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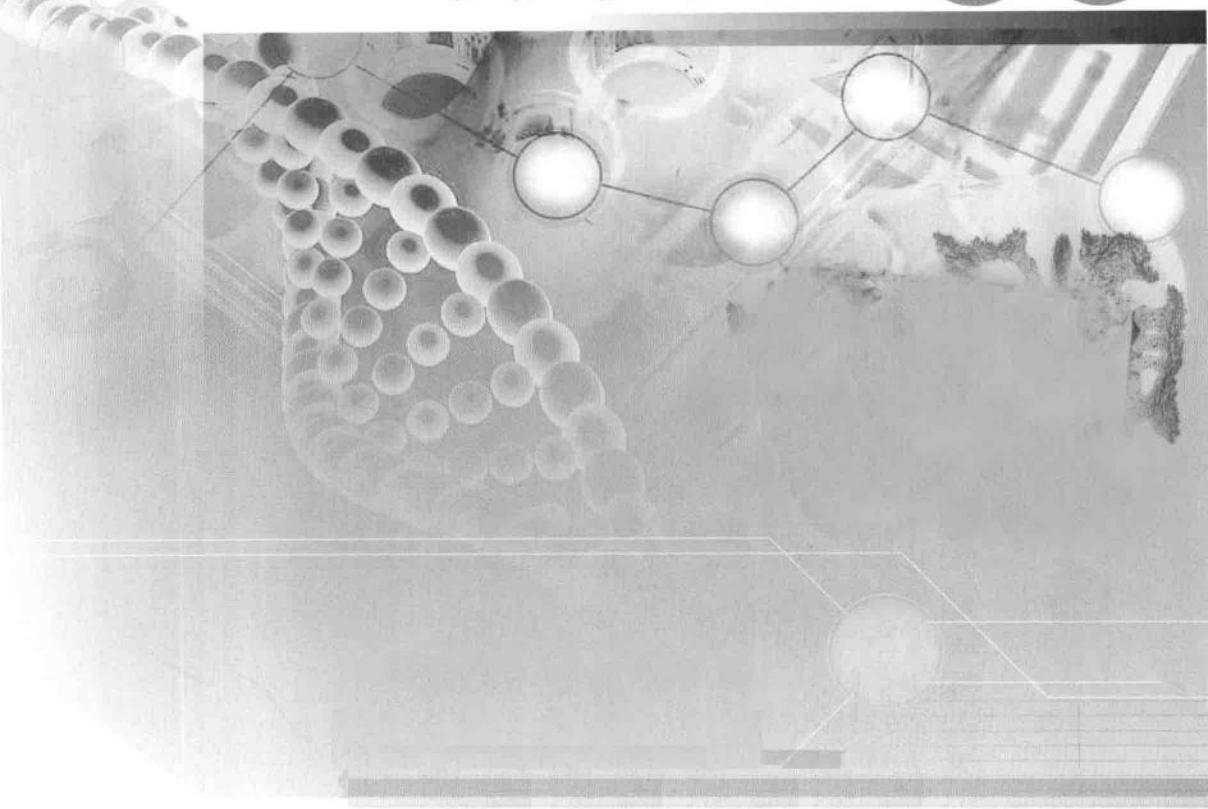


暨南大學出版社
JINAN UNIVERSITY PRESS

中国·广州

图书在版编目（CIP）数据

分子细胞生物学实验教程 = Molecular Cell Biology Laboratory Manual/邹奕，周天鸿主编. —广州：暨南大学出版社，2013.9
ISBN 978-7-5668-0734-2

Ⅰ.①分… Ⅱ.①邹…②周… Ⅲ.①分子生物学—细胞生物学—实验—高等学校—教材—英文 Ⅳ.①Q7-33

中国版本图书馆 CIP 数据核字(2013)第 200690 号

出版发行：暨南大学出版社

地　　址：中国广州暨南大学
电　　话：总编室（8620）85221601
　　营销部（8620）85225284　85228291　85228292（邮购）
传　　真：（8620）85221583（办公室）　85223774（营销部）
邮　　编：510630
网　　址：http://www.jnupress.com　http://press.jnu.edu.cn

排　　版：广州市天河星辰文化发展部照排中心
印　　刷：佛山市浩文彩色印刷有限公司

开　　本：787mm×1092mm　1/16
印　　张：8.75
字　　数：160 千
版　　次：2013 年 9 月第 1 版
印　　次：2013 年 9 月第 1 次

定　　价：19.50 元

彭磷基外招生人才培养改革基金资助

Author's Comments

Molecular cell biology involves an assortment of techniques, which are generally found difficult for undergraduate students. On reviewing courses taught by many other instructors of molecular cell biology, and giving the increasing number of overseas university students in China, it was apparent that there was a special need for the publication of a laboratory guideline in English for undergraduate lab exercises. The collection in this laboratory manual was carefully chosen to cover topics that were considered fundamental to an appreciation of modern molecular cell biology, and was modified to compromise the individual lab conditions in different Chinese institutions.

This manual is organized into eleven chapters and appendix dealing with materials added on a regular basis. It is suitable for the senior undergraduates to first-year graduate students with biology majors, with a prerequisite of general biology and some biochemistry background. Some lab exercises included in this manual are with sophisticated, expensive and time-consuming procedures. They are designed to lead toward further work at the molecular level of study and can be modified according to instructors' expertise and individual lab conditions. At some institutions that cell biology is an introductory course rather than a prerequisite, and it may be used with no laboratory components or with simple equipments to illustrate basic concepts.

The exercises are at three levels. Level I exercises present fundamental concepts and utilize minimal techniques required to study cells. It is believed that any institution should have the facilities to complete these exercises. Level II

presents techniques and procedures which require more time and energy from the students and instructors. Work at this level is appropriate to a biology major continuing to graduate and professional studies. Level III exercises combine several advanced techniques in molecular biology and are meant to encourage student research projects. Students will be required to work beyond the scheduled laboratory.

This work is supported by Peng Linji Foundation of Undergraduate Teaching Program Grant No. 51032101 from Jinan University.

Contents

Chapter 1 Microscopy

1.1 Introduction: Light Microscope—Resolution vs Magnification

The light microscope (LM) remains a basic tool of cell biologists, with technical improvements allowing the visualization of ever-increasing details of cell structure. A compound microscope is composed of two elements: a primary magnifying lens and a secondary lens system. Light is caused to pass through an object and is then focused by the primary and secondary lens. If the beam of light is replaced by an electron beam, the microscope becomes a transmission electron microscope. If light is bounced off of the object instead of passing through, the light microscope becomes a dissecting scope. If electrons are bounced off of the object in a scanned pattern, the instrument becomes a scanning electron microscope. The function of any microscope is to enhance resolution. It can be measured as the smallest distance between two points, which allows us to see the points as distinct. Therefore, resolution increases as the distance decreases.

Contemporary light microscopes are able to magnify objects up to about a thousand times. Since most cells are between 1 and 100 μM in diameter, they can be observed by light microscope, as can some of the larger subcellular organelles, such as nuclei, chloroplasts, and mitochondria. However, the light microscope is not sufficiently powerful to reveal fine details of cell structure, for which resolution—the ability of a microscope to distinguish objects separated by small distances—is even more important than magnification. Images can be magnified as much as desired (for example, by projection onto a large screen), but such magnification does not increase the level of detail that can be observed.

The limit of resolution of the light microscope is approximately 0.2 μM; two objects separated by less than this distance appear as a single image, rather than being distinguished from one another. This theoretical limitation of light microscopy

is determined by two factors—the wavelength (λ) of visible light and the light-gathering power of the microscope lens (numerical aperture, NA) —according to the following equation: Resolution = 0.61λ/NA. The wavelength of visible light is 0.4 to 0.7 μm, so the value of λ is fixed at approximately 0.5 μm for the light microscope. The numerical aperture can be envisioned as the size of the cone of light that enters the microscope lens after passing through the specimen. It is given by the equation: NA = η sin α, where η is the refractive index of the medium through which light travels between the specimen and the lens. The value of η for air is 1.0, but it can be increased to a maximum of approximately 1.4 by using an oil-immersion lens to view the specimen through a drop of oil. The angle α corresponds to half the width of the cone of light collected by the lens. The maximum value of α is 90°, at which sin α = 1, so the highest possible value for the numerical aperture is 1.4. The theoretical limit of resolution of the light microscope can therefore be calculated as follows: resolution = 0.61 × 0.5/1.4 = 0.22 um. Further improvements in this aspect of light microscopy cannot be expected.

Resolution is often confused with magnification, which refers to the size of an image. In general, the greater the magnification, the greater the resolution, but this is not always true. Resolution can be enhanced by reducing the wavelength to the ultraviolet range and yet again by levels of magnitude to the wavelengths electrons have in motion. The use of electrons as the light source gives rise to the electron microscope. UV (ultraviolet) light can not be seen directly by the human eye. Thus, these forms of microscopy rely on photography, or upon fluorescent screens.

Several different types of light microscopy are routinely used to study various aspects of cell structures. The simplest is bright-field microscopy (Figure 1.1), in which light passes directly through the cell and the ability to distinguish different parts of the cell depends on contrast resulting from the absorption of visible light by cell components. In many cases, cells are stained with dyes that react with proteins or nucleic acids in order to enhance the contrast between different parts of the cell. Prior to staining, specimens are usually treated with fixatives (such as alcohol, acetic acid, or formaldehyde) to stabilize and preserve their structures.

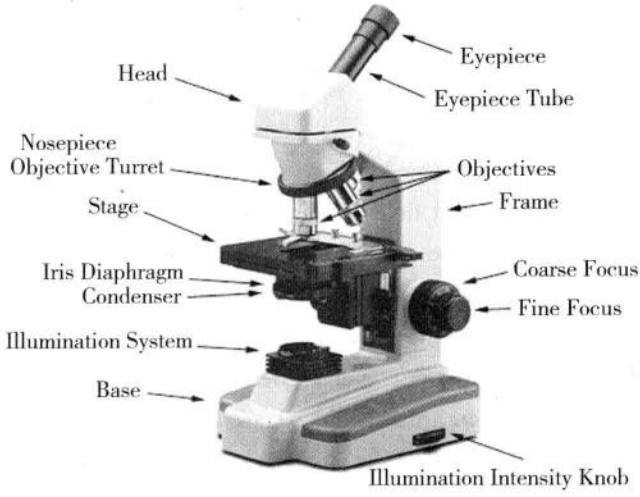


Figure 1.1 A schematic illustration of a standard Nikon binocular microscope, showing the names of various components.

1.2 Bright Field, Dark Field, Phase Contrast

The light microscope is used under bright field conditions to study the organization of cells in fixed and stained sections of tissues. The light microscope may also be used to monitor certain operations such as cell fractionation and biochemical characterization of cellular components. The microscope will also be used to quantitatively determine the number of cells in a suspension using a special device known as a hemocytometer, or cell counting chamber.

Without staining, the direct passage of light does not provide sufficient contrast to distinguish many parts of the cell, limiting the usefulness of bright-field microscopy. However, optical variations of the light microscope can be used to enhance the contrast between light waves passing through regions of the cell with different densities. In bright-field microscopy, transparent structures (such as the nucleus) have little contrast because they absorb light poorly. However, light is slowed down as it passes through these structures so that its phase is altered compared to light that has passed through the surrounding cytoplasm. If the normal phase shift is increased (usually by 1/4 wavelength), then the microscope becomes a phase contrast microscope. Phase-contrast convert these differences in phase to differences

in contrast, thereby yielding improved images of live, unstained cells. Phase contrast microscopes can be designed to have medium phase or dark phase renditions, by altering the degree of additional shift to the wavelength from 1/4 to 1/2 wavelengths, respectively.

If the light image is reversed, then the microscope becomes a dark field microscope. All standard bright field microscopes can be readily converted to dark field by inserting a round opaque disk beneath the condenser. Dark field microscopy was first utilized to examine trans-filterable infectious agents, later to be termed viruses, and to determine that they were particulate in nature. Small objects, even those below the limits of resolution, can be detected easily with dark field, as the object appears to emit light on a dark field. Look at the sky for a comparison. It is fairly easy to see stars in a dark sky, but impossible during the day. The same is true for dark field vs bright field microscopy.

Finally, if the normal light microscope is functionally turned upside down, the microscope becomes an inverted microscope. This is particularly useful in tissue culture since it allows observation of cells through the bottom of a culture vessel, without opening the container, and without the air interface normally present between the objective and the surface of the culture. By adding phase contrast optics to the inverted microscope, it is possible to monitor tissue cultures directly, without the aid of stains or other enhancements.

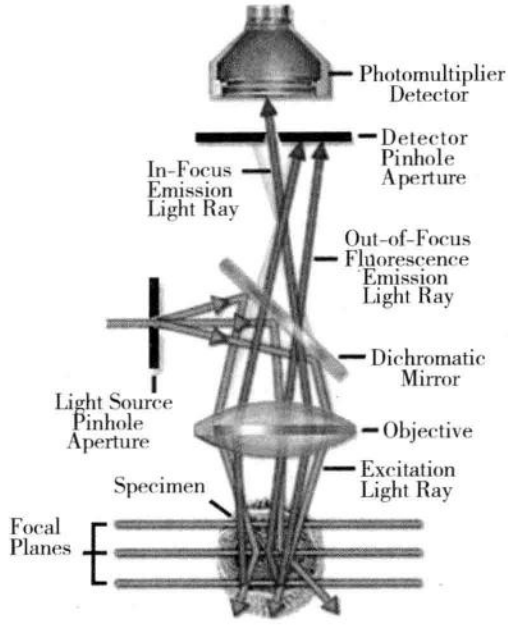
1.3 Fluorescence Microscopy and Confocal Microscopy

Light microscopy has been brought to the level of molecular analysis by methods for labeling specific molecules so that they can be visualized within cells. Specific genes or RNA transcripts can be detected by hybridization with nucleic acid probes of complementary sequence, and proteins can be detected using appropriate antibodies. Both nucleic acid probes and antibodies can be labeled with a variety of tags that allow their visualization in the light microscope, making it possible to determine the location of specific molecules within individual cells.

Fluorescence microscopy is a widely used and very sensitive method for studying the intracellular distribution of molecules (Figure 1.2). A fluorescent dye is used to label the molecule of interest within either fixed or living cells. The fluorescent dye is a molecule that absorbs light at one wavelength and emits light at a second

wavelength (see Chapter 10). This fluorescence is detected by illuminating the specimen with a wavelength of light that excites the fluorescent dye and then using appropriate filters to detect the specific wavelength of light that the dye emits. Fluorescence microscopy can be used to study a variety of molecules within cells. One frequent application is to label antibodies directed against a specific protein with fluorescent dyes, so that the intracellular distribution of the protein can be determined. Proteins in living cells can be visualized by using the green fluorescent protein (GFP) of jellyfish as a fluorescent label. GFP can be fused to a wide range of proteins using standard methods of recombinant DNA, and the GFP-tagged protein can then be introduced into cells and detected by fluorescence microscopy.

Confocal microscopy combines fluorescence microscopy with electronic image analysis to obtain three-dimensional images. A small point of light, usually supplied by a laser, is focused on the specimen at a particular depth. The emitted fluorescent light is then collected using a detector, such as a video camera. Before the emitted light reaches the detector, however, it must pass through a pinhole aperture (called a confocal aperture) placed at precisely the point where light emitted from the chosen depth of the specimen comes to a focus (Figure 1.2). Consequently, only light emitted from the plane of focus is able to reach the detector. Scanning across the specimen generates a two-dimensional image of the plane of focus, a much sharper image than that obtained with standard fluorescence microscopy. Moreover, a series of images obtained at different depths can be used to reconstruct a three-dimensional image of the sample.



(**Edited from *The Cell*: *A Molecular Approach*. 2nd edition. Cooper GM.**)

Figure 1.2 **Illustrations of fluorescence microscopy (left) and confocal microscopy (right)**

Left: fluorescence microscopy. Light passes through an excitation filter to select light of the wavelength that excites the fluorescent dye. A dichroic mirror then deflects the excitation light down to the specimen. The fluorescent light emitted by the specimen then passes through the dichroic mirror and a second filter (the barrier filter) to select light of the wavelength emitted by the dye.

Right: confocal microscopy. A pinpoint of light is focused on the specimen at a particular depth, and emitted fluorescent light is collected by a detector. Before reaching the detector, the fluorescent light emitted by the specimen must pass through a confocal aperture placed at the point where light emitted from the chosen depth of the specimen comes into focus. As a result, only in-focus light emitted from the chosen depth of the specimen is detected.

1.4 Electron Microscopy

Because of the limited resolution of the light microscope, analysis of the details

of cell structure has required the use of more powerful electron microscopy. The electron microscope (EM) can achieve a much greater resolution than that obtained with the light microscope because the wavelength of electrons is shorter than that of light. The wavelength of electrons in an electron microscope can be as short as 0.004 nm—about 100,000 times shorter than the wavelength of visible light. Theoretically, this wavelength could yield a resolution of 0.002 nm, but such a resolution cannot be obtained in practice, because resolution is determined not only by wavelength, but also by the numerical aperture of the microscope lens.

Two types of electron microscopy—transmission and scanning—are widely used to study cells. The transmission electron microscope (TEM) has a resolving power of 3Å – 10Å. The scanning electron microscope (SEM) is becoming increasingly popular with cell biologists because of its remarkable ability for quantifiable mapping of surface detail, along with improved resolution (30Å – 100Å) and its ability to show 3D structure.

The transmission electron microscope is identical in concept to the modern light microscope. It is composed of a light source (in this case an electron source), a substage condenser to focus the electrons on the specimen, and an objective and ocular lens system. In the electron microscope, the ocular lens is replaced with a projection lens, since it projects an image onto a fluorescent screen or aphotographic plate. Since the electrons do not pass through glass, they are focused by electromagnetic fields. Instead of rotating a nose-piece with different fixed lenses, the EM merely changes the current and voltage applied to the electromagnetic lenses.

Another characteristic of electron microscopes is that they are usually designed upside-down, similar to an inverted light microscope. The electron source is on top, and the electrons travel down the tube, opposite to light rays traveling up a microscope tube. This is merely a design feature that allows the operator and technicians ease of access to its various components. The newer electron microscope is beginning to look like a desk with a TV monitor on it.

Until recently, the major advantage of an electron microscope also has been its major disadvantage. In theory, the transmission electron microscope should be capable of giving a resolution of several angstroms. This would give excellent molecular resolution of cell organelles. However, as the resolution increases, the field of view decreases and it becomes increasingly difficult to view the molecular detail within the cell. Electron microscopes designed to yield high resolution have to