Quantitative morphological analysis of early mouse embryogenesis in vitro

I. Perfusion culture system, tissue preparation, sampling

By RUSSELL L. DETER

From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas

SUMMARY

To facilitate a quantitative morphological analysis of early mouse development under controlled conditions, a perfusion culture system capable of supporting embryogenesis to blastocyst stage has been developed. The use of a mesh system allows identification of individual embryos by position, and control of their orientation during culture and preparation for light and electron microscopy. Quantitative evaluation of tissue-processing procedures has permitted selection of conditions which reduce changes in linear dimensions to $-1.6 \pm 1.8\%$ in two-cell embryos. Through the definition of a coordinate system based on mesh structure and the development of a special sectioning procedure, sections can be localized within the intact embryo and three-dimensional coordinates given to any element of embryo volume.

INTRODUCTION

To extend the analysis of structural change and its relationship to the biological and biochemical events occurring during pre-implantation embryogenesis, a quantitative morphological study of early development in the mouse is being undertaken. In order to have access to embryos during their development and to control their micro-environment, embryogenesis is being carried out in vitro (Biggers, Whitten & Whittingham, 1971; Hoppe & Pitts, 1973). To correlate physiological and biochemical parameters with the structural organization of living embryos and those prepared for light and electron microscopy, culture conditions and tissue-processing procedures have been sought which (1) support normal embryogenesis in vitro, (2) permit rapid change of the embryo's microenvironment, (3) conserve the identity and orientation of individual embryos, and (4) minimize changes in embryo geometry induced by fixation and embedding. As the quantitative validity of the morphological analysis depends on the adequacy of the sampling procedures employed, a means for gaining access to the entire embryo volume and specifying the samples taken for study has been developed.

1 Author's address: Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, U.S.A.
MATERIALS AND METHODS

Embryos

Embryos used in these investigations were $F_1$ hybrids obtained by super-ovulating (Gates, 1972) immature (3–5 weeks) BALB/c females, mating them with adult 129 males and collecting the embryos at various times (beginning 44 h after hCG administration) by flushing the oviducts (Mintz, 1971). Whitten’s defined medium (Whitten, 1970) equilibrated with a $5\% O_2 - 5\% CO_2 - 90\% N_2$ gas mixture was used as the flushing solution.

Culture system

Embryo culture was carried out in the perfusion culture chamber described by Dvorak & Stotler (1971) connected to a medium reservoir (Glenco Scientific Glass Co., Houston, Texas) and a Desaga parastaltic pump (Brinkman Instruments, Westbury, N.Y.). The temperature of the medium in the chamber, monitored by a thermistor (tele-thermometer, Yellow Springs Instrument Co., Yellow Springs, Ohio) attached to the upper coverslip, was maintained at $37^\circ C$ with an air stream stage incubator (Model C300, Nicholson Scientific Co., Bethesda, Maryland). The culture chamber was mounted on the stage of a bright-field light microscope to permit continuous observation of embryo development.

The flow rate through the 0.2 ml compartment containing the embryos was kept constant at 1.0 ml/h. Whitten’s medium, equilibrated with the $O_2-CO_2-N_2$ gas mixture described previously, was used in all experiments. To prevent embryo loss or change in position, embryos were placed in compartments formed by a special mesh system (Fig. 1a). This system (0.5 x 1.0 cm) was prepared by bonding a nylon mesh (Nitex HD 3-95, Wire Tech, Houston, Texas) to a polystyrene or polyester coverslip (Lux Scientific Corp., Thousand Oaks, Calif.) with heat. For mouse embryos, a mesh with interstices measuring $\sim 90 \times 90 \mu m$ was used. The mesh system was placed in a sterilized partially assembled chamber and the collected embryos transferred to the mesh with a small amount of fluid in a micropipette. After the embryos were in their mesh compartments, the chamber was closed and filled with medium.

Microscopy

Embryo development in culture was followed using bright-field, phase, interference-contrast and modulation-contrast light microscopy, with bright-field microscopy being used most often. To permit correlative studies with transmission light and electron microscopy, embryos were fixed and embedded while still in their mesh compartments after removal of the mesh system from the chamber. To evaluate the degree of embryo distortion, two-cell embryos, orientated so that the area of contact between the blastomeres could be kept in the plane of focus (Figs. 1b, c), were photographed after each processing step.
Fig. 1. Light and electron microscopy of two-cell mouse embryos. (a) Living mouse embryos in culture as seen with bright-field microscopy, × 73. (b) An unfixed embryo is seen at higher magnification, × 370. (c) Same embryo after fixation, dehydration and embedding in VCD, × 370, as described in Table 1. (d), (e) Sections of embedded embryos prepared for transmission light ((d) × 642) and electron ((e) × 662) microscopy. In these two figures, the embryo profile (A), the plastic-coverslip junction (B) and the right-hand side of the section (C) can be seen. In the upper left-hand corner is a profile of one of the mesh fibers.
Fig. 2. Embryo coordinate system. This drawing illustrates the three-dimensional coordinate system used to specify the location of structures within the embryo. \( A \) is the polyester coverslip to which the nylon mesh, \( B \), is bonded. The mesh fiber parallel to the \( X \) axis has been removed so that the coordinate axes can be seen more clearly.

and the perpendicular distance from the center of the cleavage plane to the edge of one blastomere was measured on the projected (500 x) images of the negative obtained (Deter, 1974). These measurements were compared and the percentage change recorded. The optimal preparation procedure is given in Table 1.

To permit section localization and assignment of three-dimensional coordinates to any point within the embryo, a special sectioning procedure was developed. This procedure required the definition of a Cartesian coordinate system (Fig. 2) in which the \( X-Y \) plane is congruent with the coverslip of the mesh system, the \( X \) axis parallel to one fiber of the compartment containing the embryo and the origin in a corner of that compartment or at a point on the \( Y \) axis where a perpendicular through the point first intersects the embryo. In preparing embryos for sectioning, micrographs of the embedded embryos were used as references. A direction of approach was chosen and the mesh orientated so that the section plane was perpendicular to the mesh plane and parallel to the \( X \) axis. With a LKB Pyramitome (Model 11800, LKB Produkter AB, Bromma, Sweden), a square block containing the embryo was formed, its right-hand side lying in the \( Y-Z \) plane. The size of the block face was made large enough to encompass the maximal embryo profile and the plastic–coverslip junction. For light microscopy, 210 nm sections parallel to the \( X-Z \) plane were cut, stained with toluidine blue (Mercer, 1963) and photographed using a Leitz Ortholux microscope. Electron microscopy was carried out on 50–70 nm sections placed on Formvar-coated slot grids (0·2 × 1·5 mm) as described by Sjostrand (1967). These sections, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), were photographed in a modified (Deter, 1974) RCA 3-G or Seimens 102 electron microscope.
To localize sections within the embryo, the number and section thickness (estimated from interference colors) were recorded for all sections beginning with the first to contain a profile of the zona pellucida. The $Y$ coordinate for any given section was obtained by summing the thicknesses of all preceding sections. The $X-Y$ coordinate system used in the sectioning procedure was then superimposed on photographs of the intact embedded embryo and the origin on the $Y$ axis identified. The location of sections within the embryo could then be specified by their $Y$-coordinate values. To test this procedure, the distance from the origin to the beginning of the second blastomere in four two-cell embryos was determined using $\sim 0.2 \mu m$ sections. These values were compared with measurements of the same distances made on photographs.

**RESULTS**

*Embryogenesis in perfusion culture*

Two-cell, three-cell and four-cell embryos placed in culture 48, 53 and 53 h after hCG injection and followed for 53, 48 and 64 h developed into both morulae and blastocysts. The percentage values were 56 % (45/81), 51 % (30/59) and 15 % (3/20) for morulae and 22 % (18/81), 46 % (27/59) and 70 % (14/20) for blastocysts respectively. Development was similar to that reported previously (Biggers et al. 1971). The only problems encountered were inadequately stretched meshes, so that embryos were able to move from one compartment to another in a space between the mesh and the coverslip, and mesh compartments that were too large, so that embryos rotated and became dislodged from their compartments because interaction between the zona pellucida and the mesh fibers was inadequate. When placed in an appropriate mesh system, embryos remained fixed in position throughout the culture period.

*Preparation of embryos for transmission light and electron microscopy*

Fixation and embedding of embryos retained in the mesh required minimization of mechanical stress and adequate displacement of fluids if embryo position and orientation was to be conserved and a well-polymerized plastic obtained. Embryos appear to be particularly sensitive to mechanical displacement before fixation in glutaraldehyde and after dehydration in alcohol. During these periods movement of the mesh must be avoided if possible and the flow of liquids over the mesh should be slow and from a constant direction. Because of its large surface area and many recesses, there is a tendency for fluids to be retained in the mesh, interfering with the polymerization of the plastic. Through use of a low viscosity plastic (VCD-Spurr, 1969), extensive drainage of the mesh after each step and large exchange volumes, this problem was eliminated. However, embedding in VCD required the use of polyester instead of polystyrene coverslips as the latter became swollen and opaque in the VCD monomer. Unfortunately, polyester coverslips interfere with phase microscopy.
### Table 1
Preparation of embryos for light and electron microscopy

<table>
<thead>
<tr>
<th>Process</th>
<th>Solution*</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Glut (4%)-PO₄ (0.05 M, pH 7.4)</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>2. PO₄ (0.05 M, pH 7.4)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3. OsO₄ (2%) -PO₄ (0.05 M, pH 7.4)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>4. PO₄ (0.05 M, pH 7.4)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Dehydration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. EtOH (50%)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2. EtOH (75%)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3. EtOH (95%)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4. EtOH (100%)†</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>5. EtOH (100%)†</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Embedding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. EtOH (67%)-VCD mixture (33%)†</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>2. EtOH (50%)-VCD mixture (50%)†</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>3. EtOH (33%)-VCD mixture (67%)†</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>4. VCD mixture (100%)†</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>5. VCD mixture (100%)</td>
<td>2-3 days at 60 °C</td>
<td></td>
</tr>
</tbody>
</table>

Effect of preparation procedure on two-cell mouse embryos

The data presented below represent the percent change in the distance from the center of the cleavage plane to the edge of one blastomere observed after different steps in the tissue preparation procedure. Average values and their standard deviations are given, the number of observations indicated in parentheses.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfixed v. Glut fixed</td>
<td>7.6 ± 2.7 (20)</td>
</tr>
<tr>
<td>Glut fixed v. OsO₄ fixed</td>
<td>0.7 ± 2.2 (18)</td>
</tr>
<tr>
<td>OsO₄ fixed v. EtOH dehydrated</td>
<td>-6.4 ± 2.5 (16)</td>
</tr>
<tr>
<td>EtOH dehydrated v. VCD infiltrated‡</td>
<td>-5.8 ± 3.1 (13)</td>
</tr>
<tr>
<td>VCD infiltrated‡ v. VCD embedded§</td>
<td>2.6 ± 2.7 (13)</td>
</tr>
<tr>
<td>Unfixed v. VCD embedded§</td>
<td>-1.6 ± 1.8 (15)</td>
</tr>
</tbody>
</table>

* Solution abbreviations: Glut, glutaraldehyde; PO₄, NaH₂PO₄-NaHPO₄ buffer; OsO₄, osmium tetroxide; EtOH, ethanol; VCD mixture, vinylcyclohexene dioxide mixture described by Spurr (1969).
† These steps were carried out under anhydrous conditions.
‡ Infiltrated with the VCD mixture to the 100\% stage.
§ Following polymerization of the VCD mixture.

The procedure outlined in Table 1 gives embedded embryos which differ from unfixed embryos by an average of only 1.6\% in linear dimensions (a difference of approximately 5 % in volume). The shrinkage induced by alcohol is counteracted by swelling during fixation and polymerization. Preliminary electron-microscopic studies have revealed no artifacts that could be attributed
Morphological analysis of early mouse embryogenesis

to volume changes. To obtain well-defined membranes, an OsO₄ concentration of 2% was required (Calarco, 1968).

Sectioning

Figs. 1(d) and (e) show thick and thin sections containing the embryo profile (Fig. 1(d), (e) A) and the VCD-coverslip junction (Figs. 1(d), (e) B), the latter lying parallel to the X axis. The right-hand side of the section (Fig. 1(d), (e) C) is parallel to the Z axis. A Z-coordinate value for any point in the section can be determined by measuring its perpendicular distance from the VCD-coverslip junction. Similarly, the X-coordinate value is determined by measuring the perpendicular distance to a line perpendicular to the VCD-coverslip junction which passes through the intersection of the junction with the right-hand side of the section. The Y-coordinate value is determined from section numbers and thicknesses. In four tests of the procedure for determining Y-coordinate values, the distance across a blastomere determined from section data differed from that measured directly on negatives by -2.5%, +0.5%, +5.2% and -2.8%, respectively.

DISCUSSION

Preliminary studies suggest that the perfusion culture system described can support early embryogenesis not only of mouse but also of rabbit embryos, if the medium described by Naglee, Maurer & Foote (1969) is used.

The response of embryos to different steps in the processing procedure proved variable (Table 1), but the overall effect on embryo geometry could be minimized with proper choice of conditions. However, optimal conditions may vary with the stage of development, as preliminary studies indicate that the response of unfertilized oocytes and blastocysts differs from that of two-cell embryos. The quantitative evaluation of the tissue-preparation procedure described here is the first reported for mammalian embryos and the first for any tissue using the VCD embedding medium. It is one of the few (Kushida, 1962; Weibel & Knight, 1964; Tootze, 1964; Hayat, 1970; Bone & Denton, 1971; Eisenberg & Mobley, 1975) carried out on any tissue with any processing procedure. The overall change in volume observed in our investigations is much less than that reported in most previous studies.

Processing embryos still held in their original mesh compartment conserves individual embryo identification and orientation, thus permitting correlation of data from sections with that obtained from living embryos. However, continuous visual monitoring during each step of the procedure is essential since embryo movement, rotational as well as translational, can occur. If an embryo axis can be defined, it is relatively easy to compensate for such movements unless the rotation is in a plane perpendicular to the mesh. This has not been a major problem but could become important with embryos having a more complex geometry.
To obtain morphological data having quantitative validity, any sampling must be representative. With embryo populations, individual embryo identification makes possible the definition of more homogeneous sub-groups (based on stage of development, developmental sequence, specific morphological characteristics, etc.), while giving each embryo an equal chance to be included in the sample drawn from its subgroup. The sectioning procedure described here, which gives access to all parts of the embryo volume and provides a means for locating each section within the embryo, permits the design of an appropriate procedure for sampling sections, depending on the distribution of the object being studied (Cochran, 1963). To characterize subcellular organization, section samples must be drawn appropriately from individual blastomeres. Since blastomere profiles from the same cell appear in different sections, they must be identifiable in order to be available for sampling. An analysis of the three-dimensional coordinates of reference points and profile areas make such identifications possible (Deter & Wagner, 1975; Deter, unpublished).

The methods described are particularly appropriate for studies in which correlation of morphological organization with biochemical or physiological parameters is the principal objective. They can also be applied to electrophysiological studies of embryo development if the upper coverslip of the culture chamber is replaced with a thin sheet of transparent plastic. Embryos held in the mesh are then accessible to microelectrodes. Recent work (Rice & Deter, unpublished) has indicated that the procedure of Meller, Coppe, Ito & Waterman (1973) for combined scanning and transmission electron microscopy can be applied to embryos in mesh compartments, allowing correlation of surface as well as internal morphology with physiological or biochemical observations.

Quantitative morphological studies may make possible the identification of early structural differences in blastomere organization, heretofore undetectable until the late blastocyst stage (Calarco & Brown, 1969; Enders & Schlafke, 1965), and may provide morphological correlates for differentiation events detected by other means (Herbert & Graham, 1974). It is also possible that compartments, produced by diffusion barriers (Ducibella, Albertini, Anderson & Biggers, 1975) or functional linkage of cells through intercellular junctions (Burton & Canham, 1973), can be defined and characterized.

Finally, the data derived from morphometric studies of embryogenesis have permitted the development of a mathematical simulation of early morphogenetic events (Deter & Wagner, 1975; Wagner & Deter, 1977) that permits the testing of hypotheses concerned with the control of morphogenesis and makes possible a study of the sequence of geometrical changes which give rise to specific embryo configurations.

The author would like to thank Ms Velma Caldwell for her excellent technical assistance and Dr Roberto Vitale for his help in preparing the illustrations. This work was supported by the Center for Population Research and Studies in Reproductive Biology (NIH Grant HD 7495).
REFERENCES


(Received 12 October 1976, revised 2 March 1977)