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Microdialysis on-chip crystallization of soluble and membrane proteins with the MicroCrys platform and *in situ* X-ray diffraction case studies

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Supplementary Information

1. MicroCrys platform

1.1 Visualization and GUI

Figure S1 is a screenshot of the "Display" tab used for manipulating the parameters of visualization such as the intensity of the front and back LED lights and the zoom of the inverted uEye camera. For example, in Figure S1a, the camera zoom is set to 75 % and the intensity of both the front and back lights is set to less than 50 %. The user can adjust the focus with the scrolling wheel of the computer's mouse, whereas a more accurate control of the focus is achieved with the "Accurate focus off/on" button on the "Display" tab. The example in Figure S1a shows the "Accurate focus off/on" button clicked, indicating that rolling the mouse wheel allows for an accurate focus control, while unclicking the button leads to a rapid focus change even with a single mouse roll. This dual operation is very useful for screening the entire chip when an accurate focus mode is not essential or for screening a specific compartment of the chip like the protein reservoir where an accurate focus mode is crucial to identify the protein crystals. Finally, another functionality controlled through the "Display" tab is the spatial movement of the metallic support along the x- and y-axes by clicking the left button of the mouse on the displayed image. For instance, Figure S1a illustrates part of the protein reservoir and part of the linear fluidic channel of the chip. If the user desires to inspect the protein reservoir, then clicking on the upper point of the image moves the motorized stage accordingly (y axis) and reveals the image of the whole protein reservoir. Accordingly, the motors can be moved on either direction of the x- and y-axes by merely left-clicking on the image revealing various parts of the chip.

The "Image Record" tab of the GUI is used for handling the recording parameters, such as the directory and the frequency (s) for registering images. In Figure S1b, for example, one image is recorded every 1000 s in the defined directory. Another feature handled by the GUI is the possibility to control when the LED lights are turned on to prevent overheating and induced evaporation in the protein reservoir by continuous lighting. Specifically, the LED lights can be turned on only during image recording by selecting the "Light on only during recording" button (Figure S1b). Consequently, when this functionality is chosen, the lights will be turned on for recording even if the user has set to zero the front- and back-lights in the "Display" tab. Finally, the "Start" or "Stop" buttons can be pressed to initiate or cease image recording.

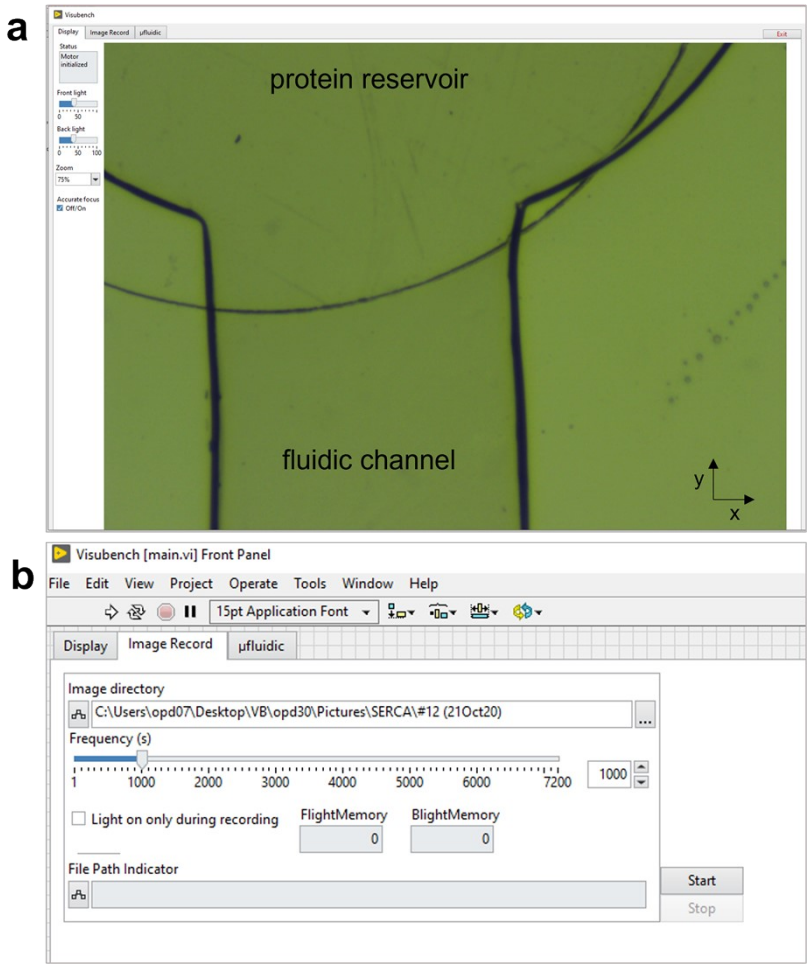

Figure S1 The GUI developed in LabVIEW for automated control of all the separate units of MicroCrys. The interface includes three tabs: (a) the "Display" tab for manipulating the parameters of visualization, (b) the "Image Record" tab for handling the recording parameters and the "µfluidic" tab for controlling the pressure-driven fluidic system (not shown).

1.2 Fluid handling

There are two operational modes for handling solution mixing and circulation when using the OB1 controller (Elveflow) with the MicroCrys platform. Figure S2a illustrates a schematic representation of the first operational mode. In this mode the crystallization solution is prepared manually by mixing the buffer and precipitant solutions in advance and one pressure channel is used to drive the solution from the pressurized container (50 mL Falcon tube) into the inlet port of the microchip. Under continuous flow operation, the crystallization solution flows from the inlet port towards the linear fluidic channel of the chip and is disposed from the outlet port to a waste container. Nucleation and subsequent growth of the protein crystals occur in the protein reservoir as the flowing crystallization solution diffuses from the fluidic channel through the regenerated cellulose (RC) dialysis membrane towards the protein reservoir. A detailed description of the chip's micro-patterns and the crystallization process through microdialysis is presented elsewhere [1]. Choosing this operational mode allows for the realization of multiple on-chip crystallization experiments since each of the OB1 pressure channels can be used to circulate pre-mixed solutions in separate chips.

As can be seen in Figures S2a and S2b, the setup for flow regulation also includes two thermal-based digital MFS4 flow sensors (Elveflow) for measuring the flow rate in a range of 1 µL min^{-1} to 1 mL min^{-1}. Flow sensors can be added at any point along the direction of the flow where accurate measurement

or regulation is necessary. For example, one of the flow sensors can be connected prior to the inlet port of the chip to regulate the flow of the crystallization solution as imposed by the pressure-driven controller (Figure S2a).

Figure S2b shows the schematic representation of the second operational mode, where the crystallization solution is not prepared manually by mixing all the components in advance, but the MUX Distributor (Elveflow) is used to automate the procedure. The distributor has six inlets and one outlet port (6 to 1 model) and can add sequentially solutions in a mixing reservoir. Solutions (salts, buffers, PEGs, etc.) are shown in Figure S2b with numbers 1 – 4 (green color) inside the pressurized reservoirs. These solutions are injected one by one through the MUX Distributor and a microfluidic valve (MPV 3/2, Elveflow) into an intermediate reservoir, in order to prepare the crystallization solution (blue color), which is finally driven into the fluidic channel through the OB1 pressure controller, as described previously. A flow sensor (MFS4) is regulating the flow rate of the solutions prior to their injection within the intermediate reservoir and a second flow sensor is connected before the inlet of the microfluidic chip to regulate the flow rate of the crystallization solution.

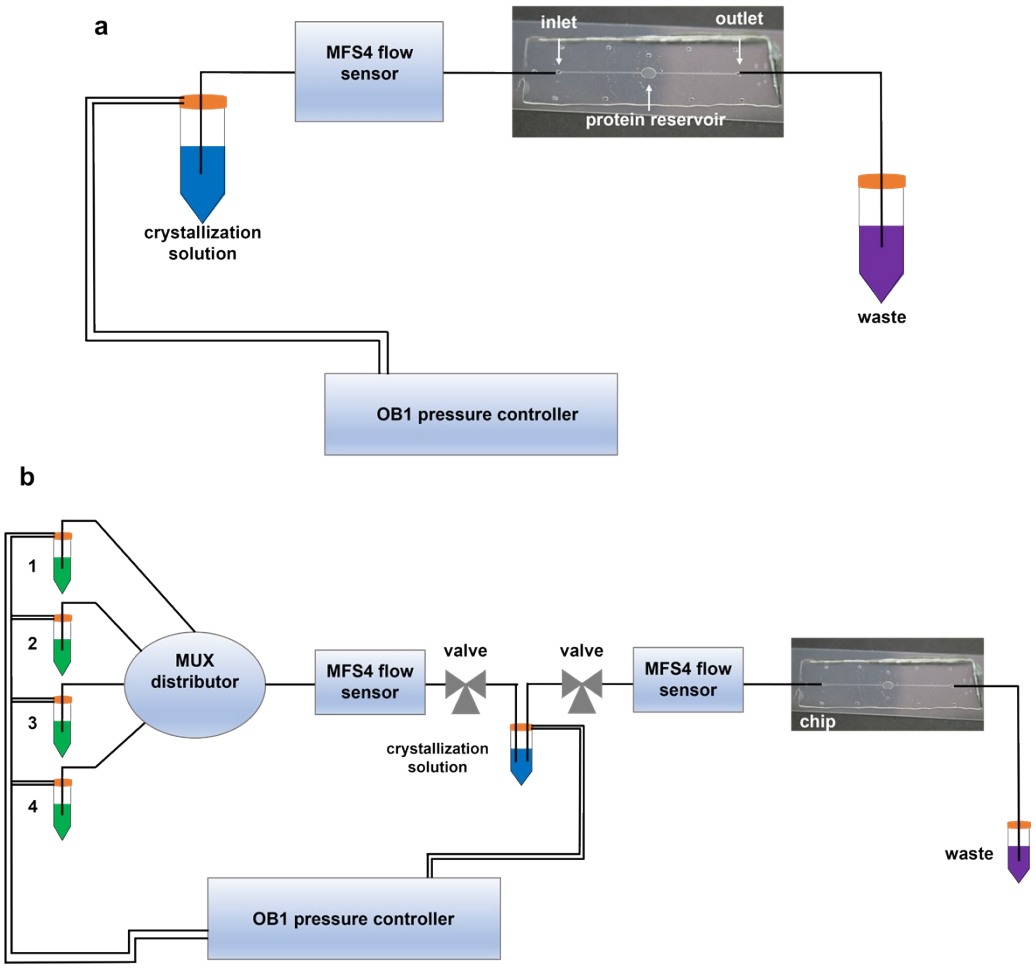

Figure S2 Schematic illustration of the two operational modes for mixing and circulating crystallization solutions in the microchips. (a) The OB1 pressure controller (Elveflow) is used for injecting pre-mixed solutions (blue) in the fluidic channel of the chip. (b) The OB1 pressure controller and the MUX distributor (Elveflow) are used for mixing components (salts, buffers, PEGs, etc. shown in green) of the crystallization solution (blue) by sequential injection into a mixing reservoir.

Elveflow has developed the ESI (Elveflow Smart Interface) software for an interactive, user-friendly control of all the connected instruments. Our goal has been to integrate the main functionalities of the ESI software in the LabVIEW program developed for MicroCrys. The "µfluidic" tab of the GUI (Figure S1a or Figure S1b) has been dedicated to the integration of the fluidic system. The first prototype of MicroCrys doesn't include yet these developments, so the user has to use the ESI. In the future, all the separate parts of MicroCrys will be integrated in a single GUI.

1.3 Thermal regulation

1.3.1 The programmable controller

The thermal regulation of the protein reservoir using only a single Peltier module was feasible by exploiting the capabilities of the TC-XX-PR-59 controller (Laird Thermal Systems), a programmable temperature controller designed for reversible control of thermoelectric assemblies like the Peltier modules. An accurate and precise regulation can be achieved for both cooling and heating through various operational modes (power, on/off, Proportional Integral Derivative (PID) or algorithm). The TC-XX-PR-59 controller is operating with a pulse-width modulation (PWM) [2] of the output and can be configured through a RS-232 interface. The base frequency of the controller is 10 kHz, while a minimum power supply of 450 W can supply 11 – 30 V voltage to the controller.

The reversible controller features two programmable fan outputs, an alarm output relay and outputs for LEDs, thermoelectric modules and NTC (negative temperature coefficient) thermistors. The connections on the output relays of the controller are shown in Figure S3. Specifically, the DC power supply and the Peltier module are connected in four flat pin terminals and the NTC temperature sensor is connected through a three-wire KK type Molex terminal. The RS-232 standard and the fan are connected to screw terminals. Moreover, the controller features three extra two-wire KK type Molex terminals for alarms, other types of temperature sensors and external potentiometers.

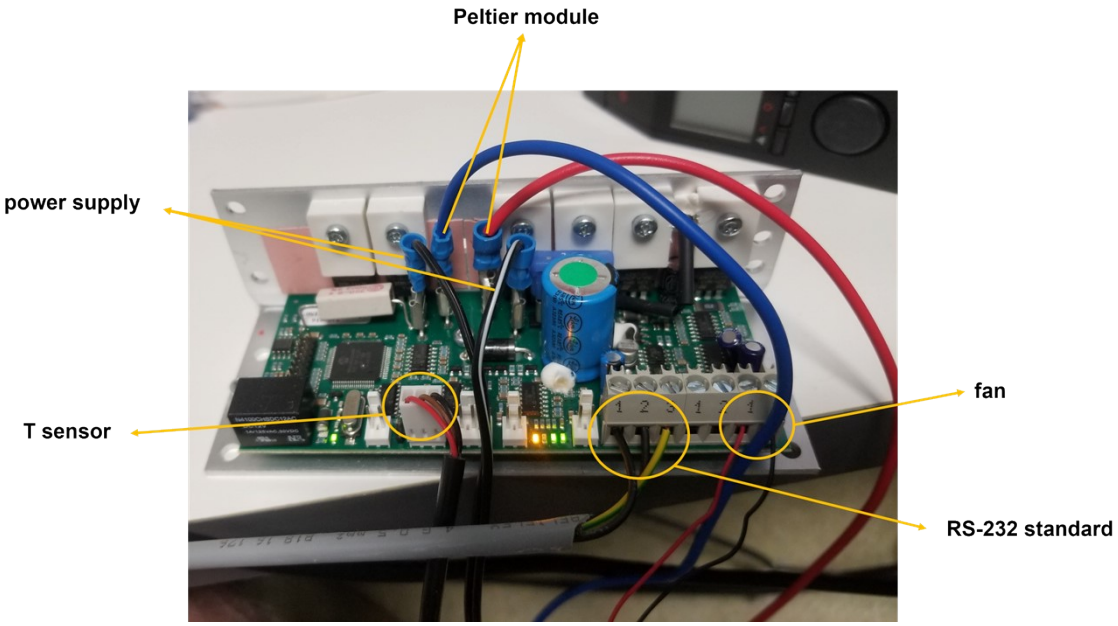

Figure S3 The TC-XX-PR-59 programmable controller (Laird Thermal Systems). The output relays feature the DC power supply, the Peltier module, the temperature (T) sensor, the fan and the RS-232 communication.

1.3.2 The temperature sensor

For on-chip protein crystallization, accurate temperature measurements are required in the proximity of the protein reservoir. Though several tests were conducted with a variety of temperature sensors, the most well-suited solution was the use of J type thermocouple (RS Components) with a probe head of 2 mm. As described in section 3.1.2 Thermal regulation and shown in Figure 2a, the thermocouple is positioned between the lid of the Peltier module and the bottom part of the microchip, near the protein reservoir. The probe head of the J type thermocouple is sufficiently small to fit in this position. Moreover, the thermocouple can measure temperatures in the range of - 50 °C to 250 °C with a ± 1.5 °C accuracy and a response time of 5 s.

In general, a thermocouple is a temperature sensor made of two wires from different metals, joined together at one end to form an electrical junction. The J type thermocouple consists of one positive, iron wire and one negative, constantan alloy (copper-nickel) wire. The thermocouple produces a voltage as result of the Seebeck effect [3] that can be interpreted to measure the temperature. For example, for the J type thermocouple by RS Components, a 5.269 mV output voltage corresponds to 100 °C.

1.3.3 The Peltier module

The Peltier effect was used for the thermal regulation of the chip's protein reservoir. Typically, the Peltier effect describes the heat absorption or release generated by an electrical current flowing through the junction of two different semiconductive materials [4]. For this application, the SH10-23-06-L1-W4.5 annular thermoelectric cooler (Laird Thermal Systems) was used because the ceramics (Al_2O_3) on both sides have a circular hole of 7.2 mm diameter in the center. Thus, the thermoelectric module can be positioned right below the chip and its hole can be aligned with the protein reservoir facilitating visualization with the inverted uEye camera of MicroCrys. The Peltier module is shown in Figure S4.

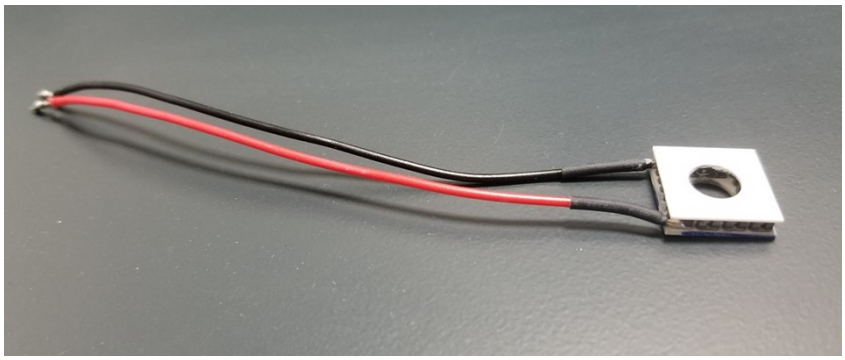

Figure S4 The Peltier element (Laird Thermal Systems) used for thermal regulation features a hole with a 7.2 mm diameter allowing visualization of the protein reservoir with MicroCrys.

Even though the combination of the SH10-23-06-L1-W4.5 Peltier module with the TC-XX-PR-59 controller demonstrates many advantages concerning the design and operation of the thermal regulation system, one of the major challenges hindering this development was the different operating voltage of the controller (12 V) and the Peltier module (3 V). The issue was eventually solved by the addition of a resistance R in serial connection to the Peltier module. A 2.7 Ω coil resistance from the RS100 series (RS Components) covered by aluminium envelope was used (Figure 2b, section 3.1.2 Thermal regulation). With this resistance value, the voltage of the Peltier module (measured with an

external oscilloscope, ISO-TECH Kunststoff) was 2.3 V, lying within the operating specifications of the manufacturer.

1.3.4 GUI for thermal regulation

The LT-Interface has been developed by Laird Thermal Systems for operating the TC-XX-PR-59 reversible controller. However, in the framework of automating and integrating the controller into MicroCrys, an assembly of input/output electronic WAGO cards has been developed (Figure S5). The J type thermocouple is connected to a two-channel analog input card WAGO 753-469 and a two-channel analog output card WAGO 753-550 is connected to the two-wire KK type Molex terminal of the controller. The communication between the WAGO cards and the computer is achieved by the MODBUS TCP/IP communication protocol through a Fieldbus Ethernet coupler WAGO 750-352 card. A power supply WAGO 787-602 card with 24 V DC output voltage and 1.3 A output current is connected prior to the Fieldbus coupler. After assembling the Fieldbus node, the end module (WAGO 750-600 card) is installed to complete the internal data circuit and ensure proper data flow throughout the system.

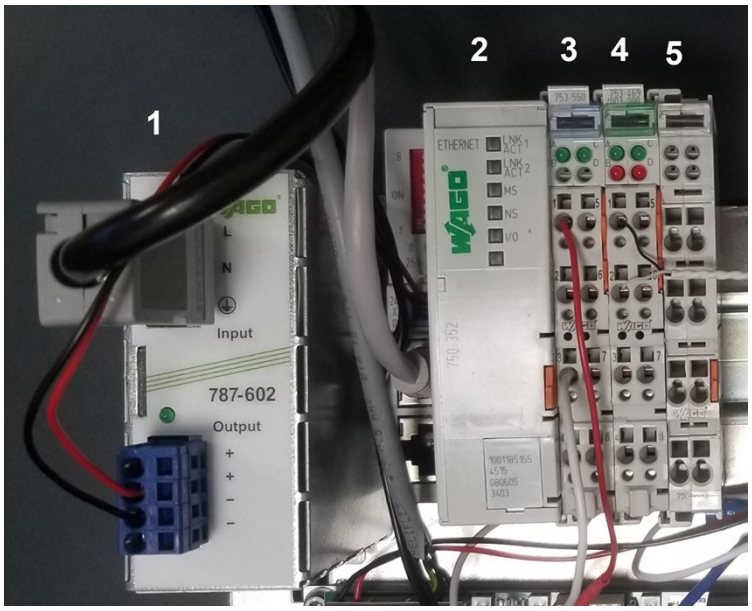

Figure S5 The assembly of electronic WAGO cards for automating the use of the programmable controller. The cards are: 1. The switched-mode power supply 2. The fieldbus Ethernet coupler 3. The analog input 4. The analog output and 5. The end module.

The data is recorded on the computer by a program written in LabVIEW and the GUI is shown in Figure S6. It should be mentioned that the GUI described here is only used with the thermal regulation system for testing and verifying the system. It shouldn't be related yet to the main GUI of MicroCrys described in section 1.1 Visualization and GUI of the Supplementary Information. Once the thermal regulation system is exhaustively tested and results are produced, we aim to merge both the GUI for visualization and the GUI for thermal regulation into one single GUI where all the separate components of MicroCrys can be managed. A serial communication protocol employing ASCII characters (Serial Command Interface, Laird Technologies) can be used for taking full command of the TC-XX-PR-59 controller. Specifically, the parameters of the internal EEPROM (Electrically Erasable Programmable Read-Only Memory) memory are copied to the runtime registers when the controller is initiated.

However, using the RW command, the master can write the runtime registers to EEPROM after ensuring that they have been changed. This communication is incorporated in the main VI of the LabVIEW program written for thermal regulation. Pressing the “Init Peltier CTRL” button on the GUI (Figure S6) verifies the proper booting of the controller as described above.

Then the user can press the “Init WAGO” button which allows the reading of temperature with the J thermocouple through the WAGO cards and the MODBUS protocol. The GUI includes a waveform chart window for visualizing temporal variations (s) of the temperature (°C) measured with the J type thermocouple. The thermocouple reads the temperature allocated by the Peltier module which is power supplied directly with a PID control integrated in the LabVIEW program. A sliding bar labelled “Voltage from WAGO to Peltier CTRL” with a value range from 0 – 5 V has been added to perform this operation. The controller has been set to power regulation mode meaning that the output power can range from – 100 % to + 100 % depending on the voltage. For example, 0 V correspond to – 100 % power, 2.5 V to 0 % power and 5 V to + 100 % power. Moreover, when performing an experiment, the user can set the desired temperature in °C in the “Set point in degree” box and turn on the “Start Peltier” and “Start Regulation” switches. The PID algorithm will regulate the voltage to adjust the temperature to the set input value. Finally, a “STOP” button allows terminating the algorithm that runs all the functions necessary for thermal regulation. In summary, when using the GUI for thermal regulation, the user must first initialize the controller and the WAGO system, then set the temperature value and finally turn on the switches for the Peltier module and the PID regulation.

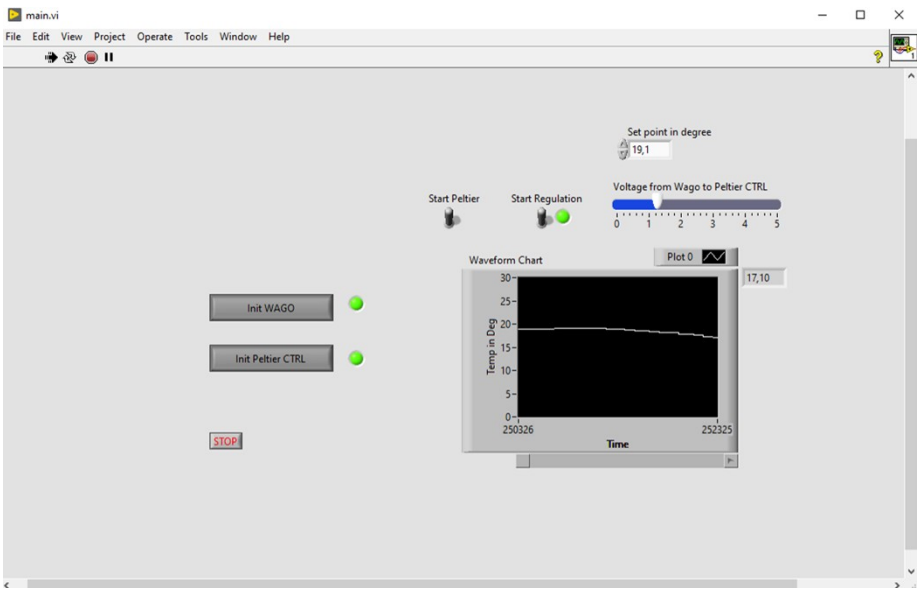

Figure S6 The GUI of the LabVIEW software developed for thermal regulation of the microchips. The interface includes buttons to initialize the controller and the WAGO system, to set the temperature (°C) and start the regulation of the Peltier module.

1.3.5 Support for heat dissipation

A support for the thermal regulation of the microchips was designed with the CAD (Computer Aided Design) software SolidWorks and manufactured from aluminium at the SERAS platform (Institut Néel, Grenoble). The main goal of fabricating the support entirely from a conductive metal was to enhance heat dissipation by cooling fins integrated in the main body of the unit. The external dimensions of the aluminium support are 12.7 cm (length) and 8.6 cm (width), fitting precisely on the metallic holder of the MicroCrys platform (Figure 1b, section 2.1.2 Sample stage). A top and a bottom view of the

support's CAD designs is shown in Figures S7a and S7b or in Figures S7c and S7d, respectively. Specifically, Figure S7a illustrates a top view of the metallic support where the cavity designed for the Peltier module is shown. The Peltier module (green color) is placed in the cavity and the design of the whole system ensures that the hole of the Peltier is aligned with the holes drilled throughout the body of the aluminium support allowing light protrusion for visualization of the chip's protein reservoir through the inverted uEye camera of the MicroCrys platform.

Moreover, a lid and a frame were designed for the Peltier module (Figure S8). These components are shown in a top view of the CAD design in Figure S7b, in brown and blue colors, respectively. The aluminium lid has a hole of 5 mm diameter for visualization through the inverted uEye camera and can be fixed on the support with screws. The dimensions of the aluminium lid and the polymeric frame are 15 x 15 mm and 29 x 19 mm, respectively.

The metallic support also features nine cooling fins fabricated within the body of the unit as shown in Figure S7c. Moreover, the bottom view of the support's CAD design in Figure S7c illustrates the cooling fan integrated within the support. The fan is an AVC axial cooling fan from an NVIDIA Quadro 4000 graphics card with a diameter of 7 cm and a speed range of 3100 to 3900 rpm. The fan operates as a turbine forcing airflow through all the cooling fins of the metallic support ensuring sufficient heat dissipation. The bottom part of the support is shielded with an aluminium lid as shown in Figure S7d, with dimensions of 120 x 72 mm. The diameter of the hole at the fan position is 48 mm. Moreover, the lid features a hole for visualizing the protein reservoir of the dialysis chip. Finally, it should be mentioned that while the upper part of the aluminium support fits accurately on the sample holder of MicroCrys, the bottom part exceeds vertically by 15 mm below the holder allowing airflow through the cooling fins.

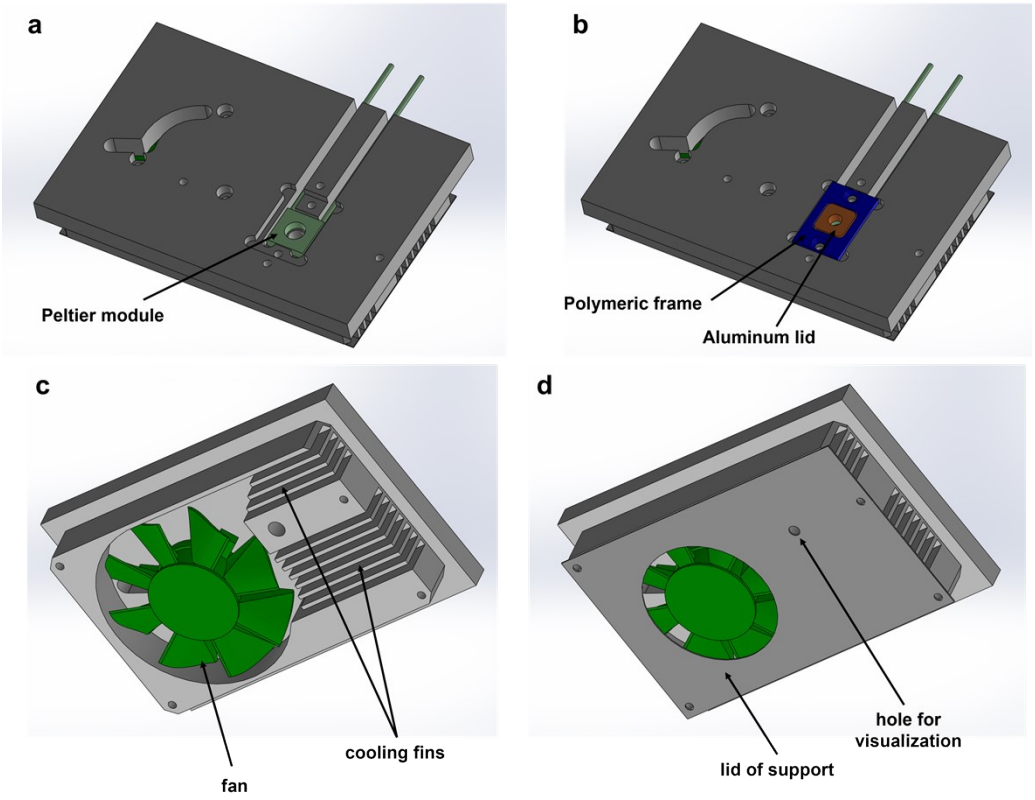

Figure S7 The CAD designs (SolidWorks) of the aluminium support developed for on-chip thermal regulation. Top view illustrating (a) the Peltier module (green) and (b) the aluminium lid (brown) and the polymeric frame (blue) designed for the Peltier module. Bottom view featuring (a) the cooling fins integrated on the main body of the support and the position for the cooling fan and (b) the bottom lid of the support.

1.3.6 Lid for the Peltier module

The aluminium support for thermal regulation (section 3.1.2 Thermal regulation) features a cavity for the Peltier module which is positioned below the chip ensuring that the protein reservoir of the chip is aligned with the hole of the Peltier module for visualization and recording during the experiment. Originally, the lid was made of aluminium but the thermal transfer between the hot part of the Peltier element and the aluminium support was too large because both units were made of the same conductive material, as shown in transverse view in Figure S8a. This problem was resolved by fabricating two distinct parts for the lid. One part was made of aluminium for direct contact with the cold side of the Peltier element and one part was designed as a frame made of a mixture of carbon fibers and epoxy adhesive for insulating the main body of the support (Figure S8b). The CAD design showing these two components is included in Figure S7b.

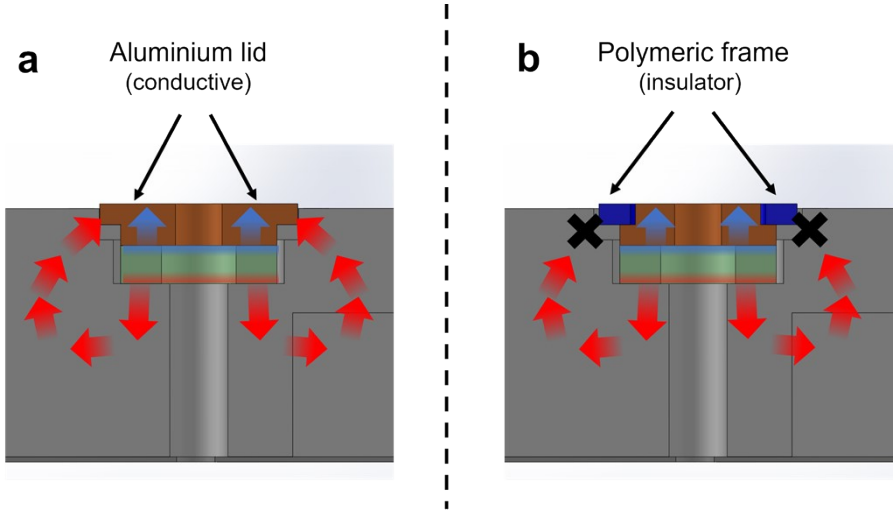

Figure S8 Transverse view of the CAD designs for the chip's support developed for thermal regulation. (a) The aluminium lid for the Peltier module (brown) and the heat transfer from the hot side of the module (red arrows) and (b) the aluminium lid (brown) with the polymeric frame (blue) used as a thermal insulator.

2. On-chip membrane protein crystallization

This section provides some preliminary results for the on-chip crystallization of two membrane proteins: the multidrug efflux transporter AcrB from *Escherichia coli* and the sodium-pumping TmPPase from *Thermotoga maritima*. Figure S9a shows AcrB crystals grown on-chip at 293 K in the presence of 10 % v/v PEG 4000, 5 % v/v glycerol, 50 mM ADA pH 6.5 and 200 mM $(NH_4)_2SO_4$. AcrB was solubilized in 0.02 % v/v DDM (Anatrace) detergent and the same concentration of detergent was added to the crystallization solution in order to avoid dialyzing out the detergent from the protein reservoir. The protein concentration was approximately 5 mg mL^{-1} and the MWCO of the RC dialysis membrane embedded within the chip was 12 – 14 kDa. The native AcrB crystals shown in Figure S9a

were tested for their diffraction quality at the BL13 - XALOC beamline (ALBA), as described in section 3.2.4 On-chip membrane protein crystallization and *in situ* X-ray diffraction experiments. The crystals diffracted at a resolution lower than 14 Å as shown in Figure S9b where the arrows indicate the few diffraction spots.

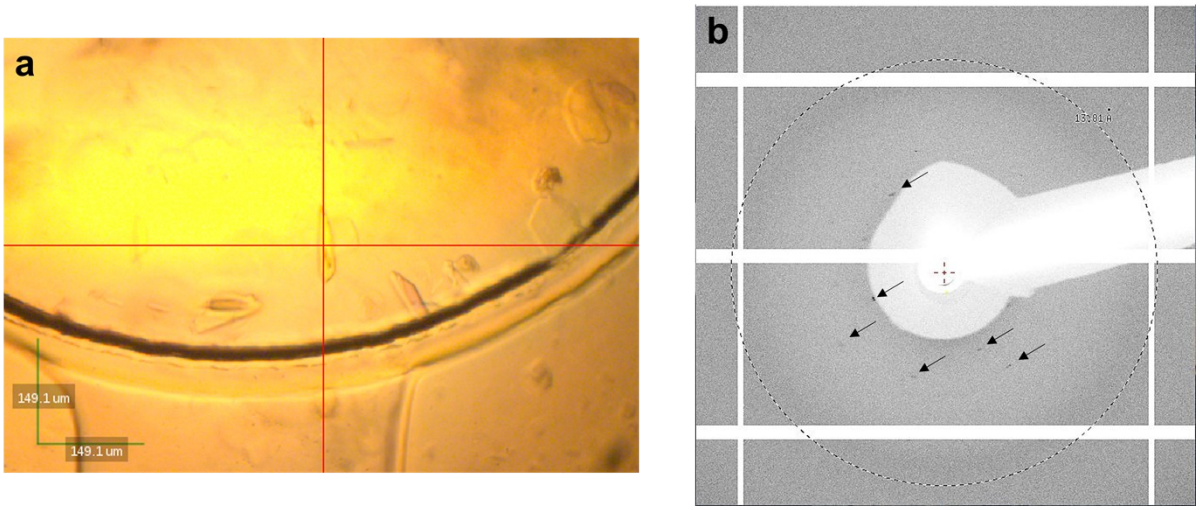

Figure S9 (a) AcrB (5 mg mL^{-1}) crystals grown on-chip in 10 % v/v PEG 4000, 5 % v/v glycerol, 50 mM ADA pH 6.5 and 200 mM $(NH_4)_2SO_4$ and 0.02 % v/v DDM at 293 K. The scale bar represents 149.1 µm. (b) Diffraction spots of AcrB crystals indicated by arrows at a resolution lower than 14 Å. The *in situ* diffraction experiment was performed at BL13 - XALOC beamline (ALBA).

TmPPase was solubilized in 0.5 % v/v octyl glucose neopentyl glycol (OGNPG) detergent and the protein concentration for the on-chip dialysis crystallization at 293 K was approximately 12.5 mg mL^{-1}. The protein solution also contained 50 mM MES-NaOH pH 6.5, 50 mM KCl, 5 mM $MgCl_2$, 3.5 % v/v glycerol, 2 mM DTT (dithiothreitol) and 4 mm Na_4IDP (imidodiphosphate sodium salt). The crystallization solution contained 28 % v/v PEG 400, 50 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 175 mM KCl, 2 mM DTT, 4 mm Na_4IDP and 0.5 % v/v OGNPG detergent. The MWCO of the RC dialysis membrane on the chip was 12 – 14 kDa. TmPPase crystals grown on-chip (Figure S10a) were tested for *in situ* X-ray diffraction at P14 beamline at PETRA III (DESY). The X-ray wavelength was 0.98 Å, the photon flux was 2 x 10^{13} ph s^{-1} and the beam size was 2 µm x 6 µm. The diffraction images were recorded with an EIGER 16M (Dectris) detector with an active area of 311 x 327 mm^2 and 18 mega pixels resolution. The TmPPase crystals diffracted at a resolution close to 15 Å as shown in Figure S10b, where the arrows indicate the weak diffraction spots.

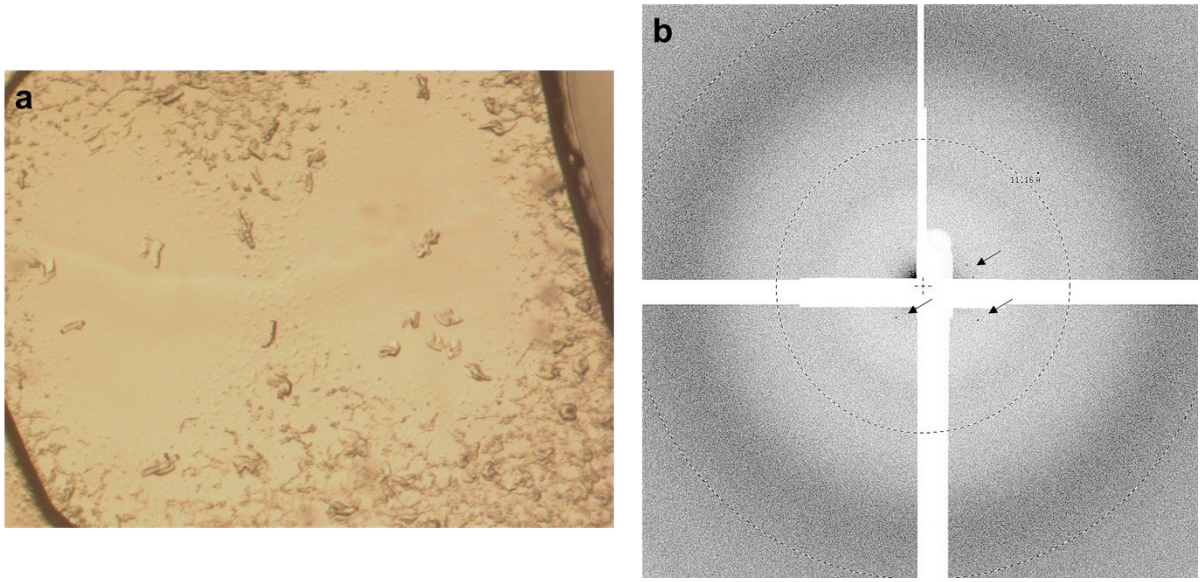

Figure S10 (a) TmPPase (12.5 mg mL^{-1}) grown on-chip via microdialysis in 28 % v/v PEG 400, 50 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 175 mM KCl, 2 mM DTT, 4 mm Na_4IDP and 0.5 % v/v OGNPG at 293 K. The scale bar represents 100 µm. (b) Diffraction spots of TmPPase crystals indicated by arrows at a resolution close to 15 Å. The *in situ* diffraction experiment was performed at P14 beamline (PETRA III).

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