

Human embryos *in vitro*: pioneer illustrations of oocyte maturation, fertilization, cleavage and blastulation

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These illustrations were taken between 1962 and 1972 when founding investigations into early human embryology were opening prospects of IVF and assisted human conception, the preimplantation diagnosis of inherited disease and the growth of embryonic stem cells using outgrowths of blastocyst cells. I am indebted to the photographic staff of the Physiological Laboratory, Cambridge University, UK, the Oldham and District General Hospital, UK and the Medical School, Johns Hopkins University, Baltimore, USA for their help in preparing these illustrations.

Oocyte maturation (Figures 1.1–1.4)

In those now far-off days, it was first essential to establish the correct conditions and timing for the *in-vitro* maturation of human oocytes from the germinal vesicle (dictyate) stage. During the maturation phase, all meiotic stages between diakinesis and metaphase II (MII) would have to be identified and timed exactly. The total duration of oocyte maturation *in vitro* would then predict the interval between the onset of the LH surge, or an injection of human chorionic gonadotrophin (HCG), and ovulation, as found in other species (Edwards, 1965a). When human oocytes in MII and with an extruded first polar body became available, studies on the fertilization of these oocytes *in vitro* could begin. In turn, analyses could then be made on embryonic pronuclear stages, cleavage and blastulation in the human embryo.

In the 1930s, Pincus *et al.* had shown how rabbit and human oocytes released from their follicles would spontaneously mature *in vitro* to MII. Unfortunately, while their rabbit analyses were sound, their analyses of human oocytes were confounded by the large numbers in their study which already possessed chromosomes when isolated from their follicles. These oocytes were presumably in MII, and had apparently been extracted from atretic follicles. Pincus and Saunders (1939) failed throughout their study to identify the exact chromosomal stages of meiosis, leading to such incorrect conclusions about maturation. The frequency of human oocytes containing chromosomes in their work peaked at 12 h in culture, indicating this was the interval required for full maturation to occur. This conclusion was erroneous. It led later investigators to inseminate human oocytes after 12 h maturation *in vitro* (Menkin and Rock, 1948; Hayashi, 1963).

The correct timing of 37 h was identified 25 years later (Edwards, 1962, 1965a,b), and is illustrated in the present series of illustrations. All the events of maturation: breakdown

of the germinal vesicle, appearance of chromosomes in diakinesis which is the last stage of meiotic prophase I, onset of metaphase I, anaphase I and telophase I, extrusion of the first polar body and movement to MII. Each stage was timed, and the whole duration of maturation required 37 h.

Fertilization *in vitro* (Figures 1.5–1.7)

During the 1960s, it was firmly believed that capacitation was essential to enable spermatozoa to penetrate through, or even attach to the zona pellucida. Moreover, capacitation could apparently only be achieved within the female tract (Dauzier and Thibault, 1956). In 1964, Yanagimachi and Chang managed to achieve the fertilization of hamster oocytes *in vitro* using epididymal spermatozoa (Yanagimachi and Chang, 1964). Even though epididymal spermatozoa were used and the embryos failed to cleave to any extent, their finding was encouraging for attempts at human fertilization *in vitro*. However, human ejaculated spermatozoa would have to be used for IVF after most seminal plasma had been removed, e.g. by mild centrifugation, and seminal plasma was believed to possess anti-capacitating factors which were presumably removed within the female reproductive tract. Early attempts to fertilize human oocytes *in vitro* after oocyte maturation *in vitro* using washed ejaculated spermatozoa produced occasional oocytes with pronuclei, but sperm tails could not be identified in ooplasm (Edwards *et al.*, 1966). Another attempt to expose inseminated oocytes to these secretions involved inserting a small chamber lined with porous Millipore filter into the uterus of women volunteers. Before insertion, it was filled with washed, ejaculated human spermatozoa (Edwards *et al.*, 1968). The intention was to obtain capacitation within the chamber, so it was inserted at mid-cycle. Unfortunately, no fertilization resulted with spermatozoa recovered from the chambers, and analyses of the fluid contents of the chamber indicated that it had seemingly caused inflammation. Emphasis was accordingly changed to the use of washed ejaculated human spermatozoa *in vitro*. An animal model was needed, and the bovine was chosen because cow oocytes could be matured *in vitro* (Edwards, 1973) and bull semen could be collected from a nearby cattle artificial insemination station. Seminal plasma was removed from the bull spermatozoa using mild centrifugation and was used to inseminate fully mature cow oocytes (Edwards, 1973). Various culture media were used including physiological saline solutions and medium 199. Several cow oocytes were found to be undergoing fertilization, which indicated that the problem of sperm capacitation had been overcome. This method was therefore repeated with human oocytes and semen. Initially, a low proportion of human

oocytes with two pronuclei was identified. With increasing skills and the use of various media, many human pronucleate oocytes were later obtained after insemination *in vitro* using washed ejaculated spermatozoa.

Cleavage and blastulation of the human oocyte *in vitro* (Figures 1.8–1.14)

When fertilization and pronucleus formation were seen to occur *in vitro*, the stage was set to culture the human embryos to the blastocyst. Patrick Steptoe was now a fellow colleague in the team, and the work was now being applied clinically in Oldham. Maturation *in vitro* was abandoned, because the resulting embryos develop very poorly. This was first shown in rabbit by Chang (1955), and confirmed in our laboratory in Cambridge where *in-vitro* matured and fertilized rabbit blastocysts were found to possess a defective inner cell mass or to lack it completely. It was necessary to collect mature human oocytes from their follicles just before they ovulated. Patients were accordingly given mild ovarian stimulation to a total of 12 ampoules of human menopausal gonadotrophin (HMG) i.e. a total of 900 IU and 5000 IU of HCG. Laparoscopy was performed 35–36 h after HCG injection, i.e. when maturing oocytes were approaching MII an hour or so before the first ovulations were expected (Steptoe and Edwards, 1970). All maturing human oocytes were now aspirated from their follicles by means of laparoscopy.

The oocytes were inseminated 2–3 h later in simple modified physiological saline solutions. The oocytes were incubated overnight and checked on the following day for the presence of pronuclei. Those already pronucleate were placed in fresh culture medium. Standard media such as Ham's F10 and F12, Waymouth's medium, medium 199 and others were tested. No obvious advantages seemed to be offered by any of these media except perhaps for Ham's F10, and this was mostly used, reinforced with the patients' own serum, some bovine serum albumin, and antibiotics. Resulting embryos were examined three or four times daily to check their growth *in vitro*. Embryos cleaved regularly to 2-, 4- and 8-cell stages within 2.5 days (Edwards *et al.*, 1970; Steptoe *et al.*, 1971). Their appearance then changed as they compacted, followed by a 'stripey' appearance as morulae formed and blastocoelic fluid began to accumulate between the constituent cells of the emerging blastocysts. Superb blastocysts were seen on days 4–5, and some were expanding a day or so later. Hatching from the zona pellucida was seen, and the oldest blastocyst had hatched and expanded and was rapidly differentiating on day 9 post-insemination (Edwards and Surani, 1978). Chromosome counts on cleaving embryos and blastocysts failed to reveal any triploids or tetraploids, but exact counts were difficult in view of the small numbers of embryonic cells in division.

Opportunities had now been opened to replace human embryos at various stages into their mother in attempts to alleviate infertility. It was thus possible to plan the preimplantation diagnosis of genetic disease, as in rabbits, by excising a

few trophectoderm cells for examination and classification (Gardner and Edwards, 1968). Sexing was 100% accurate after the blastocysts were transferred to foster mothers and delivered at full term. Later attempts to sex human blastocysts by examining them for the chromatin body or the fluorescent Y body were unsuccessful since neither of these organelles were detected in day 5 embryos (Fowler and Edwards, 1973). Studies on outgrowths of embryonic cells from blastocysts were also possible now. This would enable embryo stem cells, or differentiating tissues, to be grown *in vitro* and used for study or, one day, for grafting into sick adults. Initial studies on rabbits had shown how cells differentiated into various tissues *in vitro*, and how it was possible to prepare embryo stem cells carrying specific markers (Cole *et al.*, 1965, 1966).

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Figure Legends

Figure 1.1

Onset of the breakdown of the germinal vesicle in a human oocyte *in vitro*. The light area is the germinal vesicle. A ring-shaped nucleolus can be seen in the centre of the vesicle, associated with some chromosomes emerging into diakinesis. Other condensing chromosomes lie free in nucleoplasm. Note the elongated form of the chromosomes and their association in tetrads which is typical of the conversion from diplotene to diakinesis (Edwards, 1965b).

Figure 1.2

Diakinesis in a maturing human oocyte. The structure of the chromosome pairs and their chiasmata can be clearly seen. Those with one chiasma are arranged in a cross-like shape, and those with two chiasmata display chromosomes in a looped form. An excellent example of two chiasmata is shown lower left where the chromatids are looped together. Some chromosomes or chromatids appear to be separating, possibly due to centromere separation. Counting chiasmata in oocytes to estimate their total chiasmata count provided the first clue to identifying the mechanism of univalent formation. Premature centromere separation and univalent formation could be causes of monosomy and trisomy in human embryos. (Inset) diakinesis in a mouse oocyte maturing *in vitro*; notice the beautiful single- and double-chiasmate tetrads. Diakinesis lasts for only 1 or 2 h, so this essential stage enables an exact timing control to be established during maturation to within 1–2 h. Its identification in human oocytes 24 h after the onset of culture showed decisively that human oocytes would mature *in vitro*, and enabled a close timing of germinal vesicle breakdown. It ultimately led to the timing of all stages human maturation and ovulation.

Figure 1.3

Metaphase I in a human oocyte maturing *in vitro*. The chromosomes are now condensing rapidly, and the structure of chiasmata first seen in diakinesis can still be seen. Note the typical chromosome associations. Two chromatids (arrows) appear to be separated, and to have formed univalent chromosomes. They are probably chromosomes 21 or 22, so this illustration may indicate one form of origin of Down's syndrome. This illustration was taken ~26 h after the oocytes were placed *in vitro*.

Figure 1.4

This illustration shows a detail of MII chromosomes in the human oocyte and its first polar body. This oocyte was matured *in vitro* for 37 h. The typical structure of MII chromatids can be seen and showed that meiosis was evidently totally completed in all chromosome pairs to MII. The first polar body had been extruded, but its chromosomes became mixed with those of the oocyte in this preparation. This identification of MII oocytes set the stage for studies on the fertilization *in vitro* of human oocytes matured *in vitro*.

Figure 1.5

A pronucleate human oocyte studied in the initial phases of the work. The two pronuclei, each containing several nucleoli, can be seen clearly. The small size of the nucleoli and their absence of a polarized appearance suggests that these pronuclei are in the mid-stage of their development. There is a group of irregular extruded bodies which may include polar bodies. The oocyte had been inseminated with washed spermatozoa ~20 h earlier, but no formal identification of fertilization was made because the sperm tail was not identified within the oocyte. It is possible that the mid-piece and flagellum had been destroyed by fixing and staining the oocyte with aceto-orcein which will not preserve morphological detail.

Figure 1.6

Another oocyte examined ~24 h after insemination. The two closely adjacent pronuclei give the appearance of approaching

syngamy. The nucleoli are small and not polarized, so syngamy may have been delayed or arrested.

Figure 1.7

With increasing experience, human fertilization *in vitro* was achieved in 1969 (Edwards *et al.*, 1969). This illustration shows a composite of pictures made in Oldham in 1970 showing all the stages of human fertilization in oocytes matured and fertilized *in vitro*. Illustrations show (*upper left*) a living human oocyte with its first polar body and zona pellucida; its corona cells were removed to facilitate examination. *Upper centre*, spermatozoa have migrated through the zona pellucida and are about to enter the perivitelline space surrounding the oocyte. *Upper right*, the spermatozoon has passed through the zona pellucida and is just touching the oolemma via its acrosomal region. The sperm tail was seen to be actively beating outside the zona pellucida. This is perhaps the moment when the initial fusion of spermatozoon and oocyte occurs. *Lower left*, the spermatozoon is now within the ooplasm, and its nucleus is expanding slightly. The sperm mid-piece and flagellum can be seen. *Lower centre*, shows how the sperm nucleus is beginning to swell, and its mid-piece is beginning to disintegrate, yet still clearly visible together with its flagellum. *Lower right*, a fertilized oocyte with two pronuclei and polar bodies. The smaller pronucleus, i.e. adjacent to the polar bodies, is probably the maternal pronucleus. The larger pronucleus is probably paternal. Note that the two pronuclei have lined up with the polar bodies, suggesting they are all aligned on an axis passing diametrically across the oocyte from the location of the polar bodies. This axis is now strongly believed to establish polarity in mouse and human oocytes, and similar events presumably occur in other mammals.

Figure 1.8

Embryos allowed to cleave *in vitro* did not usually arrest in early cleavage stages, but the pronucleate oocyte shown in this illustration shows a rare oocyte apparently arrested at syngamy, after its pronuclei had condensed. The oocyte remained pronucleate and cleavage did not occur. On examination, the group of illustrated chromosomes was found, perhaps typical of an arrested syngamy and at the prophase of the first cleavage.

Figure 1.9

Within a few months, all the stages of growth from 1-cell to blastocysts were observed *in vitro*. This illustration is a composite of the successive stages of the growth of human embryos *in vitro*. It was constructed in Oldham in 1971. *Upper left*, a 4-cell embryo with crossed blastomeres, a thick zona pellucida, and many spermatozoa on or within the zona pellucida. Most 4-cell embryos possessed blastomeres with a typically crossed appearance. *Upper centre*, an 8-cell embryo is shown with spermatozoa in or on the zona pellucida, and with a small group of cumulus cells still attached to the zona pellucida. This illustration was taken after 2.5 days in culture. The blastomeres are distinct. *Upper right*, a 16–32-cell human embryo now taking the form of a morula. Fluid seemed to accumulate between its constituent cells, giving a 'stripey' appearance indicative of imminent blastulation. The zona pellucida is thinning, and spermatozoa can be seen on and in it. *Lower left and centre*, these illustrations show human blastocysts formed *in vitro* ~4–5 days after insemination. Notice the distinct cell mass, blastocoelic cavity, very thin zona pellucida and the large cells adjacent to the inner cell mass which appear to be secretory. *Lower right*, a blastocyst grown *in vitro* and prepared for cytogenetic examination on day 5 post-insemination. Notice the numerous even-sized nuclei, the relative absence of fragmenting nuclei, and the presence of many mitoses. It includes well above 100 nuclei and 13 mitoses.

Figure 1.10

This illustration shows one of the first human blastocysts ever to be seen, and was grown *in vitro* after a mature oocyte aspirated from its mother was inseminated *in vitro*. It had blastulated late on day 4 post-insemination, and this illustration was taken on day 5. Strands of cytoplasm crossed its blastocoelic cavity, as if dividing this cavity into segments. Some of the traversing membranes disappeared with ongoing culture, although others persisted to give the impression of an abnormal form of blastulation.

Figure 1.11

A human embryo cultured *in vitro* for 9 days post-insemination having reached an advanced stage normally seen just after implantation has begun (Edwards and Surani, 1978). Notice that the blastocyst has escaped from the zona pellucida, which contains fragmented material and perhaps one or two cells. The blastocyst itself is enlarged and elongated, and has a distinct embryonic disc and trophoctoderm. Endoderm was apparently migrating around the blastocoelic cavity. All stages of growth of this embryo from fertilization *in vitro* to day 9 were examined when it was fixed and prepared for electron microscopy. Many cell lines must be differentiating within the embryonic disc, since the embryos will be well implanted and probably producing haemoglobin by day 14, but follow-up studies and attempts to obtain cell lines from such embryos have only recently been reported.

Figure 1.12

The blastocyst shown in Figure 11 was fixed for electron microscopy. Some damage occurred to it in the preparative stages. Nevertheless many cells, nuclei and cytoplasmic contents can be seen, as well as indications of extraembryonic endoderm. This section passes through the embryonic disc, and large trophoctoderm cells with typical nuclei can be seen (Edwards, 1980).

Figure 1.13

The first attempts at the preimplantation diagnosis of genetic disease in mammals were made in 1968 at Cambridge University (Gardner and Edwards, 1968). The illustration shows a rabbit blastocyst on a holding pipette, and a fine aspirated pipette which has been used to draw out a small sample of trophoctoderm through the zona pellucida in a thin string-like projection. The scissors are used to sever the small piece of trophoctoderm shown, which was used to sex the embryo by scoring it for the presence or absence of the sex chromatin body. *Inset*: Notice the typical characteristics of the sex chromatin body (arrowed) in the excised tissue, showing this embryo to be female.

Figure 1.14.1

The first attempts to obtain tissue outgrowths from blastocysts and grow embryonic stem cells *in vitro* were achieved in rabbits in the mid-1960s (Cole *et al.*, 1965, 1966). The zona pellucida was removed, and the blastocysts placed *in vitro* then attached to the plastic culture dishes via a monolayer of trophoctoderm. Cells of the inner cell mass grew and differentiated on this convenient trophoctoderm layer. Various types of cell were identified including blood islands (see 1.14.2) and possibly muscle cells. This illustration, the only one still available, shows strands of connective tissue associated with small blebs; the original papers should be consulted for better figures. Colonies of rabbit embryonic stem cells grew through many passages *in vitro*, could be cryopreserved and displayed characteristic biochemical markers.

Figure 1.14.2

Blood islands identified as above. (Published with permission from Cole, R.J. *et al. Dev. Biol.* (1966) **13**, 385–307 by Harcourt.)

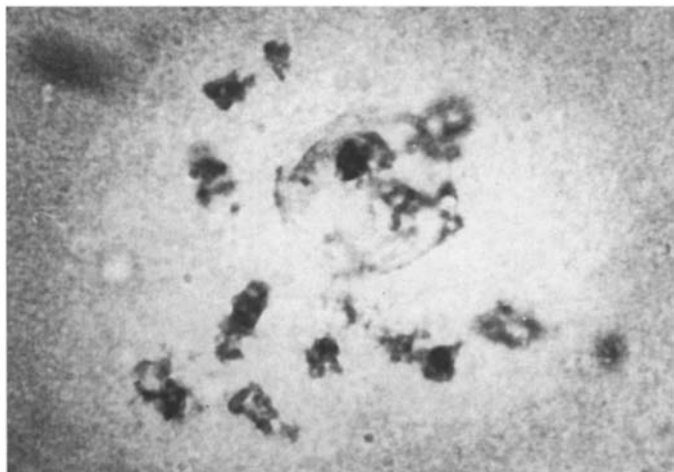


Figure 1.1

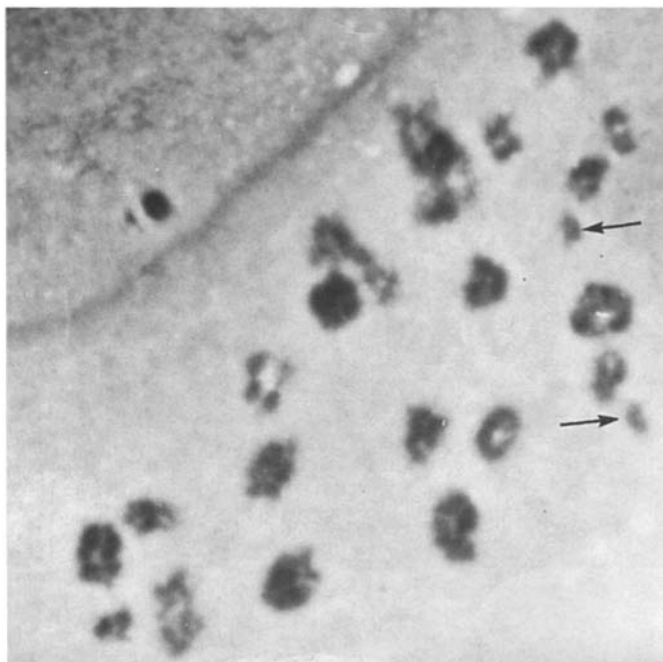


Figure 1.3

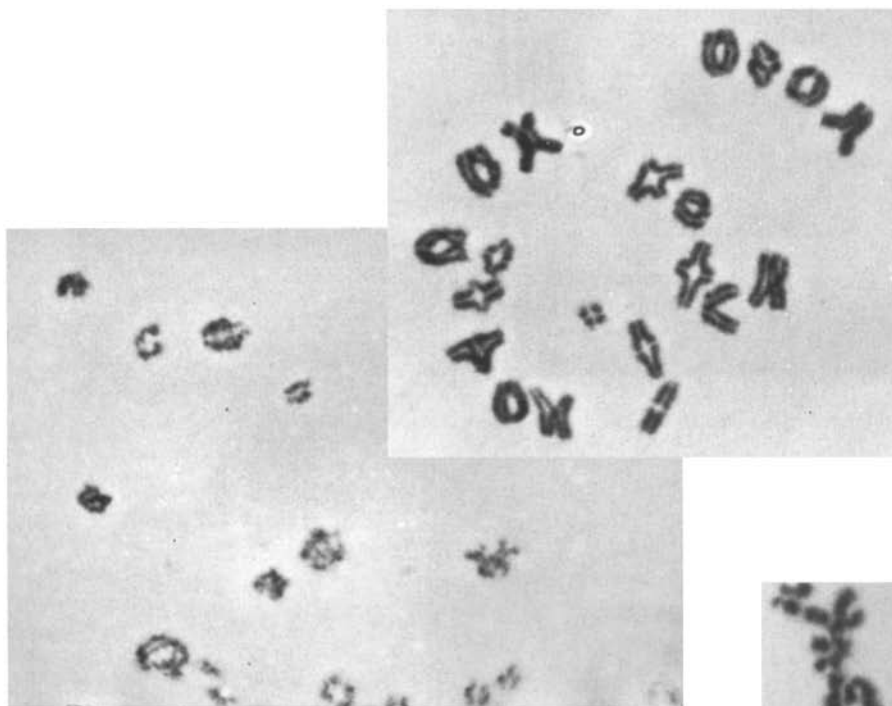


Figure 1.2

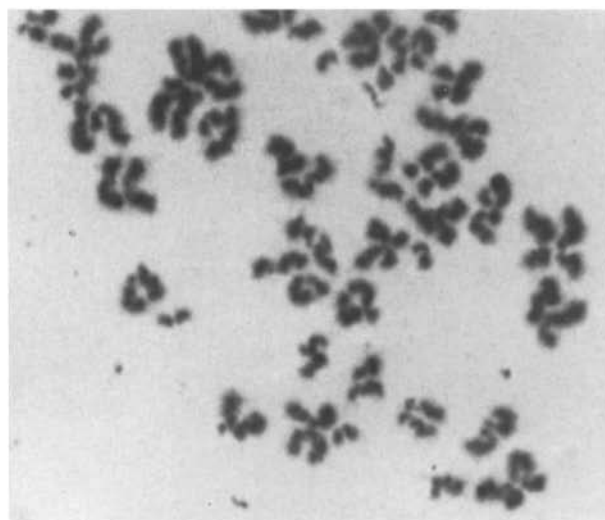


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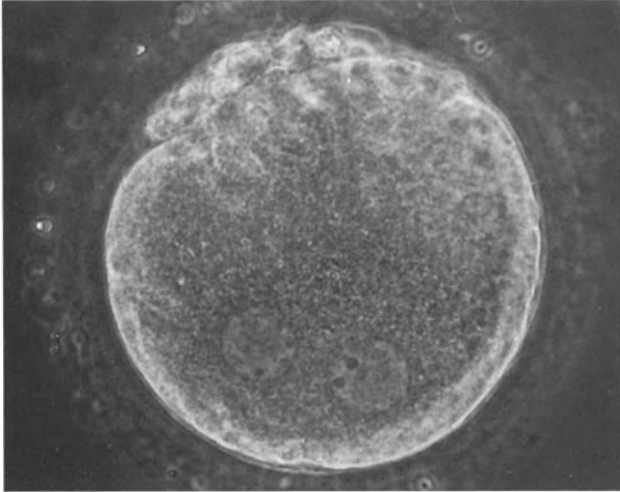


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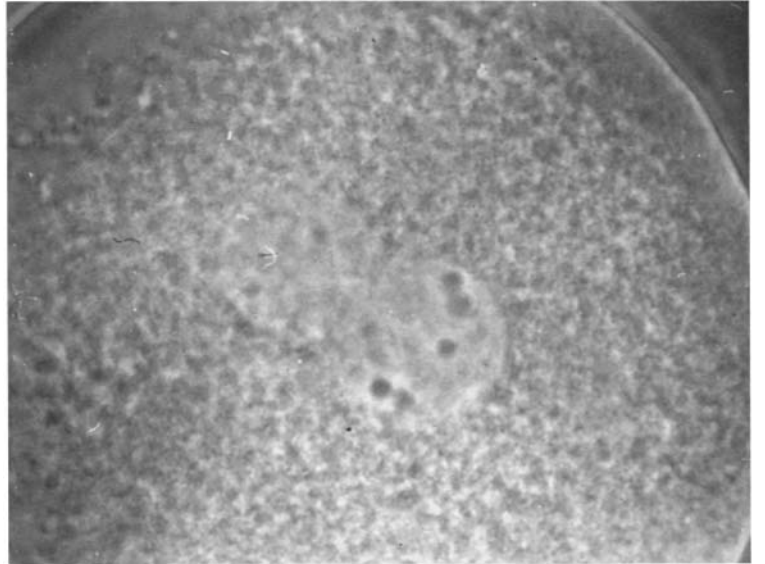


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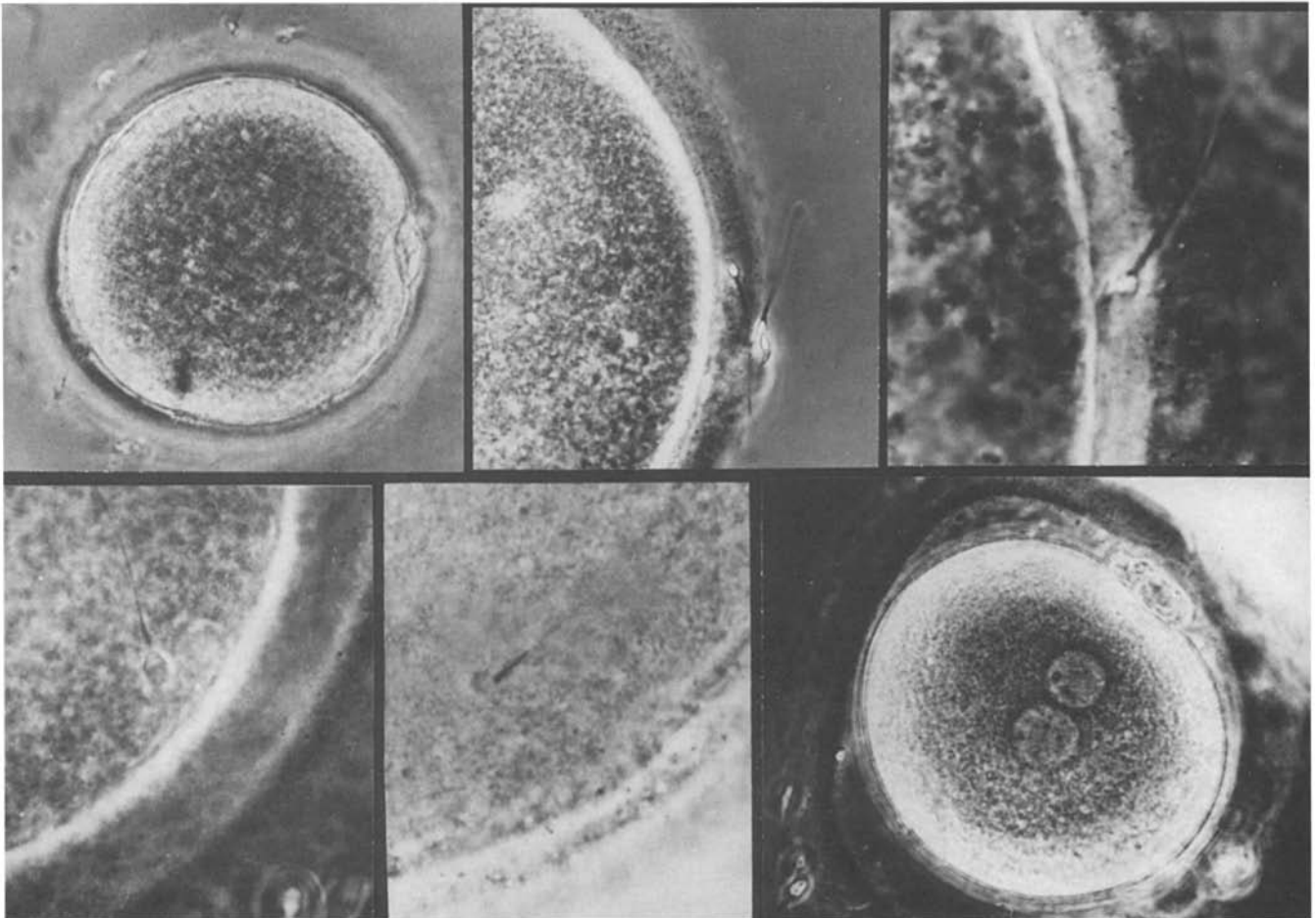


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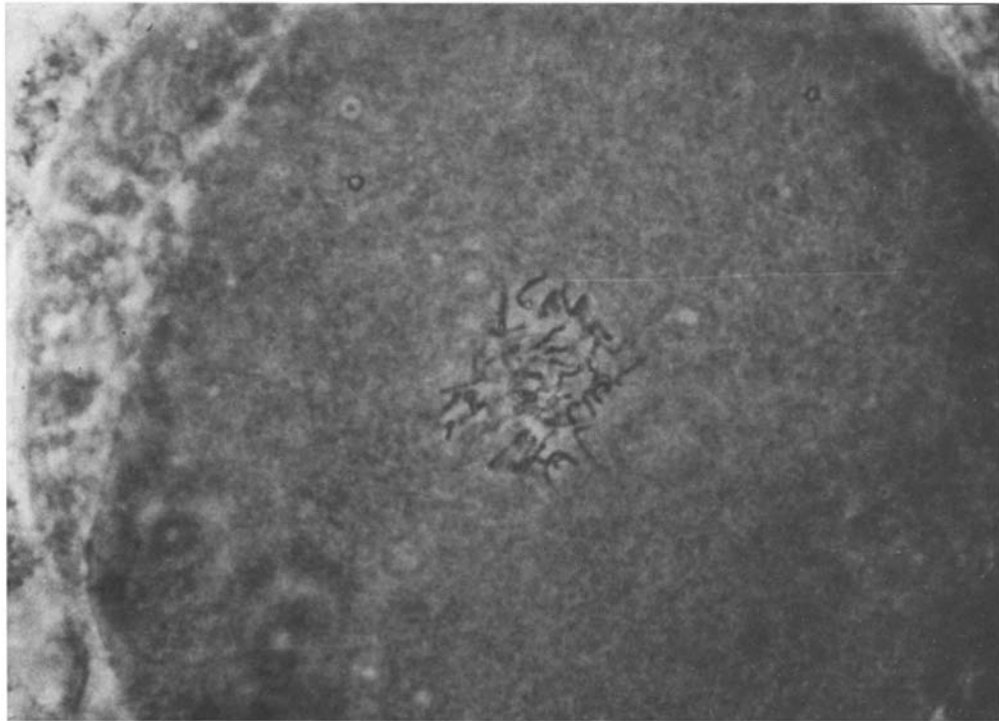


Figure 1.8

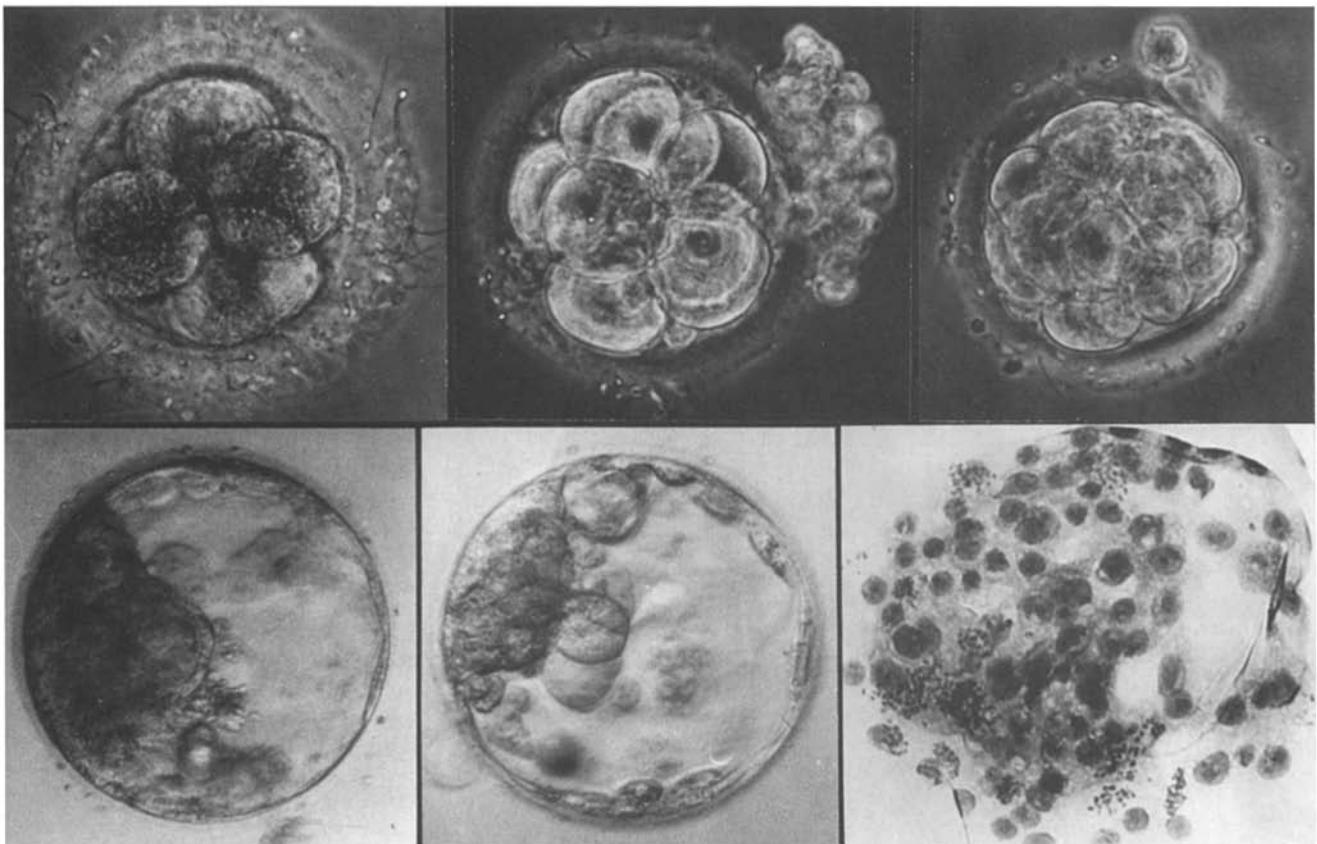


Figure 1.9

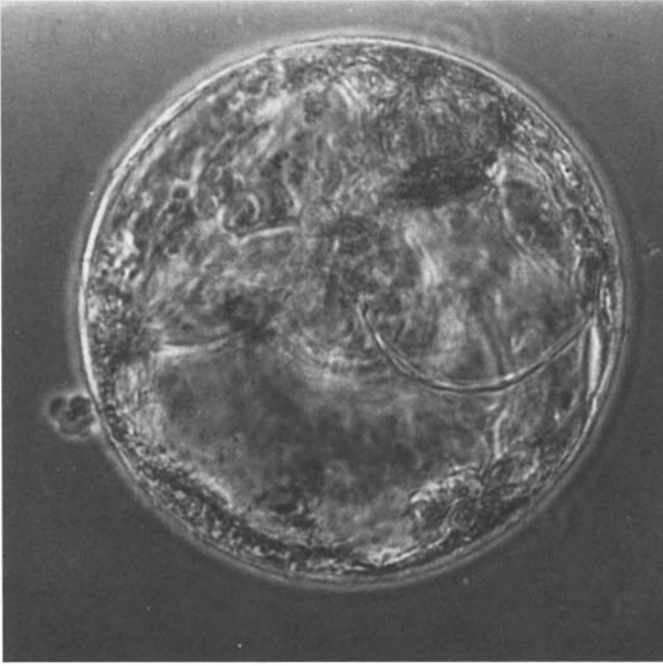


Figure 1.10

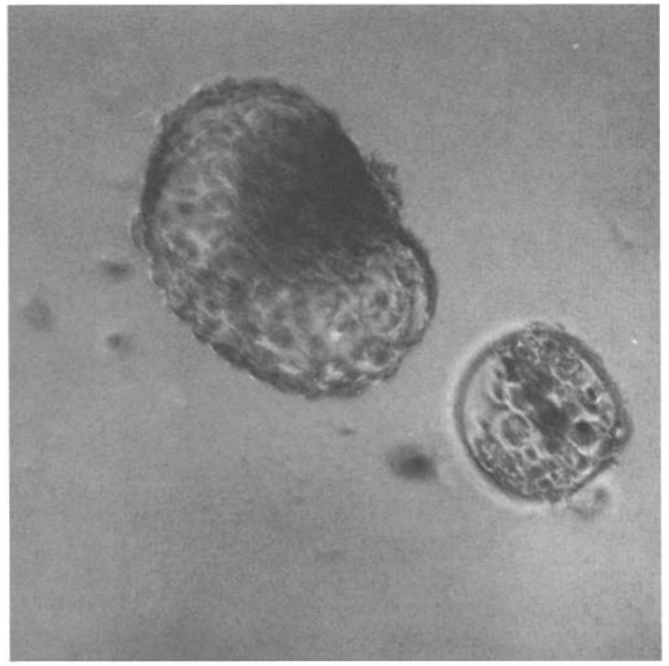


Figure 1.11

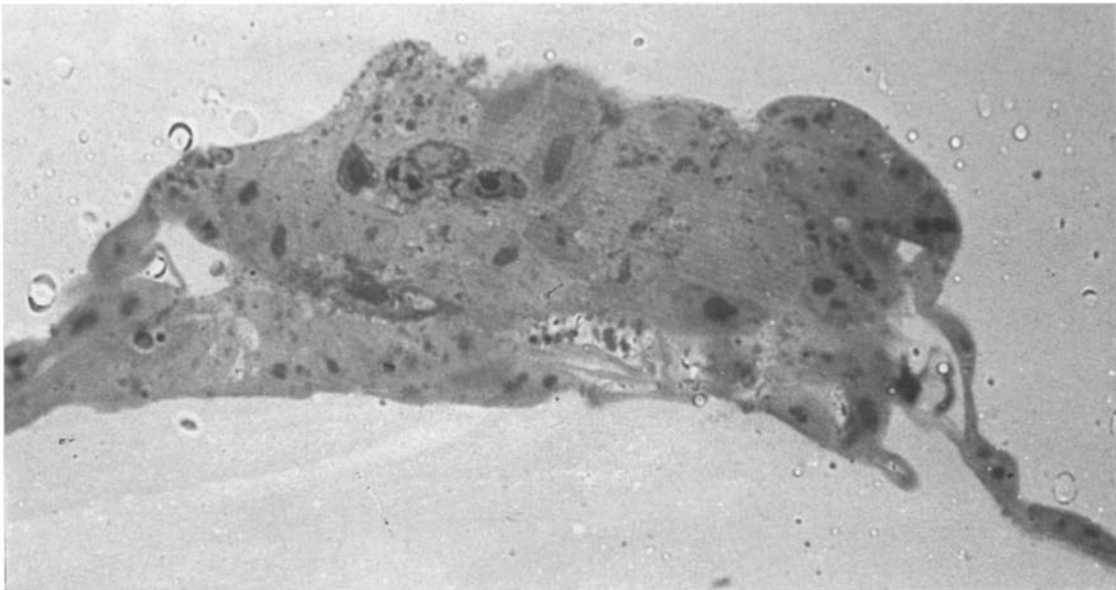


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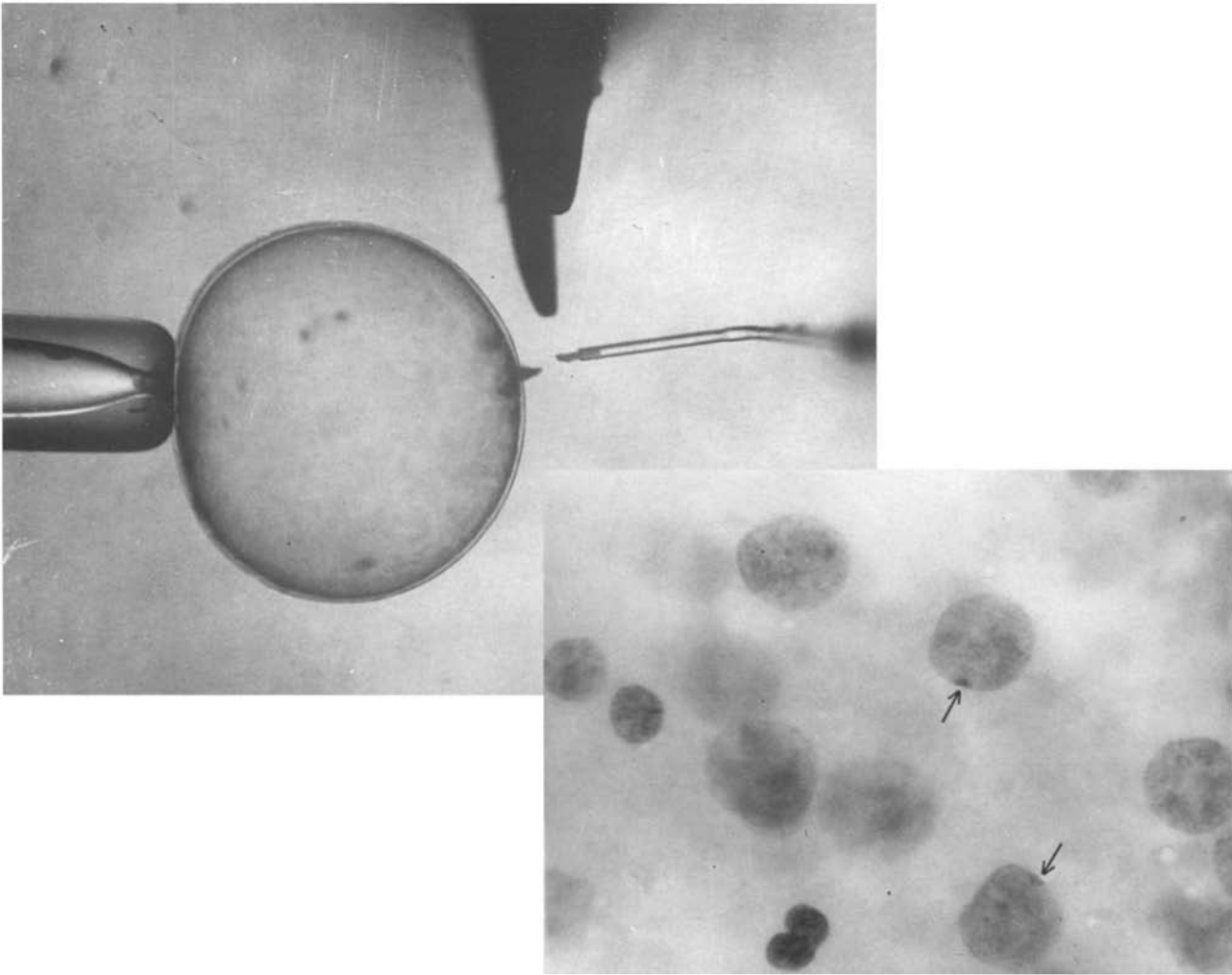


Figure 1.13

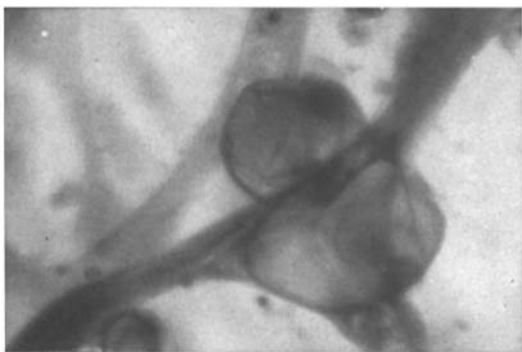


Figure 1.14.1



Figure 1.14.2