

Analyzing the ultrastructure of the oocyte to determine viability post-thaw using the CRi Oosight™ imaging system.

In the past few years, the clinical application of oocyte cryopreservation in the field of assisted reproductive technologies (ART) has advanced considerably as protocols are refined and oocyte-specific cryoprotectants are made available. Despite this progress, cryopreservation success rates remain low and oocyte viability continues to be compromised primarily because the ultrastructure of oocytes remains highly susceptible to cryodamage. Practitioners need an assay to assess viability in order to ensure that those oocytes that are used to produce clinical pregnancies are in fact healthy and structurally sound. Standard embryo scoring methods fall short in accurately detecting issues associated with frozen/thawed oocytes. The following application note describes an optical imaging method that allows the assessment of oocyte viability before and after freezing. This method can be used in addition to standard grading protocols in order to improve the overall efficiency of cryopreservation protocols, and in the process, also enhance our understanding of the basis of cryodamage.

Recently published studies have reported that both the meiotic spindle and tri-laminar zona pellucida in the oocyte undergo morphological changes during the freeze/thaw process (Chen 2004, Nottala 2007, Rienzi 2004). These structures play a vital role in fertilization and embryo development and are thus critical to overall oocyte viability (Raju 2007, Shen 2005, Shen 2006). The ability to assess biophysical changes in these structures of the oocyte post-thaw, in comparison to their original unfrozen physiological state, presents a unique and useful method to determine the viability of thawed oocytes.

The Spindle Apparatus

The meiotic spindle in mammalian oocytes is temperature-sensitive, and is known to actively depolymerize when the oocyte is not maintained at its optimum temperature, typically 37°C (Wang 2002). The use of a cryoprotectant helps to stabilize the spindle during the freezing and thawing process and is postulated to keep the structure of the spindle intact. Time-lapse imagery has shown that is not entirely true because the meiotic spindle apparatus in the oocyte ultimately depolymerizes during the washing step(s) of the post-thaw process (Rienzi 2004). All oocytes that exhibit a spindle post-thaw have undergone spindle reconstruction (Figure 1). Furthermore, it has been routinely shown that oocytes whose spindles fail to repolymerize are in fact biologically compromised (Moon 2003, Noyes 2006, Rienzi 2004). One can therefore rely on spindle reformation as an

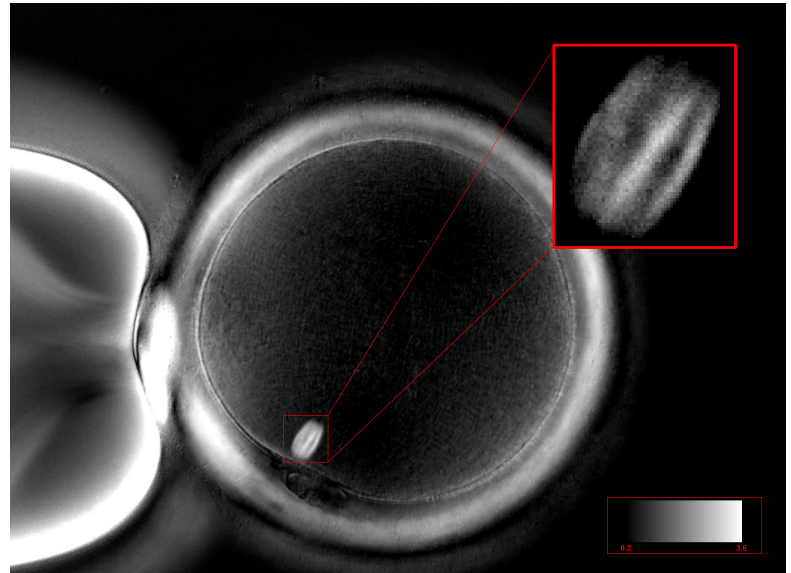


Figure 1. Human MII oocyte with a strongly birefringent, barrel-shaped spindle. Average mean retardance of the spindle is 2.5nm.

indicator of oocyte viability. Because the spindle that exists in post-thaw oocytes has been reformed, it is also important to understand whether it repolymerized to its original integrity or if it underwent cryodamage and was compromised in some way. To determine this effect quantitatively, the Oosight imaging system can be used to uniquely visualize and measure the underlying degree of molecular order of the spindle. The measured parameter, referred to as retardance (nm), is a measure of the structural density of the spindle. The mean area retardance of the spindle can be measured before and after freezing using an automated spindle finder tool in the Oosight software (Figure 2). If the measured retardance of the reformed post-thaw spindle is much lower than that of the original pre-frozen measurement, one can establish that the spindle apparatus did not reform to its original structural integrity, an indication that the viability of the oocyte is compromised (Shen 2006, Raju 2007).

The Oosight system also uniquely measures the orientation of microstructures in the spindle. During the cryopreservation process, the spindle apparatus may undergo structural changes such that the microtubules comprising the spindle become misaligned post-thaw. Spindle misalignment has been shown to lead to aneuploidy (Battaglia 1996, Chatzimeletiou 2005). The Oosight system can be used to detect and determine the misalignment of underlying structures of the spindle apparatus (Figure 3), and consequently be used to measure and determine overall viability.



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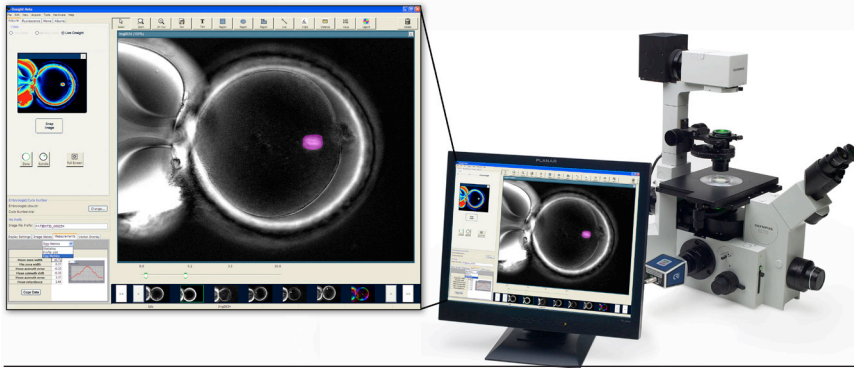


Figure 2. Oosight imaging system. Software screenshot shows the automated spindle finder tool and associated measurements.

The Trilaminar Zona Pellucida

The zona pellucida in the human oocyte is comprised of three distinct glycoprotein layers (Keefe 1997, Pelletier 2004). Electron microscopy and polarized light microscopy using Oosight technology have been used to resolve these three layers and have been used to confirm that the inner zona undergoes hardening when subjected to adverse conditions in vitro which inevitably lead to compromised viability (Kilani 2006). Nottola et al (2007) have shown that the varied sucrose concentrations used during cryopreservation result in varying degrees of hardening in the inner zona. The measured retardance of the inner zona should therefore not increase from the base measurement taken before freezing.

Additionally, a weak retardance measurement of the inner zona is also associated with compromised oocyte viability (Shen 2005). A study by Shen et al established that the pregnancy rate was reduced to less than 12% in fresh oocytes with an inner zona retardance measurement of less than 2 nm. On the other hand, the pregnancy rate skyrocketed to 90% in the study group whose oocytes exhibited an inner zona retardance measurement above 3 nm. Although this particular study did not mention zona hardening in relation to high values leading to lower rates, other unpublished reports have found this to be the case. It may be that both low and highly ordered inner zonae are indeed biologically compromised, while those that exhibit a mean value above the established 2 nm “low” range, and below the unpublished 5 nm “high” range are within the “normal” range. Additional oocyte studies are required to confirm this postulation.

Nonetheless, the Oosight system can be used to effectively assess the structural integrity of the trilaminar zona pellucida and

monitor biophysical changes in thawed oocytes. An average area retardance measurement of the inner zona can be taken before and after freezing and deviations from the baseline measurement in either direction can be used to indicate compromised structural integrity (Figure 4).

Summary

Whenever a clinical application is employed that is known to negatively alter the state of the biological material being used it is imperative that a system of checks and balances is put into place to monitor and assess the extent of that change. The Oosight system can help do this for all oocytes undergoing cryopreservation by providing a non-invasive, quantitative and reproducible method that helps to ensure that vital structures in the

oocyte are reformed to their original, pre-frozen state. Those oocytes that show changes in retardance and orientation from baseline measurements should be monitored using additional screening methods such as preimplantation genetic diagnosis (PGD) and should potentially be selected against at the time of implantation. If baseline measurements are not available, retardance and orientation measurements can still be useful in detecting potential problems. For example, a non-existent spindle, a low retardance area value and/or misalignment in the spindle apparatus can be used as cues of compromised oocyte viability. Furthermore, an inner zona measurement that is either

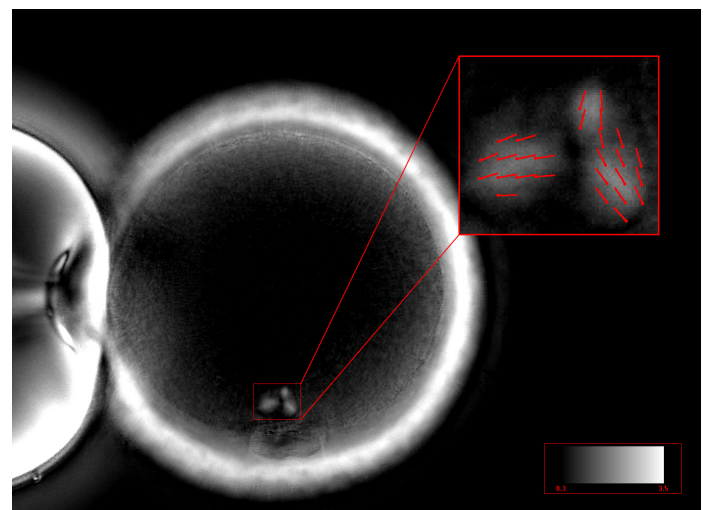


Figure 3. Human MII oocyte with a disrupted, tripolar spindle. Enlarged view shows vector map identifying the long-axis of the microtubules.

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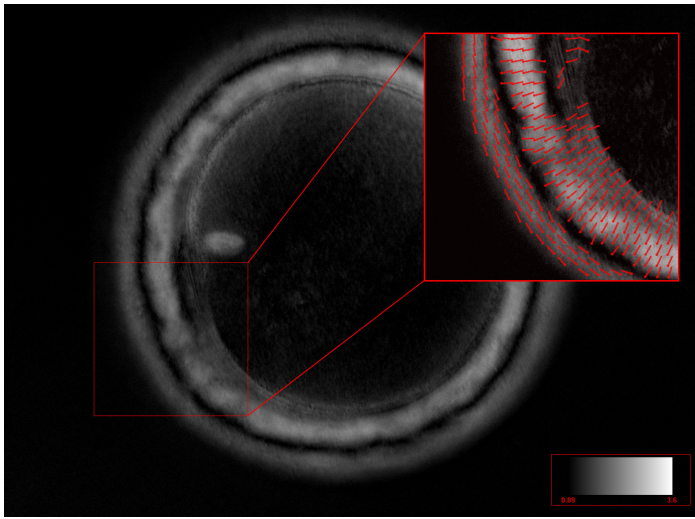


Figure 4. Human MII oocyte with a disrupted inner zona pellucida. Enlarged view shows vector map identifying the long-axis of the glycoproteins.

very high (>5 nm) or very low (<2 nm) should be taken into consideration when assessing oocyte viability.

In summary, the Oosight imaging system can be used to assess oocyte viability post-thaw by identifying oocytes that fail to reform a spindle, those that reform weak spindles and those that have microtubules that are misaligned, as well as those that have compromised structural integrity of the inner zona pellucida.

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