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# 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 Strokes 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.

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 spermatocyte (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

**Table I Glossary of terms.**

| | |
|---|---|
| Birefingence | 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 |
| Retardance | A quantitative measure of the *birefringence*, the metric unit is nanometer |
| Polarized light | 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). |
| Polarized light microscopy | 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. |
| Linear polarization microscopy | 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. |
| Circular polarization microscopy | 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. |

spindle analysis was pioneered by LaFountain and Oldenbourg (LaFountain and Oldenbourg 2004) in crane fly 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 trusty 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-intense. 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

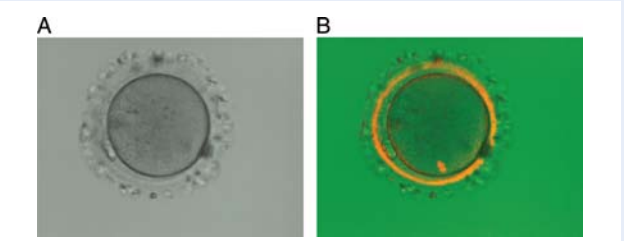


**Figure 1** Spindle and zona imaging using polarization microscopy. A human metaphase-II oocyte is shown in normal bright field microscopy (**A**) and as an overlay picture of the bright filed image in green and the polarization image in red (**B**). The structures with birefringence properties, like 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°.

development. 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.

### *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 oocytes 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

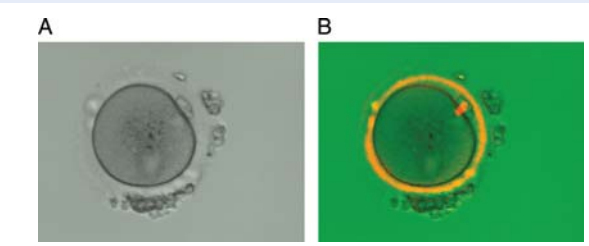


**Figure 2** Metaphase-II oocyte or not? Based on the normal microscopic image one would judge the oocyte shown to be in metaphase-II, according to the presence of a first polar body. However, polarization microscopy reveals that this oocyte is still in the transition phase of the meiotic cell cycle 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.

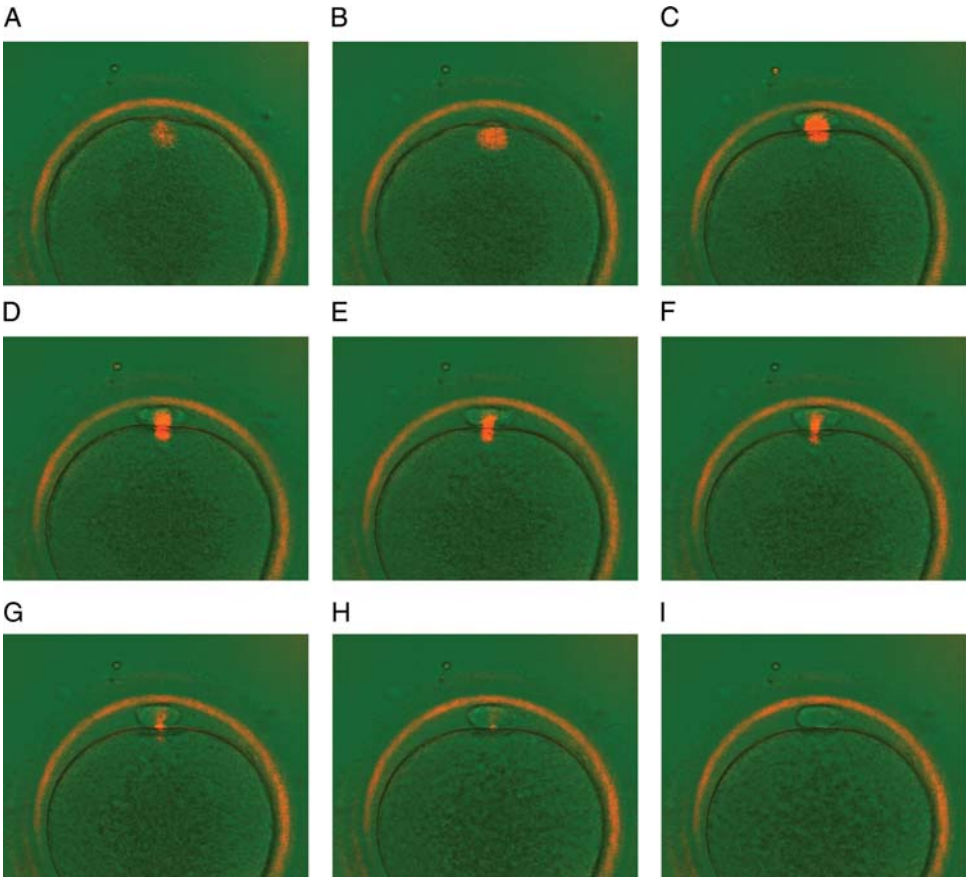


**Figure 3** Spindle dynamics during the meiotic cell cycle. The course of the meiotic cell cycle in a human oocyte is shown from the beginning of the extrusion of the first polar body up to the dissolution of the spindle. The images represent a total time scale of 160 min (**A**–**I**) and the time 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.

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 (life style 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

mouse oocytes suggests that disturbances and exposures to chemicals that interfere with microtubule dynamics in the spindle can significantly delay the maturation to metaphase-II (Vogt *et al.*, 2009). Therefore polarization microscopy might prove helpful in the long run to improve media for *in vitro* maturation or detect maturation delays associated with life style factors or occupational and environmental exposures (Shen *et al.*, 2008).

Similar to *in vivo* matured oocytes, the location of the spindle is positively correlated to the fertilization rates in *in vitro* matured oocytes (Fang *et al.*, 2007). Most importantly, the presence or absence of the spindle in *in vitro* matured oocytes was found to be dependent on external factors, the most important being temperature (Sun *et al.*, 2004), and on mitochondrial DNA and ATP contents (Zeng *et al.*, 2007). This underlines that the spindle in *in vitro* matured oocytes is probably highly 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 may be of great value in *in vitro* maturation cycles, as it allows investigators to follow the maturation process of each individual oocyte by spindle imaging and to choose the right time for ICSI or cryopreservation.

### *Spindle retardance*

Spindle retardance is directly proportional to the density of the microtubules (Sato *et al.*, 1975) and with proper settings even the retardance of individual microtubules can be measured (Oldenbourg *et al.*, 1998; LaFountain and Oldenburg, 2004). Several studies investigated the relation between spindle retardance and developmental parameters. A positive correlation was found in regard to 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). Other studies found an inverse relation between spindle retardance and maternal age (De Santis *et al.*, 2005; Shen *et al.*, 2006). A recent publication compared the oocytes from pregnant and non-pregnant women in regard to spindle retardance and density (Kilani *et al.*, 2009). Spindle density was significantly higher in oocytes that resulted in a pregnancy.

In the future, this may open a field for further research on a possible correlation between spindle characteristics and maternally derived aneuploidies in human oocytes (Keefe *et al.*, 2003; Shen *et al.*, 2008). However, at present, one has to consider that retardance measurements are dependent on the orientation of the spindle and probably also on the time of investigation. In most of the work done so far, there was a clear overlap of spindle retardance in the different groups investigated (age: De Santis *et al.*, 2005; pregnancy: Kilani *et al.*, 2009). This imposes the question of the usability of this measurement to select the best oocyte or embryo simply by retardance measurement, although the potential is there once the practical application has been developed further.

Spindle retardance can reflect changes that are associated with certain physiological events like oocyte activation (Liu *et al.*, 2000; Navarro *et al.*, 2005). Furthermore, external factors have an influence on microtubules and consequently may influence retardation measurement (Sun *et al.*, 2004; Navarro *et al.*, 2005; Shen *et al.*, 2005a). In view of all the variables contributing to spindle retardance, the use of spindle retardation measurements for therapeutic purposes and as a predictive tool in a routine IVF setting is still under debate.

Furthermore, it has been questioned whether polarization microscopy is a good tool for studies on fine changes in spindle assembly in a recent study on thawed oocytes where polymerized microtubules were visualized by fluorescence confocal scanning microscopy but not by polarization microscopy (Coticchio *et al.*, 2006). Polarization microscopy of spindles in human oocytes unfortunately does not predict alignment of chromosomes at the metaphase plate. This may also apply to fresh oocytes and it is an open discussion on the accuracy and reliability of spindle imaging by polarization microscopy.

### *Spindle visualization as a tool for quality assessment of laboratory parameters*

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 malsegregation 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 and the potential of an embryo to develop to the blastocyst stage (Rama Raju et al., 2007). Especially the work by Shen and co-worker stimulated prospective studies on the potential value of zona birefringence measurement as a prognostic tool in ART.

Using a polarization imaging system the intensity and uniformity of the zona inner 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

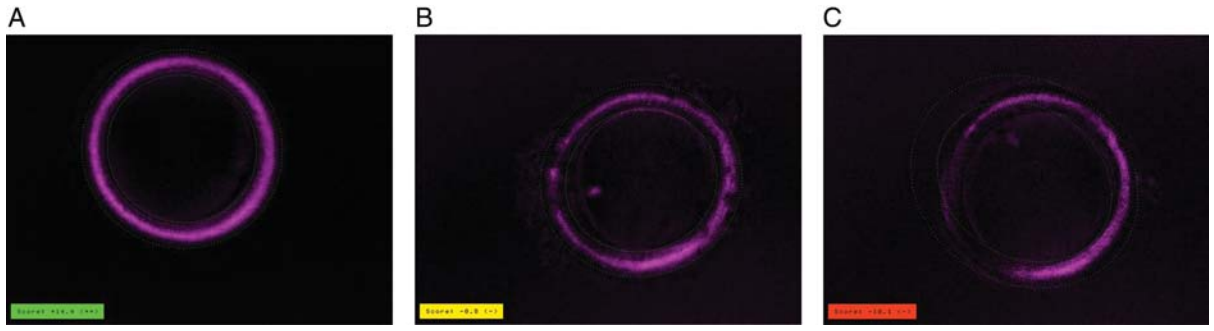


**Figure 4** Zona imaging using automatic zona detection and analysis. The inner layer of the zona pellucida can be visualized by polarizing microscopy. An automatic zona imaging system (Octax PolarAide, Octax, Bruckberg, Germany) allows for the generation of a score which is mainly based on the intensity and uniformity of the zona birefringence. The oocytes displayed show either a very uniform and strong birefringence (4**A**, score high = green), a less uniformity and birefringence (4**B**, yellow = intermediate) and a very low and extremely irregular birefringence (4**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.

formation rates 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 prognostic 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

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

| Publication | Study type | Fertilization method | Outcome observed |
|---|---|---|---|
| Shen *et al.*, 2005b | Retrospective | ICSI | High zona birefringence correlated with conception cycles |
| Rama Raju *et al.*, 2007 | Retrospective | ICSI | High zona birefringence was associated with improved embryo development and blastocyst formation rate |
| Montag *et al.*, 2008 | Prospective | ICSI | High zona birefringence correlated with embryo development on Day 3 and improved implantation and pregnancy rates |
| Ebner *et al.*, 2010 | Prospective | ICSI | Zona imaging correlated with blastocyst formation rate; zona birefringence influenced by stimulation protocol |
| Madaschi *et al.*, 2009 | Prospective | ICSI | High zona birefringence correlated with improved implantation and pregnancy rates and reduced miscarriage rate |
| Cheng *et al.*, 2010 | Retrospective | IVF | No correlation of zona birefringence after IVF with embryo development or clinical results |

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 immuncytochemical 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 (Svalander *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, KvdV and HvdV corrected the first draft and all authors were involved in the first and second revision of the manuscript.

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# References

Allen RD, Allen NS. Video-enhanced microscopy with a computer frame memory. *J Microsc* 1983;**129**:3–17.

Allen RD, Travis JL, Allen NS, Yilmaz H. Video-enhanced contrast polarization (AVEC-POL) microscopy: a new method applied to the detection of birefringence in the motile reticulopodial network of Allogromia laticollaris. *Cell Motility* 1981;**1**:275–289.

Almeida PA, Bolton VN. The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote* 1995;**3**:357–365.

Antinori M, Licata E, Dani G, Cerusico F, Versaci C, d'Angelo D, Antinori S. Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial. *Reprod Biomed Online* 2008;**16**:835–841.

Assidi M, Montag M, van der Ven K, Sirard M. Biomarkers of human oocyte developmental competence expressed in cumulus cells before ICSI: a preliminary study. *J Assist Reprod Genet* 2010;**16** [Epub ahead of print].

Baccetti B. Microscopical advances in assisted reproduction. *J Submicrosc Cytol Pathol* 2004;**36**:333–339.

Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan R. Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**:3431–3433.

Bartoov B, Berkovitz A, Eltes F, Kogosovsky A, Yagoda A, Lederman H, Artzi S, Gross M, Barak Y. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertil Steril* 2003;**80**:1413–1419.

Berkovitz A, Eltes F, Yaari S, Katz N, Barr I, Fishman A, Bartoov B. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Hum Reprod* 2005;**20**:185–190.

Berkovitz A, Eltes F, Lederman H, Peer S, Ellenbogen A, Feldberg B, Bartoov B. How to improve IVF-ICSI outcome by sperm selection. *Reprod Biomed Online* 2006;**12**:634–638.

Bianchi V, Coticchio G, Fava L, Flamigni C, Borini A. Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. *Hum Reprod* 2005;**20**:1078–1083.

Bianchi V, Coticchio G, Distratis V, Di Giusto N, Flamigni C, Borini A. Differential sucrose concentration during dehydration (0.2 mol/l) and re-hydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. *Reprod Biomed Online* 2007;**14**:64–71.

Bogner K, Hinsch KD, Nayudu P, Konrad L, Cassara C, Hinsch E. Localization and synthesis of zona pellucida proteins in the marmoset monkey (Callithrix jacchus) ovary. *Mol Hum Reprod* 2004;**10**:481–488.

Borini A, Sciajno R, Bianchi V, Sereni E, Flamigni C, Coticchio G. Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. *Hum Reprod* 2006;**21**:512–517.

Boudjema E, Magli MC, Crippa A, Baccetti B, Ferraretti AP, Gianaroli L. Correlation between sperm morphology and birefringence properties in human spermatozoa: implications for sperm selection at ICSI. *Hum Reprod* 2009;**24**(Suppl.1):i72.

Braga DP, Figueira Rde C, Rodrigues D, Madaschi C, Pasqualotto FF, Iaconelli A Jr, Borges E Jr. Prognostic value of meiotic spindle imaging on fertilization rate and embryo development in in vitro-matured human oocytes. *Fertil Steril* 2008; **90**:429–433.

Brewster D. On the laws of polarisation and double refraction in regularly crystallized bodies. *Phil Trans Royal Soc* 1818;**108**:199–273.

Chamayou S, Ragolia A, Alecci C, Storaci G, Maglia E, Russo E, Guglielmino A. Meiotic spindle presence and oocyte morphology do not predict clinical ICSI outcomes: a study of 967 transferred embryos. *Reprod Biomed Online* 2006;**13**:661–667.

Chen CK, Wang CW, Tsai WJ, Hsieh LL, Wang HS, Soong YK. Evaluation of meiotic spindles in thawed oocytes after vitrification using polarized light microscopy. *Ferti Steril* 2004;**82**:666–672.

Cheng J, Huang L, He B, Lu F, Wang X, Wu Z, Shi D. Quantitative analysis of the intensity of zona pellucida birefringence of oocytes during IVF cycles. *Reprod Fertil Dev* 2010;**22**:1167–1174.

Cobo A, Bellver J, Domingo J, Pérez S, Crespo J, Pellicer A, Remohí J. New options in assisted reproduction technology: the Cryotop method of oocyte vitrification. *Reprod Biomed Online* 2008a;**17**:68–72.

Cobo A, Pérez S, De los Santos MJ, Zulategui J, Domingo J, Remohí J. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. *Reprod Biomed Online* 2008b;**17**:350–359.

Cohen Y, Malcov M, Schwartz T, Mey-Raz N, Carmon A, Cohen T, Lessing JB, Amit A, Azem F. Spindle imaging: a new marker for optimal timing of ICSI. *Hum Reprod* 2004;**19**:649–654.

Cooke S, Tyler JP, Driscoll GL. Meiotic spindle location and identification and its effect on embryonic cleavage plane and early development. *Hum Reprod* 2003; **18**:2397–2405.

Coticchio G, De Santis L, Rossi G, Borini A, Albertini D, Scaravelli G, Alecci C, Bianchi V, Nottola S, Cecconi S. Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Hum Reprod* 2006;**21**:1771–1776.

Coticchio G, Sciajno R, Hutt K, Bromfield J, Borini A, Albertini DF. Comparative analysis of the metaphase II spindle of human oocytes through polarized light and high performance confocal microscopy. *Fertil Steril* 2010;**93**:2056–2064.

Crippa A, Magli MC, Paviglianiti B, Boudjema E, Ferraretti AP, Gianaroli L. DNA fragmentation and characteristics of birefringence in human sperm head. *Hum Reprod* 2009;**24**(Suppl. 1):i95.

De Almeida Ferreira Braga DP, de Cássia Savio Figueira R, Queiroz P, Madaschi C, Iaconelli A Jr, Borges E Jr. Zona pellucida birefringence in in vivo and in vitro matured oocytes. *Fertil Steril* 2010;**94**:2050–2053.

De Santis L, Cino I, Rabellotti E, Calzi F, Persico P, Borini A, Coticchio G. Polar body morphology and spindle imaging as predictors of oocyte quality. *Reprod Biomed Online* 2005;**11**:36–42.

De Santis L, Cino I, Coticchio G, Fusi FM, Papaleo E, Rabellotti E, Brigante C, Borini A, Ferrari A. Objective evaluation of the viability of cryopreserved oocytes. *Reprod Biomed Online* 2007a;**15**:338–345.

De Santis L, Coticchio G, Paynter S, Albertini D, Hutt K, Cino I, Iaccarino M, Gambardella A, Flamigni C, Borini A. Permeability of human oocytes to ethylene glycol and their survival and spindle configurations after slow cooling cryopreservation. *Hum Reprod* 2007b;**22**:2776–2783.

De Sutter P, Dozortsev D, Qian C, Dhont M. Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 1996;**11**:595–597.

Ebner T, Yaman C, Moser M, Sommergruber M, Feichtinger O, Tews G. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. *Hum Reprod* 2000;**15**:427–430.

Ebner T, Moser M, Sommergruber M, Tews G. Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review. *Hum Reprod Update* 2003;**9**:251–262.

Ebner T, Moser M, Tews G. Is oocyte morphology prognostic of embryo developmental potential after ICSI. *Reprod Biomed Online* 2006;**12**:507–512.

Ebner T, Balaban B, Moser M, Shebl O, Urman B, Ata B, Tews G. Automatic user-independent zona pellucida imaging at the oocyte stage allows for the prediction of preimplantation development. *Fertil Steril* 2010;**94**:913–920.

Eichenlaub-Ritter U, Chandley AC, Gosden RG. Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: an immunofluorescence study. *Chromosoma* 1986;**94**:337–345.

Eichenlaub-Ritter U, Shen Y, Tinneberg HR. Manipulation of the oocyte: possible damage to the spindle apparatus. *Reprod Biomed Online* 2002;**5**:117–124.

Engelmann TW. *Pflügers Arch* 1875;**11**:432.

Fang C, Tang M, Li WL, Zhou CQ, Zhuang GL, Leong M. Visualization of meiotic spindle and subsequent embryo development in *in vitro* and *in vivo* matured human oocytes. *J Assist Reprod Genet* 2007;**24**:547–551.

Frattarelli JL, Miller KA, Fletcher-Holmes DW, Hoyt CC, Nagy ZP, Taylor TH, Scott RT. The use of quantitative birefringence imaging to assess oocyte competence. *Hum Reprod* 2007;**22**(Suppl. 1):i166.

French DB, Sabanegh ES, Goldfarb J, Desai N. Does severe teratozoospermia affect blastocyst formation, live birth rate, and other clinical outcome parameters in ICSI cycles? *Fertil Steril* 2010;**93**:1097–1103.

Gianaroli L, Magli MC, Collodel G, Moretti E, Ferraretti AP, Baccetti B. Sperm head's birefringence: a new criterion for selection? *Fertil Steril* 2008;**90**:104–112.

Gianaroli L, Magli MC, Ferraretti AP, Crippa A, Lappi M, Capitani S, Baccetti B. Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. *Fertil Steril* 2010; **93**:807–813.

Gook D, Martic M, Borg J, Edgar DH. Identification of zona pellucida proteins during human folliculogenesis. *Hum Reprod* 2004;**19**(Suppl. 1):140.

Hardarson T, Lundin K, Hamberger L. The position of the metaphase II spindle cannot be predicted by the location of the first polar body in the human oocyte. *Hum Reprod* 2000;**15**:1372–1376.

Herrler A, Beier HM. Early embryonic coats: morphology, function, practical applications. An overview. *Cells Tissues Organs* 2000;**166**:233–246.

Hyun CS, Cha JH, Son WY, Yoon SH, Kim KA, Lim JH. Optimal ICSI timing after the first polar body extrusion in *in vitro* matured human oocytes. *Hum Reprod* 2007; **22**:1991–1995.

Inoué S. Polarization optical studies of the mitotic spindle. I. The demonstration of spindle fibers in living cells. *Chromosoma* 1953;**5**:487–500.

Inoué S. Video image processing greatly enhances contrast, quality and speed in polarization-based microscopy. *J Cell Biol* 1981;**89**:346–356.

Inoué S, Sato H. Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. *J Gen Physiol* 1967;**50**:259–292.

Isachenko V, Montag M, Isachenko E, Zaeva V, Krivokharchenko I, Shafei R, van der Ven H. Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. *Hum Reprod* 2005;**20**:492–496.

Iwayama H, Hochi S, Yamashita M. Birefringence parameter available for quantitative analysis of human zona hardness. *Zygote* 2010;**27**:1–7.

Keefe D, Tran P, Pellegrini C, Oldenbourg R. Polarized light microscopy and digital image processing identify a multilaminar structure of the hamster zona pellucida. *Hum Reprod* 1997;**12**:1250–1252.

Keefe D, Liu L, Wang W, Silva C. Imaging meiotic spindles by polarization light microscopy: principles and applications to IVF. *Reprod Biomed Online* 2003; **7**:24–29.

Kilani S, Cooke S, Kan A, Chapman M. Are there non-invasive markers in human oocytes that can predict pregnancy outcome? *Reprod Biomed Online* 2009; **18**:674–680.

Kirschner M, Mitchison T. Beyond self-assembly: from microtubules to morphogenesis. *Cell* 1986;**45**:329–342.

Kuhn JR, Wu Z, Poenie M. Modulated polarization microscopy: a promising new approach to visualizing cytoskeletal dynamics in living cells. *Biophys J* 2001; **80**:972–985.

Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Traounson A. Birth following vitrification of small number of human oocytes. *Hum Reprod* 1999; **14**:3077–3079.

LaFountain JR Jr. Spindle shape changes as an indicator of force production in crane-fly spermatocytes. *J Cell Sci* 1972;**10**:79–93.

LaFountain JR Jr, Oldenbourg R. Maloriented bivalents have metaphase positions at the spindle equator with more kinetochore microtubules to one pole than to the other. *Mol Biol Cell* 2004;**15**:5346–5355.

Larman MG, Minasi MG, Rienzi L, Gardner DK. Maintenance of the meiotic spindle during vitrification in human and mouse oocytes. *Reprod Biomed Online* 2007; **15**:692–700.

Liu L, Trimarchi JR, Oldenbourg R, Keefe DL. Increased birefringence in the meiotic spindle provides a new marker for the onset of activation in living human oocytes. *Biol Reprod* 2000;**63**:251–258.

Loutradis D, Drakakis P, Kallianidis K, Milingos S, Dendrinos S, Michalas S. Oocyte morphology correlates with embryo quality and pregnancy rate after intracytoplasmic sperm injection. *Fertil Steril* 1999;**72**:240–244.

Madaschi C, Carvalho de Souza Bonetti T, Paes de Almeida Ferreira Braga DP, Pasqualotto FF, Iaconelli A Jr, Borges E Jr. Spindle imaging: a marker for embryo development and implantation. *Fertil Steril* 2008;**90**:194–198.

Madaschi C, Aoki T, de Almeida Ferreira Braga DP, de Cássia Sávio Figueira R, Semião Francisco L, Iaconelli A Jr, Borges E Jr. Zona pellucida birefringence score and meiotic spindle visualization in relation to embryo development and ICSI outcomes. *Reprod Biomed Online* 2009;**18**:681–686.

Mansour RT, Serour MG, Abbas AM, Kamal A, Tawab NA, Aboulghar MA, Serour GI. The impact of spermatozoa preincubation time and spontaneous acrosome reaction in intracytoplasmic sperm injection: a controlled randomized study. *Fertil Steril* 2008;**90**:584–591.

Michelmann HW, Strittmatter M, Schwartz P. New aspects of sperm morphology—differential interference contrast (DIC) microscopy reveals an extreme heterogeneity and a very high percentage of aberrations in sperm-head morphology compared with several animals. In: Glander HJ, Grunewald S, Paasch U (eds). *Biology of Male Germ Cells*. Aachen: Shaker Verlag, 2009, 361–386.

Montag M, van der Ven H. Oocyte assessment and embryo viability prediction: birefringence imaging. *Reprod Biomed Online* 2008;**17**:454–460.

Montag M, Schimming T, van der Ven H. Spindle imaging in human oocytes: the impact of the meiotic cell cycle. *Reprod Biomed Online* 2006;**12**:442–446.

Montag M, Schimming T, Köster M, Zhou C, Dorn C, Rösing B, van der Ven H, van der Ven K. Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI cycles. *Reprod Biomed Online* 2008;**16**:239–244.

Montag M, Köster M, Rösing R, van der Ven K, van der Ven H. Non-invasive assessment of cryopreserved oocytes through polarized light microscopy. In: *Preservation of human oocytes*. Borini A, Coticchio G (eds). London: Informa Healthcare, 2009;174–183.

Moon JH, Hyun CS, Lee SW, Son WY, Yoon SH, Lim JH. Visualization of the metaphase II meiotic spindle in living human oocytes using the Polscope enables the prediction of embryonic developmental competence after ICSI. *Hum Reprod* 2003;**18**:817–820.

Navarro PA, Liu L, Trimarchi JR, Ferriani RA, Keefe DL. Noninvasive imaging of spindle dynamics during mammalian oocyte activation. *Fertil Steril* 2005; **83**(Suppl. 1):1197–1205.

Nikas G, Paraschos T, Psychoyos A, Handyside AH. The zona reaction in human oocytes as seen with scanning electron microscopy. *Hum Reprod* 1994; **9**:2135–2138.

Nottola SA, Machiarelli G, Coticchio G, Bianchi S, Cecconi S, De Santis L, Scaravelli G, Flamigni C, Borini A. Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations. *Hum Reprod* 2007;**22**:1123–1133.

Oldenbourg R, Mei G. New polarized light microscope with precision universal compensator. *J Microsc* 1995;**180**:140–147.

Oldenbourg R, Salmon ED, Tran PT. Birefringence of single and bundled microtubulues. *Biophys J* 1998;**74**:645–654.

Pattri HOE. Z Zellforsch Mirkoskop Anat 16:723.

Peer S, Eltes F, Berkovitz A, Yehuda R, Itsykson P, Bartoov B. Is fine morphology of the human sperm nucleus affected by in vitro incubation at 37oC? *Fertil Steril* 2007;**88**:1589–1594.

Pelletier C, Keefe DL, Trimarchi JR. Noninvasive polarized light microscopy quantitatively distinguishes the multilaminar structure of the zona pellucida of living human eggs and embryos. *Fertil Steril* 2004;**81**:850–856.

Petersen C, Oliveira JBA, Mauri AL, Massaro FC, Baruffi RL, Pontes A, Franco JG Jr. Relationship between visualization of meiotic spindle in human oocytes and ICSI outcomes: a meta-analysis. *Reprod Biomed Online* 2009;**18**:235–243.

Pickering S, Braude P, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril* 1990;**54**:102–108.

Rama Raju GA, Prakash GJ, Krishna KM, Madan K. Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: a preliminary study using polscope imaging. *Reprod Biomed Online* 2007;**14**:166–174.

Rienzi L, Ubaldi F, Martinez F, Iacobelli M, Minasi MG, Ferrero S, Tesarik J, Greco E. Relationship between meiotic spindle location with regard to polar body position and oocyte developmental potential after ICSI. *Hum Reprod* 2003;**18**:1289–1293.

Rienzi L, Martinez F, Ubaldi F, Minasi MG, Iacobelli M, Tesarik J, Greco E. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. *Hum Reprod* 2004;**19**:655–659.

Rienzi L, Ubaldi F, Iacobelli M, Minasi MG, Romano S, Greco E. Meiotic spindle visualization in living human oocytes. *Reprod Biomed Online* 2005;**10**:192–198.

Sato H, Ellis GW, Inoué S. Microtubular origin of mitotic spindle form birefringence: demonstration of the applicability of Wiener's equation. *J Cell Biol* 1975;**67**:501–517.

Schmidt WJ. *Die Bausteine des Tierkörpers in polarisiertem Licht*. Bonn: Verlag Cohen, 1924.

Schmidt WJ. *Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma. Protoplasma Monograph 11*. Berlin: Gebrüder Bornträger, 1937.

Schuh M, Ellenberg J. Self-organization of MTOCs replaces centrosome function during acentrosomal spindle assembly in live mouse oocytes. *Cell* 2007;**130**:484–498.

Sereni E, Sciajno R, Fava L, Coticchio G, Bonu MA, Borini A. A polscope evaluation of meiotic spindle dynamics in frozen-thawed oocytes. *Reprod Biomed Online* 2009;**19**:191–197.

Serhal PF, Ranieri DM, Kinis A, Marchant S, Davies M, Khadum IM. Oocyte morphology predicts outcome of intracytoplasmic sperm injection. *Hum Reprod* 1997;**12**:1267–1270.

Shen Y, Betzendahl I, Sun F, Tinneberg HR, Eichenlaub-Ritter U. Non-invasive method to assess genotoxicity of nocodazole interfering with spindle formation in mammalian oocytes. *Reprod Toxicol* 2005a;**19**:459–471.

Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U, Tinneberg HR. High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Hum Reprod* 2005b;**20**:1596–1606.

Shen Y, Stalf T, Mehnert C, De Santis L, Cino I, Tinneberg HR, Eichenlaub-Ritter U. Light retardance by human oocyte spindle is positively related to pronuclear score after ICSI. *Reprod Biomed Online* 2006;**12**:737–751.

Shen Y, Betzendahl I, Tinneberg HR, Eichenlaub-Ritter U. Enhanced polarizing microscopy as a new tool in aneuploidy research in oocytes. *Mutat Res* 2008; **651**:131–140.

Silva CS, Kapura K, Oldenbourg R, Keefe DL. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. *Fertil Steril* 1999;**71**:719–721.

Sinowatz F, Toepfer-Petersen E, Kolle S, Palma G. Functional morphology of the zona pellucida. *Anatom Histol Embryol* 2001;**30**:257–263.

Sun XF, Zhang WH, Chen XJ. Spindle dynamics in living mouse oocytes during meiotic maturation, aging, cooling and overheating: a study by polarized light microscopy. *Zygote* 2004;**12**:241–249.

Svalander P, Jakobsson AH, Forsberg AS, Bengtsson AC, Wikland M. The outcome of intracytoplasmic sperm injection is unrelated to strict criteria sperm morphology. *Hum Reprod* 1996;**11**:1019–1022.

Swann MM, Mitchison JM. Refinements in polarized light microscopy. *J Exp Biol* 1950; **27**:226–237.

Taylor TH, Chang CC, Elliott T, Colturato LF, Kort HI, Nagy ZP. Effect of denuding on polar body position in in-vitro matured oocytes. *Reprod Biomed Online* 2008; **17**:515–519.

Ten J, Mendiola J, Vioque J, de Juan J, Bernabeu R. Donor oocyte dysmorphisms and their influence on fertilization and embryo quality. *Reprod Biomed Online* 2007; **14**:40–48.

Trimarchi JR, Karin RA, Keefe DL. Average spindle retardance observed using the polscope predicts cell number in day 3 embryos. *Fertil Steril* 2005; **82**(Suppl.2):268.

Van der Ven K, Montag M, Drengner C, Köster M, van der Ven H. Differential gene expression profiles in cells of the corona radiata and outer cumulus oophorus in relation to oocyte competence. *Hum Reprod* 2009;**24**(Suppl. 1):i32.

Vogt E, Kipp A, Eichenlaub-Ritter U. Aurora kinase B, epigenetic state of centromeric heterochromatin and chiasma resolution in oocytes. *Reprod Biomed Online* 2009;**19**:352–368.

Wang WH, Keefe DL. Prediction of chromosome misalignment among *in vitro* matured human oocytes by spindle imaging with the polscope. *Fertil Steril* 2002; **78**:1077–1081.

Wang WH, Meng L, Hackett RJ, Oldenbourg R, Keefe DL. The spindle observation and its relationship with fertilization after intracytoplasmic sperm injection in living human oocytes. *Fertil Steril* 2001a;**75**:348–353.

Wang WH, Meng L, Hackett RJ, Keefe DL. Developmental ability of human oocytes with or without birefringent spindles imaged by Polscope before insemination. *Hum Reprod* 2001b;**16**:1464–1468.

Wang WH, Meng L, Hackett RJ, Oldenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. *Hum Reprod* 2001c;**16**:2374–2378.

Wang WH, Meng L, Hackett RJ, Oldenbourg R, Keefe DL. Rigorous control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates. *Fertil Steril* 2002;**77**:1274–1277.

Wassarman PM, Jovine L, Litscher ES. Mouse zona pellucida genes and glycoproteins. *Cytogenet Genome Res* 2004;**105**:228–234.

Wolinski TR. Polarization phenomena in optical systems. In: Driggers RG (ed.). *Encylopedia of Optical Engineering*, **Vol. 3**. CRC Press, 2003, 2150–2175.

Woodward BJ, Montgomery SJ, Hartshorne GM, Camplbell KH, Kennedy R. Spindle position assessment prior to ICSI does not benefit fertilization or early embryo quality. *Reprod Biomed Online* 2008;**16**:232–238.

Yoon TK, Chung HM, Lim JM, Han SY, Ko JJ, Cha KY. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated *in vitro* fertilization-embryo transfer program. *Fertil Steril* 2000;**74**:180–181.

Zeng HT, Ren Z, Yeung WS, Shu YM, Xu YW, Zhuang GL, Liang XY. Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in *in vitro* matured human oocytes. *Hum Reprod* 2007;**22**:1681–1686.