

Laser Irradiation and Raman Spectroscopy of Single Living Cells and Chromosomes: Sample Degradation Occurs with 514.5 nm but not with 660 nm Laser Light

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In Raman spectroscopic measurements of single cells (human lymphocytes) and chromosomes, using a newly developed confocal Raman microspectrometer and a laser excitation wavelength of 514.5 nm, degradation of the biological objects was observed. In the experiments high power microscope objectives were used, focusing the laser beam into a spot $\sim 0.5 \mu\text{m}$ in diameter. At the position of the laser focus a paling of the samples became visible even when the laser power on the sample was reduced to less than 1 mW. This was accompanied by a gradual decrease in the intensity of the Raman signal. With 5 mW of laser power the events became noticeable after a period of time in the order of minutes. It is shown that a number of potential mechanisms, such as excessive sample heating due to absorption of laser light, multiple photon absorption, and substrate heating are unlikely to play a role. In experiments with DNA solutions and histone protein solutions no evidence of photo damage was found using laser powers up to 25 mW. No degradation of cells and chromosomes occurs when laser light of 660 nm is used. The most plausible explanation therefore seems to be that the sample degradation is the result of photochemical reactions initiated by laser excitation at 514.5 nm of as yet unidentified sensitizer molecules or complexes present in chromosomes and cells but not in purified DNA and histone protein samples. © 1991 Academic Press, Inc.

INTRODUCTION

Progress in modern cell biology creates an evergrowing demand for data on the composition, conformation, and organization in time and space of nucleic acids, proteins, lipids, and complexes of these in cells. Raman spectroscopy has been successfully applied in many studies in this field. However, the inherently low signal

intensity due to the low cross-section for Raman scattering of most biological macromolecules has limited its applicability to the study of model systems (see e.g., [1] for reviews). With the development of a confocal Raman microspectrometer (CRM) in our laboratory, the field of investigation open to Raman spectroscopy has been significantly extended. Microscopically small intact biological objects as single cells and chromosome bands with a spatial resolution of $\sim 0.45 \times 0.45 \times 1.3 \mu\text{m}^3$ can now be studied [2–4]. Detailed molecular information from precisely defined positions in intact cells and metaphase and polytene chromosomes can be obtained in this way. However, in our first attempts to obtain Raman spectra of single biological cells and chromosomes we found that the samples were damaged by the 514.5-nm laser light commonly used in DNA/protein-Raman studies. Whereas many reports have appeared concerning the damaging effects of uv and near visible radiation on DNA and cells (see [5] for review) little literature is available on the effects of irradiation with visible laser light of high intensity. Problems with sample integrity have been reported in experiments on cell trapping with laser light of 514.5 nm [6]. Berns *et al.* [7, 8] developed a microsurgery technique using visible laser light from a pulsed laser. Using light of 532 nm they found a four-photon mechanism to be the cause of the damage to metaphase chromosomes [9], which they termed paling. However, as in our experiments the peak intensity of the laser light ($0.5\text{--}5 \text{ MW}/\text{cm}^2$) on the sample is 4 orders of magnitude lower than in their case it seemed very unlikely that a four-photon process would occur. Therefore a number of experiments were carried out to elucidate the mechanism causing sample degradation. Although our findings do not provide a definite answer, they do narrow down the range of potential mechanisms. Using laser light of 660 nm sample integrity was found not to be affected. The results presented here are not only important for the field of Raman spectroscopy but should also be of interest to other areas of research where laser microscopical techniques are used to study or manipulate living cells or other biological objects.

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MATERIALS AND METHODS

Sample preparations. Human lymphocytes were isolated from peripheral blood. The mononuclear white blood cell fraction was obtained by means of density gradient centrifugation [10]. It was depleted of monocytes by incubation in a 75-cm² tissue culture flask for 60 min at 37°C. This causes the monocytes to adhere to the surface of the flask whereas the lymphocytes remain in suspension. A few drops of this suspension were then deposited on a poly-L-lysine (Sigma P-6516)-coated fused silica substrate. During a 15-min incubation in an environment of 100% humidity the cells attach to the substrate. The substrate was then placed in a petri dish filled with phosphate-buffered saline (PBS) [0.14 M NaCl + 2.7 mM KCl + 1.5 mM KH₂PO₄ + 6.5 mM NaH₂PO₄].

Chinese hamster lung (CHL) cell (V79) metaphase chromosomes were isolated from exponentially growing cell cultures as described in [11]. The cells were grown in Eagle's minimum essential medium (MEM), without phenol red to avoid fluorescent contamination, which would cause a high background signal in the Raman spectroscopic experiments. Omission of other luminescent compounds from the growth medium (such as folic acid and riboflavin) during the last 2–10 growth cycles did not lead to further improvements in this respect.

The metaphase chromosome spread of Fig. 1A was prepared according to standard procedures, including fixation of mitotic cells in 1:3 mixture of methanol and acetic acid.

The fixed polytene chromosome sample shown in figure 1C was prepared from *Chironomus thummi thummi* salivary gland cells using standard squash preparation techniques [12]. Unfixed polytene chromosomes were obtained by a micromanipulation technique [13]. After preparation the samples were taken up in PBS with added divalent cations (1 mM CaCl₂ + 1 mM MgCl₂).

Calf Thymus DNA was purchased from Sigma (type I, Sigma D-1501) and used as received. A 25 mg/ml solution in PBS, resulting in a thick gel, was prepared. A small quantity of this gel was placed on a fused silica microscope slide and covered by a fused silica coverglass, which was sealed to the slide. A thin layer of 10 μm was thus obtained. Lyophilized calf thymus histone II-AS, purchased from Sigma (H-7755), was also used without further purification at a concentration of 50 mg/ml in PBS. This resulted in a thick but fluidic solution. The histone solution was kept between a microscope slide and a coverglass as described for the DNA-sample.

Experimental methods. The following laser sources were used in the experiments: A Coherent Innova 90 argon ion laser (457.9, 488.0, and 514.5 nm), a SIL MS 120A He–Ne laser (632.8 nm), and a Spectra Physics Model 375 B dye laser operated with DCM (Exciton) and tuned to 660 nm. The dye laser was equipped with a birefringent filter and an ultrafine etalon to produce a linewidth of ~7 GHz (~0.3 cm⁻¹). The argon ion laser was used to pump the dye laser. Raman spectra were recorded on the laboratory-built CRM mentioned before. For the measurements it was equipped with a 63X Zeiss Plan Neofluar water immersion objective (N.A. 1.2).

Absorption spectra of cell and chromosome suspensions were measured on a spectrophotometer (Perkin–Elmer 551S) equipped with a Ulbricht 45°-integrating sphere, to avoid extinction due to light scattering. Absorption spectra of glass substrates were recorded on a Beckmann DU-8 spectrophotometer. Substrates of different thicknesses were measured in order to be able to correct the data for reflection losses at the air–glass interfaces. Cell survival after laser irradiation was determined using the viability test described in [14].

Calculations. The method used to calculate heating effects due to absorption of laser light is straightforward and described in [15] and [16]. The following simplified model was used: it was assumed that the strongly focused laser light was uniformly absorbed (applying the appropriate absorption coefficients) in a half-sphere (chromosome)

or a cylinder (substrate) with the radial dimensions of the laser focus (0.25 μm), conducting the absorbed energy to their surroundings. The calculations were based on stationary conditions. In the calculation of heating of the substrate on which the samples were placed, heat conduction to the medium in which the sample was immersed was neglected. In the calculation of chromosome heating, the chromosome was treated as a DNA-protein solution with the same coefficient of heat conduction as the surrounding medium. Heat conduction to the substrate was neglected in this case. Heat conduction coefficients for water, crown glass, and fused silica were taken from [17].

RESULTS

Characteristics of the Radiation Damage

In Raman microspectroscopic experiments laser light is focused by means of a microscope objective onto a sample (Fig. 1D). In our experiments on single cells, polytene chromosomes, and metaphase chromosomes high numerical aperture (NA) objectives are used, which focus the laser light into a spot of ~0.5 μm (FWHM) in diameter. This leads to high light intensities: ~5 MW/cm² for 10 mW of laser power. For all samples investigated (cells and chromosomes) a degradation under the influence of 514.5 nm laser light was observed at all laser powers tested (0.5–20 mW). At the position of the laser focus a paling of the sample occurred (Fig. 1). This was accompanied by a gradual decrease in the intensity of the Raman signal (Fig. 2). There was no indication of excessive heating of the samples, such as a large increase in the luminescent background of the Raman signal. An interesting feature, regularly observed, was that in the Raman spectra the DNA line at 1487 cm⁻¹, mainly due to a guanine vibration involving the N7 and C8 positions, decreased in intensity more rapidly than other lines. A typical example of this is shown in Fig. 2B.

Comparison with Model Solutions of DNA and Histone Protein

For comparison it was tested whether a solution of CT-DNA would also be damaged by the laser light. A thin layer of about 10 μm of the gel-like DNA solution (25 mg/ml) was kept between two sealed coverslips. Even after 3 h of irradiation with a laser power of 25 mW on the sample the Raman spectrum remained unchanged and also upon visual inspection no proof of sample degradation was found. A similar test was done for a 50 mg/ml solution of CT histone II-AS, with the same result: no signs of degradation of the sample were found.

Wavelength Dependence of the Radiation-Induced Damage

The wavelength of the laser light turned out to be a crucial factor. Whereas at 457.9 and 488 nm the same

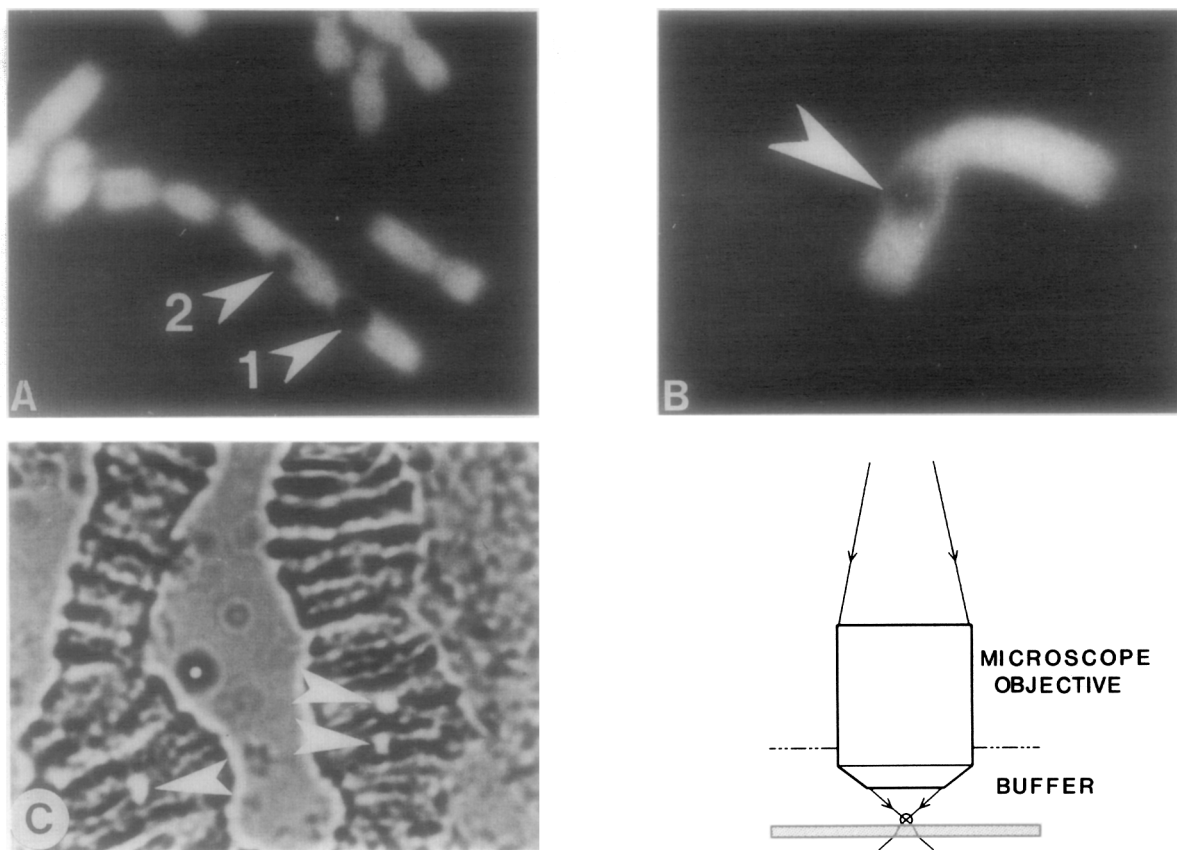


FIG. 1. Examples of the paling of biological samples in the focus of an Argon ion (514.5 nm) laser beam. The laser beam was focused into a spot of $\sim 1 \mu\text{m}$ in diameter with a 40X Nikon E Plan objective. (A) Fixed CHL-cell metaphase chromosomes after 120 s (arrow 1) and 60 s (arrow 2) of irradiation with 20 mW of laser power on the sample. The chromosomes have been stained with acridine orange after laser light irradiation for clarity (magnification: 2000 \times). (B) Isolated (unfixed) CHL-cell metaphase chromosome after a 300-s irradiation with 10 mW of laser power (magnification: 2000 \times). (C) *Chironomus thummi thummi* salivary gland polytene chromosome (from a squash preparation) after 600 s of irradiation at different locations with 5 mW of laser power on the sample (magnification: 1500 \times). (D) Focusing of the laser light on a sample.

paling effects were observed as with 514.5 nm laser light, sample damage could be avoided using laser light of a larger wavelength. No paling effects were observed using laser light of 632.8 or 660 nm. Raman spectra obtained consecutively at the same measuring position on a sample were found to be completely reproducible, using 660 nm laser light for excitation. The measurements on a polytene chromosome band, shown in Fig. 3, illustrate this. No Raman experiments were carried out with 632.8 nm laser light.

We also determined the percentage of cells (freshly isolated human lymphocytes) surviving a 5-min irradiation with laser light as a function of laser power and wavelength of the laser light. The results are shown in Fig. 3. Lymphocytes are small (diameter $<10 \mu\text{m}$) rounded cells with little cytoplasm. The laser light was focused in the center of the cells. It is mainly the nucleus that is irradiated in this way. Although the tests were done for small numbers of cells only it makes clear that

laser light of 632.8 and 660 nm is much less harmful than 514.5 nm laser light. Whereas 514.5 nm-irradiation affects cell viability already at a laser power of 5 mW no adverse effects are noticed with 632.8 and 660 nm irradiation for laser powers up to 20 mW. Above this level irradiation with red laser light also causes cell death. The tests with 632.8 nm had to be carried on a different experimental set up, the only significant difference being that a lower numerical aperture (0.65) was employed. This results in a larger diameter of the laser focus. However, a control measurement with 514.5-nm laser light with the same (NA = 0.65) objective resulted in the same curve as shown in Fig. 4 for 514.5 nm (obtained using a 1.2 NA objective). This indicates that the total integrated laser light dose on a cell is the important parameter, rather than the intensity with which it is applied, at least for the intensity range and cell type investigated here.

A number of experiments was performed to learn

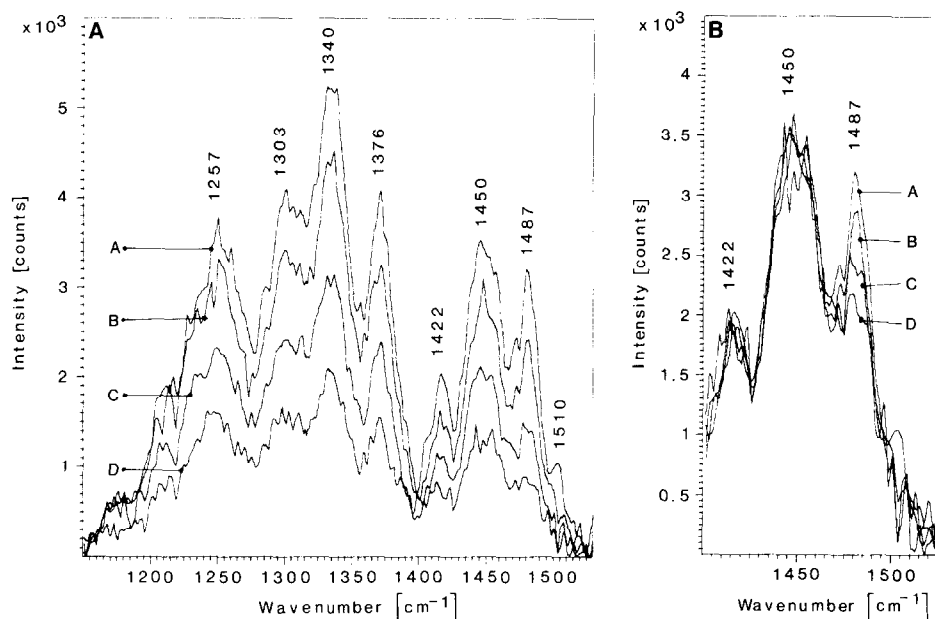


FIG. 2. Effect of laser light (514.5 nm)-induced sample degradation on the Raman spectrum. Raman line assignments are in Table 1. (A) Decrease of the intensity of the Raman signal, as recorded during measurements on isolated (unfixed) single (CHL-cell) metaphase chromosomes. Ten series of measurements were carried out on 10 different chromosomes. Each series consisted of 10 consecutive measurements at a fixed chromosome position. The spectra shown are the result of summations of corresponding measurements from the 10 series. In each measurement the laser power was 5 mW and the measuring time 60 s. Background signal from the fused silica coverslip and water have been subtracted, as well as a second order polynome representing luminescent background signal. The spectra were obtained during the (A) 1st minute of irradiation, (B) 3rd minute of irradiation, (C) 5th minute of irradiation, and (D) 9th minute of irradiation. (For further details see section about multi-photon absorption.) (B) An example of the sensitivity of the 1487 cm^{-1} guanine/adenine band to laser light irradiation. Detail of the spectra shown in A. The spectra were scaled to have equal intensity in the spectral interval 1150–1460 cm^{-1} .

more about the cause of the sample degradation induced by laser light of 514.5 nm.

Absorption of Laser Light

An absorption spectrum of a suspension of chromosomes was recorded (Fig. 5). This was done on a spectrophotometer equipped with a partially integrating sphere in order to avoid large extinction effects due to light scattering. The spectrum shows only the well-known uv absorption of DNA and proteins. Absorption at 514.5 nm (optical density (O.D.) 0.0087) was lower by roughly two orders of magnitude than at 260 nm (O.D. 1.04). On the basis of Fig. 5 and assuming that the absorption of laser light occurs uniformly over the irradiated position it can be argued that the absorption at 514.5 nm is too low to cause sample damage through heating effects. A solution of 50 μg DNA/ml has an O.D. of 1/cm at 260 nm. The DNA concentration in a CHL metaphase chromosome will be of the order of 100 mg/ml and the path-length of the laser light through a chromosome is about 0.5 μm . (A CHL mitotic cell contains 6.6 pg of DNA [18], divided over 22 chromosomes. The total added length of the chromatids of these chromosomes was estimated by light microscopic observation to be 200 μm and the diameter of a chromatid is about 0.5 μm .) It follows that at

260 nm roughly 20% of the incident radiation will be absorbed by a chromatid. This means that at 514.5 nm a chromatid absorbs a fraction of about 5×10^{-3} of the incident laser light. A calculation (Materials and Methods) shows that 10 mW of laser power (514.5 nm) would give rise to an increase in temperature of about 100°C in the chromosome at the position of the laser focus. However, there are several indications that this result is not realistic. As noted before, no damaging occurs using laser light of 660 nm, while Fig. 5 suggests that light absorption at that wavelength is not much less than at 514.5 nm. It is most likely, therefore, that the larger part of the "absorption" above ~ 300 nm is due to residual light scattering effects. Furthermore damage was found also to occur using laser powers < 1 mW, where the temperature increase would be an order of magnitude lower than with 10 mW. It is therefore not very likely that heating effects are the cause of damage in the case of chromosomes.

For cells some additional absorption can be expected. Absorption spectra (not shown) obtained from human lymphocytes showed a low intensity absorption band centered at 420 nm and an absorption band around 550–560 nm just above noise level, probably due to flavins and heme-group containing molecules (e.g., cytochromes).

TABLE 1

Raman Line Assignments for the Spectra in Figs. 2 and 3

Line position (cm ⁻¹)	Assignment (Refs. [19, 20, 21])
670	Thymine (T), Guanine (G)
681	G
729	Adenine (A)
750	T
788	DNA: O-P-O, Cytosine (C), T
832	DNA: backbone (BK) (B-conformation)
895	DNA: BK
928	DNA: BK, protein (p): α -helix
1004	phenylalanine
1094	DNA: O-P-O ⁻
1254	Amide III, A
1303	A
1340	A p: CH deformation (def)
1377	T, A, G
1421	A, G
1449	p: CH def
1487	G, A
1510	A
1577	G, A
1660	Amide I

Multi-Photon Absorption

When light intensity is high enough multiphoton processes can occur, as indicated by Calmettes and Berns

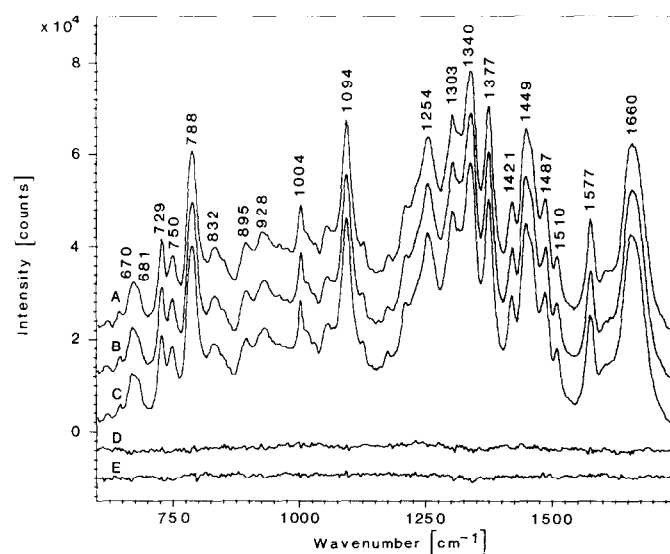


FIG. 3. Raman spectra obtained from a (dark) band of an unfixed *Chironomus thummi thummi* polytene chromosome in PBS. The spectra (A, B, C) were recorded consecutively at the same position in the chromosome band. Laser power, 10 mW (660 nm); measuring time, 600 s. Background signal from fused silica and buffer has been subtracted. An appropriate straight line has been subtracted to correct for a remaining slightly sloping background. Difference spectra: (D) A-B, (E) A-C. The spectra have been shifted along the ordinate for clarity of presentation. Raman line assignments are given in Table 1.

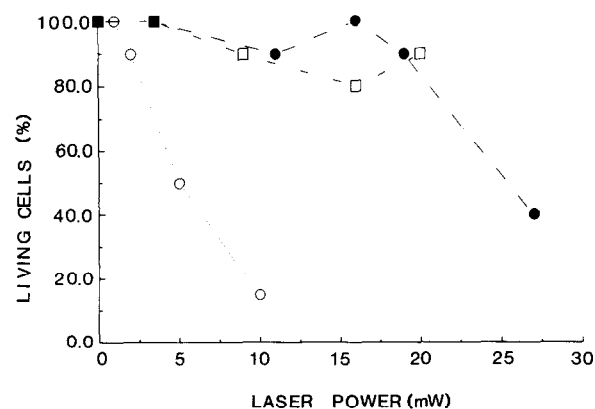


FIG. 4. Percentage of cells (human lymphocytes) surviving a 300-s laser light irradiation as a function of laser light power for 514.5, 632.8, and 660 nm. (○) At 514.5 nm; objective, 63X Zeiss Plan Neofluar water immersion (N. A. 1.2) focus diameter, $\sim 0.5 \mu\text{m}$. (□) At 632.8 nm; objective, 40X Nikon E Plan (N. A. 0.65) focus diameter, $\sim 1 \mu\text{m}$. (●) At 660 nm, objective and beam diameter are the same as that for 514.5 nm. The laser beam was focused in the center of the cells. Each point in the graph represents 10 irradiated cells (20 for 660 nm 25 mW point). Control samples contained $> 90\%$ living cells.

[8]. The energy contained in two photons with a wavelength of 514.5 nm lies exactly at the maximum of the DNA absorption band in the uv. The damaging effects of uv radiation on DNA are a well-known phenomenon. With an experiment we checked whether two or multiphoton absorption could play an important role in sample degradation. In that case not only the total laser light dose applied to a sample but also the intensity of the laser light should determine the extent of the damage. With the CRM Raman spectra of single metaphase chromosomes were recorded. A chromosome was positioned in the laser focus (without preference for type of chromosome or position on a chromosome) and a series of consecutive short Raman spectroscopic measurements was made at that position on the chromosome. The time course of the decrease in Raman signal inten-

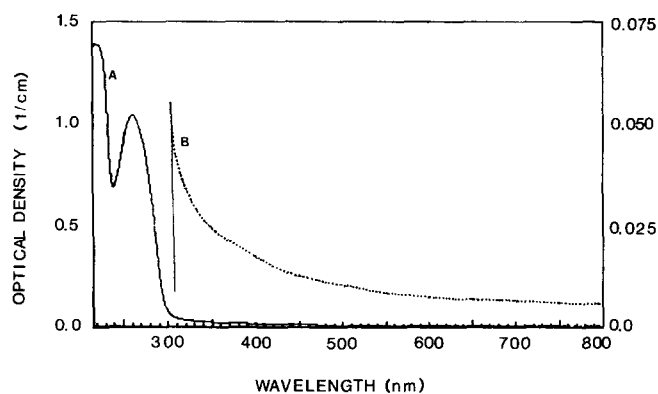


FIG. 5. (A) Absorption spectrum of a suspension of CHL-cell metaphase chromosomes. (B) 20 \times magnification of spectrum A.

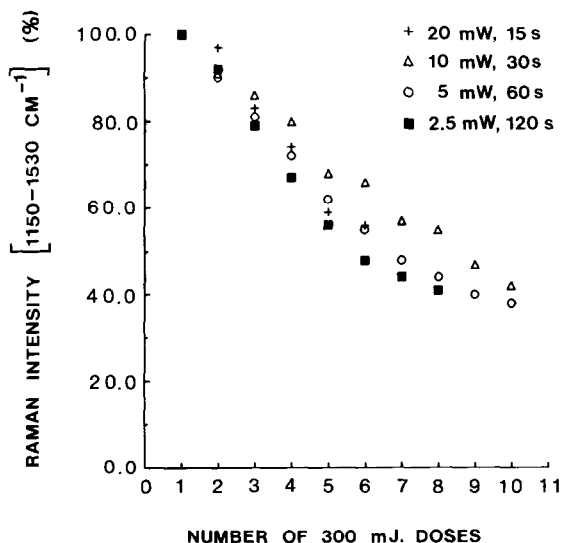


FIG. 6. Decrease in Raman signal intensity, observed in measurements on single metaphase chromosomes for different laser light powers (514.5 nm). The laser light dose in each measurement was kept constant at 300 mJ. Laser power (measuring time per measurement): +, 20 mW (15 s); Δ , 10 mW (30 s); \circ , 5 mW (60 s); \blacksquare , 2.5 mW (120 s). Every point in the graph represents the integrated Raman signal (normalized over 10 measurements). See text for further details. At every laser power 10 series of measurements were carried out.

sity was thus determined. This was done for laser powers ranging from 2.5–20 mW. For the different laser powers used the signal integration time for each measurement was adjusted in such a way that the total irradiation dose for each measurement was kept constant at 300 mJ. At every laser power 10 such measurement series were made, each on a different chromosome. The results in Fig. 2 are examples of how the Raman signal intensity decreased with time, using a laser power of 5 mW. In order to make a quantitative comparison between the results obtained at different laser powers the total Raman signal contained in the spectral region 1150–1530 cm^{-1} in the resulting spectra was integrated. The relative decrease in integrated signal intensity as a function of irradiation dose on the sample is shown in Fig. 6. Taking the decrease in signal intensity as a measure of sample degradation it follows from this figure that the amount of damage depends on the total laser light dose only and not on the intensity of the laser light. This agrees with the observation mentioned earlier that total laser irradiation dose on a cell seems to be the parameter determining cell survival and not the intensity of the laser light. Therefore the influence of two or multiphoton absorption processes is considered to be negligible.

Substrate Heating

The temperature rise of a glass substrate (O.D. 0.02/cm at 514.5 nm) due to absorption at the position of the

laser focus was calculated and found to be negligible (at most 0.1°C for 10 mW of incident laser power), indicating that it is not of any importance for sample degradation. The calculation for a fused silica substrate yields even lower values for the temperature rise, due to the fact that the transmittance (T) of this material is extremely high ($T > 0.9999/\text{cm}$, Melles Griot Optics Guide IV), while the heat conduction coefficient is the same as for the glass substrate.

DISCUSSION

From the results presented here it follows clearly that in laser experiments on (living) biological matter the choice of the wavelength of the laser light is a matter of concern. Based on experiments it was found that the use of laser light of 660 nm greatly improves the experimental conditions in that it does not affect sample integrity. When using 514.5 nm laser light a paling occurs for all types of samples investigated thus far. Although this paling resembles the findings of Berns *et al.* [7–9] no evidence was found for multiphoton absorption as in their case. Furthermore they observed that in metaphase chromosomes histones were more sensitive to laser irradiation (532.8 nm from a frequency-doubled Nd Yag pulse laser) than DNA [22]. This appears to disagree with our observation that the guanine Raman line at 1487 cm^{-1} decreases most rapidly in intensity. Most likely the effects of laser irradiation described in this paper are of a different kind than those that occur during irradiation with pulsed laser light of much higher peak intensity. Another difference in experimental conditions, that may influence the specific paling mechanism, is that in the work of Berns *et al.* [7–9, 22] chromosomes were irradiated intracellularly, whereas in our experiments isolated chromosomes were used. No definite answer concerning the cause for the reported effects of 514.5 nm (and 488/457.9 nm) laser light on biological samples has been found. Multiphoton absorption and substrate heating could be excluded as possible mechanisms. Therefore one-photon laser light absorption by the chromosomes and cells remains the only possible process involved in sample degradation.

For the case of chromosomes it is not plausible, that heating effects due to laser light absorption play an appreciable role. Thus photochemical processes should be considered to cause the radiation damage.

The experiments with CT-DNA and CT-histone have shown that two of the main chromosome constituents in their purified form are not susceptible to radiation damage (at 514.5 nm). Other compounds or complexes that can act as photosensitizers must therefore be present in chromosomes and cells. DNA bases (especially guanine) and a number of amino acids (methionine, tyrosine, tryptophan, histidine, cysteine) can be degraded in a photodynamical process, requiring the

presence of light, a sensitizer, and oxygen [23]. In such a process laser excitation brings the sensitizer via the singlet state in a long-lived triplet state. It can then react with an oxygen molecule to form singlet oxygen or a superoxide-ion. The reactive oxygen species could cause the observed radiation damage, through oxydation of the DNA bases or amino acids leading to lesions in these molecules.

The observation that the guanine Raman line at 1487 cm^{-1} loses its intensity most rapidly would agree with the fact that guanine is the DNA base that is most sensitive to this type of processes. Sensitizer triplets can also produce free radicals or radical ions through interaction with a reducing substrate, which can then react further to induce damage. In such a process the presence of oxygen is not required [23]. An experiment (not shown) in which a mixture of glucose oxidase and catalase was added to a metaphase chromosome preparation showed that damage still occurred. Such an enzyme mixture was shown [24] to be very effective in depleting a sample of free oxygen molecules. However, reactive hydrogen peroxide may be present and the enzymes contain flavines which can act as sensitizers [23]. This matter was not further pursued.

The fact that excitation with laser light of 660 nm does not induce sample degradation indicates that the sensitizer molecules do not absorb at this wavelength.

Summarizing, it can be stated that the laser irradiation-induced degradation of chromosomes and cells is probably a photochemical process mediated by as yet unidentified sensitizer molecules or complexes. These sensitizers are excited by blue and green laser light but not by the 660-nm laser light used in the CRM.

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