E 21 954 F

MICROSCOPICA ACTA

JOURNAL OF MICROSCOPIC EQUIPMENT, METHODS AND APPLICATIONS

NUMBER 1 . VOLUME 81 · SEPTEMBER 1978

S. HIRZEL VERLAG STUTTGART

Microsc. Acta

ISSN 0044-376 X

MICROSCOPICA ACTA

Journal of microscopic equipment, methods and applications

formerly

Zeitschrift für wissenschaftliche Mikroskopie und mikroskopische Technik

MICROSCOPIA ACTA publishes reviews, original papers, reports and references to literature covering the whole field of light and electron microscopy with regard to the aspect of microscopy technique.

MICSROSCOPIA ACTA veröffentlichen Übersichtsbeiträge, Originalarbeiten, Berichte und Literaturhinweise, die das Gebiet der Lichtmikroskopie und der Elektronenmikroskopie unter dem Gesichtspunkt der mikroskopischen Technik behandeln.

Contributions will be accepted in English, German or French and should be submitted to the Acting Etidor:

Manuskripte können in deutscher, englischer oder französicher Sprache abgefaßt sein. Sie sind bei der Redaktion einzureichen:

MICROSCOPIA ACTA

Prof. Dr. Louis Jenny, Bäumlihofstr. 375
CH-4125 Riehen/Switzerland

Tel. Private: (0 61) 49 59 69
Institute: (0 61) 43 44 15

Terms of delivery

The journal is published in issues combined to a volume of approximately 450 pages. Subscription rate DM 145,— per volume. Orders can be placed with any bookseller or the publisher.

Bezugsbedingungen

Die Zeitschrift erscheint in einzelnen Heften, die zu Bänden mit etwa 450 Seiten zusammengestellt werden. Preis im Abonnement je Band DM 145,—.
Bestellungen nimmt jede Buchhandlung im In- und Ausland und der Verlag entgegen.

MICROSCOPICA ACTA

JOURNAL OF MICROSCOPIC EQUIPMENT, METHODS AND APPLICATIONS

184-5

Volume 81 — 1979 — Band 81

S. HIRZEL VERLAG STUTTGART

Microsc. Acta

ISSN 0044-376 X

MICROSCOPICA ACTA

Journal of microscopic equipment, methods and applications

formerly

Zeitschrift für wissenschaftliche Mikroskopie und mikroskopische Technik

MICROSCOPICA ACTA publishes reviews, original papers, reports and references to literature covering the whole field of light and electron microscopy with regard to the aspect of microscopical technique.

MICROSCOPICA ACTA veröffentlichen Übersichtsbeiträge, Originalarbeiten, Berichte und Literaturhinweise, die das Gebiet der Lichtmikroskopie und der Elektronenmikroskopie unter dem Gesichtspunkt der mikroskopischen Technik behandeln.

Contributions will be accepted in English, German or French and should be submitted to the Acting Editor:

Manuskripte können in deutscher, englischer oder französischer Sprache abgefaßt sein. Sie sind bei der Redaktion einzureichen:

MICROSCOPICA ACTA

Prof. Dr. Louis Jenny, Bäumlihofstr. 375
CH-4125 Riehen/Switzerland

Tel. Private: (0 61) 49 59 69
Institute: (0 61) 43 44 15

The issues of Volume 80 appeared:

Number 1 September 1978
Number 2 November 1978
Number 3 January 1979
Number 4 March 1979
Number 5 May 1979

A. Authors of papers

Adams, Derek, see Williams, David

Agroskin, L. S., see Pevzner, L. Z.

Åkerman, Karl, E. O.: *Measurements of membrane potentials using the dye safranine* . 147
Bestimmungen von Membranpotentialen mit Hilfe von Safranin

Azzam, R. M. A.: *Optical detection of cell-surface changes associated with malignant transformations in vitro* 313
Optische *in-vitro*-Feststellung von Zelloberflächenänderungen in Verbindung mit bösartigen Transformationen

Bezler, Hans †, see Leder, Ortwin

Bhattacharyya, Tapan Kumar: *A differential staining technique for vertebrate histology (Short communication)* 299
Une méthode de coloration différentielle pour l'histologie des Vertébrés

Boer, Hein, and Woulter H. van Eek: *The penetration of the embedding medium methyl methacrylate in undecalcified bone* 181
Das Eindringen von Methylmethacrylat als Einbettungsmittel für unentkalkte Knochen

Borovikov, Yurii S., and Natalia A. Chernogriadskaia: *Studies on conformational changes in F-actin of glycerinated muscle fibers during relaxation by means of polarized ultra violet fluorescence microscopy* 383
Konformationsänderungen im F-Aktin im Verlauf des Erschlaffens glyzerinisierter Muskelfasern. Untersuchungen mit Hilfe von Fluoreszenzmikroskopie im polarisierten UV

Chernogriadskaia, Natalia A., see Borovikov, Yurii S.

Chun, Moonjin, see Sernetz, Manfred

Collins, Barbara Ann, and Edward Ford MacNichol, jr.: *Long term fixation for histological studies (Short communication)* 155
Langzeitfixation für histologische Untersuchungen

Collins, Barbara Ann, and Edward Ford MacNichol, jr.: *Sectioning of Epon blocks in the 20 µm to 60 µm range for histological studies (Short communication)* 227

20—60 µm dicke Schnitte von Epon-Blöcken für histologische Untersuchungen

Colman, Ondina D., and Juan C. Stockert: *Observations on the nucleolar staining by ethanolic phosphotungstic acid (Short communication)* 27

Beobachtung über die Nukleolarfärbung durch äthylalkoholische Phosphorwolframsäure

Cremer, Christoph, and Thomas Cremer: *Considerations on a laser-scanning-microscope with high resolution and depth of field* 31

Überlegungen zu einem Laser-Scanning-Mikroskop mit hoher Auflösung und Schärfentiefe

Cremer, Thomas, see Cremer, Christoph

Dewar, Catherine L., and Michael W. Wolowyk: *Scanning electron microscopy of blood cells* 209

L'examen de leukocytes et d'érythrocytes au microscope à balayage

Dimock, Ronald V., jr., see Hazen, Terry

Dutt, Mihir K.: *Cytochemical specificity of acridine red towards RNA and depolymerised DNA* 189

Cytochemische Spezifität von Akridinrot gegenüber RNS und depolymerisierter DNS

Dutt, Mihir K.: *Modified Feulgen staining of DNA with aqueous solution of pinacyanol* 195

Modifizierte Feulgen-Färbung für DNS mit wässriger Lösung von Pinacyanol

Dutt, Mihir K.: *Increased shelf-life and Feulgen staining intensity of a modified trichloracetic acid-Schiff reagent* 275

Verbesserte Haltbarkeit und Feulgen-Färbungsintensität eines modifizierten Trichloressigsäure-Schiff-Reagenz

Dutt, Mihir K.: *Hoffman's violet and Dahlia as specific stains for animal chromosomes* 293

Hoffmans Violett und Dahlia als spezifische Farbstoffe für tierische Chromosomen

Dutt, Mihir K.: *Shelf-life of citric acid-toluidine blue O-SO_2 and its influence on Feulgen staining* 367

Zitronensäure-Toluidinblau O-SO_2: Lagerfähigkeit und Einfluß auf die Feulgen-Färbung

Dutt, Mihir K.: *Detection of DNA in mammalian tissues following removal of RNA with cold phosphoric acid* 373

Nachweis von DNS im Gewebe von Säugern nach Entfernung der RNS mit kalter Phosphorsäure

Dutt, Mihir K.: *Fixation of mammalian tissues in different fixatives and its influence on the staining with methyl green-pyronin* 379

Das Fixieren von Säugergewebe in verschiedenen Fixativen und deren Einfluß auf die Färbung mit Methylgrün-Pyronin

Eek, Woulter H van, see Boer, Hein

Friebe, Bernd: *Untersuchungen zum Schwesterchromatidenaustausch bei Secale cereale* 159

Analysis of sister chromatid exchange (SCE) in *Secale cereale*

Gebhart, Josef, see Roth, Christa

Glahn-Luft, Birgit, Peter Schley und Rudolf Wassmuth: *Geschlechtsdifferenzierung von neugeborenen Kaninchen mit Hilfe der Karyotypanalyse zu Versuchszwecken (Kurzmitteilung)* 289

Sexual differentiation of newborn rabbits with karyotype analysis for experimental use

Gundersen, Hans Jørgen G.: *Estimators of the number of objects per area unbiased by edge effects* 107

Abschätzungsmethoden für die Objektflächendichte, frei von systematischen Fehlern durch Randeffekte

Gupta, A. K., and K. Singh: *Influences of primary coma on partially coherent images: Truncated bar targets* 265

Der Einfluß primärer Koma auf die partiell kohärente Abbildung: Abgestufte periodische Strichraster

Habermalz, Fritz: *Farbmetrische Untersuchungen über die mit Umkehrfarbfilmen darstellbaren Farben* 137

Colorimetrical investigations of colors produced by color reversal films applied for photomicrography

Hannibal-Friedrich, Otto, see Sernetz, Manfred

Hartmann, Horst, und Klaus Paradies: *Ein Präparationsverfahren für die Auflichtfluoreszenz-Mikroskopie (Kurzmitteilung)* 407

A simple preparation technique for incident light fluorescence microscopy

Hazen, Terry C., Gerald Smith, and Ronald V. Dimock, jr.: *A method for fixing and staining peritrich ciliates* 15

Eine Methode zum Fixieren und Färben von rundum begeißelten Ciliaten

Holländer, Horstmar: *Identification of cytoplasmic laminar bodies in neurons of the cat lateral geniculate nucleus by phase contrast microscopy* 131

Phasenkontrastmikroskopischer Nachweis von cytoplasmatischen Lamellenkörperchen in Nervenzellen des geniculatum laterale der Katze

Jacob, Helmut, Judit Nowak, Peter Veith und Heiko Wasmund: *Elektronische Datenerfassung und -verarbeitung im On-line-Betrieb bei der Mikroskop-Photometrie fester, fossiler, organischer Stoffe* 45

On-line data collection and processing in connection with the microscope photometry of solid, fossil, organic matter

Kemali, Milena: *Application of a rapid Golgi method and use of potassium permanganate in the histology of Hydra* 309

Anwendung einer modifizierten, raschen Golgi-Methode und Verwendung von Kaliumpermanganat in der Histologie von Hydra

Kraus, Herbert, see Schwarz, Jürgen

Kunt, Manfred, see Leder, Ortwin

Leder, Ortwin, Manfred Kunt und Hans Bezler †: *Pilotstudie zur Auswertung von Vaginalabstrichen in der zytologischen Hormondiagnostik* 281

The evaluation of vaginal smears in cytological hormone diagnosis: A pilot study

Lüttge, Bernhard, see Menold, Richard

MacNichol, Edward Ford, jr., see Collins, Barbara Ann

Marshall, P. N.: *Reticulation, polychromasia and stippling of erythrocytes (Review)* . . 89

Netzbildung an, Polychromasie und Tüpfelung von Erythrocyten

Martin, H.: *Zum Gedächtnis an Prof. Dr. Alfred Grabner (20. Jan. 1896—28. Jan. 1978)* 63

Mayfield, C. I., see Polonenko, D. R.

Menold, Richard, and Bernhard Lüttge: *Freeze-etching of dispersions without contamination of the fracture faces* 317

Kontaminationsfreies Gefrierätzen von Dispersionen

Mort, Elizabeth, see Williams, David

Nowak, Judit, see Jacob, Helmut

Paradies, Klaus, see Hartmann, Horst

Peters, J. Hinrich: *Zeitrafferanlage zur Lebendbeobachtung von Zellen in der Kultur* . . 217

Device for time lapse studies on living cells in culture

Pevzner, L. Z., T. G. Raygorodskaya, and L. S. Agroskin: *Quantitative microspectral evaluation of the ratio of arginine-rich to lysine-rich histones in neurons and neuroglial cells* 9

Evaluation microspectrale quantitative du ratio des histones riches en arginine ou lysine dans les neurones et des cellules névrologiques

Pietruschka, Fricke, see Zierold, Karl

Polonenko, D. R., and C. I. Mayfield: *Fluorescence probes for use as staining agents in soil* 303

Fluoreszierende Stoffe als Färbemittel für Bodenproben

Raffler, Hartmut, see Schwarz, Jürgen

Raygorodskaya, T. G., see Pevzner, L. Z.

Roth, Christa, and Josef Gebhart: *Rapid particle size analysis with an ultramicroscope* . 119

Schnell registrierendes Ultramikroskop für Schwebstoffteilchen

Schäfer, Dieter, see Zierold, Karl

Schley, Peter, see Glahn-Luft, Birgit

Schwarz, Jürgen, Herbert Kraus und Hartmut Raffler: *Methodische Untersuchungen zur quantitativen Bildanalyse an Zellen des weiblichen Genitaltraktes* 201

Methodical investigations for quantitative image analysis on cells of the female genital tract

Sernetz, Manfred, Otto Hannibal-Friedrich und Moonjin Chun: *Bestimmung radialer Dichtegradienten in Oxiran-Acrylharzperlen durch Mikrointerferometrie und Mikrofluorometrie* 393

Determination of radial concentration gradients in oxirane-acrylic beads by microinterferometry and microfluorometry

Singh, K., see Gupta, A. K.

Smith, Gerald, see Hazen, Terry C.

Spijker, H. J. D.: *A procedure for obtaining thin sections of undecalcified bone biopsies embedded in methyl methacrylate* 17

Eine Methode zur Anfertigung von Dünnschnitten Methylmethacrylat-eingebetteter, nicht-entkalkter Knochenbiopsien

Srivastava, Kanti, and Mohd. Yunus: *A modified technique to study epidermis in Verbenaceae (Short communication)* 177

Eine modifizierte Präparationsmethode für Epidermisuntersuchungen an *Verbenaceae*

Stockert, Juan C., see Colman, Ondina D.

Veith, Peter, see Jacob, Helmut

Wasmund, Heiko, see Jacob, Helmut

Wassmuth, Rudolf, see Glahn-Luft, Birgit

Williams, David, Derek Adams, and Elizabeth Mort: *A histochemical method for the detection of metals in tissues, with references to the use of surgical implants* . . . 1

Histochemischer Nachweis von Metallen in Geweben, mit Bezug auf die Verwendung metallischer Implantate

Wolowyk, Michael W., see Dewar, Catherine L.

Wurtz, M.: *9th International Congress on Electron Microscopy, Toronto, August 1—9, 1978 (Report)* 329

Yunus, Mohd., see Srivastava, Kanti

Zierold, Karl, Fricke Pietruschka, and Dieter Schäfer: *Calibration for quantitative X-ray microanalysis of skeletal muscle cells in culture* 361

Kalibrierung zur quantitativen Röntgen-Mikroanalyse von Skelettmuskelzellen in Kultur

B. Book reviews

Beyer, Hermann: *Handbuch der Mikroskopie,* 2., stark überarb. Aufl. (VEB Verlag Technik, Berlin 1977.) 169

Blaker, A. A.: *Handbook for Scientific Photography.* (W. H. Freeman & Comp., Reading/England-San Francisco 1978.) 168

Dobson, P. J., J. B. Bendry, and C. J. Humphreys (Edits.): *Electron Diffraction 1927—1977* (Institute of Physics Conference Series No. 41). (The Institute of Physics, Bristol 1978.) 358

Gaunt, W. A., and P. M. Gaunt: *Three-Dimensional Reconstruction in Biology.* (Pitman Medical, Tunbridge Wells/England 1978.) 354

Geyer, G.: *Elektronenmikroskopische Histochemie,* Teil 1: *Nachweis- und Kontrastierungsmethoden für Kohlehydrate, Proteine, Aminosäuren, Nucleinsäuren, Lipide und Mineralstoffe,* (Band I, Teil 3 des „Handbuchs der Histochemie"). (Gustav Fischer Verlag, Stuttgart 1977.) 167

Glauert, Audrey M. (Edit.): *Practical Methods in Electron Microscopy,* Vol. 5, Part I: Lewis, P. R., and D. P. Knight: *Staining Methods for Sectioned Material* / Part II: Chandler, J. A.: *X-Ray Microanalysis in the Electron Microscope.* (North-Holland Publishing Company, Amsterdam 1977.) 85

Glauert, Audrey M. (Edit.): *Practical Methods in Electron Microscopy,* Vol. 6, Part I: *Autoradiography and Immunocytochemistry* / Part II: *Quantitative Methods in Biology.* (North-Holland Publishing Company, Amsterdam 1977.) 263

Hayat, M. A. (Edit.): *Principles and Techniques of Electron Microscopy: Biological Applications,* Vol. 6. (Van Nostrand-Reinhold Company, New York-London 1976.) . 171

Hayat, M. A. (Edit.): *Principles and Techniques of Electron Microscopy: Biological Applications,* Vol. 7. (Van Nostrand-Reinhold Company, New York-London 1977.) . 263

Ibach, H. (Edit.): *Electron Spectroscopy for Surface Analysis* ("Topics in Current Physics", Vol. 4). (Springer-Verlag, Berlin-Heidelberg-New York 1977.) 86

Johannessen, J. V. (Edit.): *Cellular Pathobiology: Metabolic and Storage Diseases* (Vol. 2 of the series "Electron Microscopy in Human Medicine"). (McGraw-Hill International Book Company, New York-Düsseldorf 1978.) 353

Klieneberger-Nobel, Emmy: *Pionierleistungen für die Medizinische Mikrobiologie.* (Gustav Fischer Verlag, Stuttgart 1977.) 85

Knowles, P. F., D. Marsh, and H W. E. Rattle (Edits.): *Magnetic Resonance of Biomolecules (An introduction to the theory and practice of NMR and ESR in biological systems).* (John Wiley & Sons Ltd., Chichester/Sussex 1976.) 169

Kuhlmann, W. D.: *Ultrastructural Immunoperoxidase Cytochemistry* (Vol. 10/No. 1 of the series "Progress in Histochemistry and Cytochemistry"). (Gustav Fischer Verlag, Stuttgart 1977.) 170

Lillie, R. D. (Edit.): *H. J. Conn's Biological Stains,* 9th ed. (Williams & Wilkins Company, Baltimore 1978.) 355

Lippert, Herbert: *Das wissenschaftliche Manuskript: Fachzeitschrift — Vortrag — Dissertation — Fachbuch* (U & S Taschenbuch 1012). Urban & Schwarzenberg, München 1977.) 86

Meadows, R.: *Renal Histopathology, a light, electron, and immunofluorescent microscopy study of renal disease,* 2nd ed. (Oxford University Press, New York-Melbourne 1978.) 354

Meek, G. A., and H. Y. Elder (Edits.): *Analytical and quantitative methods in microscopy* (Soc. of Experimental Biology Seminar series No. 3). (Cambridge University Press, London 1977.) 354

Needham, G. H.: *The Practical Use of the Microscope (including Photomicrography),* 2nd printing. (Ch. C. Thomas Publisher, Springfield IL/USA 1977.) 84

Petzow, G.: *Metallographisches Ätzen.* (Gebr. Borntraeger, Stuttgart 1976.) 84

Schach, S., und T. Schäfer: *Regressions- und Varianzanalyse: Eine Einführung* (Hochschultext). (Springer-Verlag, Berlin-Heidelberg-New York 1978.) 353

Thompson, S. W., and L. G. Luna: *An Atlas of Artifacts, Encountered in the Preparation of Microscopic Tissue Sections.* (Charles C. Thomas Publisher, Springfield IL/USA 1978.) 167

Whitman, R. L. (Edit.): *Multidisciplinary Microscopy* (Vol. 104 of the Proceedings of the Society of Photo-Optical Instrumentation Engineers). (Soc. Photo-Optical Instrumentation Engineers). Bellingham WA/USA 1977.) 170

C. Subject index

List of abbreviations

AR	Autoradiography
EM	Electron microscopy
F	Text in French
G	Text in German
HC	Histochemistry
LM	Light microscopy
LP	List of publications
MG	Macrography
MP	Microprobe (X-ray, electron)
P	Preparation method
PI	Photometry, interferometry
SEM	Scanning electron microscopy
ST	Stereology, morphometry
TI	Technical information

absorption curve: acridine red stained nuclei (LM, HC) 193
— —: basic fuchsin; various TCA-Schiff solutions (LM, HC) 279
— —: Hoffmann's violet (LM, P) 295
— —: night blue; Feulgen modification (LM, HC) 376
— —: pinacyanol; Feulgen modification (LM, HC) 198
— —: silver stain, ammoniacal (LM) 12
— —: toluidine blue O-citric acid, Feulgen stain (LM, HC) 369
acetic carmine stain, Sémichon; peritrich ciliate (LM) 15
acridine orange; constitution, properties (LM, P) 97
— red; substitute for pyronin; RNA (LM, P, HC) 189
acrylic beads, see oxirane
adenosine triphosphate; membrane potential (MG, P) 149
— — (ATP); F-actin conformation (LM, P, HC) 383
Agfa-Gevaert color films; colorimetry (LM) 137-G
— — Scientia 23D56P3 (EM, TI) 66-G
ammonia; cuticle separation, epidermis (LM) 177
anaemia, blood cells (SEM, P) 209
analysis, see also image —, micro —
—, maceral; fossil organic matter (LM, PI) 45-G
—, particle size, aerosol; device, calibration (LM) 119
anilino-1-naphthalene sulfonic acid, salts; soil microorganism stain (LM) 303
anticontamination technique: freeze-etching dispersions (SEM) 317
arginine rich histones, neural cells (LM, P, PI) 9
artifacts; methyl methacrylate embedding bone (SEM) 181
autoradiography: materials, methods, applications (LP) 67, 331
azure B; constitution, properties (LM, P) 98
— II-methylene blue; fish retina study (LM, P) 228

Balanus rostratus Hock., barnacle; muscle fiber, F-actin changes (LM, P, PI) 384
bacteria, membrane potential; measurement (LM, P) 147
Balzers Union, new devices (TI) 65-G
bias, edge effect; methods free of — (ST) 107

biased, unbiased estimation of numbers (ST) 113
bio objects; laser damage, evaluation (LM) 31
biopsies, bone; undecalcified (LM, P) 19
—, tissues; metal traces by implants (LM, P, HC) 1
blood cells; morphology (SEM, P) 209
— culture: long, short term; karyotype analysis (LM, P) 289-G
bone, undecalcified; methyl methacrylate penetration (SEM, P) 181
—, —; sectioning 19
books, new 172, 359
—, reviews 84-G, 167-G, 263-G, 353-G
BrdU; fluorescence quencher, DNA (LM, P) 160-G
brilliant cresyl blue; constitution, properties (LM, P) 96

Ca ion; detectable concentration (MP) 361
calibration curves, MP; Ca, Cl, K, Na ions 365
—, particle size analysis, ultramicroscopy (LM, ST) 119
cat, nerve cells; cytoplasmic laminar bodies (LM, P) 131
celestine blue; stain sequence, vertebrate tissue (LM) 299
cell culture, in vitro study; conditions, protection (LM, P) 219-G
—, distribution, vaginal smears; hormone diagnosis (LM, P) 281-G
—, femal genital tract; automated cytology (LM, P, PI) 201-G
—, surface; ellipsometric detection, transformations (LM) 313
chromosome spreading; sex determination, rabbit (LM, P) 289-G
— staining, DNA (LM, PI) 293
ciliate, pertrich; differentiation macronucleus, zooid relaxation (LM, P) 15
cinemicrography, time lapse; cells in vitro (LM) 220-G
citric acid-toluidine blue O-SO_2 ; Feulgen stain, shelf life (LM, PI) 367
Cl ion; detectable concentration (MP) 361
Co trace in tissue, surgical implant (LM, P, HC) 1
coatings for slides (LM) 407-G
coherence, partially; diffraction image, coma (LM) 265
color matching: additive, optimal, comparison (LM) 144-G
— reversal films: colorimetry, color bodies, color reproduction limit (LM) 131-G
— variation of staining, influence fixatives (LM, PI) 381
coma, primary; formulation of influences on image (LM) 265
—, —; illuminance, diffraction image (LM) 265
concentration, detectable; Ca, Cl, K, Na ions (MP) 361
—, particles; comparison with EM determination (LM) 127
—, —; determined by ultramicroscopy (LM, P) 128
—, radial gradients; oxirane-acrylic beads (LM, PI) 393-G
—, — —; relation matrix & thickness (LM, PI) 394-G / calculation formula (LM, PM) 395-G
conformational change, F-actin; muscle fibers (LM, P) 390
counts, point, profile (LM, ST) 110
courses & meetings 87, 176, 264, 360, 440
curvature, total; estimators, unbiased (LM, ST) 111
cuticle separation, epidermis study (LM, P) 177
cytochemistry, histochemistry; applications (LP) 261
—, —; methods, principles, theory (LP) 258
cytological hormone diagnosis; vaginal smears (LM, P, ST) 281-G
cytology, automated; image analysis vaginal cells (LM, PI, ST) 201-G
cytoplasmic laminar body; geniculate nucleus, cat (LM, P) 131

dahlia; chromosome stain (LM, PI) 293
darkfield illumination, ultramicroscopy; particle sizing (LM) 119
data collection, processing; maceral analysis, organic fossil matter (LM, PI) 45-G
dehydration, drying (LP) 234, 415
—, blood cells (SEM, P) 211
densitometry (LP) 77, 349
density profile; bounded enzyme, γ-globulin (LM, P, PI) 403-G
—, radial, gradient; model calculation, sphere, shell model (LM, PI) 397-G
depth of field, high; laser-scanning-microscope (LM) 31
detection limit, particle size; ultramicroscopy (LM) 119

diffraction image; asymmetry, coma, illuminance (LM) 265
dispersions; freeze-etching, fracturing, contamination free (SEM, P) 317
DNA, aldehyde; staining (LM, HC, PI) 195, 373
—, chromosomal; staining (LM, HC, PI) 293
—, depolymerised; staining (LM, HC, PI) 189, 379
—, phosphate staining (LM, HC, PI) 195, 373
—; pinacyanol stain (LM, HC, PI) 195
drop freeze-fracturing; dispersions (SEM) 317
ducks; tissue staining by sequence (LM) 299

earth sciences; applications of EM (LP) 256, 437
— —; — — LM (LP) 250, 432
edge effect; estimating numbers per area (ST) 107, 115
electron diffraction (LP) 81
— microscope; applications to earth & materials sciences (LP) 256, 437
— —; — — life sciences (LP) 254, 434
— —; equipment, image producing methods, theory (LP) 251, 429
ellipsometry; application, cell surface changes (LM) 313
—, special optical methods (LP) 81
embedding (LP) 239, 419
— mould; methyl methacrylate, bone biopsies (SEM) 19
enzyme, immobilized; γ-globulin, density profile (LM, P, PI) 402-G
epidermis, *Verbenaceae;* cuticle separation (LM, P) 177
epon blocks; thick sections (LM, P) 227
erythrocyte, immature; morphology, historical review (LM, P) 91
—; polychromasia, reticulation, stippling (LM, P) 89
erythrosin; stain sequence, vertebrate tissue (LM) 299
estimation; object per area, unbiased (ST) 107
—; total curvature (ST) 111
etching (LP) 237, 417
evaluation images; photography, computer, digitzing (LP) 73, 343
— particle size; ultramicroscopy (LM, P) 124
— — —; — compared EM determination (LM, P) 127
—, special methods (LP) 80, 352

F-actin; conformational changes, muscle fibers (LM, P) 383
fast green; stain sequence, vertebrate tissue (LM) 299
femal genital tract, cell image analysis (LM, P, ST) 201-G
Feulgen stain; compared with Papanicolaou, genital tract cells (LM) 205-G
— —; modifications (LM, HC, PI) 195, 275, 367, 373
— —; shelf-life increased (LM, HC, PI) 275, 367
fibers, muscle; *Balanus rostratus,* rabbit; F-actin change (LM, P) 383
films, new; micrography (LM, EM, TI) 66-G
fishes; retinal study (LM, P) 155, 228
fixation (LP) 234, 415
—; depolymerized DNA-RNA (LM, PI) 379
—; influence of metal cations, stains color (LM, PI) 381
—; long term, retinal tissues, fishes (LM) 155
—; peritrich ciliates (LM) 15
—, rapid; blood cells (SEM) 209
fluorescence microscopy, incident light (P) 407-G
— —; fibrous materials, powders (P) 407-G
— —; organic fossil matter, maceral analysis analysis (P, PI) 45-G
— —; polarized UV; muscle fiber, F-actin change (P, PI) 383
— quencher (P) 390
— staining (LP) 244, 425
— —, applications (LP) 246, 426
— —; soil microorganisms (LM) 303
fluorometry (LP) 77, 349
— compared with interferometry (LM, PI) 404-G
freeze-etching, -fracturing; dispersions, contamination free (SEM) 317
freezing, freeze-fracturing (LP) 237, 417
—, — —; device for dispersions (SEM) 321
furnace, microincineration; tissues (LM, P, HC) 4

geniculate nucleus, cat; cytoplasmic laminar bodies (LM, P, PI 131
Giemsa + fluorescence technique; chromatid exchange (LM) 159-G
globulin, γ-; density profile, spheres (LM, PI) 402-G
glycerination, muscle fiber; fluorescence LM (P, PI) 383

glycerol-water emulsion; freeze-fracturing (SEM) 317
Golgi method modified; histology of Hydra (LM, HC) 309
Graber, Alfred; life 63-G
gradient, radial concentration; calculation (LM, PI) 395-G
—, — density profile; acrylic beads, sphere, matrix (LM, PI) 393-G
grids, supports (LP) 239, 419

haddock; retinal study (LM, P) 227
hardness, measurement (LP) 79, 351
heterochromatin; reactivity phosphotungstic acid (LM, P) 27
Heyder formula, size of particles (LM, PI) 125
histone, arginine/lysine rich; neurons, neuroglial cells (LM, P, PI) 9
— mixture; silver stain, absorption (LM, PI) 11
Hoffmann's violet; chromosome stain (LM, PI) 293
hologram, point; laser microscope focussing 31
hormone diagnosis; vaginal smears, cytology (LM, P) 281-G
Hydra attenuata; Golgi method modified (LM) 309

image analysis (LP) 74, 345
— —, quantitative; vaginal cells (LM, ST) 201-G
—, diffraction; asymmetry, illuminance, coma (LM) 265
— producing methods EM (LP) 251, 429
— — — LM (LP) 248, 427
implants, surgical; metals in tissue (LM, P, HC) 1
in vitro probing, cell surface; ellipsometry 313
— — study, cell culture; time lapse (LM, P) 219-G
infiltration, methyl methacrylate; bone (SEM, P) 181
interference contrast LM; retinal study (P) 155, 227
interferometry (LP) 79, 351
—, fluorometry; spheres, differences (LM) 394-G
intranuclear differentiation; Unna's stain (LM) 27
ion beams (LP) 81
— detection in MP; Ca, K, Na, Cl (SEM) 365
K ion; detectable concentration (MP) 365
— permanganate; *Hydra attenuata* (LM, P) 309
karyotype analysis; newborn rabbit, sex determination (LM) 289-G
Kodak color films; colorimetry (LM) 138-G
— Technical Pan Film SO-115 (TI) 66-G

labelling (LP) 234, 415
— protein; rhodamine isothiocyanate (LM) 403-G
laboratory technique (LP) 232, 412
lactic acid; cuticle separation, epidermis (LM) 177
laser & holography (LP) 81
— damage, bio objects; evaluation 38
— scanning microscope; project, theory 31
leukemia, lymphoma; blood cells (SEM, P) 209
life sciences; applications of EM (LP) 254, 434
— —; — — LM (LP) 250, 432
light microscopy, macro technique; applications (LP) 250, 432
— —, — —; equipment, image producing methods, theory (LP) 248, 427
lysine rich histones, neural cells (LM, P, PI) 9

maceral analysis, organic fossil matter (LM, PI) 45-G
macronucleus, differentiation; ciliates (LM, P) 15
malignant transformations, cells; ellipsometry 313
mammalian tissue; influence fixatives, stains (LM) 373, 379
materials, powdery, fibrous, fluffy (LM, P) 407-G
— science; applications of EM (LP) 256, 437
— —; — — LM (LP) 250, 432
membrane potential, bacteria; measurement (MG) 147
metal cations in fixative; influence stain (LM, PI) 381
— trace, tissues; surgical implants (LM, P, HC) 1
methyl methacrylate, embedding bone (LM) 19
— —, penetrating undecalcified bone (SEM) 181
methylene blue; retina stain (LM) 228
Micro Ion Mill AUTO MIM (TI) 65
microanalysis, X-ray; calibration (P) 361
—, —; see also microprobe

microfluorometry, microinterferometry; acrylic beads, concentration gradient (LM, PI) 394-G
—, —; immobilized enzyme, protein (LM, PI) 402-G
micrography; color films, color rendering 137-G
microincineration furnace; metal in tissue (LM, P, HC) 1
microorganisms, staining in soil (LM) 303
microphotometry; data collection, processing, organic fossil matter (LM, P) 45-G
—, scanning; cell image (LM, P) 203-G
microprobe methods; applications (LP) 70, 335
— —; equipment, preparation (LP) 69, 332
— nondestructive; ellipsometry, cell surface 313
microscopy (LP) 67, 231, 331, 411
—; general principles (LP) 231, 411
microspectrophotometry, quantitative; neural cells (LM, P) 9
mineralizing organic matter (LM, P, HC) 4
mordant for stain sequence (LM) 299
morphology, blood cell surface (SEM, P) 211
—, immature erythrocyte (LM, P) 98
morphometry (LP) 74, 345
muscle fibers; F-actin changes, glycerination (LM, P) 383
—, skeletal; culture, ion detection (MP, P) 361
mounting (LP) 239, 419
— media; refraction index (LM) 134
— sections flat (LM, EM) 20

Na ion; detectable concentration (MP) 365
— metasilicate; mineralizing organic matter (LM, P, HC) 4
nerve cell, cat; cytoplasmic laminar body (LM, P) 131
nervous, non-nervous components, Hydra (LM, P) 309
neurons, neuroglial cell; histone stain (LM, P, PI) 9
—, — —; ratio arginine/lysine, dayly rhythm (LM, P, PI) 13
new methylene blue; constitution, properties (LM, P) 96
night blue; Feulgen modification, mammalian tissue (LM) 373
Nissl stain; nucleus, laminar bodies (LM) 131
nitric acid: cuticle separation, epidermis (LM) 177
nuclear stain; Feulgen modification (LM) 373
nuclei, rat liver; Feulgen modification (LM, HC) 367
nucleolar stain; ethanolic phosphotungstic acid (LM) 27
Nomarski interference contrast; retinal tissue (P) 155
number of object per area; unbiased estimation (LM, ST) 107

object transfer function; diffraction image (LM) 265
on-line data collection; image analysis (LM, PI) 45-G
optical detection, cell surface changes (LM) 313
— methods, special (LP) 81
orange G; stain sequence, vertebrate tissue (LM) 229
organic matter; fluorescence, maceral analysis (LM, PI) 45-G
— —; mineralizing (LM, P, HC) 1
osmic acid; postfixation retinal tissue (LM) 155
— —; staining nervous components, Hydra (LM) 309
oxirane-acrylic beads; density, concentration gradients, enzymes (LM, PI) 393-G

Papanicolaou stain, comparison Feulgen; vaginal cytology (LM) 205-G
particle, refuse; incident light fluorescence (P) 407-G
— size, aerosol; device, calibration (LM) 119, 124
— sizing, counting (LP) 74, 345
patients, ill, healthy; blood cells (SEM, P) 209
peak/background ratio; ion detection (MP) 364
perfusion (LP) 234, 415
periodic bar target; object transfer function (LM) 265
pH value; Feulgen stain, Schiff-type dye (LM, HC) 367
phase contrast; nucleus, laminar body (P) 131
phosphomolybdic acid; mordant tissue, stain sequence (LM) 229
phosphoric acid; removal RNA, mammalian tissue (LM) 373
phosphotungstic acid; nucleolar stain, heterochromatin (EM) 27

photometry (LP) 77, 349
photomicrography, color; limit of color rendering (LM) 137-G
pinacyanol; modified Feulgen stain (LM, HC) 195
point counts, estimation numbers per area (ST) 110
— hologram, focussing laser microscope 40
polarized UV fluorescence microscopy; muscle fiber, F-actin changes (LM, P) 383
Pollachius virens (Pollock); retinal study (LM, P) 155
polychromasia, erythrocyte (LM, P) 89
polychrome blue stain, Unna; heterochromatin complex (LM) 27
polymerisation, methyl methacrylate; temperature course (SEM) 22
polystyrene sphere; calibrating aid, particle sizing (LM) 119
powders; incident light fluorescence (P) 407-G
preparation; equipment (LP) 234, 414
—; principal methods, laboratory technique (LP) 232, 412
probing, in vitro; nondestructive, ellipsometry 313
profile counts, unbiased (ST) 112
protein, bounded; determination (LM, PI) 393-G
— marking; rhodamine isothiocyanate, RITC (LM) 403-G
pyronin G/Y, substituted by acridine red; DNA, RNA (LM, PI) 193
— — methyl green stain; mammalian tissue; fixation influence (LM) 379

rabbit, muscle fibers; F-actin change, relaxation (LM, P) 383
—, new born; sex determination (LM, P) 289-G
radial concentration gradient, acrylic beads (LM, PI) 393-G
— density variation, calculation (LM, PI) 398-G
rat, intestine, liver, testis; Feulgen stain (LM, HC) 373
— liver, nuclei; Feulgen stain (LM, HC) 367
— —; trichloracetic acid-Schiff reagent (LM, HC) 275
— organs; stain sequence (LM) 299
refractive index, mounting media (LM) 134
refractometry (LP) 79, 351
refuse particles; mounting (LM) 407-G
Reichert Polyvar, wide field LM (TI) 65
relaxation, muscle fibers; F-actin change (LM, P) 383
—, zooid; ciliates (LM, P) 15
replicas (LP) 237, 417
resolution, high; limit, ultramicroscope 119
—, —; laser scanning microscope 31
reticulocyte; morphology, stains (LM) 89, 98
retinal tissue; long term fixation, stain, section (LM) 155, 227
rhodamine isothiocyanate (RITC); protein label (LM, PI) 403-G
RNA; fixation, stains (LM, HC, PI) 189, 373 379
— removal; phosphoric acid (LM, HC) 373
rubeanic acid; staining Co in tissue (LM, HC) 5

safranine; detection membrane potential (MG) 147
scanning microphotometry; cell image, vaginal cytology (LM, P) 201-G
Schiff-TCA solutions; spectral absorption (LM, HC, PI) 279
— -type dye; Feulgen stain (LM, HC, PI) 367
Secale cereale, sister chromatid exchange (LM, P) 159-G
section edge, influence estimating N_A (ST) 115
— flat mounting 19
sectioning (LP) 239, 419
— thick epon blocks (LM) 227
Sémichon acetic-carmine stain; ciliates (LM) 15
sex determination, rabbits, karyotype analysis (LM, P) 289-G
shelf-life, stains; increasing (LM) 275, 367
shell model; density gradient calculation, spheres (LM, PI) 398-G
silicone rubber coat; mounting powders (LM) 407-G
silver iodide dispersion; freeze-fracturing (SEM) 317
— stain; histone mixture; spectral curve (LM, PI) 9, 12
sister chromatid exchange; influences (LM, P) 160-G
— — —; Poisson distribution (LM, P) 162-G
skeletal muscle; culture, X-ray MP (SEM, P) 361
smears, vaginal; hormone diagnosis (LM, P) 281-G
soft ware, data collection, processing; maceral analysis (LM, PI) 45-G

soil, microorganisms; fluorescence stains (LM) 303
specificity, stains (LM) 9, 189
spectroscopy, spectrometry (LP) 83
spheres, acrylic; radial density, concentration (LM, PI) 303-G
— polystyrene; calibration aid, particle sizing (LM) 119
stain, acetic-carmine; ciliates, macronucleus (LM) 15
—, acridine red; substitute for pyronin G/Y (LM, PI) 193
—, batch variations; erythrocyte stains (LM) 96
—, citric acid-toluidine blue-O-SO_2 (LM, HC) 367
—, differential; heterochromatin, SCE (LM) 159-G
—, ethanolic phosphotungstic acid; heterochromatin (LM) 27
—, Feulgen compared with Papanicolaou (LM) 205-G
—, — modifications (LM, HC) 195, 275, 367, 373
—, Hoffmann's violet, dahlia; chromosomes (LM) 293
—, methyl green-pyronin: influence fixatives (LM) 379
—, night blue; mammalian tissue (LM) 373
—, Nissl; cytoplasmic body, nucleus (LM) 131
—, pinacyanol; Feulgen modified, DNA (LM, HC) 195
—, silver; histone mixture, specificity (LM, PI) 9
—, toluidine blue, methylene blue, azur II; retinal tissue (LM) 155, 228
—, Unna's polychrome blue; nucleolar stain, heterochromatin (LM) 27
staining; materials, chemicals, properties of dyestuffs (LP) 240, 412
— methods; metals in tissue (LM, HC) 4
— —; for LM & EM (LP) 240, 421
— —; — — — —, applications (LP) 243, 424
— —; reticulocyte, polychromatic, stippled erythrocytes (LM) 94, 99
stereology (LP) 74, 345
super 8-camera; time lapse in vitro study, living cells (LM, P) 217-G
surface morphology; blood cells (SEM, P) 211
tannic acid; Ti stain in tissue (LM, HC) 5
targets, periodic truncated; diffraction image (LM) 265
temperature course; polymerisation methyl methacrylate (P) 22
thick sections, epon blocks (LM) 227
thickness, measurement (LP) 79, 351
— plastic bead & matrix concentration (LM, PI) 394-G
Ti, staining in tissue (LM, HC) 1
time lapse device; living cells in vitro (LM) 217-G
tissue, mammalian; stains (LM, HC) 373, 379
—; metal traces; detection (LM, HC) 1
—, retinal; fishes (LM, P) 155, 227
—, vertebrate; staining sequence (LM) 299
toluidine blue stains (LM) 155, 228, 367
transfer function, optical; influence of coma (LM) 265
trichloracetic acid (TCA)-Schiff reagent; properties (LM, HC, PI) 275
tryptophanyls, location; muscle fibers (LM, PI) 383

ultramicroscope; particle analysis, resolution, calibration (LM) 119
Unna's polychrome blue stain; heterochromatin complexes (LM) 27
uridine, bromdesoxiuridine, fluordesoxiuridine; influence chromatid exchange (LM) 160-G

vaginal cytology, (LM, P) 201-G
— smears; sampling, hormone diagnosis (LM, P) 281-G
Verbenaceae, epidermis study (LM, P) 177
veronal-osmium oxide postfixation; retinal tissue (LM) 155
vertebrate tissue; sequence stain (LM) 299
video recording; time lapse in vitro study, living cells (LM, P) 220-G
vital stain; immature erythrocyte (LM) 98

washing (LP) 234, 415

X-ray methods (LP) 81
— — microanalysis; muscle skeletal, ions (SEM, P) 361

yeast cell dispersion; freeze-fracturing (SEM) 317

zooid relaxation; peritrich ciliate (LM, P) 15

MICROSCOPICA ACTA
VOL. 81 · NUMBER 1
September 1978
Pages 31—44

Key words: *Laser-scanning microscope — theory — possibilities of development — high resolution — high depth of field — point-hologram for focusing — evaluation of biological object damage by laser.*

Considerations on a laser-scanning-microscope with high resolution and depth of field

Christoph Cremer [1]) and Thomas Cremer

Institute of Human Genetics, University of Freiburg, Federal Republic of Germany

Received on 6 February 1978, english version on 6 April 1978

Summary

In conventional light microscopy, the depth of focus is severely limited. This limitation might be overcome by a light optical scanning procedure. In this procedure, the specimen surface is scanned point for point by a focused laser beam. The image of the specimen surface is generated by an electronic system, similar to the procedure used in the scanning electron microscope.

Possibilities to develop a "laser-scanning-microscope" on the basis of available techniques (laser microirradiation, miniprocessors, light detecting systems, automatic focusing, holographic focusing etc.) are discussed.

On account of its possibility to form images of high resolution and depth of focus, a laser-scanning-microscope might become a valuable tool in addition to conventional light microscopy and scanning electron microscopy.

Überlegungen zu einem Laser-Scanning-Mikroskop mit hoher Auflösung und Schärfentiefe

Die bei der herkömmlichen Lichtmikroskopie unvermeidliche, starke Beschränkung der Schärfentiefe könnte durch ein lichtoptisches Scanning-Verfahren überwunden werden, bei der die Objektoberfläche von einem fokussierten Laserstrahl punktweise „abgetastet" wird und das Bild der Objektoberfläche ähnlich wie beim Rasterelektronenmikroskop auf elektronischem Wege zusammengesetzt wird.

Der konstruktive Plan eines „Laser-Scanning-Mikroskopes" auf der Grundlage der heute gegebenen technischen Voraussetzungen (Lasermikrobestrahlung, Miniprozessoren, Lichtnachweissysteme, automatische Fokussierung, holographische Fokussierungsverfahren etc.) wird beschrieben und Probleme einer technischen Realisierung werden erörtert.

Aufgrund seiner besonderen Darstellungsmöglichkeiten könnte das Laser-Scanning-Mikroskop-Verfahren eine wertvolle Ergänzung herkömmlicher lichtmikroskopischer sowie rasterelektronenmikroskopischer Verfahren werden.

1. Introduction

One of the fundamental limitations of conventional light microscopy is due to the fact that with increasing magnification and numerical aperture the depth of focus becomes very low [10]; at high magnification and numerical aperture it is

[1]) Dipl.-Phys. Dr. Christoph Cremer, Institut für Humangenetik der Universität Freiburg, Albertstrasse 11, D-7800 Freiburg i. Br., Bundesrepublik Deutschland / Federal Republic of Germany.

only a fraction of a micron. This limitation results from the laws of geometrical and wave optics and therefore principally cannot be surmounted in conventional light microscopy. If it is desired to obtain an image of a specimen surface at a magnification corresponding to a high numerical aperture in light microscopy, but with high depth of focus, nowadays a scanning electron microscope is used. In this case, however, an observation of living biological objects usually is not possible. Furthermore, the light microscopical image of an object contains important informations, which are only difficult or even not obtainable by using the scanning electron microscope.

Our contribution deals with the problem whether the severe limitation of the depth of focus which is inevitable in conventional light microscope, might be removed by a light optical scanning procedure in which the image of the object is formed point by point whereas in conventional light microscopy all points of the image are formed simultaneously.

The present state of laser technique and of electronics suggests that the realization of such a laser scanning microscope might be feasible. Recently, scanning methods have been used to obtain images with high resolution (1 μm) of biological objects by means of focused acoustic waves ("scanning acoustic microscope") [2, 19].

The laser-scanning-microscope-method proposed here is intended to solve two problems:

1) A high resolution image of a specimen surface characterized by a high degree of unevenness has to be formed, the unevenness being too large to allow the forming of a sharp image of the whole specimen surface by conventional light microscopy.

To save trouble, in the following only objects with a fluorescent surface are considered. Furthermore, it is assumed that the fluorescence of the surface differs in at least one wavelength λ_{Fl} from the fluorescence emitted by the interior of the object. This assumption does not restrict very much the class of objects which are accessible to an imaging in the laser-scanning-microscope, because in objects without a sufficient specific natural fluorescence in many cases fluorochromes may be attached to the surface. E. g., in biological objects fluorochrome-conjugated antibodies may be used [20].

2) Simultaneously with the imaging of the topographical details of the specimen surface (problem 1), the fluorescence distribution on the surface is to be recorded. In addition to an imaging of the surface, this gives light optical informations which are in principle difficult or even not obtainable with scanning electron microscopy.

2. Elements of a laser-scanning-microscope

Fig. 1 shows the basic design of a laser-scanning-microscope. A laser system (1) emits coherent light of appropriate wavelength(s) λ_E [22] with small beam divergence [12]. The wavelength λ_E has to be selected in such a way, that at least at a given wavelength λ_{Fl} only the specimen surface contributes considerably to the fluorescence emission.

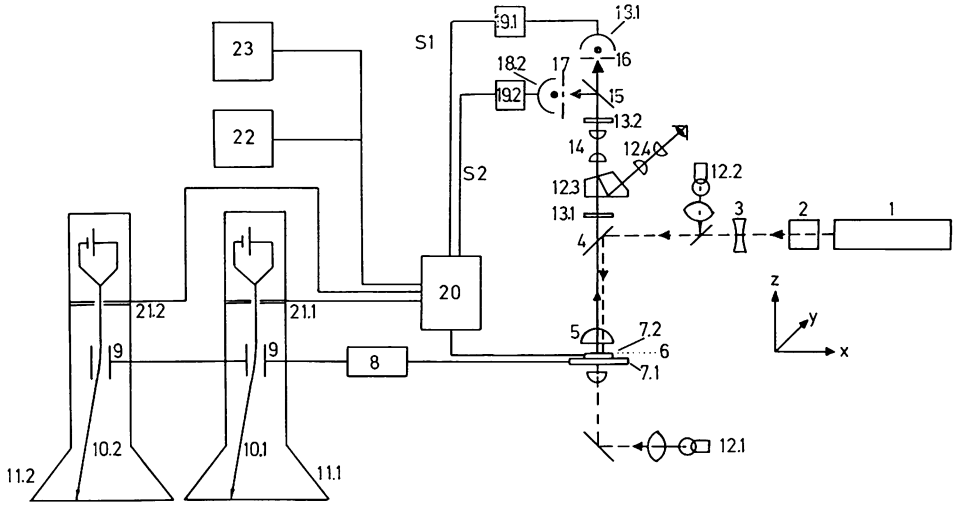

Fig. 1: Schematic design of a laser-scanning-microscope.
1) laser system (continuous wave laser); **2**) electro-optic modulator; **3**) adjusting lens; **4**) selecting mirror; **5**) microscope objective for focusing of the laser beam. The objective is simultaneously used for observation and collection of the fluorescent light; **6**) object plane;
7) scanning stage:
7.1: mechanism for horizontal displacement (X, Y) of the object,
7.2: mechanism for vertical displacement (Z) of the object;
8) scan generator; **9**) deflecting systems;
10) recording beams:
10.1: recording beam for topographical display of the specimen surface,
10.2: recording beam for display of the fluorescence distribution;
11) TV-screens:
11.1: TV-screen for topographical display of the specimen surface,
11.2: TV-screen for display of the fluorescence distribution;
12) optical system for visual observation:
12.1: system for transmitted light illumination,
12.2: system for incident light illumination,
12.3: beam splitter,
12.4: eye piece;
13) filters:
13.1: barrier filter for elimination of the exciting laser light (wavelength λ_E),
13.2: narrow band filter for selection of the fluorescence light (wavelength λ_{FI});
14) magnifying system; **15**) beam splitter (50%); **16**) circular measuring diaphragm (image plane B1); **17**) annular measuring diaphragm (image plane B2);
18) photomultipliers:
18.1: photomultiplier for measuring the luminous flux $\Phi 1$ in the circular measuring diaphragm **16** (signal S1),
18.2: photomultiplier for measuring the luminous flux $\Phi 2$ in the annular diaphragm **17** (signal S2);
19) amplifier circuits:
19.1: amplifier for photomultiplier **18.1**,
19.2: amplifier for photomultiplier **18.2**;
20) electronics of the Automatic Focusing System;
21) intensity control for recording beams:
21.1: control for beam **10.1**,
21.2: control for beam **10.2**;
22) facility for the twodimensional numerical display of the specimen surface and of the fluorescence distribution, respectively (facultative); **23**) facility for the threedimensional display of the specimen surface and of the fluorescence distribution, respectively (facultative).

By means of gas lasers presently available it is possible to obtain a large number of discrete laser lines in the ultraviolet and visible spectral region. Dye lasers allow it to obtain coherent light of any wavelength in the whole visible and near ultraviolet region. For reasons of power stability, it is advisable to use a continuous wave laser system [12], the beam intensity being controlled externally with an electro-optic modulator (2).

The coherent beam of wavelength λ_E enters into the scanning microscope and passes an adjusting lens (3). Then the beam is reflected by a selecting mirror (4) in such a way that it becomes collinear with the optical axis (Z) of the microscope. The selecting mirror (4) reflects the excitation light (wavelength λ_E) maximally while it transmits maximally the emitted fluorescent light (wavelength λ_{Fl}). A microscope objective (5) of high numerical aperture focuses the laser beam into the object plane (6). Using a laser beam of small divergence and an appropriate adjustment of the adjusting lens (3), it is possible to obtain in the object plane an almost diffraction limited focus.

The optical arrangement described here is used in a number of laser microbeams [4, 5, 12]. E. g. using coherent ultraviolet light of 257.3 nm we succeeded in limiting the object field irradiated to an area with a diameter of 0.4 μm [12]. In this case, an objective 100 : 1/1.25 was used. Using an objective 32 : 1/0.4, the minimum diameter of the irradiated area was approx. 1 μm [25].

The object is placed on a special scanning stage (7) (see below). The horizontal movement [(X,Y)-direction] (7.1) is coupled to the deflecting systems of two recording beams (10.1) and (10.2) of two TV-screens in connection with a scan generator (8). In addition to the scanning stages presently used [displacement in (X,Y)-direction only] there exists a mechanism (7.2) which allows it to displace the object also very rapidly in direction of the optical axis (Z) of the microscope.

The fluorescence excited by the laser microirradiation in the specimen surface is collected by the objective (5). A barrier filter I (13.1) eliminates the rest of the excitation light (wavelength λ_E). A portion of the fluorescence light is used for visual observation by the help of a beam splitter (12.3). The visual observation is made through the eye piece (12.4). To diminish fading, the visual observation can be also done using transmitted light (12.1) or incident light illumination (12.2) with other wavelengths than λ_E.

A narrow-band filter II (13.2) transmits only light of wavelength λ_{Fl}. Having passed a magnifying lens system, the fluorescence light is splitted by a 50% beam splitter (15). The image planes of the lens system (14) are B1 and B2, respectively. In the plane B1 a *circular* measuring diaphragm (16) is located the diameter of which is equivalent to the diameter (in the B1-plane) of the principal maximum of the diffraction pattern of a self-luminous point source situated in the object plane (luminous flux $\Phi 1$). In the plane B2 an *annular* measuring diaphragm (17) is located in such a way that only the luminous flux $\Phi 2$ passes which corresponds to the first secondary maximum of the diffraction pattern of the self-luminous point source. Having passed the measuring diaphragms (16) and (17), the fluorescence light enters into the photomultipliers (18.1) and (18.2), respectively. The signals S1 and S2 resulting after amplification units (19.1) and (19.2) are processed in the electronics (20) of the "Automatic Focusing System" (AFS).

The basic functions of the Automatic Focusing System (AFS) are:

1) By way of the signals S1 and S2 the Z-coordinate $Z_0(X,Y)$ is determined which corresponds to the exact focusing of the laser beam on the specimen surface at a given (X,Y)-site. Then the AFS generates a signal Σ by which the intensity of the recording beam (10.1) is controlled proportional to $Z_0(X,Y)—Z_M$, Z_M being a fixed intermediate position. If this procedure is performed for all coordinates (X,Y) of the specimen surface, then on the TV-screen (11.1) an image of the specimen surface is generated which is formed by different gray values. For visual observation, the display has a long-persistence phosphor coating; a higher resolution may be obtained with a short-persistence phosphor coating and photographic recording. The image contrast is generated only by electronic means on the basis of the Z_0-values determined by measurements of S1(Z) and S2(Z).

This procedure allows an optimal adaptation of the image contrast to the special requirements of the object.

2) If the laserbeam is focused exactly on the specimen surface, then the signal $S1(Z_0)$ produced by the aid of the photomultiplier (18.1) corresponds to the fluorescence signal in conventional microfluorometry [20] of objects with an even surface.

After determination of Z_0, the AFS has to perform simultaneously the following procedures:

(i) to control the intensity regulation (21.1) of the recording beam (10.1) proportional to $Z_0 - Z_M$;

(ii) to control the intensity regulation (21.2) of the recording beam (10.2) synchronized with (10.1) proportional to $S1(Z_0)$.

By this procedure the distribution of fluorescence on the specimen surface is shown on the TV-screen (11.2).

An alternative to the use of two seperated TV-screens to display specimen surface and fluorescence distribution is the use of a colour-TV-monitor, on which specimen surface and fluorescence distribution are shown in different colours.

A numerical representation of the specimen surface and the fluorescence distribution, respectively, may be obtained by means of the numerical display (22).

Furthermore, in connection with a computer system, it is possible to realize on a TV-screen a perspective image of the specimen surface under various optical angles (23).

3. Considerations on a technical realization

3.1. The "Automatic Focusing System"

As pointed out above, it is necessary to determine for each (X,Y)-site:

(i) the Z-coordinate $Z_0(X,Y)$ at which the laser microbeam is focused exactly on the surface;

(ii) the fluorescence signal S1 corresponding to the (X,Y,Z_0)-site.

This problem might be solved by means of an "Automatic Focusing System": In addition to the displacement of the object in (X,Y)-direction, the object is moved in Z-direction in a continuous mode (sinusoidal vibration). The period of

this oscillation has to be short compared to the duration of the displacement in (X,Y)-direction from one point to the next: the amplitude has to be larger than the height of the maximum elevation of the specimen surface. At a given (X,Y)-site, for each Z-value the signals S1(Z) and S2(Z) are registered and stored in the electronics (20) of the AFS. It is assumed that S1(Z) and S2(Z) are proportional to the luminous fluxes Φ_1 and Φ_2 entering into the photomultipliers (18.1) and (18.2), respectively.

From optical considerations it follows that the quotient $\Phi 1/\Phi 2 = S1/S2$ is a maximum if the laser microbeam is focused exactly on the specimen surface: If $Z = Z_0$, the photomultiplier (18.1) registers the luminous flux $\Phi 1$ corresponding to the principal maximum of the diffraction pattern of the image of a small fluorescent spot induced by the laser microirradiation in the specimen surface. The photomultiplier (18.2) registers the luminous flux $\Phi 2$ corresponding to the first secondary maximum. Under favourable conditions, $\Phi 2$ is only a small percentage of $\Phi 1$. For $Z = Z_0$ this results in a high contrast S1/S2. For $Z \neq Z_0$ (i. e. if the laser beam is defocused) the contrast is smaller.

From all S1/S2-values obtained at a given (X,Y)-site, the electronics (20) selects the maximum value max. S1/S2 and determines the corresponding Z-value Z_0. Simultaneously, the S1-value corresponding to max. S1/S2 is determined. Both informations are used in the way described in section 2 and represent the elevation and the fluorescence intensity, respectively, of the given (X,Y)-site. This procedure is performed for all (X,Y)-coordinates of the surface and eventually results in an image of the specimen surface with high depth of focus and of the fluorescence distribution on the surface, respectively.

In principle, also other ways of automatic focusing are conceivable. E. g., instead of the object itself one could displace the adjusting lens [(3), Fig. 1], in order to change the relative position of the laser focus to the specimen surface. In this case however, the displacements of the adjusting lens necessary are much larger than if the object itself is moved. In consequence, mechanical difficulties occur because the displacement has to be performed very rapidly. Therefore we feel that a vertical displacement of the object itself would be considerably easier to realize.

We would like to point out that a determination of Z_0 only by means of the maximum of the luminous flux $\Phi 1$, using objects with large differences in the elevations of the surface, is only possible, if the differences in absorption and fluorescence efficiency at neighbouring sites of the specimen surface are small.

3.2. Electronic and mechanic requirements

It is decisive for the practical utility of the proposed scanning procedure, that the determination of $Z_0(X,Y)$ and $S1(X,Y,Z_0)$ and the horizontal displacement of the scanning stage [(7.1), Fig. 1] is performed so rapidly that the object may be scanned in a sufficient short time. The requirements to be satisfied are illustrated in the following example: The surface of an object with a 100 μm × 100 μm lateral extension and maximum differences in elevation of the surface of 10 μm has to be displayed with a resolution of approx. 0.5 μm. Then a focus diameter of 0.5 μm is required. The step length in (X,Y)-direction has to be 0.5 μm, too.

In Z-direction, for each (X,Y)-site 20 measurements of S1 and S2, respectively, have to be made. $Z_0(X,Y)$ and $S1(X,Y,Z_0)$ have to be determined for a total of 4×10^4 (X,Y)-sites and to be transferred to the TV-screens.

If an image of the specimen surface has to be formed within 10 seconds, the average velocity of the scanning stage has to be 2 mm/s. Compared to the velocities of scanning stages presently available for light microscopy, this velocity is very high. It may be attained, however, by means of a continuous vibratory motion. In this manner, a scanning velocity of 5×10^4 measuring points within only one second has been realized in a scanning acoustic microscope [19].

The object itself may be located in a special observation chamber [13] under defined ambient conditions, allowing the use of an objective with a small working distance (high numerical aperture). The chamber may be mounted on piezoelectric elements which allow a rapid displacement in Z-direction. In order to obtain stereopairs, such a chamber may be tilted between successive exposures.

To determine $Z_0(X,Y)$ and $S1(X,Y,Z_0)$, to transfer these signals to the TV-screens, and to adjust the next (X,Y)-point a time of 250 μs is available, if the total scanning time is 10 seconds.

If the scanning stage is moved continuously, the time for determination of Z_0 and $S1(Z_0)$ has to be so short (e. g. 25 μs) that during this time the displacement of the object in (X,Y)-direction is negligibly small. If during this time 20 signals S1, S2 as a function of Z have to be recorded, one signal S1 and S2 respectively, has to be recorded within approx. 1 μs. To realize this requirement, the following procedure is proposed: The vibration of the piezoelectric crystal effecting the Z-displacement of the object is controlled by an alternating voltage with a frequency of 20 kc which generates a sinusoidal Z-displacement of the object with the same frequency and an amplitude of 6 μm measured from an intermediate value Z_M. If sensitive objects have to be scanned, the frequency has to be reduced, if necessary, to avoid a damage due to the acceleration forces generated by the vibration. In this case, of course, the duration of the horizontal scan has to be prolonged, too.

The twenty S1 and twenty S2-values measured during a half oscillation period (25 μs) are recorded (see above). To register and to store these signals, a time of 1 μs is available for each S1/S2 pair. The present state of electronics allows to realize this requirement provided that the irradiation power density at the specimen surface is high enough.

Following the transfer of the signal Σ (corresponding to Z_0-Z_M) to the TV-screen [(11.1), Fig. 1] and of the signal $S1(Z_0)$ to the screen [(11.2), Fig. 1], the data (20×Z, 20×S1, 20×S1/S2), from which the both signals Σ and $S1(Z_0)$ have been determined, may be erased. Therefore, only a small storage capacity is required in the AFS.

3.3. Requirements concerning the light source

The irradiation power density necessary depends on the energy absorbed per second, the quantum efficiency of fluorescence and the sensitivity of the photomultiplier systems. It is assumed that within a measuring time of 1 μs at least 10^3 quanta have to be received by the photomultiplier [(18.1), Fig. 1] to produce a sufficient high signal.

N_0 incident quanta per second yield $N_A = \alpha N_0$ absorbed quanta per second. These N_A quanta induce the emission of $N_F = \beta N_A = \alpha\beta N_0$ quanta per second having the wavelength λ_{Fl}. Furthermore, absorption, reflection and beam splitting in the microscope have to be considered. If the total transmission of the system between specimen surface and photomultiplier is assumed to be 10%, the condition $N_0 \geqq \frac{1}{\alpha\beta} 10^{10}$ (s^{-1}) has to be satisfied. E. g., for $\alpha = \beta = 10^{-2}$ the condition $N_0 \geqq 10^{14}$ quanta per second follows.

If light in the ultraviolet and visible spectral region is used, $N_0 = 10^{14}$ quanta per second are equivalent to a power of several 10^{-5} Watt in the focus. Assuming a spot diameter of 0.5 μm, the incident power density lies in the order of magnitude of 10^4 Watt/cm².

The typical power of continuous wave lasers with emission in the ultraviolet and visible region lies in the range of 1 mW to several Watt. It is possible to focus the greater part of this power to a spot with a diameter in the range of a wavelength [3, 21]. From this it follows that the focal power densities obtainable are as high as $10^5 - 10^8$ Watt/cm². Even if more unfavourable conditions are assumed than in the example given above, a sufficient high power density can be provided. Much higher power densities are available by means of pulsed laser sources [5, 24]. They have, however, the disadvantage that at high power the pulse repetition rate is relatively low (up to several kc [24]). Furthermore, it is difficult to realize the high power stability necessary.

Similar problems occur if incoherent flash lamps [9] are used. On the other hand, using incoherent light sources with continuous emission [9], in general the power densities required are not available.

3.4. The problem of damage induced by the scanning procedure in biological objects

The power densities of $10^5 - 10^8$ Watt/cm² which are available by using continuous wave lasers are far below the threshold for dielectric breakdown and frequency doubling [4].

Severe damage in the object by "microexplosions" as it is observed if pulsed laser sources of high power are used [15, 16], may be avoided. Therefore, in our discussion concerning the damage of the object by the scanning beam we shall consider only thermal and photochemical effects.

At first the temperature rise to be expected is estimated. It is assumed, that

a) the object is irradiated continuously,
b) the energy absorbed per second, Pabs, is uniformly distributed in the whole object and completely transformed into heat,
c) the object is cylindrical with height H and radius R,
d) dissipation of heat occurs only perpendicular to the cylinder axis, which is coaxial with the optical axis of the laser beam.

Then in the equilibrium, i. e. after a long irradiation time, the following temperature distribution may be calculated [13]:

$$T(r) = T(R) + \frac{\text{Pabs}}{4\pi k R^2 H}(R^2 - r^2) \quad \text{for } r \leqq R$$

$$T(r) = T(R) + \frac{\text{Pabs}}{2\pi k H} \ln(R/r) \quad \text{for } r \geqq R$$

(T = temperature, r = distance from the cylinder axis).

The heat conductivity k is assumed to be equal in object and ambient medium. If the diameter of the object is 100 μm and the height is 10 μm and assuming the heat conductivity of water, a temperature rise, [T(0) = T(1 mm)]

$$\Delta T = 7 \times 10^{-3} \times \text{Pabs}\ (°\text{C}), \qquad \text{Pabs in erg/s},$$

is calculated. In the derivation of this formula it is assumed that the temperature rise for $r = 1$ mm is negligibly small. If an incident power density of 10^4 Watt/cm² in a focus of 0.5 μm diameter and an absorption of 1% (see section 3.3) is assumed, the temperature rise of the whole object is 1.4×10^{-2} °C.

Of course, the temperature rise of the whole object says little about the local temperature rise induced at a given (X,Y)-site by the laser microirradiation. The present knowledge is small concerning the temperature distribution at the irradiation site in case of a short time impact of light with high power density [5]. A first estimate, however, may be obtained from measurements [14] of the temperature pattern of a focused ruby laser beam ($\lambda = 694.3$ nm). If it is assumed that the temperature distribution obtained in these experiments using pulse durations of 2.2×10^3 μs:

(i) is valid also for the shorter duration of irradiation [approx. 25 μs at a given (X,Y)-site] in the laser-scanning-microscope, at least concerning the order of magnitude;
(ii) is approximatively proportional to the absorbed energy,

then for the example considered above a local temperature rise of only 10^{-4} °C per measuring site compared to the given ambient temperature follows. Even if the actual local temperature rise induced should be higher by several orders of magnitude, this would not result in a damage in biological objects. Concerning this point, however, further systematic investigations have to be done.

At an incident power density of 10^4 Watt/cm² in the focus (diameter 0.5 μm) the incident energy per second is 200 ergs. The irradiation dose can be diminished to 20 erg/s if the irradiation is not continuously performed, but only during the measuring times of 25 μs per (X,Y)-site. The rapid switching on and off of the irradiation required in this case may be realized by means of an electro-optical modulator [(2), Fig. 1]. A scanning of living objects with the irradiation power and the velocity indicated should be possible, if there is no enrichment of target molecules absorbing very strongly the exciting laser light, and if there is no induction of strongly damaging photochemical processes. E. g., the microirradiation of mammalian cells with ultraviolet light ($\lambda = 257$ nm) induces cell death at much lower doses than assumed in our example [13]. On the other hand, using light in the ultraviolet region it may be expected that a sufficient fluorescence is induced at much lower irradiation power densities. Besides the selection of the most appropriate wavelength in the case to be investigated (perhaps in combination with vital

dyes), a very sensitive light detecting system for $\Phi 1$ and $\Phi 2$ (Fig. 1) is a prerequisite for a nondestructive scanning of a living object. One should consider the possibility to use photon-counting light detecting systems. For the scanning of nonliving objects, in many cases a higher power density may be used, so that the use of expensive photon-counting systems is not necessary.

3.5. The resolving power of the laser-scanning-microscope

In principle, the resolving power of the proposed laser-scanning-microscope is given by the spot diameter of the laser microbeam: The fluorescence of two object sites can be measured separately from each other if the sites can be separately "illuminated" by the laser focus. In practice, spot diameters as small as 0.5 μm have been obtained by laser microirradiation in the ultraviolet and visible region, using objectives of high numerical aperture. In principle, this corresponds to a resolving power of 0.5 μm in the laser-scanning-microscope. Using plane aplanatic optical systems, a focusing to a diameter of less than approx. $\lambda/2$ is not possible for physical reasons [1]. The working distance of microscope objectives of high numerical aperture (N.A $\geqq 1$) is small and usually lies in the range of several hundred micrometers at maximum. If a much larger working distance is required by the special features of the object, objectives of larger working distance but smaller resolving power have to be used. A possibility to avoid this difficulty in the laser-scanning-microscope is offered by the use of a point-hologram [17] instead of the microscope objective [(5), Fig. 1]. By means of point-holograms, a focusing to a spot diameter of approx. $\lambda/2$ has been realized [17]. Since it is possible to choose the distance of the focus from the hologram and the diameter of the hologram independently from each other, a much larger working distance can be achieved without reducing resolving power than it is possible with lens objectives of high numerical aperture. With regard to a use of point-holograms to focus coherent light, two interesting points may be mentioned:

(i) it is not necessary that the Abbe-condition [7, 8] is fulfilled in point-holograms,
(ii) if non-plane holograms are used, a considerably larger spherical angle may be used for focusing than in plane optical systems.

In this case, the conventional theory of the focusing of light by plane aplanatic optical systems [1] seems not applicable without modification. The theoretical and practical consequences of holographic focusing [17] with respect to the resolving power of a laser-scanning-microscope are difficult to predict. Some remarks on this problem will be given in the appendix.

Appendix

Some remarks on the holographic focusing of coherent light

The propagation and diffraction of light is described by Maxwell's electromagnetic theory. For homogeneous isotropic dielectric media the principal solutions are given by periodic electromagnetic fields which fulfill the Sommerfeld condition [23]. Then, according to the theory of partial differential equations [18], the following problem has only one solution: Consider a region G in the three-dimensional space. On the closed envelope F of G the electric ($\vec{E}$) and magnetic ($\vec{H}$)

field strength is known for each point of F. $\vec{E}$ and $\vec{H}$ are continually differentiable functions and represent in G solutions of the wave equation. Then the original electromagnetic field in G can be reconstructed by a superposition of monochromatic waves of a fixed frequency ω. which are propagated from each element of the closed envelope F. This principal possibility of a single-valued exact reconstruction of an electromagnetic field is a consequence of the time invariance of the homogeneous Maxwell equations. A consideration of the quantized Maxwell field yields analogous results [6].

Light sources with a diameter of $0 < r \leqq \lambda$ (decay of the intensity to a fraction $1/e$ in a distance r from the geometrical centre of the light source) are physically possible. The electromagnetic fields produced by these light sources obey the above mentioned conditions. Then the exact reconstruction of such a field by superposition of monochromatic waves of a fixed frequency, but with different incident angles should also be a physically possible process. In principle, this process requires the superposition of light waves with all incident angles possible in the three-dimensional space (closed envelope). It seems conceivable to realize this requirement by means of a holographic procedure [8, 17]. According to the above mentioned principles, a hologram may be regarded as the boundary condition the fulfillment of which yields the reconstructed waves.

At first, we consider the more simple case of a plane quadratic hologram with a lateral length D. It is assumed that the hologram has no image defects as spherical aberration, coma, astigmatism [17]. Then the lateral diameter of an image spot formed in the middle axis of the hologram in the distance b of the hologram is given [17] by

$$d \approx 0.89 \times \frac{b}{D} \lambda \tag{1}$$

λ being the wavelength of the reconstructed wave. Since it is possible to chose b and D independently from each other, the spot diameter as far as it is limited only by the diffraction at the edge of the hologram may be made smaller than the values obtainable in good lens systems [17]. According to formula (1) it would be possible to produce by means of such a point-hologram a spot with a diameter which would be considerably smaller than the wavelength used. We do not know, however, how far formula (1), which has been derived from scalar wave theory, describes the actual spot diameter appropriately at least in a qualitative respect for values $b/D \ll 1$, because using very high numerical apertures, considerable deviations are found between the scalar and the electromagnetic treatment [1]. But even if a calculation by means of Maxwell's theory should show that it is not possible to focus coherent light to a diameter $d \ll \lambda$ using plane holograms, it seems conceivable that this aim might be achieved by means of a so-called "4π-point-hologram" [11]. This is defined as a point-hologram which is characterized by the postulate that it forms an envelope closed as far as possible.

It is beyond the scope of this appendix to discuss further details concerning form and size of such a 4π-point-hologram, its production, material problems, or direction, polarization, amplitudes and coherence of the incident and the reconstructed waves, respectively. The schematic design in Fig. 2a is only intended to indicate some points:

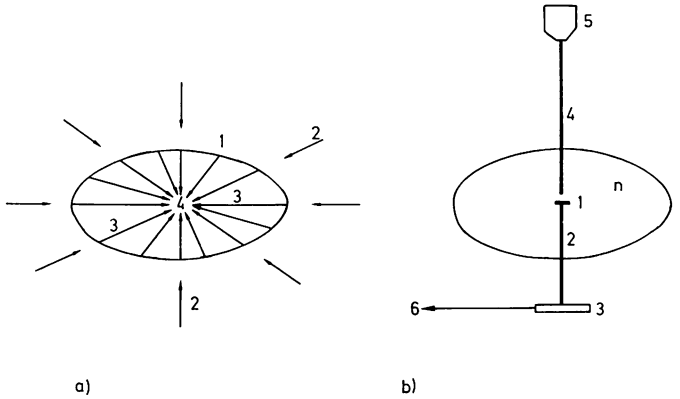

Fig. 2: Focusing by "4π-point-holograms".

a) focusing by means of a 4π-point-hologram (principle):
1) surface of the 4π-point-hologram (ideally a closed envelope); **2)** incident waves; **3)** reconstructed waves; **4)** "focus" of the 4π-point-hologram.

b) application of a 4π-point-hologram in a laser-scanning-microscope:
1) specimen with a fluorescent surface located in the "focus" of a 4π-point-hologram; **2)** holding device (same refractive index n as the specimen and the immersion fluid); **3)** scanning stage to displace the specimen; **4)** light conductor; **5)** microscope objective for the collecting of the fluorescence light; **6)** connection to the scan generator and to the Automated Focusing System (see Fig. 1).
n: refractive index of the immersion fluid.

1) The coherent light used for the reconstruction of the hologram, i. e. for focusing, has to be of an appropriate amplitude distribution, and ideally is to be propagated from all directions (total spherical angle 4π). Since Abbe's condition [7, 8] has not to be fulfilled, the amplitudes and the incident angles of the coherent waves falling on the 4π-point-hologram can be varied almost independently from each other, if the same wave configuration has been used before in the production of the hologram or if the hologram has been produced for this wave configuration by synthetic methods.

2) The usable dimensions of the 4π-point-hologram have to be large. This implicates a high coherence of the incident waves.

3) Edges and strong curvatures have to be avoided as far as possible in order to reduce the influence of diffraction waves [23] occurring at these sites. To what extent this aim can be realized, remains to be investigated.

4) Possibly, it might be favourable, in analogy to formula (1) derived for a quadratic hologram, to choose also in the 4π-point-hologram a small ratio of the focal length to the diameter of the hologram. For geometrical reasons, this is possible for one focal plane only. It would be already advantageous, however, for the application envisaged, if in this way only one focal diameter could be reduced to a minimum.

Fig. 2b shows the principal design of a laser-scanning-microscope with a 4π-point-hologram in place of a conventional microscope objective. The object (1) with a fluorescent surface is located in the centre of the 4π-point-hologram. The

diameter of the hologram is assumed to be large compared with the dimensions of the object. The object is placed on a holding device (2), the refractive index n of which is equal to that of the object itself. The interior of the hologram is filled with an immersion fluid of refractive index n. A small hole in the 4π-point-hologram serves to connect the holding device (2) to a scanning stage (3). The fluorescent light emitted by the object is collected by a light conductor (4) which is connected to a microscope objective (5). The light signal is processed in the same way as in the laser-scanning-microscope designed in Fig. 1. It is of importance that the geometry of the light conductor is chosen in such a way that the part of the specimen surface which contributes to the luminous flux collected by the light conductor is as small as possible. It is assumed that by means of the holographic focusing the excitation light is focused on an area of the surface with a diameter which is considerably smaller than the wavelength used, and that the absorbance by the holding device and the object is small. Since the excitation light is incident from all directions, also the object surface outside the focus is excited to fluoresce. This background fluorescence is estimated to be small compared with the signal, if the thickness of the surface layer contributing to the fluorescence is considerably smaller than the "focus diameter".

The use of plane point-holograms for focusing the exciting beam in the laser-scanning-microscope seems to be realizable without difficulties and suggests to be advantageous at least with respect to the much larger working distance available compared with conventional microscope objectives of high numerical aperture. Whether it might be possible to produce point-holograms in which a larger spherical angle than approx. 2π is used for focusing, and whether such holograms might be really used in a laser-scanning-microscope, remains to be investigated. Perhaps the use of non-plane point-holograms might result in a better focusing even if the spherical angle is considerably smaller than 4π.

The above-said speculations on focusing by "4π-point-holograms" are only intended to allude to a method which perhaps might be used to enhance the resolving power of a laser-scanning-microscope by means of holographic focusing. To our knowledge, a detailed investigation of the principal theoretical and practical limitations of holographic focusing with non-plane point-holograms does not exist. We feel this to be an interesting subject, independently of the field of application envisaged here.

Acknowledgement

We wish to thank Dr. J. S. Ploem, University of Leiden (The Netherlands), for valuable discussion.

References

[1] Barakat, R.: *The intensity distribution and total illumination of aberration-free diffraction images.* In: E. Wolf (Edit.): *Progress in Optics,* Vol. I, pp. 67—108. North-Holland Publ. Co., Amsterdam 1965.

[2] Bennett, S. D.: *The scanning acoustic microscope.* In: G. A. Hay (Edit.): *Medical Images: Formation, Perception and Measurement,* pp. 122—135. (The Institute of Physics.) John Wiley & Sons, Chichester-New York 1976.

[3] Bereiter-Hahn, J.: *Laser als Mikromanipulator in Biologie und Medizin: 1. Grundlagen der Anwendung von Laserlicht zur Mikrobestrahlung biologischer Objekte.* Microsc. Acta **71,** 225—241 (1972).
[4] Bereiter-Hahn, J.: *Laser als Mikromanipulator in Biologie und Medizin: 2. Methodik und Anwendung der Laser-Mikrobestrahlung biologischer Objekte.* Microsc. Acta **72,** 1—33 (1972).
[5] Berns, M. W.: *Biological Microirradiation. Classical and Laser Sources.* Prentice-Hall, Inc., Englewood Cliffs, N.J. 1974.
[6] Bjorken, J. D., und S. D. Drell: *Relativistische Quantenfeldtheorie.* Bibliographisches Institut, Mannheim 1967.
[7] Born, M.: *Optik. Ein Lehrbuch der elektromagnetischen Lichttheorie,* 2. Aufl. Springer-Verlag, Berlin-Heidelberg-New York 1965.
[8] Born, M., and E. Wolf: *Principles of Optics. Electromagnetic Theory of Propagation, Interference and Diffraction of Light.* 4th Edition. Pergamon Press, Oxford-London 1970.
[9] Carlson, F. E., and C. N. Clark: *Light sources for optical devices.* In: R. Kingslake (Edit.): *Applied Optics and Optical Engineering,* Vol. I, pp. 43—109. Academic Press, New York and London 1965.
[10] Claussen, H. C.: *Mikroskope.* In: S. Flügge (Hrsg.): *Handbuch der Physik,* Bd. XXIX *(Optische Instrumente),* pp. 343—425. Springer-Verlag, Berlin-Heidelberg-New York 1967.
[11] Cremer, C., und T. Cremer: *4π-Punkthologramme: Physikalische Grundlagen und mögliche Anwendungen.* In: Patentschrift P 21 16 521.9 *(„Verfahren zur linsenfreien Erzeugung von Kugelwellen",* Anm. C. Cremer und T. Cremer). Deutsches Patentamt, Dienststelle Berlin, Patentschriftenvertriebsstelle 1971.
[12] Cremer, C., C. Zorn, and T. Cremer: *An ultraviolet laser microbeam for 257 nm.* Microsc. Acta **75,** 331—337 (1974).
[13] Cremer, C., T. Cremer, C. Zorn, and L. Schoeller: *Effects of laser uv-microirradiation (λ = 2573 Å) on proliferation of Chinese hamster cells.* Radiat. Res. **66,** 106—121 (1976).
[14] Daniel, J. C. jr., and K. Takahashi: *Selective laser destruction of rabbit blastomeres and continued cleavage of survivors in vitro.* Exp. Cell Res. **39,** 475—482 (1965).
[15] Egner, O., und J. Bereiter-Hahn: *Laser-Strahlenstichversuche an Fisch-Melanophoren.* Z. wiss. Mikrosk. **70,** 17—22 (1970).
[16] Hillenkamp, F., R. Kaufmann und E. Remy: *Der Laser als Instrument der Zellforschung im Mikro- und Submikrobereich.* LASER **3** (4), 40—42 (1971).
[17] Kiemle, H., und D. Röss: *Einführung in die Technik der Holographie.* Akademische Verlagsges., Frankfurt a. Main 1969.
[18] Leis, R.: *Vorlesungen über partielle Differentialgleichungen zweiter Ordnung.* Bibliographisches Institut, Mannheim 1967.
[19] Lemons, R. A., and C. F. Quate: *Acoustic microscopy: biomedical applications.* Science **188,** 905—911 (1975).
[20] Ploem, J. S.: *Quantitative fluorescence microscopy.* In: G. A. Meek, and H. Y. Elder (Edits.): *Analytical and Quantitative Methods in Microscopy,* pp. 55—89. Cambridge University Press, Cambridge 1977.
[21] Rempel, R. C.: *Optical properties of lasers as compared to conventional radiators.* Spectra-Physics Laser Technical Bulletin **1,** 1—9 (1963).
[22] Rounds, D. E.: *Laser applications to biology and medicine.* In: *Laser Handbook,* Vol. II, pp. 1863—1890. North Holland Publ. Co., Amsterdam 1972.
[23] Rubinowicz, A.: *Die Beugungswelle in der Kirchhoffschen Theorie der Beugung.* Państwowe Wydawnictwo Naukowe, Warszawa 1957.
[24] Tradowski, K.: *Laser.* Vogel-Verlag, Würzburg 1968.
[25] Zorn, C., T. Cremer, C. Cremer, and J. Zimmer: *Laser uv microirradiation of interphase nuclei and post-treatment with caffeine.* Hum. Genet. **35,** 83—89 (1976).