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Predicting sample heating induced by cantilevers illuminated by intense light beams

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ABSTRACT

Hybrid microscopy based on Atomic Force Microscopy (AFM) and fluorescence microscopy represents a commonplace experimental approach to study cell biology processes in liquid media at physiological temperature. However, many types of experimental artifacts can arise depending on the fluorescence illumination and detection technique utilized. For example, fluorescence excitation light gets absorbed by AFM cantilevers inducing local heating provoking undesirable as well as uncontrollable cantilever deflections. Here we present a numerical modelling approach based on a Finite Element Model (FEM) to predict sample heating in liquid media quantitatively, depending on illumination wavelength, illumination pattern, and cantilever shape and composition. Modelling results indicate substantial local temperature increases in-line with temperature increases derived from experimental cantilever deflections induced by fluorescence excitation light. We predict temperature increases of ~0.05 – 0.5 °C for wide-field illumination and ~5 – 15 °C for confocal illumination within the boundary conditions established, which could, for example, induce local protein conformational changes. We conclude that sample heating is an important issue requiring consideration in experimental set-ups involving intense light illumination of AFM cantilevers, especially when conducting single molecule investigations.

Introduction

Shortly after the invention of the AFM in 1986, cantilever application and control in liquids was demonstrated enabling the investigation of biological processes at the nanometric scale in physiological conditions [1]. Since AFM is a surface characterization technique in which a cantilever approaches samples from the top, complementing AFM experimental capacities with a fluorescence technique in an epi-illumination from below is considered a powerful extension [2,3]. With the recent developments of combined AFM/fluorescence microscopy platforms [3], there is a need to understand how different types of cantilever illumination can affect sample temperature locally. This is especially relevant in the biological sciences area, where temperature control is critical not to disturb sample properties.

When operating both AFM and fluorescence imaging techniques simultaneously artefacts induced by mechanical vibrations or cantilever illumination can occur [4], complicating the observation of dynamical biological processes [5]. Mechanical vibrations are typically mitigated by appropriate experimental design and the use of vibration isolation platforms, while artefacts induced by cantilever illumination are often avoided by acquiring AFM and fluorescence images sequentially rather than simultaneously, thereby avoiding cantilever illumination altogether [6,7]. However, sequential data acquisition might not be appropriate for the study of dynamic processes or observation by one imaging technique might disturb the observation by the other one.

Typical AFM cantilevers are micron-sized beams that come in various shapes including rectangles and triangles and consist of Si_3N_4 or SiO_2 being attached to a silicon wafer, engineered to resonate at certain frequencies. Since both beam materials are oxides that hardly reflect light in the visible and near-infrared range, a thin layer of a metallic material is added to the beam to efficiently reflect the AFM laser onto a quadrant photodiode, as illustrated in Figure 1. Gold is the material of choice for this thin layer as it is known to be chemically stable in liquid environments. It should be noted that uncoated cantilevers are nonetheless utilized but they require substantially more powerful AFM lasers to compensate the lack of cantilever reflection of the AFM laser. Hence,

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they cannot be readily used in commercial AFM systems, even though they have found applications in e.g. sub-pN force measurements [8].

Timoshenko developed an analytical model for obtaining bending and heating of a bimorph structure under illumination based on a two-dimensional model [9]. Thermal heating of cantilevers by laser illumination has been studied shortly after the invention of the AFM [10,11]. In our previous work we studied AFM cantilever distortion induced by fluorescence excitation light on a combined AFM and Differential Spinning Disk hybrid microscope system [5]. In this work the effects of illuminating a Pyrex Nitride Probe-TRiangular (PNP-TR) type A cantilever and a qp-BioAC cantilever type CB2 were evaluated. It was found that fluorescence excitation light is capable to introduce cantilever bending disturbing AFM operation, especially for PNP-TR cantilever type where the gold layer spans the entire cantilever beam. Much smaller deflection values were obtained for qp-BioAC cantilever type whereby the gold coating only partly covers the AFM beam.

Here we report on local heating induced by cantilevers of various shapes and different types of illumination based on the results of a Finite Element Model (FEM) by linking optical and thermal simulations to study local heating. For the simulations we have considered cantilevers of triangular and rectangular shapes and different types of illumination applied in wide-field and confocal fluorescence microscopy.

Methods

Finite element analysis

FEM simulations have been executed within the Lumerical (Ansys Canada Ltd., Vancouver, BC, Canada) software suite using a desktop PC (Intel Xeon W3550 3.07 GHz 4 cores, 8 GB RAM 1333 MHz DDR3); optical and thermal FEM simulations have been respectively carried out by the Finite Difference Time Domain (FDTD) [12] and HEAT solvers [13] within the DEVICE suite for photonic multiphysics simulation. 2D (FDTD) and 3D (HEAT) models of a simulation region incorporating different AFM cantilevers have been defined in a Computer Aided Design (CAD) environment.

Hybrid microscopy set-up

Figure 1 represents an experimental configuration of the FEM study reported here: a cell immersed in a cell culture dish with a glass coverslip is observed with AFM and fluorescence microscopy in a single set-up. We are contemplating a temperature ideal for live-cell observations, maintained by placing the cell culture dish in thermal equilibrium at 37 °C using a Proportional–Integral–Derivative (PID) controller.

AFM cantilevers studied include Pyrex-Nitride Probe – Triangular Cantilevers (PNP-TR, NanoWorld® AG, Neuchâtel, Switzerland) and qp-BioAC (NANOSENSORS^{TM}, Neuchâtel, Switzerland). 2D models (represented in Figure 3) have been considered to reduce computational complexity - convergence tests have been carried out to validate results obtained from 2D simulations. A multi-coefficient model for accurate material modelling of cantilever constituents (Au, Si_3N_4, SiO_2) is applied.

Confocal-type illumination profiles have been simulated with a Gaussian beam source approximating a focused beam, while a broadband plane-wave source between 350 – 700 nm is used to simulate a fluorescence wide-field illumination pattern. Heat sources have been defined in the HEAT solver based on the illuminated fraction of the AFM cantilever gold coating, with a matching power derived from the total illumination power multiplied by the material absorption coefficient, as

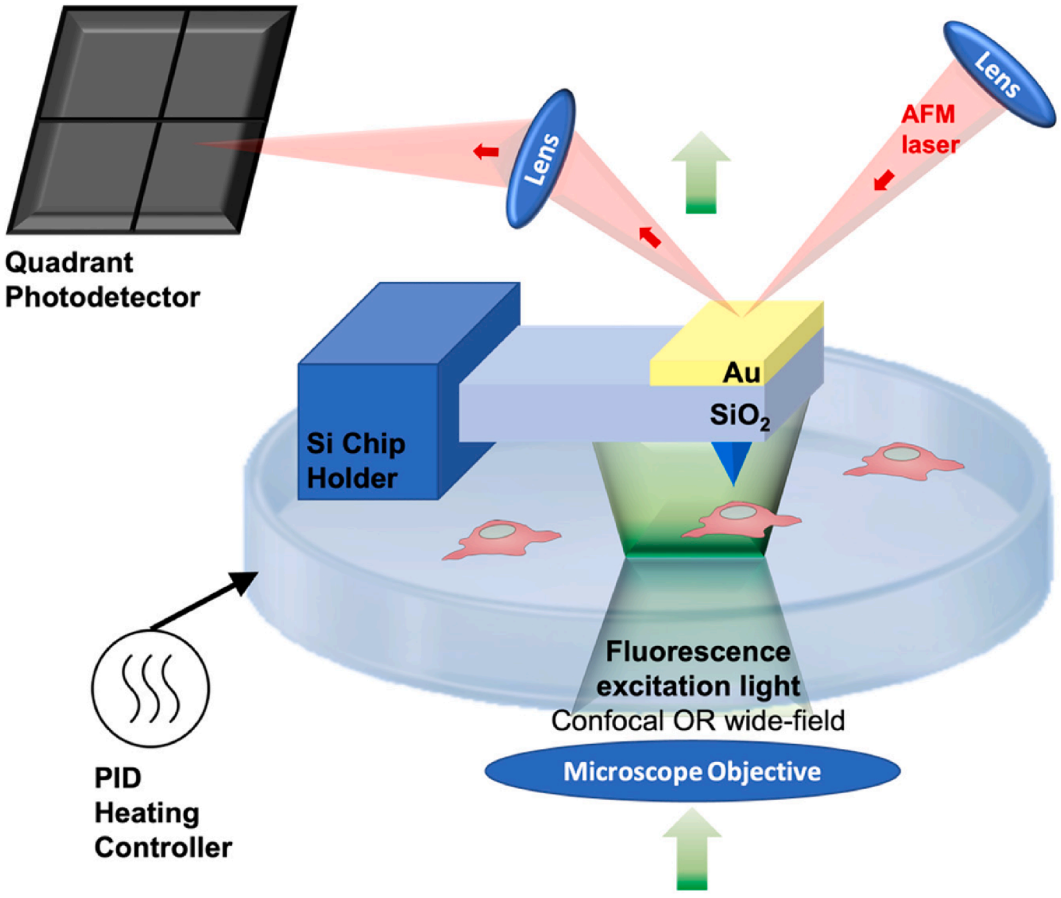

Figure 1. Conceptual configuration considered in this work: a hybrid AFM and fluorescence microscopy set-up for living cells immersed in a physiological buffer and temperature. The coverslip is in contact with a thermal heating element and can therefore be considered as a surface in thermal equilibrium at 37 °C.

obtained from the FDTD solver.

Simulation results

Here we are reporting heating induced by PNP-TR and qp-BioAC cantilevers illuminated in a confocal as well as wide-field fluorescence microscopy configuration whereby we consider the heating induced by the AFM laser negligible compared to the fluorescence illumination. In a first step, a FDTD model is applied to calculate the fraction of illumination power that gets absorbed by the gold (Au) layer of the AFM cantilever. The absorbed light power is then transferred to a thermal FEM whereby a thermal equilibrium surface is fixed between 1 and 9 μm from the cantilever. Computational memory limitations restrict the calculation of heating profiles induced with a thermal equilibrium distance < 10 μm away from the cantilever, i.e. consistent with thicknesses of adhered cells reported [14]. Note that we impose a steady state condition since we are interested in studying the system at a fixed operation point. Investigations of transient periods as, for instance, when the laser is switched off would require of a different FEM model where the initial conditions for both temperature and electric field at the confocal spot position should be established. When considering the impact of placing the thermal equilibrium further away from a PNP-TR tip undergoing confocal (left) or wide-field (right) fluorescence excitation illumination our results indicate that one achieves a saturation regime with a ~10 μm distance for the confocal illumination regime, while wide-field fluorescence excitation illumination has a tendency for further temperature rises as the thermal equilibrium distance d to the cantilever increases (Figure 2). This suggests that placing a thermal equilibrium barrier in close contact with the AFM cantilever is optimal to mitigate local temperature heating, something which however cannot always be maintained, especially when imaging the topography of adhered living cells by AFM.

Figure 3 details heating induced in PNP-TR (left) and qp-BioAC (right) cantilevers upon illumination in a wide-field configuration using a Differential Spinning Disk (DSD) fluorescence module utilizing a 60x oil immersion objective (60x NA1.4 Plan Apo VC DIC, Nikon, Japan) and an illumination wavelength band centred at 560 nm, chosen to allow for comparison with experimental results [5]. Exact surface area specifications of PNP-TR cantilever 1 (resonance frequency 67 kHz; force constant 0.32 N/m) were provided by the manufacturer (Nano-World® AG, Neuchâtel, Switzerland), resulted in a total area of 2332 μm^2, indicating we expect 0.3 mW impinging on the cantilever [5]. Optical FEM modelling indicates a total power absorption of 20 % at 560 nm as illustrated in Figure 2 b, indicating the gold layer of the PNP-TR cantilever 1 acts as a uniform heating source with 60.4 μW. Scaling the latter to the gold surface dimensions of the 2 cantilevers of the qp-BioAC chip results in a uniform heat source of 19.4 μW; since the gold surface area of CB1-3 cantilevers is very similar, FEM results do not vary significantly depending on the qp-BioAC cantilever chosen. Heating profiles only indicate a modest variation along the central cantilever beam axis, but the placement of the thermal equilibrium barrier plays a significant role in the temperature increase attained. We have reported earlier a temperature increase of 0.33 °C illuminating a PNP-TR cantilever at ambient temperature (20 °C) with ~175 μW 560 nm light, derived from an experimental observation of 9.1 nm cantilever deflection via the 2D analytical Timoshenko model [5,9]. We note that our more precise FEM predictions are congruent with these experimental observations.

Likewise to Figure 3, Figure 4 reports heating increases obtained for 1 mW 405 nm confocal fluorescence excitation light, a wavelength commonly applied in biological confocal microscopy and close to the local absorbed power fraction maximum observed in Figure 2 b. Simulation results indicate that focusing the confocal laser near the surface of the cantilever has a significant impact on the maximal temperature increase, implying that it is not trivial to observe fluorescence originating near the tip of an AFM cantilever beam without inducing significant heating in the volume where the fluorescence signal is recorded. We have modelled confocal image formation at different depths by setting up the confocal spot at different distances t below the cantilever of 0, 3.5 and 7 μm. To this end a Gaussian beam source was defined in the FDTD model with a beam profile of waist $w_0 = 125$ nm at position t below the cantilever. The injection plane is situated at 1 μm from the beam waist which corresponds to a divergence angle of 50.4 degrees. This implies that experiments aiming to observe AFM and fluorescence data from a single molecule simultaneously, are at augmented risk of excessive sample heating.

Figure 5 reports the variation of maximal temperature increases obtained along the central cantilever axis for different fluorescence excitation wavelengths for different confocal focusing distances t below the cantilever; the decrease in maximal temperature increases for increasing wavelength is in-line with the decreasing power absorption reported in Figure 2 b.

Discussion

Illuminating AFM cantilevers with a focused confocal laser beam of 1 mW, for example, at 405 nm, a wavelength commonly applied for the observation of the DAPI (4′,6-diamidino-2-phenylin-dole) DNA-specific fluorescent probe [15], leads to temperature increases in the range of ~5 – 15 °C depending on cantilever type (PNP-TR or qp-BioAC), focusing of the confocal laser beam below the AFM cantilever tip and thermal equilibrium distance. Henceforth, a dynamical process under investigation can be inadvertently modified through sample heating. Namely, research on the treatment of malignant diseases by administering heat in various ways, referred to as hyperthermia, has indicated that cell survival drops sharply above 43 °C to below 1 % for incubation

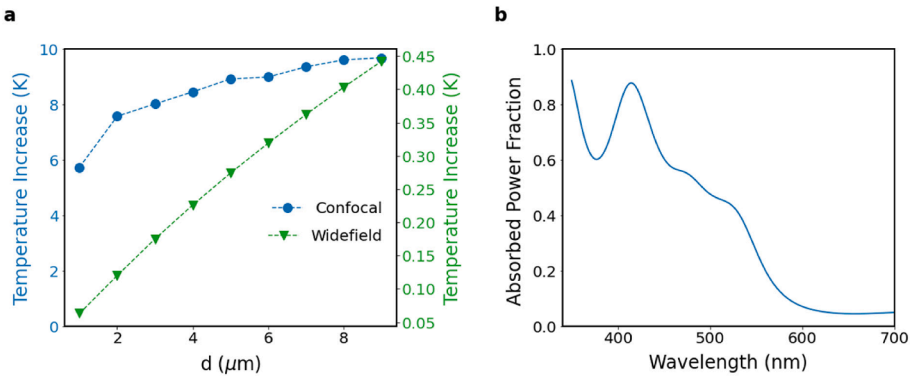

Figure 2. Temperature increase obtained by PNP-TR cantilever initially at thermal equilibrium of 37 °C after illumination with 1 mW 405 nm in confocal microscopy (left y-axis) or 0.3 mW 560 nm (right y-axis); (b) fraction of total power absorbed by the Au layer of the cantilever.

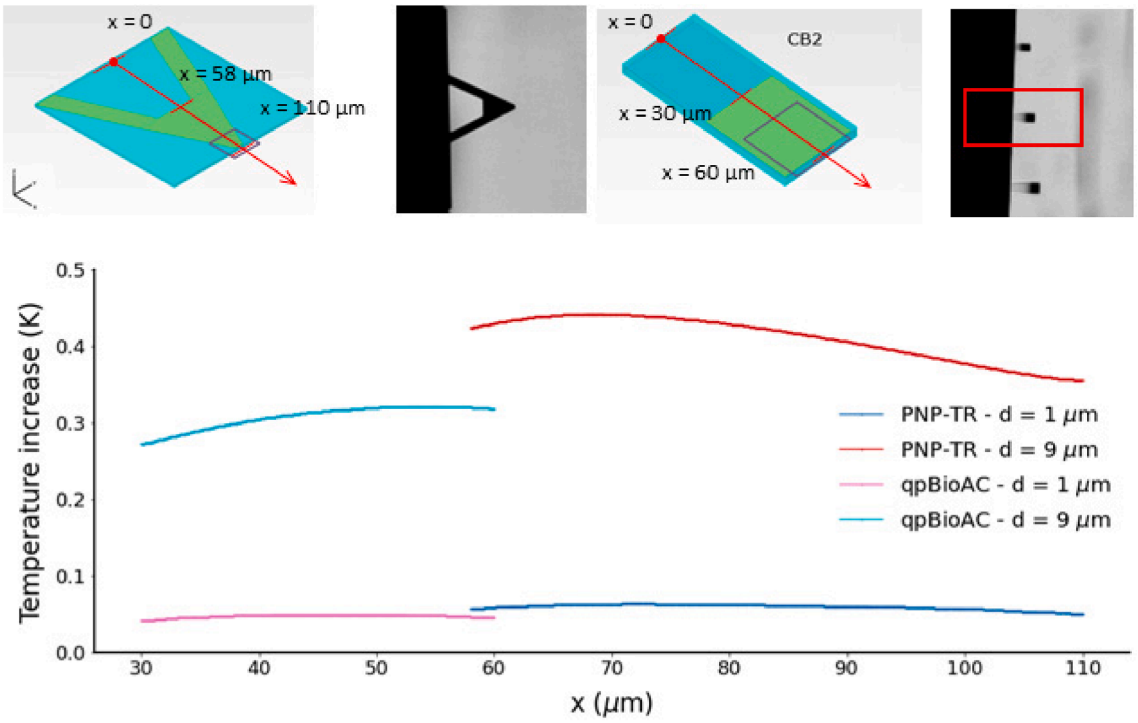

Figure 3. Temperature increase along the central cantilever beam axis induced by 0.3 mW 560 nm light through wide-field illumination on PNP-TR and CB2 qp-BioAC cantilevers. Results for d = 1 and 9 µm are represented.

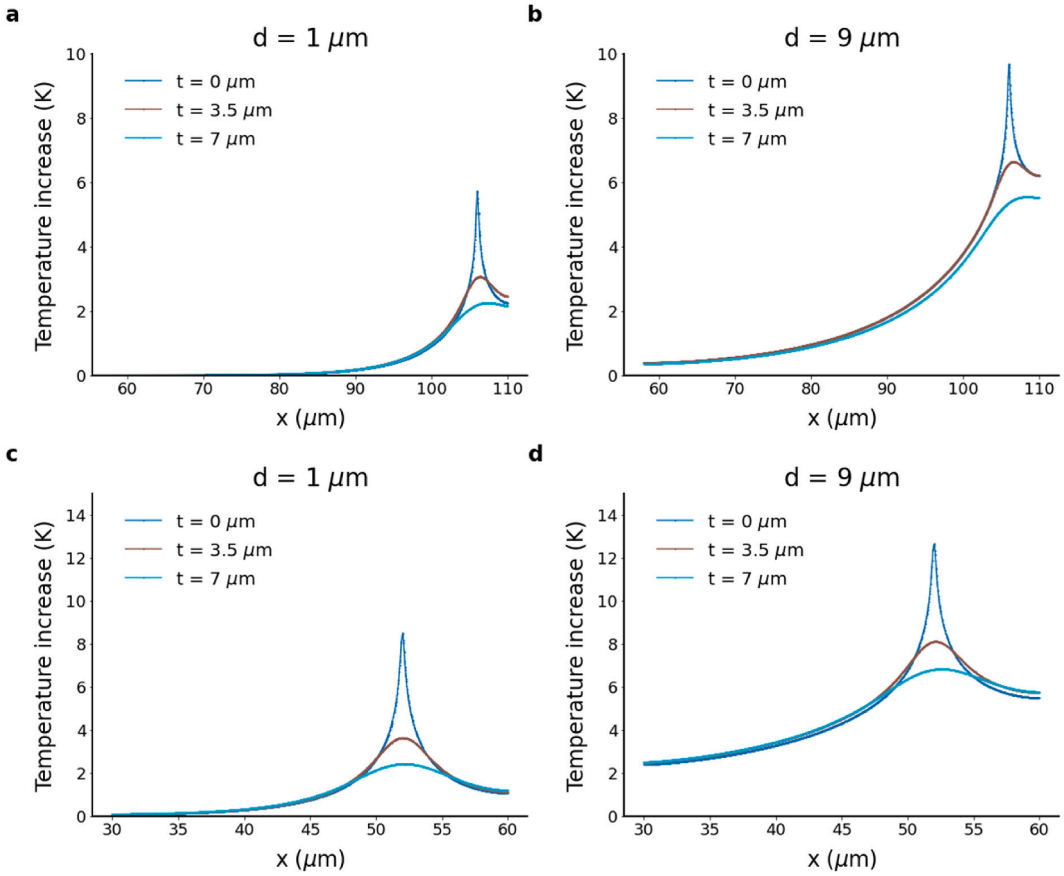

Figure 4. Temperature increase along the central cantilever beam axis induced by 1 mW 405 nm light through confocal illumination on PNP-TR (a,b) and CB2 qp-BioAC cantilevers (c,d). Results for d = 1 and 9 µm are displayed top and bottom respectively with each figure indicating heating for t = 0 µm, 3.5 µm and 7 µm. The focal centring of the spot is indicated by the blue rectangle in Figure 3.

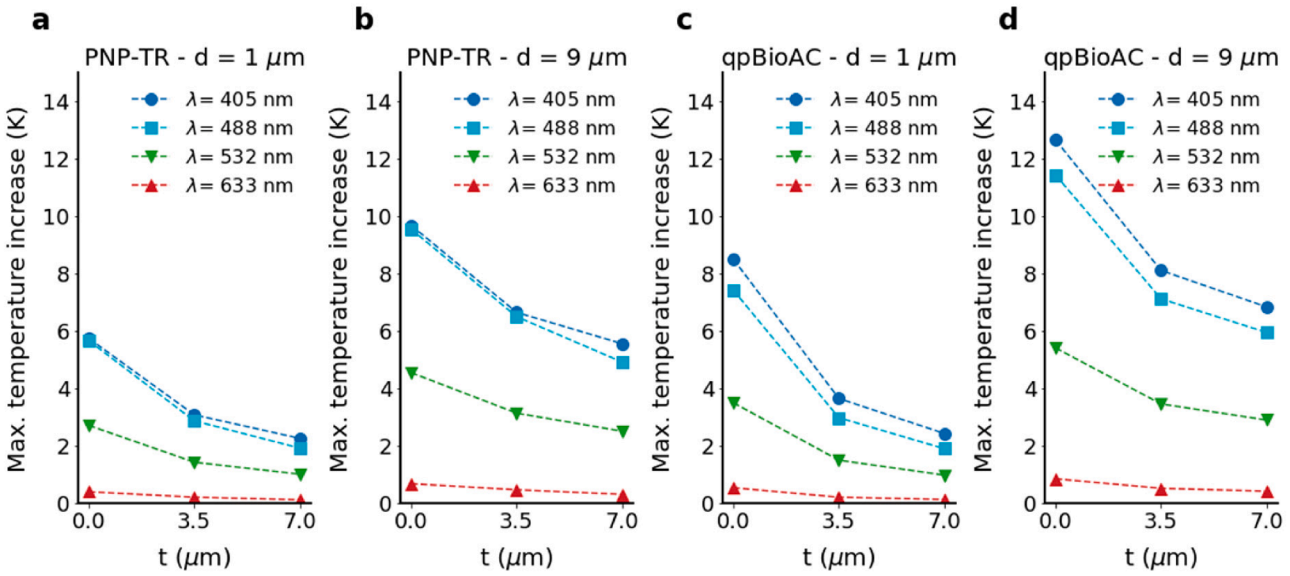

Figure 5. Maximal temperature increase observed along the central cantilever beam axis induced by 1 mW light of different wavelengths (405 nm, 488 nm, 532 nm and 633 nm) through confocal illumination on PNP-TR (a,b) and CB2 qp-BioAC (c,d) cantilevers. Results for d = 1 and 9 μm are reported with each figure indicating heating for t = 0 μm, 3.5 μm and 7 μm. The focal centring of the spot is indicated by the blue rectangle in Figure 3.

times > 100 min of FsaII cells [16]. Aside from cell death, hyperthermia has significant effects on proteins including unfolding, exposing hydrophobic groups and aggregation with proteins not directly altered by hyperthermia [17].

There are many hybrid AFM set-ups that expose AFM cantilevers to intense illumination aside from the AFM laser light. Examples include AFM/super-resolution fluorescence microscopy system integrations, in particular AFM combined with STimulated Emission Depletion (STED) [18] and Photo- Activated Light Microscopy (PALM) or equivalents thereof [19], where intense focused laser beams are typically required to induce emission depletion and fluorescence on/off switching respectively. Photothermal excitation of cantilevers represents another example where AFM cantilevers are exposed to intense (5 – 50 mW at ~650 nm) albeit sinusoidally modulated light beams [20,21], and has been applied for the study of single proteins [22]. A 50 mW beam sinusoidally modulated carries the same energy as a Continuous Wave (CW) beam of $\frac{\int_0^{2\pi} |\sin t| dt}{2\pi} * 50 = \frac{4}{2\pi} * 50 = 31.83$ mW. In our study, illuminating a CB1 cantilever of the qp-BioAC set with 31.83 mW at 633 nm would lead to a temperature increase of 16.2 °C (see Figure 5) considering linear extrapolation of the results obtained and a thermal equilibrium barrier 1 μm away. Should one place the thermal equilibrium barrier 9 μm away, this temperature increase would rise further to 29.6 °C. Clearly, due consideration must be made for potential heat damages to the samples one is investigating as is illustrated by the observation of selective unfolding and inactivation of proteins, referred to as molecular hyperthermia, when illuminating gold nanoparticles near proteins with nanosecond laser pulses [23].

Conclusion

Sample induced heating originating from AFM cantilevers illuminated with intense light beam depends strongly on illumination wavelength and configuration (wide-field vs confocal), AFM cantilever shape and materials, and, maybe surprisingly, distance of a thermal equilibrium barrier to the AFM cantilever. Since experimental parameters strongly depend on the type of set-up under consideration, we recommend performing bespoke FEM studies to evaluate if temperature increases can invoke undesired heat damage to the samples under investigation.

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CRediT authorship contribution statement

Frederico Tremoço: Investigation, Formal analysis. **Ana I. Gómez-Varela:** Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Adelaide Miranda:** Investigation, Writing – review & editing. **Martin Lopez-Garcia:** Investigation, Writing – review & editing. **Ana G. Silva:** Formal analysis, Writing – review & editing. **Pieter A. A. De Beule:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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