METHODS FOR GENTLE, DIFFERENTIAL HEATING OF PART OF A SINGLE LIVING CELL

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ABSTRACT

The fabrication and use of resistance wire microheaters is described and the results obtainable with cells are illustrated. The microheaters permit localized warming of a small area, wherever desired, within a single living cell. This is directly shown by the production of localized structural changes in the mitotic spindle. Thus in this case, subcellular areas respond independently to the local temperature. The method is applicable to a variety of cellular processes, providing clues to cellular control mechanisms.

INTRODUCTION

Experimental alteration of the rate of a cellular process is a common route to characterizing the process and its regulation. The methods described here permit localized rate alterations where desired in living cells. The experimental variable is temperature. All cellular processes are temperature sensitive, typically exhibiting a rate increase greater than twofold for a temperature increase of 10°C. Thus, if the temperature of one area within a cell can be elevated above the rest, yet remain within the physiological temperature range, then two questions can be asked concerning a given cell function: what is the most temperature-sensitive area, and what is the cellular response to local temperature elevation at this and other sites? For instance, the assembly of large structures such as the mitotic spindle could be examined. Here, putative nucleation sites might be located by a unique temperature sensitivity. In any case, the production of unusual spindle shapes, because of heightened assembly in warmer areas, would be of interest. Thus some degree of local control over assembly would be disclosed and questions about chromosome motion in such spindles would be raised.

Temperature gradients that are entirely within the physiological range, yet are steep relative to cellular dimensions and the temperature sensitivities of cellular processes, have been successfully produced. The fabrication and calibration of the microheaters presently used are described here, and the results of locally warming cells are illustrated. A brief account of the results has been presented previously (Nicklas, 1973). In the sole earlier report of differential, physiological warming of cells (Ishizaka, 1969), a different method for producing the temperature gradient was described, involving close apposition of a macro-heater and a macro-cooler. This method is uncomplicated and useful for some purposes, but the attainable gradient is very much lower (3°C across a cell 50 μm in diameter) than that achieved with the method described here and cannot be used for warming the center of a cell relative to the periphery. A suggestion for still another, as yet untested, method is offered in the present report, especially since it may be of value also for a different purpose—the localized destruction of cell components with a laser microbeam.

MATERIALS AND METHODS

The temperature gradient is produced by a resistance wire heater of very small diameter. In outline, the diameter of an already thin wire is locally reduced to

a micrometer or two. The thinnest part of the wire can readily be placed in intimate contact with the cellular region to be heated. More significantly, the local diameter reduction causes the thinnest part to be far warmer than the rest when an electrical current is passed along the wire. This arises because the electrical resistance of the wire determines the temperature and resistance increases as the square of the decrease in diameter.

Fabrication of Microheaters

A glass support (Figs. 1 a and b) is prepared with a microburner from 1 mm diameter tubing. A 30–50 mm length of 12.7 μm diameter Chromel "A" wire is then attached to the glass support with dentists' "sticky wax" (the wire is available from Omega Engineering, Inc., Stamford, Conn., and the wax from any dental supply firm). Small patches of wax, previously applied to opposite sides of the support, are warmed with heated forceps and the wire is pressed into the warm wax. The wire is attached first on one side near the leads, led along that side down to the tip, attached seriatum to the wax patches (see Fig. 1 a), then bent into a U shape around the tip (Fig. 1 b) and attached to the wax patches on the opposite side of the support in reverse order, from the tip toward the leads. The one important part of this procedure is formation of a narrow U shape at the tip without crimping the wire or leaving unresolved stresses in the wire (which would lead to shape distortion or breakage after diameter reduction). A smooth U-shaped band is produced by pulling the wire around a glass needle of about 50 μm diameter, and unresolved stress is avoided by bending the free end of the wire to its final position before attachment to the support. Finally, the two sides of the wire near the tip are cemented rigidly to each other and to the support with Epoxy cement (see Fig. 1 b; the "5-minute Epoxy" of Devcon Corporation, Danvers, Mass., obtained locally, is convenient for this purpose).

The diameter of the wire at the tip is then reduced by electrolysis. Both the tip of the wire and a platinum electrode contact an approximately 2:1 mixture of saturated aqueous $NaNo_2$ (Harding, 1939) and 95% ethanol, and a current is passed between them. Electrolysis is easily restricted to the very tip of the wire by limiting contact to one fiber at the torn edge of a piece of lens paper moistened with the $NaNo_2$-ethanol mixture. The wire is positioned, via the glass support, with a coarse manipulator, and the process of diameter reduction is viewed with a microscope. Initially 2–2.5 v AC is applied for up to 1 min; during the final stages 1 v or less is applied for only a few seconds between inspections. The residues which form during electrolysis are periodically removed by dipping the wire first into 5% aqueous HCl and then into distilled water. The process is stopped when the wire is 1.5–2.0 μm at the thinnest point. Still thinner heaters can be fabricated but almost always break too soon to be very useful. Heaters as large as 3 μm in diameter may be useful for some purposes and are very easily produced. For 1.5- to 2 μm-heaters, the method does not always work, but it is much the best of a large number of tested alternatives involving various types of wires and electrolysis solutions and also a different approach described by Whitaker (1929). The present method is certainly adequate, however. After a little practice, at least one useable heater can be produced in four attempts requiring less than 3 h in all, and one such heater was used

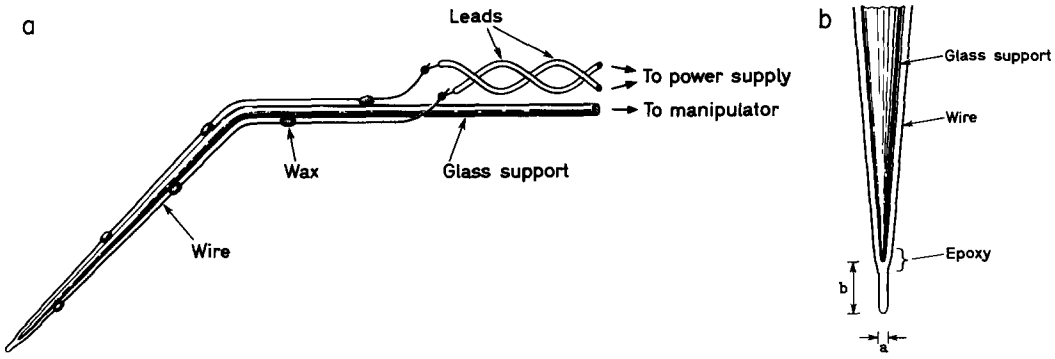

FIGURE 1 Diagrams of the microheater. Not to scale. (Fig. 1 *a*) Side view. For clarity, the wire is shown rotated slightly from its true position, actually the two limbs of the wire would appear as superimposed in this view. The glass support is approximately 100 mm long and 4.5 mm or less in height, measured vertically; the angle of the bend is 45°. (Fig. 1 *b*) Front view of the tip at greater enlargement. The position of the epoxy cement mass is indicated but the cement itself is not represented. Approximate dimensions: "a", 100 μm, "b", 500 μm; at the very tip, the wire is electrolytically reduced to 1.5–2.0 μm in diameter over a length of 2–10 μm; elsewhere the wire is 12.7 μm in diameter.

longer than a month in experiments on more than 50 cells.

Use of Microheaters

The completed heater is positioned by a micromanipulator over the cell to be warmed. The only sensitive control required is in the vertical direction, hence an inexpensive manipulator suffices (e.g., model MS—V of Brinkmann Instruments, Inc., Westbury, N. Y.). An inverted microscope with a long working distance condenser is used. The cell culture chamber (Nicklas and Staehly, 1967) is open at the top. The cells lie in a thin layer of medium covered with a 0.5–1 mm thick layer of fluorocarbon oil (Kel-F 10 oil was used but is no longer available; see Forer and Koch, 1973, for an available equivalent). This technique was designed for insect spermatocytes but is equally useful for tissue culture cells covered with their normal medium. The electrical circuit is very simple, consisting of a sensitive AC milliammeter in series with the wire, with voltage regulation by a variable transformer connected directly to the 115 v AC main. This gives adequately sensitive regulation of the current through the wire because of the large voltage drop across the ammeter (0–10 ma AC: Triplett Corp., Bluffton, Ohio).

An adequately steep temperature gradient can only be produced if ambient temperature around the cells is lowered to between 5° and 12°C. The simple expedient of placing the whole apparatus in a refrigerated room has been adopted. This poses no problems for observations by phase contrast optics but commonly induces strain in optics for polarization microscopy. I presently use a condenser (Nikon P 0.4 NA/20X objective lens) and an objective (Zeiss Pol Neofluar 0.6 NA/25X) which remain adequately free of strain at 10°C, but a simple technique for warming only the objective and condenser has been elaborated; details are available upon request.

The cell to be studied is centered in the field of view, and the precentered wire is lowered until it lies vertically at the middle level of the cell and horizontally at the area to be warmed. This produces some, but easily tolerated and reversible, mechanical distortion of the cell. Occasionally a small but disastrous amount of electrolysis occurs when the wire first touches the medium around the cells unless the leads to the wire are shorted until the power is turned on and heating is begun. Dirty wires are readily cleaned by overnight immersion of the tip in a concentrated NaOH solution, followed by brief immersion in distilled water. The use of *two* heat sources within one cell is sometimes of interest. This has been achieved by mounting two heaters of about the same diameter on separate manipulators but electrically connected in series in a single circuit.

Standardization and Calibration

The desired temperature of the wire is near, but safely below, the upper temperature limit of the cells to be warmed. A convenient standardization procedure is necessary so that the electrical current producing this temperature is readily determined for a given wire and ambient temperature. A usable standard for the insect spermatocytes studied to date is the current required to melt a wax of melting range 47°–49°C, in the usual cell culture chamber and covered with Kel-F 10 oil. The standardization is conservative; current values 10–15% above the standard are routinely used without damage to these cells (their upper temperature limit is 35°C). This is a relative standardization only—the actual wire temperature when cells are warmed is much lower than 47°–49°C because the layer of aqueous medium around the cells has more efficient heat transfer properties than Kel-F 10 oil. The required current ranges from 5 to 7 ma for wires of 1.5–1.8 μm diameter.

Absolute calibration of the thermal gradient has been attempted by three routes: heat-transfer theory, chemical temperature indicators, and biological temperature indicators.

The theoretical expression for thermal conduction from a warm cylinder (e.g., Carslaw and Jaeger, 1959) permits calculation of the temperature gradient from measured values of the thermal flux (the electrical current and wire resistance are known) and the wire radius, together with estimated values for the temperature at the wire and the coefficient of thermal conductivity. Additional heat transfer by convection can only produce a steeper gradient than expected. Therefore the calculation is usefully conservative; it is otherwise reliable to the extent that the estimates of wire temperature and conductivity coefficient are realistic.

The chemical indicators most used are one substance with a defined melting range and two temperature-indicating liquid crystals; each is placed where the cells would be in a standard culture chamber and covered with distilled water. The temperature at various distances from the heater may thus be directly visualized, using typical heaters at various current settings and ambient temperatures. The one usual melting-range standard in the temperature region of interest is 2-ethoxynaphthalene (Eastman Organic Chemicals Div., Eastman Kodak Co.; melting range: 35.5°–37°C). More elegant determinations are possible with certain liquid crystals—cholesterol esters which undergo structural transitions at defined temperatures made visible by selective reflection of light from the surface (see, e.g., Fergason, 1968). The reflected light runs through the spectrum from red light at some temperature to blue light at a higher temperature. Commercially available materials

cover completely the temperature region of interest here, with practically unlimited choice of the lowest temperature indicated and the total range covered in the red to blue color transition (e.g., from a "narrow" range of 3°C or less to a "broad" range of 20°C or more). Under the test conditions used, all these materials show some deviation from the nominal color-temperature relationship provided by the supplier and hence have been recalibrated under water in the cell culture chambers. Again because of these conditions, not all materials are adequately sensitive and brightly reflective. The best results to date have been obtained with specially prepared, broad range materials: LCI 20–40 and LCI 40–60 (Liquid Crystal Industries, Inc., Turtle Creek, Pa.). Other products, especially the narrow range indicators available in a kit covering many temperatures between 0° and 50°C (Liquid Crystal Industries, Inc.), warrant further trials.

Biological temperature indicators in principle provide the best gradient calibration. The idea is simply to capitalize on the very thermal sensitivity it is desired to probe experimentally. Rates of processes or amounts of substances are determined in cells from whole cultures at various temperatures and a graph of rate or amount versus temperature is prepared. Then, in actual experiments with a microheater, the rate or amount at various distances from the wire is determined and converted into temperature using the standardization graph. Two "biological thermometers" have been used to date: the rate of chromosome movement in anaphase and the amount of spindle birefringence. Both are useful but imperfect indicators of temperature over a range of 12°–32°C in the insect spermatocytes studied.

RESULTS

The results obtainable are illustrated by spindle birefringence in a grasshopper spermatocyte warmed with a microheater in various positions. The cell is shown after cooling to the ambient temperature but before experiments began in the first, "4 min" print of Fig. 2. The heater was then placed at the equator on the left side of the spindle as shown by the "star" on the 11-min print (in the prints, the wire is obscured by scattered light but it was clearly visible to the experimenter). The heater was turned on from 6 until 14 min. This short experiment produced no obviously localized birefringence increase; the prints are included to show the increased chromosome separation that resulted as well as the slight elevation in birefringence between the chromosomes and each pole (compare the 11- and 17-min prints with the 4 min print). Next, the heater was placed near the lower pole (21 min print) and turned on from 20 until 30 min. The striking increase in birefringence produced at the lower, warmed pole is shown both in normal and reversed compensator settings (30.3- and 30.5-min prints). Equally striking is the absence of much increase at the opposite pole. Finally, the heater was placed with its tip at the equator, over the mass of mitochondria on the right side of the spindle (38 min print) and turned on from 38 until 50 min. This produced a high birefringence associated with this mitochondrial mass, but very little change in the mass on the other side of the spindle (51- and 52-min prints). This birefringence may be due to the microtubules found amongst the mitochondria at this stage in crane fly (e.g., Fuge, 1973) and *Dissosteira* (Roos and Nicklas, unpublished) spermatocytes. Such birefringence does not develop in control cells until late anaphase, and localized heating at mid-anaphase produced no increase in mitochondria-associated birefringence (Fig. 2, 17 min print). Note the increase in chromosome separation between 4 and 51 min; a normal division was completed.

These are entirely typical results (cf. Nicklas, 1973, and in preparation). They unequivocally show that some significant measure of localized heating is attained wherever the experimenter chooses to place the heater.

DISCUSSION

The method for localized warming presented here produces an indefinitely stable, precisely controllable temperature without expensive apparatus, and can be adapted with minimal effort to a variety of cell types and experimental purposes. Against these advantages must be reckoned the necessity for some practice in fabricating the wires and the optical effects and mechanical deformation of the cell caused by the wire. A more elegant method would be warming by a laser microbeam of visible light. Visible-light microbeams certainly can be used to produce very small lesions in cell components, caused chiefly by locally very high temperatures (review: Berns and Salet, 1972). The lesion is produced by direct energy absorption by the cell component or associated molecules. This approach would not succeed for gentle, precisely controlled warming because the amount of energy absorbed, and hence the temperature, would vary intolerably with irradiation of chromosomes, the spindle, or mitochondria, for instance. An easy, but as yet untested, solution might be to use a standardized energy absorber: a microneedle fabricated from black glass and positioned where desired by a micromanipulator. The absorption of visible light

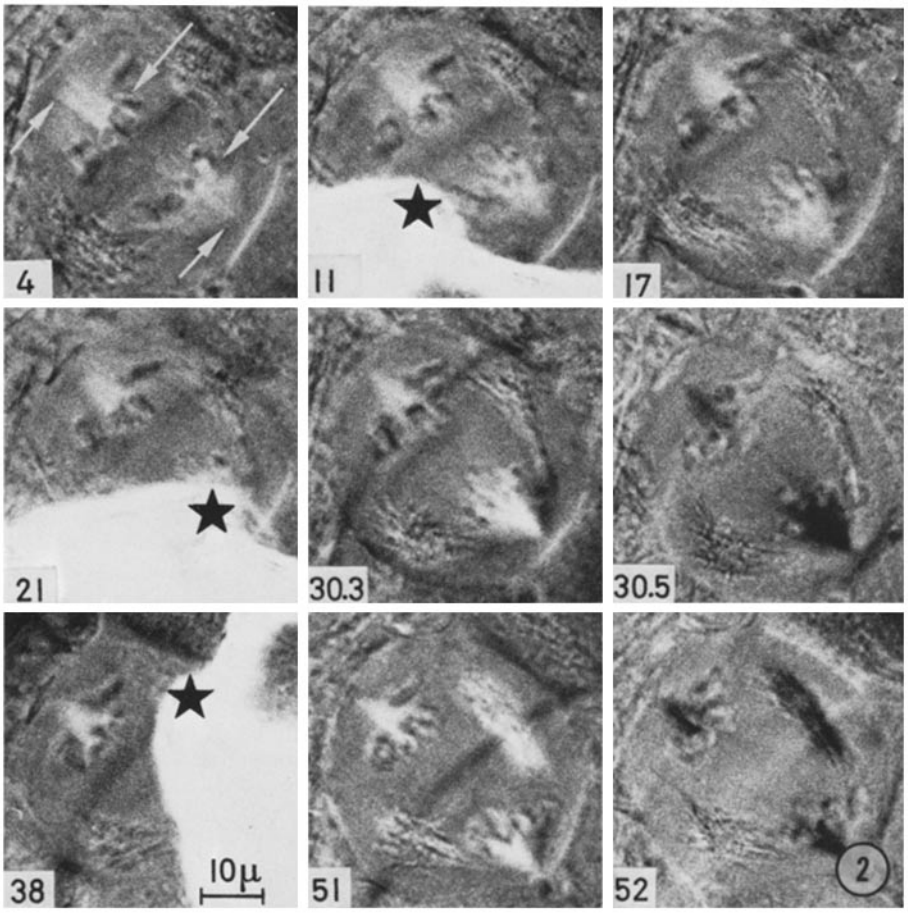

FIGURE 2 Anaphase in a *Dissosteira carolina* primary spermatocyte warmed locally with a microheater. The time in minutes is given on each of these prints from a cinematographic record made by polarized-light microscopy. Poles (short arrows) and chromosomes (long arrows) are indicated on the 4 min print, before the experiments were begun. Three separate experiments were performed with the wire positioned as indicated by the "star" on the 11-, 21-, and 38-min prints, respectively. After the first experiment (17 min) no differential birefringence was seen, but after the second (30.3, 30.5 min) and the third (51, 52 min), birefringence was strikingly and locally increased where the wire had been (details in the text; prints 30.3 versus 30.5 and 51 versus 52 are at opposite compensator settings). Ambient temperature 11°C. × 820.

by cell components is so low that absorption by the microneedle would determine the temperature obtained. Indeed, the absorption is so low that until very recently (Berns, 1972) vital staining was usually necessary to produce microlesions even with fairly high-energy lasers. This suggests that use of a black microneedle in the laser microbeam might also be useful for selective destruction of cell components. Of special interest is the prospect that the size of the induced lesion would not be limited by optical diffraction effects but rather by the size of the microneedle, and tip diameters smaller than 0.1 μm can easily be produced.

Actually, calibration of the temperature gradient is more difficult than production of the gradient. None of the three approaches used (see "Methods") is free of problems. A thorough discussion is deferred until presentation of all the observations on cells (report in preparation) since these results both contribute to gradient calibration and depend upon the calibration for interpretation. Some indication of the steepness of the gradient can be given here, however. Spindle birefringence is highly sensitive to temperature (see Stephens, 1973, and references therein) and therefore provides a direct measure of the gradient produced in living cells. Consider the birefringence nearer the (formerly) warmer versus the cooler

spindle pole in the 30.3- and 30.5-min prints of Fig. 2. From retardation measurements it can be shown that the birefringence at the warmer pole is equal to that found in cells at a culture temperature of 25°–30°C, that at the cooler pole to 15°C. The spindle length of 38 μm is the maximum distance over which this 10°–15°C temperature drop occurs. This generally agrees with values from all other estimates. A conservative overall estimate is that the temperature falls 10°C within 10 μm from the microheater, and 15°C within 50 μm. These values apply to the current experiments, at an ambient temperature of 12°C and an estimated heater temperature of 30°C. This is by no means the limit obtainable, however. Thus the gradient steepness can be increased by decreasing the ambient temperature (while holding the heater temperature constant) and by increasing the heater temperature, permissible if cells with a higher upper temperature limit are used (e.g., mammalian cells).

But calibration is not necessary to show that the temperature gradient already achieved is steep enough to affect differentially a typical temperature-sensitive process—maintenance of spindle structure—within a single, locally warmed cell (Fig. 2). At a minimum, the production of spindles with novel organization poses interesting questions about chromosome motion under such circumstances (Nicklas, 1973). Earlier, even with a much flatter temperature gradient, Ishizaka (1969) produced cells with the spindle shifted slightly away from the warmer side, presumably due to differential aster growth on that side, as the author suggests. The cell division furrow was also affected differentially, permitting significant insight into both the control of furrow position and the mechanism of furrow progression. Further applications to problems of the intracellular control of structure and function are readily suggested, for instance in microtubule assembly and particle transport in nerve axons and in the axopodia of heliozoans (e.g., *Raphidiophrys:* see Tilney, 1971). The kinetics of events at the inception of warming may provide additional insight into control processes. The present methods produce a precisely controllable, yet quite fast and localized, temperature jump under physiological conditions in living cells.

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