Lock-in by molecular multiplication

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A lock-in amplifier is physically realized at the level of fluorescent dye molecules. It is based on the general property that the emission of a fluorescent dye is the product of quantum efficiency and illumination intensity. For each pixel of a microscopic image, we measure in amplitude and phase an environment property of the dye, such as conformation, membrane voltage, or temperature. This lock-in implementation is highly parallel and reaches the ultimate photon shot noise limit. Using fast temperature oscillations, we apply it to measure the opening/closing kinetics of a molecular beacon (DNA hairpin) at 5 μs resolution. © *2003 American Institute of Physics.*
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Fluorescent dyes perform a multiplication between their quantum efficiency q and the illumination intensity I_{ex} and report the result in their emission intensity I (Fig. 1). We use this multiplication to realize a lock-in at the level of single fluorescent molecules. It allows one to record, in amplitude and phase, an oscillating quantum efficiency for each pixel of a charge-coupled device (CCD) camera. Recording the quantum efficiency q of the dye means imaging a wide variety of oscillating environment properties $E(q)$, such as pH, calcium ion concentration, molecular conformation, membrane potential, redox potential, or temperature.[1] As in any lock-in, we enhance the signal to noise ratio. The main advantage of our approach, however, is the recording of an amplitude and phase image. We demonstrate this for a DNA hairpin under temperature oscillations and reveal its opening/closing kinetics for each pixel independently.

Our approach should not be confused with situations where the illumination alone is modulated. In these cases, it has been shown that photon counters with their high gain amplification are superior to analog lock-in approaches.[2,3] Our method also has to be distinguished from situations of a modulated quantum efficiency under constant illumination where a lock-in approach proved useful.[4–6] What we present here is a combination of both: we use a comodulation of illumination and environment. This allows us to drop the requirement of a fast detector. We use a CCD camera with exposure times much slower than the modulation frequency.

The lock-in works as follows. We oscillate the environment property E at circular frequency ω. The goal is to measure the unknown relative amplitude A and phase φ of the quantum efficiency $q(E)$, which we parametrize by $q=q_0[A\sin(\omega t+\varphi)+1]$. Requiring that $q(E)$ is a linear function for the given oscillation of E, we can infer the amplitude and phase of $E(q)$. We illuminate the dye using the upper half of a sine given by $I_{ex}=\Delta I_{ex}\Theta[\sin(\omega t-\alpha)]\sin(\omega t-\alpha)+I_{ex}^{(0)}$ with different phase lags α. Note that $I_{ex}^{(0)}$ is an experimental illumination offset and $\Theta(t)$ the Heaviside function. A full sinusoidal illumination would only increase the number of photons cycled through the dye without enhancing measurement information on A and φ. We record the emission intensity I_α at phase shifts $\alpha=0°$, 90°, 180°, and 270° with a slow detector, integrating over times larger than the oscillation period $1/\omega$. Additionally we record the emission I_{back} under constant illumination $I_{ex}=I_{ex}^{(0)}$. Allowing for the experimental recording background I_{const}, we expect the following emission intensities I:

$$I_\alpha=\frac{A\pi\Delta I_{ex}q_0}{2}\cos(\alpha+\varphi)+2\Delta I_{ex}q_0+2\pi q_0 I_{ex}^{(0)}+I_{const},$$

$$I_{back}=2\pi q_0 I_{ex}^{(0)}+I_{const}. \quad (1)$$

Detailed analysis of Eq. (1) reveals that we can recover from the five recordings the unknown amplitude A and phase φ of the quantum efficiency $q(E)$ using the following expression:

$$Ae^{i\varphi}=\frac{4}{\pi}\left[\frac{I_{0°}-I_{180°}}{I_{0°}+I_{180°}-2I_{back}}+i\frac{I_{270°}-I_{90°}}{I_{270°}+I_{90°}-2I_{back}}\right]. \quad (2)$$

We apply this lock-in to monitor the opening and closing of a DNA hairpin, also called a molecular beacon. Quenching between Cy3 and Dabcyl at the ends of the DNA is used to report whether the hairpin is opened or closed[7] [Fig. 2(a)]. The conformational change is induced by fast temperature oscillations from a focused infrared source with sinusoidal heating power at frequency 170 Hz in a 10 μm thick chamber [Fig. 2(b)]. As a detector we use a slow CCD camera (12 bit, SensiCam, PCO) through a microscope (100× oil immersion, Axioskop, Zeiss). The modulated epifluorescence illumination is performed with a high-power light-emitting diode ($\lambda=505\pm30$ nm, lag<100 ns, cyan, LXHL-LE5C, Luxeon) which replaced the conventional

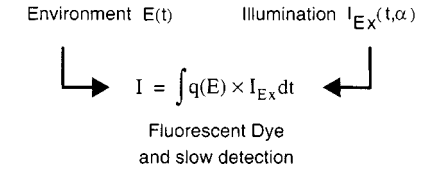

FIG. 1. Lock-in from molecular multiplication. The emission I of a fluorescent molecule is the product of quantum efficiency q and illumination intensity I_{ex}. We use this property to perform a lock-in measurement of q at the molecular level. Many fluorescent dyes report environment properties E by changes in quantum efficiency $q(E)$.

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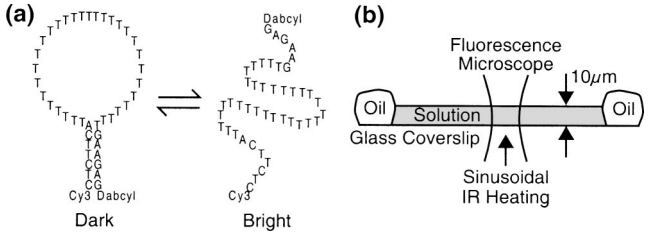

FIG. 2. Experimental setup. (a) The DNA hairpin has a Cy3 dye and a Dabcyl quencher at opposing ends: 5′-Cy3-CTCTTCATTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTGAAGAG-Dabcyl-3′. It forms either a dark closed seven-base stem with a 30-base loop or a bright random coil. The 25 μM hairpin was dissolved in 10 mM TRIS, pH 7.8, and 100 mM NaCl. (b) Temperature oscillations are applied by infrared heating in a 10-μm-thick water film between 170-μm-thick glass coverslips, sealed with immersion oil. Sinusoidal heating from a 1480 nm laser (Ref. 11) (85 mW, FOL1402PJX-317, Furukawa) deposits 2.1 mW within a Gaussian profile of 25 μm radius. The cooling from the chamber walls allows sinusoidal temperature oscillations up to 10 kHz.

halogen lamp of the microscope. It is driven by a laser current source (ILX Lightwave 3525) and a function generator (Agilent 33120A). The sinusoidal output was calibrated against a counting photomultiplier.[5] To be sure that linear bleaching does not perturb the results, we add image duplicates of the recording sequence $(I_{0°}, I_{180°}, I_{270°}, I_{90°}, I_{\text{back}}, I_{\text{back}}, I_{90°}, I_{270°}, I_{180°}, I_{0°})$ using LABVIEW[8] (National Instruments).

The resulting five images are shown in Fig. 3(a). From Eq. (2) we infer for each pixel independently the amplitude A and phase φ of the quantum efficiency q [Fig. 3(b)]. A representative central pixel recorded $I_{0°}=31820\pm240$, $I_{90°}=30930\pm210$, $I_{180°}=23340\pm170$, $I_{270°}=23620\pm160$, and $I_{\text{back}}=4400\pm70$. The intensities are given in photon counts based on camera specifications that one 12-bit count equals five detected photons. From Eq. (2) we infer amplitude $A=31.5\pm0.7\%$ and phase $\varphi=318.9°\pm1.2°$. Errors are standard deviations from a 10×10 pixel grid. Both amplitude and phase fall off toward the periphery of the cell due to heat transfer within water and the chamber [Fig. 3(c)].

Earlier we showed that a photon counting lock-in reaches the photon shot noise with absolute amplitude error $100\%/\sqrt{P}$ and absolute phase error $360°/\sqrt{P}$, where P is the number of detected photons.[5] With the above $P=115\,000$ photons, we expect errors of 0.3% and 1.1°. The phase reaches the photon shot noise limit despite camera read-out noise, but the error of the amplitude is twofold higher. We attribute this to the I_{back} term which affects amplitude more strongly than phase. An error propagation calculation based on Eq. (2) and Poisson distributions confirms this by yielding 0.8% and 1.4°.

A photon counting lock-in[5] recorded from a central 1200-pixel region an amplitude $A_{\text{PMT}}=30.6\pm0.14\%$ and a phase $\varphi_{\text{PMT}}=316.1°\pm0.36°$. This independently confirms the values of the molecular multiplication lock-in. The 1200-fold higher detection area, 20-fold shorter illumination time, and fourfold lower quantum efficiency account for the $\sqrt{1200/(20\times4)}\approx4$-fold smaller error. Both lock-in techniques show equal results over a wide range of amplitude and phase values in a frequency scan [Fig. 3(d)].

We apply the lock-in to detect the opening/closing time constant of a DNA hairpin. We calculate a reaction transfer

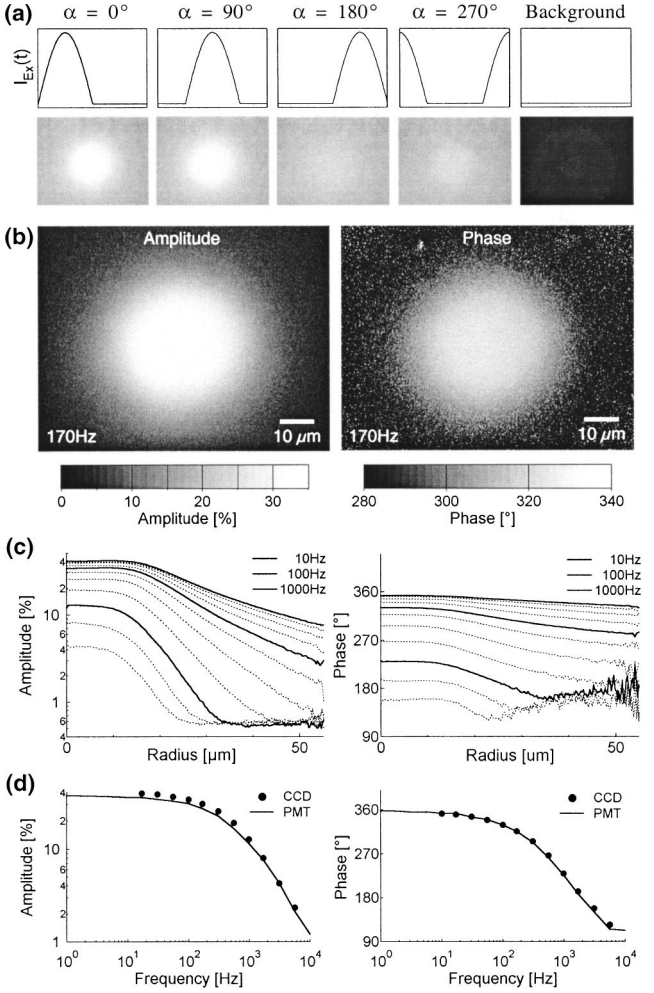

FIG. 3. Lock-in imaging using molecular multiplication. (a) Sinusoidal illumination is locked to a 170 Hz temperature oscillation. It induces oscillations in the opening/closing equilibrium of a DNA hairpin which is recorded by quenched fluorescence. Four images I_α are recorded with phase shifts $\alpha=0°$, 90°, 180°, and 270° together with a background image. (b) Derived lock-in images of amplitude and phase using Eq. (2). (c) A radial average shows how amplitude and phase drop toward the unheated periphery due to heat transfer. (d) Amplitude and phase of a central pixel drop as a function of frequency as both temperature and DNA hairpin lag behind the heating oscillation. This reproduces independent measurements using a photon counting lock-in recorded from a 1200-pixel central area (solid line) (Ref. 5).

function $h(\omega)$ over frequency based temperature jump[9,10] derivations of a two-state equilibrium between the concentration of closed and opened DNA hairpins [C] and [O] with the rate constants k_+ and k_-:

$$\mathrm{C}\underset{k_-}{\overset{k_+}{\rightleftarrows}}\mathrm{O}, \quad \frac{d}{dt}[\mathrm{O}]=k_+[\mathrm{C}]-k_-[\mathrm{O}]. \tag{3}$$

Since we perturb only the equilibrium, we use a first-order expansion around the equilibrium to relate normalized concentration changes $\Delta[\mathrm{C}]/[\mathrm{C}]$ and $\Delta[\mathrm{O}]/[\mathrm{O}]$ to the normalized temperature change $\Delta T/T$. Since closed and opened states of the hairpin have different quantum efficiencies α and β, both with a temperature sensitive component χ, we expect a fluorescence signal $F=(\alpha[\mathrm{C}]+\beta[\mathrm{O}])[\chi(T-T_0)+1]$. Based on this, we find the reaction transfer function $h(\omega)$:

$$h=\frac{\Delta F}{F}\Big/\frac{\Delta T}{T}=\frac{c}{1+i\omega\tau}+d,$$

$$c=\frac{\beta/\alpha-}{1+K\beta/\alpha}\frac{K}{1+K}\frac{\Delta H}{RT},\quad d=\chi T. \tag{4}$$

Three fitting parameters are needed: the opening/closing time constant $\tau=(k_+ + k_-)^{-1}$, an amplitude c, and a temperature background d. They can be expressed by the gas constant R, absolute temperature T, equilibrium constant $K=[\mathrm{O}]/[\mathrm{C}]$, and reaction enthalpy ΔH.

The measurements so far have recorded only the DNA hairpin fluorescence $\Delta F/F=A\exp(i\varphi)$. In the following paragraph, we show how to record in a second fluorescence color channel the temperature change $\Delta T/T=A_T\exp(i\varphi_T)$. We can then infer for each pixel the reaction transfer function $h(\omega)$, in amplitude and phase, by dividing the two lock-in measurements. Fitting to the theory in Eq. (4) will reveal the opening/closing time constant τ. The measurement of the temperature allows us to distinguish the lag of the DNA hairpin from the lag of the temperature oscillation.

We measure the temperature oscillation by including 25 μM BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein, B-1151, Molecular Probes). The quantum efficiency q of this dye records changes in pH which in turn are triggered by a change in temperature in the TRIS buffer (Tris(hydroxymethl)Aminomethane) used.[11] This reaction cascade is estimated to be faster than 10 ns and shows a temperature sensitivity of $\chi=(\Delta q/q)/(\Delta T)=-0.96\%/\mathrm{K}$. These measurements show at low frequencies a temperature amplitude of 5.3 K with 0° phase, decreasing to 0.9 K and −85.2° phase at 5600 Hz. The heat transfer of water and chamber creates a lag between temperature and heating which becomes increasingly apparent at higher frequencies.

For each pixel, the amplitude and phase of $h(\omega)$ is measured over frequency. We show the result for a single central pixel in Fig. 4(a). Equation (4) fits the measurement well and gives $\tau=330\pm15$ μs, $c=770\pm35\%$, and $d/c=-0.22\pm0.03$. The opening/closing relaxation time constant of 330 μs lies realistically between measurements on smaller hairpins in free solution[12,13] and longer hairpins bound to a surface.[14–16] Since we can perform such a fit for every pixel, we can calculate an image of reaction time constants [Fig. 4(b)]. Averaging over 10×10 central pixels results in $\tau=320$ μs with an error of the mean of 5 μs [Fig. 4(c)]. The method can be shown to be insensitive to averaged amplitude and phase profiles across the chamber, low quenching β/α of the hairpin, and diffusion of the hairpin in the chamber. It can be used to detect kinetics of surface-bound specimen. The lock-in could image over many microfluidic chambers, revealing kinetic data for each chamber independently, yielding an interesting approach for high-throughput screening applications.

To conclude, we showed that the molecular multiplication of a fluorescent dye can be used to implement a lock-in amplifier at the molecular level. This approach has several advantages. (1) The parallel imaging of millions of amplitude and phase values from a single measurement is highly parallel. In particular, the phase image reports insightful delay information. (2) Signal to noise reaches the photonic shot noise limit and compares well with photon counting lock-ins.[5] (3) Slow response times of detectors do not limit measurements at high frequencies. (4) The approach corrects for inhomogeneous illumination and bleaching. (5) Any environment that is reported by the brightness of fluorescent dyes can be measured by the lock-in. We therefore expect diverse applications in biology, neuroscience and high-throughput screening.

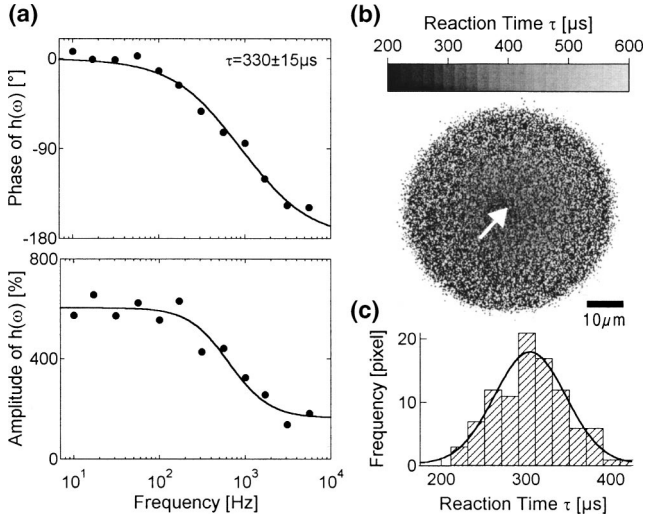

FIG. 4. Opening/closing kinetics of a DNA hairpin. (a) The reaction transfer function $h(\omega)$ is recorded for each pixel by dividing lock-in measurements of the hairpin and the temperature dye BCECF (dots). Each data point is based on only 80.000 recorded photons. It is well fitted with a first-order theory and an opening/closing time constant of $\tau=330\pm15$ μs (solid line, error from fit). (b) A map of opening/closing time constants can be obtained. (c) A 100-pixel average in the center reveals $\tau=320$ μs with a standard deviation of 47 μs and an error of the mean of 5 μs.

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