The cleavage stage embryo

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Introduction

A precise embryo quality evaluation is of paramount importance to sustain a successful in vitro fertilization (IVF) program. In most IVF clinics around the world, this quality assessment relies mainly on the morphological evaluation of cleavage stage embryos. Embryologists should be able to correlate the features observed at the optical microscope with the implantation potential of each particular embryo (Alikani et al., 2000; Ebner et al., 2003; Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

To achieve this goal, many scoring systems based on the morphological features of the dividing embryo have been developed (Giorgetti et al., 1995; Veeck, 1999; Fisch et al., 2001; de Placido et al., 2002; Baczkowski et al., 2004; reviewed by Rienzi et al., 2005; Torelló et al., 2005; Holte et al., 2007). These embryo classification systems are based on the evaluation of the number of blastomeres, the degree of fragmentation, the symmetry of the blastomeres, the presence of multinucleation and the compaction status. It is very important that the features related to implantation potential are assessed accurately and similarly. The purpose of this chapter is to illustrate morphological aspects useful for the evaluation of the implantation potential of the embryos.

Cleavage stage embryos range from the 2-cell stage to the compacted morula composed of 8–16 cells. The number of blastomeres is used as the main characteristic with the highest predictive value (Van Royen et al., 1999; Alikani et al., 2000; Fisch et al., 2001). Good quality embryos must exhibit appropriate kinetics and synchrony of division. In normal-developing embryos, cell division occurs every 18–20 h. Embryos dividing either too slow or too fast may have metabolic and/or chromosomal defects (Edwards et al., 1980; Giorgetti et al., 1995; Ziebe et al., 1997; Van Royen et al., 1999; Leese, 2002; Munné, 2006; Magli et al., 2007). Recent time-lapse studies indicate that not only the timing of cleavage, but also the time between each cell division is of importance. If all blastomeres divide in exact synchrony, only 2-, 4- or 8-cell embryos would be observed. However, it is frequent to observe 3-, 5-, 6-, 7- or 9-cell embryos, which is an indication of asynchronous development (Scott et al., 2007; Lemmen et al., 2008; Wong et al., 2010; Meseguer et al., 2011; reviewed by Kirkegaard et al., 2012). Time of scoring with respect to the insemination event has to be precisely established for a correct evaluation of the kinetics of cell division (Scott et al., 2007). However, it is also important to keep in mind that the environment in the specific laboratory, such as culture media and temperature, influences the kinetics of development.

Very frequently, the mitosis of embryos leads to externalization of parts of the cell cytoplasm, resulting in the presence of anuclear fragments surrounded by a plasma membrane (Antczak and van Blerkom, 1999). The size and distribution of fragments inside the space surrounded by the zona pellucida (ZP) are variable (Alikani et al., 1999). The amount of fragments is widely used to predict the implantation potential of the embryos and fragmentation has been related to aneuploidy (Ebner et al., 2001; Ziebe et al., 2003; Munné, 2006). If fragmentation does not reach 10% of the total embryo volume it is agreed that it does not have an impact on the embryo’s developmental potential (Van Royen et al., 2001; Holte et al., 2007).

Mitosis in blastomeres should produce two equally sized daughter cells. When the division is asymmetric, one of the blastomeres of the next generation will inherit less than half the amount of cytoplasm from the parent blastomere, leading to a defective lineage in the embryo. For example, 4- and 8-cell embryos with equal cell sizes have been shown to have lower multinucleation and aneuploidy rates and increased implantation rates (Hardarson et al., 2001; Van Royen et al., 2001; Hnida et al., 2004; Scott et al., 2007). After two cleavages, the zygote becomes a 4-cell embryo. The four cells of the embryo are normally arranged in a tetrahedron in the spherical space provided by the ZP. However, in some cases, the blastomeres are located close to a single, spatial plane produced by an incorrect orientation of the division axes. This can be associated with altered embryo polarity (Edwards and Hansis, 2005).

Each embryo blastomere should have a single nucleus. Multinucleation has been described to be associated with genetic disorders of the embryo (Kligman et al., 1996; Hardarson et al., 2001). It impairs cleavage rates and the implantation potential of human embryos.
(Pelinck et al., 1998; Van Royen et al., 2003; Moriwaki et al., 2004) and has been associated with an increased abortion rate (Meriano et al., 2004). Multinucleation can be evaluated on Days 1, 2 and 3 of development. On Day 3, however, this characteristic is more difficult to evaluate due to the more complex structure of a Day 3 embryo (Van Royen et al., 2003). Different morphological anomalies are often associated with each other, and uneven cleavage has been shown to be related to multinucleation (Hardarson et al., 2001) and fragmentation (Hnida et al., 2004).

A clear homogeneous cytoplasm is acknowledged as normal for cleavage stage embryos. The presence of anomalies such as an abundance of vacuoles and aggregation of organelles resulting in clear and granular cytoplasmic regions has to be considered in any embryo quality assessment (Veeck, 1999; Desai et al., 2000; Ebner et al., 2003).

After the embryo reaches the 8-cell stage, the blastomeres begin to show an increase in cell–cell adherence due to the spread of intercellular tight junctions. This is the start of compaction. The process of compaction advances during the next division until the boundaries between the cells are barely detectable (Veeck, 1999). If some of the blastomeres are excluded from this compaction process, the embryo may have a reduced potential for becoming a normal blastocyst (Tao et al., 2002). In a proposed grading system compaction can be classified using the following criteria: the proportion of blastomeres undergoing compaction and the morphology of the compacted embryo (Tao et al., 2002). The validity of this grading system remains to be confirmed.

This cleavage stage chapter seeks to illustrate the morphological aspects discussed in the Istanbul consensus workshop on embryo assessment (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). The aim is to introduce a more accurate and widespread comprehension of the nomenclature applied to the characterization of cleavage stage embryos.

A. Cell numbers

The developmental stage of an embryo, defined as the number of blastomeres on Days 1, 2 or 3 after insemination is an essential predictive factor for subsequent implantation and pregnancy rates (1 cell to >10 cells; Figs 211–222). For assessment of embryo cleavage (numbers of blastomeres), the currently accepted observation schedule for optimal cleavage rates was defined at the Istanbul consensus workshop to be: Day 1 (26 ± 1 h post-ICSI, 28 ± 1 h post-IVF), 2-cells; Day 2 (44 ± 1 h), 4-cells and Day 3 (68 ± 1 h), 8-cells (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Early cleavage (Figs 211 and 212), i.e. the first mitosis occurring before 26 ± 1 h (ICSI) and 28 ± 1 h (IVF) respectively, has been shown to correlate with numbers of good quality embryos, blastocyst development and pregnancy rates (Lundin et al., 2001; Fenwick et al., 2002). A number of studies have shown that the transfer of 4-cell embryos on Day 2 of culture (Fig. 215) results in significantly higher implantation and pregnancy rates compared with the transfer of embryos with either lower (Figs 213 and 214) or higher (Fig. 216) cell numbers (Thurin et al., 2005; Holte et al., 2007; Scott et al., 2007).

Correspondingly, several studies have shown that for Day 3 transfers, implantation and live birth rates are positively correlated with an increase in cell number on Day 3, with the 8-cell stage (having been a 4-cell embryo on Day 2) having the highest rates (van Royen et al., 1999; Racowsky et al., 2011). The cleavage stage of the embryo at the time of transfer also seems to have a role in predicting early pregnancy loss. Hourvitz et al. (2006) found that five or...
less blastomeres in the best embryo transferred on Day 3 was correlated with early pregnancy loss. A correlation between cell numbers at distinct observation time points and chromosomal errors has also been reported. It was shown by Munné (2006) that Day 2 embryos with 4 cells had the lowest rate of chromosomal errors, while Magli et al. (2007) showed the same to be true for embryos with 7- to
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B. Fragmentation

Small portions of cytoplasm enclosed by a cell membrane but usually not containing DNA are often formed during cell division. Fragmentation is therefore defined as the presence of anucleate

Figure 220 A cryopreserved 9-cell embryo, warmed on Day 3. One blastomere is slightly larger and one blastomere is slightly smaller than the others. It was generated by ICSI and transferred but failed to implant.

Figure 221 A 10-cell embryo with visible nuclei in some blastomeres. It was generated by ICSI and cryopreserved.

Figure 222 An embryo with more than 10 cells on Day 4. This embryo has not compacted which is unusual at this late stage. Generated by ICSI and cryopreserved.

Figure 223 Day 2 4-cell embryo with <10% fragmentation and evenly sized blastomeres. It was generated by ICSI but not transferred.

Figure 224 Day 2 4-cell embryo with <10% scattered fragmentation and evenly sized blastomeres. It was generated by IVF but not transferred.

Figure 225 Day 2 4-cell embryo with <10% fragmentation and evenly sized blastomeres. Fragments are concentrated in one area of the perivitelline space (PVS). It was generated by ICSI and transferred but the outcome is unknown.

8 cells on Day 3 (Figs 218 and 219). The same pattern was observed by Finn et al. (2010) who described a higher rate of euploidy in embryos with seven to eight blastomeres on Day 3 compared with both six (Fig. 217) or less than six blastomeres and nine (Fig. 220) or more than nine (Figs 221 and 222) blastomeres.
Figure 226 A 4-cell embryo with 10–15% scattered fragmentation, evenly sized blastomeres and a single nucleus per blastomere. It was generated by ICSI, transferred and implanted.

Figure 227 A 4-cell embryo with 10–15% scattered fragmentation, evenly sized blastomeres and a single nucleus in some blastomeres. It was generated by IVF and cryopreserved.

Figure 228 A 4-cell embryo with 10–20% concentrated fragmentation, evenly sized blastomeres and no visible nuclei. It was generated by ICSI, transferred and implanted.

Figure 229 A 4-cell embryo with 10–20% concentrated fragmentation, evenly sized blastomeres and no visible nuclei. It was generated by ICSI, transferred and implanted.

Figure 230 A 4-cell embryo with 15–20% fragmented, one of which is a large fragment and the others small and scattered. The blastomeres are evenly sized with no visible nuclei. It was generated by ICSI and cryopreserved.

Figure 231 A 4-cell embryo with 15–20% scattered fragmentation, unevenly sized blastomeres and no visible nuclei. It was generated by IVF and cryopreserved.
Figure 232 An 8-cell embryo with around 15–20% scattered fragmentation, evenly sized blastomeres and visible nuclei in some blastomeres. It was generated by IVF but was not transferred.

Figure 233 Three views of the same embryo at different focal planes. It is a 4-cell embryo with 20–25% fragmentation which roughly corresponds to the size of one cell. Note the importance of assessing the embryo at different focal planes in order to establish the degree and type of fragmentation (scattered in this case). The blastomeres are evenly sized and have visible nuclei. It was generated by ICSI and cryopreserved.

Figure 234 An 8-cell embryo with 25% scattered fragmentation and evenly sized blastomeres. It was generated by ICSI and transferred but failed to implant.

Figure 235 A 2-cell embryo with 20–25% fragmentation on Day 2 (slow development). The blastomeres are evenly sized but binucleated. It was generated by ICSI but was not transferred.

Figure 236 A 7-cell embryo with 30% fragmentation. Fragments are scattered in the PVS. It was generated by ICSI but was not transferred.
**Figure 237** A 6-cell embryo with 30–40% fragmentation and unevenly sized blastomeres. Fragments are predominantly concentrated in one area. It was generated by ICSI but was not transferred.

**Figure 238** A 6-cell embryo with 30–40% concentrated fragmentation and a thick ZP. It was generated by ICSI but was not transferred.

**Figure 239** A 4-cell embryo with around 40% fragmentation which is scattered throughout the embryo. The blastomeres are unevenly sized. It was generated by ICSI but was not transferred.

**Figure 240** A 3-cell embryo with around 40% scattered fragmentation and unevenly sized blastomeres. It was generated by ICSI but was not transferred.

**Figures 241** (a–c) Three views of an embryo with >50% fragmentation. In one focal plane (a) three to four cells can be seen but in the other two focal planes only one to two cells can be seen (b and c). The embryo was generated by ICSI but was not transferred.
structures of blastomeric origin (Keltz et al., 2006) and evaluation of the degree of fragmentation is included in almost every embryo scoring system. The degree of fragmentation is most often expressed as the percentage of the total cytoplasmic volume. The relative degree of fragmentation is defined as mild (<10%, Figs 223–225), moderate (10–25%, Figs 226–235) and severe (>25%, Figs 236–242).

It is often difficult to make the distinction between a large anucleate fragment and a small (nucleated) cell. Johansson et al. (2003) showed that portions of cytoplasm that were <45 μm in diameter on Day 2 and <40 μm in diameter on Day 3 did not contain DNA, and the authors suggested a standardization of defining fragments as all structures below these sizes.

It has been shown that a high degree of fragmentation correlates negatively with implantation and pregnancy rates (Racowsky et al., 2000), while the presence of minor amounts of fragmentation has no negative or possibly even a positive impact (Alikani et al., 1999). Two distinctly different types of fragmentation have been documented by time-lapse analysis in human embryos: definitive fragmentation, characterized as stable persistent fragments clearly detached from blastomeres and pseudo-fragmentation, characterized by a transient appearance during, or shortly after, cell cleavage, but not detected during later development (Van Blerkom et al., 2001).

Increasing fragmentation also results in reduced blastocyst formation and can influence allocation of cells during differentiation (Hardy et al., 2003). The spatial distribution of the fragments in the perivitelline space (PVS) can be differentiated into two patterns, i.e. scattered (Figs 224, 226, 227, 230–236, 239 and 240) or concentrated (Figs 223, 225, 228, 229, 237 and 238). The scattered appearance was found to be correlated with an increased incidence of chromosomal abnormality (Magli et al., 2007). The higher the degree of fragmentation, the more difficult it is to differentiate between scattered and concentrated fragmentation (Figs 241 and 242). Fragmentation is considered to be an essential parameter to include in the evaluation of developing embryos, as embryos with very strong and persistent fragmentation are less likely to be viable.

C. Blastomere size: ‘stage specific’ versus ‘non-stage specific’

It has been shown that a high degree of regularity in the blastomere size in embryos on Day 2 is related to increased pregnancy outcome following assisted reproduction treatments (Giorgetti et al., 1995; Ziebe et al., 1997; Hardarson et al., 2001; Holte et al., 2007). Uneven cleavage, i.e. a cell cleaving into two unequal sized cells, may result in an uneven distribution of cytoplasmic molecules, e.g. proteins and mRNAs, and has been shown to be correlated with a higher incidence of multinucleation and aneuploidy (Hardarson et al., 2001; Magli et al., 2001).

The relative blastomere size in the embryo is dependent on both the cleavage stage and the regularity of each cleavage division (Diagrams 1 and 2). The blastomeres of 2-, 4- and 8-cell embryos should be equal (stage-specific embryos, Figs 243–245) rather than unequal in size (non-stage-specific embryos, Figs 246–252). In contrast, blastomeres of embryos with cell numbers other than 2, 4 and 8 should have different sizes as there is an asynchrony in the division of one or more blastomeres (Figs 253–256). A 3-cell embryo should preferably have one large and two small blastomeres (Fig. 253); a 5-cell embryo, three large and two smaller blastomeres (Fig. 254); a 6-cell embryo, two large and four smaller blastomeres (Fig. 255) and a 7-cell embryo, one large and six smaller blastomeres (Fig. 256). These embryos are thereby also considered to be stage specific. However, a 4-cell embryo with one or two blastomeres much larger than the others (Figs 248–251), a 3-cell embryo with all blastomeres even in size (Fig. 257), a 5-cell embryo with two large and three smaller blastomeres (Figs 258 and 259) or one small and four larger blastomeres (Fig. 260), a 6-cell embryo with all blastomeres even in size (Fig. 261) or extremely

**Figure 242** A Day 3 embryo with >50% fragmentation. It was generated by IVF but was not transferred.

**Diagram 1** A diagram illustrating the expected cell size of a cleavage stage embryo: a human 2-cell embryo should contain two equal blastomeres of the size of the 2-cell stage and are thereby stage specific. Unequal blastomeres at the 2-cell stage (>25% difference in the diameter size of the smallest cell, i.e. less than a 1:4 proportion) are not 2-cell stage specific. The same rule can be applied to 4- and 8-cell embryos. The numbers show proportions of diameter size.
Diagram 2 A diagram illustrating the concept of stage-specific versus non-stage-specific cleavage patterns. The dark green color indicates stage-specific cleavage stage embryos, whereas the light green color indicates non-stage-specific cleavage stage embryos.

Figure 243 A 2-cell embryo with evenly sized blastomeres and no fragmentation on Day 2. The blastomeres are stage-specific cell size. The embryo was transferred but did not result in a pregnancy.

Figure 244 A 4-cell embryo with evenly sized blastomeres and no fragmentation on Day 2. The blastomeres are stage-specific cell size. Notice the clover shape arrangement of the blastomeres. It was transferred and implanted.
Figure 245 An 8-cell embryo with evenly sized blastomeres and no fragmentation on Day 3. The blastomeres are stage-specific cell size. It was transferred and resulted in a pregnancy.

Figure 246 A 2-cell embryo with unevenly sized blastomeres on Day 2. The blastomeres are not stage-specific cell size.

Figure 247 A 2-cell embryo with unevenly sized blastomeres and up to 10% fragmentation on Day 2. The blastomeres are not stage-specific cell size.

Figure 248 A 4-cell embryo with unevenly sized blastomeres on Day 2, with the cell to the right being 25% smaller than the cell to the left. The blastomeres are therefore not stage-specific cell size. The embryo was transferred and implanted.

Figure 249 A 4-cell embryo with unevenly sized blastomeres on Day 2. One blastomere is indistinct in this view. The blastomeres are not stage-specific cell size.

Figure 250 A 4-cell embryo with unevenly sized and irregular blastomeres with two blastomeres being larger than the other two. The blastomeres are not stage-specific cell size. Note that the ZP of this embryo is elongated.
Figure 251 A 4-cell embryo with unevenly sized blastomeres. The blastomeres are not stage-specific cell size.

Figure 252 An 8-cell embryo with unevenly sized blastomeres. The blastomeres are not stage-specific cell size. The embryo was transferred but did not result in a pregnancy.

Figure 253 A 3-cell embryo with one large and two small blastomeres on Day 2. The blastomeres are stage-specific cell size. The embryo was transferred but failed to implant.

Figure 254 A 5-cell embryo with three large and two small blastomeres. The blastomeres are stage-specific cell size.

Figure 255 A 6-cell embryo with two large and four small blastomeres. The blastomeres are stage-specific cell size.

Figure 256 A 7-cell embryo with one large and six small blastomeres. The blastomeres are stage-specific cell size.
Figure 257 A 3-cell embryo with three blastomeres of the same size at 26 h after insemination. The blastomeres are not stage-specific cell size.

Figure 258 A 5-cell embryo with two large and three small blastomeres instead of three large and two small blastomeres; therefore, not stage-specific cell size.

Figure 259 A 5-cell embryo with two large and three small blastomeres instead of three large and two small blastomeres; therefore, not stage-specific cell size.

Figure 260 A 5-cell embryo with four large and one small blastomeres instead of three large and two small blastomeres; therefore, not stage-specific cell size.

Figure 261 A thawed 6-cell embryo with six blastomeres of the same size rather than two large and four smaller blastomeres; therefore, not stage-specific cell size.

Figure 262 A 6-cell embryo with two very large and four very small blastomeres. The extreme size difference between the large and small blastomeres makes this embryo not stage specific.
different in size (Fig. 262) and a 7-cell embryo with three large and four smaller blastomeres (Fig. 263) would not be considered to have normal blastomere sizes in relation to cell numbers and are therefore not considered to be stage specific (for further clarification see Diagrams 1 and 2).

D. Nucleation

The nucleation status is defined as the presence or absence of nuclei in the blastomeres of the cleavage stage embryo. Ideally, the nucleation status of each blastomere in the embryo should be evaluated as a single nucleus per blastomere (Figs 264–266), no nuclei visible or multinucleation (Figs 267–270).

The most studied nucleation status is multinucleation, which is defined as the presence of more than one nucleus in at least one blastomere of the embryo (Jackson et al., 1998; Van Royen et al., 2003). Multinucleation can be evaluated both in the early cleaved Day 1 (26–28 ± 1 h post-insemination), Day 2 (44 ± 1 h post-insemination) and Day 3 (68 ± 1 h post-insemination) cleavage stage embryos, although the assessment of a Day 3 embryo may be more complicated due to the smaller cell size and the larger number of cells (Van Royen et al., 2003). Embryo quality has been shown to correlate with multinucleation, and 4-cell embryos on Day 2 and 8-cell embryos on Day 3 show reduced multinucleation compared with the other cell stages observed on these days (Van Royen et al., 2003; Ziebe et al., 2003).

Multinucleation is predictive of a decreased implantation potential (Jackson et al., 1998; Pelinck et al., 1998; Van Royen et al., 2003; Moriwaki et al., 2004) and multinucleated embryos are associated with an increased level of chromosome abnormalities (Pickering et al., 1995; Hardarson et al., 2001; Agerholm et al., 2008) as well as an increased risk of spontaneous abortion (Scott et al., 2007). Multinucleation is more frequent in blastomeres originating from embryos with uneven cleavage compared with embryos with evenly cleaved blastomeres (Hardarson et al., 2001).

Multinucleation can also be divided into binucleation (two nuclei per cell, Figs 268 and 269) or multi/micronucleation (more than two nuclei per cell, Fig. 270). These appearances probably have different origins (Meriano et al., 2004). Multinucleated embryos are usually excluded from transfer. However, it has been shown that binucleated cells on Day 1 can cleave into chromosomally normal cells (Staessen and Van Steirteghem, 1998). On the other hand, severe multinucleation is probably not compatible with normal cell cleavage.

Multinucleation evaluation should be included in any embryo assessment protocol to select the highest quality embryo for transfer, and although these embryos do give rise to live births, they should be
excluded from selection for embryo transfer if an alternative embryo is available.

The absence or presence of a single nucleus per blastomere has been shown to be a predictor of embryo implantation potential (Moriwaki et al., 2004; Saldeen and Sundström, 2005). Visualization of four mononucleated blastomeres in a 4-cell embryo (Figs 265 and 266) predicted a higher implantation rate than in cases where zero (Fig. 266) to three mononucleated blastomeres (Fig. 268) were seen (Saldeen and Sundström, 2005). However, other studies have found that grading embryo nuclear score on Day 2 had no additive value for the prediction of implantation rate above that predicted by Day 3 embryo morphology (Bar-Yoseph et al., 2011).

E. Cytoplasmic anomalies

The cytoplasm of cleaving embryos is normally pale, and clear or finely granular in appearance (Hartshorne, 2000). Cytoplasmic anomalies, such as cytoplasmic granularity, cytoplasmic pitting and the presence of vacuoles, occur occasionally and can also be scored in the morphological assessment of Days 2 and 3 embryos. However, a possible predictive value of these features to embryo quality or implantation potential is unclear.

Cytoplasmic pitting (Figs 271 and 272) is characterized by the presence of numerous small pits with an approximate diameter of 1.5 µm on the surface of the cytoplasm (Biggers and Racowsky, 2002). Although cytoplasmic pitting in Day 3 embryos seems to be associated with improved blastocyst formation, the appearance of cytoplasmic granularity has no prognostic value to embryo quality (Rienzi et al., 2003) or to pregnancy (Desai et al., 2000). Other studies have shown that culture conditions may induce cytoplasmic pitting (Biggers and Racowsky, 2002; Ebner et al., 2005b) which in extreme cases may result in an increased risk of early loss of gestational sacs (Ebner et al., 2005b).

The cytoplasm of blastomeres may be excessively darkened with centralized granularity associated with a cortical halo, as cytoplasmic organelles retract toward the center of the blastomere (Fig. 273). It was suggested that these embryos have reduced implantation potential or are destined for degeneration (Veeck, 1999). Similarly, embryos with alternating areas of granularity and clear zones within the blastomeres are even more likely to degenerate (Fig. 274).

Cytoplasmic vacuolization is probably the most common cytoplasmic dysmorphism in human oocytes/embryos. Vacuoles vary in size and in number (Figs 275–280). They are membrane-bound cytoplasmic inclusions filled with fluid that are virtually identical with the perivitelline fluid (Van Blerkom, 1990). Whereas vacuoles have been well studied and described in human oocytes, very little is known about their incidence.
and role in developing embryos. Beside vacuoles visible at the time of oocyte collection and those created artificially by ICSI, vacuoles may also arise at the compaction stage (Ebner et al., 2005a). De novo

**Figure 271** An 8-cell embryo with equally sized blastomeres showing cytoplasmic pitting. Numerous small pits are present on the surface of the cytoplasm.

**Figure 272** An 8-cell embryo with equally sized blastomeres showing cytoplasmic pitting. Numerous small pits are homogeneously distributed in the cytoplasm. The 5 blastomeres in focus are arranged in one spatial plane.

**Figure 273** A 2-cell embryo with a clear halo in both blastomeres, characterized by centralized granularity associated with an absence of organelles in the peripheral cortex.

**Figure 274** A 4-cell embryo on Day 2 with an abnormal distribution of organelles leading to differential granular and smooth zones inside each cell.

**Figure 275** An 8-cell embryo with one blastomere showing a small vacuole (arrow).

**Figure 276** A 5-cell embryo with two small and three large blastomeres. There is a small vacuole in each of the two smaller blastomeres.
formation of vacuoles on Day 4 is related to developmental arrest with a detrimental effect on blastocyst formation (Ebner et al., 2005a). It is believed that the occurrence of a few, small vacuoles (Figs 275 and 276) is not of importance (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), but in cases of extensive vacuolization (Figs 277–280) it may be detrimental, mainly to spatial development, and the assessment should be added to the selection score.

**F. Spatial distribution of cells**

Human oocytes are polarized from their earliest stages of formation and consist of an animal and vegetal pole (Antczak and Van Blerkom, 1997, 1999; Edwards and Beard, 1997). This animal and vegetal gradient is distributed differently to specific 4-cell blastomeres via the combination of meridional and equatorial cleavage divisions (Gulyas, 1975). The first cleavage occurs meridionally and results in two nearly identical daughter blastomeres each inheriting similar polarities of animal and vegetal cytoplasm. In the second cleavage, one cell divides meridionally while the other cell divides equatorially which results in four cells with different polarity (Diagram 3). The two daughter cells resulting from the meridional cleavage have inherited full polarity, while the two daughter cells from the equatorial cleavage differ in polarity with one cell containing mostly animal cytoplasm and the other cell containing mostly vegetal cytoplasm (Gulyas, 1975; Edwards and Hansis, 2005). These cleavages lead to a typical pyramidal or tetrahedral arrangement of three blastomeres with animal cytoplasm associating with the polar body; and one blastomere, inheriting only vegetal cytoplasm, located distant to the polar body (Figs 281 and 282; Edwards and Hansis, 2005). Other ways of meridional or equatorial cleavage divisions may lead to a different distribution of animal and vegetal poles in the daughter cells and may result in non-tetrahedral or ‘clover’ shaped 4-cell stage embryos (Figs 283–285). The clover shape can be maintained in the following division (Fig. 286).

Another feature sometimes seen is ovoid embryos, originating from ovoid oocytes (Figs 287–290). In these embryos, the spatial distribution of blastomeres is necessarily abnormal.

In summary, the cleavage planes are thought to determine various aspects of later development. Elucidation of the fundamental aspects of the genetic regulation of the cleavage divisions may play an important role in understanding their impact on the developmental capacity and the implantation potential of an embryo in vitro (Edwards, 2005).
Diagram 3 Diagram showing the dividing planes of the second mitotic cleavage, where one cell cleaves equatorially and one cell cleaves meridionally, giving rise to four daughter cells with different polarity.

Figure 281 A 4-cell embryo with a typical pyramidal or tetrahedral structure. The embryo was generated by IVF and was transferred and implanted.

Figure 282 A 4-cell embryo with a typical pyramidal or tetrahedral structure. The embryo was generated by IVF and was transferred but failed to implant.

Figure 283 A 4-cell embryo with a non-tetrahedral or clover structure. It was generated by ICSI and was cryopreserved.

Figure 284 A 4-cell embryo generated by ICSI with a non-tetrahedral or clover structure. It was transferred but failed to implant.
Figure 285 A clover-shaped 4-cell embryo with an expanded ZP. The relative size of the blastomeres with respect to the ZP is smaller than usual in this embryo.

Figure 286 A double clover-shaped 8-cell embryo. The clover shape of the 4-cell embryo was maintained in the subsequent division.

Figure 287 An ovoid embryo showing four blastomeres arranged in a clover shape.

Figure 288 A 7-cell, ovoid embryo showing blastomeres predominantly arranged in the one spatial plane. The ZP is septate.

Figure 289 An ovoid embryo showing eight blastomeres arranged in one spatial plane.

Figure 290 An ovoid embryo showing seven blastomeres arranged in one spatial plane. The ZP has an irregular shape.
**G. Compaction**

The human embryo appears as an indistinguishable mass of cells on Day 4 of development, the morula. A good quality morula is composed of 16–32 blastomeres and all of the blastomeres should be included in the compaction process (Tao et al., 2002).

The increase in cell-to-cell adherence should begin at the 8-cell stage and then progress rapidly with time (Figs 291–293). The cell ad-

![Figure 291](image1) **Figure 291** An 8-cell embryo that shows no signs of compaction. Cells are evenly sized and barely touching.

![Figure 292](image2) **Figure 292** An 8-cell embryo showing signs of initial compaction. The cells are tightening their contact.

![Figure 293](image3) **Figure 293** An 8-cell embryo showing signs of moderate compaction. Individual blastomeres are becoming difficult to identify.

![Figure 294](image4) **Figure 294** A clover shaped 4-cell embryo showing signs of very early compaction. A single nucleus is clearly visible in each cell.

![Figure 295](image5) **Figure 295** A 7-cell embryo showing signs of early compaction. This embryo was transferred but failed to implant.

![Figure 296](image6) **Figure 296** An embryo with more than 12 blastomeres showing no signs of compaction. With this number of cells it is very unusual that the embryo has not yet initiated compaction.
Adhesion protein E-cadherin changes in distribution from the cytoplasm to the cell membrane. Cell junctions between cells, in particular tight junctions, begin to spread (Alikani, 2005). This process has been linked to activation of the embryonic genome (Desai et al., 2000).

**Figure 297** A morula of good quality. All blastomeres have been included in the compaction process and individual cells are no longer evident.

**Figure 298** A fair quality morula. Some cell boundaries are still visible and a few small cells (or fragments) are not completely incorporated into the compaction process.

**Figure 299** A fair quality morula. Cell boundaries are still visible and an occasional cell is not completely incorporated into the compaction process.

**Figure 300** A poor quality morula with several cells and fragments excluded from the main mass of compacted cells.

**Figure 301** A fair quality morula. Cell boundaries are still visible and an occasional cell is not completely incorporated into the compaction process.

**Figure 302** A poor quality morula with several cells and fragments excluded from the main mass of compacted cells.

**Figure 303** Embryo showing early cavitation with an initial blastocoele cavity beginning to appear.

**Figure 304** Embryo showing early cavitation. An initial blastocoele cavity is beginning to appear. The ZP is broken after blastomere biopsy.
therefore considered to be a good sign of the developmental capacity of the embryo. Culture media composition and other environmental conditions can also play a role in the kinetics of an early compaction event.

Compaction can be observed in embryos before the 8-cell stage (Figs 294 and 295), while embryos with more than 10 blastomeres that do not show signs of compaction are uncommon (Fig. 296). A study by Składas et al. (2006) showed that early compacted embryos had an increased implantation potential; however, this was only true for good quality embryos (<10% fragmentation).

The Istanbul consensus has established that for a Day 4 embryo to be considered of good quality, all cells must be included in the morula (Fig. 297), while those embryos in which some cells or fragments are excluded from the compaction process (Figs 298–300) have a decreased probability of implantation.

The outer cells of compacted embryos have probably lost their totipotency as they are bound to form the trophectoderm (Cauffman et al., 2009). The next phase of development is the beginning of cavitation that leads to the formation of the blastocyst (Figs 301 and 302).

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The cleavage stage embryo


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