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A CONTINUOUS-WAVE ULTRAVIOLET LASER MICROBEAM AND
ITS USE FOR STUDIES IN SOMATIC CELL GENETICS

by

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The long-term aim of our experiments is to investigate whether it is possible to produce cell clones of mammalian cells with limited damage to a small part of the genetic material. Our approach involves micro-irradiation of one chromosome of a cell during mitosis and growing this cell up to a clone, which then can be investigated for the effects caused by the absence of a part of the genetic material. From a comparison of cells with a small deficiency in the genetic material and normal cells, one should learn something on function and regulation of those genes in which these cells differ.

Interesting questions, for example, are whether a cultured cell can do without some part of the genetic information and whether the second copy of each information, in a diploid cell present on the homologue chromosome, will compensate for the defect. It has often been suggested that the heterochromatin is an inactive part of the genetic material, so one wants to know whether a cell can really do without it. If an enzyme is known to be coded on a certain chromosome - an increasing amount of such information is now provided by the cell hybridisation technique - one might be interested in a more exact location of the corresponding gene locus on this chromosome. For an experimental approach to all these questions, the deviation from the normal genome should be limited to a definable part of the genome, preferably to a single chromosome in an otherwise normal karyotype.

There are natural sources of cells with small defects or loss of single chromosomes, but they are rather limited, mainly because most of these abnormalities are not compatible with the development of an entire organism. A cell in tissue culture apparently tolerates a much greater loss of genetic material. Most chromosomal abnormalities found, for example, in spontaneous human abortus material involve an excess of chromosomes (trisomies, triploidies and tetraploidies) or the absence of an X-chromosome, while structural abnormalities of autosomes and autosomal monosomies are very rare.[1] Tumour cells are another natural source with alterations of the karyotype. But these alterations are often unstable and involve several chromosomes.

Therefore, we are interested in producing cell populations with small structural abnormalities of single chromosomes and partial and total monosomies.

To produce such abnormalities, we set up a laser-UV-microbeam. Laser light can be almost completely focused on a spot, the diameter of which is mainly limited by diffraction at the aperture of the focusing lens. A small irradiation area and high power density are easily obtainable. We use coherent UV light with a wavelength of 257 nm, which is in the absorption maximum of DNA. In this way, the disadvantages of photosensitizing agents which have been often applied when working with visible laser light,[2,3] can be avoided. On the other hand, UV light is absorbed not only by the DNA of the chromosomes, but also by RNA, proteins and their precursors. So first of all, we had to clear up whether these side effects could be tolerated by the cells. The answer to the question whether a cell can grow to a clone after microirradiation with UV energies sufficient to produce damage to the DNA is a fundamental one for the approach.

Apparatus

A coherent UV beam is generated from a frequency-doubled argon-ion laser beam and focused on the target by a microscope lens.[4] Figure 1 shows the basic design. A continuous-wave argon-ion laser beam with a wavelength of 514.5 nm (from a Coherent Radiation model B 52-A (1)) is trans-

mitted through an ammonium dihydrogenphos-
phate crystal (Coherent Radiation model
440 (2)). Frequency doubling occurs and
coherent UV light with a wavelength of
257.3 nm is emitted. A prism (3) sepa-
rates the UV beam from the remainder of
the green beam. A beam splitter (5)
diverts a small fraction of the UV light
to a detector system (6) for UV power
monitoring. The duration of irradiation
is controlled by a photographic shutter
(7). At a selecting mirror (8), the UV
beam is joined with the red beam of a
low power He-Ne laser (2 mW) and the two
beams are aligned collinearly. The
red beam can then be used as a pilot
light in the irradiation microscope
(12 - 19). A mirror (11) reflects both
beams to the irradiation microscope,
which is located in an adjoining room
where cell culture work can be performed.

Fig. 1. Diagram of laser-uv-microbeam:[4] 1) argon-ion-
laser; 2) UV generator; 3) separating prism; 4) mirrors;
5) beam splitter; 6) UV detector; 7) shutter; 8) select-
ing mirror; 9) He-Ne laser; 10) shutter; 11) adjustable
mirror; 12) dispersing lens; 13) selecting UV mirror
(Balzers); 14) object plane; 15) microscope objective;
15a) photodiode; 16) ocular system; 17) TV system;
18) camera system; 19) illumination system.

The optical arrangement in the
microscope is similar to that used in a
fluorescence incident light microscope
--UV beam and pilot beam pass through a
dispersing lens (12) (to bring the focus
into the object plane (14) of the micro-
scope), are reflected by a selecting
mirror (13) and focused on the target by a quartz microscope objective (15) of high aperture and mag-
nification (Zeiss Ultrafluar 100x). The objective (15) can be replaced by a calibrated photodiode
(15a) to measure the power incident into the aperture of the objective.

Simultaneously, the objective serves for observation of the cells by an ocular or a television
system either by phase contrast (Ultrafluar 100/0.85 Glyc Ph) or normal transmittance light illumina-
tion (Ultrafluar 100/0.85 Glyc, or Ultrafluar 100/1.25 Glyc). Continuous observation is made possible
by means of the selecting mirror (13) that reflects 80% of the UV light (257 nm) used for irradiation
and transmits 90% of the visible light for observation in the region from approximately 450 nm to 750
nm. This mirror was developed by Balzers AG/Liechtenstein (Dr. H. Pulker and Dr. E. Ritter).

Two different procedures have been used for aiming the UV beam on the cells:

1)   The area chosen for irradiation is brought under the focus of the red pilot beam
     by means of the fine adjustable microscope stage. UV irradiation then occurs at
     the marked region.

2)   The UV focus was observed by fluorescence and adjusted by means of the dispersing
     lens (12) under a cross hair in the ocular. The cross hair then marks the irradia-
     tion site in the cell.

Spot Size and UV power

The apparatus parameters that are most important for our experiments are the irradiation spot
size, i.e., the effective diameter of the focus, and the irradiation power delivered into this focus.
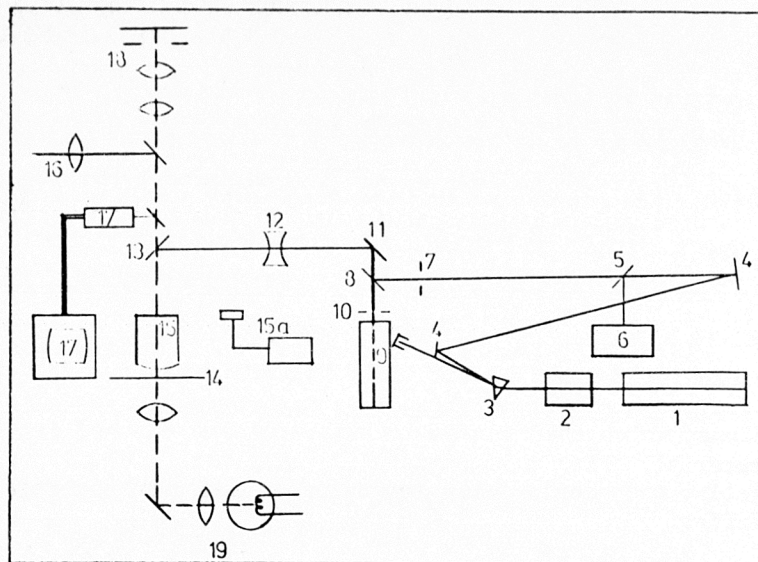
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The smallest effective spot size was determined by three procedures:

1) Irradiation on the lines of an object micrometer produce a fluorescent spot. Figure 2 shows such an experiment. The distance between two lines is 10 μm; the fluorescent spot has a diameter of approximately 0.4 μm.

2) In stained cell preparations, bleaching was induced by UV irradiation. The resulting spot diameters were approximately 0.5 μm.

3. In unstained organelles of living cells, lesions were produced that were immediately visible as darkening spots in phase contrast (Fig. 3). The diameter of these darkening spots was approximately 0.5 μm. A spot diameter of less than 1 μm can be reproduced easily in routine experiments.

Fig. 2. Producing of a fluorescent spot in the object plane by laser-UV-microirradiation. 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.[4]

By means of a calibrated spectroradiometer system, we measured a maximum UV power of 1 mW immediately after the ADP-crystal, the exciting power being 1.4 W of green laser light. Due to losses in mirrors and lenses, the irradiation power in the focus is reduced to a value of 10 μW to 100 μW, depending on the aiming system and the objective, etc., used. To determine the actual power in the focus, an image of the focus was formed on the diffusor disc of the spectroradiometer system by means of a second Ultrafluar objective that was optically connected to the focusing objective by glycerine immersion. The data given for the irradiation experiments are corrected for absorption in the material between objective and cell target.

Irradiation Experiments

Irradiation of Interphase Cells

Our first irradiation experiments were intended to provide some information on whether the laser-UV-microbeam is able to cause damage to the DNA of mammalian cells without killing them or preventing further proliferation because of side effects caused by absorption in the cytoplasm. For this purpose, we seeded cells of a line of the Chinese hamster (V79-122D1) from a single cell suspension into petri dishes containing small pieces of cover-glass. On these pieces, single cells settled down and formed clones. When the clones had reached a size of 4 to 12 cells, cover-glass pieces containing one colony were chosen and transferred into an irradiation chamber. Then all cells of a clone were irradiated either in the nucleus or in the cytoplasm with an UV spot of approximately 0.5 μm diameter. Control clones were sham-irradiated. After irradiation, the coverslips were placed back into petri dishes with fresh medium (Eagle's MEM containing nonessential amino acids and 15% foetal calf serum) and growth of the clones was determined by cell counting in an inverted microscope. So far, 16 clones were irradiated in the cytoplasm, 29 clones in the nucleus, and 52 clone served as controls.

Fig. 3. Laser-UV-microirradiation of an unstained living Chinese hamster cell: a) prior to irradiation; b)-d) subsequent micro-irradiation of nucleolus. The lesions are visible as dark spots; time between irradiations is 2 minutes. Phase contrast, objective ultrafluar 100/0.85 Glyc Ph. (used for irradiation and observation). Magnification 3 300x.[4]

Figures 4 to 6 show some characteristic fates of these clones. Plotted is the cell number of one colony in logarithmic scale against time after irradiation in hours. Controls showed exponential growth. Cells irradiated with 5 ergs in the nucleus (Fig. 4a) died. Irradiation in the cytoplasm with the same energy caused the death of some cells. But after a lag phase in growth of 20 to 30 hours, the remainder divided with a growth rate similar to that of the controls. Cells cytoplasmic-irradiated with 1.4 ergs (Fig. 4b) showed only a lag phase and normal growth afterwards - no cell death occurred - whereas, all cells nuclear-irradiated died.

Fig. 4. Application of UV energies between 1.4 and 5 ergs. a) ●: irradiation of the nucleus (5 ergs); o: irradiation of the cytoplasm (5 ergs); Δ: unirradiated control group. b) ●: irradiation of the nucleus (1.4 ergs); o: irradiation of the cytoplasm (2.2 ergs); Δ: unirradiated control group.

Abscissa: time after irradiation (hours). Ordinate: cell number in logarithmic scale. Growth curves of Chinese hamster cells (V-79-122Dl) irradiated either in the cytoplasm (c) or in the nucleus (n). The size of the irradiated area was approximately 0.3 μm^2. +: is time at which all cells of an irradiated group were classified as dead by light microscopic examination.

Irradiation with lower energies (Fig. 5a, b) showed no effect at all on the growth curve when only the cytoplasm was irradiated. But nuclear-irradiated groups either died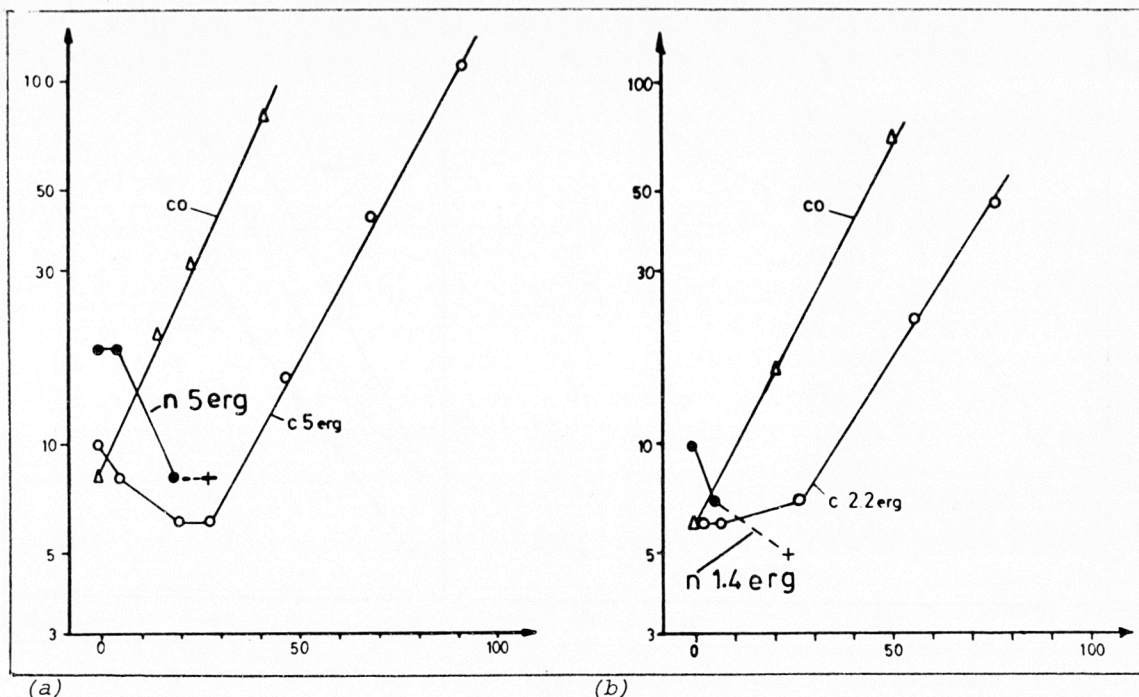 off or showed a growth rate obviously decreased when compared to the controls. We think that the different fates of the two clones nuclear-irradiated with 0.2 ergs (Fig. 5a) might be caused by a slightly heterogeneous UV sensitivity of different clones of the heteronuclear cell line, since the same result was found in three similar experiments.

One can see from the plots that the sensitivity of the cytoplasm and the nucleus to long term effects such as cell death and lower growth rate brought about by a UV spot of approximately 0.5 μm diameter differs by an order of magnitude. The absorption of UV light (λ = 260 nm) in these cell compartments, however, does not differ by more than a factor of two.[5] Therefore, the different sensitivity cannot be interpreted by a different absorption.

Figure 6 shows another effect of nuclear irradiation. Pycnotic cells appeared not only among the irradiated cells but also in later generations. From 24 apparently healthy cells, counted 76 hours after irradiation, 12 became pycnotic and showed no further division. This number is larger than the number of 8 irradiated cells - clear evidence that at least some of these cells stem from a daughter generation. Such delayed lethal effects have also been found by Puck and Marcus after x-ray irradiation[6]. They attribute them to induced genetic defects. This fits well with the fact that we saw no such effects after cytoplasmic irradiation.

Fig. 5. Application of 0.2 ergs UV energy. a) •: irradiation of the nuclei of a group I; o: irradiation of the nuclei of a group II; Δ: control group. b) •, o: irradiation of the cytoplasm; Δ: control group. Group I died after 40 hours, while group II showed a markedly decreased growth rate compared with the controls. Cytoplasmic irradiated cells had a growth rate not recognizibly different from the controls.

Fig. 6. Nuclear irradiation with UV energies between 0.07 and 0.7 ergs. a) o: irradiation with 0.1 ergs; Δ,▲: controls. 76 and 127 hours after irradiation, cells with pycnotic nuclei appeared. These cells were excluded from the plotted cell number. b) o: irradiation with 0.07 ergs; o: irradiation with 0.3 ergs; ⊖: irradiation with 0.7 ergs; Δ: control.

Using irradiation energies up to a few ergs, we did not observe an effect in an irradiated part of a cell immediately after irradiation. Only when much higher energies (10^2 to 10^3 ergs) were delivered to the nucleus, a dark spot could be observed at the irradiation site (Fig. 3).

Irradiation Of Cells In Mitosis

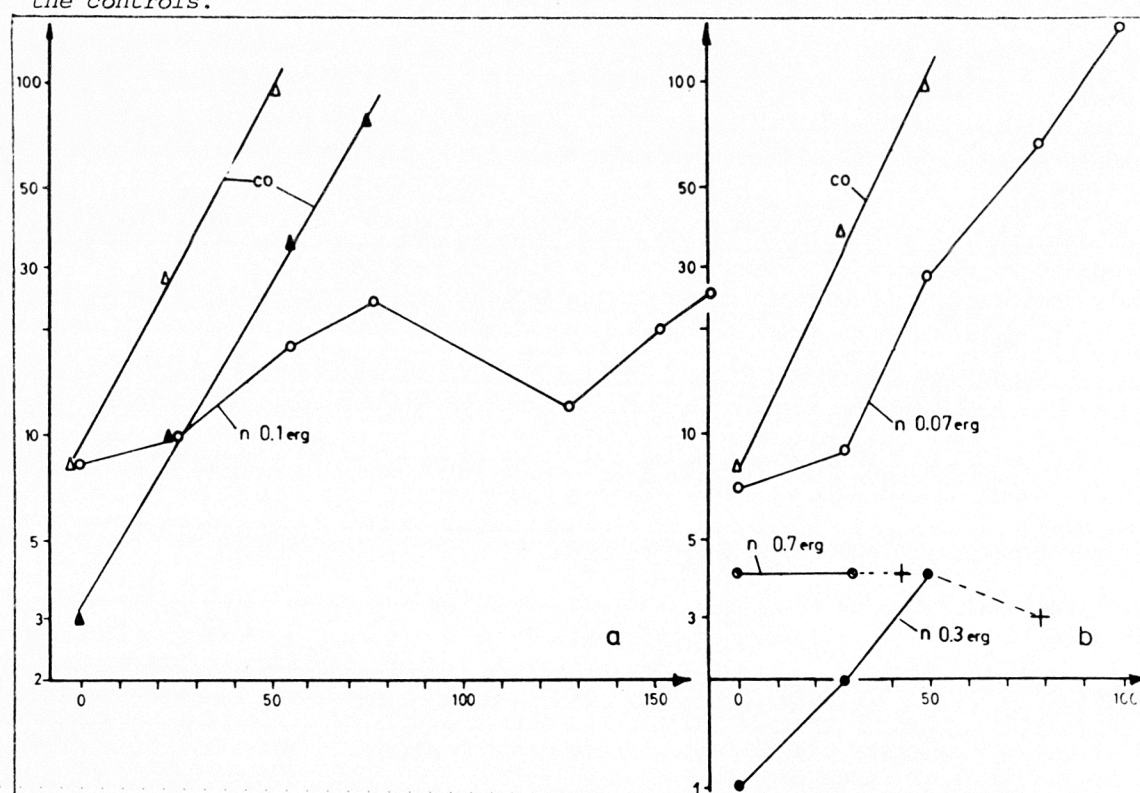Observation and irradiation of chromosomes are complicated by the fact that cells in mitosis normally round up. Hösli found that such rounded cells can be slightly pressed and so flattened reversibly[7]. One can see the chromosomes far better then.

Figure 7 shows a diploid fibroblast of the Chinese hamster, flattened in metaphase. Because we have to use quartz optics, the optical quality under irradiation conditions is worse, but even then, single chromosomes can be distinguished.

After irradiation with up to 1 erg, delivered to chromosomes in metaphase and anaphase, cells were usually able to complete mitosis and form nuclei. A darkening at the irradiated site of a chromosome could be observed in phase contrast only when higher energies (10^2 to 10^3 ergs) were used. Mitosis was stopped in every case after application of such a high energy.

Fig. 7. Metaphase of a diploid fibroblast of Chinese hamster.

In some cases, cells that were irradiated during metaphase or early anaphase showed a separation of a small cell-segment near to the irradiated area. This effect was found already when relatively low energies were applied, especially after microirradiation of metaphases at several sites.

Figure 8 shows an example. A cell irradiated during metaphase on four sites (4×10^{-4} ergs each) showed an atypical arrangement of its chromosomes some minutes after irradiation within four clumps (a), then formed two daughter cells and two extra segments (b). Later on, both cells and one segment formed one cell with five micronuclei, leaving outside one segment with one micronucleus (c). We suppose that the abnormal chromosome distribution may be due to an effect of the UV light on the spindle apparatus. Perhaps mitotic nondisjunction can so be induced by UV microirradiation.

(a)

(b)

(c)

Conclusions

From our experiments, we draw two conclusions. The first is a relative one. The great difference in the UV spot sensitivity of cytoplasm and nucleus makes it very reasonable to assume that our approach is not limited by the consequences of absorption in the cytoplasm. It is difficult to draw certain conclusions on the absolute energy used in the irradiation experiments. Measurement of an irradiation density at the irradiation site, and especially determination of the energy absorbed at a cell target, is object of many sources of systematic errors. Therefore, a comparison with data given in the literature must be performed with caution. By use of an electron microscope, Moreno found DNA lesions after nuclear irradiation of KB-cells with an
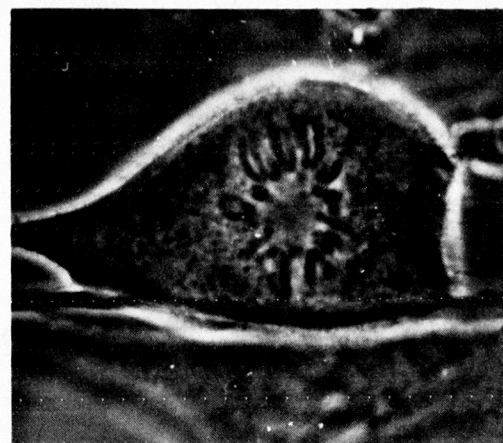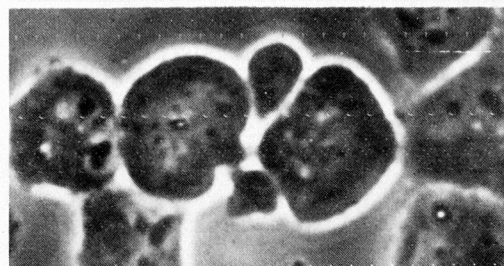
Fig. 8. Formation of micronuclei following UV microirradiation in metaphase at four sites. See text for details.

irradiation density of o.l ergs/ μm^2 (λ = 275 nm).[8] According to our measurements of absolute UV-power, areas of nuclei could be irradiated with these irradiation densities without all the cells loosing the ability to divide and form colonies. So it seems reasonable to assume that DNA lesions were produced in these experiments, especially since lethal segregation in daughter generations of nuclear irradiated cells was observed. But, direct evidence is necessary to confirm this.

Summary

A laser-UV-microbeam (λ = 257 nm) is described that allows irradiation of organelles of tissue culture cells that can be seen in phase contrast microscopy with a focus of 0.5 μm diameter and an UV power of 100 ergs/second. The coherent UV light is produced by frequency doubling the 514.5 nm line of continuous-wave argon-ion laser, and it is focused by a quartz microscope objective. A comparison of effects after irradiation of nucleus and cytoplasm in interphase cells of Chinese hamster (V79-122D1) and irradiation experiments on chromosomes give some indication that DNA lesions caused by microirradiation and further growth are compatible. Microirradiation of cells in meta-phase indicates that chromosomal nondisjunction can be induced.

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