—Mini Review—

Morphological Analysis of Human Embryonic Development Using Time-Lapse Cinematography

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Abstract: *The dynamic observation of human gametes and the early stage of embryos is of paramount interest for clarifying the physiological events during the fertilization process. We present our morphological analysis of human embryonic development after both intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) using time-lapse cinematography. The results of this study indicate that non-invasive imaging, time-lapse cinematography, is useful not only for elucidating the morphological events of fertilization in humans, but also for evaluating the physiological importance of these events during the early stages of human embryonic development. In addition, analysis of cellular activity and quality during fertilization and embryogenesis using this system will contribute to the future improvement of the clinical results of assisted reproductive technology (ART).*

Key words: *Time-lapse cinematography, ICSI, IVF, Human embryonic development*

Introduction

Assisted reproductive technology (ART) in humans has been successfully developed to become the most important tool for infertile couples who desire to have their own children. The development of this technology over two decades ago has allowed the morphological events during early human embryonic development to be observed microscopically [1–3]. These observations have contributed significantly to the great success of ART programmes in humans. It has remained difficult, however, to evaluate the dynamic changes occurring during embryonic development using these observational methods. Payne *et al.* [4] developed time-lapse video cinematography to overcome the limitations of intermittent observation. This technique provides high resolution, continuous imaging, in which the various components of the cell can be recognized and followed during the recording period. In a preliminary study, Payne *et al.* observed that oocytes that had undergone intracytoplasmic sperm injection (ICSI) developed up to the stage of pronuclei formation. Their study revealed that this method is useful for probing the early events required for successful fertilization.

We attempted to develop a new system for time-lapse observation in which optimal stable culture conditions on the microscope stage are maintained for long periods of time. Recently, we succeeded in developing a new system of time-lapse cinematography, which can take more than 2,000 successive images of human gametes and embryos [5].

This paper presents the results of our morphological analysis of human embryonic development using time-lapse cinematography.

Time-Lapse Cinematography

In 1997, Payne *et al.* [4] first developed a time-lapse video recording system mounted on the stage of an inverted microscope. In this system, the inverted microscope was fully covered by a large chamber and the space between the body of the microscope and the edge of the chamber was sealed. The large chamber was maintained at 37° C with a humidified atmosphere of 5% carbon dioxide (CO_2) in air. Using this system, time-lapse recording was performed on a single, randomly-selected oocyte for 17 to 20 hours after intracytoplasmic sperm injection (ICSI). Although we attempted to apply this system in our laboratory, we

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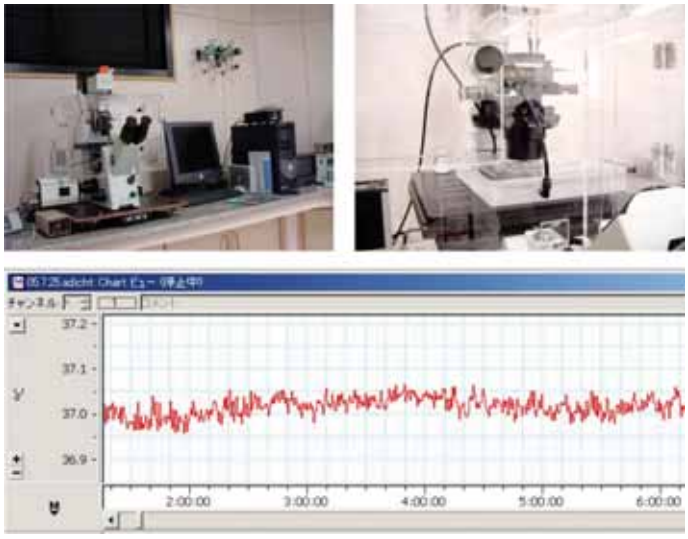

Fig. 1. Time-lapse cinematography.

failed to maintain stable culture conditions.

To overcome these problems, we developed a new system for time-lapse cinematography based on the original report of Payne *et al*. [4]. An inverted microscope (IX-71, Olympus) with Nomarski differential interference contrast (DIC) optics (Olympus, Japan) and a micromanipulator (Narishige, Japan) was covered by a handcrafted chamber made of acrylic resin. An air heater was placed in the corner of the chamber to maintain optimal temperature. Our system also contained a small acrylic chamber (15 cm × 15 cm × 3 cm) surrounded by a small water bath on the stage of the microscope. The glass Petri dish containing the embryonic cultures was placed in the center of the small chamber. Humidified CO_2 gas was infused into the chamber through the water bath. The volume of CO_2 flow and the temperature in the chamber were adjusted to optimal conditions (temperature: 37 ± 0.3° C and pH: 7.45 ± 0.03). To obtain ideal culture conditions in the 3-μl microdrop of culture medium covered by mineral oil (SAGE, USA), the system required settings of CO_2 flow, 40 ml/min, temperature in the big chamber, 38.0° C, and on the thermo-plate on the stage of microscope, 41.8° C (Fig. 1).

The inverted microscope was equipped with a CCD digital camera (Roper Scientific Photometrics, USA) connected to a computer and display through MetaMorph (Universal imaging Co., USA). Digital images of the cultured embryos were acquired every two minutes for approximately 40 hours using an exposure time of 50 ms. In total, 2,000 to 2,500 images were taken during the observation period.

After receiving informed consent from couples, a single, randomly-selected oocyte was prepared for time-lapse cinematography after ICSI or *in vitro* fertilization (IVF). After the embryos used for time-lapse observations had reached the 2- to 4-cell stage, good quality embryos, identified morphologically, were cryopreserved for future clinical use.

The Sequence and Timing of the Morphological Events during the Early Stages of Human Embryonic Development

We observed embryonic development after ICSI for approximately 40 hours. Out of 65 oocytes used in this study, 55 (84.6%) were fertilized normally and cleaved. Forty-two of these oocytes (76.4%) developed as good quality embryos (GQE; grade 1 or 2), classified by the modified Veeck's morphological classification.

As there were no significant differences in the serial events and time courses between morphologically GQE and poor quality embryos (PQE), we analysed the data obtained from all oocytes observed after ICSI (Fig. 2).

All of the oocytes had cytoplasmic waves in the region in which the granular area moved around the ooplasm. This phenomenon ceased after extrusion of the second polar body (PB). Although the identity and composition of the granular cytoplasmic area remains unknown, the observed movement appears to be related to the extrusion of the second PB (Fig. 3-A, 3-B).

The second polar body (PB) was extruded at a

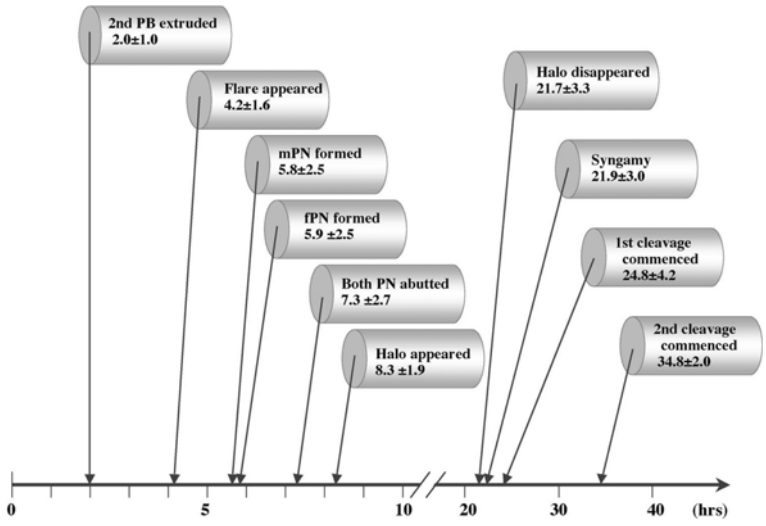

Fig. 2. Time course and events during the early stages of embryonic development after ICSI.

median time of 2.0 hours after sperm injection. The majority of second PB (81.1%) were located within 20 degrees of the first PB (Fig. 3-B).

At 2.2 hours (median time) after extrusion of the second PB, we observed a glassy cytoplasmic region in the center of the ooplasm, the cytoplasmic flare, that radiated from the site of sperm injection toward the periphery. A small male PN (mPN) then formed at the site at which the cytoplasmic flare began (Fig. 3-C, 3-D, 3-E, Fig. 4-A).

Either simultaneously with the cytoplasmic flare or shortly thereafter (approximately 5 to 6 minutes), a female PN (fPN) appeared near the second PB (Fig. 3-F, Fig. 4-A). Both PN contained a number of small nucleolar precursor bodies (NPB), which moved rapidly throughout the nucleoplasm (Fig. 3-G, 3-H, 3-I, 3-J, 3-K, 3-L, Fig. 4). The fPN was then drawn centrally towards the mPN, together with the glassy cytoplasmic region (Fig. 3-F, 3-G, Fig. 4-B, 4-C, 4-D, 4-E). After 2.4 hours (median time), the fPN abutted the mPN at the center of the ooplasm (Fig. 3-H, Fig. 4-F).

According to a large number of studies examining the molecular and cellular mechanisms of fertilization [6–9], a radial microtubule array organized from the sperm centrosome, the sperm aster, plays an essential role in detecting the fPN and bringing both PN into contact during the fertilization process in most mammals. Movement of the sperm aster can be evaluated morphologically by time-lapse imaging (Fig. 3-C, 3-D, 3-E, 3-F, Fig. 4).

After the apposition of both PNs, cytoplasmic organelles were drawn from the cortex towards the center of oocyte. Consequently, a translucent zone, dubbed a halo, appeared in the peripheral ooplasm (Fig. 3-I). The halo was first observed one hour (median time) after PN abutment (8.3 hours after sperm injection) (Fig. 3-I). The halo persisted (about 13.4 hours) in the peripheral ooplasm, disappearing just before syngamy (21.7 hours after sperm injection) (3-J, 3-K, 3-L). Although there are several hypotheses regarding the generation and function of the halo, the details of this phenomenon are still poorly understood [3,4,10-12]. In a previous study, we observed that the appearance of the halo was closely related to the morphological quality of embryos; poor quality embryos often lacked the halo [5].

The first cleavage division (C1) occurred approximately three hours after syngamy (Fig. 3-N, 3-O). Thirty minutes were required for the completion of C1 (Fig. 3-P). Of 55 oocytes with normal fertilization, 28 (50.9%) underwent the second cleavage division (C2) within the time-lapse observation period of 40 hours, occurring 34.8 hours (median time) after ICSI (Fig. 2).

Fertilization Process Observed in Human Gametes after IVF

We next tried to analyse the fertilization process in humans using IVF as a more physiological representation of fertilization events.

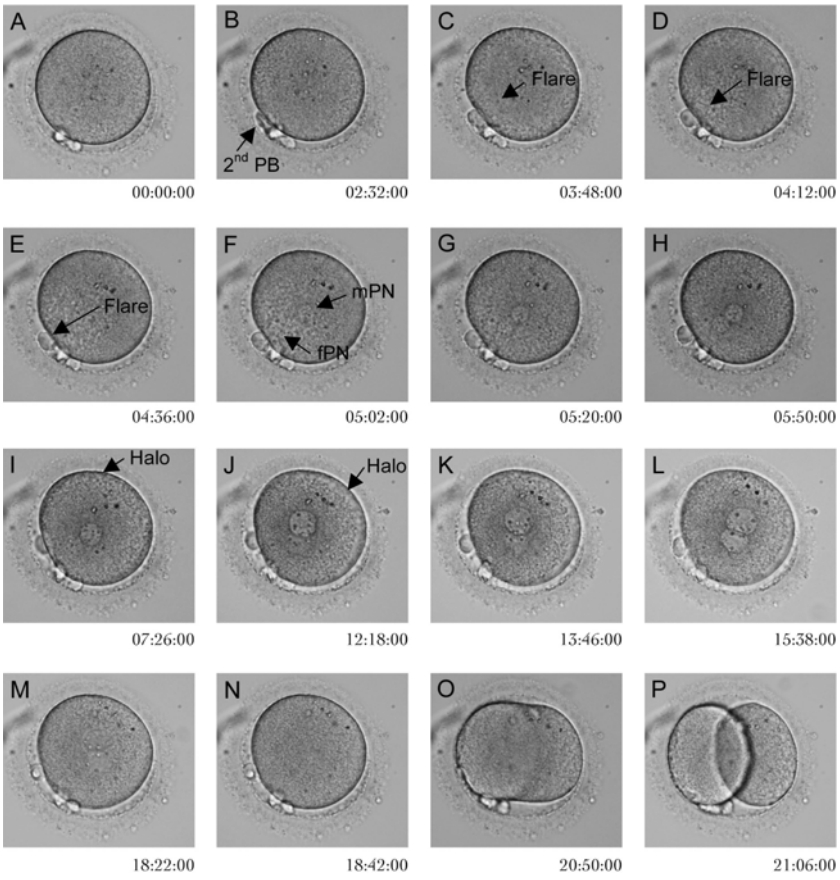

Fig. 3. A sequence of images taken during the early stage of embryonic development by time-lapse cinematography of an oocyte after ICSI. A–C: The cytoplasmic wave was seen prior to extrusion of the second polar body (PB) seen next to the first PB. D–E: We clearly observed a glassy change in the ooplasma, which radiated from the center of the oocyte toward the cortex (cytoplasmic flare). F–H: Just after the cytoplasmic flare, the male pronucleus (mPN) appeared at the center of the oocyte where the sperm had been injected. The female pronucleus (fPN) formed in the cytoplasm adjacent to the second PB, then was subsequently drawn towards the mPN, together with the cytoplasmic flare. Finally, the fPN abutted against the mPN. J–L: As both pronuclei gradually increased in size, the translucent zone (halo) appeared in the peripheral ooplasm during pronuclear growth. M–N: Just before or concurrent with syngamy, the halo disappeared as the cytoplasmic organelles moved towards the cortex. O–P: The first cleavage division began three hours after syngamy.

This study included data from 25 oocytes. Each oocyte was inseminated with 50,000 of the husband's sperm in the usual IVF manner. One hour after insemination, the cumulus cells were gently removed, so as not to damage the tail of the sperm that had penetrated the zona pellucida. The oocyte was transferred into 3 μl of culture medium for the time-lapse observation. We carefully identified the site of sperm penetration of the zona pellucida using a micromanipulator. Once we distinguished the sperm that had entered most deeply into the zona pellucida, we focused the microscope on that sperm; we then commenced time-lapse observation at 10-second intervals until the sperm reached the surface of the oocyte membrane. Thereafter, we acquired images every 2 minutes. It was difficult, however, to detect the point at which the sperm penetrated the zona pellucida. We were able to observe the process of sperm

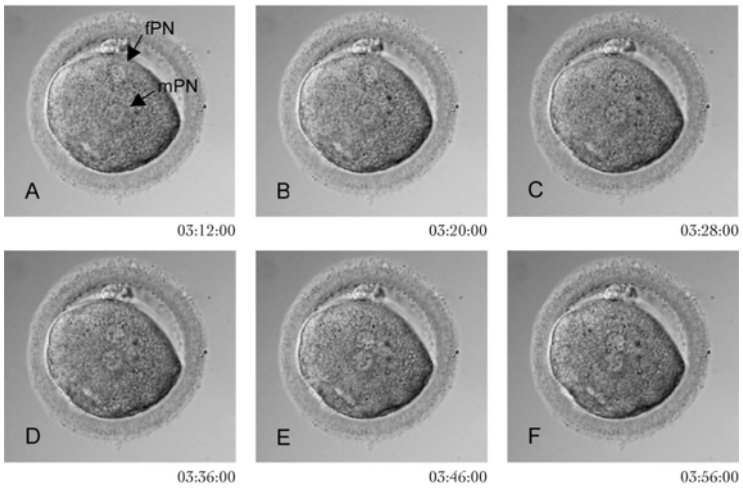

Fig. 4. Movement of the fPN towards the mPN. A: The mPN appeared in the center of ooplasm. Shortly after, the fPN was formed in close proximity to the second PB. B–E: The fPN then moved centrally towards the mPN, as both PN became gradually larger in size and the vigorous movement of nucleolar precursor bodies (NPB) began. F: The fPN abutted on the mPN at the center of the ooplasm approximately 2.5 hours after appearance of the fPN.

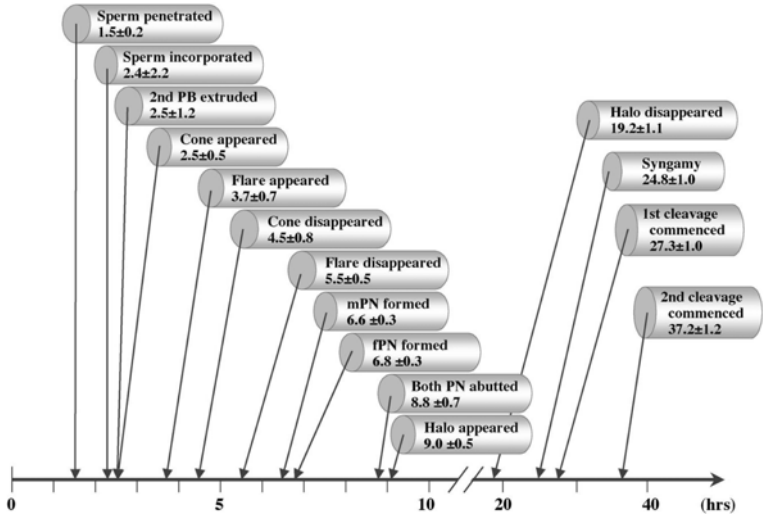

Fig. 5. Time course and events of the fertilization process in human embryos after IVF.

penetration in only 28% (7/25) of oocytes. In three oocytes, the sperm had already entered and reached the oocyte surface at the commencement of time-lapse observation. Although we could not detect sperm entry, the remaining 15 oocytes exhibited subsequent normal fertilization and cleavage. As we determined that there were no statistically significant differences in the occurrence or time course of subsequent events between GQE and PQE, we analysed the data obtained from 25 normally-fertilized oocytes (Fig. 5).

Sperm penetrated the zona pellucida 1.5 hours after insemination, approximately 30 minutes after

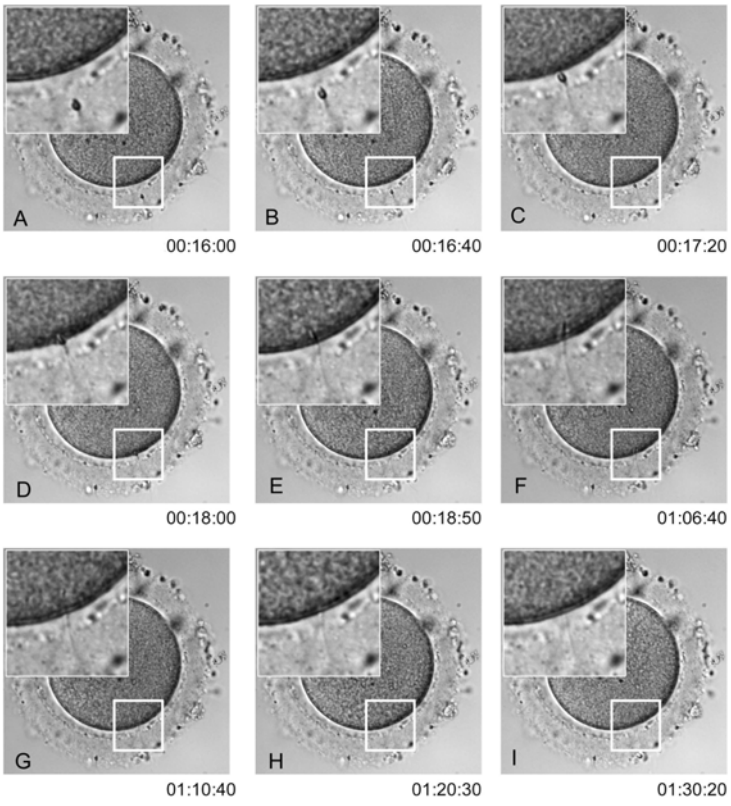

Fig. 6. Sequential images of a human embryo from sperm penetration to sperm fusion. Each image also includes a higher magnification. A: The sperm penetrated the outer layer of the zona pellucida, prior to entry into the inner layer of the zona. B–D: The sperm passed through the zona, quickly traversed the perivitelline space and attached to the oocyte membrane. E: The sperm remained bound to the membrane for approximately 50 minutes. F–I: The sperm was gradually incorporated into the oocyte, beginning with the equatorial segment of the sperm head and finally the tip of the sperm head was engulfed. The sperm tail remained on the outside of the oocyte.

commencement of time-lapse observation (Fig. 5, Fig. 6).

After penetrating the zona pellucida, the sperm quickly moved across the perivitelline space and attached to the surface of the oocyte membrane, remaining there for 40 to 50 minutes (Fig. 6-A, 6-B, 6-C, 6-D, 6-E). Thereafter, the equatorial segment of the sperm head initiated incorporation of the sperm into the oocyte membrane (Fig. 6-F), as described [13]. The mid-piece of the sperm head sank under the oocyte membrane; finally, the tip of the head disappeared (Fig. 6-G, 6-H, 6-I).

Approximately 2.5 hours (median time) after insemination, the second PB was extruded shortly after sperm fusion (Fig. 5, Fig. 7-A, 7-B).

In this study, we uncovered a previously undescribed phenomenon occurring in human oocytes. Concurrent with or shortly after extrusion of the second PB, a protrusion of the oocyte membrane, called the fertilization cone, appeared (Fig. 7-C). This phenomenon persisted for only two hours (Fig. 7-D, 7-E). In mouse studies, the fertilization cone was observed to arise at the sperm entry position (SEP) [14, 15]. We noted that after ICSI a number of oocytes exhibited a transitory protrusion of the membrane, which was similar to the fertilization cone seen in IVF embryos. This observation suggests that the sperm may not be injected in the proper position by the ICSI

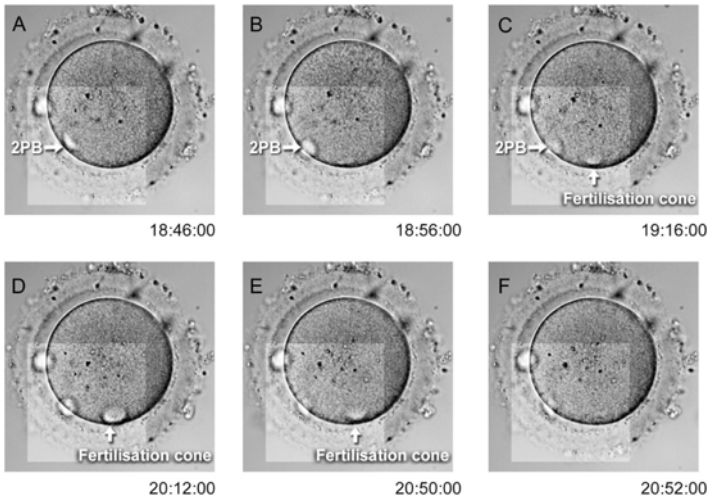

Fig. 7. Sequential images showing the extrusion of the second polar body and the appearance of the fertilization cone in a human embryo. A–B: Approximately 10 minutes after sperm fusion, a protrusion of the oocyte membrane was followed by extrusion of the second PB into the perivitelline space. C–E: Ten minutes after second PB extrusion (in this case), a protrusion of the oocyte membrane at the sperm entry point (SEP) was observed. This disturbance gradually enlarged, reaching the size of the second PB (fertilization cone). The fertilization cone persisted at the oocyte membrane for just two hours. F: The site at which the fertilization cone was seen became hollow for a few minutes after the disappearance of this structure.

procedure in a subset of oocyes. This finding also supports the idea that generation of the fertilization cone may be related to the interaction between the mid-piece of the sperm head and the oocyte membrane. Although it remains controversial if the fertilization cone has a physiological importance in early embryonic development [14–19], the observance of this phenomenon in this system may facilitate further study of the cytokinesis and development of human embryos.

As seen in ICSI embryos, we observed the appearance of the cytoplasmic flare (3.7 hours after insemination; median time) at the SEP simultaneous with or shortly before the disappearance of the fertilization cone (Fig. 5, Fig. 8-A, 8-B, 8-C). We believe that the cytoplasmic flare represents a visual manifestation of the sperm aster. There were only small differences in the timing of events during the early stages of embryonic development between embryos generated by ICSI and IVF. After mPN formation, the fertilization process progressed in an orderly manner, as shown in the ICSI embryos (Fig. 2, Fig. 5, Fig. 8-D, 8-E, 8-F, 8-G, 8-H). Although the duration of the halo was shorter in IVF embryos than that in ICSI embryos, the period from sperm entry into the oocyte to C1 and C2 in IVF embryos was the same as that seen in ICSI embryos, considering that 1.5 hours (median time) were required for sperm penetration in IVF (Fig. 2, Fig. 5).

Conclusions

Here we have presented our morphological analysis of human embryonic development after both ICSI and IVF. This study indicates that non-invasive imaging, time-lapse cinematography, is useful not only for elucidating the morphological events of fertilization in humans, but also for evaluating the physiological importance of these events during the early stages of human embryonic development. In addition, analysis of cellular activity and quality during fertilization and embryogenesis using this system will contribute to the future improvement of the clinical results of ART. Several new research projects are now in progress to address these points.

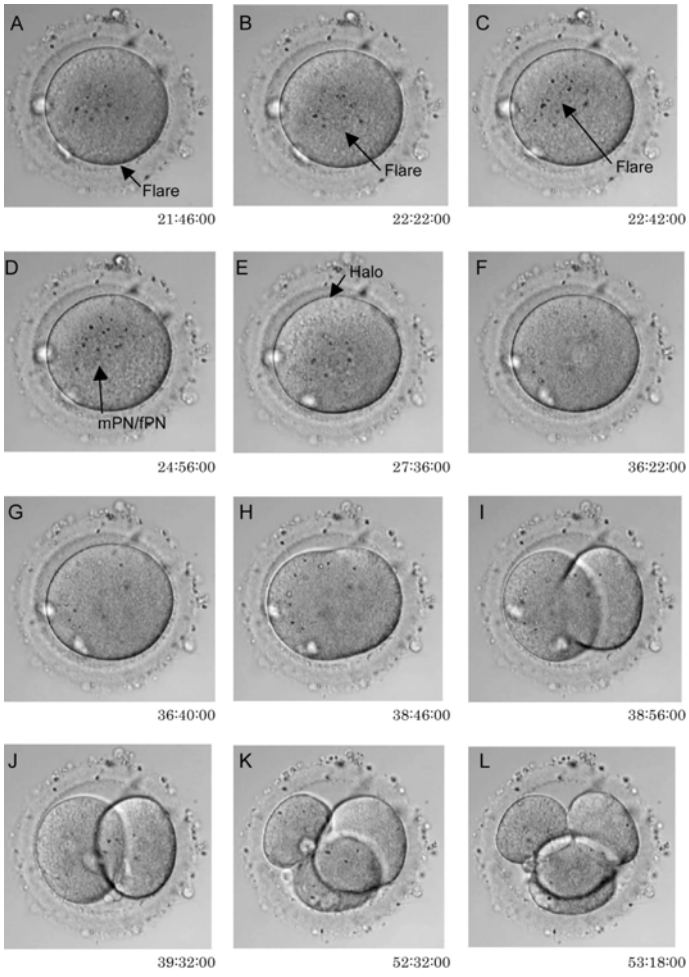

Fig. 8. Sequential images spanning from the cytoplasmic flare to the second cleavage division. A–C: Shortly after disappearance of the fertilization cone, the cytoplasmic flare appeared at the SEP, radiating from the SEP towards the center of the ooplasm. D: Both the mPN and fPN were visible at the center of ooplasm. E–F: After appearance of both PN, the halo was clearly identified concurrent with the growth of both PN. Prior to syngamy, the halo disappeared rapidly. G–J: Approximately 2.5 hours after the syngamy, the first cleavage division began at a position perpendicular to the long axis of the oocyte; this process was completed within 40 minutes. K–L: Approximately 10 hours after the first cleavage division, the second cleavage division occurred in an asynchronous manner. The blastomere on the left-hand side cleaved first, followed by the cleavage of the remaining blasotomere approximately one hour later.

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