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Altered Drug Resistance of Microtubules in Cells Exposed to Infrared Light Pulses: Are Microtubules the "Nerves" of Cells?:

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This article describes the first quantitative assay of the response of an entire population of cultured mammalian cells to a pulsating near-infrared signal. The assay measures the change of resistance to nocodazole of reconstituted cytoplasmic asters of irradiated cells. Using this assay on CV1 cells, I obtained results suggesting that pulsating near-infrared signals of 1 s pulselength reduced the stability of the radial microtubules around the centrosome. The results are consistent with the interpretation that the centrosome responded to the light by sending signals along its radial array of microtubules whose stability was then altered. The results may be an example of a more general function of the centrosome to integrate exogenous signals and send response signals along microtubules to various sites within the cell. Cell Motil. Cytoskeleton 40:183–192, 1998. © 1998 Wiley-Liss, Inc.

Key words: near-infrared; CV1 cells; centrosome microtubules, nocodazole

INTRODUCTION

Previous Work on the Possibility of a Central Signal Integrating System of Cells

Although my work of the past two decades had raised the startling possibility that the cytoplasm of mammalian cells may contain a signal integrating system analogous to the central nervous system of higher organisms [Albrecht-Buehler, 1978, 1985, 1990], the experiments fell short of suggesting suitable ways to explore its unknown mechanisms. The situation changed after I found that 3T3 cells and CV1 cells were capable of locating and reaching out to distant near-infrared light sources [Albrecht-Buehler, 1991, 1995]. The finding promised to offer an experimental approach for the study of this putative cellular signal integration system. On the basis of earlier theoretical considerations [Albrecht-Buehler, 1981] subsequent experimentation [Albrecht-Buehler, 1991] led to the postulate that the integration system is closely related to the centrosome, while the pair of centrioles plays the role of some kind of a cellular "eye" embedded inside it.

What Structures, If Any, May Play the Role of Cellular "Nerves"?

A next required step toward the concept of an 'intelligent' cell was to identify the specific structures and mechanisms that mediated between the light detection at the cell center on one hand and the extension of specific pseudopodia at the peripheral cellular cortex on the other. The mediator mechanism could not be explained by diffusible chemical signals. Such signals would seem to arrive from every possible direction and, thus, would not be able to specify a particular direction for the extension of a pseudopodium. Therefore, the signals had to be confined to individual tracks that connected the cell center with specific locations of the cell periphery. The most promising candidate for this function seemed to be the microtubules [Albrecht-Buehler, 1992]. This led to the following question: Are any signals, indeed, propagated along the microtubules to the cell cortex in response to pulsating near-infrared light? If so, how can they be detected?

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Experimental Strategies to Identify Changes of Microtubules During a Putative Signal Transmission

Signal transmission is unlikely to drastically change the microtubule structure. If microtubules, indeed, conduct such signals one could hardly expect them to cause structural changes of the microtubules drastic enough to be visible in a microscope. Such an expectation would be analogous to the search for structural changes of the optical nerve every time the retina transmits images to the brain. Nevertheless, for several years I tried, but failed to find any direct effects of pulsating near-infrared light signals on microtubules or other cytoskeletal components.

Signal transmission may alter the effectiveness of antimicrotubular drugs. Consequently, I took an indirect approach. If the putative signals themselves had no direct effect on the structure of microtubules, I hypothesized that they might enhance or diminish the effects of some other agent that was known to change the structure of microtubules. For example, it seemed possible that the traveling signals were strong enough to alter the speed of disassembly of microtubules that were exposed to an antimicrotubular drug. Therefore, I measured the stability of cytoplasmic microtubules in the presence of nocodazole while exposing the entire cell culture to pulsating near-infrared signals.

Necessity to use cells with freshly assembled microtubules. Most cells stabilize over time a large subset of their microtubules and detyrosinate their α-tubulin (formation of so-called Glu microtubules) [Gundersen et al., 1984] (Fig. 1b). Such subsets of microtubules with widely differing degrees of stability would make it hard to detect small, light-induced shifts of microtubule stability, because their varying stability would greatly increase the scatter of the data.

Furthermore, cultured cells are usually exposed to considerable light intensities from the room lights during the handling of the cultures. Such exposures to light would increase the scatter of the data of the "dark"-control experiments, especially if the planned experimental light intensity is much lower than that produced by the room lights.

In order to erase any possible imprints of past irradiation of the cells and also to increase the degree of uniformity of microtubule stability in the cells, I used cells that contained only freshly assembled microtubules: I first disassembled the cytoplasmic microtubules of the cells by an antimicrotubular drug and subsequently allowed them to re-grow in the absence of the drug. This step in the procedure required to use the most readily reversible antimicrotubular drug, nocodazole, for the study.

After removal of the nocodazole the cells formed new radial arrays of cytoplasmic microtubules with the centrosome at their center [Osborn and Weber, 1975]. In the following discussion, I shall call them "cytoplasmic asters" in order to distinguish them from "mitotic asters." With the exception of a few drug-insensitive microtubules, the cytoplasmic asters contained microtubules no older than 30 min. Their degree of stability could be assumed to be considerably more uniform, and to carry no imprints of past illumination periods. Consistent with this expectation, the cytoplasmic asters contained no Glu microtubules (Fig 1c,d).

Altered Stability of the Cytoplasmic Asters of Near-Infrared Irradiated CV1 Cells

Using CV1 cells with new cytoplasmic asters, I found that pulsating near-infrared signals in the presence of a second addition of nocodazole, indeed, promoted the disassembly of the cytoplasmic asters of the cells. The results point to a mechanism that sends signals along microtubules that mediate between light reception at the level of the centrosome and extension of specific pseudopodia at the cell periphery.

RESULTS

Disassembly of Cytoplasmic Asters of CV1 Cells

Absence of Glu-microtubules in reconstituted cytoplasmic asters. Before the cells were exposed to any anti-microtubular drugs, they showed a dense and highly centralized array of cytoplasmic microtubules (Fig. 1a) that contained a large number of stabilized microtubules as shown by anti-Glu-microtubule staining (Fig. 1b).

By contrast, the radial organization of the reconstituted cytoplasmic asters appeared less perfect (Fig. 1c), but they contained no Glu microtubules (Fig. 1d). In addition to a well-organized centralized array, the cells contained many microtubules that appeared to have polymerized without any connection with the centrosome. Over time, these microtubules can be expected to disappear because they are more likely to be spontaneously disassembled by the mechanism of the so-called "dynamic instabilities" [Mitchison and Kirschner, 1984; Mitchison et al., 1986] than are microtubules that are capped on the minus end by the centrosomal MTOCs [Gould and Borisy, 1977]. The nonradially arranged microtubules seemed to disappear more rapidly than the radially arranged microtubules after a second incubation in nocodazole, which was required by our assay (see under time course of the disassembly of cytoplasmic asters, below). Therefore, all the following illustration show again mostly radial arrays of microtubules.

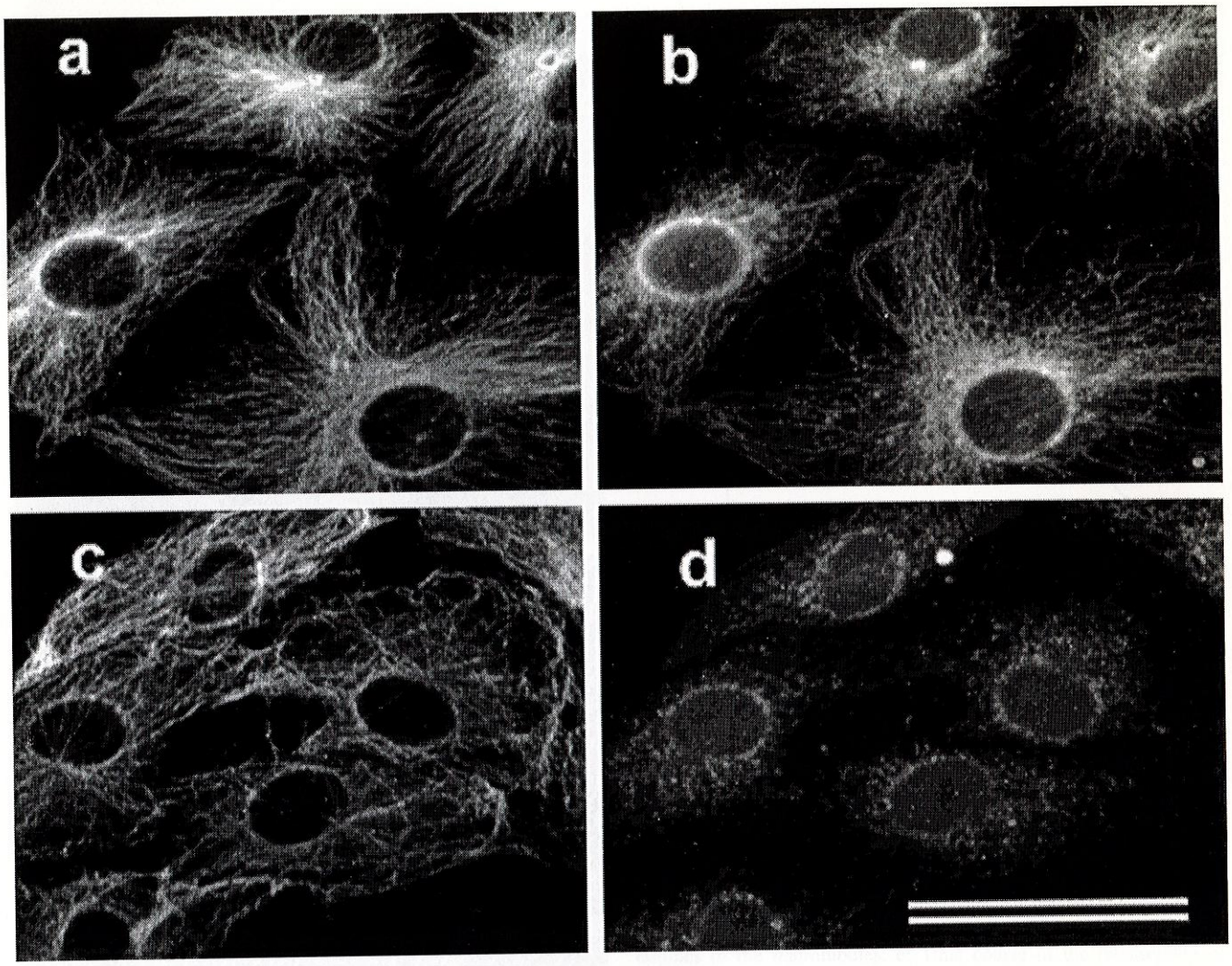


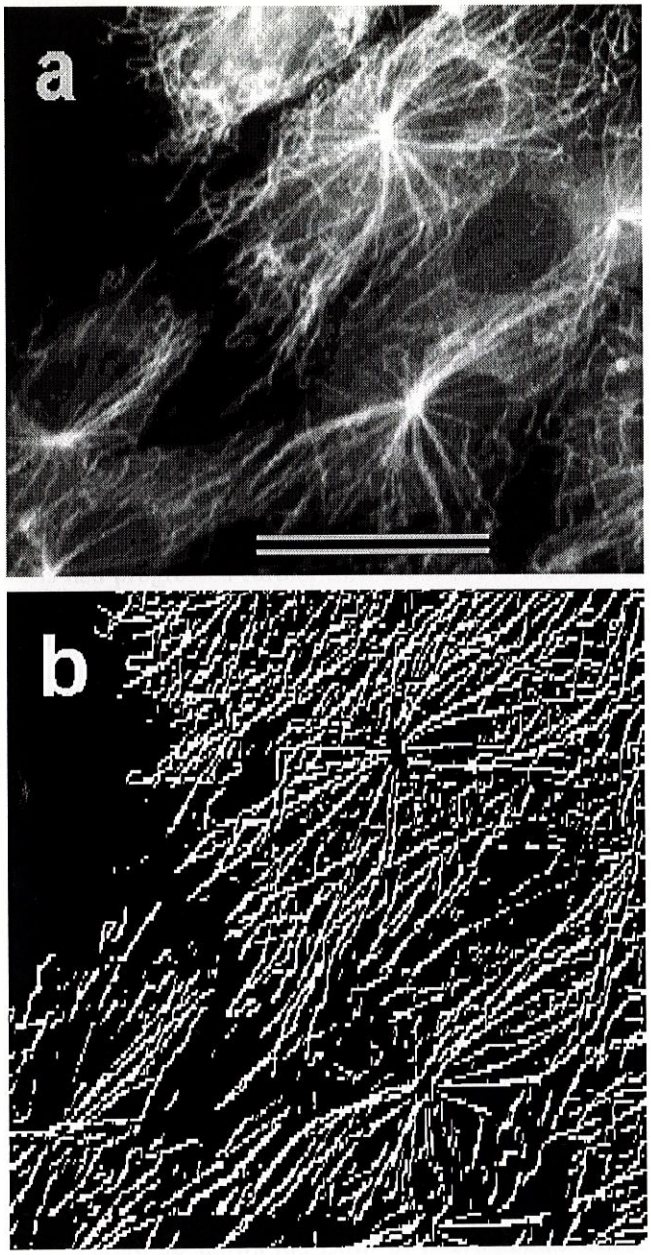
Fig. 1. Cytoplasmic asters of CV1 cells lacking Glu-tubulin. Bar = 50 μm. **a,b:** Immunofluorescence pattern of α-tubulin **(a)** and Glu-α-tubulin **(b)** of the cytoplasmic microtubules of the same field of CV1 cells. A large subset of the microtubules shown in **a** stain also anti-Glu-α-tubulin antibody. **c,d:** Immunofluorescence pattern of α-tubulin **(c)** and Glu-α-tubulin **(d)** of the cytoplasmic asters of the same field of CV1 cells. The cells show only background staining for Glu-α-tubulin.

Quantitative description of reconstituted cytoplasmic asters. In order to measure the number of reconstituted microtubules I developed for a digital camera special software that processed the immunofluorescent images of cytoplasmic asters stained with anti-α-tubulin. The details of the procedure are described under Materials and Method. Figure 2 illustrates a typical field of cytoplasmic asters (Fig. 2a) and the processing action of the program (Fig. 2b) in selectively highlighting the microtubules and counting the number N of highlighted pixels, while suppressing the various levels of diffuse background staining. Since cultures of CV1 cells contained very few multinucleated cells and each interphase cell contained only one centrosomal area the number C of cells in the field were counted manually by mouse-clicking the number of nuclei or cytoplasmic asters visible in the staining patterns. Subsequently, the computer calculated the number N of highlighted pixels per cell:

$$A_m = \frac{N}{C} \text{ pixels/cell} \qquad (1)$$

The following discussion uses this ratio A_m as a measure for the total amount of microtubules per cell.

The reproducibility and uniformity of the tubulin staining patterns and consequently, their quantitation depended critically on the plating density and culture age. If the cells were plated too sparsely, cell sizes became quite variable, counting became too time-consuming, and



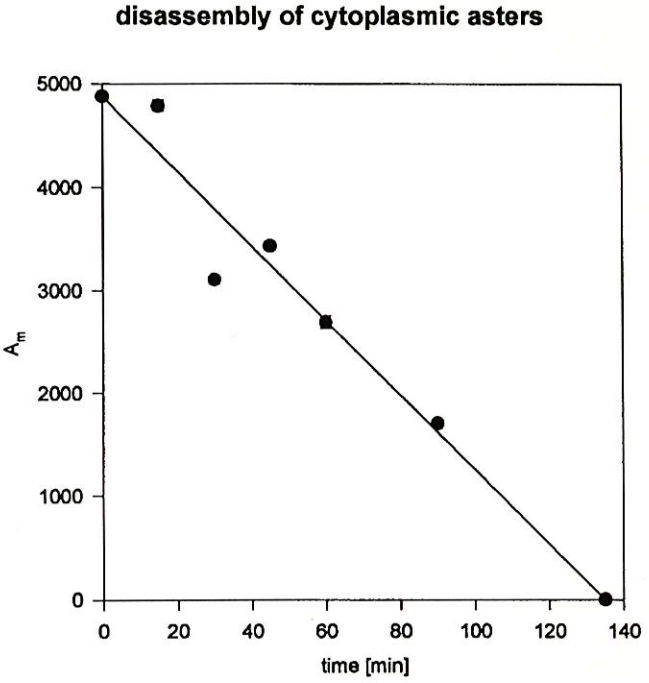


Fig. 2. Computerized measurement of the total amount of stained microtubules. **a:** Immunofluorescent micrograph of DCAs showing various levels of background staining. (Bar indicates 30 [μm]). **b:** The same image field after image processing by the computer program hicount.exe (see under Materials and Methods) showing only the outlines of the microtubules. **c:** Time course of the disassembly of cytoplasmic asters. Using the computerized method shown in **b,** the number of microtubules per cell (ordinate: A_m) (see eq. 1) was measured as the number of highlighted pixels per cell and plotted as a function of the duration in [min] of the second exposure of the cells to 20 μM nocodazole. The individual data points represent on average measurements of 850 cells each (range, 314–2422 cells). The errors of the mean for each data point are smaller than 2%. The line represents the 95% confidence linear regression line.

it was difficult to accumulate adequate sample sizes. If they were plated too densely, the staining patterns became quite diverse generating very large variances of the counts. I found that a plating density of 5,000–7,500 cells per 35-mm dish and a culture age of 2–3 days gave results that were optimal in terms of reproducibility and uniformity of the staining patterns.

Time course of the disassembly of cytoplasmic asters. As one would expect, a second exposure of the cells to culture medium (DMEM) containing 20 μM nocodazole at 37°C disassembled the cytoplasmic asters again. In the following discussion, I call this process the "disassembly of cytoplasmic asters (DCA)."

Figure 2c shows the time course of the DCA using the quantitative method described above based on an average of 850 cells per data point. Measuring time t in min, the number of microtubules/cell fits a linear regression.

$$A_m = A_{m0} - a \times t \qquad (2)$$

with the initial amount of microtubules per cell: A_{m0} = 4,881 pixels/cell, the disassembled microtubular counts

per cell and minute: a = 36 pixels/cell/min, and t = time (min), with a regression coefficient of 0.92. They extrapolate to a disassembly time of 135 min.

Sensitivity of DCA to Pulsating Near-Infrared Light Signals

Using the apparatus described in Figure 5, I tested whether the DCA was sensitive to the same near-infrared signals that had previously been shown to cause 3T3 cells and CV1 cells [Albrecht-Buehler, 1991, 1995] to extend pseudopodia toward microscopic light sources emitting them. Therefore, I exposed cultures of CV1 cells inside the experimental bin to such light signals (λ = 835 nm; pulse shape: rectangular; pulse length 1.0 s; total light power 4–16 μW for 30 min) while shielding the identical control preparations inside the control bin from the light.

Figure 3b shows that the DCA of the experimental cells had progressed further than the DCA of the control cells (Fig. 3a), suggesting that the exposure to the near-infrared light signals had decreased the stability of their cytoplasmic asters in the presence of nocodazole. Furthermore, it appeared that the diffuse staining of the experimental cells was increased compared with the control cells as if the cells had somehow stored the disassembled tubulin in a diffuse form.

Quantitative Description of the Dependence of DCA on the Characteristics of the Light Signals

In order to quantify the change of microtubule stability, CMS, I measured the amounts of microtubules per cell in irradiated and control cells as described in equation (1) and calculated the relative difference.

$$\mathrm{CMS} = \frac{\overline{A_m^{\mathrm{control}}} - \overline{A_m^{\mathrm{irradiated}}}}{\overline{A_m^{\mathrm{control}}}} \qquad (3)$$

where the bar above the variables indicates the average value.

Figure 3e shows the CMS of microtubules whose cells were exposed to near-infrared light signals (λ = 835 nm; intensity = 4 − 16 μW/cm^2; pulse length = 1 s) as a function of time, based on the average of 1,779 cells for each data point (range 634–5,554 cells).

Obviously, at the beginning of the second exposure of the cells to nocodazole (t = 0 min), as well as after completion of the disassembly (t = 135 min) there cannot be a difference between irradiated and control cells. Therefore, the CMS values at times t = 0 and t = 135 min were set to zero.

The results show that after 45 min of exposure to 20 μM nocodazole, the cytoplasmic asters of irradiated cells had disassembled approximately 25% more than control cells (Fig. 3e). Applying a two-sample t-test assuming unequal variances to the time point t = 45 min the difference of 25% between experiment and control is significant on a level of $P < .004$.

Figure 4 shows the wavelength dependence of the CMS of CV1 cells. Similar to the action spectrum of single 3T3 cells reaching out to microscopic near-infrared light sources [Albrecht-Buehler, 1991], which showed a peak at 900 nm, the wavelength near 835 nm had the strongest effect on the DCA of CV1 cells. The slight difference between the locations of the peak sensitivities may be attributable to the difference of cell lines. Therefore, the stability-altering signals along the microtubules described here may be involved in the extension of pseudopodia toward near-infrared light sources in the earlier single-cell experiments.

DISCUSSION

Pulsating near-infrared signals of 1 s pulselength seemed to accelerate the nocodazole-induced disassembly of new cytoplasmic asters in entire populations of CV1 cells. They also yielded a spectral sensitivity curve of the DCA (Fig. 4), which was very similar to the action spectrum of the behavior of single cells exposed to pulsating near-infrared signals [Albrecht-Buehler, 1991]. Therefore, the extension of specific pseudopodia by single cells exposed to the light signals appears to be closely related to the reduced stability of the radial microtubules of an entire population of cells exposed to similar irradiation.

The mechanism by which the light signals altered the stability of the radial microtubules remains unknown. However, for the following reasons, one can exclude heat and light sensitivity of microtubules and nocodazole as explanations.

Exclusion of Trivial Explanations of the Results

Exclusion of heat effects as the explanation of the results. During irradiation in the experimental bin, the cells and their cover slips were submersed in a volume of 50 ml of culture medium inside the "experimental bin." Any heating effect of the cells would have been buffered and dispersed by it, unless the culture medium itself (i.e., essentially water) was heated by the irradiating light. There are two major reasons to exclude heating of the medium as an explanation for the results:

1. The irradiation did not supply enough energy to heat the volume of the bin to any measurable extent. During its duration of 45 min, the typical experiments delivered the total energy of 5.4 mcal to the 50-ml large bin volume of water, which could heat it by no more than 10^{-4}°C.

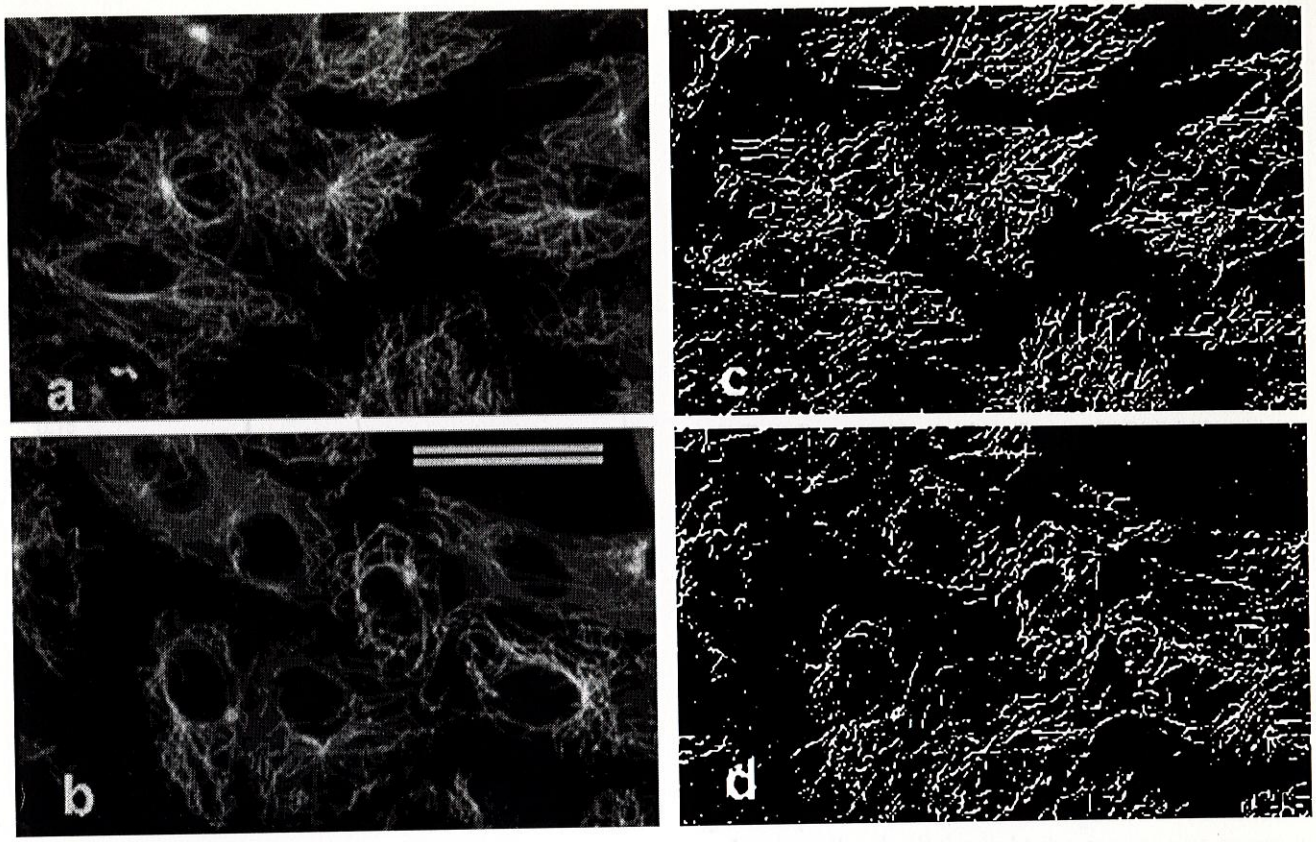


Fig. 3. Disassembly of cytoplasmic asters (DCA) after 30 min in the absence and presence of near-infrared light. Bar = 50 μm. **a:** Control: Immunofluorescent micrograph of a typical DCA of CV1 cells after 30 min in the absence of near-infrared light. Compared with the experimental cells (**b**) the diffuse staining of the cells is less pronounced and the number and of microtubules radiating from the centrosomal area is increased. **b:** Typical DCA of CV1 cells illuminated with near-infrared light for 30 min (λ = 835 nm; pulse length 1.0 s; total light power 4 μW). The cells are rather diffusely stained; compared with controls, there are fewer microtubules radiating away from the centrosomal area. **c,d:** The corresponding images after image processing by the computer program hicount.exe (see under Materials and Methods), which highlights the microtubules and counts the total number of highlighted pixels. In the example shown, the counts were 44314 pixels/8 cells (control; **c**) and 33500 pixels/8 cells (experiment; **d**). **e:** Destabilization of nocodazole treated cytoplasmic asters exposed to near-infrared light signals (ordinate: CMS; see eq. 3); λ = 835 nm; intensity = 4–16 μW/cm^2; pulse length = 1 s as a function of time of the second incubation in nocodazole. After 15–60 min of exposure of the cells to 20 μM nocodazole, the cytoplasmic asters of irradiated cells disassemble faster than control cells leading to a stability ratio (ordinate) that is smaller than 1. The individual data points represent on average measurements of 1,970 cells each (range, 634–6188 cells). The CMS values at t = 0 and t = 135 [min] are extrapolated values. Error bars = errors of the mean.

2. Water has a continuously rising absorption coefficients in the range of the wavelengths used in the experiments [Albrecht-Buehler, 1992]. Therefore, the alleged heat effect should rise with increasing wavelengths in contrast to the results which show that the change of stability, CMS, decreases steeply at wavelengths λ >900 nm (Fig. 4).

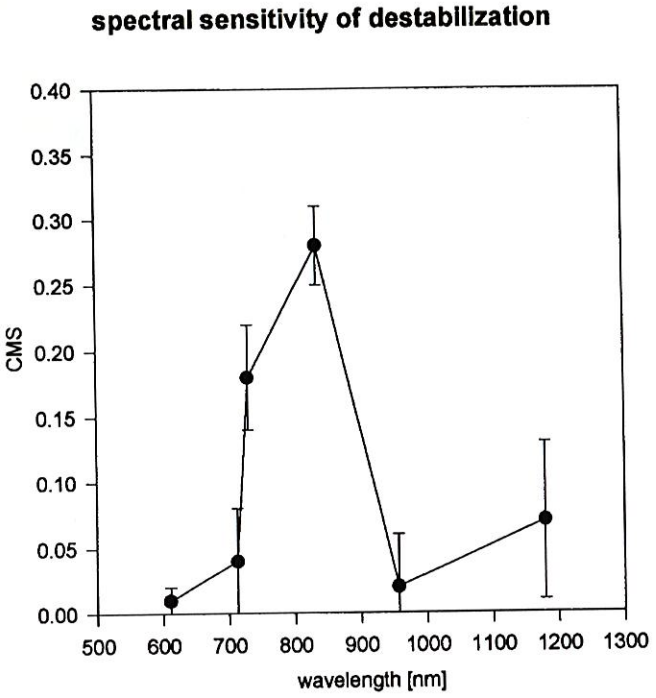


Fig. 4. Wavelength dependence of the destabilization of the microtubules of CV1 cells (ordinate: CMS; see eq. 3); rectangular pulses; pulse length = 1 s; intensity = 4 μW; average sample size: 2340 cells/data point (range, 1,270–5,060 cells/data point). Error bars = errors of the mean.

Exclusion of microtubules and nocodazole as the light sensitive factors. It is well known that nocodazole is not sensitive to visible light let alone to near-infrared light, which has an even lower photon energy. In fact, this insensitivity to light is one of the reasons that cell biologists widely prefer the drug to the light-sensitive colchicine that preceded it historically.

As to the light sensitivity of microtubules, practically all tubulin biochemistry has traditionally been carried out on open benches in well-lit laboratories without showing any effect of the illumination of tungsten filament and fluorescent lamps that emit copious amounts of visible and near-infrared light.

The earlier, single-cell experiments showed that direct irradiation of cytoplasm that included microtubule-rich domains did not prevent the extension of special pseudopodia towards near-infrared light sources as long as the centrosome itself was not irradiated [Albrecht-Buehler, 1994].

Arguments for the Propagation of Stability Altering Signals from the Centrosome to the Plus-End of the Astral Microtubules

In the absence of infrared light sensitivity of the microtubules, their observed alteration of stability must have been an indirect effect on the microtubules. Thus, the cells appeared to contain components that are infrared-light-sensitive and are connected to the microtubules whose stability they changed.

The most likely candidate for one such a component seems to be the centrosome. It is sensitive to infrared light [Albrecht-Buehler, 1994], and in animal cells it contains the centrioles whose geometric properties are consistent with the hypothesis that they are cellular instruments to locate light sources [Albrecht-Buehler, 1981, 1992]. The centrioles, in turn, are associated with the nucleating microtubules organizing centers (MTOCs) of the cytoplasmic microtubules [see references in Albrecht-Buehler, 1992].

For these reasons, the observed destabilization of the aster microtubules in the presence of nocodazole was likely to be initiated at their minus-end near the centrosome [Gould and Borisy, 1977]. In order to affect the disassembly process by nocodazole it had to propagate to the opposite end (i.e., plus-end) which is the preferred site of assembly and disassembly of tubulin dimers [Bergen and Borisy, 1980; Kirschner, 1980]. Consequently, I conclude that the stability altering signals from the centrosome were propagated along the length of the microtubules in response to the pulsating light signals. Single-cell experiments showed that the cells will eventually attempt to reach out to the light sources. In other words, upon "seeing" the light the centrosome appeared to send signals along its radial array of microtubules that eventually initiated specific movements at the distant cell periphery. Thus, one of the functions of microtubules may be to play the role of cellular "nerves."

MATERIALS AND METHODS

The experimental procedure and the evaluation program described here depend critically on a "reasonable" number of microtubules that are left in the control cells to count. If there are too few or too many microtubules left in the control cells the difference between controls and experiment may not be large enough to exceed the level of experimental variations. Therefore, each new project should begin with an exploratory series of experiments to determine the optimal nocodazole concentration and incubation times.

Culture Conditions and Chemicals

Stock cultures of CV1 cells were grown on plastic culture dishes in Dulbecco's modified of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a tissue culture incubator at 37°C in an atmosphere of 8% CO_2 and saturated humidity. Experimental cells were plated into 35-mm plastic dishes containing a 22 × 22 mm^2 glass coverslips. The initial plating density was 5,000–7,500 cells per 35-mm dish.

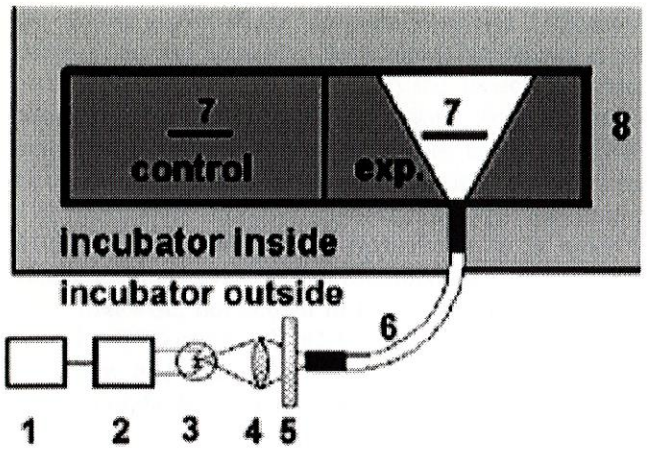


Fig. 5. Basic experimental setup for the DCA assay. 1, timer (sets pulsation pattern); 2, power supply; 3, filament bulb; 4, lens; 5, interference filter (selects illumination wavelength); 6, fiberoptic cable to transmit the light into tissue culture incubator; 7, bins filled with 50 ml of DMEM + 20 μM nocodazole at 37°C. The control bin is lined with opaque Lucite and is closed with a light, but not gas-tight, cover; 8, inside space of the tissue culture incubator.

Before being used in the experiments the cells were grown for 2–4 days under the above growth conditions. Nocodazole (methyl-(5-[2-thyenylcarbonyl]-1H-benzimidazole-2-yl)carbamate (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) in stock solutions of 2 mg/ml and added to DMEM for the final dilution.

Disassembly of Cytoplasmic Asters

Although the cells were grown under sterile conditions, the apparatus used in the DCA assay (see Fig. 5) could not be kept entirely sterile. Therefore, all DMEM-derived incubation solutions contained 0.01% Na-azide in order to reduce bacterial growth. Because DMEM contained 500 mg/L glucose and, because all incubation times were less than 30 min, it was not expected that such low Na-azide concentrations would affect the CV1 cells; indeed, I could not detect any effect of the Na-azide concentrations used on the results.

First nocodazole incubation. Two identical sets of coverslips ("control" and "experimental") were placed vertically in white porcelain staining racks, lowered into a beaker containing DMEM + 20 μM nocodazole at 37°[C] (pH = 7.4) and placed inside the dark tissue culture incubator at 37°C (8% CO_2) for 30 min.

Recovery. The racks with the cover slips were rinsed in DMEM and placed in a beaker containing DMEM at 37°C (pH = 7.4) and placed inside the dark tissue culture incubator at 37°C (8% CO_2) for 30 min.

Second nocodazole incubation and light exposure. The control and experimental racks were placed in either the "exposure bin" or the "control bin" of the light exposure apparatus (see Fig. 5). During the light exposure, both bins were covered with plastic plates that allowed gas exchange. The cover of the "control bin" was opaque, while the cover of the 'exposure bin' was transparent for the wavelengths used. Typical data of the light exposure were: exposure time 45 min; wavelength = 835 nm; pulse shape = rectangular; pulse length = 1.0 s; total light power at end of fiber optics cable = 4 μW; distance between the end of the fiberoptics and the coverslip = approx. 2 cm; medium between fiberoptic cables and test coverslips was DME (containing standard concentrations of phenol red);

Fixation. In order to reduce the diffuse fluorescent background of the preparations, the coverslips were incubated for 5 min in an extraction fixative (3.5% formaldehyde, 0.1% nonionic detergent Nonidet P-40 (Sigma) in Pipes buffer at pH = 7.0) and postfixed in −20°C methanol for 5 min. Subsequently, they were rinsed in phosphate-buffered saline (PBS) for 10 min. Cells to be stained for Glu-tubulin were fixed only in −20°C methanol for 5 min. After methanol fixation, the coverslips tend to be quite water repellent, which interferes with the subsequent antibody staining. Therefore, the methanol fixation was followed by a 30 s washing of the coverslips in PBS containing 0.1% NP-40.

Staining. The coverslips were stained with a mouse monoclonal anti-α-tubulin antibody (Amersham International, Little Chalfont, UK) (monoclonal anti-α-tubulin N356, batch 90653, dilution 1:50) for 10 min, then rinsed 3× in PBS. Subsequently, they were post-stained with rhodamine-labeled goat-antimouse antibody for 10 min (Southern Biotechnology, Birmingham, AL) (Cat. No 1010-03; lot: BO85-U265B) and rinsed 3× in PBS. Finally, the coverslips were rinsed in distilled water, dried with a hair dryer, and mounted with wax around the edges. The drying process had no significant effect on the fluorescence patterns of the preparations, but it reduced their speed of fading and reduced the background staining because the embedding medium could not slowly extract the secondary antibody. The rabbit anti-Glu-tubulin antibody was a kind gift of Dr. Gregg G. Gundersen (Department of Anatomy and Cell Biology, College of Physicians and Surgeons of Columbia University).

Light Exposure Apparatus

During their exposure to near-infrared light, the preparations were placed inside the bins of the apparatus illustrated in Figure 5. The timer (Gralab 451, Thomas Scientific, Swedesboro, NJ), lamp, and filters (Edmund Scientific, Barrington, NJ) were kept outside the tissue culture incubator in order to exclude the exposure of the preparations to any other than the selected wavelengths of light and in order to prevent any heating of the atmosphere around them. Therefore, small holes were cut into

the sealing gasket of the incubator door and 2 fiberoptic cables (Edmund Scientific, Barrington, NJ) (5-mm diameter) were fed through them into the incubator, which conducted the light to the preparations. For the sake of simplicity, Figure 5 shows only one fiberoptic cable. However, in the experiments described in this paper, I fed two such cables into the "experimental bin" that illuminated the preparations from opposite sides in order to generate a more even field of illumination.

Likewise, not shown in Figure 5 was an infrared-sensitive CCD camera (EDC-1000 CCD camera; Electrim, Princeton, NJ) (196 × 165 pixels × 256 colors), encased in a gas-tight housing, placed inside the incubator, and trained on the "experimental bin." The light scattering by the white porcelain rack in which the preparations were placed offered a convenient way to monitor the pulsation rate, the level of intensity and the spatial distribution of the irradiating light. The software to control the CCD camera was written in C++ by the author.

During experiments that determined the time course of the DCA, it was necessary to prevent exposure of the preparation to the light from the ceiling lamps of the laboratory. Therefore, the room lights were turned off before the incubator doors were opened to transfer the preparations to another bin, or to retrieve preparations for fixation. These operations required some light; nevertheless, the inside of the incubator was illuminated continuously with low levels of light previously been shown not to affect the ability of cells to reach out to pulsating near-infrared sources [Albrecht-Buehler, 1991]. Therefore, I fed a fiberoptic cable into the incubator that illuminated its inside continuously (source intensity: 100 $\mu W/cm^2$; wavelength: 600 nm). The light intensity at the level of the experimental preparation was approximately 0.3 $\mu W/cm^2$ at 600 nm (not shown in Fig. 5). In all other experiments, this inside illumination of the incubator was turned off.

Selection of wavelength and intensity. Different wavelengths of the light were selected by using different interference filters (labeled 5 in Fig. 5). Their nominal wavelengths were 600, 700, 730, 800, 900, and 1060 nm. The actual transmission characteristics of the filters were measured with a Beckman DU 650 spectrophotometer (Beckman Instruments, Fullerton, CA): 600 nm = 611 ± 18 nm; 700 nm = 713 ± 100 nm; 730 nm = 742 ± 7; 800 nm = 835 ± 33 nm; 900 nm = 957 ± 43 nm; 1060 nm = 1180 ± 100 nm. Therefore, I used the measured peaks of transmission in the text and the graphs.

Different light intensities were adjusted by changing the output of the power supply (labeled 2 in Fig. 5) and measured as the total light power emitted by the distal end of the fiberoptic cable (labeled 6 in Fig. 5) using a Tektronix J16 digital photometer with a J6502A probe (probe cross section: 1 cm^2) (Tektronix, Beaverton, OR). For the calibration, the end of the fiberoptic cable was placed approximately 1 cm away from the probe so that it illuminated the entire probe area. Consequently, the reading of the photometer was calibrated in $\mu W/cm^2$ and used directly to characterize the intensities used in the experiments. The intensities to which the cells were exposed were approximately 10 times lower because the preparations were placed about 3 cm away from the ends of the fiberoptic cables. Different pulse lengths were set by the timer (labeled 1 in Fig. 5).

The intensities of the present experiments can be estimated to be on the order of 450 nW/cm^2 at the level of the preparations. They were approximately 15 times stronger than the intensities used in the single cell observations of our previous publications, which generated intensities I had estimated to be on the order of 30 nW/cm^2 at the level of the cells near a microscopic light source (Albrecht-Buehler, 1991). (For comparison, the intensity of normal sunlight at sea level is about 80 mW/cm^2, or about 180,000 times stronger than the experimental intensities used in the present experiments. The increased light levels were chosen for the present approach in the hope of eliciting a stronger response of the microtubular system, while of course avoiding any light damage to the cells.

Evaluation of Results

The preparations were viewed in a Zeiss Photomicroscope III using a 40× oil immersion lens with an N.A. of 1.0. The fluorescent images were recorded with a EDC-1000HR CCD camera (Electrim) 753 × 244 pixels × 256 colors. Evaluation of the data used the program hicount.exe, written in Borland C++ by the author. A copy of the program can be downloaded from the website: http://www.basic.nwu.edu/g-buehler/hicam.htm. This program suppresses the diffuse background of the images, highlights the microtubular outlines, and counts the total number of pixels occupied by the stained microtubules (Fig. 2). It only counts objects that form continuous lines, but not large stained areas. For example, the centers of the asters are not counted, whereas the radial microtubules are. Subsequently, the user counts the number of cells in the image, and the program calculates and records the number of highlighted pixels per cell—a direct measure of the total length of microtubules per cytoplasmic aster.

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