

Newer Methods and Practical Applications of Tissue Culture

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The question of whether tissue culture is an art, a technique or a science has been debated, and like so many similar controversies this argument revolves around one's own definition of terms. If, however, one thinks of a science as study based on well established underlying principles, the conventional methods of tissue culture are found to be somewhat lacking in these fundamentals. Much of tissue culture work, including some of the newer procedures which are to be discussed at this time, is conducted by empirical methods and rule of the thumb laws, but it is felt, however, that steps in the right direction are being made. It is not intended to discredit anyone or any procedure, for much information has been gained through the years by investigators in various fields of biological science who have employed an assortment of tissue culture techniques. It is intended, however, to point out the limitations of the customary plasma clot culture method and to discuss some of the advances that have been made in order to overcome these inadequacies of the conventional method.

Some of the more serious shortcomings of the plasma clot culture are physical in nature, which include (1) the restriction on size of cultures, (2) the excessive time involved in preparing and maintaining cultures, (3) the fact that the cells cannot be removed entirely from the clot in the form of a suspension permitting accurate quantitative studies, (4) the handicap to visual observation and differentiations, and (5) the distortion of cell morphology. An equally serious objection to the plasma clot is recognized from the chemical standpoint because it tremendously complicates metabolic or nutritional studies.

Numerous investigators have struggled with the problem of improving and increasing the applications of tissue cultures through the elimination of the plasma clot. Some have done this by substituting simpler materials, others by attempts to free the cells from the clot. Outstanding among such investigators have been Earle and his associates at the National Cancer Institute. These workers first were able to dispose of the plasma clot by replacing it with a substrate of perforated cellophane (1) which allowed the cells to cover a much greater area, and in a uniform pattern rather than spreading out from a small clump of cells in the center of the flask. Of even greater significance, it was possible to scrape, or shake, these cells loose from the cellophane and pipette them as a suspension to new flasks, thus establishing serial cultures. Such cultures are maintained very much like those of the bacteriologist. These cultures, in which all the cells can be removed as a suspension, have greatly increased the possibilities for true quantitative handling of cell structures as this has been realized through the development of a method for planting replicate cultures (2) and for the enumeration of nuclei as an index of cell proliferation (3). It has further been possible in several instances to

isolate a single cell from these cultures and to initiate a clone from it, thereby yielding the first unequivocally pure cell cultures (4, 5).

Evans and Earle's (1) use of perforated cellophane as a substitute for the plasma clot has worked admirably well for certain cell types, especially embryonic connective tissue elements, but unfortunately has remained inferior to the plasma clot as a substrate for initiating cultures of more specialized cells from either embryonic or adult tissues. They have, however, found that some of these cells, which are more difficult to handle, can be induced to grow on cellophane if it is embedded in a plasma clot and the cells exposed to it while growing on the more favorable plasma substrate. Subcultures can sometimes be made then to other flasks having only a cellophane substrate. By these methods Earle's group has established several lines of connective tissue cells, notably among them Strain L (6), and a mouse liver culture. Strain L is at present twelve years old, while the liver cells have now been carried in serial culture for approximately five years. After these cultures had been carried for a while on cellophane it was found that they could be adapted to growth directly on the glass floor of the flask and thereby the cellophane was also eliminated.

Another technique for establishing and maintaining cell cultures directly on glass has been developed in our laboratory and has, to date, supplied us with six lines of cells (7). Plasma clot roller tube cultures were used for the original explants, but were slightly modified as only a thin line of plasma, about one-fourth inch wide, was streaked down one side of the tube in place of the customary coating of the entire inner surface. The explants were seated in this strip of plasma, which was clotted by a drop of chick embryo extract, and by forty-eight hours, a zone of outgrowth could be seen in the plasma clot around the explants. As growth continued, however, the cells came out of the thin line of plasma onto the glass surface and by two to three months had completely covered the entire lower portion of the tube. Thus, a dense population of cells growing directly on glass and free of plasma had been obtained. The area of growth in each roller tube covered at least 3000 sq. mm. When the cells in this area were scraped loose and transferred to a Carrel D 3.5 flask with a floor area of 948.6 sq. mm., a three-fold increase in population density was obtained which favored survival and continued growth of the transplanted cells. The first attempt to subculture cells by this scraping and pipetting method is the most crucial step in the procedure as more experiments fail at this point than any other. Following sufficient proliferation in the first D 3.5 flask subcultures, the cells have been scraped loose and transferred to the larger Carrel D 5.0 flasks and from these were carried over eventually to the large T-60 (60 sq. cm.) flask of Earle's in which they are being maintained as stock cultures.

Four of the six strains of cells were established in our laboratory by this procedure. Included in these are two malignant ones, the mouse sarcoma 180 and the Walker rat carcinoma 256, and two apparently different cell types grown from normal embryonic mouse skin.

The remaining two cell strains were obtained by slightly different procedures. A very proliferative strain of normal adult mouse connective tissue cells was obtained by scraping the cells loose from an area on a Carrel flask where the glass had been exposed by lysis of the clot and

pipetting them to a similar vessel devoid of plasma. These cells have been maintained in serial culture now for over a year, still are in a state of rapid proliferation and are apparently quite healthy. This culture will be identified in the literature as LLC-M1.

The sixth line of cells being carried in our laboratory was started from the Ehrlich ascites tumor cells of mice. This culture, now in its ninth month of existence, was started by pipetting the cells, which can be removed from the animal in the form of a suspension, directly to the glass surface of Carrel D 3.5 flasks. Growth in, or on a plasma clot, on cellophane, or in roller tubes has not occurred.

The advantages of working with these large glass substrate cultures are many, both from the standpoint of facility and from experimental competency. By maintaining stocks of each cell strain in the large T-60 flasks, as many as thirty Carrel D 3.5 or T-12 flasks can be uniformly inoculated with a cell suspension prepared by scraping the T-60 flask. Furthermore, if so planted, these thirty subcultures will be both quantitative and qualitative replicate cultures, and it can be expected that all thirty will be usable, at least to the same degree of reliance as is anticipated in dealing with bacterial cultures. This entire procedure can be accomplished in a matter of about twenty minutes, in contrast to more than one or two hours required to put on thirty plasma clot cultures. Of the latter there is very little assurance that all thirty cultures will grow, in fact few investigators would expect it. Then, too, there is frequently a considerable difference in the amount of growth in each primary culture as well as the possibility of having several different cell types present in the same or in sister cultures. This could and does have considerable effect on the comparison of results obtained from such cultures. A further advantage of serial tissue culture is that one set of experimental data can be re-examined with the same strain of cells, thereby establishing a base line which can be only roughly approximated in primary explant cultures. One objection raised to this method of tissue culture is that these cells maintained for long periods outside the body in a state of rapid proliferation become so altered that they are no longer satisfactory for studies in which it is desired to make some *in vivo* correlation. This thesis has some validation in that several such strains of cells have spontaneously undergone malignant changes, while others which were malignant have been observed to lose their ability to produce tumors.

The actual technique for planting replicate cultures is relatively simple and although requiring several items of special equipment, does not necessitate an excessive expense. The one prerequisite is that the cultures to be studied must be of the type which can be grown on glass or cellophane substrates. After the cells are scraped loose into the fluid medium, this suspension is passed through a double sieve which has two platinum irridium gauzes, of 80 and 150 meshes respectively. This breaks up, or removes, the cell clumps from the suspension making it more uniform in nature. The suspension is then placed in a special stirring burette, from which it is dispensed in equal volumes to the new culture flasks.

The quantitative determinations, likewise, can be done with a minimal amount of effort and outlay for equipment. Replicate cultures, of course,

are a prerequisite for this procedure and the technique for obtaining them has just been discussed. Cultures are prepared for nuclei enumeration by the following procedure: the cells are scraped loose from the glass, citric acid solution is added and the flasks are returned to the incubator for two or more hours. The citric acid digests away the cytoplasm and loosens from the glass any cells missed at the time of scraping. This suspension of nuclei is centrifuged, part of the supernatant fluid removed, and additional citric acid solution containing 0.1 per cent crystal violet added. The stained nuclei are then suspended in a known volume of a methocellulose-citric acid solution in 10 ml. graduate cylinders. Aliquot portions are taken up in special pipettes, and after agitation the nuclei are counted in a hemocytometer. By making such counts at the time a series of cultures are planted and during the course of an experiment, it is possible to obtain a very accurate evaluation of the effect of some added substance or some condition on the experimental cultures in respect to counts made on appropriate controls.

The practical applications of these long-term glass substrate serial cultures and the methods of handling them are both many and obvious. A variety of viral agents have been successfully cultivated in one cell strain or another, in situations where the cell population was under control and where it was possible to repeat tests under exactly the same experimental conditions. A very uniform viral infection can be induced in the culture by adding the agent to the cell suspension while it is being mixed in the stirring burette prior to inoculation of the cells into the culture flasks. These cultures can also be used in screening or studying the mode of action of possible antiviral drugs or antibiotics. Many such agents reported in the literature as having some inhibitory effect on virus multiplication in tissue culture probably could be eliminated by re-examining them with quantitative studies of their effect on inhibition of cell growth. The difference between no inhibition of cell growth, and that amount which the eye can accurately detect by microscopic observation is considerable, and it is in this range that possible errors in interpretation of results, of tests for viral inhibitors, are made.

Earle and his associates are using these methods of tissue culture and are finding answers to age old disputes and are replacing some of the arbitrary measures generally employed with new established facts (8). Among these are the determinations supported by quantitative data of the best concentrations of the ingredients of culture media, the effect of inoculum size on the probability of successful subculture (9), and many other such factors which had previously been guessed at and still are by many. Outstanding among the contributions of these investigators has been the development, first, of a means of sterilizing embryo extract (10), second, of the preparation of a protein free ultrafiltrate of embryo extract (11), third, the determination of the chemical composition of the extract (12), and fourth, the suggestion that the role of embryo extract is that of a potentiator for the serum which is actually the main source of food in the culture medium (8). These studies were possible only because these workers had a quantitative control over their system for evaluating the effect of the various factors on cell proliferation.

Further uses for these methods can be found in the screening of many compounds for various purposes. The use for antiviral agents has already been mentioned. With cultures of both normal and malignant cells, substances can be evaluated quantitatively for any differential inhibitory effect they might have on tumor cells as opposed to normal ones. General cellular toxicity studies can also be made where it is desirable, for example, to compare two lots of a drug, or two competing products of the same pharmacological action.

The application of this type of tissue culture, or cell culture, as it should more appropriately be called, to practical problems in various research fields, control and testing functions in industry or elsewhere, can be steadily increased by the establishment of more and varied strains of cells and by more investigators taking up this approach to cell cultivation.

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