possibility of cross-contamination of colonies by floating cells, it is advisable to place a cloning cylinder (of glass or plastic; height, 10 mm; internal diameter, 6 mm; Fisher Scientific Company) over the presumptive hybrid colonies as soon as they are observed. The medium is removed from the culture dish, and the cylinder positioned over the colony, adhering to the dish by means of silicone grease. Care should be taken not to disturb the colony when renewing the medium in the cylinder.

When colonies contain a few hundred cells, they can be transferred to new culture dishes or bottles. This is done by removing the medium from the cylinder, detaching the cells from the dish by treatment with a few drops of a trypsin solution or other solution which is normally used for detaching the particular cells employed, and transferring the detached cells using a sterile disposable Pasteur pipette inserted into a latex tube adapted for oral manipulation. The tip of the Pasteur pipette should be bent at a 45° angle and drawn to capillary diameter. If the cells are not too firmly attached, the enzyme treatment can be omitted and the cells detached simply by scraping and oral manipulation with the special Pasteur pipette.

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[55] Basic Methodology for Cell Culture—Cell Transformation

By JOSEPH SAMBROOK and ROBERT POLLACK

The word "transformation" is used to describe the permanent acquisition of new characters by cultured cells. Of the many different types of transformation that are known, the best studied is neoplastic transformation. Here, cells that have been exposed to certain viruses, chemicals (or X-rays) change their social behavior and take on many of the attributes of tumor cells (see reviews of Eckhart,[1] Dulbecco,[2] Green,[3] Temin,[4] and Sambrook.[5]

[1] W. Eckhart, *Physiol. Rev.* **48**, 513 (1968).
[2] R. Dulbecco, *Science* **166**, 962 (1969).
[3] M. Green, *Annu. Rev. Biochem.* **39**, 701 (1970).
[4] H. M. Temin, *Sci. Amer.* **226**, 24 (1972).
[5] J. Sambrook, *Advan. Cancer Res.* (1972, in press).

COMMONLY USED CELL LINES

Cell line	Derived from	Properties	Uses
BHK21	Hamster embryo fibroblasts	Shows some degree of growth control *in vitro;* nonpermissive for polyoma, human adenoviruses	Transformation by polyoma; studies of incomplete infection by polyoma and human adenoviruses
NIL2	Hamster embryo fibroblasts	As BHK21	As BHK21
3T3	Mouse embryo fibroblasts from outbred Swiss mice	Shows high degree of growth control *in vitro;* permissive for polyoma, nonpermissive for polyoma, nonpermissive for SV40	Transformation by SV40; studies of incomplete infection by SV40; lytic growth of a plaque assay of polyoma virus
3T12	Mouse embryo fibroblasts from outbred Swiss mice	Shows low degree of growth control *in vitro;* "spontaneous transformant"	
BALB/c3T3	Mouse embryo fibroblasts from inbred BALB/c mice	Shows high degree of growth control *in vitro;* permissive for polyoma; nonpermissive for SV40. Isogenic with BALB/c3T12 cells, permissive for mouse sarcoma/leukemia viruses	Transformation by SV40, lytic growth and plaque assay of polyoma virus. Isogenic with BALB/c3T12, growth and transformation by mouse sarcoma/leukemia viruses
BALB/c3T12	Mouse embryo fibroblasts from inbred BALB/c mice	Shows low degree of growth control *in vitro;* "spontaneous transformant," isogenic with BALB/c3T3	Isogenic with BALB/c3T3

BSC-1, VERO, CV-1, MA-134	African green monkey kidney cells	Permissive for SV40	Plaque assay and production of SV40 stocks
KB	Human carcinoma of nasopharynx	Permissive for human adenoviruses	Growth of adenovirus stocks
Primary cells			
Mouse embryo fibroblasts		Permissive for polyoma, mouse sarcoma/leukemia viruses	Growth and transformation by mouse sarcoma/leukemia viruses; growth and plaque assay of polyoma virus
Baby mouse kidney cells		Permissive for polyoma	Growth and plaque assay of polyoma virus
Hamster embryo fibroblasts		Nonpermissive for polyoma and some human adenoviruses	Adenovirus transformation and incomplete infection
Rat embryo fibroblasts		Nonpermissive for polyoma and some human adenoviruses	Adenovirus and polyoma transformation and incomplete infection
African green monkey kidney cells		Permissive for SV40	Growth and plaque assay of SV40
Human embryo kidney cells		Permissive for human adenoviruses	Plaque assay of human adenoviruses
Chick embryo fibroblasts		Permissive for avian sarcoma/leukosis viruses	Growth and transformation by avian sarcoma/leukemia viruses

All mammalian cells are maintained in culture by the periodic replacement of medium and serum. Populations of untransformed cells divide while they remain sparse, but as the culture becomes more crowded, the growth rate decreases dramatically; in fact the cells of some lines stop dividing altogether once they have formed a confluent monolayer. It is not understood how this growth control is mediated. At one time it was thought that the growth of cells in tissue culture is controlled entirely by "contact inhibition"—the individual cells in a culture responding to the close proximity of other cells by ceasing to multiply.[6] It is now clear, however, that although cell with cell contact plays a role in inhibiting cell division, some as yet undefined factors in serum also have an effect in regulating the extent to which cells multiply in culture.[7] In any case, after untransformed cells are infected with one of a variety of tumor viruses, or exposed to X-rays or carcinogens, some of the cells may be transformed and will no longer respond to all the controls which regulate the multiplication of untransformed cells in culture: the transformants continue to divide in conditions of high cell density and/or low serum concentration, which severely limits the growth of untransformed cells, and it is this differential multiplication which provides the basis for the commonly used transformation assays.

DNA Viruses (Papovaviruses and Adenoviruses)

DNA tumor viruses show two distinct sorts of interactions with cells. Either there is a lytic response which results in production of virus progeny and death of the infected cells, or an incomplete (abortive) infection during which very little or no progeny virus is produced and the cells survive. Which response will predominate depends on the exact combination of virus and cell-type used; some of the most commonly used pairs are set out in the table. Some abortively infected cells are stably transformed by the virus, and as a general rule *only* abortively infected, nonpermissive cells are transformed. The proportion of stably transformed cells increases with increasing multiplication of infections. It is also possible, at least in the case of polyoma virus and SV40, to isolate transformed cells from populations of productively infected permissive cells. For example, mouse 3Te cells transformed by polyoma virus and African green monkey kidney cells transformed by SV40 have been isolated. These transformants are thought to arise either from permissive cells which have been infected by a defective virus particle that cannot complete its replication but is capable of transforming, or from variant cells

[6] M. Stoker and H. Rubin, *Nature (London)* **215**, 171 (1967).
[7] R. Dulbecco, *Nature (London)* **227**, 802 (1970).

in the population which cannot support a productive infection. In any case, this type of transformant is very rare and consequently most studies of transformation have concentrated on the infection of nonpermissive cells where the response is not obscured by the massive cell death found in lytic infections.

Transformation of Nonpermissive Cells by SV40 and Polyoma

Assays for the titer of transforming virus on nonpermissive cells can be either *selective* or *nonselective*. Selective assays permit only transformed cells to grow, and all colonies that are recovered after about a dozen cell divisions will contain transformed cells. Nonselective assays permit all cells to grow after infection, and a subjective determination of the fraction of colonies which are transformed must be made, by comparison with mock-infected cultures.

As examples, agar assay for transformation of BHK21 hamster cells by polyoma virus is selective, while the dense colony assay for transformation of 3T3 mouse cells by SV40 virus is nonselective.

Polyoma-BHK

BHK21/13 is a pseudodiploid hamster cell line derived from baby hamster kidney cultures.[8] As it grows, the cells line up in swirling parallel assays, so that the colonies are stellate. BHK21 needs to be attached to a solid surface in order to divide in the presence of calf serum; if the cells are suspended in agar they will not grow. However, polyoma-transformed BHK cells *will* grow in agar, to form large spherical colonies, typically 0.1 mm or more in diameter after 1 week.

Method (after Macpherson and Montagnier[9])

Prepare 1.25% Bacto-Agar (Difco) in water suitable for cell growth (deionized, or glass distilled, or both). Autoclave at low temperature.

Prepare 2-fold concentrated Dulbecco's Eagle's Medium (2× medium) and sterilize by pressure-filtration through 0.22-μm Millipore filters. Powdered media are available from Grand Island Biologicals, New York, and should be diluted in one-half the recommended volume of water. Dulbecco's modification of Eagle's medium is suitable for BHK21 cells.

Preparation of Plates. A *base* layer of agar is necessary to keep BHK cells from adhering to the bottom of the dish, where they will divide. To make it, melt 80 ml of agar and cool it to 45° in a water bath. Warm

[8] M. Stoker and I. Macpherson, *Nature (London)* **203**, 1355 (1964).
[9] I. Macpherson and L. Montagnier, *Virology* **23**, 291 (1964).

100 ml of 2× medium and 20 ml of calf serum to 45° in the bath. Mix together the serum, 2× medium and agar in a prewarmed flask, and pipette 7-ml lots into 5-cm diameter petri dishes. Permit the base layer to harden.

Infection. A high titer of virus is necessary, as transformation is about 100 times less efficient than plaque formation.

Melt 0.5% agar for top layer and cool it to 45°. Remove medium from a plate of BHK21 cells, inoculated 1–2 days earlier at 2 to 5 × 10^5 cells/plate. Add 0.5 ml of virus suspension in serum-free medium to the plate and incubate it at 37° in 100% humidity, 10% CO_2, and 90% air for 1 hour to permit virus adsorption. After adsorption, wash the cells twice with serum-free 1× medium, suspend them in 2 ml of 0.1% trypsin, and pipette the trypsinized cells to make a suspension of single cells. Centrifuge the suspension at 1000 rpm for 5 minutes, and resuspend in 1× medium with serum. Count the cells in a hemacytometer and adjust the concentration to 10^5/ml, then make 1:10 and 1:100 dilutions in 1× medium with serum. Add 1 volume of cells to 2 volumes of 0.5% agar and immediately pipette 3-ml aliquots of the mixture onto base layers, being careful not to penetrate the base agar, so that no cell reaches the bottom of the dish. This will yield dishes with 10^5, 10^4, and 10^3 infected cells in the top agar. Always set up mock-infected dishes with identical numbers of BHK cells, to get a measure of the BHK cells that are growing in agar spontaneously. These should be few, and the colonies should be small. If many BHK21 cells grow into colonies in agar, the line is useless for polyoma transformation and should be replaced.

After 7–10 days, large, round polyoma-BHK colonies can be counted under a dissection microscope. The number of colonies in agar should be proportional to input virus titer (PFU/ml) up to about 500 PFU/cell. To determine the frequency of transformation, count a plate of a cell dilution (10^5, 10^4, or 10^3) which has less than 100 agar colonies. The transformation frequency is colonies in agar/cells inoculated. On plastic dishes, both BHK and polyoma-BHK have a plating efficiency of at least 50%, so this transformation frequency will be no less than half the frequency of transformed cells/colony-forming cells. The transformation frequency is usually determined as colonies in agar/colonies on plates, to compensate for the fact that only about one BHK cell in two will form a colony on plates at low inoculation density.

Recovery. Remove polyoma-BHK colonies from the agar by aspiration with a Pasteur pipette. Transfer the colony to a tube with 1 ml of medium plus serum; disperse it and plate the suspension into a single dish with medium plus serum. Recovered colonies should be recloned

to exclude the descendents of untransformed BHK21 cells which might have been carried along in the initial aspiration of the colony.

SV40-3T3

3T3 is a subtetraploid mouse line derived from noninbred Swiss mouse embryo cultures by a passage protocol that resulted in a line with a high degree of contact inhibition of cell division.[10] 3T3 cells will grow only until a density of about 4×10^4 cells/cm^2 is reached, then they will cease dividing and remain as a thin monolayer of viable cells until they are separated from one another (by trypsinization, dilution and replating, or by wounding of the monolayer). SV40-transformed 3T3 cells do not stop dividing at this low density; they pile up at least ten times more cells/cm^2 before cell division slows. In this nonselective assay, SV-3T3 colonies are recognized as dense foci on a background monolayer of 3T3 cells.

Method (Based on Todaro and Green[11]*)*

Infection. Inoculate two plates (5 cm, Falcon) with $1\text{–}2 \times 10^5$ cells per plate in Dulbecco's modified Eagle medium (Gibco) plus 10% calf serum (Gibco). After 3–4 days the plates should be confluent and should contain few ($<10^{-3}$) cells in mitosis.

Remove the medium, wash the monolayer with serum-free medium and infect one plate with 0.5 ml of SV40 virus in serum-free medium, and mock-infect the other plate with 0.5 ml of serum-free medium, as a control. Incubate the plates in 100% humidity, 37°, 10% CO_2, 90% air for 2 hours. Wash off the unadsorbed virus with medium containing serum, add fresh medium with serum, leave the plates overnight, or proceed to next step immediately.

Transfer. Suspend infected and control plates in 2 ml each of 0.01% trypsin. Count cells with hemacytometer; there should be 0.8 to $1\text{–}2 \times 10^6$ per plate; more cells means that the 3T3 line has spontaneously lost contact inhibition; fewer, that the infected plates were damaged in infection. In either case, the assay will not be reproducible. Add 1.5 ml of cells ($\sim 5 \times 10^5$) to 30 ml of medium plus serum and inoculate six plates with 4 ml each. These plates will each have one-tenth of the infected cells; label them "10^{-1}, infected," transfer 3 ml into 27 ml of medium plus serum, and repeat serially to get six plates each at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Repeat for mock-infected control dish.

Incubate all dishes ($6 \times 5 \times 2 = 60$) for 10–14 days, changing

[10] G. Todaro and H. Green, *J. Cell Biol.* **17**, 299 (1963).
[11] G. Todaro and H. Green, *Virology* **23**, 117 (1964).

medium 3 times a week. When colonies on the 10^{-4} and 10^{-5} plates are about 5 mm in diameter, fix four plates of each dilution by removing the medium, washing with PBS, and adding formalin plus PBS (1:10) for 15 minutes. Pour off the formalin and stain the dishes with Harris hematoxylin for 1 hour, then wash with tap water, 0.1% ammonium hydroxide, and water again, and let dry overnight.

Count all colonies on the 10^{-5} and 10^{-4} plates: there should be at least three or four colonies on the 10^{-5} plates in both the infected and control cultures, otherwise the 3T3 plating efficiency is subnormal. Plating efficiencies of the infected and control plates should be the same.

Examine the 10^{-1}, 10^{-2}, and 10^{-3} plates. Control cultures should be even light blue monolayers. Infected cultures will have dark circular areas: these are SV40-transformed colonies—count them and calculate transformation frequency as transformed colonies per total colonies.

Recovery. With cloning cylinders, pick transformed regions from the two remaining infected plates of the dilution series which has about ten distinct transformed colonies. Grow up in medium plus serum, then reclone these colonies *twice,* to be free of untransformed cells.

Presumptive polyoma-BHK and SV-3T3 transformed clones should have viral T-antigen in all nuclei. This may be visualized with the appropriate fluorescein-conjugated antibodies.

Adenoviruses

The best-studied system of adenovirus transformation is that described by McAllister and Macpherson.[12] They used adenovirus type 12 and the NIL-2 line of hamster cells, but the method seems to work with other types of adenovirus and cells. The critical factor for success seems to be low calcium concentration in the medium.

Wash semiconfluent monolayers of MIL-2 cells in 50-mm petri dishes with Tris-saline solution and infect with dilutions of the virus ranging up to about 200 PFU per cell. After 3 hours at 37° in a humidified atmosphere of 10% CO_2 in air, remove the virus and replace with 2.5 ml of calcium-free Eagle's medium supplemented with 5% dialyzed calf serum, 2% fetal bovine serum, 0.1 m*M* calcium chloride, 2-fold vitamins and amino acids and containing 0.5% agar.

After 2–3 days at 37° in humidified 10% CO_2 in air 2.5 ml of the same medium without agar is added and the incubation is continued with changes of the fluid medium every 2–3 days.

After 28–45 days the monolayers can be stained with Giemsa after the agar has been removed carefully: the transformed cells are seen as

[12] R. McAllister and I. Macpherson, *J. Gen. Virol.* **2**, 99 (1968).

dense areas against a lighter background of untransformed cells. If a line of transformed cells is to be established, a clone can be picked before staining by aspiration through the agar layer, plated in fluid medium, and recloned in the usual way.

RNA Tumor Viruses

By contrast with the DNA tumor viruses, the RNA tumor viruses do not kill susceptible cells; virus multiplication and transformation can occur in the same cell. There are three general classes of interaction between a cell and an RNA tumor virus. (a) The virus multiplies in a cell which is not transformed. (b) The virus multiplies in a cell which is concomitantly transformed. (c) The cell is transformed, but the virus fails to multiply.

Because the result of any particular infection depends on the phenotype as well as the genotype of both the cell and the virus, several types of *in vitro* assay have been devised for the RNA tumor viruses. The one most commonly used for sarcoma viruses is the focus-forming assay which is described below.

The leukemia viruses do not cause focus formation and they are usually titrated by immunological techniques which detect virus-specific antigens in infected cells, by assay for the particle-associated enzyme RNA-dependent DNA polymerase, or by interference assays. Interference assays are based on the fact that cells infected with leukemia viruses often will not support the growth of a superinfecting sarcoma virus, and the reduction of the yield of the sarcoma virus can be used as a measure of the amount of leukemia virus present in a stock.

As the complexity of the genetic and nongenetic interactions between RNA tumor viruses has become appreciated, assays for the different viruses have become more complex and tailored to suit individual circumstances. However, the focus-forming assay as described originally by Manaker and Groupé[13] remains the main method of assaying transformation by RNA tumor viruses.

Focus-Forming Assay for Rous Sarcoma Virus (RSV)[13-15]

Although transformation of mammalian cells by certain strains of RSV has been reported, most workers still use primary cultures of chick cells. It is essential to check that the cells are genetically sensitive and do not carry any interfering viruses. This is best done by using cells from individual chick embryos and checking a small aliquot of the cells in a focus-

[13] R. Manaker and V. Groupé, *Virology* **2**, 839 (1956).
[14] H. Temin and H. Rubin, *Virology* **6**, 669 (1958).
[15] M. G. P. Stoker and I. Macpherson, *in* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), p. 313. Academic Press, New York, 1967.

forming assay. While the sample of cells is tested the main lot can be stored frozen as described by Dougherty and Rasmussen[16] or held in sparsely seeded petri dishes.

Plate about 1–2 × 10^6 cells in plastic 50-mm dishes in Eagle's or NCI medium supplemented with 2-fold vitamins and amino acids and containing 10% tryptose phosphate broth plus 7% calf serum.

Make dilutions of RSV in the same medium and add 100–1000 focus-forming units (FFU) in 0.1 ml to the cell suspensions in each dish. Incubate the cultures overnight in 10% CO_2/air to permit the cells to spread out into a sparse monolayer. Aspirate the medium and replace with the same medium containing 0.5% agar.

Continue incubation for a further 5–6 days. If the cultures turn yellow, the CO_2 should be reduced to 5%. On day 5, the cultures should be fed by addition of 2.5 ml of the same medium. On day 10, foci of cells showing morphological alterations and different growth properties will be visible at 100× by phase contrast illumination. These foci can be picked by aspiration through the agar layer or counted by staining the monolayer with Giemsa after removal of the agar overlays.

Finally, it is important to realize that the agents used to cause transformation of cells in culture also produce tumors in experimental animals and should be treated with caution. Stringent safety precautions, such as those described in Hellman's safety booklet published by NCI should be enforced. Most of the rules are no more than common sense.

[16] R. M. Dougherty and R. Rasmussen, *Nat. Cancer Inst. Monogr.* **17**, 337 (1964).

[56] Cell Synchronization

By ELLIOTT ROBBINS

It is possible to obtain large numbers of relatively well synchronized cells in the G_1, S, and G_2 and mitotic phases of the cell cycle, thus allowing biochemical and morphological studies which potentially may elucidate the mechanisms that regulate cell division. Factors that must be considered in the choice of a particular method for synchronization are: the number of cells required, the specific phase in which optimal synchrony is desired, and the possible interference of chemical synchronizing drugs with projected analyses.

The two basic methods most commonly used for cell synchronization are: (1) chemical blockage of the cell's progression through its cycle at a