

Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography

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In this study, we have used time-lapse video cinematography to study fertilization in 50 human oocytes that had undergone intracytoplasmic sperm injection (ICSI). Time-lapse recording commenced shortly after ICSI and proceeded for 17–20 h. Oocytes were cultured in an environmental chamber which was maintained under standard culture conditions. Overall, 38 oocytes (76%) were fertilized normally, and the fertilization rate and embryo quality were not significantly different from 487 sibling oocytes cultured in a conventional incubator. Normal fertilization followed a defined course of events, although the timing of these events varied markedly between oocytes. In 35 of the 38 fertilized oocytes (92%), there were circular waves of granulation within the ooplasm which had a periodicity of 20–53 min. The sperm head decondensed during this granulation phase. The second polar body was then extruded, and this was followed by the central formation of the male pronucleus. The female pronucleus formed in the cytoplasm adjacent to the second polar body at the same time as, or slightly after, the male pronucleus, and was subsequently drawn towards the male pronucleus until the two abutted. Both pronuclei then increased in size, the nucleoli moved around within the pronuclei and some nucleoli coalesced. During pronuclear growth, the organelles contracted from the cortex towards the centre of the oocyte, leaving a clear cortical zone. The oocyte decreased in diameter from 112 to 106 μm ($P < 0.0001$) during the course of the observation period. The female pronucleus was significantly smaller in diameter than the male pronucleus (24.1 and 22.4 μm respectively, $P = 0.008$) and contained fewer nucleoli (4.2 and 7.0 respectively, $P < 0.0001$). After time-lapse recording, oocytes were cultured for 48 h prior to embryo transfer or cryopreservation. Embryo quality was related to fertilization events and periodicity of the cytoplasmic wave, and it was found that good quality embryos arose from oocytes that had more uniform timing from injection to pronuclear abutment and tended to have a longer cytoplasmic wave. In conclusion, we have shown that time-lapse video cinematography is an excellent tool for studying fertilization and early embryo development, and have demonstrated that human fertilization comprises numerous complex dynamic events.

Key words: fertilization/ICSI/polar body/pronuclei/time-lapse video

Introduction

In-vitro fertilization (IVF) technology has allowed us to view and analyse the early events of human fertilization and embryogenesis. However, concern for the well-being of the embryo has limited the intensity of observation, and the analysis of growth rates and morphological changes has been deduced largely from the morphological appearance of embryos at discrete time points, often many hours apart. Such ‘freeze-frame’ images of the dynamic processes of growth and change necessarily limit the information available to the observer, and some short-lived processes may be missed entirely. In addition, the gradual nature of change in cellular morphology can obscure some processes which become obvious when condensed into a coherent, moving image.

Nagy *et al.* (1994) described the time-course of fertilization of human oocytes after intracytoplasmic sperm injection (ICSI). Oocytes were observed every 2 h post-injection, but these researchers conceded that there were problems in identifying the first and second polar bodies and the male and female pronuclei (PN) during the course of observation. Furthermore, the exact timing of events such as sperm head decondensation, polar body (PB) extrusion and PN formation is not possible using this system of fixed time-point observation.

Time-lapse video cinematography overcomes the limitations of intermittent observation by providing a higher resolution and continuous image in which the various components of the cell can be recognized and followed during the entire course of the recording period. We have developed a system in which we maintain oocytes and embryos under conventional culture conditions and record high quality time-lapse video images using Nomarski differential interference contrast (DIC) optics. Oocytes undergoing ICSI are ideal for studies of this nature as the cumulus cells have been removed prior to injection, cytoplasmic features of the oocyte are clearly visible and the exact time of sperm penetration is known. This paper presents our preliminary observations on oocyte activation, PB extrusion and PN formation in human oocytes after ICSI.

Materials and methods

Patients

A total of 50 oocytes were studied from 42 patients who fulfilled one of the following criteria: (i) semen was too poor for routine IVF (<500 000 motile spermatozoa recovered); (ii) 0–25% fertilization

in routine IVF; or (iii) surgical sperm recovery. This study has the approval of The Queen Elizabeth Hospital Ethics of Human Research Committee (Woodville, Australia) and the Reproductive Technology Council of South Australia.

Ovarian stimulation, oocyte preparation and semen preparation

The procedures for ovarian stimulation, oocyte preparation and sperm preparation have been described fully in Payne (1995) and Payne and Matthews (1995); hence only brief details are provided here. Ovarian stimulation was achieved by desensitization in the previous luteal phase using leuproline acetate (Lucrin; Abbott Australasia, Kurnell, Australia) followed by stimulation with human menopausal gonadotrophin (Humegon; Organon, Lane Cove, Australia; Pergonal; Serono, Frenchs Forest, Australia). Ovulation was induced with 5000 IU human chorionic gonadotrophin (Profasi; Serono), and oocytes were collected by transvaginal oocyte recovery.

Oocytes were cultured for 2–4 h in human tubal fluid (HTF) medium (Quinn *et al.*, 1985) containing 5% maternal serum prior to removal of the cumulus cells with 1 mg/ml ovine hyaluronidase (Type III; Sigma Chemical Co., St Louis, MO, USA). They were then washed in fresh HTF containing serum, and residual coronal cells were removed using a fine-bore glass pipette. Denuded oocytes were cultured for up to 2–3 h prior to microinjection. All oocyte preparation steps were performed at 37°C in an atmosphere of 5% CO₂ in air.

Ejaculated semen samples were produced by masturbation, while spermatozoa were obtained from the rete testis or proximal epididymis of some patients by micro-epididymal sperm aspiration (MESA). Motile spermatozoa were isolated from most samples using discontinuous Percoll gradients (Payne *et al.*, 1991) or mini-Percoll gradients (Ord *et al.*, 1990). However, when only occasional motile spermatozoa were present, the sample was centrifuged at 500 g, resuspended in 2 ml HTF, centrifuged three times at 300 g and finally resuspended in 200–300 µl HTF. Washed sperm preparations were stored at 37°C in an atmosphere of 5% CO₂ in air until required.

Intracytoplasmic sperm injection

ICSI was performed essentially as described by Van Steirteghem *et al.* (1993a,b). The exact methodology employed in our laboratory for the manufacture of glass instruments and ICSI has been described in Payne (1995). Briefly, an inverted microscope equipped with Nomarski DIC optics and Narishige micromanipulators was used for ICSI. The microscope stage was enclosed by a perspex environmental chamber which was maintained at 37°C and had a humidified atmosphere of 5% CO₂ in air. Only morphologically normal, mature oocytes were injected. A live spermatozoon was immobilized and then drawn up into an injection pipette. An oocyte was held firmly against the holding pipette and the injection pipette was pushed gently into the oocyte. The oocyte plasma membrane was drawn back into the injection pipette until it ruptured, and the spermatozoon was then carefully injected into the oocyte. The exact time of injection for each oocyte was recorded.

Time-lapse video recording

A single, randomly selected oocyte was prepared for time-lapse video recording shortly after injection by placing it in a pre-equilibrated 5 µl drop of HTF containing serum under 4 ml mineral oil (Sigma Chemical Co.). Recording commenced within 30 min of injection.

Standard culture conditions were maintained during time-lapse recording by culturing the oocytes on the stage of an IX-70 inverted microscope (Olympus Optical Co., Tokyo, Japan) equipped with a perspex environmental chamber which was maintained at 37°C and had a humidified atmosphere of 5% CO₂ in air. The chamber was

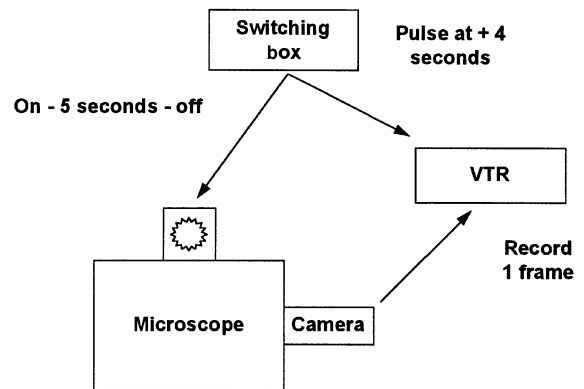


Figure 1. Set-up for time-lapse video recording.

fully covered during the course of recording to exclude extraneous light, and most of the recording occurred during the hours of darkness. Nomarski DIC optics and glass Petri dishes (Glaswerk, Wertheim, Germany) were used to ensure the highest image quality.

A purpose-built switching box turned the microscope lamp on for 5 s every minute (Figure 1). The colour temperature of the lamp stabilized after 4 s, at which time a pulse was sent from the switching box to signal the video recorder (S-VHS time-lapse video cassette recorder, AG-6730; Panasonic, Tokyo, Japan) to capture one frame. The time at which each frame was captured was appended automatically to the recorded image. The microscope light was then switched off. This sequence was repeated every minute for the duration of the recording period (17–20 h). Images were captured using a TK 1280E low-light CCD colour video camera (JVC, Tokyo, Japan), which ran continuously and allowed the recording of a suitable image at a light intensity of only 6 lux. Immediately after the completion of recording, oocytes were returned to conventional culture for an additional 48 h prior to transfer or cryopreservation.

Scoring of embryo quality

For simplicity, embryos selected for embryo transfer and freezing were scored as good embryos; those not suitable for embryo transfer or freezing were scored as poor embryos. Our scoring criteria were based on cell number, the shape and size of blastomeres, granularity of the cytoplasm, integrity of the membranes and percentage fragmentation. Embryo grading and selection were performed by an embryologist with no prior knowledge of which embryos underwent time-lapse recording and which were cultured in a conventional incubator.

Data collection and statistical analysis

Video sequences of each oocyte were viewed at least 10 times to ascertain the precise order of events. All timings were taken relative to the time of injection and are expressed as hours and minutes (h:min). For analysis of the time-lapse sequences, the initial magnification of $\times 200$ was augmented to $\times 1064$ (validated by an objective micrometer) on a Trinitron KX-14CPI monitor (Sony Corporation, Tokyo, Japan). Each oocyte was measured within 30 min of injection, at PN abuttal and again at 17 h post-injection. Male and female PN were measured at abuttal and at 17 h post-injection. Each measurement was taken from the monitor and was calculated as the average of the longest and shortest diameters.

Fisher's exact test and Student's *t*-test were used to analyse differences. A *P* value < 0.05 was considered to be significant.

Results

A total of 50 oocytes were recorded, of which 38 (76%) formed two PN and two PB, four (8%) developed one PN and

Table I. Outcome of recorded oocytes compared with sibling oocytes cultured in a standard incubator

	Recorded	Sibling	Fisher's exact test
No. of oocytes	50	487	
0 PN	7 (14)	119 (24)	NS
1 PN	4 (8)	17 (3)	NS
2 PN	38 (76)	343 (70)	NS
3 PN	1 (2)	8 (2)	–
No. transferred or frozen	22 (58)	221 (64)	NS

Values in parentheses are percentages. PN = pronucleus; NS = not significant.

two PB, one (2%) formed three PN and one PB, and seven (14%) remained unfertilized. Of 487 sibling oocytes from the same cohorts which were injected and then cultured in a conventional incubator, 343 (70%) fertilized normally, 17 (3%) formed one PN and two PB, eight (2%) formed three PN and one PB, and 119 (24%) were unfertilized (Table I). At 72 h post-injection, embryos were selected for transfer or cryopreservation according to our standard laboratory protocols. Embryos resulting from 22 of the 38 (58%) normally fertilized, recorded oocytes were transferred or frozen compared with 221 of 343 (64%) of the normally fertilized, sibling oocytes cultured in a standard incubator. There were no significant differences in the fertilization rates or embryo quality between the two groups of oocytes (Table I).

Description of morphological events

Two pronuclei, two polar bodies

Overall, 35 of the 38 normally fertilized oocytes had a slightly granular area located in the cortical two-thirds of the ooplasm, which comprised ~20% of the total cell volume (Figure 2A). This granular area moved in a circular manner around the ooplasm (cytoplasmic wave), either clockwise or anticlockwise, making two to 10 complete circuits during the course of recording (Figure 2B). During the course of the granulation of the ooplasm, the sperm head decondensed (Figure 2C) and, shortly afterwards, the second metaphase plate became visible (Figure 2D). The cytoplasmic wave continued throughout this phase. The second PB was extruded (Figure 2E) and the cytoplasmic wave then ceased. The second PB was not always extruded adjacent to the first PB, and in one extreme case the angle subtended between the two PB was 120°. The second PB in Figure 2 was extruded immediately adjacent to the first PB and closest to the observer. There was a brief contraction of organelles away from the cortex of the oocyte, accompanied by a glassy cytoplasmic appearance which radiated out from the centre of the oocyte (Figure 2E). We have called this phenomenon a cytoplasmic flare. Immediately thereafter, a small male PN appeared centrally, accompanied, simultaneously or shortly thereafter, by the appearance of a female PN close to the second PB (Figure 2F). Both PN had small nucleoli which moved rapidly within the nucleoplasm. The female PN was then drawn centrally towards the male PN (Figure 2G) until the two PN abutted. The next phase was characterized by growth of the PN, coalescence of some nucleoli and

withdrawal of cytoplasmic organelles from the cortex towards the centre of the oocyte (Figure 2H). The PN moved slightly and continually within the cytoplasm, and the nucleoli were continuously mobile within the PN.

One pronucleus, two polar bodies

Four oocytes formed one normal PN and two PB (Figure 3). In each case, the PN was of female origin. In two of these oocytes, the sperm head decondensed (Figure 3A) and a small male PN of ~5 µm diameter formed (Figure 3B) but failed to increase in size. In these oocytes, the female PN was drawn to the centre of the oocyte (Figure 3B) until it abutted the tiny male PN (Figure 3C). In the other two oocytes in which only a female PN formed, the PN moved randomly within the ooplasm. One of these oocytes cleaved to a normal-appearing 4-cell zygote but was discarded. In all four oocytes, the female PN increased in size until, at the end of the recording period, it had an average diameter of 25 µm (Figure 3D). The oocytes were recorded for a further 24 h but there was no development of additional PN during this time.

Three pronuclei, one polar body

One oocyte formed three PN and one PB. This oocyte exhibited no cytoplasmic waves prior to decondensation of the sperm head and its subsequent transformation into the male PN. Two female PN formed in the cytoplasm near the first PB at the same time as the male PN formed, and together they migrated to and abutted the male PN in the centre of the oocyte. At the end of the recording period, the female PN were each 18.6 µm in diameter and the male PN was 21.3 µm in diameter.

Unfertilized oocytes

Of the seven unfertilized oocytes, four exhibited cytoplasmic waves of granulation that were similar in appearance to the fertilized oocytes, but continued unabated during the entire course of recording with a constant, specific periodicity but gradually diminishing intensity.

Timing of morphological events

The timings of PB extrusion, male and female PN formation and PN abutment were calculated relative to the time of injection in 38 normally fertilized oocytes. In 30 of the 38 oocytes, each time point could be determined; however, in the other eight oocytes, either the second PB was out of focus at extrusion and one or both the PN were out of focus. Data are shown in Table II and in Figure 4. Times from injection to extrusion of the second PB, the appearance of the male and female PN and abutment varied markedly between oocytes. Despite these differences, however, all fertilized oocytes had a similar morphological appearance at the conclusion of the recording period.

Of the 37 oocytes in which PB extrusion could be timed, none had extruded the second PB by 1 h post-injection, five (14%) had extruded the second PB by 1–2 h post-injection, 19 (51%) had extruded the second PB by 2–3 h post-injection and the remaining 13 (35%) extruded the second PB by 3–8 h post-injection. The median time for PB extrusion was 2 h 39 min.

In 32 oocytes both the male and female PN were in focus at the time of their formation and could be timed. Of the

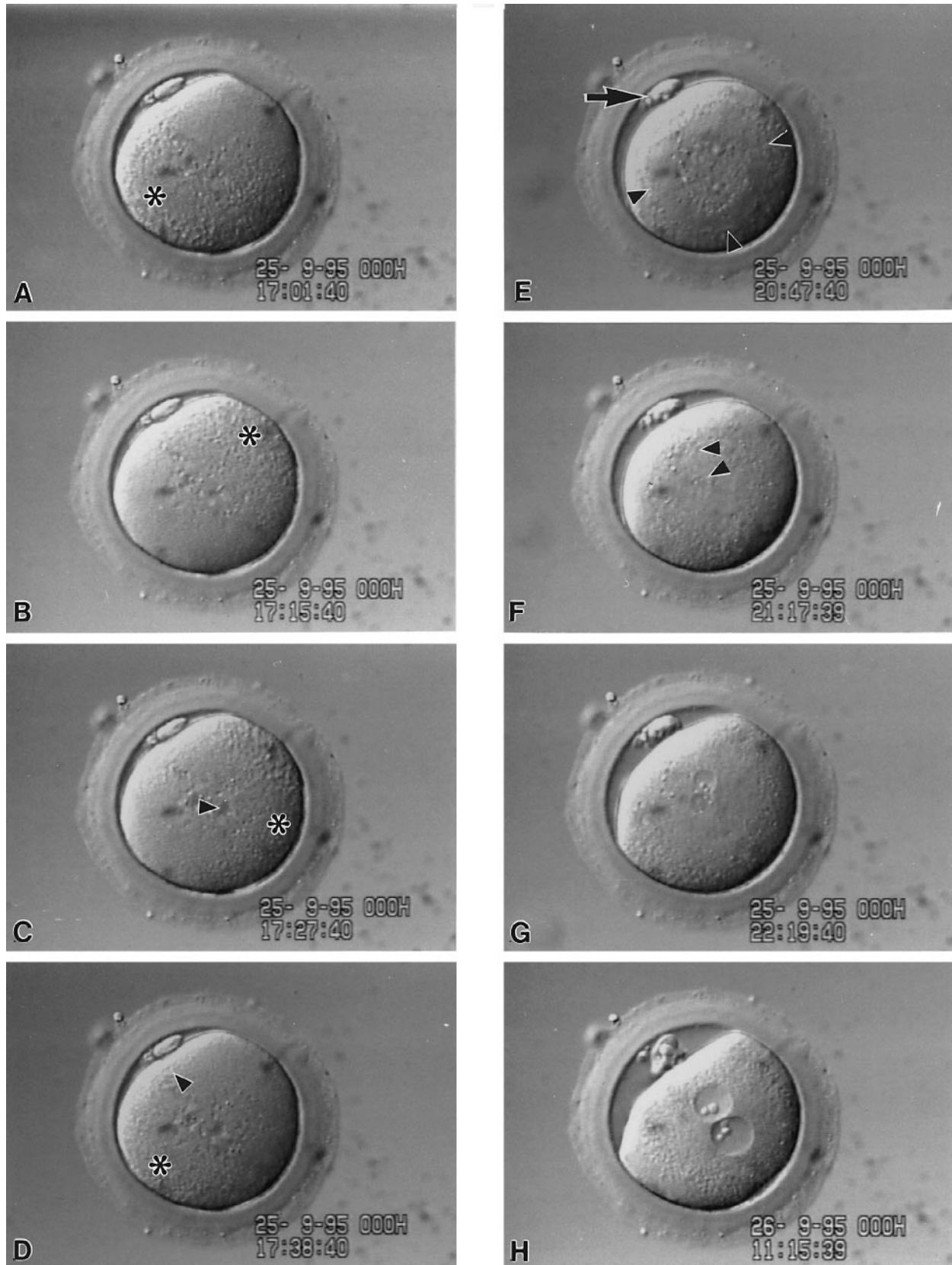


Figure 2. A sequence of images from a time-lapse recording of a normally fertilized oocyte. The region of granulated cytoplasm is indicated by the asterisks in (A)–(D). In (C), the arrowhead denotes the decondensing sperm head. In (D), the metaphase plate is indicated by the arrowhead. (E) The cytoplasmic flare (arrowheads) and the second PB (arrow) lying above the first PB (compare A–D). The appearance of male and female PN (arrowheads) is shown in (F), and pronuclear abutment and enlargement are shown in (G) and (H) respectively. It is emphasized that these images were printed from video tape sequences and are therefore not as clear as those seen on a video monitor. This particularly applies to (D), in which the metaphase plate is difficult to see but which nevertheless is very clear in video sequences.

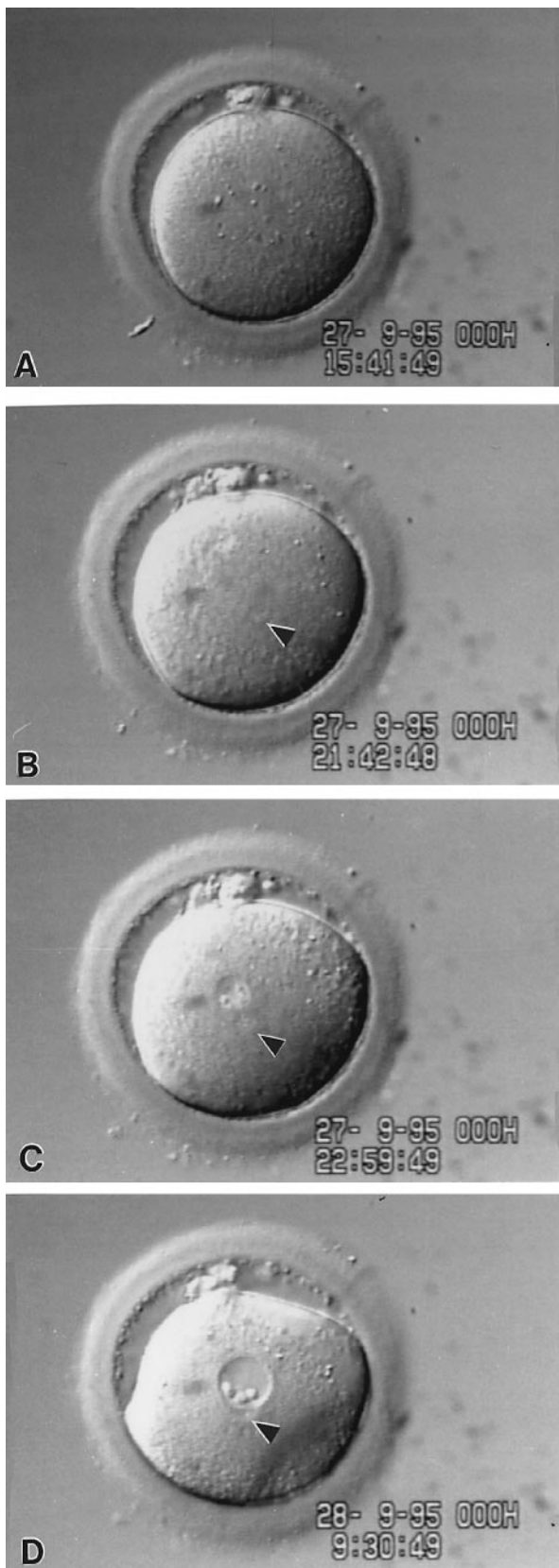


Figure 3. A sequence of images from a time-lapse recording of an oocyte which developed a female PN and a rudimentary male PN (arrowhead) that failed to enlarge and develop.

remaining six oocytes, both the male and female PN were out of focus and were unable to be timed in two oocytes, the male PN was out of focus in one oocyte and the female PN was out of focus in three oocytes.

Male and female PN were visible as early as 2 h 51 min post-injection. By 5 h post-injection, 51% of the oocytes had formed PN. In 20 (63%) of the 32 fully timed oocytes, the appearance of the male and female PN was simultaneous, while in six (19%) oocytes the time between their appearance was ≤ 10 min. In two (6%) oocytes the time difference was between 11 and 30 min (12 and 29 min), and in only four (13%) oocytes was the time difference >30 min (31 min, 42 min, 1 h 23 min, 2 h 15 min). In the 12 oocytes with asynchronous appearance of the male and female PN, the male PN always appeared before the female PN, and the average time between the appearance of the two PN was 31 min.

Association of fertilization events with embryo quality

The time from sperm injection to PB extrusion, then to male and female PN formation and PN abuttal, in 30 fertilized oocytes was related to embryo quality on day 3 (Figure 5). In the other eight fertilized oocytes, either the PB or one or both PN was out of focus at the critical time points, and these oocytes were therefore excluded from this analysis. Embryos were grouped according to whether they had good morphology (transferred or frozen) or poor morphology (not suitable for transfer or freezing) on day 3. Differences between the time the various developmental stages were reached in individual oocytes were wide and were not associated with embryo morphology. However, the variability in time taken to achieve various stages of PN development was significantly different between embryos of good and poor quality (Table III).

Morphometry of oocytes and pronuclei

Measurements of oocyte diameter and PN diameter in normally fertilized oocytes are presented in Table IV. Oocytes decreased significantly ($P < 0.0001$) in diameter from 111.6 ± 3.6 to 106.0 ± 4.6 μm during the 17–20 h recording period. Male PN increased significantly ($P < 0.0001$) in diameter from 16.5 ± 2.7 μm at abuttal to 24.1 ± 2.2 μm at the end of the recording period. Over the same time period, female PN increased in size from 15.3 ± 2.5 to 22.4 ± 2.3 μm ($P < 0.0001$) and were significantly smaller than male PN at the end of the recording period ($P = 0.008$). There were significantly fewer nucleoli in the female PN than in the male PN (7.0 ± 2.8 versus 4.2 ± 0.3 , $P < 0.0001$) at the end of the recording period.

Cytoplasmic waves

The presence or absence of cytoplasmic waves was recorded in each oocyte. Of the 50 recorded oocytes, 43 had cytoplasmic waves and the periodicity could be determined in 36 of them (Table V). Of those oocytes that extruded a second PB, 93% had a discernible cytoplasmic wave (35/38 two PN and 4/4 one PN). In contrast, of those oocytes that did not extrude the second PB, only 50% exhibited cytoplasmic waves (4/7 no PN and 0/1 three PN). There was no detectable relationship between the periodicity of the wave and PN status. We also

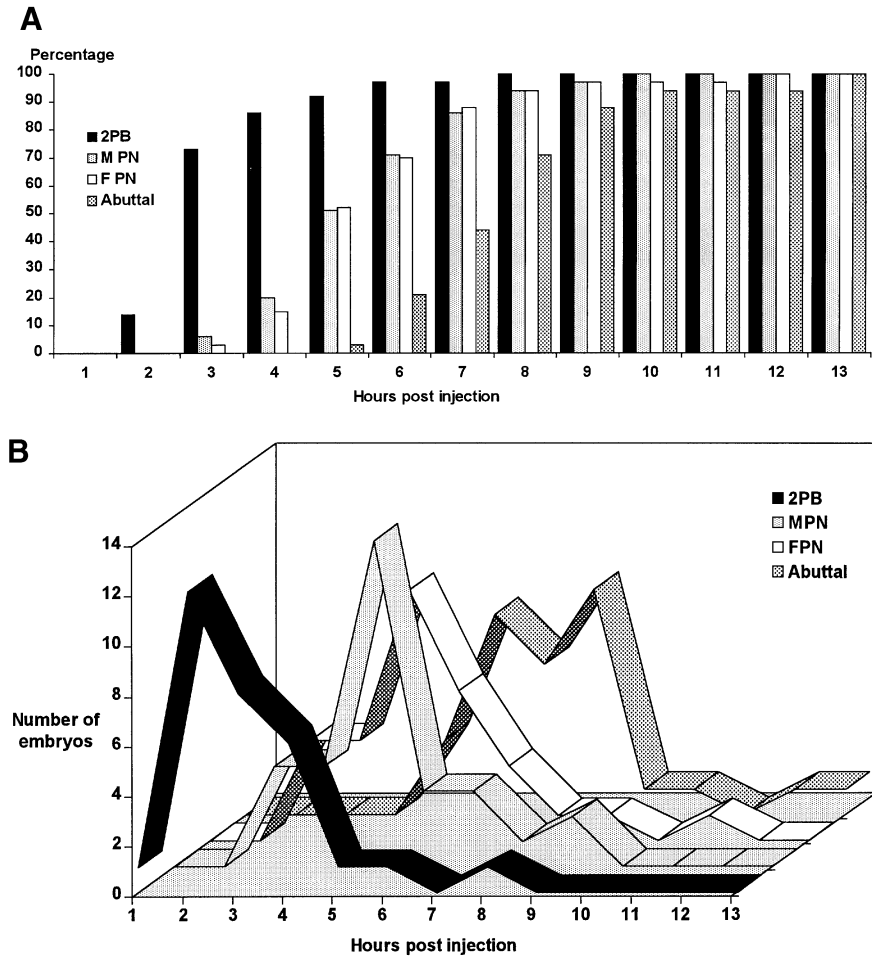


Figure 4. (A) Time at which fertilized oocytes reached specific stages of fertilization in 30 oocytes. FPN = female pronucleus; MPN = male pronucleus; 2PB = second polar body. (B) Progressive attainment of specific stages of fertilization in 30 oocytes.

Table II. Time course of second polar body (PB) extrusion and pronucleus (PN) formation and abuttal in a total of 38 normally fertilized oocytes

	<i>n</i>	Median	Minimum	Maximum
Oocyte pick-up to injection	38	6:36	4:37	9:52
Injection to PB	37	2:39	1:24	8:00
Injection to male PN	35	4:59	2:51	9:11
Injection to female PN	33	4:59	2:51	11:26
Injection to abuttal	34	7:09	4:50	12:48

Values are h:min post-injection; *n* indicates the number of observations of each event as not all events could be timed for each oocyte.

observed that, in three oocytes donated to research that were denuded of their cumulus cells and then recorded for 17 h without injection, each oocyte exhibited a cytoplasmic wave with a periodicity similar to that of the injected oocytes.

The period of the cytoplasmic wave could be timed in 29 normally fertilized oocytes, and this was related to subsequent embryo quality (Figure 6). Good quality embryos tended to have a longer periodicity than poor quality embryos; however, the difference ($4 \text{ min} \pm 8 \text{ s}$ versus $35 \text{ min} \pm 9 \text{ s}$ respectively) was not statistically significant ($P = 0.075$).

Identification of the female pronucleus

Time-lapse recording enabled positive identification of the female PN. Three criteria were important: diameter, number of nucleoli and location relative to the second PB. The female PN was closest to the second PB in 92% of the fertilized oocytes, and it was smaller, had fewer nucleoli or was closest to the second PB in 100% of the fertilized oocytes. In 40% of the oocytes, the female PN met all three criteria. If two of the three criteria were used to identify the female PN, the misidentification rate was only 5%.

Discussion

In this study, we have developed a novel method for observing and recording oocyte activation, PB extrusion and PN formation in human oocytes after ICSI. The system incorporates high quality DIC optics, standard culture conditions and a very low light intensity. We have shown that time-lapse video recording does not affect the fertilization rate or subsequent development of the embryos. This is perhaps not surprising, given that mouse and rabbit oocytes and hamster and bovine embryos exposed to $>3000 \text{ lux}$ for hours up to several days had similar fertilization rates, cleavage rates and development to live

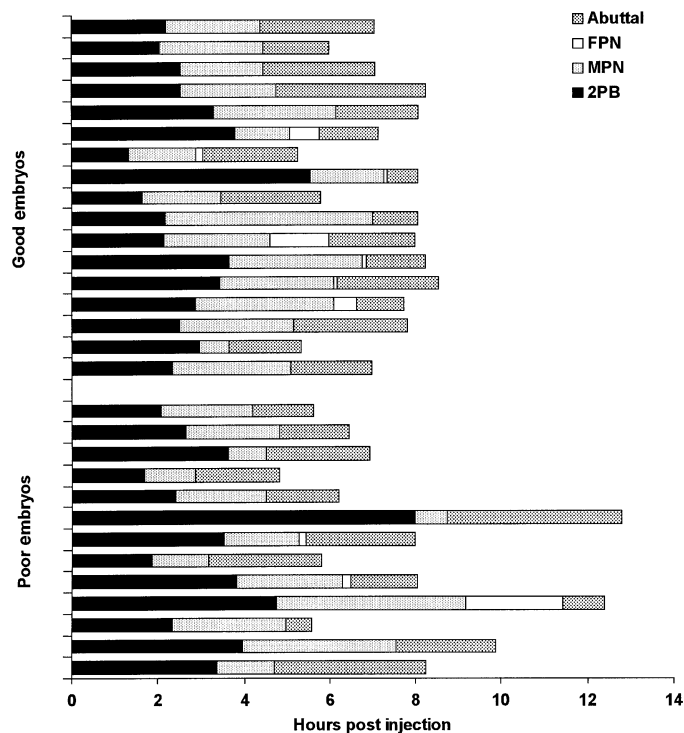


Figure 5. Comparison of the time between various stages of fertilization in 30 good quality and poor quality embryos. FPN = female pronucleus; MPN = male pronucleus; 2PB = second polar body.

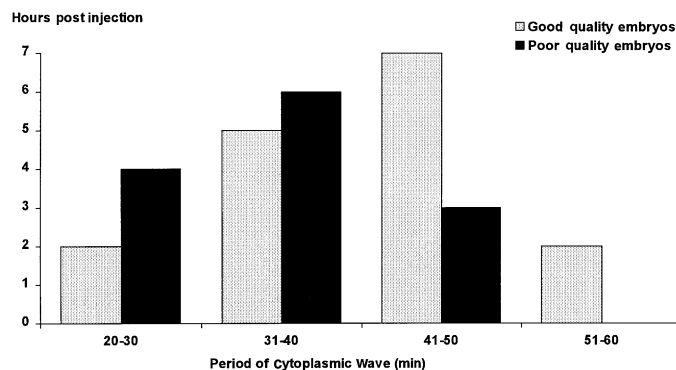


Figure 6. Periodicity of the cytoplasmic wave in oocytes which resulted in good quality ($n = 16$) and poor quality ($n = 13$) embryos.

Table III. Times taken for various stages of fertilization to be reached in good quality (transferred or frozen) and poor quality (not suitable for transfer or freezing) embryos

	Good quality embryos ($n = 17$)	Poor quality embryos ($n = 13$)	Comparisons of SD
Injection to PB	2:45 ± 0:59	3:23 ± 1:40	$P = 0.03$
PB to male PN	2:22 ± 0:55	2:04 ± 1:04	NS
Male PN to female PN	0:11 ± 0:22	0:12 ± 0:37	$P = 0.03$
Female PN to abuttal	1:56 ± 0:43	2:00 ± 0:52	NS
Injection to abuttal	7:14 ± 1:04	7:39 ± 2:34	$P < 0.001$

Values are h:min post-injection, expressed as means ± SD. PB = second polar body; PN = pronucleus; NS = not significant.

young compared with control oocytes and embryos not exposed to light (Bedford and Dobrenis, 1989; Barlow *et al.*, 1992; Grisart *et al.*, 1994; Gonzales *et al.*, 1995). Furthermore, Van den Bergh *et al.* (1995) did not demonstrate any adverse effects of repeated exposure to light on human oocytes that had undergone ICSI. In our studies, we have deliberately restricted the light exposure to 6 lux for 5 s every minute, resulting in a total light exposure of only ~30 000 lux during a 17–20 h recording period. This represents only a 1–3% increase in the total light exposure of oocytes during culture, and therefore does not represent a major departure from standard practices.

This technology has enabled us, for the first time, to describe accurately and time the exact events of human fertilization. We have been able to follow differences in the sequence and/or timing of events in individual oocytes and relate these to the eventual development of embryos. Furthermore, we can accurately measure oocytes and cytoplasmic structures at any stage during the course of fertilization. Our observations have revealed novel features of human oocytes and fertilization, such as the cytoplasmic wave of granulation and the cytoplasmic flare. We have also established that sperm nuclear decondensation precedes extrusion of the second PB, and we have plotted the sequence and timing of the formation, abuttal and growth of the male and female PN.

Nagy *et al.* (1994) reported that 52% of injected oocytes had extruded the second PB by 2 h post-injection. Van den Bergh *et al.* (1995) observed that 29% of oocytes had a second PB within 1 h of injection and 76% of oocytes had two PB within 3 h of injection. Dozortsev *et al.* (1995) observed that 4% of activated and fertilized oocytes had extruded the second PB 2–3 h post-ICSI. In this study, we found that 14% of the fertilized oocytes had extruded the second PB by 2 h post-injection. The earliest time of extrusion was 1 h 24 min, and 65% of the oocytes had extruded the second PB by 3 h post-injection. Thus, there appear to be slight differences between our results and those of the aforementioned studies. In this study, the exact time of injection was recorded for each oocyte, and real time was appended to the video image during the course of recording; hence we were able to calculate the exact time from injection to PB extrusion for each individual oocyte. The methodology employed by Nagy *et al.* (1994), Van den Bergh *et al.* (1995) and Dozortsev *et al.* (1995) may have precluded such precision.

The first appearance of male and female PN occurred as early as 2 h 51 min post-injection; by 5 h post-injection, 51% of the fertilized oocytes had already formed two PN. The male PN appears centrally, while the female PN forms in the ooplasm adjacent to the second PB. Pronuclei first appeared as small bodies ~5 mm in diameter and, as such, they could easily be missed during routine, sporadic observations. This probably accounts for the much longer time of 6 h to the first sighting of PN by Nagy *et al.* (1994). In contrast, PN are relatively obvious at the time of abuttal, and 21% of oocytes had reached PN abuttal by 6 h post-injection, which agrees more closely with the observations of Nagy *et al.* (1994).

In 63% of the oocytes, the appearance of male and female PN was simultaneous, and in only 13% of the oocytes was

Table IV. Size of oocytes and pronuclei (PN) in 38 normally fertilized oocytes

	At injection	At PN abuttal	At 17 h post-injection	Difference (%)
Oocyte diameter (μm)	111.6 \pm 3.6 ^a	108.4 \pm 3.4	106.0 \pm 4.6 ^b	-5
Male PN diameter (μm)	-	16.5 \pm 2.7 ^c	24.1 \pm 2.2 ^d	+49
No. of nucleoli	-	8.0 \pm 2.4	7.0 \pm 2.8 ^e	
Female PN diameter (μm)	-	15.3 \pm 2.5 ^f	22.4 \pm 2.3 ^g	+46
No. of nucleoli	-	5.9 \pm 0.6	4.2 \pm 0.3 ^h	

Values are expressed as means \pm SD.

^{a,b} $P < 0.0001$; ^{c,d} $P < 0.0001$; ^{f,g} $P < 0.0001$; ^{c,f}not significant; ^{d,g} $P = 0.008$; ^{e,h} $P < 0.0001$.

Table V. Pronuclear (PN) status and periodicity of the cytoplasmic waves of granulation

Oocytes	No. of oocytes with waves	No. of oocytes timed	Periodicity (min) median (range)
0 PN	4/7 (57%)	3	33 (22-43)
1 PN	4/4 (100%)	4	46 (36-46)
2 PN	35/38 (95%)	29	38 (20-53)
3 PN	0/1	-	-
Uninjected	3/3 (100%)	3	40 (38-43)

The number of oocytes timed represents the number of oocytes in which we were able to time accurately the periodicity of the cytoplasmic waves.

the time difference >30 min, with a maximum of 2 h 15 min in one oocyte. In this regard, our results disagree with those of Nagy *et al.* (1994), who reported that ~50% of normally fertilized oocytes showed asynchronous PN development at 6 h post-injection. Similarly, Dozortsev *et al.* (1995) found that at 4-5 h post-ICSI, 50% of oocytes had a female PN, whereas only 22% had a male PN. Their cytogenetic study described nuclear morphology following ICSI as determined by Giemsa staining, whereas our study described the chronological appearance of PN over a continuous time interval. There may be some difference in interpreting the initial appearance of the nuclear structures, especially as Dozortsev *et al.* (1995) were not able to visualize the structures with the light microscope at the beginning of PN formation 5 h after injection. The asynchronous appearance of a second PN at 4-6 h after fertilization check (Staessen *et al.*, 1993) was not observed in any oocytes during this study. In addition, the four oocytes in which a single PN was seen at 17 h post-ICSI were recorded for an additional 24 h, and there was no development of a second PN during this time. We also did not see PN fusion (Levron *et al.*, 1995) in any of the 43 oocytes which developed PN.

Dieguéz *et al.* (1995) reported that one PN was significantly larger than the other PN at 16-18 h post-insemination. We are now able to confirm that the larger of the two PN in normally fertilized human oocytes is the male PN. Furthermore, the male PN always had significantly more nucleoli and was usually the most distal of the two PN from the second PB. Using any two of three criteria (diameter, nucleoli and location), we were able to correctly identify PN as male or female 95% of the time.

Four oocytes developed a prominent female PN and two PB. However, two oocytes were seen to have a decondensing sperm head and subsequent formation of a small male PN of

<5 μm diameter which failed to develop further. These preliminary observations agree with previous studies of one PN oocytes after routine insemination and ICSI, in which 52 and 51% respectively contained a single sperm nucleus (Van Blerkom *et al.*, 1994; Flaherty *et al.*, 1995). Furthermore, the observation that the female PN was drawn to the non-developing male PN indicates that the centrosome of the injected spermatozoon had organized microtubules into an aster (Schatten, 1994). In contrast, in the two oocytes in which the sperm head did not decondense, the female PN moved randomly within the oocyte cytoplasm, indicating that a functional aster had not formed.

The existence of a cytoplasmic wave in the ooplasm was unexpected and, to our knowledge, a novel and unique observation. The granular area, which, as it moves around the ooplasm, comprises the cytoplasmic wave, was thought to be the area from which the first PB was extruded (Palermo *et al.*, 1995). However, in this study we have shown this not to be the case because the metaphase plate is clearly visible and stationary while the granular area moves. The identity and composition of the granular cytoplasmic area is unknown; however, it does not occur in response to piercing the cytoplasm during ICSI because we demonstrated that uninjected oocytes also exhibit this phenomenon for a prolonged period after denudation. As such, the cytoplasmic wave is probably not associated with activation of the oocyte. However, it appears to have some involvement with extrusion of the second PB, as 95% of the oocytes that extruded the second PB exhibited a well-defined cytoplasmic wave, whereas 50% of the oocytes that did not extrude the second PB had no discernible cytoplasmic wave. In addition, the cytoplasmic wave ceased after extrusion of the second PB, and was not observed again during PN formation and development. Taken together, these data suggest that oocytes which do not activate after ICSI (Flaherty *et al.*, 1995) have an inherent defect, reflected in many of them by the absence of a cytoplasmic wave.

Polar body extrusion is a specialized form of asymmetric cell division. The distinctive nature of the cytoplasmic wave in oocytes suggests that it might contain a localized structural feature which either forms and unforms in a cyclical manner in different regions of the ooplasm, or physically moves around the oocyte. Indeed, the granular area might stabilize the cytoplasm of the oocyte so that cytokinesis occurs only in the immediate vicinity of the metaphase plate, thus restricting loss of cytoplasm in the PB. Casual observations in our laboratory concerning the fluidity of the ooplasm during ICSI have

indicated that the granular area is more viscous than the surrounding cytoplasm (unpublished data). The cytoplasmic wave passes the metaphase plate several times before the second PB is extruded, and it might therefore act as an internal primer so that PB extrusion is timed and/or primed to occur at the correct time. The relationship between the cyclicity of the cytoplasmic wave and embryo quality approaches statistical significance ($P = 0.075$), and oocytes that had a longer cytoplasmic wave seemed to give rise to better quality embryos. Though preliminary, these data further support the contention that the cytoplasmic wave is a vital component of oocyte physiology and may be an important indicator of oocyte viability. Further ongoing studies will clarify this.

Another novel finding in this study was the occurrence of a cytoplasmic flare, consisting of a glassy change in the appearance of the ooplasm which radiated out from the centre of the oocyte towards the cortex, immediately prior to the appearance of the male and female PN. We speculate that the cytoplasmic flare may be related to the Mazia cell body, which is an area of cytoplasm organized by the centrosome and includes microtubule motor proteins such as the sperm aster. During fertilization, an aster of microtubules polymerizes and radiates out from the sperm centrosome and draws the female PN to the male PN (Schatten, 1994). The cytoplasmic flare occurs at precisely this stage of fertilization and may represent a visual manifestation of this microtubule polymerization. Furthermore, the Mazia cell body may be involved in the migration of organelles towards the centre of the oocyte, an event which occurs after the cytoplasmic flare and during the PN growth phase. Further work will elucidate the nature and significance of the cytoplasmic flare.

Sperm head decondensation was observed clearly in some oocytes. It occurred quickly (2–3 min), and always preceded extrusion of the second PB. The decondensed sperm nucleus formed a round body ~5 µm in diameter which remained unchanged for some hours, until the cytoplasmic flare occurred, after which it transformed rapidly into a male PN. No decondensing sperm heads were observed in the seven unfertilized oocytes. However, this may have been due to their being out of focus during recording. Flaherty *et al.* (1995) stained unfertilized ICSI oocytes with Hoechst 33342 and found that 71% had a partially decondensed sperm head within the cytoplasm. Hence, as this time-lapse study continues, we expect to see decondensing spermatozoa in some unfertilized oocytes.

The second metaphase plate was not always directly adjacent to the first PB, and this concurs with our previous observation that ~20% of fertilized oocytes have a second PB which is displaced by $>10^\circ$ from the site of the first PB (unpublished data). Hence, the location of the first PB is not always a reliable marker of the location of the oocyte chromosomes in denuded oocytes. This view is supported by Palermo *et al.* (1995). Other than the transient appearance of the second metaphase plate just prior to second PB extrusion, we were unable to determine a reliable morphological feature which gave 100% certainty as to the location of the oocyte chromosomes. It is our view that rough removal of the coronal cells prior to ICSI, by squeezing the oocyte through a denuding pipette which is too narrow, causes distortion of the oocyte

and can displace the first PB from its original position. This is undesirable if the oocyte is destined for ICSI, because at present the only means of identifying the area containing the oocyte chromosomes is by locating the area of cytoplasm adjacent to the first PB. Furthermore, this may lead to injection of the spermatozoon into the metaphase spindle (Flaherty *et al.*, 1995).

In conclusion, we have shown that time-lapse video cinematography is an exciting, powerful and non-invasive technique for observing cellular activity during fertilization and embryogenesis in a coherent, uninterrupted manner, not otherwise available by analysis in real time. This study has elucidated the complete sequence and timing of morphological events up to and including the formation of PN in human oocytes. While the results are preliminary, our ongoing studies will clarify the relationship between the cytoplasmic wave, the timing of various fertilization events and embryo quality.

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