



# A Study of the Multilaminar Structure at the Time of Fertilization Using Digital Image Processing

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## Abstract

The zona pellucida (fertilization) is a glycoprotein coat that envelopes the oocyte and embryo, binds sperm during fertilization and facilitates transfer of the embryo through the Fallopian tube. Several lines of evidence suggest that the zona is multilaminar. We hypothesized that the multilaminar structure of the zona speaks could be imaged non-destructively with the polarized light microscope. A recent modification of the polarized light microscope (pol-scope), which combines innovations in polarization optics with novel image processing software, allows measurement of birefringence at all points of the image. Hamster metaphase II oocytes were placed on glass cover slips which replaced the bottom of culture dishes, imaged under differential interference contrast (DIC) and pol-scope optics, then digitized and processed to measure birefringence magnitude and orientation. The polscope revealed the zona to be divided into outer and inner layers separated by a zone of low retardance. This ending is consistent with speaks in the outer layer oriented tangentially and in the inner layer oriented radially. The multilaminar structure of the mammalian zona suggested by differential lectin binding and by scanning electron microscopy could be imaged non-destructively with the polscope. Because the polscope provides a non-destructive method to identify macro-molecular organization of the zona, it may prove useful in developmental studies of test tube babies and for development.

**Keywords:** Multilaminar , DIC, Hamster metaphase.

## Introduction

The mammalian egg is encapsulated by an acellular glycoprotein coat called the zona pellucida (zona) (Wassarman, 1987). The zona recognizes and binds spermatozoa at fertilization, and during preimplantation development prevents dispersion of blastomeres, protects the embryo and facilitates the passage of the embryo through the Fallopian tube. Escape of the embryo from the zona, called hatching, must occur before the embryo can implant into the endometrium. Although the zona appears homogeneous when imaged under conventional bright field, Nomarski differential interference contrast (DIC) or Hoffman optics, scanning electron microscopy suggests a more complex structure of the zona. The internal layer of the zona (closest to the oolemma) has an irregular, punctate surface, while the outer layer of the zona appears more lattice-like. Lectin binding, which is widely used to determine cell surface structure and dynamics because it identifies specific saccharide sequences in oligosaccharides, also suggests distinct layers of the zona.

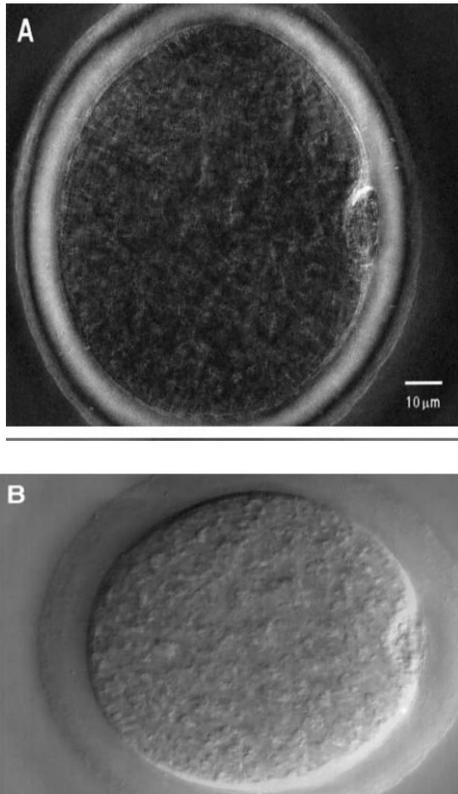
The structure and function of the zona are clinically important because hatching of the blastocyst from the zona may be a limiting step after in-vitro fertilization (IVF) in humans. Prolonged exposure of embryos to artificial culture conditions probably impairs their ability to hatch. Zona morphology, particularly its thickness and degree of irregularity, predicts subsequent implantation. Moreover, artificial zona thinning (assisted hatching) may improve the ability of some human embryos to hatch *in vitro* and to implant after transfer. Assisted hatching has been performed with acid Tyrode's solution, by

dissection with microneedles, and via laser vaporization. Despite promising initial results, assisted hatching also has been associated with detrimental outcomes, including degeneration of blastomeres, reduced implantation rates, trapping of the embryo within the zona, and increased monozygotic twinning. Hence, improvement in the safety and efficiency of assisted hatching is needed.

In order to develop a more rational approach to the clinical measurement of zona thickness and to assist hatching, we sought to improve non-destructive imaging of the zona. Because of suggestions of its multilaminar structure, we postulated that the zona would exhibit optical anisotropy, and that the filaments in the inner and outer layers would exhibit different orientations of their slow axes, and therefore could be distinguished by the polarized light microscope. A recent modification of the polarized light microscope (pol-scope), which combines innovations in polarization optics with novel image processing software (Oldenbourg and Mei, 1995), was used.

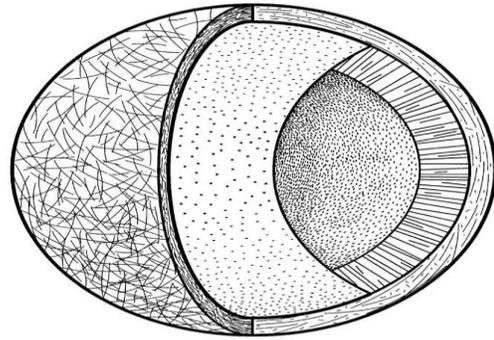
## Materials and methods

Oocytes ( $n = 21$ ) were obtained from sexually-mature, female golden hamsters (*Mesocricetus auratus*), 150 g, maintained on 14 h light:10 h dark schedule. Ovulation was stimulated in hamsters ( $n = 5$ ) by an i.p. injection of 25 IU pregnant mare's serum gonadotrophin (Diosyath B.V., Oss, The Netherlands), followed by 25 IU human chorionic gonadotrophin (HCG; Sigma, St Louis, MO, USA). Oocytes were collected with polarized light microscope identifiers. Inner zona pellucida



**Figure 1.** Hamster oocyte, imaged with the pol-scope (A) and with Nomarski differential interference contrast (DIC) optics (B) using a 360/1.4 NA Plan Apo oil immersion objective and 1.4 NA condenser. In the pol-scope image (A) the zona pellucida is divided into outer and inner layers, each layer displaying birefringence, separated by an isotropic layer. The slow axis of birefringence in the outer layer is oriented tangentially while in the inner layer the slow axis orientation is radial. The likely explanation for the zona birefringence is ordered, submicroscopic arrays of filaments. In the outer layer, filaments would be preferentially oriented tangentially, while in the inner layer filaments would have preferred radial orientation. Filaments in the intermediate layer would be ordered randomly. In contrast to the pol-scope image in the DIC image (B), the zona pellucida appears uniform on the morning after HCG administration by flushing the excised reproductive tracts with ~1 ml of equilibrated Tyrode's culture medium. Metaphase II oocytes were affixed to glass slides mounted in media and covered with a cover slip before viewing.

Oocytes were imaged using the pol-scope, which has been described previously (Oldenbourg and Mei, 1995). The pol-scope employs circularly polarized light and incorporates a precision universal compensator made from two liquid crystal variable retarders. The novel universal compensator measures anisotropy at all points within the specimen, without regard to orientation of the specimen's birefringent axis. A video camera and digital image processing software provide fast measurements of specimen anisotropy (retardance magnitude and orientation) at all points of the image. The images document fine structural and macromolecular organization within a thin optical section of the specimen. The sensitivity of the current instrument is 0.02 nm of the egg.



**Figure 2.** A schematic of the structure of the zona, based on the pol-scope. The inner and outer zona are arranged in distinct layers with their filaments oriented perpendicular to one another. The filaments of the inner zona appear to course radially from the oocyte, while filaments from the outer zona course tangentially about the oocyte. The intermediate layer exhibits no optical anisotropy, suggesting random orientation of the zona filaments in this region. specimen retardance, measured with data gathered in 0.43 s at all 6403480 image points. The same microscope set-up was used with differential interference contrast optics by inserting standard Wollaston prisms into the optical path.

Thickness of the zona and each of its layers were measured in four quadrants of metaphase II oocytes using NIH Image (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

## Results

Under polarization optics, using the pol-scope, the zona of all oocytes appeared to be subdivided into two birefringent layers separated by an anisotropic layer (Figure 1A). The birefringence of the outer layer had its slow axis oriented tangentially, hence parallel to the oolemma. The birefringence of the inner layer had its slow axis oriented perpendicular to the outer layer, in an orientation radial relative to the oocyte (Figure 2). The thickness of the three layers (inner, isotropic, and outer) was measured in randomly selected oocytes ( $n = 21$ ); the thickness of the respective layers was  $6.30 \pm 1.60$ ,  $1.87 \pm 0.59$  and  $4.96 \pm 0.80 \mu\text{m}$  (mean  $\pm$  SD). The average retardance magnitude in the inner layer was 1.4 nm, in the outer layer was 0.7 nm, while the black layer had no retardance. Under DIC optics the zona appeared uniform (Figure 1B).

## Discussion

The zona pellucida is a matrix comprised of three glycoproteins ZP1, ZP2 and ZP3. Imaging the architecture of the zona glycoproteins using transmission electron microscopy or scanning electron microscopy was technically challenging because their highly hydrated state complicates the process of fixation with conventional agents, such as ethanol or glutaraldehyde, and mechanical stress introduced during freezing and drying creates artefacts by alternating alignment of zona filaments. A number of modifications to standard fixation protocols have been introduced in order to minimize the effects of fixation artefact. Ruthenium Red has been used to stabilize structural glycoproteins and polyanionic carbohydrates and detergents have been employed to solubilize zona proteins. When this technique of fixation was applied to oocytes, and the zona evaluated by scan-

ning electron microscopy, thin filamentous structures appeared in a regular arrangement throughout the zona, while the outer surface of the zona appeared fenestrated (Familiari *et al.*, 1992a,b). Indeed, most investigators agree that the zona glycoproteins form fibrillar structures since solubilized zona, sedimented and processed for transmission electron microscopy, demonstrates long fibrils (Wassarman and Mortillo, 1991).

More controversial has been the question of whether zona filaments are organized homogeneously or in concentric layers. Backscattered electron imaging fracture label, an adaptation of the fracture-label method for scanning electron microscopy, provides information about the three-dimensional organization of antigens and receptors over the cell. Application of this technique to the zona of hamster oocytes demonstrated uniform distribution of lectin binding sites in the zona matrix, and thus did not support the proposal that the zona of golden hamsters is ultrastructurally divisible into distinct layers but rather that glycoproteins are uniformly distributed across the zona (Kan and Zalzal *et al.*, 1994). Similarly, freeze substitution of hamster oocytes revealed an overall porous configuration throughout the zona (Yudin and Cherr *et al.*, 1988). Capacitated hamster spermatozoa with intact acrosomes were found throughout the matrix.

These findings contrast those of Phillips and Shalgi (1980), who viewed the outer surface of the hamster zona using scanning electron microscopy and found it fenestrated, with a lattice-like appearance, while the internal surface of the zona appeared irregular and particulate. The layered structure of the zona also was suggested by Yanagimachi and Nicolson (1976), who reported differential lectin staining throughout the hamster zona. The most likely explanation of the results of pol-scope evaluation of the zona is that the zona is arranged in two separate layers, each with an ordered array of filaments oriented perpendicular to one another (Figure 2), and separated by an intermediate layer exhibiting random orientation of zona filaments. The inner filaments course radially from the oocyte, while the outer filaments run circumferentially around the oocyte. Filaments in the intermediate layer are isotropic, that is they display random orientation relative to one another. Our multilayered model of the structure of the hamster zona pellucida, based on studies with the pol-scope, is consistent with that proposed by Phillips and Shalgi (1980) and Yanagimachi and Nicolson (1976).

The pol-scope offers an advantage over previous methods to evaluate the structure of the zona because it is non-destructive, and thus enables us to view the structural orientation of zona filaments without fixing or otherwise destroying them. Such an approach facilitates developmental and clinical studies of the zona, and avoids structural artefacts introduced by fixation. Thus, the pol-scope can be used to study the zona in individual embryos throughout preimplantation development, and in oocytes during fertilization. The non-destructive feature of the pol-scope is especially salient for the study of human preimplantation embryos, since ethical guidelines preclude their intentional destruction. Moreover, the improved resolution of embryo morphology raises the possibility that the pol-scope might prove useful in clinical embryo morphology grading.

Finally, the improved resolution of zona anatomy, rendered non-destructively, may resolve the controversy over the clinical utility of assisted hatching. Indeed, future studies should examine separately the anatomy of each of the zona's layers during hatching *in vitro* as well as *in vivo*.

## References

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