Gamete competence assessment by polarizing optics in assisted reproduction

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BACKGROUND: The purpose of this study was first to give an overview of the historical development of polarization microscopy, second to describe the various applications of this technique in assisted reproduction techniques (ART) and third to discuss the potential benefit of polarization microscopy as a predictor for IVF success.

METHODS: The history of polarization microscopy was undertaken by performing a backward search in the scientific literature using Google and internet sites of several Societies for Microscopy and Cell Biology. Studies of polarization microscopy in ART were identified by using a systematic literature search in PubMed and Scopus.

RESULTS: A total of 62 articles were identified by the direct search and further relevant articles were found by screening the cited literature in these articles. The topics relevant for assisted reproduction were spindle and zona imaging in combination with IVF success, meiotic cell cycle progression, pharmaceutical studies and cryopreservation. A separate topic was the use of sperm birefringence in ART.

CONCLUSIONS: The majority of studies are observational studies and were not performed in a randomized manner and there is no direct comparison of techniques using other gamete selection markers. Despite this, most studies show that polarization microscopy may help us to further increase our knowledge on gametes and meiosis. Whether certain applications such as spindle or zona imaging may lead to an increase in IVF success is unclear at present. Publications on the use of polarization microscopy on sperm are still very limited.

Key words: polarization microscopy / spindle / zona / oocyte / sperm birefringence
Introduction

Historical overview of polarization microscopy in cell biology

In 1808, Etienne-Louis Malus discovered that, after the passage through a calcite crystal, light shows a certain distinct orientation, and in analogy to magnetic bodies he called this type of light as polarized light. Around 1811–1815, Sir David Brewster proposed a formula that allowed calculating the angle at which light must strike a substance for maximum polarization, which became known as Brewster’s angle or Brewster’s law (Brewster, 1818). This—together with the thoughts and inventions of other physicists such as Fresnel, Arago, Biot, Nicole and Faraday—resulted in Stokes parameters (1852), which describe mathematically the polarization properties of light (in: Wolinski, 2003). A glossary of relevant terms is provided (Table I). These early contributions still prove useful in numerous practical applications, from adjusting radiosignals to the development of fibre optics and lasers. At that time, however, polarized light was mainly used in microscopy and the first commercial polarized light microscope was produced in 1834 by Henry Fox Talbot using Nicole’s prism.

Undoubtedly, light microscopy was a catalyst for the newly forming field of cell biology in the 19th century, although the use of polarized light to study animal and plant cells was rare. In 1875 Engelmann reported that, in the frog, the sperm tail shows birefringence, whereas the sperm head does not (Engelmann, 1875), and this is probably the first published application of polarized light in male gametes. A first systematic study of living animal cells and tissues was presented in 1924 by Schmidt. He described the structure and development of skeletal and cellular components in animal cells (Schmidt, 1924) by the use of polarized light. And in 1937, he published a book, which contains images from sea urchin zygotes. These images are believed to represent the very first photographic record of the spindle and astral birefringence, at least in embryos.

Table I Glossary of terms.

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<th>Term</th>
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<tr>
<td>Birefringence</td>
<td>Also called double refraction. The decomposition of a ray of light into two rays when it passes through certain anisotropic materials, such as crystals of calcite, parallel bundles of microtubules, inner layer of the zona pellucida</td>
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<td>Retardance</td>
<td>A quantitative measure of the birefringence, the metric unit is nanometer</td>
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<td>Polarized light</td>
<td>Light that travels as a wave and within a certain field. Polarized light can be linear if the orientation of that field comprises only one plane (e.g. using a single band filter called linear polarizer), or circular if the orientation of the field does change and comprises all planes (e.g. using a circumpolar polarizer).</td>
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<td>Polarized light microscopy</td>
<td>A standard light microscope that uses two polarizing filters in the light beam: a polarizer and an analyser. The polarizer is positioned beneath the specimen stage and in case of a linear polarizer can be rotated through 360°. The analyser is placed behind the objectives and can be moved in and out of the light path as required.</td>
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<td>Linear polarization microscopy</td>
<td>Uses a set-up with linear polarizing filters where both the analyser and polarizer are positioned at right angles to each other. In this position they are said to be crossed and no light can pass through the system. However, the presence of an anisotropic object in the light beam will cause refraction. By fine adjustment of the angle between the polarizer and the analyser, an indirect image of the structures causing the refraction can be visualized provided that these structures are properly orientated in regard to the polarized light.</td>
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<td>Circular polarization microscopy</td>
<td>In the polarization microscope systems used in assisted reproduction, the linear polarizer is replaced with a circumpolar polarizer, which allows spreading the polarized light on any structure within an object. The conventional analyser is replaced by rotating liquid crystals, which allow detecting refraction at any plane. The image is visualized by digital image processing where the information sampled from all different planes is added into one image.</td>
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The pioneering work by Schmidt was later taken up by others (Swann and Mitchison, 1950; Inoué, 1953). Especially the work done by Shinya Inoué at the Marine Biological Laboratory at Woods Hole resulted in numerous publications dedicated to the study of spindle dynamics in living cells by polarization microscopy. In 1953, he showed for the first time the filamentous nature of the live mitotic spindle by using time-lapse movies recorded with the polarizing microscope (Inoué, 1953). His subsequent work resulted in landmark papers; he showed that spindle fibres shorten and elongate in an ordered manner and that a great deal of spindle dynamics is due to microtubule polymerization and de-polymerization (Inoué and Sato 1967). He and co-workers also demonstrated the relationship between spindle retardance and microtubule density for the first time (Sato et al., 1975). Then in 1972, LaFountain performed one of the first non-invasive studies of the dynamics of the meiosis I spindle and chromosome behaviour in a living animal spermatoocyte (LaFountain, 1972).

Another step forward was the use of video-enhanced microscopy, which employed image-processing techniques for background subtraction and greatly improved the sensitivity of polarized light microscopy (Allen et al., 1981; Inoué, 1981). This approach enabled the visualization of individual microtubules, which were reconstituted in solution (Allen and Allen, 1983). Although the value of computer technology in the field of microscopy was already recognized, it took another decade before polarization microscopy was fully computerized. A new type of polarization microscope system used liquid crystals to modulate the polarization state (Oldenbourg and Mei, 1995) and its realization was only possible due to employing circularly polarized light, development of algorithms and improvements in computer technology. The underlying principle enables not only real-time visualization of birefringent structures but also real-time calculation of polarization parameters on a pixel-by-pixel basis (Oldenbourg et al., 1998). More recently, this approach was further refined in a prototype instrument using the so-called modulation polarization microscopy, which allows visualization of cytoskeletal elements in living cells, including stress fibres and vesicular structures travelling along the cytoskeleton (Kuhn et al., 2001). Furthermore, qualitative and quantitative...
spindle analysis was pioneered by LaFountain and Oldenbourg (LaFountain and Oldenbourg 2004) in crayfish spermatocytes.

Real-time visualization of meiotic spindles, independent of orientation, allowed for the first time to investigate spindle properties in large cells such as oocytes. The technique was immediately adapted to study the relevance of the presence and location of the spindle in human metaphase-II oocytes during ICSI (Silva et al., 1999). The assessment of spindle dynamics in living human oocytes was a further step to investigate the timing of the meiotic cell cycle (Montag et al., 2006). In addition, the birefringence properties of the zona pellucida were studied (Pelletier et al., 2004). Several groups reported independently that zona birefringence assessment of the inner layer of human oocytes is of clinical relevance and may serve as a prognostic marker for oocyte quality. Recently, polarization microscopy was applied to select human spermatozoa for ICSI (Gianaroli et al., 2008).

This review summarizes the current role of polarization microscopy in assisted reproduction.

**Methods**

A systematic literature search was performed in PubMed and Scopus. The following key words were applied: polarization microscopy OR birefringence AND oocyte AND spindle (yield: 44 publications); polarization microscopy OR birefringence AND oocyte AND zona (yield: 13 publications); birefringence AND sperm (yield: 68 publications). All retrieved literature citations were included in the evaluation, except for the search on birefringence and sperm where all 68 publications were screened and 63 were excluded as they were not relevant to the topic of interest. In addition, the literature cited by the 62 remaining publications was searched for references that escaped the PubMed search, and this identified another 25 articles that were either directly or indirectly linked to the topics and were included. Literature related to the history of polarization microscopy was identified by performing a backward search in the scientific literature as well as using Google and internet sites dealing with the development of Microscopy and Cell Biology. Original articles were not available, but citations could be established through defined and trustworthy sources on optical engineering.

**Results and Discussion**

**Adaption of polarization microscopy in embryology**

The traditional approach of polarization microscopy using a polarization filter and a crossed compensator allow visualization of only those structures that were properly orientated in regard to the polarized light. Although this was widely used in embryological research—mainly on sea urchin oocytes, which like the mouse, exhibit a very large spindle and also possess a very clear cytoplasm—it was by no means acceptable in a therapeutic setting like in human IVF. Furthermore, in the relatively large three-dimensional human oocyte, the metaphase-II spindle is comparatively small and the standard polarization technique was not a convenient method to be applied to search for birefringent structures simply because it was time-intensive. All studies published until 1995 on the fate of the meiotic spindle, for example, during transient cooling used immunohistochemical methods that involved fixation of the oocytes (Pickering et al., 1990; Almeida and Bolton, 1995) and was not compatible with subsequent therapeutic treatments.

A novel polarization microscope, presented in 1995 (Oldenbourg and Mei, 1995), was the first to enter the field of applied embryology. The inventors overcame the problem of the unknown specimen orientation by replacing the single band polarizer with a circumpolar polarizer, thus allowing spreading the polarized light on any structure within an object. The traditional analyser was replaced by rotating liquid crystal retarders and the digital image finally revealed birefringent structures independent of the specimen orientation. The first publications on embryological specimen described the two elements within the mammalian oocyte that are birefringent: the zona pellucida (Keefe et al., 1997) and the meiotic spindle (Silva et al., 1999) (Fig. 1).

**Spindle imaging by polarization microscopy in oocytes**

**Spindle detection: presence and location of the spindle and its relevance for IVF/ICSI**

Following the introduction of ICSI, a discussion arose about a possible risk of damaging the spindle during the injection procedure and that such potential damage may cause severe problems for further development. When spindle imaging by polarization microscopy became available, it seemed as if this technique has been long awaited for in order to answer some of these specific questions (discussed in Eichenlaub-Ritter et al., 2002).

One of the first investigations using polarization microscopy is aimed at locating the spindle during ICSI (Silva et al., 1999). It was found that the first polar body, which is usually thought to be located in an ooplasmic region underneath the polar body, is not a reliable predictor of the location of the metaphase-II spindle and may sometimes show a deviation from the position of the first polar body (Fig. 1). This observation was supported by immunohistochemical studies of spindles involving anti-tubulin antibodies in in vitro and in vivo matured human oocytes (Hardarson et al., 2000). Later, Rienzi et al. (2003) studied the relevance of the location of the spindle in relation to the first polar body (PB) as a prognostic tool. These authors 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, but that spindle position had no effect on embryo development.

**Figure 1** Spindle and zona imaging using polarization microscopy.
A human metaphase-II oocyte is shown in normal bright field microscopy (A) and as an overlay picture of the bright field image in green and the polarization image in red (B). The structures with birefringence properties, like the metaphase-II spindle (located at 6 o’clock) and the inner layer of the zona pellucida can be clearly seen in the red/green image but not in bright field. Note that in this oocyte the spindle is not located underneath the polar body but shows a deviation of 90°.
Spindle dynamics to assess the maturation process of oocytes

In view of the heterogeneity of the published data on spindle imaging by polarizing microscopy, one should take into account that spindles were recently found to be absent during the maturation process (Eichenlaub-Ritter et al., 2002). Therefore the discussion on the presence or absence of spindle and its influence for assisted reproduction techniques (ART) is also related to timing and spindle dynamics, and most of the studies mentioned earlier did not take this into account.

During the final maturation of an oocyte, the spindle is a highly dynamic structure. On the basis of unpublished observations of the course of the meiotic cell cycle by Eichenlaub-Ritter et al. (2002), several authors have reported that for a considerable time the spindle disappears during the transition process in telophase I (Rienzi et al., 2004; De Santis et al., 2005). The course of the meiotic cell cycle was investigated in detail in metaphase-I oocytes derived from stimulated cycles and were matured to metaphase-II in vitro (Montag et al., 2006; Shen et al., 2008). The transition from metaphase-I to metaphase-II was documented by video cinematography in combination with polarization microscopy. Time lapse studies showed that following the extrusion of the first polar body, the spindle still formed a connective strand (presumably representing the remnant of the central spindle midbody) between the first polar body and the ooplasm for ~75–90 min prior to complete spindle disassembly (Figs 2 and 3). For a time period of another 40–60 min, no spindle was detectable in the oocytes, followed by formation of the metaphase-II meiotic spindle which typically appeared underneath the first polar body ~115–150 min after polar body extrusion from the oocyte. Therefore, some oocytes classified as metaphase-II based on the presence of a first polar body by conventional light microscopy can actually be in early telophase I with spindle remnants linking the polar body and the ooplasm (Fig. 2). It was reported, that injection of oocytes in telophase I will result in a high proportion of three-pronuclear oocytes (Montag et al., 2008).

As only microtubules of the midbody between the oocyte and polar body can be visualized non-invasively while the presumably unordered microtubules of the prometaphase-II spindle are not detected with the system, it is not possible to distinguish between an aberrant oocyte without spindle and one in late telophase I/early prometaphase-II, which may eventually mature to metaphase-II, and one in which the spindle becomes depolymerized due to ageing (Shen et al., 2008). Therefore, even a single observation with a spindle imaging system immediately prior to the ICSI procedure, as performed in most studies mentioned above on spindle presence, appears not to be adequate. Thus, it was shown that ~50% of oocytes showing no spindle during a first examination display a spindle when they are observed after an additional 2 h in culture (Montag et al., 2006). Interestingly, the

Figure 2 Metaphase-II oocyte or not? Based on the normal microscopic image one would judge the oocyte shown to be in metaphase-II, according to the presence of a first polar body. However, polarization microscopy reveals that this oocyte is still in the transition phase of the meiotic cell cycle and must be classified as telophase I. ICSI of such an oocyte may result in the formation of three pronuclei due to a disturbance of the meiotic cell cycle and the failed extrusion of the second polar body.

Spindle dynamics to assess the maturation process of oocytes

In contrast, Cooke et al. (2003) found in a similar study a positive effect of oocytes with PB-aligned spindles on embryo development. The whole concept of spindle imaging for ICSI was challenged by a study that showed that the highest fertilization rates and good quality embryos were derived from oocytes with spindles in a vicinity close to the injection zone (Woodward et al., 2008).

However, the discussion on the relevance of spindle deviation is still unclear, as the daily routine work in the laboratory indicates that this phenomenon is mainly due to manipulation and stress caused during oocyte denudation (Taylor et al., 2008). Consequently, the same stress could affect the complete cytoskeleton of an oocyte and be responsible for the lower fertilization rates and, to certain extent to, impaired embryo development. Although in the mouse it is known that postovulatory ageing causes disorganization and eventual detachment of the spindle from the cortex and its centripedal translocation (Eichenlaub-Ritter et al., 1986), there are no reliable data on possible time-dependent shifts in localization of the spindle after oocyte retrieval in the human.

Several studies reported on the importance of the presence of a spindle in human oocytes, although the results in these studies are sometimes contradictory. In some studies, oocytes with a spindle showed significantly higher fertilization rates (Wang et al., 2001a, b; Cohen et al., 2004; Rienzi et al., 2005; Shen et al., 2006; Rama Raju et al., 2007; Madaschi et al., 2008), whereas two studies (Moon et al. 2003; Fang et al., 2007) did not find a significant difference in fertilization rates. Embryonic developmental competence on Day 3 was superior in some studies (Wang et al., 2001b; Cooke et al., 2003; Moon et al., 2003; Shen et al., 2006; Rama Raju et al., 2007; Madaschi et al., 2008) but not in others (Rienzi et al., 2003; Cohen et al., 2004). Two studies reported significantly higher blastocyst formation rates from oocytes with a detectable spindle (Wang et al., 2001b; Rama Raju et al., 2007). In regard to pregnancy and implantation rates, only one study reported a positive correlation with spindle presence (Madaschi et al., 2008) but another study did not (Chamayou et al., 2006). However, it should be mentioned that the latter study had a low percentage of oocytes with birefringent spindles compared with other reports. This may reflect technical difficulties in identification of the spindle, which could contribute to the failure to detect a correlation.

A recent meta-analysis investigated the influence of the meiotic spindle visualization in human oocytes on the outcomes after ICSI (Petersen et al., 2009). The authors included ten published trials, although there was heterogeneity among some of the studies. The overall results showed statistically higher fertilization rates, cleavage rates and embryo developmental rates up to the blastocyst stage for oocytes with a detectable spindle. So far there is no prospective study on the absence of spindle and rate of spontaneous abortion.
fertilization rates in oocytes that had no spindle in the first examination but presented with a spindle 2 h later were not different from those of oocytes having a spindle right from the beginning. However, there are no data on embryo quality and implantation rate in cases where such oocytes were used for further culture and transfer.

There are only few reports linking clinical data and spindle imaging. Madaschi and colleagues recently reported (Madaschi et al., 2009) that spindle visualization was negatively influenced by the total FSH dose given during stimulation but not by age or follicles present at hCG administration and also not by the stimulation protocol used (agonist versus antagonist).

Another clinical study revealed that the time elapsed from HCG administration could have an influence on spindle imaging and hence on oocyte maturity (Cohen et al., 2004). In practice one may always find differences in the maturation progress between different oocytes among the cohort of all follicles and respective oocytes from one patient. It is likely that at time intervals closer to HCG administration, more oocytes are still in the final transition process to metaphase-II. Therefore proper timing of ICSI could be crucial especially for oocytes showing no spindle at a certain time point, where it may be indicated to wait for some time until that oocyte is in a state that supports further development. Delayed maturation may also be an indicator of an intrinsic disturbance in the patient (lifestyle related, mutation, handling, or exposures, as for instance demonstrated in the mouse model (Shen et al., 2008) or an inappropriate stimulation protocol. Definitely this needs to be addressed in a proper study.

The possibility to follow the maturation process by polarization microscopy attracted researchers working on in vitro maturation of oocytes derived from small follicles at the germinal vesicle (GV) stage (Fang et al., 2007; Hyun et al., 2007; Braga et al., 2008). Hyun et al. (2007) applied spindle imaging to in vitro maturation and showed that it is a good tool to decide on the optimal timing for ICSI in in vitro matured oocytes. The underlying reason being that oocytes derived from GV stage oocytes are not in synchrony during the in vitro maturation process and thus an optimized treatment by ICSI might benefit from the knowledge about the exact timing of the cell within the meiotic cell cycle. However, compared with the timing reported for the progression through the meiotic cycle after polar body extrusion in oocytes from stimulated cycles (Montag et al., 2006), in vitro matured oocytes do show a different time course and develop faster (Hyun et al., 2007). Following the time course of anaphase I progression and first polar body formation in

**Figure 3** Spindle dynamics during the meiotic cell cycle. The course of the meiotic cell cycle in a human oocyte is shown from the beginning of the extrusion of the first polar body up to the dissolution of the spindle. The images represent a total time scale of 160 min (A–I) and the time difference from one image to the following is 20 min. Note the increase in spindle birefringence during the phase of polar body extrusion (A–C) and the presence of a spindle bridge, which connects the first polar body to the ooplasm for another 60–80 min before the spindle is completely dissolved (D–I). Pictures were taken out of a video sequence which is available online as Supplementary data.
The basic structures of spindles are the microtubules and these are in a dynamic state of polymerization depolymerization, especially in oocyte spindles (Kirschner and Mitchison, 1986; Schuh and Ellenberg, 2007) as well as dynamic instability in response to parameters such as pH and temperature. These biochemical properties of spindle fibres make them an ideal tool to assess the proper setting of these important laboratory parameters.

It was shown that human spindles start to disintegrate at a temperature of 33°C (Wang et al., 2001c; 2002). Once a spindle has disintegrated, its reassembly depends on the minimal temperature and how long it was exposed to that particular temperature (Pickering et al., 1990). Whether or not improper spindle re-assembly may also lead to deficiencies in spindle function and enhance chromosome segregation is unknown. If the temperature dropped below 25°C a successful spindle-reformation is very unlikely (Wang et al., 2001c). Therefore one may use polarization microscopy to monitor effects of cooling and heating of oocytes during in vitro manipulation.

Like temperature, pH is another source for spindle disassembly. It was shown that the exposure of oocytes in un-buffered culture medium to ambient air does cause spindle disassembly within 8–10 min (Montag and van der Ven, 2008). Whether spindle reformation after a pH shift is also dependent on a certain threshold is not known at present. Altogether these findings imply that if a laboratory consistently fails in visualizing a spindle in all or a large fraction of living human oocytes, it may be a good idea to carefully monitor the laboratory conditions and especially to check the actual state and fluctuations of temperature and pH in all equipment and media involved.

Zona imaging by polarization microscopy

When polarization microscopy was applied to mammalian oocytes, one of the first publications did not focus on the most prominent birefringent structure, namely the spindle, but reported on the birefringence properties of the zona pellucida (Keefe et al., 1997).

Zona birefringence and zona architecture

Under polarized light, the zona pellucida of hamster oocytes shows a multi-layer architecture where three layers within the zona pellucida can be distinguished by their birefringent properties (Keefe et al., 1997). The inner zona layer exhibits the highest amount of birefringence, followed by a thin middle layer that is completely devoid of birefringence. The outer layer usually shows a faint birefringence.
The same characteristic three-layer pattern was also found in the zona pellucida of human oocytes (Pelletier et al., 2004).

However, it is still an unresolved question of how the multi-laminar structure of the zona pellucida as revealed by polarization microscopy (Keefe et al., 1997; Pelletier et al., 2004; Shen et al., 2005b) does actually form on the basis of the known components of the zona pellucida, namely the zona proteins (ZP) and the embedded glycoproteins and polysaccharides. The paracrystalline network structure of the zona pellucida (Wassarman et al., 2004) is formed during the follicular maturation mainly by the oocyte (Nikas et al., 1994) and, to a lower extent, by the granulosa cells (Sinowatz et al., 2001; Bogner et al., 2004; Gook et al., 2004). Therefore it appears that the extent of birefringence of the inner zona layer is primarily an indication of the degree of order of the contributing structures within the zona during oocyte growth and maturation, and since most ZPs are produced during the oocyte growth phase, may reflect follicular integrity and oocyte health.

Zona birefringence in relation to outcome of ART
The birefringence of the inner layer of the zona pellucida was found to show variations in intensity among different oocytes. Based on this, (Shen et al., 2005a, b) carried out a retrospective study and observed that differences in zona birefringence intensity of the inner zona layer could be linked to conception versus non-conception cycles. Another retrospective study found a correlation between zona birefringence intensity and uniformity of the inner zona layer's retardance was assessed in unfertilized metaphase-II oocytes by a non-invasive single observation prior to ICSI treatment (Montag et al., 2008). This served as the main distinguishing parameter and allowed classification of oocytes by high or low birefringence of the zona pellucida. Based on zona birefringence as the only selection criterion, two fertilized oocytes, preferably derived from oocytes with high birefringence, were selected for further culture and transfer. The resulting implantation, pregnancy and live birth rates were significantly different between cycles where the transferred embryos were derived from oocytes with high versus low birefringence. If only high birefringent cells were transferred, the implantation and pregnancy rates were double that of transfers of only low birefringent cells. A unique result of this study was that embryo development was superior in embryos derived from high birefringent oocytes, which showed a better embryo development on Day 3 but not on Day 2 compared with embryos from low birefringent oocytes. This initial study was solely based on the subjective evaluation of a single investigator and as such not suitable for generalization. However, the zona pellucida is an optimal target for an automatic sampling of measurement values, and two different approaches for automatic zona imaging have been presented (Frattarelli et al., 2007; Montag and van der Ven, 2008).

One approach is to detect the radial orientation of glycoproteins in the inner zona layer (Frattarelli et al., 2007). If the inner zona layer is disrupted or less uniform the angular deviation of the radial orientated structures is greater and hence a characteristic for a presumably suboptimal oocyte. Data from prospective clinical studies evaluating this theory are not yet available.

Another measuring device is based on the automatic detection of the inner zona layer due to its birefringence (Montag and van der Ven, 2008) (Fig. 4). 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. With this approach, an objective and user-independent score of the corresponding oocyte can be obtained within a short observation time. In a prospective study, the results of this automatic zona imaging were comparable to the data from the subjective study mentioned previously (Montag et al., 2008).

In another prospective study, Ebner et al. (2010) further explored the relationship between the birefringence of the inner zona layer and preimplantation development. They used automatic zona imaging at the oocyte stage prior to ICSI and subsequent culture up to the blastocyst stage. When the automatic detection of the birefringence of the inner zona layer in the oocytes failed, the corresponding embryos showed significantly lower compaction rates, blastocyst

**Figure 4** Zona imaging using automatic zona detection and analysis. The inner layer of the zona pellucida can be visualized by polarizing microscopy. An automatic zona imaging system (Octax PolarAide, Octax, Bruckberg, Germany) allows for the generation of a score which is mainly based on the intensity and uniformity of the zona birefringence. The oocytes displayed show either a very uniform and strong birefringence (A, score high = green), a less uniformity and birefringence (B, yellow = intermediate) and a very low and extremely irregular birefringence (C, red = score low) of the inner zona layer. A video sequence is available online (Supplementary data), which shows the detection of the zona pellucida birefringence by an automatic module.
formations and were significantly less involved in the initiation of a pregnancy. Overall they found that the automatic zona score was a strong predictor of blastocyst formation but not for embryo quality and pregnancy.

Another recent study reported a positive correlation between zona pellucida birefringence score and implantation and pregnancy rates (Madaschi et al., 2009). These authors were the first to show that the miscarriage rate was higher in embryo transfer cycles where the transferred embryos were exclusively derived from oocytes with a low zona birefringence score.

Interestingly, assessment of the zona pellucida by conventional microscopy and without the information of polarization microscopy cannot be used as a predictive factor for the success of ICSI (Ten et al., 2007). However, it is difficult to explain what makes the prog nostic value of zona birefringence imaging in ART and why a high and uniform birefringence of the inner zona layer is associated with better success rates. One possible explanation is that regular structural integrity of the zona pellucida may reflect an optimal cytoplasmic potential of an oocyte and its various cellular and molecular structures; oocytes with high birefringence have the best developmental competence for embryonic growth and implantation. Preliminary data indicate that oocyte competence assessed by polarization microscopy correlates with different expression profiles of certain candidate genes in subpopulations of the cumulus-oophorus complex (Van der Ven et al., 2009; Assidi et al., 2010). Whether or not other exogenous factors (Herrler and Beier, 2000) have an influence on oocyte competence is still an open question (for discussion see Shen et al., 2005b).

Recent data suggest that the zona birefringence does change during maturation (De Almeida Ferreira Braga et al., 2010) and that immature oocytes possess in general a higher overall birefringence compared with mature ones. This observation was already noted by Ebner et al. (2010) and these authors further reported that zona scores are different in oocytes derived from antagonist protocols or from long protocols. Taken together, these data indicate that during the maturation of the oocyte, changes occur at the level of the zona pellucida, which may be influenced by the stimulation and which can be traced by birefringence measurements.

Summarizing the available data on the use of zona imaging does suggest that the technique might have a benefit for IVF success, although the benefit is not uniform in all publications on the topic (Table II). A common finding is that a uniform and strong zona birefringence characterizes oocytes that might have a better potential to develop up to the blastocyst stage than others. In view of extended culture to the blastocyst stage, which is performed in numerous laboratories worldwide nowadays, one may critically ask whether zona imaging has its justification or is simply not necessary compared with blastocyst culture. However, this argument needs to be applied to nearly every prognostic parameter such as pronuclear scoring or early cleavage. At present we do not have studies that use single embryo transfer of individual oocytes traced from the beginning up to blastocyst stage with all the prognostic parameters being recorded, including polarization microscopy. Therefore, it is hardly possible to give the potential benefit of an individual prognostic parameter in comparison with other techniques.

### Spindle and zona imaging during cryopreservation of oocytes

#### Spindle imaging during cryopreservation of oocytes

As mentioned earlier, changes in temperature affects the integrity of a metaphase-II spindle. Consequently, cryopreservation and/or vitrification of oocytes are interventions, which interferes with spindle integrity during freezing as well as during thawing/warming.

In view of this, polarized light microscopy has been used to study the effect of cryopreservation on metaphase-II oocytes. In the very first study (Rienzi et al., 2004), spindle positive metaphase-II oocytes were subjected to a standard slow freezing/rapid thawing protocol. During the thawing process, 37% of all oocytes presented with a spindle, however, these oocytes completely disappeared within the subsequent washing steps and after incubation for another 3 h, spindles finally reappeared in 57% of all thawed oocytes. These data were confirmed by another study on slow freezing of human metaphase-II oocytes, where spindle re-organization occurred within 3–5 h after thawing (Bianchi et al., 2005).

In the past few years, numerous publications reported on the optimization of the freezing solutions used for slow freezing and the methodology has improved, especially by raising the sucrose content in the freeze/thaw solutions (Borini et al., 2006; Coticchio et al., 2006; De Santis et al., 2007a). A recent study showed that the use of zona imaging could be considered as a significant predictor of pregnancy and a valuable parameter for selecting high-quality oocytes prior to ICSI.

#### Table II Overview of the effectiveness of zona imaging in assisted reproduction.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Study type</th>
<th>Fertilization method</th>
<th>Outcome observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shen et al., 2005b</td>
<td>Retrospective</td>
<td>ICSI</td>
<td>High zona birefringence correlated with conception cycles</td>
</tr>
<tr>
<td>Rama Raju et al., 2007</td>
<td>Retrospective</td>
<td>ICSI</td>
<td>High zona birefringence was associated with improved embryo development and blastocyst formation rate</td>
</tr>
<tr>
<td>Montag et al., 2008</td>
<td>Prospective</td>
<td>ICSI</td>
<td>High zona birefringence correlated with embryo development on Day 3 and improved implantation and pregnancy rates</td>
</tr>
<tr>
<td>Ebner et al., 2010</td>
<td>Prospective</td>
<td>ICSI</td>
<td>Zona imaging correlated with blastocyst formation rate; zona birefringence influenced by stimulation protocol</td>
</tr>
<tr>
<td>Madaschi et al., 2009</td>
<td>Prospective</td>
<td>ICSI</td>
<td>High zona birefringence correlated with improved implantation and pregnancy rates and reduced miscarriage rate</td>
</tr>
<tr>
<td>Cheng et al., 2010</td>
<td>Retrospective</td>
<td>IVF</td>
<td>No correlation of zona birefringence after IVF with embryo development or clinical results</td>
</tr>
</tbody>
</table>
of a very efficient protocol for slow freezing of metaphase-II oocytes does support spindle reformation in more than 80% of the frozen thawed oocytes within 1 h after thawing (Sereni et al., 2009).

An alternate technique for cryopreservation of immature and mature human oocytes is vitrification, which has steered much interest due to the success that had been reported by various authors (Kuleshova et al., 1999; Yoon et al., 2000; Isachenko et al., 2005; Cobo et al., 2008a). Polarization microscopy studies showed 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 two hours (Chen et al., 2004). Larman et al. (2007) reported that during the vitrification process, metaphase-II oocytes spindles remained present and did not disappear. However, a temperature drop below 37°C resulted in spindle depolymerization, whereas maintaining the temperature at a physiological point left the spindle intact and unaffected. Although these authors had great success with vitrification protocols, the meiotic spindle was not preserved in their hands when they used a slow freezing protocol instead.

Based on the published data, it is difficult to reach any conclusion regarding the advantage of one or the other cryopreservation technique, especially considering the differences in vitrification or slow freezing techniques from laboratory to laboratory (Borini et al., 2006; Bianchi et al., 2007; De Santis et al., 2007b). Besides the variations in temperature that are used during the equilibration steps in the freezing or thawing solutions in both methods, there are numerous variations in terms of the composition of these solutions. One should also bear in mind that the visualization of a spindle with polarization microscopy during or shortly after freezing and thawing may be compromised due to unknown factors (Coticchio et al., 2006, 2010). This was extensively discussed especially in view of the role of the cryoprotectants, whose presence in the cytoplasm of the oocyte may have altered the optical characteristics in a way that—under certain circumstances—does not allow visualizing a still-existing spindle (De Santis et al., 2007b). Interestingly, a recent publication showed that in metaphase-II oocytes, the reformation of the spindle was comparable irrespective of the cryopreservation protocol used (vitrification/slow freezing; Cobo et al., 2008b). However, this work was also done by immunocytochemical visualization of the spindle in fixed oocytes rather than by polarization microscopy in live oocytes.

In view of time needed for freeze/thawing procedures, it will be important to compare spindle presence for each method and in individual centres in order to determine the optimal time for ICSI.

**Zona imaging during cryopreservation of oocytes**

The prognostic value of zona imaging has mainly been examined in fresh metaphase-II oocytes. Thus far, only a preliminary study on the use of polarization microscopy for zona imaging of metaphase-II oocytes during freezing and thawing has been presented (Montag et al., 2009).

This study shows that the initial zona score of oocytes assessed prior to freezing did change during the freezing and thawing process in a slow cryopreservation protocol. Three to 4 h after thawing, the score was higher compared with the initial value but with time did return to the initial score. Therefore, exposure of oocytes to a cryoprotectant has an impact on the zona and in particular on the birefringence of the inner zona layer. The exact biophysical nature of these changes is still unknown; however, a change in the zona score is a sign for a re-arrangement of the filamentous structures within the zona, and a rising score indicates a shift towards a state with a higher order. Probably this change does reflect a hardening of the zona, a phenomenon that is frequently discussed in regard to cryopreservation of oocytes and embryos. Based on an electron microscopic analysis, Nottola and colleagues (2007) reported that 3–4 h after thawing, human metaphase-II oocytes showed signs of zona hardening, which is in accordance with the observations by polarization microscopy. However, the available data suggest that the zona may undergo further changes and that the state present 3–4 h after thawing is not premanent (Montag et al., 2009).

As the cryopreservation procedure also impacts the zona structure, the prognostic benefit of zona imaging can only be used whether the imaging is done at a proper time point. At present it is unclear whether the changes of the zona structure observed during freezing/thawing can be used as another indicator for the quality of the oocyte or for the quality of a freezing protocol. However, this question may be answered soon based on the findings of a recent study that established an algorithm based on the value of retardance and zona thickness allowing for quantitative assessment of zona hardness (Iwayama et al., 2010).

**The use of polarization microscopy for selecting spermatozoa**

More than a century ago, Engelmann described the birefringence properties of the sperm tail from the frog, but he saw no birefringence in the sperm head (Engelmann, 1875). The studies by Schmidt (1924, 1937) and Pattri (1932) were the first to show a negative birefringence of the sperm head, which could be attributed to the orientation of the chromatin in the sperm head. Since then, the field of microscopy has evolved and the instrumentation used today is much more sensitive compared with the microscopes that were available in earlier times. In addition, the instrumentation used for detecting birefringence in the sperm head differs in its working mode from the instrumentation that had been described earlier in this manuscript for spindle and zona imaging. The major differences being that (i) the objectives used for sperm work need a higher magnification and a higher numerical aperture and (ii) the detection module for the analysis of the polarized light was adapted for the lower magnitude of sperm head birefringence (Gianaroli et al., 2010). Inoué was the first to show that polarization microscopy can be used to study the acrosome reaction in spermatozoa (1981). The natural birefringence in the sperm head can be attributed to the state of acrosome reaction, where non-reacted spermatozoa do possess birefringence over the entire sperm head compartment, whereas reacted sperm only show a birefringence in the postacrosomal region (Baccetti, 2004).

In two recent publications, the potential benefit of sperm birefringence as a selection criterion was assessed (Gianaroli et al., 2008; 2010). The authors concluded that the technique was suitable for the treatment of severe oligoasthenoteratozoospermia sperm samples and for testicular spermatozoa, as in both situations the technique did help in identifying the most competent spermatozoa (Gianaroli et al., 2008). In a prospective randomized study the injection of acrosome-reacted versus non-acrosome reacted spermatozoa, which were selected based on the birefringent analysis, had no
effect on the fertilization rate, but implantation, pregnancy and delivery rates were significantly higher with reacted spermatozoa (Gianaroli et al., 2010). However, the concept of a potential benefit for ICSI outcomes while using acrosome-reacted spermatozoa is still under debate (Mansour et al., 2008) and further prospective studies from other groups will hopefully clarify this in the near future.

Preliminary studies were already undertaken to evaluate the relevance of other sperm parameters with birefringence properties. According to these data, sperm birefringence is more likely to be present and to characterize spermatozoa with normal morphology (Boudjema et al., 2009). It was also reported that selection of sperm with birefringence in the sperm head does increase the likelihood of DNA strand integrity, as birefringent sperm were found to possess less DNA fragmentation compared with non-birefringent sperm (Crippa et al., 2009). In summary, it is important to note that the use of polarization microscopy for selecting spermatozoa must be considered as an emerging technique. Further studies need to be performed in order to be able to judge the full potential of this selection marker, especially in comparison with other existing techniques.

Conclusions

Assessing the competence of gametes in assisted reproduction attracts more and more interest.

For over a decade, oocyte competence was judged by the appearance of certain morphological markers such as the presence of vacuoles, granules, refractive bodies and shape of the polar bodies (De Sutter et al., 1996; Serhal et al., 1997; Balaban et al., 1998; Loutradis et al., 1999; Ebner et al., 2000, 2003, 2006). For spermatozoa, the possible benefit of morphological examination has also attracted much interest (Bartoov et al., 2003; Berkovitz et al., 2005, 2006; Antinori et al., 2008) although the extent of its relevance is discussed (Svlander et al., 1996; Peer et al., 2007; French et al., 2010; Michelmann et al., 2009). Nowadays polarization microscope systems are available, which can be easily implemented in the routine daily laboratory work. Therefore, the technique can be considered to be an important new tool in characterizing the developmental potential of oocytes.

Polarization microscopy allows for the visualization of structures and details that are otherwise inaccessible. The information that can be obtained is new in the sense that we can map the state of a cell within a dynamic process, like the stage of maturation of an oocyte within the meiotic cell cycle. And we can learn something about the history of an oocyte, for example by looking at the birefringence pattern of the zona pellucida, which may tell us something about the maturation and the follicular environment of the corresponding oocyte prior to puncture. In the future we should aim to better understand the underlying history that is responsible for the variations observed, for example in spindle and zona retardance. According to the data reported so far in observational studies, spindle presence and zona birefringence does help in selecting gametes with a higher chance to achieve fertilization and an embryo that may develop to blastocyst. At present there is no general consensus that investigating the potential of gametes by polarization microscopy does contribute to viable implantations and pregnancies. This still needs to be verified by controlled studies. Comparable studies on the benefit of polarization microscopy in the andrological field are on their way.

Supplementary data

Supplementary data are available at http://humupd.oxfordjournals.org/.

Authors’ roles

All authors contributed to the manuscript: MM and MK wrote the first draft, KvD and HvD corrected the first draft and all authors were involved in the first and second revision of the manuscript.

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