Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59799R2
Full Title:	Fabrication of Microscope Stage for Vertical Observation with Temperature Control Function
Keywords:	Microscope, Microscope stage, Vertical observation, Temperature control, Diatom, Floating Diatom, Rubber heater, Smartphones, Petri dish, Glass slide
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tokyo, Japan

TITLE:

Fabrication of Microscope Stage for Vertical Observation with Temperature Control Function

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KEYWORDS:

microscope, microscope stage, vertical observation, temperature control, diatom, floating diatom, rubber heater, smartphones, Petri dish, glass slide

SUMMARY:

Presented here is a protocol using a temperature-controlled microscope stage that allows a sample container to be mounted on a vertical microscope.

ABSTRACT:

Samples are usually placed onto a horizontal microscope stage for microscopic observation. However, to observe the influence of gravity on a sample or study afloat behavior, it is necessary to make the microscope stage vertical. To accomplish this, a sideways inverted microscope tilted by 90° has been devised. To observe samples with this microscope, sample containers such as Petri dishes or glass slides must be secured to the stage vertically. A device that can secure sample containers in place on a vertical microscope stage has been developed and is described here. Attachment of this device to the stage allows observation of sample dynamics in the vertical plane. The ability to regulate temperature using a silicone rubber heater also permits observation of temperature-dependent sample behaviors. Furthermore, the temperature data is transferred to an internet server. Temperature settings and log monitoring can be controlled remotely from a PC or smartphone.

INTRODUCTION:

Optical microscopy is a technique employed to increase observable details via magnification of a sample with lenses and visible light. In optical microscopy, light is directed onto a sample, then transmitted, reflected, or fluorescent light is captured by magnifying lenses for observation. Various types of microscope are available that differ in design to accommodate different uses and observation methods. The different designs include an upright microscope, which is structured to illuminate a sample from below for observation from above, and an inverted microscope, which illuminates the sample from above for observation from below. Upright microscopes are the most common and widely used design. Inverted microscopes are often used to observe samples that cannot allow a lens close in distance from above, such as cultured cells adherent to the bottom of a container. Many research groups have reported observations in a wide range of fields using inverted microscopes[1-7]. Many additional devices have also been developed that take advantage of the features of inverted microscopes[8-13].

Currently, in all conventional microscope designs, the microscope stage is horizontal and is therefore unsuitable for the observation of samples producing movement in the vertical plane, (due to gravity, buoyancy, motion, etc.). To make these observations possible, the microscope stage and light path must be rotated to vertical. The vertical stage is required to vertically mount glass slides or sample containers such as a Petri dishes to the stage. To address this, a sideways inverted microscope tilted by 90° has been devised. However, attaching samples with tape or other adhesives does not yield the necessary long-term immobility. Described here is a device that can achieve the necessary stability. This device permits observation over time of sample movement in the vertical plane. Mounting of a silicon rubber heater has also made it possible to observe the influence of temperature variation on sample behavior. Temperature data is transferred to an internet server by Wi-Fi, and temperature settings and log monitoring can be controlled remotely from a PC or smartphone. To our knowledge, the stage attached to a sideways tilted microscope tilted by 90° has not yet been reported in previous studies.

The microscope stage is composed of three aluminum plates. The middle aluminum plate is mounted to the lower aluminum plate that attaches to the stage. The silicone rubber containing the temperature sensor is attached between the middle and upper aluminum plates. Rubber bands are used to affix the sample. Claws are attached in the left and right four points of the upper aluminum plate to secure the rubber bands. The control circuit of the temperature regulator receives a signal from the temperature sensor embedded in silicone rubber and modulates electric power by the pulse width modulation (PWM) method. The temperature can be gradually increased to 50 °C in 1 °C increments. This device is useful for applications in which vertical sample motions may be temperature-dependent.

This report provides examples of temperature effects on the floating phenomenon of diatoms. As examples of diatom observation studies, measurements of sedimentation velocity of cell clusters, motion analyses, ultrafine structure studies, etc. have been reported[14-23]. The specific gravity of diatoms floating in water with photosynthetic organisms is slightly higher than that of water, so they tend to sink; however, they will rise if even slight convection is occurring. To study this phenomenon, a glass slide is affixed vertically to a microscope stage, and the effects of increasing temperature on diatom vertical motion are observed.

PROTOCOL:

1 Design

1.1 Fabrication of aluminum plates

1.1.1 Cut a 101 mm hole in the center of an aluminum plate of dimensions 150 mm x 200 mm x 2 mm to be used as the forefront plate with a laser processing machine. Machine claws at eight points to affix two rubber bands across the length, or two across the width of this plate (see **Supplemental Figure 1A** and **Supplemental Figure 2A)**.

1.1.2 Cut a 130 mm hole in the center of another 150 mm x 200 mm x 5 mm aluminum plate to be used as the middle upper plate with a laser processing machine. Machine eight notches for attaching rubber bands at two points across the length, or two across the width of this plate (see **Supplemental Figure 1B** and **Supplemental Figure 2B)**.

1.1.3 Cut a 130 mm hole in the center of a 150 mm x 200 mm x 4 mm aluminum plate to be used as the middle lower plate with a laser processing machine (see **Supplemental Figure 1C** and **Supplemental Figure 2C)**.

1.1.4 Cut a 30 mm hole in the center of a 150 mm x 200 mm x 1.5 mm aluminum plate to be used as the base plate (see **Supplemental Figure 1D** and **Supplemental Figure 2D)**.

1.2 Fabrication of two aluminum pedestal

1.2.1 Cut a 30 mm hole in the center of the aluminum plate (100 mm diameter, 3 mm thickness) and make a notch from one side with the dimensions 42 mm wide x 30 mm deep (see **Supplemental Figure 3A)**.

1.2.2 Cut a 30 mm hole in the center of the plate in an aluminum plate (100 mm diameter, 4 mm thickness) and drill three 3 mm holes located 25 mm from the center, spaced 120° from each other (see **Supplemental Figure 3B**).

1.3 Fabrication of three pressed cork disc

1.3.1 Cut a 20 mm hole in the center of the pressed cork disc (100 mm diameter, 2 mm thickness) with a water jet cutting machine. Make one cut 42 mm across x 30 mm deep, then one cut 4 mm wide x 5 mm deep (see **Supplemental Figure 4A**).

1.3.2 Cut a 20 mm hole in the center of the pressed cork disc of dimensions 100 mm diameter, 1 mm thickness with a water jet cutting machine. Make a cut 42 mm across x 30 mm deep, a cut 4 mm wide x 40 mm deep (see **Supplemental Figure 4B**).

1.3.3 Cut a pressed cork plate from a 100 mm diameter disc with a 42 mm width and 30 mm depth. Two sheets of 1 mm thickness and one sheet of 2 mm thickness are required (see **Supplemental Figure 4C**).

1.4 Fabrication of silicone rubber heater

1.4.1 Fabricate a heater using a 100 mm diameter disc of 2.5 mm thick silicon rubber with built-in Nichrome wire and cut a 20 mm hole in the center of the disc (see **Supplemental Figure 5**).

1.5 Assemble parts described in steps 1.1–1.4 by stacking them as shown in **Supplemental Figure 6.**

1.6 To construct a microscope stage, refer to **Supplemental Figure 6**, cross-section of the microscope stage. Fix ① and ②, then ③ and ④ with screws. Fix ④ and ⑥ with screws. Fix ② and ③, ⑤ and ⑥, ⑤ and ⑦, ⑦ and ⑨, and ⑥ and ⑧ with adhesive.

2 Hardware design outlines

2.1 Prepare a “power supply and programming circuit” as shown in **Supplemental Figure 7**. Supply 12 V DC to the heater controller from the J4 terminal connected to the AC adapter. Decrease the voltage from 12 V DC to 3.3 V DC for the circuit power supply using a regulator because the CPU supply voltage is 3.3 V DC.

NOTE: USB 1 is a terminal for 5 V DC and serial signal of development PC. Although 5 V DC is not essential, it is used as the power source to program the CPU. This is also converted to 3.3 V DC by the regulator. J1 is a control signal terminal at the time of programming. This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.2 Prepare a “heater control circuit” as shown in **Supplementary Figure 7**. Switch to 12 V DC with Q5 (P channel MOS FET) and supply it to the heater. Q5 is a switching element that controls 12 V DC with PWM to adjust the amount of power supplied to the heater.

NOTE: The circuit includes an LED to visually confirm that voltage is being supplied to the heater. This drive signal (HEATER_C) is a PWM signal from the CPU. When an overheat signal is detected by the protection circuit, the BREAKER signal switches to LOW, and the operation of the MOS-FET stops. This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.3 Prepare a “connector circuit for heater unit” as shown in **Supplementary Figure 7**. Install a USB connector for connection with the heater section.

NOTE: This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.4 Prepare a “connector circuit for temperature sensor” as shown in **Supplemental Figure 7.** Mount the connector (Euroblock receptacle 2P) to connect the temperature sensor.

NOTE: This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.5 For a “A/D converter” as shown in **Supplemental Figure 7**, use ADS 1015 as an AD conversion device.

NOTE: AD conversion device converts the values of the temperature sensor and overheat detection sensor from voltage to digital values. This is a 12-bit multiplexer AD conversion device and is connected to the CPU with the I2C interface. This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.6 Make a “protect circuit” as shown in **Supplemental Figure 7** by connecting the overheat detection sensor (OHS) signal to the inverting input of the OP amp. Compare this signal with the voltage of the trimmer resistor connected to the noninverting input.

2.6.1 Ensure that when the voltage becomes lower than the voltage of the trimmer resistor, the output of the OP amplifier goes HIGH, the connected NPN transistor Q2 turns ON and the BREAKER signal goes LOW.

2.6.2 Ensure that at the same time, Q4 turns ON and the connected overheat indicator LED D6 lights up.

NOTE: This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.7 For a “display section” as shown in **Supplemental Figure 7**, use 192 x 64 dots for OLED. Connect with the CPU via the I2C interface.

2.7.1 Reset the OLED by separating the GND of the OLED by the CPU signal IO0 using an NPN transistor Q1 connected to the GND of the OLED.

NOTE: This OLED displays various types of information. This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.8 For a “LED & rotary encoder with push switch” in **Supplemental Figure 7**, mount a rotary encoder by solder that functions as a push switch and incorporates two LEDs.

2.8.1 Connect one LED to VCC for use as a power LED. The other is connected to the CPU for use as an indicator during heater operation.

2.8.2 Use a push switch contact for heater START/STOP that is connected to CPU. Connect the A and B outputs of the rotary encoder to the IO input set in the CPU interrupt.

NOTE: This circuit is housed in the controller case shown in **Supplemental Figure 8**.

2.9 For the CPU in **Supplemental Figure 7**, use the CPU of WROOM - 02D.

2.9.1 Output from IO12, IO13 to the “display unit” because the interface of the display is I2C standard. Connect IO0 to "display unit" and reset the OLED.

2.9.2 Connect IO15 to "heater control unit" and control the power supplied to the heater by PWM output.

2.9.3 Connect IO2 to "LED & rotary encoder with push switch" and light the START LED. Connect IO4 and IO14 to "LED & rotary encoder with push switch" and receive the signals (REA and REB) from the rotary encoder to determine the set temperature. Connect IO5 to "LED & rotary encoder with push switch" and start/stop the heater.

3 Software design outline

3.1 Use Arduino CORE for WROOM - 02D for the CPU as the controller for this system.

NOTE: As input devices, the start/stop switch, rotary encoder, temperature sensor (thermistor) are used. As output devices, an LED, character display (OLED), and heater are used. The communication device uses Wi-Fi.

3.2 Outline of the operation

3.2.1 Detect the operation of the rotary encoder as shown in the LED & rotary encoder with the push switch in **Supplemental Figure 7**, store it as the set temperature, and display it on the OLED. Set the input terminal of the CPU to which the phase terminals REA and REB are connected as an interrupt input terminal and process the rotation (forward and reverse) of the rotary encoder by interrupt. Set it to +1 for forward rotation and -1 for reverse rotation. Write the set temperature to the global variable and use it for the heater temperature control. At the same time, update the set temperature display of the OLED.

3.2.2 Identify start and stop by CPU IO 5 by start/stop switch (SW-S) as shown in the CPU of **Supplemental Figure 7**. The state of the start/stop switch is a timer interrupt process every 50 ms.

NOTE: Since the switch is a momentary switch, it reverses the state of start/stop when it is pushed and released. This state is stored in the global variable.

3.2.3 Use a thermistor for the temperature sensor. Read the measured values from the sample sensor (refer to “connector circuit for heater connection” in **Supplemental Figure 7**) into the CPU after A/D converter (refer to “A/D converter” in **Supplemental Figure 7**). Supply the current to the heater by turning on the IO15 port in the “CPU” of **Supplemental Figure 7**.

NOTE: There are two types of temperature sensors. One is used to measure the temperature of the sample and control the heater on the set temperature, and the other is attached to a heater and used for heat prevention. Connect the thermistor to 3.3 V via a resistor and record the change in resistance as a change in voltage. Remove a noise by the moving average method.

3.2.4 Use a thermistor for temperature prevention temperature sensor. Overheat detection is performed using a thermistor (R2) (“connector circuit for heater connection” in **Supplemental Figure 7**), and when the set value is exceeded, the heater current is shut off (“protect circuit” in **Supplemental Figure 7)**.

NOTE: This sensor is incorporated into a circuit and not through the CPU. This sensor is independent from the CPU and compared with the resistance value set by the resistor trimmer by a differential amplifier in an analog manner. When it detects that the temperature has exceeded the set value, it intervenes in the FET switch, which controls the current to the heater and forcibly stops the current supply. The purpose is to prevent the temperature of the heater from exceeding a certain level even in a situation where the CPU does not work properly.

3.2.5 Turn on the LED in the “LED & rotary encoder with push switch” of the S**upplemental Figure 7** by the CPU (in the “CPU” of **Supplemental Figure 7**), when the equipment is in operation.

3.2.6 Display the set temperature and measured value to OLED in the "display section" of the **Supplemental Figure 7** by the CPU (in the "CPU "of **Supplemental Figure 7**).

3.2.7 Drive the FET switch in the "heater control circuit" of **Supplemental Figure 7** with PWM from CPU to control the heater.

3.2.8 Control the heater by PID, based on measured temperatures acquired by the temperature sensor. Use Arduino's pid_v1.h library for PID processing.

NOTE: The CPU calibrates the time, communicates with the server, transmits data, and receives instructions from the server. When the sensor temperature exceeds the set temperature, the current to the heater is set to 0, and overshoot is suppressed.

3.2.9 Use the built-in Wi-Fi connection function of the CPU and connect to the Internet. Transmit set the temperature, heater temperature, etc. to the designated server by Wi-Fi.

4 System configuration

4.1 Build the system according to **Supplemental Figure 9**.

4.2 Use a thermistor as a sensor for temperature measurement. Connect the thermistor wire to the "SENSOR" terminal on the controller case. Receive the temperature signal measured by the thermistor.

4.3 Connect a microscope stage incorporating the rubber heater and the "HEATER" of the controller case with a dedicated cable. Control the current to the rubber heater.

4.4 Change the set temperature with the knob on the controller.

NOTE: Temperature log monitoring, temperature setting can be operated remotely from a PC or smartphone.

4.5 Equip a Wi-Fi with the controller.

4.6 Transfer the measured temperature, set temperature, and time information at measurement from the controller to the server via the internet. The data measurement cycle time is 5 s and cycle time for data transfer to the server is 1 min.

4.7 Access the server from the controller side at regular intervals and transfer the measurement data stored in the CPU of the controller to the server for analysis and graphing.

4.8 Refer to the supplementary material for how to operate the server.

5 Design of the sideways inverted microscope

5.1 Fix two aluminum plates of 15 mm in thickness vertically with screws to create a basic mount.

5.2 Attach a jig (one place) to the horizontal part of the base mount.

5.3 Place the microscope stage part vertically, attach the jigs (two places) to the vertical part of the base stand and fix the bottom of the microscope to the base stand.

5.4 Fix the microscope stage with screws.

6 Method of operation

NOTE: Here, the sample used is a mixture of Bold Modified Basal Freshwater Nutrient Solution liquid culture medium, sodium metasilicate, vitamins, and sterile water. 800 µL of this sample is diluted in 10 mL of fresh water medium.

6.1 Observation method

6.1.1 Inject 1000 µL of the prepared sample into a self-made glass chamber.

NOTE: The self-made glass chamber arranges two slide glasses in parallel and fixes them with an adhesive. A normal Petri dish has a large thickness and cells escape in the depth direction in the chamber, making it difficult to observe with a microscope. To prevent this, the chamber with a small depth direction is made, which makes it possible to prevent the cells from escaping in the depth direction in the chamber. A low temperature curable epoxy resin adhesive is used to bond around the glass to prevent the sample dropping from the chamber.

6.1.2. Attach a separately prepared video camera to the microscope. Connect a video camera using the microscope's dedicated lens adapter and shoot the sample.

6.1.3. Use a microscope with a 10x eyepiece and 200x objective.

6.1.4. Attach the vertical microscope stage to a microscope in four locations with 4 mm screws.

NOTE: Refer to **Supplemental Figure 1A** and **Supplemental Figure 2A** for design drawings of aluminum plates. In this experiment, an inverted microscope was used. This was tilted by 90°, and the fabricated microscope stage was affixed with screws. Refer to **Figure 1**.

6.1.5. Place a sample on a microscope stage perpendicular to the ground surface. Secure the sample with two rubber bands using the four claws made lengthwise.

6.1.6. Set the temperature to 40 °C with the controller shown in **Supplemental Figure 8**. Turn the controller knob to set the temperature. Check the set temperature on the display. Press the knob to start temperature control, and the blue LED will light up. Press the knob again to turn off the LED and stop temperature control.

NOTE: The measured temperature is displayed in real-time, and the heater is controlled to reach the set temperature. When the temperature control starts, the blue LED lights up and remains so while the heater is in operation. When the heater overheats, the red LED lights, and the heater automatically stops.

6.1.7. Refer to "The Server Operation Manual" in the supplementary information for server operation.

NOTE: A server for data storage is required. The server's database uses My-SQL.

7 Measurement of surface temperature distribution of rubber heater

7.1 Measure the distribution of the rubber heater surface temperature by thermography to check the temperature uniformity.

7.2 Attach the microscope stage which incorporated a rubber heater with a stand.

7.3 Change the setting temperature of the rubber heater surface to 35 °C, 45 °C, 55 °C, and 65 °C, and measure by the thermography from the front (refer to **Supplemental Figure 10**).

8 Temperature response test

8.1 Start temperature control by setting the sample set temperature to 30 °C. Wait until the measurement value reaches 30 °C and stabilizes. Increase the preset temperature stepwise by

5 °C from 30 °C to 50 °C and wait until the measured value stabilizes following the respective preset temperature.

8.2 Decrease the preset temperature stepwise by 5 °C from 50 °C to 30 °C and detect the tracking ability of the measured value.

REPRESENTATIVE RESULTS:

Figure 2 shows the temperature distribution of the rubber heater. The surface temperature of the rubber heater was uniform at each temperature. **Figure 3** shows the responsiveness of the measured temperature to set temperature changes. The orange line shows the set temperature and blue line shows the change of the sample temperature. The overshoot of the measured value to the setting change is small and the tracking is quick.

Diatom cells were observed to provide a specific example of the use of this device. The trajectory analysis of moving diatom cells is a useful approach to evaluating the motility of diatom cells. However, although a normal inverted microscope observes the sample horizontally, it is not suitable for observation of the influence of gravity or floating movement in the vertical direction.

In this experiment, the microscope stage with temperature controller was attached to an inverted microscope which had been rotated 90°. Temperature-dependent vertical motion of diatoms was successfully recorded. With this method, the locus of vertical motion of diatoms was detected as shown in **Figure 4.** As a result of observing with 100 individuals of diatoms, the average speed was 7.01 µm/s at room temperature and 470.1 µm/s at 40 °C. The effects of thermal convection on the vertical floating phenomenon of diatom cells were visualized by direct observation.

FIGURE & TABLE LEGENDS:

Figure 1: Photograph of the device fixed to the microscope stage. Appearance of the device fixed to the microscope stage. The device is fixed to the microscope stage with four screws.

Figure 2: Temperature distribution of rubber heater. The distribution of rubber heater as measured by thermography. The heater temperature was changed stepwise from ambient temperature to 35 °C, 45 °C, 55 °C, and 65 °C. The temperature was uniformly distributed across

the heater at each temperature.

Figure 3: Responsiveness of temperature signal. This shows the response when the set temperature is raised from 30 °C to 50 °C and lowered from 50 °C to 30 °C. The set temperature was changed in increments of 5 °C. In the stable state, the measured temperature is within ± 1.5 °C of the set value.

Figure 4: The locus of diatom movement. The vertical trajectories of diatom motion due to temperature changes have been plotted. The blue lines show trajectories of diatom cells at 25 °C for 27.06 s and at 40 °C for 0.2 s.

Supplemental Figure 1: Design drawing of aluminum plates (with dimensions). (**A**) The plate is 2 mm thick x 150 mm wide x 200 mm long, with a centered 101 mm diameter hole to allow insertion of the rubber heater. Each plate edge has two machined claws to which rubber bands may be attached to secure samples onto the stage. To attach this vertical stage to a microscope with 4 mm screws, 4.2 mm screw holes are drilled at four locations symmetrically surrounding the central hole. (**B**) The plate is 5 mm thick x 150 mm wide x 200 mm long, with a centered 130 mm diameter hole. Machine notch locations to match claw locations on forefront plate to permit attachment of sample-securing rubber bands across the stage. For attachment of the stage to a microscope, four 4.2 mm screw holes are drilled in matching locations to those in the forefront plate. (**C**) The plate is 4 mm thick x 150 mm wide x 200 mm long, with a centered 130 mm diameter hole. A 30 mm span is cut out of the center of the right 200 mm face of the plate, to the depth of the central hole. This purpose of the cut-out is to allow attachment of the heater connector on the right side. In the same positions as in the forefront plate, four 4.2 mm screw holes are drilled for attachment of the stage to a microscope. (**D**) The plate is 1.5 mm thick x 150 mm wide x 200 mm long, with a centered 30 mm diameter hole. In the same positions as in the forefront plate, four 4.2 mm screw holes are drilled for attachment of the stage to a microscope.

Supplemental Figure 2: Design drawing of aluminum plates (without dimensions).

Supplemental Figure 3: Design drawing of aluminum pedestals. (**A**) To be installed on the upper side: diameter is 100 mm, thickness is 3 mm. A 30 mm diameter hole is drilled in the center and a cutout of 42 mm width x 30 mm depth is made on one side. (**B**) To be installed on the lower side: diameter is 100 mm, thickness is 4 mm. A 30 mm diameter hole is drilled in the center, and three 3 mm holes have been placed at 120° to each other at a distance of 25 mm from the center.

Supplemental Figure 4: Design drawing of pressed cork discs. (**A**) To be installed on the upper side between the silicon rubber heater and the upper aluminum pedestal: diameter is 100 mm, thickness is 2 mm. A 20 mm diameter hole is drilled in the center, and two cuts (42 mm wide x 30 mm deep, 4 mm wide x 40 mm) are made at right angles to each other in sides of the disc. (**B**) To be installed on the lower side between the silicon rubber heater and the lower aluminum pedestal: diameter is 100 mm, thickness is 1 mm. A 20 mm diameter hole is drilled in the center. (**C**) This support is 42 mm wide × 30 mm deep, and cut from the circumference of a 100 mm diameter disc.

Supplemental Figure 5: Specification of silicone rubber heater. The diameter is 100 mm and the thickness is 2.5 mm. A 20 mm diameter hole is drilled in the center. The power supply is 12 V, with 18 W load capacity. The heater consists of Nichrome wire, with a lead wire connected to the electrode.

Supplemental Figure 6: Cross-section of the microscope stage. This is a sectional view of the microscope stage. The aluminum pedestal is attached to the backside aluminum plate and the rubber heater is installed on the outermost surface. The pressed cork is installed for insulation between the rubber heater and aluminum pedestal.

Supplemental Figure 7: Details of the circuit diagram. This indicates the circuit built in the controller. The circuit diagram is divided into nine parts according to individual functions.

Supplemental Figure 8: Design drawing of plastic controller case. Dimensions are 143.9 mm length x 85.3 mm depth x 25 mm width. The temperature setting knob, operating/overheating lamp, and indicator are located on the plastic controller case. The temperature can be set while watching the indicator by turning the set knob. Pushing this knob starts the temperature controller. The measured temperature is displayed in real-time, and the heater is controlled so that it reaches and holds the set temperature. When the temperature controller is turned on, the blue LED lights up and remains lighted while the heater is in operation. When the heater overheats, the red LED comes on and the heater automatically stops. Pressing the temperature controller knob again will stop it.

Supplemental Figure 9: System configuration. The microscope stage with an incorporated controller is connected to rubber heater with a dedicated cable. Measured sample temperature signals are received, and current to the rubber heater is transmitted by the controller. Measured signals from the controller are wirelessly sent to the server via the Internet router. The server compiles measurement data for analysis and graphing. Temperature log monitoring and temperature settings can be controlled remotely via a PC or smartphone.

Supplemental Figure 10: Temperature distribution measurement by thermography.

DISCUSSION:

Trajectory analysis of moving diatom cells is a useful approach to evaluating diatom motility. However, while a normal inverted microscope observes samples horizontally, it is not suitable for observations of the influence of gravity or floating movement in the vertical direction. Developed and described here is a vertical microscope stage with temperature control and attached to an inverted microscope, which has been rotated by 90°. This microscope stage with temperature control allows observation of temperature-dependent vertical motion of diatom cells.

A critical step within the protocol is the controller circuit design. A breaker circuit was implemented to ensure safety. When the sensor is disconnected from the sample or the microcontroller does not operate properly, the current to the heater is cut off by a circuit different from the microcontroller.

Since the control system adopted the PID system to control the current of the heater, a technique for finding the optimum parameter of the PID is required. Compared with the existing method, remote operation and monitoring are possible by Wi-Fi function, data collection on a server, and the temperature setting function. As the structure of the stage part attached to the microscope is complicated, simplification of this structure warrants a future study.

This equipment uses a heater to raise the temperature, but cooling is unpowered; therefore, the set temperature cannot be below room temperature. Cooling samples to temperatures lower than room temperature will require a complicated cooling device, which is under consideration for future work.

ACKNOWLEDGMENTS:

The authors have no acknowledgements.

DISCLOSURES:

The authors have no conflicts to disclose.

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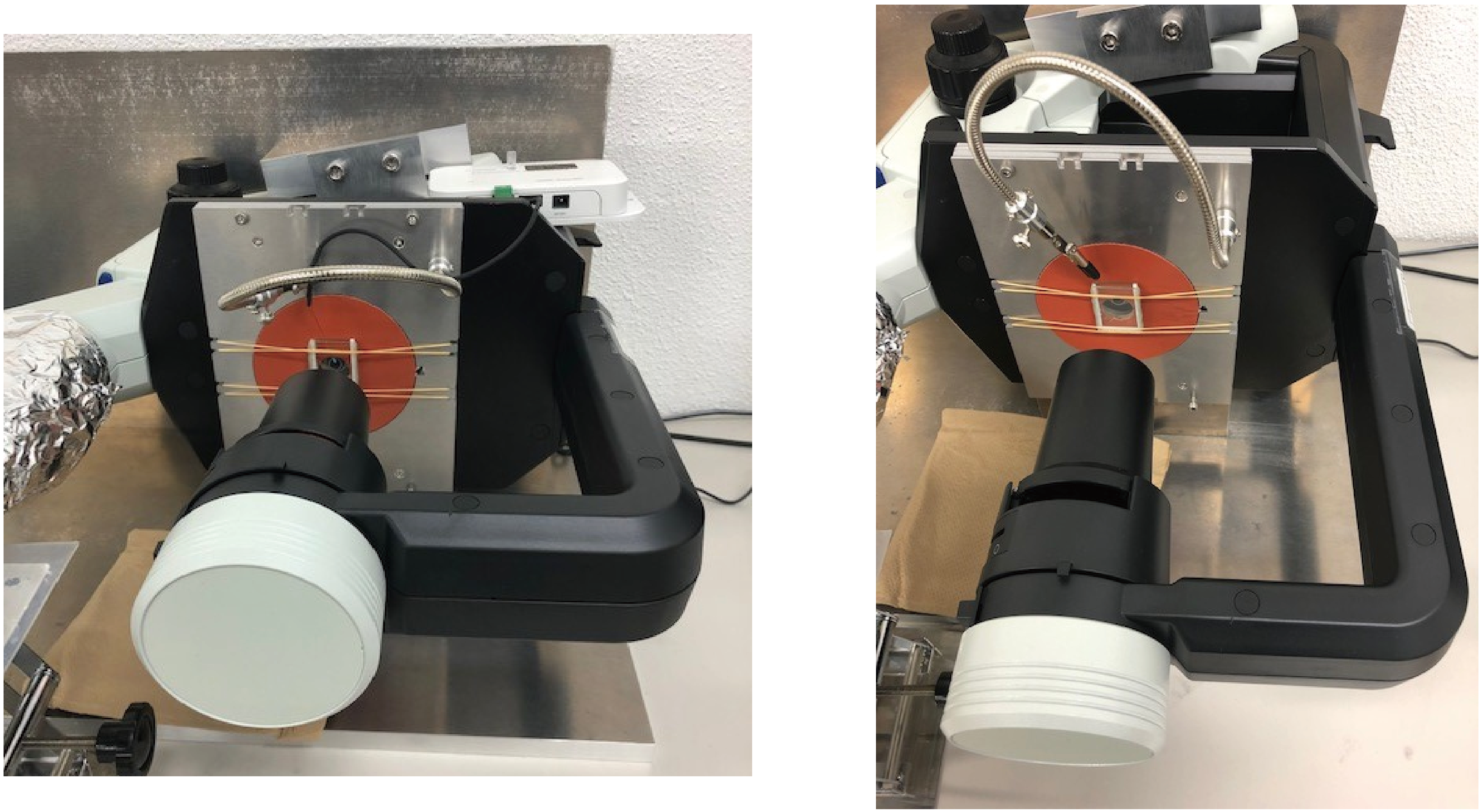
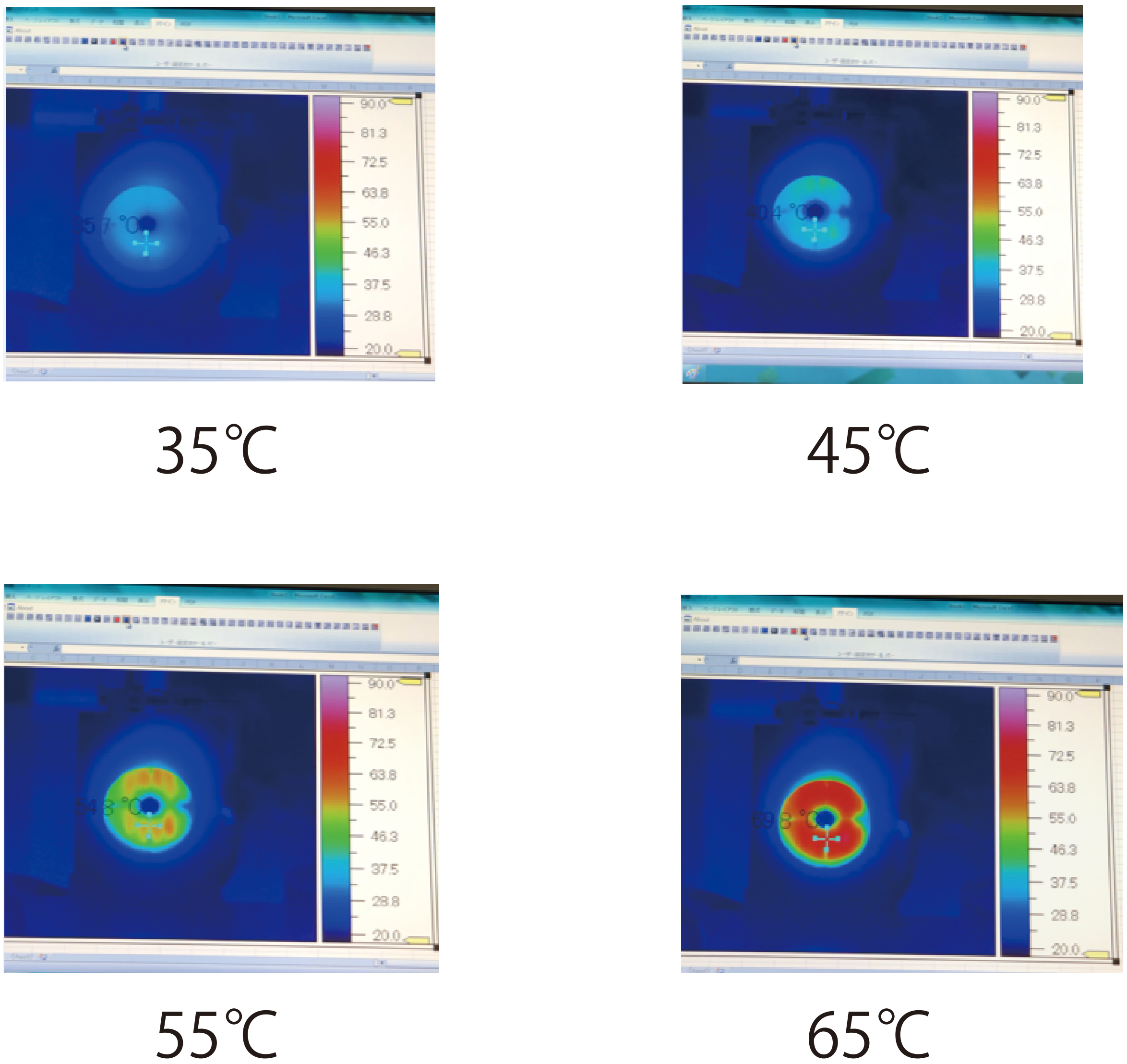
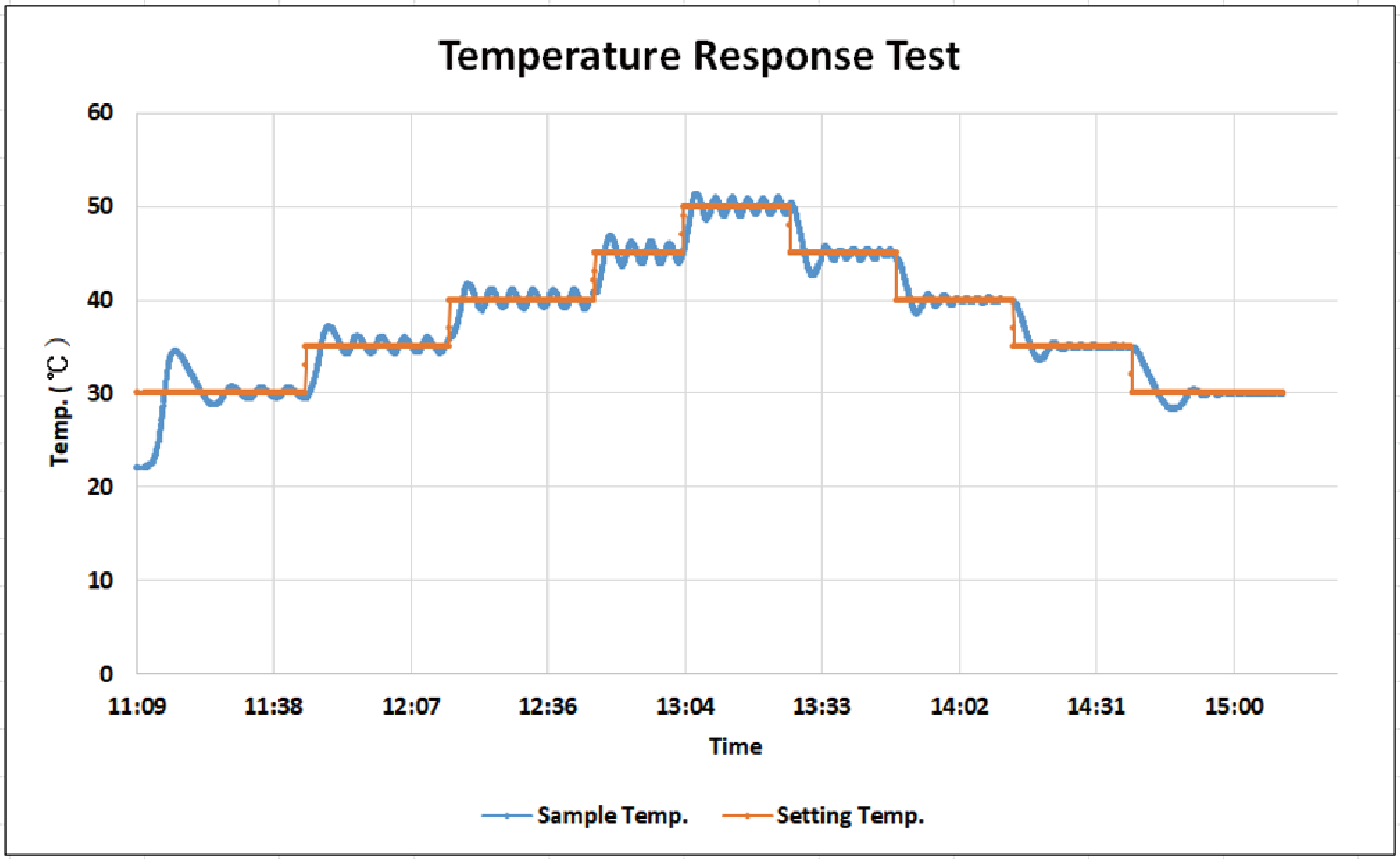


Figure2




Temperature Response Test
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40
30
20
10
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Temp. (℃)
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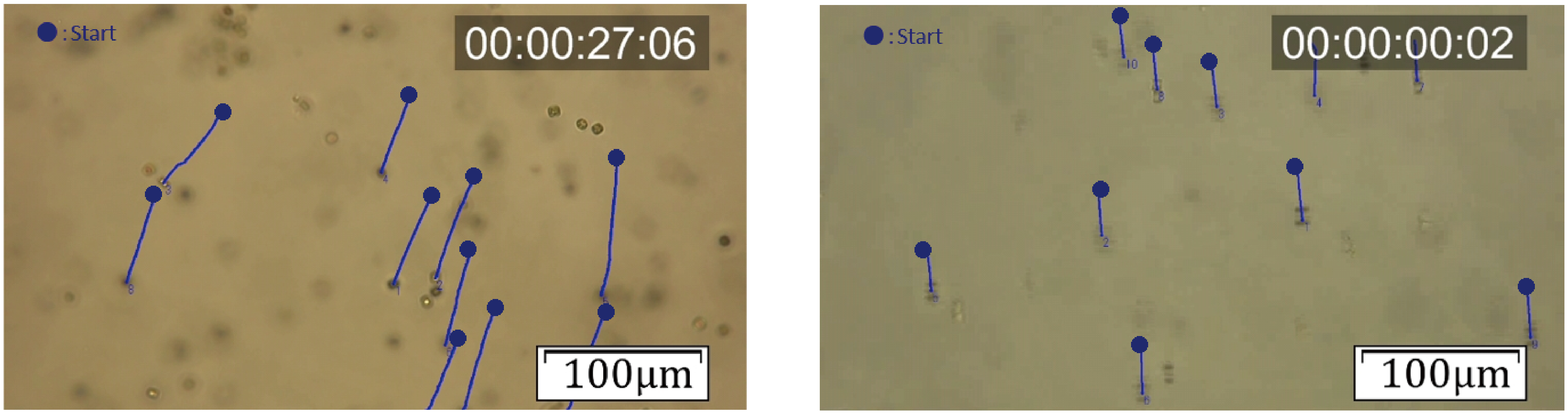
13:33
14:02
14:31
15:00
Time
Sample Temp.
Setting Temp.

Figure4



25 ℃ (27.06 seconds later)

40 ℃ (0.2 seconds later)

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AC adapter 12V2A	Akizuki Denshi Tsusho Co., Ltd.	AD-D120P200	Tokyo, Japan
ADS1015 Substrate	Akizuki Denshi Tsusho Co., Ltd.	adafruit PRODUCT ID: 1083	Tokyo, Japan
Alminium Plate (Back Side Plate)	Inoval Co., Ltd.	W 150mm×L 200㎜×T 1.5mm	Gifu, Japan
Alminium Plate (Forefront Plate)	Inoval Co., Ltd.	W 150mm×L 200㎜×T 2mm	Gifu, Japan
Alminium Plate (Middle Lower Plate)	Inoval Co., Ltd.	W 150mm×L 200㎜×T 4mm	Gifu, Japan
Alminium Plate (Middle Upper Plate)	Inoval Co., Ltd.	W 150mm×L 200㎜×T 5mm	Gifu, Japan
Aluminum Pedestal (Lower Plate)	Inoval Co., Ltd.	D 100mm×T 3mm (30Φ)	Gifu, Japan
Aluminum Pedestal (Upper Plate)	Inoval Co., Ltd.	D 100mm×T 3mm (30Φ)	Gifu, Japan
Bold Modified Basal Freshwater Nutrient Solution	Sigma-Aldrich Co. LLC	B5282-500ML	St. Louis, USA
Controller Case	Marutsu Elec Co., Ltd.	pff-13-3-9	Tokyo, Japan
CPU	Akizuki Denshi Tsusho Co., Ltd.	ESP-WROOM-02D	Tokyo, Japan
Inverted microscope	Olympus Corporation	CKX 53	Tokyo, Japan
Low temperature hardening epoxy resin adhesive	ThreeBond Co., Ltd.	TB2086M	Tokyo, Japan
Multi-turn semi-fixed volume Vertical type 500	Akizuki Denshi Tsusho Co., Ltd.	3296W-1-501LF	Tokyo, Japan
OLED module	Akihabara Inc.	M096P4W	Tokyo, Japan
Pressed Cork (For supporting electrode)	Tera Co., Ltd.	W 42mm×L 30㎜	Ishikawa, Japan
Pressed Cork (Lower Disk)	Tera Co., Ltd.	D 100mm×T 0.5mm (20Φ)	Ishikawa, Japan
Pressed Cork (Upper Disk)	Tera Co., Ltd.	D 100mm×T 2.5mm (20Φ)	Ishikawa, Japan
Rotary encoder with switch with 2 color LED	Akizuki Denshi Tsusho Co., Ltd.	P-05772	Tokyo, Japan
Silicone rubber heater	Three High Co., Ltd.	D 100mm×T 2.5mm (20Φ)	Kanagawa, Japan
Substrate	Seeed Technology Co., Ltd.	mh5.0	Shenzhen, China
Temperature sensor	Akizuki Denshi Tsusho Co., Ltd.	NXFT15XH103FA2B050	Tokyo, Japan
Three-terminal DC / DC regulator 3.3 V	Marutsu Elec Co., Ltd.	BR301	Tokyo, Japan

Universal Flexible Arm	Banggood Technology Co., Ltd.	YP-003-2	Hong Kong, China
USB cable USB-A - MicroUSB	Akizuki Denshi Tsusho Co., Ltd.	USB CABLE A-MICROB	Tokyo, Japan
Video Canera	Sony Corporation	HDR-CX590	Tokyo, Japan

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Department:	Department of Physics
Institution:	Tokyo University of Science
Title:	Fabrication of Microscope Stage for Vertical Observation with Temperature Control Function

Signature: Y. Matsukawa Date: Feb. 04, 2019

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Response to Editorial comments

We are greatly appreciated for your thoughtful and supportive comments. We have revised our manuscript in response to your suggestions and hope that this improved manuscript is acceptable for publication.

1. We corrected the manuscript according to the instructions of the editor.
2. The manuscript was corrected according to the editor's specific comment described in the manuscript.
3. The representation of the protocol section has been made imperative tense.
4. We removed some of the subheadings and combined few shorter steps in the protocol section.
5. In the protocol section, we have described as specific as possible how the steps are performed.
6. We removed personal pronouns from the Protocols section.
7. The theme of this research is the fabrication of a microscope stage with temperature control. This is a device attached to a microscope tilted by 90 degrees to observe the vertical behavior of the sample. The experiment using diatoms is an application example of the microscope stage to the last. Therefore, it is difficult to deeply discuss the experimental results. However, using this device, we were able to directly observe and visualize the effect of thermal convection on the vertical floating phenomenon of diatom cells. This is described in REPRESENTATIVE RESULTS.
8. We described the details of the glass slide in 5.2.1 in the manuscript.
9. We have submitted to "journal Microscopy and Microanalysis". It is currently under review but the acceptance date is unclear. Therefore, we deleted the journal name.
10. Figures from the previous publication are not used.
11. All figures are uploaded in illustrator format.
12. The materials table is sorted in alphabetical order.

[Reviewer #1]

Major Concerns

We observe using a self-made slide glass, and a petri dish on the market. The self-made slide glass is attached two slide glass with a width and is reinforced with an adhesive around it to make it a leakproof structure. This self-made slide glass will post a thesis in the near future. When we observe samples using a commercially available petri dish, close around the petri dish with a special plumbing tape.

A. Initially, we used an acrylic board as a preliminary experiment to make a simpler structure. However, to raise the sample temperature to 50 ° C, it needed to raise the temperature of the rubber heater to around 70 ° C. At this time, a situation occurred that the acrylic board was slightly deformed by the temperature. Therefore, we changed the material to aluminum. Although heat resistance has been improved by using aluminum as the material, aluminum has the problem of large heat dissipation and a difficulty in raising the temperature. Therefore, we considered the use of insulation and used cork with good processability as insulation.

   As you pointed out, we think it is possible to simply attach a rubber heater to the surface of the aluminum plate. We think that it will be the structure which prepares two aluminum boards in order to store a rubber heater and store a rubber heater in the upper board. In this case, since there is no room for storing the heater wires and sensor wires, it is fixed to the aluminum surface with tape. We thought that this was not good in appearance and also in safety. Given the effect of aluminum heat dissipation on the microscope, it cannot be directly attached to the microscope stage. Therefor we attached the insulation. Furthermore, in order to secure a space for mounting the heater and sensor board, the aluminum plate was divided into multiple sheets. We made the current structure to meet these conditions. As you point out, we think that simplification is necessary, and we will consider it as a future issue.

B. Since Figures 1-1 to 1-4 have been changed in number to Figure S1 A to D, the explanation will be given with new numbers. The four holes (4-M3 countersink) on the plates in Figure S1 A and B are for screwing Figure S1 A and B. Figure S1 A and B are screwed from the surface of A. Figure S1 C and D are screwed from the back of D. The method of assembly has been added to 1.11. The design drawings contain information on production. In order to make it easy to understand, we added a drawing without lines to the supplementary information S2 and added it to the protocol.

C. As you point out, the observation is mainly on a slide glass or petri dish made vertical, but the temperature distribution of the rubber heater is relatively uniform, so it is possible to observe samples within the diameter of the rubber heater.

D. Similar products are available on the market. However, the products on the market do not have Wi-fy function, Log management on server, or temperature setting function. In addition, we made a dedicated controller because we could add our own improvement in the future. The temperature range of this device is 80 ° C as a design value, but as you point out, it is considered unnecessary for biological systems. In our laboratory, the temperature used for floating observation of diatoms is up to 40 ° C. Therefore, we changed the temperature range to 50 ° C. Along with this, the description of Introduction has changed.

As you pointed out, we think it is possible to control temperature using a single aluminum plate. However, as mentioned in Answer A, it is difficult with one plate because it is necessary to storage the heater electrode and fix the heater and sensor wires. There is also a way to fix them with tape, but there is also the risk of inducing trouble. If we don't consider the appearance, there will be an inexpensive way. However, this will be an issue for the future.

[Reviewer #2]

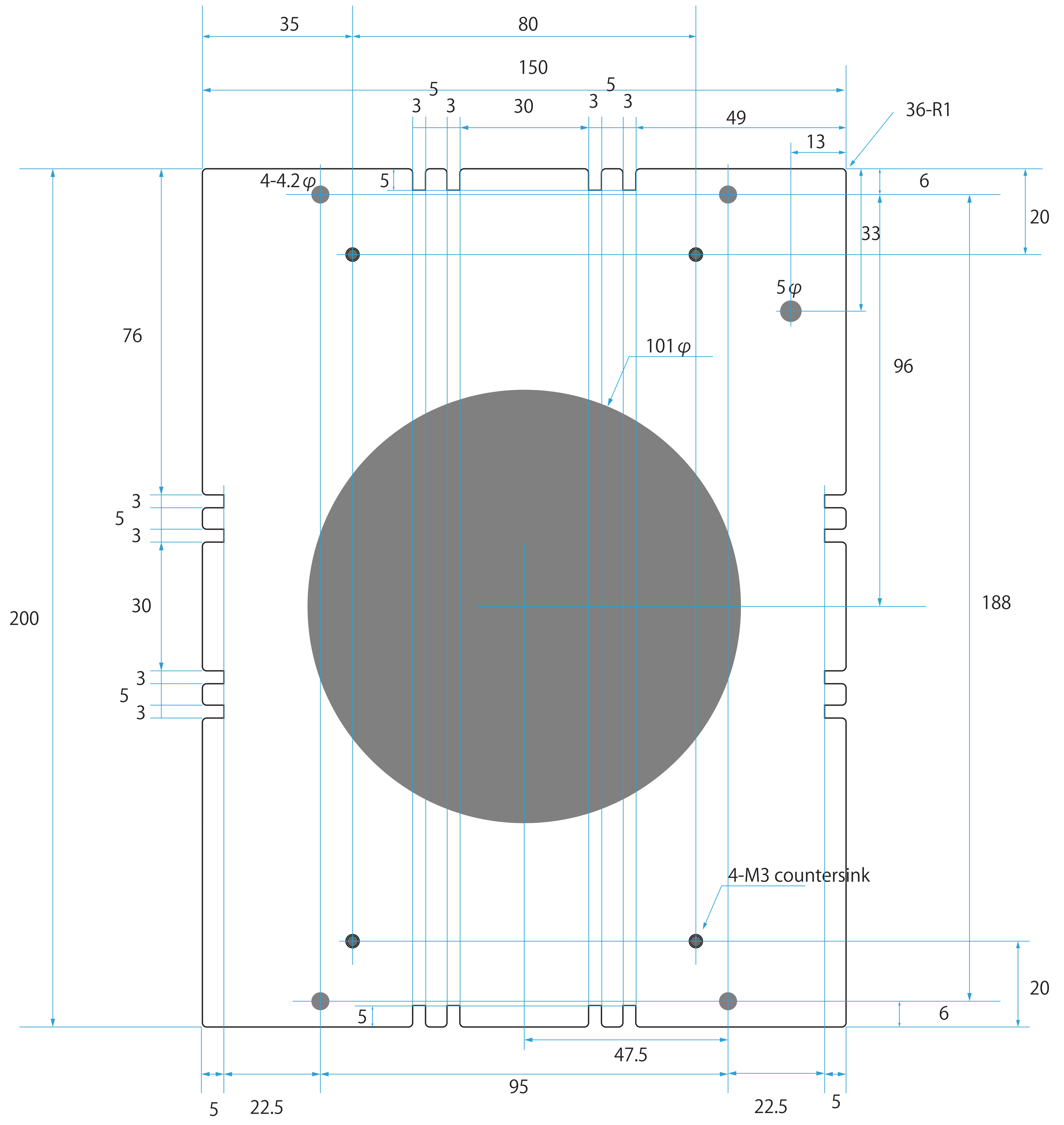
Major Concerns

1. Basically, it is not a dissertation on diatom observation, so it will not be described in detail, but the method of preparation of diatoms, the observation method, and the conditions of the microscope are added to 5.1.1, 5.1.2 and 5.1.3 respectively. The result of measurement data has been added to REPRESENTATIVE RESULTS. Details of these experiments will post a thesis in the near future.
2. As it is not a paper of the sideways inverted microscope, it will not be described in detail, but an outline has been added in protocol 6 “Design of the sideways inverted microscope”. The 90-degree tilted inverted microscope is being posted in the journal "Microscopy & Microanalysis" with the title "Design of sideways inverted microscope stand to observe mobility of diatom cells".

Minor Concerns

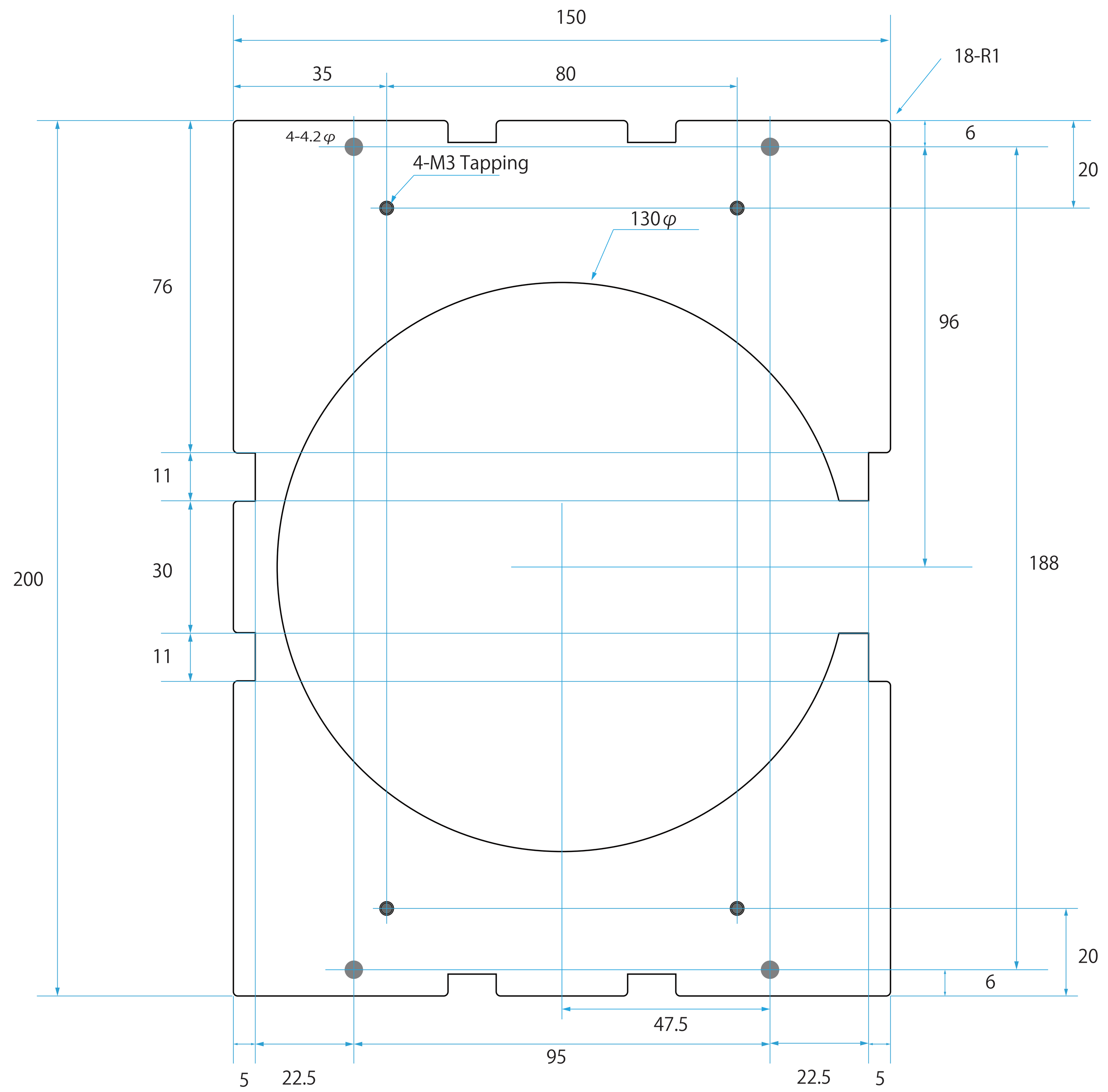
1. We thank you for your advice. However, this paper relates to the hardware of the microscope stage with temperature control function. In the future, we would like to conduct experiments as pointed out using this device and submit a paper.

2. "Responce" in Figure 10 has been modified to "Response" in Figure 2 with the new number.
3. Figure 11 was a mistake in Figure 10, but it was changed to Figure 2.



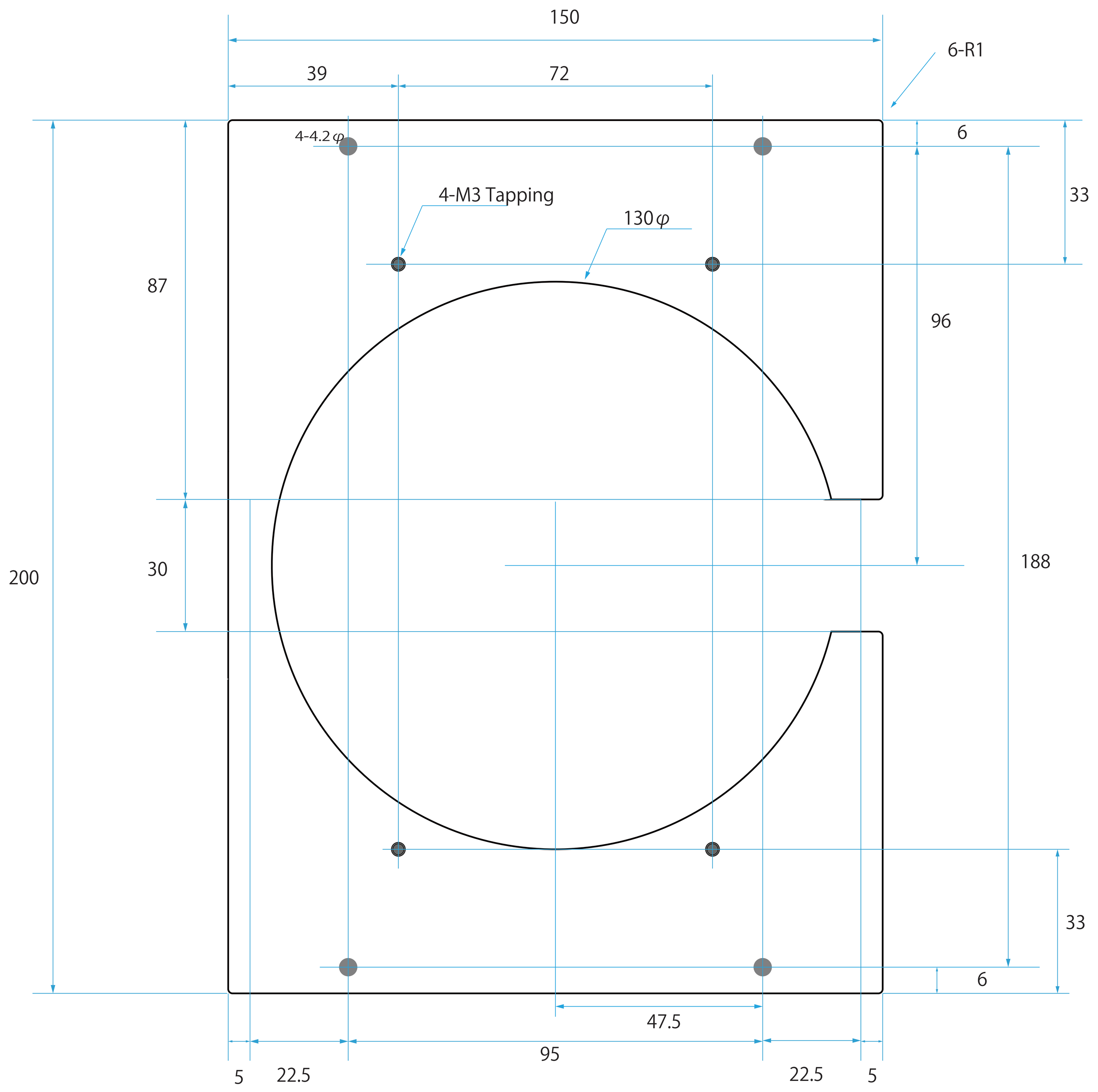
(Forefront plate)

[1A]



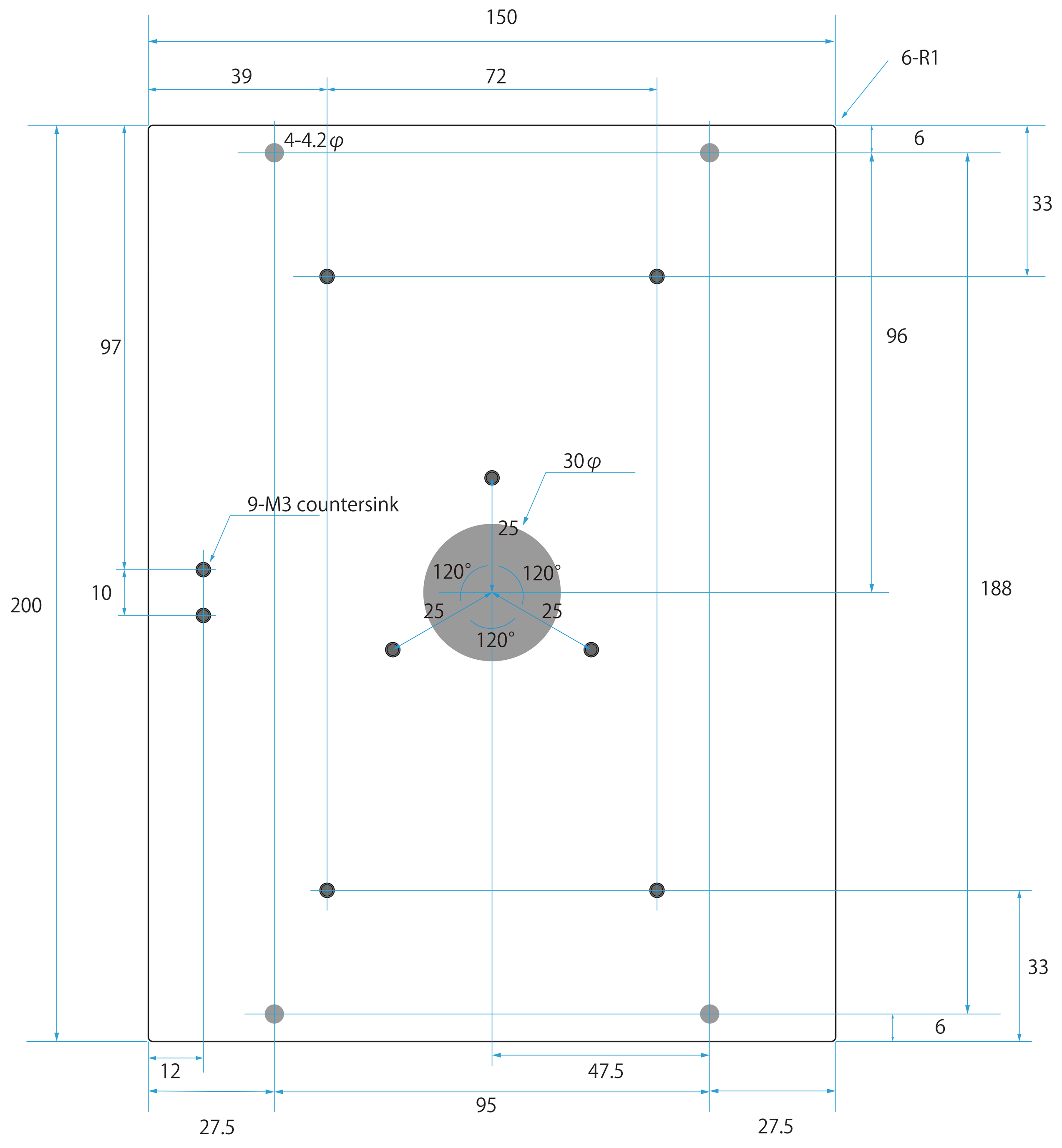
(Middle upper plate)

[1B]



(Middle lower plate)

[1C]



(Backside plate)

[1D]

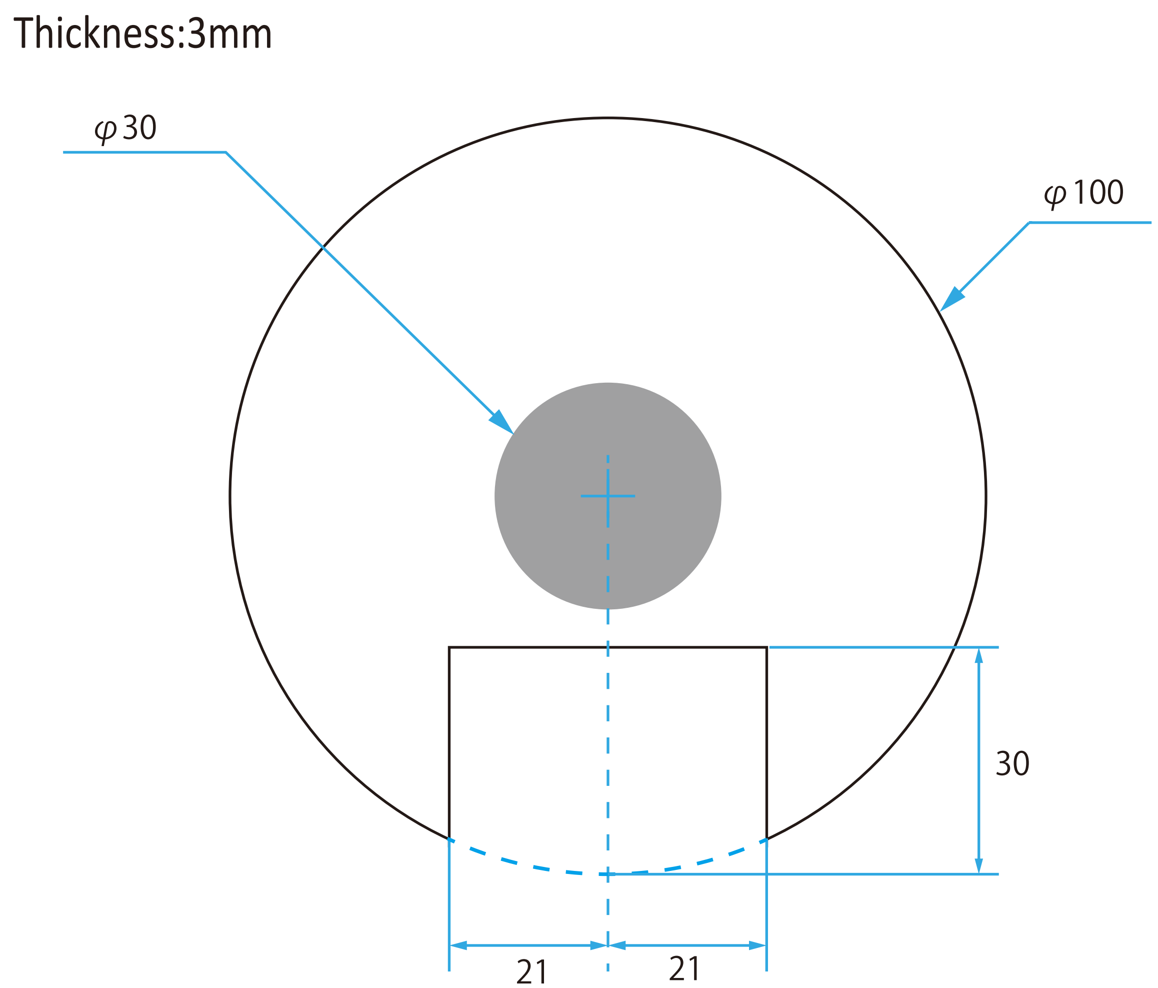
Supplemental Figure2

（Forefront plate ） [2A]

（Middle upper plate ） [2B]

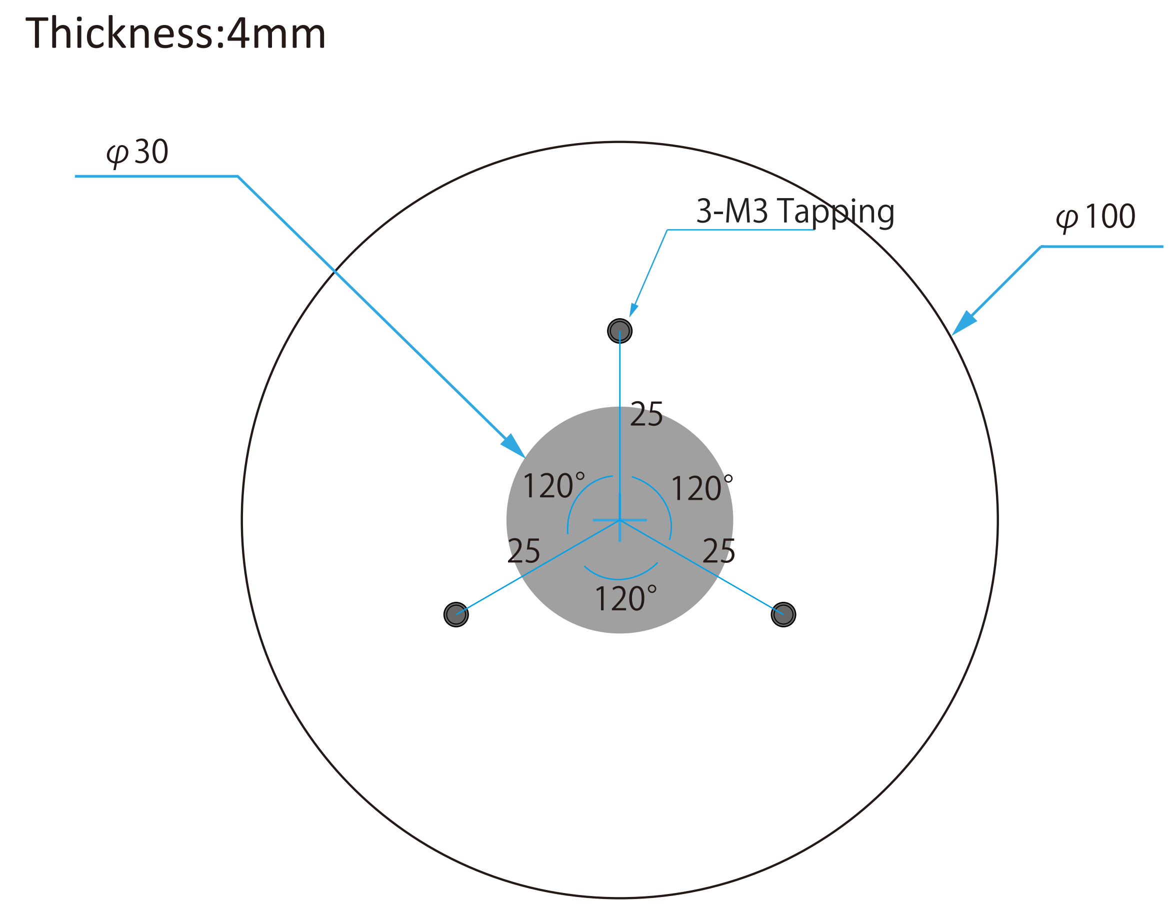
（Middle lower plate ） [2C]

（Backside plate ） [2D]



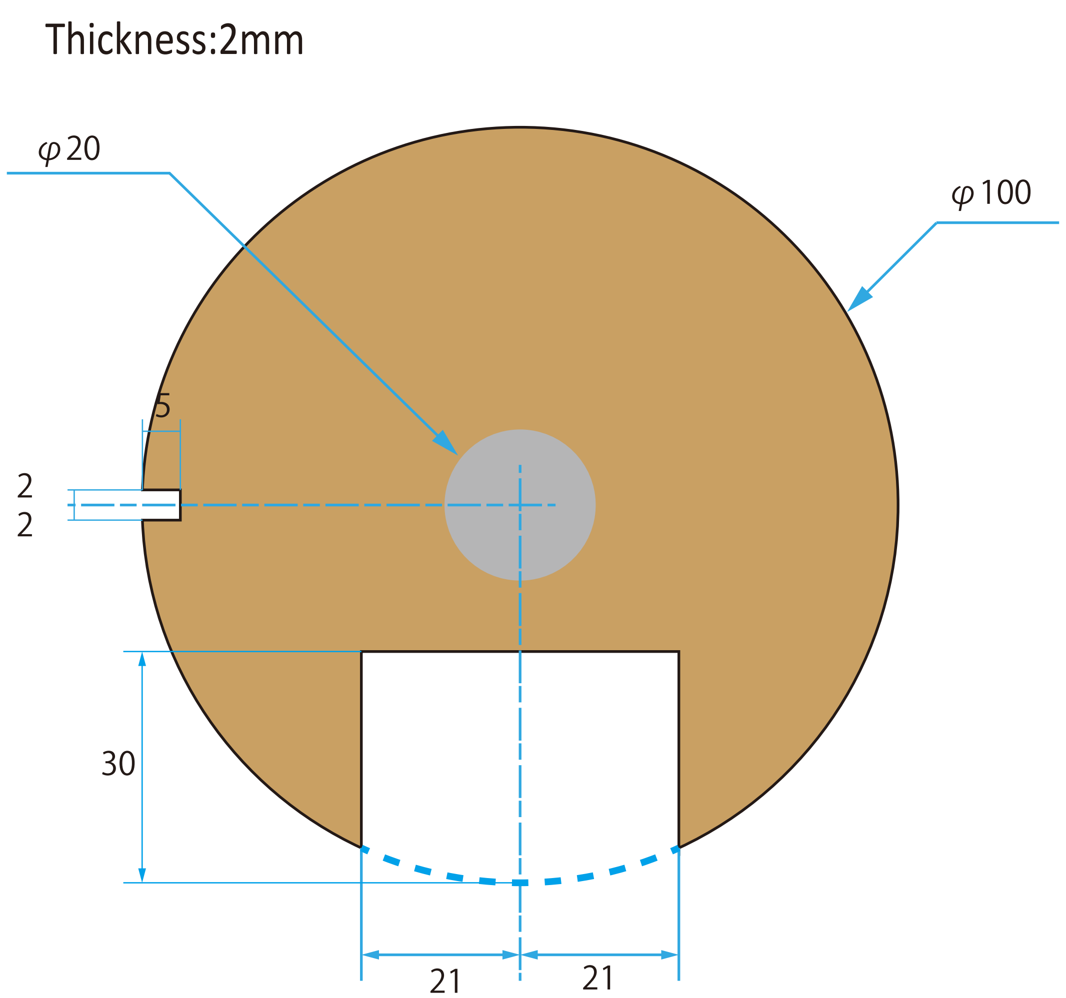
(Upper plate)

[3A]



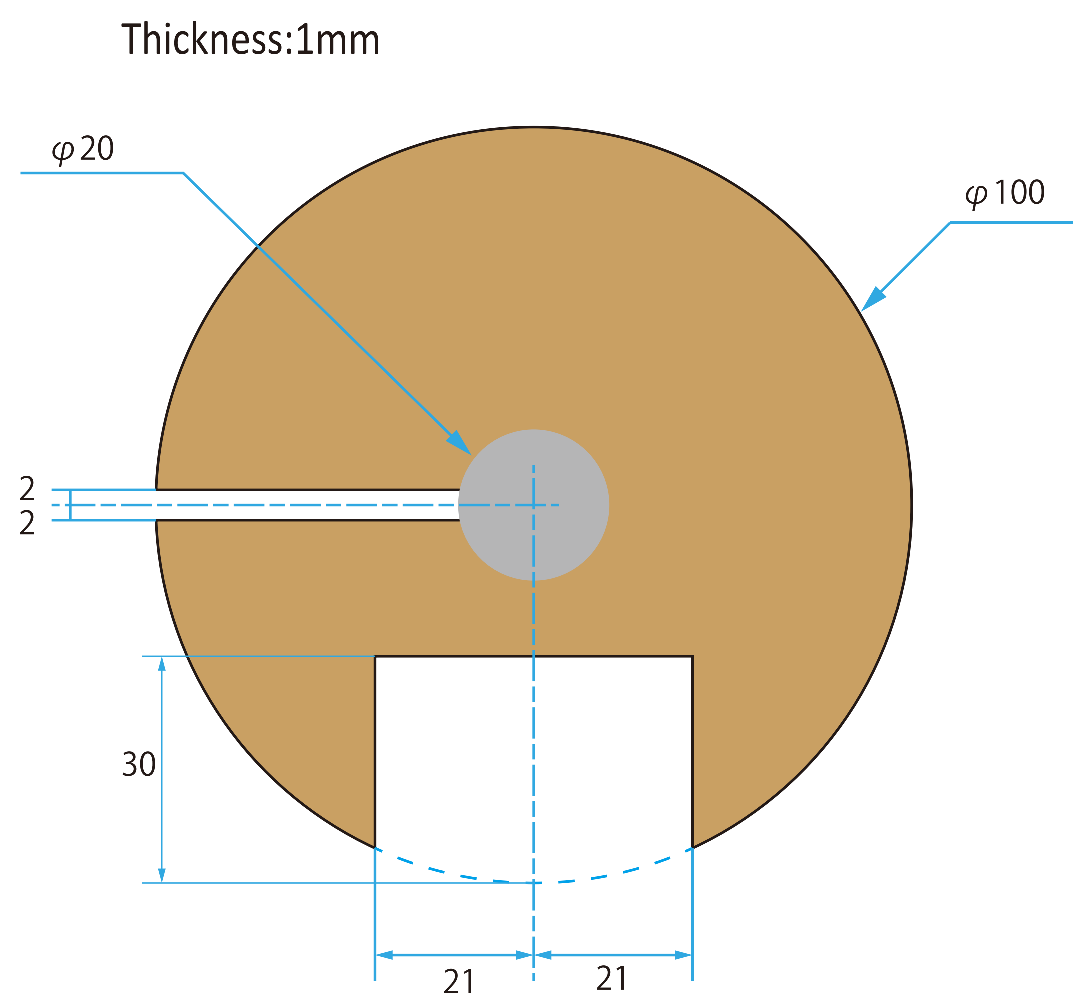
(Lower plate)

[3B]



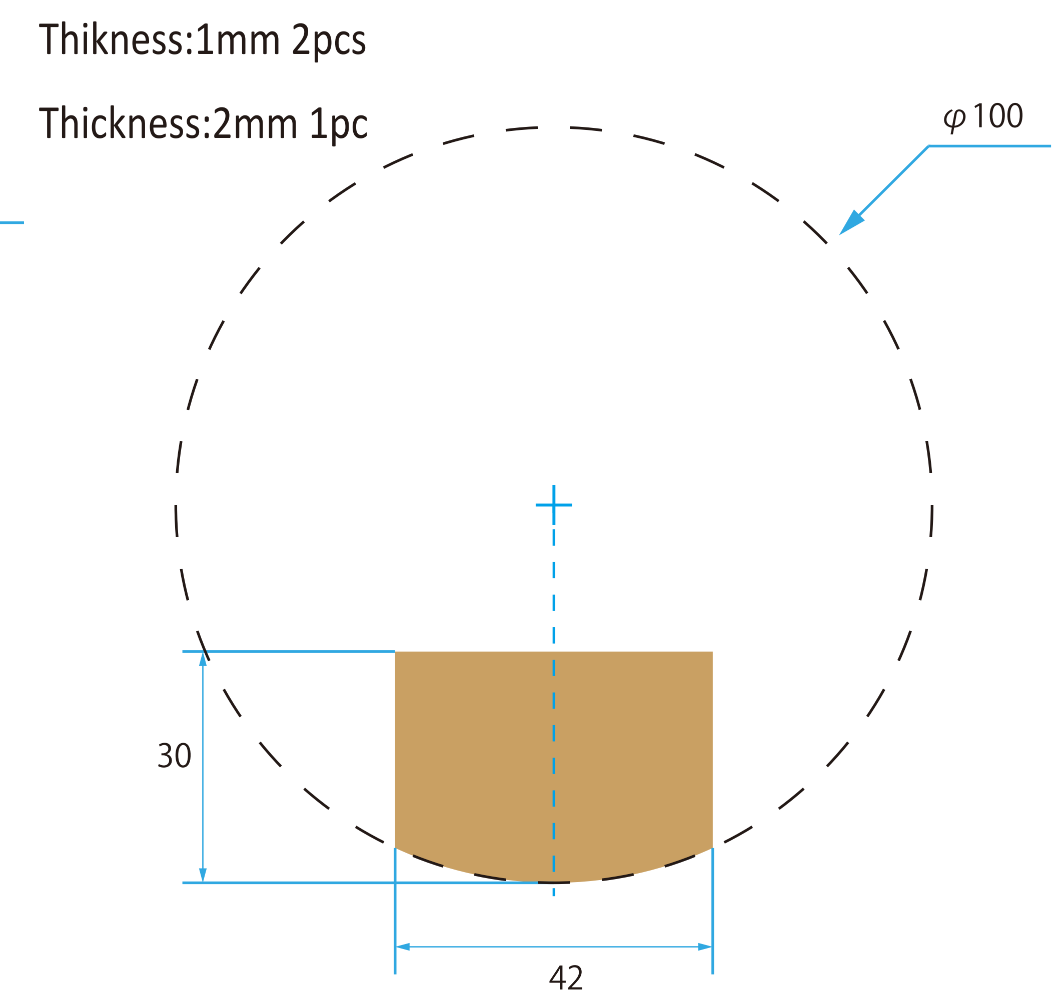
（Upper disc）

[4A]



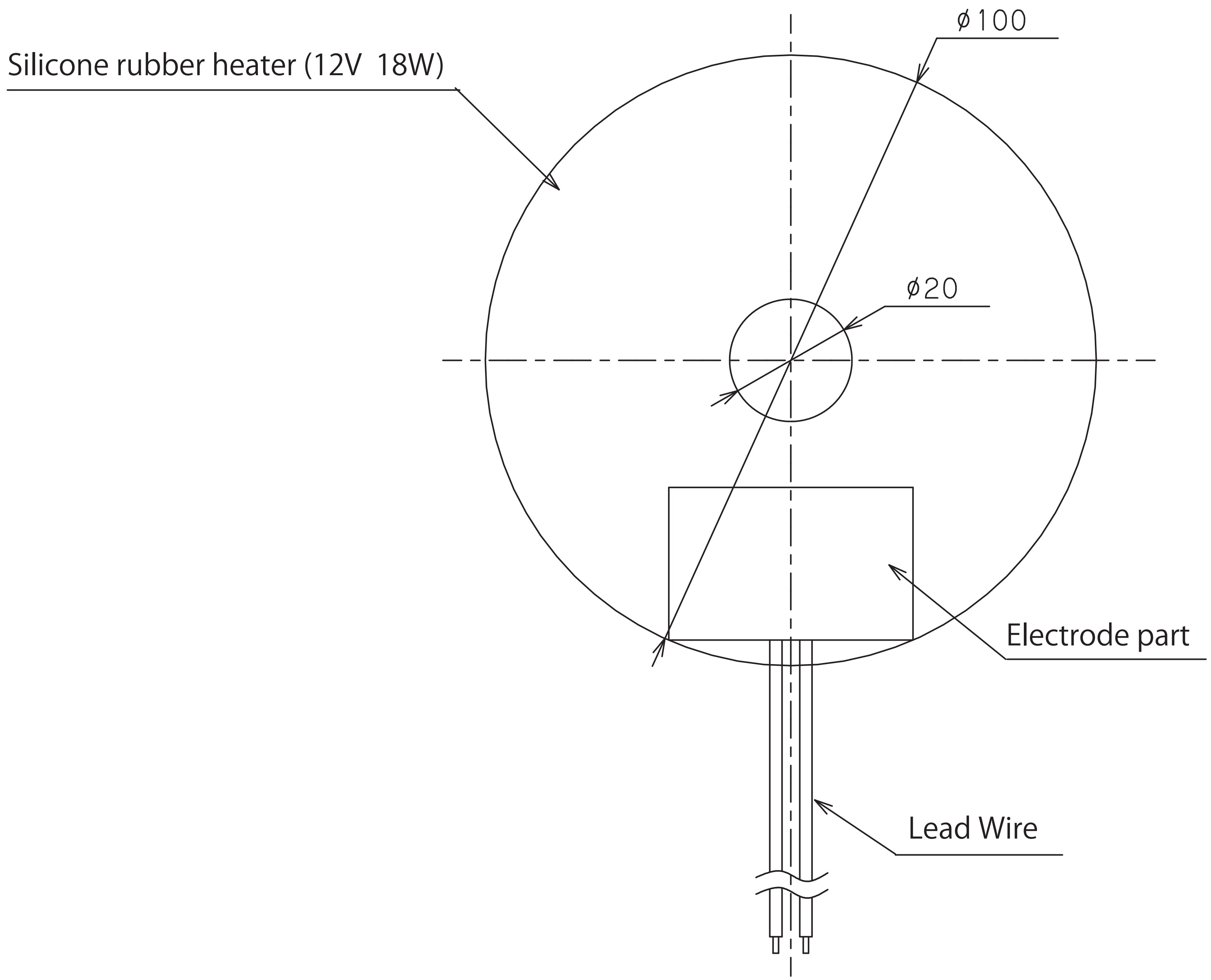
（Lower disc）

[4B]

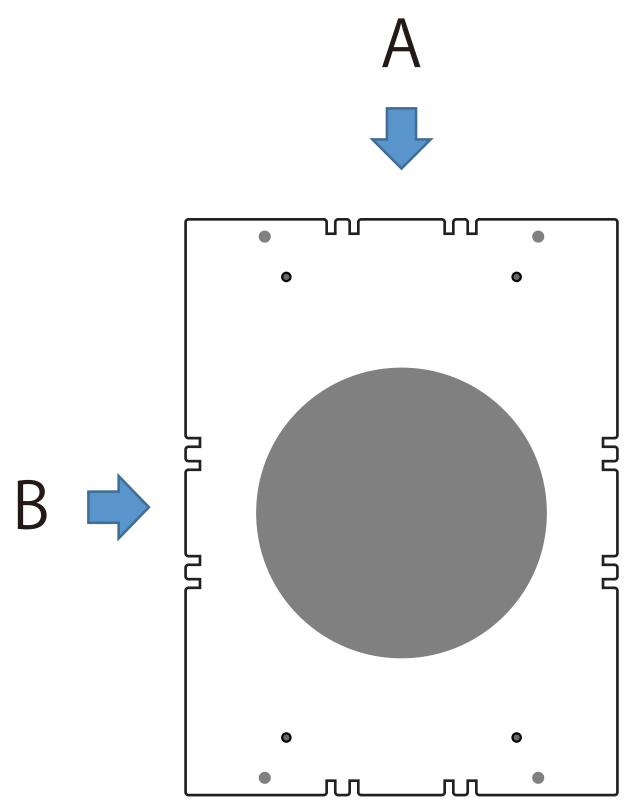


（For spporting electrode part of heater）

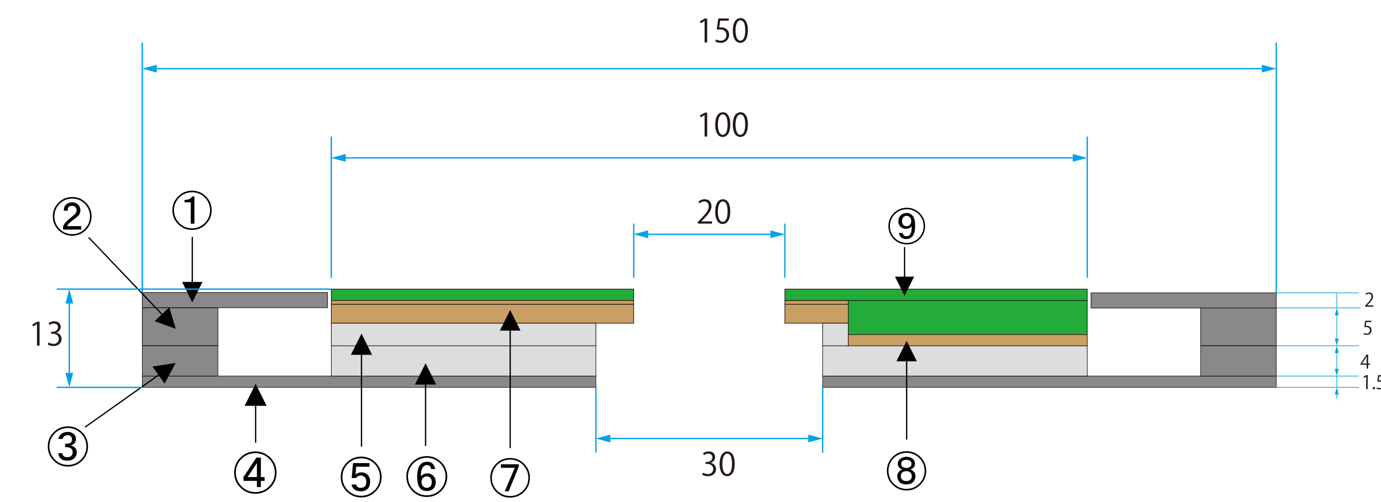
[4C]



Silicone rubber heater (12V 18W)
ϕ100
ϕ20
Electrode part
Lead Wire

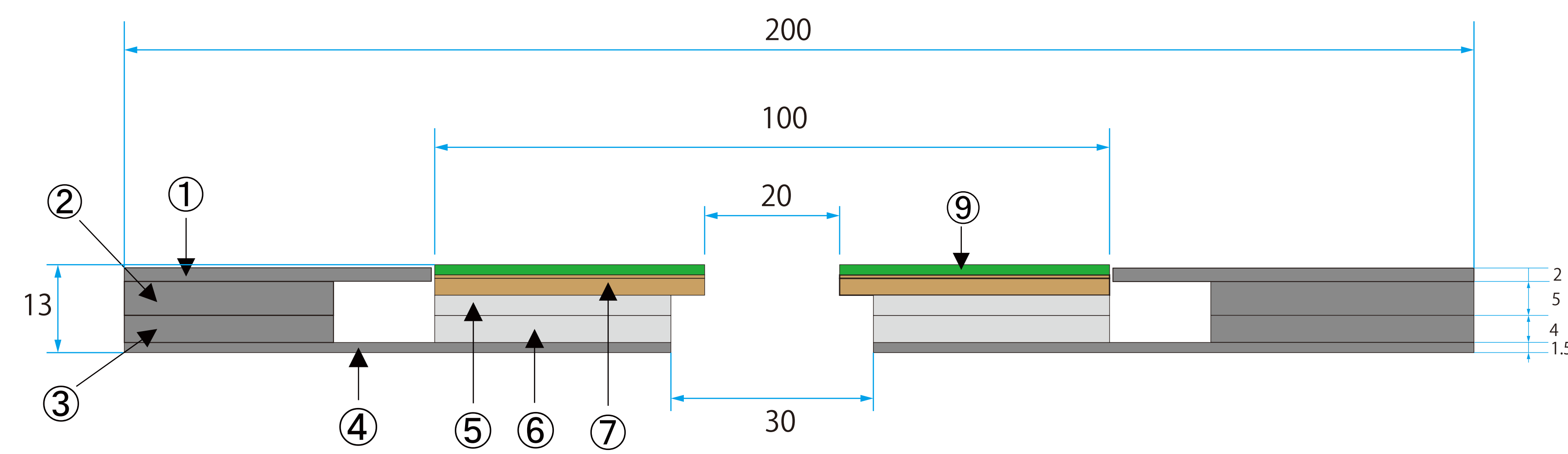


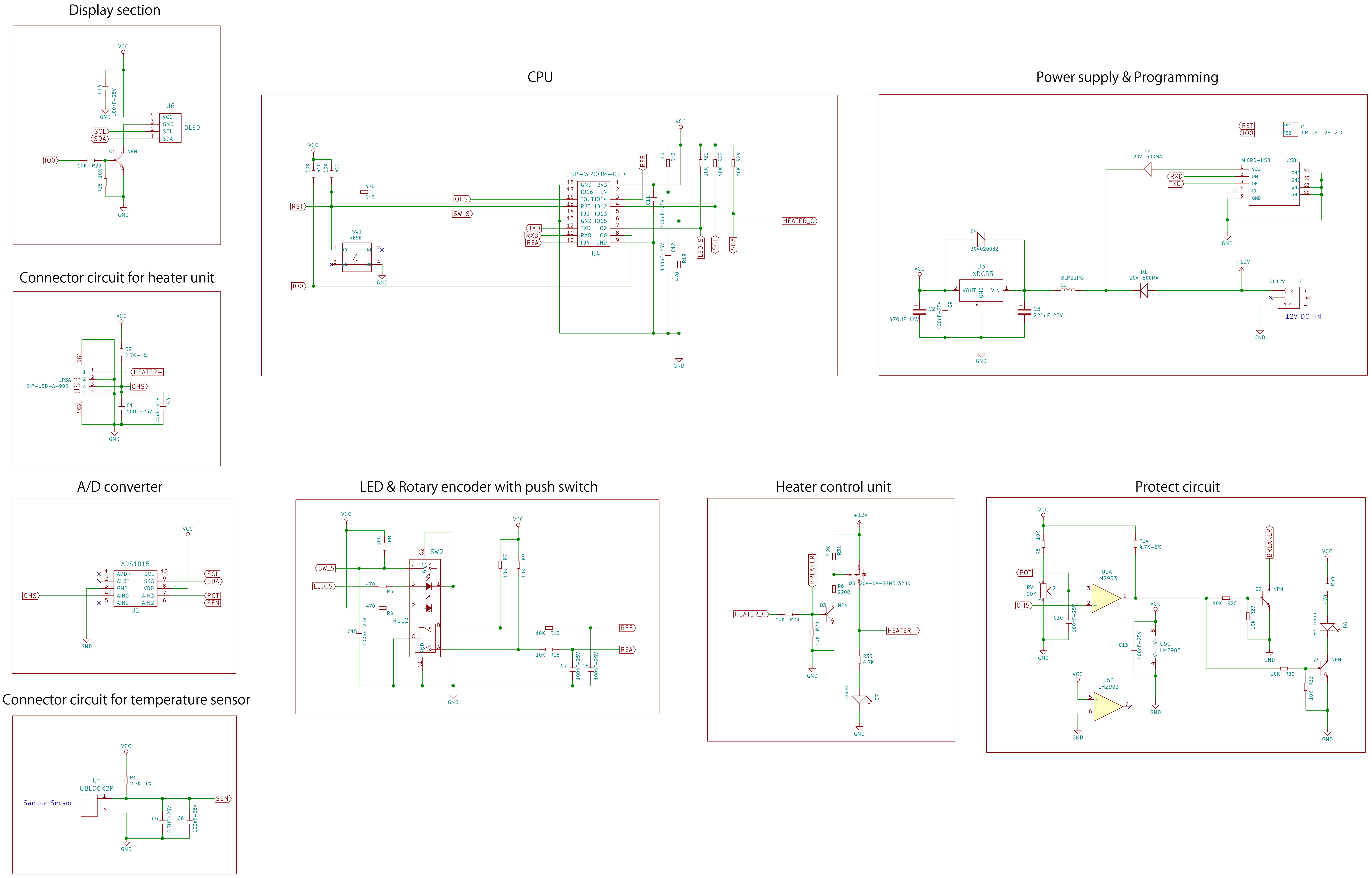
A Cross-sectional view as seen from short side direction

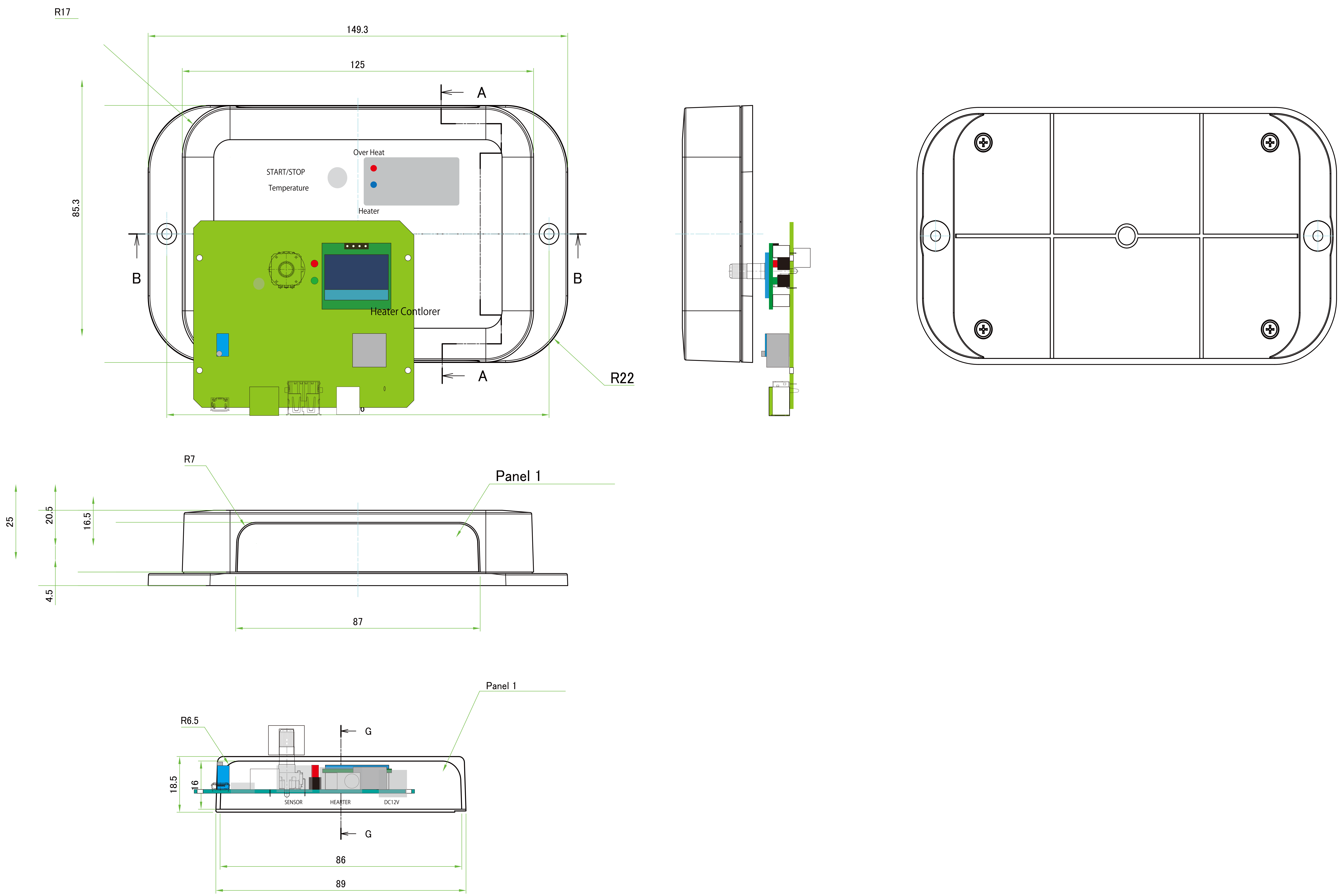


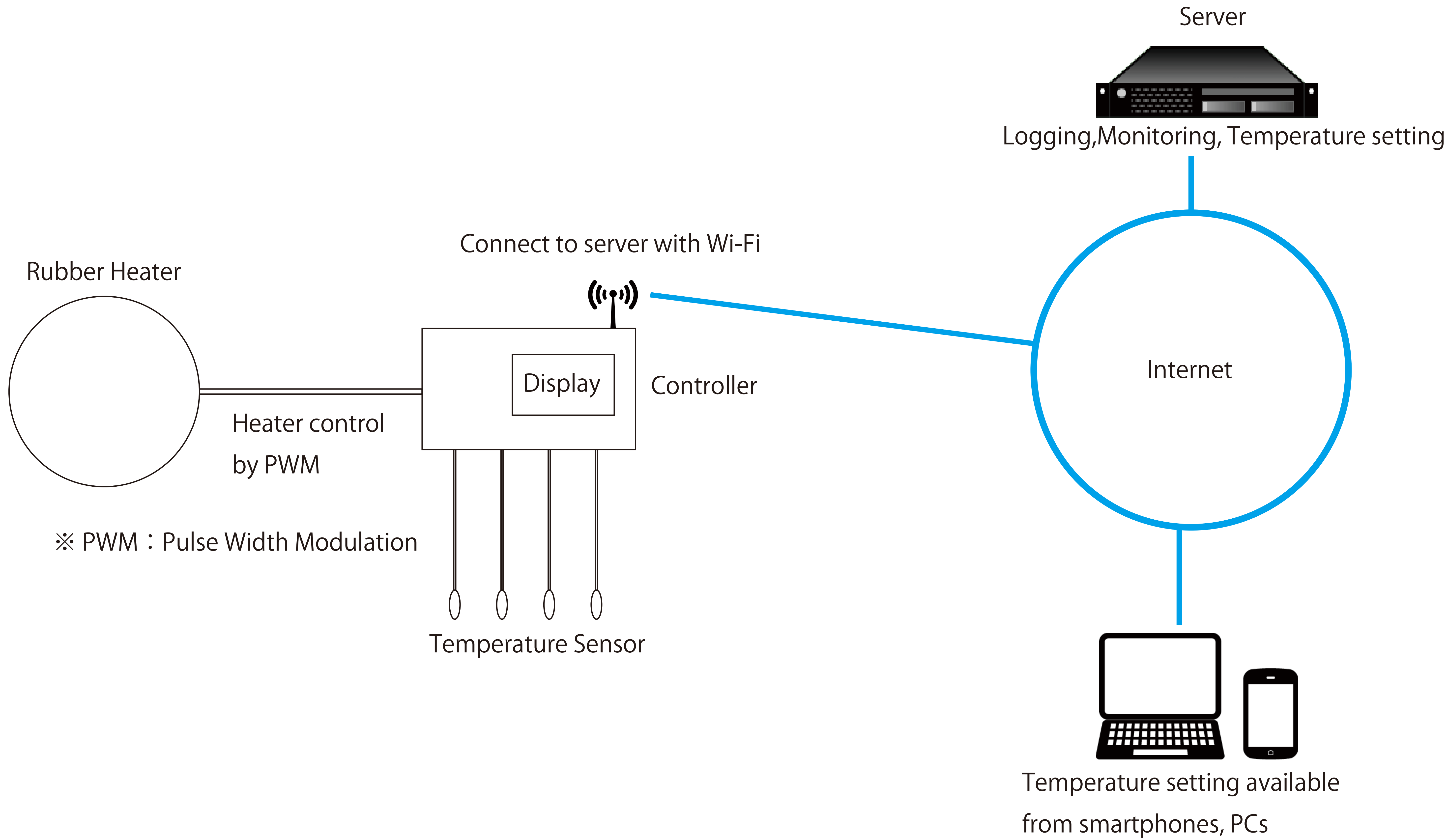
① Forefront plate → Supplemental Figure [1A]
② Middle upper plate → Supplememntal Figure [1B]
③ Middle lower plate → Supplememntal Figure [1C]
④ Backside plate → Supplememntal Figure [1D]
⑤ Upper side pedestal → SupplementalFigure [3A]
⑥ Lower side pedestal → Supplemental Figure [3B]
⑦ Cork disc → Supplemental Figure [4A],[4B]
⑧ Cork disc → Supplemental Figure [4C]
⑨ Silicone rubber heater → Supplemental Figure 5

B Cross-sectional view as seen from long side direction

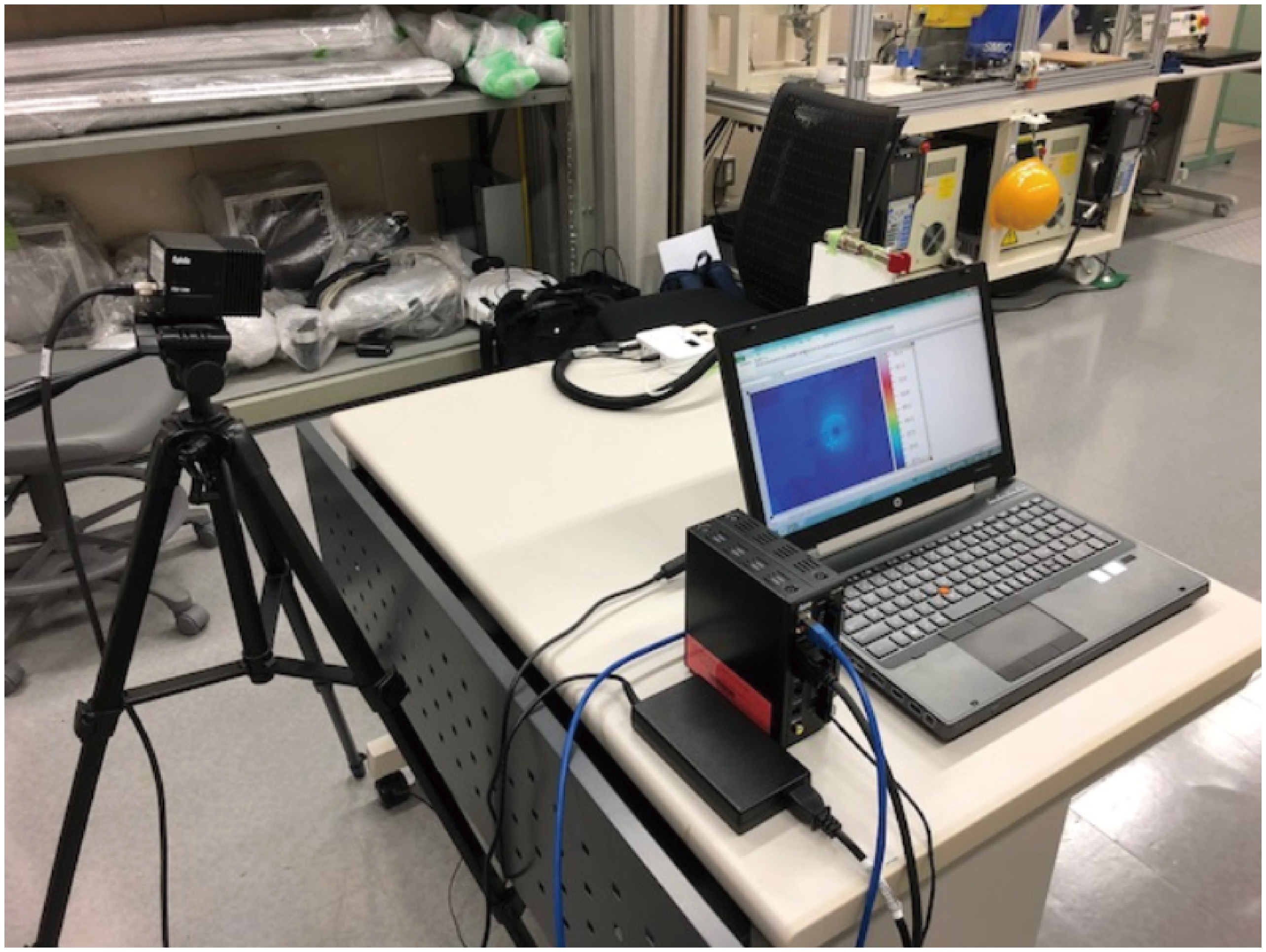



Display section
CPU
Power supply & Programming
Connector circuit for heater unit
A/D converter
LED & Rotary encoder with push switch
Heater control unit
Protect circuit
Connector circuit for temperature sensor
ESP-WROOM-02D
LXDC55
ADS1015
LM2903
Sample Sensor
12V DC-IN


R17
149.3
125
A
85.3
B
Over Heat
START/STOP
Temperature
Heater
B
Heater Contlorer
R22
A
R7
Panel 1
25
20.5
16.5
4.5
87
Panel 1
R6.5
G
18.5
16
SENSOR
HEATER
DC12V
G
86
89


Server
Logging,Monitoring, Temperature setting
Connect to server with Wi-Fi
Rubber Heater
Display
Controller
Internet
Heater control
by PWM
※ PWM：Pulse Width Modulation
Temperature Sensor
Temperature setting available
from smartphones, PCs

Supplemental Figure10



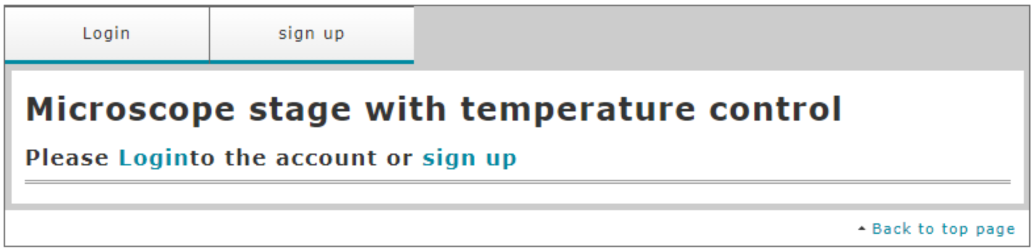
[Supplementary information]

The server operation manual

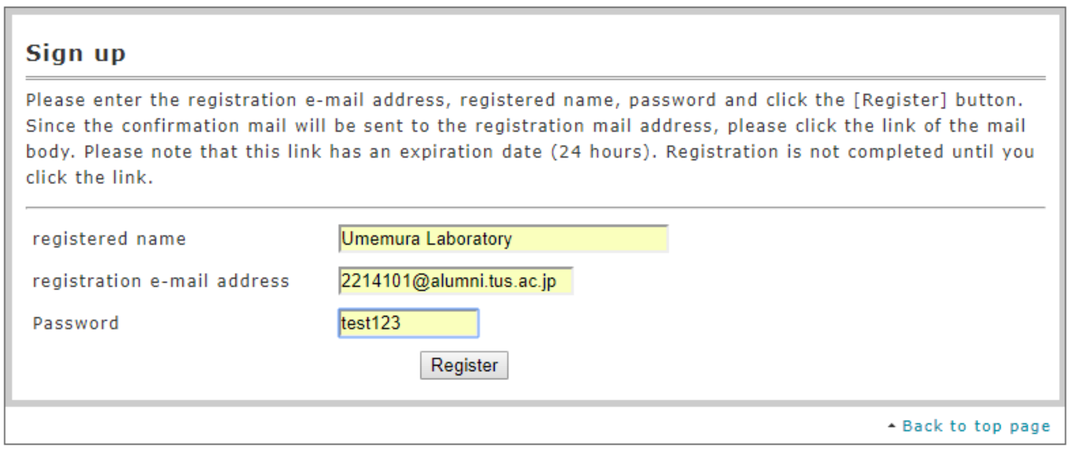
1. Sign up

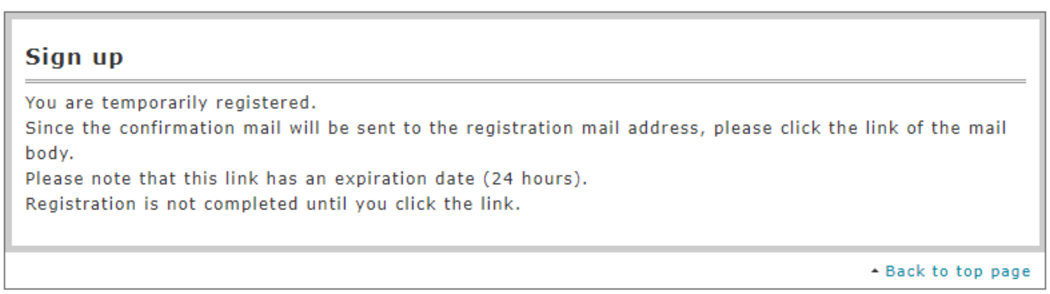
1.1 User Registration

1.1.1 Display the server login screen at the login URL.



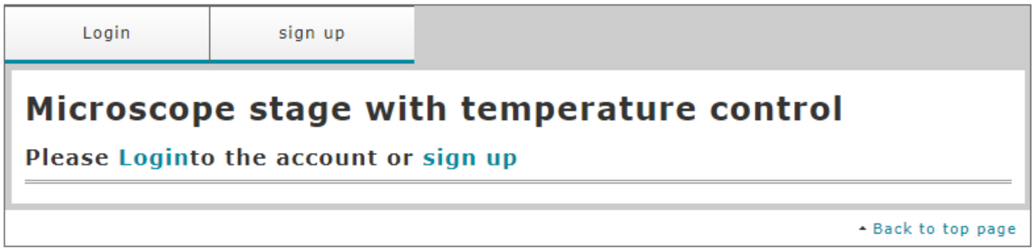
1.1.2 Click [sign up] button. Enter necessary items according to the message.



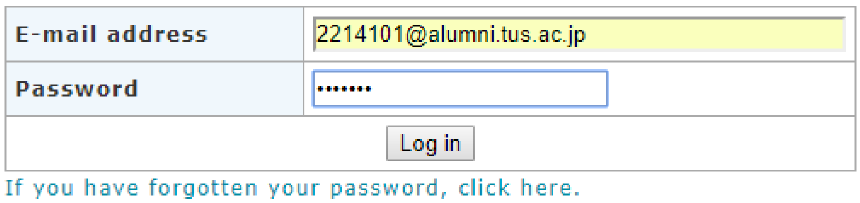


1.2 Terminal Registration

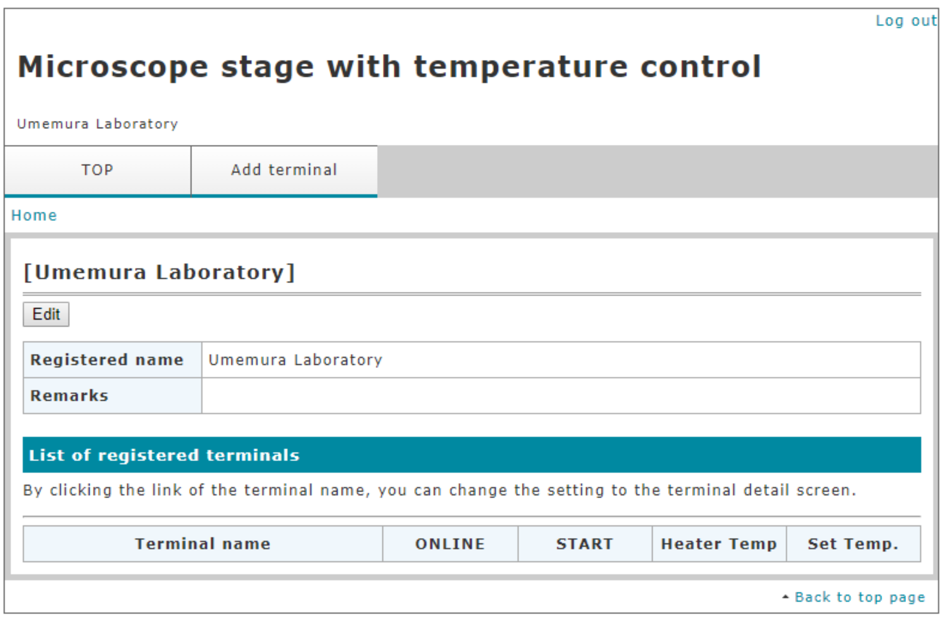
1.2.1 Display the server login screen at the login URL.



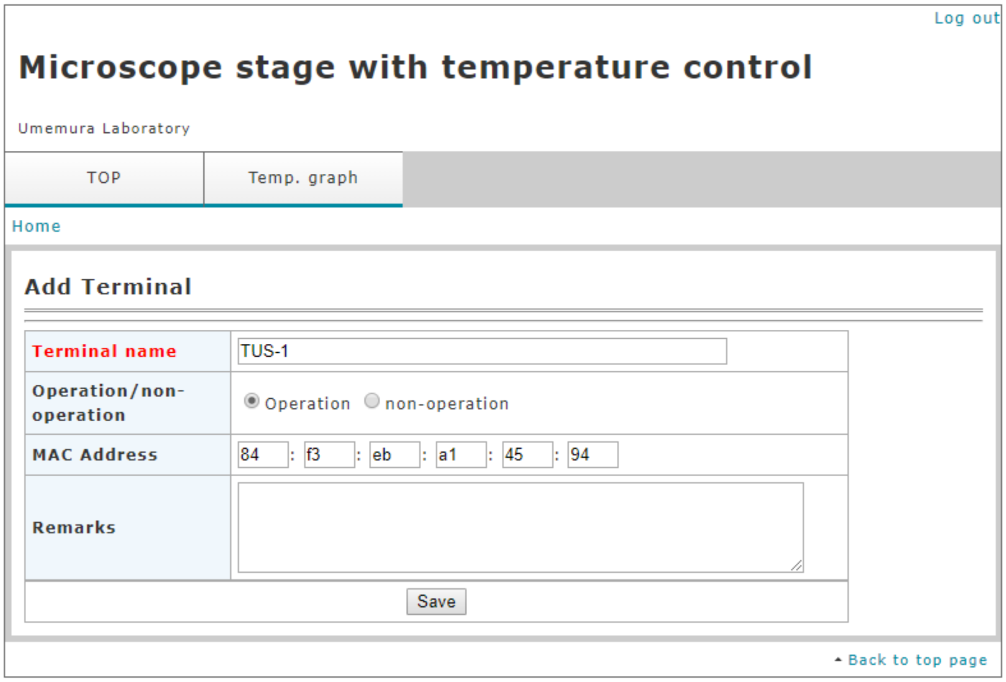
1.2.2 Click [Login] button. Enter e-mail address and password.



1.2.3 Click the [Login] button to display the next screen.



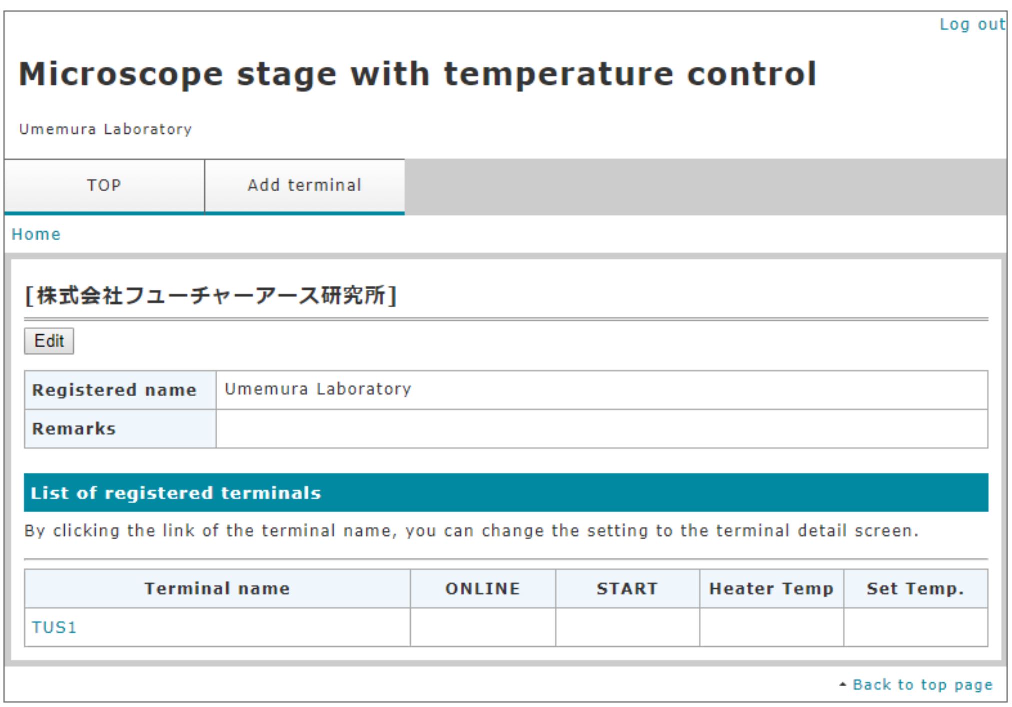
1.2.4 Click [Add terminal] button to display the next screen, and enter necessary information.



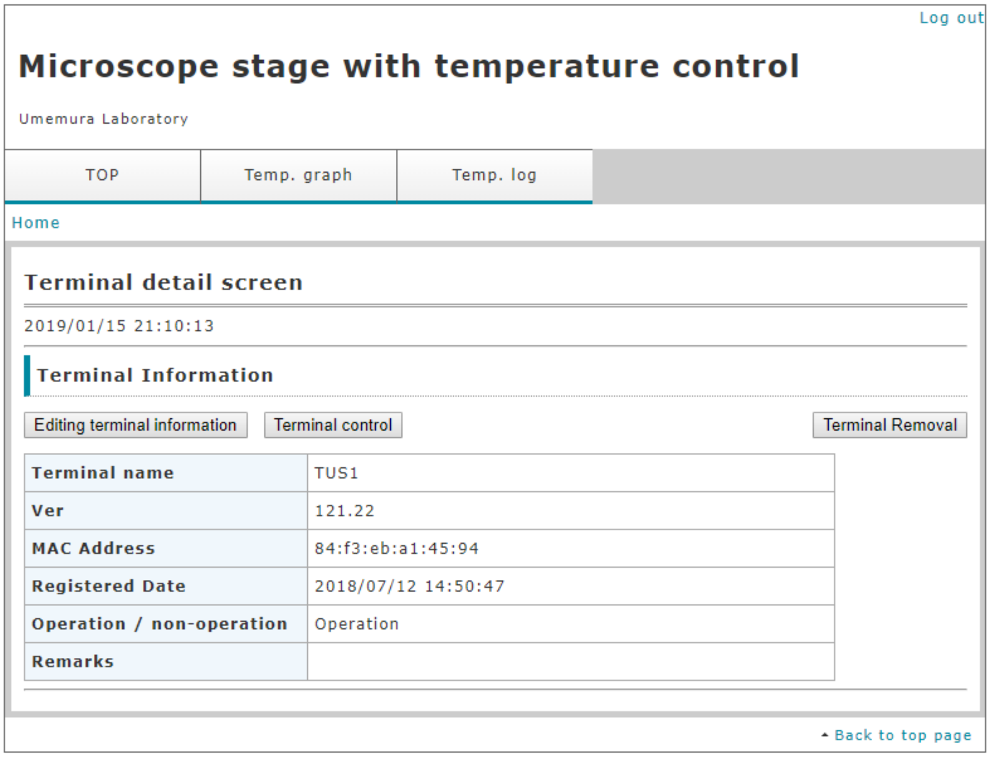
2. Operation

2.1 Operation Status

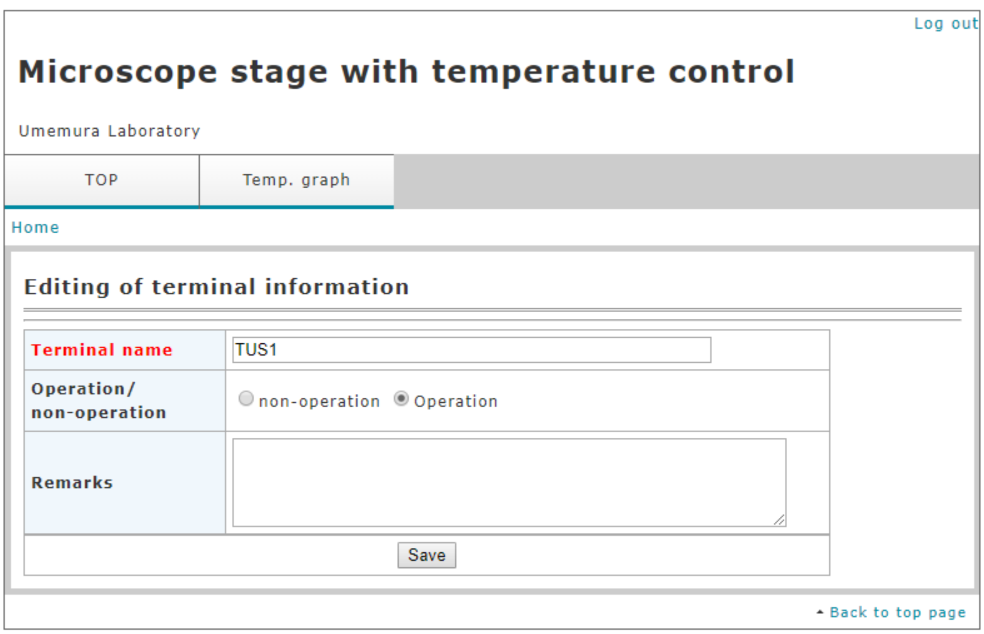
2.1.1 Log in and call up the next screen.



2.1.2 Click [Terminal name] button to display the next screen and confirm terminal information.

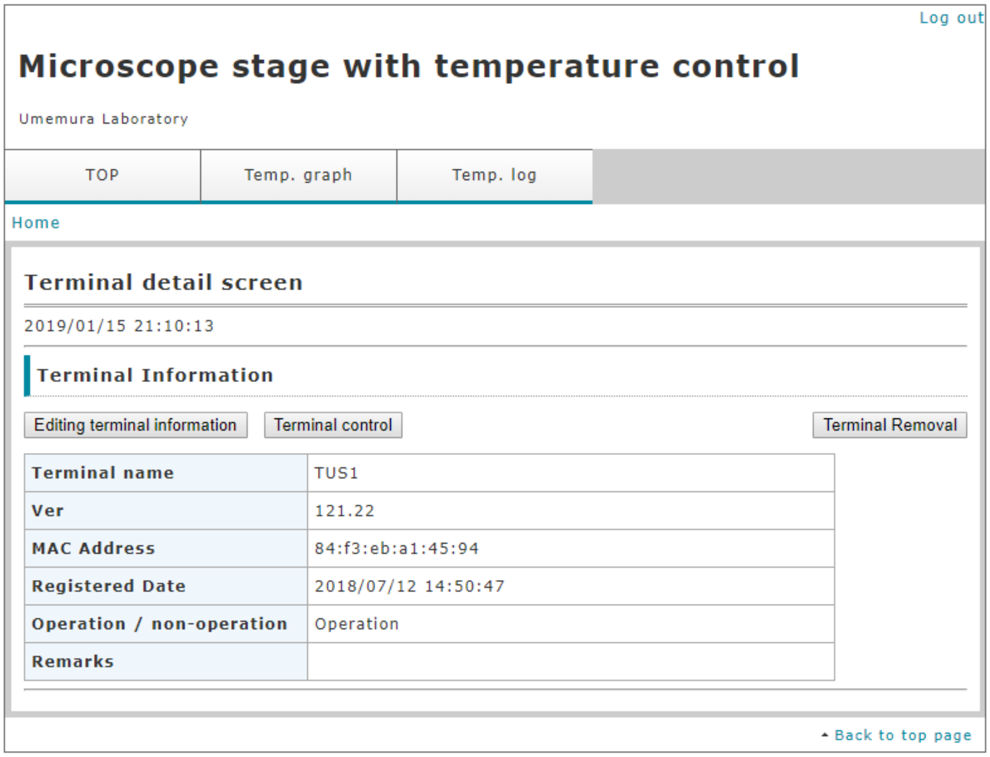


2.1.3 Click [Editing of terminal information] button to display the next screen and change the operation status.

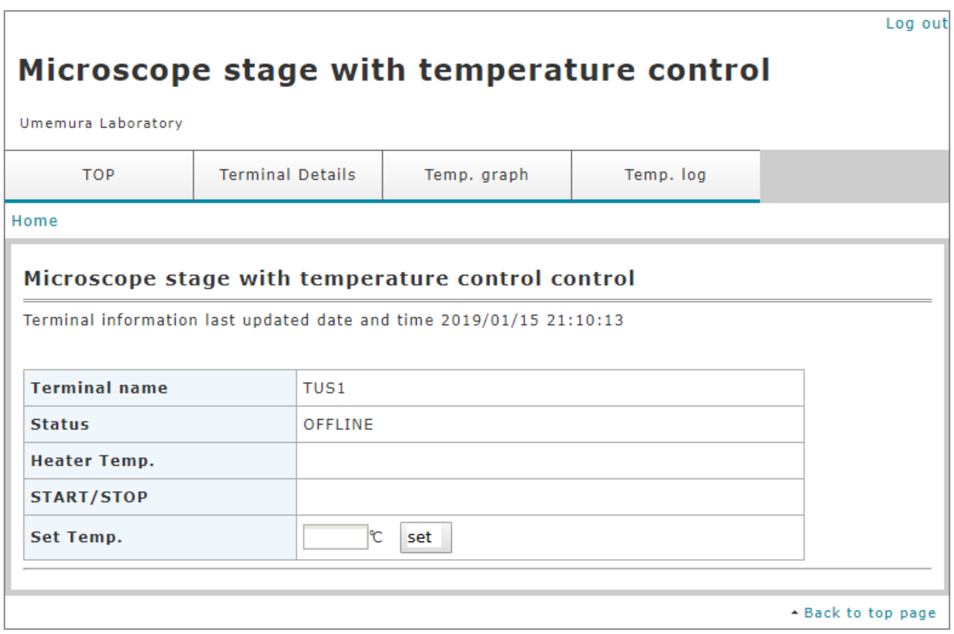


2.2 Temperature setting

2.2.1 Call up the next screen.

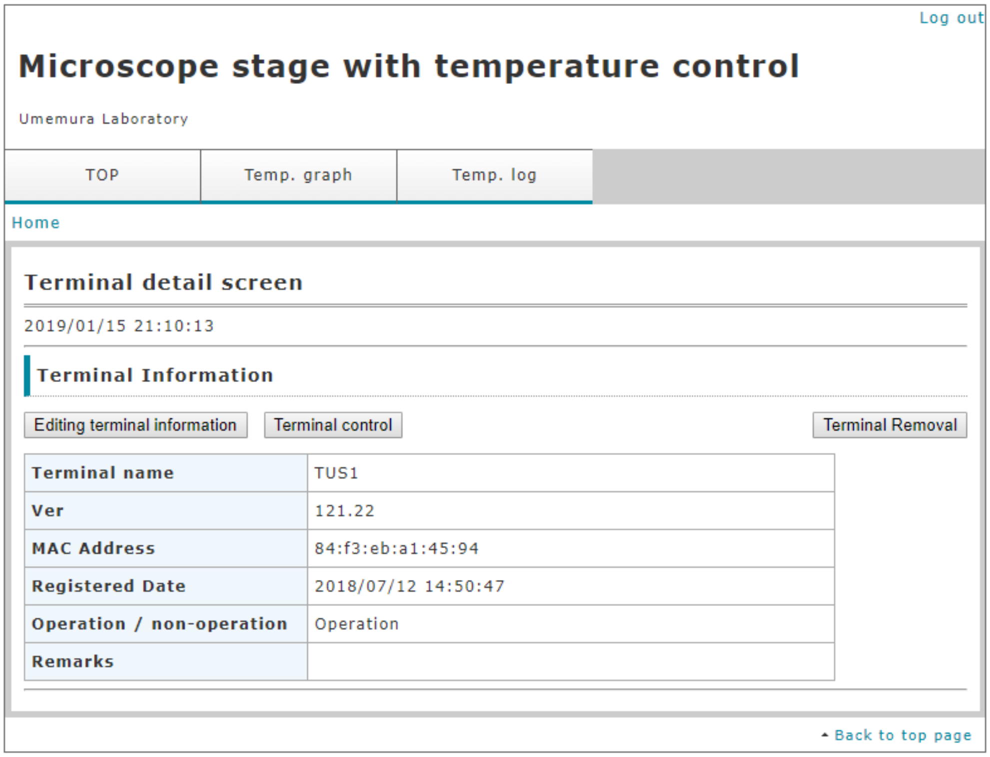


2.2.2 Click [Terminal control] button to display the next screen and set the target temperature.

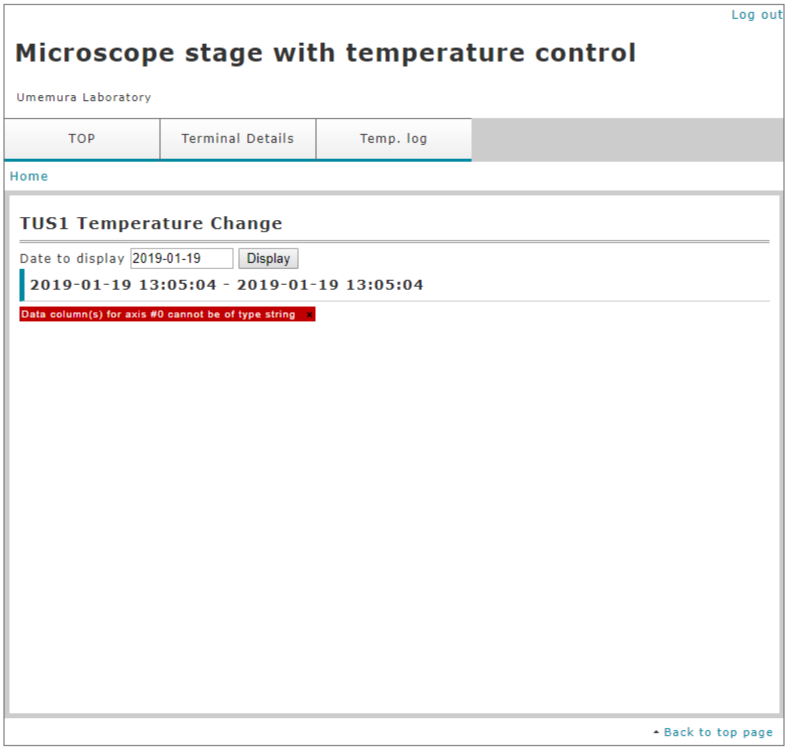


2.3 Display of temperature graph

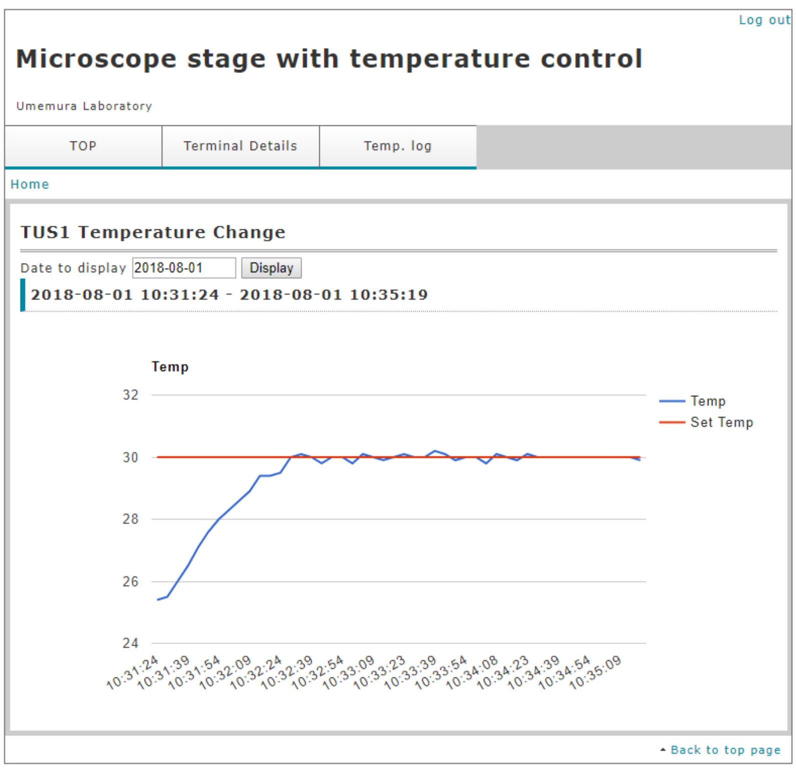
2.3.1 Call up the next screen.



2.3.2 Click [Temp. graph] button on the terminal details screen. The following screen is displayed.

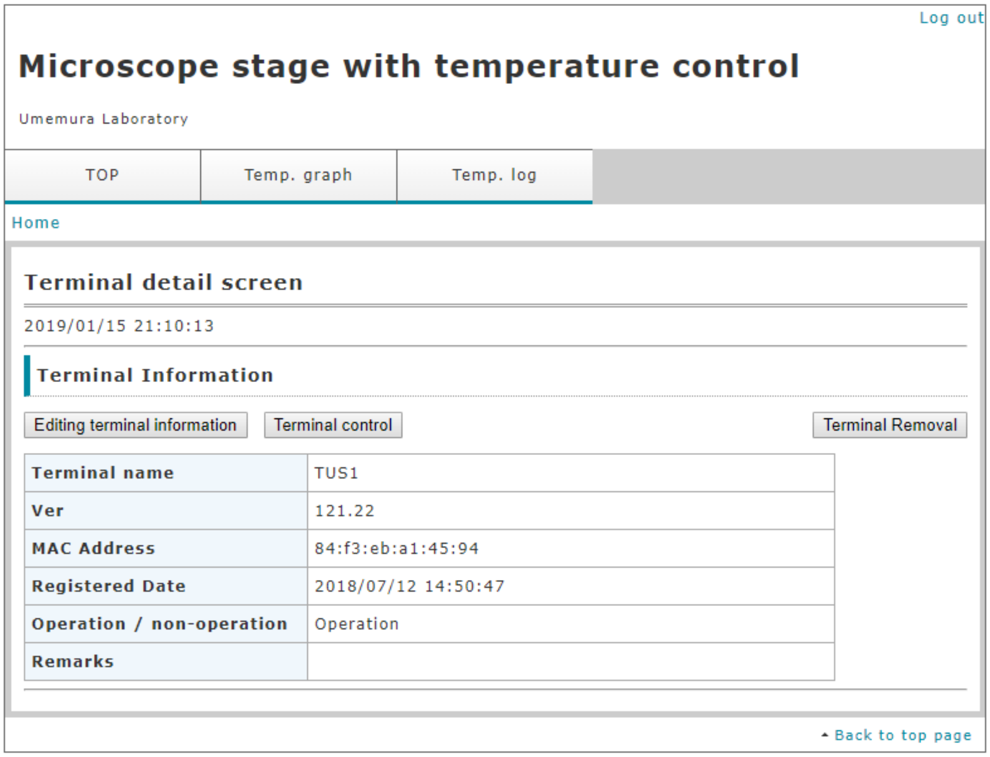


2.3.3 Specify the date to be displayed and click [display] button. The following graph is displayed.

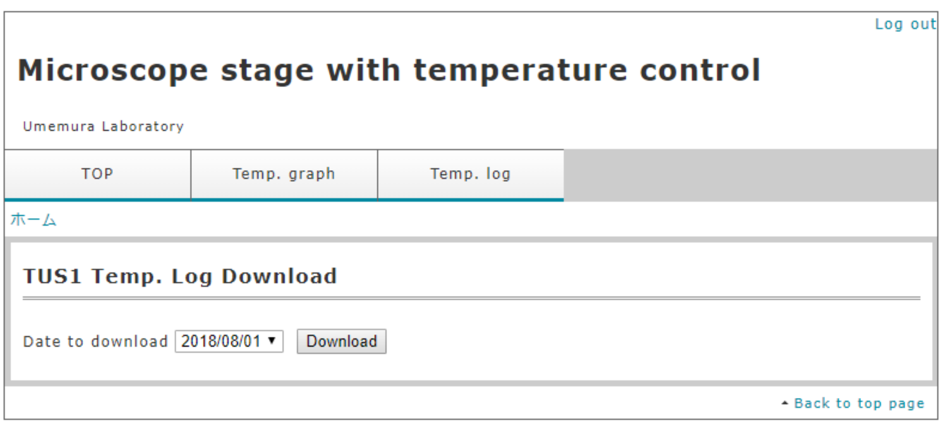


2.4 Download of temperature log data.

2.4.1 Call up the next screen.



2.4.2 Click [Temp. log] button on the terminal details screen. The following screen is displayed.



2.5 Specify the date to be displayed and click [download] button. The following CSV data is downloaded.

	A	B	C	D
1	Time	Heater Temp	Setting Temp	Heater Voltage
2	10:31:24	25.4	30	191.76
3	10:31:29	25.5	30	198.86
4	10:31:34	26	30	192.65
5	10:31:39	26.5	30	173.06
6	10:31:44	27.1	30	163.33
7	10:31:49	27.6	30	160.22
8	10:31:54	28	30	151.6
9	10:31:59	28.3	30	145.81
10	10:32:04	28.6	30	138.76
11	10:32:09	28.9	30	131.07
12	10:32:14	29.4	30	114.01
13	10:32:18	29.4	30	115.58
14	10:32:24	29.5	30	103.62
15	10:32:29	30	30	0
16	10:32:34	30.1	30	0
17	10:32:39	30	30	0
18	10:32:43	29.8	30	100.26
19	10:32:49	30	30	0
20	10:32:54	30	30	0
21	10:32:58	29.8	30	100.62