Journal of Visualized Experiments

Human egg maturity assessment and its clinical application

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60058R1
Full Title:	Human egg maturity assessment and its clinical application
Keywords:	in vitro fertilization; assisted reproduction; human oocyte maturation; immature oocytes; timing of ICSI; polarized light microscopy; embryology
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Brno, Czech Republic

TITLE:

Human Egg Maturity Assessment and its Clinical Application

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KEYWORDS:

in vitro fertilization, assisted reproduction, human oocyte maturation, immature oocytes, timing of ICSI, polarized light microscopy, meiotic spindle

SUMMARY:

We provide an outline of the clinical protocol for non-invasive assessment of human egg maturity using polarized light microscopy.

ABSTRACT:

The optimal timing of intracytoplasmic sperm injection (ICSI) is of a serious concern for fertility programs because untimely sperm entry diminishes the egg's developmental competence. Presence of the first polar body (PB) together with the meiotic spindle indicates completion of the oocyte maturation and the egg's readiness for fertilization. In clinical practice, it is customary to assume that all oocytes displaying a PB are mature metaphase (MII) oocytes. However, PB extrusion precedes the formation of the bipolar MII spindle. This asynchrony makes the mere presence of PB an unreliable marker of oocyte maturity. Noninvasive spindle imaging using polarized light microscopy (PLM) allows quick and easy inspection of whether the PB-displaying oocyte actually reassembled a meiotic spindle prior to ICSI. Here, we present a standard protocol to perform human egg maturity assessment in the clinical laboratory. We also show how to optimize the time of ICSI with respect to the oocyte's developmental stage in order to prevent premature sperm injection of late-maturing oocytes. Using this approach, even immature oocytes extruding PB in vitro can be clinically utilized. Affirmation that MII spindle is present prior to sperm injection and individual adjustment of the time of ICSI is particularly important in poor

prognosis in-vitro fertilization (IVF) cycles with a low number of oocytes available for fertilization.

INTRODUCTION:

To become a fertilizable haploid egg, the diploid oocyte has to extrude half of its genetic information into an adjacent cell, called the first polar body (PB), and align chromosomes in the equator of the bipolar metaphase II (MII) spindle. While PB can be clearly observed by conventional light microscopy, detection of genetic material and cytoskeletal structures typically requires invasive preparatory procedures that are incompatible with the oocyte's further use for fertility treatment. Hence, in clinical practice, the presence of the PB is regarded as a hallmark of oocyte maturity. However, live imaging of microtubule and chromosome dynamics during human oocyte maturation revealed that PB becomes visible a couple of hours before bipolar MII spindle is assembled and chromosomes are aligned[1]. Nevertheless, in the transmitted light, the MII arrested eggs are indistinguishable from the oocytes which just entered the process of chromosome segregation. Thus, a cohort of oocytes, classified as MII oocytes based only on the PB presence, might contain late-maturing oocytes that have not yet completed their development and thus are not ready for fertilization.

The delay of oocyte maturation is likely to affect the population of poor responders with a low number of MII oocytes and a high proportion of immature oocytes collected unexpectedly in stimulated cycles[2]. In vivo, only a single, best-quality egg achieves maturity and becomes ovulated. In in-vitro fertilization (IVF) cycles, controlled ovarian hyperstimulation is used to recruit multiple oocytes for maturation. Gonadotropin surge triggers a resumption of the meiotic program and egg progenitors are supposed to reach the MII arrest stage within 36 hours[3]. However, oocytes retrieved from preovulatory follicles often constitute an assortment of PB-displaying MII oocytes and immature oocytes, either at metaphase I (MI) or germinal vesicle stage (GV) (**Figure 1**). Only MII oocytes are subjected to intracytoplasmic sperm injection (ICSI) while immature oocytes are typically discarded. Yet, when cultivated in vitro, MI oocytes are commonly observed to extrude PB in vitro. Despite their general inferiority, late-maturing oocytes which spontaneously completed the first meiotic division during overnight culture have been successfully used as last resource oocytes, and live births have been reported[4-8]. Hence, the untimely injection of the sperm might be a primary reason underlying poor developmental outcomes of late-maturing oocytes reported in previous studies[9-11].

Polarized light microscopy (PLM) combined with an image processing software allows for non-invasive visualization of the meiotic spindle in the live oocyte. The double reflection is generated by the interaction of polarized light beam with the highly ordered assembly of microtubules building the bipolar spindle. Since the polarized light is of normal intensity, the technique could be safely used in clinical settings to view the dynamics of the division apparatus[11-14]. The presence of the MII spindle birefringence within the oocyte has been identified as a marker of an egg's developmental competence[9,15-23]. Thus, it has been suggested that non-invasive meiotic spindle imaging could be used for egg quality control in clinical practice[11,14,20].

Since the timeline for the microtubular dynamics during oocyte meiosis has been resolved[1], the observed PLM pattern can be better related to the time course of MI to MII transition. Shortly

after PB emission, the nascent MII spindle becomes undetectable by PLM. However, if the oocytes are kept in the culture, the birefringence signal may emerge later when the bipolar MII spindle reassembles[9-11]. Thus, in oocytes extruding a PB in vitro, the absence of the spindle might be only temporary corresponding to the physiological transition of the late-maturing oocyte from MI to MII stage. If the MII spindle signal is undetectable, sperm injection can be deferred to a later time point providing extra time for the MII spindle formation. PLM-aided optimization of ICSI time maximizes the chance of late-maturing oocytes to be clinically utilized and make a difference for poor prognosis patients[9].

Below, we provide a step-by-step protocol of how to perform non-invasive spindle imaging in human oocytes. We also demonstrate how PLM can be employed to avoid the risk of premature fertilization of late-maturing oocytes.

PROTOCOL:

This protocol describes the clinical procedure which is an 'add-on' to standard IVF treatment. It should be performed by experienced personnel in compliance with good laboratory practice and clinical guidelines[24,25]. Obtaining written informed consent of from the eligible patients is recommended. This protocol was approved by the institutional Ethics Committee.

1. Egg retrieval and denudation

1.1. Induce ovarian stimulation using conventional stimulation protocols[3]. Adjust the dose to individual response. When two or more follicles, visualized by ultrasound scan, reach a diameter of 18 mm, induce oocyte maturation with application of 250 µg human chorionic gonadotropin (hCG). Schedule oocyte pick-up (OPU) at 35−36 h post hCG injection.

1.2. Collect retrieved cumulus-oocyte complexes (COCs) in CO_2-independent handling medium (**Table of Materials**). Following a short incubation period (10−15 min) in a CO_2-independent incubator, briefly (up to 30 s) expose collected COCs to hyaluronidase solution (**Table of Materials**). Under a stereomicroscope, mechanically remove cumulus-corona cells by gently pipetting COCs with a 200 µL filter tip.

1.3. Using denudation micropipettes with a gradually decreasing diameter (200 µm, 180 µm and 150 µm), gently strip the oocytes from the remaining follicular cells and wash the oocytes 3x in handling medium.

1.4. Assess the number and developmental status of the denuded oocytes according to the presence or absence of the nucleus and first PB (**Figure 1**).

1.5. Check the inclusion criteria for PLM examination. Carry out an egg maturity assessment if there is (1) an unexpected poor response to conventional stimulation with fewer than 6 MII oocytes collected at OPU and (2) a history of previous fertilization failure or oocyte immaturity.

1.6. Place GV, MI and MII oocytes into separate wells in an IVF dish, each containing 500 µL of

preequilibrated CO_2-dependent culture medium (**Table of Materials**) covered with mineral oil (**Table of Materials**).

1.7. Incubate for an additional 3−4 h at 37 °C in a humidified atmosphere of 5% O_2 and 6% CO_2.

2. Preparation for PLM examination and subsequent ICSI

NOTE: The protocol provided here describes the PLM assessment performed using the OCTAX Polar AIDE system (**Table of Materials**). Alternatively, other commercial spindle view system can be used.

2.1. Prepare plates for embryo cultivation.

2.1.1. Depending on the number of oocytes to be examined (total of MII oocytes, and MI oocytes extruding a PB during preincubation period), prepare either a 4-well plate or a 12-well plate, fill each well with 500 µL or 30 µL of culture medium, respectively, and cover with previously equilibrated mineral oil.

2.1.2. Ensure that both culture medium and mineral oil have been equilibrated in the CO_2 incubator overnight. Keep the prepared dish in the CO_2-dependent incubator for at least 2 h. Number the wells if necessary to track the developmental fate of individual oocytes.

2.2. Prepare the ICSI dish.

2.2.1. Use assigned plastic dish and make 5 µL droplet of prewarmed handling medium for each PB-displaying oocyte and one extra droplet for needle washing. Make an additional droplet of polyvinylpyrrolidone (PVP) solution (**Table of Materials**) for sperm immobilization prior to ICSI (add sperm just before ICSI) and overlay with prewarmed mineral oil.

2.2.2. Keep the prepared ICSI dish in the CO_2-independent incubator for at least 20 min. Number the wells if necessary to track the oocytes after ICSI.

2.3. Prepare the PLM examination dish.

2.3.1. Use assigned glass bottom dish and make 5 µL droplets of prewarmed handling medium for each PB-displaying oocyte. Overlay with prewarmed mineral oil.

2.3.2. Keep the dish in the CO_2-independent incubator for at least 20 min. Number the droplets if necessary to track the oocytes after PLM examination.

2.4. Set the microscope ready for PLM examination.

2.4.1. Switch the heated stage on the inverted microscope on well in advance to achieve correct warming temperature. Ensure that the settings are accurately adjusted to maintain 37 °C in the

handling medium droplets in the PLM/ICSI dish during micromanipulation procedures.

2.4.2. Fit the sterile holding and ICSI needle into microinjection holders and bring them into focus. Alternatively, use a hatching needle.

2.4.3. Select the appropriate objective (20x and 25x are the most suitable) and ensure that the condenser is in bright field position.

2.4.4. Insert green interference filter (the light turns green) and set the liquid crystal analysis slider into working position.

2.4.5. Set the shutter to ~50% and adjust circular polarizer to decrease background noise.

2.5. Set the computer ready for PLM examination.

2.5.1. Launch the imaging software.

2.5.2. Select **Video | Video Source | polarAIDE** in the video menu in the top menu bar.

2.5.3. Switch to live video by going to the **Video** page and activate spindle and zona analysis by hitting on the icon (**Supplemental Figure 1**) in the video toolbar.

2.5.4. Select display mode for dynamic scaling during spindle imaging: (1) red (birefringence)/green (background) combined view, or (2) white (birefringence)/and black (background) view. The dynamic scoring mode is used for autoscoring of zona pellucida.

3. Examination of egg maturity

3.1. After the preincubation period (3−4 h), perform a PLM examination revealing the oocyte maturity status at the standard time of ICSI (39−40 h after hCG trigger).

3.2. Transfer all oocytes into individual droplets on the PLM dish and place it under the inverted microscope prepared for spindle imaging. Remember to remove the plastic lid from the glass bottom dish before starting the examination.

3.3. Bring the first oocyte into focus. If it is difficult to search for the cell under green light, pull out the green filter temporarily. Make sure the green filter is inserted before analysis.

3.4. Observe the detected oocyte birefringence image (red/orange on green background) as it is computer-processed and displayed in real time on the computer screen. Remember that the signal is not visible in the eyepiece.

3.5. If the message announcing light exposure is too low/high pops up, adjust the brightness to the suitable intensity using the microscope's light intensity knob.

3.6. Use a holding and ICSI needle to turn the oocyte so the PB is in the 12 o'clock position and focus to the PB.

3.7. If the spindle birefringence is not visible at first sight in the vicinity of PB, gently turn the oocyte around each axis by slightly touching the zona pellucida to ensure the alignment of the polarized light with the array spindle fibers (**Supplemental Video**). Declare the absence of the MII spindle as long as the oocyte fails to show the spindle signal despite rigorous rotation.

3.8. Based on the observed birefringence pattern, classify the oocytes into the following categories (**Figure 2**): (A) oocytes with bright signal of bipolar barrel-shaped MII spindle with clearly delineated boundaries and even distribution of birefringence; (B) oocytes with dysmorphic, apolar and translucent MII spindle with irregular boundaries and uneven distribution of the signal; (C) oocytes with no detectable MII spindle birefringence in ooplasm; (D) anaphase I/telophase I oocytes, showing a microtubular bridge (a connective strand between first PB and the oocyte) instead of MII spindle.

NOTE: Oocyte with detectable MII spindle signal (grade A or B) are suitable for immediate ICSI.

3.9. Take a snapshot (F9) or record video for a report and/or subsequent image analysis. Keep documentation of image pattern to reduce subjectivity of the operator.

3.10. Move to the position of a next oocyte and repeat steps 3.6−3.9.

4. Optimizing ICSI timing

4.1. Transfer all spindle-positive oocytes (grade A or B, step 3.8) into the ICSI dish and subject them to ICSI according to standard protocols[24,25].

4.2. If the oocytes show no detectable MII spindle signal, place the PLM dish into the CO_2-independent incubator and shift the ICSI into a later time.

4.3. Perform PLM re-examination ~2−3 h later following steps 3.3−3.9. If some oocyte(s) still lack a MII spindle, further delay ICSI for additional 1−2 h.

4.4. Transfer all oocytes into the ICSI dish and inject them according to standard protocols[24]. If the PLM dish is compatible with the bending angle of the microinjection needle, perform ICSI immediately in the PLM dish after switching to a bright field mode.

4.5. Upon ICSI, transfer oocytes to prepared plates for embryo cultivation and culture until the blastocyst stage.

REPRESENTATIVE RESULTS:

Polarized light microscopy makes it possible to instantly inspect whether the oocyte completed nuclear maturation and assembled MII spindle prior to ICSI. Due to its non-invasive character, it can be used to safely assess the human egg's readiness for fertilization in clinical settings[11-14]. Spindled oocytes are more likely to give rise to viable embryos than oocytes without a spindle[9,15-23]. Also, oocytes featuring a distinct bipolar spindle appear to have a higher developmental competence than oocytes with dysmorphic spindles[9,21,22]. Taken together, spindle imaging can serve as a tool to identify the oocytes with the greatest potential to be successfully fertilized, undergo preimplantation development and support full-term pregnancy[9,15-23].

The reported incidence of the spindle varies (44−97%) reflecting the diversity of the studied population[9,15-23,26-28]. In vivo matured oocytes from normal responders typically show bipolar MII spindle at 40 h after hCG trigger[9,18,27,29]. However, in slow responders, the pool of oocytes retrieved from preovulatory follicles is affected by maturation delay[9,27]. Here, spindle imaging should be carried out to discriminate the oocytes arrested in the MII stage from those undergoing important maturational transition (**Figure 3**). We recommend the removal of follicular cells immediately upon retrieval, and the incubation of MI and MII oocytes in separate wells to be able to distinguish the In vivo matured oocytes and late-maturing oocytes extruding PB in vitro, shortly before ICSI. In case the denudation is performed just before ICSI, these two populations of oocytes are indistinguishable based on the PB appearance.

The phase-specific changes of the PLM pattern should be interpreted in the context of knowledge of the spindle dynamics during meiotic maturation. During the interkinesis, division apparatus undergoes extensive structural reorganization and PLM signal transiently disappears[1,9-11]. Thus, in developmentally delayed oocytes extruding PB in vitro, the absence of the MII spindle signal may not necessarily reflect cellular disturbance but it could indicate the oocyte's progression from MI to MII stage (**Figure 3**). If the sperm is injected at an unphysiological time point, the egg's developmental potential is compromised. Postponing ICSI provides late-maturing oocytes with more time to assemble an MII spindle whose presence is associated with better clinical results[9-11]. In the clinical study involving slow/poor responders, nearly 60% of initially spindle-negative oocytes developed spindle signal before PLM re-examination approximately 2 h later[9]. Depending on the developmental stage and overall fitness of late-maturing oocytes, the MII spindle appearance after PB extrusion typically takes 2−6 h (unpublished observation). The oocytes exhibiting a microtubule bridge (anaphase/telophase, grade D) during the first PLM examination require a longer time to develop MII spindle signal than oocytes with no visible spindle (emerging spindles, grade C). Individual adjustment of the time of ICSI to the egg's developmental stage prevents premature activation of the oocyte and results in an improved fertilization rate and successful embryo development[9].

Although late-maturing oocytes extruding PB in vitro generally have a lower developmental competence than In vivo matured oocytes, the rate of embryo development is acceptable if they manage to assemble a detectable spindle prior to delayed ICSI (blastulation rate of 41.32%)[9]. Using this approach, even immature oocytes, which are normally rejected for fertility treatment, can be clinically utilized, produce transferable embryos, and give rise to full-term pregnancies. Evaluation of the maturational stage of each individual oocyte is especially beneficial in poor

prognosis cycles yielding a small number of MII oocytes and/or after performing a rescue in vitro maturation of immature oocytes[9].

Apart from egg maturity assessment, spindle imaging is also recommended prior to PB biopsy in order to avoid spindle destruction in anaphase I/telophase oocytes. In experimental embryology, visualization of the meiotic spindle is used during egg enucleation and/or spindle transfer procedure[13].

FIGURE LEGENDS:

Figure 1: Human oocyte maturation. In vivo matured oocytes (MII) exhibit PB at the time of retrieval. Immature oocytes (GV, MI) can spontaneously complete maturation in vitro. Scale bar = 20 µm.

Figure 2: Oocyte classification based on PLM-detected pattern. Representative examples of oocyte grades (A-D) based on the appearance of the spindle signal. PLM-detected pattern: (**A**) prominent signal of bipolar spindle, (**B**) translucent apolar MII spindle spindle, (**C**) no detectable spindle, and (**D**) microtubule bridge. Scale bar = 20 µm.

Figure 3: Stages of MI to MII transition in oocyte maturation. The appearance of oocytes in bright field (top row), combined with a fluorescence signal of chromosomes (cyan) and microtubules (magenta) (middle row), and in polarized light (bottom row) is shown. Each oocyte was first PLM-examined and immediately fixed. Fixed oocytes were (immuno)labeled with Hoechst (DNA) and anti-α-tubulin antibody (microtubules). The yellow arrow indicates the presence of PB and the white arrow highlights the position of birefringent microtubules. Scale bar = 20 µm. This figure has been modified from Holubcová et al.[9].

Figure 4: Correlation of chromosome-microtubule organization with birefringence pattern detected by polarized light microscopy. The appearance of oocytes in the bright field combined with a fluorescent signal for chromosomes (cyan) and microtubules (magenta) (top row), and in polarized light (bottom row) is shown. Each oocyte was fixed immediately after PLM-examination. DNA (Hoechst) and microtubule (α-tubulin) were stained. (**A-C**) oocytes with PLM-detectable spindle yet misaligned chromosomes (**A**, **B**) or loosely focused spindle poles (**C**); (**D-F**) abnormal oocytes without PLM-detectable MII spindle showing underdeveloped (**D**), apolar (**E**) or no spindle (**F**). The white arrow highlights the position of birefringent microtubules, and asterisk (*) indicates the position of decondensed chromatin. Scale bar = 20 µm.

Figure 5: Birefringence structures in human oocytes. Representative examples of (**A-D**) birefringent structures detected by PLM in human oocytes. ZP = zona pellucida; PM = plasma membrane (oolema); RB = refractile bodies, vacuoles. White arrow, meiotic spindle in different stages of maturation. (**E**) MII spindle misalignment with polar body (PB). (**F**) Spindle detachment from plasma membrane. Scale bar = 20 µm.

Supplemental Figure 1: Desktop layout of the image analysis software.

Supplemental Video: PLM examination procedure. Rotation of the oocyte is required to exclude the presence of the spindle (arrow). Scale bar = 20 μm.

DISCUSSION:

The acentrosomal meiotic spindle in human oocytes is highly dynamic and a delicate structure[1]. Under suboptimal conditions, the microtubule fibers quickly depolymerize and meiotic spindle disassembles[30-33]. Hence, it is critically important to ensure that the oocyte culture and micromanipulation conditions, namely temperature and pH are in the optimal range. To minimize the risk of spindle disruption, the oocytes should be kept in a temperature-controlled environment maintaining 37 ± 0.5 °C in the oocyte media. Use of microscopes and microinjection setup equipped with a heated stage is strictly required. All manipulation with oocytes outside of the incubator must be carried out in HEPES/MOPS buffered media to avoid pH fluctulations. To avoid potential adverse effects of excessive oocyte handing in ambient condition, the total time of PLM examination should not exceed 10 minutes. The oocytes have to be analyzed on the glass bottom dish with the plastic lid removed because positioning of a standard plastic in the beam path impairs image analysis. Because plastic and glass bottom dishes have different thermal characteristics, the temperature should be checked directly in the droplets of handling medium in the examination dish.

In addition to laboratory conditions, procedural differences and operator's micromanipulation skills could have an impact on PLM examination accuracy. Only highly assembled bipolar spindles can be noninvasively visualized. Observed signal is proportional to the degree of structural organization of the spindle. Nascent, apolar, loosened or disarrayed spindles display only blurred birefringence or none at all (**Figure 3** and **Figure 4**). Besides, imperfect alignment of polarized light with microtubule arrays produces only a translucent and ill-defined birefringence despite the actual presence of well-developed bipolar spindle (**Figure 4**). Unless properly oriented, the spindle signal might be easily missed and oocyte maturity misdiagnosed (**Supplemental Video**). For optimal spindle imaging, the oocyte has to be properly turned around each axis. Since birefringence is not visible in eyepieces, the operator has to watch the computer screen while performing rotation of the oocyte. Previous experience with micromanipulation and training of spindle imaging in surplus in vitro matured oocytes is desirable before switching to clinical application.

Besides the spindle, the cumulus cells surrounding the oocyte, oolema, the inner layer of the zona pellucida and some cytoplasmic structures (e.g., refractile bodies, vacuoles) exhibit birefringence (**Figure 5A-D**). Unless thoroughly removed from the oocyte before imaging, tightly attached follicular cells produce background noise and compromise meiotic spindle detection. Be careful not to mistake a large refractile body for the spindle. Many cytoplasmic inclusions residing in the cytoplasm display high brightness which sharply contrasts with the dark background. Meiotic spindle, on the other hand, exhibit only moderate signal, blurred boundaries and typically attaches to the oolema beneath or adjacent to the first PB. Occasionally, PLM examination reveals a gross spindle deviation from its standard position (**Figure 5E,F**). If there is a major misalignment between the division apparatus and the PB, PLM helps to orient the oocyte to avoid the risk of

spindle injury during microinjection. The relative position of the spindle within the oocyte does not appear to influence the developmental potential of the resulting embryos[17,34]. However, when the spindle competely detaches from the oolema, fertilization abnormalities occur. Interestingly, some poor-quality oocytes undergo chromatin decondensation immediately after PB extrusion instead of starting microtubule nucleation (**Figure 4F**). Using conventional light microscopy and the PB as the only marker of oocyte maturity, such sub-competent oocytes would be considered as fertilizable. Thus, on top of the routine morphological assessment, spindle imaging adds important information about the egg's maturity and can serve as an indirect marker of its quality.

The incidence of spindled MII oocytes is likely to be affected by characteristics of the study population (e.g., genetic background, medical condition, maternal age). In addition, the proportion of developmentally delayed oocytes within the cohort will influence the actual number of detected spindle-negative oocytes[9,27]. Meiotic spindle visualization makes it possible to clearly identify fertilizable oocytes arrested in the MII stage. Furthermore, the second inspection at a later time may reveal whether the spindle-negative oocyte is abnormal or has only recently progressed through MI/MII transition[9-11]. When allowed to develop a spindle prior to ICSI, even immature oocytes, which are routinely discarded, can produce viable embryos[9]. In cycles with very few oocytes available for fertilization, finely-timed ICSI of oocytes extruding PB can serve as a rescue strategy and alternative to cycle cancellation.

Nevertheless, extending the preincubation time should not be generalized to all oocytes. In vivo matured oocytes from normal responders typically exhibit an MII spindle[9,20,21,27]. Here, the chance of successful spindle imaging decreases with time as a consequence of post-ovulatory in vitro aging[35]. If possible, ICSI should be performed on the day of retrieval and should not exceed 9 hours (45 hours post hCG), the period associated with a decline in resulting embryo quality[36,37]. Oocytes displaying distinct bipolar spindles ought to be subjected to ICSI without further delay. In summary, individualized optimization of ICSI timing is worthwhile in poor-prognosis patients to exclude any risk of premature sperm injection. However, it is unnecessary, too time-consuming and laborious to be performed in all IVF cycles.

PLM analysis reveals whether the oocyte reached the MII stage. However, noninvasive visualization of the meiotic spindle provides no information about chromosome organization. There might be severe chromosome misalignment and/or maternal age-related chromatid splitting in oocytes featuring a bipolar spindle (**Figure 5**). Various other factors have significant impact on reproduction success (e.g., sperm factor, mitochondria, embryonic genome activation, irregular cleavage, epigenetics, endometrium, maternal immunity). Therefore, detection of the MII spindle per se, does not guarantee a positive clinical outcome of the IVF procedure.

ACKNOWLEDGMENTS:
We thank the embryology laboratory of Reprofit International.

DISCLOSURES:
The authors have nothing to disclose.

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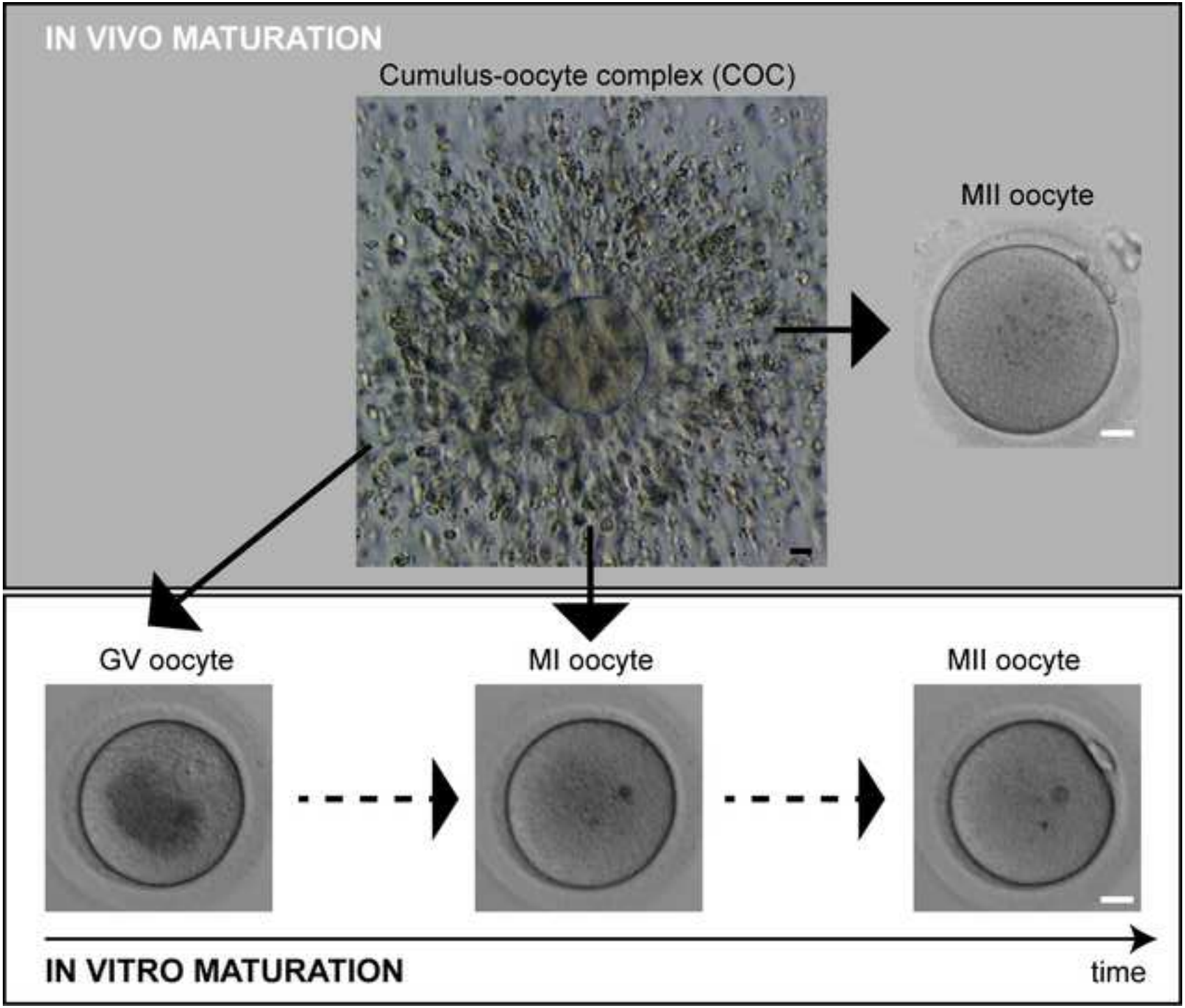
Figure



Figure

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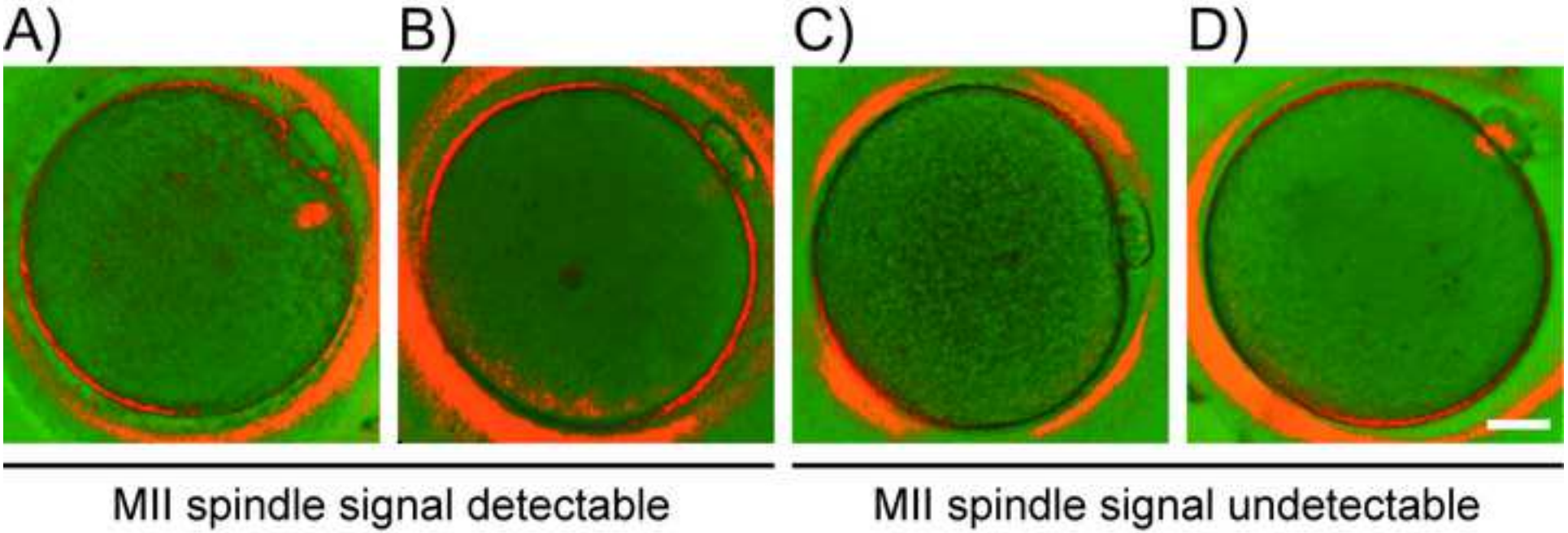

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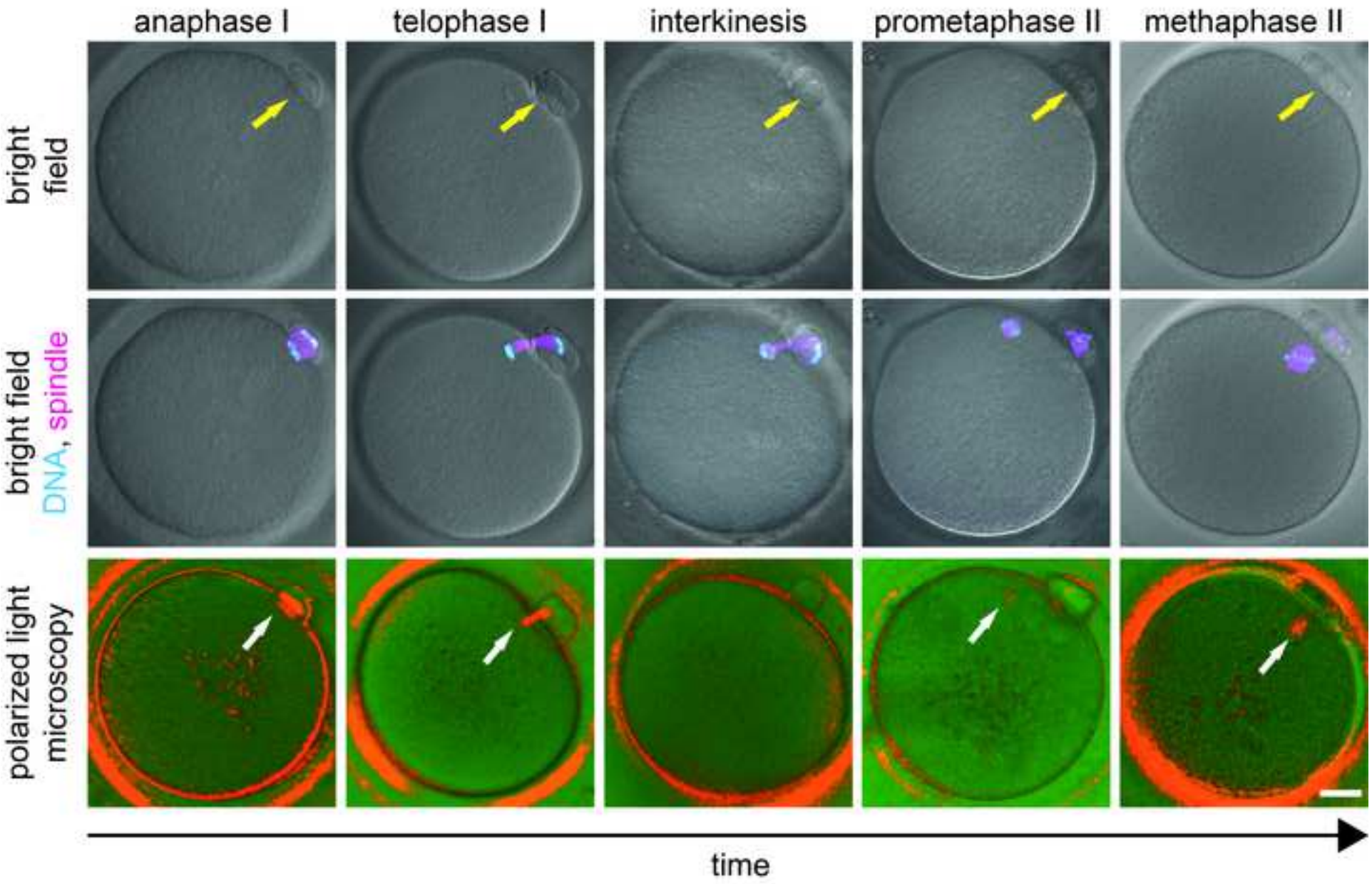

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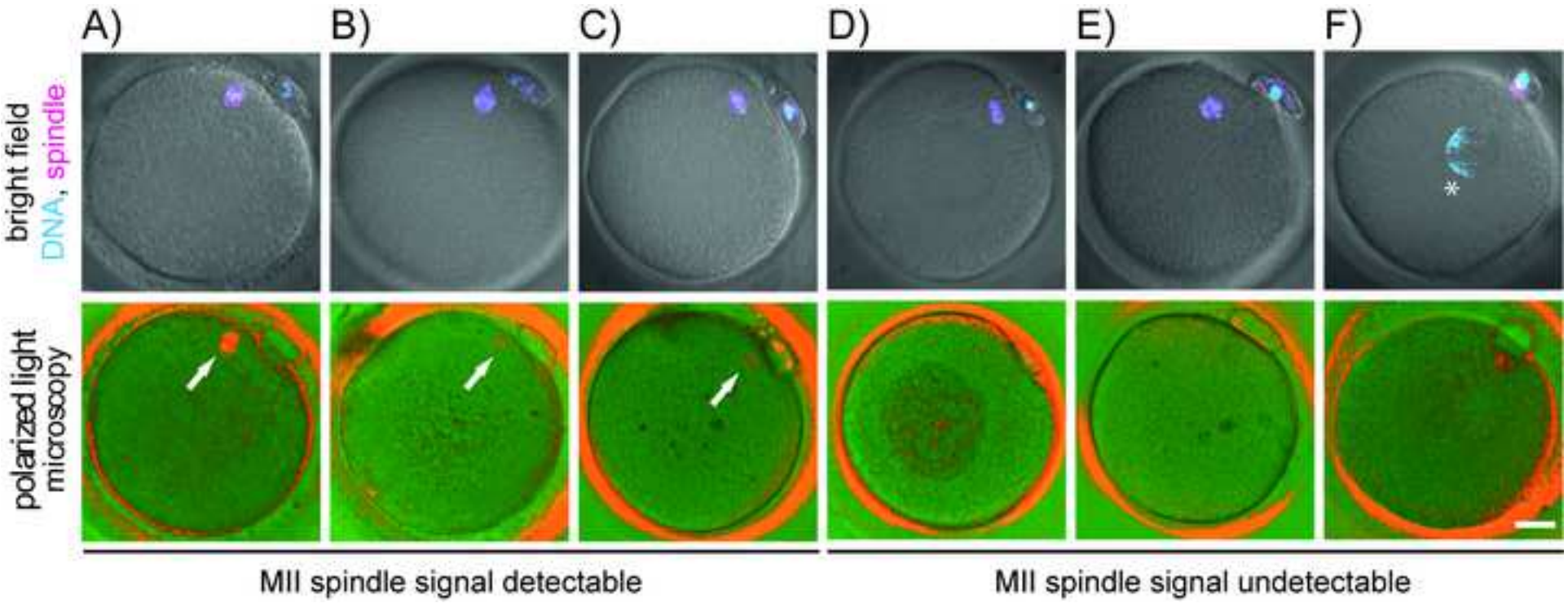

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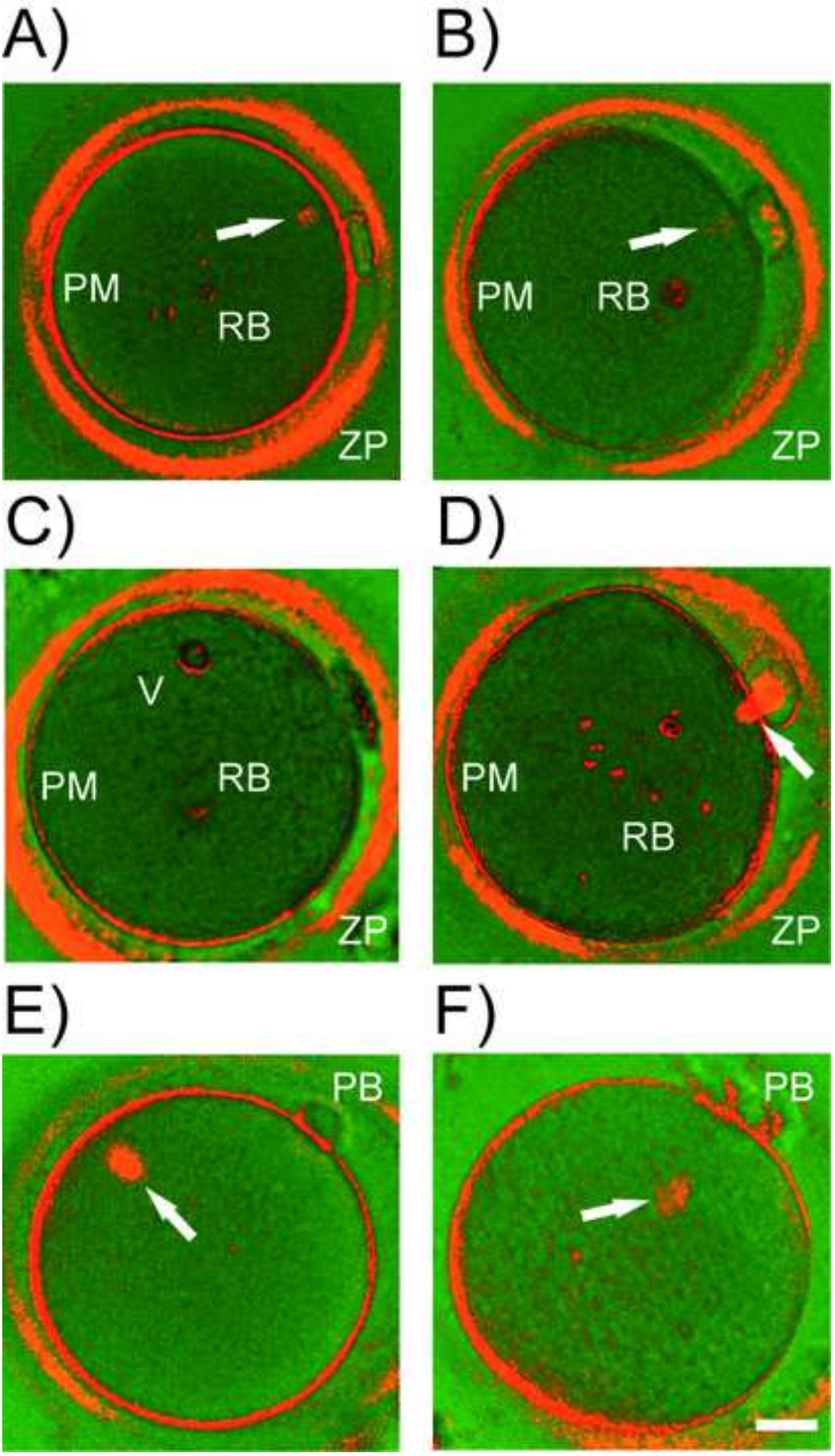

Supplemental Video

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Video or Animated Figure

Supplementary video.avi

Name of Material/Equipment		Company	Catalog Number
Continuous Single Culture Complete with Human Serum Albumin		Irvine Scientific	90164
Denuding micropipette	150 µm	Microtech IVF	005-150C
Denuding micropipette	180 µm	Microtech IVF	005-180B
Denuding micropipette	200 µm	Microtech IVF	005-250-A
FluoroDish		World Precision Instruments	FD 5040-100
Holding micropipette		Microtech	001-120-30
Hyaluronidase solution		Irvine Scientific	90101
ICSI micropipette		Microtech	002-5-30
Micro Droplet Culture Dish		Vitrolife	16003
Multipurpose **Handling Medium** (MHM) with Gentamicin		Irvine Scientific	90163
Nikon Eclipse TE 2000-U		Nikon	
Nunc IVF Petri Dish, 60 mm		Thermo Fisher Scientific	150270
Nunc non-treated 4-well IVF dish		Thermo Fisher Scientific	179830
OCTAX polarAide		MTG	
Oil for embryo culture		Irvine Scientific	9305
Polyvinylpyrrolidone		Irvine Scientific	90123

Comments/Description
bicarbonate based single-step **culture medium** for embryo culture
suitable for oocyte transfer between dishes
suitable for oocyte transfer between dishes
glass-bottom dish (alternative: WillCo-dish GWST-5040 WillCo Wells)
sterile glass microneedles for oocyte denudation
sterile glass microneedles
12-well plate for embryo culture
handling medium, MOPS/HEPES buffered
inverted microscope with heated stage
plastic ICSI dish
4-well plate for embryo culture
integrated PLM system
oil for overlay
for sperm immobilization prior to ICSI

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Title of Article: HUMAN EGG MATURITY ASSESSMENT AND ITS CLINICAL APPLICATION

Author(s): ZUZANA HOLUBCOVÁ, DRAHOMÍRA KYJOVSKÁ, MARTINA MARTONOVÁ, DARJA PAŘÍZKOVÁ, TEREZA KLENKOVÁ, SOŇA KLOUDOVÁ

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April 30, 2019

Dear Editor, Dr. Xioyan Cao,

On behalf of my co-authors, I thank you for the opportunity to revise and resubmit our manuscript entitled "**Human egg maturity assessment and its clinical application**" for publication in *JoVE*.

Thank you for the thorough review and the detailed comments provided by you and the selected peer reviewers. We have incorporated the suggested changes into the manuscript, which has benefited from these insightful revision suggestions.

Please find our specific answers to each of your questions and comments in italics below the corresponding comment.

Sincerely,
Dr. Zuzana Holubcová

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The revised manuscript has been proofread by several readers including a native (U.S.) speaker.

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Explicit copyright permission is being submitted along with the revised manuscript. It contains both the letter from the editor and the link to the editorial policy statement regarding previously published article. The Figure legend for the Figure 3 has been amended in compliance to editor´s request.

3. Please revise lines 76-77 to avoid previously published text.

The sentence has been rephrased.

4. Please use the micro symbol µ instead of u.

5. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

6. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., H2O,

7. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
8. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.”

- 4-8: requested corrections have been made.

9. Please provide the composition of handling medium/cultivation medium, etc. If it is purchased, please cite the Table of Materials.

Both media are commercially supplied. The 'cultivation medium' has been renamed 'culture medium' as requested by Reviewer #3. Citation of the Table of Material has been added at the point of the first use of the term.

10. 2.2: Please break into sup-steps.

Step 2.2 represents one action – preparation of ICSI dish - which is necessary to be performed in one go. Small droplets must be covered by oil immediately to avoid evaporation. Splitting the text into several sub-steps would increase the risk of undesirable delay and would be in conflict with standard laboratory practice in IVF settings. The authors would prefer to keep step 2.2 in one paragraph.

11. 3.4, 3.8: Please write the text in the imperative tense or include it as “NOTE”.

Amendments have been made.

12. Please remove the embedded figure between lines 162 and 167 from the manuscript and upload it separately to your Editorial Manager account.

Figure submitted separately (“desktop layout.jpg“).

13. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Short steps described in 2.4.2. to 2.4.7 were combined in 2.4.2 to 2.4.4

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14. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

Amendments have been made.

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Critical steps of the protocol have been highlighted.

18. Figure 4: Please include a scale bar. Please describe panels A-F in the figure legend.

The missing scale bar has been added and figure legend extended.

19. Please describe Figure 4 and Figure 5 in the representative results section.

Figure 4 and Figure 5 are referenced in Discussion section as they are related to assessment interpretation and troubleshooting.

20. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

The discussion has been restructured based on editors and reviewer #2 comments. It consists of 6 paragraphs covering:

a) Critical steps within the protocol – paragraphs 1 and 2
b) Any modifications and troubleshooting of the technique – paragraphs 2 and 3
c) Any limitations of the technique – paragraph 6
d) The significance with respect to existing methods – paragraphs 4 and 5
e) Any future applications of the technique – paragraph 4

21. References: Please do not abbreviate journal titles.

Full names of journals are shown.

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Reviewers' comments:

Reviewer #1:
The manuscript "Human egg maturity assessment and its clinical application" by Holubcová et al., aims at describing a standard protocol to perform human egg maturity assessment in a clinical laboratory by noninvasive spindle imaging using polarized light microscopy that allows quick and easy inspection of whether the PB-displaying oocyte actually reassembled a meiotic spindle prior to ICSI.
The manuscript is very well set up and illustrates in detail and effectively, step by step, the procedures for the characterization of the meiotic spindle in the mature oocyte. This procedure has important practical implications since it addresses one of the delicate aspects in the selection and manipulation procedures of oocytes subjected to IVF cycles. This aspect reflects on the effectiveness of assisted reproduction techniques and, above all, on the health of patients who are subjected to hormonal treatments at each IVF cycle.

Title and abstract are appropriate. The authors discuss the potential application of this approach and the procedures described guarantee the achievement of the indicated results.
The procedure is clearly explained, and the critical steps are highlighted with insights when appropriate.

Minor
Since these procedures must be conducted by highly experienced personnel, it is recommended to indicate reference to IVF standard laboratory procedures, manuals and guidelines. The inclusion of these additional information would be useful.

The reference to standard IVF protocols has been provided in the original manuscript (reference 3 and 27). In the revised version, we have added additional information at the very beginning of the protocol section including relevant citations of current clinical guidelines and routine IVF protocols.

Reviewer #2:
Manuscript Summary:
The manuscript JoVE60058 describes the protocol for microscopy of oocytes to check their maturity. The authors used the old technique of polarized light microscopy for observation of oocyte's meiotic spindle. In their previous study, they have shown that the extrusion of polar body in human oocytes is not a reliable indicator of oocyte's maturity. Many such oocytes have not completed the assembly of second meiotic spindle yet and are frequently prematurely injected with a sperm. In this and their previous study the authors tried to determine the optimal time of sperm injection into the oocyte with respect to the meiotic spindle assembly.

Although the authors' previous study has already described the technique, this video paper could clarify some technical details that are important to use the technique successfully in routine practice.

Dr. Zuzana Holubcova | Research Scientist | Masaryk University, Faculty of Medicine, Department of Histology and Embryology | Kamenice 3, building A1 | CZ-62500 Brno | zholub@med.muni.cz, www.med.muni.cz/histology/zuzana-holubcova/

Major Concerns:

Page 2, line 113: Write the COCs incubation time from OPU to cumulus cells removal. It seems this was done soon after OPU.

The revised version of the manuscripts states the time between OPU and oocyte denudation in step 1.2 of the protocol (10-15 minutes).

Page 2, line 128: Why were COCs denuded 3-4 hours before ICSI? In ESHRE guidelines for good practice in IVF laboratories (2015) it is writen: Since denuded oocytes are more vulnerable to pH changes, the timing of denudation should be kept close to the timing of injection. The authors should explain why did they use different timing.

The authors are aware of the recommendation of ESHRE 2015 guidelines. However, we would like to point out that the timing of denudation/ICSI remains a subject of controversy in the ART field. There is currently no general consensus between IVF clinics on optimal time of cumulus cells removal and ICSI. Multiple studies attempted to address the impact of timing of these key procedures but the data reported in the literature is conflicting. Some studies reported the beneficial effects of preincubation of intact COCs (e.g. Hassan 2001, Zhang et al 2012, Mizuno et al, 2018) while others found no influence of the timing of oocyte denudation on ICSI cycle outcome (e.g. Van de Velde et al., 1998; Yanakida et al 1998, Barcena et al 2016, Naji et al., 2018). Some clinics favour keeping oocytes in COCs until the time of ICSI in the belief that the oocyte can take advantage of the interaction with the somatic cell compartment. However, the role of the cumulus-corona in the final stage of oocyte maturation is uncertain, since gap-junctions, ensuring communication between the developing oocyte and its surrounding cells, are already closed when meiosis is resumed. The necessity of cumulus cells in maintaining the right pH is also questionable since MOPS/HEPES buffered media has been evidenced to maintain a stable pH that is optimal for oocyte in vitro culture. On the contrary, results of murine studies suggest that the presence of cumulus cells could induce apoptotic changes and accelerate oocyte aging in vitro (Kong et al 2018, Zhu et al 2015, Miao et al 2005). Hence, more well-controlled studies are needed before a generally accepted conclusion on this issue can be drawn.

Based on our experience, the early oocyte denudation does not compromise the success rates of fertility treatment. In our previously published study (Holubcova et al 2019), oocyte denudation undertaken shortly after OPU allowed us to distinguish between 1) in vivo matured MII oocytes and 2) MI oocytes which extruded PB in vitro (MI/MII) shortly before ICSI. Our data confirmed previous findings that the two subpopulations differ in the incidence of PLM-detectable MII spindle and their developmental potential (Rienzi et al., 2003). In case the denudation is performed just before ICSI, MII and MI/MII oocytes are indistinguishable based on PB appearance. The information about the number of in vivo matured (MII) oocytes is pivotal for making the decision whether to perform PLM examination or not. For instance, if there are 8 MII oocytes available at the time of retrieval, in our opinion, the PLM examination is not necessary. But if only (up to) 5 out of 8 oocytes exhibit a PB and some oocytes extrude PB in vitro it is likely that the whole pool of oocytes is delayed in maturation. Particularly the patients with a low number of in vivo matured oocytes available for ICSI can benefit from for egg maturity assessment the most. Our approach is explained in the second paragraph of the results section (lines 295-302) as well as in discussion section (paragraphs 4 and 5, lines 429-448) Nevertheless, in principle, the egg maturity assessment described here could be carried out even upon late denudation.

Page 5, line 242: The authors should comment and cite the following studies:
Mizuno et al. (2018). The timing of cumulus cell removal for intracytoplasmic sperm injection influences the capability of embryonic development. Reprod Med Biol. Nov 20;18(1):111-117.

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Pujol et al. (2018). Is there a relation between the time to ICSI and the reproductive outcomes? Hum Reprod May 1;33(5):797-806.

Mizuno et al (2018) reported significantly higher developmental competence of oocytes which were kept in intact follicles during the preincubation period when compared to oocytes denuded soon after OPU. However, this is a small prospective study, comparing only 21 vs. 33 IVF treatment cycles. Moreover, there are two recent large-scale studies (Barcena et al 2016, Naji et al 2018) which found no effect of denudation to ICSI timing on IVF outcomes. Both latter studies used a large data set (3986 and 2051 cycles, respectively) collected using operator-independent, automated, tracking and evaluated the data with advanced statistical methods with respect to confounding factors.

The study by Pujol and colleagues suggests limiting the time of incubation of mature oocytes to avoid the detrimental effect of oocyte aging in vitro (reference 34 in the first version of manuscript, 36 in revised version). However, the linear regression presented in this paper shows that the likelihood of achieving the pregnancy/live birth declines only after an extended cultivation period (~9 hours). These results are representative for the unselected IVF population, while we are specifically focusing on slow/poor responders with a low number of in vivo matured eggs and a large proportion of late-maturing oocytes. Here, postponement of ICSI is beneficial allowing oocytes to complete maturation as demonstrated in our previous study.

The current manuscript does not provide a complete overview of the studies addressing the denudation/ICSI timing. A separate review paper would be appropriate to cover this hotly-debated topic. Following editorial instruction, we have designed this manuscript as purely methodological, focusing on the technique of non-invasive spindle imaging and optimizing the time of ICSI with respect to the developmental stage of the oocyte.

Minor Concerns:
Page 2, line 108: The authors should write at what follicle size was the ovulation triggered and what was the dose of hCG?

Information added in step 1.1 of the protocol.

Page 3, line 135: To get the right pH, the Petri dish with culture medium and oil should be preincubated for 5-6 hours. Two hours is not enough.

We thank the reviewer for this critical note. We fully agree that incubating in a CO_2 atmosphere for only two hours only would not be sufficient to achieve the optimal pH. In the revised manuscript (step 2.1), it has not been emphasized that both mineral oil and culture medium have to be prequilibrated in CO_2 incubator overnight. In addition, the prepared culture dish should be kept in the CO2 incubator for an additional 2 hours to ensure that the temperature and pH are in an optimal range.

Page 4, line 188 and page 6, line 297: Zona Pellucida should be written with small letters.

Corrections have been made throughout the manuscript.

Page 5, line 222: Insert a special warning that the drop in the temperature during manipulation of oocytes outside incubator can cause a depolarization of microtubules and meiotic spindle disassembly.

The importance of temperature and pH control during oocyte micromanipulation has been highlighted as a critical step of the protocol. Not only transient cooling (Wang et al 2001) but also

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overheating (Sun et al 2004) causes disruption to the meiotic spindle in human oocytes. We have added the latter reference and further emphasized the importance of optimizing temperature and pH control during oocyte micromanipulation in the first paragraph of discussion and in step 2.4.1 of the protocol.

Reviewer #3:

Manuscript Summary:

In the manuscript entitled "Human egg maturity assessment and its clinical application", Holubcová et al describe in detail a protocol for assessing the presence of a meiotic spindle in human oocytes retrieved for assisted reproduction using polarized light microscopy. They provide examples of oocytes where the spindle cannot be seen, and also of others that contain an abnormal spindle. They also propose the steps to follow for thorough determination of oocyte quality, including utilizing oocytes that are delayed in meiotic maturation at the time of retrieval.

Major Concerns:
I do not have any major concern.

Minor Concerns:

- The metaphase II egg is actually not haploid. It will be haploid only after completing meiosis II. So, I suggest removing the terms haploid and diploid from line 50.

By definition, the haploid cell contains a single set of chromosomes. In meiosis I, one set of homologous chromosomes of the diploid MI oocyte is eliminated into a polar body. Hence, MII oocyte bearing a single set of chromosomes fulfills the definition of a haploid cell. Each of its 23 chromosomes (haploid set in humans) is composed of two chromatids which are being segregated only when meiosis II is resumed by activating factors delivered by the sperm. Similarly, a normal somatic cell is still called diploid even if each of its 46 chromosomes is composed of two chromatids in G2 stage of the cell cycle. According to multiple sources (e.g. Junqueira´s Basic Histology 15th Ed (2018) or https://www.nature.com/scitable/definition/haploid-309), the term haploid is commonly used for an unfertilized egg.

- Please provide information about the polarized microscope used in your studies.

The authors followed the editorial instructions for the authors requesting to avoid company brand names in the manuscript text. Instead, the type of spindle imaging system, as well as type of inverted microscope, have been stated in Table of Materials. Besides, the type of polarized light microscopy system in now stated in the note immediately at the end of the protocol part (line 275).

- What is the difference between Figs 3 and 4?

Figure 3 illustrates MI to MII transition. Bright field/ PLM/immunofluorescence images of the same oocyte, each representing different timepoint of the final stage of oocyte maturation, are shown.

Figure 4 is shown to remind the reader about limitations of spindle imaging which are discussed in the text e.g. presence of the spindle but severe chromosome misalignment (A ,B) or loose spindle poles (C), actual spindle/chromatin configuration in oocytes with no PLM-detectable spindle (D-F). A detailed description of examples patterns in Figure 4 has been added to the figure legend.

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- Please clarify what is green and what is red in the polarized microscopy images.

The explanation that green is the background and red represents the birefringence signal has been included into steps 2.5.4 and 3.4 of the protocol.

- The full name of metaphase II should be indicated in line 36, which is the first time the MII abbreviation is used.
  Line 93: PLM-navigated: I'd change it to PLM-aided
  Please replace cultivation with culture in several parts of the manuscript.
  Line 139: sperm, not sperms
  Line 185: rephrase to "using the microscope's light intensity knob."
  Last word of line 229: than (not then)
  Line 276: translucent apolar MII spindle.

All requested corrections have been made. We thank the reviewer for critical reading.

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Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);desktop layout.jpg

Title: Egg maturity assessment prior to ICSI prevents premature fertilization of late-maturing oocytes

Author: Zuzana Holubcová, Drahomíra Kyjovská, Martina Martonová et al

Publication: Journal of Assisted Reproduction and Genetics

Publisher: Springer Nature

Date: Jan 1, 2019

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