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An ultraviolet Laser microbeam for 257 nm *)

Eine Laser-UV-Mikrobestrahlungsapparatur für 257 nm

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Summary

A laser-uv-microbeam is described for the wavelength 257 nm which allows microirradiation of preselected sites of living cells with an effective spot size of approx. 0.5 μm in diameter, as measured by fluorescence experiments and by uv-induced lesions in stained cell specimens and in unstained living cells. The maximum irradiance power density is approx. 10^2 erg/(s·μm²). The ultraviolet light is produced by frequency-doubling of the 514.5 nm line of a continuous wave argon-ion-laser. The optical arrangement in the irradiation microscope is similar to that used in a fluorescence incident light microscope. Focusing and observation are done by means of the same quartz-objective (×100).

Zusammenfassung

Eine Laser-UV-Mikrobestrahlungsapparatur wird beschrieben für die Wellenlänge 257 nm. Sie gestattet eine gezielte Mikrobestrahlung lebender Zellen mit einem effektiven Fokusdurchmesser von ca. 0,5 μm, bestimmt aus Fluoreszenzexperimenten und aus UV-induzierten Läsionen in gefärbten Zellpräparaten und in ungefärbten lebenden Zellen, bei einer maximalen Bestrahlungsstärke von ca. 10^2 erg/(s·μm²).

Das ultraviolette Licht wird erzeugt durch Frequenzverdoppelung der 514,5 nm-Linie eines kontinuierlichen Argon-Ionen-Lasers. Die optische Anordnung im Bestrahlungsmikroskop ist ähnlich derjenigen in einem Fluoreszenzauflichtmikroskop. Fokussierung und Beobachtung erfolgen durch dasselbe Quarz-Objektiv (100 : 1).

1. Introduction

UV-microbeams for partial cell irradiation have been used for more than 60 years [11]. The large effective spot size ($\geqq 2\,\mu m$) and the low irradiance power density (10^{-2} erg/(s·μm²)) obtainable with conventional uv-sources [8], however, have limited their applicability in producing small defined lesions on cell organelles mov-

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ing relatively fast: The irradiation damage is widespread and the required irradiation times range between several seconds and a few minutes.

Recently, pulsed laser sources have been used to avoid these disadvantages [2, 5, 9]. So far, no device for partial cell irradiation has been described, which makes use of a continuous laser-uv-source with a wavelength in the absorption maximum of the DNA in connection with a focusing objective of high aperture (n.a. $\approx$ 1.).

In this report, a cw-laser-uv-microbeam is described for the wavelength 257 nm which allows microirradiation of organelles of living cells with an effective spot size of 0.5 μm in diameter and an irradiation power density of 10^2 ergs/(s·μm²). Our group has set up this device to study possibilities of producing defined lesions on chromosomes in mammalian cells. Experiments were undertaken to obtain information on whether by using a laser-uv-microbeam focal lesions of the DNA could be produced in nuclei of Chinese Hamster cells without preventing further cell proliferation [4].

2. General description

The basic design of the device is represented diagrammatically in figure 1:

A continuous wave (cw) laser beam with a wavelength of 514.5 nm, emitted by an argon-ion-laser (1), is transmitted through an ammonium dihydrogen phosphate (ADP) crystal (2). Thus, due to nonlinear optical effects in the ADP-crystal, frequency doubling of the 514.5 nm laser light occurs and coherent uv-light with a wavelength $\lambda = 257.3$ nm is emitted, being collinear with the exciting laser beam. The uv-beam is separated from the exciting green laser beam by a prism (3) and reflected by two mirrors (4) to a beam splitter (5) which diverts a small fraction of the uv-light to a detector system (6) for power measurement. The duration of irradiation is controlled by a photographic shutter (7). By means of a selecting mirror (8), reflecting the uv-light while transmitting the red light, a red pilot beam ($\lambda = 632.8$ nm) emitted by a low power He-Ne-laser (9) is aligned collinearly to the uv-beam. A mirror (11) reflects the beams into the irradiation microscope (12 – 19). To facilitate sterile conditions, the irradiation microscope is located in an adjoining room, and both beams are transmitted there through a quartz window. The optical arrangement in the irradiation microscope is similar to that used in a fluorescence incident light microscope: uv-beam and pilot beam pass a dispersing lens (12) with a negative focal length of approx. 5 cm. After reflection by a selecting mirror reflecting the uv-light to approx. 80% and visible light to 10%, the beams are focused into the object plane (14) by an immersion objective of high magnification and aperture (15). To measure the uv-power falling into the aperture of the objective, it can be replaced by a photodiode (15 a). Simultaneously, the focusing objective serves for observation of the specimen by either an ocular system (16) or a television monitoring system (17). Photographs of the specimen are made with the attached camera system (18). The specimen can be observed also during irradiation because the selecting mirror (13) transmitts the ligth produced by the illumination system (19) to approx. 90% in the whole visible spectrum.

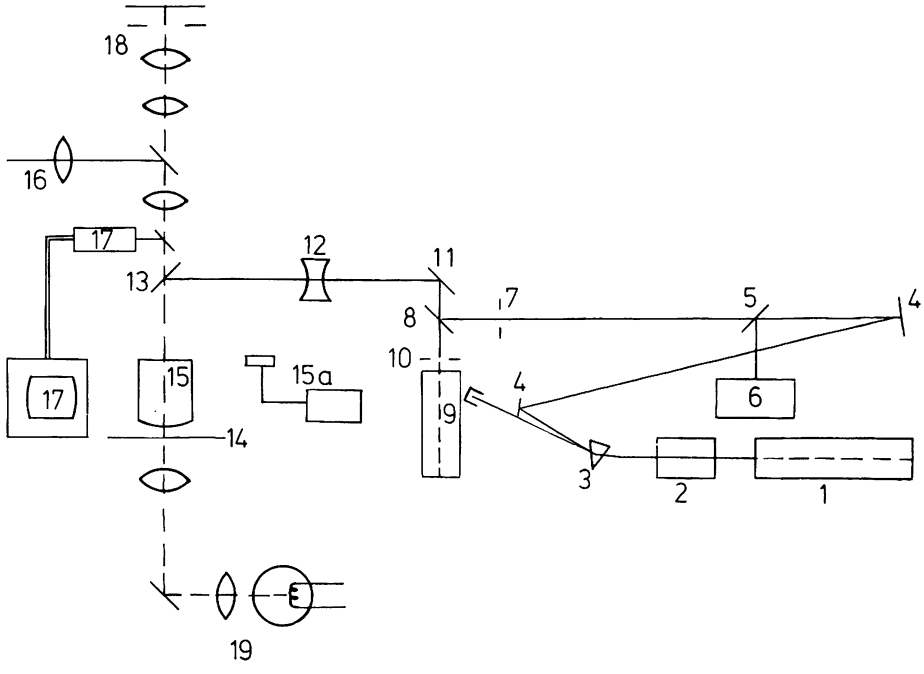

Fig. 1: Diagram of laser-uv-microbeam:

1 = argon-ion-laser
2 = uv-generator
3 = separating prism
4 = mirrors
5 = beam splitter
6 = uv-detector system
7 = shutter
8 = selecting mirror
9 = He-Ne-laser
10 = shutter
11 = adjustable mirror
12 = dispersing lens
13 = selecting uv-mirror (Balzers)
14 = object plane
15 = microscope objective (either Zeiss Ultrafluar ×100/0.85 Glyc Ph or ×100/1.25 Glyc)
15 a = photodiode
16 = ocular system
17 = TV-system
18 = camera system
19 = illumination system

To microirradiate living cells, a special tissue chamber (Fa. Tecnomara) designed by P. HÖSLI is used, which allows slight squeezing of the cells. It is placed on a fine adjustable thermoregulated microscope stage. The area selected for microirradiation can be marked by two different procedures:

a) The selected region is placed into the axis of the pilotbeam focus, which is visible as a red spot through the ocular or on the televison screen. UV-microirradiation then occurs at the selected region marked by the spot.
b) The selected region is marked with a cross-hair located in the image plane of the objective. With help of the dispersing lens (12, Fig. 1), the uv-beam is adjusted in such a way that the fluorescing spot produced by the focussed uv-light on a test specimen in the object plane is congruent with the intersection of the cross-hair.

3. Details of construction

The argon-ion-laser is a model 52 Be-A from Coherent Radiation Lab., with a prism wavelength selector, which selects the 514.5 nm line in TEM_{00} mode (beam diameter 1.4 mm, beam divergence 0.8 mrad). The cw-output power (max. 1.4 W) is regulated by varying tube current and measured by a calibrated internal power meter. The frequency doubling is performed with a uv-generator model 440 from the same company. Prisms, mirrors, optical benches, mirror mounts etc. are standard ones. The selecting mirror (13, Fig. 1) in the irradiation microscope was developed by Balzers AG/Liechtenstein. All other elements of the irradiation microscope are from Zeiss/Oberkochen. The focusing microscope objectives used were Ultrafluar 100/0.85 Glyc. Ph and Ultrafluar 100/1.25 Glyc. The laser-uv-microbeam apparatus stands on heavy tables to protect it against vibrations. Due to separation of the microscope table from the laser table, working with the irradiation microscope (12 – 19, Fig. 1) does not disturb the optical arrangement for uv-generation and measurement (1 – 11, Fig. 1).

4. Measurements

Absolute uv-powers were measured with a calibrated spectroradiometer system (Model 585, EG + G). After careful adjustment of position and temperature of the ADP-crystal, a maximum uv-output of 1.1 mW was measured behind the separating prism (3, Fig. 1), the power of the exciting beam being 1450 mW. The uv-output is regulated by varying the power of the exciting laser beam. Due to losses in lenses. objectives and mirrors the irradiation power in the focus is reduced to 1 to 10% of the uv-power measured immediately behind the prism. To measure the irradiation power in the object plane, an image of the focus was transferred onto the diffusing disk of the spectroradiometersystem by means of a second ultrafluar-objective, the front lenses of the two objectives being optically connected by glycerine. To determine the smallest effective spot size in the object plane, (14, Fig. 1), three procedures were used:

a) A quartz object micrometer, whose lines consist of an organic compound, was placed into the object plane. By directing the uv-microbeam on a line, the organic compound was excited to fluorescence, and the fluorescent area was microphotographed. Measurement of the spot sizes thus produced gave a diameter of the fluorescent area of 0.4 μm (Fig. 2). Variation of the exposure time between 15 and 120 s did not significantly alter the spot size.
b) In homogeneously stained nuclei paling was induced by uv-microirradiation, the irradiation times being approx. 10^{-1} s. The resulting spot diameters obtained from photomicrographs were approx. 0.5 μm.
c) In unstained cell organelles (nucleoli) of living cells, lesions were produced with a phase contrast objective (Ultrafluar 100/0.85 Glyc Ph), they became immediately visible after microirradiation as darkening spots, the smallest diameter of the spots being approx. 0.5 μm (Fig. 3), irradiation time 1 s. From our experience it can be said that an effective spot size with a diameter smaller than 1 μm can be reproduced easily in routine experiments.

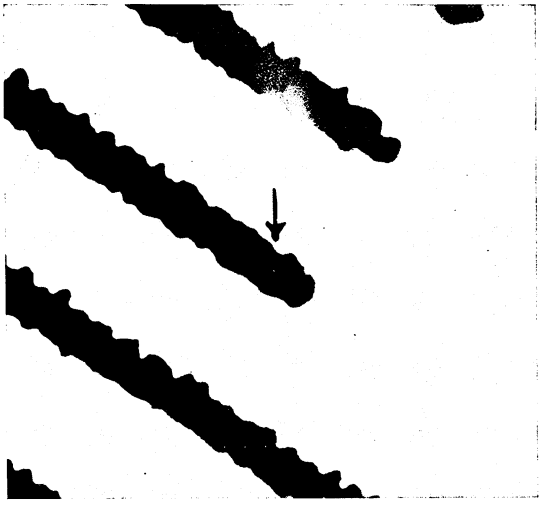

Fig. 2: Production of a fluorescing spot in the object plane by Laser-uv-micro-irradiation. The objective is a Zeiss Ultrafluar 100/1.25 Glyc, and the specimen a Zeiss quartz object micrometer, on which the fluorescing spot is produced. The distance between two lines matches 10 μm.

5. Discussion

5.1. uv-output

The power of the uv-beam generated in the ADP-crystal is proportional to the square of the power of the exciting laser beam [7]. The proportionality factor between the uv-power and the square of the laser power is specified by the manufacturer to be 4×10^{-3}/Watt. The experimentally found values were approx. 1×10^{-3}/Watt.

5.2. Focusing

The f-number of the focusing objective being very small, theoretical analysis of focusing has to use electromagnetic vector theory of diffraction to get an exact solution of the focusing problem [1]. For qualitative consideration use of scalar wave theory should be sufficient [3]. On this basis, calculations for laser beams are available [6]. Comparing the experimental results with these calculations, we conclude that 80 to 95% of the total energy delivered into the focus plane should fall into an area within the spot diameter observed. Furthermore, due to the strong convergence of the focused beam, high irradiation power density is achieved only in a region the extension of which along the optical axis is comparable with the spot diameter [6]. Thus it can be expected that the effects of uv-microirradiation outside the focus can be kept relatively small.

5.3. Comparison with other uv-microbeams

The smallest effective spot diameter applied in biological microirradiation experiments on living cells with the purpose of producing defined lesions in unstained cell organelles, using conventional sources, was reported to be approx. 2 μm [8]. Irradiation times varied in these cases from 5 s to 480 s, the maximum irradiation power densities being approx. 10^{-2} erg/(s·μm²). A comparison with the data of the laser-

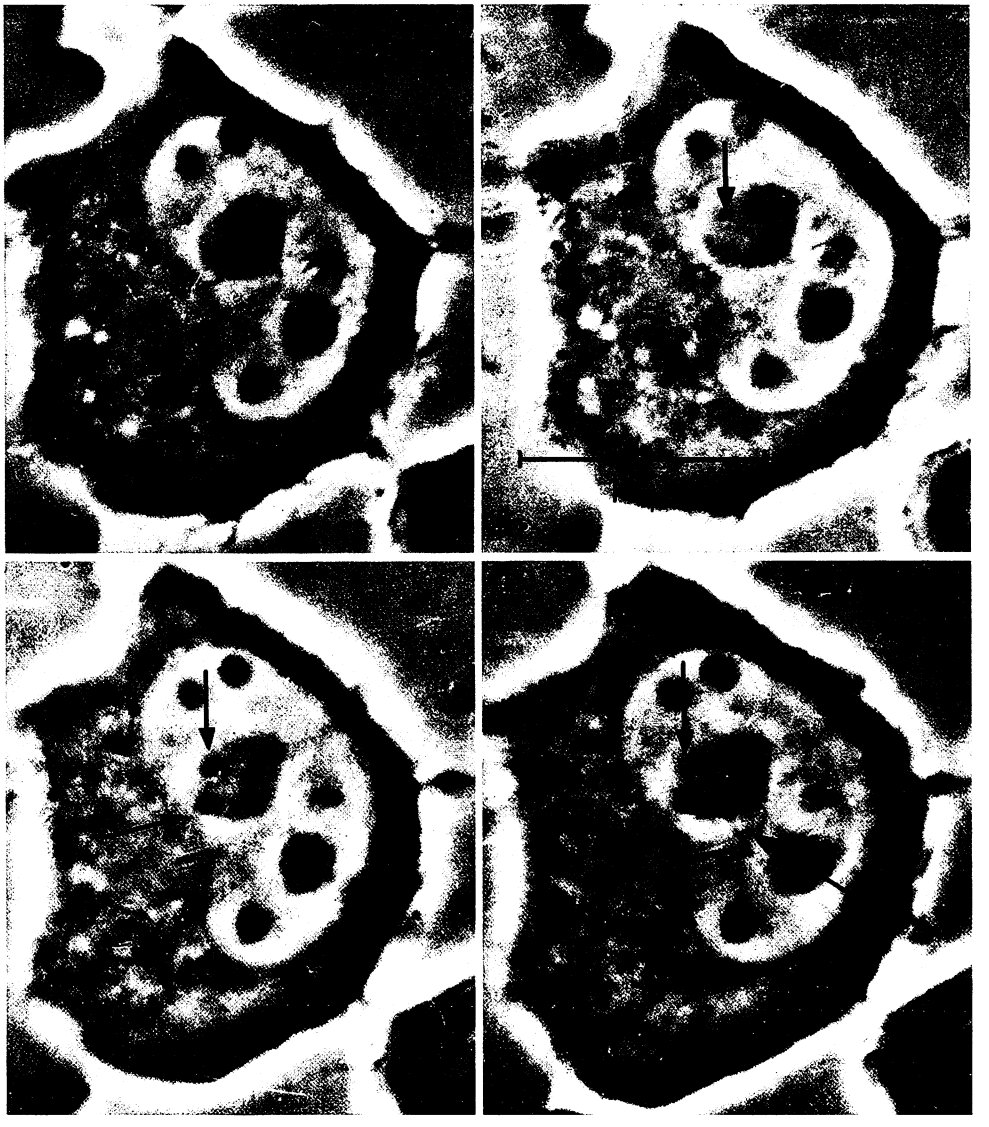

Fig. 3: Laser-UV-Microirradiation of an unstained living Chinese Hamster cell. — a) Prior to irradiation. — b) — d) Subsequent microirradiations of nucleolus, visible as dark spots; time between irradiations: 2 min. Phase contrast, objective Ultrafluar 100/0.85 Ph Glyc (used for irradiation and observation). Magnification 3200 : 1.

uv-microbeam described above shows the improvement available by using a coherent uv-source: The laser-uv-microbeam has an effective spot diameter four times smaller ($\approx 0.5\,\mu m$), and the irradiation power density of max. $10^2\,erg/(s\cdot\mu m^2)$ is several orders of magnitude higher than in the conventional device. MORENO using a conventional uv-source with a wavelength of $\lambda = 275\,nm$ observed focal lesions in KB cell

nuclei irradiated with an energy density of 10^{-1} erg/μm^2 [10]. With our apparatus it is possible to apply such an uv-energy density within a few milliseconds. This short irradiation time facilitates an irradiation of defined segments of moving cell organelles. Our results indicate that using the small uv-laser focus a considerably higher energy density can be delivered to cell nuclei without preventing further proliferation [4].

Laser-microbeams for biological research using ultraviolet light emitted by pulsed laser sources (20 ns to 50 µs) have been reported for 351 nm, mixed with green and blue laser light [2], for 347 nm [5], and for 265 nm [9].

Contrary to pulsed laser sources, a continuous wave laser source offers the possibility to vary irradiation power density and irradiation time independently from each other. Since $\lambda = 257$ nm is in the absorption maximum of DNA, a continuous wave laser uv-microbeam as described in this paper may be especially useful for microirradiation of chromosomes in living metaphase cells.

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The selecting mirror in the irradiation microscope was developed by Fa. Balzers AG/Liechtenstein (Drs. H. PULKER and TH. RITTER).

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