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Morphological Selection of Gametes and Embryos: 2PN/Zygote

Martin Greuner and Markus Montag

Introduction

The question of the start of live is as old as live itself and nobody can really answer it.

> Live is the search from nothing for something.
>
> **— Christian Morgenstern**

The pronuclear (PN)-stage embryo is the first stage of live where the genetic material of both female and male is visible as pronuclei and when fertilization is initiated. Although it is possible to judge the morphology of the oocyte and the spermatozoa by itself, the PN stage is the first event that allows for morphology rating of the new live by various small details that are indicative for the future of the embryo.

> The basis of all is small. "Omnia rerum principia parva sunt."
>
> **— Marcus Tullius Cicero (106–43)**

There are numerous possibilities for noninvasive markers to evaluate the developmental potential of an embryo, but studies on transcriptomics [1], proteomics [2], and metabolomics [3] are still largely experimental. Thus, light microscopic morphological examination of the cells is still the most common routine in the laboratory for examining development from the oocyte to the embryo.

The challenge is that in the *in vitro* fertilization (IFV) program, only about 10% of the collected oocytes have the potential to develop into an embryo that can implant [4,5]. About 50%–70% [6] of the oocytes are aneuploid [7], and about 70% of the embryos, 44 hr after fertilization, present aneuploid blastomeres [8]. With reference to the success or failure of an assisted reproductive technique (ART) therapy, selection based on microscopic evaluation of morphology is essentially still the preferred method and essential.

After ovulation or ovum pick-up in therapy, the human oocyte has to be arrested in the second meiotic division (metaphase-II), usually characterized by the presence of a first polar body. The entry or the injection of the sperm into the oocyte initiates the biochemical activation of the oocyte by a sperm-specific protein called phospholipase C zeta [9,10] and leads to a change in the membrane potential, a rise of the intracellular Ca^{2+} level [10], and the second meiotic division. The second polar body containing the chromatids from one haploid chromosome set is extruded, and the female pronucleus is formed (Figure 6.1). During this process, the ooplasm rotates in a periodical way, and in parallel the sperm chromatin decondensates. The sperm cell also delivers the centriole that has a leading role in further development and control of microtubules that are important for the symmetry of the developing embryo [11]. These microtubules pull the haploid female pronucleus toward the male pronucleus. Both pronuclei finally migrate to the center of the cell and align [12–14]. Microtubules also mediate the organization of a mitochondrial clustering to the center of the cell in the area around the pronuclei. This could be the result of the different metabolic needs of the new life. The mitochondria are where ATP generation takes place, thus they are essential for further embryo development [13,15].

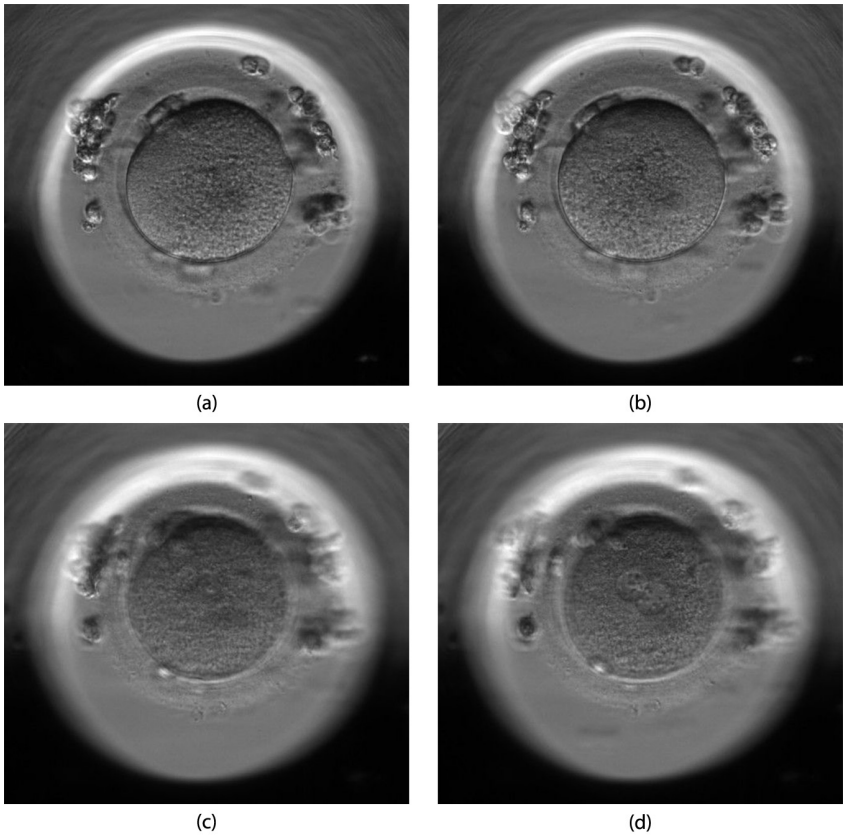


FIGURE 6.1 Time-lapse sequence showing the course of polar body extrusion. (a) 2.3 hr post-ICSI (pI). (b) 3.6 hr pI. (c) 6.7 hr pI. First appearance of pronuclei in the periphery. (d) 11.3 hr pI. Centralization of the pronuclei.

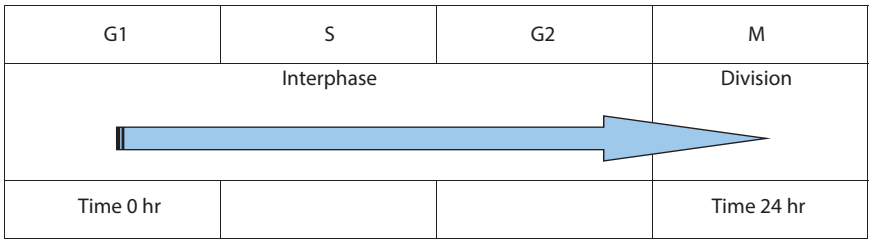
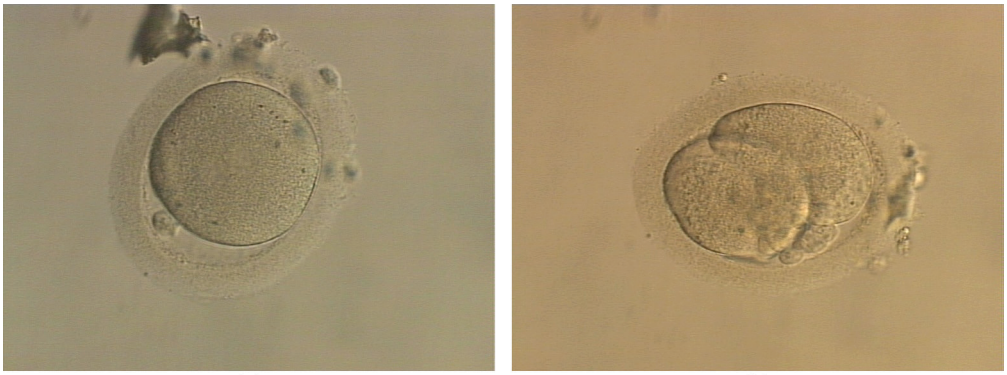


FIGURE 6.2 Cell cycle of an eukaryotic cell is divided into four stages. (From Alberts B et al., *Molekularbiologie der Zelle,* Zweite Auflage VCH Verlagsgesellschaft, Weinheim, 1990.)

The G1 phase starts approximately 2–3 hr after sperm entry, and pronuclei appear after 4–6 hr. This process is finished 18–22 hr after sperm entry or injection. Figure 6.2 [16] illustrates this cell cycle of an eukaryotic cell. A routine microscopic morphological examination [17–19] of the pronuclei is performed 16–18 hr after sperm entry. The judgment is done with reference to the number, size, and the symmetry of the pronuclei. Nuclear precursor bodies are formed in the pronuclei and constitute the nucleoli. Nucleoli are where pre-rRNA synthesis takes place. The symmetry and synchrony of the nuclear precursor bodies in the pronuclei can be used to evaluate the potential of the developing embryo [20–22].



(a) (b)

FIGURE 6.3 The pronuclear membrane breakdown (a) and early cleavage (b).

Anomalies in the cytoplasm of the PN-stage oocyte, such as vacuoles and refractile bodies, can negatively influence development of the embryo [23–25]. The appearance of a peripheral cytoplasmic translucency is seen in the majority of PN-stage oocytes and is discussed in the literature as another potential marker [14,26,27]. These criteria are most commonly used to evaluate the PN-stage oocyte.

The identical reduplication of the haploid chromosome set of each pronuclei takes place in the S phase within 6 hr after the G1 phase, followed by the onset of PN membrane breakdown and syngamy. With the fusion of the male and female genetic material, the fertilization cascade is finished, and embryo development starts by cellular division cycles. Figure 6.3 demonstrates this fusion and the start of the cellular division cycle.

The PN-stage judgment comprises (1) number of pronuclei; (2) size of pronuclei; (3) number, size, and distribution of nucleoli; (4) cytoplasmic halo; and (5) recommendations.

Number of Pronuclei

The occurrence of the pronuclei signals the initiation of the fertilization cascade. The number of pronuclei is an important indicator for aneuploidy. A normal fertilized cell presents with two centrally positioned, juxtaposed pronuclei.

A regular fertilized oocyte shows, in general, two pronuclei.

Existence and Number of Pronuclei

One day after oocyte retrieval (16–18 hr after sperm injection), it is possible to examine the pronuclei. Not all cells exhibit two pronuclei; there is the possibility of the appearance of only one or three and more pronuclei (Figure 6.4).

The judgment of the number of pronuclei is not always simple due to the three-dimensional spherical structure, with pronuclei being located somewhere inside this structure. To determine exactly the number and position of pronuclei, it is important to use a suitable optical mode, such as the Hoffmann modulation contrast optic, at a sufficiently high magnification (e.g., 40× objective). A simple stereomicroscope does not give sufficient resolution. Sometimes, it is very important to rotate the oocyte to identify pronuclei that are positioned above one another. Figure 6.5 illustrates this problem, where two pronuclei are positioned exactly above each other so that it looks like one PN cell. The same problem could appear by looking at a cell with three pronuclei (Figure 6.6). Depending on the orientation, such a cell could be judged as normal when looked at just once without turning.

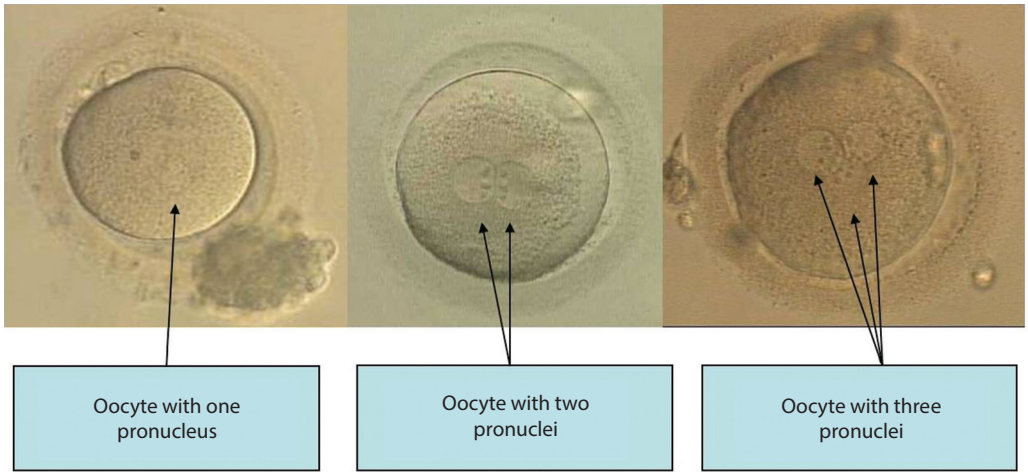


FIGURE 6.4 Oocyte with one, two, and three pronuclei.

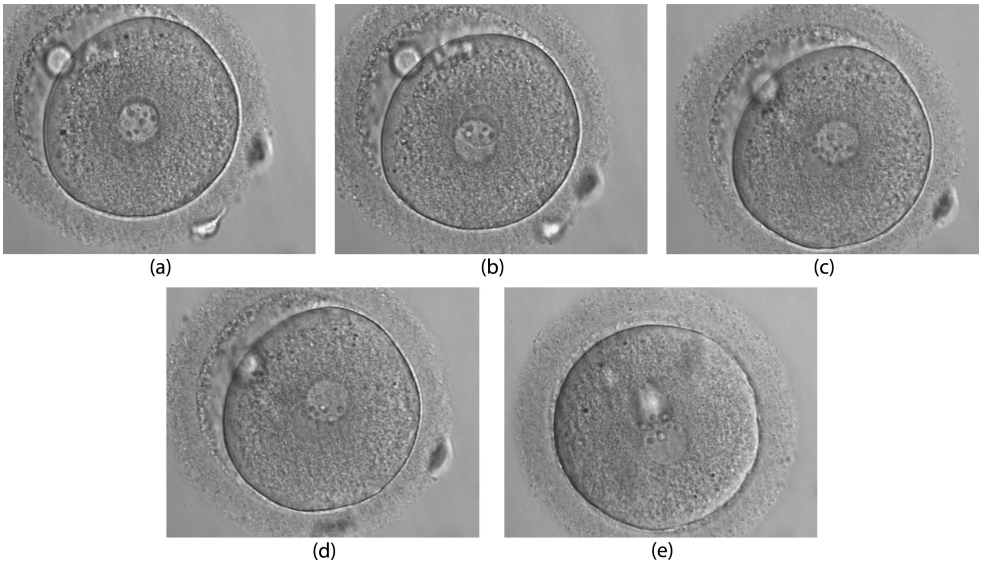


FIGURE 6.5 One cell seen in different positions, illustrating the difficulty in identifying the number of pronuclei. (a) This could be a cell with one pronucleus. (b-e) Rotation reveals the presence and position of two pronuclei.

Oocytes with One Pronucleus

An oocyte exhibiting 1PN has to be judged in a different way. Time-lapse sequences can show the development from PN appearance up to fusion. This allows identification of whether pronuclei appear in asynchrony or whether one pronucleus disappears earlier than the other pronucleus or whether pronuclei fuse prematurely. Also, cells that develop from the beginning with only one pronucleus can clearly be identified. Looking only once may not enable a correct judgment, and the potential of 1PN-stage oocytes depends on the fertilization method that determines, for example, the risk of aneuploidy.

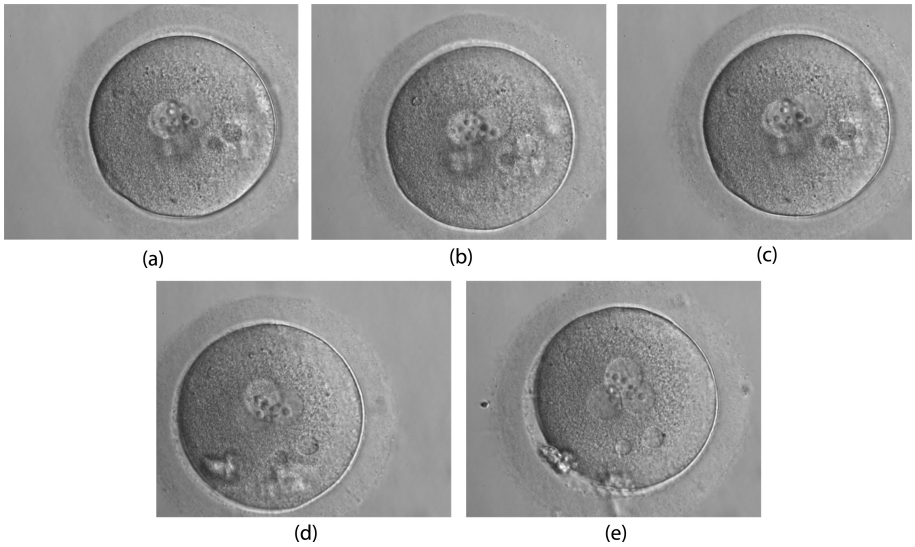


FIGURE 6.6 One cell in different positions to identify correctly the number of pronuclei. (a) This could be a cell with two pronuclei. (b-e) The same cell rotated to identify clearly three pronuclei.

In Vitro *Fertilization*

Oocytes with just one pronuclei (1PN stage) that develop after insemination of the cumulus-oocyte complex are not always aneuploid. Plachot et al. [28,29] showed that between 46% and 69% of these cells are haploid and that between 13% and 29% are diploid. Staessen et al. [30] analyzed even more cells (80.3%) to be diploid and found only 12.5% to be diploid. The asynchronous development of the cell can be a reason for 1PN-stage cells (early appearance or disappearance of one pronucleus) but parthenogenetic activation also could be a reason. Another possibility is the entry of a sperm that did not decondense or two pronuclei that fused. Balakier et al. [31] reported after fluorescent *in situ* hybridization (FISH) analyses that 50% of 1PN-stage oocytes were diploid. These results lead, in the routine work, to the question how to handle 1PN-stage oocytes after IVF when no other cells are available. A solution is to discuss with the patient the possible risk of aneuploidy of 1PN-stage oocytes after IVF. If the patient agrees to it, it is possible to have an embryo transfer. Staessen et al. [32] reported repeated birth from healthy children after transfer of 1PN-stage oocytes from IVF.

Intracytoplasmic Sperm Injection

The appearance of 1PN-stage cells after intracytoplasmic sperm injection (ICSI) is different from that of cells after IVF. Sultan et al. [33] could show that only 9.5% of these cells were diploid in comparison with 61.9% after IVF. Literature reveals that 1PN-stage oocytes after ICSI can be diploid but that the chance is just between 5.3% and 27.9% [30,34,35]. The risk of chromosomal anomalies (aneuploidy) in cells with just one pronucleus after ICSI is clearly higher than after IVF, leading to the recommendation not to transfer or freeze these cells.

Use of PN-stage oocytes with just ONE pronucleus	
IVF	ICSI
Diploid, 13%–80.5%	Diploid, 5.3%–27.9%
When no other cell is available, further culture and transfer can be performed after informing the patient about the possible risks	Further culture and transfer not recommended
No cryopreservation	No cryopreservation

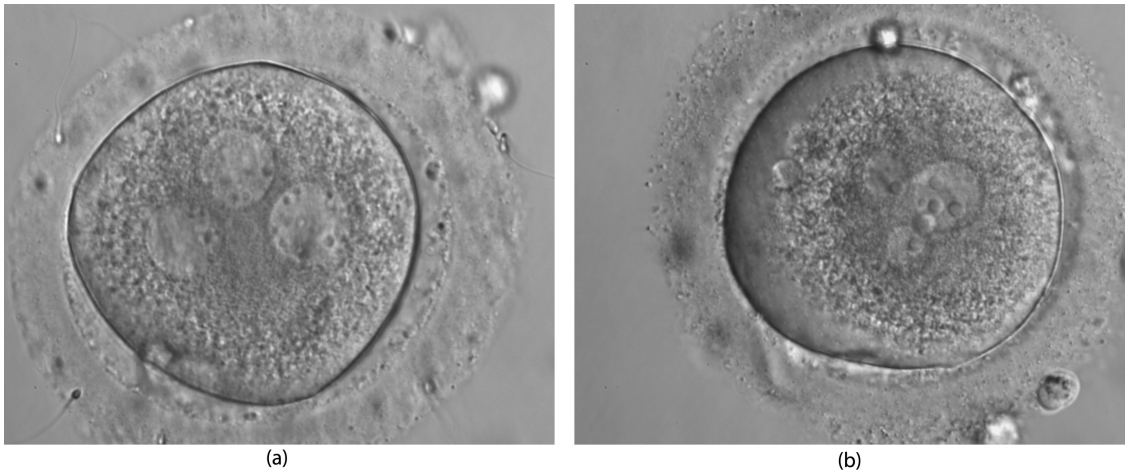


FIGURE 6.7 Cells with irregular fertilization or development with three and four pronuclei. (a) Oocyte with three pronuclei. (b) Oocyte with four pronuclei.

Three or More Pronuclei in an Oocyte

The appearance of three or more pronuclei in one oocyte (Figure 6.7) usually indicates an underlying error that has occurred during the developmental process. These cells are at high risk of being polyploid.

Three PN-stage cells have three haploid chromosome sets in comparison with the regular case with two haploid chromosome sets. If the redundant chromosome set originates from the maternal side, it is called digyny; if it is from the paternal side, it is called diandry. A triploid cell does normally not result in a pregnancy or miscarriage. In case of a pregnancy, the child dies after birth [36]. In IVF, the reason for diandry can be due to entry of two sperms into the oocyte [37]. The entry of a diploid sperm is very unlikely but possible. A possible explanation for digyny is failure of extrusion of the second polar body, and this is the most common reason for the presence of three pronuclei after ICSI [38].

PN-stage oocytes with three pronuclei or more after IVF or ICSI should be discarded and not be used for transfer or cryopreservation.

PN-Stage Cells with Two Pronuclei

PN Size and Position

After the entry or the injection of the sperm into the oocyte, the fertilization cascade is initiated. Within the next 2.5–4.5 hr, the second polar body is discharged and the PN formation starts. The sperm chromatin is very tightly packed with protamines, and these have to be exchanged with histones that are part of the decondensation process. At the same time, the maternal pronucleus is formed; it is slightly smaller than the paternal pronucleus. Both pronuclei grow in size during their development and move together in the center of the cell (Figure 6.8). The position is very important because the first cleavage furrow goes through the PN axis [39].

In most cases, variation in this sequential event shows a lower developmental potential. When one pronucleus is clearly smaller, as shown in Figure 6.9, the corresponding cells have a poor prognosis for initiating a successful pregnancy [17,40].

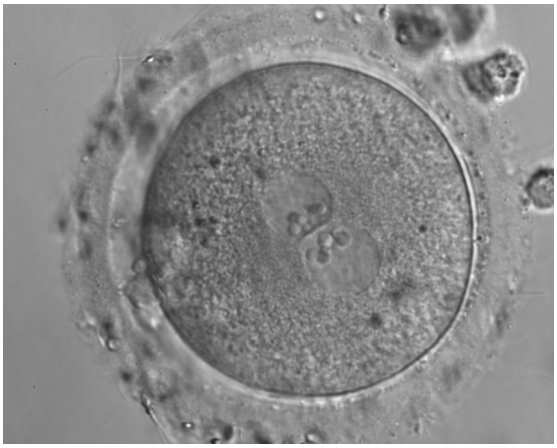


FIGURE 6.8 Pronuclear (PN)-stage oocyte with two regular pronuclei centrally positioned and of equal size (paternal pronucleus slightly larger).

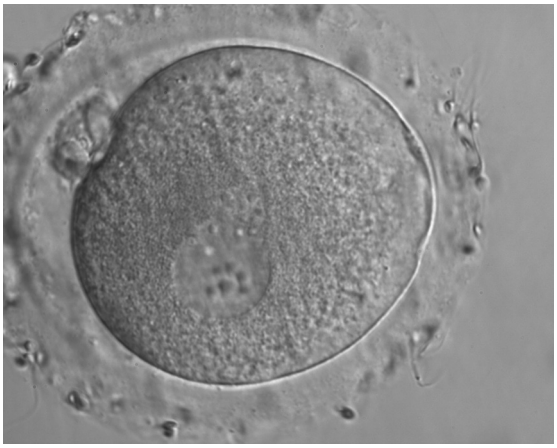


FIGURE 6.9 Pronuclear stage with uneven size of pronuclei.

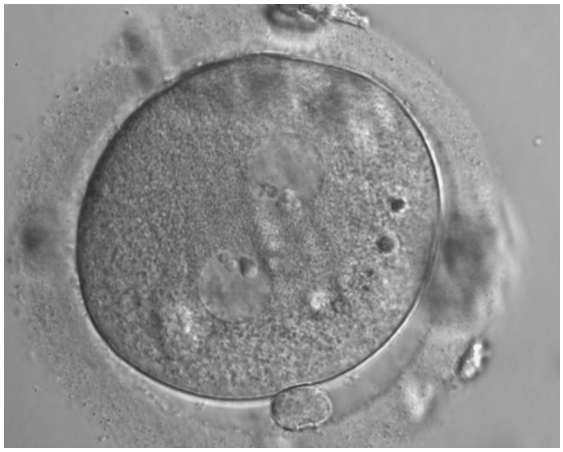


FIGURE 6.10 Pronuclei stage oocyte with two pronuclei not aligned in the center.

The pronuclei should be located side by side in the center of the cell. If this is not the case and if pronuclei are not aligned, as shown in Figure 6.10, further development is usually slow and irregular.

PN stage with two pronuclei that are not of the same size or that are not aligned at the center of the oocyte show a reduced development potential.

A reason for the low development potential could be the asymmetry in the cell. The first cleavage furrow is generally formed in the PN plane, and if pronuclei are located toward the edge of the oocyte, this can lead to problems and can be a potential reason for poor development [41]. In lower order animals, such as insects, worms, and reptiles, polarity is a well-established fact. In mammals, it is discussed whether it is asymmetry or polarity. Invertebrate and vertebrate oocytes present with a pronounced asymmetry in the position of the

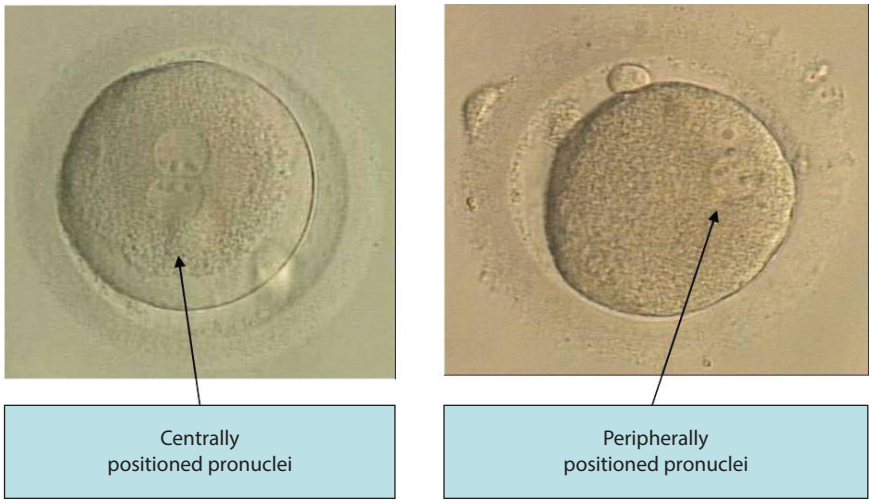


FIGURE 6.11 Location of the pronuclei in the oocyte: centrally or peripherally.

organelle distribution. If this is a manifestation of structural and molecular events and is invariable as well as irreplaceable, it is called polarity. The fact that it is possible to remove a blastomere from a human embryo shows that the polarity is not as strict as in lower order animals. Nevertheless, although in the human PN-stage asymmetry occurs, nonalignment of the pronuclei or asymmetry of the nucleolar precursor bodies is not a good sign.

However, it is very important to remember that PN scoring and PN appearance and disappearance is a time-dependent development and only time-lapse imaging can really see the dynamic structure of the development [42]. This dynamic can be the reason for the different opinions in the literature when pronuclei are located in the periphery of the cell, as demonstrated in Figure 6.11. Ebner et al. [27,43] showed a lower developmental potential for these cells. The relatively rare phenomenon of peripheral pronuclei could be a reason why it is not often found in the literature. Garello et al. [44] reported that this phenomenon is associated with failure or retarded embryo development, but his observations were only based on 19 cells. The problem of peripheral pronuclei is that the first cleavage furrow goes through the PN axis [39], and when the PN are peripherally located, this can result in an abnormal cleavage pattern, eventually leading to uneven two-celled embryos. Nevertheless, we also observed live birth after transfer of embryos derived from such oocytes. The prognostic value with regard to biological processes is not necessarily 100% correct. Still, it is important to identify the cells with the best statistical chances for achieving a pregnancy.

Number and Distribution of Nuclear Precursor Bodies (Nucleoli)

During the development of the pronuclei, the nucleolar precursor bodies appear. These are regions where rRNA synthesis and processing take place. They bind at repeated rDNA sequences of certain chromosomes and are responsible for the active transcription, processing, and protein packaging of ribosomal RNAs [45]. It is possible to determine the number and distribution of the nucleolar precursor bodies in pronuclei using Hoffmann contrast optics at 400× magnification. The underlying symmetry gives information about the development potential of the cell. Symmetry and balance are very important. The Alpha and ESHRE consensus paper (2012; [20,21]) has tried to define and classify PN scoring based on nucleolar precursor bodies. The consensus paper defined a good prognosis, when the nuclear precursor bodies show a symmetrical picture (range 1), but they mention that there are more specialized systems, such as Z-scoring [39]. Nonsymmetrical or other arrangements, including peripherally sited pronuclei, are rated as range 2, and cells with pronuclei with absent or just a single nuclear precursor body are rated abnormal.

The Alpha and ESHRE paper is a consensus and reflects the diversity in this topic in the literature. In routine work, it is often a problem that we just look once at a cell and we do not look at the development. This static observation can be the reason why different results from the same scoring system are reported. It has to be mentioned that some studies found no correlation between PN scoring and the developmental potential of the resulting embryo [46–51]. Nucleoli appear and disappear in a time-dependent way [52], and it is a good example how fast and symmetric development can be. This means that nucleolar patterns do rapidly change in a highly time-dependent manner in most oocytes during the alignment with the cleavage furrow between the two pronuclei and the gradual reduction of the number of nucleoli [53]. This can also been seen in time-lapse monitoring [42]. With this knowledge, it is obvious that the time of entry of the sperm into the oocyte and the maturational state itself play an important role. Nonsymmetrical formation of nucleolar precursor bodies reflects problems in the oocyte, but it does not necessarily mean that the cell cannot solve this problem. For the biologist, it is important to record and assess the right signals and symmetries to be able to compare the cells of one patient to identify those that are best suited for embryo transfer.

With ICSI, we know the exact time of entry of the sperm. Early development after IVF is in most cases slower because the sperm themselves have to find the way into the oocyte. In routine work, this is estimated to cause a delay in the range of 2 hr.

In our opinion, the Z-scoring model proposed by Scott et al. [14,17,39] is an easy scoring system that covers most of the other systems and variations presented in the literature [27,54–61]. According to Scott's work, the symmetric forms Z1 and Z2 show a high potential for initiating a pregnancy. It is very similar to the scoring system presented in the Alpha and ESHRE consensus paper (2011) [20,21].

PN Scoring According to Scott et al. [17,62]

Z1: Both pronuclei with equal numbers of nuclear precursor bodies (nucleoli) aligned at the PN junction

Z2: Both pronuclei with equal numbers of nuclear precursor bodies (nucleoli) scattered symmetrically

Z3: Both pronuclei with inequality of numbers or alignment of nuclear precursor bodies (nucleoli)

Z4: Both pronuclei of different size or not aligned in a central position in the cell

PN Scoring According to the Alpha and ESHRE Consensus Meeting 2011

Range 1: Symmetrical	Equivalent to Z1 and Z2
Range 2: Nonsymmetrical	Other arrangements, including peripherally sited pronuclei
Range 3: Abnormal	Pronuclei with 0 or 1 nuclear precursor body

Examples of these scoring systems are shown in Figure 6.12 [14,17,20,21,62].

The appearance and development of the nucleolar precursor bodies are time-dependent events and show how far the cell has progressed in the cell cycle. Symmetries are important in the development of cells, and the symmetric view of the Z1 and Z2 scores characterizes a PN stage with high potential. Figure 6.13 shows the time-dependent formation of the nucleoli in the pronuclei. For selection of the cells for transfer, a symmetric formation and pronounced progression in the cell cycle warrant a positive choice.

Timing of the development of the PN stage is influenced by the culture medium and by the culture conditions. This can be another reason for explaining the reported differences in the literature. However, for the decision of which cell has the greatest potential, it is important to compare all cells from one patient in a given cycle to others so as to choose the best cells. Asymmetric behavior of the cells can lead to a change in the stimulation regimes or the external conditions in the next cycle to reach better results.

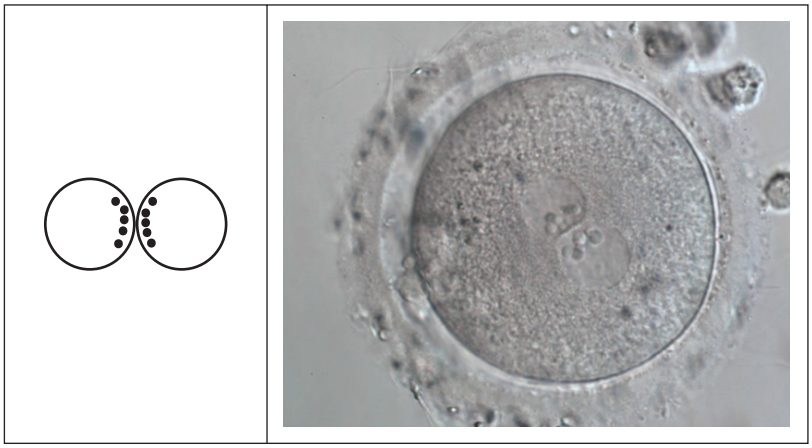
Symmetry and Polarity

In nonmammalian species, the animal and the vegetal pole can be distinguished [44,63]. In the development of the mammalian cells, symmetries and polarization play an important role [41], but this role is not clearly understood yet. This is also reflected in the PN score, but it looks like symmetry and polarity

are essential in allvdevelopmental steps. It starts in the oocyte and continues through fertilization events until embryo development. Symmetry and polarity of the orientation of the polar body can play a role in embryo quality [44]. Polar bodies that are not situated next to each other and those that display a large angle between them are suggested to be of poorer quality [64]. A reason for this reduced quality could be a suboptimal orientation of the pronucleus that leads to cytoplasmic turbulence or uneven cleavage and fragmentation [22]. However, at least the first polar body is not affixed to the oolemma, hence timing can play an important role.

Presence of the So-Called Halo

The outer coat of the oocyte, the zona pellucida, is transparent. This is the reason why one can see the pronuclei as well as intracytoplasmic structures such as vacuoles and inclusion bodies. A cytoplasmic halo is formed when mitochondria and cytoplasmic components are pulled by microtubules from the periphery to the more central region [14]. Because of this pulling, the periphery gets cleared of granular structures, forming the so-called halo [65,66], as shown in Figure 6.14.



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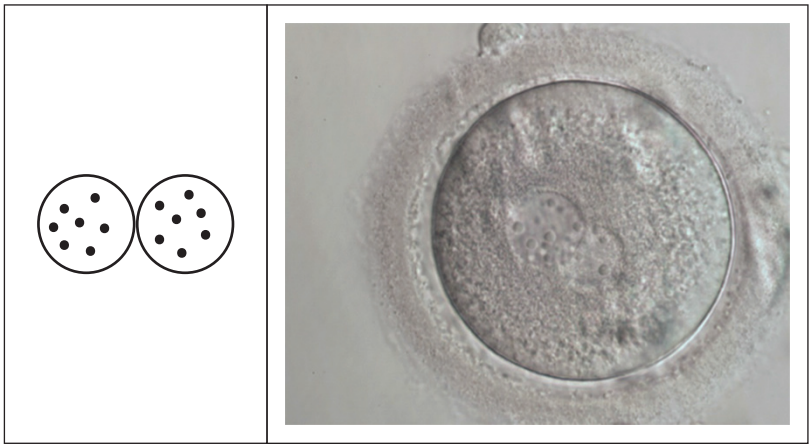
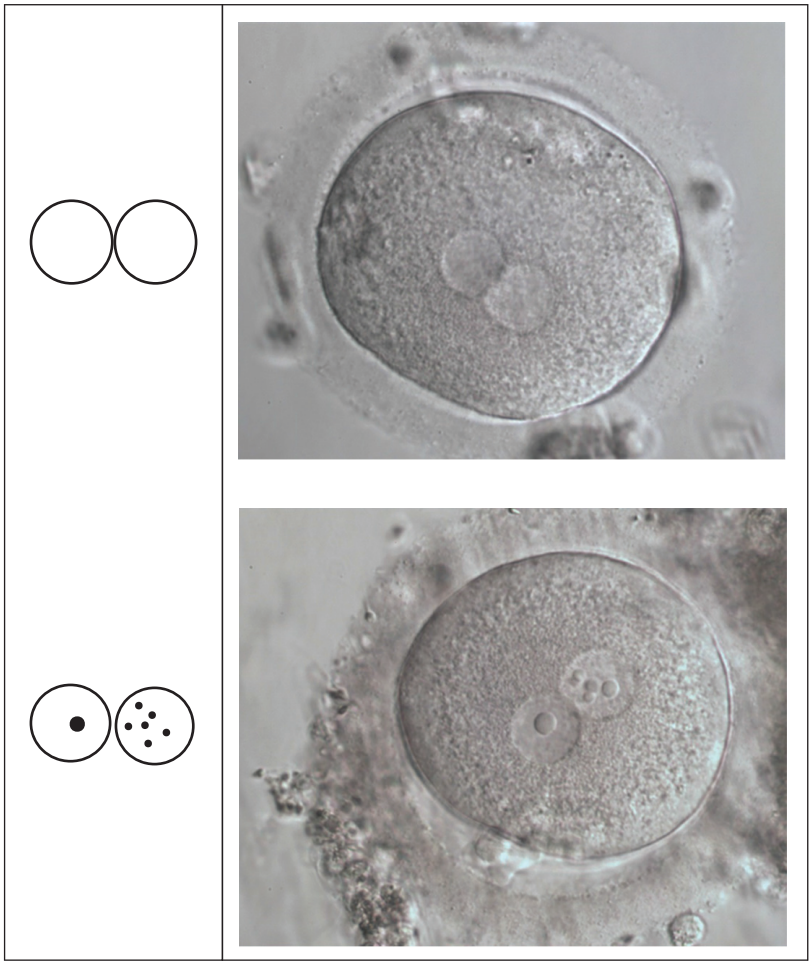


(12b)

FIGURE 6.12 Scoring systems for pronuclear (PN) stages. (a) PN stage with Rang 1 (Z1) score (symmetrical). (b) PN stage with Rang 1 (Z2) score (symmetrical).

(12c)

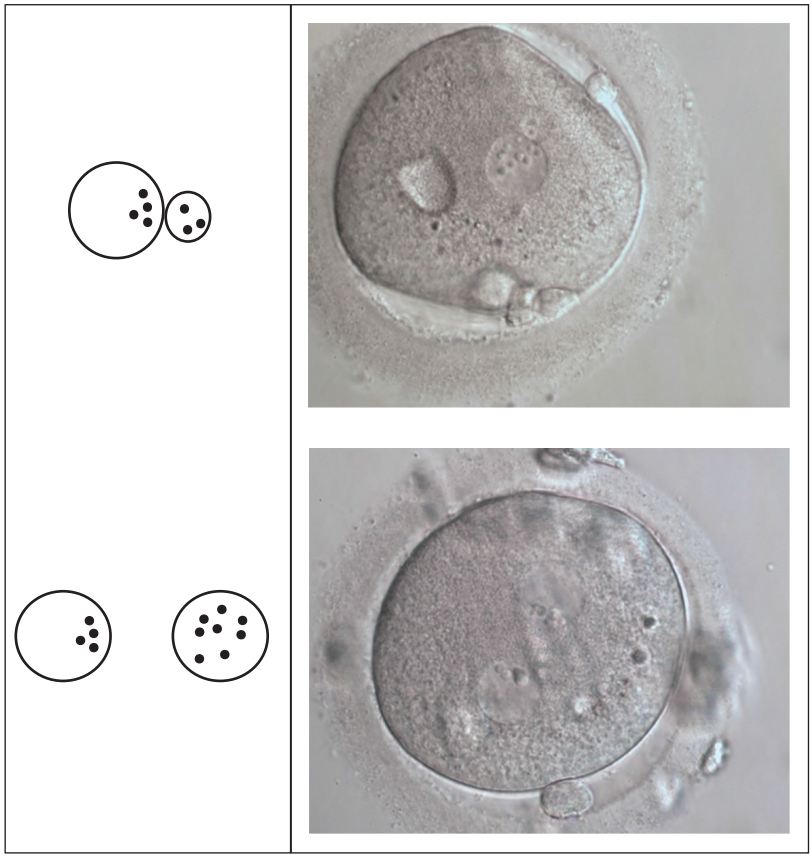
FIGURE 6.12 (*Continued*) Scoring systems for pronuclear (PN) stages. (c) PN stage with Rang 2 (Z3) score (nonsymmetrical).



(12d)

FIGURE 6.12 (*Continued*) Scoring systems for pronuclear (PN) stages. (d) PN stage with Rang 3 (abnormal, 0 or just 1 nuclear precursor body).

The phenomenon of a subplasmalemmal zone was first mentioned by Payne et al. [67]. Different reports exist about the importance and significance of this region in relation to the potential of the corresponding PN stage. Most of the cells (about 67.7%–88.7%) display a halo but in a different specificity [47,65,66]. We know that it appears because of the transport from mitochondria and cell toward the pronuclei [15], but the importance and the meaning of symmetrical and polar halos are not yet understood [14,47]. Ebner et al. [65] evaluated the halo as a positive sign, whereas Salumets et al. [47] found no influence and Zollner et al. [61] differentiated between normal halo as positive and concentric or extreme halos as negative. Our own study [68,69] found no influence by just looking to see whether there is a halo or not, but it could be that the halo also appears and disappears and it is just a momentum picture in the development of the cell. This could be the reason why the question toward the potential of the halo cannot be simply answered as yes or no. Time-lapse imaging should enable to gain more information in this regard.



(12e)

FIGURE 6.12 (*Continued*) Scoring systems for pronuclear (PN) stages. (e) PN stage with (Z4) score. PN of different sizes are not aligned in a central position in the cell. (Modified from Scott L, Smith S, *Hum Reprod* 13, 1003–1013, 1998; Scott L et al., *Hum Reprod* 15, 2394–2403, 2003; Scott L et al., *Hum Reprod* 22; 230–240, 2007; Alpha Scientists in Reproductive Medicine, ESHRE Special Interest Group of Embryology, *Reprod Biomed Online* 22, 2011, 632–646; Alpha Scientists in Reproductive Medicine, ESHRE Special Interest Group of Embryology, *Hum Reprod* 2011, 1–14.)

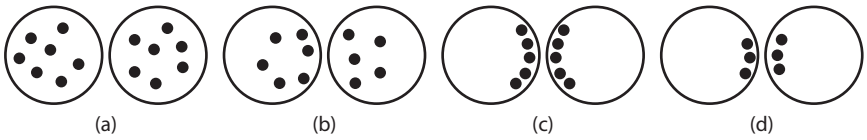


FIGURE 6.13 Time-dependent progression of the orientation of the nucleoli in the pronuclei of the pronuclear stage from (a) dispersed to (b) moving towards centre to (c) alignment at side of pronuclear contact to (d) fusion of nucleoli and further alignment.

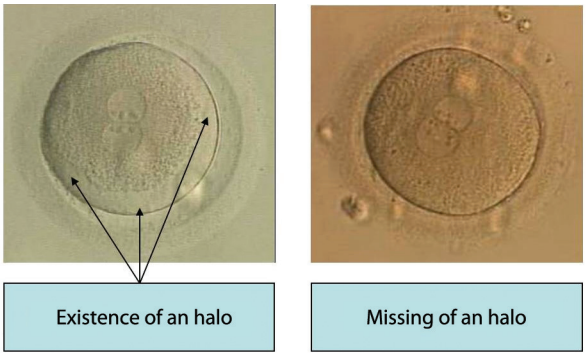


FIGURE 6.14 Occurrence of the halo effect.

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