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RESPONSE OF INFUSORIAN CELLS TO INJURY CAUSED BY A LASER MICROBEAM

MORAVČÍK Z.[1], JANISCH R.[1], JEŽEK J.[2], ZEMÁNEK P.[2]

[1]Department of Biology, Medical Faculty, Masaryk University, Brno
[2]Institute of Scientific Instruments, Academy of Sciences, Brno

A b s t r a c t

The effects of pulse laser irradiation on the cortex of *Paramecium caudatum* and *Blepharisma undulans undulans* were studied. The character and extent of cortical injury was video recorded and subsequently analyzed. Destruction of the cytoskeleton by laser irradiation was detected by immunofluorescence staining. A difference in the development and healing of the wound was observed between *Paramecium* and *Blepharisma* cells. A more immediate reaction was recorded in *Blepharisma* cells containing blepharismin, a red pigment, known to absorb light energy. The damage to the infusorian cortex due to laser irradiation was compared with that produced by mechanical devices.

K e y w o r d s

Pulse laser irradiation, Microsurgery, Optical scissors, Laser microbeam, *Paramecium*, *Blepharisma,* Cell cortex damage

INTRODUCTION

The infusorian cell has a complex surface structure termed the cortex, which makes it a very convenient model for the study of cell surface reactions to various kinds of damage. Local defects in the surface are particularly well discernible and the progress of their repair can be recorded in terms of both morphology and physiology and compared with intact surfaces. Injury to the cell surface can be induced by either mechanical or photonic devices. The former, such as a mechanical microscalpel, were first made of glass fibers and later of metal, and both types have frequently been used in experimental cytology (for review see Balamuth (*1,2)*. The latter are based on pulse UV irradiation and are called "laser scissors". Even before lasers were introduced in microsurgical techniques, light energy in the form of a UV microbeam was used to produce targeted damage to cell structures (*3*). Laser scissors, based on a focused UV laser beam (microbeam), came into intensive use more than 30 years ago (for review see *4,5*). They have been shown to damage organic biopolymers without thermal destruction of the surrounding structures (*6*). Pulse lasers are also often used to make holes in the cell surface in order to (i) facilitate transfer of various materials

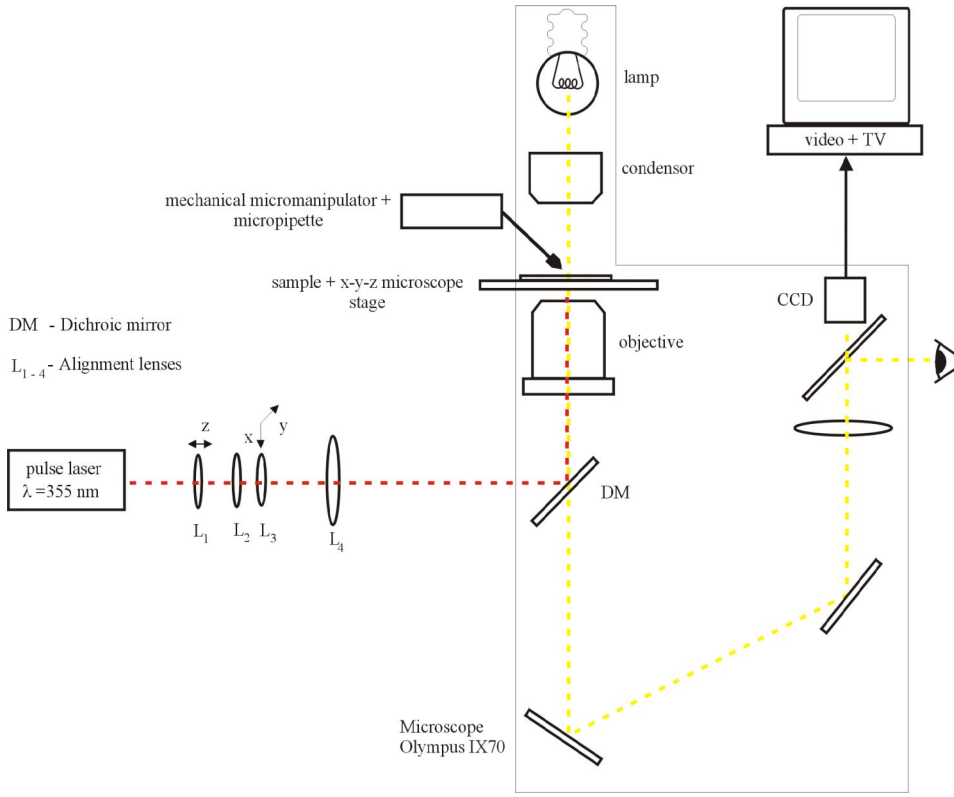

Fig. 1

A schematic drawing of the laser scissors used

such as organelles, DNA molecules or foreign particles into the cell interior (*7*), (ii) prepare the egg surface in assisted reproduction or (iii) damage specific cellular structures (*8*). In any deliberate microsurgical interference with the cell surface there is always concern that the invasiveness of the procedure be minimal. *Berns et al.* (*9*) described a procedure for the chemical analysis of microscopic amounts of ionized gas produced in the minute focal volume of laser scissors.

Cell surface reactions to mechanical damage by a metal microscalpel have been extensively studied in *Paramecium* cells and the process of wound healing and changes in the behavior of the damaged cell have been reported (*10–14*). In this study, the appearance of damage produced in the cell surface by laser scissors and reactions of the infusorian cortex are described and compared with the previous findings. The results reported here provide the first information on the effect of pulse laser irradiation on the cortex of *Paramecium* and *Blepharisma* cells.

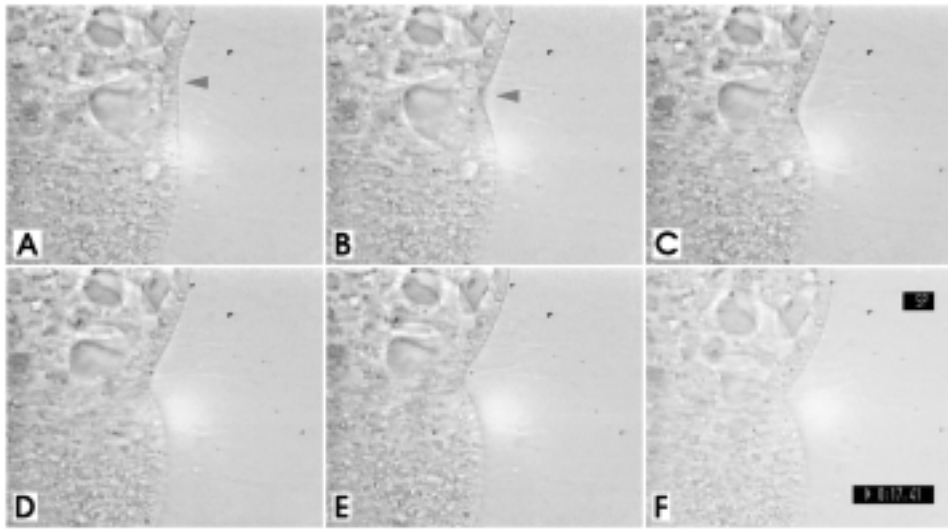

Fig. 2

Effect of tangentially directed laser beam on the *Paramecium* cell

MATERIALS AND METHODS

Specimens

The clones used included *Paramecium caudatum* Ehrenberg (*15*) isolated from the local environment and *Blepharisma undulans undulans* Stein (*16*) obtained from the Centre de Biologie Cellulaire, Clermont-Ferrand, France. Both species were grown, in pure culture, in Villeneuve-Brachon's (*17*) medium containing wheat grains and a *Klebsiella aerogenes* inoculum at room temperature.

Treatment of cells

1.1 Cell immobilization. For irradiation by pulse laser, a single infusorian cell was drawn into a micromanipulation capillary and thus immobilized. The procedure was carried out in a drop of water on a microscopic slide. We used a TransferMan NK mechanical micromanipulator with a CellTram Air micropipette (Eppendorf, Germany).

Pulse laser irradiation. Laser Minilite II (Continuum) was used to generate UV laser pulse with the following properties: wavelength, λ=355 nm; maximal pulse energy, 8 mJ; pulse length, 5 ns. The laser beam was enlarged by a system of lenses, directed to an Olympus IX70 microscope by dichroic mirrors and focused by Olympus Plan 20X and Plan 40X microscope objectives (*Fig. 1*). The position of the beam in the viewing field of the microscope was adjusted laterally and longitudinally by movable lenses L_1 and L_3 (*18, 19*). Specific areas of the surface or selected structures close below the surface of an immobilized cell were irradiated by laser pulses of energy ranging from 4 to 15 μJ.

Video-enhanced microscopy image processing

Images viewed in the microscope were recorded on video, digitized and transferred to a computer. These images were analyzed by software, developed in our laboratory, which automatically processed several images simultaneously. The program allowed us to enhance image quality by contrast expansion, adjustment of brightness, etc.

Visualization of microtubules stained by immunofluorescence

Cell treatment. The cells of *Blepharisma undulans undulans* were fixed and permeabilized in 1 % para-formaldehyde (Sigma, Germany) with 1 % Triton X-100 in PHEM buffer at pH 6.9 for 90 min. The fixative was removed by PHEM buffer (3 x 10 min). The cells were incubated with a TU-01 primary antibody (Exbio, Czech Republic) diluted 1:200 in PHEM and 2 % bovine serum albumin for 60 min. They were freed of excess antibody with PHEM and 2 % Tween 20 (Sigma, Germany) (3 x 10 min) and incubated with a SWAM/FITC secondary antibody (Sevac, Czech Republic) diluted 1:100 in PHEM for 45 min. After rinsing with PHEM and 1 % Tween 20 (4 x 10 min), the cells were mounted in Vectashield (Vector, USA) with DAPI. They were viewed in a direct fluorescence microscope to detect microtubules.

PHEM buffer consisted of 60 mM PIPES (10.389 g), 25 mM HEPES (2.603 g), 10mM EGTA (1.902 g), 2 mM $MgCl_2$ (0.095 g) and redistilled water up to 500 ml; pH was adjusted to 6.9.

Fluorescence microscopy image processing. Images viewed in a direct fluorescence microscope (Leitz Labor Lux S, Jena, Germany) were photographed by a cooled CCD digital camera (ST-8, Santa Barbara Instruments, USA). The camera had a mechanical shutter and exposure times ranged from 0.11 to 3 600 sec. The camera produced 16-bit-wide digital data.

The data were captured and processed by special software for fluorescence microscopy image analysis.

RESULTS

Cortex injuries in *Paramecium caudatum*

Damage to the cortex of a *Paramecium* cell immediately after pulse laser irradiation varied in appearance, as illustrated in two selected series of images in *Figs 2* and *3*. This appearance was related to irradiation intensity and the site of the damage. A laser beam directed tangentially produced a wound involving depression and contraction of the surface (*Fig. 2*). The surrounding cortex and the cytoplasm below remained unaffected.

A laser beam targeted close below the cortex had a more destructive effect (*Fig. 3*). Within a fraction of a second, due to the sudden rise in temperature at the irradiated site, organic molecules turned into gas molecules and produced gas bubbles of about 3 μm in diameter which in turn, caused the cytoplasm with organelles, mitochondria and trichocysts to "explode" out of the cell. The exploded cytoplasm was destroyed to such an extent that it produced no membrane-bound bodies. The cell itself reacted with a sudden contraction along its longitudinal axis, which moved it away from the viewing field. The wound in the cortex had a necrotic appearance and was free of the plasma membrane; a sharp, protruding rim of the wound was formed by the plasma membrane. Exploded trichocysts were visible as rod-shaped bodies (arrows) in the close vicinity of the cell (*Fig. 8*). After 20 seconds, the wound edge shrunk concentrically and the cortex preserved

around it retracted towards the cell center. This crater-like appearance was characteristic of wounds produced by a laser beam and was easily distinguished from injuries caused by a mechanical microscalpel (*12*).

Cortex injuries in *Blepharisma undulans undulans*

Exposure of the *Blepharisma* cortex to a laser beam had effects similar to those observed in *Paramecium* cells, only the contraction of the wound circumference was less distinct. The cytoplasm at the wounded site also burst out but left only a shallow depression in the surface (*Fig. 4*). This was clearly seen at 2 sec but, by 8 sec after irradiation, the surface had smoothed out. The spilled cytoplasm sometimes produced spherical bodies, each with a sharp outline, apparently a newly-formed plasma membrane. The edge contraction and depression in the center are presented in *Fig. 5*.

Cell immobilization in a capillary made it possible to focus a laser beam on a predetermined site (*Fig. 6*). A tangential, superficial laser beam focused on the caudal part of the cell released a small amount of cytoplasm. Within fractions of a second, the edge of the wound had contracted. When damage to the caudal part was highly destructive and the cell pole became disrupted, the wound, nevertheless healed within a few seconds through concentric contraction of its edge.

Wounds in the *Blepharisma* cortex due to laser irradiation distinctly affected longitudinal bundles of cortical microtubules. Immunofluorescence staining showed that the arrangement of bundles was severely affected; some of the bundles were either partially or completely destroyed (*Fig. 7*).

Series of images in *Figs 2–6* and *8* are also available as animations on the www address *http://med.muni.cz/biologie/thesis/moravcik/fb*

DISCUSSION

Reactions to injury produced in the cortex of a *Paramecium* cell by mechanical devices, such as a metal microscalpel, have been studied in detail (for review see *13*). Should either end part be severed, the defect is covered within seconds with a newly-formed plasma membrane continuous with the plasma membrane under the intact cell surface. This new membrane-covered surface of the wound protrudes above the remainder of the surface. Development of the membrane and its properties are related to the presence of calcium ions (*20*). The wound edge, formed by the preserved cortex, begins to contract after a period of several minutes.

The cortex response to laser-induced injury within the first few minutes of irradiation is different. Our results showed that the wound circumference in the undamaged cortex strongly contracted immediately after irradiation. This gave the damaged site a characteristic crater-like appearance, with necrotic cytoplasm in the wound center.

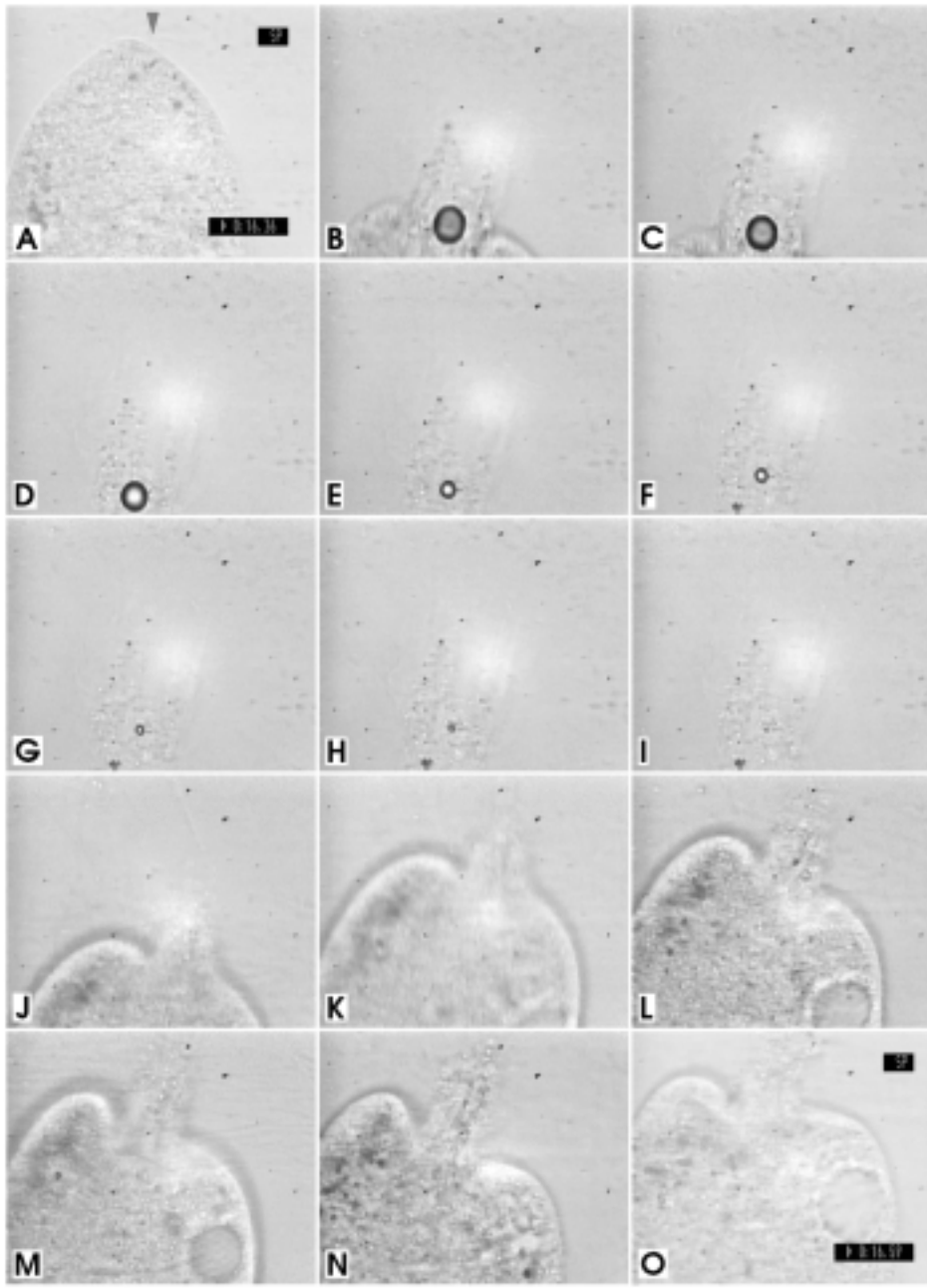

Fig. 3
A more destructive effect of laser beam targeted close below the cortex

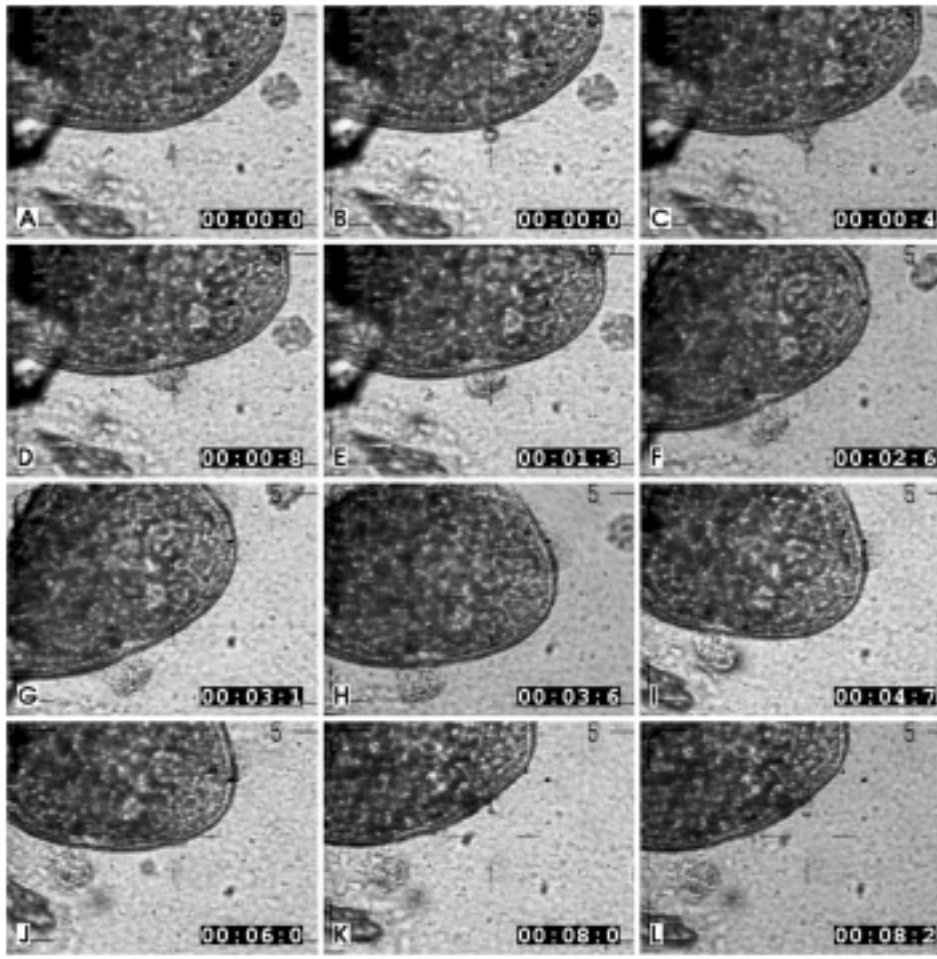

Fig. 4
Exposure of the *Blepharisma* cortex to a laser beam in the front part of the cell

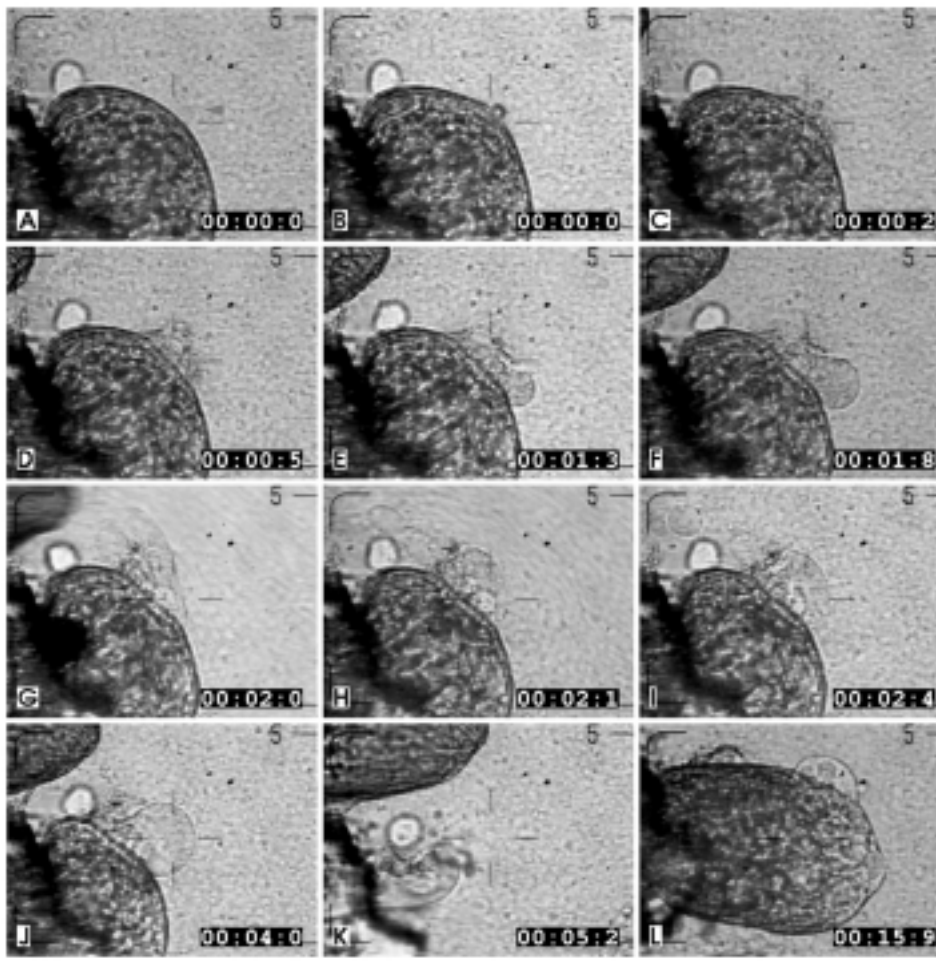

Fig. 5
The spilled cytoplasm after irradiation of *Blepharisma* cell by laser beam

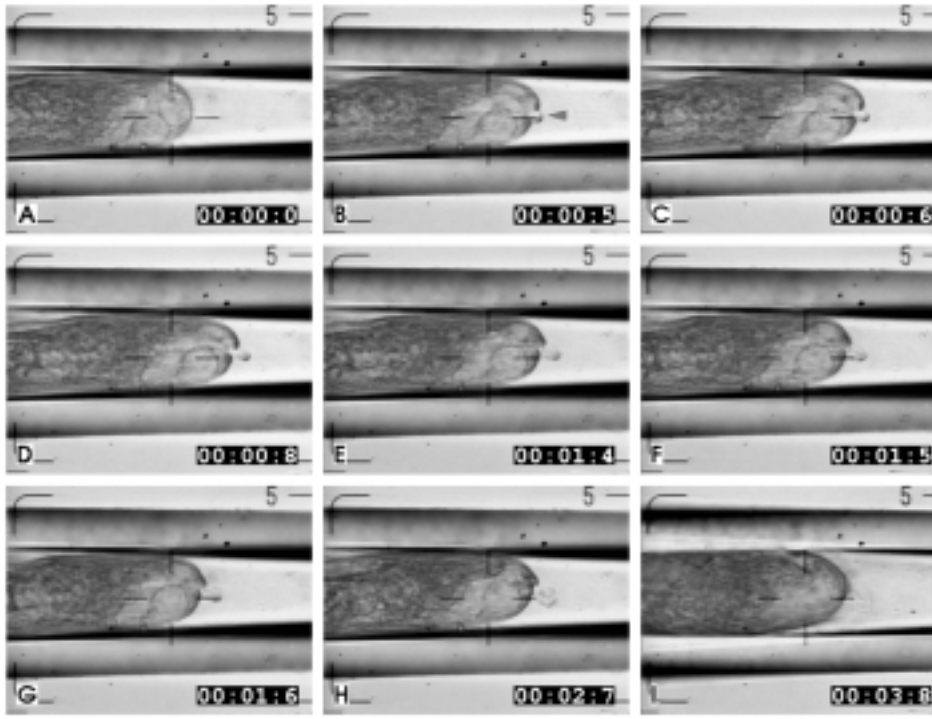

Fig. 6
Blepharisma cell immobilized in capillary and irradiated by a laser at caudal end

The *Paramecium* cortex is composed of subpellicular alveoli and a complex cortical cytoskeleton; its main components are microtubular structures involving kinetosomes continuous with ciliary axonemes, postciliary and transverse microtubules and, during cytokinesis, suprakinetodesmal microtubules also called the cortical cytospindle (*21, 22*). The other elements of the cortical cytoskeleton include a granulofibrillar network known also as the outer lattice, striated fibrils, kinetodesmal fibres and an infraciliary lattice situated at the deepest cortical level (*23, 24*). The infraciliary lattice was first described in 1937 by *Gelei (25*) in silver-coated specimens and later studied in detail with the use of electron microscopy by *Sedar and Porter* (*26*) and *Allen* (*23*). This irregular mesh is made up of bundles, 70 to 100 nm thick, consisting of parallel fibrils 3 to 4 nm in diameter. *Garreau de Loubrese et al*. (*27, 28*) identified two polypeptides (23 kD and 24 kD) responsible for the contraction of these fibrils in the presence of calcium ions. Evidence suggests that the infraciliary lattice is the only component of the cortical cytoskeleton with contractile abilities and is probably involved in the healing of any wound in the cell surface by facilitating contraction of wound circumference.

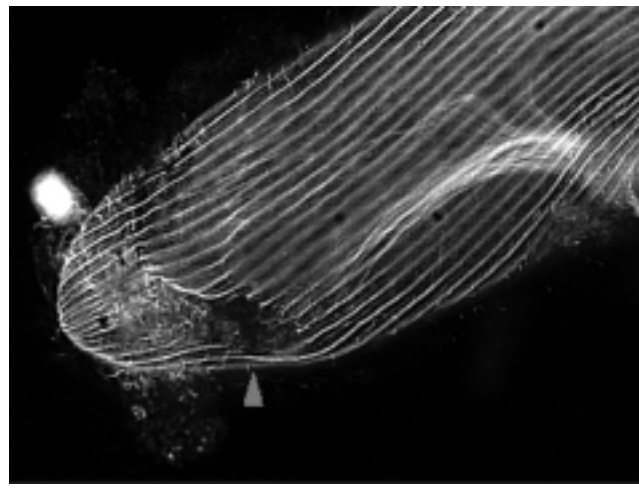

Fig. 7
Laser-induced wound in the *Blepharisma* cortex visualized by immunofluorescence staining

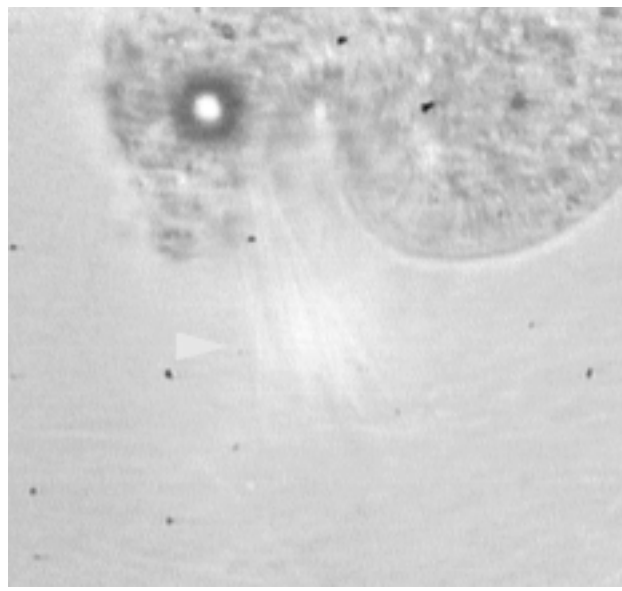

Fig. 8
Exploded trichocysts visible in the close vicinity of the cell

The different reaction of the *Paramecium* cortex damaged by a laser pulse, as compared to mechanical injury, can be explained by a local rise in temperature due to laser irradiation. This may have produced substantial changes in phospholipid and protein molecules that are available for an immediate repair of the plasma membrane in a mechanically damaged cortex. The absence of a membrane resulted in a higher loss of cytoplasm than is seen in mechanically produced wounds. The temperature rise may also have affected membrane vesicles whose large numbers in the cytoplasm are available as source material for a new membrane that, in a mechanically induced wound, develops on the exposed cytoplasmic surface (*12*).

In the *Blepharisma* cortex, the injury caused by pulse laser and the subsequent reactions showed different features. Contraction of the cortex preserved around the wound occurred almost immediately after irradiation, without the formation of a crater-like depression in the center.

The high elasticity of the *Blepharisma* cells was demonstrated by their behavior in capillaries whose diameter was smaller than the cell diameter. It is attributed to the system of longitudinal cortical microtubules visualized by immunofluorescence staining. A laser beam produced damage that varied according to the site of irradiation. In their cortical layer *Blepharisma* cells contain granules with a red pigment called blepharismin that are believed to be photoreceptor organelles with a defensive function (*29*). These granules apparently absorb more laser energy than the surrounding cytoplasm or other cell structures. When these granules are irradiated, the developing defect is more extensive and the consequences more serious. In *Blepharisma,* no cortical cytoskeletal structure similar to the infraciliary lattice of *Paramecium* has been found (*29*). Nevertheless, when an injury was extensive and the loss of cytoplasm was high, the wound edge was able to contract immediately, particularly on the cell poles.

The highly differentiated structures of the infusorian cortex permit a detailed study of both the defects produced by microsurgery and the subsequent processes leading to wound repair. Characteristic changes developing in different protozoan species in response to defined types of intervention make it possible to distinguish between the effects of microsurgery carried out with mechanical microscalpels and those caused by photonic devices. In the latter the heat released, after laser irradiation had been absorbed by the affected structures, plays the main role. Although the immediate, destructive thermal effect is limited to a very small region (about 1 to 3 μm in diameter), the injury spreads and involves the surrounding cortical areas; this is associated with morphological features such as a crater-like depression in *Paramecium.* A thorough study of the destructive action of laser irradiation at the ultrastructural level may help to gain a deeper insight into the adverse side effects of this method that is commonly used in cell biotechnologies.

A c k n o w l e d g e m e n t

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Moravčík Z., Janisch R., Ježek J., Zemánek P.

REAKCE BUNĚK NÁLEVNÍKŮ NA POŠKOZENÍ LASEROVÝM SKALPELEM

S o u h r n

Byl studován vliv ozáření pulzním laserem na kortex prvoků *Paramecium caudatum* a *Blepharisma undulans undulans*. Charakter a rozsah kortikálního poranění byl zaznamenáván na video a analyzován. Destrukce cytoskeletu ozářením laseru byla detekována imunofluorescencí. Rozdíl ve vzniku a hojení poranění byl sledován u buněk *Paramecium* a *Blepharisma*. Buňky *Blepharisma* obsahující červený pigment blepharismin, o kterém se ví, že absorbuje světelnou energii reagovaly lépe. Poškození kortikálního kortexu nálevníků po ozáření laserem bylo srovnáno s mechanickými vlivy.

REFERENCES

1. *Balamuth W.* Regeneration in Protozoa: a problem of morphogenesis. Quart Rev Biol, 1940; 15: 290-337.
2. *Tartar V.* Morphogenesis in Protozoa. In: Research in Protozoology, Chen TT, ed. Oxford, London, Pergamon Press, 1967; 2, 1–116.
3. *Hanson ED.* Inheritance and regeneration of cytoplasmic damage in Paramecium aurelia. Proc. Natl. Acad. Sci. U.S.A. 1955; 41: 175–194.
4. *Berns MW, Aist J, Edwards J et al.* Laser microsurgery in cell and developmental biology. Science 1981; 213: 505–513.
5. *Berns MW.* Laser surgery. Sci Am 1991; 264: 84–90.
6. *Srinivason R.* Ablation of polymers and biological tissue by ultraviolet lasers. Science 1986; 234: 559-565.
7. *Tao W, Wilkinson EJ, Stanbridge EJ, Berns MW.* Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane. Proc Natl Acad Sci USA 1986; 84: 4180–4184.
8. *Tao W, Walter RJ, and Berns MW.* Laser-transected microtubules exhibit individuality of regrowth, however most free new end of the microtubules are stable. J Cell Biol. 1988; 107: 1025–1035.
9. *Berns MW, Wright WH, Wiegand-Stebing R.* Laser microbeam as a tool in cell biology. Internat Rev Cytol. 1991; 129: 1–44.
10. *Janisch R.* Sub-microscopic structure of injured surface in Paramecium caudatum. Nature 1964; 204: 200–201.
11. *Janisch R.* Regeneration of Surface in Paramecium caudatum. Acta Protozool. 1965; 3: 363–367.
12. *Janisch R.* Formation of the plasma membrane during regeneration of Paramecium caudatum. Protoplasma 1985; 125: 94–102.
13. *Janisch R.* Biomembranes in the life and Regeneration of Paramecium. Acta Fac Med Univ Brun 1987; 93: 160 pp.
14. *Janisch R.* Response of the cortical cytoskeleton of Paramecium caudatum to merotomy. Arch Protistenk. 1991; 140: 321–333.
15. *Ehrenberg CG.* Die Infusionstierchen als volkommene Organismen. Leipzig 1838.
16. *Stein F.* Der Organismus der Infusionsthiere. Leipzig 1859–1878.
17. *Villeneuve-Brachon S.* Récherches sur les Ciliés heterotriches. Arch Zool Exp Gén. 1940; 82: 1–100.
18. *Zemanek P, Sramek L, Jonas A, Moravcik Z, Janisch R, Liska M.* Standing wave trap and single beam gradient optical trap - experiments and biological applications. In: Proceedings of the Wave and Quantum Aspects of Contemporary Optics (Proc. SPIE 3820), Hrabovsky M, Strba A, Urbanczyk W. eds., SPIE-The International Society for Optical Engineering, Washington. 1999; pp. 401–410.

19. *Jezek J, Jonas A, Liska M, Jedlicka P, Lukasova E, Kozubek S, Zemanek P.* Combined system for optical cutting and multiple-beam optical trapping. Proc SPIE, 2000; 4016: 303–308.
20. *Janisch R.* The mechanism of the regeneration of surface structures of Paramecium caudatum. Folia Biol. 1966; 12: 65–74.
21. *Cohen J, Adoutte A, Grandchamp S, Houdebine LM, Beisson J.* Immunocytochemical study of microtubular structures throughout the cell cycle of Paramecium. Biol Cell. 1982; 44: 35–44.
22. *Delgado P, Romeo MR, Torres A.* Microtubular system of Paramecium in Division: Pattern of cytospindle assembly. J Protozool. 1990; 37: 182–186.
23. *Allen RD.* Fine structure of membranous and microfibrillar systems in the cortex of Paramecium caudatum. J Cell Biol. 1971; 49: 1–20.
24. *Wichterman R.* The biology of Paramecium, 2nd ed.,. Plenum press, New York 1986, 599pp.
25. *Gelei G von.* Ein neues Fibrillensystem in Ectoplasma von Paramecium. Arch Protistenk. 1937; 89: 133–162.
26. *Sedar AV, Porter KR.* The fine structure of cortical components of Paramecium multimicronucleatum. J Biophys Biochem Cytol. 1955; 1: 583–604.
27. *Garreau de Loubresse N, Keryer G, Vigues B, Beisson J.* A contractile cytoskeletal network of Paramecium: the infraciliary lattice. J Cell Sci. 1988; 90: 351–364.
28. *Garreau de Loubresse N, Klotz C, Vigues B, Rutin J, Beisson J.* Ca^{2+} -binding proteins and contractility of the infraciliary lattice in Paramecium. Biol Cell. 1991; 71: 217–225.
29. *Matsuoka T, Tsuda T, Ishida M.* Presumed photoreceptor protein and ultrastructure of the photoreceptor organelle in the ciliated protozoan, Blepharisma. Photochem Photobiol. 1994; 60: 598–604.
30. *Miyake A, Harumoto T, Salvi B,Rivola R.* Defensive function of pigment granules in Blepharisma japonicum. Europ J Protistol. 1990; 25: 310–315.
31. *Dembitzer HM, Hirshfield HI.* Some new cytological observation in the heterotrichous ciliate, Blepharisma. J Cell Biol. 1966; 30: 201–207.