TEMPERATURE MODULATION AND PHASE SENSITIVE IMAGING TO DETECT POINT MUTATIONS

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ABSTRACT

Temperature oscillations allow one to modulate the concentration of reagents and products with given amplitude and phase shift, both parameters depending on the dynamics of the chemical system under study. We here rely on thermal excitation in a microdevice, associated with fluorescence video microscopy as well as image filtering protocols, to selectively enhance the signal issued from a labeled probe that hybridizes with a targeted oligonucleotide. Furthermore, by simply tuning the stimulation parameters, focus can be put either on the perfect match sequence or on some other one that bears a point mutation.

KEYWORDS: Kinetic measurements, SNP, Oligonucleotide, Fluorescence microscopy, Heat transfer.

INTRODUCTION

Species detection in a biological medium is often achieved through titration: the target binds a probe introduced by the experimentalist. However, such equilibrium approach is not very selective. For instance, on-chip SNP analysis has to rely on sophisticated hybridization buffers and washing protocols. Thus, we here propose a homogeneous technique to distinguish between a match target, C19, and a mismatch one, M19 – both pairing with a 9mer fluorescent probe P. More precisely, we modulate the mixture temperature T at pulsation ω using a microfabricated resistor (Fig. 1a and b) [1,2], which results in oscillations of the analyte concentrations [2,3]. For a given probe/target couple, characterized by the k_{on} and k_{off} hybridization and dissociation rate constants, the out-of-phase response of the chemical system displays a sharp maximum when the following "resonant" conditions are satisfied: $\omega_R/2 = k_{on}$ [target] $= k_{off}$ [4]. Thus, selective addressing of the sought for sequence can be performed implementing an appropriate thermal excitation and a delayed video acquisition.

EXPERIMENTAL

The various solutions to be analyzed are introduced in four microchannels, 30 µm large and 10 µm high, molded in PDMS and closed by a glass wafer (Fig. 1). A 250 µm large indium tin oxide (ITO) resistor, fabricated on the latter substrate, enables thermal excitation while still allowing epifluorescence imaging [1,2]. Injection of current at the angular frequency $\omega/2$ results in Joule heating and periodic modulation of the sample temperature at ω.

Before performing any selective detection, duplex formation has been thoroughly characterized in order to determine the "resonant" conditions associated with each target. First, the microscope stage temperature is ramped to yield a thermal denaturation curve from which the association thermodynamic constant K can be inferred at any T [5]. Second, the reactive mixture is periodically forced out-of-equilibrium relying on Joule heating in the ITO element. Thereby, because the probe label is quenched upon pairing, the observed fluorescence oscillations report on the hybridization extent (Fig. 2a). Scanning ω should then provide the chemical system transfer function. However, for a given AC current, the magnitude of the induced temperature modulation may vary with the angular frequency, according to the thermal transfer function of the microsystem [1]. Hence, we here normalize the amplitude of the green-emitting probe/target response by the one of a red-emitting molecular beacon, MBr, that has previously been mixed to the samples. In fact, the latter species acts as a thermometer which has such a small characteristic time that it provides at any ω a signal proportional to the temperature change [5,6]. The ratio of the fluorescence amplitudes is next fitted by $R^{g/r} = R_0 / [1 + (\tau_\chi\omega)^2]^{1/2}$ to extract the reaction relaxation time τ_χ (Fig. 2b) [2,3]. Eventually, the rate constants are computed using $K = k_{on} / k_{off}$ and $1/\tau_\chi = k_{on}$ [target] + k_{off} (Table 1).

Selective imaging of a "resonant" reagent necessitates to measure the amplitude of the green fluorescence oscillations with a $-\pi/2$ phase delay with regard to the thermal modulation [4]. More mathematically, if the reactive mixture temperature varies as $T = T_R + \beta T_R \sin(\omega_R t)$, its emission follows $F = F_0 + \beta[F_{1sin} \sin(\omega_R t) + F_{1cos} \cos(\omega_R t)]$, from which one has to extract the F_{1cos} term because it is, in first approxiamation, proportional to the concentration in targeted species [4]. Such latter operation is in fact easily achieved thanks to phase sensitive detection [7]: the video movie of the microchannels is multiplied by $\cos(\omega_R t)$ and averaged over several periods in order to yield $F_{1cos} = \beta/2 \langle F \times \sin(\omega_R t)\rangle_t$. Importantly, the exact phase of the excitation was determined by using MBr, the fluorescent thermometer that instantaneously responds to any temperature modification.

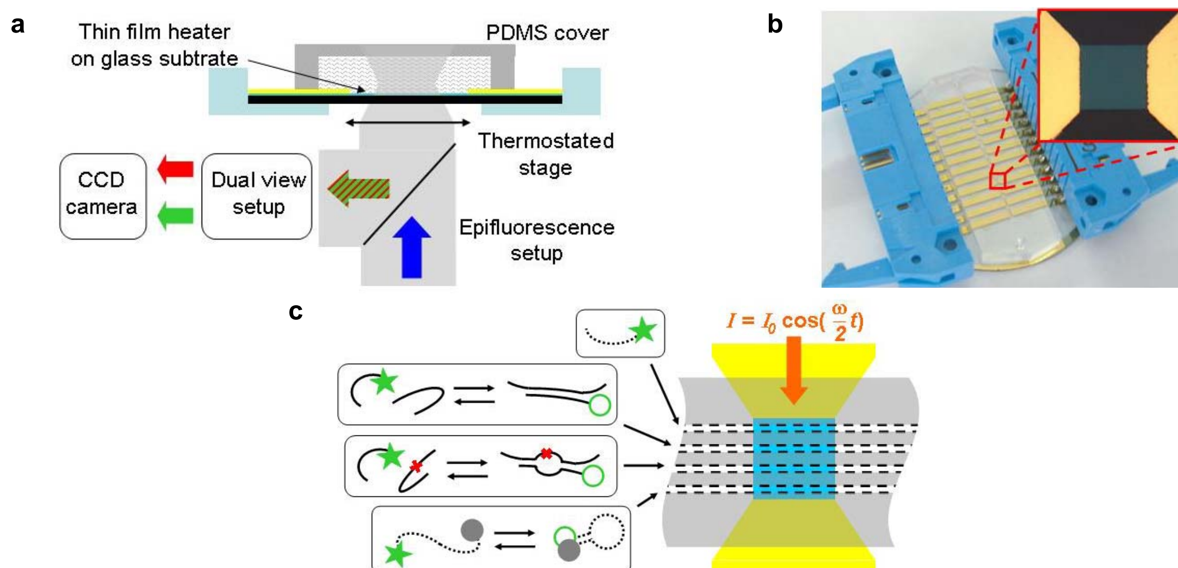

Figure 1. (a) Layout of the experimental setup, which includes the microchip and a dual-view microscope for simultaneous imaging in two colors. (b) Picture of the microdevice, with a zoom on the 250 µm heater. (c) Corresponding scheme indicating the composition of the different oligonucleotide solutions used for the selective imaging experiments. All the Rhodamine-Green labeled strands are at 1 µM in $Mg(OH)_2$ 1.25 mM NaOH 25 mM Hepes 55 mM buffer, pH 7.5 at 20 °C, supplemented with 0.1% PDMA ; the unlabeled targets are at 2.3 µM. The first and fourth channels correspond to species simulating contaminants, the non-reactive dT_{13} sequence and a rapidly exchanging molecular beacon, MB [5]. Stars (★) hold for emitting fluorescent dyes and circles (○) for quenched ones. To determine the thermal excitation phase and amplitude, we added to all the samples 2 µM of a Texas-Red labeled molecular thermometer, MBr [5,6].

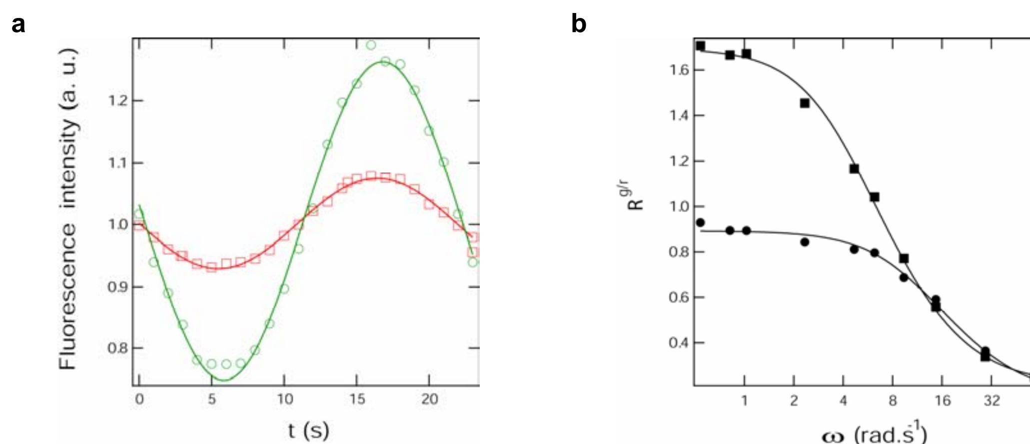

Figure 2. (a) Temporal evolution of the fluorescence intensity issued from the Rhodamine-Green labeled probe P, which here interacts with the M19 target, (○) and from the reference Texas-Red labeled molecular beacon MBr (□). A thermal excitation of amplitude 2.4 °C was applied at 0.25 rad.s^{-1}around 23 °C. Buffer and concentrations are similar to the ones given in the Figure 1 caption, except that [P] = 3 µM. (b) Frequency dependence of the normalized modulation amplitude $R^{g/r}$ at 23 (■) and 28 °C (●).

RESULTS AND DISCUSSION

Each target is introduced with the probe in a microchannel. Furthermore, to mimic contaminants, slow and fast reactive species are injected in side positions (Fig. 1c). The stage is subsequently thermostated in order to oscillate around the average temperature T_R, characterized by $K = 1$, and the thermal excitation is tuned at ω_R. Relying on the above phase sensitive acquisition protocol, the chip is eventually imaged in quadrature with respect to the temperature modulation (Fig. 3). As expected, depending on whether we use "resonance" conditions associated with C19 or M19, the signal from the chosen target is enhanced. For example, although the match to mismatch signal ratio, I_{C19}/I_{M19}, was 0.52 in standard fluorescence microscopy, we could reverse the contrast to selectively emphasize on C19 with $I_{C19}/I_{M19} = 2.19$ (Table 1). Noticeably, the emission from the two model interfering species was extinguished.

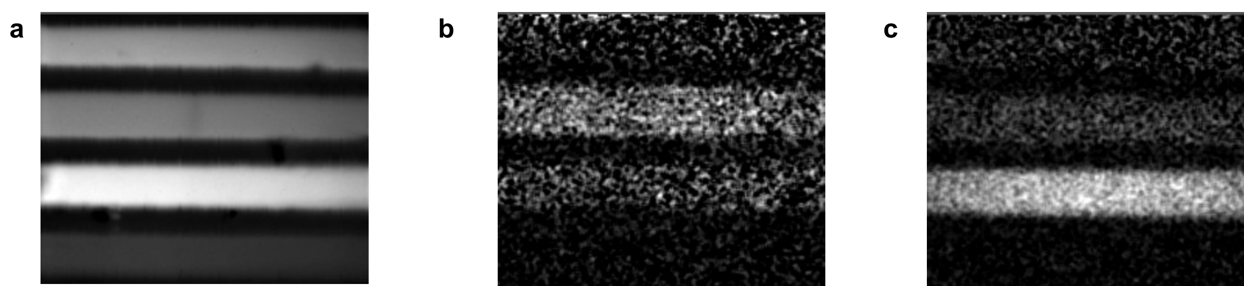

Figure 3. Images of the device filled with the oligonucleotide mixtures described in Fig. 1c. (a) No thermal excitation is applied, the fluorescence emission is steady. (b – c) Temperature is modulated with a 1 °C amplitude and fluorescence is detected in quadrature. The average temperature T_R and the excitation angular frequency ω_R are chosen to emphasize either on match (b) or on mismatch (c) – see Table 1 for details.

Table 1. Ratio of the match to mismatch fluorescence signals, I_{C19}/I_{M19}, obtained from the Figure 3 experiment. Results are presented for "resonance" conditions, T_R and ω_R, that favor the detection of either C19 or M19. When using standard fluorescence detection (Fig. 3a), we have $I_{C19}/I_{M19} = 0.52$. The hybridization and dissociation rate constants, k_{on} and k_{off}, for the two duplexes are also reported at both T_R.

Target	T_R (°C)	ω_R (rad.s^{-1})	I_{C19}/I_{M19}	k_{on} (10^6 M^{-1}s^{-1})	k_{off} (s^{-1})
C19	35.0	15.2	2.19	3.47	7.55
M19	27.5	5.9	0.21	1.26	2.95

CONCLUSIONS

Whereas traditional imaging provides a global intensity value that can hardly be related to the presence of a given species, our "temperature modulation and quadrature detection" approach enables unambiguous sequence detection. Indeed, the signal associated with the perfectly complementary strand always increases when shifting from the mismatch to the match "resonance" conditions. Conversely, the signal corresponding to the mutant always follows the opposite trend.

Moreover, the contribution of the fluorescent contaminants is cancelled out. Therefore, applied to microarrays or equivalent hybridization-based techniques, the present analytical protocol would significantly reduce the false-positive number and increase the assay sensitivity. Additionally, detection can here be achieved with various non-complementary fluorescent strands in solution: our technique is homogeneous and does not require a separation step to wash away unpaired species. As a result, there is no more need for the complex procedures necessary to immobilize oligonucleotides on solid supports.

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