Symposium: Innovative techniques in human embryo viability assessment

Oocyte assessment and embryo viability prediction: birefringence imaging

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Abstract

Embryo viability is a key element for success in assisted reproduction. Since the beginning of the era of assisted reproduction treatment, embryo viability was mostly considered to be a function of developmental progression during the preimplantation phase. In the last decade, several morphological criteria of oocytes and embryos were evaluated with regard to their potential for predicting embryo viability. The introduction of polarization light microscopy systems in assisted reproduction has enabled the detection of structures within oocytes that possess a natural birefringence. Birefringence imaging of the meiotic spindle and the zona pellucida in living animal and human oocytes represents a new approach in the assessment of oocyte and embryo viability. The technique was applied in several studies to select oocytes in order to improve treatment success. This review will summarize the present knowledge of birefringence imaging. The various applications in basic and clinical research as well as in clinical treatment will be presented, especially with regard to their effect on assisted reproduction.

Keywords: *embryo, human oocyte, polarization microscopy, spindle, viability, zona pellucida*

Polarization microscopy: from the past to assisted reproduction techniques

Polarized light was being used a century ago to investigate the structure and development of skeletal and cellular components in animal cells (Schmidt, 1924). Fifty years ago, polarized light microscopy was used to visualize the microtubule-dependent birefringence of the mitotic spindle (Swann and Mitchison, 1950). In 1953, Inoué showed for the first time the filamentous nature of the mitotic spindle in living cells using time-lapse movies recorded with the polarizing microscope (Inoué, 1953). With time, the sensitivity of polarized light microscopy was improved by the use of video-enhanced microscopy, which employs image-processing techniques for background subtraction (Allen *et al.*, 1981; Inoué, 1981). Inoué and colleagues demonstrated that microtubules are responsible for spindle birefringence and established a relationship between spindle retardance and microtubule density (Sato *et al.*, 1975). More recently, a new type of polarization microscope system was developed using liquid crystals to modulate the polarization state (Oldenbourg and Mei, 1995). With improvements in computer technology, this principle enabled not only real-time visualization of birefringent structures but also real-time calculation of polarization parameters on a pixel-by-pixel basis. The non-invasive nature of polarization microscopy also attracted researchers in the field of assisted reproduction. Soon it was discovered that polarized light microscopy identified a multilaminar structure within the zona pellucida of hamster oocytes (Keefe *et al.*, 1997). Furthermore, it was reported that in hamster oocytes the site of the first polar body was not a good predictor for the location of the metaphase II spindle (Silva *et al.*, 1999). These findings were verified in human oocytes (**Figure 1**) and stimulated further studies in human assisted reproduction on the value of spindle and zona imaging with regard to the evaluation of oocyte viability and a possible relation to embryo viability (De Santis *et al.*, 2005;

Chamayou *et al*., 2006; Shen *et al*., 2006; Madaschi *et al*., 2007; Rama Raju *et al*., 2007).

Spindle imaging

Initial studies on spindle imaging focused on the presence of the spindle, because a spindle could not be detected in all oocytes. However, spindle visualization requires a proper insight into polarization microscopy techniques and of the biochemical properties of spindle fibres. First of all, polarized light is adversely altered with respect to its polarization properties when passing through plastic dishes. Therefore it is mandatory to use glass-bottom dishes for spindle imaging. Furthermore, a characteristic feature of the spindle is the dynamic instability of the microtubules involved in spindle formation (Kirschner and Mitchinson, 1986). Microtubule turnover may be one reason for the sensitivity of the spindle to temperature and pH. Therefore, the most important aspect in regard to spindle visualization is the proper set-up of the microscopic system to control essential factors like temperature and pH, which may influence the visibility of the spindle.

The pH can easily be controlled by using appropriate buffered media in order to allow longer working time outside the incubator without the oocyte being affected by pH changes. The use of non-buffered culture media even in a droplet covered with mineral oil will result in changes of the pH within minutes (**Figure 2**).

Another most important factor is the temperature. Human spindles start to disintegrate at a temperature of 33°C (Wang *et al*., 2001a, 2002). Spindle reassembly depends on the minimal temperature in the culture system and the exposure time to that particular temperature (Pickering *et al*., 1990). Below a certain threshold of 25°C the spindle will never form again (Wang *et al*., 2001a) and it is tempting to speculate that partial spindle recovery may result in the formation of a deficient spindle, which may not separate chromosomes properly and give rise to aneuploid oocytes. Therefore, temperature-induced spindle disassembly may have a dramatic impact on fertilization and embryo development. Most IVF laboratories are nowadays equipped with heated working surfaces and microscope tables in order to allow temperature stability of dishes, media and oocytes. The appropriate temperature should be adjusted by measurements in the culture medium within the dish.

Therefore, the temperature and pH sensitivity of spindle imaging seems to be a good tool for quality control. In laboratories with reduced visualization of spindles in living human oocytes, the laboratory conditions should be carefully monitored.

Spindle presence and location

Numerous studies have investigated the effect of the presence and location of the spindle on fertilization rates and embryo viability. Oocytes presenting a spindle showed significantly higher fertilization rates in many studies (Wang *et al*., 2001b; Cooke *et al*., 2003; Rienzi *et al*., 2003, 2005; Cohen *et al*., 2004; Madaschi *et al*., 2007; Rama Raju *et al*., 2007) except in one investigation (Moon *et al*., 2003).

The location of the spindle in relation to the first polar body may also serve as a prognostic tool. Rienzi *et al*. (2003) reported that oocytes with a deviation of the spindle location from the position of the polar body of more than 90° showed lower fertilization rates (also see **Figure 1**). However, the discussion on the relevance of spindle deviation is still unclear, as the daily routine work in the laboratory indicates that this phenomenon could be due to manipulation and stress caused by oocyte denudation. Fang *et al*. (2007) showed that the use of polarized light microscopy in detecting spindle deviation in oocytes did not lead to higher fertilization rates compared with a control group without spindle imaging, although numbers were low in this paper. Therefore, further data are needed to demonstrate if spindle deviation is a sign of a physiological deficiency or an effect of manipulation and if it has an influence on embryo viability.

The studies on the presence of the meiotic spindle do not investigate directly embryo development but relate the presence of meiotic spindle to embryo development, which is a key aspect of embryo viability. In oocytes with a spindle, subsequent embryonic developmental competence on day 3 was superior in most studies (Wang *et al*., 2001b; Cooke *et al*., 2003; Moon *et al*., 2003; Madaschi *et al*., 2007) but not in others (Rienzi *et al*., 2003; Cohen *et al*., 2004). Two studies reported significantly higher blastocyst formation rates when a spindle could be visualized in the oocyte (Wang *et al*., 2001b; Rama Raju *et al*., 2007). The majority of authors do agree that the presence of the spindle in a mature oocyte can predict fertilization rates, cleavage rates and embryo quality. However, so far, the outcome in terms of pregnancy and implantation rates was only assessed in two studies. The results of these studies were contradictory, as one study reported a correlation between spindle presence and higher pregnancy and implantation rates (Madaschi *et al*., 2007) but another study did not (Chamayou *et al*., 2006).

A possible explanation for some of the contradictory observations in studies on spindle presence could be the dynamics of spindle formation during oocyte maturation. Recently the transition from metaphase I to metaphase II in human oocytes was assessed by video-cinematography (Montag *et al*., 2006). It could be demonstrated that the human spindle behaves highly dynamically during this important phase of meiosis and that the timing of spindle imaging seems to be of utmost clinical importance (**Figure 3**). Some oocytes classified as metaphase II, based on the presence of a first polar body by conventional light microscopy, were in early telophase I with spindle remnants linking the polar body and the ooplasm as seen by polarization microscopy. Video-cinematography revealed that, for a considerable time, the spindle disappears in telophase I and reforms usually within 40–60 min.

This phenomenon has been already mentioned by others (Rienzi *et al*., 2004; De Santis *et al*., 2005) and was partially outlined on the basis of unpublished observations of the course of the meiotic cycle by Eichenlaub-Ritter *et al*. (2002). Therefore, a single observation of the spindle appears to be inadequate to identify if an oocyte without a visible spindle is abnormal (absence of spindle) or has just entered late telophase I. In the later case the probability of spindle formation within 2 h is very high (Montag *et al*., 2006). Accordingly, quite a large number of oocytes showing no spindle during a first examination can display a spindle when observed after another 2–3 h in culture. In our laboratory, it has been found that oocytes that acquired a

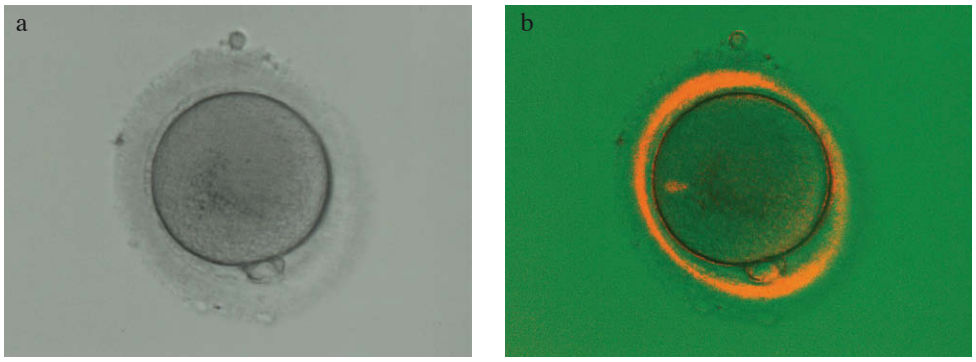

Figure 1. A human oocyte is shown by conventional light microscopy in (**a**) bright field and (**b**) as a composite image, combining bright field (green) and the polarized light image (red; OCTAX PolarAIDE™, OCTAX Microscience GmbH, Altdorf, Germany). The spindle as well as the inner layer of the zona pellucida show birefringence. It can be seen that the spindle is not located underneath the first polar body, but presents with a deviation of approximately 100°. Original magnification ×250.

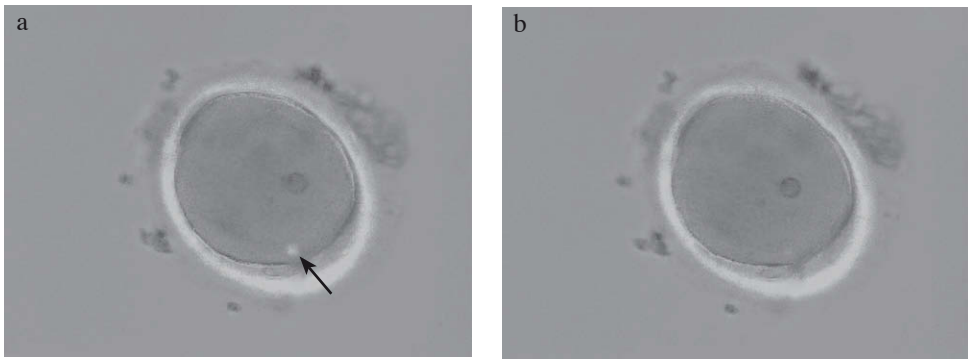

Figure 2. A human oocyte with a metaphase II spindle was exposed to normal atmosphere in a dish containing non-buffered medium droplets covered by mineral oil. (**a**) At the beginning of this time-lapse study, the spindle can be clearly seen (arrow; OCTAX PolarAIDE™). (**b**) Exposure to ambient air for 16 min causes a shift in pH, which results in almost complete disassembly of the spindle. Original magnification ×250.

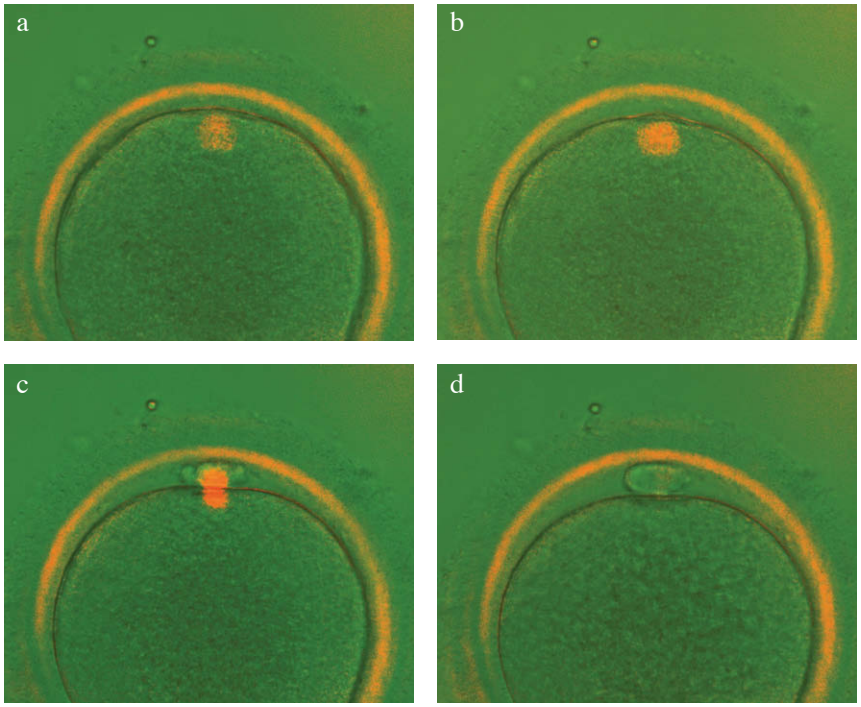

Figure 3. A time-lapse series shows the spindle appearance at different stages of first polar body extrusion (OCTAX PolarAIDE™). (**a**) In a typical metaphase I oocyte, the spindle is clearly visible. (**b**) During extrusion of the first polar body the intensity of the spindle increases. (**c**) After complete extrusion a spindle bridge remains in place between the first polar body and the oocyte. (**d**) Disassembly of the spindle bridge prior to formation of the metaphase II spindle (not shown). Original magnification ×400.

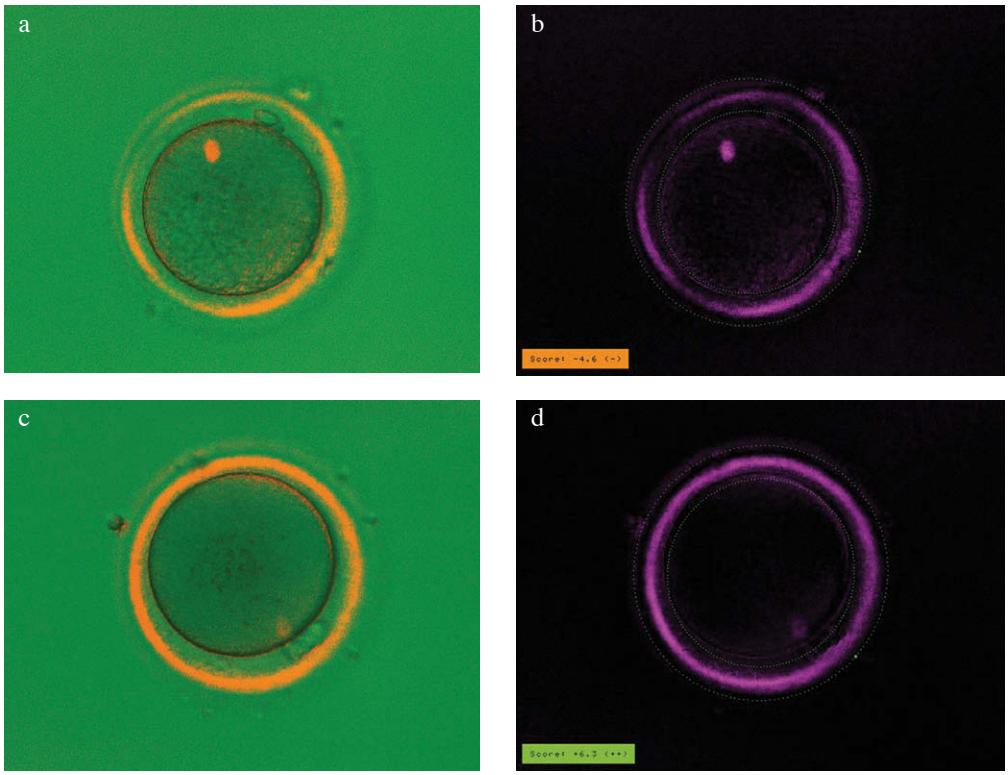

Figure 4. Two human metaphase II oocytes are shown (OCTAX PolarAIDE™). (**a**,**b**) Oocyte characterized by a very uneven birefringence of the inner zona layer distribution, with variations in intensity and thickness, characteristic for an oocyte with a lower implantation potential. The red/green composite (**a**) allows for subjective evaluation only, whereas an automatic scoring module (**b**) gives an objective and user-independent score. (**c**,**d**) Oocyte representative of an oocyte with a high implantation potential due to a uniform and very intense birefringence of the inner zona layer. The red/green composite (**c**) and the automatic scored image (**d**) are shown. Original magnification ×250.

spindle between the first observation and a second observation 2 h later showed the same fertilization rates as those having a spindle already at the time of the first observation (78.3% versus 78.4%). Only oocytes without a spindle during the first and second examination showed significantly lower fertilization rates (64.0%; $P < 0.05$).

Interestingly, if an oocyte in late anaphase or telophase I (showing a spindle bridge between the first polar body and the ooplasm; **Figure 3b**) is injected with a spermatozoon, that oocyte will be activated but at a non-physiological time point. This has been documented for oocytes in our routine intracytoplasmic sperm injection (ICSI) programme, where spindle observation was performed after ICSI. Some of these oocytes did not react at all and remained unfertilized, some oocytes formed a second polar body but did not progress further, and the majority of oocytes reacted but showed three pronuclei and a missing second polar body 16–20 h after ICSI (authors' unpublished data).

Spindle retardance

Spindle retardance is directly proportional to the density of the microtubules (Sato *et al.*, 1975) and even the retardance of individual microtubule fibres can be measured (Oldenbourg *et al.*, 1998). Some studies have investigated the value of spindle retardance measurements as a predictive factor in assisted reproduction. Two studies reported a trend for an inverse relation between spindle retardance and maternal age (De Santis *et al.*, 2005; Shen *et al.*, 2006), although the number of oocytes investigated in both studies was small. A positive correlation was also found between spindle retardance and pronuclear score after ICSI (Shen *et al.*, 2006), embryo development on day 3 (Trimarchi *et al.*, 2005) and blastocyst formation rate (Rama Raju *et al.*, 2007). Several publications showed that the influence of external factors on microtubules may affect retardation measurement (Sun *et al.*, 2004; Navarro *et al.*, 2005; Shen *et al.*, 2005a) and these authors suggest the use of spindle retardance for assessing such factors. However, as mentioned earlier, spindles are also dynamic structures, e.g. retardance measurements are orientation dependent (unpublished data, M Montag and H van der Ven) and an increase in spindle retardance can be observed during certain physiological events like oocyte activation (Liu *et al.*, 2000; Navarro *et al.*, 2005). Therefore, the use of spindle retardance measurements as a predictive tool is still under debate.

Spindle imaging of cryopreserved metaphase II oocytes

Polarized light microscopy has been applied in the cryopreservation of spindle positive metaphase II oocytes. Following cryopreservation of human oocytes by a slow-

freezing–rapid-thawing protocol (Rienzi *et al*., 2004), spindles were present in 37% of all oocytes after thawing; however, they all disappeared within the following washing steps and after further incubation a spindle finally reappeared within 3 h in 57% of all thawed oocytes. Another study on slow freezing of human metaphase II oocytes also confirmed that, following thawing, a reorganization of the spindles occurs within 3–5 h (Bianchi *et al*., 2005). However, these data are in contradiction with a study on thawed oocytes where polymerized microtubules were visualized by fluorescence confocal scanning microscopy, although the spindle was not organized in a normal way (Coticchio *et al*., 2006). Whether or not the presence of cryoprotectants in the cytoplasm of the oocyte may have altered the optical characteristics in a way that did not allow visualizing a still-existing spindle is unclear. This topic is extensively discussed in a recent review (De Santis *et al*., 2007). In contrast to the slow-freezing protocol, Chen and co-workers reported that, after vitrification and warming of mouse metaphase II oocytes, spindles were found in 50% of the warmed oocytes and in another 25% the spindle appeared within the following 2 h (Chen *et al*., 2004). However, in contrast to the slow, freezing–rapid, thawing protocol, spindles remained present and did not disappear.

Spindle imaging of in-vitro matured oocytes

The fact that spindle dynamics during the meiotic cell cycle has an effect on the success of ICSI suggests applying spindle imaging to in-vitro maturation. As mentioned above, the presence of the first polar body is not a good indicator for the completion of maturation. In addition, maturation of human oocytes from the germinal vesicle stage to metaphase II *in vitro* is not synchronous. Similar to in-vivo matured oocytes, the location of the spindle is positively correlated to the fertilization rates of in-vitro matured oocytes (Fang *et al*., 2007). Furthermore, the use of spindle imaging makes it possible to follow the maturation process as was recently reported by Hyun and co-workers (2007). Their data show that spindle imaging is a good tool to decide on the optimal timing for ICSI in in-vitro matured oocytes. Others found that the presence or absence of the spindle in in-vitro matured oocytes is dependent on external factors, the most important being the temperature (Sun *et al*., 2004), and on mitochondrial DNA and ATP content (Zeng *et al*., 2007). This underlines that the spindle in in-vitro matured oocytes is probably very sensitive and may react by structural changes. This may also explain the high frequency of chromosome misalignments in in-vitro matured oocytes, which can be partly detected by polarization light microscopy (Wang and Keefe, 2002).

Consequently, the use of polarized light microscopy is of great value for in-vitro maturation cycles, as it is possible to follow the maturation process of each individual oocyte by spindle imaging and to choose the right time for ICSI or cryopreservation.

Zona imaging

Besides the visualization of the spindle, zona imaging was proposed as another valuable predictive marker of oocyte/embryo quality. Assessment of the zona pellucida by conventional microscopy cannot be used as a predictive factor for the success of ICSI (Ten *et al*., 2007). However, polarization microscopy allows the distinction of three layers within the zona pellucida of human oocytes. The inner zona layer exhibits the highest amount of birefringence (Pelletier *et al*., 2004). In a retrospective study, Shen *et al*. (2005b) observed that zona birefringence of the inner zona layer was different in conception versus non-conception cycles. Embryos transferred in conception cycles were primarily derived from oocytes with high magnitude of light retardation caused by the inner layer of the zona pellucida. In a retrospective evaluation, Rama Raju *et al*. (2007) found a correlation between zona birefringence and the potential of an embryo to develop to the blastocyst stage.

In order to see if zona imaging is a reliable and robust technique, a prospective study has been conducted using subjective zona imaging (Montag *et al*., 2008). Using a polarization imaging system, unfertilized metaphase II oocytes were classified by a non-invasive single observation prior to ICSI treatment with special emphasis on the intensity and uniformity of the inner zona layer's retardance (**Figure 4**). Subjective zona evaluation (**Figure 4a**,**c**) was the only selection criterion and two fertilized oocytes were selected for further culture and transfer on day 3. Implantation, pregnancy and live birth rates were significantly higher between transfers of embryos derived from oocytes with a high and uniform inner zona birefringence, compared with embryos derived from other oocytes.

In contrast to spindle imaging, zona imaging is more suitable for an automatic sampling of measurement values allowing an objective and user-independent scoring of the corresponding oocyte. Two devices are at present available that allow for automatic zona imaging.

One such measuring device is based on the automatic detection of the birefringence of the inner zona layer. Once detected, a software module automatically starts to calculate and display in real time a zona-score based on the intensity and distribution of the birefringence at 180 measuring points (**Figure 4b,d**). The results of this approach (Montag *et al*., 2007) were comparable with the data from the subjective pre-study, which were mentioned above (Montag *et al*., 2008).

Another approach presented by Frattarelli *et al*. (2007) focused on the radial orientation of glycoproteins in the inner zona layer (**Figure 5**). This system investigates the angular deviation, which is greater if the inner zona layer is disrupted or less uniform. However, data from a clinical study using this device are not yet available.

The background for both systems is the paracrystalline network structure of the zona pellucida (Wassarman *et al*., 2004). A high and uniform birefringence of the inner zona layer appears to be primarily an indication of an optimal formation of this ordered structure during oocyte maturation. Therefore, zona imaging by polarization light microscopy is an indirect measure of oocyte quality. Initial data from an ongoing study show that oocytes with a high and uniform birefringence of the inner zona layer have a higher potential to develop to the blastocyst stage after ICSI compared with other oocytes (personal communication; T Ebner, Women's General Hospital, Linz, Austria). These preliminary data are in accordance with the

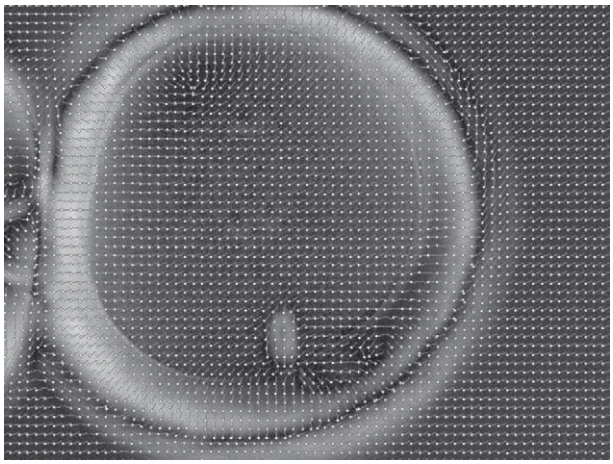

Figure 5. Picture of an oocyte generated by polarized light microscopy (Oosight™; CRI, Woburn, MA, USA). The grey lines indicate the direction of the molecules within the oocyte and the zona pellucida. (Courtesy of Peter Nagy and Tyl Taylor.)

report that embryo development on day 3, but not on day 2, was superior in embryos derived from oocytes with high and uniform birefringence of the inner zona layer (Montag *et al*., 2008). Therefore, zona imaging is a good prognostic factor of embryo viability, provided that the evaluation is performed at the oocyte stage because zona birefringence increases with prolonged culture (Kilani *et al*., 2006). At least at the blastocyst stage the expanding embryo induces pressure on the zona, which in turn causes an increase in zona density (Montag *et al*., 2000a,b) and may explain the high birefringence at that stage. The effect of zona pellucida architecture changes due to in-vitro culture conditions remains unclear.

The physiological basis of differences in zona imaging is still not fully understood. A high birefringence of the inner zona layer is primarily an indication of an optimal formation of the ordered structures of biomolecules in the zona pellucida during oocyte maturation. However, it may reflect the conditions to which an oocyte was exposed during follicular growth and maturation. An oocyte with a high and uniform zona birefringence may have had better conditions compared with an oocyte with an 'un-ordered' zona structure. It is tempting to speculate that a regular structural integrity of the zona pellucida may reflect an optimal cytoplasmic potential of an oocyte resulting in better developmental competence for embryonic growth and implantation. This complex deserves further attention and research.

Conclusions

Birefringence imaging of the spindle and of the inner layer of the zona pellucida is a new tool in assisted reproduction. Data from numerous studies indicate that birefringence imaging can improve the selection of oocytes that can develop into embryos with high viability. The technique is non-invasive and can easily be applied in an IVF programme. For certain applications, like zona imaging, a single observation can significantly improve oocyte selection and consecutive implantation and pregnancy rates. Birefringence imaging may prove to be a valuable add-on technique for sampling as much information as possible in order to identify the oocyte that has the highest chance to develop into an implantation-competent embryo. It is another step towards achieving a pregnancy following single embryo transfer. The spread of this technique will soon result in new applications and new insights in basic research as well as in clinical assisted reproduction.

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