Optical Measurement of Temperature in Tissue Culture Surfaces under Infrared Laser Light Excitation at 800nm using a Fluorescent Dye

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Abstract: The use of infrared laser light (IRLL) for biomedical applications has gained momentum the potential applications in humans. The use of IRLL presents some limitations due to the dangerousness of these radiations when exceeding some safety thresholds depending on the target tissue. This position paper describes step by step a well-known technical method usually applied for microfluidics but here applied for the first time to measure the temperature and the heat evolution in a cell culture environment under IRLL excitation at 800 nm. The measurement of temperature is based on the property of Rhodamine B (RhB), a fluorescent dye whose fluorescence intensity decreases linearly with temperature increases, illustrated by preliminary microscopic measurements of temperature in cell culture dishes containing RhB solution under the IRLL excitation from 0 mW to 300 mW.

1 INTRODUCTION

Infrared laser light (IRLL) in the near infrared range (NIR) has become an indispensable tool for biological studies with multiple applications, including multiphoton microscopy (Hoover and Squier, 2013), photodynamic therapy (Li et al., 2009) and neural stimulation (Richter and Tan, 2014). A recurring question often appears in the context of IRLL excitation of biological targets (Welch 1984): what is the potential impact of an IRLL excitation on tissue health with respect to hyperthermic stress, cell or DNA damage? This issue is particularly important in the case of a tool dedicated to a clinical use on humans. The evaluation of the short term or long term effects of IRLL excitation on tissues is a complex problem, including a large number of parameters to consider: excitation parameters (average power, wavelength, repetition rate, duration of exposition and power or energy density). The first consequence of IRLL excitation to be considered is the modification of local temperature in the exposed tissue and the range of temperature increase obtained (Liljemaln et al., 2013).

The effects of different exogenous heating sources, such as the IRLL excitation, have been investigated with wide variety of microscopy techniques for mapping temperature at the single-cell level (Baffou et al., 2014). Among them, several were applied to cultured cell lines, as in the Fluorescence-lifetime microscopy based cellular measurements of temperature (Shang et al., 2013). Fluorescence lifetime depends on other parameters like the pH, the viscosity or the solvent of the target making this method uncertain for temperature measurement above a limit of 37°C (Okabe et al., 2012). The anisotropy of the fluorescence from green fluorescent protein (GFP) has also been used for intracellular temperature mapping in cultured cells exposed to focal and bulk liquid heating (Donner et al., 2012). This technique is highly promising, but requires the use of established cell lines stably expressing fluorescent proteins, making it unsuitable for neurons or primary tissue cultures that are difficult to transfect. Fluorescent quantum dot sensors have also been used for intracellular temperature measurements induced by external heat sources in a single living cell (Martinez Maestro et al., 2010) or the Er^{3+}-doped nanoparticle spectrum (Vetrone, et al. 2010). Other methods include use of the temperature sensitivity of the fluorescence spectra of the rare earth EuTTA fluorescence spectrum (Zohar et al. 1998) or the

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doped silica nanoparticles containing these substances (Yang et al. 2014). Despite the nanometric size of these particles, their application are hindered by their repartition in the sample and differential uptake into organelles and membranes, perturbing their biochemical properties. The pioneering studies using NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) and Laurdan showed the potential of small organic fluorescent probes as optical thermometers in living cells (Chapman et al., 1995). Indeed, it was shown that NBD fluorescence lifetime recordings were suitable for monitoring ultrafast phenomena as the impact of short pulse microirradiation on single cells. Unfortunately the temperature resolution provided by this method was approximately 2°C.

Rhodamine B (RhB), is a fluorescent, water soluble dye, with an absorption peak at 554 nm, an emission peak at 576 nm, and a temperature dependent fluorescence quantum yield (Ferguson et al., 1973). This quantum yield decreases linearly with an increase in temperature (Kubin et al., 1982). When the temperature is reduced, the value of the quantum yield of spontaneous luminescence sharply increases and makes certain that any possible negative effects due to intermolecular relaxation processes are overridden (Ali et al., 1990). RhB is highly temperature sensitive, negligibly pressure sensitive, and nominally pH independent above a pH of 6 (Shah et al., 2009), and has been used to measure the temperature in a wide variety of fluidic applications (Shah et al., 2007; Gui et al., 2008; Ross et al., 2002; Low et al., 2008; Sakakibura et al., 1999).

Interestingly, RhB has been also used as a mitochondrial probe for measuring mitochondrial membrane potential in cells (Reungpatthanaphong et al., 2003). This work suggested that RhB can distribute across biological membranes in response to the transmembrane potential. This property lead us to think that RhB dye could be useful in making temperature measurements on small biological compartments at the subcellular level. The sensitivity of this technique was shown in the measurement of temperature in rat tail tendon samples exposed to radiofrequency electromagnetic fields (Chen et al., 2009), and used to rule out any mean temperature increases in cells exposed to nanosecond pulsed electromagnetic fields (Kohler et al., 2013).

In this position paper, we present preliminary work describing the method for the temperature measurement in microscopic environment relevant to the culture of biological cells under IRLL excitation at 800 nm using the temperature-sensitive fluorescent dye RhB. The calibration procedure is detailed and an application for the measurement of temperature of a cell culture surface containing RhB under an IRLL excitation at 800 nm from a Titanium-Sapphire laser delivered through an optical fibre is shown.

2 EXPERIMENTAL SETUP

The experimental setup is composed of two distinct parts located in a temperature experimental room at 23.0°C. The global setup is presented in Figure 1.

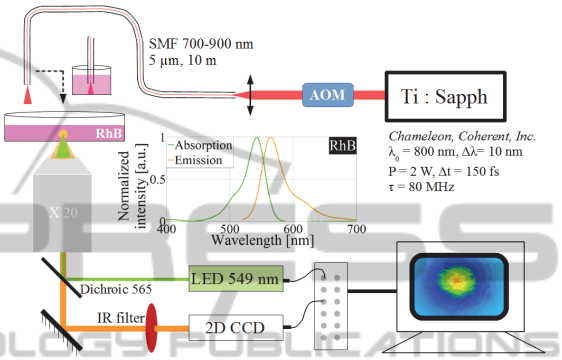

Figure 1: Setup schematic for temperature measurements of a RhB solution after heating with IRLL excitation from a commercial Titan Sapphire laser system.

2.1 Setup for RhB Level of Fluorescence Measurement

The local heating of the solution of RhB was tested with a concentration of 50 µmol/L. The whole experimental setup was placed inside a wide field fluorescent microscope coupled with a light-emitting diode (LED)-based light engine (Spectra7, Lumencorp) and a combination of dichroics and emission filters (Chroma or Semrock). The light from the LED at 549 nm with 50 mW of average power was focused at the surface of a petrie dish containing RhB solution by a 20x microscope objective. The backward emitted fluorescence from RhB was collected in epifluorescent detection, with an electron multiplied CCD with 512x512 pixels registered at 16 bits precision (Evolve 512, Photometrics). Both the LED and CCD were controlled and synchronized by a computer using freely available software (Winfluor, Strathclyde University). The LED excitation and the resulting RhB fluorescence emission were thus measured at a frame rate of 0.198 s.

2.2 Conversion of the RhB Fluorescence Intensity into Temperature

The sensitivity of RhB fluorescence in measuring temperature was previously shown to be 0.2°C (Kohler et al., 2013) using two calibration steps. The first calibration was made using a fibre optic thermometer (Luxtron) with analogue voltage output that was sampled with an analogue to digital board (USB-6229-BNC, National Instruments) interfaced with Winfluor, measuring the temperature of a dish of water progressively heated with a metallic ring containing heating resistance (Warner Instruments). This step evaluated the linear relation between the temperature of the target, measured with the thermometer, and the voltage output of the fibre thermometer, as read by Winfluor. Figure 2a presents the evolution of the water temperature in the dish and the voltage recorded by software. This defined the calibration curve of the thermometer. Next, the petri dish was filled with 2.5 ml of RhB solution and its temperature was manipulated by the heating resistance chamber. For each steps of 0.198 s when the RhB solution temperature increased, the fluorescence level was recorded. Figure 2b shows the evolution of the temperature as measured with the level of fluorescence detected by the CCD pixels with a grayscale encoded on 16 bits.

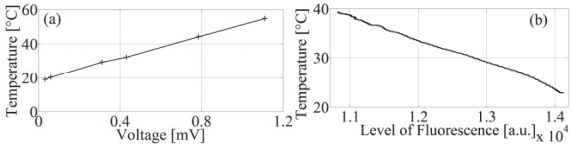

Figure 2: a. Calibration of temperature measured by the fibered thermometer as a function of its voltage measured by Winfluor. b. Calibration of the temperature as a function of the level of RhB fluorescence encoded in a grayscale on 16 bits measured by the CCD interfaced with Winfluor.

As expected, the level of RhB fluorescence decreased linearly with the increase of the temperature of the RhB solution. All the further results of measurements will be expressed using this conversion of fluorescence to temperature.

2.3 Infrared Laser Light Excitation

The focal excitation of temperature in the RhB solution was realized by a femtosecond infrared Titan-Sapphire (Ti: Sapph) laser. As this system is very bulky, the IRLL was injected into a 10-m-long fibre deporting the laser source wherever required in the experimental room. The single mode fibre (SMF) from Fibercore (reference SM750) was chosen for delivering the IRLL with a mode field diameter of 5 µm and a numerical aperture (NA) equal to 0.12. Figure 1 shows the whole experimental setup.

The IRLL came from a Ti: Sapp laser (Chameleon, Coherent, INC.) delivering 140 fs pulses at 800 nm, 10 nm of spectral bandwidth at the full width half maximum (FWHM) at the repetition rate of 80 MHz. Before the SMF input, an acousto-optical modulator was inserted (Figure 1), allowing the rapid modulation of average power before the IRLL injection into the SMF. At the SMF output, the maximum of average power stabilized at around 300 mW. After the propagation through a 10-m-long fibre, the spectrum was broadened from 10 nm FWHM to 100 nm FWHM resulting from nonlinear effects and the pulse duration was increased from 140 fs to 10 ps due to dispersion effects. In the first instance, in the context of the measurement of temperature evolution of a solution of RhB, the femtosecond properties of the pulses were not a critical point in this experiment, although it might be relevant and pursued in future experiments on biological sample in the condition of imaging with a two photon microscope.

3 RESULTS AND DISCUSSION

The optical fibre was placed 1 mm above the bottom of the petri dish containing the RhB solution. Three series of experiments were performed to illustrate the use of RhB as a fluorescent dye allowing the temperature measurement of a solution in response to IRLL heating. The first experiment was carried out with a continuous IRLL excitation. Next, the effect of a periodic, intermittent IRLL excitation on RhB fluorescence was investigated. Finally, the evaluation of the temperature distribution of the RhB solution in 2D was studied in the cone of IRLL from the optical fibre.

3.1 First Experimental Determination of the Temperature Increase of a Solution of RhB under a Permanent IRLL Excitation

For the first set of experiments, the RhB fluorescence was recorded on an area containing the entire region illuminated by the IRLL. Figure 3a represents the position of the optical fibre realized when delivering few microwatts of IRLL at 800 nm, insufficient to changing the RhB solution fluorescence. The

recorded image is shown in pseudo-colour without the IR filter (Figure 1) in front of the CCD. This step was dedicated to the fibre positioning in the field of view of the CCD camera and the physical delimitation of the ROI and selection of fluorescence measurements by Winfluor. No LED excitation was used to achieve this picture.

The objective of this part was to determine the averaging of temperature in this ROI, resulting from the continuous IRLL excitation at 800 nm over a total duration of one min. This first experiment was done with an average power of IRLL excitation of 200 mW at the fibre output. Figure 3b shows the resulting temperature measurement, determined after the conversion of fluorescence to temperature, as previously described.

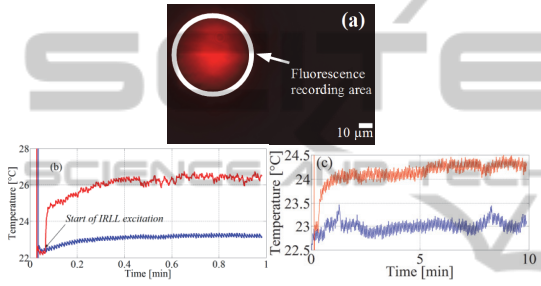

Figure 3: a. Localisation of the optical fibre and ROI delimitation of the measured level of fluorescence under 200 mW of IRLL excitation. b. Resulting temperature measured in the ROI, blue: temperature measured without excitation, red: temperature measured under the IRLL excitation. c. Temperature measured in the ROI with 100 mW of IRLL excitation using exposure time of 10 min.

First, to compare the local heating resulting from the IRLL excitation with a reference, we have measured the temperature in the ROI without IRLL (Figure 3b, 3c blue curves). Then, the temperature of the ROI under IRLL excitation was measured and is shown on the same graph representing the temperature measurement without IRLL. The fluorescence measurements, switching on the LED at 549 nm, were started after 2 s of recording, so as not to miss a starting event. Then, the IRLL excitation thought the optical fibre was started 3 s after the start of fluorescence acquisition.

When the IRLL was started, the modification of temperature was immediately visible and characterized by the strong increase in the red curve compared to the blue one (Figure 3b, 3c). In Figure 3b, the rapid deviation in the curve represents a temperature increase of 1.7°C with a rise time less than the 0.192 s between each point of the measure. In the next seconds and until the half of the measure, the local temperature increases until asymptoting to 26.3°C. The temperature was then stabilized at 26.5°C until the end of the recording. The decline of the increase in temperature reveals the presence of a steady state reached, illustrated also with the Figure 3c with an illumination duration of excitation of 10 and 20 min, respectively. The limitation in temperature increase presumably results from the large volume of RhB solution of 2.5 mL in the petri dish, compared to the small volume under IRLL excitation. By a simple calculation considering the optical fibre NA, the index of the medium equal to 1.33 and the distance of 1 mm between the fibre tip and the bottom of the dish, only $8.6.10^{-6}$mL of the RhB solution was heated by IRLL excitation which represents less than 0.01% of the total volume. Therefore, an averaging of the temperature of the ROI, by temperature exchanges with the whole solution of RhB, are limiting focal elevation in temperature. Further, the constancy in the temperature especially in the case of the 10 min of IRLL excitation, suggests that there is not a significant photobleaching effect in the limit of the duration of the measurement.

3.2 RhB Fluorescence Characterization under an Alternative IRLL Excitation

The objective was to investigate the possible effects of an intermittent IRLL excitation compared to a permanent illumination. The experimental protocol implements three phases of 1 min of fluorescence recording in the same ROI as the one defined in Figure 3a. During the first phase of 1 min, the RhB fluorescence without IRLL was recorded in order to have a reference value. Next, the RhB fluorescence was measured under IRLL excitation with an average power of 150 mW. Finally, the influence of alternating between IRLL excitation at 150 mW and switching off was studied.

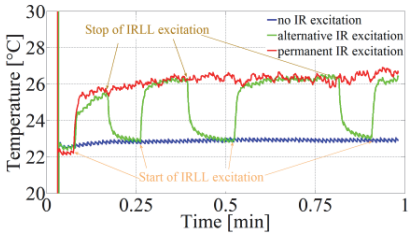

Figure 4: Intermittent IRLL excitation in the RhB fluorescence. Blue line: without IRLL. Red line: constant IRLL excitation. Green line: intermittent IRLL excitation.

The points of start and stop of the IRLL excitation are explicitly identified. Globally, during the intermittent

IRLL excitation on and off (Figure 4 green curve), the RhB solution was excited with IRLL for a total of 38 s. Different durations of IRLL excitation were applied from 5 s until 18 s. The delay between two IRLL excitations was constant and set at 6 s. The green curve is alternatively very well merged with the red line and with the blue line, respectively, corresponding to the IRLL excitation or the absence of IRLL (Figure 4). When the IRLL starts, in the second and third start of excitation, the time necessary for the green curve to be superimposed with the red one was 1.5 s. In the case where the IRLL stopped, the time delay for the RhB fluorescence was higher than for the start. For the first stop, it was 3 s; for the second, it was 3.6 s, as it was for the third one. These long times of stabilization of more than 1 s, regardless of high or low state status, may result from the relatively small volume of the IRLL excitation highly sensitive to temperature exchanges between the excited volume and the whole volume of RhB solution with a constant temperature equal to the room temperature at 23°C.

3.3 Temperature Mapping

The optical fibre delivers the IRLL vertically as a cone of light, without focusing or collimation of the beam. The apex angle of the cone depends exclusively on the numerical aperture of the fibre core and the index of the medium approximated at water at 1.33. The excitation is therefore spatially non uniform leading to a not homogenous temperature of the RhB solution in the whole petri dish. Thus, as the level of RhB fluorescence is measured on a 2D plane, a decrease in temperature distribution was observed when moving away from the centre of the IRLL illumination cone. Figure 5a details the position of the 6 ROIs. All of the 6 ROIs was touched together in a line. Figure 5b reveals the resulting measurement of temperature mapping in the 6 ROIs with and without excitation.

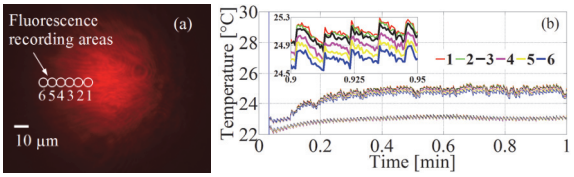

Figure 5: a. Positions of the 6 ROIs in the cone of light from the optical fibre; 6 areas in a line with a common limit. b. Temperature mapping inside the cone of IRLL with and without excitation. Inset: temperature evolution in the 6 ROIs between 0.90 and 0.95 min.

For all the measurement with or without IRLL, each of the 6 ROIs fluorescence levels were recorded in the same time. The first record without IRLL has defined the reference temperature at 23°C. Then, the IRLL was used to heat the target cone of RhB solution with an average power of 70 mW. The temperature of the 6 ROIs followed the same evolution law during the rise time. During the following time, and until the end, the temperature was stabilized, similar to that of the first experiment (Figure 3c and 3d).

4 CONCLUSIONS

We have presented a method for measuring the local temperature of a biological sample thanks to an optical method using the temperature sensitive dye Rhodamine B. The experimental setup was built around a fluorescence microscope, with a light-emitting diode illumination system for measuring the level of fluorescence of RhB. We have first shown the immediate influence of the infrared laser light excitation at 800 nm at 200 mW caused a focal increase of temperature of 1.7°C and the process of temperature stabilisation for a recording duration between 1 min and 10 min. Then, we have shown that the fluorescence of the RhB can be also used in the case of an intermittent IRLL excitation, with a good reproducibility. Finally, the distribution of temperature inside the cone of infrared laser light was measured.

The important interest of this setup lies in its simplicity of implementation, first requiring RhB, a very standard and sensitive fluorescent dye with a high sensitivity to temperature changes and resolution of 0.2°C. Then, the insertion of the global setup for temperature measurement around a fluorescent microscope requires few simple modifications of the microscope itself. Finally, as biological observations are often made using fluorescent microscopy, the additional tool of temperature analysis allows the correlation of biological processes with the modification of temperature.

For our future experiments, we will test this method on the temperature modification of cells under the effect of IRLL. The influence of illumination and temperature on cell properties like plasma membrane permeability under IRLL illumination will be used to define a threshold of optical parameters of the excitation not to be exceeded to maintain cell viability in experiments.

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