

A Description of the Technique for Nuclear Transplantation in *Xenopus laevis*

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WITH PLATE

INTRODUCTION

A METHOD by which nuclei can be successfully transplanted into Amphibian eggs was first worked out by Briggs & King (1952) for the eggs of *Rana pipiens*. *Xenopus laevis* is an atypical Anuran since its eggs can be obtained throughout the year, and the resulting embryos can be reared to maturity within 12 months under laboratory conditions. Because of these advantages we have used *Xenopus* for nuclear transplantation experiments. Though the principle of Briggs & King's technique has been followed, differences between the eggs of *Rana* and *Xenopus* have made it necessary to modify their technique before it can be satisfactorily applied to the eggs of *Xenopus*. The purpose of this publication is first to give technical details of these modifications, and secondly to discuss the extent to which they might affect a direct comparison of the results of transplantations in *Rana* and *Xenopus*.

TECHNIQUE

Apparatus

A low-power binocular microscope giving a magnification of between $\times 25$ and $\times 50$ has been found suitable for transplanting nuclei. A syringe capable of delivering small quantities of fluid is required. We have used an 'Agla' micrometer syringe (Burroughs Wellcome & Co., London), in which a fairly strong spring was fitted to the shaft of the plunger, controlling the uptake of fluid. The needle of the syringe is connected to a micropipette by polythene tubing of about 1-mm. bore. The micropipettes are constructed as follows. Lengths of thin-walled glass tubing are cleaned chemically and thoroughly washed out. They are then drawn out to an internal diameter of 20 to 50 μ as required. The syringe, tubing, and micropipette are all filled with liquid paraffin (B.D.H., Ltd.); this has been found to give superior control to that given by air or water. It does not appear to have any harmful consequences on either the donor cell

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or recipient egg, with which it should not in any case come into contact. The pipette is held and controlled by a Singer 'Microdissector' (Plate, fig. A). This instrument reduces all hand movements by a factor of about 4, and does not invert movements in any direction. Direct movement is particularly valuable in the vertical plane.

The source of ultra-violet irradiation which we have used for inactivating the egg pronucleus is a 100-W medium-pressure mercury arc lamp made by Hanovia Ltd., Slough, England. This source gives an appreciable emission at all wavelengths from 2,100 Å to 3,200 Å, as well as visible light. The lamp is mounted horizontally, giving a downwardly directed beam on to the eggs, which are placed 13 cm. below the bulb. A quartz condenser, 4 cm. above the eggs, focuses the beam on to an area about 2 cm. in diameter.

The nuclear marker

A nuclear marker (Elsdale, Fischberg, & Smith, 1958; Fischberg & Wallace, 1960) has proved most helpful for our nuclear transplantation experiments. Various uses to which this marker has already been put have been described in recent publications from this laboratory (Fischberg, Gurdon, & Elsdale, 1958;

TABLE 1

The use of the Xenopus nuclear marker for determining the origin of nuclei in transplant-embryos

Donor nuclei from the 1-nucleolated strain are transplanted into recipient eggs from normal 2-nucleolated individuals

| <i>Ploidy</i> | <i>Number of nucleoli</i> | | |
|---------------|--|---|---|
| | 1 | 2 | 3 |
| Haploid | Parthenogenetic development of haploid egg pronucleus. Less than 1 per cent. | | |
| Diploid | Development of transplanted nucleus only. 60-100 per cent. | Parthenogenetic development of diploid egg nucleus. Less than 1 per cent. | |
| Triploid | | Haploid egg nucleus combined with diploid transplanted nucleus. Less than 1 per cent. | |
| Tetraploid | | Doubled transplanted nucleus. Up to 40 per cent. (Gurdon, 1959). | Diploid egg nucleus combined with transplanted nucleus. Less than 1 per cent. |

Gurdon, 1960; Blackler, 1960). Diploid nuclei of wild-type *Xenopus* each have two nucleolar organizers, and usually have two nucleoli; however, a variable proportion of such nuclei (average 30-50 per cent.) have only one nucleolus

resulting from fusion of the nucleolar material of the two organizers. The nuclear marker consists of a mutation causing inhibition or loss of the nucleolar organizer, of which there is one in every haploid set of chromosomes. Thus nuclei of diploid individuals heterozygous for this mutation have only one nucleolus, and may therefore be easily distinguished from nuclei of normal diploids, the majority of which have two nucleoli. In all other respects individuals heterozygous for this mutation are entirely normal. Animals homozygous for this mutation possess no proper nucleoli, but have small droplets of nucleolar substance which are of irregular size and number. However, homozygotes are inviable and die before feeding.

For nuclear transplantation experiments it has been customary to transplant marked donor nuclei into eggs of unmarked female frogs. Assuming that the ploidy and nucleolar number of a transplant-embryo is known, the origin of its nuclei is immediately apparent (see Table 1). The nuclear marker can be rapidly identified under the phase-contrast microscope.

The preparation of donor cells

The developmental stages of donor embryos have been identified according to the Normal table for *X. laevis* of Nieuwkoop & Faber (1956).

The dissociation of donor cells has been carried out by the methods of King & Briggs (1955). We usually use Barth's saline solution 'X' (Barth & Barth, 1959) instead of Niu and Twitty's solution, but the results are very little, if at all, affected by this change. Many tissues, especially those of younger embryos, can be dissected away from the rest of the embryo without the help of trypsin. The tissue is placed in Barth's solution 'X', in which calcium and magnesium are replaced by 5×10^{-4} M versene. The time required to bring about dissociation in versene increases as the age of the donor embryo increases. For early stages such as blastulae, 10 minutes of versene is sufficient. Longer periods of 20 minutes or more are needed for later stages. Dissociated cells can be kept without harm in normal strength Barth solution, phosphate-buffered to about pH 7.7, for over half an hour before being transplanted. When dissociated they are transferred to an agar-covered slide with at least 1 ml. of Barth solution on it. To prevent evaporation altering the strength of the saline solution, the slide carrying the donor cells should only be placed in the beam of the microscope lamp when drawing the cell into the pipette.

The preparation of recipient eggs

Adult females of *Xenopus* can be induced to ovulate several times a year by injection of gonadotropic hormone. We have used the commercial product 'Gonan' (B.D.H., Ltd.) or 'Pregnyl' (Organon Laboratories, Ltd.), and find that a dose of about 300 units is suitable for females and one of 150 units for males (the latter should have black nuptial pads on the forearm before injection).

The dose is varied according to the size of the animals and their condition. The presence of a male is not necessary to induce a hormone-injected female to lay eggs. It has been shown (Gurdon, 1960*b*) that eggs used as recipients for nuclear transplantation give results which vary not only from one frog to another, but also from one ovulation to the next of the same frog. No way has yet been found of improving the quality of eggs, and we have no evidence that the eggs of frogs imported from Africa give consistently better results than the eggs of those reared in the laboratory.

Before transplanting a donor nucleus into an egg, it is necessary to remove or inactivate the egg pronucleus. In frogs of the genus *Rana* and in newts this can be reliably done by lifting the egg nucleus and a little surrounding cytoplasm out of the egg with a needle (Porter's technique, 1939). This method is used by Briggs & King for their nuclear transplantation experiments with *Rana*. The great strength and elasticity of the vitelline membrane of *Xenopus* eggs prevents this method being used both reliably and harmlessly for the eggs of this species (Gurdon, 1960*a*). We have found that a short exposure (less than 1 minute) of the animal pole of an egg to ultra-violet irradiation inactivates the egg pronucleus but does not damage the egg in any other respect. Another very considerable advantage of this ultra-violet treatment is that it renders the vitelline membrane penetrable to a micropipette without destroying it, and at the same time destroys the jelly over the animal pole of the egg. It may also sterilize the injection area. Unless weakened in this way, the vitelline membrane cannot be penetrated by a pipette without damaging the egg. The effects of ultra-violet irradiation on *Xenopus* eggs form the subject of another publication (Gurdon, 1960*a*), which describes the fate of the irradiated egg nucleus and considers the evidence that the ultra-violet does not damage the egg cytoplasm. The main reason for this latter belief is that a considerable increase in u.v. dose does not increase the abnormalities of transplant-embryo development.

Best results are obtained if eggs are not used for transplantation immediately after laying, but are left in water for about 15 minutes (Gurdon, 1960*b*). Eggs to be irradiated are placed dry, about six at a time, on a glass slide. Free water must be removed so that the egg remains with its animal pole facing upwards. The slide carrying the eggs is then placed under the ultra-violet beam for the appropriate amount of time (between 20 and 50 seconds according to the particular ovulation). The eggs are now ready for the injection of the donor nucleus.

The transplantation procedure

The disaggregated donor cells on an agar-covered slide can be seen very easily if all light coming from below the slide is cut out and strong illumination from above is used (Plate, fig. B). There is some variation in the size of cells from various regions of a donor embryo, and a cell is chosen which is just too big to fit into the end of the pipette, so that the cell-wall is broken when the cell is sucked up (Plate, fig. B). The cell cytoplasm should still completely surround

the nucleus, which must not be greatly distorted by this treatment. This part of the technique follows the technique of Briggs & King (1952).

The slide carrying the irradiated eggs (still not surrounded by fluid) and that carrying the donor cells can conveniently be placed on a larger glass slide. This enables the donor cells or recipient eggs to be quickly placed in the field of view as required. Before the donor cell is drawn into the pipette, a small air bubble should be sucked into it so as to separate the paraffin from the saline solution (Plate, fig. B). This allows the passage of fluid out of the pipette to be accurately observed when the donor cell has passed out of sight (Plate, fig. C). The position of the air bubble must, of course, be adjusted each time a new donor cell is sucked up. The pipette is inserted into the egg so that its tip which contains the donor cell comes to lie somewhere between the centre of the egg and its animal pole. While watching the air bubble the syringe control is turned so that the donor cell and a small amount of saline solution (but not the air bubble) is deposited near the centre of the egg. If this is done carefully and if the u.v. dose has been judged correctly, the egg will heal up very quickly after withdrawal of the pipette (Plate, figs. D, E). It is desirable to inject a small amount of saline solution with the donor cell because it helps to ensure that the cell is not damaged by the end of the pipette, whilst at the same time it does not affect development. During the operation the pipette has been controlled with the help of the microdissector. The average time required for the injection of one egg with a nucleus is about 1 minute. Thus, if six eggs on one slide are irradiated and injected, the eggs will remain uncovered with fluid for about 10 minutes. If the operation takes longer, fewer eggs should be done at one time. If eggs are left uncovered by water for too long, the jelly and vitelline membrane may dry on to the egg surface, causing abnormal development or preventing development altogether.

The culturing and scoring of transplant-embryos

As soon as the eggs on one slide have all been injected with a nucleus, the slide is placed in a small Petri dish containing full-strength Niu & Twitty's solution. The eggs are left in this solution for a few hours because this facilitates the healing of the injection wound. This solution is replaced by one-tenth strength Niu & Twitty's solution some time during cleavage. Shortly before hatching the embryos are placed in tap-water from which excess chlorine has been allowed to evaporate. When tadpoles are ready to feed they are placed in 50-ml. tubes and fed on a weak suspension of powdered nettles (*Urtica* sp.). They are changed to a larger bottle (about 3 tadpoles per litre of water) as they grow, but are fed on nettle-powder until metamorphosis, which occurs about 2 months after laying. We experience very little mortality between the beginning of feeding and metamorphosis, and have not found it necessary to use antibiotics at any stage. After metamorphosis frogs are fed on *Tubifex*, and later on liver.

Many transplant-embryos develop abnormally, and the following procedure

is followed when it is clear that an embryo will not differentiate any further. It is drawn under a camera lucida; then a small part of it is lightly squashed on a slide under a coverslip. The number of nucleoli per nucleus can be quickly counted under the phase-contrast microscope (Elsdale, Fischberg, & Smith, 1958). Chromosome numbers can be determined after leaving the tissue in aceto-orcein for about 1 minute before squashing. The rest of the embryo may then be fixed for sectioning. If an embryo is not required for sectioning, the whole of it may be squashed so that the ploidy and nucleolar number can be estimated in different regions. If the embryo is old enough to have a tail, part of this may be removed and stained in haemalum, which shows the nucleoli and chromosomes clearly.

THE MAIN DIFFERENCES IN THE NUCLEAR TRANSPLANTATION TECHNIQUES FOR *XENOPUS* AND *RANA*

The most obvious difference between Briggs & King's technique for *Rana* and our modifications of it for *Xenopus* concerns the method of recipient egg enucleation. With *Rana* the egg nucleus is removed from the egg, while in *Xenopus* it is irradiated with ultra-violet and then allowed to degenerate in the egg cytoplasm. The fate of the irradiated egg nucleus in *Xenopus* has been followed, and there is no reason to believe that this or the other consequences of the ultra-violet treatment affect the development of the transplanted nucleus in any way (Gurdon, 1960a); this question is discussed further by Briggs & King (1960) and Gurdon (1960c).

Another modification which we have introduced concerns the *Xenopus* nuclear marker. The presence of this marker is not associated with any abnormalities, but gives proof that the nuclei of transplant-embryos are derived from the injected nucleus alone.

Other differences also exist which are of considerable practical importance but which do not affect the results. For instance, *Xenopus* eggs are left uncovered by saline solution for about 10 minutes during transplantation; this only causes damage if the eggs are allowed to become dry on the surface; but eggs in which this has happened can easily be recognized and thrown away.

When comparing nuclear transplantation experiments in *Rana* and *Xenopus*, variation in the quality of recipient eggs must be considered. We have found that nuclei from the same donor embryo, when transplanted into the eggs of different females, can give very different results. It seems that some of our abnormal transplant-embryos may be attributed to poor quality of recipient eggs; this might be due to the fact that it is not possible to give *Xenopus* the same conditions in the laboratory as they enjoy in the wild.

One may conclude that, with the possible but unlikely exception of egg nucleus degeneration in *Xenopus*, none of the differences in technique between *Rana* and *Xenopus* influence the results of experiments with these species.

Differences in the results should therefore represent real differences between the species and not differences in technique. This conclusion applies only in principle, and does not take account of variation due to any inconsistency with which the technique is carried out on different occasions or by different workers. The effect of varying certain parts of the technique, more than would happen under normal circumstances, is described elsewhere (Gurdon, 1960b).

SUMMARY

1. Technical details are given of a method of transplanting embryonic nuclei in *X. laevis*.

2. This technique is based on Briggs & King's method for *R. pipiens*. Owing to certain differences between the eggs of *Rana* and *Xenopus*, their technique has been modified for *Xenopus*.

3. The main modification for *Xenopus* concerns the use of ultra-violet irradiation instead of enucleation by a needle.

4. Differences between transplantation techniques for *Rana* and *Xenopus* are unlikely to account for the differences in experimental results obtained with these species.

RÉSUMÉ

Description de la technique de transplantation du noyau chez Xenopus laevis

Des détails techniques sont donnés sur une méthode de transplantation des noyaux embryonnaires chez *Xenopus laevis*. Cette technique est basée sur la méthode employée par Briggs & King chez *Rana pipiens*. A cause de certaines différences entre les œufs de *Rana* et de *Xenopus*, la technique a été modifiée pour *Xenopus*. La principale modification concerne l'utilisation des rayons ultra-violet à la place de l'énucléation par une aiguille. Les différences entre les techniques de transplantation utilisées chez *Rana* et *Xenopus* n'expliquent vraisemblablement pas dans les différences entre les résultats obtenus chez ces deux espèces.

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EXPLANATION OF PLATE

FIG. A. Photograph of the transplantation apparatus, showing a 'Singer' microdissector, a microscope, and an 'Aglá' syringe with polythene tubing. Illumination is provided by a small spot-lamp (not shown).

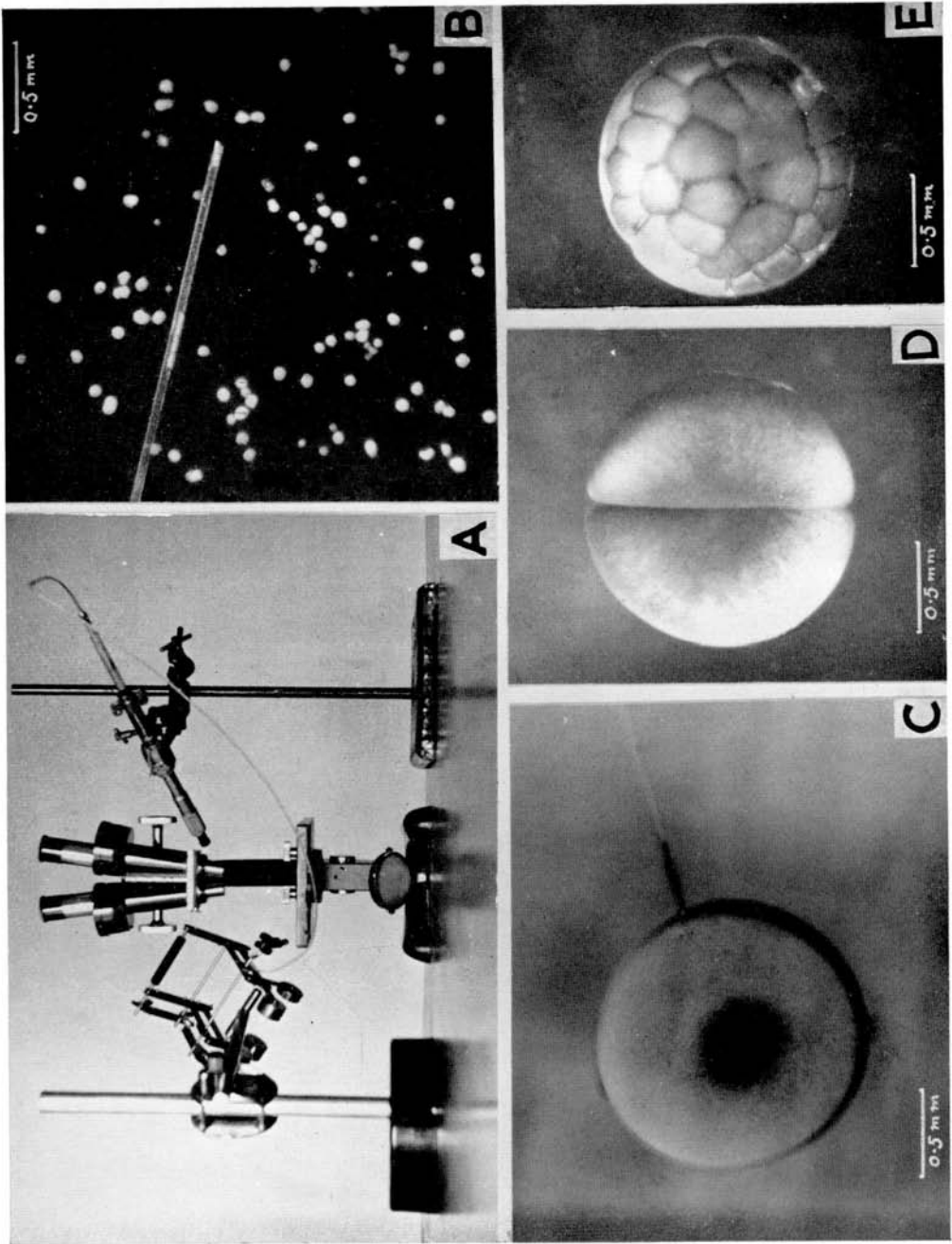
FIG. B. Disaggregated cells of a *X. laevis* late blastula, one of which has just been sucked into the end of the micropipette; the air-bubble marker can be seen in the shaft of the pipette. $\times 25$.

FIG. C. The micropipette is seen inserted into the recipient egg. The donor cell has been injected and the air-bubble marker (dark) is seen where the shaft of the pipette penetrates the egg. $\times 25$.

FIG. D. A 2-cell stage of a successfully transplanted egg. The penetration wound has completely healed and can no longer be seen. $\times 25$.

FIG. E. Another transplant-embryo at the morula stage. The small exovate from the injection (lower right of picture) has separated from the surface of the 'egg', and will not interfere with further development. $\times 25$.

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