Michael Hoppert

Microscopic Techniques in Biotechnology

Michael Hoppert

Microscopic Techniques in Biotechnology

Michael Hoppert

Microscopic Techniques in Biotechnology

Author

Dr. Michael Hoppert
Georg-August-Universität-Göttingen
Abteilung Strukturelle Mikrobiologie
Grisebachstrasse 8
37077 Göttingen
Germany

Library of Congress Card No.:
Applied for.

British Library Cataloguing-in-Publication Data:
A catalogue record for this book is available from the British Library.

Bibliographic information published by Die Deutsche Bibliothek
Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the Internet at <http://dnb.ddb.de>

Printed in the Federal Republic of Germany

Printed on acid-free paper

Composition Mitterweger & Partner, Kommunikationsgesellschaft mbH, Plankstadt
Printing betz-druck GmbH, Darmstadt
Bookbinding Litges & Dopf Buchbinderei GmbH, Heppenheim
ISBN 3-527-30198-4

Preface

Among the instruments used for the investigation of biological objects, only microscopes provide a real image and not just an abstract data set. Interpretation and analysis of the image allows in one determination the exploration of a variety of important features, such as the total quantity of the objects, cell volume and volume of compartments, the presence of contaminants or infective agents, the status of the cell cycle, and topography. Microscopic techniques are prerequisites for localization of subcellular fractions and proteins, differentiation of organisms, microspectrometry, and all micromanipulation techniques.

Over the past 50 years, electron microscopy has opened new pathways for biologists to understand structure-function interrelations at the subcellular level. New light-based optical techniques involving the detection of fluorescent molecules have introduced a new dimension of imaging into light microscopy, enabling cellular events to be studied in vivo down to molecular scales.

In fact, microscopes of any kind are now user-friendly, easy-to-operate instruments, and most of the preparation techniques are standardized and easily applicable. Though the book provides just a short glimpse into all these techniques, it is written for the reader seeking a quick start in key microscopic techniques.

Aim and Scope of this Book

This book is devoted to microscopic applications in biotechnology. Of course, most of the tools described do not differ from the instrumentation used in other fields of life sciences. The book seeks to convey the potential of microscopy for biotechnological applications.

The introductory sections give general overviews of the biological objects, which should also allow non-biologists (chemists, technical engineers, or computer software specialists) working in the field of biotechnology to understand the potential of microscopic imaging. The sections describing useful applications contain short and straightforward introductions into the structures of the objects described in terms of the view in the instrument used. The non-biologist will find a description of biological objects that is far from complete, but comprehensive enough to give an idea of the objects structures. In the “useful applications” sections, selected problems are described in

more detail. These problems are illustrative for a broad range of biotechnological applications and demonstrate the possibilities of the techniques. These sections contain only incidental information concerning the technical procedures, which are described in greater detail in the "Preparation Techniques" sections. These contain short introductions to the apparatus used and describe the technical procedures in detail. Representative procedures are included as step-by-step protocols. Of course, a selection of protocols may be critical and the reader may miss a protocol for her or his special needs. Here, the extended reference list is helpful. In addition, some manufacturers provide excellent and detailed protocols especially adapted to their products. Those protocols are, of course, not included here. One has to consider that the number of protocols naturally widely exceeds the scope of a single book covering such a wide field of microscopic techniques.

Recently developed or developing techniques are summarized in a separate chapter. These techniques may come to be (or are already) of utmost importance for biotechnology, but are not (yet) available for a large range of users.

Dr. Michael Hoppert
Göttingen, February 2002

Acknowledgements

The protocols and figures for a book such as this are necessary to draw from a wider field than is encountered in our single laboratory. Thus, I would like to thank numerous colleagues for allowing me to take benefit from their helpful advice as well as from their published micrographs and illustrations. I would also like to thank all former and present members of the Structural Biology department for their diverse contributions, and Jan Hegermann (University of Göttingen) for his help during manuscript preparation. I am especially grateful to Prof. Dr Frank. Mayer for his essential foundations, without this book could not have been written.

Michael Hoppert

Concerning the contributions to Chapter 2.4.8.9, I would like to thank Drs. O. Bertrand (INSERM U76, Paris) and J.-L. Rigaud (Institut Curie, Paris) for helpful discussions and suggestions. Part of the ideas for this chapter were put on paper during my Visiting Professorship at the University of Marburg in the Institute for Virology, and I am grateful to Prof H.-D. Klenk and Dr. E. Bogner for inviting me. The collaboration with Dr. Bertrand was sponsored by The British Council (PN 99.121).

Andreas Holzenburg

Foreword

This book was initiated by Wiley-VCH after the remarkable success of the series BIOTECHNOLOGY. In volume 1 of this series, cell structure was described. The publishers felt that a more detailed treatment of this subject may be of interest for scientists engaged in research and development in biotechnology. They managed to convince one of the most competent scientists in the field of imaging of biological samples, Michael Hoppert, to do the writing. The outcome is presented here.

A first glimpse at the table of content gives an idea on the breadth of the work. Further inspection reveals that the book is indeed an in-depth compendium. The first part concentrates on representative examples of biological material to be investigated in the context of biotechnology, and on instructions for their preparation. This part is also combined with descriptions of the theoretical basis, applications, and limitations of multiple kinds of methodologies of light microscopical imaging, starting from the most basic light microscopy, and encompassing sophisticated light microscopical techniques, such as state-of-the-art fluorescence laser scanning microscopy in various modifications for qualitative and quantitative analysis of samples.

In the second part, imaging by application of electron microscopy is treated with the same accuracy. Descriptions of representative samples, ranging from isolated macromolecules and unicellular organisms, many kinds of differentiated cells of eukaryotic organisms, to complex tissue, are presented, together with careful but nevertheless straight-forward instructions on how to prepare these samples.

In the final section, Michael Hoppert describes specialized approaches of imaging not usually dealt with in conventional books on imaging techniques for biological samples, for example, total internal reflection microscopy, multiphoton excitation microscopy, fluorescence resonance energy transfer, fluorescence correlation spectroscopy, soft X-ray microscopy, microscopic imaging with photons and heavy ions, just to name a few of them.

Carefully selected illustrations, together with the references given at the end of each of the sections of the book, simplify the search for the technique of choice needed for a specific problem.

Michael Hoppert's book carries features that may make it the reference book *par excellence* in the field of imaging of biological samples, with a special emphasis on biotechnology.

I trust that this book will have the success it deserves.

Prof. Dr. Frank Mayer
Göttingen, Januar, 2003

Contents

1
Light Microscopy

1.1
Applications and Limitations in Light Microscopy

Light microscopes are the most widely used instruments for visualization of structures down to the scale of a few micrometers. The instruments are available in numerous variants, suitable for detection of phase contrast, fluorescence, changing polarity of light, etc. The big advantage of light microscopes is their mechanical stability, allowing operation under varying environmental conditions, and their ease of operation. The fact that fully hydrated specimens may be visualized under natural environmental conditions (room temperature, atmospheric pressure etc.) remains the most important feature of light microscopic applications in biomedical research. Visible light as an imaging medium does not usually induce artificial changes in the specimen (a big disadvantage of the shorter-wavelength radiation). With no other microscopic technique is such a broad range of well established staining procedures, additional observation facilities, manipulating tools, etc. available for visualization of fully hydrated specimens in a natural surrounding. Although alternative techniques may become applicable for routine research in the near future, only light microscopy currently allows the elucidation of dynamic processes such as growth, cell movement, vesicle transport, etc.. Biologists, though, have to take account of the fact that the resolution limit in light microscopes, essentially represented by the wavelength of the light, allows depiction of whole organisms and large subcellular compartments, but none of even the largest macromolecules. Although direct imaging of viruses, protein molecules, etc. is impossible, they may be *localized* by use of probes with specific binding affinities (antibodies, enzyme substrates, oligonucleotides, etc.). These techniques, especially in conjunction with fluorescent markers, allow the detection of small quantities of the specifically marked targets. Depending on the individual binding affinities, a minimum number of targets have to be present for a detectable signal to be emitted. Nevertheless, specialized fluorescent techniques allow detection down to one molecule. At the cellular level, it is – in principle – possible to detect one single cell in a specimen (e.g., a single pathogen in a wood sample), but this of course requires automated systems. The lower detection limit in a non-automated system is around 50 (bacterial) cells in a volume of 100 μl. The sample must be thin enough to allow transmission by the light beam or detection of the fluorescence signal (this is also true

for confocal microscopes). Nevertheless, when the structures are large enough, and the sections are optically transparent (i.e., the multiple layers do not blur the final image), up to 0.5 mm are acceptably thin for an overview. This allows the cutting of, for example, wood samples with a simple razor blade. The lower limit of specimen thickness is around 200 nm.

The ease of specimen handling in the operation of the light microscope has facilitated the development of numerous microtechniques, from closed flow cells for circulation of liquid samples (for the observation of biofilm development, for example) to micromanipulators, optical tweezers, etc. that allow handling of single cells. The image information itself may be quantified by image processing, spectroscopic analysis of the transmitted light, volumetric measurements, and so on.

Essentially, all microscopes only reveal the properties of the specimen that produce alterations in the imaging medium (light, X-ray, electron beams, etc.). This is also true for light microscopy, and allows numerous intrinsic properties of the specimens to be detected. Upon interaction with the specimen, light may change its intensity – regularly at a specific wavelength – leading to contrast and color. Variations in the optical density of the specimen change the phase properties, leading to phase contrast. The arrangement of molecules in the specimen may turn the polarization plane. Visible light induces excitation of certain molecules to produce fluorescence, regularly applied in connection with fluorescent probes. Light absorption, induction of fluorescence, changes in the phase properties, and polarization are all used for construction of light microscopes. Raman imaging may be viewed as a microscopic technique that helps to elucidate the chemical composition of a specimen with the aid of visible light. However, the interaction of light with the specimen, unlike that of X-rays or electron beams, does not convey information on the elemental composition.

The essential drawback of optical light microscopy is the resolution limit, with 0.16 μm as a theoretical value, but no better than 0.3 μm under optimum conditions under the microscope. However, optical near-field microscopy is a microscopic technique also based on visible light as imaging medium that breaks the resolution limit. Instrumentation and applications are currently under development. This technique is presented in detail below (see Section 1.4.11).

1.2 Useful Applications

1.2.1 Cellular and Tissue Structure

1.2.1.1 Prokaryotic cell structures at the light microscopic level

All bacteria, irrespective of their degree of cellular complexity in various physiological or taxonomic groups, have an envelope consisting of a cytoplasmic membrane and, in most cases, a rigid wall. The envelope encloses the cytoplasm and interacts, at its exposed layer, with the environment. The cytoplasm contains, in most cases without obvious compartmentation, nucleic acids, ribosomes, storage materials, enzymes,

and low-molecular weight components within the cytosol. Their size and structural simplicity restricts cytological investigation of bacterial structures by light microscopy to the detection of large cellular inclusions. Specialized staining methods allow localization of small intracellular targets, but do not help to elucidate their fine structures. Nevertheless, light microscopy for bacteria is indispensable when living cells or processes inside living cells have to be monitored.

The cell envelope

Simple negative staining allows detection and determination of the dimensions of spacious bacterial capsules, sheaths, or slimes (often termed "extracellular polymeric substances" or EPSs) as depicted in Fig. 1.1 a. When organized into a regular morphologically structured component, this is usually referred to as a capsule, glycocalyx, or sheath. When loosely organized, or of variable consistency, it is designated as a slime. Capsules, sheaths, or slimes represent the outermost layer of most free living Gram-positive and Gram-negative bacteria. Common sugars making up the polysaccharides are rhamnose, galactose, glucosamine, galacturonic acid, or hyaluronic acid; their relative amounts vary. Though usually composed of polysaccharides, EPSs composed of polypeptides or cellulose have also been described. EPSs are important virulence factors of pathogenic bacteria, because they counteract host defense mechanisms. Some of the more loosely attached EPSs (slimes) are of industrial interest; *Leuconostoc* converts sucrose into dextran gel. EPSs connect bacteria to each other and their substratum, thus allowing them to build up consortia, such as flakes or films. EPSs protect cells against sudden changes in environmental parameters (pH, water content, antibiotic agents). Under optimized conditions, in laboratory cultures, bacterial strains often lose the ability to synthesize EPSs. The EPSs appears to be bound to the cell wall not by covalent bonds, but by electrostatic interaction with the outer wall polymers.

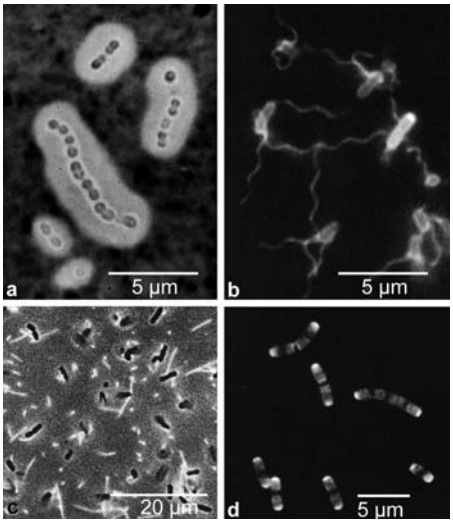


Fig. 1.1 Structures outside the bacterial cell wall: (a) India Ink preparation of encapsulated pneumococci. The cells appear slightly gray within the bright capsules. The dark ink particles do not penetrate the capsule material (Gillies and Dodds, 1977).
(b) *Escherichia coli* cells labeled with an amino-specific Alexa Fluor dye, examined in a fluorescence microscope. The wavy flagella are attached to the fluorescent cell surfaces (Linda Turner and Mary A. Nilsson, Rowland Institute for Science, Cambridge, Mass.).
(c) *Escherichia coli* K-12 Hfr H; visualization of the F-pili. A fluorescence marker is coupled to the pilus-specific bacteriophage MS 2 (Jarchau, 1985; Mayer, 1986).
(d) Immunofluorescence of D-cysteine residues in sacculi from *E. coli* cells labeled with D-cysteine and chased in the presence of cell division inhibitors (de Pedro et al., 1997)

Though the swimming of bacteria in liquid media is easily to observe under any standard light microscope, specific stains must be applied for visualization of bacterial flagella by bright-field microscopy. Recording of fluorescently labeled flagella also reveals details of their motion (Fig. 1.1 b). In addition, the localization of pili and fimbria by application of fluorescence techniques is possible (Fig. 1.1 c). These structures, together with the EPSs, are mainly involved in interactions between cells and their surroundings. They take part in the initial stages of biofilm formation, the primary attachment of a cell on a surface. During the attachment process, the cells often have to penetrate a layer of repulsive surface charges. Pili penetrate this layer, thus allowing contact between cell and surface. The contact between pili and other cell surfaces may be highly specific, during conjugation or in symbiotic and pathogenic cell-cell interaction, for example.

Since the thickness of the next, innermost layers of the cell envelope are below the detection limit of light microscopes, no direct imaging of these layers is possible. Differentiation between cell wall types by the use of specific stains (most commonly the Gram stain) is possible, though no direct information on the wall structure can be deduced from these methods. However, the light microscope also permits differentiation of larger labeled areas on the cell surface. Figure 1.1 d depicts the differentiation between old and newly synthesized parts of the murein sacculus in *E. coli*. Although, from the image alone, the cell wall itself is invisible, the intensities of the fluorescence signals represent the quantities of a cell wall component (here introduced artificially) and allow conclusions to be drawn on the distribution of old (brighter) and newly synthesized (darker) portions of the cell wall.

Structures inside the prokaryotic cell

Direct visualization of large intracellular inclusions (storage granules and endospores, Fig. 1.2 a, e, and f) is possible without any pretreatment. Endospores form typical, highly refractive inclusions (Fig. 1.2 a) and are important taxonomic markers for the genera *Bacillus* and *Clostridium*. A variety of other inclusions in bacteria (gas vacuoles, magnetosomes, chlorosomes, phycobilisomes, glycogen and polyphosphate granules, protein crystals) are at best very difficult to discriminate. Reliable structural analysis is only possible with electron microscopic techniques. Nevertheless, detection and quantification of the large inclusions may be possible with differential stains. Some bacterial polymeric inclusions are raw materials of biotechnological relevance. Polyhydroxyalkanoates (PHAs) form inclusion bodies, functioning as carbon and energy storage materials, accumulated in a variety of prokaryotes. These inclusions usually appear to be more or less spherical. The hydrophobic nature of these inclusions allows hydrophobic agents to be used as stains. Figure 1.2 b shows cells of *Pseudomonas* sp. stained with the fluorescent Nile Blue. The image shows a composite photograph of a phase contrast (i.e., bright-field) and fluorescence image. Though application of fluorescent stains is not necessary to visualize PHA inclusions, the two separate images (fluorescent and bright-field) allow easy quantification of the relative amount of PHA when the surface area of the whole cells (bright-field) is compared with the area of the fluorescence signal.

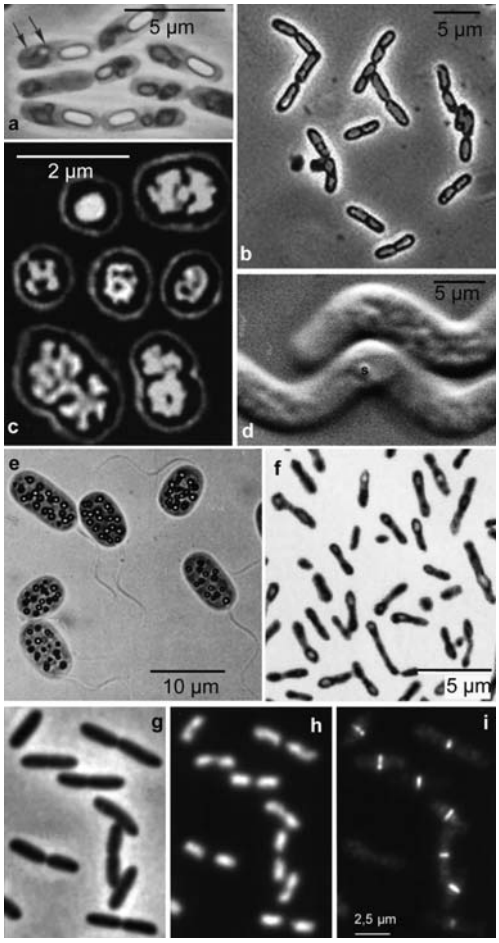


Fig. 1.2 Intracellular bacterial structures:
(a) *Bacillus cereus* var. schwetzova forms, besides a spore (bright, oval cellular inclusion), a diamond-shaped and a spherical parasporal body (arrows) (Gould and Hurst, 1969).
(b) Polyhydroxybutyrate (PHB) granules in *Pseudomonas*. Overlay of a bright-field and a fluorescence image of *Pseudomonas* cells. The phase contrast provides natural contrast between the cell and the background, but small PHB granules are not readily visible. The cells were therefore stained with Nile Blue A, a fluorescent stain that binds to PHB. The PHB can easily be seen as bright yellow granules (micrographs taken by William Ghiorse, Section of Microbiology, Cornell University).
(c) Nucleoid structure as revealed by DAPI staining of *Methanococcus jannaschii* cells (Malandrin et al., 1999).
(d) Two spirillum-shaped cells shown by differential interference contrast. Small sulfur globules (S) are visible (Guerrero et al., 1999).
(e) Large sulfur globules (here in *Chromatium okenii*) are highly refractive and have a characteristic appearance in the phase contrast image (Pfennig and Trüper, 1992).
(f) *Pseudomonas* cells containing aggregates of a recombinant protein. The aggregates deform the normal cellular shape (Carri and Villaverde, 2001).
(g, h, i) Localization of the FtsZ protein in *Escherichia coli*: (a) bright-field image (phase contrast), (b) DAPI-stained fluorescence image (staining of DNA), (c) immunofluorescence of FtsZ. The ring-like structure is depicted in side-view, as a short line at the site of cell division (Den Blaauwen et al., 1999)

Localization and the overall structure of the nucleoid may be visualized by fluorescence microscopy of DAPI-stained cells. The presented figure (1.2 c) implies a coralline shaped structure of the nucleoid. Since the preparation procedure requires a chemical fixation, artificial alterations of the nucleoid may not be excluded, but it becomes clear that the nucleoid shape varies during the cell cycle. Again, the fluorescent signal correlates with the DNA content and allows rough quantification of DNA in individual cells.

Though the relevance of bacteria in biogeochemical cycles is rarely visible, intracellular deposition of elemental sulfur is an exception. The highly refractive granules are deposited intercellularly in chemolithoautotrophic bacteria capable of oxidation of reduced sulfur compounds. The presence of sulfur globules is a reliable indicator of this metabolic feature. Though small sulfur globules are sometimes difficult to discriminate from other inclusions (Fig. 1.2 d), large and numerous sulfur granules have a characteristic appearance under the light microscope (Fig. 1.2 e). Large protein inclusion bodies commonly occur in recombinant bacteria upon gene overexpression. The inclusions are sometimes big enough to influence the cell shape, as depicted in Fig. 1.2 f.

The cell cycle in eukaryotes may have been the first complex dynamic event to have been elucidated by light microscopy. In recent years, events in the bacterial cell cycle have also been documented. This application illustrates that macromolecular assemblies inside prokaryotic cells may very well be detectable under the (fluorescent) light microscope. In Fig. 1.2 i, the protein FtsZ – an important component of the division apparatus in prokaryotes – is located by immunofluorescence. The protein plays a key role during septum formation and forms a ring-like polymer at the site of cell division.

1.2.1.2 Eukaryotic cell structures at the light microscopic level

Fungal and plant cells

Since eukaryotic cells are normally larger than prokaryotic cells and compartmented by membraneous organelles, discrimination of their large intracellular structures is easier than in bacterial cells. Nevertheless, not all compartments are easy to detect in any cell.

Of all the types of eukaryotic cells, plant cells, due to their size and their rigid, highly refractive walls, are the most appropriate objects for visualization by light microscopy. In Fig. 1.3 a, meristematic cells from the root tip of *Vicia faba* provide an overview of the structures that may be differentiated with light microscopes. The cell wall, vacuoles, and the nucleus are the most conspicuous structures. It is difficult to differentiate smaller compartments: proplastids, possibly mitochondria, and cisternae of the endoplasmic reticulum (see also Fig. 1.3 b)

Because of their apparent simplicity, yeast cells are often used to serve as model eukaryotes. Cellular structures other than the wall, the vacuole, and the nucleus are very difficult to distinguish without application of fluorescent dyes (Fig 1.3 c). Many yeasts also produce capsular material, usually phosphomannans, β-linked mannans, heteropolysaccharides containing pentoses, glucuronic acid residues, and D-galactose. The cell wall itself consists of several layers that cannot be resolved by light

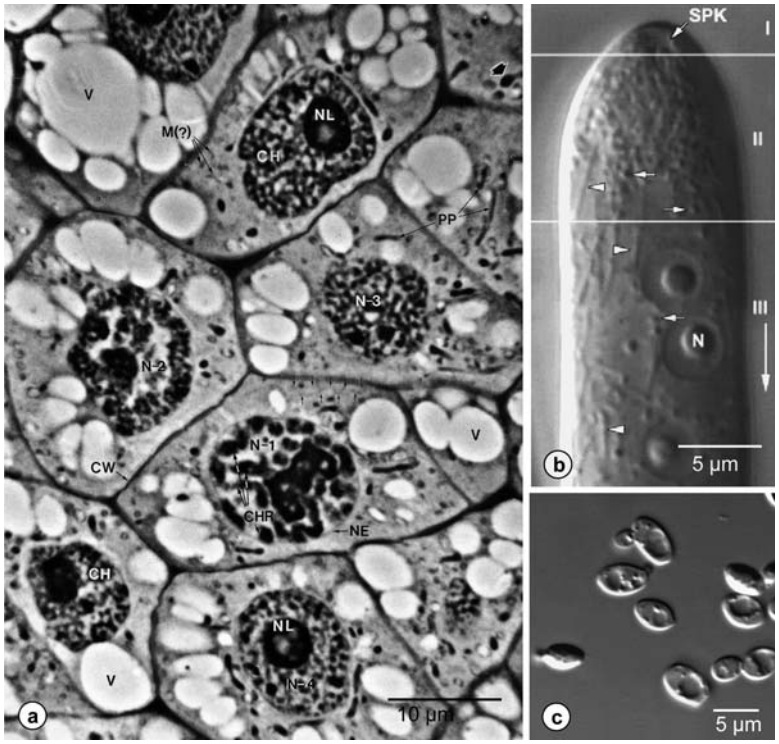
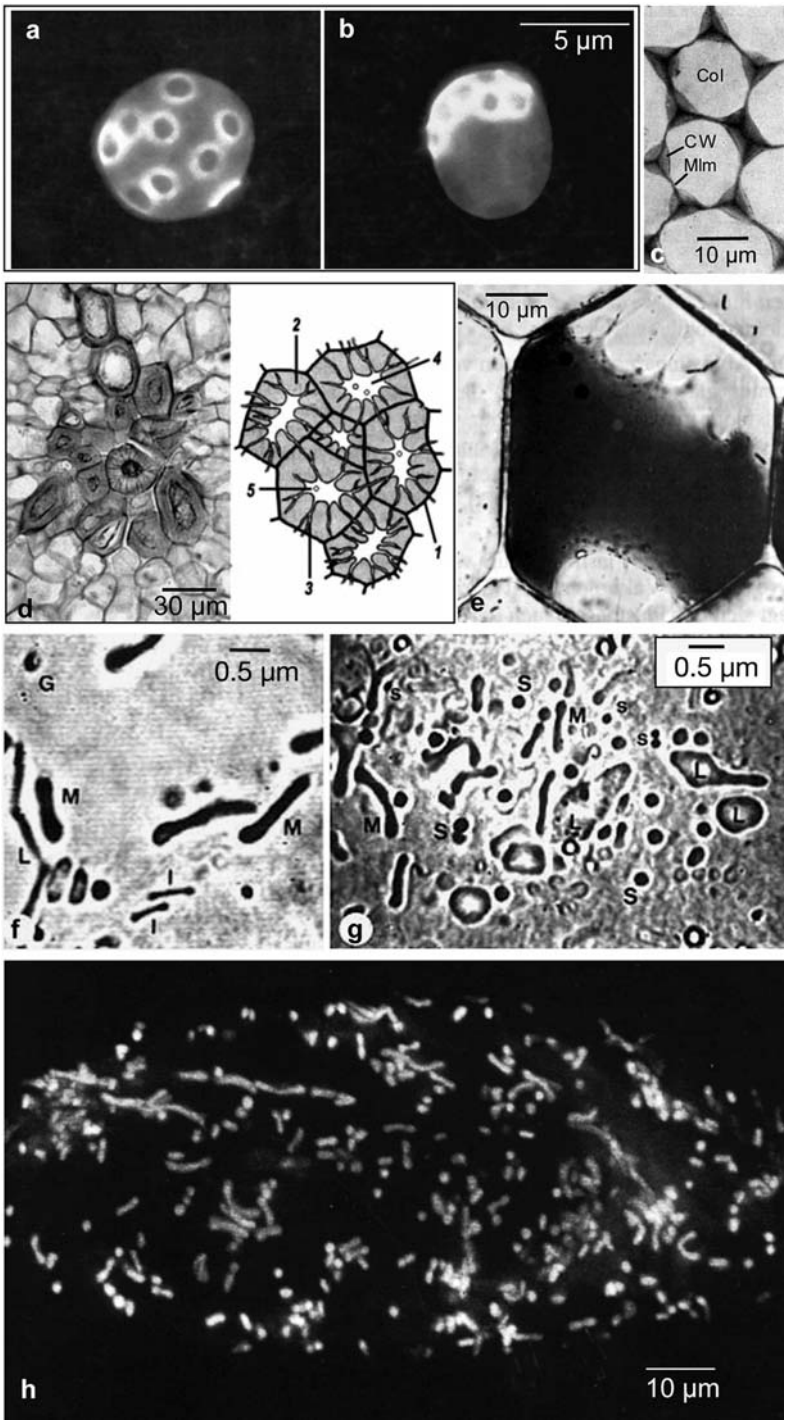


Fig. 1.3 Fungal and meristematic plant cells (overview):
(a) Thin section from the root-tip meristem of *Vicia faba*, showing the visible structures in plant cells (CW = cell wall, N1-N4 = nuclei, NL = nucleoli, NE = nucleus envelope, CH = chromatin, CHR = chromosomes, PP = proplastids, M? = mitochondria?, V = vacuole, small arrows depict thin membranous or filamentous structures at the resolution limit) (Gunning and Steer, 1996).
(b) Tip of an *Allomyces macrogyna* hypha. Region I of the hypha contains the Spitzenkörper (SPK), responsible for cell wall synthesis. Region II is characterized by numerous vesicles (arrows) and mitochondria (arrowhead). Region III contains nuclei (N), mitochondria and vesicles are less prominent (McDaniel and Robertson, 2000).
(c) Differential interference contrast image of yeast (*Saccharomyces*) cells. Large vacuoles fill 1/3 to 1/2 of the cell (the IN-VSEE Project, Arizona State University)

microscopy. Nevertheless, characteristic bud scars are visible, especially after fluorescence staining, as depicted in Fig. 1.4 a.

In most fully developed plant cells, the walls appear thick and multi-layered. Combinations of staining solutions also allow rough chemical differentiation of wall layers. Pectinous middle lamellae, cellulose walls with various degrees of lignification, may be discriminated (Fig. 1.4 c, d).

The large vacuoles appear bright and homogeneous. While vacuoles in meristematic plant cells are small and numerous (see Fig. 1.3 a), a single vacuole fills more than 50 % of the cellular volume in a mature cell. The tonoplast separates the vacuolar lumen from the cytoplasmic exterior. Vacuoles act as storage containers for carbohydrates, protein, lipids, and secondary metabolites, as well as compartments of osmoregula-


a
b
5 µm
Col
CW
Mlm
c
10 µm
d
30 µm
1
2
3
4
5
10 µm
e
0.5 µm
G
M
L
I
f
0.5 µm
S
M
L
O
g
10 µm
h

◀ **Fig. 1.4** Compartments in fungal and plant cells – cell walls, vacuole, mitochondria:
(a, b) Fluorescence images of yeast cells stained with Calcofluor White. Bud scars appear bright. Figure a depicts a mutant cell with random distribution of bud scars on the cell surface, (b) the wild-type cell (Chant et al., 1991).
(c) Angular collenchyma in *Cucurbita pepo*. The middle lamella appears as a dark line after staining with hemalaun (Mlm = middle lamella, Col = lumen of the cell, CW = cell wall).
(d) Isodiametric sclereids (stone cells) with very thick lignified cell walls, and numerous (branched) pits in peach (*Pyrus communis*), stained with phloroglucinol (stained red due to the presence of lignin). The sclereids are embedded in the fruit ground tissue (1 = primary wall, 2 = secondary wall, 3 = branched pit, 4 = cell lumen, 5 = cross-sectioned pit) (John Cheeseman, Univ. Illinois; diagram modified from Weier et al., 1974).
(e) Plasmolysis of an epidermal cell from *Rhoeo spathacea*. The vacuole, filled with colored sap, occupies nearly the total volume of the cytoplasm (from Nultsch and Grahle, 1983).
(f, g) Components of the cytoplasm in an epidermal cell of *Allium cepa*. The cells of the inner epidermis of the onion were examined with a UV microscope at a wavelength of 310 nm. The higher resolution of this microscope compared to conventional light microscopes allows a clearer image of small organelles (f1: M = mitochondrion, l = small, mitochondria-like inclusions, L = leucoplast, G = Golgi apparatus (?). f2: Legend as in f1, S = Large sphaerosomes, ss = small sphaerosomes (membrane-surrounded vesicles) and particles with unknown function (from Gunning and Steer, 1996).
(h) *Nicotiana* cell with mitochondria, viewed by fluorescence microscopy after staining with Rhodamine-123 (from Gunning and Steer, 1996)

tion and intracellular protein degradation. Vacuoles sometimes contain various visible inclusions such as polymetaphosphate (volutin) granules. In higher plants, the vacuoles are often filled with water-soluble pigments (Fig. 1.4 e).

Although mitochondria are visible as faint structures under the standard light microscope, high resolution or staining with fluorescence markers provide clearer images of mitochondrial shape and distribution. Large mitochondria of the *Allium cepa* epidermis are visualized by use of short-wavelength ultraviolet light. (Fig. 1.4 f, g). Specific staining is also possible with fluorescent agents. The vital stain Rhodamine-123 is predominantly accumulated in mitochondria. The fluorescent image (Fig. 1.4 h) illustrates the shape and distribution of the organelles in cultured *Nicotiana* (tobacco) cells.

Plastids, especially pigmented plastids (chloro- and chromoplasts), are the most characteristic compartments of plant cells. Under appropriate conditions, thylakoidal grana structures (i.e., thylakoid membrane stacks) are visible as small disks inside chloroplasts (Fig. 1.5 a). Autofluorescence of the photosynthetic pigments allows detection of plastids by fluorescence microscopy without application of any additional stain (Fig. 1.5 b). Like mitochondria, plastids contain DNA that may be visualized under the fluorescence microscope by staining with DAPI (4',6-diaminidino-2-phenylindole) (Fig. 1.5 c). The differentiation of plastids may not only lead to photosynthetically active chloroplasts. Chromoplasts contain carotenoids and xanthophylls, responsible for some orange-reddish fruit colors. Mature chromoplasts are regularly spindle-shaped (Fig. 1.5 d). The loss of pigments (by mutation, for example) in certain tissue types may lead to colorless plastids (leucoplasts). Sometimes, plastids with storage function are also termed leucoplasts. Among these, the starch-containing amyloplasts are most important and are widespread in plant storage tissue. The development of starch grains in plastids is especially easy to visualize after iodine (I/KI) staining of

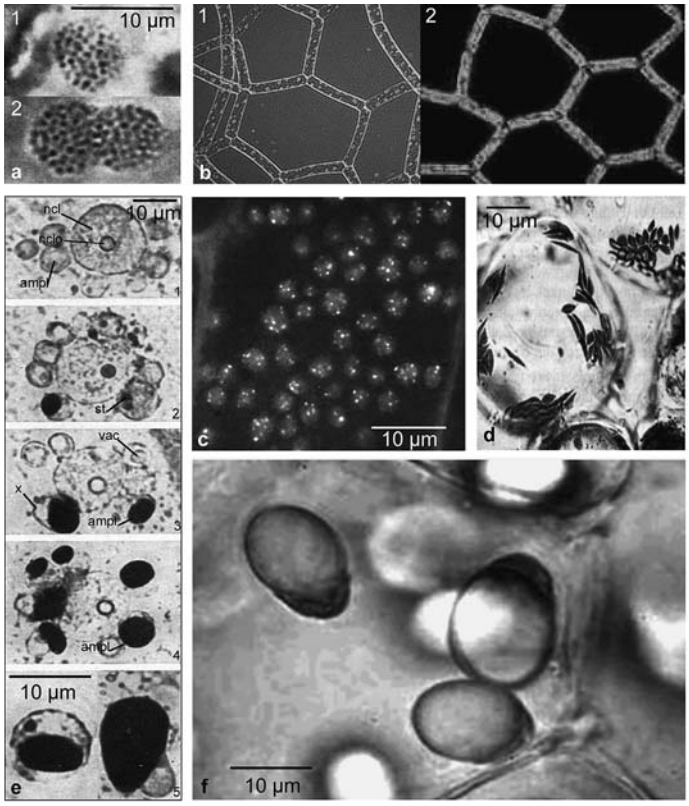


Fig. 1.5 Compartments in plant cells – plastids:
(a) The lens-shaped chloroplasts in cells from *Spinacia oleracea* are depicted in top view. Grana stacks of the thylakoid membrane are visible as small, dark, disk-shaped dots in the lumen of the chloroplast. The bright stroma is located among the grana. 1 = single chloroplast; 2 = dividing chloroplast (from Braune et al., 1983)
(b) *Hydrodictyon reticulatum*. Bright-field (1) and fluorescence (2) images. Use of chlorophyll autofluorescence is a simple method for selective imaging of chloroplasts in plant cells (Peter V. Sengbusch – b-online).
(c) Fluorescence microscopic image of chloroplasts of *Elodea*. Plastid DNA (nucleoids) are stained with the DNA-specific fluorescence marker *DAPI (4',6-diaminidino-2-phenylindole)*. Each fluorescent dot is equivalent to one nucleoid (from Kleinig and Meier, 1999).
(d) Spindle-shaped chromoplasts in the mesocarp cells of *Rosa rugosa* (from Nultsch and Grahle, 1983).
(e) *Solanum tuberosum*. Development of starch granules in the amyloplasts (1–4). Starch appears dark after treatment of the sample with iodine solution. Nucleus (ncl), nucleolus (nclo), amyloplast (ampl), starch (st), vacuole (vac), (x) shows an amyloplast with the stroma displaced by a growing starch granule. 5: Two amyloplasts with starch granules. Magnification in 2–4 is the same as in 1 (from Braune et al., 1983).
(f) Massive starch accumulation in the chloroplasts of *Pellionia* (Urticaceae). The crescent-shaped "cap" is still green and photosynthetically active. Though small starch granules regularly occur in chloroplasts, voluminous granules in photosynthetically active plastids are exceptional (Peter V. Sengbusch – b-online)

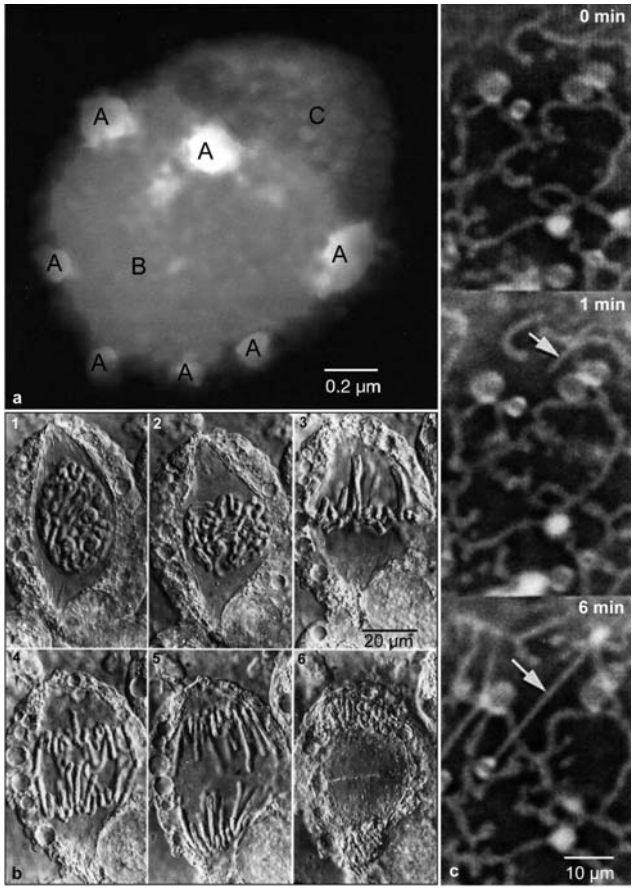


Fig. 1.6 Cellular compartments: nucleus and endoplasmic reticulum.
(a) Organization of sub-compartments of the yeast nucleus as visualized by indirect immunofluorescence (from Wente et al., 1997).
Three functional "compartments" have been differentially marked within the nucleus. One contains the majority of the chromatin (B), stained red with ethidium bromide; the nucleolus (C) is stained blue with a nucleolus-specific antibody and the fluorescent marker CY5; telomers (A), are stained green with another specific antibody (FITC as fluorescent marker).
(b) Mitosis phases in an endosperm cell of *Haemanthus katharinae*. Differential interference contrast (from Kleinig and Mayer, 1999).
1. Prophase: Polar caps and nucleus envelope are clearly visible; nucleoli and condensed chromatin are in the nucleus.
2. Prometaphase: Nucleus envelope is fragmented, centromers move to the equatorial plain.
3. Metaphase: Spindle apparatus is completed, chromosomes form the metaphase plate.
4. Anaphase: Kinetochores of the chromatides move polewards.
5. Late anaphase.
6. Telophase: forming of the daughter nuclei, beginning of cell division.
(c) Confocal scanning laser microscopy images of details in the peripheral area of a living tobacco BY-2 cell. Endoplasmic reticulum and other membrane systems were stained with rhodamine-123. The images were taken after 1 min and 6 min after the initial image (0 min). Arrows indicate a changing structure (from Gunning and Steer, 1996)

starch, as depicted in Fig. 1.5 e. Finally, the starch grain fills most of the volume of the mature amyloplast. Photosynthetically active chloroplasts also regularly contain starch. Exceptionally, large starch grains also occur in this plastid type (Fig. 1.5 f).

The large nucleus and (condensed) chromatin are easily detectable in most eukaryotes. Most fundamental discoveries of structural features during the cell cycle have been achieved by (bright-field) light microscopy. Though classical staining methods for bright-field microscopy (see Fig. 1.3 a) are fully sufficient to resolve the nuclear structure (nucleolus, stages of chromatin condensation), the use of fluorescent probes reveals additional features. The organization of an inter-phase yeast nucleus is depicted in Fig. 1.6 a. Fluorescent dyes specifically stain the chromatin (the majority of the volume) and the nucleolus, where ribosomal DNA is transcribed and ribosomes are assembled, as well as the repressor activator protein 1, an abundant nuclear protein that binds to the repetitive sequences found at all yeast telomers. Bright spots mark the positions of the telomeric sequences. Inside an inter-phase nucleus, nucleoli are visible as more highly condensed entities. The use of interference contrast and differential interference contrast microscopes allows the condensed chromatin to be visualized without additional staining. Thus, some dynamic events (such as chromosome segregation) may be directly observed without damaging the sample (Fig. 1.6 b).

The Golgi apparatus, the endoplasmic reticulum, and cytoskeletal elements are also visible by light microscopy when fluorescent stains are applied. The fine structures of these compartments remain unresolved, but the dynamics of these compartments (membrane flow, transport) are visible. Vital staining of the endoplasmic reticulum in the cell periphery of cultured plant cells (the cortical endoplasmic reticulum) reveals the extensive movement in a network of fine vesicles and tubules (Fig. 1.6 c).

Localization of Golgi stacks and dynamics of the Golgi apparatus are depicted in Fig. 1.7. A suitable marker system for this compartment is an antibody specific for a carbohydrate residue of a glycoprotein family predominantly localized in the Golgi apparatus of plants. The fluorescently labeled antibody reveals the position of Golgi stacks in intact cells. In cells treated with the fungal metabolite brefeldin A, the Golgi

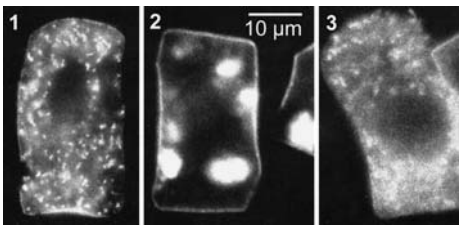


Fig. 1.7 Cellular compartments: Golgi apparatus.
Fluorescence microscopic images of osmotically intact cells from the root tip of *Zea mays*. The Golgi apparatus is labeled by immunofluorescence (from Gunning and Steer, 1996).
1. Normal state. The dark area in the center of the cell is the unlabelled nucleus.
2. Cell treated with the antibody brefeldin A, which inhibits a variety of membrane transformations. Cisterna stacks are dispersed because the transport from the ER to the Golgi apparatus is interrupted. Golgi membranes form big clusters.
3. Cell treated with brefeldin A as in 2, after 2 h recovery

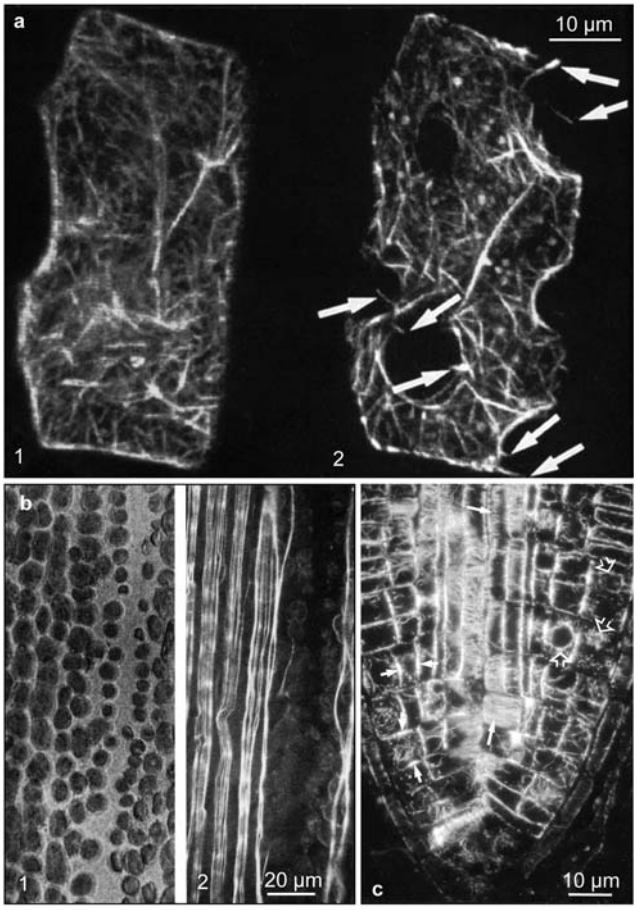


Fig. 1.8 Cytoskeletal elements in plant cells:
(a) Phalloidin, marked with rhodamine, was injected into this epidermal cell of *Tradescantia* for detection of actin filaments. The actin cytoskeleton is visible. After gentle plasmolysis the cytoplasmic membrane is detached from the cell wall. Arrows indicate actin filaments that extend from the protoplast to the cell wall (from Gunning and Steer, 1996).
(b) Detail of an internodial cell of *Chara* in bright-field (left) and fluorescence (right) image. Actin filaments associated with the rows of chloroplasts are labeled with a fluorescence-marked anti-actin-antibody. The actin-free zone separates the two oppositely directed cytoplasma streams (from Kleinig and Meier, 1999)
(c) Median longitudinal section from the apical root meristem of *Azolla*. Microtubules were visualized by immunofluorescence microscopy. In elongating cells, cortical microtubules perpendicular to the long axis of dividing cells can be seen marking the division plane (long arrows). Short arrows indicate preprophase ribbons, which appear in cells shortly before division. Some cells are forming mitosis spindles (wide arrows) (from Gunning and Steer, 1996)

apparatus becomes disassembled. After termination of brefeldin treatment, the stacks become redistributed.

Although plant cells are stabilized by rigid cell walls, they have complex cytoskeletons, especially involved in intracellular transport of subcellular compartments, chromosome segregation, and cell wall formation. Staining with phalloidin-rhodamine, a fluorescent complex that binds to actin, reveals the presence of the actin cytoskeletal network. Figure 1.8 a shows the presence of actin in an epidermis cell of *Tradescantia* before and after plasmolysis. In plasmolyzed cells, some thin filaments clearly protrude from the cytoplasmic membrane to the cell wall. Actin filaments are responsible for the transport of chloroplasts. The large internodial cells of the green alga *Chara* contains streaming rows of chloroplasts, obviously attached to actin filaments. The filaments are localized with anti-actin antibodies, coupled to actin, and visualized by fluorescence microscopy. Microtubules indirectly determine the cellular size by determination of the sites of synthesis of cellulose fibrils. The microtubular network is depicted in a root tip of the aquatic fern *Azolla*, stained with fluorescently labeled anti-tubulin antibodies. The distribution of microtubules in cells depends on the stage of the cell cycle (Fig. 1.8 c).

Animal cells

Animal cells exhibit the same degree of complexity as cells from higher plants. Numerous staining techniques for cytological differentiation in biomedical research have been developed for staining of compartments. Most of these techniques are only relevant for medical diagnostics, and so are not presented in detail here. Again, fluorescent techniques reveal localization and dynamics of cytoskeleton, mitochondria, ER, and Golgi apparatus. At the light microscopic level, the most characteristic features of animal cells are the lack of a thick, possibly multi-layered, wall and the absence of plastids and large vacuoles. The outermost layer of a typical animal cell exposed to the environment is formed by the glycocalyx, oligosaccharide chains that usually contain sialinic acid and produce a negative surface charge. Extracellular glycoproteins and polysaccharides have been found to be associated with the oligosaccharides.

The role of the intracellular stabilizing structure is therefore more pronounced. In the absence of an outer wall, the cellular shape is directly determined by the cytoskeletal elements. These are detectable by use of stains or specific antibodies. Although the structures of all single cytoskeletal fibers are below the resolution limit of light microscopes, the dynamics of these systems have been extensively investigated by (time-resolved) microscopic fluorescence techniques. Thick fiber bundles are also visible in phase or interference contrast. Besides their involvement in intracellular transport, the cytoskeletal elements also play key roles in cell movement.

Intermediary filaments, which are absent in plant cells, are involved in maintenance of the overall cellular shape. Localization of these filaments is again routinely performed with the aid of specific antibodies, as shown for bundles of cytokeratin and vimetin filaments in cultured animal cells (Fig. 1.9 a, b).

Actin filaments (F-actin) are composed of actin monomers, representing the most abundant cellular protein. The filaments are responsible for cellular movements, including gliding, contraction, and cytokinesis. The association of actin filaments with

the protein myosin is responsible for muscle contraction. Phase contrast images of certain cell types allow visualization of these elements. The distribution of "stress fibers", as shown in Fig. 1.9c, correlates with the immunofluorescent image of actin, as depicted in Fig. 1.9d. The fibers are composed of numerous actin filaments.

Microtubules also act as scaffolds to determine cell shape, and provide "tracks" for cell organelles and vesicles to move on. Microtubules also form the spindle fibers for separating chromosomes during cytokinesis. Localization of actin, myosin, and tubulin (as microtubule monomer) is depicted in Fig. 1.10a. Though in principle quite similar, cytokinesis in animal cells differs from that in plant cells in some aspects.

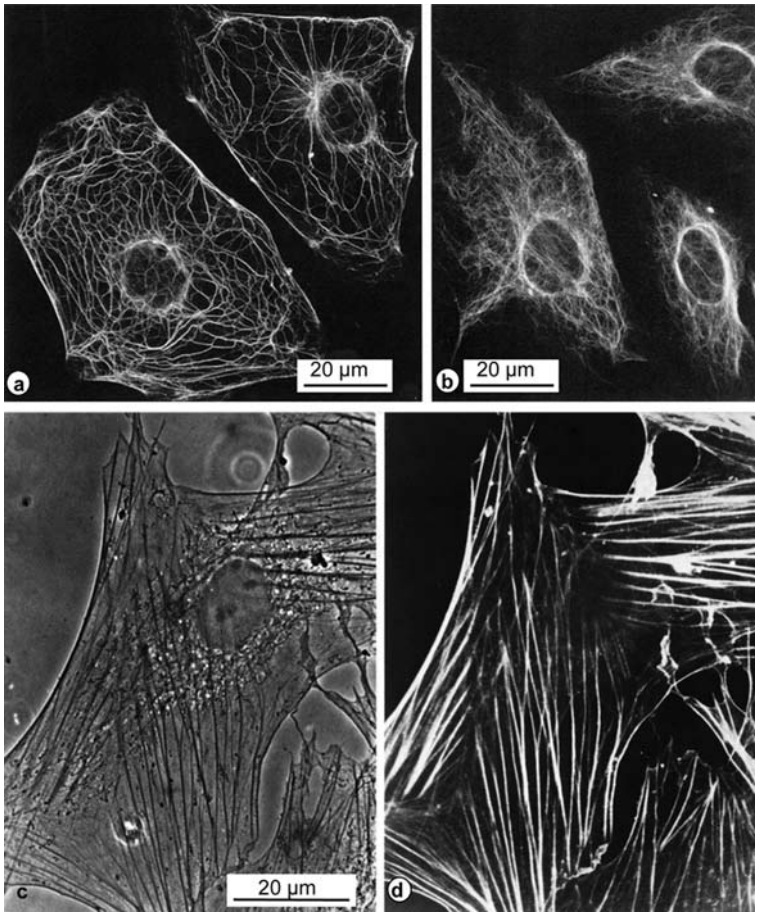


Fig. 1.9 Specialized features in animal cells: cytoskeletal elements.
(a, b) Intermediate filaments of kidney cells from the rat kangaroo visualized by indirect immunofluorescence (a, cytokeratin filaments; b, vimentin filaments) (from Kleinig and Meier, 1999).
(c, d) Actin filaments (stress fibers) in rat cells. The same detail seen in phase contrast (b) and by indirect immunofluorescence (c) (from Kleinig and Meier, 1999)

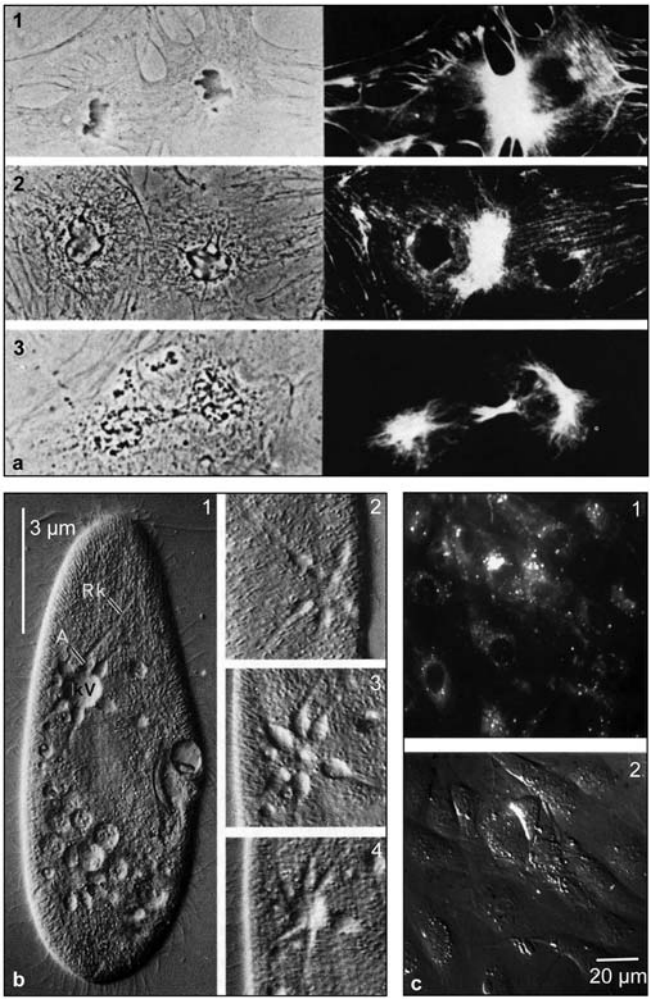


Fig. 1.10 Specialized features of animal cells: cytokinesis and contractile vacuoles in ciliates:
(a) Cytokinesis of animal tissue culture cells (PtK2-cells). Detection of actin (1), myosin (2), and tubulin (3) at the plane of division by indirect immunofluorescence (right; phase contrast images are shown at the left half of the figure) (from Kleinig and Sitte, 1986).
(b) Contractile vacuole of the ciliate *Paramecium*. 1: Cell with contractile vacuole (kV), ampullae (A), and radial canals (Rk). 2–4: Stages of the filling of the vacuole. Interference contrast (from Kleinig and Meier, 1999).
(c) Punctate fluorescence of a marker after endocytosis by cultured cells. (1) Cells observed in epifluorescence. (2) Same field of cells as in A, observed in interference contrast. Dye is distributed in a punctate pattern throughout the cytosol (from Niles and Malik, 1999)