Non-contact fluorescent bleaching-independent method for temperature measurement in microfluidic systems based on DNA melting curves†

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This report introduces a bleaching-independent temperature measurement method based on the analysis of the fluorescence emitted during the melting of DNA molecules with the SYBR-Green I intercalator, in a microvolume where the strong non-linearity of the signal is used to eliminate the photobleaching effect as well as to determine the heat transfer rate between a heater and the sample and the temperature non-uniformity within the sample.

The recent expansion in the development of micro total analysis systems (μTASs) and lab-on-a-chip platforms (LOCs)[1] has generated a new family of miniaturized devices operating with sub-microlitre sample volumes. These systems have significantly impacted applications such as drug discovery and point-of-care diagnostics, where the reduction in volumes not only allows economies of scale and portability, but also harnesses specific physical phenomena restricted to small samples, such as unique mass and heat transfer properties. The control of temperature in these devices is often crucial for a number of different applications, such as nucleic acids hybridization, micro-polymerase chain reaction (PCR), melting curve analysis (MCA)[2] and scanning differential fluorimetry.[3] The temperature of the sample can be determined directly by inserting a small temperature probe next to the sample.[4] However, the heat capacitance of the temperature probe should be significantly smaller than that of the sample to minimize its influence on the sample temperature. This requirement is difficult to fulfill once the sample volume is in the nanolitre range or even smaller.

Non-contact measurement techniques can provide a solution for the problems associated with small sample sizes. A widespread method is infrared thermometry, which is based on detecting the amount of emitted radiation in the 8–12 μm wavelength band.[5] Its precision depends on the exact determination of the sample emissivity, which is a limiting factor. Moreover, the conventional glass capping of microfluidic chips is not transparent for these wavelengths, restricting the application of this method to less practical materials. Another option is to introduce temperature sensitive compounds such as liquid crystals,[6] and thermochromatic[7] or fluorescent dyes.

Among those, fluorescent probes are the most commonly employed, either through the measurement of temperature dependent fluorescence intensity[8] or using molecular beacons,[9] *via* the improved versions measuring fluorescence lifetime,[10] its intensity ratio[11] or its coherent anti-stokes Raman scattering (CARS) signal.[12] However, in addition to the complex equipment needed (photocounting detectors or dual lasers), the bleaching of the molecule's fluorescence with time can lead to a considerable variation on the temperature reading.

Here we demonstrate the bleaching-independent non-contact measurement of temperature in a microdroplet, based on the melting curve analysis of DNA[2] using the fluorescent intercalator SYBR Green I. We show that an analysis of the fluorescence emitted by the sample as the temperature is scanned allows determining various parameters related to the temperature profile created. The DNA melting temperature T_m is defined as the temperature at which half of the double stranded DNA (dsDNA) melts into single stranded DNA (ssDNA). This temperature can be determined analytically and it depends only on the concentrations of DNA and salt in the sample,[13] making it independent of photobleaching. We further show that an analytical study of the inflexion point of the melting curve provides access to information on the temperature non-uniformity within the sample at the melting temperature.

By analyzing the melting profile of a reference set of DNA molecules (the melting temperature of which is known) in a microsystem, the local temperatures within the microsystem can be derived as well as the temperature non-uniformity within the sample. In a space domain system, such as a flow-through device, this method can be used to measure heat transfer. The understanding of the temperature variations obtained will have important implications in the control of a wide range of chemical and enzymatic reactions.

We used a previously developed virtual reaction chamber (VCR)[14] as an example to demonstrate the applicability of the methodology to LOC devices. A micromachined silicon chip integrated with thin film metal heaters/sensors was mounted on a custom-made printed circuit board (PCB). The temperature was controlled by a pulse-width modulation (PWM) with a closed-loop feedback system with proportional integrated derivative (PID) controlled by a LabView Professional v. 8.0 software (National Instruments, Inc.). The DNA sample was covered with mineral oil (M5904, Sigma-Aldrich, Inc.) to prevent evaporation and was separated from the heaters on the silicon chip by a 0.17 mm thick cover slip, silanised to obtain a hydrophobic/oleophobic surface (FOTS, 667420 Sigma-Aldrich, Inc.).

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The PCB was placed under an Axiotron II (Zeiss, Inc.) microscope equipped with a fluorescein isothiocyanate (FITC) fluorescent cube (49002, Chroma, Inc.). An X-Cite 120 PC metal halide light source (EXPO Photonic Solution, Inc.) was used for illumination and a photomultiplier tube (PMT) H5784-02 (Hamamatsu Photonics, K.K.) was employed to collect light emitted from the sample. The PMT gain was adjusted in the range from 6×10^4 to 8×10^5 by controlling its input voltage in the range from 0.6 V to 0.8 V. Fig. 1A shows a schematic of the set-up, while the actual VRC is shown in Fig. 1B.

The amplitude of the emitted fluorescence F from the DNA-intercalator indicates the ratio of dsDNA to all DNA in the sample. Here it was recorded as a function of applied temperature T. The complex analytical equation[13] can be here replaced by an empirical formula as shown previously,[14] when we adopted a modified sigmoidal function to fit the curves obtained to accommodate for the temperature sensitivity of SYBR Green I as well as its photobleaching. A similar function was used in this work (eqn (1)):

$$F = \frac{A_0 + A_1 \times T}{1 + e^{-(T - T_m/s)}} + A_2 \quad (1)$$

where T_m is the DNA melting temperature. A_0, A_1 and A_2 are correction factors for the photobleaching, the temperature sensitivity of the fluorescence and the background fluorescence, respectively. They are evaluated by non-linear curve fitting (Origin Version 7.5, MicroCal Corp.) and used to normalize the data to do away with the dye's sensitivity to temperature. S is a parameter defining the slope of the normalized curve $F(T)$ at the temperature, T_m. It can be derived numerically from the normalized peak of the first derivative at T_m according to the equation:

$$\frac{dF}{dT}(T = T_m) = -1 \times \frac{1}{4 \times S} \quad (2)$$

The slope S is given by the melting properties of DNA (DNA concentration and free enthalpy and entropy of the reaction[13]), as well as the sample temperature non-uniformity (Fig. 1C). Fig. 1C shows a finite element analysis (ANSYS, Inc.) to visualize the extent of the non-uniformity within the droplet.

Fig. 2A shows the melting behavior of a 0.2 μL DNA sample obtained after real time PCR (see ESI† for sequences and melting temperatures determined on a LightCycler—Roche, Inc.) and its analysis. The effects of photobleaching can be seen as the melting is repeated for the same sample nine times at 5 minute intervals. The fluorescence signal dropped exponentially (time constant of 14.5 min) over the repeats to less than 10% of the original value. However, this effect had little influence on the determination of both T_m and S (below 0.25% variation for T_m and less than 10% for S, Fig. 2B), confirming that both T_m and S are independent of photobleaching. Thus T_m can be used to ascertain the temperature of the sample at one point of the temperature scan, corresponding to one value of the heater driving parameter (such as dissipated power) and calibrate the system, while S determines the temperature non-uniformity within the sample. This method requires that the melting temperature of the DNA molecules used is known. This temperature depends on the concentrations of DNA as well as the sodium ions in the buffer. Models have been developed to calculate it but measurements typically provide more accurate results. The common system that we have used to obtain these reference temperatures (LightCycler, Roche Diagnostics, GmbH) has a reported standard deviation of 0.18 °C.[15] This error has to be taken into account when analyzing the temperature measurement resulting from this method.

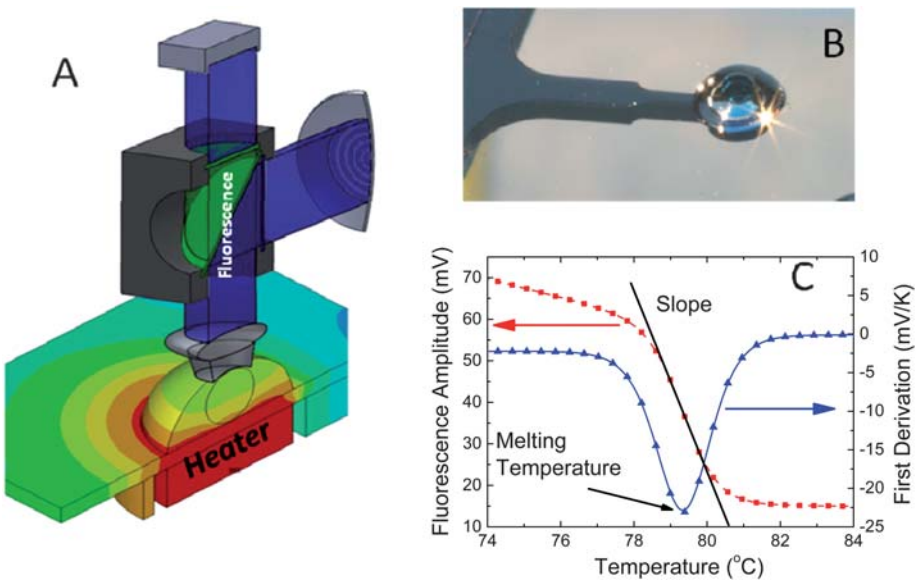

Fig. 1 (A) Schematic drawing of the measurement set-up. The DNA-based sample was placed on a microscope cover slip and covered with mineral oil forming a virtual reaction chamber (VRC). The sample was illuminated with filtered light at a wavelength 450–490 nm and the emitted light in 500–550 nm range was collected by a photomultiplier tube (PMT). The VRC was heated from 74 to 94 °C by the heater below it. The finite element analysis model clearly shows the non-uniform distribution of the temperature during the transient regime. (B) Photograph of the VRC with a 0.2 μL sample covered with 1.2 μL of mineral oil. (C) Melting curve analysis of a DNA (see ESI†) showing a sharp fluorescence drop at the melting temperature T_m (left) defined as the temperature at the peak of the first derivation. The peak amplitude is the slope value at T_m and is related to the temperature non-uniformity across the sample.

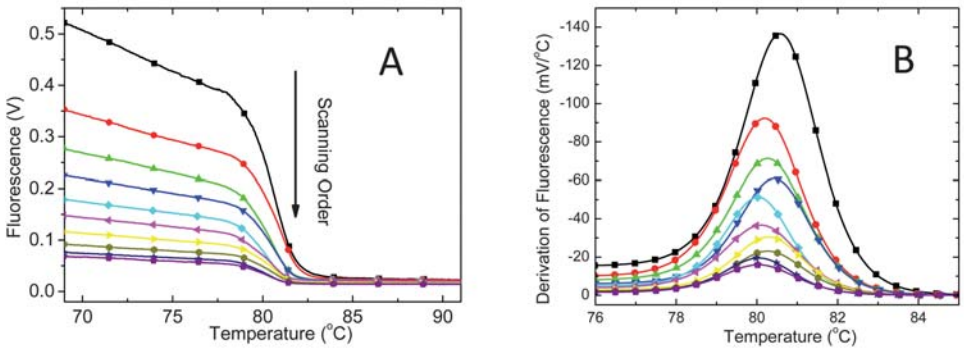

Fig. 2 (A) Melting curves of the DNA sample. The 0.2 μL DNA droplet was covered with 1.8 μL mineral oil and was warmed 10 times consecutively (5 min interval) from 60 °C to 100 °C with a scanning rate of 0.1 °C s^{-1}. The readings from the PMT were taken every 0.05 °C (400 data points for each curve). Within 45 min, the fluorescence amplitude dropped below 10% of its original value due to bleaching. (B) In spite of the drop in signal amplitude, the first derivation of the curves with respect to temperature showed mean value of melting temperature T_m of 80.29 °C with only ±0.18 °C fluctuation (standard deviation). This is similar to the errors obtained on conventional systems (LightCycler for example gives a standard deviation of 0.18 °C as well), and could be attributed to inherent tolerances of the instrumentation used. The parameter S behaves similarly (0.62 mV °C^{-1} ± 0.065 mV °C^{-1}) showing that extracted values are independent of photobleaching. The square markings on the curves are for visualization only.

The measurement described above revealed that the sample temperature was delayed with the respect to the heater temperature. This phenomenon arose from the speed of the heat transfer from the heater to the liquid sample, linked to the thermal conductivity of the system. This temperature difference can be suppressed by using a slower scanning rate (from 5 °C s^{-1} to 0.1 °C s^{-1} as shown in Fig. 3A). Alternatively the measured (extracted) melting temperature as a function of the scan rate can also be used to determine the heat transfer rate between the system and the sample.

The heat transfer time was extracted from the melting temperature shift as a function of the scanning rate (see Fig. 3B). Its amplitude depends on the sample heat capacity (volume and composition). As the volume decreases, the heat transfer becomes faster, resulting in faster analysis without compromising the readouts.

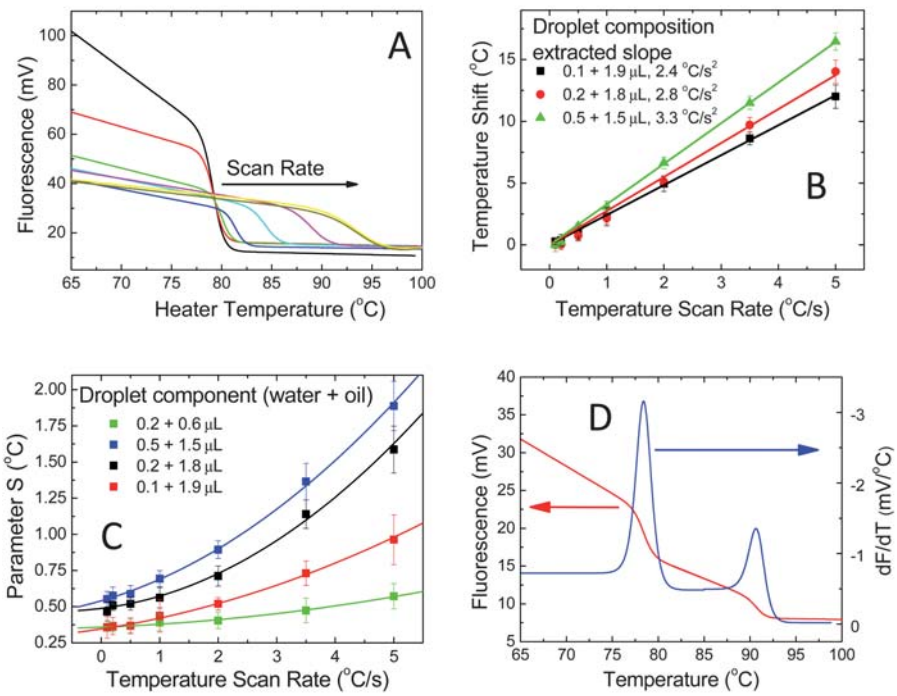

Fig. 3 (A) Melting curve analysis of a 0.2 μL sample covered with 1.8 μL of a mineral oil with temperature scan rate as parameter, from 0.1 °C s^{-1} to 5 °C s^{-1}. An increased scan rate caused delay in the fluorescence drop which appears as an increase in the DNA melting temperature. (B) Extracted melting temperatures as a function of the scan rate for different sample compositions (volume of DNA and volume of oil). (C) Slope S as a function of the scanning rate showing that the temperature non-uniformity within the sample increased with the scan rate. (D) A mixture of two DNA samples, each with a different melting temperature, demonstrated the temperature measurement at two points, in the same VRC.

The parameter S (Fig. 3C) shows the extent of the temperature non-uniformity in the sample at T_m and it is the function of both the scan rate and the heat capacity of the sample. This is expected since the stationary regime (uniform temperatures in the drop after the transient regime) is longer to establish when the temperature difference is more abrupt.

Single point temperature measurements might not be sufficient to perform a solid calibration of the temperature behavior of a microfluidic system. The sharp non-linear transition observed at the melting temperature within a few degree centigrade allows deconvoluting the melting behaviors of more than one DNA molecule, provided that the melting temperatures are different. Fig. 3D shows that two DNA molecules (T_m = 78.5 °C and 90.7 °C as measured by LightCycler) can be mixed together without compromising the analysis. Two distinguishable peaks of the fluorescence amplitude derivation can be observed. It increases the robustness of the temperature analysis.

In conclusion, we have demonstrated a method for non-contact temperature measurements using the fluorescence signal from DNA melting reactions. This method's precision is not affected by the photobleaching effect, making it very robust and suitable to measure temperatures within microfluidic systems. It can be used in a temperature range from 50 °C to 90 °C, since it requires the melting of DNA molecules. This range could be extended by monitoring the unfolding of proteins.

Although we have here used a VRC in a droplet, the same principles can be easily transcribed to microfluidic channels with an optical access to their contents. The technique was also used to study the heat transfer rate between the heater and the sample. This variant could be applied to determine internal flow rates inside a microchannel by observing the shift in the location of the DNA melting point inside the channel. Finally we also demonstrated that the sample temperature non-uniformity can be monitored. Certain reactions, such as PCR, require precise temperature control and the method shown here allows determining both temperature as well as temperature uniformity within the sample.

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