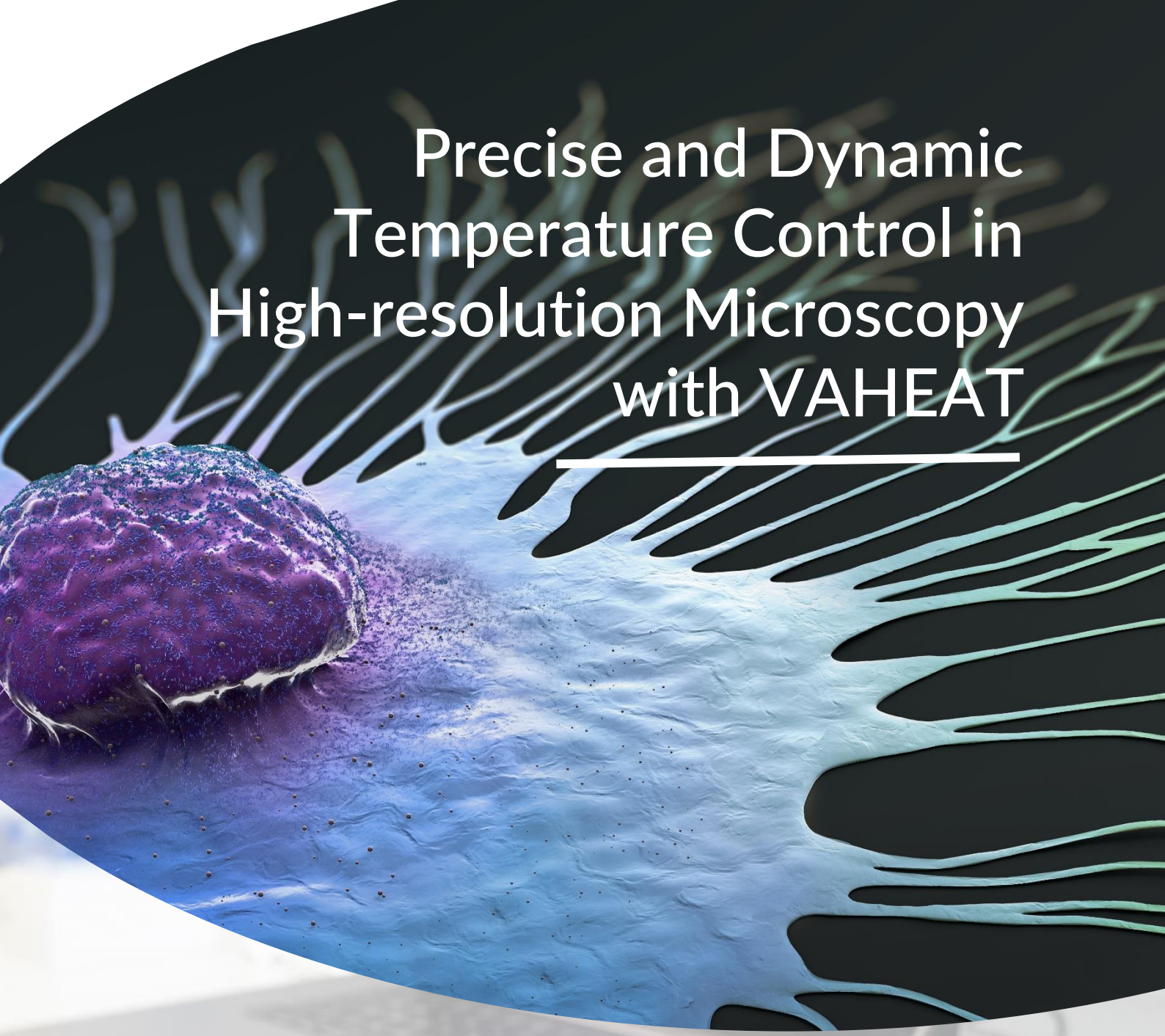
Precise and Dynamic Temperature Control in High-resolution Microscopy with VAHEAT



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INTERHERENCE

HIGH SENSITIVITY MICROSCOPY

Keywords

temperature control

live cell imaging

stage top incubator

temperature sensing

VAHEAT

Abstract

Temperature is a key parameter in most biological and physical systems. It is, however, an inherent technological challenge to precisely track and control the temperature of small sample volumes when using high- and super-resolution microscopes. Various effects can cause a deviation of the sample temperature from its expected value, specifically in the observed volume or field of view. These effects include direct thermal contact of the objective lens and the coverslip via immersion medium, illumination induced heating, evaporative cooling or triggered phase transitions. In this paper, we shed light on these phenomena and introduce our newly developed device VAHEAT, which follows a microscopic approach for controlling and measuring the sample temperature. Our system overcomes traditional limitations of temperature sensing and controlling in high sensitivity microscopy applications, leading to more reproducible experimental results and conquering new experimental parameter ranges.

Introduction

Many biological systems require physiological conditions in order to behave naturally or simply to survive during the experiment. Important environmental parameters include salt concentration, pH, atmosphere and temperature. In solution, salt concentration and pH can be easily adjusted and maintained on a macroscopic level either using perfusion or robust buffer systems. Atmospheric conditions can be set by changing the composition of the surrounding air with an incubation system. Temperature control, however, is a challenging task, especially when working with high resolution optical microscopes. The main reason for this is the sample volume, which typically ranges from a few to hundreds of microliters. The associated heat capacity (10 – 500 mJ/K) is four to five orders of magnitude smaller than the heat capacity of surrounding elements such as the microscope objective (approx. 5 – 15×10^4 mJ/K). The temperature of the sample volume is accordingly prone to every slight change in net energy input or output (e.g. illumination induced heating), any thermal link to its surrounding (e.g. objective lens), or internal energy releasing or consuming processes (e.g. phase transitions).

An often observed but unwanted change of temperature within the field of view (FOV) occurs when using high numerical aperture objectives that require an immersion medium. In this configuration, the immersion medium establishes a direct thermal link between the objective and the coverslip. The FOV, thus, adopts a temperature close to the objective's (see Figure 1a and 1b). The precise temperature is, however, an unknown even when working in a thermally stabilized enclosure or incubator as it depends on many experimental variables including the sample volume, the imaging modalities and the type of immersion medium. Additionally, other effects such as laser induced heating, evaporation or exchange of medium can also lead to a change of temperature by several degrees Celsius. This huge parameter space makes it nearly impossible to have precise knowledge about the temperature in the field of view without measuring and actively controlling it.

Conventional sample heating approaches build up on the belief that macroscopic heating of the entire microscope setup or at least of the objective in combination with the stage insert will ensure a certain temperature in the sample volume. These systems suffer, however, from slow equilibration, insufficient temperature precision, limited temperature range and bulky design. Furthermore, the large amount of heat transferred to the microscope can lead to mechanical drift and limits the range of applications. Prior calibration with an external temperature probe is often necessary as the built-in temperature probes are sensing close to but not within the sample volume. It turns out that even external calibration with a separate probe is challenging because most temperature probes that are available on the market exhibit similar heat capacities (typ. 10 – 30 mJ/K) as the sample volume. Inserting an external probe into the sample volume can thus substantially change the overall heat capacity and affect the read out temperature (neglecting the additional thermal link of the electrical wires and sensing current). Only a temperature sensor with negligible heat capacity and good thermal insulation from the surrounding would be capable of measuring the correct and unbiased sample temperature.

Here, we present our VAHEAT device, which is based on microscope coverslips incorporating an optically transparent thin film heating element made of indium tin oxide and a microfabricated platinum temperature probe (Smart Substrates).

The probe is less than 100 nm thick and adds a heat capacity of less than 130 nJ/K which is more than five orders of magnitude smaller than the typical heat capacity of a sample volume. Mounting of samples directly onto these coverslips restricts the heating to the sample volume and thermally decouples it from the environment. This local heating and temperature sensing enables fast and precise temperature changes with heating rates of up to 100˚C/s, precision higher than 0.1°C and the possibility to program arbitrary temperature profiles similar to PCR thermocyclers. The fast, feedback driven thermal response of our coverslips will counteract any external disturbances introduced by e.g., perfusion systems or changes in the room temperature. Very localized heating also reduces the overall heat load transferred into the setup and leads to tolerable warming up of the objective (Figure 1c and d) and minimal thermal drift. Overall, this technology has the potential to enable new types of temperature-sensitive experiments and to help increase the accuracy and reproducibility of imaging studies.

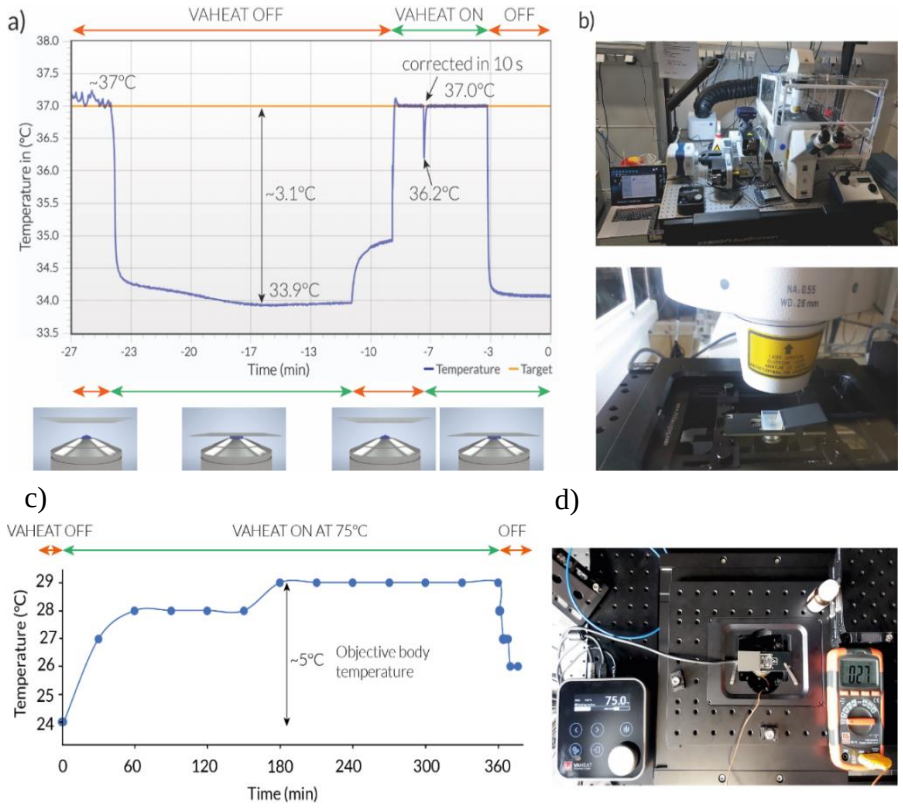

Figure 1: The microscope environmental chamber (PeCon) was equilibrated at 37°C for 4 hours prior to the experiment. The instrument was used first as a temperature probe. After the immersion oil objective (63×/1.4 Plan Apochromat) touched the Smart Substrate, the temperature decreased to 33.9°C. Removing the objective from the cover slip at t-12 min led to a slow temperature recovery. Switching heating ON at t-9 min led to immediate correction to 37°C. Repeating the experiment with heating ON led to only a 10 s dip in temperature and quick recovery to 37°C. b) Photo of the spinning disk confocal microscope at the Optical Imaging Centre Erlangen, where the experiment was performed. c) The VAHEAT device was used with a 100×/1.46 TIRF oil objective and was heated to 75°C while the temperature of the objective was measured. The objective warmed up by a maximum of 5°C and cooled down again within minutes after the heating was switched off. d) Photo of the experimental setup with external temperature probe measuring the temperature of the objective body.

Methods and Materials

The components

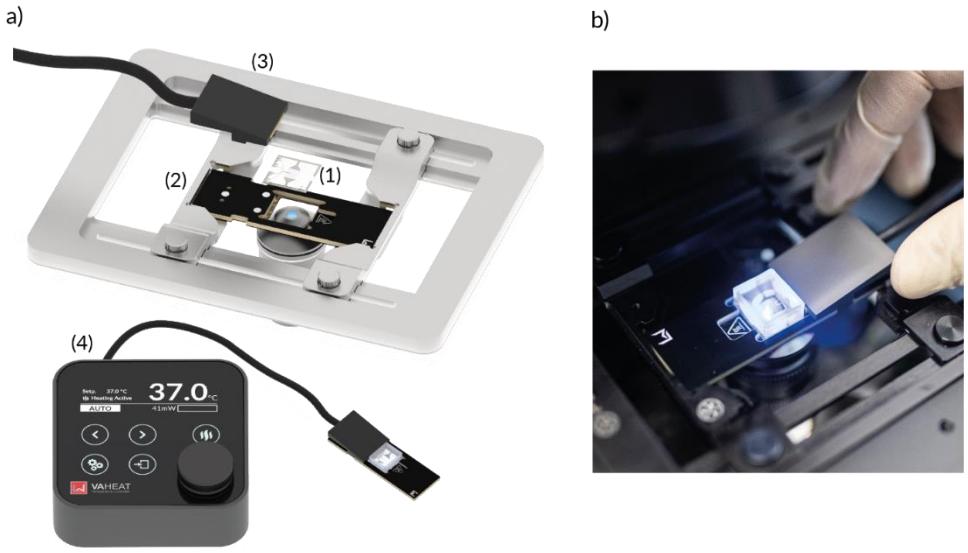

Figure 2: a) Rendering of the four instrument parts. (1) Smart Substrate, (2) microscope adapter, (3) probe head, (4) control unit. b) The instrument mounted on a microscope. The version of the Smart Substrate with reservoir for liquid samples is used.

The device comprises of four components, namely (1) the smart substrate, (2) the microscope holder, (3) the probe head and (4) the control unit as depicted in (Figure 2a). The core piece is the smart substrate, an exchangeable part, that incorporates a precise four-point temperature probe and a transparent, thin film heating element.

(1) Smart substrates

The Smart substrates are designed to replace the conventional coverslips in the experimental workflow and are made of borosilicate glass with ultra-low autofluorescence. With the dimensions of 18×18 mm and a thickness of 170 +/- 5 µm (#1.5H standard), they are designed for high-resolution imaging (Figure 3a). The temperature probe is made of platinum and is less than 100 nm thick. It is attached to the upper side of the substrate and covered by a thin layer of glass, such that it is in direct thermal contact with the sample but electrically isolated. The transparent heating element is made of a less than 50 nm thick layer of indium tin oxide (ITO) applied on the lower side. The ITO layer is 5×5 mm large to ensure a homogeneously heated sample volume in the FOV even when an oil-immersion objective is used for imaging (Figure 3b). The Smart Substrates are supplied in three different versions – standard range up to 100°C, extended range up to 200°C and a version with reservoirs for liquid samples (Figure 3c). With the possibility to attach custom-made microfluidics or other PDMS reservoirs (Figure 3d), the prototypes demonstrate the capability to promptly change experimental design using rapid prototyping and PDMS.

In an inverted microscope, the samples are imaged through the ITO layer (Figure 2b), which has a transmittance in the visible spectrum comparable to standard coverslips, resulting in a negligible impact on the imaging

quality. The temperature probe within the smart substrate measures the temperature 80 times per second and is connected through a PID controller to the heating element. This feedback allows to compensate for slow, as well as fast external temperature variation induced by e.g., air flow or fluid exchange. The temperature response of each substrate is characterized after manufacturing and the resulting two parameters R25 and TKR are supplied to the user, who must enter them before the measurement for high precision experiments. The smart substrates can be single-use but can be also washed by most of the conventional organic and water-based solvents and cleaned by sonication. The substrates withstand oxygen plasma cleaning (also applicable for bonding PDMS-based microfluidics) as well as thermal and UV sterilization methods but should not be autoclaved or exposed to low pH.

(2) Microscope adapter

The microscope adapter supports the smart substrates and electrically connects the heating element. Its footprint of 75×25 mm^2 and height of approx. 2 mm resembles a standard microscope slide ensuring broad compatibility with various microscope stages. It is best fixed on microscope stages with metal clips. It can be cleaned using the same protocols as for the Smart Substrates. The opening on the bottom side of the adapter with a size of 16×16 mm^2 ensures unhindered optical access with high numerical aperture objectives.

(3) Probe head

The probe head connects via a magnetic clamp mechanism to the microscope adapter. It holds the smart substrates in place and ensures the electrical connection of the substrates and adapter to the control unit. It is designed to allow optical access from top when working with upright microscopes or applying white light illumination in transmission.

(4) Control unit

The control unit allows to precisely set and read out the temperature of the sample. A computer interface can be used to synchronize temperature data with e.g., camera frames. The integrated turning knob enables real-time temperature control when studying in-situ temperature dynamics. The sample temperature will be displayed on the controller as soon as the smart substrate is positioned in the cavity of the microscope adapter and the probe head is connected. The control unit offers various heating modes namely ‘Auto’, ‘Shock’, ‘Direct’ and ‘Profile’. The conventional operational mode is ‘Auto’. Here, the temperature of the sample volume is automatically feedback controlled, compensating for environmental influences. The mode is activated by pressing ‘heat’ while the set point temperature can be adjusted using the turning knob. An application programming interface (API) is available to enable device control and parameter readout via a USB-interface. Other operating modes enable direct control of the heating power ‘direct’, to send well defined heat pulses ‘shock’ or to run more complex temperature protocols ‘profiles’.

Sample preparation

Depending on your needs, the sample is prepared on the substrate either before or after connecting it to the control unit. The latter allows to already set the correct temperature of the smart substrate before placing the sample. Cells can be seeded on the Smart substrates and they adhere normally, not showing preference for any part of the substrates. As the surface pointing towards the sample is made of pure glass, it can be functionalized or passivated by using established protocols.

Key features of the device

Local heating

The heat transferred into the system is kept to a minimum. This reduces thermal drifts, tensions and preserves the optical imaging quality.

Extended temperature range

Tune the temperature of your sample volume between ambient and 200˚C.

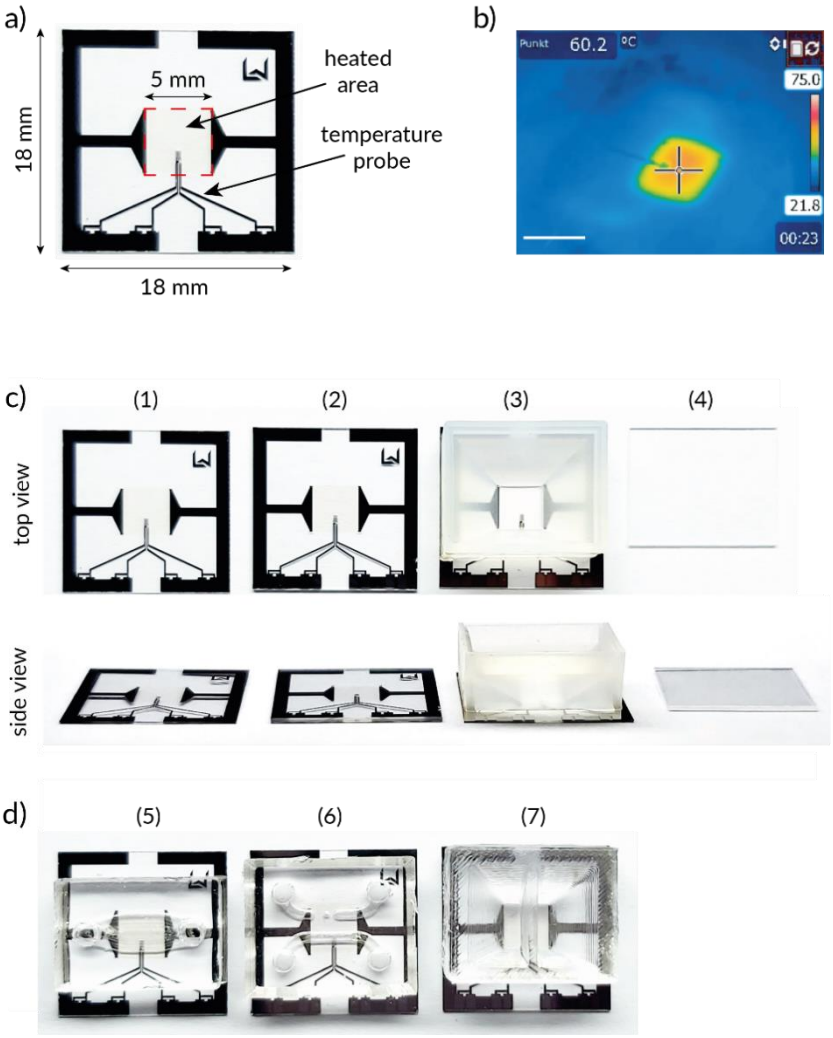

Figure 3: a) Smart Substrate with the ITO thin-film heating element and the temperature probe highlighted. b) Thermal camera image of substrate being heated while in contact with an oil-immersion objective. This demonstrates homogeneous heating of the FOV. Scale bar = 5 mm. c) Commercially available Smart Substrates. (1) Standard range up to 100°C, (2) extended range up to 200°C, (3) standard range with reservoir for up to 600 µl liquid samples, and (4) a glass cap to close the reservoir. d) Prototypes of (5) one-channel and (6) two-channel microfluidic chips and a (7) split reservoir with two 1.8 × 5 mm chambers for simultaneous observation of two samples.

Direct sensing

A precise and fast temperature sensor in the FOV measures the temperature 80 times per second. This allows to counteract external temperature changes coming e.g., from perfusion systems, strong illumination or air conditioning.

Fast dynamics

Heating rates of up to 100˚C/s and direct feedback allow precise temperature stabilization (Figure 4a and 4b). Well defined temperature profiles can be arbitrarily set.

Compatibility

VAHEAT is compatible with all conventional inverted microscopes and many upright setups. There is no need of prior preparation, installation or calibration. The system can be even employed in various incubation or vacuum chambers.

Analogue electronics

Both the heating and the sensing elements are all analogue devices to minimize the electronic noise in the sample volume. This opens up the possibility to combine VAHEAT with electronically sensitive measurement schemes such as atomic force microscopy.

Results

Thermal and optical performance

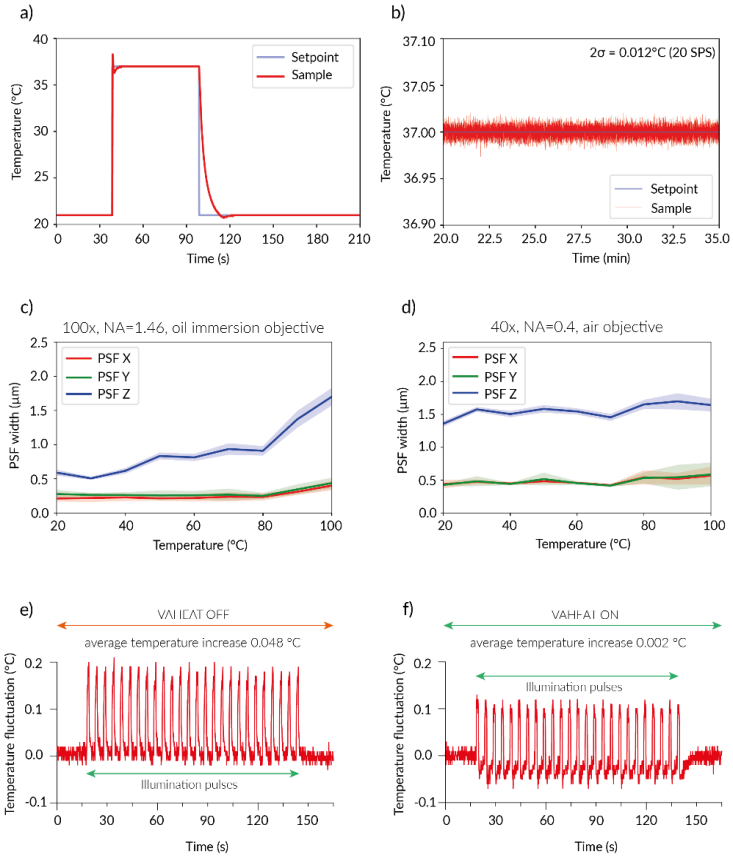

Figure 4: a) An example temperature recording from our instrument running a simple temperature profile with an empty Smart Substrate. The local heating and feedback mechanism enable well-controlled, fast temperature changes in the FOV. Heating rates up to 100°C/s are achievable for samples with small heat capacity, for example, thin films. For liquid samples, heating rates can reach 30°C/s. b) Excerpt from a longer measurement demonstrating the precision achievable with VAHEAT, which reaches down to 0.01°C (rms) over hours to days. c) Experimentally observed PSF of 100 nm green fluorescent beads recorded with an immersion objective (100×, 1.46 NA) and immersion oil. PSF elongation in axial direction is induced at higher temperatures mainly due to change of the refractive index of the immersion oil. d) PSF of beads imaged with a confocal microscope from room temperature to 100°C using VAHEAT and an air objective (40×, 0.4 NA). Spherical aberrations are minimal when working without immersion medium. Data were recorded at the Max Planck Institute for the Science of Light in Erlangen, Germany. e) Use of VAHEAT as a temperature probe to capture illumination-induced heating. An empty Smart Substrate was illuminated with a 1 s 488 nm laser pulse (at 50% laser power) 25 times with 4 s breaks. A 63×/1.4 objective was used for illumination. f) When the heating (feedback loop) is switched on, it compensates, and the resulting net overheating is close to 0°C. Experiment was performed in an environmental imaging chamber (PeCon) set to 37°C (heating OFF) and 38°C (heating ON).

We experimentally evaluated the temperature step response of our heating stage under standard operating conditions with no thermal load attached (Figure 4a). Changing the setpoint from room temperature to 37 °C yielded heating rates of up to 100 °C/s. This rate is strongly dependent on the thermal load (sample volume, objective etc.) attached to the smart substrates. Typical thermal loads of 400 µL water in combination with an immersion objective result in heating rates of up to 30°C/s. The maximal cooling rate depends on the thermal environment but can be as large as -10 °C/s. At typical temperature

of 37 °C, we were able to stabilize the sample temperature down to a two-sigma variation of 0.01°C with a sample rate of 20 Hz (Figure 4b) demonstrating the fast compensation of external thermal effects.

When performing high- or super-resolution microscopy, the optical performance of the imaging system is crucial for the quality of the obtainable data. Therefore, we measured the three-dimensional point-spread function (PSF) of 100 nm fluorescent beads embedded in a gel using a commercial microscope (Nikon Eclipse Ti-2). The temperature of the sample was set between 20°C and 100°C using our device. Figures 4c and 4d show the measured PSF when imaging with an immersion objective (1.46 NA) and an air objective (0.4 NA). We could show that the lateral PSF (x-y plane) stays diffraction limited up to 80 °C. Above that temperature, the lateral PSF increases most probably due to diffusion of the fluorescent beads. The axial component (z direction) of the PSF exhibits a characteristic elongation with temperature, when working with immersion oil (Figure 4c). Here, the effective optical path length changes with increasing temperature because the immersion oil has a non-negligible temperature dependence of its refractive index. This leads to a non-optimized optical path length and deteriorated optical resolution (spherical aberrations). Spherical aberrations at elevated temperatures can be minimized by choosing a special immersion oil or adjusting the correction ring on the objective. An alternative approach at the expense of a lower numerical aperture is to use dry (air) objectives (Figure 4d).

Applications requiring precise temperature adjustment and recording

There are various applications where precise temperature control is essential in optical microscopy. In the following, we discuss representative examples covering various scientific fields:

a) Setup calibration

Determining and controlling the sample temperature in a complex optical setup is not trivial. If absolute temperature reference and/or a precision better than 3°C is required, separate thermal calibration steps are typically necessary. Besides these static temperature variations, the illumination of your sample can cause local heating due to absorption by the sample or imaging medium (Figure 4e and 4f). Furthermore, triggered phase transitions, mixing enthalpies or injection of fluid into a microfluidic device can also lead to a change of temperature in the sample volume relative to the global setting. Highly sensitive probes inside the FOV are therefore essential to track and characterize those changes and, if needed, to trigger compensation with the heating element.

b) Live-cell imaging

It is of special importance to ensure a well-defined temperature when working with live cells (1). A drop or increase in temperature during observation can activate stress responses (2,3), change the properties of the cytoskeleton (4) and overall change cellular processes. Here, our device can be used to ensure a constant temperature in the sample volume nearly regardless of the environmental conditions during imaging and also while manipulating the samples. Here, we provide examples of live-cell imaging in mammalian cells at 37°C to visualize lysosomes (Figure 5a) or cytoskeleton (Figure 5b) using our device. Besides static temperature control, our device can be used to study temperature dependencies of cellular behavior covering a large dynamic range from room temperature up to 100˚C. It is particularly suitable for inducing heat shock and simultaneous imaging of the sample. Such approach was used for studying meiosis with temperature sensitive mutants in yeast (5). Other examples include calcium imaging in heat-sensitive neurons (unpublished) or time lapse imaging of thermophilic microorganisms

(6). Our device has been quickly adopted by the community of microbiologists studying the cell biology of Archaea, which is a group of organisms containing many extremophiles that are notoriously hard to image live (7).

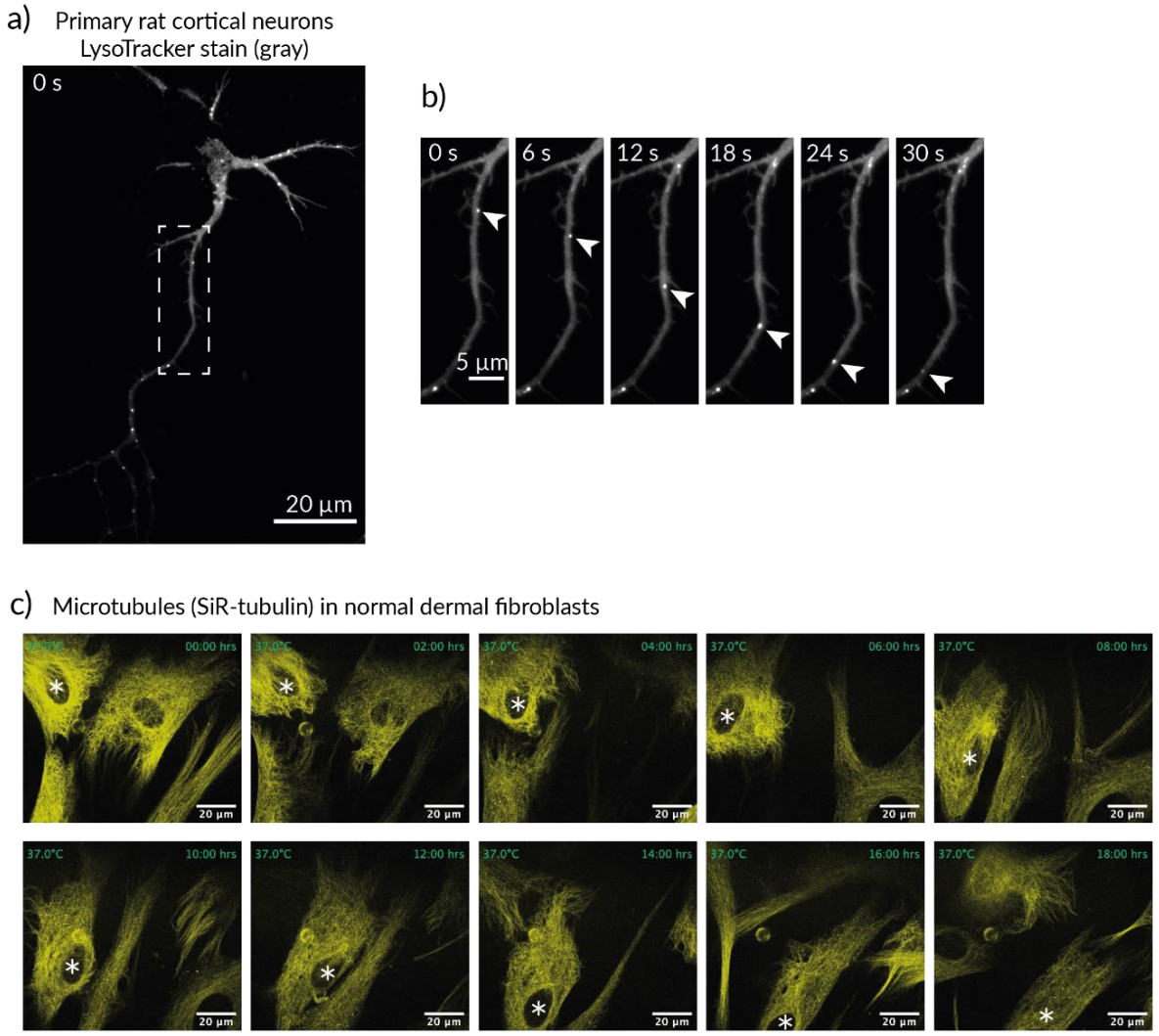

Figure 5: a) Primary rat cortical neurons were cultured on poly-D-lysine-coated Smart Substrates and stained with LysoTracker™ Green (ThermoFisher), which stains acidic compartments in the cell. The neurons were imaged on a TIRF microscope for 2 min every 0.5 s using the Leica PL APO 100×/1.47 NA oil objective and ORCA-Flash 4.0 V3 CMOS camera (Hamamatsu). Temperature was maintained at 37°C by our instrument, and cells were incubated in 5% CO2 humidified atmosphere. b) Enlarged area from the box in a) showing tracking of vesicle movement in a neurite at selected time points from the movie. c) Long-term live-cell imaging of normal dermal fibroblasts (NDHF) that were stained with SiR-tubulin (Spirochrome). The cells were incubated at 37°C using the Smart Substrate with reservoir and were continuously imaged for more than 18 hours. Images were acquired at the Optical Imaging Centre Erlangen.

c) Phase transitions

Phase transitions are an important subject of research in material and increasingly also in life sciences. Dynamic and precise temperature control is beneficial as it allows to steer, trigger and study crystallization or liquid-liquid phase separation in situ. Here, the following topics are of current interest: Liquid-liquid phase separation of proteins and RNA in cells is speculated to be one of the driving mechanisms behind compartmentalization of cells and maintenance of non-membranous organelles in the cytoplasm such as P-granules and stress granules or compartments in the nucleus such as nucleoli or sites of active transcription (8). The appearance or dissolution of these structures is typically sensitive to temperature and is best studied by high resolution microscopy (9). Using our device would allow to obtain the whole phase diagram easily.

Biological membranes are composed of many different constituents and exhibit a complex phase diagram depending on composition and environmental conditions. Measuring the dynamics of lipid membranes on a microscopic level is a key component for understanding the behavior of transmembrane proteins, membrane organelles and ultimately the complex systems such as cells.

Temperature control close to the melting point of organic and inorganic substances plays an important role in material analysis, sample preparation and quality control. Our device is ideally suited to study for example the melting point of micro crystals, polymer films or to steer the self-assembly and crystallization behavior of colloids (10).

d) Single Molecule Imaging and DNA science

Biological molecules such as proteins are highly sensitive to temperature in their function, interactions or aggregation (11). Especially, when tracking single molecules and studying their interactions it becomes important to have precise knowledge and control of the sample temperature. In one such study our device was used to characterize a macromolecular transport system made out of DNA origami (12). Precise temperature control in these single-molecule TIRF measurements was important for reproducibility.

Hybridization of DNA strands is temperature sensitive. Typical melting temperatures range between 50°C and 90°C depending on the number and composition of base pairs. This temperature regime is usually not accessible with conventional heating systems for high-resolution microscopes and opens up new experimental options when imaging DNA based systems. Especially in context of spatially resolved transcriptomics, temperature dependent binding and unbinding rates can be exploited to increase the throughput when investigating large samples with methods such as MERFISH (13). Also, in DNA PAINT the dissociation rates are considerably influenced by changes in temperature by only a few degrees Celsius (14).

e) Diffusion processes

Diffusion coefficients strongly depend on temperature. A branch of current research deals with the diffusional behavior of single nanoparticles in complex systems. A well-controlled temperature in the FOV is markedly important in order to extract and model system relevant parameters. In a recent preprint, (15) developed a method based on iSCAT microscopy to evaluate complex mixtures of nanoparticles. Our device was used to characterize temperature dependence of diffusion constant of gold nanoparticles.

f) Nanophotonic structures

Temperature control of nanophotonic structures, such as gold nanoparticles or photonic crystals, is useful to tune their resonance frequency or even change their shape (16,17). Local instead of global

temperature control increases the achievable temperature range, long-term temperature stability, enables fast resonance tuning and allows even adoption in vacuum chambers.

Discussion

Designing a general-purpose temperature control system that is suited for imaging large (e.g., multi-well plates) as well as small sample volumes (e.g., single wells) proves to be impossible. Macroscopic approaches for heating and sensing the temperature might be suited for some experiments but only allow for slow heating rates, limited temperature range and low precision. On the other hand, in high- and super-resolution microscopy, the researcher investigates small sample volumes with a distinct local temperature that can substantially deviate from the one set globally. Miniaturizing the sensing and heating circuitry as well as restricting it to the sample volume brings precision, speed and an extended temperature range.

Although our system follows the miniaturization approach, it still faces minor restrictions in its current implementation. For example, it does not take care of gas or humidity control. However, our device can be combined with many commercial or custom-made incubators. The working principle of heating and temperature control is independent of the present atmosphere and also works in vacuum, which is relevant for many single molecule experiments. Another limitation is that the lid for the reservoirs is not heated. This can result in condensation and thus lead to a disturbed illumination profile in transmission. A simple but powerful strategy to avoid condensation is adding mineral oil on top of the sample volume, an approach common in mammalian embryo cultivation.

Other strategy employed by some users is enclosing the sample in a glass capillary placed on the substrate. Yet another method to avoid evaporation and condensation is to use microfluidic chip, with which the substrates are well compatible. However, having no lid as an essential component of the system brings the advantage of easy access for manipulating the sample during experiments.

Some experiments require cooling the samples below ambient temperature. At this point our system does not have active cooling capability, however due to the small heated volume, the passive cooling from maximal back to ambient temperature takes only seconds to minutes depending on the sample volume. To reach below ambient temperature, this device can be combined with a cooled enclosure around the microscope adapter.

Lastly, the heated area on the substrates is limited to 5×5 mm^2, which can be small for some large samples and microfluidic chips. This design is however motivated by maintaining maximum precision and temperature homogeneity across the whole heated area. It is straightforward to manufacture substrates with larger heated area e.g., 13×10 mm for applications, which do not require higher than 0.1°C absolute precision. Due to the modular design of the device, independent upgrades to the substrates, microscope adapter, probe head or the control unit can be made in the future. It will be straightforward to accommodate requirements of researchers and new experimental method

Conclusions

Measuring and controlling the temperature of small sample volumes during imaging is non-trivial, as the sample heat capacity is minuscule compared to the one of the measurement apparatus. We showed that introducing a microscopic temperature probe on a coverslip with negligible heat capacity enables for the first time to track the sample temperature during experimentation with high precision. Combining this temperature probe with a transparent thin film heating

element (smart substrate) results in a potent heating stage, which is easy to use and is well suited for a large variety of temperature sensitive imaging studies. The fast feedback loop between temperature sensing and local heating ensures temperature stability better than 0.1 °C and allows programming of complex temperature profiles similar to a thermal cycler. Using standard sized coverslips and a microscope adapter ensures compatibility with most of the inverted and many upright light microscopes. This system opens up new possibilities for designing experiments in high-resolution microscopy starting in biology and reaching out to chemistry and material science, wherever precise temperature control is crucial for reproducible and physiologically meaningful results.

Acknowledgements

We would like to thank the Optical Imaging Center (OICE) of the Friedrich Alexander University Erlangen- Nuremberg (FAU) and the Max Planck Institute for the Science of Light (MPL) in Erlangen for extensive discussions and their help with various experiments

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