Open-dish incubator for live cell imaging with an inverted microscope

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Here we describe the design and fabrication of an inexpensive cell culture incubator for the stage of an inverted light microscope for use in live cell imaging. This device maintains the temperature of the cell culture at 37°C with great stability and, after reaching equilibrium, provides focal stability of an image for 20–25 min with oil-immersion lenses. We describe two versions of the incubator: one for use with standard 60-mm plastic culture dishes, and the other version for imaging of cells on glass coverslips. Either can be made for less than $400. Most components are widely available commercially, and it requires only simple wiring and 3 h to assemble. Although the device is generally useful for live cell imaging on an inverted microscope, it is particularly suitable for work in which instruments are introduced into the culture, such as electrophysiology or micromanipulation. The design is based on the principle that control performance is limited by the lag time between detection and response. The key element of the design is a heated, temperature-controlled aluminum ring serving as a mini-incubator surrounding the culture vessel. For this reason, we call our design a "ringcubator."

INTRODUCTION

Live cell imaging for extended periods is a requirement for several physiological and cytological studies including electrophysiology (1), micromanipulations (2,3), and observations of cellular motility (4). Such imaging has seen explosive growth accompanying the introduction of green fluorescent proteins (GFPs) to allow visualization of target proteins in living cells (5,6). The most frequent subjects of live cell imaging are cultured mammalian cells, which must be maintained at 37°C on the stage of the microscope during the course of observations. Although microscope stage incubators of various designs are commercially available, most cost more than $1500 and have drawbacks for work that involve the introduction of instruments into the cell culture. For example, we introduce glass needles into cultures of neurons to apply mechanical stimuli to cells over many hours (3). Our dissatisfaction with the cost and limitations of the commercially available incubators stimulated the design of an inexpensive microscope stage incubator that is well

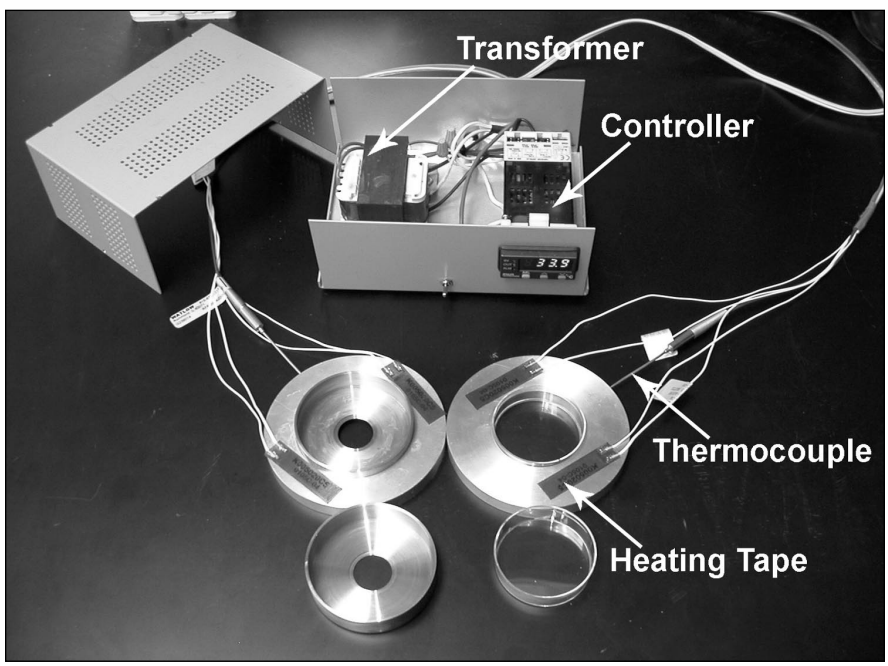

Figure 1. Ringcubator devices for plastic culture dishes and for glass coverslips. The chassis box containing the transformer and the controller is shown at center and is identical for both heated units. The heated and temperature-controlled ring on the left accommodates a custom stainless steel culture dish for glass coverslips. On the right is a similar temperature-controlled ring designed to accommodate standard 60-mm culture dishes. Note that in both designs, the rings, not the culture dishes, are directly heated and measured for temperature.

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suited for micromechanical work and for live cell imaging in general. The total cost is less than $400 and requires fewer than 3 h to assemble from commercial parts and one or two custom-machined parts available from any university or commercial machine shop. One version controls a standard plastic culture dish, and another accommodates a custom culture dish for cells grown on glass coverslips. The design feature that makes temperature control particularly stable is the use of a heated aluminum ring, whose temperature is directly controlled. This aluminum ring serves as a kind of "solid-state water bath" (i.e., constant temperature incubator) surrounding the culture dish. For this reason, we call our device a "ringcubator."

MATERIALS AND METHODS

Components

As shown in Figure 1, there are two versions of the ringcubator, and both are composed of six main parts: an incubator or temperature controller and a 120 to 28 V transformer, which are housed in a ventilated chassis box, two 5 W heating tapes, a T-type thermocouple, and a custom-machined aluminum ring, which accommodates the 5 W heaters and the thermocouple. The only additional components are an on-off switch, various wiring, and miscellaneous small parts, such as grommets and heat-shrink tubing. The devices shown in Figure 1 are durable and robust. The transformer, controller, heaters, and thermocouple are all in light-duty use in this application, and none of our four devices has required a repair in 1 year of use.

Thermocouples, heating elements, and incubators come in a bewildering variety of types from numerous manufacturers. For these specialized temperature-control components, we ascribe some importance to the particular parts specified here, insofar as other similar parts did not work as well. The incubator is a Fuji Electric PXV3-TAY2-4V fuzzy-logic controller (Fuji Electric, Tokyo, Japan), which can be obtained in versions to accept various line voltages for worldwide use. This device is widely available from sales firms specializing in process control (e.g., http://www.ttiglobal.com or http://www.alphacontrols.com). The thermocouple input to the controller is a T-type thermocouple (Cat. no. AFEDOTA020G8030; Watlow, St. Louis, MI, USA). This catalog number includes several important specifications for ruggedness and sensitivity. Local sales agents can be located from this manufacturer's Web site (http://www.watlow.com). The heating tapes are adhesive-backed, 28 V, 5 W kapton "flexible heaters" obtained directly from Omega Engineering (Cat. no. KHLV-0502/5-P; Stamford, CT, USA; http://www.omega.com). These accept AC current, as here, or DC (e.g., to avoid introducing 60 cycle noise into electrical recordings). In the latter case, an AC-DC stepdown transformer must be substituted for the AC type specified here. The remaining commercially available components are simpler, and we think any equivalent components would work well. The transformer is a 1.5 amp 120 to 28 V AC power transformer (Cat. no. TX2815; All Electron-

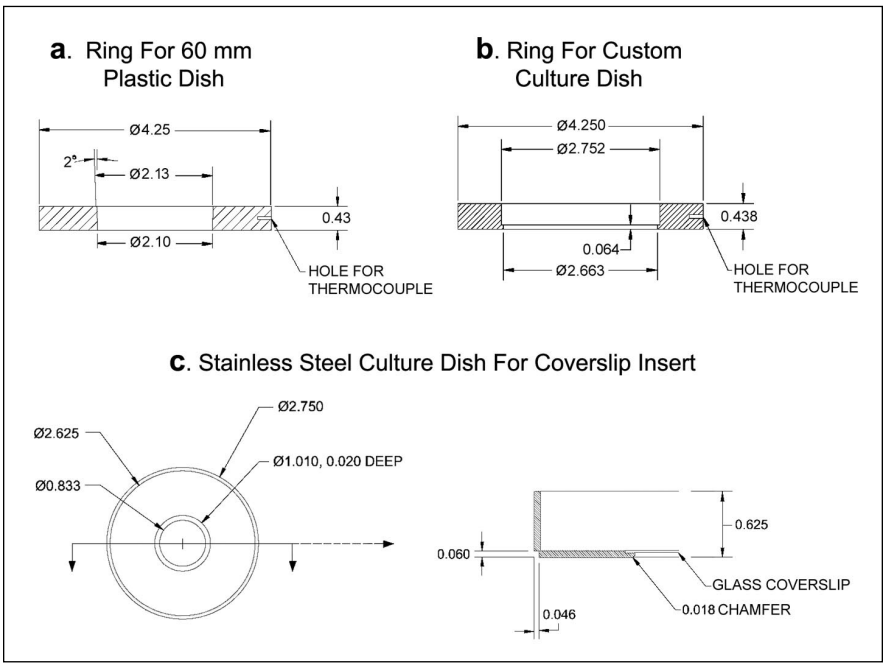

Figure 2. Drawings of the custom machined metal parts (all dimensions are in inches).

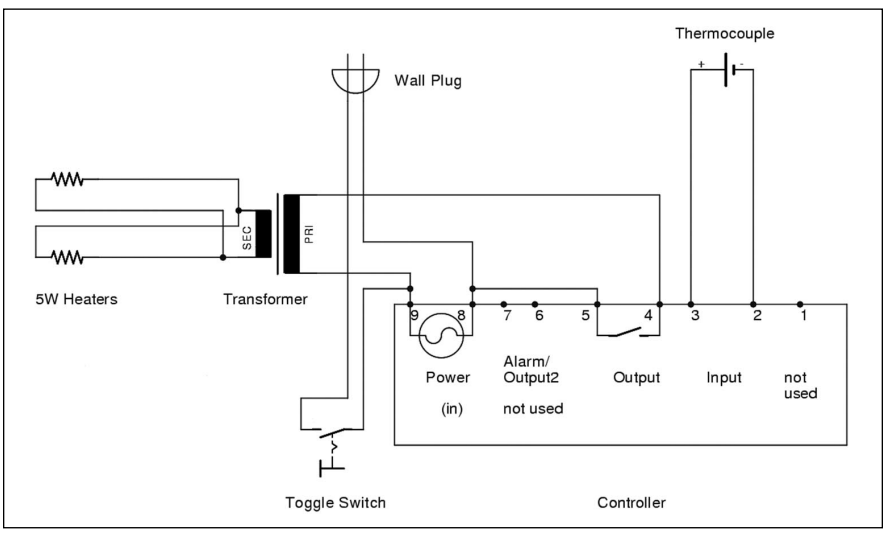

Figure 3. Wiring diagram for both ringcubators. The two heating tape elements are adhesive-backed and should be attached to the top surface at opposite sides of the aluminum ring, with all wires extending in the same direction as the thermocouple wires, as shown in Figure 1. Both input leads should be twisted together, and similarly for both output leads, and each twisted pair is then connected to each of the transformer's 28 V output wires (coded red in the specified transformer) by lengths of insulated 20-gauge stranded copper wire. The thermocouple has two output wires that are connected directly to the controller. Conveniently, the wiring sockets at the back of this controller are numbered. The red wire of the thermocouple is connected to wiring socket no. 2 (which is marked negative, "-") at the back of the incubator, while the blue thermocouple wire is connected to socket no. 3 (labeled positive, "+"). To prevent entanglements of the six wires leading from the aluminum ring to the chassis box, all the wires are bundled into a length of heat-shrink tubing. For 120 V currents, power from the laboratory wall socket is used to power the incubator and is also passed through to the transformer by the incubator serving as a temperature-controlled on-off switch. Accordingly, one 120 V input wire of the transformer (white) is connected with one incoming power lead from the lamp cord, and both are inserted into wiring socket no. 9 at the back of the incubator. The other power lead of lamp wire is connected to the user-operated external on-off switch shown at the front of the chassis box as in Figure 1. The other side of this switch is then connected to wiring socket no. 8 of the incubator, and socket no. 8 is then bridged to socket no. 5. (Lengths of lamp wire are used throughout to carry all 120 V currents.) The remaining 120 V input wire of the transformer is then connected directly to socket no. 4 of the incubator.

ics Corp., Van Nuys, CA, USA; http://www.allelectronics.com). The perforated chassis box (7″L × 5″W × 3″H) is from LMB/Heeger (Cat. no. Perf-145; Commerce, CA, USA). This box and the wires, switches, and other minor parts for the device are available at local electronic supply houses.

The heated ring for 60-mm plastic culture dishes (Figure 2a) is composed of 6061 aluminum, 1 cm thick and 4.25″ outer diameter (o.d.). The dish well is machined to a 2° taper to provide a tight fit for Falcon® and Corning culture dishes. Investigators should provide their machinists with a culture dish to work from. The hole for the thermocouple is placed in the side of the ring and is sufficiently tight that the probe cannot be removed easily without tools. Including the cost of machining this ring, this version of the ringcubator for standard culture dishes cost us less than $250 to fabricate.

For higher resolution observations of cells on coverslips, we designed a similar ring accommodating a large diameter stainless steel (304 alloy) culture plate as shown in Figure 2, b and c. These components require careful machining, in that the dish must fit as tightly as possible in the ring, but still move freely enough to avoid spilling contents during placement and removal. The large diameter was predicated on the need to introduce instruments from the side at relatively shallow angles (our microscope condenser prohibits steep angle positioning). As shown, this culture dish has a central hole and recessed ledge on which to mount a 25-mm round glass coverslip. This mounting ledge is also convenient for confining a small-volume water droplet over the coverslip for analyses following live cell imaging: fixations, immunostaining, etc. It thus helps conserve antibodies and other expensive reagents.

We affix the coverslip to the ledge using Dow Corning No. 7 Release Compound (Neely Industries, Arlington, TX, USA; http://www.neelyindustries.com), which is a silicone grease intended for thermo-molding operations. This grease has a near optimum viscosity, in that it dispenses easily from a syringe. Coverslips can be removed intact if care is used, and it provides a highly reliable seal for long periods. We dispense the grease in a thin bead (like caulk) around

the ledge from a 10-mL syringe to which we attach a standard tip for adjustable micropipets (Eppendorf® tip). Placing the coverslip onto the ledge spreads the grease into a waterproof seal between glass and steel plate.

Assembly and Wiring

The wiring is the same for both units, is shown schematically in Figure 3, and is described in detail in the legend. The basic circuit is conceptually simple, however. Based on temperature input from the thermocouple, the controller is serving as an on-off switch providing temperature-controlled 120 V output to the transformer, which in turn provides 28 V power to the two heaters.

Incubator Program

The specified incubator is more complex than required for the relatively simple, PID (proportional, integral, derivative) temperature regulation that we use. In addition to a value for the set point (37°C), this device can be programmed for more than two dozen parameters using three buttons on its face, as described in the supplied booklet of instructions. However, we programmed only seven variables, all found in the second menu layer, which appears after pushing the Select button for 7 s. All remaining variables were left at factory default. Programming is straightforward and similar to setting a multifunction digital watch or programming a VCR. Each programmed parameter is associated with an acronym code shown on the LED display on the face. In the description of the programming input to follow, the parameter display code is shown in parentheses following the numerical value. For these parameters, we used settings of 0.7 (P) for proportional bandwidth, 120 s (I) as the setting for integral time, and 35 s (D) for derivative time. These three parameters control the temperature range around the set point over which control is exercised, the rate of heat output in approaching the set point, and how fast the temperature is changing in the ring. Three other important parameters with standard settings are to specify: (*i*) the use of a T-type thermocouple, 7 (P-n2); (*ii*) a temperature readout to one decimal point, 1 (P-dp); and (*iii*) a value of 17 (Ar) for auto-reset windup (a refinement of integral control).

Only one other parameter requires a change from factory settings, but this important parameter value requires user input based on the particular properties of each unit. This is the process variable offset (PVOF in the device code). This value reflects a mutual temperature calibration between a given thermocouple and a given controller and also conditions of heat loss by the dish. By setting an appropriate value for the PVOF, the temperature on the LED display on the front of the controller will closely match (±0.2°C) the temperature in the dish, and a set point of 37.0 will maintain 37°C in the dish. Given our experience with laboratory microscopy, we think most use of the ringcubator will occur on 1 or 2 microscopes in the same laboratory. Under these conditions, no resetting of PVOF has been required on any of our ringcubators over the course of months. Also, recalibration has not been required for use in different rooms and different buildings if accuracy of ±1°C is

acceptable. In our hands, the PVOF required resetting if the desired temperature set point was changed by more than 2°C and when our laboratory ventilation system changed from heating to cooling modes with the change of season.

The procedure for determining process value offset is basically one of trial-and-error using a good thermometer directly and completely immersed in the dish. We suggest starting the trials with a PVOF of -6. Allow the reading on the controller to reach the set point (37°C) and wait an additional 30 min before checking the thermometer in the dish. If the water temperature is above 37°C, increase the PVOF by half the value of the temperature difference (e.g., -5.0 for 39°C), if below 37°C, decrease by half the difference (e.g., -7.0 for 35°C). Wait to reach equilibrium and repeat adjustments until the water temperature remains stable at 37°C. If this procedure does not produce good temperature control around 37°C, we suggest checking the variables putatively left at factory default to confirm true default values, which are listed in a table at the back of the instruction booklet.

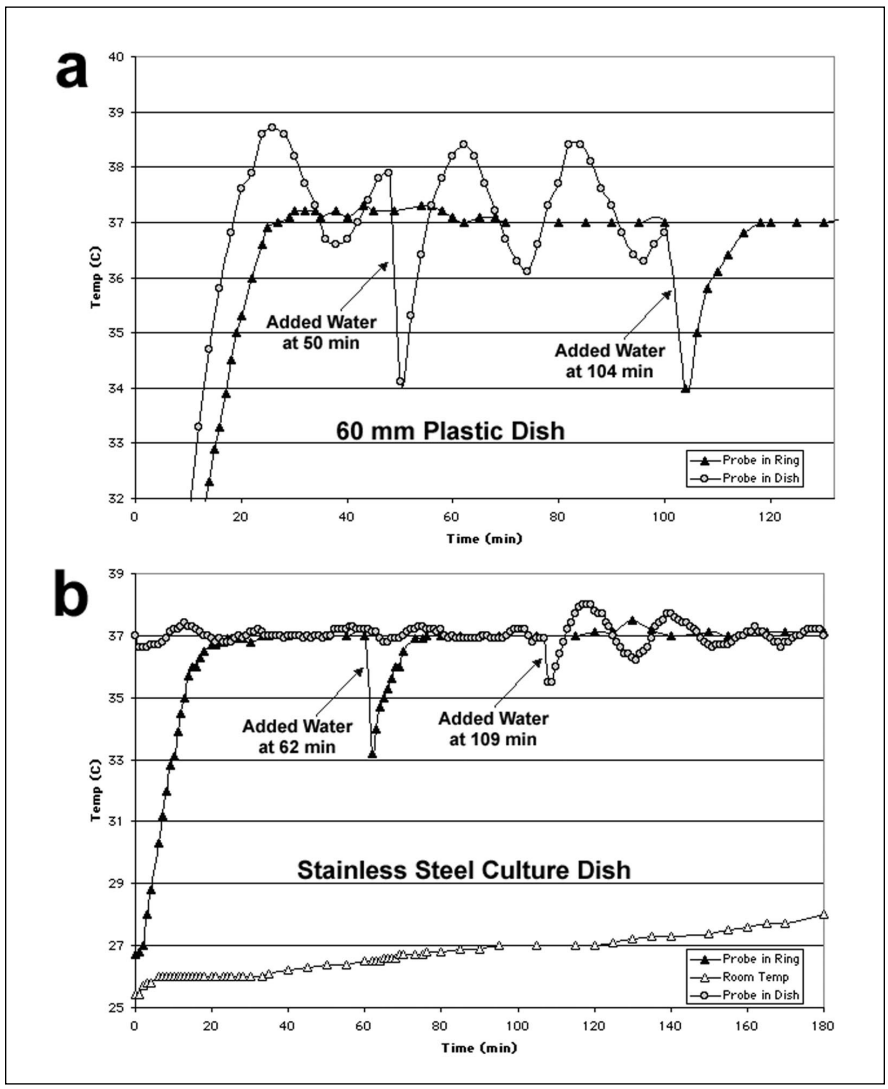

Figure 4. Control by the ringcubator designs of culture dish temperature as a function of time. (a) Temperature at the center of a standard 60-mm plastic culture dish as a function of time after turning on the ringcubator with the thermocouple in the ring (-▲-) or with the thermocouple in the culture dish (-o-). Room temperature water was added to the probe-in-dish sample at 50 min. With this control geometry, temperature in the dish fluctuated by as much as 3°C both before and after perturbation. In contrast, with the probe in the ring and having set an appropriate process variable offset (PVOF), very stable temperature control is achieved in the culture dish before and after perturbation at 104 min. (b) These trials are similar to those of panel a, except temperature was measured just above the coverslip in the stainless steel dish design, and an additional perturbation was introduced (i.e., the room was gradually heated). Once again, note the superior temperature control performance with the thermocouple in the ring after addition of cool water at 62 min, compared to the fluctuations after adding water at 109 min to the probe-in-dish sample.

Measurement of Temperature Stability

Temperature stability was assessed by measuring the temperature of medium or water in the culture dish with a mercury or digital thermometer. Both standard plastic and custom steel dishes were assessed, and all components were equilibrated to room temperature at the beginning of the observations. After assembling the appropriate components, the ringcubator was turned on, and temperature measurements were taken every 2 min. In each trial, cool water was added to the dish to determine the response to temperature perturbation in the dish, and in some trials, room temperature was varied slightly.

Effect of Incubator on Focal Stability

Focal stability of images from a DMIRB microscope (Leica Microsystems, Bannock, IL, USA) was measured as the length of time an image remained in excellent to good focus without manipulating the microscope (i.e., time-to-poor focus of an initially sharp image). Trials were conducted at 37°C as for ordinary use and equilibrated at room temperature as a control for aspects of focal stability not dependent on temperature control. REF52 (rat embryo fibroblast) cells expressing GFP-actin (2) were fixed briefly in cold methanol (-20°C) to control for changes due to the dynamic actin array itself, not focus per se. The cells were cultured on glass coverslips and observed within the stainless steel culture dish shown in Figure 1. This allowed the use of high resolution, short focal depth, oil-immersion objectives. A trial began by initially obtaining a sharp focus on particular actin stress fibers within the imaged cell and then recording images automatically every 60 s for 30 min using the automatic features of the Open*lab* Imaging system (Improvision, Lexington, MA, USA). This allowed the cells to be illuminated (by UV) only for

the brief time of the exposure, minimizing fluorescent photobleaching. These time-lapse images were then subjectively assessed for focus by the first author. Conditions for achieving 20- to 25-min periods of good-to-excellent focus without refocusing are presented in the Results.

RESULTS

We perceive only two parameters indicative of the functional performance of a microscope-stage incubator: (*i*) stability of temperature control at 37°C for the cells, and (*ii*) focal stability of the image. Figure 4 shows typical data on the temperature stability within culture dishes maintained by the ringcubator and the importance of having the thermocouple in the ring, rather than in the dish, which we think would be intuitive for most biologists. Figure 4a shows the temperature within a plastic tissue culture dish with the thermocouple in the ring (filled triangles), as designed, or immersed in the dish (open circles). Room temperature water was added to the dish to perturb the temperature for each probe placement. As shown, temperature stability with the probe in the ring was notably superior to that with the probe directly in the dish. With the thermocouple in the ring, temperature was maintained at 37° ± 0.5°C during periods without perturbation and required about 10 min to restore the set temperature after addition of cooler water at 104 min. In contrast, placement of the probe directly into the culture dish resulted in sinusoidal fluctuations of temperature of 2° around the 37°C set point, with and without perturbation.

As shown in Figure 4b, similar results were obtained for trials with the stainless steel culture dish. Here, two temperature perturbations were introduced: (*i*) addition of room temperature water, and (*ii*) changing the temperature in the laboratory 2° to 3°C (i.e., the maximum our laboratory building permits). With the probe in the ring, the temperature in the stainless steel dish in the absence of perturbation is remarkably stable, presumably owing to the larger thermal mass (30 mL of medium) and better heat transfer of the steel dish compared to the standard 60-mm plastic culture dish. With the thermocouple in the ring, the temperature returned to the set point following perturbations without overshoot. With the thermocouple in the dish, in contrast, addition of water at 109 min again produced significant sinusoidal temperature fluctuations damping out slowly, as predicted by control theory (see Discussion).

Focal stability was assessed with REF52 cells expressing GFP-actin (2), because they are characteristic of live cell images, and they provide sharp lines of actin stress fibers, which serve as a critical marker for depth of focus. In order to assess focal changes per se, rather than changes in the highly dynamic actin array of the sample, we used fixed cells. With ordinary dry lenses, focal stability was maintained for more than 40 min (data not shown). However, we regard images obtained with oil-immersion objectives as the most challenging test of focal stability, both because of the narrow depth of focus of such lenses and because the immersion oil provides for better heat transfer and local temperature perturbation than an air gap. We compared the focal stablity of such images in fixed cells maintained at 37°C with samples equilibrated at room temperature. Such ambient observations provide a control for limitations of the microscope and/or of room temperature control in laboratories (i.e., focal shifts that would be seen without the ringcubator). For both conditions, we found that good focus was maintained for about 20–25 min once heat transfer equilibrium was reached. Figure 5a shows images from a trial at 37°C, in which a high-resolution image was maintained for 25 min after a 90-min equilibration period in situ on the microscope stage. Similarly, Figure 5b shows such a trial at room temperature, in which all the components, except the cell-containing coverslip itself, were allowed 3 h to come to room temperature prior to assembling and observing the sample. Even here, a 25-min equilibration period on the stage was required to obtain focal stability for more than 5–10 min. Thus, once heat transfer equilibrium is reached by the ringcubator, we found that focal stability was limited by factors other than the performance of the ringcubator (perhaps temperature control of the laboratory or creep in the microscope). For normal use at 37°C, the time to reach thermal equilibrium with oil-immersion lenses can be shortened to about 20 min if all components, including the objective lens, are prewarmed to 37°C.

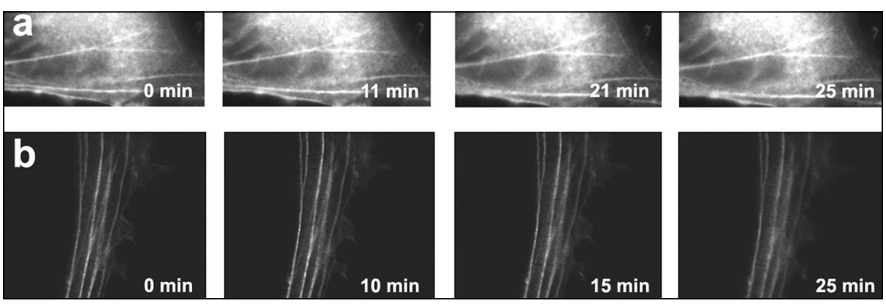

Figure 5. Photographs of focal stability with the ringcubator, using a 63× 1.32 N.A. (numerical aperature) oil-immersion objective. (a) Ninety minutes after turning on a fully assembled ringcubator culture (i.e., after heat transfer equilibrium is reached at 37°C as for intended operation), these fluorescent images of green fluorescent protein (GFP)-actin stress fibers in fixed REF52 cells were obtained without adjusting the focus of the microscope. (b) Similar fluorescent images without focal adjustment obtained from fixed REF52 cells maintained at room temperature with the ringcubator off as a control. These images were obtained after 3 h of nominal equilibration of all components at room temperature and 20 min after equilibration on the microscope stage. The same 20–25 min of stability of focus was seen whether the ringcubator was on or off, indicating that once equilibrium is reached, fine focal stability is limited by the microscope or room temperature change, not the ringcubator.

DISCUSSION

As shown in Figures 4 and 5, the ringcubator design provides excellent stability of the temperature of the culture medium and of the focus of a cell culture on an inverted microscope. The ringcubator achieves this performance as a result of the very short time lag between heat output and temperature change in the ring, not the culture dish. This characteristic time lag is the key

factor in the control of temperature for a small volume of water near room temperature. That is, there is a time lag between the temperature actually changing in the dish and the heater going on (and off). The longer the time lag, the more heat is gained or lost by the sample after the set point is reached, thus producing a subsequent unwanted overshoot. For temperature control of a nonstirred culture dish (the normal state of affairs), this characteristic time lag, τ(s), is related to geometry and material by the following thermal diffusion equation:

$$\tau = r^2/\pi\alpha \qquad \text{[Eq. 1]}$$

where r is the distance between the heat source and the thermocouple, $\pi = 3.14$, and α is the thermal diffusivity of the heated material [α(cm^2/s) = thermal conductivity (cal/[cm][s]°C) divided by heat capacity (cal/cm^3°C)]. By placing the heaters on an aluminum ring (very high thermal diffusivity) and attaching the thermocouple within a few centimeters to the same ring, a short time lag is achieved, thus achieving tight temperature control. As shown by the trials in Figure 4, placing the thermocouple in the unstirred dish markedly increased time lag for control because of the low thermal diffusivity of water/medium. The longer time lag under these conditions produces sloppy control, because the thermocouple does not immediately sense changes at the heater or the environment. With the thermocouple in the ring, however, the controller "knows where it is" faster and so achieves significantly better control. Consider if automobiles only responded to a driver's commands after a 10-s or 1-min delay. A more accurate, if fantastic, analogy would be if light required 1 or 10 s to reach the driver. Equation 1 should also provide information regarding an investigator's efforts to modify the ringcubator for his/her particular use. For example, changing the thickness of the aluminum ring should have little effect, assuming sufficient area and mass for heat transfer, because α and r are unchanged. Increasing the diameter would increase lag time and diminish control, while a smaller diameter ring (e.g., for a 35-mm culture dish) should work better.

Our rings have a quick characteristic time, somewhat <30 s, while the medium has a time lag τ of about 4 min because of the low α. The relatively rapid temperature oscillations in the ring will die out before reaching the center of the dish. Therefore, the ring serves as a kind of constant temperature "bath" surrounding the culture. The difference in response times between the dish and the ring also allowed us to use an inexpensive controller with a 30-s mechanical (rather than a faster solid-state) relay and to program this low-cost controller to sense change and adjust the heating rate quickly without any significant temperature oscillations appearing in the dish. While this design achieves exceptional control stability, a slight disadvantage is uncertain control accuracy (i.e., a true set point of 37°C would not produce 37°C in the dish). Thus, accurate temperature control with this set-up requires empirical setting of the PVOF for each unit, as discussed in the procedure for programming the controller. This offset varies from unit to unit and is straightforward to determine experimentally, as described in the Materials and Methods. Once set, our experience indicates that the unit is quite stable over time on the same or similar microscopes. If temperature accuracy of ±1°C is acceptable to maintain cultures, our experience is that no resetting is required for use on different microscopes, in different rooms, or in different buildings, assuming ordinary room temperature ventilation control.

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