

Birefringence imaging of spermatozoa, spindle and zona pellucida

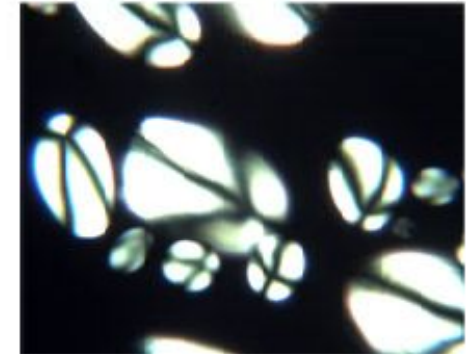
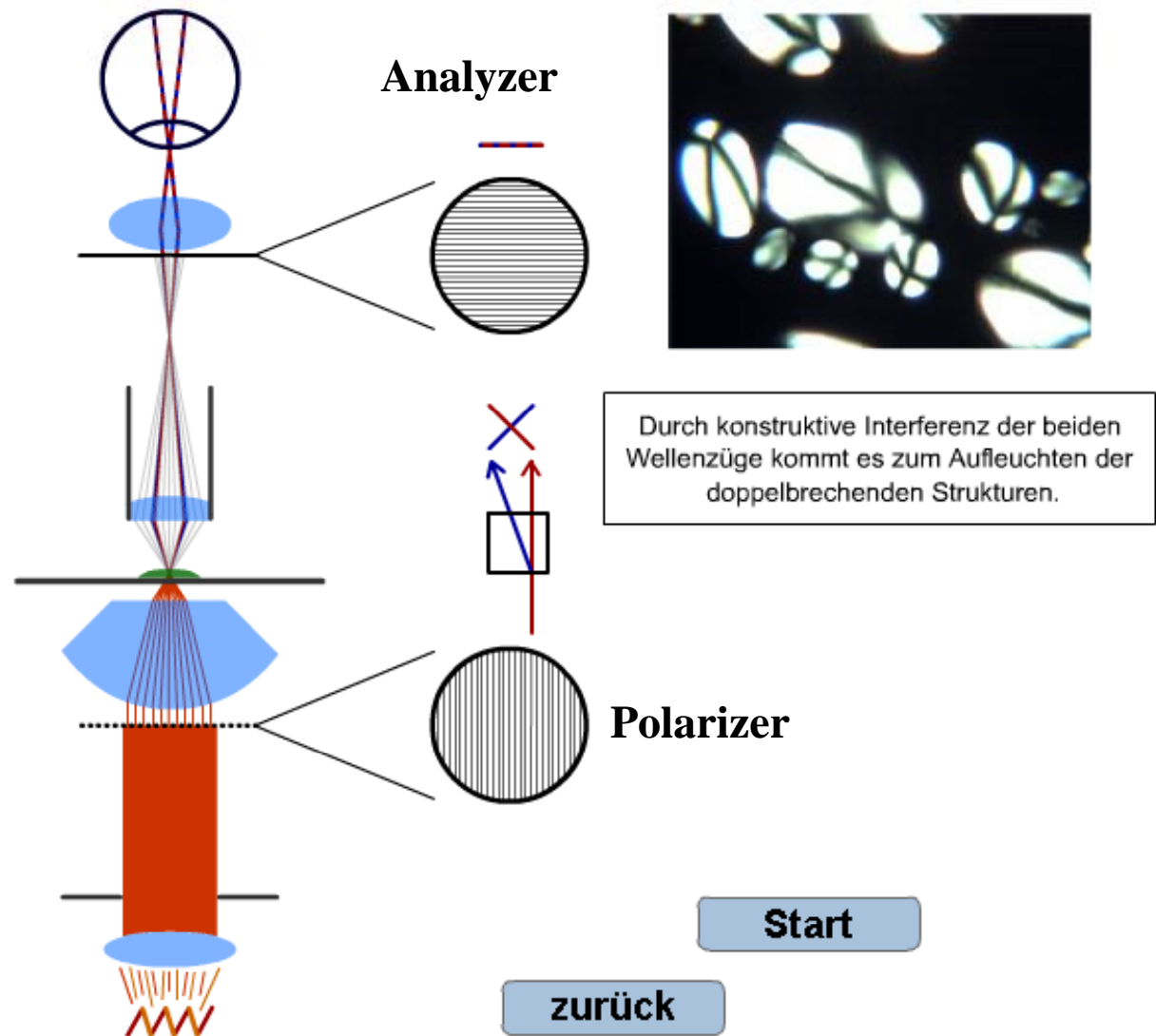
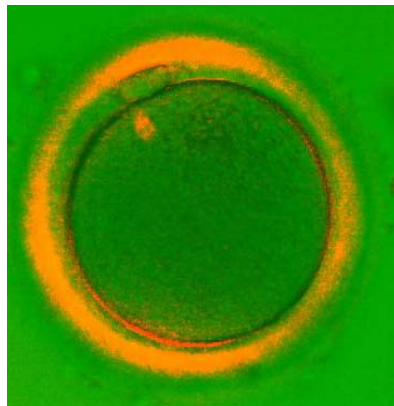
**Markus Montag
Dept. Gynecological Endocrinology & Reproductive Medicine
University of Bonn, Germany**

History of polarization microscopy

- 1808: Malus discovers „Polarization“
- 1815 / 1852: Brewster's law / Stokes parameter are basics for the application of polarization
- 1834: first commercial polarization microscope
- 1875: Engelmann discovers birefringence in the sperm from frog
- 1924: Schmidt describes cytoskeletal elements
- 1953-1981: Inoué & Allen publish various ground breaking articles on microtubules and spindle

Principle of polarization microscopy

- Shows structures with birefringent properties



Durch konstruktive Interferenz der beiden Wellenzüge kommt es zum Aufleuchten der doppelbrechenden Strukturen.

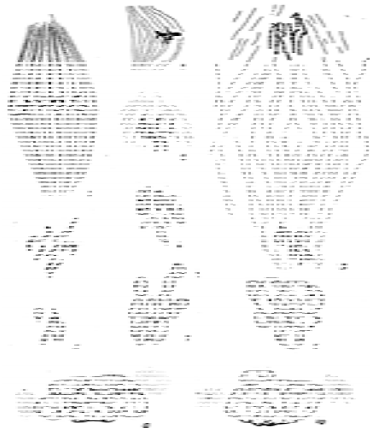
Birefringence imaging in sperm

- First report on birefringence of the sperm tail by Engelmann, 1875
- Negative birefringence of the sperm head related to chromatin orientation (Schmidt, 1924, 1937; Patti 1932)
- Inoué was the first to show that polarization microscopy can be used to identify acrosome-reacted spermatozoa (1981), further refined by Baccetti, 2004

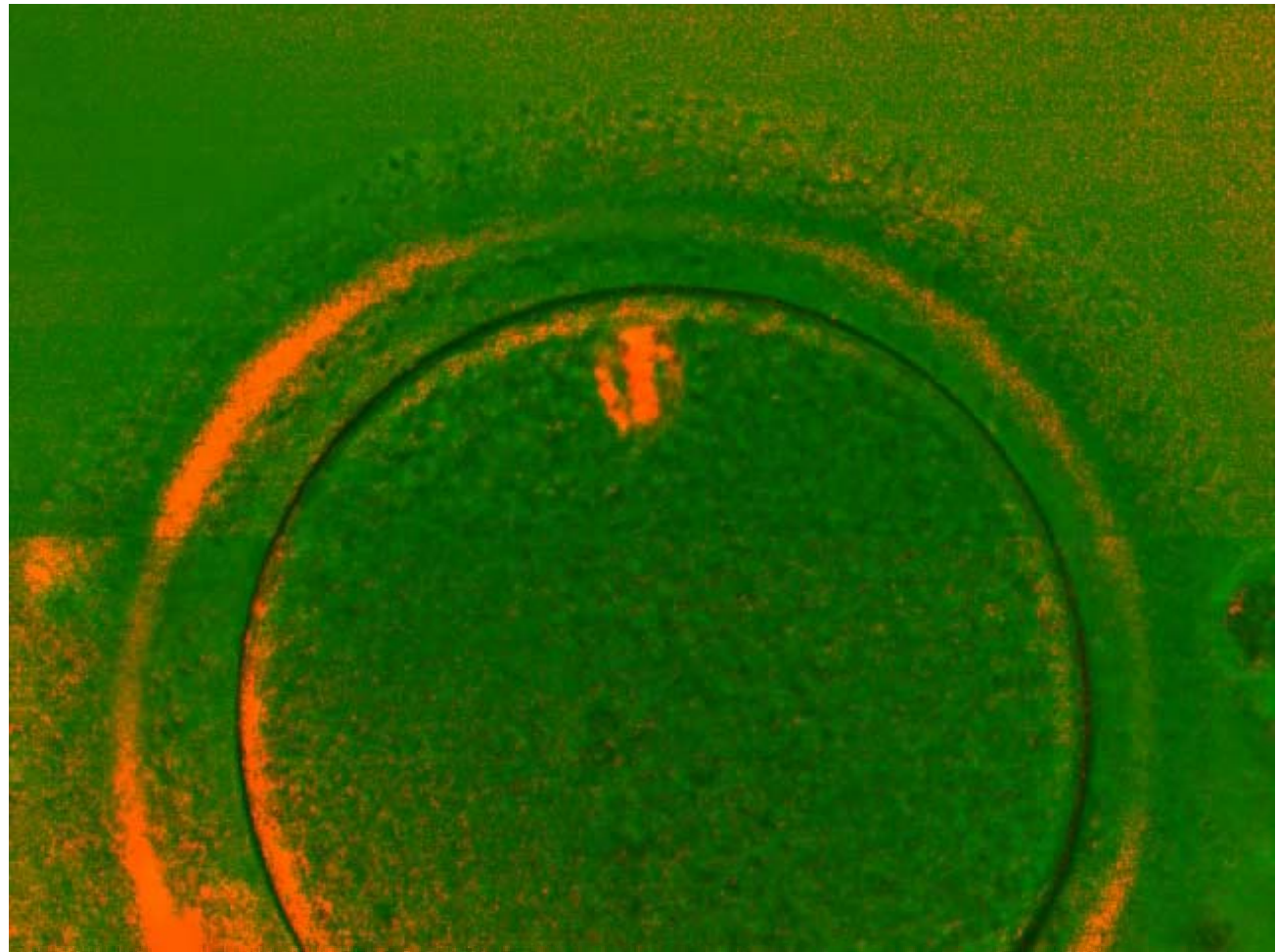
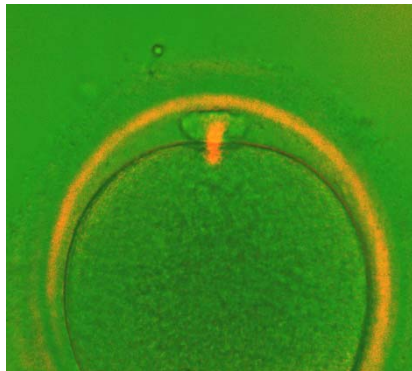
Sperm birefringence as a selection criterion in ART

- Gianaroli et al., 2008 a/b:
 - Use of acrosome-reacted spermatozoa (identified by polarization microscopy) raises implantation and pregnancy rates for severe Oat and testicular spermatozoa
- Boudjema et al., 2009:
 - Sperm birefringence is more likely to characterize spermatozoa with normal morphology
- Crippa et al., 2009:
 - Sperm birefringence does increase the likelihood of DNA strand integrity (less DNA fragmentation)

Birefringence imaging in oocytes



Richards, Biol Bull 1917

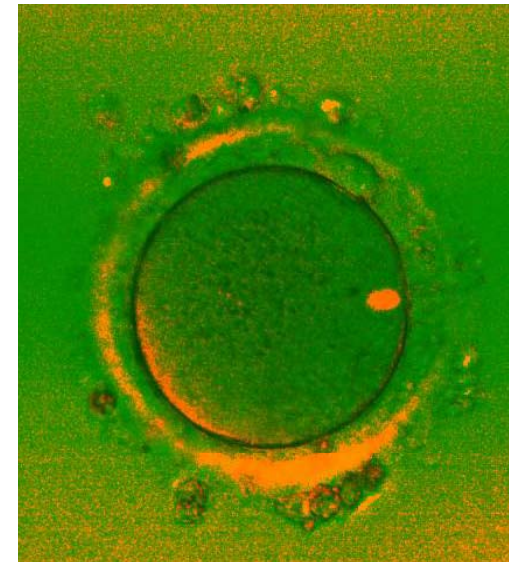


Polarization microscopy for detecting spindle and zona birefringence in oocytes:
- Spindle imaging / Zona imaging -

Where is the spindle

- **Spindle at ICSI**

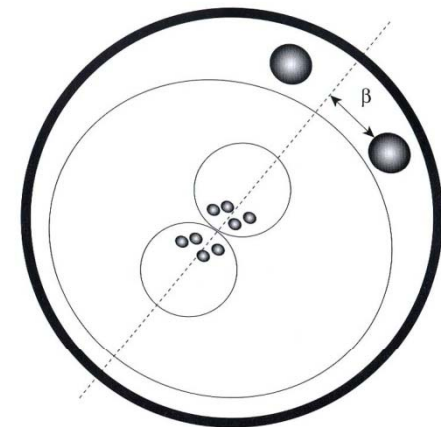
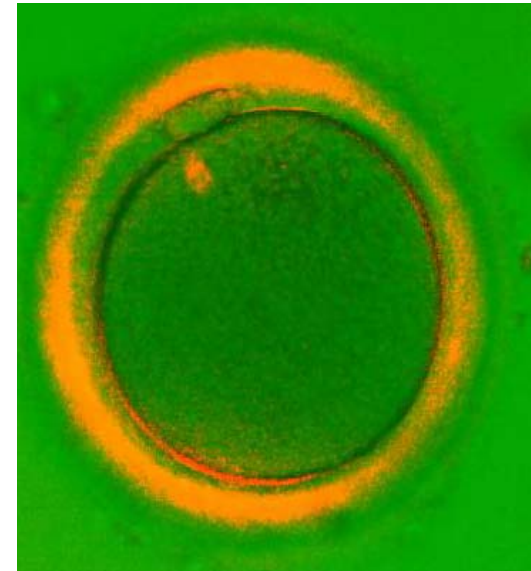
- First report that spindle is not always where it should be: Silva et al., 1999
- Report that visualization of the spindle position has no benefit at all: Woodward et al., 2008



Where is the spindle

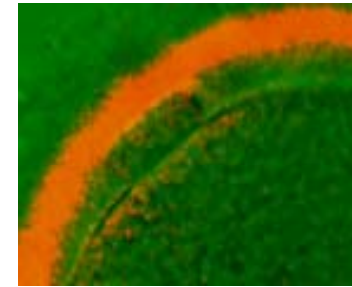
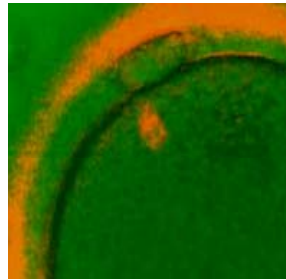
- **Spindle- / polar body-relation**

- Rienzi et al. 2003
 - Negative effect of fertilization if PB and spindle are dislocated, but no effect on embryo development
- Cooke et al., 2003
 - Positive effect if PB and spindle are not dislocated on embryo development
- Taylor et al., 2008
 - Dislocated PB are the result of a too harsh pipetting mode during hyaluronidase preparation
- Polar bodies do move: Scott, 2008



What if we cannot see the spindle

No. of publications on the prognostic value



Spindle visible

Spindle not visible

Fertilization

4 x ↑

2 x Ø

Embryo dev. day 3

5 x ↑

2 x Ø

Blastocyste rate

2 x ↑

Preg.-/Impl.-rate

1 x ↑

1 x Ø

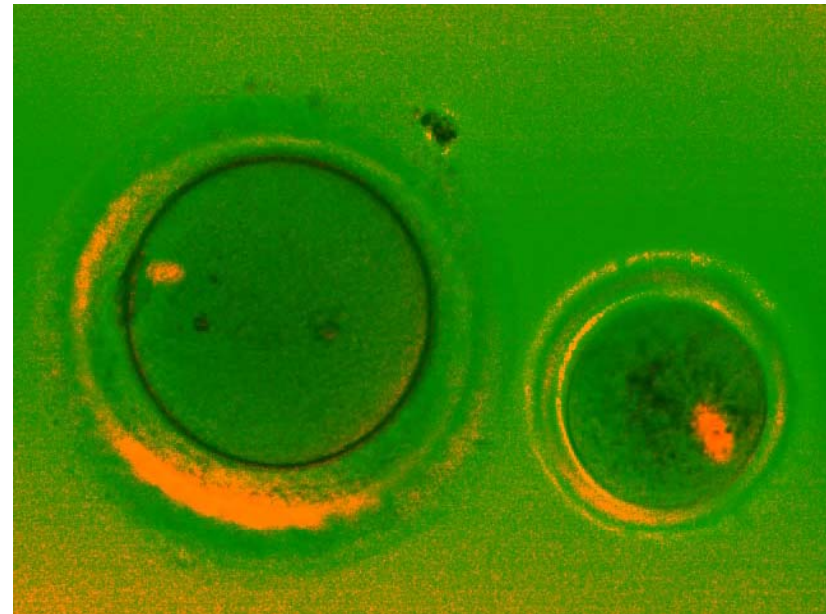
More information needed: Petersen et al., 2009 RBMOnline

When is the spindle visible

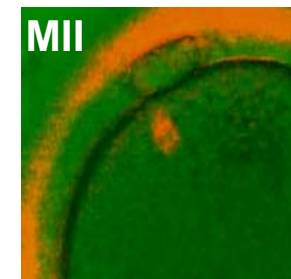
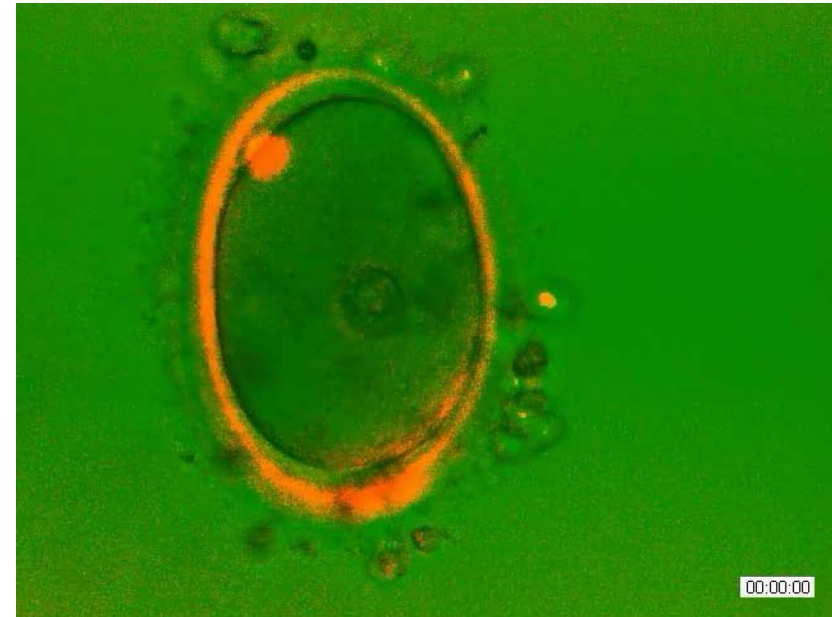
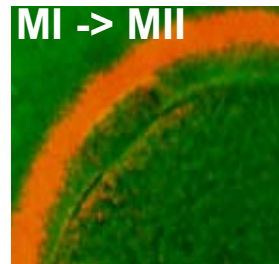
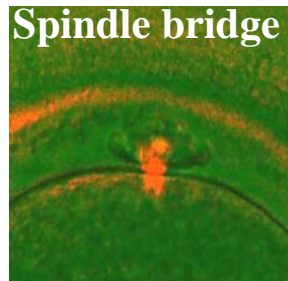
Temperature

- Critical temperature:
33°C
- Culture medium
- Heated surfaces:
 - Stereo microscope
 - Injection microscope
- Incubator

pH of the medium



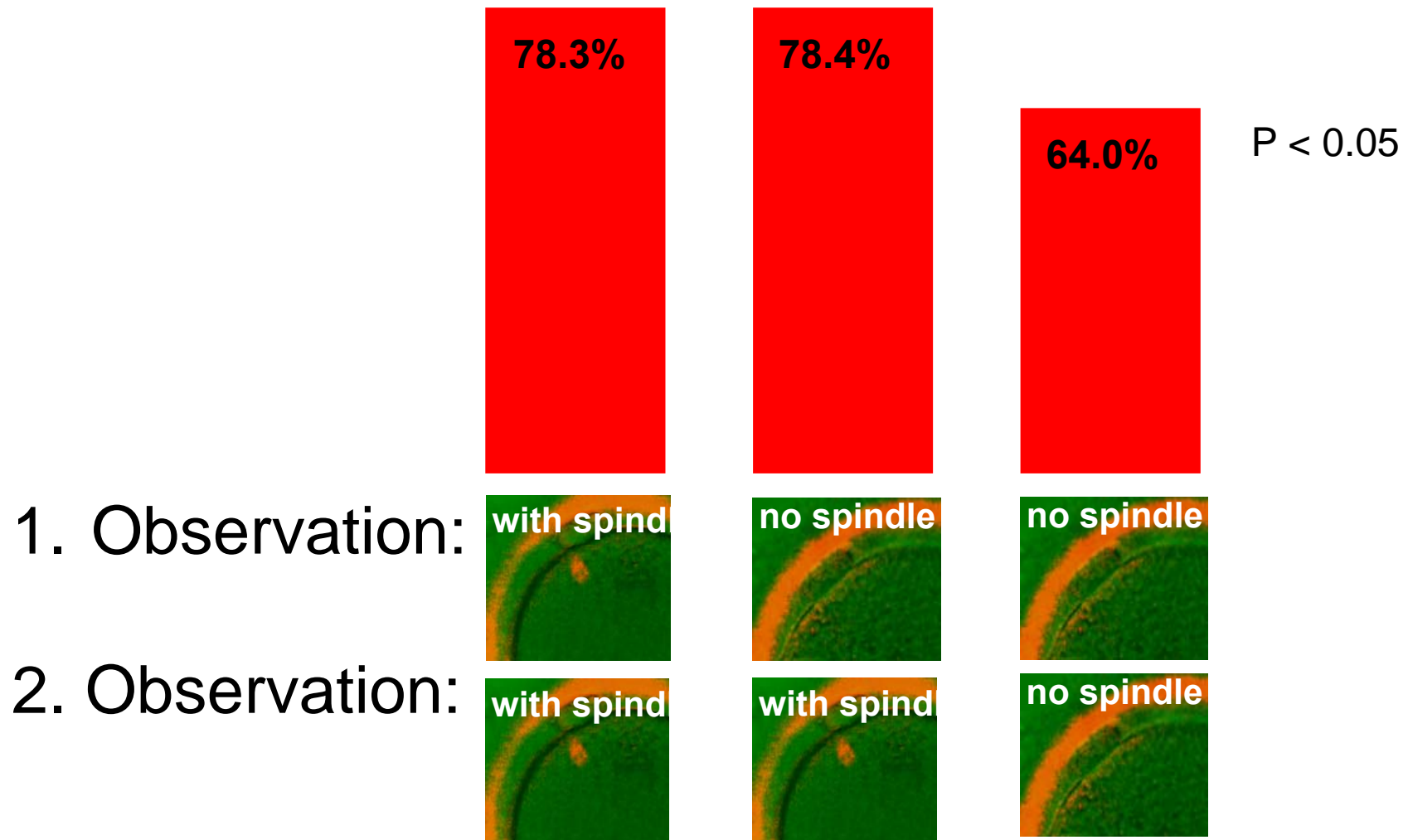
Course of the meiotic cell cycle



Cave: Different timing in oocytes from IVM / unstimulated cycles

Presence of the spindle and fertilization rates

Fertilization rates



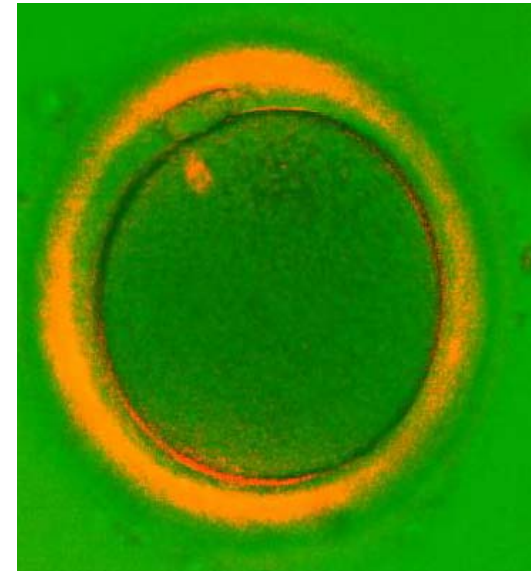
Summary spindle imaging

- **Spindle-Imaging**

- Localisation during ICSI (Silva et al. 1999)
- Enucleation (Liu et al. 2000)
- Spindle-/polar body relation (Rienzi et al. 2003)
- Chromatin aberration (Wang et al. 2003)

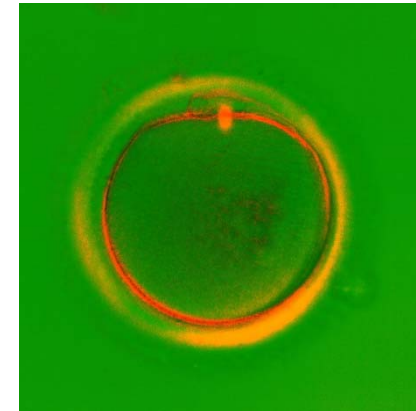
- **Spindle dynamics**

- Temperature sensitivity (Wang et al. 2001)
- Meiotic cell cycle (Montag et al. 2006)



Spindle imaging – conclusions

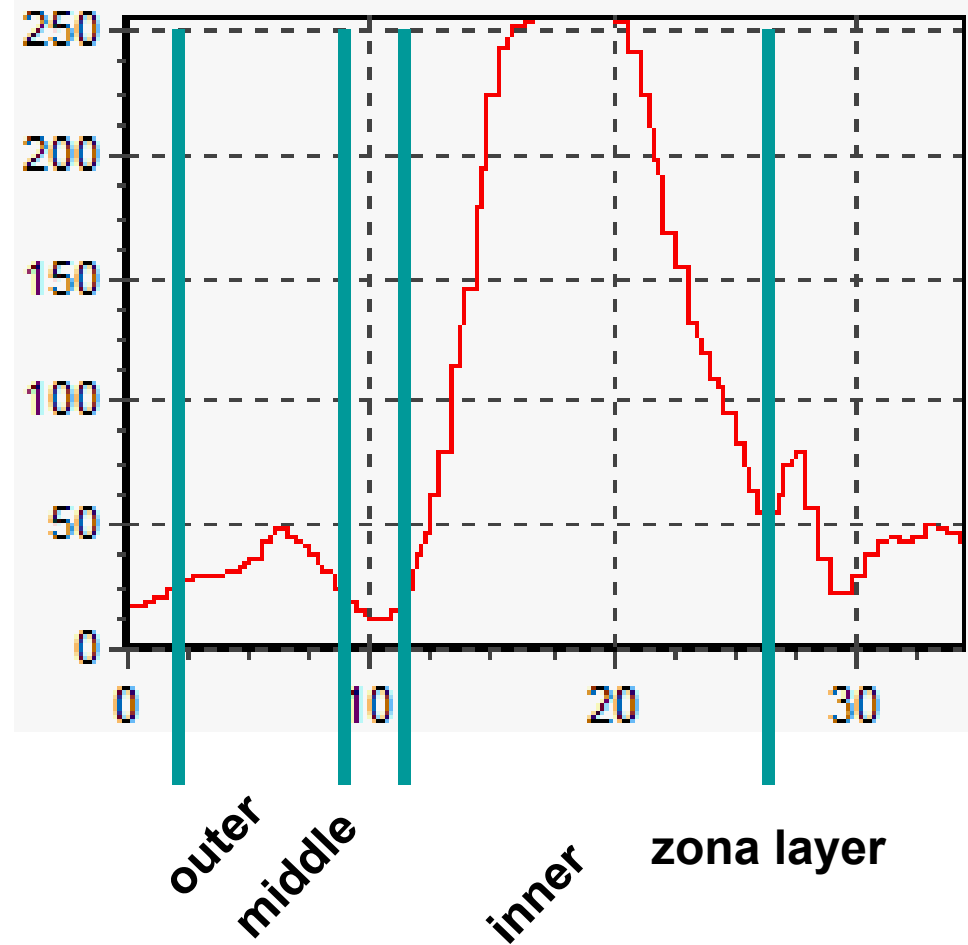
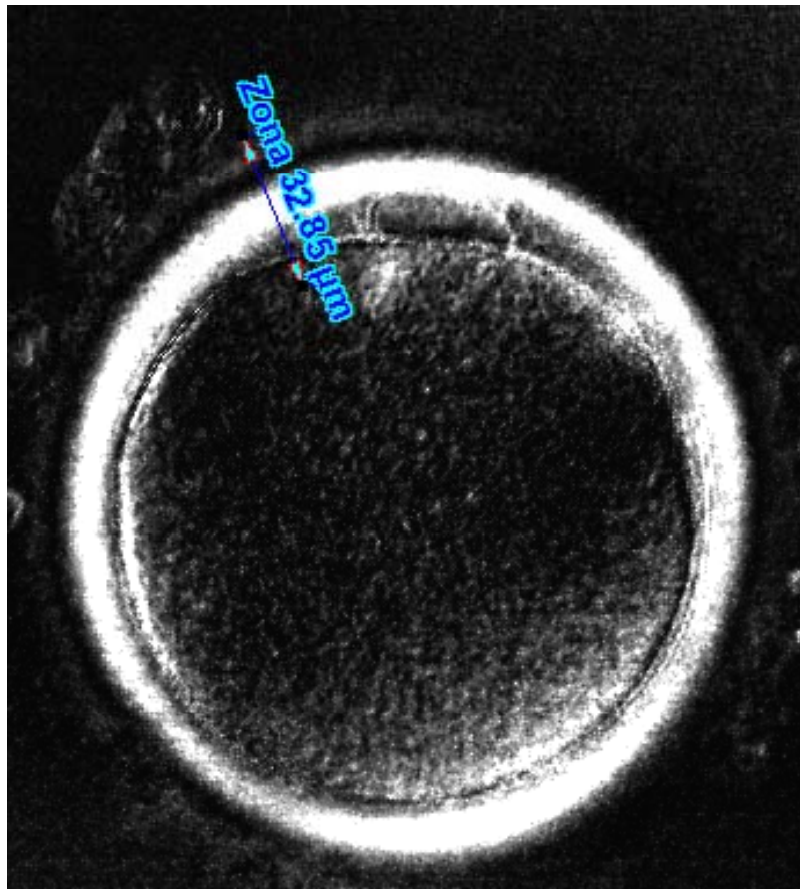
- The benefit of spindle imaging is:
 - To get better insight into the meiotic time course
 - To determine oocyte maturity
 - To allow for better timing of ICSI
 - To avoid oocytes with 3PN after ICSI



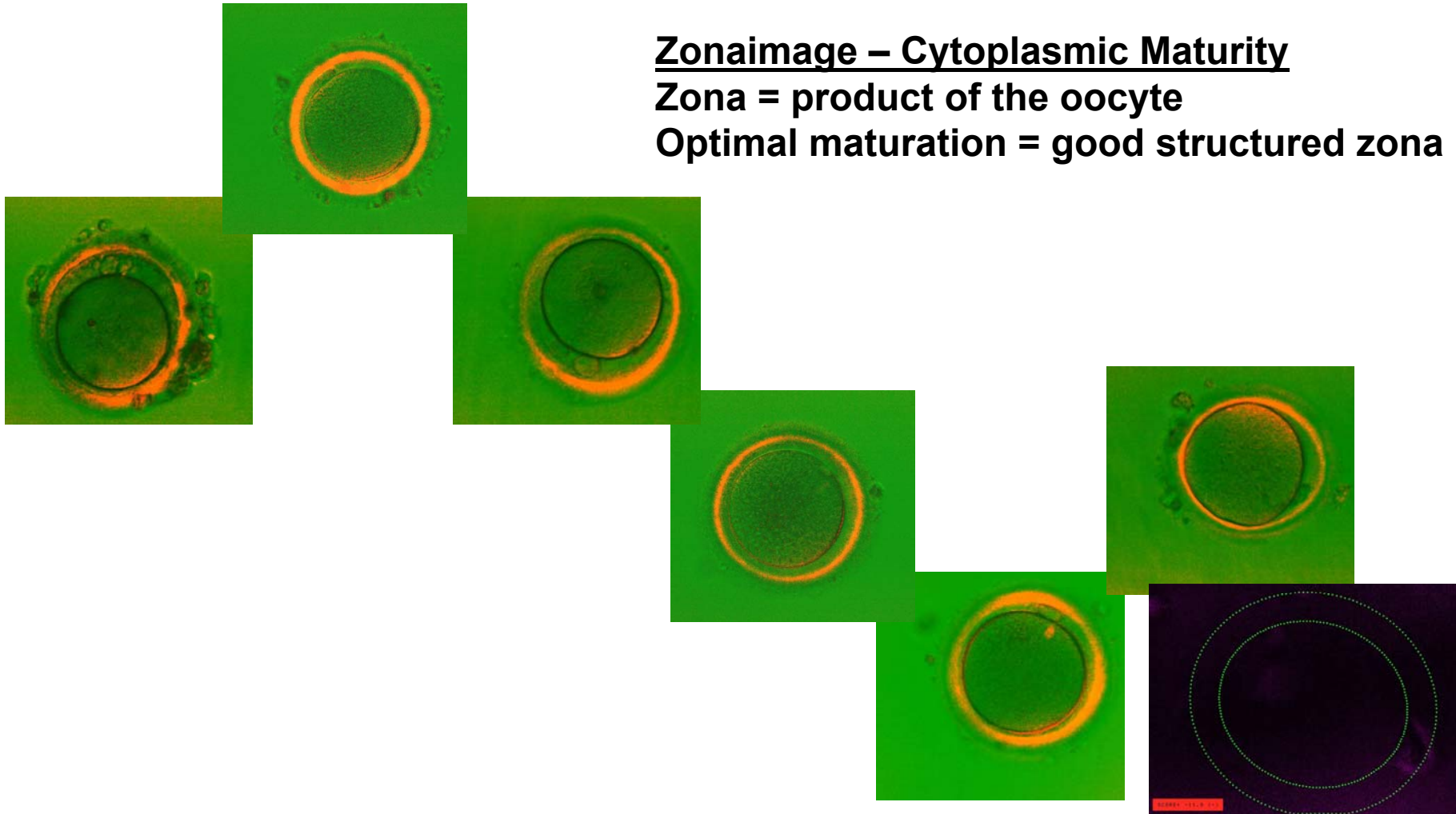
Spindle imaging during cryopreservation

- **Slow freezing:**
 - Rienzi et al. 2004 / human:
 - 37% oocytes with spindles present after thawing, but disappear later
 - 57% of oocytes show a spindle after 3h incubation
 - Bianchi et al. 2005 / human:
 - spindle appears in 3-5h after thawing
 - Coticchio et al. 2006 / human:
 - Confocal microscopy shows aberrant spindles
 - Sereni et al., 2009 / human:
 - Spindle reformation in >80% with optimised protocols
- **Vitrification:**
 - Chen et al., 2004 / mouse:
 - Spindles present in 50% of oocytes after warming
 - Larman et al., 2007 / human + mouse:
 - Spindles are constantly present

The multilaminar structure of the human zona pellucida



Zona-imaging – a possible parameter for cytoplasmic maturity?

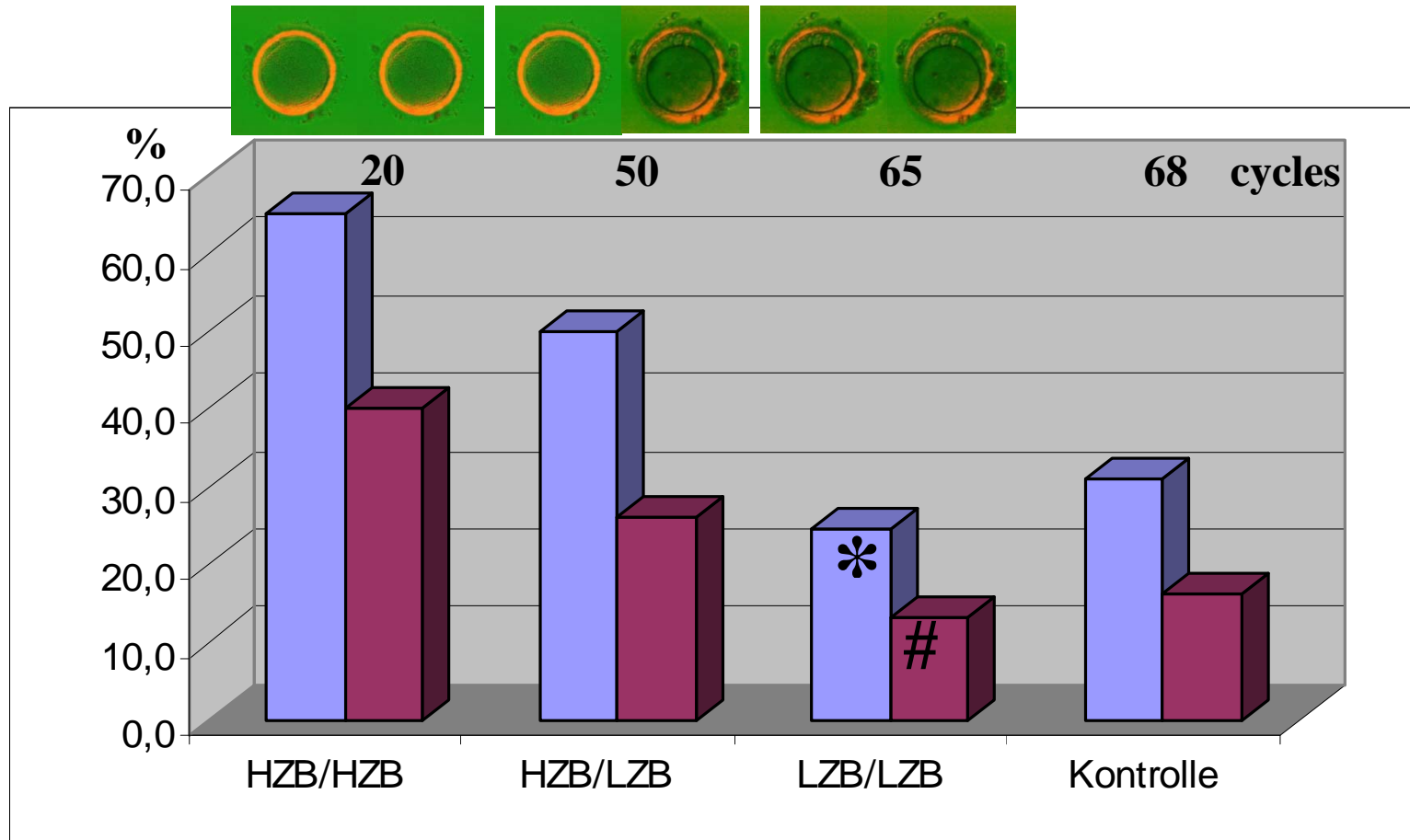


Zonaimage – Cytoplasmic Maturity

Zona = product of the oocyte

Optimal maturation = good structured zona

Results of zona-imaging



LZB/LZB versus HZB/LZB and HZB/HZB

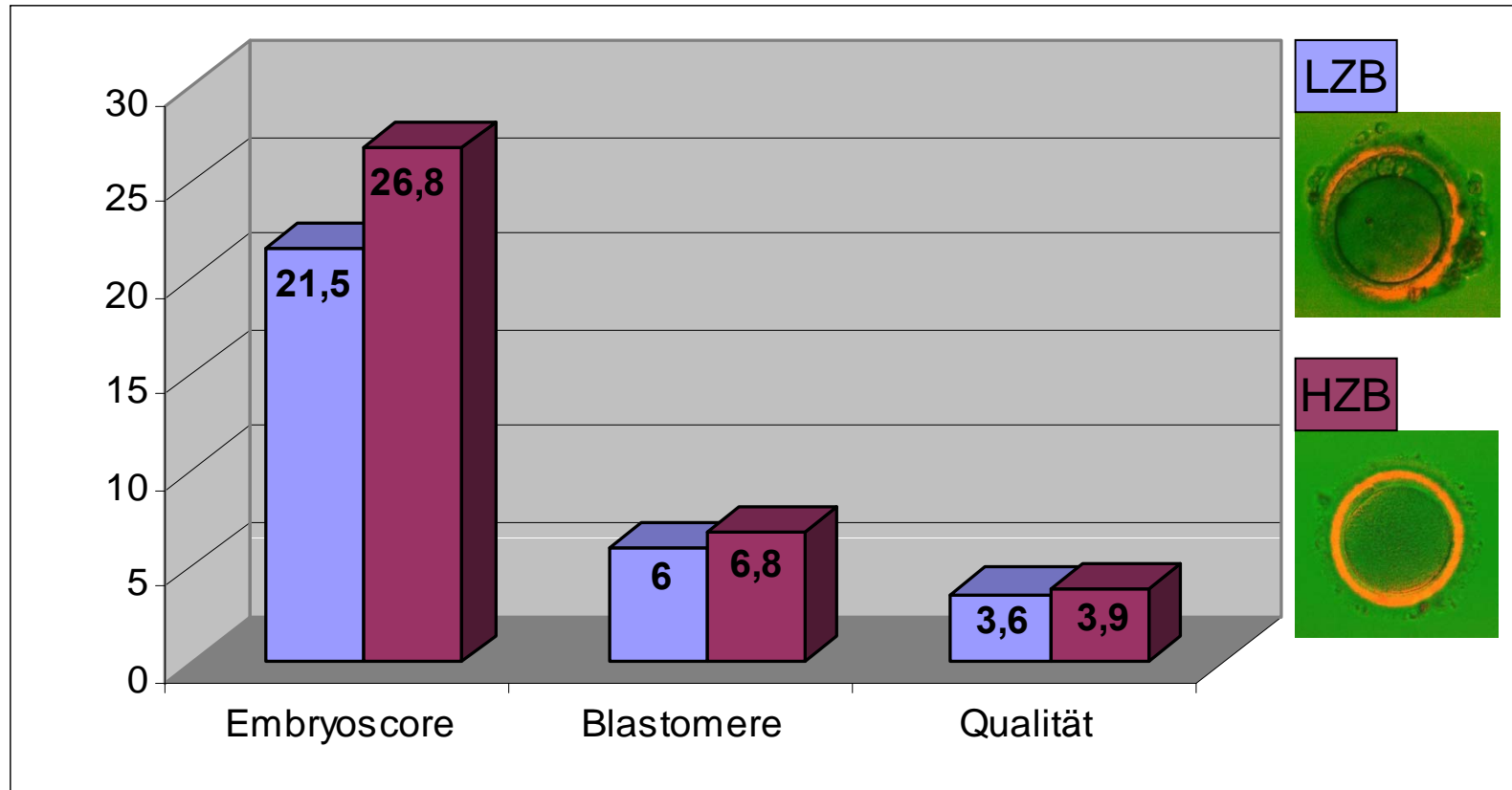
* $P < 0.005$

$P < 0.025$

Pregnancy rate

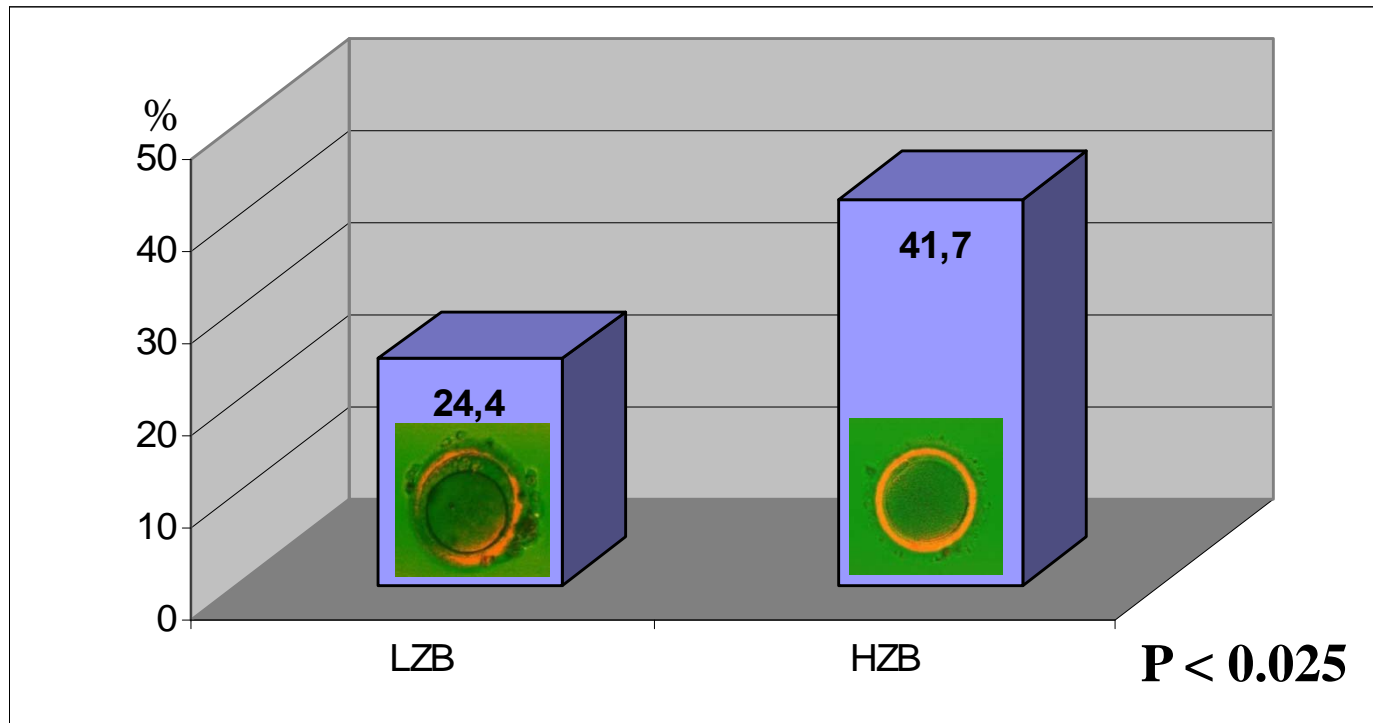
Implantation rate

Embryo development on day 3



Day 3: Embryoscore $P < 0.025$
No. of blastomeres $P = 0.089$
Embryo quality $P < 0.001$

Number > 8-cell grade A on day 3



Ebner et al.: Zona-Imaging correlates with blastocyst formation
Fertility & Sterility 2010

Correlation Zona-Image and ART

- | | | |
|-------------------------|---------------|--|
| • Shen et al., 2005 | Retrospective | Preg.-rate |
| • Raju et al., 2007 | Retrospective | Embyo development
Blastocyste formation |
| • Montag et al., 2007/8 | Prospective | Impl./Preg.-rate
Embryo development |
| • Ebner et al., 2009 | Prospective | Blastocyste formation |
| • Madaschi et al., 2009 | Prospective | Impl./Preg.-rate
Abortion rate |
| • Cheng et al., 2010 | Retrospective | No clear effect in IVF |

The zona pellucida as a marker for oocyte quality?

- The zona pellucida is formed by the oocyte during follicular growth
- An optimal follicular environment supports the formation of a well-ordered and structured zona
- A good zona is an indirect characteristic for an oocyte with a good follicular development

Gene expression in cumulus cells as indicator of oocyte competence

Cumulus and granulosa cells:

regulate oocyte growth and acquisition of oocyte developmental competence

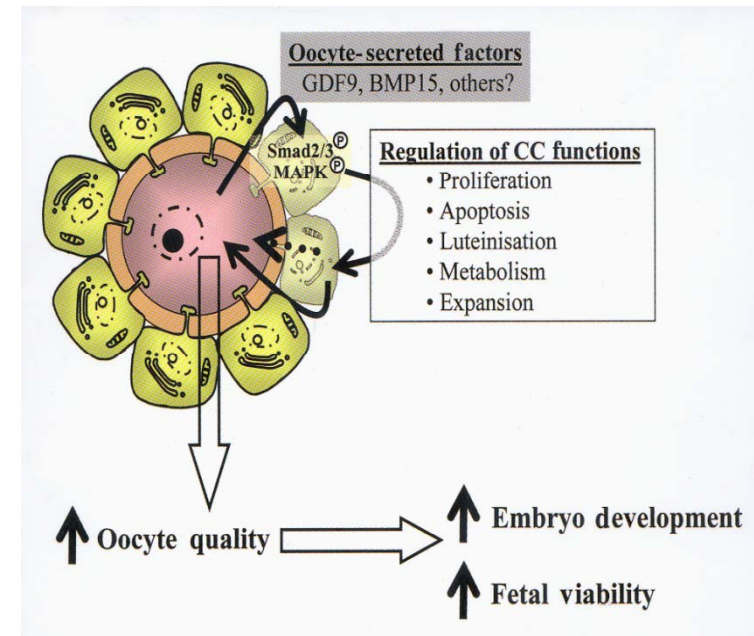
Oocyte secreted factors:

direct differentiation and function of cumulus cells

Bidirectional communication

via transzonal projections with formation of gap junctions

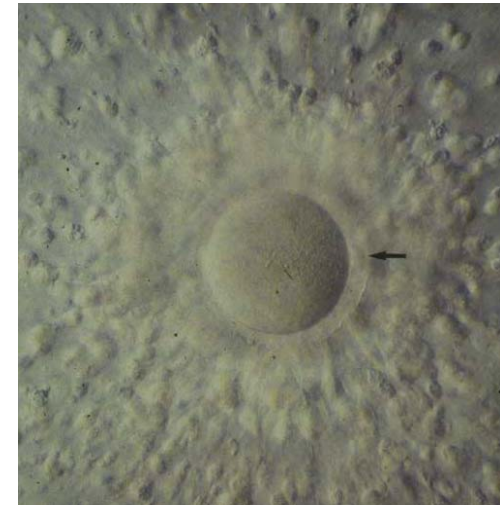
Oocyte – cumulus cell regulatory loop



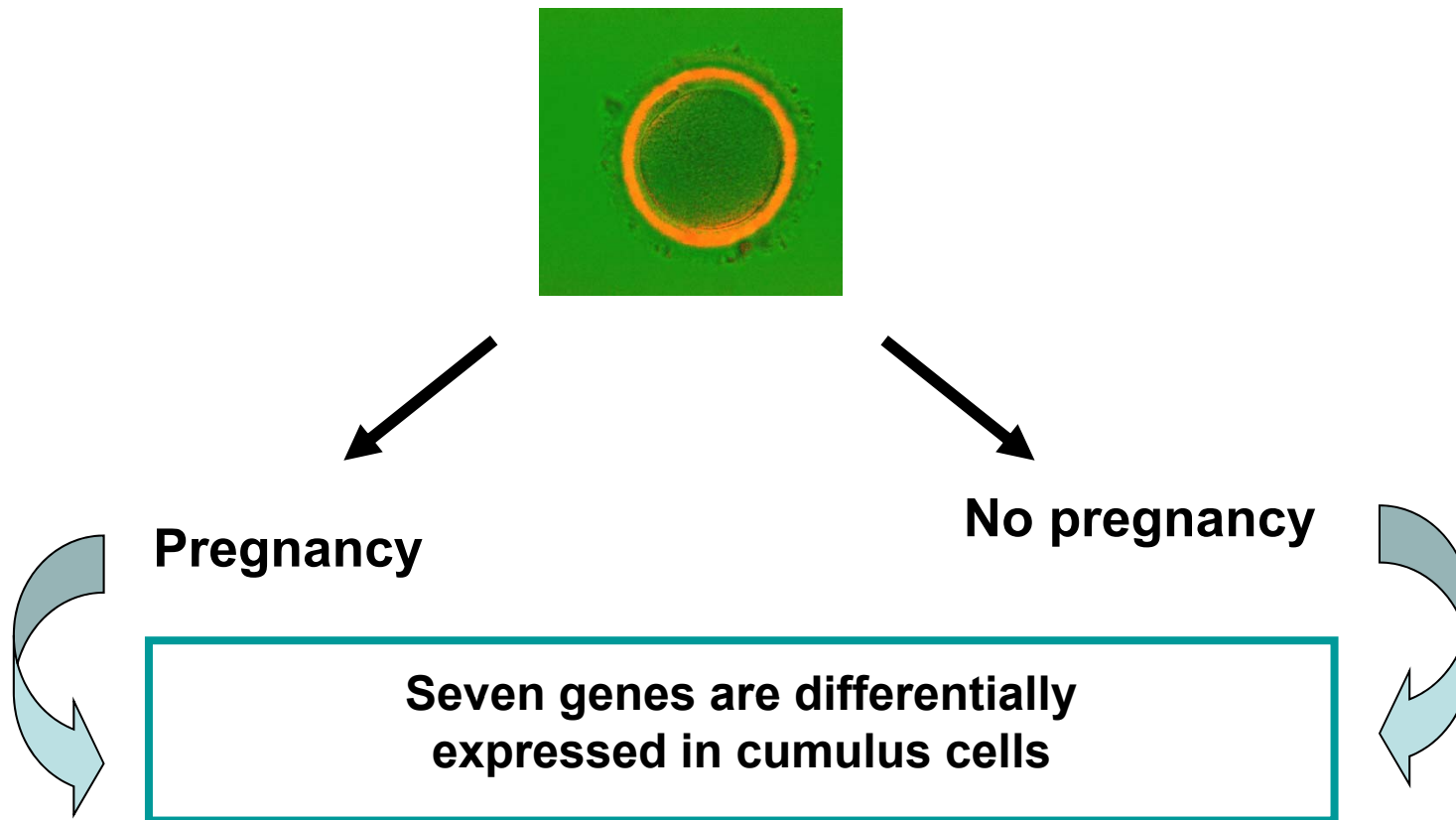
From: Gilchrist et al., HRU 14, 2008

Expression of candidate genes in cumulus cells relative to the zona score

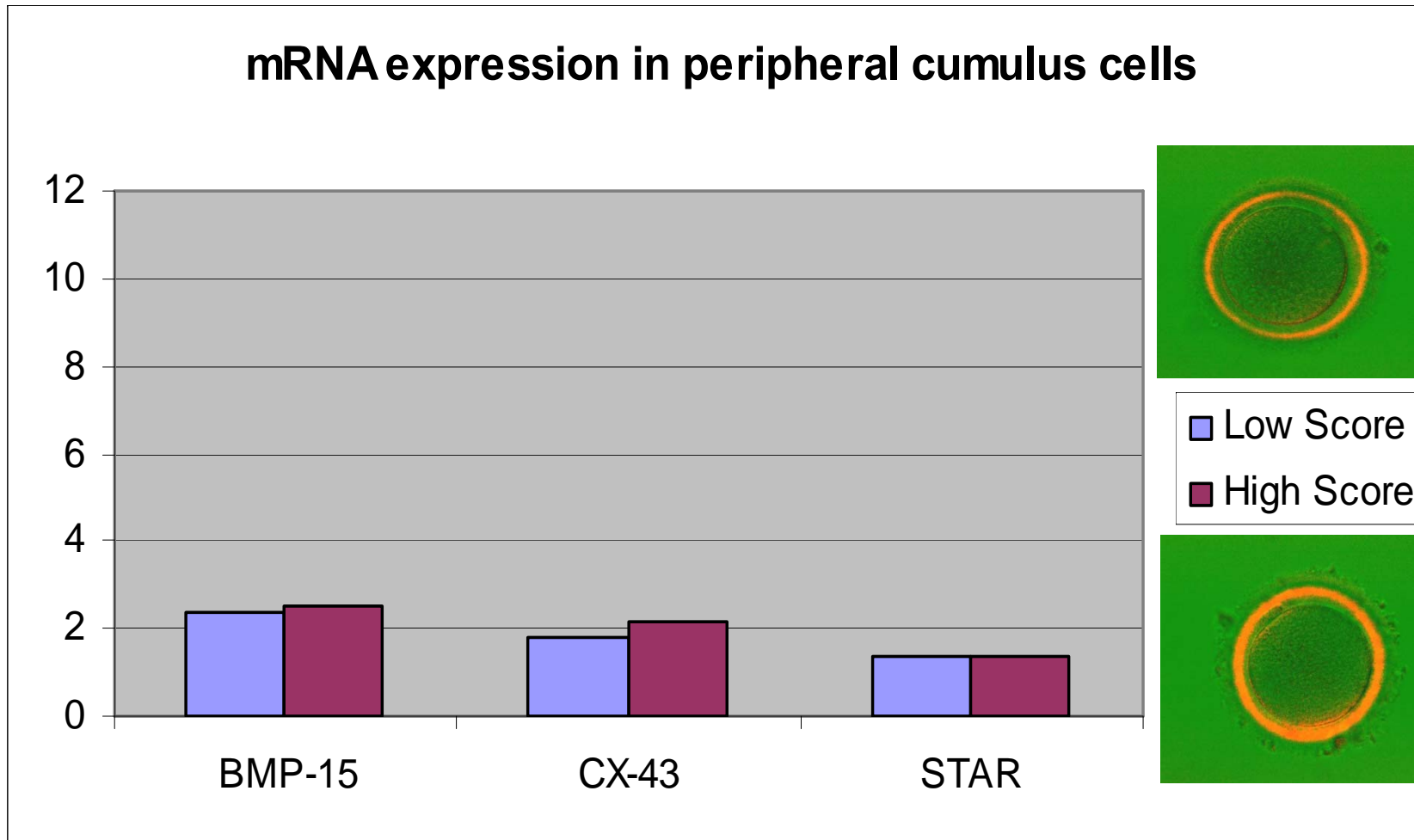
- **BMP15** (Bone morphogenetic protein 15)
 - oocyte secreted
 - central regulator of cumulus/ granulosa cell differentiation
- **CX43** (Connexin 43)
 - major protein of gap junctions
 - formation of transzonal projections
- **STAR** (Steroidogenic Acute Regulatory Protein)
 - critical in steroidogenic activity
 - expressed in cumulus cells after LH-surge
- **RPL-19** (Ribosomal protein L19)
 - housekeeping gene / - reference gene



Differences in gene expression in oocytes with a good zona score

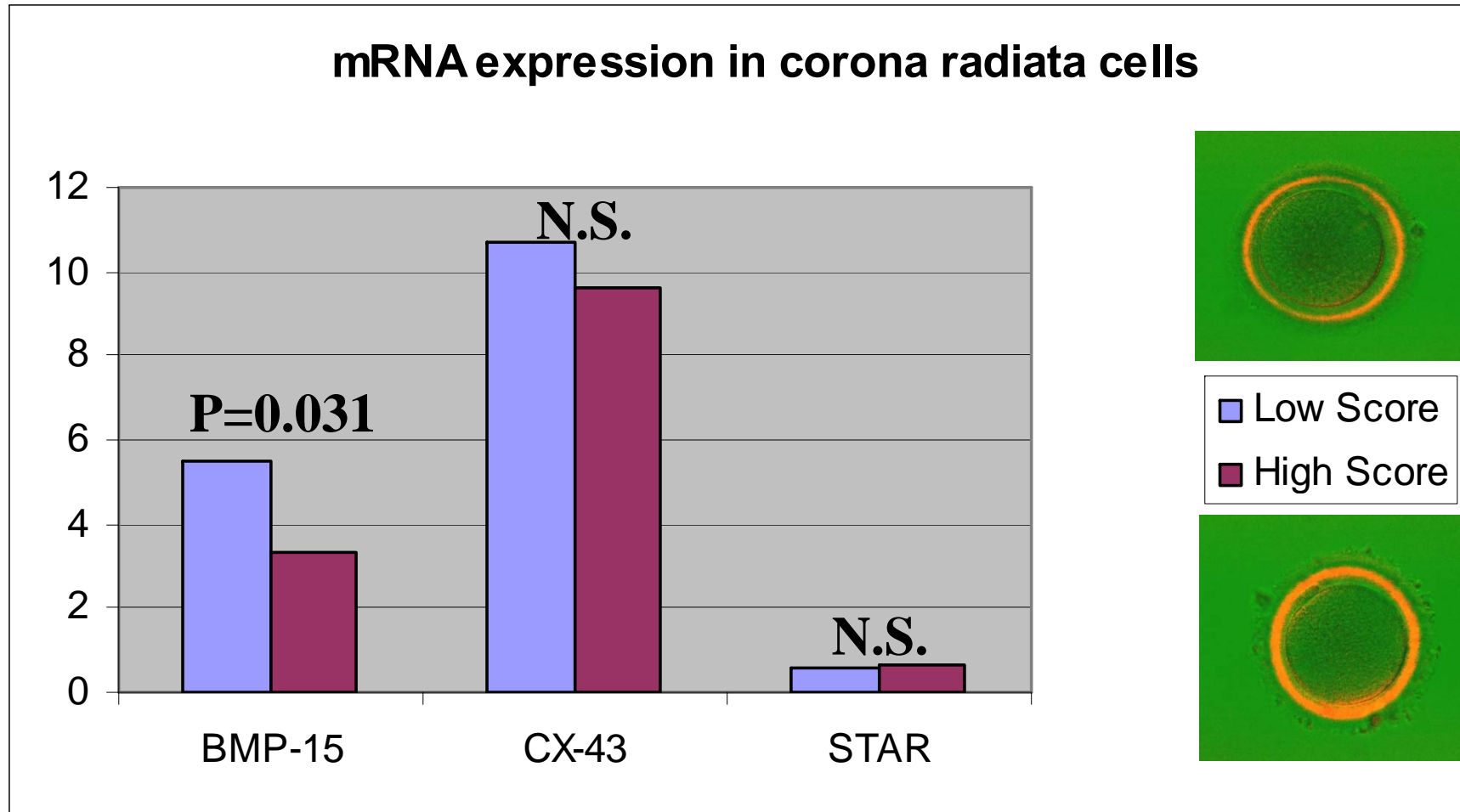


Oocytes with high versus low zona score



Values indicated as Means / Statistical comparisons done by ANOVA

Oocytes with high versus low zona score



Values indicated as Means / Statistical comparisons done by ANOVA

Conclusions

- Polarization microscopy is a good tool to judge the quality of lab parameters (temperature / pH)
- Spindle- and Zona-imaging are prognostic criteria in an ART cycle (oocyte maturity / embryo-potential)
- No substitute for aneuploidy testing
- Basic research to fully understand the underlying mechanisms is still needed

