J. Embryol. exp. Morph. Vol. 24, 2, pp. 249–255, 1970
Printed in Great Britain

Methods of transplanting nuclei from single cultured cells to unfertilized frogs' eggs

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SUMMARY

Two methods of transplanting single nuclei from monolayers of cultured cells to unfertilized eggs of *Xenopus laevis* are described, illustrated, and tested.

The detached-cell method is simpler and quicker to operate and is suitable for homogeneous populations of cells which are easily removed from the substrate on which they are growing. The other, attached-cell, method is technically more elaborate, but is applicable to cells whose properties can be individually determined under the phase-contrast microscope and to cells which are not readily dissociated from other cells or from their substrate.

INTRODUCTION

Reasons for wishing to transplant nuclei from cultured cells have been specified elsewhere (Gurdon & Laskey, 1970). The technical problems which affect attempts to transplant such nuclei arise from the small size of cultured cells compared to embryonic cells. Thus the nucleus of a cultured cell is more likely to be exposed to the medium, or to be damaged in some other way, than is the nucleus of the much larger cells so far used successfully for nuclear transfers. It is therefore particularly important to demonstrate the harmlessness of methods used to transplant nuclei from cultured cells. This is most convincingly done by showing that normal development can result from their application. Of the two methods described below, one is suitable for homogeneous populations of cells which can be detached from their substrate and handled as single cells; the other is applicable to cells which remain attached to the substrate on which they are growing.

THE DETACHED-CELL METHOD

The principle of the method is similar to that commonly used for embryonic cells of *Xenopus* (Elsdale, Gurdon & Fischberg, 1960). The preparation and culture of monolayers, together with their release from the substrate by trypsin, is described elsewhere (Gurdon & Laskey, 1970). A single cell is drawn into the end of a micropipette of internal diameter slightly less than that of the cell, so as to break or weaken the cell wall while disturbing as little as possible the cytoplasm which surrounds the nucleus, a method devised by Briggs & King (1952).

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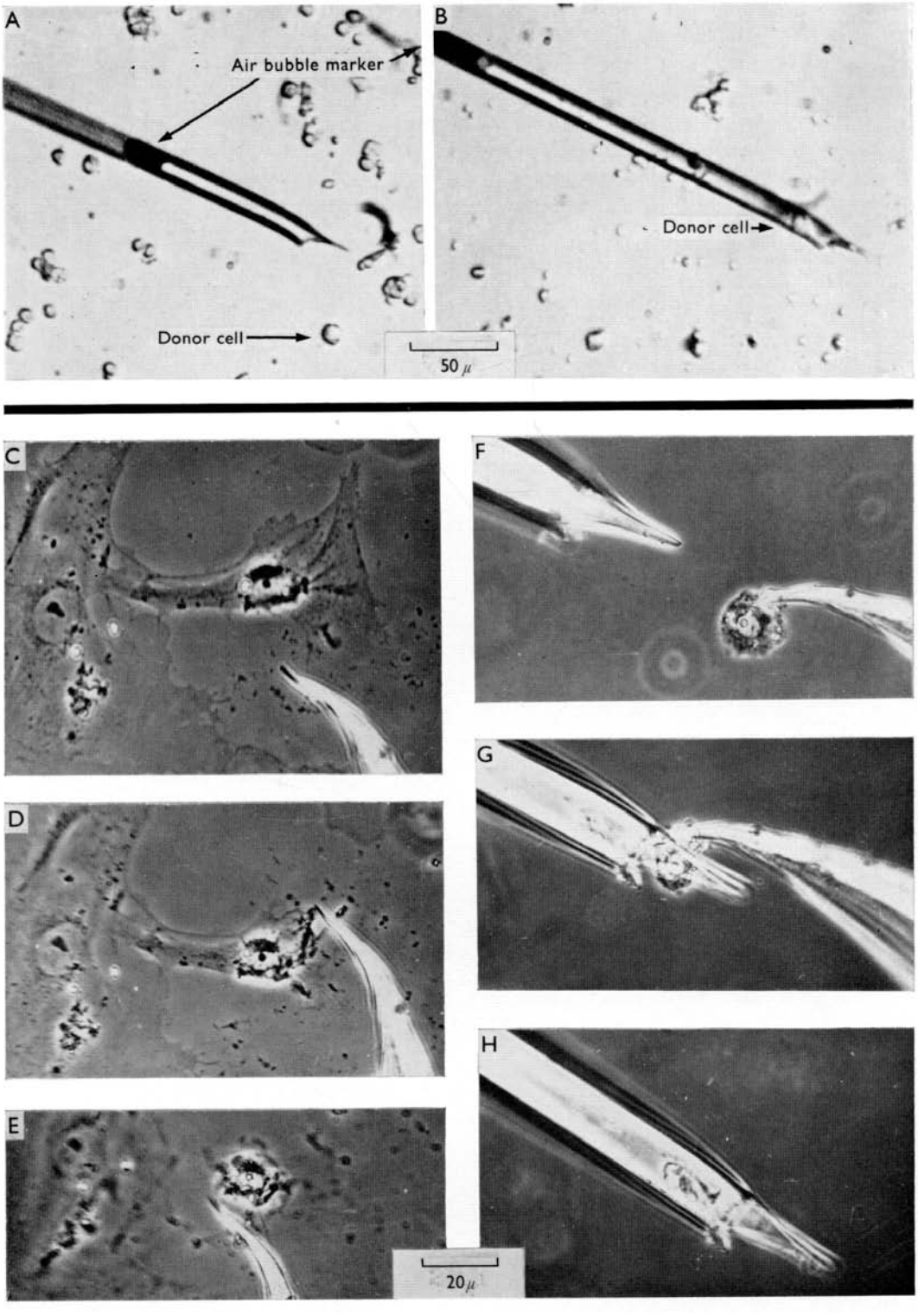
A
Air bubble marker
Donor cell
50 μ
B
Donor cell
C
D
E
20 μ
F
G
H

The whole broken cell is injected into an unfertilized egg, the animal pole of which has been exposed to a dose of u.v.-irradiation sufficient to inactivate the egg chromosomes (Gurdon, 1960*a*).

Attention to the following details is necessary to ensure satisfactory operation of the method. Adequate control of the fluid in the tip of the fine pipettes required for these experiments is provided by the use of a non-extensile material such as stiff plastic tubing, and an incompressible medium such as paraffin oil, for connecting the syringe and glass micropipette. The tip of the micropipette needs to be sharpened with a microforge so as to penetrate an egg without damage, but must also have a smooth rim so as to permit the donor cell to pass freely into and out of the pipette without catching on the edge of the pipette orifice (Fig. 1 A). The presence of Ca^{2+} and Mg^{2+} causes the donor cells to stick to their substrate, to each other, or to the injection pipette. Divalent cations are therefore omitted from the modified Barth's saline solution (Gurdon & Laskey, 1970) in which the donor cells are stored before nuclear transfer, and it is then unnecessary to use an agar-covered glass surface for the detached cells. Their small size and lack of yolk causes the donor cells to be hard to see, and we consider diffuse substage lighting, such as that provided by the Watson 5:1 zoom stereomicroscope, to be essential (Fig. 1 A). The nuclear transfer procedure is greatly accelerated if the same microscope magnification can be used for drawing the donor cell into a pipette and for inserting the pipette into the recipient egg. We find that this can be achieved, with practice, at a total magnification of 30 times.

Variation in two conditions is known from previous experience (Gurdon, 1960*b*) to have an important effect on the success of nuclear transfer experiments in *Xenopus*: the nature of the donor cell dissociation procedure, and the degree of donor cell distortion in the injection pipette. The monolayer of epithelial cells used for these experiments could be detached from the surface on which they were grown either by a 30 min exposure to 5×10^{-4} M-EDTA (ethylenediamine-tetra-acetic acid), or by a 10 min exposure to 0·5 % trypsin at 25 °C, both made up in Ca^{2+} and Mg^{2+}-free saline solution. Table 1 compares the cleavage and development of nuclear transfers made from cells prepared by EDTA or trypsin dissociation. In view of the very limited postblastula development promoted by first transfers of cultured-cell nuclei, later stages of development were tested with swimming tadpole endoderm nuclei. Within the limits of these tests, cell

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Fig. 1 (A, B). Detached-cell method of transplanting nuclei. A single donor cell (A) is sucked into a micropipette. The air bubble marker moves sufficiently far up the pipette shaft (B) to serve as a guide when the pipette tip and donor cell have been immersed in the recipient egg. (C–H) Attached-cell method of transplanting nuclei. The edges of a well-spread cell are cut with a fine glass needle (C, D). The cytoplasm spreads evenly round the nucleus, and sticks to the glass needle (E, F). The cell (with an incomplete cell membrane) is sucked from the needle into a wide-bore micropipette (G, H), and is then injected into a recipient egg in the usual way.

dissociation by trypsin seems no more harmful than that by EDTA. Evidently any trypsin which remains associated with donor cells after washing is not necessarily harmful to recipient eggs into which it is injected. The effect of varying the degree of donor cell distortion, the second variable investigated in these experiments, is shown in Table 2. Genuine cleavage is dependent upon the insertion of a single ruptured cell. The injection of many ruptured cells, of a single unbroken cell, or of saline solution without a cell, all fail to promote genuine cleavage. Together these results constitute important evidence that the transplanted nucleus and not the egg nucleus is responsible for the cleavage of injected eggs, a conclusion supported by several other kinds of results (Gurdon & Laskey, 1970).

Table 1. *The effect of different cell detachment procedures on nuclear transfer success*

(A) Monolayer of cultured cells

Detachment procedure	Total transfers	Pattern of cleavage (%)				
		Deficient	Abortive	½ cleaved	¾ cleaved	Complete
EDTA	74	63	8	22	7	—
Trypsin	44	52	16	25	7	—

(B) Stage 40 tadpole endoderm cells

Detachment procedure	Total transfers	Partial blastulae	Complete blastulae	% of complete blastulae achieving			
				Gastrula	Neurula	Muscular response	Heart-beat or beyond*
EDTA	125	77	34 (100 %)	44	14	6	12
Trypsin	105	77	20 (100 %)	35	15	10	15

* In this experiment 4 'EDTA-transplants' became entirely normal feeding tadpoles, while none of the 3 'trypsin-transplant' tadpoles developed normally, but we doubt if this difference is significant or reproducible.

In Expts. (A) and (B), the recipient eggs of two females were used to a similar extent for EDTA- and trypsin-experiments. EDTA (at 5×10^{-4} M) and trypsin (at 0·5 %) were made up in a full strength saline solution (see Gurdon & Laskey, 1970) lacking Ca^{2+} and Mg^{2+} ions.

The success of this method can be judged by the development of nuclear-transplant eggs (Gurdon & Laskey, 1970). At least some nuclei must be undamaged by the procedures used, because a few entirely normal, and several nearly normal, tadpoles have been obtained—usually by the serial transplantation of nuclei from partial blastulae, which arise from about one-quarter of all injected eggs. The remaining 75 % of injected eggs either fail to cleave at all, or cleave abortively. These cases could be most simply explained by a failure to

rupture the donor cell, that is by a failure to expose the cultured cell nucleus to egg cytoplasm. This possibility has been excluded by use of donor nuclei whose DNA had been previously labelled with [^{3}H]thymidine. Cultured cells were plated in Petri dishes containing coverslips and incubated with 1 μCi/ml of [^{3}H]thymidine for 36 h. Fixation and autoradiography of the cells attached to the coverslips showed that 70 % of the cell population had labelled nuclei. The cells remaining in the Petri dishes were detached by 0·5 % trypsin and their nuclei were transplanted into eggs. After fixation and autoradiography, 70 % of the eggs which were fixed within 1 h of nuclear transfer were found to contain a labelled (therefore transplanted) nucleus which was well mixed with egg cytoplasm and free from the donor cell membrane. Most of the other 30 % of injected eggs contained one or two unlabelled nuclei, one of which was probably the transplanted nucleus. In conclusion, the nuclei of nearly all donor cells are satisfactorily deposited in the cytoplasm of recipient eggs, and at least in some cases transplanted nuclei are shown to be undamaged because they support normal development.

Table 2. *Tests showing that recipient egg cleavage depends upon the injection of a single broken cell*

Each recipient egg injected with	Total transfers	% of transfers which cleaved	
		Partially	Completely
No injection	34	0	0
Saline solution	143	0	0
Single whole cell (no cell distortion)	62	0	0
Single cell (normal distortion; detached-cell method)	3406	28	3·5
Many cells per egg (normal distortion)	67	0	0
Single cell (attached-cell method)	35	23	3

THE ATTACHED-CELL METHOD

A monolayer of epithelial cells, such as those used for these experiments, is viewed under a phase-contrast microscope at a magnification of about 150. Under these conditions, the nucleus, nucleolus, and cell outline are clearly visible (Fig. 1 C). By use of an appropriate micromanipulator, a glass needle is used to cut one of the cytoplasmic extensions by which a cell is attached to its substrate (Fig. 1 D, E). Since the cells are attached primarily or exclusively at these points, they are quite easily peeled away from the surface to which they adhere, while remaining stuck to the point of the glass needle (Fig. 1 F). The

whole cell is now drawn into a pipette, large enough to avoid further cell disruption (Fig. 1G, H) and is then injected into a recipient egg.

The main technical difficulty in operating this method is the need to reduce substantially the microscopic magnification from that used to draw the donor cell into the pipette (×150), to the much lower enlargement suitable for injection into the recipient egg (not more than ×40); for the latter it is necessary to include in the field of view not only a large part of the egg surface, but also the air-bubble marker in the pipette shaft (Fig. 1A). The use of a Vickers ×40, 15 mm working distance, phase-contrast objective facilitates the handling of donor cells, but stereoscopic vision is not available with this lens. The only stage in the procedure which requires any manipulative dexterity is the early handling of donor cells. This is much facilitated by using the point of the sharpened micropipette as well as a glass needle, both being held in micromanipulators. To prevent excessive stickiness of the donor cells during handling, donor cells may be kept in Ca^{2+} and Mg^{2+}-free saline solution. Tests have shown that the cleavage promoted by nuclei which are transplanted in this way is similar whether the donor cells are handled in serum or saline and with or without divalent cations.

The success of this method has been compared to that of the detached-cell method in respect of the cleavage promoted by transplanted nuclei. There is no significant difference in the results obtained when the same sample of donor cells and recipient eggs were used for both transplantation procedures (Table 2). Although this method has not so far been tested on a large scale, it appears to give similar results to the detached-cell method. It is noteworthy that when the donor cell membrane is known to have been broken in every case, as in attached-cell nuclear transfers, the proportion of injected eggs which fail to cleave is no higher than in the detached-cell experiments.

APPLICATION OF THE TWO METHODS

The relatively greater convenience of operating the detached-cell method is indicated by the fact that it is possible, under favourable conditions, to achieve over 100 nuclear transfers/h; on the other hand it would be hard to achieve as many as ten transfers/h by the attached-cell method. The detached-cell method should therefore be used whenever possible, but this requires (*a*) that the cells can be released from their substrate by procedures known to be harmless, and (*b*) that the donor cell population is homogeneous in respect of the particular property being investigated by the nuclear transfer experiment. These conditions do not necessarily pertain to all cell cultures. For example, some collagen-producing cells and muscle cells are very hard to isolate by trypsin, and other enzymes are not known to be harmless for nuclear transfer experiments. Even clones of cells are heterogeneous in some respects, and if it were necessary to carry out nuclear transfers from cells expressing a certain type of specialization, or from cells individually characterized in some other way, the detached-cell method could

not be used. By the attached-cell method it is possible to transplant a nucleus from a single cell whose individual properties can be observed directly under the phase-contrast microscope.

RÉSUMÉ

Méthodes de transplantation individuelle de noyaux issus de cellules en culture, dans des œufs vierges de Xenope

Deux méthodes utilisées pour la transplantation individuelle de noyaux provenant de cellules cultivées en couche monocellulaire, dans des œufs vierges de *Xenopus laevis* sont décrites, illustrées et éprouvées.

La méthode de la cellule-détachée, qui est la plus simple et la plus rapide à pratiquer, peut être utilisée dans le cas de populations cellulaires homogènes, pouvant être aisément séparées du substrat sur lequel elles sont cultivées.

La seconde technique, de la cellule-attachée est techniquement plus élaborée. Elle est applicable aux cellules dont les propriétés peuvent être déterminées individuellement par l'étude au microscope à contraste de phase. Elle est applicable également à des cellules qui ne peuvent être facilement séparées des autres cellules ou encore de leur substrat.

The authors are indebted to V. A. Speight for expert technical assistance, and to the Medical Research Council for supporting this work.

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(Manuscript received 12 January 1970)