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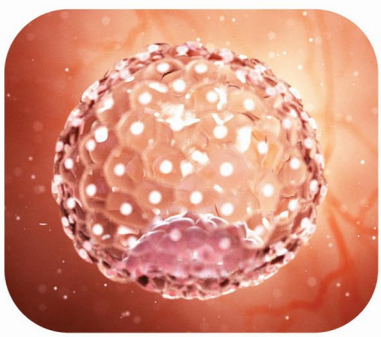
Journey to Inception of Life

A Guide to ART Laboratory Techniques





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CHAPTER 11

Intracytoplasmic Sperm Injection

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INTRODUCTION

Intracytoplasmic Sperm Injection (ICSI) has revolutionized the field of assisted reproductive technology, offering hope to patients with severe male infertility. First introduced in 1992 by Palermo and colleagues, ICSI involves the direct injection of a single sperm into an oocyte's cytoplasm, bypassing natural barriers to fertilization. This technique quickly became the cornerstone of in vitro fertilization (IVF) treatments, addressing conditions such as low sperm count, poor motility, and abnormal morphology. Over the years, advancements in micromanipulation tools and laboratory protocols have enhanced the precision and success rates of ICSI. This chapter delves into the evolution of ICSI, its technical intricacies, and its critical role in achieving successful fertilization and embryo development.

The first successful intracytoplasmic sperm injection (ICSI) embryo and pregnancy were reported in 1992 when Palermo and colleagues accidentally injected a human spermatozoon into the ooplasm of a human oocyte during a subzonal sperm injection (SUZI) procedure.[1] Initially, ICSI only marginally improved fertilization outcomes compared to SUZI. Today, ICSI is a highly effective procedure widely used in in vitro fertilization (IVF) laboratories around the world.

Before 1992, severe male infertility cases were often untreatable, leading to a fertilization failure rate of up to 30% in IVF treatments for male infertility. Artificial insemination with donor sperm was the standard treatment. ICSI has revolutionized this field, offering a significant chance for these patients to fertilize oocytes and develop healthy embryos. It is now the first-line treatment for a variety of qualitative or functional sperm disorders.[2]

First-line applications for ICSI:

- IVF fertilization failure
- Sperm agglutination
- Severe oligozoospermia and cryptozoospermia
- Severe asthenozoospermia (e.g., Kartagener syndrome)
- Teratozoospermia, including globozoospermia and pinhead sperm
- Immunological factors such as antisperm antibodies
- Azoospermia, both obstructive and nonobstructive, including cases of paraplegia
- Ejaculatory dysfunction, such as retrograde ejaculation
- Impaired spermatogenesis or spermiogenesis, including immature spermatozoa and various stages of spermatids and spermatocytes
- Oncology patients with cryopreserved sperm before chemotherapy or radiotherapy
- Hepatitis C positive men
- Diagnostic ICSI to evaluate oocyte quality at retrieval

- Rescue ICSI after failed IVF
- Prevention of polyspermy in patients with a history of repeated polyspermy
- Preimplantation genetic diagnosis (PGD)
- In vitro matured oocytes

Intracytoplasmic sperm injection requires the temporary breaching of both the oolemma (egg membrane) and the sperm plasma membrane. Success is indicated by the free flow of ooplasm and permanent immobilization of the sperm tail, making ICSI a technically demanding procedure.[3] ICSI bypasses several upstream events in natural fertilization, such as sperm motility acquisition, capacitation, acrosome reaction, and zona pellucida binding. Instead, a selected and immobilized spermatozoon is directly injected into the cytoplasm of an oocyte after rupturing the oolemma.

PREPARATION AND TECHNIQUE

Preparation for Intracytoplasmic Sperm Injection

Handling of oocytes: Mature oocytes are highly sensitive to environmental changes, particularly deviations in pH and temperature. The type of buffer used in the culture medium and its temperature are therefore critical factors. Even if these conditions do not immediately impact fertilization rates, they can have long-term effects on embryo quality, potentially leading to lower pregnancy rates.[4]

The media used for flushing and aspirating oocytes are typically buffered with bicarbonate and/or HEPES and may include antibiotics and heparin to prevent contamination and clotting.

Micromanipulation procedure: Micromanipulation procedures, including ICSI, are performed outside of the incubator over the objective lens of an inverted microscope **(Table 1)**. Gametes are placed into media drops that are overlaid with light mineral oil, using shallow, sterile, tissue–culture–grade plastic Petri dishes. The microtools used for these procedures are constructed from borosilicate glass capillary tubes, which are essential for holding oocytes and aspirating or injecting spermatozoa.

Micromanipulation systems attached to an inverted microscope, requiring specific equipment and conditions due to the setup's limited working distance include:

- *Precision micromanipulators:* Micromanipulation systems, either hydraulic or motorized, provide high precision in the movement of microinjection and holding pipettes. Hydraulic micromanipulators are preferred for their smooth, backlash-free movement, ensuring fine control during ICSI.
- *Objective lens and working distance:* The inverted microscope's objective lens has a limited working distance, necessitating the use of low-profile micromanipulation tools. This space constraint demands specialized, finely designed pipettes that can operate efficiently within the microscope's field without obstructing visibility or movement.
- *Microenvironment maintenance:* During micromanipulation, maintaining optimal conditions is critical. A heated stage is used to regulate the temperature of the gametes and culture media at physiological levels (37°C), which minimizes temperature-induced stress and ensures the viability of the oocyte and spermatozoa throughout the procedure.
- *High-resolution imaging:* The inverted microscope is typically equipped with advanced imaging systems, such as differential interference contrast (DIC) or Hoffman modulation contrast, which enhance the visibility of cells

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TABLE 1: Overview of micromanipulator systems and microinjectors used in intracytoplasmic sperm injection (ICSI).

Type of micromanipulator system	*Control system*	*Scientific points*
Motorized micromanipulator	Motorized	• Utilizes electric motors for precise control • Suitable for complex and automated movements • Can be integrated with digital systems for enhanced precision
Manual micromanipulator	Manual	• Requires manual adjustment for movement • Provides high control but is user-dependent • More suitable for basic procedures with fewer complexities
Automatic micromanipulator	Automated	• Features computer-controlled automation • Ensures high reproducibility and precision • Ideal for high-throughput ICSI procedures
Piezo micromanipulator	Piezoelectric control	• Uses piezoelectric crystals for movement • Offers rapid and precise movements with minimal vibration • Highly effective for delicate procedures like ICSI
Hydraulic microinjector (holding/injecting)	Hydraulic	• Utilizes fluid pressure for holding and injecting cells • Provides smooth, precise control • Requires regular maintenance to prevent fluid leakage
Pneumatic microinjector (holding/injecting)	Pneumatic	• Operates using air pressure for holding and injecting cells • Offers high precision with minimal mechanical interference • Less sensitive to vibrations

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and subcellular structures, improving the precision of microinjections and manipulations during ICSI.

- *Use of specialized pipettes:* Micropipettes used in ICSI, including injection and holding pipettes, are designed with specific dimensions to suit the oocyte's structure. The injection pipette's diameter, typically 5–10 µm, allows for smooth sperm insertion, while the holding pipette stabilizes the oocyte without causing mechanical damage.
- *Vibration control:* To enhance precision, micromanipulation systems are often placed on anti-vibration tables, which minimize external mechanical disturbances that could affect the accuracy of the micromanipulation process. Vibrations, even at low frequencies, can lead to errors during sperm injection or oocyte handling.
- *Three-dimensional control:* The micromanipulator system allows for precise three-dimensional movement, controlled via joystick or precision knobs, enabling the operator to manipulate sperm and oocytes accurately. This level of control is essential for minimizing oocyte damage

and increasing the success rates of fertilization in ICSI procedures.

- *Operator skill:* The success of micromanipulation procedures like ICSI is highly dependent on the skill and experience of the operator. Proper handling of the micromanipulation system requires extensive training to ensure accurate sperm injection and minimize potential damage to the oocyte membrane.
- *Micromanipulation environment:* The entire process occurs in an open system, outside the controlled environment of an incubator. Therefore, the microscope stage is heated, and media are supplemented with essential nutrients and stabilizers to mimic in vivo conditions as closely as possible, ensuring the maintenance of cell viability during the procedure.

Intracytoplasmic sperm injection technique: The same media used for oocyte preparation are employed during micromanipulation. However, when procedures extend outside the incubator for longer durations, a HEPES-buffered medium supplemented with penicillin, pyruvate, and human serum albumin (HSA) is recommended. Polyvinylpyrrolidone (PVP) is commonly used for sperm manipulation due to its capacity to reduce sperm motility, making the spermatozoa easier to inject.

The ICSI involves the precise injection of a morphologically normal sperm into an oocyte. To ensure success, the laboratory environment must be free from vibrations to prevent disruptions during the procedure.

Manipulation of the spermatozoon: The ICSI process is conducted using a micromanipulator attached to an inverted microscope. The spermatozoon must be completely immobilized, which is achieved by damaging its tail. This step is crucial and is done by aligning the sperm with the injection needle and intentionally breaking the tail. The sperm is then aspirated and positioned at the tip of the injection needle for subsequent injection **(Figs. 1 to 4)**.

Manipulation of the oocyte: The oocyte is prepared for injection by positioning its polar body at either the 12 o'clock or 6 o'clock position **(Fig. 5)**. The spermatozoon is carefully placed at the tip of the injection needle, and the geometric center of the oocyte is identified by gently indenting the zona pellucida **(Fig. 6)**. The needle is then pushed horizontally through the zona pellucida and vitelline membrane, creating an invagination **(Fig. 7)**. A precise

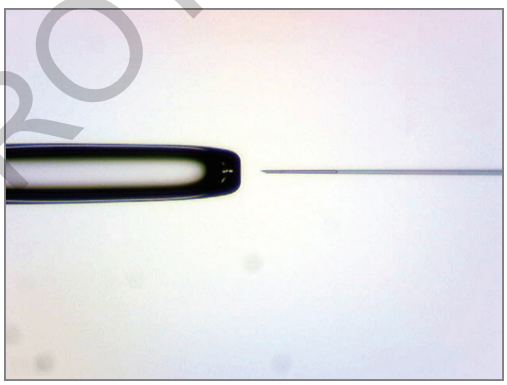

Fig. 1: Precisely aligned holding and injection pipettes.

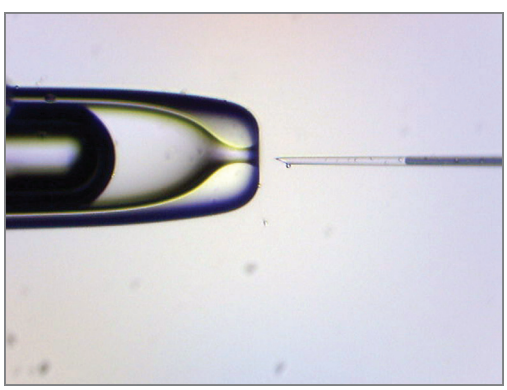

Fig. 2: Pipettes immersed in media drop for washing and pressure calibration.

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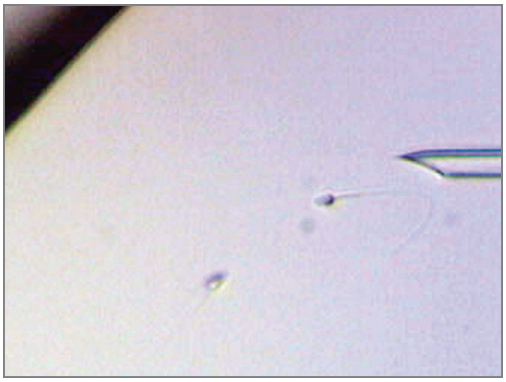

Fig. 3: The sperm tail is slashed to partially immobilize and assess morphology.



Fig. 6: Secure the oocyte firmly and position the sperm precisely at the tip for injection from the 3 o'clock position.

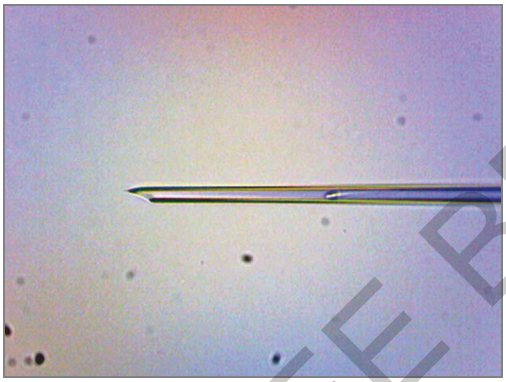

Fig. 4: Carefully aspirate the tapped sperm into the micropipette.



Fig. 7: Inject the oocyte until the pipette reaches the 9 o'clock position, aspirate some cytoplasm until breakage is observed, and then deposit the sperm.



Fig. 5: Position the oocyte so that the polar body is at either the 6 o'clock or 12 o'clock position.

jab from the 3 o'clock position is used to break the membrane, and a small amount of cytoplasmic material is aspirated to ensure the needle is correctly positioned within the cytosol. The spermatozoon is then deposited at the 9 o'clock position **(Fig. 8)**.

Following injection, the oocytes are transferred to preincubated culture medium layered with oil and incubated at 37°C in 5% CO_2 for 18–20 hours. Fertilization is assessed post incubation using an inverted microscope. Normally fertilized oocytes will exhibit two pronuclei and two polar bodies.

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The cleavage of these fertilized oocytes is evaluated on the second day following ICSI.[2]

TROUBLESHOOTING

Troubleshooting during ICSI is essential for overcoming challenges that can occur during the delicate sperm injection process. By quickly identifying and resolving issues like pipette blockages, improper sperm immobilization, or difficulty penetrating the oocyte membrane, embryologists can improve the chances of successful fertilization and achieve better clinical outcomes **(Table 2)**.



Fig. 8: Withdraw the microinjection pipette and gently release the oocyte.

CONCLUSION

The ICSI procedure, while intricate and demanding, is a critical component of assisted reproductive technology. The success of ICSI not only hinges on the precision of the micromanipulation techniques but also on the delicate handling and preparation of oocytes and spermatozoa. Ensuring optimal environmental conditions and using appropriate media throughout the procedure are vital for enhancing fertilization outcomes and improving embryo quality, ultimately

TABLE 2: Common intracytoplasmic sperm injection (ICSI) issues and troubleshooting steps.

Issue	*Description*	*Troubleshooting steps*
Difficulty immobilizing spermatozoa	Spermatozoa continue to move after tail damage, complicating the injection process	• Ensure complete tail severance with additional force • Use polyvinylpyrrolidone (PVP) to reduce sperm motility • Check the sharpness and alignment of the injection needle
Problems with oocyte handling	Oocytes are difficult to hold or manipulate due to slippage or movement	• Adjust holding pipette suction strength • Reposition the holding pipette for a better grip • Ensure proper medium viscosity to prevent movement
Difficulty penetrating the zona pellucida	Injection needle fails to penetrate the zona pellucida smoothly	• Confirm needle sharpness and inspect for deformities • Adjust the angle of entry to be perpendicular • Apply consistent pressure during insertion
Improper positioning of the oocyte's polar body	Polar body is not correctly aligned, risking oocyte damage	• Rotate the oocyte using the micromanipulator • Adjust microscope optics and lighting for better visualization • Reposition the holding pipette

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Issue	*Description*	*Troubleshooting steps*
Inconsistent needle insertion	Injection needle does not enter the cytoplasm smoothly, leading to failed injection	• Ensure micromanipulator calibration and smooth operation • Practice gentle, controlled needle movements • Reattempt insertion with proper alignment if resistance is felt
Difficulty aspirating cytoplasmic material	There is trouble confirming needle placement in the cytoplasm	• Check suction settings on the micromanipulator • Use a new, clean needle to avoid blockages • Adjust needle depth to ensure it is in the cytoplasmic region
Oocyte degeneration post-ICSI	Oocytes degenerate after the ICSI procedure, leading to failed fertilization	• Review the procedure for potential sources of trauma • Handle oocytes quickly and return them to optimal conditions • Ensure media quality and avoid contamination
Vibration interference during ICSI	Vibrations in the laboratory affect micromanipulation stability	• Use vibration isolation platforms • Regularly maintain the micromanipulator • Minimize movement around the workstation during the procedure

contributing to higher pregnancy rates. As such, continuous advancements in the protocols and techniques used in ICSI are essential for optimizing the success of this procedure in clinical practice.

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CHAPTER 12

Fertilization Assessment and Embryo Grading

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INTRODUCTION

Fertilization assessment globally follows standardized protocols, typically involving the identification of two pronuclei and polar bodies within 16–18 hours post insemination, ensuring normal fertilization. Embryo grading systems, such as those from the ESHRE (European Society of Human Reproduction and Embryology), ASRM (American Society for Reproductive Medicine), and Istanbul Consensus, emphasize parameters like cell count, symmetry, fragmentation, and blastocyst development to uniformly assess embryo quality for transfer, cryopreservation, or further culture. These grading systems aim to optimize embryo selection for successful implantation and pregnancy outcomes worldwide **(Table 1)**.

FERTILIZATION ASSESSMENT AND PRONUCLEAR SCORING

Optimal fertilization of oocytes is critical for successful embryo development. An ideally fertilized oocyte should be spherical, contain two polar bodies, and have two centrally located, juxtaposed pronuclei of equal size with distinct membranes.[1] The pronuclei should have equal numbers and sizes of nucleolar precursor bodies (NPBs), ideally aligned equatorially at the region where the membranes meet.

Fertilization check: At the fertilization check, both the size and location of the pronuclei should be evaluated. Features such as widely separated pronuclei, pronuclei of significantly different sizes, and the presence of micronuclei are considered severely atypical. If smooth endoplasmic reticulum (sER) disks are observed in normally fertilized oocytes (especially after conventional In vitro fertilization (IVF) rather than ICSI), these oocytes should not be used for transfer.

Second day-1 assessment: A second assessment on day 1 may be conducted for quality control or prognostic reasons, focusing either on syngamy or early cleavage.[1]

Pronuclear scoring: Pronuclear scoring provides additional information to the fertilization check **(Figs. 1A to D)**. It categorizes pronuclei as symmetrical, nonsymmetrical, or abnormal. Abnormal pronuclei include those with no NPBs (ghost pronuclei) and those with a single NPB (bull's eye pronuclei), which are associated with poor outcomes.[2]

CLEAVAGE-STAGE EMBRYOS

Assessment of cell number: Embryos should be at specific developmental stages at set times post insemination: 4 cells on day 2 and 8 cells on day 3. Deviation from these rates is linked to reduced implantation potential.[1]

Fragmentation: Fragments, defined as extracellular membrane-bound cytoplasmic structures, are categorized by severity: Mild (<10%), moderate (10–25%), and severe (>25%). Fragment size also varies by the embryo's stage: <45 μm in diameter on day 2

TABLE 1: Fertilization assessment and embryo grading as per various consensus worldwide.

Grading system	*Year*	*Criteria*	*Grades*	*Description*
Zygote scoring (Z-Score)	1999	Pronuclei alignment, nucleolar precursor bodies (NPBs)	Z1 to Z4	• *Z1:* Perfect alignment of pronuclei and NPBs • *Z2:* Slight deviation in alignment • *Z3:* Pronuclei and NPBs are not aligned • *Z4*: Pronuclei and NPBs are highly misaligned
Day-3 cleavage-stage grading	1988	Cell number, symmetry, fragmentation	1–4	• *Grade 1:* 6–8 cells, no fragmentation, symmetric • *Grade 2:* 6–8 cells, <10% fragmentation, slightly asymmetric • *Grade 3:* 6–8 cells, 10–25% fragmentation • *Grade 4:* <6 cells, >25% fragmentation, highly asymmetric
Day-5 blastocyst grading	1999	Expansion, inner cell (ICM) mass, trophectoderm (TE)	1–6 (expansion), A to C (ICM and TE)	• *Expansion (1–6):* 1 = Early blastocyst, 6 = Hatching blastocyst • *ICM (A to C):* A = Tightly packed, B = Loosely packed, C = Few cells • *TE (A to C):* A = Many cells, B = Few cells, C = Very few cells
SART (Society for Assisted Reproductive Technology) grading	2000	Cell number, fragmentation, symmetry	Excellent, good, fair, poor	• *Excellent:* 6–8 cells, <10% fragmentation, symmetric • *Good:* 6–8 cells, 10–20% fragmentation, slightly asymmetric • *Fair:* 5–8 cells, 20–50% fragmentation, moderately asymmetric • *Poor:* <5 cells, >50% fragmentation, highly asymmetric
ASRM (American Society for Reproductive Medicine) Grading	2000	Cell number, fragmentation, symmetry, multinucleation	1–4	• *Grade 1:* 6–8 cells, <10% fragmentation, symmetric, no multinucleation • *Grade 2:* 6–8 cells, 10–25% fragmentation, slightly asymmetric, no multinucleation • *Grade 3:* 5–8 cells, 25–50% fragmentation, moderately asymmetric, possible multinucleation • *Grade 4:* <5 cells, >50% fragmentation, highly asymmetric, multinucleation present
Gardner's blastocyst grading	2000	Expansion, ICM, TE	1–6 (expansion), A to C (ICM and TE)	• *Expansion (1–6):* 1 = Early blastocyst, 6 = Fully expanded • *ICM (A to C):* A = Many cells, B = Several cells, C = Few cells • *TE (A to C):* A = Many cells forming a cohesive epithelium, B = Few cells forming a loose epithelium, C = Very few cells

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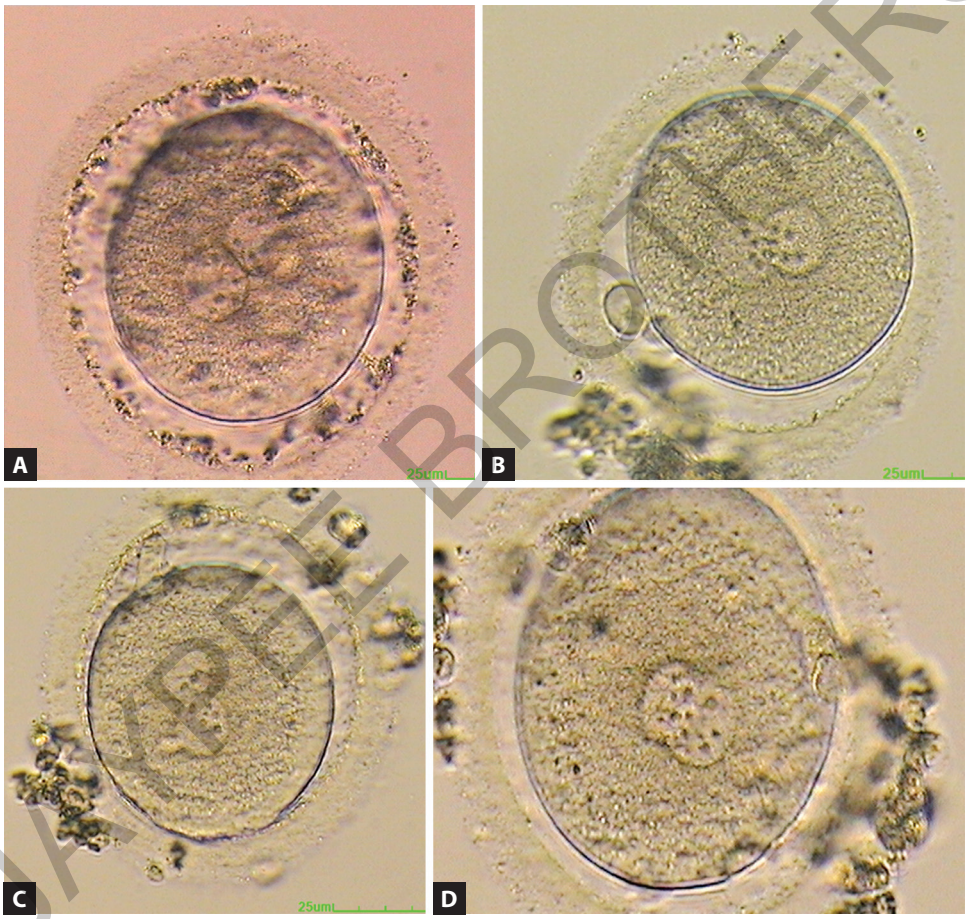
Grading system	Year	Criteria	Grades	Description
Alpha Scientists and ESHRE (European Society of Human Reproduction and Embryology)	2011	Comprehensive morphology evaluation	Different categories	This evaluates cumulus–corona complex, cytoplasm, polar body, and overall morphology to categorize oocytes into different grades



Figs. 1A to D: Fertilization assessment: Confirmation of normal fertilization with presence of 2 pronuclei.

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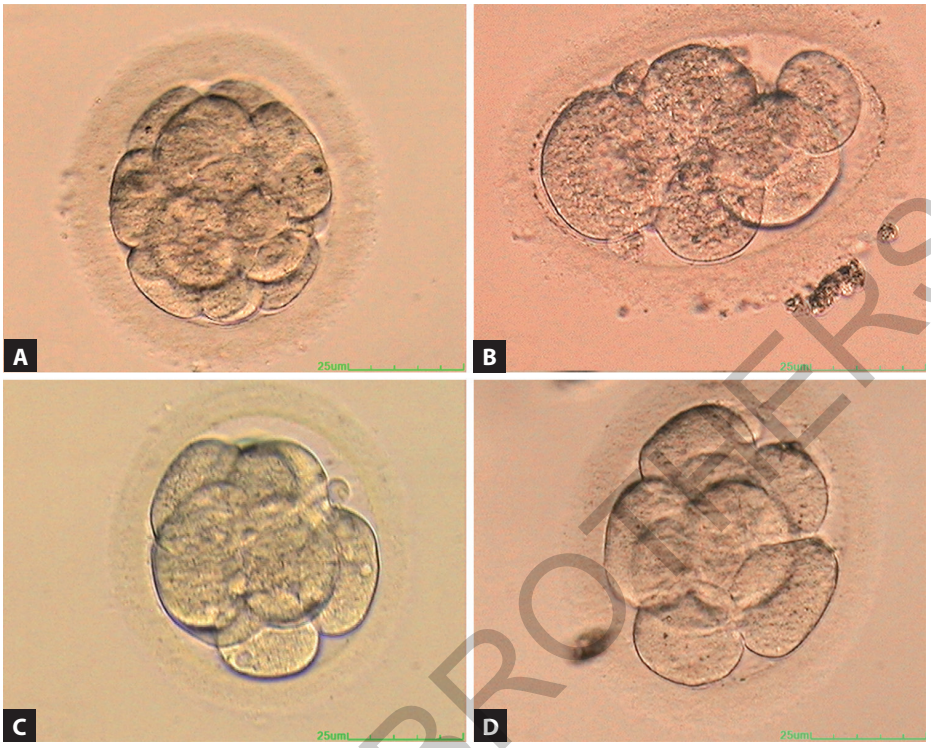
and <40 µm on day 3. The impact of fragment localization is not clearly defined due to its dynamic nature within the embryo.

Multinucleation: Multinucleation, the presence of more than one nucleus in a blastomere, is linked to decreased implantation potential and increased chromosomal abnormalities. Multinucleated embryos have a higher risk of spontaneous abortion. Assessment is recommended on day 2



Figs. 2A to D: Day-3 cleavage-stage embryo assessment revealing embryos at the 6–8 cell stage.

(44 ± 1 h post insemination). Recording the incidence of multinucleation in each embryo is essential for accurate evaluation.[1]

Cell size and other morphological features: For embryos at the 2-, 4-, and 8-cell stages, blastomeres should be evenly sized. Other stages may show size differences due to incomplete cleavage phases. Cytoplasmic granularity, membrane appearance, and vacuoles are also assessed, though their impact on implantation potential requires further research.

Cleavage-stage embryo scoring system: An optimal day-2 embryo (44 ± 1 h post insemination) should have four equally sized mononucleated blastomeres in a three-dimensional tetrahedral arrangement, with <10% fragmentation. For day-3 embryos (68 ± 1 h post insemination), an optimal embryo should have eight equally sized mononucleated blastomeres with <10% fragmentation **(Figs. 2A to D)**.[1] The scoring system for cleavage-stage embryos includes cell number, grade, and reason for the grade.

DAY-4 AND DAY-5 ASSESSMENTS

Day-4 assessment (morula stage): A day-4 embryo should be compacted or compacting, entering the fourth round of cleavage. Compaction should involve nearly all the embryo volume **(Figs. 3A and B)**. The consensus scoring system for day-4 embryos includes grades for good, fair, and poor embryos based on the extent of compaction.[3]

Day-5 assessment (blastocyst stage): At this stage (116 ± 2 h post insemination),

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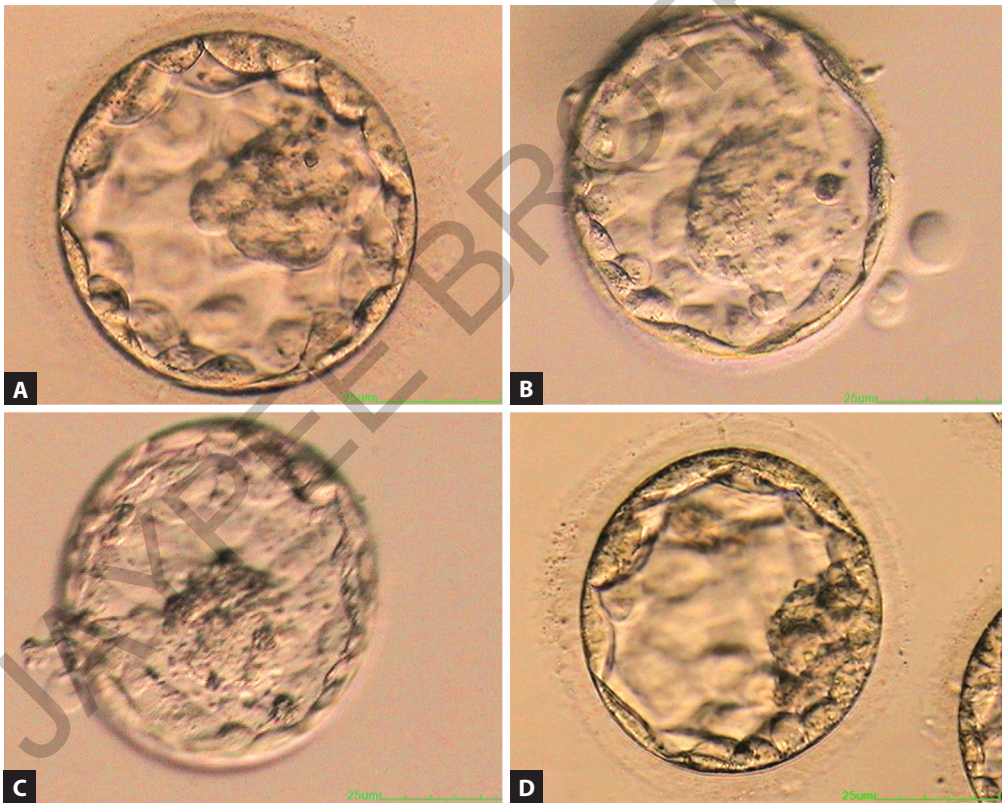
Figs. 3A and B: Day-4 assessment: Morula stage embryos.



Figs. 4A to D: Day-5 assessment: Expanded blastocyst observed.

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an optimal blastocyst should be fully expanded to hatched, with a prominent, easily discernible inner cell mass (ICM) and a cohesive trophectoderm (TE).[4] The ICM and TE are crucial for implantation and fetal development **(Figs. 4A to D)**. Variants

like cytoplasmic strings or structures in the perivitelline space have unknown significance and require further investigation.

Definition of a nonviable embryo: A nonviable embryo is defined as one where development has arrested for at least 24 hours, or where all cells have degenerated or lysed.[1]

These consensus points aim to standardize the assessment of oocyte and embryo morphology worldwide, fostering a common language among embryologists.

CONCLUSION

Paying close attention to how we assess fertilization and grade embryos is crucial for the success of assisted reproductive technology (ART) procedures. By following consistent guidelines for evaluating each stage—from pronuclei to blastocysts—embryologists can better predict, which embryos are most likely to result in a successful pregnancy. This approach requires not only precision but also a willingness to adapt to new research and techniques. By doing so, ART laboratories can improve their success rates, offering better outcomes and hope for patients looking to start or grow their families.

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Journey to Inception of Life

A Guide to

ART Laboratory Techniques

This book serves as a comprehensive guide to the intricate world of assisted reproductive technology (ART), offering both foundational knowledge and advanced techniques in embryology. Covering a wide range of topics, from laboratory set-up and semen analysis to advanced procedures such as intracytoplasmic sperm injection (ICSI) and embryo biopsy, this book provides invaluable insights for both experienced professionals and those new to the field. With a focus on practical applications, techniques, and best practices, it serves as an essential resource for anyone involved in ART.

Rich with scientific depth and practical guidance, this book not only highlights technical precision but also reflects the authors' deep commitment for helping families to achieve their dreams of parenthood. Whether you are an embryologist, a medical professional, or a student of reproductive technology, this book offers a roadmap to excellence in the ever-evolving landscape of ART.

Chandan Vijay Salunke PhD with over 20 years of experience in the field of embryology, he has become a highly respected figure in assisted reproductive technology (ART). He is the Managing Director, Kasturi ReproAssist Concepts LLP, Panjim, Goa, India. He has collaborated with more than 26 in vitro fertilization (IVF) centers across the country, contributing his expertise in the embryology aspects of ART treatments. He holds a PhD in Environmental Sciences and an MSc in Microbiology from the University of Pune, Maharashtra, India. His deep understanding of embryology has helped countless couples realize their dream of parenthood. His dedication to continuous improvement and research ensures that his work remains at the cutting edge of reproductive science. He is committed to mentoring the next generation of embryologists and improving ART practices worldwide.

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