

Cells

A LABORATORY MANUAL

Volume 2: Light Microscopy and Cell Structure

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Cells
24 A Laboratory Manual

28
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Volume 1: Culture and Biochemical Analysis of Cells
Volume 2: Light Microscopy and Cell Structure
Volume 3: Subcellular Localization of Genes and Their Products

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Printed in the United States of America
Interior design Emily Harste

Project Managing Editor Kaaren Janssen
Project Coordinator Mary Cozza
Production Editor Patricia Barker

Desktop Editor Susan Schaefer
Cover Design Emily Harste and Ed Atkeson

Volume 2 front cover: Optical section obtained by confocal laser scanning microscopy of the nucleus of the unfertilized egg of the surf clam, *Spisula solidissima*. This late prophase premeiosis I nucleus contains condensed chromosomes and a prominent nucleolus labeled with a human autoantibody directed against DNA (green). The red ring at the edge of the nucleus is the nuclear lamina labeled with an antibody directed against the major clam nuclear lamin protein. This beautiful system has played an important role in studies of the cell cycle and is being used to study the mechanism of attachment of the chromosomes to the nuclear lamina. (Photo provided by S. Khoun, A. Goldman, R. Moir, T. Spann, and R. Goldman, Northwestern University Medical School, Chicago, and the Marine Biological Laboratory, Woods Hole.)

Library of Congress Cataloging-in-Publication Data

Spector, David L.
Cells : a laboratory manual / David L. Spector, Robert D. Goldman,
Leslie A. Leinwand.

p. cm.
Includes bibliographical references and index.
Contents: v. 1. Culture and biochemical analysis of cells -- v.

2. Light microscopy and cell structure -- v. 3. Subcellular
localization of genes and their products.
ISBN 0-87969-522-6 (cloth : alk. paper). -- ISBN 0-87969-521-8
(comb : alk. paper)

1. Cytology--laboratory manuals. I. Goldman, Robert D.
II. Leinwand, Leslie A. III. Title
QH583.2.S64 1997
571.6--dc21
DNLM/DLC
for Library of Congress

97-25924
CIP

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Observation of Live Cells in the Light Microscope

INTRODUCTION

The direct observation of the dynamic properties of known molecular species in vivo has been made possible through a variety of molecular, biochemical, immunological, and imaging techniques. For example, the now widespread use of GFP tags (see Chapter 78) permits the direct observation of specific cellular constituents in vivo. However, in order to observe live and healthy mammalian cells in the microscope field, there are technical problems with respect to the maintenance of temperature, pH, etc., of the culture medium. This is not a significant problem when observing non-mammalian or plant cells.

Typically, live-cell experiments can be classified into two categories: developmental studies to establish natural behavior, or induced change to study the effects of a controlled factor. The correlation between in vitro and in vivo phenomena is paramount for mammalian live-cell investigations. It is of the utmost importance to simulate the host conditions of the isolated specimen accurately during live-cell microscopy. This chapter describes multiple approaches for the micro-observation of live cells.

History

Specimens used for early microscopy were often live, or at least temporarily live. Techniques for fixing and staining were developed along with advances in microscopy. As microscope optics improved, the limitations of the optical system defined the necessity for specimen preparation. Microscopists played a game of give and take based on optical constraints. Magnification, numerical aperture, resolution limits, working distance, and correction for chromatic aberration were recognized as interrelated factors. In addition, methods of illumination to include bright-field, dark-field, phase, and fluorescence were brought into mainstream microscopy. With these forms of microscopy available, it was accepted that the specimen needed to be prepared to meet the optical limitations of the microscope. These preparations most often required the fixing of the specimen.

Cameras using film were combined with microscopes to document images. Conventional film-based photomicroscopy was suitable for most bright-field, dark-field, and fluorescence images because exposure time was of no concern. Movies were made of live cells in laboratories that had the necessary specialized equipment. Most of these movies were made using bright-field modes because film required higher light levels. Live-cell fluorescence movies were not possible due to the toxicity of the dyes and phototoxic effect of long exposures.

Although fixed cells yield less information in comparison to live specimens, there are some advantages. Fixed cells are flatter and last longer. They don't move or require constant care, and they can be stained with a variety of compounds to selectively enhance contrast. Most anti-fade solutions are compatible with fixed (but not live) cells.

When images of live cells were recorded on conventional film, the microscopist

was limited by the sensitivity and resolution of the film, which also limited the kinds of experiments that could be done. Film has many limitations when compared to modern, intensified, or cooled CCD cameras and photomultiplier scanning techniques. Consequently, early live-cell chambers were inherently limited by the associated technology. Early microscopes did not have the image-contrasting enhancements that modern microscopes have. Therefore, the live-cell microscopy that was done was either poor by today's standard or limited to high-contrast specimens.

Recent Technical Developments

The following developments have made live-cell microscopy easier:

- Imaging-contrast enhancement optical techniques, such as DIC, Hoffman, Phase, and Varel, fluorescence, and confocal microscopy (see Sections 9 and 10).
- Video cameras and quantitative CCD cameras capable of electronic contrast stretching.
- Desktop computers bringing quantitative imaging capabilities into mainstream science.
- Advances in the development of dyes and labeling techniques.
- Micro-environmental control systems developed to keep pace with the imaging technology.

Today these technologies have provided the instrumental foundation for conducting live-cell experiments, requiring less time for engineering equipment, and leaving the researcher more time for biology (Pentz and Schulle 1981; Webb 1986; Shotton 1988).

Cells can be maintained in an incubator, but putting the microscope in an incubator is impractical. The logical extension is to duplicate incubator conditions on the stage of the microscope. Cells need certain known regulated conditions such as nutrients, removal of waste products, carbon dioxide, a growth surface matrix or lattice in the case of tissue, and temperature control. In order for cells to be visualized, they must be in an environment that is both conducive to their viability and compatible with the microscope's optical limitations.

Microenvironmental Chambers

There are two basic forms of specimen containment and micro-environmental control. In applications where single-cell analysis is appropriate, the specimen must be contained in an optical enclosure. The two most commonly used methods of micro-observation are the open culture dish in which fluids are open to the atmosphere, and the closed system, which is sealed.

Typical open culture dish systems are the simplest form of live-cell observation and are generally well suited for short-term experiments or applications where physical access to the cells is necessary. The traditional method of growing cells in a container, then observing the cells on a peripherally heated microscope stage is adequate for investigations requiring only basic modes of relatively low-resolution microscopy. For more advanced investigations requiring high-resolution imaging using fluorescence, DIC, and/or confocal modes, a more advanced environmental control system should be used.

Various types of simple culture chambers were developed for observing live cells

under the microscope. Some of the more popular types include the Maximow chamber, the Romicron chamber, and the Rose chamber. The Maximow chamber was the simplest early chamber, consisting of a thick micro-culture depression slide made of heavy glass. An earlier version of a closed system was constructed by placing a gasket between a coverslip and a slide or two coverslips. The Romicron chamber is also made of plate glass, but with an optically ground cylindrical depression centered in the slide so that the bottom of the depression is optically clear. A double-coverslip technique is used to cover the chamber. This consists of a larger (usually) rectangular slide upon which is placed a smaller circular coverslip with the primary explant or cells of interest in medium. The smaller coverslip is held onto the larger slide by capillary action. This double cover is then inverted to span across the cylindrical chamber and sealed (typically with beeswax). This sort of chamber is appropriate for use with experiments where perfusion is not needed and the experiment is of short duration so that pH, CO₂, temperature, and the buildup of waste products are not common problems. The Rose chamber is a more elaborate version of a simple closed chamber, consisting of a thick plate glass slide having a circular opening through the slide to form the chamber. The top and bottom of the chamber are sealed, typically with coverslips placed above and below the opening through the slide, often with a rubber O-ring or gasket between the slide and a coverslip to allow perfusion into and out of the chamber. This sandwich of coverslip, O-ring, slide, coverslip is held together by aluminum rims on the top and bottom of the sandwich that may be screwed together to form a tight seal. Although these simple chambers may be suitable for many purposes, the more sophisticated chambers described in this chapter generally provide a more advanced environmental control system, appropriate for more complex experimental observations.

Why a Chamber?

A chamber can be any structure with optical, fluid, and temperature control capabilities. This may be a simple culture dish or flask, or a complex dedicated live-cell environmental system.

- Observation of single cells in organisms with traditional, diffraction-limited optics is difficult and sometimes impossible. Therefore, cells have to be studied in an isolated culture.
- The specimen needs to be studied on a flat transparent surface.
- Cells also need a fluid containment structure for liquid media of a suitable volume.
- Perfusion may be needed to maintain control of pH, for removal of waste products, and to add reagents that will alter cellular physiology.

Types of Environmental Control Techniques

Aside from the optical and physical embodiment of a chamber, a number of methods of temperature control are used. This is especially important in studies of mammalian cells. Thermal regulation devices employ a variety of techniques, including air-stream specimen warmers; electro-resistive elements; Peltier effect; pumped, thermally controlled fluids; infrared illumination; and first-surface thermal transfer.

Temperature control factors include range, accuracy, speed, and recovery. In an effort to meet these requirements, it may be necessary to take drastic steps. Over the years, temperature has been controlled using extreme measures, such as placing the entire microscope in an incubator with only the eyepieces exposed. This is very expensive and detrimental to the microscope. Another option has been to encase the stage of the microscope with a Plexiglas chamber while pumping in heated air. The most popular option is to use a heated plate or stage warmer to surround the culture dish or coverslip chamber. The least desirable techniques are warm air blowers and/or infrared lamps surrounding the stage. This is because these devices involve maintaining temperature by switching heaters on and off continuously, frequently causing focal drift and contamination. An alternative solution to the above techniques utilizes first-surface thermal transfer to regulate cell temperature. An optical cavity or coverslip can be temperature controlled by passing a current through an electrically conductive coating on one of the optical surfaces that defines the optical cavity. This technique has proved to have many advantages over traditional methods.

The following is a list of some of the typical difficulties encountered with traditional chambers:

- Coverslip breakage that occurs during assembly.
- Leakage onto and sometimes into the microscope.
- Lack of flexibility to accommodate varied volumetric requirements.
- Fixed flow characteristics.
- Flow rates slower than biological events to be recorded.
- Minimal volumes larger than required by protocol.
- Inadequate temperature control.
- Lack of compatibility with all modes of microscopy.

SELECTING A MICRO-ENVIRONMENTAL SYSTEM

Microscope Factors

Generally, inverted microscopes are used for cultured cells, but in some cases, an upright microscope is appropriate. When using an upright microscope, some concessions have to be made depending on the intended modes of microscopy. A major component of the microscope that needs special consideration is the objective. Special care must be taken in determining the magnification, working distance, and numerical aperture, all of which are related to the depth of field. It is also important to realize that immersion-type objectives will need to be temperature controlled when used with mammalian specimens. If a transmitted light condenser will be used, allowance must be made for the working distance, physical size, and proximity to the chamber. The geometry of the stage also must be matched or mated to the environmental chamber.

Micro-environment Physical Characteristics

When using an open system, the following characteristics need to be addressed: volume, clear aperture, material, bottom thickness, geometry, evaporation and condensation, ambient light, view angle, and biocompatibility.

When selecting a closed system, the following characteristics need to be addressed: fixed or variable volume, separation of optical surfaces, perfusion flow

characteristics, volume exchange rate, laminarity, shear stress, and flow channel geometry.

Typical Culture Dish Observation

In applications where cells are being observed with low numerical aperture (N.A.) objectives, an open culture dish and phase contrast or DIC microscopy are frequently used. Today's sophisticated experiments, however, are far more demanding on the cell's environment, especially as it relates to microscopy. For example, during long-term development or induced-change studies, it is necessary to accurately simulate the host conditions on the microscope stage without limiting the resolution of the microscope and to maintain the simultaneous flexibility to change variables.

This simulation should include control of temperature, medium, pH, and environmental toxicity. Medium and pH can be controlled by regulated perfusion. The toxicity of the artificial environment must be evaluated against compatibility with the specimen (Inoue 1986). Presuming the artificial environment is nontoxic or biologically inert, one of the most difficult factors to control has been temperature. With all the relevant papers on the importance of accurate temperature control and its effect on data, it should be readily apparent that temperature is a critical factor in most mammalian live-cell experiments (Taylor and Wang, 1989; Taylor et al. 1994).

When using applications requiring high N.A. objectives, most investigators use carefully supported coverslips to attain optical compatibility and peripheral stage warmers to simulate the host environment. This frequently means sacrificing the accuracy of thermal control, and/or adequate perfusion of the cells, resulting in either inaccurate data, due to compromised cells, or poor images.

The characteristics and limitations of traditional methods of open dish micro-observation are summarized here (see Fig. 75.1).

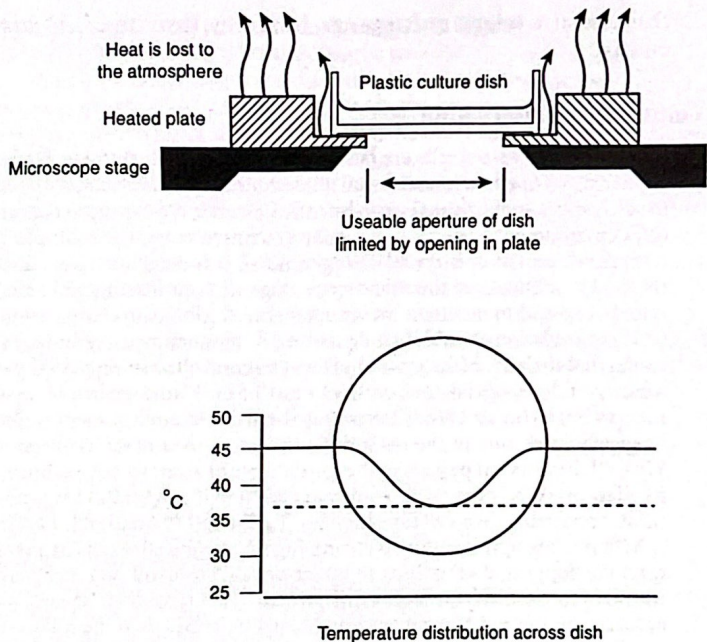
Conventional culture dishes are made of plastic, which is known to have the following characteristics:

- Nonuniform optical surface, which degrades the image.
- Strain in the optical surface due to the injection molding process, which prohibits the use of optical systems employing polarized light, including DIC (see Section 9).
- Thickness of the bottom surface, which prohibits the use of high N.A. objectives.
- Inefficient heat transfer from the source to the cells, which results in long temperature-stabilization cycles.

Traditional culture dish warmers are peripheral heating devices that must radiate heat through two poor, thermally conductive media (air and plastic), therefore resulting in:

- Nonuniform temperature distribution.
- Excessive thermal transfer time during thermal recovery (on the order of minutes).
- Thermal expansion of the metal surface causing vertical displacement apparent during 3D imaging or confocal applications.

If these factors do not present a problem, a stage-warmer-based system is adequate and preferable. However, if the experiment requires more accurate temperature control or optical compatibility, the techniques described in the following section ought to be considered.

**FIGURE 75.1**

Traditional open culture dish microscopy. The traditional technique is limited by slow and inefficient stage heaters, resulting in nonuniform temperature distribution. In addition, plastic dishes are poor conductors of heat; thus the temperature does not recover quickly during or after perfusion. Finally, the usable aperture of the dish is limited by the opening in the heat-transfer plate.

Advances in Culture Dish Live-cell Systems

An alternative solution to these problems is to use a micro-environmental system that utilizes a first-surface thermal transfer technique. This technique utilizes a thin-film coating of a transparent, electrically conductive coating applied to the bottom surface of a glass substrate, which is then incorporated into a hybrid disposable culture dish structure optimized for live-cell imaging. This technique provides temperature control directly to the cells through a thermistor feedback loop which applies an electrical current through the coated underside of the glass substrate, without greatly affecting its transmission spectrum. The thermal response using this technique is as fast as 0.1°C/sec. Because of this fast response time, the controller can regulate the current control within seconds, making it possible to compensate for temperature changes that occur in the dish due to surface evaporation or perfusion. This fast response time enables the addition of high-speed safety circuitry to protect the cells in the event of error. This technique offers high-resolution imaging capabilities through a uniform glass surface free of strain and available in a variety of thicknesses, including the popular No. 1.5 coverglass for high N.A. applications.

The dish environment is compatible with all modes of microscopy including, but not limited to, bright-field, dark-field, phase, DIC, fluorescence, reflection interference, and confocal.

The first-surface thermal transfer technique, introduced on a coverslip in 1993, provides the basis for many adaptations of the basic principle. Since that time, numerous applications and accessories have been employed. After becoming familiar with the accuracy, ease of use, and superior optical imaging obtainable in a first-surface thermal transfer system, it is easy to envision and implement numerous derivations for specific applications other than isolated cell culture. The following are examples of some of these uses, accessories, and benefits, based on the Delta TC3 System (see Fig. 75.2; Bioptechs, Inc.).

- Tissue slice specimens, natural or artificial, can be easily suspended in an optical reference plane in the dish at the appropriate focal distance from the objective for ease of observation.
- Transmembrane migration or diffusion of cells or ions can be dynamically observed during isolated perfusion of inhibitors or changes of ion concentration to the basilateral surface.
- A hinged perfusion adapter set provides a convenient and inexpensive method of positioning perfusion needles in the culture dish. Typically, these are used to maintain low-volume perfusion during long-term experiments.
- A coverglass lid is available for use during microscopy to define an optical surface above the specimen and on the surface of the liquid in the dish. When a series of images are acquired in transmitted light mode, there is a changing fluid interface

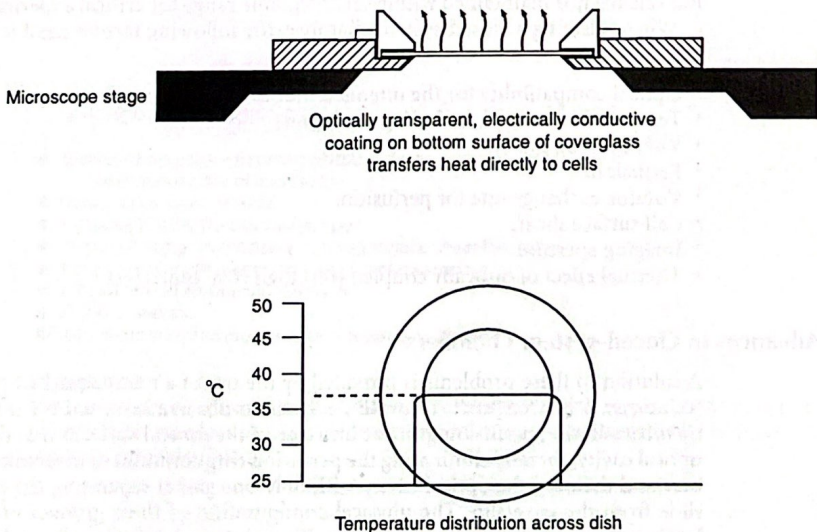


FIGURE 75.2

First surface thermal transfer. Some advantages of the system are the highly accurate temperature control, fast thermal recovery, and uniform temperature distribution.

between air and the surface of the medium, causing image contrast shifts. Forming an optically flat surface on top of the medium eliminates this problem.

- An electrically heated lid eliminates condensation from forming on the under surface of the cover. The heated lid is reusable and powered by a low-voltage source.
- A cooling ring which circulates an isolated refrigerant fluid within the dish can be used to reduce the specimen temperatures to below ambient.

Traditional Closed-system Chambers

A closed-system chamber is required when there is a need either for complete isolation of the specimen from the outside world (DeBlasio et al. 1987; Farkas et al. 1993, 1995) or for the use of more advanced microscopic methods that are incompatible with the use of open dishes. In this case, cells are placed in a temperature-controlled, perfusable, optical cavity or plated onto a coverslip which is part of this cavity for microscopy. There are several traditional configurations of these closed-system chambers commercially available. Nearly all of them utilize the same basic characteristics (Fig. 75.3).

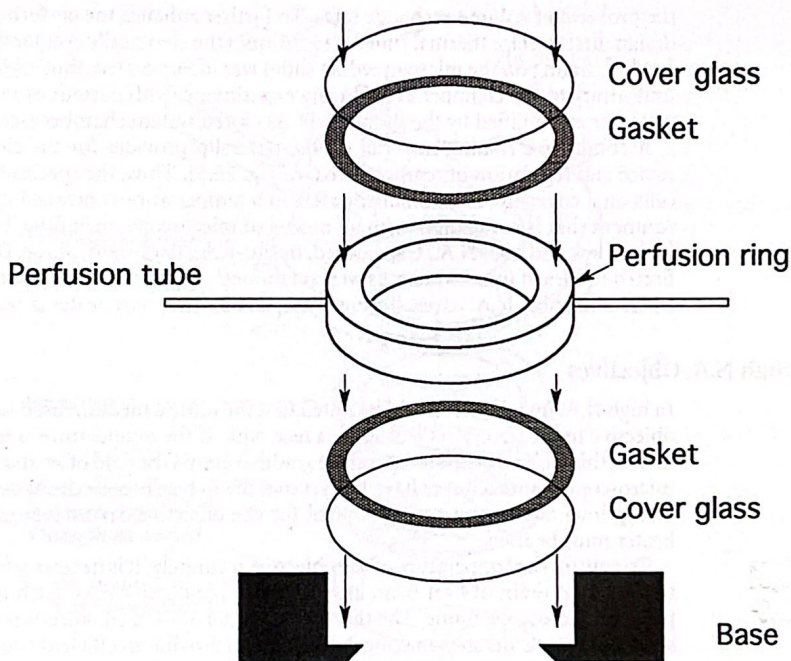
Traditional closed-system chambers provide two optical surfaces separated by a perfusion ring sealed with gaskets. This sandwich is then clamped together by several other structures. With this type of enclosure, the perfusion rate, volume, and optical suitability for various modes of microscopy are interrelated. In most cases, perfusion with this configuration may result in turbulence that can dislodge cells, and there is a trade-off for optical compatibility with all modes of microscopy. Furthermore, temperature control is usually achieved through the use of peripheral heaters or warmed air blown across the stage. In either case, temperature control is not reliable nor maintained within an acceptable range for critical experiments.

When selecting a closed-system chamber, the following factors need to be considered:

- Optical compatibility for the intended modes of microscopy.
- Temperature control, including uniformity.
- Volume of chamber.
- Perfusion.
- Volume exchange rate for perfusion.
- Cell surface shear.
- Imaging aperture.
- Thermal effect of optically coupled (oil) high N.A. objectives.

Advances in Closed-system Chambers

A solution to these problems is provided by the use of a microaqueduct perfusion technique. A microaqueduct flow live-cell micro-observation chamber is prepared by incorporating perfusion grooves into one of the optical surfaces that define the optical cavity, thereby eliminating the perfusion ring common to most other chambers and defining the optical cavity with only one gasket separating the perfusion slide from the coverslip. The physical configuration of these grooves produces a laminar flow region in the optical cavity. The single gasket design allows the user to define the size, volume, thickness, and shape of the optical cavity. In addition, microaqueduct perfusion provides large aperture flow inputs and outputs, eliminating



INHERENT SHORTCOMINGS

- Excessive separation between optical surfaces limits condenser N.A. in transmitted modes of microscopy
- Fixed optical cavity volume
- Requires lengthy fluid exchange rates
- Turbulent perfusion produces high cell surface shear/flow rate
- Not compatible with certain modes of light microscopy
- Difficult to load and secure with cells
- Prone to leakage
- Temperature control requires use of a warm air blower

FIGURE 75.3

Traditional closed-system chamber. Some disadvantages of this system are fixed optical cavity volume, production of turbulence with perfusion, and incompatibility with certain modes of light microscopy.

the problem of volume exchange rates. To further enhance the performance of this design, first-surface thermal transfer technique (the electrically conductive, thermal control coating on the microaqueduct slide) was incorporated, thus adding thermal uniformity to the chamber even during experiments with periods of no flow. This system is exemplified by the Biopotech FCS2 closed system chamber (see Fig. 75.4).

A conductive coating material on the coverslip provides for the close maintenance and regulation of temperature (see Fig. 75.5). Thus, the specimen, adherent cells on a coverslip, is maintained safely in a temperature-controlled optical environment that is compatible with all modes of microscopy, including, but not limited to, low and high N.A., transmitted, bright-field, dark-field, phase, DIC, and reflected modes of fluorescence, as well as confocal. For optimal temperature control when using high N.A. lenses (immersion), an objective lens heater is required.

High N.A. Objectives

In high N.A. imaging, it should be noted that the optical medium used to couple the objective to the coverglass will act as a heat sink. If the temperature is not also regulated, this will result in a temperature gradient across the field of as much as 5°. The microscope manufacturers have not yet met the live-cell researchers' needs by providing integrated temperature control for the objective, so an external objective heater must be used.

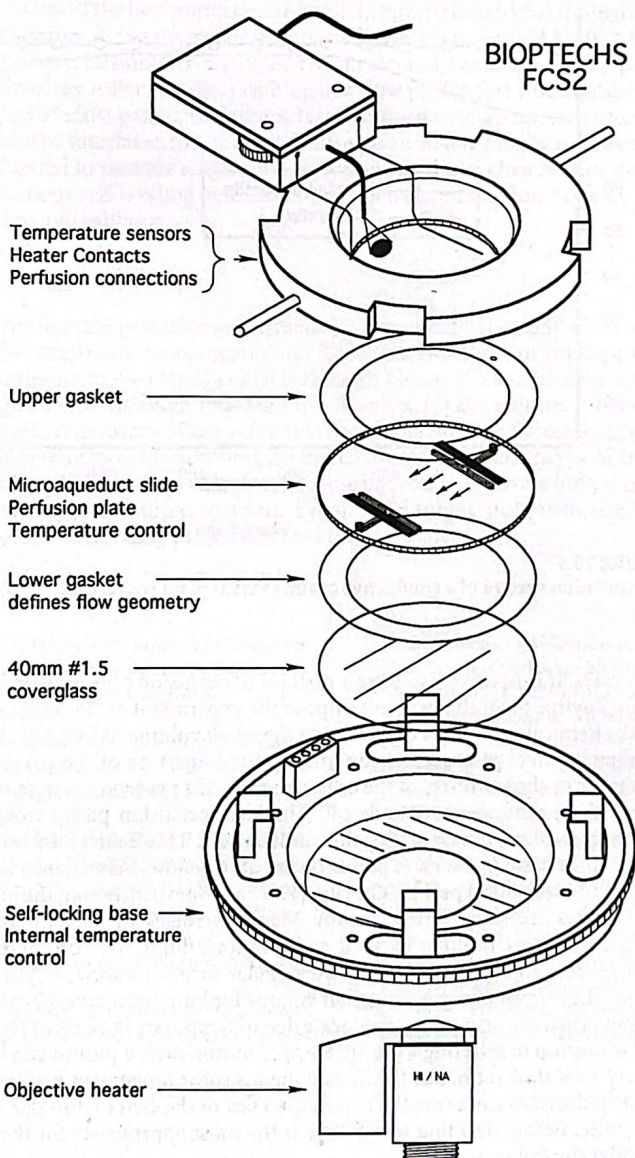
To control the temperature of the objective accurately, it is necessary to overcome the constant drain of heat from any thermally conductive mass such as the nose-piece or microscope frame. The thermal characteristics of all objectives and microscopes vary considerably, making it necessary to provide an efficient transfer of heat to the objective with an intelligent controller. Such a system would sense the temperature of the objective at a point close to the specimen and regulate the heat applied to the objective, while taking into account the thermal mass of the objective and the ambient conditions. A device for this purpose, which particularly surrounds the upper portion of the objective central tube with a heating band and measures the thermal transfer in a gap formed between the ends of the heating bands, is available from Biopotech, Inc. The controller regulates the heater current in such a manner that it maintains the temperature to within 0.1°C.

Although the objective heater is designed to fit most objectives, it does not fit all objectives. Some objectives, especially the water-coupled ones, are particularly difficult to work with. The main problem is their large diameter and lack of adequate surface area on which to transfer heat. Sometimes their size makes it difficult, if not impossible, to have sufficient working room to focus on the specimen.

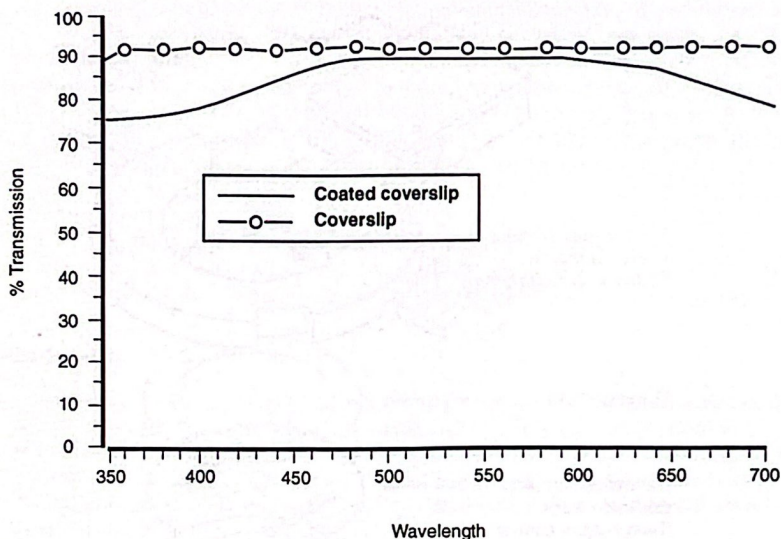
The microscope manufacturers have agreed that heating of the objectives to 37°C will not harm the objectives, but they have not made any comments about daily heating. It is recommended that the objective be stored in a 37°C enclosure when not in use to reduce the possibility of detrimental effects of thermal cycling between physiological and room temperatures.

Perfusion

Perfusion is necessary for one of the following reasons: Either experiments on live cells take place over a time span greater than cells will tolerate without fresh media, or the experiment is one in which chemical change is induced through perfusion. In

**FIGURE 75.4**

Microaqueduct slide technique. This system is characterized by sophisticated temperature control, high-volume perfusion rates, and compatibility with standard microscopy techniques.

**FIGURE 75.5**

Transmission spectra of a conductive coating versus plain coverglass.

both cases, it is necessary to have a method of perfusing cells in their micro-observation environment that will not impede the experiment or the acquisition of electrical, chemical, or optical data. Due to the small volume of live-cell chambers, the diaphragm effect produced at the unsupported aperture of the coverslip, and the sensitivity to shear stresses of the cells, selection of a perfusion system requires great care and, in some cases, a trade-off. The ideal perfusion pump would provide a closely controlled, purely analog flow indefinitely. This is currently not available.

The three basic methods of perfusion are gravity flow, manual injection with a syringe, and mechanical pumps. Gravity flow is very inexpensive but difficult to control at flow rates necessary for microscopy. Manual syringes are ideal for adding growth factors, inhibitors, or other periodic small-volume fluids. Mechanical pumps are the most reliable and are available in two popular forms, motorized syringe and peristaltic. The syringe pump is limited in volume for long-term experiments and subject to flow variations on a micro-flow scale due to temporary sticking of the plunger.

Use caution in selecting a pump: Stepper motor-driven pumps can be controlled to very slow flow rates, but the instantaneous rotor movement produces hydrodynamic pulses that can cause the coverslip to flex or the cells to dislodge with the sudden pulse. Before deciding which type is the most appropriate for the application, consider the following:

- Flow rate.
- Uniformity of flow rate.
- Range of flow rate.
- Quality of flow.

One of the best pumps is the Lambda pump (Bioprotech) for perfusion during microscopy. It is a peristaltic pump small enough to be held in the palm of one hand. It uses a tachometer-regulated DC motor and a multistage, step-down gear box to drive the roller spindle, resulting in a flow profile free from sudden pulsations typical of most peristaltic pumps. It is equipped with an internal speed control. It can also be interfaced to a computer through an analog interface. The pump can be configured to provide single tube perfusion for closed chambers or dual tube for continuous self-leveling perfusion in an open chamber. Flow rates are adjustable from 2 to 180 ml/hour.

Tubing

For live-cell perfusion experiments, it is prudent to use USP Class 6 certified tubing for maximum biocompatibility. There are two types of tubing that meet this requirement: PharMed (available through Fisher, VWR, and other suppliers) and Tygon 2275. Although PharMed is well suited for cell culture, it has one main drawback: It is opaque. The perfusate is not visible through the tubing; therefore, bubbles that may form in the tubing are hidden from view. This is a major concern when using a closed-system chamber, because bubbles in a narrow optical chamber become dislodged by surface tension. Tygon 2275 tubing in $\frac{1}{16}$ inch size is clear and not prone to leaching plasticizers into the perfusate.

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