Technical Note

Sample temperature control for single molecule experiments with the MicroTime 200

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Introduction

The MicroTime 200 [1] is a high-end time-resolved confocal fluorescence microscope with outstanding sensitivity for experiments even down to the single molecule level. It can be used for a wide range of fluorescence lifetime based analysis methods like Fluorescence Lifetime Imaging (FLIM) but is also suited for techniques such as Fluorescence Resonance Energy Transfer (FRET), Anisotropy or Fluorescence Correlation Spectroscopy (FCS, FCCS, FLCS). Typical applications for the MicroTime 200 include studies of molecular interaction and localisation, analyte concentration, mobility and diffusion processes with a wide range of samples. For several of the studied samples it is known that their dynamics are affected by the sample temperature and it is therefore sometimes advantageous to equip the MicroTime 200 with a sample temperature control system.

A variety of sample temperature controller systems for microscopes are commercially available today, which are designed for different purposes and various specimen geometries. In this technical note we present and discuss the implementation of a sample temperature control system from Bioptechs [2], which is based on a microfluidic flow cell. This geometry is especially well suited for experiments with freely diffusing samples in a well confined aqueous volume or immobilised objects which can be prepared in an aqueous environment on a coverslip surface.

MicroTime 200 requirements

The only important prerequisite for the implementation of the temperature controlled sample chamber from Bioptechs is that the MicroTime 200 has to be equipped with the objective scanning option. In this configuration the sample is fixed and confocal scanning is carried out by moving the microscope objective horizontally with the piezo scanner. This option allows the use of a variety of specimen holders including a microfluidic flow cell and its associated temperature control periphery. A PiFoc for axial scanning (e.g. for 3D image stacks) can be added as an option.

Fig. 1: Assembled Microfluidic flow cell (FCS2, above) and microscope objective heater (below) along with their corresponding controllers

The Bioptechs sample temperature control system

The system includes a heatable microfluidic flow cell (FCS2), used as the sample chamber, a heating element for the microscope objective and the corresponding control electronics to set, display and monitor the sample temperature (see Fig. 1). An objective heater is used to eliminate the heat sinking effect of optical coupling medium (oil, glycerin or water) from specimens at physiological temperatures. As all objectives have different thermal profiles, the objective heater requires a separate dedicated temperature controller.

Temperature-controlled microfluidic flow cell (FCS2)

The flow cell used is the Focht Chamber System 2 (FCS 2) [5], which includes:

- A microfluidic flow cell acting as the sample chamber (see Fig. 2). The sample volume between two glass slips is defined by a gasket, that can have any custom desired geometry and any thickness between 0.1 mm to 1.0 mm. The temperature sensor can be seen in Fig. 2 pointing from above onto the upper surface of the flow chamber. All necessary parts can be purchased by Bioptechs. Further details about the design and working principle can be found on the Bioptechs website [5].
- An adapter to mount the chamber on the microscope stage. A suitable stage adapter is readily available for the inverted Olympus microscope bodies IX71 and IX81. The installation on the MicroTime 200 is therefore possible with no particular modification (see Fig. 8 and Fig. 9.)
- The control electronics (see Fig. 1) to set and control the chamber temperature.

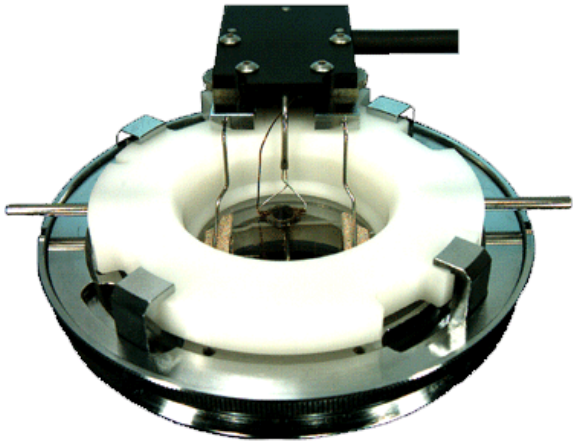

Fig. 2: Assembled FCS 2 stage

Microscope objective heating unit

The heating system [3] for immersion microscope objectives consists of:

1. an adjustable band that surrounds the objective and allows to heat the objective
2. a surface probe thermal sensor which is positioned in the gap between the ends of the heating band. This surface probe thermal sensor measures the temperature of the objective
3. a small unit for the whole heating and sensor assembly, which fits easily to all objectives ranging in diameter from 17 mm up to 35 mm
4. a objective thermal insulator

As advised by Bioptechs, an additional thermal insulation spacer was used along with an inverted heater configuration (see Fig. 3). In this configuration the heating band is placed below the temperature sensor and the fixing band. This configuration achieves a great thermal isolation and sufficiently avoids heat transfers from the microscope objective to the microscope body. Bioptechs can provide those thermal spacers [4] as standard. As a side effect, the objective rises in its axial position by the insulation spacer, which must be compensated by elevating the sample stage with suited elevation bars that can be obtained from Bioptechs.

Note: Bioptechs has recently developed and machined a dedicated thermal insulator for the PiFoc, which eliminates the need to raise the sample stage.

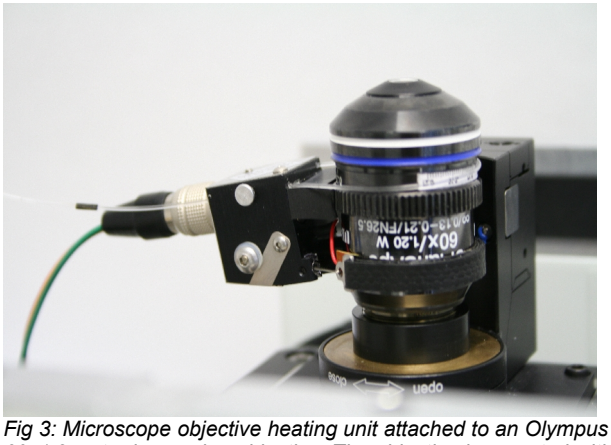

Fig 3: Microscope objective heating unit attached to an Olympus 60x1.2 water immersion objective. The objective is mounted with a spacer acting as thermal insulator on top of a piezo element for axial fine positioning (PIFOC from Physik Instrumente, Karlsruhe, Germany)

Testing and operation

Raster scanning with the objective heater in objective scanning configuration

In the objective scanning configuration the sample is kept in place and the raster scanning is performed by moving the objective with the piezo scanner. As the installation of the objective heater adds an additional weight of approx. 25 g onto this piezo scanner, it was necessary to determine if this additional weight affects the scanning movement and positioning accuracy. For this purpose several images of sub-micron structured plant samples and dye stained latex beads were recorded using monodirectional and bidirectional scanning

patterns. All acquired images showed no distortions or influences of the additional weight, proving that the additional weight of the objective heater has no influence the objective scanning accuracy.

Set temperature accuracy

The desired temperatures can be set with the temperature controller. However, as all objectives have different thermal profiles, a slight mismatch is induced between the programmed temperature and the actual temperature at the sample location. This error is compensated by introducing an offset to the control loop. A thermistor probe is supplied with the system so that a reference reading can be taken from immersion fluid placed on top of the objective at the specimen plane. This allows precise adjustment of the control loop.
A comparison between measured and set temperatures at the FCS 2 and the objective heater showed a slight offset which could be corrected for by a calibration curve (see Fig. 4). Applying this correction to the set temperature at the controllers, it was possible to adjust the temperature in the sample compartment within + 0.2 °C of the desired temperature.

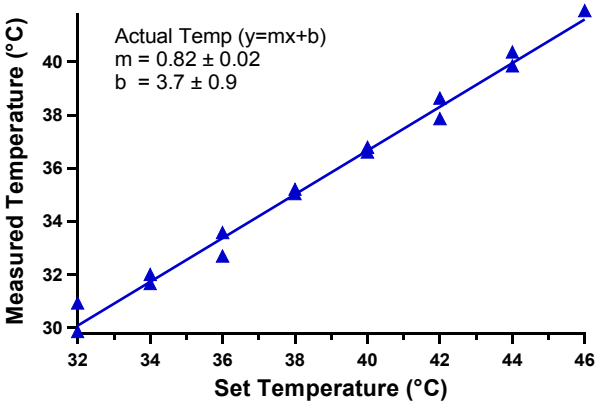

Fig. 4: Temperature calibration curve. The “Set Temperature” was programmed at the objective heater and FCS 2 chamber controller. The “Measured Temperature” was determined with the temperature sensor placed within the immersion fluid. Using this calibration curve the appropriate “Set Temperature” for the desired sample temperature can be found.

the maximal temperature the objective can bear without damage. This information is available from the objective manufacturer. The maximal temperature for the Olympus UPLSAPO 60XW is for example 50°C.

Additional comments

Temperature control

The tested FCS 2 system and the objective temperature control are only designed for heating and not for chilling. Bioptechs also provides products for cooling the sample as well as the objective (not tested).

Ease of use

The FCS 2 sample chamber is easy to clean and sterilize. All parts of the FCS2 that have contact with the specimen can be autoclaved. The FCS 2 is compatible with all standard microscopy illumination modes (like transmitted illumination, phase contrast, etc.).

Single Molecule Spectroscopy

The inside volume of the tested chamber is about 31 microliter with the provided gasket (0.1 x 14 x 24 mm). This is almost too large for single molecule experiments, where typically about 10-20 microliter are desired. The real limiting volume factor was, however, the size of the access tubings to the gasket, which added around 80 microliter additional volume.
For single molecule spectroscopy experiments other gasket geometries that are available from Bioptechs should be used. There is e.g. a 3 x 22 x 0.1 mm gasket available with an internal volume of only 6.6 microliter. There are also special FCS2 ports for low dead volume access tubing (29 microliter or even 12 microliter).
A further important point is that that the sample preparation (for example for immobilised molecules on a surface) must be compatible with the flow cell design.

Application examples

Temperature dependent RNA folding studied via FRET

Conformational changes and folding, e.g. of RNA or proteins, is an intensively studied topic in single molecule spectroscopy. Surprisingly only little attention is paid up to now to the fact, that a huge number of these processes is also affected by the ambient temperature. Conducting these experiments at well defined different temperatures can therefore improve the accuracy and reproducibility as well as open up new insights into thermally activated processes.

In the following example the temperature dependency of the conformational changes of a specific RNA motif was studied in collaboration with the group of David Nesbitt (JILA, University of Colorado, Boulder, CO 80309, USA). This Tetraloop-Receptor (see Fig. 5) features two different conformations so called “undocked” and “docked” geometries [6,7]. For visualisation purposes the DNA was specifically labeled with the dyes Cy3 and Cy5, that show Fluorescence Resonance Energy Transfer (FRET) depending on their distance. In the undocked geometry the FRET

efficiency is lower than in the docked geometry due to the larger distance between acceptor and donor molecule. The RNA molecules where freely diffusing in buffer solution and the individual FRET efficiency were recorded for every single RNA strand passing the confocal investigation volume [compare 8].

The resulting FRET efficiency histograms in Fig. 6 show pronounced differences when the temperature is increased by 10 K. The decrease of the docked population at higher temperature indicates that heat impedes docking of the RNA chain. Based on these values the reaction enthalpy for the docking process can be calculated and was found to be negative supporting the initial finding, that the the undocked state is favoured at higher temperatures.

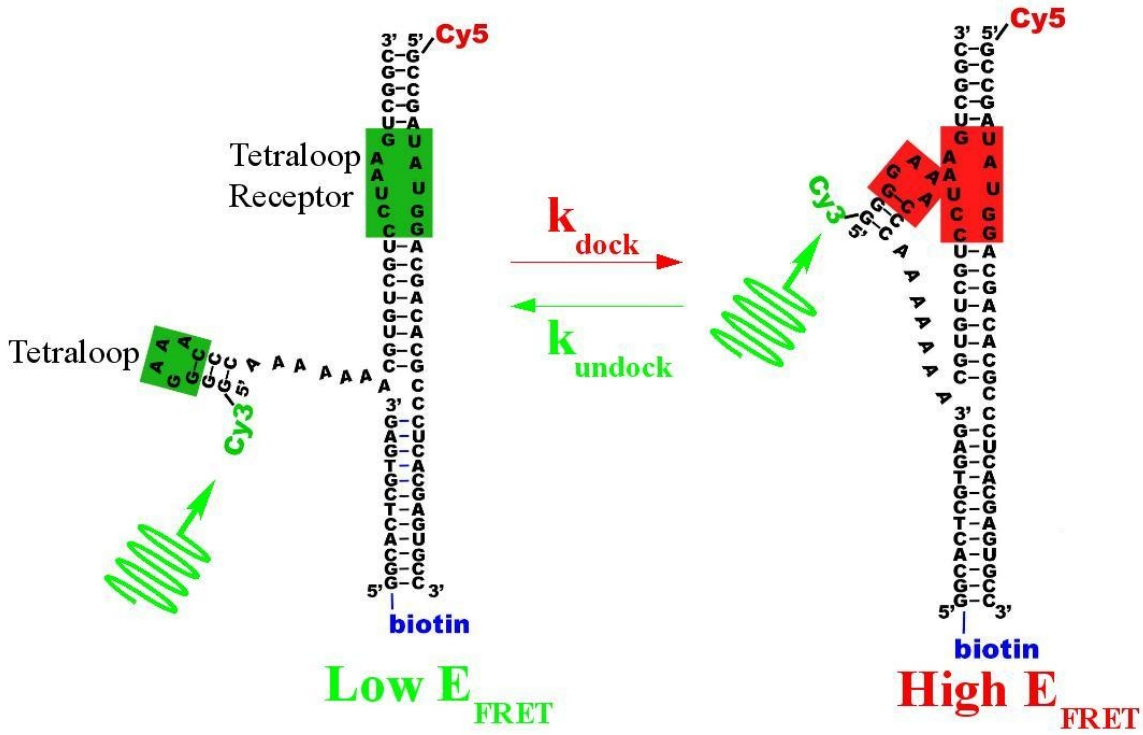

Fig. 5: Undocked (left) and docked (right) configuration of the Tetraloop-Receptor. The undocked conformation shows a lower FRET efficiency than the docked conformation

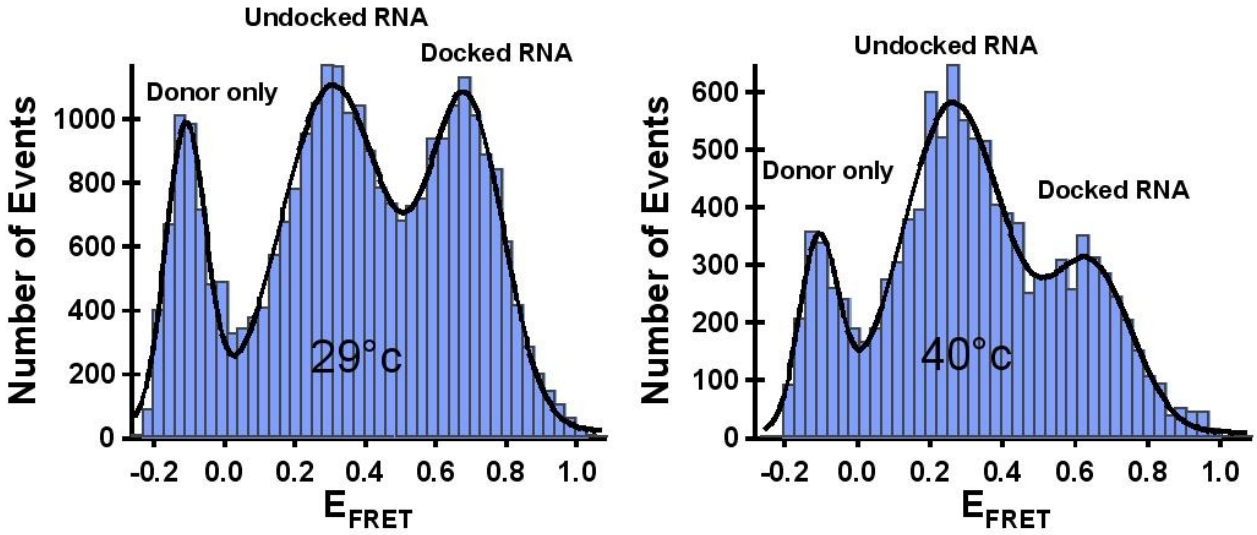

Fig. 6: FRET efficiency histograms for freely diffusing DNA strands at 29°C and 40°C. The first peak at low FRET efficiencies corresponds to donor only species, the second peak at about 30% FRET efficiency corresponds to the undocked RNA and the third peak at about 70% corresponds to highly FRET efficient docked RNA. The decrease of the docked population at higher temperature is clearly visible and indicates that heat impedes docking of the RNA chain.

Mobility of diffusing single molecules

The measurement of the diffusion coefficient can be used to investigate the size or size changes of molecules in order to e.g. detect conformation changes of proteins or to identify and quantify species differing in their hydrodynamical radius. The diffusion coefficient is, however, temperature dependent and a temperature control that controls and stabilizes the temperature over the time period of the measurement at the sample location is therefore beneficial. In order to investigate the influence of the temperature on the diffusion coefficient, the diffusion coefficient of a dye molecule (Atto655-(COOH), Atto-Tec GmbH Germany) diffusing in water upon temperature changes was studied. As this measurement requires a very accurate and quantitative diffusion coefficient determination, two focus Fluorescence Correlation Spectroscopy (2fFCS) was applied [9].

The result of the measurement is shown in Fig. 7 and clearly indicates the temperature dependance of the diffusion coefficient. The discrepancy between measurement and theory is most certainly due to the fact that for this measurement no objective heater was used. The heat flow towards the objective makes in this case quantitative temperature measurements difficult and one can therefore conclude that an objective heater is an imperative for such measurements.

Conclusion

The integration of the Bioptechs sample temperature control system into the MicroTime 200 is straightforward and requires no special modifications of the system. All necessary parts can directly bought from Bioptechs. The temperature control is easy to use and allows a temperature control with an accuracy of ± 0.2 °C from room temperature up to the limit at which the objective can be heated without damage.

The performance of the MicroTime 200 in terms of scanning stability or optical resolution is also not affected. Any single molecule analysis available as a standard feature (such as FLIM, FCS, FRET...) is therefore also possible under temperature control, which is relevant in cases were the dynamic of molecules is strongly affected by the temperature.

The only restrictive condition for the tested FCS 2 flow cell system is that the sample preparation must be compatible with the flow cell design.

The MicroTime 200 can also be equipped with a Bioptechs Delta T® micro-environmental system. This system provides a temperature controlled, convertible open or closed system optical cavity that is compatible with high magnification, high numeric aperture lenses where fluid perfusion, and humidified CO_2, can be controlled.

Beyond pure temperature control, the MicroTime 200 can also be equipped with a complete incubation chamber if further control of the air conditions and composition like CO_2 and humidity for life cell measurements are needed. For this application the Olympus cell^cubator is supported [10].

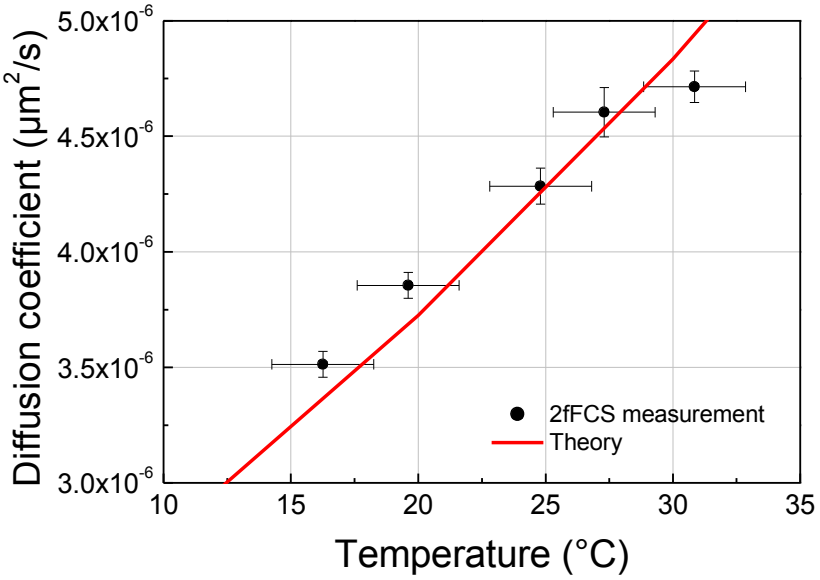

Fig. 7: Diffusion coefficient of Atto655-(COOH) measured at temperatures ranging from 16 °C up to 31 °C. The red line displays the theoretically expected values.

Acknowledgement

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Further reading

[1] PicoQuant GmbH – MicroTime 200 Inverse Time-resolved Fluorescence Microscope Brochure. Available online: http://www.picoquant.com/dl_datasheets/MicroTime200_Brochure.pdf

[2] http://www.bioptechs.com/

[3] http://www.bioptechs.com/Products/OBJ_HTR/obj_htr.html

[4] http://www.bioptechs.com/Products/OBJ_HTR/Options/objoptions.html#thermiso

[5] http://www.bioptechs.com/Products/FCS2/fcs2.html

[6] Christopher D. Downey, Julie L. Fiore, Colby D. Stoddard, Jose H. Hodak, David J. Nesbitt, and Arthur Pardi, Biochemistry 2006, 45, 3664-3673

[7] Jose H. Hodak, Christopher D. Downey, Julie L. Fiore, Arthur Pardi, and David J. Nesbitt, PNAS July 26, 2005 vol. 102 no. 30 10505–10510

[8] Application Note: FRET analysis with pulsed interleaved excitation using the MicroTime 200 Benedikt Kraemer, Felix Koberling, PicoQuant GmbH, Martin Roos, RKI, Berlin Steffen Rüttinger, PTB, Berlin, March 2005. Available online: http://www.picoquant.com/products/microtime200/appnote_pie_fret.pdf

[9] Application Note:Two-Focus Fluorescence Correlation Spectroscopy, Thomas Dertinger, University of California Los Angeles, USA, Benjamin Ewers, Benedikt Krämer, Felix Koberling, PicoQuant GmbH, Germany, Iris v.d. Hocht, Forschungszentrum Jülich, Germany; Jörg Enderlein, Eberhard Karls Universität Tübingen, Germany, February 2008. Available online: http://www.picoquant.com/products/microtime200/appnote_2fFCS.pdf

[10] http://www.microscopy.olympus.eu/microscopes/Life_Science_Microscopes_scan_R_Key_Features.htm

Appendix

Installing the objective heater

The following images (see Fig. 8) show the main steps of the installation of the heat isolator and the objective heater on the MicroTime 200.

Briefly, first the thermal insulation spacer is fixed on the PIFOC, then the objective is installed and at the end, the objective heater is mounted on the objective.

A closer view of the objective (a Olympus UplanSApo 60x water immersion, NA 1.2.) with the added heater is shown in Fig. 3.

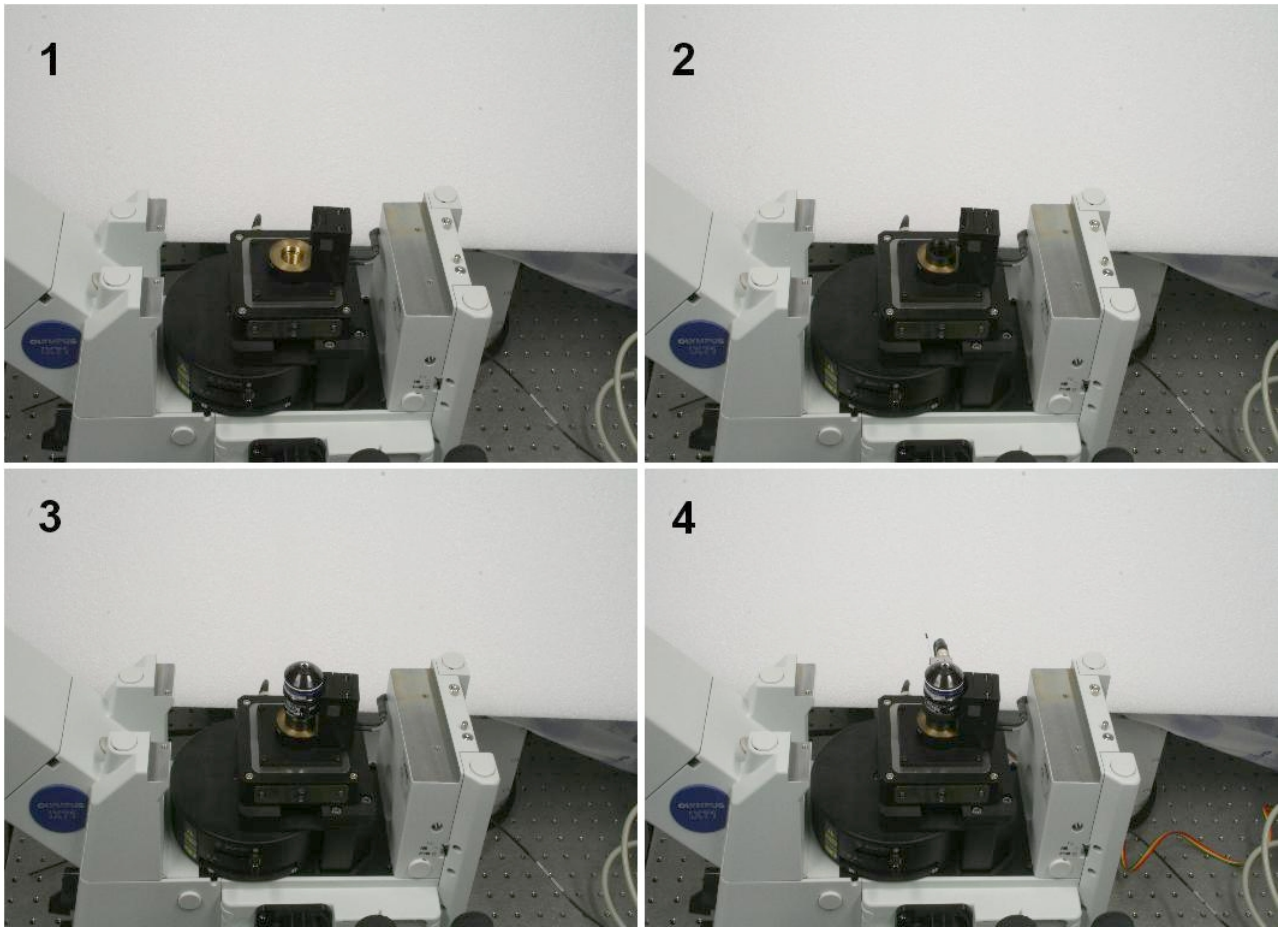

Fig.8: 1-Modified IX71 body with objective scanning and PIFOC stage 2- Thermal insulation spacer installed on PIFOC 3- Objective installed on heat spacer 4-Heater installed on the objective

An important aspect is to be noticed: since the thermal insulation spacer raised the objective, it is necessary to correct for this by introducing two bars that elevate the sample stage by 8 mm (see Fig. 9). Elevation bars can be obtained from Bioptechs for the Olympus IX71 and IX81 microscope body.

Assembly of the sample temperature controller

The assembly of the sample temperature controller is straightforward and shown in Fig. 9. Briefly, first the elevation bars are installed to correct for the raise of the objective due to the thermal insulation spacer. Then the stage with stage adapter is mounted and finally the FCS 2 is placed upon the adapter.

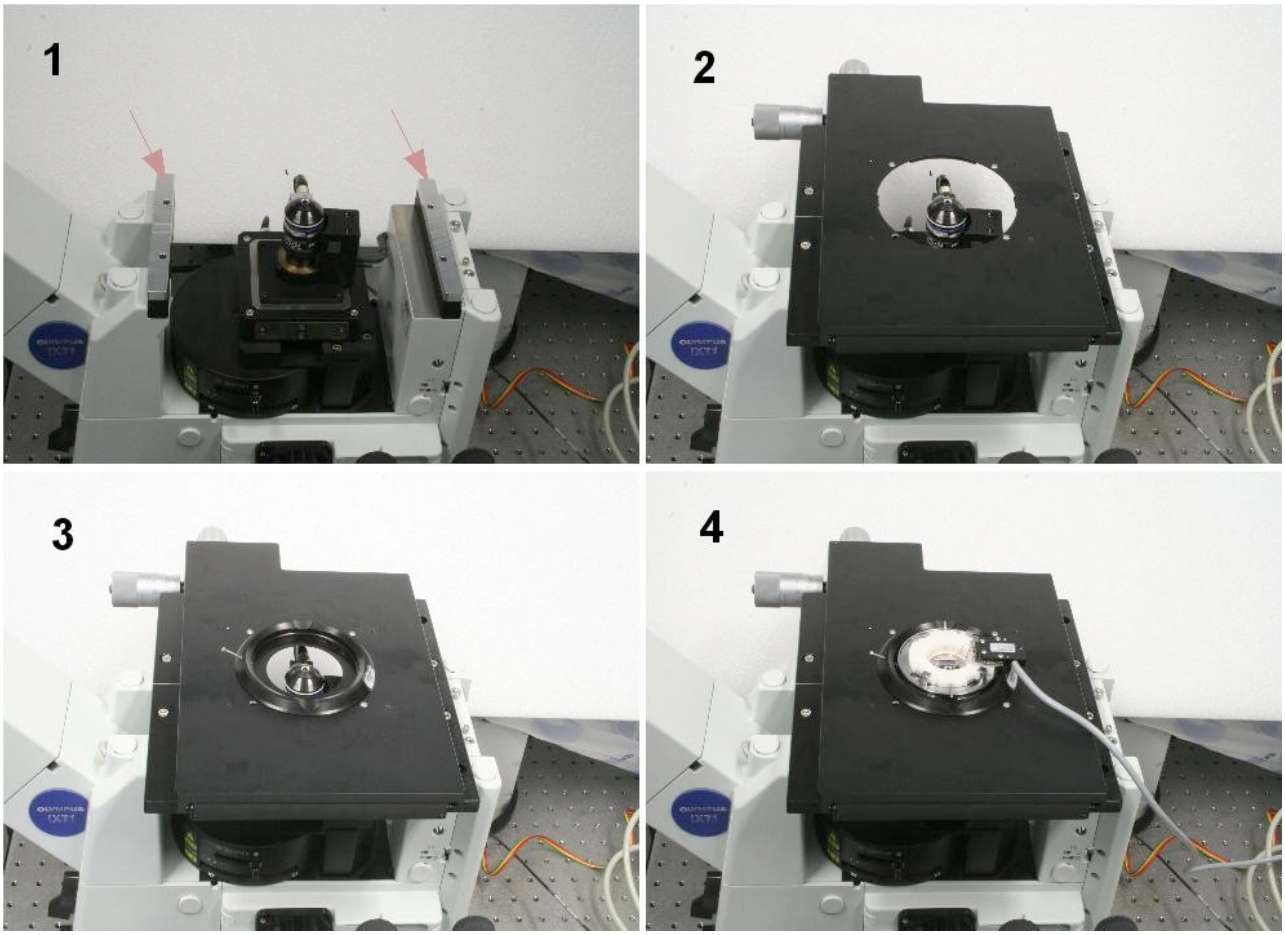

Fig. 9: 1- elevating bars (red arrows) 2- MicroTime 200 XY stage 3- Bioptechs stage adaptor 4- Bioptechs temperature controlled sample chamber

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