Chapter 12

Culturing Cells on the Microscope Stage

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I. Introduction

Keeping live, healthy cells on the microscope is crucial for many types of experiments, in particular, fluorescence microscopy of dynamic processes. Although cultured cells may be observed for a short period of time without any special device, a culture system is usually necessary for observations which last for more than 10 minutes. Since the development of tissue culture techniques, many different approaches for this purpose have been taken. In this chapter, we will review the requirements which govern the design of microscope culture systems and discuss in detail several different systems, including one designed and used successfully in this laboratory. We will, however, not describe simple chambers for short-term maintenance of living cells (see, e.g., McNeil, Chapter 10; Chen, Chapter 7; and Swanson, Chapter 9, this volume, for several designs of simple observation chambers).

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II. Requirements of the Microscope Culture System

A. Requirements for Maintaining the Cell

The major requirements for maintaining the cell are temperature, pH, osmolarity, and nutrients. Cultured mammalian cells should generally be maintained at a temperature close to 37°C. However, with the exception of temperature-sensitive mutants, most cells can tolerate a slightly lower temperature (e.g., 32–34°C), as well as slight variations in the temperature (±1°C).

Generally, pH of the medium should be maintained between 7.2 and 7.4. Although cells may be kept in a nonbicarbonate-based buffer system for a limited period of time, the bicarbonate system is still the ideal choice for long-term cell culture. However, its use requires either sealing the chamber or providing the chamber with CO_2 in order to maintain the proper pH. Sometimes HEPES is added to the medium to improve the buffering capacity. It slows down and decreases the extent of the change in pH, but does not eliminate entirely the requirement of CO_2 (Freshney, 1983).

The osmolarity of the medium must also be maintained at a physiological level. Drift in osmolarity is usually caused by evaporation. Cells are also sensitive to rapid changes in osmolarity, which may be induced, for example, by a sudden replacement of evaporated medium with fresh medium.

Finally, nutrients and minor components (e.g., growth factors) must be supplied to the cell by perfusion or periodic replacement of the culture medium. Although normally each change of medium can last for 24 to 72 hours, more frequent replacement may be required for microscope culture chambers with a very small volume.

The required performance of a microscope culture system depends on the period of observation, the nature of the experiment, and the property of the cell. On the one hand, for short-term observations, the best design may be simply a sealed cover slip. On the other hand, for long-term experiments of highly sensitive cells, precise monitoring of the environment coupled to automatic perfusion of solutions may be necessary. For most purposes, the optimal solution is likely to be a compromise between the two extremes.

B. Practical Requirements

The culture system should also fulfill various requirements of the experiments. First, any culturing system should be easily sterilized. Once sterilized, there should be little or no manipulation required on the substrate for cell culture. The container should be easily covered or sealed during observation to minimize the exposure to sources of contamination. However, it

should also offer good accessibility if the experiment involves microinjections or physical manipulations of the cell. Access to the medium is also important for the addition of external agents in some experiments. If the experiment involves cell cloning or preparation for immunofluorescence after microscopic observations, the culture system should also allow such tasks to be easily performed.

The dimensions of the culture chamber, including the overall size, the surface area for cell culture, and the depth of the chamber are important considerations for many experiments. The culture container should fit easily onto the microscope stage, yet large enough to accommodate a sufficient number of cells in easily accessible and observable areas. The depth should be shallow enough to ensure good optical quality for transmitted light and adequate accessibility of cells, but large enough to provide enough medium around cells.

Good optical quality is essential for microscopic observations. Plastic tissue culture dishes are excellent in many respects, but they cannot be used with high resolution objectives. Not only is their thickness incompatible with the short working distance of most objectives for fluorescence optics, the strong fluorescence emitted by the plastic material makes fluorescence microscopy essentially impossible. In order to obtain high-quality images, glass cover slips must be used. They should be washed thoroughly and treated with acid–alcohol or extracellular matrix proteins, such as collagen or fibronectin, to promote the attachment of cells. Although most objectives are designed for No. 1 cover slips, No. 2 cover slips can often be used here to provide a higher mechanical strength of the chamber without seriously compromising the quality of images.

Finally, different culture systems may vary widely in terms of ease of setting up, the versatility, the sturdiness and reliability, and the cost. While a highly sophisticated and expensive setup may be ideal for certain experimental protocols, frequently it is more productive to have a large number of simple culture dishes. It should also be clear that there is no perfect system for all purposes, and the strength and weakness of each system must be evaluated in relation to the specific experiments. In the following sections, we will discuss a small number of representive culture systems.

III. Cell Culture Chambers

The most sophisticated chambers incorporate mechanisms for the control of various parameters, without the need of a separate microscope

incubator. The Leiden Culture System (Fig. 1; Ince *et at.*, 1983, 1985; commercially available from Medical Systems Corp., Greenvale, New York) is such an example. A round glass cover slip of 24-mm diameter is assembled with a Teflon ring to form a dish, which may be placed in a regular CO_2 incubator during the initial plating of cells. The dish is inserted into a microincubator before setting up on the microscope. The microincubator maintains the temperature by activating a heating coil and by directing warm CO_2 across the surface. With the temperature probe immersed directly into the medium, the temperature can be controlled precisely (see Section IV, B). Evaporation is controlled by layering mineral oil across the surface, an effective technique used by electrophysiologists for many years. Otherwise, the chamber remains open and allows access of microelectrodes, although the actual area accessible is limited by the hindrance of the rim of the chamber and the small diameter of the cover slip. Direct replacement of medium is also difficult due to the presence of oil. In addition, the culture chamber is connected to a number of tubings and cables, which may be a nuisance. Finally, the system is designed primarily for the inverted micro-

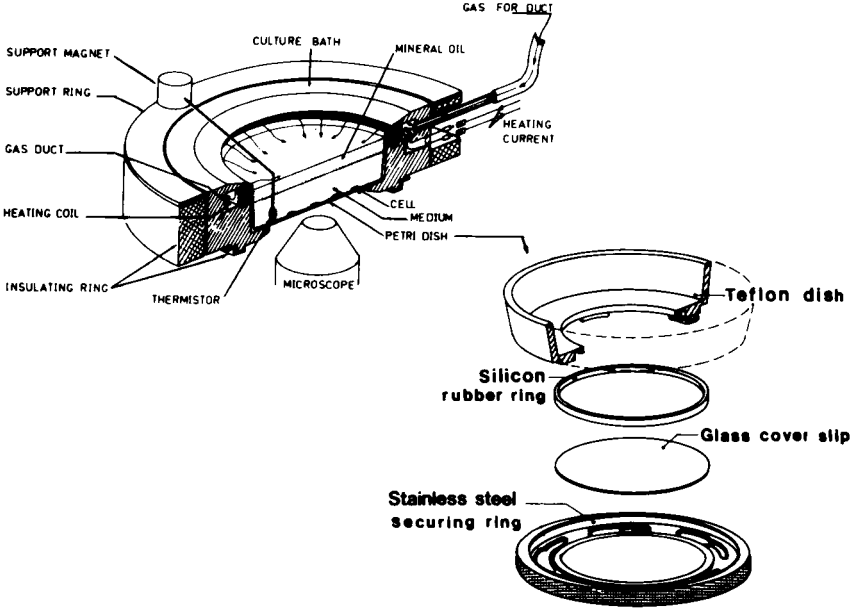

FIG. 1. Diagram of the Leiden Culture System (kindly supplied by Medical Systems Corp.).

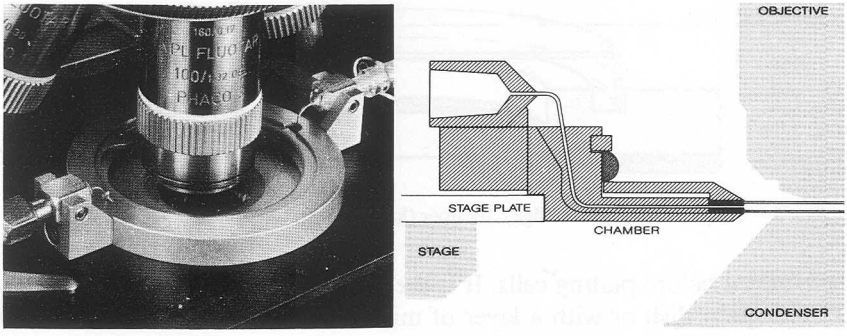

FIG. 2. Diagram and photograph of the Dvorak-Stotler Chamber (kindly supplied by Nicholson Precision Instruments).

scope; both the oil and the rim interfere with the use of objectives in upright microscopes.

The Dvorak-Stotler Chamber (Fig. 2; Dvorak and Stotler, 1971; commercially available from Nicholson Precision Instruments, Inc., Gaithersburg, Maryland) represents one of the sealed chambers with ports for perfusion. Unlike the Leiden Culture System, both the top and bottom of the chamber consist of cover slips. The two cover slips are separated by a very short distance, 1.2 mm. The most important advantage of the shallow depth is the ideal optical qualities for transmitted illumination in both upright and inverted microscopes. It allows the use of condensers of very short working distances, such as those for high-resolution Nomarski optics. However, cells are inaccessible for physical manipulations and are subject to fluid shear unless the rate of perfusion is very slow. Setting up the chamber without introducing air bubbles may also require practice. The condition of the medium is maintained by the sealed chamber and by a continuous perfusion of medium. Since the chamber has no temperature control system, it must be heated with a separate incubator (such as an air curtain incubator, discussed later). The relatively high price prohibits the acquisition of multiple systems. Therefore, when more than one cover slip is involved in the experiment, they must be placed in plastic culture dishes during initial cell culture, removed before observation, and mounted into the chamber: a tedious process with risks of contamination.

Less complicated culture chambers are also available. The Gabridge Chamber (also called Chamber/Dish; Fig. 3; Gabridge, 1981; commercially available from Bionique Laboratories, Inc., Saranac Lake, New York) is basically just a cover slip held tightly in place against a Teflon plate with the help of a metal pressure plate and an O-ring. The chamber is assembled and

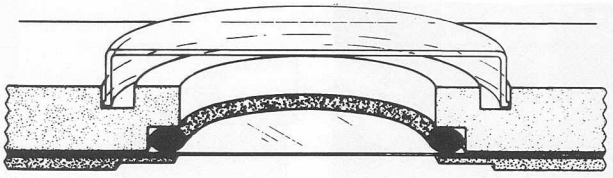

FIG. 3. Diagram of the Gabridge Chamber (kindly supplied by Bionique Laboratories).

autoclaved before plating cells. It is then covered with the top of a regular 35-mm petri dish or with a layer of mineral oil as for the Leiden Culture System. The former allows direct replacement of the medium and avoids any possible effect of mineral oil. However, it is not as effective as the oil in preventing evaporation and sometimes collects condensation on its lower surface. Because of the open nature, the chamber allows cells in the center and distal areas to be manipulated, although a significant fraction of cells hide under the edge of the Teflon core and are inaccessible to microelectrodes. Like the Leiden System, Gabridge chambers are designed primarily for the inverted microscope in conjunction with condensers of relatively long working distances. The chamber also requires the use of a separate device for the maintenance of temperature and pH. However, one major advantage of the chamber is the low cost, which makes it practical to acquire multiple sets in a regular laboratory.

The culture chamber used in our laboratory represents a simplification of the Gabridge Chamber (Fig. 4). It consists of a 70 × 50 × 6 mm plexiglass or Teflon plate with a 35-mm diameter annulus drilled through the center. These dimensions can be varied to suit individual circumstances. One side

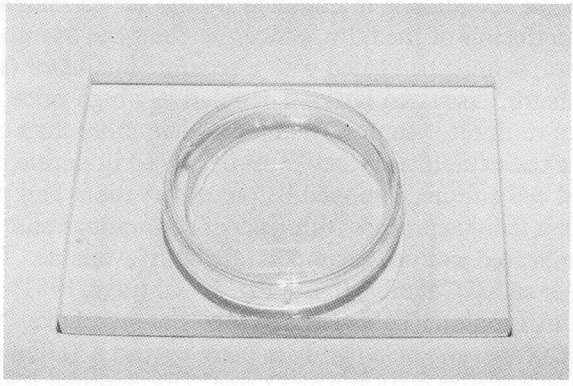

FIG. 4. Photograph of the culture chamber used in our laboratory.

of the annulus is covered by attaching a 45 × 50 mm cover slip with a continuous strip of Dow-Corning high-vacuum grease around the annulus. The assembled chamber is autoclaved and used much like a Gabridge Chamber. Because of the similarity to the Gabridge Chamber, most comments about the Gabridge chamber also apply here.

IV. Microscope Incubators

For most culture chambers, it is necessary to maintain the culture condition, as discussed previously, with separate devices. However, few microscope incubators are commercially available, and those available often require the addition of extra components to cover all the requirements. Therefore, we will discuss pH, osmolarity, and temperature controls separately.

A. Maintenance of pH and Osmolarity

For a sealed culture chamber with perfusion ports, these parameters are normally maintained by continuous perfusion. A syringe pump is usually used to maintain a constant flow.

For open chambers, even with an oil overlay, the pH of the bicarbonate buffer must be maintained by a continuous supply of CO_2. Although the actual requirement of CO_2 is about 5%, we found a gentle stream of pure CO_2 more effective due to the leakiness of the microscope incubation system in general. A simple system may be constructed with a gas flow regulator, which controls the flow of CO_2 up to 1000 ml/minute, and a tubing held near the culture chamber. The flow is adjusted to maintain the pH in an optimal range, as judged by the color of phenol red in the medium. The required flow of CO_2 varies with the design and the manipulation involved. A higher rate is required if the microscope is not enclosed or if the enclosure is open for manipulations.

For a more precise regulation of CO_2 concentration, an enclosure (see next section) with a CO_2 sensor may be built. A solenoid control valve is then regulated based on the CO_2 concentration detected inside the enclosure. Such devices are available from manufacturers of incubators. However, in a leaky enclosure the triggering of the control solenoid may be frequent and annoying. In addition, since it is difficult to obtain a uniform distribution of CO_2 inside the enclosure, the location of the probe may be critical.

Maintaining the osmolarity in an open dish is difficult, especially if hot

air is used for temperature regulation (see section below). The problem may be minimized by covering or sealing the chamber as much as possible, by making a special cover with slots matching the requirement of accessibility (Albrecht-Buhler, 1987), by placing a dish of water at the outlet of hot air to humidify the air (see next section), by periodic replacement of the medium, and by the oil overlay technique as mentioned above.

B. Temperature Regulation

Essential components for temperature regulation are a temperature sensor, a heater, and a controller which regulates the heater according to the signal from the sensor.

The most direct and precise way for monitoring temperature is immersing a sensor into the medium (Bright *et al.*, 1987). Its disadvantage is also clear: risks of contamination and/or extra efforts in setting up. Placing the sensor anywhere else introduces the possibility that the temperature of the medium may be significantly different from the set temperature. For example, since the culture chamber is in contact with both the air and the microscope stage, unless the two are identical in temperature, neither is ideal for placing the probe. In addition, unless air temperature is regulated both above and below the culture chamber, their differences in temperature will both make the medium temperature unpredictable and cause warping of cover slips, which leads to drifts in focusing.

One possibility to solve these problems is to enclose the stage and the entire air space around the chamber within an insulated incubator (see below). The probe may then be located on the stage while still allowing a precise control of the temperature. In a more open system, consistent results may also be obtained if (1) enough time is allowed for the system to reach a steady state before use; (2) the medium temperature is calibrated as a function of the set temperature; (3) a constant amount of medium is used in all experiments; and (4) a constant temperature is maintained in the laboratory.

The controller for the heater must be of the proportional type. At steady state, it will maintain the heater at a steady and low power. Inexpensive on–off types alternate only between the fully on or off states, and the fluctuation in temperature may be very large.

There are several types of heaters. Those incorporated into the microscope stage are most convenient, but available only for a limited type of microscopes. In addition, unless the cover slip is directly in contact with the stage, the heating may not be efficient. A second type, referred to as "air stream" or "air curtain" incubators, simply blows warm air at the specimen (Orion Research, Cambridge, Massachusetts; and Nicholson Precision In-

struments; it may be built simply by combining an appropriate hair dryer with a proportional controller and a probe). It can be positioned conveniently with little interference to the use of the microscope; however, the temperature of the medium may vary with the positioning and continual warming and cooling can strain high-quality objectives. In addition, if the warm air is directed at an open dish, evaporation and loss of CO_2 may be rapid. A variation of the warm air blower uses an infrared source (Opti-Quip, Highland Mills, New York), thus reducing the problem of air stream. However, the infrared radiation is easily picked up by most electronic detectors, such as low light level video cameras.

The warm air blower may be used in conjunction with an enclosure incubator for the microscope, so that the air in the enclosure is recirculated continuously through the heater. This has several advantages: the enclosed air mass serves as a temperature buffer and reduces the air flow required for maintaining a steady state; the CO_2 concentration is better maintained; and warm air does not have to be directed at the culture chamber. The disadvantage, physical hindrance caused by the enclosure, may be minimized with a proper design incorporating one or more sliding or hinged doors.

While enclosure incubators are difficult to design for the upright microscope, they are commercially available for Nikon and Leitz inverted microscopes. Such enclosure incubators, however, appear overpriced and may not suit the particular experimental needs. Therefore, we designed and built our own enclosures with plexiglass.

Our incubator is built to rest on the edges of the gliding stage of a Zeiss IM35 microscope (Fig. 5). With a mechanical stage, a simple frame has to be added under the incubator to avoid interference with the moving sample carrier. The sides of the enclosure are insulated with foam pads. Access to the chamber is provided by sliding doors on its left- and right-hand sides. Two round plexiglass ports at the rear wall permit connection to the inlet and outlet of a warm air blower, which is a compact, adjustable hair dryer set at a power of 600 W. Flexible aluminum pipes (2-in. Volkswagon preheating duct) are used to bring warm air from the blower into the enclosure, in order to isolate any vibration. Humidification of the air is achieved by placing a dish of water at the outlet of warm air (where it enters the enclosure). The hair dryer is plugged into a proportional temperature controller (Yellow Springs Instruments, Yellow Springs, Ohio; Model 72), which receives signals from a surface probe (YSI Mode 421) attached to the stage with silicone glue.

Due to the dissipation of heat around the objective, the temperature of the medium is usually 1–2°C lower than the set temperature. The drift in focusing may also be severe if a large difference in temperature exists between air masses inside and outside the enclosure. For experiments where

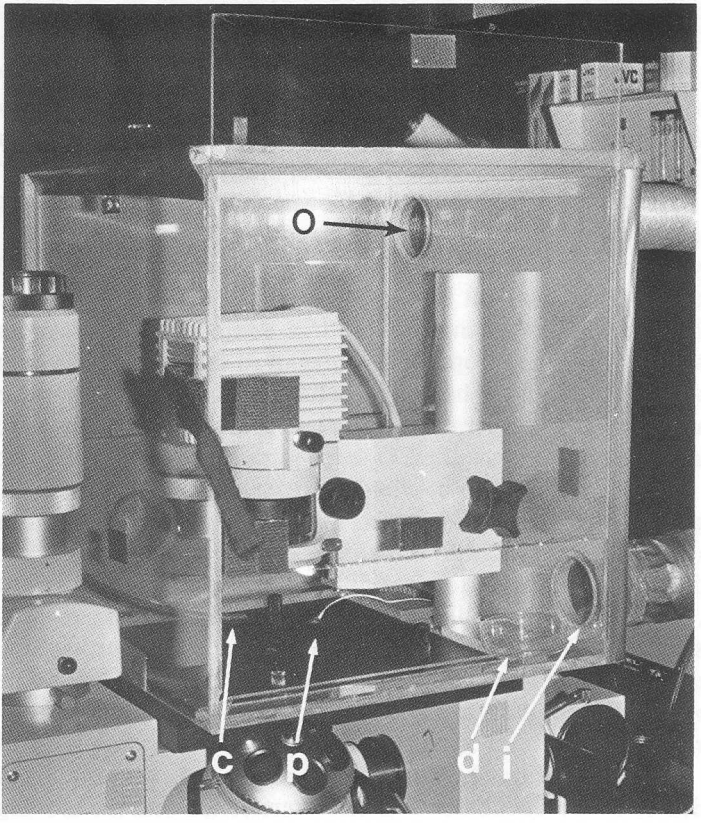

FIG. 5. Photograph of the microscope enclosure used in our laboratory. i, Warm air inlet; o, air outlet; d, dish with water; p, temperature probe; c, CO_2 inlet. Insulations for the door and the front side are not installed.

a precise control and a high stability are required, the area under the sample and around the microscope nosepiece (lower chamber) may be enclosed with plates attached to the sides of the microscope. In this case, warm air is pumped into one side of the lower chamber and directed to the main body of the incubator through a piece of flexible, insulated (using a wool sock with the tip cut off) pipe attached to the opposite side. This configuration is capable of holding the medium temperature to within 0.1 °C of set temperature. Through a combination of temperature regulation, CO_2 infusion, and medium replacement, we have succeeded in culturing cells for more than 3 days in our microscope incubators.

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