

Studying the temperature-dependent events of live cells under confocal and epi-fluorescence microscopy using a solid-state heating/cooling system

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1. Introduction

Temperature has a very profound influence on the physiology of living organisms. At the molecular and cellular levels, this influence is due to the diverse effects of temperature changes on the functions of ion channels, ion-binding proteins and ion transporter, on the activities of enzymes, and on the physical properties of biomolecules, such as the diffusion rates of ions and macromolecules in the cytoplasm or extracellular space, the fluidity of biomembranes and the polymerization -depolymerization equilibrium of cellular cytoskeleton [1-6]. For mammals, the body temperature is usually kept within a narrow range when the environmental temperature fluctuates by expending metabolic energy. However, upon exposure to temperatures outside the normal range that is most suitable for survival, many animals are able to survive by entering in an inactive or torpid state [4].

To study how cells undergo changes in response to variations in the temperature of their environment, one would need a system to continuously monitor the changes of cells while the temperature of the medium surrounding them is changed in a controlled manner. To monitor the morphological changes of cells, microscopy is one of the most commonly employed and one of the most powerful tools. Recent advances in microscopy technology, including the development of confocal and multi-photon microscopes [7, 8], and the use of fluorescent proteins and labeling techniques [9-11], have enabled us to study various target molecules in live cells. A number of commercially available systems have been developed for maintaining cells in an incubator-like environment while the fluorescent target molecules in cells are monitored continuously by fluorescence microscopy. However, most of these systems are not suitable for studying rapid temperature-dependent changes in cells because of their slow temperature changing rates.

Here, we report a temperature control system for monitoring the fluorescence target molecules in live cells that allows the temperature of the medium surrounding the cells to be changed rapidly. Using this system, we present a temperature-induced, reversible movement of microtubules from the core to the peripheral regions of the growth cones of cultured rat hippocampal neurons. Growth cones are highly mobile enlargements at the ends of growing neurites. Growth cones are known to follow the guidance of a wide spectrum of attractive or repulsive molecules in their surroundings and lead neuronal axons to reach, usually via tortuous routes, the regions containing their appropriate targets [12-14]. Both the microfilament and microtubule cytoskeletons, respectively, are enriched in the peripheral and core regions of growth cones and contribute to the movement of growth cones [15-17]. The stabilization of some microtubules extending into the peripheral region is further believed to be related to the turning

behavior of growth cones [18]. Our observation of cold-induced invasion of microtubules into the peripheral regions of growth cones will be useful in the future as a tool to study how the formation of microtubules in the peripheral region of growth cones is regulated.

2. Experiment

2.1 Design and implementation of a temperature control system

The temperature control system reported here consists of three major components (Fig. 1A). They are described separately as following.

The microchamber plate. This component is a 75 x 25 x 2 mm (length x width x thickness) acrylic slide containing two concentric circular openings, respectively of 18 mm in diameter/0.15 mm deep and 10 mm in diameter/1.5 mm deep. These openings accommodate the glass coverslip (18 mm in diameter and 0.12 mm in thickness, Matsunami, Japan) and medium, respectively, used in culturing rat hippocampal neurons (as described below). A third opening (5 mm in diameter and 0.5 mm deep) is made next to the shallower opening, and this opening helps with the tasks of mounting and removing the glass coverslips to and from the chamber. Another hole (hole 2) is drilled through to the deeper opening and allows the placing of the thermocouple probe (SA1XL-K,

Omega Engineering Inc., USA) in the middle of the chamber, and the probe is in turn connected to a temperature meter (Model HHM1, Omega Engineering Inc., USA). Two more holes (holes 1 and 3) made on the side of the deeper opening can serve as the inlet and outlet for circulating fresh medium in the chamber when needed. These latter holes were not used in this study because cultured rat hippocampal neurons could be kept in the abovementioned chamber for at least 30 minutes at 37°C without exhibiting any abnormal changes in their shape or microtubule arrangement.

The cooling/heating plate. This component is a commercially available 7W thermoelectric cooler/heater (TECI-3103, Centenary Materials, Taiwan) (20 x 20 x 4 mm). Its function as a heater or cooler is selected by switching the polarity of the input DC current. Its cooling/heating output is controlled by adjusting the DC current from a transformer (SWE0320 (3V 2A), Kaming, Taiwan).

The circulating plate. This component consists of an aluminum plate (75 x 40 x 6.5 mm) containing an open channel (5 mm wide and 4.5 mm deep), with the open side facing the bottom, and a piece of soda lime glass (0.55mm thick, Bionic Technology Co., Taiwan) is glued to the bottom side of the aluminum plate. Two holes (Fig. 1, holes 4 and 5) are made and serve as the inlet and outlet of ice-cold water which is pumped in and pulled out of circulating plate by a peristaltic pump when the system operates in the cooling mode. When in the heating mode, the flow of ice-cold water through the circulating plate is stopped.

Two pieces of aluminum blocks (29 x 15 x 8 mm) are placed between the microchamber and circulating plates for a better support. The abovementioned microchamber and circulating plates and supporting aluminum blocks were made by the Scientific Instrument Center of National Tsing Hua University (Hsinchu, Taiwan). Fig. 1B shows a cartoon illustrating how the temperature control system (Fig. 1A) is connected to its auxiliary parts, including a peristaltic pump for pumping ice-cold water through the circulating plate, the transformer and adjuster for current supply and the temperature meter for temperature measurements of the medium inside the microchamber. A photograph of the temperature control system attached to a confocal microscope is shown in Fig. 1C.

2.2. Preparation of neuronal cultures, transfection and fluorescence microscopy

Neurons were prepared from a number of hippocampus dissected from Sprague-Dawley rat fetuses at embryonic day 18 by a previously reported procedure [19]. Briefly, each hippocampus was dissected from the rat embryo, pooled and treated with papain (10 units/ml). Afterwards, the dissociated cells were washed and suspended in MEM supplemented with 5% horse serum and 5% fetal calf serum (Gibco, Frederick, MD, USA). Cells, at the density of 5 or 10×10^4 cells/cm², were then plated onto glass coverslips (18 mm in diameter, Matsunami, Japan) precoated with poly-D-lysine, placed in the wells of 12-well culture plates, and incubated at 37°C in a humidified incubator with 5% CO₂/95% air for 3 days. Thereafter, the cells were treated with 5 μM cytosine 1-β-D-arabinofuranoside for 1 day. The culture medium of hippocampal neurons was replaced with NB/B27 (with additional 0.025 mM glutamate, Gibco) on DIV 1 and subsequently replaced with NB/B27 every 4 days.

Plasmids encoding YFP-α-tubulin and DsRed1-N1 were transfected into the cultured rat hippocampal neurons on 4-6 DIV using a ProFection[®] Mammalian Transfection Systems-Calcium Phosphate kit (Promega Corporation, PA, USA). Briefly, cells were placed in fresh conditioned culture media 1 hr prior to transfection. The plasmid/calcium phosphate precipitate was prepared by mixing one volume of plasmid in 2 M calcium chloride with an equal volume of 2× HBS (280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM Hepes at pH7.10). After being kept at room temperature for 30 min, the precipitate were added drop-wisely to each well and mixed gently. After incubation in the incubator for 1 hr, cells were washed with fresh culture medium, placed in fresh culture medium and then returned to the incubator at 37°C. On DIV 9, the cells on the glass coverslip were rinsed with PBS, and the coverslip was mounted on top of the microchamber described in Fig.1 with the surface containing neurons facing the interiors of the chamber. The temperature control system was then placed under the water-immersion object lens of a confocal microscope (LSM 5 PASCAL, Zeiss) or an epi-fluorescence microscope (LABOPHOT-2, Nikon).

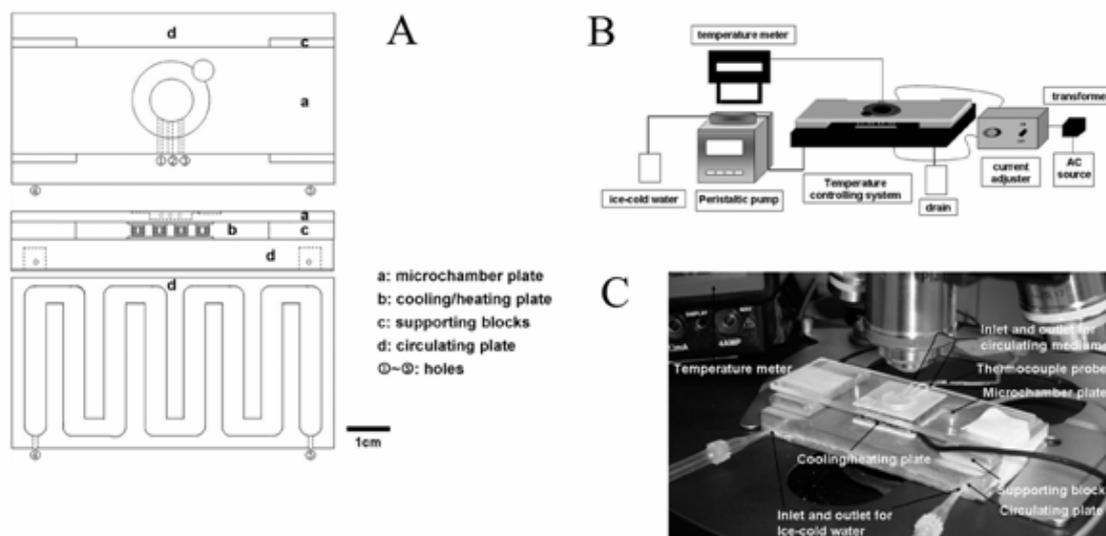


Fig. 1. (A) Schematic outlines of the temperature-control system. The top, middle and bottom pictures are the system viewed from the top, front and bottom, respectively. Scale bar: 1 cm. (B) The cartoon illustration of the connections between the various parts of the temperature-control system as described in (A) with auxiliary parts, including a transformer, a current adjuster, a peristaltic pump and a temperature meter. (C) Photograph of the temperature-control system placed under the lens of a confocal microscope.

3. Results and discussion

3.1 Performance characterization of the temperature control system

The temperature control system with its chamber filled with PBS and covered with a glass coverslip without neurons on it was placed under a dry lens of a confocal microscope in order to test its performance. When the fluorescent light was turned on, the temperature of the medium in the chamber could be heated to higher than 60°C and cooled to lower than 0°C by controlling the amplitude and polarity of DC current to the cooling/heating plate. The temperature of the medium in the chamber could be rapidly and reversibly set between 4°C and 37°C with the heating and cooling rates of 1 and 0.5°C/sec, respectively (Fig. 2A). When the same experiment was performed with a water-immersion lens with water being placed between the glass coverslip and the lens, the temperature of the medium in the chamber could still be heated to higher than 60°C and cooled to 8-9°C (Fig. 2B). The temperature of the medium could also be set between 37°C and 8-9°C rapidly and reversibly with heating and cooling rates of 0.8 and 0.2°C/sec, respectively. That the medium of chamber could not be cooled below 8°C in the later experiment is most likely due to bidirectional heat dissipation between the microscope and the temperature control system as mediated by the water between them.

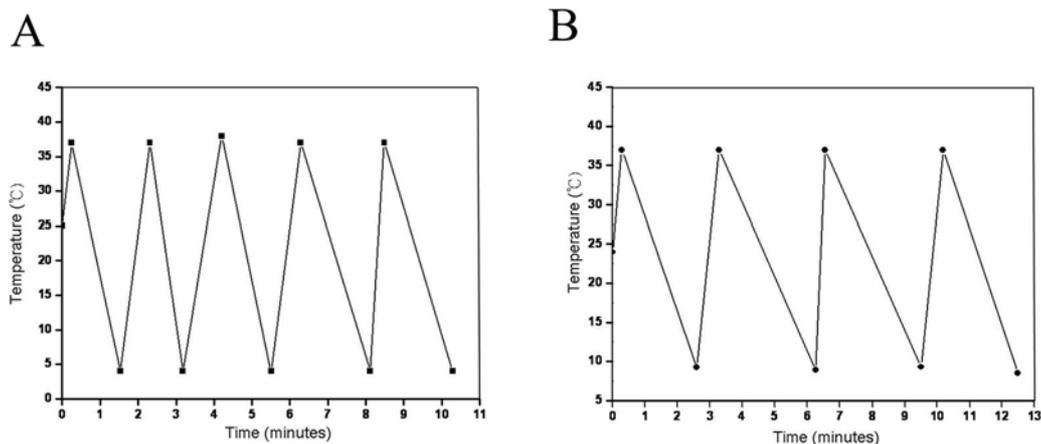


Fig. 2. Characterization of the temperature change performance of the temperature-control system when operated under (A) a dry and (B) a water-immersion objective lens. During the heating phase, the circulation of ice-water in the circulation plate of the system was stopped, and it was resumed during the cooling phase. The results are from a single experiment that is representative of a total of 3-5 experiments.

3.2 Cold-induced protrusion movements of microtubule bundles in growth cones

After being maintained under culturing conditions for 9 days, cultured rat hippocampal neurons expressing both DsRed1-N1 and YFP- α -tubulin were examined under a confocal microscope using a water-immersion lens. Since DsRed1-N1 could efficiently diffuse into all parts of the cells, including dendrites, axons and even very smaller structures such as dendritic spines, the cellular structure of the neurons expressing DsRed1-N1 could be clearly seen under a fluorescence microscope. When the neurons were kept at 37°C, fan-shaped growth cones were frequently found at the tips of the slender processes of neurons (an example of a fan-shaped growth cone is shown in Fig. 3, A3). YFP- α -tubulin was found to be enriched at the base of the growth cone (Fig. 3, A1, A2 and A3). After the medium surrounding this neuron was cooled to 8.1°C for 3 min, two YFP- α -tubulin bundles were detected in the peripheral region of the growth cone that was originally devoid of YFP- α -tubulin at 37°C (arrows in Fig. 3, B2). After warming up to 37°C again, these green fluorescent bundles disappeared completely or partially (Fig. 3, C1, C2 and C3).

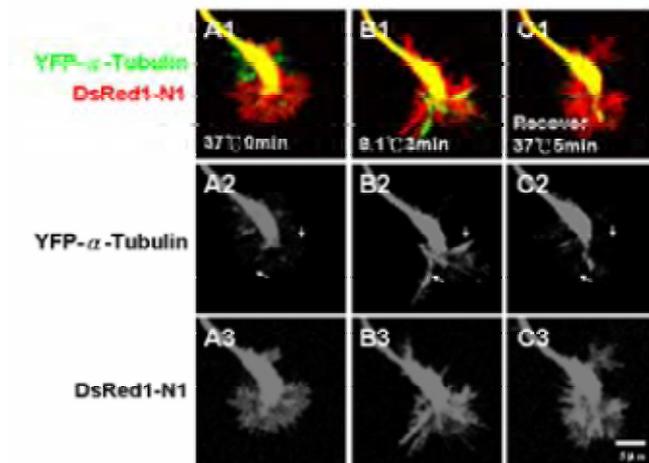


Fig. 3. Temperature-dependent changes in the YFP- α -tubulin distribution in a growth cone. A glass coverslip with cultured rat hippocampal neurons expressing both DsRed1-N1 and YFP- α -tubulin growing on the surface was mounted on the temperature-control system and examined under a confocal microscope with a water-immersion objective lens. A, B and C are respectively the fluorographs of a growth cone taken at 37°C, three minutes after the temperature of the medium was cooled to 8.1°C and 5 min after the medium was warmed to 37°C again. Arrows indicated the cold-induced tubulin bundles appearing in the peripheral region of the growth cone. Scale bar: 5 μ m.

The transient appearance of YFP- α -tubulin bundles in the peripheral regions of growth cones at lower temperatures were found in most of the growth cones encountered in this study (12/14) (more examples were shown in Fig. 4). The results indicate that a sudden drop in the temperature of the medium would seem to activate some signaling paths in growth cones, thereby leading the microtubules originally residing in the core region to grow into the peripheral regions where they are seldom found at 37°C.

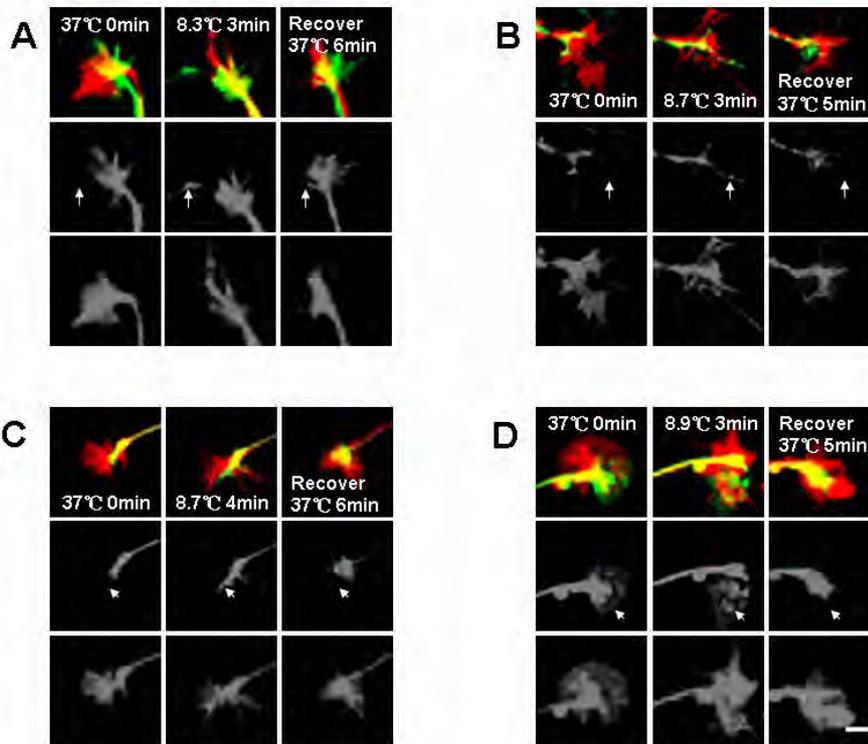


Fig. 4. Temperature-dependent changes in the YFP- α -tubulin distribution in growth cones. The data were obtained from cultured rat hippocampal neurons co-expressing DsRed1-N1 and YFP- α -tubulin with the temperature-control system by the same procedures described in Fig. 3. Scale bar: 5 μ m.

A wide spectrum of attractive and repulsive molecules has been found to guide the movements of growth cones, and these molecules are able to activate an array of intracellular signaling paths in the growth cone [20-22]. These intracellular signals ultimately affect the dynamics of the membrane, cytoskeleton and adhesion of the growth cones, thereby leading to the growth, retraction and turning behavior of the growth cones. Based on the effects of local application of microtubule-stabilizing or -destabilizing reagents on growth cones, it has been hypothesized that the turning behavior of growth cones results from the microtubules extending into the peripheral region being preferentially captured by the F-actin in the filopodia facing the attractive cue and hence they are stabilized. These stabilized microtubules may lead to the intracellular transportation of various materials to the side facing the attractive cue, which then leads to growth cone turning [23-26]. Our results indicate that a brief exposure to cold results in the extension of microtubules from the core to the peripheral regions of growth cones. This simple system may be used in the future in studying the mechanism(s) underlying the extension and stabilization of microtubules from the core to the peripheral regions of neuronal growth cones.

The major difference between the temperature control system reported here and other commercially available ones is that the formal system does not rely on a bright field optical microscope to examine the cell shape. Instead, the cell shape is outlined by the red fluorescence of the DsRed1-N1 expressed in the cytoplasm of the neurons. Many other fluorescence proteins, such as GFP, tTomato, and lipophilic dyes, such as DiI and DiO, may also be used for the same purpose. When cells are expressing the above fluorescence proteins or labeled with the abovementioned fluorescent dyes, their shapes can be examined in detail in a chamber that is transparent on only one side because the incident and reflected lights in upright confocal and epi-fluorescence microscopes go through the same path by way of the objective lens. The trade-off of spending the extra effort on making cells fluorescent is that one is able to place

non-transparent parts of the setup on the lower side of the chamber. The temperature control system described here, which is able to efficiently change the temperature of the medium surrounding the cells, can thus be used to study a broad range of target cells using fast heating and cooling rates; this exemplifies the above trade-off.

4. Summary

Here, we report the design and implementation of a temperature control system which could be used to study cellular changes in responses to temperature changes. This temperature control system is suitable for studying cells that are expressing at least two fluorescence proteins, one for outlining the cell shape and the other for tagging the protein of interest. This system can be used with upright confocal and epifluorescence microscopes. This system was used to discover that a brief exposure to cold induces the microtubules originally enriched in the core region of cultured rat hippocampal neuronal growth cones to invade the peripheral regions of the growth cones. After warming up, these cold-induced microtubule bundles would retract back to the core region again. In the future, experiments like these will help us study the mechanism(s) underlying how microtubules may contribute to the turning and growth behavior of neuronal growth cones.

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