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A culture vessel for maintaining cells at high density

Robert J. Klebe, Anne M. Grant & George M. Grant
Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX, USA

Summary. The Sykes-Moore and Rose chambers have been used for many years in time lapse studies of subconfluent cell cultures. Unfortunately, use of a Sykes-Moore chamber for the time lapse studies of cells maintained at very high cell density was found to be unsatisfactory even if a medium perfusion system was employed. First, CO_2 produced by cells led to the formation of gas bubbles which often obstructed the optical path. Second, the specimen regularly went out of focus due to gas pressure build up which caused the top and bottom glass coverslips to warp and occasionally break. Third, after several days, cells underwent degenerative changes apparently due to lack of oxygen. A simple culture chamber is described here which permits cells to be maintained at very high cell density and observed at high magnification for a week or more.

Key words: Cell culture, Methods, Microscopy, Morphogenesis, Perfusion, Time lapse cinemicrography

1. Introduction

Time lapse cinemicrography studies usually involve observation of individual cells at high magnification. In such studies, cells are maintained at very low density since many microscopic features of cells are obscured under confluent conditions. For such studies, the Rose [1] and Sykes-Moore chambers [2] have proven to be quite satisfactory. We attempted to use the Sykes-Moore chamber for time-lapse studies of cells maintained at very high cell density and found that several problems occurred even when we used a peristaltic pump to perfuse a Sykes-Moore chamber with medium several times a day. The chief problems encountered were the production of CO_2 gas bubbles which (a) interrupted the optical path and (b) resulted in constant defocussing of the system due to the deformation of the coverslips which serve as the top and bottom of the chamber. We have devised a simple culture vessel for long term observation of cells at extremely high cell density which permits an unobscured view of cells for over a week. The chamber consists of a standard plastic T-flask equipped with a constant level device which maintains culture medium at a constant level at any perfusion rate.

2. Materials

A. Equipment
- Peristaltic pump, model G-07521-50[1]
- Four Easy-Load pump heads, model G-07518-00[1]
- Inverted phase microscope, model M[2]
- Television camera, model TC2511U8[3]
- Time lapse tape recorder, model AG-6030-P[4]
- Clock operated switch (6 events/day, 1 minute resolution), model 1454[5]
- CO_2-incubator, model 3341-3[6]
- 37 °C warm room[7] (or similar devise for maintaining cultures at 37 °C during time lapse observations)
- Hack saw[8]
- Small vise[8]
- Hand operated drill[9]
- Touch-O-matic Bunsen Burner, 03-975[9]

B. Supplies
- 100 ml wide mouth bottles, 1395-100[10]
- PharMed peristaltic pump tubing, size 13, G-06485-13[1]
- PharMed peristaltic pump tubing, size 14, G-06485-14[1]
- Miniature barbed polypropylene fitting, 06365-22[1]
- Miniature barbed polypropylene fitting, 06365-66[1]
- Tissue culture flasks, Corning 25 cm^2, model 25103-25[10]
- 18 gauge needles, 30519611[11]
- Glue, Duco cement[8, 12]
- Glue, silicone rubber, No. 8641[8, 13]
- Marking pen, No. 1441[14]
- IV locking head tubing fitting, No. 4481[18]

C. Media
1. Chemicals
- Ham's F12 medium, N6760[15]
- Dulbecco's medium, D5648[15]
- Newborn calf serum, N0515[15]
- Sodium bicarbonate, S5761[15]
- Penicillin-G, potassium salt, P7794[15]
- Streptomycin sulfate, S9137[15]
- Gentamicin sulfate[19]

95% ethanol[16]
Chlorox[16]

2. Growth medium
   50% Dulbecco's MEM/50% Ham's F-12 containing 10% newborn calf serum plus 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml Gentamicin sulfate.

D. Cell lines
   MDCK (canine kidney cell line, ATCC CCL34)[17]

3. Procedure

A. Preparation of liquid level control for culture flask
   1. Cut off the Luer-lock plastic fitting from three 18 gauge needles. Hold the needles with a small vice and cut the plastic fitting off with a hack saw. Bend the needles by placing them in a vise and tap carefully with a hammer. Bending the needles aids in preventing the accidental dislodgement of the needles once they are glued in place.
   2. Mark the side of the flask at the intended liquid level with a marking pen.
   3. Using a red hot 18 gauge needle, poke four holes in the sides of a Corning 25 cm^2 flask (well above the intended liquid level) (see Figure 1 insert). One hole was placed in the top of the flask to accept the liquid level control needle (see Figure 1 and text).
   4. Three 18 gauge needles prepared as described in step #1 above were inserted into the three side holes in the flask such that the beveled opening of the needle faced the bottom of the flask. The needle should be inserted such that the needle opening will be constantly submerged at the intended liquid level. Glue the needles in place with airplane glue (Duco cement).
   5. Insert the cut end of an 18 gauge needle into the top hole of the flask such that the end of the needle is exactly at the intended liquid level and glue the needle in place with airplane glue.
   6. A pressure relief is inserted into the fourth hole. The pressure relief consists of an 18 gauge needle with a small piece of cotton inserted into its hub. To sterilize the pressure relief, the cotton is saturated with 70% alcohol and allowed to dry.
   7. A 100 ml bottle is prepared to serve as the medium reservoir (see Figure 1, item D). Two holes are drilled in the cap and two 15 cm lengths of PharMed tubing, size 13, are inserted into the holes and glued in place with silicone rubber glue. Two miniature barbed fittings are inserted into the protruding tubing to accept the PharMed tubing that goes to the culture vessel.
   8. A 100 ml waste bottle (Figure 1, item E) was prepared by inserting a rubber stopper into the bottle. A single hole in the stopper was used to receive the two pieces of tubing that withdraw medium from the chamber.

B. Planting vessel with cells
   1. After the glue has dried, sterilize the flask by rinsing once with Chlorox, once with 70% ethanol, and twice with sterile water (10 ml per wash in that order).
   2. Plant cells at the desired cell density in 10 ml of culture medium.
   3. Connect the needles together with two sterile pieces of PharMed tubing to prevent contamination during the remaining steps.
   4. Place the flask in a CO_2-incubator to allow cells to attach and spread.
   5. To remove a few dead cells that might inadvertently obstruct the field of view, give the flask a complete medium change just before the start of the time lapse study.

C. Connection of flask to the peristaltic pump (see Figure 1)
   1. Connect the needle in the top of the flask (C) to PharMed size 14 tubing such that medium will be withdrawn from the flask through this needle. An IV locking head tubing fitting is used to make this connection.
   2. Connect one needle in the side of the flask (B) to PharMed size 13 tubing such that medium will be withdrawn from the flask through this needle.
   3. Connect the other two needles (A, A) in the side of the flask with PharMed size 13 tubing such that medium will be pumped into the flask through these needles from the medium reservoir bottle.
   4. Add medium to the medium reservoir bottle. Allow medium to equilibrate with the desired CO_2-in-air mixture prior to starting the experiment.

D. Time lapse cinemicrography
   1. To avoid possible interference of observations by cell debris, focus the microscope on a region of the flask near an inlet needle.
   2. Turn on the video camera and television monitor.
   3. Focus the microscope and adjust lighting conditions.
   4. Start the videotape recorder at the desired time lapse setting.
   5. Program the timer to deliver a desired volume of medium at predetermined intervals. The system is calibrated by determining the flow rate from each piece of tubing at a given pump setting. Once the flow rate is estab-

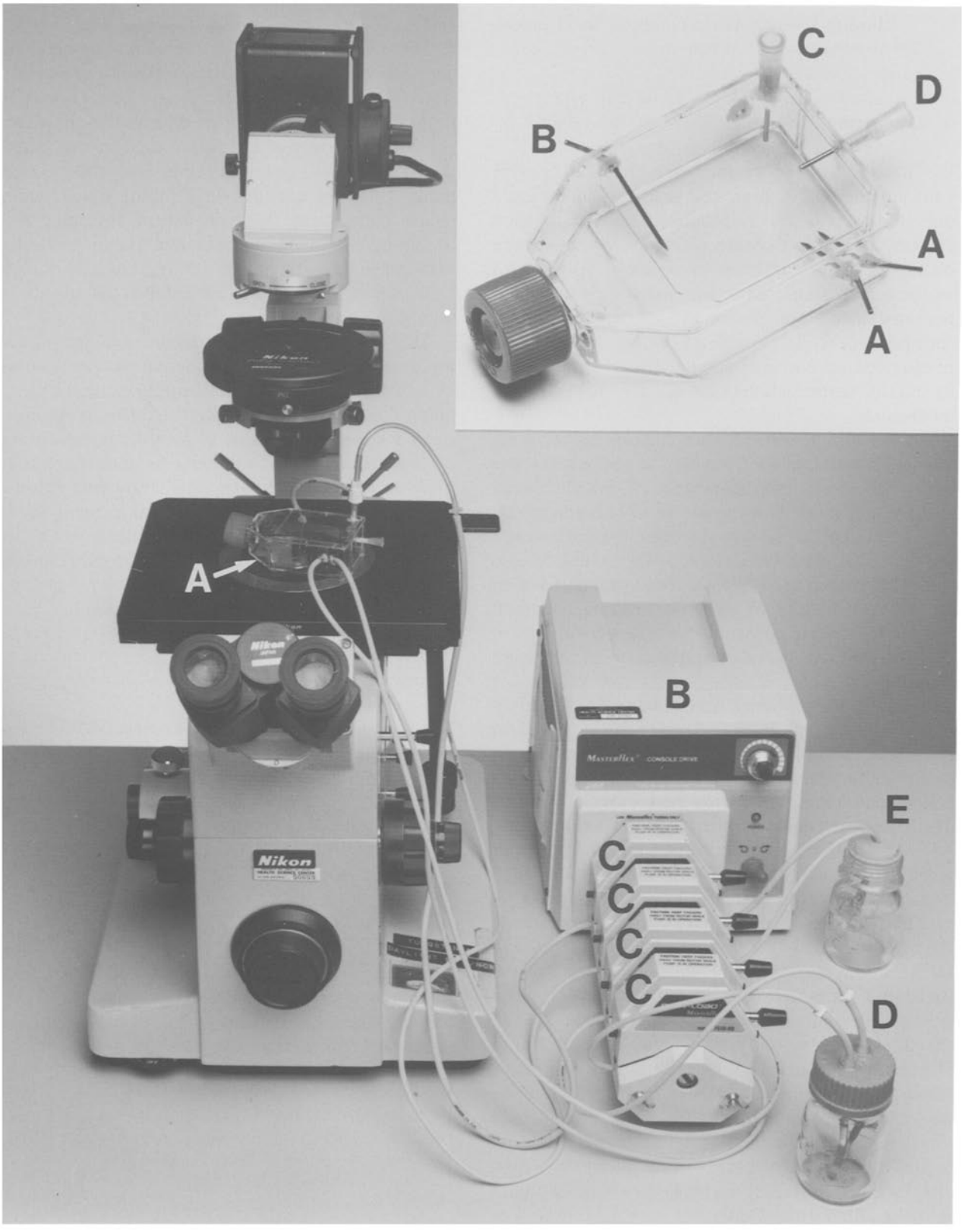

Figure 1. Connection of time-lapse chamber to peristaltic pump. Note that two pieces of tubing deliver medium from the medium reservoir bottle to the chamber. A = time lapse chamber; B = peristaltic pump; C = four independent pumping heads; D = medium reservoir bottle; E = waste bottle.
Insert: Device for automatically feeding cultures and maintaining a desired liquid level. A = inlet ports to be connected to size 13 tubing; B = outlet port to be connected to size 13 tubing; C = liquid level control outlet, connected to size 14 tubing; D = pressure relief.

lished, a desired volume can be delivered by turning the peristaltic pump on for a predetermined period of time.

4. Discussion

We wished to observe morphological changes in cells maintained at high cell density and initiated our studies with Sykes-Moore chambers. In such studies, MDCK cells were planted in Sykes-Moore chambers that had been completely filled with medium and connected to a peristaltic pump which perfused the culture. We soon found that gas bubbles formed once cells became confluent and that the microscope appeared to continually go out of focus in spite of several attempts to bolster the stage lock mechanism.

By sealing all orifices with silicone rubber glue, we determined that the formation of gas bubbles was not due to a leak in the chamber itself. We concluded that the gas bubbles were due to CO_2 produced by the cells. The constant problem of the microscope going out of focus was determined to be due to CO_2 pressure deforming the glass coverslips that make up the top and bottom of the Sykes-Moore chamber. Indeed, on several occasions, the glass coverslips of the Sykes-Moore chamber split due to gas pressure build-up.

To solve the problems noted above, we modified a standard 25 cm^2 culture flask such that medium could be perfused into the flask without altering the liquid level. Maintenance of a constant liquid level was essential in order to keep the specimen in focus. The constant level device that we devised operates as follows. Medium was pumped into the chamber through two narrow bore (size 13) pieces of PharMed tubing. Medium was withdrawn from the chamber by one narrow bore size 13 tubing and one piece of larger bore (size 14) tubing *which was connected to the needle in the top of the flask.* At the rpm of the peristaltic pump that we selected (setting 4.0), each size 13 tubing had a flow rate of 2.6 ml/min while the size 14 tubing had a flow rate of 10.1 ml/min. Hence, the two size 13 inlet tubings had a net flow rate of 5.2 ml/min while the size 13 plus size 14 outlet tubing had a maximum flow rate of 12.7 ml/min. Liquid level was maintained at the bottom of the top needle for two reasons. First, since two size 13 pieces of tubing were used to pump medium into the chamber and only one size 13 tubing was used to pump from the bottom of the chamber, medium could never be entirely depleted from the chamber. Second, medium could never go above the level of the top outflow needle since the maximum inflow rate was 5.2 ml/min while the maximum outflow rate was 12.7 ml/min. Note that the size 14 tubing port only withdrew medium from the chamber on an intermittent basis. Hence, this simple system can maintain a constant liquid level.

There are several types of peristaltic pump heads currently on the market. A peristaltic pump which is similar in design to that described here (Figure 1, item C) is essential for this application. Since two different sizes of tubing are used by one pump, it is important that the pump use independent pump heads for each piece of tubing rather than a single pump head that can accept several pieces of tubing. By using independent pump heads for each piece of tubing, one can individually set the flow rates through two different sized pieces of tubing.

The times at which the pump turns on and off and the interval during which it is active can easily be set by a household timer which supplies current to the pump during preset time intervals. The inexpensive timer we used allowed us to turn the pump on and off six times/day for intervals as short as 1 min. Thus, it was possible to automatically feed cultures with any desired volume of medium as many as six times/day. We have used the device described above to observe cells maintained at tight confluence for over a week without the specimen going out focus even when a 20x phase objective was used.

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Notes on suppliers

1. Cole-Parmer instrument Co., Niles, IL, USA
2. Nikon, Japan
3. RCA, Lancaster, PA, USA
4. Panasonic, Japan
5. Woods, Taiwan
6. Precision Scientific Inc. Chicago, IL, USA
7. Made to order, University of Texas Health Science Center, San Antonio, TX, USA
8. Home Depot, San Antonio, TX, USA
9. Fisher Scientific, Houston, TX, USA
10. Corning, Corning, NY, USA
11. Becton Dickenson, Franklin Lakes, NJ, USA
12. Devon Corp, Wood Dale, IL, USA
13. Dow-Corning, Midland, MI, USA
14. Precision Dynamics, San Fernando, CA, USA
15. Sigma Chemical Co., St. Louis, MO, USA
16. General Stores, University of Texas Health Science Center, San Antonio, TX, USA
17. American Type Culture Collection, Rockville, MD, USA
18. Abbott Laboratories, North Chicago, IL, USA
19. SoloPak Laboratories, ElkGrove Village, IL, USA

References

1. Rose GA (1954). A separable and multipurpose tissue culture chamber. Texas Rep Biol Med 12: 1074–1083.
2. Sykes JA, Moore EB (1959). A new chamber for tissue culture. P.S.E.B.M. 100: 125–127.

Address for correspondence: Robert J. Klebe, Department of Cellular and Structural Biology, University of Texas Health Center, San Antonio, TX 78284, USA
Phone: (210) 567 3828; Fax: (210) 567 3803