Remote temperature measurements in femto-liter volumes using dual-focus-Fluorescence Correlation Spectroscopy

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Received 9th May 2008, Accepted 9th January 2009
First published as an Advance Article on the web 6th February 2009
DOI: 10.1039/b807910b

Remote temperature measurements in microfluidic devices with micrometer spatial resolution are important for many applications in biology, biochemistry and chemistry. The most popular methods use the temperature-dependent fluorescence lifetime of Rhodamine B, or the temperature-dependent size of thermosensitive materials such as microgel particles. Here, we use the recently developed method of dual-focus fluorescence correlation spectroscopy (2fFCS) for measuring the absolute diffusion coefficient of small fluorescent molecules at nanomolar concentrations and show how these data can be used for remote temperature measurements on a micrometer scale. We perform comparative temperature measurements using all three methods and show that the accuracy of 2fFCS is comparable or even better than that achievable with Rhodamine B fluorescence lifetime measurements. The temperature dependent microgel swelling leads to an enhanced accuracy within a narrow temperature range around the volume phase transition temperature, but requires the availability of specific microgels, whereas 2fFCS is applicable under very general conditions.

Introduction

Precise remote temperature measurements within femtoliter-small volumes play a key role in customizing biological, biochemical and chemical techniques to lab-on-a-chip (LOC) devices.

To measure temperature in LOC devices with direct contact to sample is a common and easy technique, using *e.g.* resistance of platinum whose relation to temperature is nearly linear over a wide temperature range.[1] In cases of measuring in a very small volume, the sample cannot be directly contacted with a platinum resistance, therefore we focus on contactless methods for precise temperature determination.

The two most frequently used fluorescence-based techniques for remote temperature measurements are based on: (i) employing the temperature dependence of the fluorescence intensity of specific materials,[2,3] and (ii) measuring the temperature dependence of the fluorescence lifetime of specific fluorescent dyes. Group (i) uses effects such as thermochromicity of liquids,[4] special properties of Ni, *e.g.* Ni(II) high-spin/low-spin interconversion[5] or NiCr/Ni-films,[6] temperature-dependent conductivity of semiconducting nanoparticles[7] or thermo-responsivity of some polymers,[8,9] as well as photo bleaching of fluorescent dyes.[10] Group (ii) employs temperature-dependent fluorescence lifetime changes of Rhodamine B[11,12] which are induced by temperature-dependent conformational changes of sidegroups.[13,14] All these approaches are appropriate for particular applications but they are dependent on the availability of highly specific chemicals or materials.

Here, we present a more generally applicable technique which is based on the absolute measurement of diffusion of small fluorescent dyes. Thermally induced Brownian motion of molecules and particles in solution is a fundamental property that is macroscopically described by the diffusion coefficient. The Stokes–Einstein equation[15] relates the diffusion coefficient of spherical objects to their hydrodynamic radius (R_h). For particles or molecules with known hydrodynamic radius, the measured diffusion coefficient enables one to determine either temperature or viscosity (which itself depends on temperature) of the solvent. On one hand the temperature can be measured in a solvent with known temperature dependent viscosity and on the other hand, the viscosity of the solvent can be measured if the temperature of the sample is known.

Recently, a new technique, dual-focus Fluorescence Correlation Spectroscopy (2fFCS), was introduced which allows for *absolute* and precise measurements of diffusion coefficients of small fluorescing molecules close to the infinite dilution limit (typically nanomolar concentration). The achievable accuracy of the method was estimated to be better than ±2–4%, and the spatial resolution of the method is below one micrometer.

Applications of FCS and dual-focus/dual beam FCS-measurements in microchannels and LOC devices are well established in the literature.[16–21] The 2fFCS technique described here, can be used with the same sample chambers that are employed in studies with chip-devices. As the 2fFCS technique is characteristically different from the above mentioned dual-focus/dual beam experiments, we don't focus here on the device itself, but *a fortiori* to the novel 2fFCS technique.

In the study presented here, we use 2fFCS for determining the temperature within femtoliter volumes by measuring the diffusion of fluorescing molecules with known size and, using the Stokes–Einstein relation, converting the diffusion coefficient into a temperature value. We compare the accuracy of the 2fFCS

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approach with temperature measurements based on fluorescence lifetime changes of Rhodamine B and with measurements based on diffusion changes of thermosensitive particles. In contrast to the last two methods, 2fFCS is generally applicable and does not depend on the availability of specific materials.

Temperature dependent measurements require a precise temperature control in the sample environment. The technical implementations of such a sample environment *e.g.* in a microfluidic chamber[22] or a sealed microscope sample cell is far beyond the scope of this contribution. Recently, we have reported on a temperature controlled and sealed sample cell for fluorescence microscopes.[23] As already mentioned above, the optical requirements of the 2fFCS technique are comparable to other optical techniques and thus 2fFCS is well suited for LOC devices.

Methods and materials

Theoretical background

Here, we briefly recall the theoretical background of 2fFCS.[24] In 2fFCS, two overlapping detection volumes are generated, which are identical in shape but shifted to each other perpendicular to the optical axis. This is achieved by using one (or two) pulsed lasers in such a way that the polarization of each laser pulse is turned by 90° with respect to the preceding pulse. When sending this excitation light through a Nomarski prism as used in conventional differential interference contrast (DIC) microscopy, each laser pulse is slightly deflected according to its polarization. After focusing the light through a water-immersion objective with high numerical aperture, this generates two foci with small lateral shift between them. To distinguish which fluorescence photon was generated by which laser pulse (*i.e.* in which focus), one measures the photon detection times with picosecond accuracy using time-correlated single-photon counting (TCSPC) and associates each detected photon with the latest preceding excitation pulse. This allows for calculating the autocorrelation function (ACF) for each focus as well as the cross-correlation function (CCF) between both foci. Because the lateral shift between both foci is precisely known (it only depends on the properties of the Nomarksi prism), a global analysis of both the ACFs and CCF allows for determining an *absolute* value of the diffusion coefficient of fluorescent molecules or particles in solution.

As was shown in detail in ref. 24, adequate model functions for the diffusion-related part of the ACF or CCF (neglecting for a moment any photophysics-related fluorescence fluctuations) are given by eqn (1):

$$\tilde{g}(t,\delta,\mathbf{v}) = \frac{c}{4}\sqrt{\frac{\pi}{Dt}}\int dz_1 \int dz_2 \, \frac{\kappa(z_1)\kappa(z_2)}{8Dt + w^2(z_1) + w^2(z_2)} \exp\left[-\frac{(z_2 - z_1)^2}{4Dt} - \frac{2\delta^2}{8Dt + w^2(z_1) + w^2(z_2)}\right] \quad (1)$$

where t is the lag-time of correlation, δ is the lateral distance between the detection volumes, ε_1 and ε_2 are factors proportional to overall excitation intensity and detection efficiency in each focus, c is the concentration of fluorescent molecules or particles, and D their diffusion coefficient. Here, the functions $\kappa(z)$ and $w(z)$ are given by eqn (2)–(4):

$$w(z) = w_0\left[1 + \left(\frac{\lambda_{ex} z}{\pi w_0^2 n}\right)^2\right]^{1/2} \quad (2)$$

and

$$\kappa(z) = 2\int_0^a \frac{d\rho\rho}{R^2(z)} \exp\left(-\frac{2\rho^2}{R^2(z)}\right) = 1 - \exp\left(-\frac{2a^2}{R^2(z)}\right) \quad (3)$$

with

$$R(z) = R_0\left[1 + \left(\frac{\lambda_{em} z}{\pi R_0^2 n}\right)^2\right]^{1/2} \quad (4)$$

where λ_{ex} and λ_{em} are excitation and centre emission wavelengths, n is the sample refractive index, a is the confocal pinhole radius, and w_0 and R_0 are fit parameters. For calculating the ACF of each focus, one has to set, in eqn (1), δ to zero, and to replace $\varepsilon_{1,2}$ by either ε_1^2 or ε_2^2, respectively. The integration in eqn (1) has to be performed numerically. Fitting of experimental data is done globally for both the ACFs and the CCF, where one has fit parameters $\varepsilon_1 \cdot c^{1/2}$, $\varepsilon_2 \cdot c^{1/2}$, w_0, R_0, and D.

As was recently shown in ref. 25, the absolute fitted values of w_0 and R_0 can become rather arbitrary when working under optical conditions with strong aberration. However, as was also shown in ref. 25, the fitted value of diffusion coefficient is still remarkably exact. In other words, the absolute values of w_0 and R_0, are not significant for the calculation of the diffusion coefficient and therefore they are not reported.

The distance δ between detection volumes is a setup constant and has to be determined only once for a given measurement system. This can be done for example by determining the diffusion coefficient of fluorescently labelled polymer particles with known size. This calibration procedure has been described in detail in ref. 26.

More technical details concerning the setup can be found in ref. 24.

Experiments

Dynamic light scattering (DLS) measurements were performed on a standard ALV 5000 system, equipped with a laser of 633 nm wavelength. Scattering intensity was detected at angles of 60°, 90°, and 120°, respectively, and the hydrodynamic radius was calculated with a second order cumulant fit using the Stokes–Einstein relation. The measurement system was equipped with a temperature controlled water bath giving a precision in sample temperature stabilization of ±0.2 K.

The 2fFCS measurement system was based on a MicroTime200 Fluorescence Spectroscopy and Microscopy System (MT200, PicoQuant GmbH, Berlin, Germany) as described in ref. 26 and 27 with a dual-focus modification as presented in ref. 24 and Fig. 1. The setup is equipped with two identical 470 nm lasers (LDH-P-C-470B), two identical 635 nm lasers (LDH-P-635), as well as one 532 nm laser (PicoTA530N), whose beam is split into two mutually time-delayed pulse trains. All lasers are linearly polarized in such a way that, after combining the light of all lasers, one obtains for each wavelength pulse trains with alternately switching polarization. Pulse width is ~50 ps, and

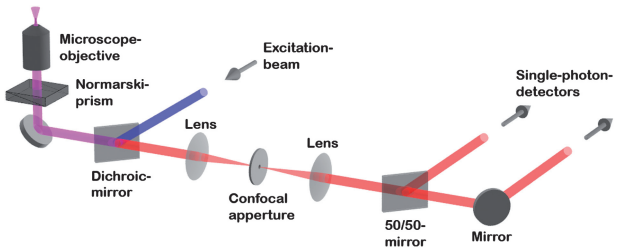

Fig. 1 Principle of a confocal two focus experiment.

pulse repetition rate is either 20 or 40 MHz, adjustable to the specific fluorescence lifetime of the measured fluorophore.[28] The lasers are coupled into a polarization maintaining single mode fibre for optical cleaning, and, after re-collimation, reflected towards the microscope's objective (UPLAPO 60x W, 1.2 N.A., Olympus Europa (Hamburg, Germany) by a major triple-band dichroic (z470/532/638rpc, AHF-Analysentechnik, Tübingen, Germany). Before entering the objective, the laser beam is passed through a Nomarski prism (U-DICTHC, Olympus Europe, Hamburg, Germany) to deflect pulses according to their polarization into two slightly inclined directions. After focusing through the objective, this generates two overlapping foci. Fluorescent light is collected by the same objective and focused onto a single circular aperture (diameter 200 μm). After passing several emission filters (HQ505/30m for $\lambda_{ex} = 470$ nm, HQ580/70m for $\lambda_{ex} = 532$ nm, and HQ687/70m for $\lambda_{ex} = 635$ nm, all purchased from AHF-Analysentechnik, Tübingen, Germany), the light is split by a non-polarizing beam splitter cube and focused onto two single photon avalanche diodes (SPAD, PDM series, Micro Photon Devices, Bolzano, Italy). A dedicated single-photon counting electronics (PicoHarp 300, PicoQuant Company, Berlin, Germany) is used to record single-photon detection events with 4 ps temporal resolution (time tagged time resolved or TTTR detection mode[29]). Using the picosecond detection times of the fluorescence photons, one determines by which laser pulse and thus in which focus the photon was generated.[24] Using this information, ACFs and CCF are computed with a custom-built MatLab routine.[30] When calculating all correlation functions, only photons from different SPAD detectors are correlated to eliminate afterpulsing and dead time effects of the detectors.

The typical spatial resolution in the direction of the optical axis is below one micron; the resolution in the perpendicular plane is given by the diffraction limit and is typically half a micron.

The robustness of 2fFCS against refractive index mismatch was experimentally demonstrated in ref. 24, where the diffusion of the dye Atto655 in aqueous solutions of varying concentrations of guanidinium hydrochloride up to 6M had been measured, not finding any deviation of the determined diffusion coefficient from its theoretically expected value. In ref. 24, an extensive theoretical study of the performance of 2fFCS under different optical and photophysical conditions was presented, showing the exceptional robustness of 2fFCS even under refractive index mismatch corresponding to the introduction of an additional slab of glass of ten micrometer thickness between the objective and the sample (corresponding, for example, to the impact of a sample refractive index of 1.52 when focusing at 10 μm above a chip's bottom glass surface, or the impact of a sample refractive index of 1.4 when focusing at 27 μm).

A data acquisition time of 30 minutes per temperature was employed both for the lifetime and diffusion measurements.

Temperature is controlled by a custom-made temperature regulation[23] with an absolute temperature accuracy of ±0.05 K (within the detection volume). The achievable temperature range for 2fFCS and lifetime measurements is 5 to 65 °C. During measurements, the sample chamber was sealed to prevent solvent evaporation and convection.

Materials

All samples are prepared using LiChrosolv water for chromatography (No. 115333, Merck KGaA, Darmstadt, Germany). For 2fFCS experiments we used Atto655-maleimid (No. AD 655-4, ATTO-TEC, Siegen, Germany). Rhodamine B (Bio-Chemika, No. 83689) was obtained from Sigma-Aldrich (Seelze, Germany). 2fFCS single molecule experiments were carried out with nanomolar dye concentrations. Fluorescent labelled poly-n-isopropylacrylamide ($PNIPAM^{Rh-B}$) microgel was synthesized following standard protocols as published in ref. 31–36 using a mixture of unlabelled and labelled monomers with a molar ratio of approx. 1 : 0.016. Labelled monomers (methacryloxyethyl-thio-carbamoyl-rhodamine B, No. 23591) were purchased from Polysciences. (400 Valley Road, Warrington, PA). DLS and 2fFCS experiments are performed at same sample concentrations of 0.05 wt% microgel solution.

We studied the effect of bleaching on 2fFCS measurements in ref. 24 and ref. 37, while investigating the robustness of 2fFCS with respect to optical saturation of fluorescence. When the excitation intensity is becoming so large that bleaching starts to impact the measurement, it leads to an apparent increase in the diffusion coefficient. 2fFCS cannot compensate for that and consequently one has to employ excitation intensities where bleaching does not yet affect the measurement. In this study 2fFCS measurements were performed at different excitation intensities and it was ensured that the experimental results were independent of excitation intensity.

Results and discussion

Lifetime measurements on Rhodamine B

We first measured the lifetime dependence of Rhodamine B in aqueous solution and in methanol. In Fig. 2, a comparison of single molecule lifetime data of Rhodamine B (crosses) with literature values from ref. 11 and 14 (dots and circles) is presented. We could perfectly reproduce the published values for water.

However, in contrast to the report in ref. 11, we find a different temperature dependence of the Rhodamine B lifetime in methanol (stars). Taking into account that the lifetime change is caused by a different rotational flexibility of the dye molecule which is again dependent on the solvent's viscosity, a different temperature dependence of the fluorescence lifetime in water and methanol, respectively, can be expected. This illustrates a disadvantage of the method: besides being dependent on a specific dye (Rhodamine B), the exact temperature behaviour of the lifetime

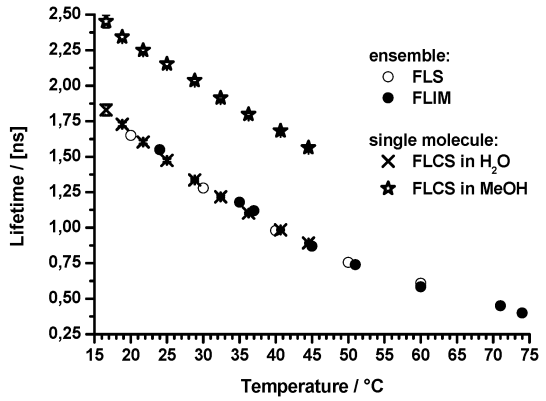

Fig. 2 Fluorescence lifetime correlation spectroscopy (FLCS) measurements of Rhodamine B. Fluorescent lifetime measurements obtained from single molecule experiments in aqueous solutions (crosses) and in methanol (stars) compared with published fluorescence lifetime obtained from fluorescence lifetime spectroscopy (FLS)[14] and fluorescence lifetime imaging (FLIM),[11,14] as a function of temperature in aqueous solution (dots and circles).

has a complex solvent-specific dependence. On one hand the chemical environment of the fluorescent dye (*e.g.* pH, salt) influences the lifetime, on the other hand any contamination that potentially quenches the fluorescence will change this dependence. This restricts the general applicability of the lifetime method, in contrast to the diffusion measurement where only the solvent specific temperature dependent viscosity needs to be known.

2fFCS measurements of temperature

2fFCS offers the possibility of using any fluorescent dye for temperature measurements. 2fFCS allows for determining the diffusion coefficients of dye molecules with an absolute accuracy of ±2–4%. Because for most solvents, the temperature dependence of their viscosity is well known, one can use the measured diffusion coefficient to directly derive a temperature value using the Stokes–Einstein equation.

In Fig. 3 *(upper plot)*, we present the raw autocorrelation—and cross-correlation—functions of a measurement of Atto655_imid, as well as the fitted model described in eqn (1)–(4).

In Fig. 3 *(lower plot)*, we show as an example the measured diffusion coefficient of the dye Atto655-maleimid as a function of temperature. The data (crosses) are fitted with the Stokes–Einstein equation, showing perfect agreement between predicted and determined behaviour of diffusion coefficient with temperature. It should be emphasized that the focal volume where the diffusion is measured is on the order of femtoliters.

Thus, 2fFCS-based diffusion measurements are an ideal tool for determining absolute temperature values with sub-micrometer spatial resolution.

It should also be noted that this method will rather not work when using conventional FCS: because the refractive index of any liquid is a function of temperature, varying temperature will introduce refractive index mismatch between solution and an objective's immersion medium, leading to aberrations in laser focusing and fluorescence detection. As was pointed out in detail

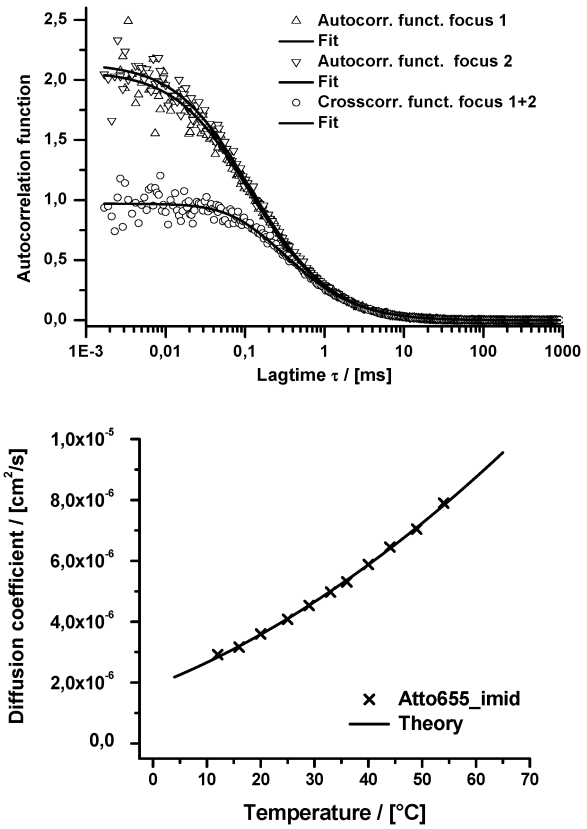

Fig. 3 *(top)*: 2fFCS measurement of Atto655_imid, with a concentration of 10^{-9} mol/l, autocorrelation functions, and 2f-cross-correlation function fitted with the 2fFCS-model, as described in eqn (1)–(4). *(bottom)*: Temperature dependent measurement of Atto655_imid fluorescence dye. Diffusion coefficient from 2fFCS is in perfect agreement with Stokes–Einstein equation.

in ref. 38, this will change the detection volume and temporal decay of an ACF, leading to an apparently slower diffusion coefficient in conventional FCS. The important point is that 2fFCS is insensitive to such aberration effects and can thus be used over a wide temperature range without suffering from the changing optical aberrations at different temperature values. Moreover, the method will be insensitive to effects such as partial fluorescence quenching which will deteriorate the performance of any fluorescence-lifetime based temperature determination.

Microgel swelling

The last method we studied was using the temperature-dependent swelling of microgel particles for determining temperature. The used particles were labelled PNIPAM^{Rh-B} microgels. The diffusion coefficient of the particles can be measured either with DLS or with 2fFCS. In contrast to the "pure dye" experiments of the preceding subsection, where one uses only the dependence of solvent viscosity on temperature, thermosensitive microgel particles change also their intrinsic hydrodynamic radius R_h with temperature. They exhibit a very pronounced decrease in R_h that takes place within a very narrow temperature range and can thus be used for highly accurate temperature measurements within this transition region.

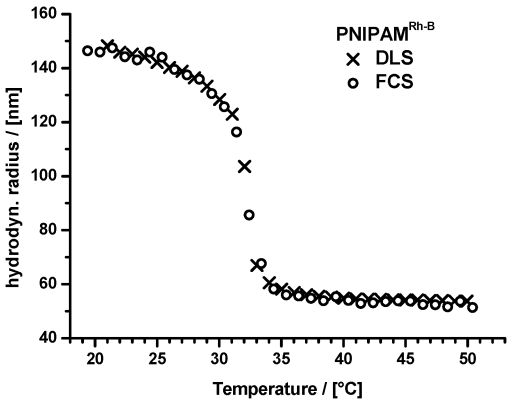

Fig. 4 Labelled PNIPAM^{Rh-B} microgels: DLS and 2fFCS measurements of thermosensitive microgel, with VPTT around 32 °C.

In Fig. 4, the temperature dependency of the hydrodynamic radius R_h of labelled PNIPAM^{Rh-B} microgel particles as determined by dynamic light scattering (DLS) and by 2fFCS is shown. The volume phase transition temperature (VPTT), defined as the inflection point of the curve, is approximately 32 °C. Depending on the synthesis, it is possible to adjust the VPTT of such polymers as desired within a broad temperature range.

Again, the comparison of ensemble data from DLS and results from single molecule 2fFCS shows a perfect agreement, showing the high performance of 2fFCS. Thus, 2fFCS together with temperature-dependent microgel swelling allows for highly accurate temperature measurements on the sub-micrometer length scale.

Comparison of methods and estimate of achievable precision

In the preceding sub-sections, we have presented measured data on the temperature dependence of (i) fluorescence lifetime of Rhodamine B, (ii) the diffusion coefficient of Atto655-maleimid, and (iii) the hydrodynamic radius of labelled PNIPAM^{Rh-B} microgel particles as measured *via* their diffusion coefficient. To estimate and compare the accuracy of the different techniques, we discuss model calculations with the following assumptions: (i) the absolute precision of lifetime determination within a femtoliter volume at reasonable dye concentration and measurement time is estimated to be between 50 and 100 ps; (ii) the accuracy of a typical 2fFCS diffusion measurement is assumed to be between 2% and 4%; (iii) the temperature dependence of microgel swelling is behaving as shown in Fig. 4, *e.g.* 32 °C; and (iv) particle radius changes approximately by a factor of 2 within the transition region of the VPTT.

Using all these assumptions, Fig. 5. shows a comparative estimate of the achievable accuracy of temperature determination for all three methods. Shown are regions bound by the estimated lower and upper accuracy of each method (50–100 ps for lifetime measurements, 2–4% for diffusion measurements). These accuracies are given by the experimental setup (as *e.g.* time resolution) but are independent of the temperature. Thus the accuracy of the temperature determination is given by the accuracy of the lifetime/diffusion data and can be calculated *via* the first derivative of lifetime/diffusion with respect to temperature.

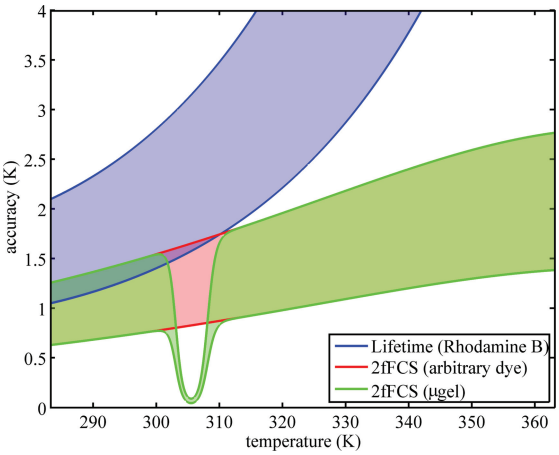

Fig. 5 Comparison of achievable temperature accuracy between lifetime and 2fFCS diffusion measurements. *Blue*: Lifetime measurement of Rhodamine B. *Red*: 2fFCS on arbitrary fluorescence dye. *Green*: 2fFCS on thermosensitive material with VPTT around 32 °C. It should be emphasized that the temperature measurement based on molecular diffusion and microgel diffusion give for most of the temperature region (outside the microgel swelling transition region) identical confidence intervals, as indicated by the shading where both regions overlap.

As can be seen, the lifetime-based method (blue band) has the smallest accuracy, which quickly deteriorates with increasing temperature. This is due to the fact that the relative lifetime change becomes smaller with increasing temperature. In contrast, 2fFCS measurements of dye diffusion (red band) show a higher accuracy with a much smaller dependence on absolute temperature. The microgel–particle diffusion method provides the highest accuracy of ±0.2 K. The transition temperature of thermosensitive polymers can be varied over a broad temperature range[32,33,35,36,39] and also the core–shell microgels with two transitions can be prepared.[34,40] However, this high accuracy is limited to the VPTT. At other temperatures one has the same accuracy as that of the dye-diffusion method.

As can be seen in Fig. 5, the achievable accuracy of temperature measurements based on diffusion measurements with 2fFCS is better than that achievable with conventional fluorescence lifetime measurements on Rhodamine B, in particular at high temperature values, where the relative change of lifetime as function of temperature becomes increasingly smaller. Concerning the technical complexity of a 2fFCS set-up, it should be mentioned that it is based on a standard confocal research microscope with DIC capability and requires only moderate modification of such a system. It is certainly not much more complex than a fully-fledged TCSPC system. One limitation of 2fFCS is the requirement of measuring on microchips with a thin cover plate so that the high-NA objective is still able to focus and detect light inside the chip. However, when aiming at micrometer spatial resolution, one has always to use high-NA objectives with correspondingly limited working distance (above glass surface) of *ca.* 150–250 micrometers.

Conclusions

In this contribution we have compared three different methods for remote temperature measurements in microfluidic chips with

sub-micrometer spatial resolution: fluorescence-lifetime based, dye diffusion based, and VPTT based methods. A crucial point for the diffusion-based measurements was the availability of the recently introduced 2fFCS technique that allows for absolute diffusion measurements within femtoliter volumes without being affected by aberration effects as introduced by the temperature-dependent refractive index mismatch between sample solution and objective's immersion medium.

The comparison of the different methods shows that diffusion measurements on labelled PNIPAM^{Rh-B} microgel particles achieves the highest accuracy in temperature determination, but only within the narrow temperature range of the VPTT. However, this can be of high interest for special biological applications, where one is interested in maximum accuracy but only within a narrow temperature range. 2fFCS measurements of dye diffusion show a rather uniform accuracy of temperature determination of *ca.* 1–2 K. The lowest accuracy is achieved when using the temperature-dependence of the fluorescence lifetime of Rhodamine B. Of all considered methods, the 2fFCS measurements of dye diffusion offers the greatest versatility, being independent of any specific dye or material properties.

Acknowledgements

We are indebted to PicoQuant Company (Berlin, Germany) for technical support. Financial support by the Deutsche Forschungsgemeinschaft (SPP1259) and Deutsche Volkswagenstiftung is gratefully acknowledged. We thank B. Brugger for the preparation of the labelled microgel sample BB010.

References

1 E. E. Krommenhoek, M. v. Leeuwen, H. Gardeniers, W. M. v. Gulik, A. v. d. Berg, X. Li, M. Ottens, L. A. M. v. d. Wielen and J. J. Heijnen, *Biotechnology and Bioengineering*, 2008, **99**, 884–892.
2 R. Fu, B. Xu and D. Li, *International Journal of Thermal Sciences*, 2006, **45**, 841–847.
3 R. Samy, T. Glawdel and C. L. Ren, *Anal. Chem.*, 2008, **80**, 369–375.
4 V. N. Hoang, G. V. Kaigala and C. J. Backhouse, *Lab on a Chip*, 2008, **8**, 484–487.
5 M. Engeser, L. Fabbrizzi, M. Licchelli and D. Sacchi, *Chemical Communications*, 1999, 1191–1192.
6 W. Yan, H. Li, Y. Kuang, L. Du and J. Guo, *Journal of Alloys and Compounds*, 2008, **449**, 210–213.
7 S. P. Wang, S. Westcott and W. Chen, *Journal of Physical Chemistry B*, 2002, **106**, 11203–11209.
8 S. Uchiyama, Y. Matsumura, A. P. de Silva and K. Iwai, *Analytical Chemistry*, 2004, **76**, 1793–1798.
9 K. Iwai, Y. Matsumura, S. Uchiyama and A. P. de Silva, *Journal of Materials Chemistry*, 2005, **15**, 2796–2800.
10 L. Gui and C. L. Ren, *Applied Physics Letters*, 2008, **92**, 024102.
11 R. K. P. Benninger, Y. Koc, O. Hofmann, J. Requejo-Isidro, M. A. A. Neil, P. M. W. French and A. J. deMello, *Anal. Chem.*, 2006, **78**, 2272–2278.
12 D. Ross, M. Gaitan and L. E. Locascio, *Anal. Chem.*, 2001, **73**, 4117–4123.
13 S. Ishizaka and N. Kitamura, *Bulletin of the Chemical Society of Japan*, 2001, **74**, 1983–1998.
14 N. Kitamura, Y. Hosoda, C. Iwasaki, K. Ueno and H. B. Kim, *Langmuir*, 2003, **19**, 8484–8489.
15 A. Einstein and R. Fürth, *Investigations on the theory of the Brownian movement*, Dover Publ., New York, NY, 1956.
16 M. Brinkmeier and R. Rigler, *Exp. Techn. Phys.*, 1995, **41**, 205–210.
17 M. Brinkmeier, K. Dorre, J. Stephan and M. Eigen, *Analytical Chemistry*, 1999, **71**, 609–616.
18 H. Blom, M. Johansson, M. Gösch, T. Sigmundsson, J. Holm, S. Härd and R. Rigler, *Appl. Opt.*, 2002, **41**, 6614–6620.
19 P. S. Dittrich and P. Schwille, *Analytical Chemistry*, 2002, **74**, 4472–4479.
20 D. J. LeCaptain and A. Van Orden, *Analytical Chemistry*, 2002, **74**, 1171–1176.
21 M. L. Plenert and J. B. Shear, *Proceedings of the National Academy of Sciences of the United States of America*, 2003, **100**, 3853–3857.
22 A. I. K. Lao, T. M. H. Lee, I. M. Hsing and N. Y. Ip, *Sensors and Actuators A: Physical*, 2000, **84**, 11–17.
23 C. B. Müller and W. Richtering, *Colloid & Polymer Science*, 2008, **286**, 1215–1222.
24 T. Dertinger, V. Pacheco, I. von der Hocht, R. Hartmann, I. Gregor and J. Enderlein, *ChemPhysChem*, 2007, **8**, 433–443.
25 T. Dertinger, A. Loman, B. Ewers, C. B. Müller, B. Krämer and J. Enderlein, *Opt. Express*, 2008, **16**, 14353–14368.
26 C. B. Müller, K. Weiß, W. Richtering, A. Loman and J. Enderlein, *Opt. Express*, 2008, **16**, 4322–4329.
27 M. Böhmer, F. Pampaloni, M. Wahl, H. J. Rahn, R. Erdmann and J. Enderlein, *Review of Scientific Instruments*, 2001, **72**, 4145–4152.
28 B. K. Müller, E. Zaychikov, C. Bräuchle and D. C. Lamb, *Biophysical Journal*, 2005, **89**, 3508–3522.
29 D. V. O'Connor and D. Phillips, *Time-correlated single photon counting*, Academic Press, London, Orlando, 1984.
30 J. Enderlein and I. Gregor, *Review of Scientific Instruments*, 2005, **76**, 033102.
31 H. Senff and W. Richtering, *The Journal of Chemical Physics*, 1999, **111**, 1705–1711.
32 R. Pelton, *Advances in Colloid and Interface Science*, 2000, **85**, 1–33.
33 M. Keerl and W. Richtering, *Colloid & Polymer Science*, 2007, **285**, 471–474.
34 I. Berndt and W. Richtering, *Macromolecules*, 2003, **36**, 8780–8785.
35 J. F. Lutz, O. Akdemir and A. Hoth, *Journal of the American Chemical Society*, 2006, **128**, 13046–13047.
36 S. Huber and R. Jordan, *Colloid and Polymer Science*, 2008, **286**, 395–402.
37 A. Loman, T. Dertinger, F. Koberling and J. Enderlein, *Chem. Phys. Lett.*, 2008, 18–21.
38 J. Enderlein, I. Gregor, D. Patra, T. Dertinger and B. Kaupp, *ChemPhysChem*, 2005, **6**, 2324–2336.
39 X. Guo, T. Lu and X. Huang, *Colloid & Polymer Science*, 2008, **286**, 469–473.
40 W. Blackburn and L. Lyon, *Colloid & Polymer Science*, 2008, **286**, 563–569.