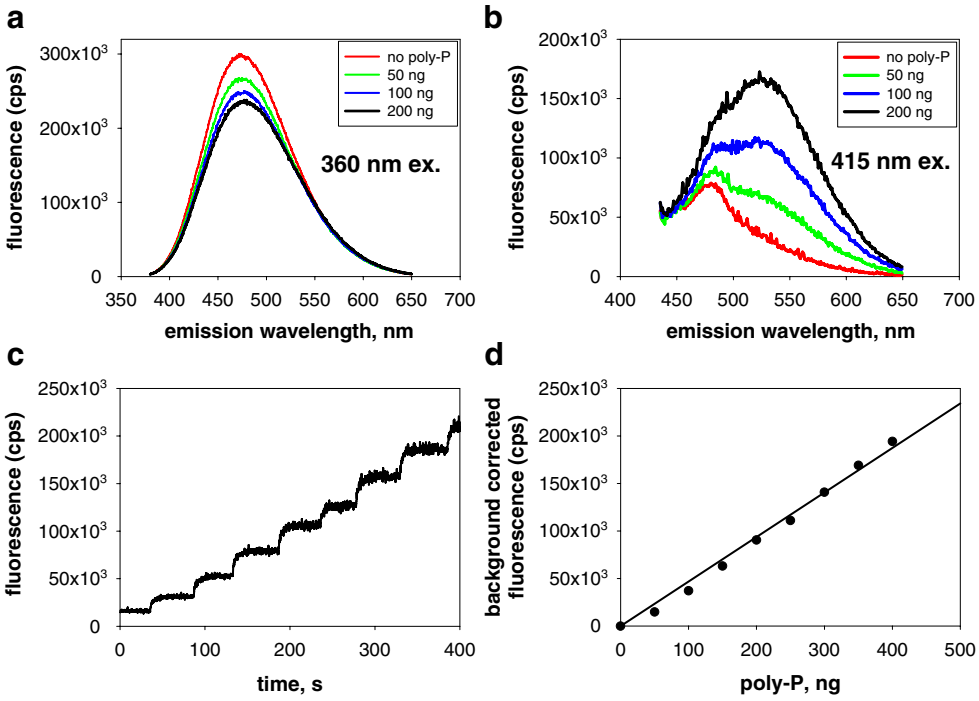

Fig. 2 DAPI-polyP fluorescence for lower concentrations of poly-P. **a** Emission spectra of 10 μM DAPI in the presence of 0, 25, 50, and 100 ng/ml poly-P. Fluorescence was excited at 360 nm. **b** Emission spectra of 10 μM DAPI in the presence of 0, 25, 50, and 100 ng/ml poly-P. Fluorescence was excited at 415 nm. **c** Change of fluorescence intensity of DAPI excited at 415 nm and collected at 550 nm in response to a series of additions of 50 ng aliquots of poly-P-75. **d** Data from **c** with fluorescence plotted as a function of total poly-P added

range of 50 to 400 ng poly-P/2 ml sample buffer the fluorescent signal demonstrates nearly linear dependence on the poly-P concentration (Fig. 2d), making this protocol ideal for the quantitative measurement of the poly-P polymer in this range. We have found the linear relationship is maintained up to 1.5 μg/ml of poly-P with further increase in poly-P leading to signal saturation at a molar ratio of PO_3:DAPI of ~3:1.

DAPI-poly-P fluorescence in the presence of DNA and ATP

Given that DAPI is a fluorescent probe widely used for DNA staining we tested the effect of DNA on the DAPI-poly-P signal. When excited at 415 nm, the addition of 50 ng/ml of poly-P to sample buffer containing 10 μM DAPI resulted in similar increases in fluorescence emission in the absence and presence of 0.5 μg/ml DNA (Fig. 3a). Thus, the presence of 0.5 μg/ml of DNA does not interfere with the poly-P assay in the tested sample when [DAPI]= 10 μM, indicating high specificity of the signal for poly-P even in the presence of DNA. As can be seen from Fig. 3b presence of 0.5 μg/ml of DNA does not alter the linear relationship between fluorescence and poly-P concentration when 415 nm excitation wavelength is used and data is collected at 550 nm.

In addition, we tested the effect of ATP on DAPI fluorescence using excitation at 415 nm. We found that the addition of up to 800 μg/ml of ATP to the cuvette caused only moderate increase in DAPI fluorescence intensity at 550 nm compared to addition of 0.250 μg/ml of poly-P to 2 ml of the sample buffer (Fig. 3c). At this emission wavelength, the presence of more than 1000-fold excess by weight of ATP over poly-P leads to a fluorescence signal increase of only ~15%. Thus we expect that the presence of up to 100 times more ATP compared to poly-P will not interfere with the quantitative assay of poly-P.

Kinetics of the DAPI-poly-P fluorescence increase in the presence of polyphosphate of different sizes

We investigated the dependence of the DAPI fluorescence signal on the length of the poly-P chain. These experiments used poly-P standards with average polyphosphate chain length of 15 (poly-P-15) and 130 P (poly-P-130); these were kindly provided by Dr. T. Shiba (Matsumoto Dental University, Japan). As can be seen in Fig. 4a, the rate of the fluorescence increase in the presence of 10 μM poly-P was dramatically slower when poly-P-15 was added than the rate of increase when an equal amount of poly-P-130 was added (Fig. 4b). On the other hand, the steady-state fluorescence intensity for both samples (1 μg of poly-P each) was the same. Thus the steady-state fluorescence appears to depend on the weight of poly-P added (which is proportional to the number of units of PO_3) but not on the chain length.

Since the rate of development of maximum fluorescence depends on chain length this indicates that this rate may be used to indicate the poly-P chain length in a sample. The slower rate of development of maximum fluorescence intensity for shorter chain length polyphosphates also

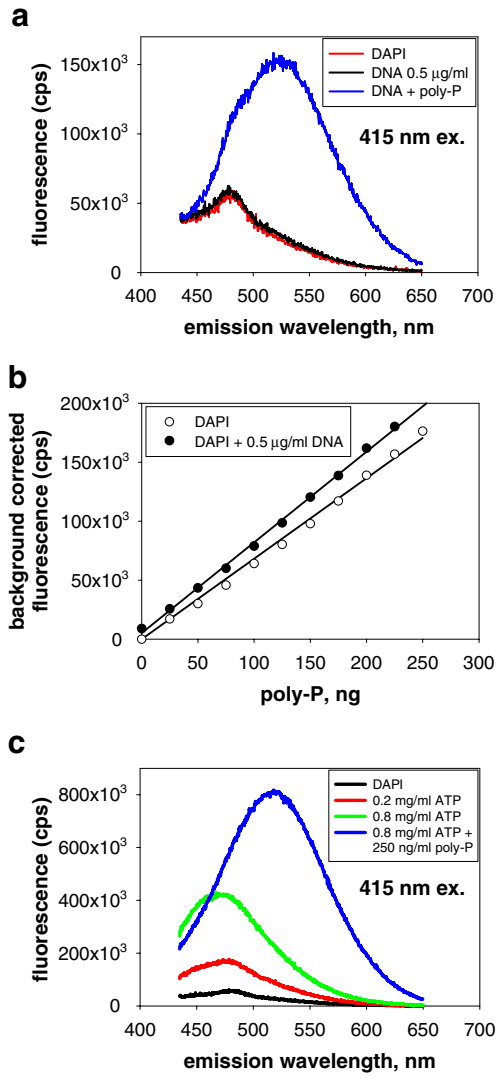

Fig. 3 DAPI-poly-P fluorescence in the presence of DNA. **a** Fluorescence emission spectra for 10 µM DAPI in the absence and presence of DNA and DNA + poly-P. Fluorescence was excited at 415 nm. Note: with 415 nm excitation the fluorescence of DAPI is not altered by the presence of 0.5 µg/ml DNA. **b** Sequential addition of 25 ng/ml of poly-P results in an increase in fluorescence similar to that seen in the absence of DNA. **c** Effects of addition of ATP on DAPI fluorescence

suggests that, for in vitro assays, the DAPI-poly-P sample incubation time may need to be optimized for different types of samples.

Fluorescent measurement of polyphosphatase enzymatic activity

In order to estimate the specific activity of poly-P metabolizing enzymes highly sensitive methods of poly-P detection are required. Here we demonstrate that our protocol can be used as a means of detecting the poly-P hydrolytic activity of purified exopolyphosphatase from yeast (scPPX) [21]. In these experiments, 1µg of purified scPPX was added to 30 µl of sample solution containing 15 µg of poly-P-75. The amount of poly-P was estimated from the maximal change of DAPI fluorescence intensity in response to the addition of 2 µl samples taken from the reaction mixture at different time-points (Fig. 5a). As can be seen from Fig. 5b, maximal DAPI fluorescence progressively decreased as a function of incubation time reflecting the kinetics of poly-P hydrolysis by scPPX.

Dependence of DAPI poly-P fluorescence on calcium in vitro, and on poly-P complex formation in intact *E. coli* cells

When using DAPI for measurement of poly-P concentration, consideration should be given to the composition of the buffer, mainly the presence of divalent cations. We have found that excitation at 415 nm and fluorescence emission measured at 550 nm, the DAPI-poly-P fluorescence decreases with an increase in calcium concentration above 10 mM (data not shown). Fluorescence of free DAPI does not change in the presence of such concentrations of calcium. Thus, the fluorescence decrease is caused, presumably, by complex formation between calcium and poly-P which reduces the formation of DAPI-poly-P complexes. Therefore, quantitative estimation of poly-P in a test sample

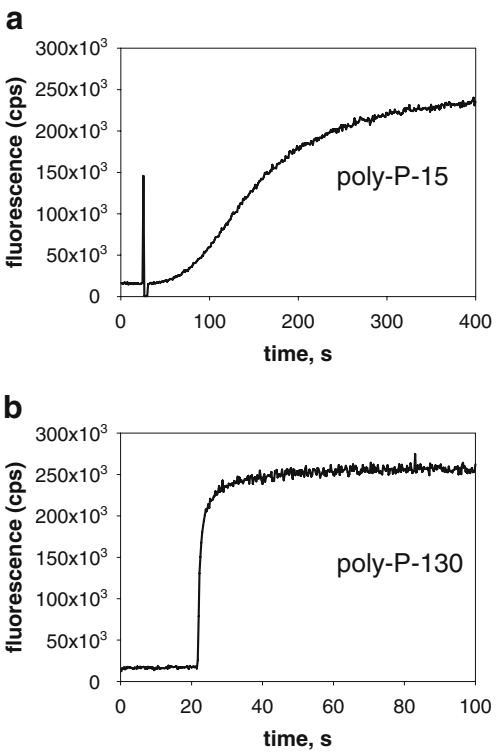

Fig. 4 The rate of the DAPI-poly-P fluorescence increase depends on the length of poly-P polymer. Fluorescence was excited at 415 nm and collected at 550 nm. In both panels, 0.5 µg/ml of poly-P was added: in **a** poly-P-15, and in **b** poly-P-130, was added to 10 µM DAPI

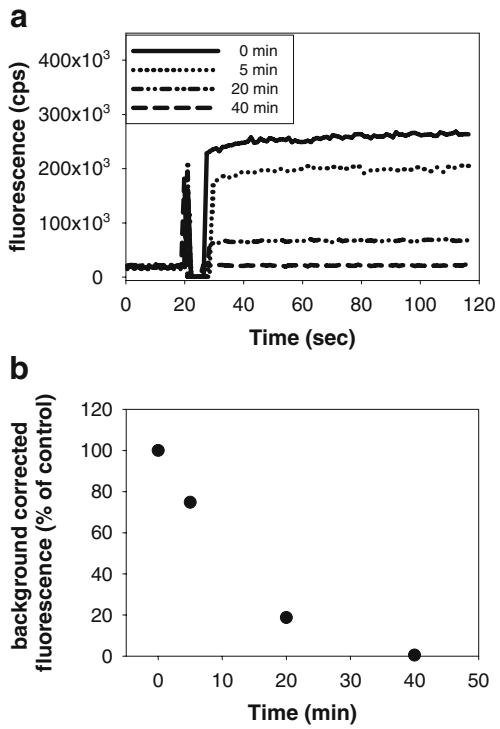

Fig. 5 Time dependent decrease of DAPI fluorescence in response to treatment with poly-P hydrolyzing enzyme, scPPX. A reaction mixture containing 30 μl of recording buffer, 15 μg of poly-P-75 and 1 μg of scPPX was incubated at 37 °C and 2 μl aliquots were taken at indicated time points and added to separate cuvettes each containing 2 ml of recording buffer and 10 μM DAPI. Fluorescence was excited at 415 nm and collected at 550 nm. **a** Traces showing changes in fluorescence intensity as a function of time in response to addition of aliquots of the reaction mixture incubated for increasing periods of time to allow progressive hydrolysis of the poly-P. **b** Time course of the maximum DAPI-poly-P fluorescence measured for the series of aliquots of the reaction sample

should be done by comparison of the test fluorescence intensity with fluorescence of poly-P standards prepared in the same buffer. For this reason, caution should be exercised if attempting to use our method as a quantitative assay in vivo.

However, the dependence of DAPI-poly-P fluorescence on the state of poly-P opens the possibility for use of this method in studies of complex formation between poly-P and calcium and/or other molecules. We tested this possibility by measuring DAPI-poly-P fluorescence in intact competent *E. coli* cells. In competent *E. coli*, poly-P is known to form complexes with polyhydroxybutyrate and calcium [22]. In this form, poly-P complex may not be available for binding to DAPI. However, poly-P can be released from the complex by heating the cells to 60 °C [23]. We measured the intensity of DAPI fluorescence in response to addition of equal aliquots of heated and non-heated competent *E. coli* cells. We have found that DAPI-poly-P fluorescence in heated competent cells is 2.7±0.7 (n=3, P<0.005) times greater compared to non-heated competent *E. coli* cells. This confirms that DAPI fluorescence can be used as an indicator of the relative amount of free poly-P in live cells.

Discussion

Poly-P is a polymer that plays important roles in the functioning of many organisms. It has been demonstrated, in both prokaryotes and lower eukaryotes, that poly-P contributes to energy storage [24, 25], provides a reserve pool of inorganic phosphate [26, 27], participates in the regulation of gene expression, protects cells from heavy

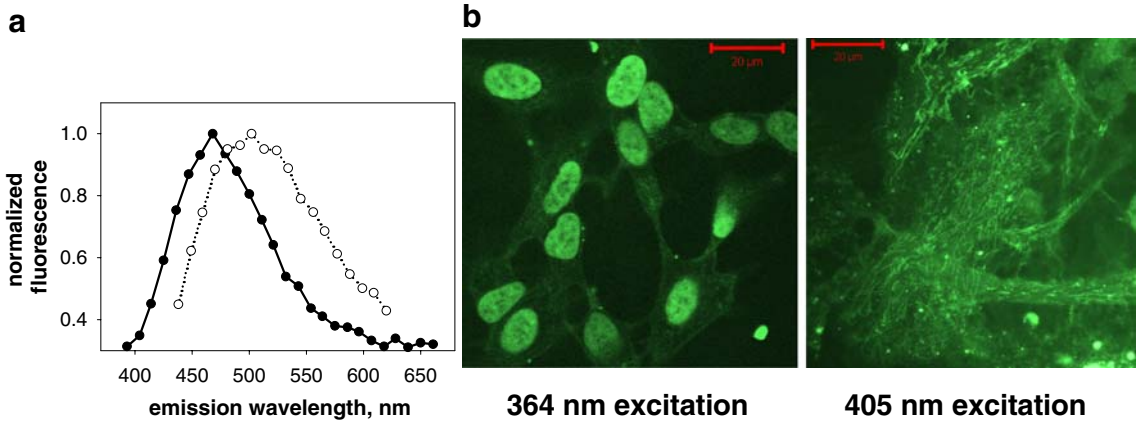

Fig. 6 **a** Differing DAPI fluorescence emission spectra recorded from the mitochondrial regions of two representative, primary cultured neurons (one for each excitation laser) using excitation wavelengths of 364 nm (*filled symbols*, *solid line*) and 405 nm (*open symbols*, *dotted line*). Note the shift in the spectrum excited at 405 nm, which resembles the typical shift seen for poly-P in vitro (see Fig. 2a,b). **b** Confocal images of different coverslips of co-cultured neurons and astrocytes collected using 364 or 405 nm lasers for excitation. Note the strong nuclear signal when the 364 nm laser was used, with a much weaker signal from the surrounding mitochondrial region. With 405 nm excitation, the nuclear signal was much weaker, and the diffuse signal from the surrounding area, which includes the mitochondria, predominated (*scale bars*, 20 μm)

metal toxicity by forming complexes with heavy metals [28] and participates in channel formation by forming complexes with calcium and polyhydroxybutyrate [29]. Recent reports in mammalian systems highlight roles for poly-P in mitochondrial function and sensory signaling (see "Introduction").

In spite of a long history of poly-P research, few analytical methods are currently available for quantitative assays of poly-P. In this paper, we describe a novel protocol, for quantitative fluorescent measurements of the low amounts of poly-P, which has a number of advantages over other poly-P assays. Introduction of a highly sensitive method of poly-P assay is particularly important for studies of its physiological roles in higher eukaryotes, where amounts of this polymer are significantly lower [3], making its detection difficult.

Two other methods are currently available for assays of ng/ml amounts of poly-P. One method is based on the conversion of poly-P into inorganic orthophosphate by treatment with hydrochloric acid and subsequent estimation of the amount of released P_i [8]. Although low amounts of orthophosphate can be measured, this method is relatively non-specific due to possible contamination of the signal with phosphate of non poly-P origin (e.g. DNA or ATP), which can be a source of significant error when low levels of poly-P are present. In our protocol, on the other hand, DAPI-poly-P fluorescence does not depend substantially on the presence of these polyanions in the sample. In fact, accurate measurements can be done even in the presence of the up to ten fold higher (by weight) amounts of DNA and up to hundred fold higher (by weight) amounts of ATP, compared to poly-P. This suggests that our method could be used for the in vitro assay of poly-P from relatively crude samples.

A second method for poly-P estimation, which is highly sensitive and specific for polyphosphates, is based on the enzymatic assay of poly-P using polyphosphate kinase (PPK). PPK, is an enzyme that converts ADP into ATP using poly-P as a substrate. The amount of ATP produced by PPK corresponds to the number of orthophosphate groups cleaved, and thus to the quantity of poly-P in the sample. This assay was used to demonstrate the existence of poly-P in higher eukaryotes [3]. Although this method allows accurate detection of small amounts of poly-P, it can not be applied for measurements of short-chain (less than 60) poly-P [9], whereas our new protocol allows detection of poly-P as short as poly-P-15. Shorter poly-P, with its lesser net negative charge, appears to require a longer time for equilibration of DAPI binding (Fig. 4).

Although previously DAPI has been successfully used for poly-P staining in live cells using confocal and fluorescence microscopy with excitation at 364 nm [15], we believe that under certain conditions the use of longer excitation wavelengths can be beneficial, specifically in imaging of eukaryotic cells with relatively low levels of poly-P. Recently, we used confocal microscopy with 405 nm laser excitation to detect poly-P in mitochondria of live mammalian tissue culture cells [6]. In these experiments, the emission spectrum from the mitochondrial region resembled the spectrum of free poly-P seen in vitro. Importantly, DAPI fluorescence was decreased in cells over-expressing polyphosphatase confirming that DAPI can be used in vivo to monitor changes in poly-P. We have found that, when a 364 nm laser is used, the spectrum collected from the mitochondrial region of primary cultured of neurons does not display the typical poly-P shift (Fig. 6a), suggesting that, when 364 nm excitation is used, most of the emission signal originates from the mitochondrial DNA and/or DAPI binding to other components [11]. Figure 6b shows images of co-cultured neurons and astrocytes collected using 364 nm and 405 nm lasers when the fluorescent signal was integrated at >580 nm. Note that when 405 nm laser was used the signal from nuclei was undetectable, whereas when excited at 364 nm DAPI-poly-P signal from mitochondria overlaps with signal from DAPI-DNA from nuclei.

In summary, our fluorimetric method, based on excitation at 415 nm and measurement of emission at 550 nm, provides a practical approach for routine quantitative assays, based on its simplicity and speed, its high sensitivity, and its ability to detect poly-P of a wide range of chain lengths. Finally, our results provide a rigorous and informed basis for wider use of DAPI for in vivo, fluorescence microscopy.

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