a CooperSurgical Company

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NEXUS

Indian Fertility Society & ORIGIO India Initiative

ICSI - NUANCES

Dr M Gouri Devi
President - IFS

It is a great privilege for me to write this message for the E-bulletin of IFS "Nexus". Their is a wide spread application of Intracytoplasmic sperm injection (ICSI) in IVF centers, more so in cases of TESA, TESE, PESA etc. It is time we revisit the technique and understand its nuances. This bulletin is going to help you all to understand the procedure and its intricacies.

It is an IFS-Origio initiative and these E-bulletins are highly appreciated by IFS members. I congratulate Dr. Pankaj Talwar for the great efforts he puts in to keep the Nexus going.

Prof (Dr) Pankaj Talwar
Secretary General - IFS
Editor NEXUS

At the very onset, the editorial team would like to thank all of you for positively appreciating our previous bulletins of NEXUS. The bulletin has been named NEXUS which means bridging gaps.

Such bulletins are the call of the day and enormously bridge the gap between the existing knowledge and recent advances. The aim of bringing this bulletin is educating us about the finer aspects of ICSI and its nuances.

Both IVF and ICSI are the assisted reproductive techniques, ICSI being the variation of IVF technique. However, most of the patients get foxed by these two terms and confuse one with the other. They aren't too sure as to which one they should use and what its benefits will be. The only difference between the two is that in IVF technique, the sperm are left to fertilize the eggs in a Petri dish while in ICSI; we give the sperm the boost they require to get into the oocyte- this is done with the use of a micromanipulator. In most of the cases, ICSI can be used to overcome severe male infertility or to treat infertility in women over the age of 40 as advanced age is a limiting factor to the success of fertility treatment.

Through this E-bulletin we would make an endeavor to simplify the process of ICSI for the beginners and experienced alike and hope the bulletin would help you to improve your results.

Guest Editors for the Edition

It is great honor and pleasure to be a part of this NEXUS bulletin on ICSI. I am thankful to the team of Indian Fertility Society for such an initiative step of ART Booklet which helps reproductive biologists to enhance success rate.

The purpose of this bulletin is not only highlight theory but also to offer practical information of each micromanipulator available commercially. It has to be noted that there are intricacies in implementing the ICSI procedure.

This handbook will be immensely beneficial for all the lab practitioners to fine tune the result to a great extent. Most importantly this booklet will help everyone to formulate not just the now- how but also they'll know why.

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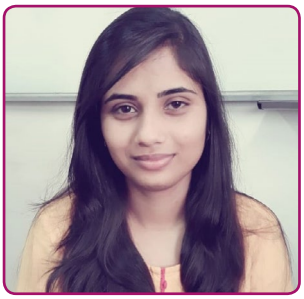

The nexus is a complete guideline for the beginners as well as the practitioners' because it covers various ICSI machine along with their working in detail. The entire making of the nexus was a learning experience for me and it's an honor to be a part of it. It may be recommended when there is a reason to suspect that achieving fertilization may be difficult or with male infertility factors.

Through this edition, we have tried to summarize all the information available to enhance our understanding of the conundrum associated with ICSI. I would also like to thank Mr. Krishnendu Maity, Mr. Rajesh Gurjar, Mr. Bharat Singh and Mr. Venkatesan for helping me in designing the bulletin.

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B.Tech (Biotechnology)

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PART-1

Intracytoplasmic Sperm Injection (ICSI) - Basic concepts

Sub-Index

Part 1

1. Introduction:

In the past, different Assisted Reproductive Techniques such as Artificial Insemination - AI, In Vitro Fertilization - IVF were used according to tubal patency of woman and andrological profile of the man.
Palermo et al – 1992 introduced technique of **Intra Cytoplasmic Sperm Injection [ICSI]** and from then paternal influence in embryogenesis has gained substantial attention. ICSI is first choice for male sub fertility. Pregnancies could be achieved by ICSI not only with the ejaculates of extremely poor quality, but also with spermatozoa from the epididymis and sperm retrieved from testicular tissue after biopsy.

ICSI requires the use of micromanipulator to inject single morphological normal sperm into the ooplasm. The success of ICSI procedure depends upon variety of factors: equipment used for procedure, experience and expertise of user, quality of oocytes and sperm, rupture of oolemma and risk of disturbing the spindle during injection.

2. Indication for ICSI procedure:

1: Ejaculated spermatozoa
- -Oligozoospermia
- -Asthenozoospermia
- -Teratozoospermia
- -Globozoospermia
- - Fertilization failure or poor fertilization in previous IVF cycle
- -Auto conserved frozen sperm from cancer patients in remission
- -Ejaculatory disorders (eg, electro-ejaculation, retrograde ejaculation)

2: Epididymal spermatozoa
- -Congenital bilateral absence of the vas deferens
- -Failed vaso-vasvasostomy
- -Obstruction of both ejaculatory ducts

3: Testicular spermatozoa
- -All indications for epididymal spermatozoa
- -Azoospermia
- -Necrozoospermia
- -For man with high sperm DNA fragmentation

4: For cryo preserved oocytes
5: For couple scheduled for PGS/PGD

3. Micromanipulation equipment

Companies dealing with Micromanipulators are

(I) Research Instruments – RI
(II) Narishige
(III) Eppendorf

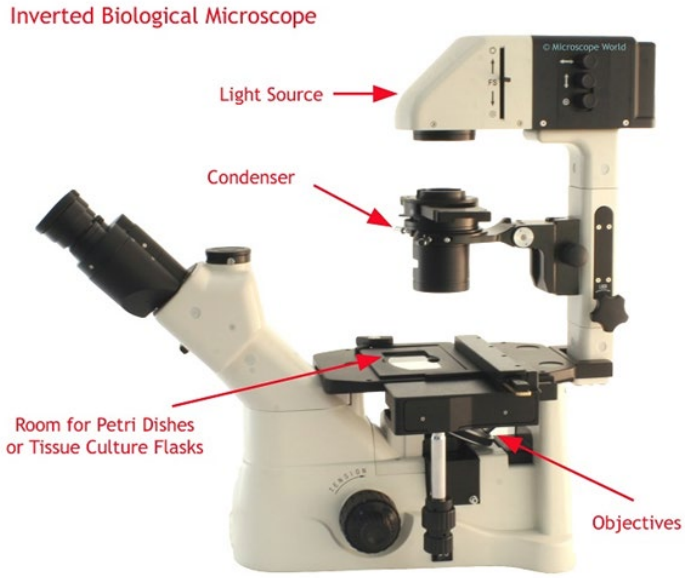
The standard parts of the equipment are:

a. Inverted Microscope:

A basic inverted microscope can be from **Nikon** or **Olympus**

The inverted microscope is designed with **the light source** and the **"condenser"** lens above the specimen. The condenser lens concentrates the light.
The **"objective"** and turret of the microscope is on the bottom. The objective focuses the light to produce a real image. Also, the inverted microscopes permit the use of Kohler Illumination which is a great option as well as Differential interference contrast (DIC) and phase contrast optics (creating different shades of brightness) greatly enhancing the image for better viewing.

John Lawrence Smith, of Tulane University, invented the inverted microscope.



A standard inverted microscope with binocular eyepieces of 10 X or 15 X magnification is commonly used for micromanipulation. The objective lenses vary from a combination of 4 X, 10 X, 20 X, 40 X and 100 X, and other magnification objectives may be available on request. It is useful to have the objectives fitted in ascending order on the objective turret so that a clockwise turn of the thumb wheel will increase the magnification in a step-wise fashion. Objectives should be completely screwed into the objective turret to provide optimal magnification.

Condensers may be fitted as a turret at the top part of the microscope. Hoffman optics accentuates optical gradients so that the resulting image has different shades of grey. The modulated lighter and darker areas produce a 3-D image, which facilitates micromanipulation.

Hoffman modulation optics has an accessory lens (Bertrand Lens) which can be inserted into the light path above the analyzer. When determining vibration directions or interference figures using parallel or convergent polarized light, this lens is used to bring the image of the interference figures into focus. It is essential to use the appropriate filter for each objective, and each set must be tuned by using the **Bertrand filter.**

Microscopes with **integrated modulation contrast (IMC)** are set up so that the orientation of the contrast (direction of the shadow in the 3-D image) is identical for all objectives. This ensures that the spatial impression of the specimen stays the same when changing objectives or magnification. Adjustment of the slit diaphragm is not necessary with IMC.

Nomarski interference contrast optics (also known as differential interference contrast, DIC) uses an illumination technique to enhance contrast in unstained, transparent samples. Light is polarized by a polarizer situated below the condenser, and the polarized light passes through a prism, which causes slight displacement. A second prism and polarizer provide interference contrast and the resulting shading results in a 3-D image. However, despite the introduction of high-quality plastics, this system can only be used in conjunction with glass-based dishes, and therefore has largely been replaced by the Hoffman system.
If gametes or embryos cannot be clearly visualized in 3-D, the optical modulation settings may need to be adjusted.

Heated stage:

Different types of heated stages are available: **opaque stages** allow passage of light through them or **metal stages** with an aperture for the light source.
The stage temperature should be set to maintain microdroplet temperature (and hence the gametes/embryos within them) at 37.0 °C, validated using a thermocouple placed within the microdroplet.
A single temperature setting will suffice only if a single type of dish is used for all micromanipulations. When different types of dishes are used, especially if the material of the dish base differs (e.g. plastic/glass), the heated stage should be reset and revalidated for each dish type.
A flow of warmed air below the stage has recently been introduced as an extra feature by some micromanipulation equipment companies, as a means of potentially improving the consistency of heated stage temperature. As with all new innovations, microdroplet temperature must be validated prior to use, to confirm that the air flow does not cause additional evaporation from microdroplets. Since the air flow is below the dish, this should not be an issue as long as sufficient oil overlay is used.

b. Micromanipulator:

Micromanipulator is fit onto the inverted microscope, and has the following parts.

I. Pipette holders:

Pipette tool holders (PTHs) secure the pipettes in the system; taking the PTH apart is a useful exercise, in order to understand how it fits together. The PTH is usually loosened at the tip by a counter-clockwise turn in order to fit a pipette. If oil syringes are used, ensure that a meniscus of oil can be seen exuding from the tip before inserting the micropipette. Pass the micropipette gently through the initial opening, and then through an internal "O" ring. There should be a slight resistance as it passes through the "O" ring; does not use excessive force, as this may cause damage.

— **Resistance and blockage:** Take the PTH apart and check for remnants from a previous pipette that may have broken inside. Passing a small-gauge needle through the lumen should ensure that the lumen is free. Once the pipette is in position, the tip can be tightened via a clockwise rotation. A pipette may sometimes break during installation, in this case the holders must be cleaned and checked to make sure that no glass particles are left inside; these will impair the gentle control of fluid flow required during manipulations. Oil systems can be flushed with the oil in the micro-injectors; air systems can be cleaned by pushing a fine metal wire through the entire length of the holder until the particles are removed.

The pipette tips must be flush with the base of the dish, and the angle in the pipette is therefore crucial. Commercial manufacturers provide Quality Assurance for the angles of their pipettes, but the angles may need slight adjustment for optimal performance in your system. Fitted angle guides for PTHs can help in setting the tool holder angle, but they should be used as a guide only, as the angle may need further adjustment. Angle guides can also be made in-house to facilitate pipette alignment.
Some PTHs are designed so that the complete holder can be moved laterally/outwards from the area of manipulation; this design facilitates pipette set-up and provides easy access to the dish. When familiarizing yourself with this type of micromanipulation station, be careful not to accidentally catch your hand on any fitted pipettes when removing the dish from the stage.
Double tool holders have often used for PGD or fragment removal. These are usually set to the same angle; the manufactured angle of the pipettes may differ slightly, and some can be set independently.
The angle of the injection pipette (IP) must be absolutely precise; the tip of the holding pipette (HP) is larger and can tolerate a wider variation in angle. Spending time to make sure that angles are accurately adjusted will make the micromanipulation procedure easier.

II. Manipulators:

Manipulators can be controlled mechanically, hydraulically (usually via oil) or electronically. Each type provides movement in all directions, operating via screws. Familiar system is essential, in particular, knowledge of how each part of the manipulator links to the next. All systems can provide excellent micromanipulation, but each requires appropriate training. Switching between systems may involve an initial period of adjustment, but this should be relatively simple if the basic skills have been learned.

Manipulators position the pipette tips precisely, in the X–Y–Z plane. Their joystick controls can be either hanging or upright. The coarse control allows larger movements, and fine control allows the microscopic movements required during the micromanipulation procedure. The level of control in oil-hydraulic systems can be changed by adjusting the movement ratio. All controls should initially be centred to the midpoint of their scales, to allow leeway in all planes. Complete familiarity with the manipulator controls is essential before starting any micromanipulation procedure.
Oil-hydraulic systems have better control, but may be prone to drift, particularly if there is any strain on the tubing. Always make sure that the controls are centred before each new procedure, with a little slack in the tubing.
More recent models include memory buttons that can auto-store pipette position and individual microdroplet position in the dish; these "extras" can potentially reduce the time needed for each micromanipulation. Digital images and procedure timing can also be recorded. **Piezo-assisted ICSI** has been reported **(Takeuchi et al., 2001)**, but its use to date is more applicable for animal models.

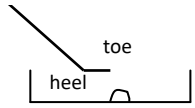
III. Syringes:

Pneumatic (air) and **hydraulic (oil) syringes** are commonly used for micromanipulation in ART, and each system has its own advantages/disadvantages. Oil systems must be completely free of bubbles, from the syringe and throughout the tubing. The syringes are easily filled and topped-up via a two-way screw.

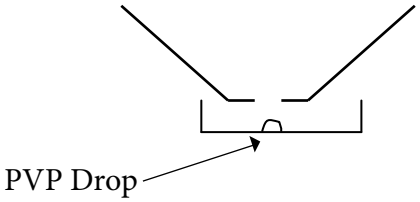
IV. Micropipettes:

Micropipettes vary in angle and lumen size, and tip may **be spiked** or **not spiked**; the type of pipette used is a personal choice. All micropipettes are very fragile, and great care is needed in removing them from their packaging. Make sure that you are familiar with the safest way to do this. Check each pipette to ensure it is not broken: breakages most frequently occur at the tip. Companies do their best to ensure safe packaging and delivery, but there may be a batch with frequent breakage. This must be reported to the company so that the source of the damage can be investigated. Never use a broken pipette, even if the damage is minor.

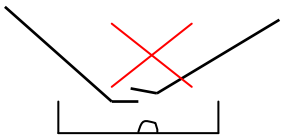
C. Mounting of pipette:



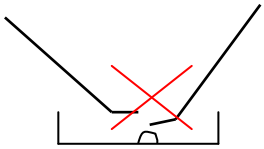
The pipette is mounted with the tip parallel to the media droplet/bottom of dish.



Holding-and injection pipettes are mounted with the tip parallel to the media droplet/bottom of dish, so that tip of injectionpipette can be moved into holding pipette(see picture below).



Pipette mounted **incorrectly**



Pipette mounted **incorrectly** toe will hit bottom

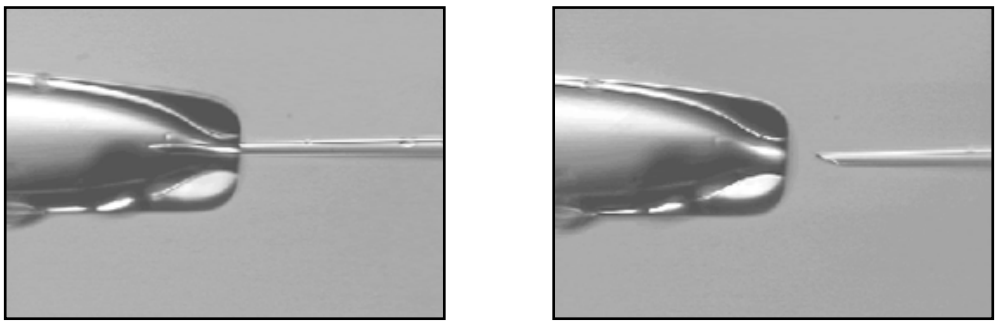


Fig: Correctly mounted injected and holding micro pipette. The contact of the pipettes should be avoided as it may damage the injecting pipette tip.

- The holding pipette is fitted into the left tool holder and the injection pipette is fitted into the right tool holder for righ handed embryologist.
- When focusing the microscope both pipettes must be in focus at the same time.

4. Preparation the day before the Oocyte pick-up:

a. Culture dishes:

4-well culture dishes or centre well dish are prepared and filled with IVF medium and kept under oil cover. The number of wells must match the number oocytes after injection.

Another 4-well culture dish is prepared with IVF medium under oil to maintain the oocytes during aspiration and until the ICSI procedure is started.

b. Oil:

Oil is incubated in the CO_2 incubator in a bottle or tube.

5. Preparation on the ICSI day:

a) Hyaluronidase:

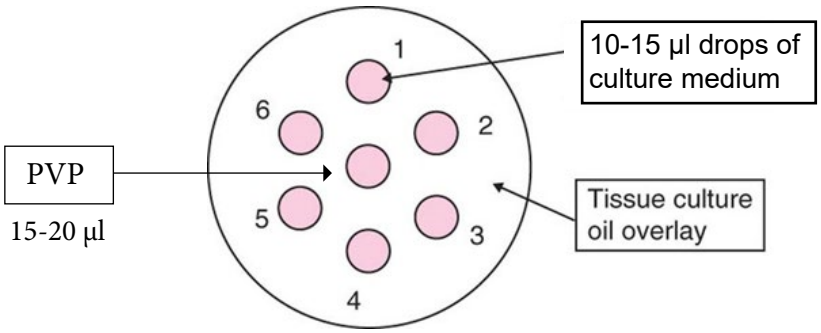
Generally 80 IU of Hyaluronidase enzyme is used for denudation. Two hours incubation is enough before denudation.

b) ICSI dish:

ICSI dish is prepared about an hour prior to performing ICSI.

ICSI is done in a flat Petri dish of dimension **50 x 09 mm**

1. Take a sterile ICSI dish and label with the name of patient using marker pen at the edge of the dish.
2. Now dispense 15-20 µl (10% w/v PVP (must be at room temperature) at the centre of the dish (for Sperm to slow down their movement)
3. Surrounding the PVP droplet dispense 6-8 droplets of HEPES buffered media of 10 µl each (for holding oocytes).
4. Cover the droplets with 5-6 ml of equilibrated sterile oil
5. Place back the lid and keep in an incubator till used.
6. Just prior use take denuded oocytes in the oocyte holding droplets and add processed sperms about 1-2 µl to the PVP droplet under stereo zoom microscope. Now your ICSI dish is ready for performing ICSI



c. Oocyte denuding:

1. The cumulus-oocyte complexes are kept in 4-well dishes with IVF medium from aspiration until denudation for approximately 2 hours and then do ICSI [**Alpha consensus- 2012**]

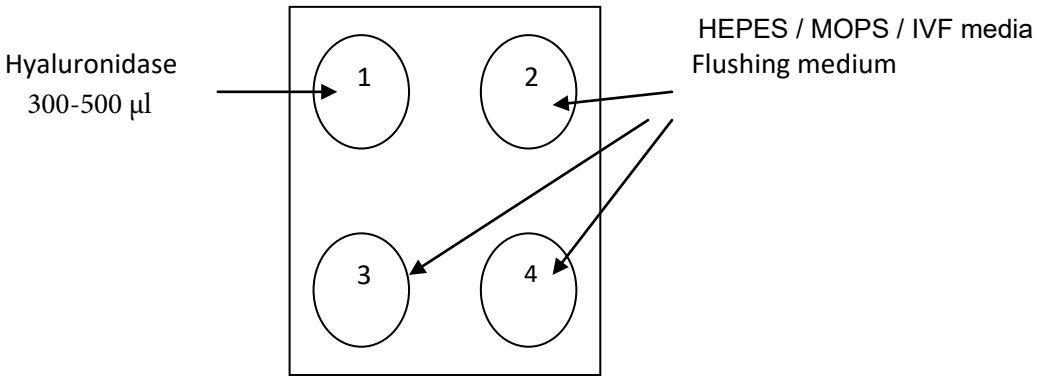

Human Reproduction, Vol.26, No.6 pp. 1270–1283, 2011
Advanced Access publication on April 18, 2011 doi:10.1093/humrep/der037

human reproduction ORIGINAL ARTICLE *ESHRE pages*

The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting†

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology

2. Preparation of denudation dishes.



The dish is placed in the CO_2 incubator until denudation if using IVF media.

The oocytes are denuded following these instructions:

a. 4 –8 oocytes are placed in Hyaluronidase in well no. 1 for maximum 30 seconds. The removal of the cumulus and corona cells is enhanced by aspiration of the complexes up and down with a pipette. (140-170 µl)

b. The oocytes are moved to well no. 2. Here the oocytes are rinsed for the remaining cumulus cells by using a pipette. The cleaned oocytes are moved to well no. 3.

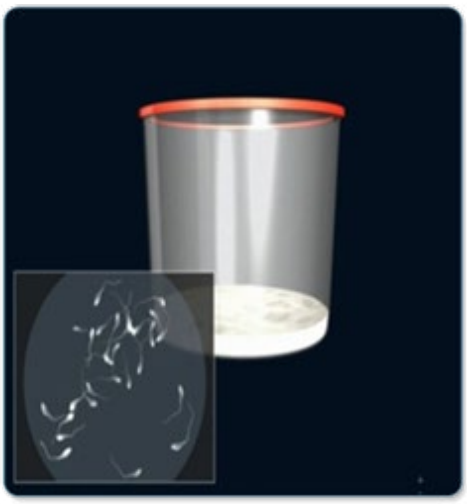
c. Move cleaned oocytes to the IVF medium dish until ICSI is performed.

6. Sperm preparation:

a. Semen collection:

Male partner should collect semen sample by masturbation.

Semen sample should be collected either at room near andrology laboratory or if collected at home should be brought to the laboratory within in 30 min.



WHO 2010 parameters

Parameters	Lower Reference Limit
Semen volume (ml)	1.5
Sperm concentration (10^6/ ml)	15
Total sperm number (10^6/ ejaculate)	39
Progressive motility (PR %)	32
Total motility (PR + NP %)	40
Vitality (live sperms %)	58
Sperm morphology (NF %)	4
pH*	>/= 7.2
Leukocyte* (10^6/ ml)	<1
MAR/ Immunobed test* (%)	<50

7. Sperm preparation method:

The density gradient is preferred method for oligospermic sample. Swim up is the choice of method for normozoospermic sample.

a. Density Gradient method:

Principle: Separates sperm cells based on their density

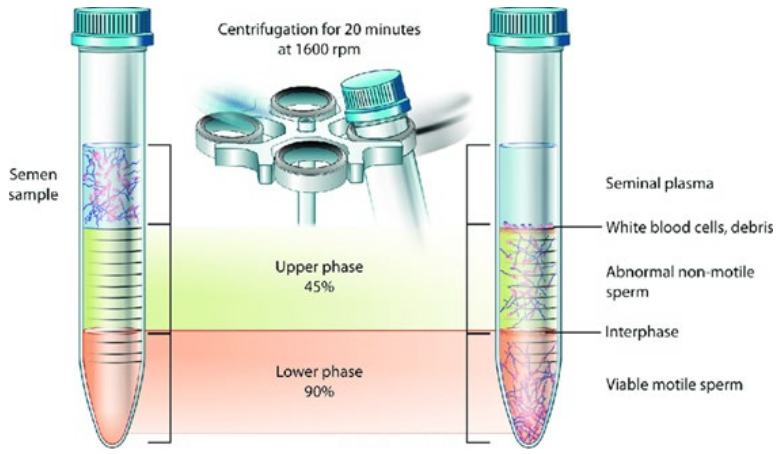
I**nterphase between 45-90%** - Leukocytes, cell debris and morphologically abnormal sperm with poor motility are trapped here and it minimises the production of ROS by leukocytes and non-viable sperm cells

Continuous density gradient - Density gradually increases from the top of a continuous gradient to its bottom

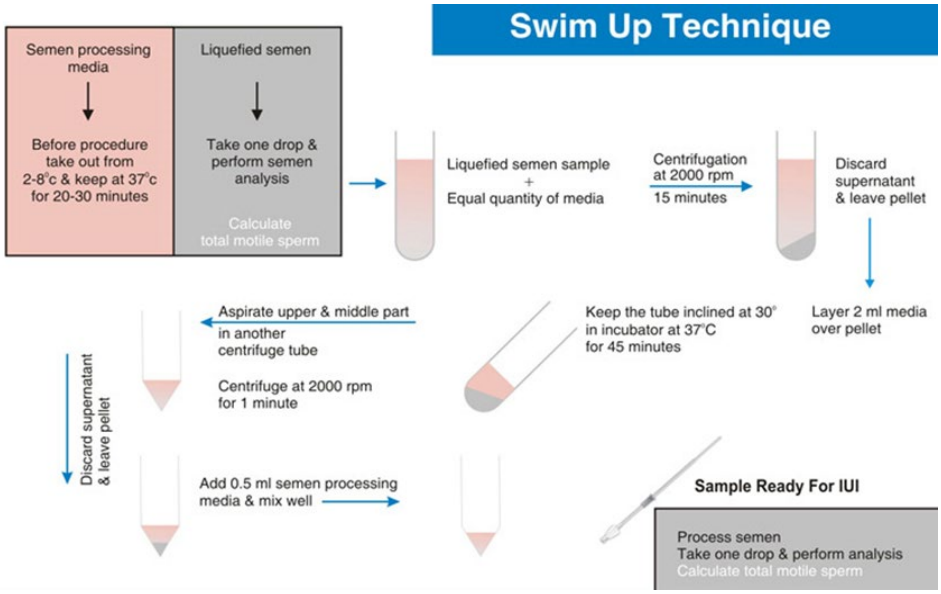
Discontinuous density gradient - Number of layers of decreasing density are placed on top of each other

STEPS:

1. Gently pipette 90% solution followed by 45% solution in a 15 ml coical test tube. Two visible layers should be seen.
2. Layer 2 ml of semen on top of the column.
3. Centrifuge for 10 min at 600g to assists the sperms in reaching the bottom layer.
4. All supernatant is removed leaving the sperm pellet, behind.
5. The pellet is transferred to a new tube and resuspended in 2.0 ml of HTF medium.
6. The tube is centrifuged again for 5-7 min at 250g in order to remove the silica particles found in gradient solutions.
7. Remove all supernatant leaving the pellet and resuspend it with 0.4 ml of media.
8. This method can be used to harvest motile sperms from poor quality samples. Usually, a two gradient column is adequate for the processing of most semen samples.

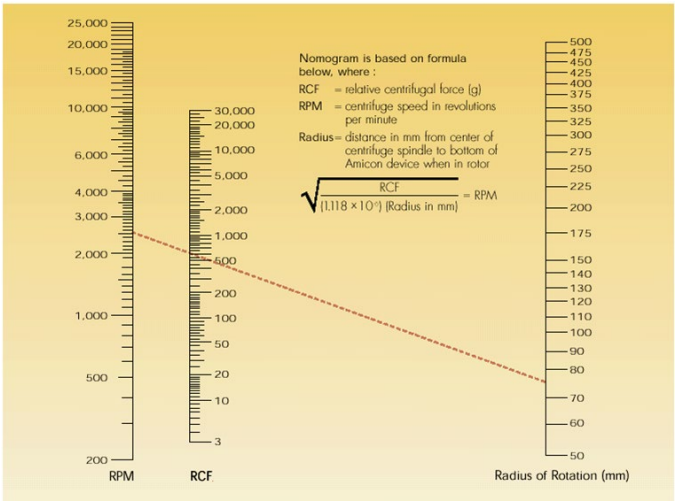


b. Swim up method:



Advantages	Disadvantages
- easy to perform - very cost-effective - usually recovery of a very clean fraction of highly motile spermatozoa	- restricted to ejaculates with high sperm count and motility - low yield - spermatozoa can be massively damaged by reactive oxygen species - significant decrease of the percentage of normally chromatin-condensed spermatozoa

- **The concept of Centrifugal force:** Each laboratory should determine the centrifugal force and centrifugation time necessary to form a manageable sperm pellet. When sperm numbers are extremely low, it may be necessary to modify the centrifugal force or the time, in order to increase the chances of recovering the maximum number of spermatozoa.



c. Sperm preparation for Retrograde Ejaculation:

Retrograde ejaculation occurs whereby the semen is ejaculated into the bladder. The acidity of the urine will kill sperms quickly. Alkalinization of the urine is very important in order to recover live motile sperms.

The patient is instructed to alkalinize his urine by intake of sodium bicarbonate, 3g (two table spoons) dissolved in a glass of water in the evening before bed. In the morning, the patient must empty his bladder completely and drink another glass of sodium bicarbonate before coming directly to the laboratory.

Ask the patient to partially empty the bladder, menstrubate and immediately urinate in multiple semen collection jars.

STEPS

1. The urine is divided into tubes and centrifuged for 10 min at 1500 rpm.
2. The supernatant is removed leaving behind the pellet. The contents of all tubes are pooled and semen analysis is done.
3. Add 2-3 ml of culture medium before proceeding with a gradient column.

d. Surgically retrieved sperm preparation method:

At the clinical level, different procedures of testicular sperm retrieval (conventional TESE, micro-TESE, FNA/TESA, MESA, PESA) are being performed, the choice is mainly based on the cause of azoospermia.

At the level of the IVF laboratory, mechanical procedures to harvest the sperm from the tissue may be combined with enzymatic treatment in order to increase the sperm recovery rates.

Mechanical method:

Different mechanical methods are used in order to maximize sperm recovery from testicular biopsies. Testicular tissue pieces can be processed by rough shredding, fine mincing, vortexing and crushing in a grinder with pestle.

Shredding and fine mincing of the tissue with rupture of the seminiferous tubules is so far the preferred and most widely used tissue processing procedure immediately after retrieval.

Enzymatic method:

The enzyme solution consists of buffered medium with 1000 IU/ml collagenase IV, 25 µg/ml DNase and 1.6 mM $CaCl_2$. A ready-to-use product is commercially available (GM501 Collagenase, Gynemed).

After exposure of the tissue at 37 °C for 1 h to 1 ml collagenase solution while regularly shaking the tubes, the reaction is stopped by addition of 10 ml buffered medium. The digested tissue solution is gently centrifuged at 50xg for 5 min in order to remove residual non-digested pieces or debris. The supernatant cell suspension is washed twice with buffered medium and the resuspended pellet is used to prepare droplets under oil.

If testicular aspiration or tissue has lots of RBCs than Erythrocyte-lysing buffer can be used either before or after enzymatic treatment.

Note: Simple wash is the choice of method for surgically retrieved sperm.

Preparation of RBC lysis buffer:

i. Measure 0.829 g Ammonium chloride (NH4Cl), 0.100 g Potassium bicarbonate (NaHCO3), 0.074 g Ethylenediaminetetraacetic acid (EDTA).
ii. Dissolve all components in 100 ml of tissue culture-grade water and adjust the pH to 7.2
iii. Sterilize the buffer using a 0.2-µm syringe filter and store in the refrigerator at 4°C for up to four weeks.

Note: Simple wash is the choicest method for surgically retrieved sperm.

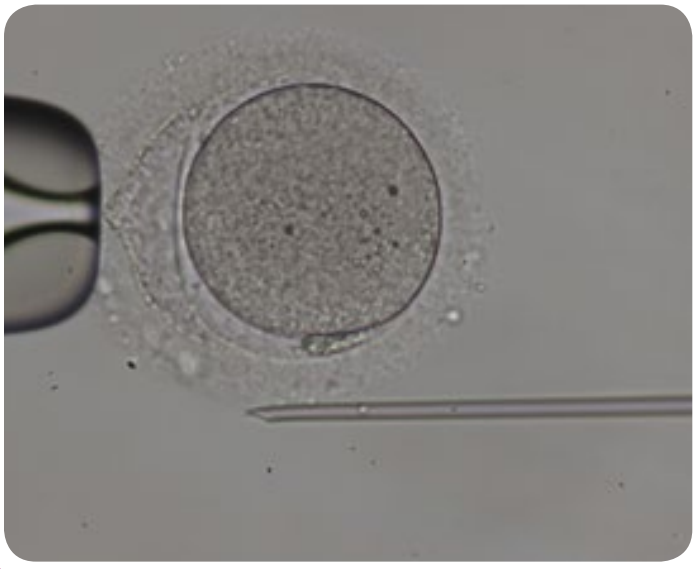


Figure 1 : The oocyte is held onto the holding pipette with the first polar body placed at the 6 o'clock position.
The sperm is moved along the injection pipette and brought to rest at its tip.

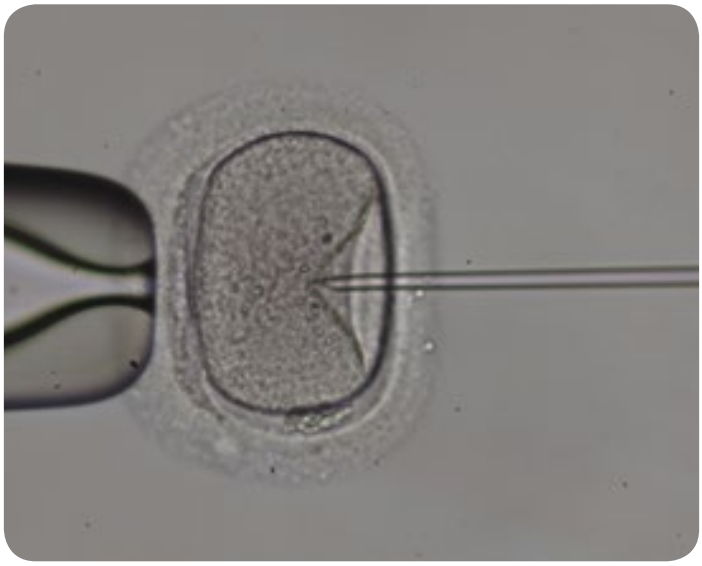


Figure 2 : Injecting pipette is pushed through the zona pellucida of the oocyte, causing an indentation (Funnel like pattern) of the oolemma.

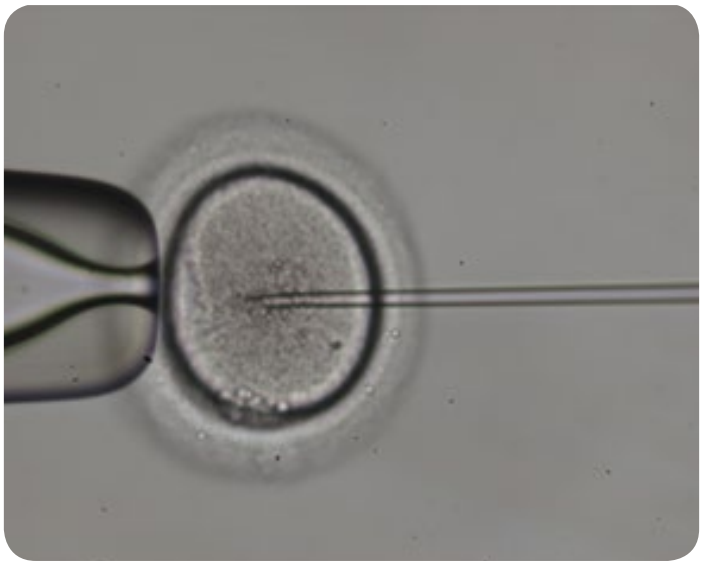


Figure 3 : The injection pipette is advanced half way into the ooplasm of the oocyte, and the ooplasm is aspirated into the injection pipette. The aspirated ooplasm plus the spermatozoon is deposited well towards the centre of the oocyte

- ICSI is done approximately 40 hours post hCG injection. An oocyte is held with the help of holding pipette keeping polar body either at 12 or 6 o'clock position.
- Sperm is immobilized by injuring on its tail and then aspirated into an injecting pipette.
- Sperm is injected into an egg keeping injecting needle at 3 o'clock position. Before depositing sperm into cytoplasm, aspirate cytoplasm into the injecting needle and along with cytoplasm deposit the sperm into an egg gently.
- Gently withdraw the injecting pipette from an egg.
- Wash the injected egg in media and further culture.
- After 12 - 18 hours check for fertilization.

a. When performing ICSI with PESA/ MESA/ TESA/ TESE sperms:

1. You can create sperm reservoir droplet where in the processed sample is taken directly into the dish.
2. Sperms from the reservoir are aspirated and moved to the PVP droplet under inverted microscope using micromanipulator and then proceed for the immobilization and ICSI.
3. Also in case of TESA samples where you cannot see any motility, the reservoir can be of HOS Media from where you select the sperms showing the coiling of tail and then proceed for ICSI like explained above.
4. In case of immotile sperm, one can also use motility enhancer like pentoxifylline or theophylline.

Note: 5 mM solution of pentoxifylline can be prepared by dissolving 1.391 mg pentoxifylline [sigma cat no - P -1784] in 1 ml of HEPES buffered culture medium.

b. Artificial oocyte activation:

The average normal fertilization rate in ICSI is approximately 80- 90%, however complete or virtually complete fertilization failure occurs in 1% to 3% of ICSI cycles.

The literature reveals fertilization failure after ICSI may be explained by defects in the oocyte, sperm or ICSI technique.

i. Oocyte factors:

Total Fertilization Failure after ICSI cycles is mostly due to

- low number of mature oocytes or morphological abnormal oocytes
- oocyte activation failure
- Poor ovarian reserve

ii. Sperm factors:

- Viability, Abnormal chromatin status
- The inability of sperm to activate oocytes (PLC zeta isoform virtual or complete deficiency) (TFF)

Human oocyte activation suggests that the spermatozoa activate the oocyte both by acting at a receptor on the oocyte surface and by releasing a soluble factor into the ooplasm. The latter can eventually assume the totality of the task, provided it is aided by artificial stimuli brought about by the ICSI procedure. After penetration by the fertilizing spermatozoon, an acrosomal-derived soluble factor (oscillogen, oscillation) activates the repetitive calcium ion transient release.
Before considering gamete donation, artificial activation of the oocytes may be proposed as an option to couples experiencing repeated failed fertilization cycles after ICSI.
There are **two methods** available for oocyte activation. These include:-

- Activation with electrical stimulation
- Chemical activation (Activation with calcium ionophore)

After ICSI, oocytes were washed three times with HTF and were exposed to 100 µl of HTF medium containing 5 µm of calcium ionophore A23187 (Sigma; stored at 1 mg/mL in Dimethyl Sulphoxide at – 20°C) under paraffin oil on a warm plate (37°C) for 5 minutes.
Sigma product Ionomycin calcium salt - I 3909 can also be used, 5 µl can be added to 0.7 ml fertilization media covered with oil.

9. The issue with ICSI:

a) Causes of oocytes damage:

- Having a holding needle that is too small, this may cause the oocytes to be pushed off in the injection procedure.
- If the oocytes are held on the holding needle too tightly, the opposing membrane to the injection side becomes stretched and damaged. Gentle suction with a large holding needle will avoid these problems.
- Any injection procedure that touches the opposing membrane will often result in degeneration of oocytes. So stay away from it.
- Movement of the injection needle in an up or down inside the oocyte will cause excessive damage.
- Excessive aspiration of cytoplasm during sperm injection may result in degeneration.
- The vibration of the injection needle may cause damage.
- Bad needle design will cause damage.

b) Possible reasons for Fertilization Failure:

- Non-breaking of the inner membrane
- Improper sperm immobilization
- Injection of an oocyte with misaligned polar body causes damage to the meiotic spindle.
- Sperm deposited in vesicles within the oocyte.

c) Possible Reasons for Poor Embryonic Development:

- Bad Micro injection technique
- Excessive time used for microinjection resulting in a significant decrease in temperature
- Poor culture media or out dated culture media
- Unknown oocyte abnormalities.

d) Indicators of a good ICSI:

- The Oocyte survival marked by intact ooplasm, healed oolemma, no leak of ooplasm from the site of injection and homogenous appearance like before injecting.
- Time per oocyte for injection should be less than 4 min (inclusive of sperm immobilization and oocyte injection) and totally a patient with oocytes should have been injected with in 30 min.
- Proper 2 PN and 2 PB seen at the end of 12-16 hrs with nucleoli well aligned in each pronucleus. At least 80% injected M II should have fertilized (ideally > 90% is expected).
- Good embryo formation on day 2 and further (at least 70% of fertilized oocytes should form embryos)
- Finally, it is the pregnancy rate and implantation rate which should be at most same like your conventional IVF.

e) Indicators of Bad ICSI:

- Oocyte going into necrosis immediately after ICSI – marked by central browning, swollen ooplasm, Cytoplasmic degeneration.
- Time taken for 10 oocytes to be injected is more than 45 min and all the oocytes were exposed to outer environment simultaneously (You can instead perform ICSI in batch of 5 oocytes per plate reducing the exposure of uninjected / injected oocytes to external environment)
- Fertilization rate and Embryo formation rate is less than 70%.
- Pregnancy rate, implantation rates are less than that of your conventional IVF treatment cycles.

10. Troubleshooting:

Source	Trouble	Error	Solution
Injection pipette	Extreme difficulties in penetrating the zona & the Oolemma	Needle tip not sharp	Change the pipette
	Small oil bubbles on the outside of the injection needle	The incorrect technique used in lowering the needle into the PVP	At the beginning of ICSI insert the needle into the PVP from the side
	Immotile sperm does not stay in position but moves up the pipette	Leakage in the injection system	Check & tighten all sealing.
	Tip of the pipette is clogged with dirt or little droplets of media		Blow the whole content out of the pipette into the oil which covers the ICSI dish until air bubbles appear
Holding pipette	Oocytes & pipette are not in focus at the same time	The pipette is installed in an upward instead of downward angle	Change pipette holder so that the tip of the pipette points towards the bottom of the petri dish
	The oocyte is pushed away from the pipette during injection	The pipette is not located in the equatorial segment of the oocyte	Lift the holding pipette slightly until oocyte is not longer in contact with the bottom
Microscope	Micromanipulators and /or sperm are not visible inside oil or medium	Objectives are wrong and/or don't fit the condenser	Change objective so they are compatible with the condenser (ask your dealer)
	No fertilization after ICSI because of oil and medium temperature	Stage warmer is too hot	Measure the medium temperature in the micro droplets (max 37˚C)
Microscope	No fertilization after ICSI because of oil and medium temperature	Stage warmer is cold	Measure the medium temperature in the micro droplets (max 37˚C)
Micromanipulators Micro tool holder	Oil is leaking out of the sealing of the micro tool holders	No sealing material inside the holders or is made up of wrong material	Sealing material must be made from silicone (not Teflon)
Micropipettes	The pipette cannot be filled with oil or media	Broken glass pieces inside the needle holders	Cleaning of the holders with a straight piece of wire
Injection pipette	Not possible to hit the sperm with the tip of the needle	The incorrect angle between pipette and the bottom of the Petri dish	Change the angle of the pipette till the tip points towards the bottom of the dish
	The delayed reaction of the liquid flow inside the pipette	Air bubbles inside the tubes or pipette filled with oil	Refill the tubes with light white paraffin oil
Microinjection	The sperm swims out the oocyte after injection	The micro needle did not penetrate the oolemma	After inserting the needle, some ooplasm must be sucked into the pipette to break the oolemma
	Movement of the sperm inside the pipette is not constant and controlled	Faulty tubing material	Use extremely stiff tubing (TFE)

11. Polyspermy:

- The formation of triploid zygotes differs in origin, depending on whether they are generated by ICSI or IVF.
- Only 1% of oocytes after ICSI result in tripronuclear zygotes and are digynic due to failure in extrusion of the second polar body

12. Fertilization check:

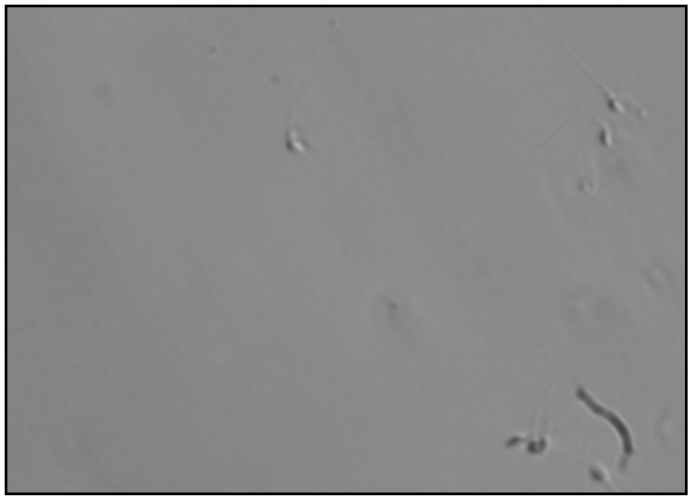
- Normal Fertilization: Presence of two pronuclei at 12- 15 hrs after ICSI
- Extrusion of second polar body

13. Sperm quality and ICSI:

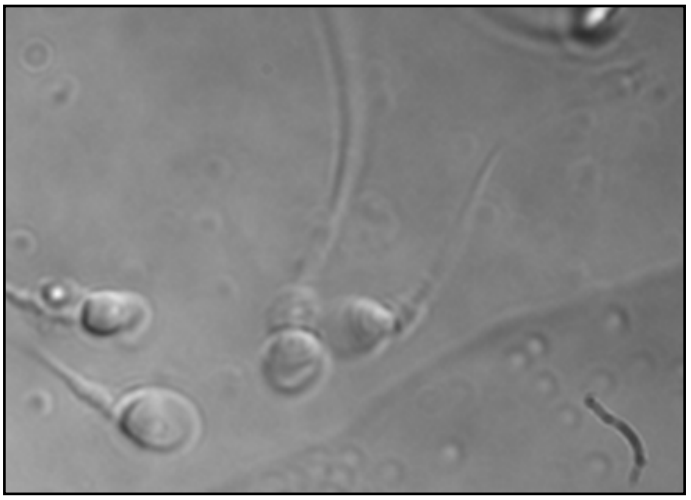
Success rates of intracytoplasmic sperm injection are independent of basic sperm parameters. Sperm can be selected with the use of following:

a. Real-time FINE SPERM MORPHOLOGY ASSESSMENT (400 X)

b. IMSI (intracytoplasmic morphologically selected sperm injection) is a new method based on motile sperm organellar morphology examination (MSOME) performed with an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification of 6600X.



400 X



6600 X

MSOME Criteria for the morphological normalcy of the sperm nucleus were defined as below:

- SMOOTH
- SYMMETRIC
- OVAL CONFIGURATION
- HOMOGENEITY OF THE NUCLEAR CHROMATIN MASS (no more than one vacuole / less than 4% of the nuclear area)

The average length and width limits in 100 spermatozoa with a normal looking nucleus, are estimated as follow:

- LENGTH: 4.75 ± 0.28 µm
- WIDTH: 3.28 ± 0.20 µm

14. Sperm binding assay:

a. Hypothesis:

Mature spermatozoa may selectively bind to Hyaluronan -HA.

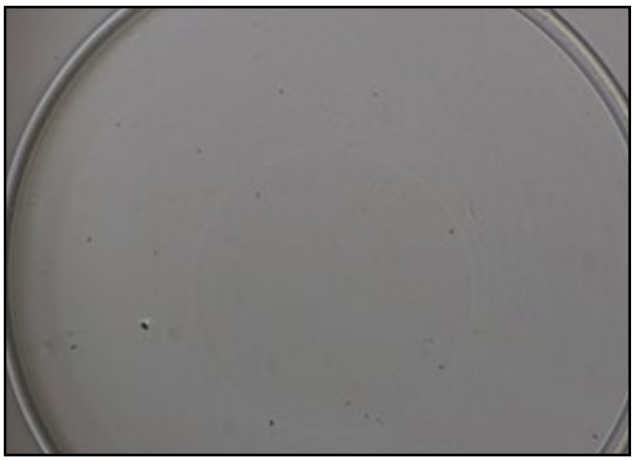
– Diminished sperm maturity (failure of spermatogenetic membrane remodelling) may be related to increased levels of chromosomal aberrations.

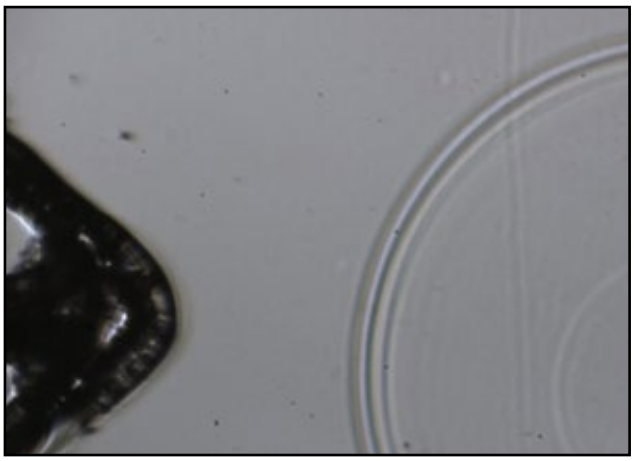
– Solid-state HA binding would facilitate the selection of individual mature sperm with low levels of chromosomal aneuploidies.

b. HBA assay basics:
– Contains thin layer of Hyaluronan
– Selected spermatozoa bind to solid state HA

c. Sperm preparation:
– A drop of washed spermatozoa is placed close to the edge of the HA spot (in the special Petri dish)
– Spermatozoa are allowed to migrate spontaneously
– HA-bounded spermatozoa are aspirated and ICSI carried out.

d. PETRI DISH ICSI (PICSI)





The **PICSI Sperm Selection Device** is intended for use in the treatment of infertile couples by ICSI; PICSI is indicated for the selection of mature sperm for injection.

STEPS

- Take out a PICSI dish, Sperm Preparation Medium and a diluted sperm suspension.
- Hydrate the Hyaluronan microdot with approx. 10 µl Sperm Preparation medium or other suitable sperm diluent. Overlay with oil and incubate for 5-10 min.
- Gently place 5-10 µl prepared sperm next to the hydrated Hyaluronan drop and make sure the two drops make contact. (Recommended concentration: 100.000 mature sperm / ml)
- After 5-15 minutes - Place the dish in the light of the inverted microscope and aspirate bound and highly motile sperm head first into the injection pipette.

- Lower the injection pipette into the center of the micro-dot.
- Aspirate only sperm from the middle of the PICSI droplet.
- Do not aspirate low motility sperm.
- Do not aspirate "hopping" sperm – indicating low binding ability.
- Select and transfer around 40-50 bound motile sperm and transfer the sperm to the injection dish into a PVP or SpermSlow droplet.

- Perform ICSI.

15. ICSI with spindle view:

The meiotic spindle plays an important role in the oocyte during chromosome alignment and separation at meiosis.

Spindle abnormalities in oocytes during meiosis increase with age in women over 40 years, which is a contributing factor to the aneuploidy in embryos after fertilization (Battaglia et al., 1996) and poor embryo

During ICSI, the location of the metaphase II (MII) spindle is commonly assessed in relation to the location of the first polar body. The ICSI needle must avoid the metaphase spindle during sperm injection.

Absorbed by birefringent structures within cells such as microtubules of the spindle

Up to 180º shift from Polar body

Use PB to adjust injection site thereby avoiding spindle damage during injection (less aneuploidy)

Use for selection purposes: Higher developmental competence of eggs and embryos where spindle can be seen (Wang et al 2001)

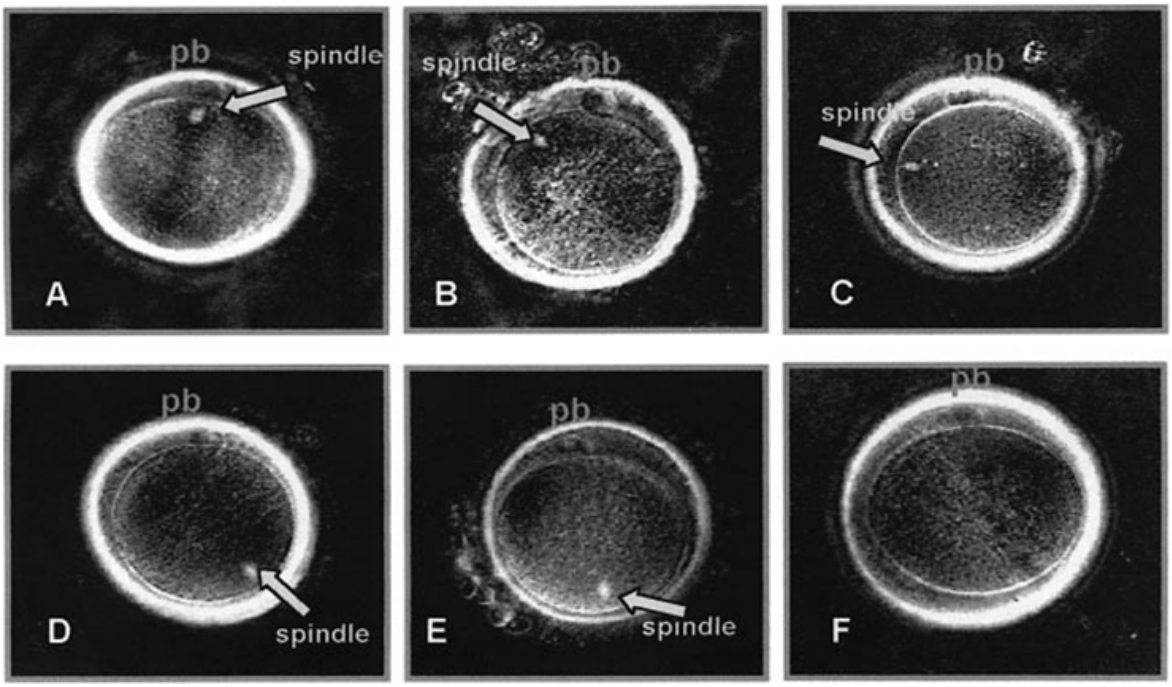


Figure: Birefringent spindles in living human oocytes imaged at metaphase II-stage using a Polscope just before ICSI. (A) Oocyte had the spindle located under the first polar body. (B) Oocyte had the spindle located between 0° and 60° relative to the polar body. (C) Oocytes had the spindle located between 60° and 120° relative to the polar body. (D) Oocytes had the spindle located between 120° and 180° relative to the polar body. (E) Oocytes had the spindle located exactly at 180° relative to the polar body. (F) Oocytes had no visible spindle. Original mag, 3200. PB = Polar body; arrow = spindle; bar = 50 mm

16. Round Spermatid Nucleus Sperm Injection [ROSNI] & Round Spermatids Sperm Injection [ROSI]

Men without spermatozoa or elongating spermatids in their testes have been considered sterile and are advised to consider using a sperm donor. However, these men may have round spermatids. We have been able to accurately identify these cells based on their structural and physical characteristics (verified by karyotyping and FISH)

ROSI techniques have the following theoretical advantages over ROSNI procedures:

i. Ooplasmic injection of the intact round spermatid cell ensures the transfer of all the cytoplasmic components of the male gamete into the female gamete;
ii. ROSI techniques are less time-consuming than ROSNI techniques; and
iii. Mechanical or chemical manipulations of the nuclear matrix and envelope of the male gamete are avoided when ROSI procedures are applied.

17. ICSI key points

PART 1: Sperm immobilization

Aim: To immobilize 10 sperm in 60 seconds

Step 1:- Set up injection pipette and sperm-drop in PVP + couple of micromanipulation drops
Step 2:- Check pipette works
Step 3:- Start timer
Step 4:- Immobilize 10 sperm
Step 5:- Stop timer
Step 6:- Record time on the datasheet
Step 7:- Repeat twice on five separate occasions

PART 2: Sperm pick-up and control (straight after part 1)

Aim: Immobilize sperm, orientate it, pick it up, move to new drop and release slowly. 10 in less than 5 minutes

Step 1:- Set up as above
Step 2:- Immobilize 1 sperm, orientate it and pick-up with injection pipette
Step 3:- Move to another drop and release sperm slowly
Step 4:- Repeat 10X. Stop timer
Step 5:- Record result on data-sheet
Step 6:- Repeat twice on five separate occasions

PART 3: Oocyte orientation

Aim: Orientate a completely denuded oocyte using a holding pipette. 6 in 2 minutes

Step 1:- Use 1 or 2 spare MII from either IVF or ICSI failed cycle
Step 2:- Set up 2 manipulation drops
Step 3:- Set up holding the pipette
Step 4:- Star timer
Step 5:- Use fine controls, X and Y shift and pressure to rotate oocyte and pick-up in orientation used for injection
Step 6:- Release slowly
Step 7:- Repeat but with polar body on opposite side of the oocyte 6 times
Step 8:- Stop timer
Step 9:- Enter results on the datasheet
Step 10:- Repeat twice on 5 separate occasions

PART 4: Oocyte injection (Can use set-up from part 3)

Aim: Put parts 1 -3 together and inject oocytes. 5 oocytes in 10 minutes

Step 1:- Set up pipettes and manipulating drops
Step 1:- Start timer
Step 1:- Inject as per clinical protocol
Step 1:- Repeat 5X with the same oocyte
Step 1:- Stop timer
Step 1:- Record time on the datasheet
Step 1:- Repeat twice on five occasions

PART-2

Frequently Asked Questions (FAQs)

Part 2

1. FAQs on Instrumentation

a. What is Hoffman module?

Hoffman modulation contrast microscopy (HMC microscopy) is an optical microscopy technique for enhancing the contrast in unstained biological specimens.

b. Which heating stage is preferred for ICSI procedure, linkam metal stage or Tokai heat glass stage?

Metal heated stage is from the outset whereas glass heated stage came into use later. Indeed the polyamide, which is good heat conductor with high heat resistance is sandwiched between two glasses and it ensures uniform heat conduction. But over a period the glasses may get scratches. It is a user's choice.

c. Which manipulator is superior?

Selection is based on features, function, ease of use, pipette set up and exchange, injectors, ease of installation, upgradation possibility, service and price.

d. What is spindle imaging system and what are the benefits of it?

Studies on polarized light microscopy revealed a correlation between the presence of a spindle in human metaphase II meiotic oocytes and the fertilization rate following intracytoplasmic sperm injection (ICSI). Using a spindle imaging system, it is possible to visualize the spindle image and the conventional light microscopic view of the oocyte simultaneously.

e. Which injecting holder is better pneumatic injection holder or hydraulic injecting holder?

Injecting holder should have features like the easy insertion of the injecting pipette, easy for Pressure release, operational responsiveness according to our preference and helps to achieve a wide range of movement. Again it will be a user's choice.

f. Why micro manipulator is advised to locate on vibration-free table?

Vibration free table or anti-vibration table are specially developed for micromanipulation in IVF labs. Vibration free table features an anti-vibration mechanism for passive dampening of the microscope. It helps smooth operation during injection.

2. FAQs on Microtools

a. Which is preferred injecting and holding pipette for an ICSI procedure?

Micro manipulation pipettes should have controlled dimensional specifications and sterilized by gamma irradiation and tested for single-cell MEA survival. Angle and diameter is a matter of choice.

3. FAQ on disposables and media

a. Can ICSI be done on normal plain petri dish instead of specially designed ICSI dish?

Plain dish can also be used for ICSI if it is tested for sterility and IVF use. The users should be comfortable with its use with out disturbing the alignment of micro pipette.

b. What is PICSI dish and what are the advantages?

PICSI is a method of selecting the best possible sperm for fertilization in the IVF protocol. PICSI is based on the concept of Hyaluronan binding of normal sperm.

c. Which media should be used to handle oocytes during ICSI procedure?

Oocytes can be placed in simple pre-warmed HEPES media droplet or gamete handling media which is commercially available.

d. In which media oocytes should be cultured after ICSI procedure?

If IVF lab is using single step media than after ICSI oocytes should be cultured in IVF media and for sequential stage media, oocytes can be transferred into cleavage stage media after an ICSI procedure.

4. FAQs on pre ICSI procedure:

a. When should one can prepare ICSI dish and what is the ideal incubation time before an ICSI procedure?

ICSI dish should be prepared with pre warmed media, PVP and oil, ideally two hours before procedure.

b. How long one can keep prepared sperm before ICSI procedure?

Fresh or frozen sperm should be prepared after an egg pick up and it can be stored for one to two hours depending upon the media in which it is prepared. If hepes is used then it can be stored at room temperature and if it is bicarbonate media then CO_2 incubator is used for storage.

c. What will be the ideal time to do ICSI after oocyte retrieval?

Ideally ICSI should be done 40 hours post HCG injection.

d. Why PVP is used for ICSI?

Polyvinylpyrrolidone (PVP) is a synthetic copolymer with a molecular weight of 360,000 Daltons. PVP is used for immobilization of sperm in ICSI procedure.

e. Why ICSI is advisable with testicular sperm for a man with high DNA fragmentation having sperm in ejaculate?

DNA damage occurs at the post-testicular level, hence testicular sperm may have a better DNA integrity than ejaculated sperm.

f. What is the reason for doing ICSI for frozen oocytes?

Freezing lead to premature exocytosis of cortical granules consequent to a zona pellucida molecular mechanism that inhibits natural sperm penetration hence ICSI is a method of choice.

g. What is rescue ICSI?

Fertilization failure is a known complication of conventional IVF cycles. Rescue ICSI was introduced in 1993 to overcome this catastrophe of failed fertilization but it remains controversial to date.

h. What is IMSI?

Intracytoplasmic morphologically selected sperm injection (IMSI) is a variation of ICSI that uses a high power microscope to select sperm. This allows embryologists to look at the sperm in greater detail at 6600X magnification.

i. It is difficult for complete denudation even after using hyaluronidase enzyme solution, what should be done?

Ensure that hyaluronidase has been warmed to 37ºC degrees before use. If the cumulus mass is sticky, repeated pipetting with pulled Pasteur pipette can help.

j. What should be done if during denuding oocyte get stuck at the end of the pulled Pasteur pipette?

With gentle use of a hypodermic needle free the oocyte from the tip of a pipette.

k. How do we prepare testicular sperm which has lots of RBCs?

If testicualr aspirate or tissue has lots of RBCs than Erythrocyte-lysing buffer can be used.

5. FAQs on during ICSI procedure:

a. Why polar body is placed at 12 or 6 o'clock position during ICSI procedure?

Studies have shown that the meiotic spindle is aligned beneath the first polar body (PB) in metaphase II human oocytes and to avoid hurting spindle polar body is placed either at 12 or 6 o'clock position.

b. Can ICSI be done with sperm without the tail as many times while immobilization fragile tail got detached from the head

This might happened with fragile sperm, ICSI should be avoided with head only as lack of sperm integrity can compromise normal mitotic division of the embryo, resulting in chromosomal mosaicism.

c. Why sperm preparation from a surgically retrieved sample some time forms a sticky and viscous interface between the medium and the overlaying oil?

It may happen due to release of proteins into medium during dis aggregation of the biopsied sample, creating a colloid like solution. Aspiration of the media under the oil and washing with the fresh medium solve the problem or new dish can be prepared.

d. Why some time no spermatozoa are seen after loading the PVP drop with an aliquot of sperm preparation?

It may happen if spermatozoa have not yet had time to swim to the bottom of the PVP drop or too few sperms have been added. Allow the sperms sufficient time to swim up or add more sample.

e. Why some time sperm got stuck to the tip of injection pipette while attempting to immobilize them?

Sperm can be removed by rapidly moving the tip of the pipette against the bottom of the micro injection dish or use the course manipulator up and down to release the stuck sperm.

f. What should be done if sperm got to stick to the bottom of the ICSI dish following immobilization?

Aspirate the sperm head into microinjection pipette and then move forward rapidly to dislodge tail from the bottom of the dish.

g. Why some time sperm rush into the injecting pipette despite repeated attempts for stabilization?

It may happen if the oil or medium in the injection pipette has become mixed with air bubbles, compromising hydraulic control. Replace the pipette if it fails to resolve the problem

h. Why sometimes it is difficult to penetrate through ZP?

It may happened if the injecting pipette is blunt or ZP is extremely though. Blunt needle should be replaced and faster approach can solve the issue of though ZP.

i. Why some time oolemma fails to rupture following its aspiration into the injection pipette?

It may happen if oolemma is tough and elastic. Advance the injection pipette upto two-thirds in to ooplasm and deposit the sperm.

j. What should be done if there is an excessive aspiration of ooplasm or excessive outflow of medium following sperm deposition is observed?

This can happen if injection pipette is partly blocked or the oil or medium has become mixed with air bubbles, compromising hydraulic control. In such case change the injection pipette.

k. Why some time we observe that oocyte shrunk following injection?

If the oil overlay is not covering completely the drops of media, resulting in evaporation and increased osmolarity. Top up or replace the oil with the fresh batch.

l. What is the advantage of the use of pentoxifylline ?

Pentoxifylline, a methylxanthine derivative, enhances the quality and quantity of sperms. It increases cellular levels of cAMP and in turn, stimulate a cAMP-dependent kinase which induces sperm tail protein phosphorylation with subsequent increase in sperm motility.

m. When one can use HOS solution?

Hypo Osmotic Solution - HOS helps to select viable sperm from the immobile population of sperms. Viable sperm tail will coiled in HOS solution, such sperm after wash can be used for an ICSI.

6. FAQ on post ICSI procedure:

a. What treatment will benefited after ICSI for globozoospermic , severe OAT or cryptozoospermic sample?

Fertilization failure after ICSI may be explained by defects in oocyte, sperm, or the ICSI procedure.
Oocyte factors includes inherited genetic defects and oocyte activation failure. Sperm factors includes abnormal chromatin status or inability of sperm to activate oocytes.
Artificial activation of the oocytes may be proposed as an option to such couples.

There are two methods available for oocyte activation. These include activation with electrical stimulation and activation with calcium ionophore.

After ICSI , oocytes were washed three times with HTF and were exposed to 100 µL of HTF medium containing 5 µM of calcium ionophore A23187 (Sigma; stored at 1 mg/mL in dimethyl sulfoxide at −20°C) under paraffin oil on a warm plate (37°C) for 5 minutes.

Sigma product Ionomycin calcium salt - I 3909 can also be used , 5 ul can be added in 0.7 ml fertilization media covered with oil.

7. General question - the slippery slope of ICSI

a. If a busy lab has more than five ICSI cases in a row, do embryologists need to change injecting and holding pipette after every procedure?

Performing intracytoplasmic sperm injection (ICSI), it is customary to use
one injection pipette for sperm selection(immobilization and injection) and one holding pipette (to hold an egg) per patient.

Embryologist should change the micro tools after each ICSI for following reasons:

- Mechanical immobilization of the spermatozoa before injection plays an important role in achieving consistently high fertilization rates .Immobilization is commonly performed by pressing the sperm tail against the bottom of the injection dish using the tip of the injection pipette. After injecting into an oocyte, aspiration of ooplasm (cytoplasm of oocyte) is another important factor for oocyte activation and fertilization. Sharpness of needle will help to immobilize sperm , to break the oolemma and to aspirate the ooplasm. Hence needles should be changed.

- Every couple undergoing an IVF cycle desire a normal healthy baby and we as professionals are committed towards it. In using the same micro tools for holding - injecting dganetes, we might compromise the genetic makeup of the progeny. so it is must to change micro tools as it hardly takes two minutes.

b. Is ICSI valid for all?

The indication to perform intracytoplasmic sperm injection (ICSI) in the earlier days was male factor infertility. Today, this have been expanded to all most all the cases because of the fear of fertilization failure after conventional IVF insemination.

Mostly ICSI is indicated for the:

a. Oligoasthenoteratozoospermia

b. Patient with previous failed fertilisation.

c. Patient whose fertilization rate have been unexpectedly poor.

d. Patient with surgically retrieved spernatozoa (TESA, PESA and MESA) eather for azoospermia or man with high DNA fragmentation.

e. Patient who want to go for PGS or PGD.

The basic difference between two techniques is that in conventional IVF 50,000 - 100000 prepared motile spermatozoa are placed around the oocyte in a dish while ICSI involves injection of single spermatozoa into a mature oocyte.

In indicated cases for ICSI there is no choice, however in men with normal sperm count (as per WHO 2010 semen criteria) and no obvious other reason, which insemination method will be a right option? So here comes a debate for Needle Vs dish. To do ICSI for all or only for indicated cases?

Lets start with advantages and disadvantages of each method based on different studies done globally.

The advantage of conventional IVF insemination includes:

- As motile spermatozoa are placed around the oocytes with cumulus for few hours there is a chance that metaphase I oocyte can also fertilized after reaching metaphase II stage.
- Reported studies on sibling oocyte shows an advantage in terms of number of oocyte fertilize, implantation with, clinical pregnancy rate, or live birthrate as compared to ICSI over conventional IVF.
- Comparative Studies done on young donor oocyte model for insemination method (both conventional IVF as well as ICSI) to shows no advantage on standard insamination
- There are evidences that the conventional IVF exhibits advantages over the ICSI method, infertility indipendent of male factor for advance age patients with fewer (≤ five) oocytes retrieved.

Few disadvantage of conventional IVF insemination are

- Sperm with DNA fragmentation or if used in higher concentration may increase oxidative stress in media if kept for prolong period in dish. This might be over come by changing the media after 5-6 hours of insemination.
- Approximately 10% cases can not get fertilized due to female, male or “un explained nature”.

Pros and Cons of ICSI:

- ICSI is a procedure which effectively eliminates male infertility by introducing sperm cell directly into an egg, hence increases chances for fertilization for male factor.

The performance of ICSI for male factor infertility may be associated with:
Chromosomal abnormalities of embryo (deletion of genes) which might increase the insidence of miscarriages, birth defect and potential increase in male infertility of the male offsprings (later in life).
Above may not be true if ICSI is done for non male factor infertility.

- ICSI is only choice of method for azoospermic men to become father.
- TESA / TESE ICSI is also preferred for men with high sperm DNA fragmentation, azoospermia and for men with the variable count per ejaculate from a standard laboratory.

Few important points to be considered for an ICSI cycle:
a. There are approximately 3% chances of fertilization failure due to defects in oocytes, sperm or the procedure itself.

b. Needs a complete knowledge of micromanipulator

c. Need a skilled embryologist

d. It is a time consuming procedure

Take home message from this will be careful evaluation of couple is very essential before undergoing conventional IVF insemination, ICSI or split of both
Every lab should maintain their fertilization,implantation, pregnancy and take home baby rate to evaluate and study their data.

PART-3

Setting up of Micromanipulator

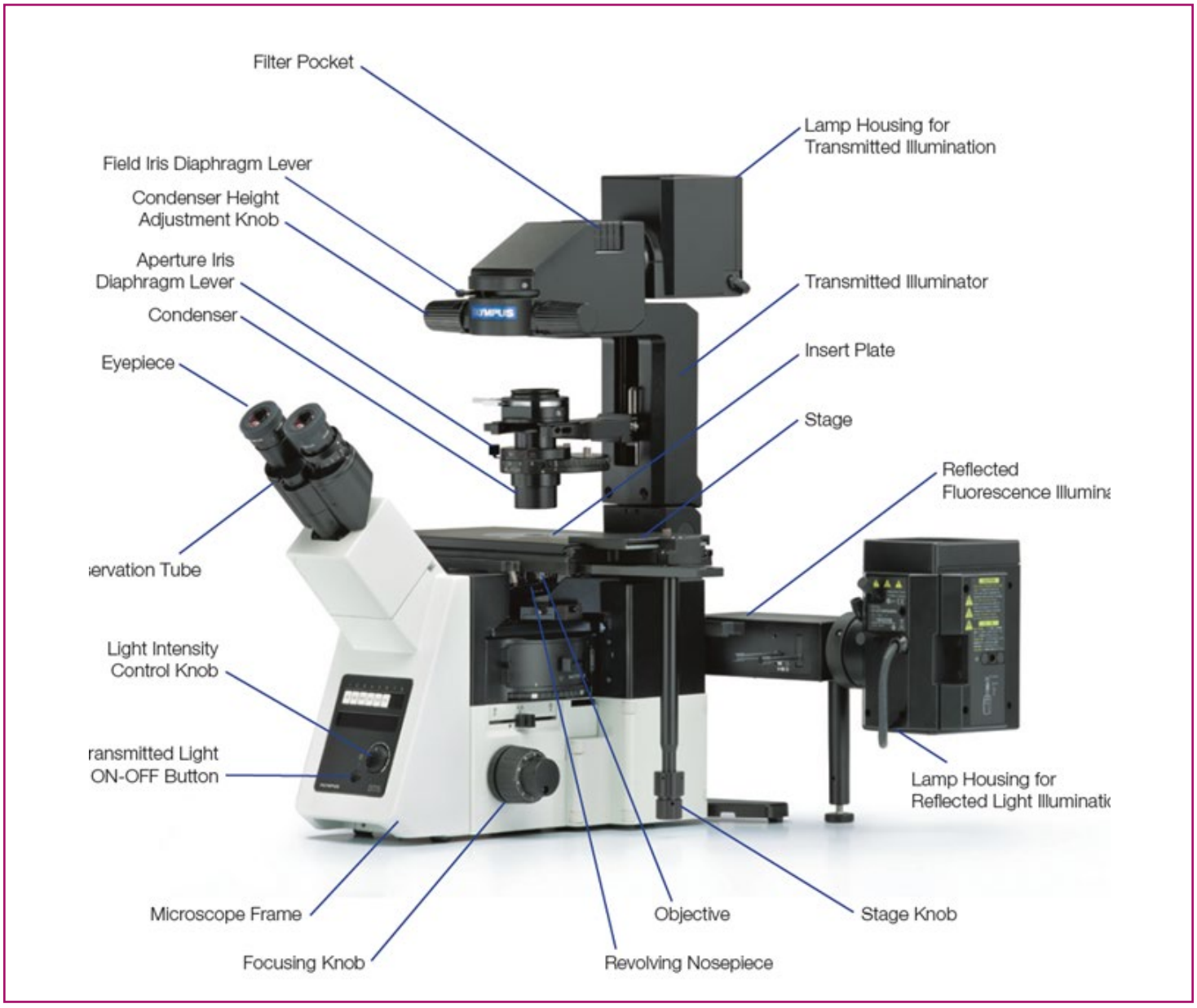
Part 3

Setting up of Micromanipulator

1. Micromanipulation equipment

Micromanipulation is the technique whereby sperm, eggs and embryos can be handled on an inverted microscope stage attached with the micromanipulator.

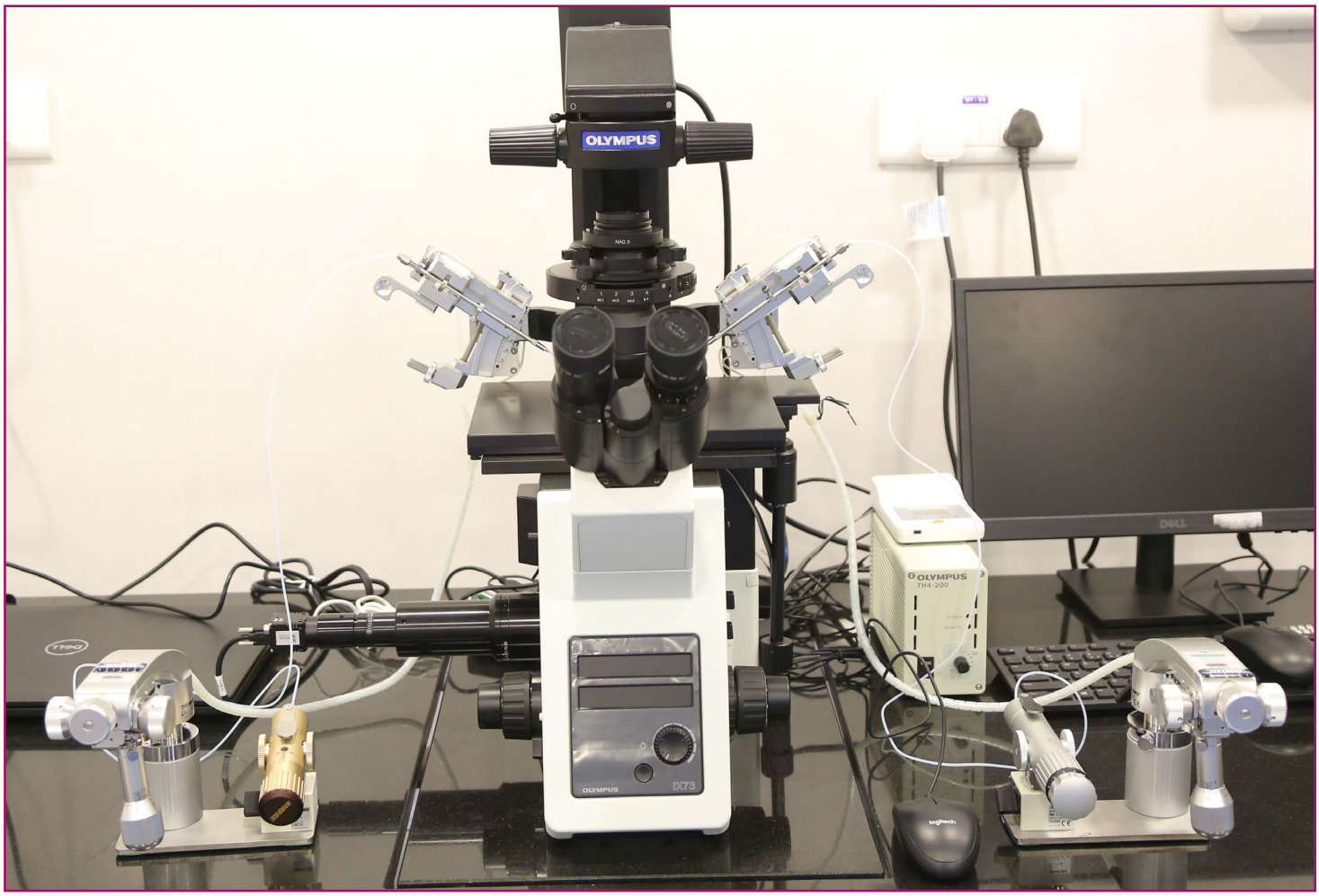
2. Inverted microscope:



- An inverted microscope is a microscope with its light source and condenser on the top, above the stage pointing down, while the objectives and turret are below the stage pointing up. It was **invented in 1850 by J. Lawrence Smith, of Tulane University.**
- The stage of an inverted microscope is usually fixed, and focus is adjusted by moving the objective lens along a vertical axis to bring it closer to or further from the specimen. The focus mechanism typically has a dual concentric knob for coarse and fine adjustment.

- Depending on the size of the microscope, four to six objective lenses of different magnifications may be fitted to a rotating turret known as a nose piece.
- Inverted microscopes are used in micromanipulation applications where space above the specimen is required for manipulator mechanisms and the microtools they hold, and in metallurgical applications where gametes can be placed on top of the stage and viewed from underneath using reflecting objectives.

3. ICSI with Narishige manipulator



- **Elichi Narishige established Narishige scientific instrument laboratory in Tokyo in 1953 and first three axes hydraulic micromanipulator was produced in the early 1980s.**
- Narishige company distributed through four big microscope companies Leica, Zeiss, Olympus and Nikon. Nikon - Narishige jointly launched three axes, oil hydraulic, joystick controlled micromanipulators.

3.1 Narishige workstation installation

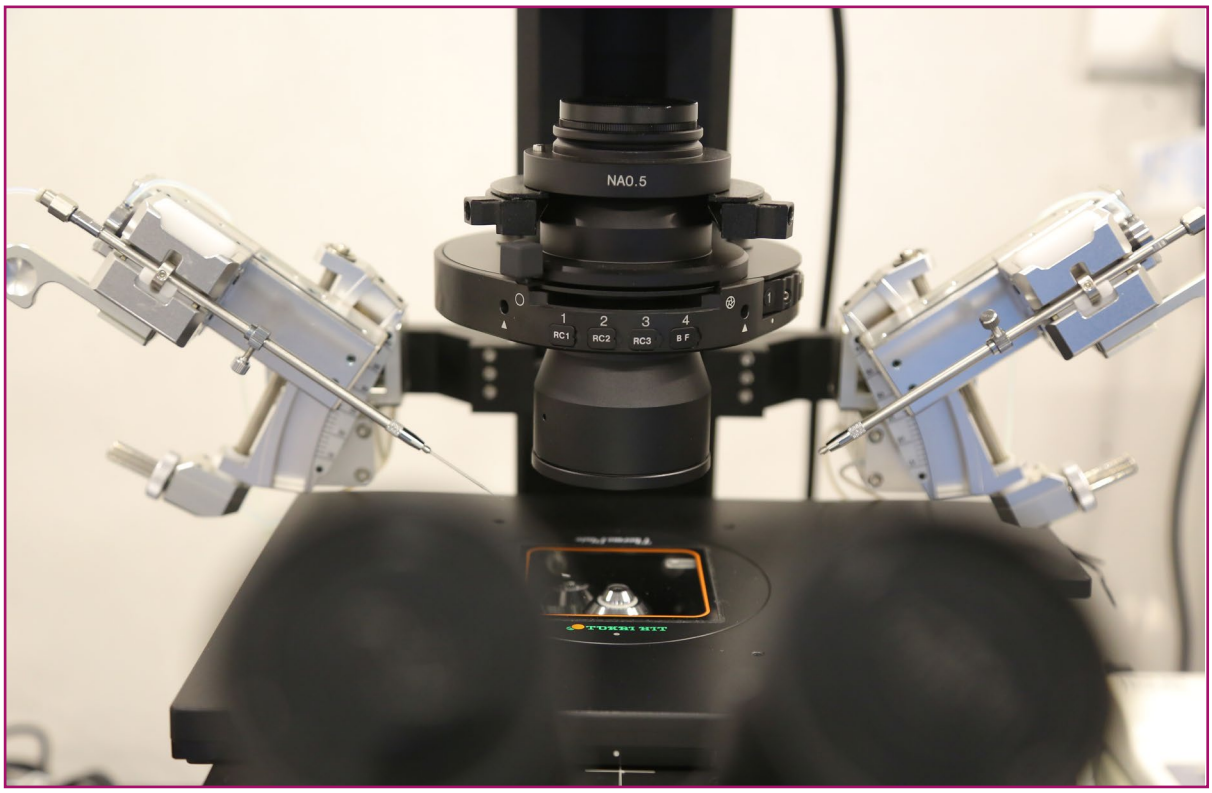
Narishige workstation is modular in construction with following parts.

Microscope with

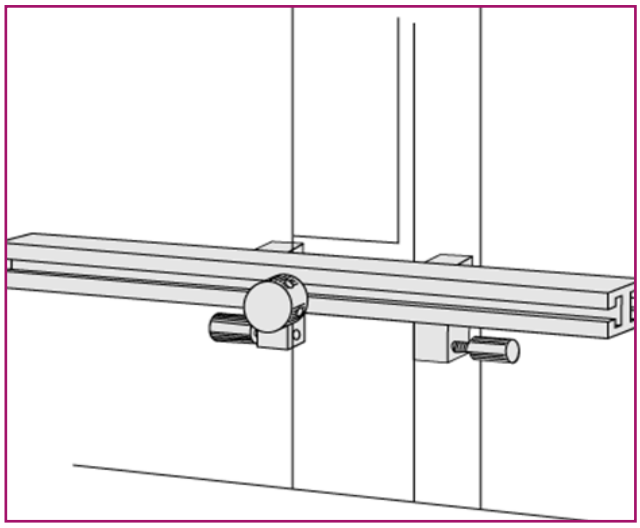
- Mounting chamber
- Course manipulator
- Micromanipulator with universal joints
- Micro injectors including tool holders

Generally, workstation installation is done by the company engineer.

a. Mounting chamber installation:



- This allows the manipulators to be mounted on the microscope, usually on the illumination support pillar.
- Mounting adapters are available with or without vertical bars for mounting mechanical or motorized course manipulators.



b. Course manipulator installation

- They are fitted by means of two large screws onto the slot in the vertical pillar of the mounting adaptor

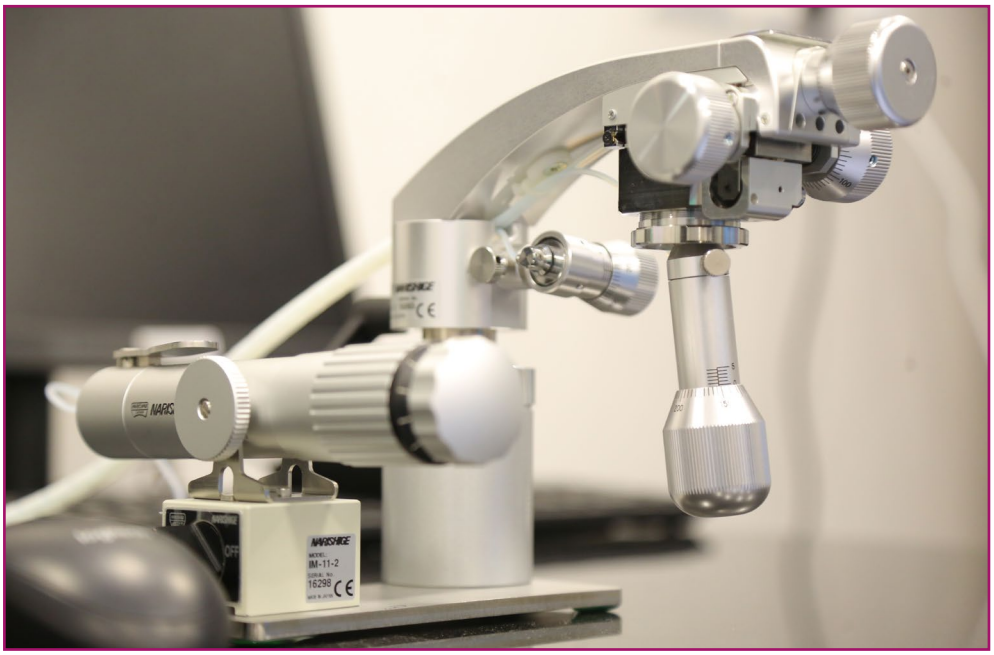
c. Micro manipulator installation

- Narishige micro manipulators are controlled via a hydraulic or pneumatic system.
- A master cylinder with a rolling rubber diaphragm is connected via a tubing to a slave cylinder of equal size.
- The system is oil filled or air and closed to the outside.
- At the master cylinder end, movements of the hand controller [joystick] push a short rod into the diaphragm, which displaces the hydraulic oil and moves out the diaphragm at the slave cylinder by an equal amount.
- The displacement of the slave diaphragm thus pushes a rod attached to the slide on the head stage and moves the pipette holder on three axes.

d. Universal joints:

The swing and tilt universal joints with a hydraulic or pneumatic control that allows the pipette to be raised and lowered. (Fig d)

Picture courtesy: Yashoda Fertility and Research Institute



(Fig d)

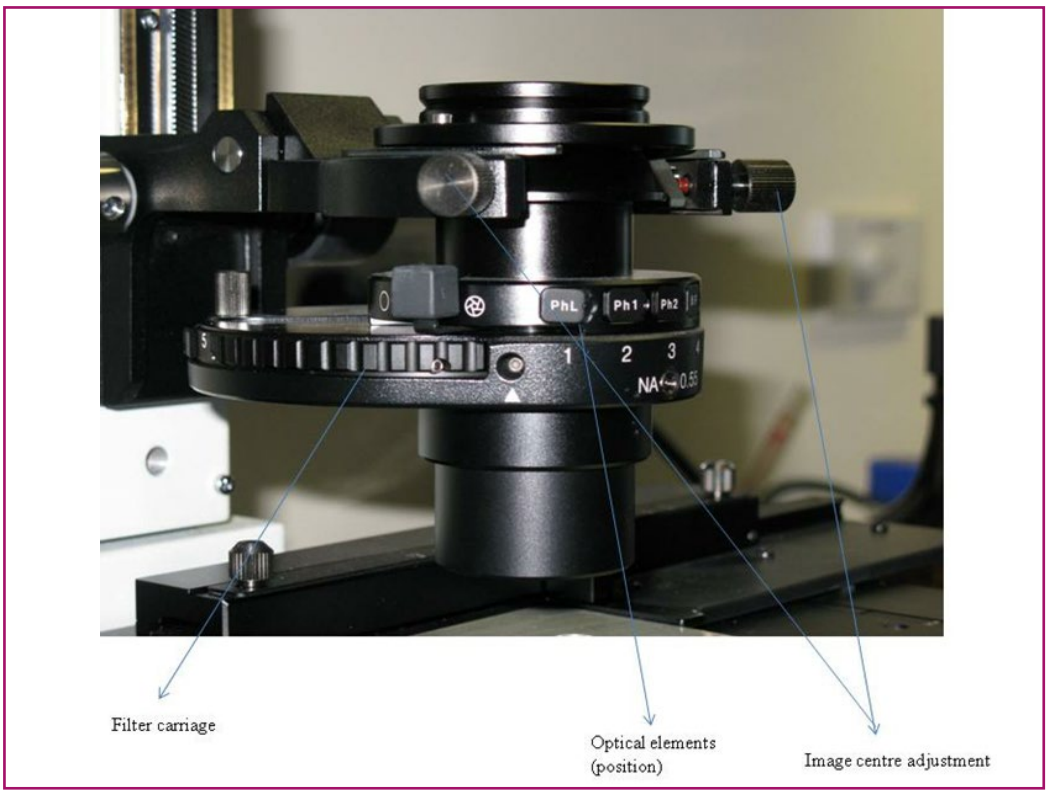
e. Micro Injectors:

Microinjector syringe of hydraulic manipulators is of glass barreled and the plunger are tipped either with a Teflon plug or metal, with two silicon 'O' rings (Fig. e)

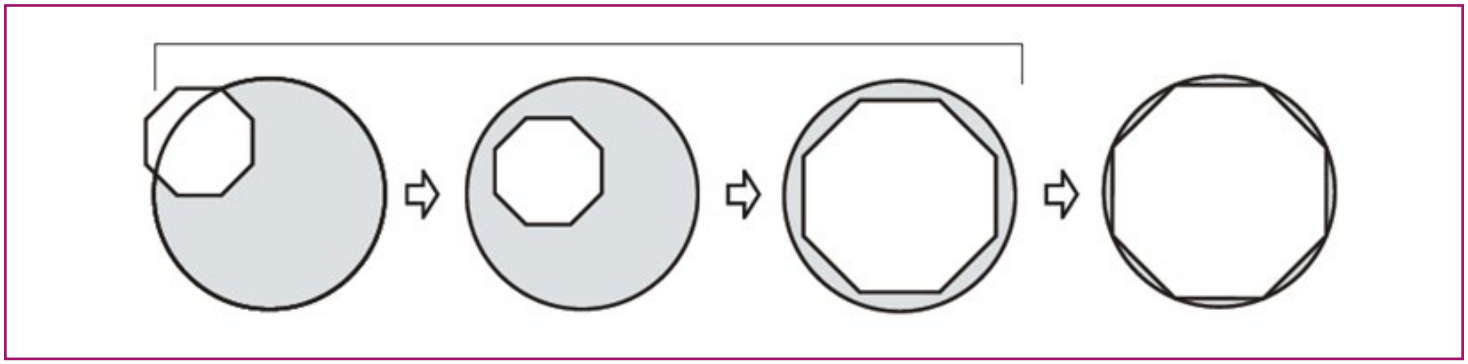
(Fig e)

f. Inverted microscope alignment:

- Switch on the inverted microscope. Get the mechanical stage to its centre X-axis '0' and Y-axis '0'
- Keep the light intensity to the comfortable level; select the lowest power objective in bright field mode.
- Close the diaphragm of the light source completely, open the condenser diaphragm completely. Now you can see an Octagon in the microscopic field.



- If the Octagon is not clear bring it to crisp focus by using the Condenser height adjusting knob, but, do not use the microscope focus knob.
- The octagon should be in the centre of the field, if not then bring it to the centre of the field by working simultaneously on both condensers centring screws.



- Open the light source diaphragm such that the edges of the octagon just touch the edge of the microscopic field. Readjust the light intensity to your comfort level.
- Turn to 10x objective remove one of the eyepieces of your microscope and look through. You will see the objective HMC/RC/DIC depolarizes.

Objectives

- Differential Interference Contrast (DIC)

It is suitable for viewing colourless and transparent specimens and live cells.

- Relief Contrast (RC)

It helps to observe the conversion of irregularities on the specimen into bright/dark contrast and specimens appear three dimensional

- Hoffman modulation contrast (HMC)

- It is an optical microscopy technique for enhancing the contrast in unstained biological specimens.
- Turn the condenser filter wheel to position the HMC/RC/DIC filter corresponding to the 10X objective.
- Using the filter positioning screws / Allen keys rotate and adjust the filter on the condenser to match with the objective depolarizer. Now your 10X object with its corresponding filter is aligned properly.
- Whenever you select the 10X objective you should turn to 10X filter.
- Repeat this alignment with each filter corresponding to each objective like 20X, 40X separately (Except 4X which doesn't have an HMC/RC/DIC filter corresponding to it).
- Now your microscope is properly installed and ready to use with its condenser and filter aligned and centred.

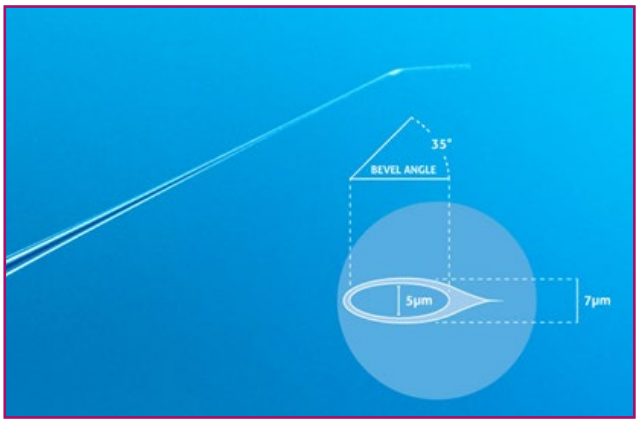
g. Alignment of Micromanipulators

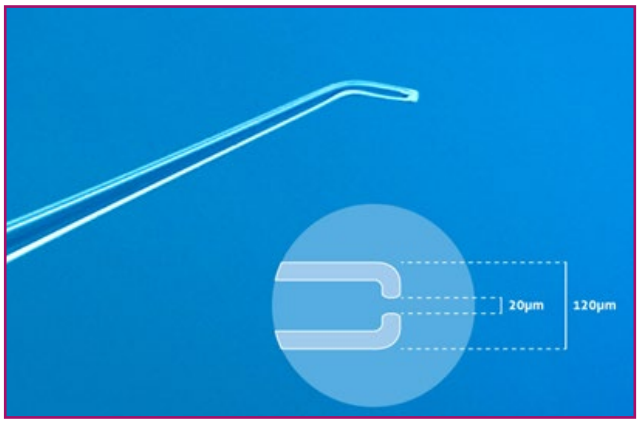
This method holds good for Narishige micromanipulators

- Switch on both micro manipulators
- Bring the coarse manipulator to its centre using the motorized joystick of the coarse manipulator such that X-axis '0', Y-axis '0' and z-axis '0'.
- Work on to the fine manipulator controls and bring the fine manipulator axes to their centre X-axis '5', Y-axis '5' and Z-axis '5'.
- Adjust the micro tool holder at 35^0 angle, depending upon angle of injecting or holding needle.
- Adjust the micro tool holder at the centre of the microscope field.
- Insert the holding and injecting pipettes into the microtool holders and attach the loaded micro tool holder to the micromanipulator.
- Now switch on the microscope, select 4X objective in Bright Field (BF) mode.
- Take a dish and make a scratch mark on to the base of the dish.
- Using microscope focus, bring the scratch mark to its crisp focus and do not change the focus from now on till alignment is done. Remove the dish from the stage.
- Align the pipettes.

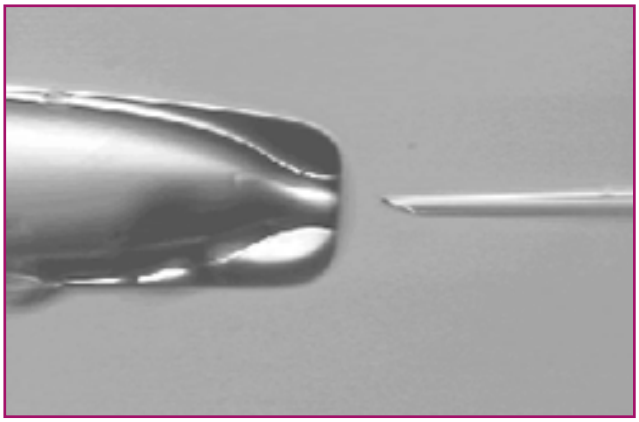
h. Loading of micro tools: [Injecting and Holding pipette]

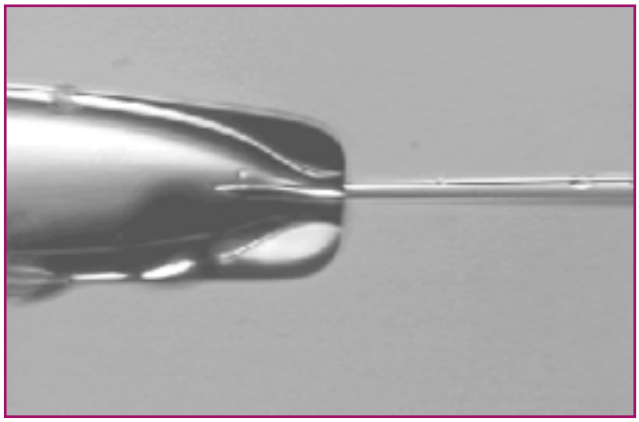
- Remove the microtool holder from the Universal holder joint.
- Loosen the tip of the microtool holder gently by unscrewing (anticlockwise) 2-3 turns.
- Take out a sterile angled micro tool and gently insert into the lumen of the microtool holder through the loosened tip. You will feel a slight resistance when you are passing through the silicon gasket in the microtool holder. Advance further till the resistance is lost and then tighten the tip by screwing action (clockwise). Now your micro tool is secure and loaded.





- When using an oil syringe first blow out some oil till oil starts freely flowing. Then insert the microtool in through the tip of the microtool holder. After the resistance is lost tighten the tip to secure the micro tool.





i. Filling of oil in an Oil injector:

- Detach the Teflon tube from the hub of the syringe.
- Fill oil completely to the full capacity of the syringe, through the lower hub of syringe taking care to avoid air intake.
- Attach the Teflon tube with the micro tool holder and gently start expelling oil through the Teflon tube. Take care to see that the tip of the micro tool holder is loosened.
- When oil starts freely flowing through the tip of the micro tool holder and no air bubbles are seen in the Teflon tube, insert the micro tool holder with the loosened tip into a tube filled with oil and start aspirating the oil into the syringe. Avoid aspirating the air.
- Fill the syringe to 80% of its capacity; tighten the tip of the micro tool holder and wipe off excess oil using a tissue, now the oil filled injector is ready for use.

j. Equilibrating an Air syringe:

- Before using the air syringe and micro tools (with the media in which the manipulation is being done) should be equilibrated for optimum efficiency and safety of the gametes.
- First, align the micro manipulator as per instructions precisely.
- Take about 30µl of pre-warmed media for holding pipette or room temperature warmed PVP by injecting pipette into a sterile dish.
- Tighten up the equilibration screw (Narishige Only) and start aspirating the media into the micropipettes by turning the injector anticlockwise to about 25/30 turns.
- Leave for about 2-3 min, and then loosen the equilibration screw (Narishige only).
- This will equalize the intra tubal pressure with that of the atmosphere.
- Now your air syringe is ready for use.

3.2 Troubleshooting

a. Issues with motorized course manipulators:

Issue 1: One axis of motorized manipulator moves intermittently

Probable reason: Fixing screws may be loosened spontaneously.

Suggested solution: The motor spindle needs to be realigned and tightened

Issue 2: One of the motorized manipulators does not work in one axis.

Probable reason: One of the control cables has become jammed between the motor and mounting adapter and microscope.

Suggested solution: Switch off the manipulator and gently free the cable, possibly by undoing its fixing screws and removing it from the mounting adapter.

Issue 3: One of the motorized manipulators does not work in any axis.

Probable reason: maybe because of loose wire or electrical fault. Need to call service engineer for this issue.

b. Issues with Hydraulic joystick operated micro manipulator

Issue 1: The pipette moves in the opposite direction to the movement of the hydraulic joystick control in X and Y axis

Probable reason: The ratio adjustment of the hydraulic joystick control has been adjusted past the zero position.

Suggested solution: Directly below the joystick pivot is an adjustment ring with graduations. Loosen the adjacent ring, which allows the joystick to move freely in its socket. Now hold the joystick so that it does not spin, rotate the ration ring clockwise until it will not turn any further. Then, rotate the ring slightly so that the infinity symbol ά is found. Rotating the ring anti clockwise should result in a larger displacement of the tooltip for a given movement of the joystick. Unscrewing the ring results in the ratios moving through ά, 600:1, 300:1 , 200:1, 150:1,100:1, 86:1, 75:1 and 66:1
Adjust the ratio ring until the movement of the pipette tip at normal working magnification seems appropriate. The ratio is personal preference.

Issue 2: The pipette moves in the opposite direction to the movement of the hydraulic joy stick.

Probable reason: The heels of the bent micro tool rubbing on the bottom of the dish cause this phenomenon.

Suggested solution: Realign the pipette.

Issue 3: The pipette hardly move in response to commands from the hydraulic joystick.

Probable reason: The ratio setting is too fine.

Suggested solution: Readjust the ratio

Issue 4: The pipette either hardly moves or fails to move in one axis.

Probable reason: Either the rotary control of the X or Y axis has been turned out too far, or the cartridge diaphragm for that axis is ruptured.

Suggested solution: Need to contact to service engineer.

Issue 5: Purple coloured precipitate in the hydraulic lines of the micromanipulator.

Probable reason: old age and cumulative effects of UV light.

Suggested solution: To replace with new.

c. Micro injectors

Issue 1: Oil leaks from the syringe plunger or tip

Probable reason: Because of narrow diameter and pressure built up inside the syringe.

Suggested solution: To fill the oil again with the care that no air gap should be there

Issue 2: Delay between the movement of the injector control and movement of the sperm in the pipette

Probable reason: Due to backlash in the injector mechanics and may be due to poor hydraulic caused by air bubbles in the system.

Suggested solution: Backlash should be addressed by company engineer and for poor hydraulic - fill the oil again.

Issue 3: Oil fails to exit the tool holder when the injector screw is turned clockwise

Probable reason: There may be a block in the tool holder.

Suggested solution: Check the patency of tube holder by inserting the big wire into it and again check for oil drop from the tip of the tool holder.

Issue 4: Injecting pipette cannot be inserted past the sealing ring of the tool holder

Probable reason: There may be the remnants of a broken micro pipette inside the sealing ring.

Suggested solution: Inspect the condition of the sealing ring inside the tool holder, clean it. If found damage then replace with the new.

Issue 5: The range of the course manipulator is insufficient to position micropipette in central vicinity of the mounting stage

Probable reason: The micropipette has been pushed too far into the tool holder or tool holder has been placed too far into universal joint

Suggested solution: Loosen the universal screw and check the position of the micro pipette in the tool holder

Issue 6: Micromanipulator warm stage is too far away to allow the pipette tips in the microscope field of view

Probable reasons: The pipette holder is positioned close to or far away from the microscope stage.

Suggested solution: It is only with the practice one can learn the setting.

4. Setting up of RI Micromanipulator

Research Instruments (RI) offers Two Micromanipulator systems.
a. The Integra Ti
b. The Integra 3 (Latest)

4.1 THE INTEGRA Ti

The Integra Ti is the previous model to be offered by Research Instruments and represents a move towards a more integrated design.

S. No.	Equipment	Description
1	**Micromanipulators**	Movement of the micropipettes provided by Research Instruments' TDU500 is purely mechanical. They incorporate both coarse and fine controls in one compact unit. Unlike the Narishige manipulators, the Research Instruments units are not controlled remotely. The manipulator joysticks extend downwards from the Integra stage. Semicircular hand-rests are supplied, which greatly assist ergonomics. Like the Narishege micromanipulators, rotating the fine control knob actuates the z-axis movement. The movement is proportional and very similar in feel to Narishige. Being based on simple mechanical components, the Research Instruments micromanipulators are also highly reliable and do not require routine servicing and maintenance. The reduction ratio can be altered from 100: 1 down to 500: 1, the total maximum displacement being 5 mm.
2	**Quadruple heated stage**	The Integra Ti is supplied with four independently controlled heated surfaces or, as an upgrade option, a glass heated central stage. The second and third additional heated stages are designed to maintain the temperature of additional Petri dishes while others are being worked on. The fourth heating channel can be used to heat an external plate such as a stereo microscope.
3	**Pipette set-up**	All Research Instruments tool holders have a special function to instantly move the micropipette vertically by 16 mm. The home function, consisting of a special objective and spacer, allows the micropipette to be set up 16 mm above the Petri dish and then lowered rapidly to the desired position. This system is extremely quick, taking just ten seconds to load a pipette and locate it within the microscope's field of view.
4	**Screw- actuated syringe injectors**	The **Screw- actuated syringe (SAS)** air injectors are the only air syringes designed specifically for air injection of sperm, eliminating the need for oil. Due to their distinctive design, some users have nicknamed them 'the mushroom'. The addition of an equilibration button allows the micropipettes to be equilibrated with the external atmospheric pressure at any time, a very useful feature. The SAS-SE has a very smooth rotation.
5	**Micrometre syringe MSHD**	Suction/injection is achieved by using the rotary actuator with a fine micrometre-thread which has a 15 mm movement range. A Hamilton 1 ml gas-tight glass syringe with a Luer taper is incorporated. A Luer-taper hypodermic needle is mounted on the taper of the Hamilton syringe, the syringe is connected by hard polythene tubing to a micropipette, which in turn, may be fitted to a micromanipulator or similar instrument. The micrometer-actuated syringe is a complete unit, mounted on a small base. Its convenient dimensions enable it to be ergonomically positioned next to the control lever of a micromanipulator to facilitate single-handed operation of both instruments.

4.2 SET UP

a. Setting up Micropipettes

The following protocol demonstrates the microtool set-up of a Research Instruments system. It assumes that installation is complete and that the PL30 tool holders are attached and adjusted to the bend angles of the micropipettes in use.

- Take an empty Petri dish, lightly scratch a cross in its centre, on the upper surface
- Place the dish in the centre of the microscope stage (avoid having a 'foot' on the leading edge of the dish which could engage on the raised centre of the WIS insert). Centralize the cross by using the lever.
- Select the 4 X objective (without spacer), focus on the scratched cross on the upper surface. During the set-up procedure ***do not adjust the microscope focus again during this setup.***
- Select the 4 X objective with the spacer.
- Raise fully, the lever. Insert a holding pipette into the left-hand side micropipette holder. Insert the micropipette holder into the tool holder of the micromanipulator. Repeat the above steps to set up the injection pipette.
- **Centralize all the controls of the micromanipulator by ensuring that both fine and coarse control levers are vertical.**
- Select the 4 X objective (**without** spacer). The scratch on the Petri dish should still be in focus.
- Lower the lever fully. The micropipette will come into view slightly above the dish surface. (If the pipette is repeatedly found to be too high or too low, the distance of travel can be adjusted using a small screwdriver to adjust screw (H)). Fine adjustments can be made using the micromanipulator fine control lever .

b. Injector set-up

The air syringes of the Integra system must be equilibrated before sperm injection. The holding side is not so critical, but it should be equilibrated nonetheless. To equilibrate the sperm injector SAS, place a drop of medium or PVP in the Petri dish. It is not necessary to cover this drop of medium with oil, providing that you are working in an aseptic environment. Lower the micropipette into the equilibrating drop. From its lowest position, rotate the SAS anticlockwise (i.e. upwards) a number of turns (approximately 75% of its travel). Leave to equilibrate for approximately five minutes; the PVP/media should rise up the micropipette until it almost reaches the unpulled shank of the micropipette. Press the equilibration button to neutralize the air pressure. For older models without an equilibration button, to equilibrate the pressure within and outside the micropipette it is necessary to disconnect and reconnect the tubing between the SAS and tool holder. Lift the pipette using the PL30 vertical movement lever and exchange the equilibration dish for the injection dish.

c. SAS INJECTOR SET-UP (Step by Step)

Assembly: TO FIT THE HARD POLYTHENE TUBING

- Look underneath the base.
- Insert the end of the hard polythene tubing through the 'hole' in the side of the base.
- Ease the tubing over the 'spigot'; ensure that the tubing is securely fitted.

(If the tubing becomes worn at this connecting point it could be a source of air-leaks: - remove the tubing, cut-off approx 1cm, then reconnect the tubing.)

- The syringe is then connected by the hard polythene tubing to a micropipette, which in turn, may be fitted to a micromanipulator or similar instrument.

Operation

Suction or Injection is obtained by turning the metal coloured 'rotator' on-top of the syringe:
Injection: Rotate clockwise (the rotator will go down)
Suction / Aspiration: Rotate **anti-clockwise** (the rotator will go up)

Equilibrating Screw- Actuated Syringe

- Place a fairly large drop of PVP/media in the centre of a Petri dish, (or a dish-lid).
- Rotate the 'rotator' **CLOCKWISE**, until fully down.
- Press the **'E-button** (equilibrating button)'.
- Using the micromanipulator controls, lower the micropipette into the media/PVP drop.

- Rotate the SAS anticlockwise (i.e. upwards) approx, 75% of its travel (i.e. approx 75 mm).
- Leave to equilibrate for approx 5 minutes, the PVP should rise up the micropipette until it almost reaches the un-pulled shank of the micropipette.
- Press the 'E-button' and continue to press the button while rotating the SAS clockwise (down); leave approx 25mm of the travel for adjustments during the injection procedures.
- Lift pipettes. Exchange Petri-dishes.

d. MSHD MICROMETER SYRINGE (Step by Step)

Assembly

- Place the Hamilton syringe, with the coupling attached in the clamp. Tighten the two syringe clamp screws.
- Push the micrometre actuating screw into the socket of the micrometre syringe body and tighten the micrometre clamp screw firmly.
- Rotate the micrometre actuating screw thimble so that the spindle enters the hole in the micrometre coupling. Continue rotating until the coupling begins to move forward.
- Tighten the micrometre coupling screw very firmly. The syringe plunger should now rotate with the micrometre actuating screw and move in or out with the spindle.

Operation

a. Topping-up the system with oil

- Unscrew the conical tip from the micropipette holder.
- Place the end of the micropipette holder into the oil.
- Aspirate oil by rotating the micrometre-screw "S" anti-clockwise.

b. Inserting the micropipette into the Micropipette holder:

When inserting the micropipette the oil must **NEVER** reach the tip of the micropipette (even temporarily) as this causes it to be 'sticky' and makes it almost impossible to control the sperm. It is essential to have a 30-40 mm air-gap between the oil and the medium. If you have a messy/problem micropipette – change it for a new one.

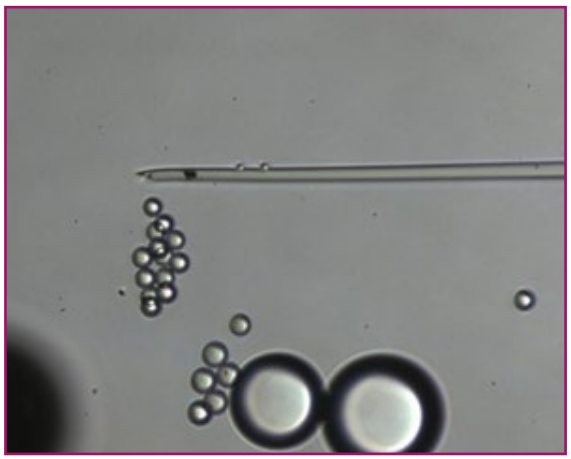
4.3 Troubleshooting

a. Issues with injecting pipette

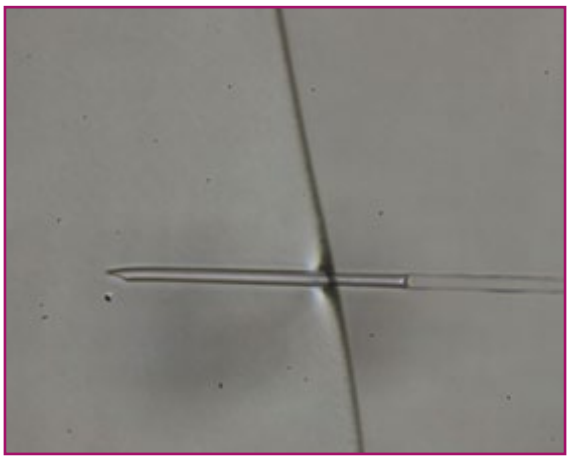
Issue 1: Injection pipette aligns in the air but not on the Petri dish.
Probable reason: Pipette alignment from the fine control of microscope.
Suggested solution: Fine control (Red lever) adjustment of the ICSI machine. (Fig a)

(Fig a)

Issue 2: Small oil bubbles on the outside of the injection needle. (Fig a)
Probable reason: Incorrect technique used in lowering the needle into the PVP drop.
Suggested solution: At the beginning of ICSI insert the needle in to the PVP from the side. (Fig b)

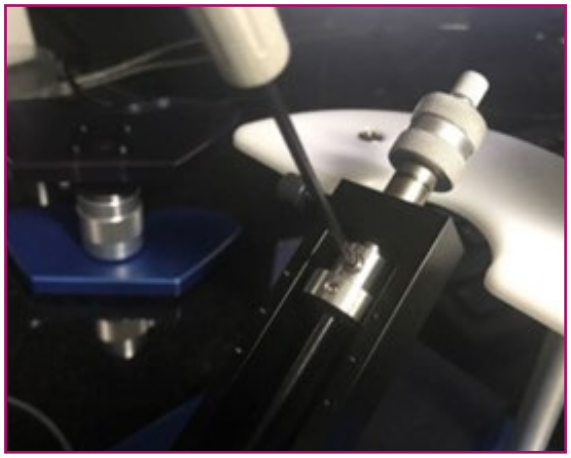


(Fig a)



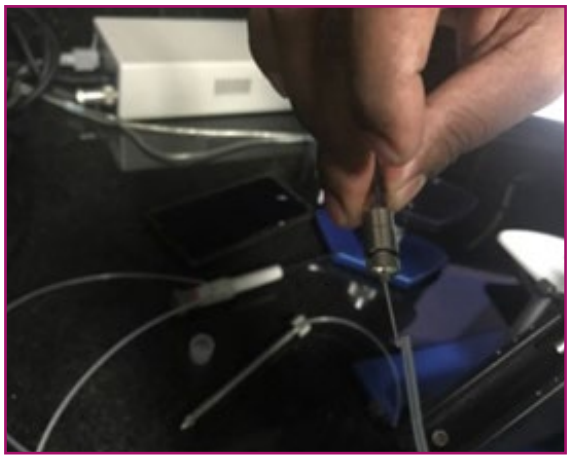
(Fig b)

Issue 3: Immotile sperm does not stay in position, but moves up the pipette. Air bubbles inside the tube.
Probable reason: Leakage in the injection system.
Suggested solution: Check & tighten all sealing (Fig a, b & c) and flush out the bubbles from the oil syringe (Fig d) by detaching it completely. Also check the 'O' ring (Fig e), if it is faulty then need to be replaced.

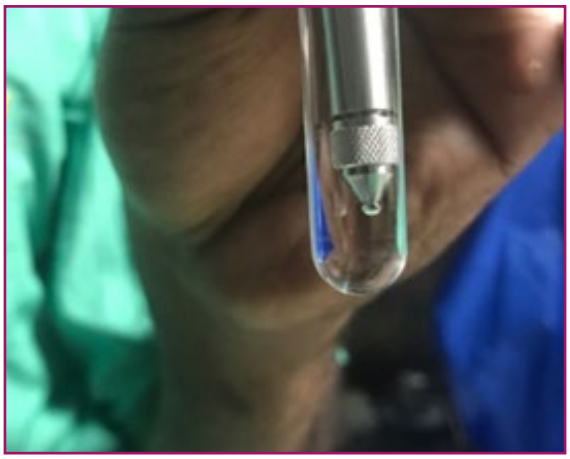


(Fig a)

(Fig b)



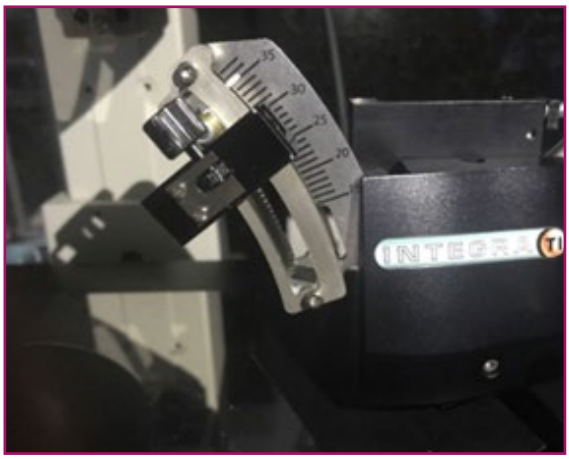
(Fig c)



(Fig d)

(Fig e)

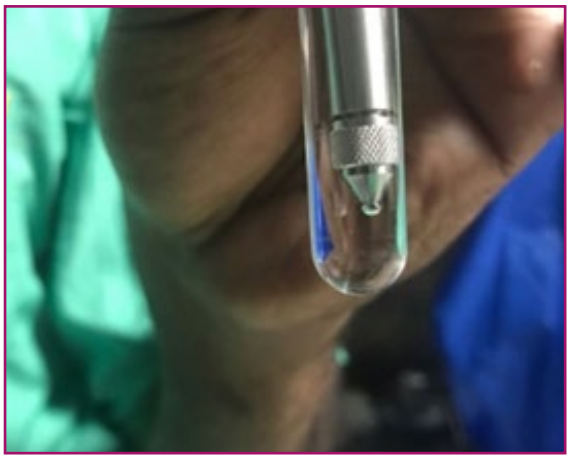
Issue 4: Tail crushing of sperm doesn't happen with the injection pipette
Probable reason: Angle of PL 30 MPH holder is not being matched with the angle of pipette
Suggested solution: Angle should be equal or higher (maximum 3˚) than the angle of pipette. (Fig a)



(Fig a)

Issue 5: Injection pipette not getting inserted in the MPH (Micropipette holder)
Probable reason: Broken part of the pipette blocks the place where the pipette is being inserted
Suggested solution: Broken part should be removed first. (Fig a)
Also check the 'O' ring, if it is faulty then it should be replaced. (Fig b)

(Fig a)



(Fig b)

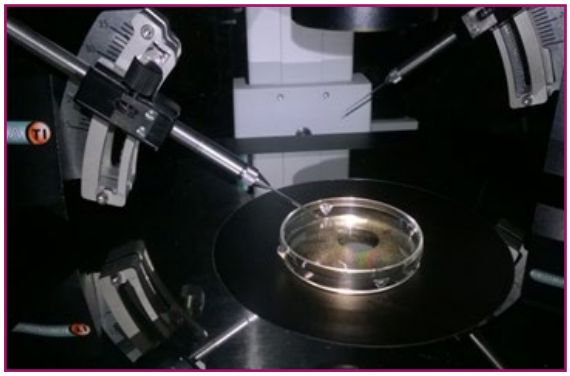
Issue 6: No fine z-movement (up-and-down) on micromanipulator. (Fig a)
Probable reason: Lever locked in one position because it has been rotated too much either up or down.
Suggested solution: If the height gauge is not showing a green section, then the fine movement has been locked in the lower position. If there are two red sections and a green section, then it is locked in the upper position. Rotate the fine control clockwise or anticlockwise to free the locked movement dependant on whether the micromanipulator has been locked in the upper or lower position or loose it from the screws. (Fig b)

(Fig a)

(Fig b)

b. Issues with Holding pipette

Issue 1: Oocytes & pipette are not in focus at the same time
Probable reason: Alignment of holding pipette is not being done properly
Suggested solution: Alignment should be done by putting an empty scratched Petri dish on the centre stage of ICSI machine (Fig a). Focusing of pipette should be aligned from fine control of ICSI as well as microscope (Fig b & c).



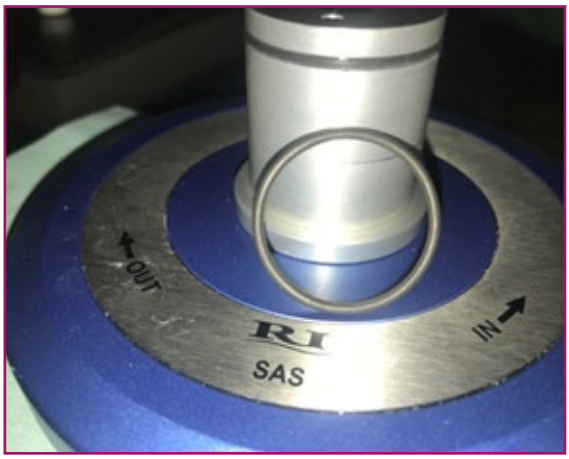
(Fig a)

(Fig b)

(Fig c)

Issue 2: Oocyte is pushed away from the pipette during injection.
Probable reason: Pipette is not located at the equatorial segment of the oocyte.
Suggested solution: Lift the holding pipette slightly until oocyte is not longer in contact with the bottom.

Issue 3: Oocyte movement stucks automatically in the pipette
Probable reason: De-flush the button of Air Syringe.
Suggested solution: De-flush the air syringe as well as check the 'O' ring if it is faulty then need to be replaced. (Fig a)



(Fig a)

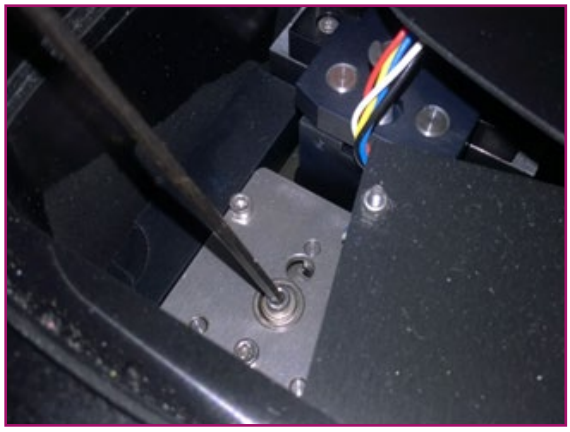
Issue 4: Holding pipette normally stucks in the MPH (Micropipette holder)
Probable reason: Broken part of the pipette blocks the place where the pipette is being inserted. (Fig a)
Suggested solution: Broken part should be removed first (Fig a). Then check the flow of the oil and after that insert the pipette. Check the 'O' ring, if it is faulty then it should be replaced. (Fig b)

(Fig a)

(Fig b)

Issue 5: No fine z-movement (up-and-down) on micromanipulator. (Fig a)
Probable reason: Lever locked in one position because it has been rotated too much either up or down.
Suggested solution: If the height gauge is not showing a green section, then the fine movement has been locked in the lower position. If there are two red sections and a green section, then it is locked in the upper position. Rotate the fine control clockwise or anticlockwise to free the locked movement dependant on whether the micromanipulator has been locked in the upper or lower position or loose it from the screws. (Fig b)

(Fig a)



(Fig b)

c. Issues with Microscope

Issue 1: Mismatch in focusing of the eye piece and display monitor
Probable reason: Adjustment from the coarse/fine control of Microscope.
Suggested solution: Focusing in the eyepiece is being from the coarse/fine control of Microscope and then adjust the camera accordingly. (Fig a)

(Fig a)

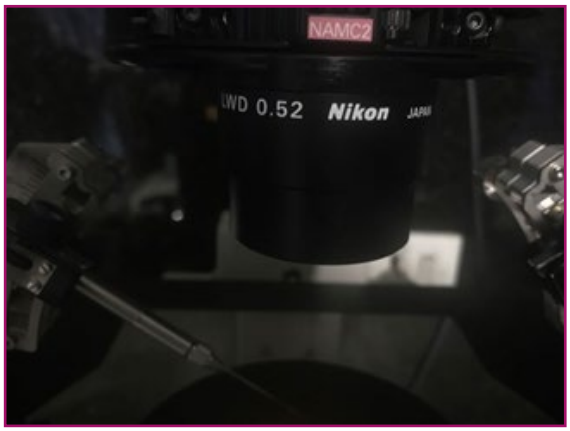
Issue 3: Microscope light fused off (Fig a)
Probable reason: Check all electrical connections to the microscope, replace the bulb.
Suggested solution: If the bulb is not available then can use torch on that place for the time being. (Fig b)

(Fig a)

(Fig b)

Understanding Contrast Modulation in RI System

NAMC (Nikon Advanced Modulation Contrast) enhances contrast by converting optical gradients into variations in light intensity in both stained and unstained specimens in the same way like Hoffman Modulation Contrast (HMC). The NAMC system uses newly lined Plan Fluor objectives **(S Plan Fluor ELWD NAMC 20X and 40X)** with a full 360˚ rotating modulator and a convenient 'stop' mechanism. NAMC accessories include condensers, turrets and modules and five specifically designed NAMC objectives, including Super Plan Fluor ELWD 20X and 40X, and Achromat 10X, LWD 20X and 40X.



Principle: The modulation contrast microscopy adopts the same optical system as the ordinary microscopes, but with some additional parts that convert the transparent specimen into variation of light intensity. These additional parts modulate the amplitude of the light that pass through the specimen, thus changing the intensity of light making up the visible image.

In differential interference contrast and phase contrast microscopy, the phase objects are converted into the variation of light intensity by the phase change that occurs when light passes through the specimen.

4.4 THE INTEGRA 3

The Integra 3 is the latest model being Offered Research Instruments Ltd UK– The World's Most Advanced Micromanipulation System. Every detail of the Integra 3 is geared up towards one goal – to ensure you by creating the best possible conditions for ICSI and sample manipulation.

S. No.	Equipment Feature	Description
1	**Thermosafe Heating** (Fig 4.1)	I. Eliminates cold/hot spots II. Maintains 37° much better than glass/standard metal heating systems The new Integra 3 comes with Thermosafe which, by passing a gentle stream of air under the Petri dish, maintains sample temperature across the dish. A 3.5 inch colour touch screen display allows you to check and control temperature and track procedure time.
2	**Micromanipulator**	Mechanical Micromanipulator I. Long Lasting II. Easy to install/maintain/repair III. Natural movement/no delay
3	**Oil Syringes** (Fig 4.3)	**SOS Oil Syringe** Integra 3 comes with advanced SOS Oil Syringe. SOS is a screw actuated syringe for the precise transfer and injection of spermatozoa. Oil refilling kit also comes with SOS oil syringe. Oil capacity is more and bubble removal is easy as compared with MSHD oil syringe.
4	**Shortcut Keys** (Fig 4.4)	**User Friendly built-in features** I. Take photos, video, count and time things all from the Integra stage II. Under-stage lights; useful in dimly-lit labs III. Stopwatch to time your procedures If you need to keep track of samples or procedures, just hit the shortcut buttons to film or photograph any stage of the process. There's a counter button and an on-screen stopwatch as well A motion triggered light illuminates under the stage as soon as we place our hand to change objectives which helps in selection of objectives. (Fig 4.2)
5	**RI Viewer Software**	Integra 3 can be used without a computer or camera. However, if you wish to connect it to a computer, RI Integra 3 is being supplied with RI Viewer Software which allows you to use all the features by just pressing shortcut keys available on the stage. It also offers line measurement tools and a built-in simulator for training and demonstration purposes.
6	**Safe**	Stage temperature control status indicator light and alarm monitors every second of the process. A log is kept of every alarm triggered

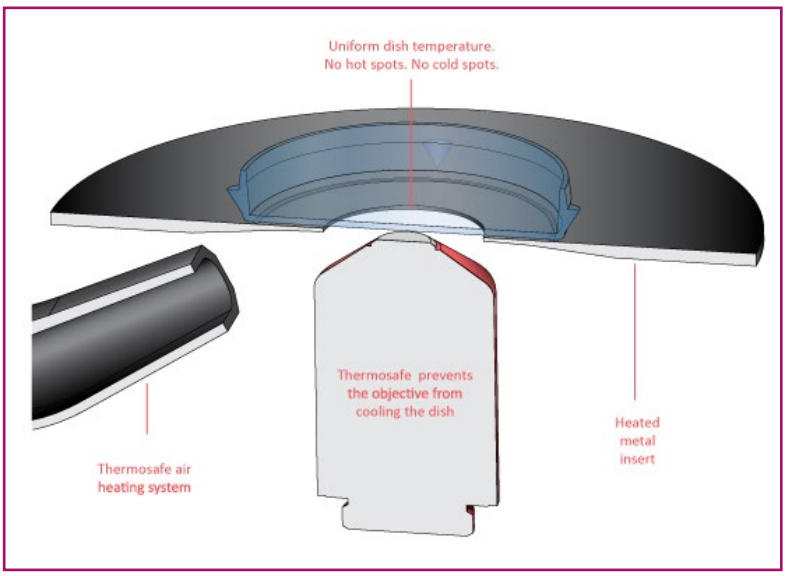


Fig 4.1: Thermosafe heating system

Fig 4.2: Motion triggered light

Fig 4.3: SOS oil syringe

Fig 4.4: Integra 3 shortcut keys

TROUBLESHOOTING SPECIFIC TO INTEGRA 3

a. Manipulation

Problem	Possible cause	Solution
The stiffness of the Fine Control Lever is too tight	Lever stiffness not adjusted	Select the green-ended hexagon driver from the tool kit provided.
Or too loose		Loosen the locking screw, then adjust the lever's resistance by turning anti-clockwise to loosen or clockwise to tighten.
The stiffness of the Coarse Control Lever is too tight or too loose		Select the green-ended hexagon driver from the tool kit provided. Insert the driver into a screw head located around the Coarse Control Lever and slightly tighten or loosen the screw (less than one turn). Test the lever action. If it is not to your liking, insert the driver into a different screw head and loosen or tighten.
Objectives hit Heated Stage Insert when changing objectives or adjusting focus	Objectives are focused too high	Refocus on the top surface of a Petri dish
	The specimen is too close to the edge of the hole in the insert, causing the objective to foul the side of the hole	Position the specimen as close as possible to the centre of the hole in the insert
	Short working distance objective fitted	Please contact RI for more information

b. Temperature Controller

Problem	Possible cause	Solution
Heated Stage Insert or Heated Stage Plate too hot or too cold	Insufficient settling time	After switching on the system should be left for the display to show a constant temperature before use
	Difference in temperature between specimen and plate Surface	Adjust the set point to allow for the difference
	Temperature set point incorrect	Touch the screen on the icon corresponding to the affected heating system. Change if necessary then press Save

PART-4

ICSI protocol followed at our centre

Part 4

I. INTRODUCTION

The intracytoplasmic sperm injection (ICSI) process involves the placement of a single spermatozoon directly into the ooplasm of the denuded mature oocyte, thus circumventing the zona pellucida and the plasma membrane. The fact that ICSI technology can result in higher fertilization and pregnancy rates irrespective of sperm numbers makes it an attractive micromanipulation procedure.

A common indication for ICSI is, patients with poor sperm counts, or in azoospermic men where spermatozoa are surgically retrieved from the testes or epididymis. Oocytes from a poor harvest should also undergo ICSI as denudation carried out before the microinjection guides us about the oocyte maturity and has prognostic significance. ICSI is also recommended when oocytes are to be subjected to preimplantation genetic diagnosis (PGD) technology.

II. ICSI MEDIA AND PIPETTES

The ICSI medium is a buffer system, supplemented with antibiotics, proteins and an energy source. Similar medium, supplemented with Hyaluronidase in desired concentration can be used for oocyte denudation. These media are best stored in a refrigerator at 4-8˚C and aliquot for use as required within the expiry period as per the manufacture's recommendation.

1. It is essential to pre-warm all media to 37˚C in an incubator before use. Carbon dioxide equilibration of the PVP and Hyaluronidase media is not recommended by most manufacturers, except with Cook's Hyaluronidase media, which requires overnight equilibration in the CO_2 atmosphere. The media vials once warmed should be used immediately. These should not be refrigerated again.
2. For ICSI procedure, small droplets of buffered media are, as a rule, placed into a microinjection dish and overlaid with pre-equilibrated mineral oil at 37˚C. These are then placed in the incubator. Therefore, the preparation of the microinjection dishes done before oocyte denudation, which takes 8-10 minutes, allows sufficient time for the media droplets to warm to the desired temperature.
3. PVP is used to slow down sperm progression and facilitate the ICSI procedure. It is also best stored in a refrigerator. Since this is also prepared as a droplet in the microinjection dish; it can be handled similarly to the ICSI medium, though it is much more viscous.
4. All other culture media for embryo cultures are to be equilibrated in the CO_2 atmosphere.
5. The holding and injecting pipettes, which are to be fixed on the micromanipulator, are both made from borosilicate glass capillary tubes. A spike is present in the injection pipette for non-traumatic injection of the sperms into the oocyte. Both pipettes are bent, 1 mm from the tip at an angle of approximately 35 °, to enable injection procedure to be performed with the tips positioned horizontally in a falcon ICSI dish.

III. PREPARATION OF ICSI DISHES

1. The most important consideration when preparing dishes for microinjection is that the droplets of the medium should be small and covered completely with oil. Falcon 1006 ICSI dishes are used. Each droplet must be labelled and numbered so that there is no confusion while working under the microscope.
2. Small microinjection droplets (10-15 micro L) of medium and PVP are placed onto the microinjection dishes first and then overlayed with oil. These small droplets stick to the bottom of the plate and are therefore more stable. The distance between each droplet should be adequate so that they don't coalesce with each other. To avoid spilling of oil while handling the plate, droplet should be small so that they are easily covered with the minimal quantity of oil.
3. The choice of the buffered media for the microinjection droplets will depend upon the experience of the ICSI operator. If the operator is experienced then, manipulation may be performed in microdroplets of bicarbonate buffered media e.g. Vitrolife's IVF, Cook's Cleavage or Medicult's Universal media. With this media, the operator has approximately 5-10 minutes to conduct the microinjection before the pH is altered. If the operator needs more time Vitrolife's MOPS /Cook's Gamete buffer (HEPES) /Medicult's flushing media may be used. Here the operator has nearly 20 minutes per dish.
4. The droplet of PVP in which spermatozoa are manipulated is fundamental to the procedure and should be located easily and quickly. This could be placed in the dish as a long narrow line of the medium. PVP droplet may also be placed centrally with small droplets of buffered media placed around them at a close distance.
5. Sometimes when the semen sample is oligospermia or sperms have been surgically extracted a larger droplet of buffered media (20 micro l) may be made in the microinjection dish as a reservoir for spermatozoa. Sperms are aspirated from this 'sperm drop' and moved to the PVP droplet for manipulation prior to injection.

6. It is recommended that not more than five oocytes are injected at one sitting, as this will minimize the total exposure of each oocyte to the changes in temperature and pH outside the incubator. These changes may result in failure to cleave or, generate fragments in the embryos. Thus if more than five mature oocytes are to be injected, it will be essential to prepare two microinjection dishes. The rationale for this is that while one dish is being used for microinjection, the other can be kept warm and equilibrated inside an incubator.
7. Cook/ Swemed/ Midatlantic 140 microns pipette is preferred to make buffered media microinjection droplets. To dispense PVP, which is very dense and viscous, falcon polystyrene pipette's (3ml) may be used. Small micropipette tips may be used for the same. Polycarbonate pipettes 140 Microns after cutting the narrow tip with sterile blade or scissors are used. This gives complete accuracy while making the droplets or narrow line of the PVP media. Equilibrated and warm oil is now pipetted over them until the drops are completely covered. This can be confirmed by examining the interface between the medium and oil under stereo zoom microscope.

IV. DENUDATION

Hyaluronidase is used to remove the cumulus cells surrounding the oocyte to enable correct alignment of the oocyte with respect to the polar body prior to sperm injection.

Hyaluronidase is used at the concentration of 80IU/ml. Before starting the procedure keep 140/170 microns pipettes preheated and ready. Cumulus-oocyte complexes are initially placed in the droplet of 300 µl of Hyaluronidase solution media for 20 seconds and then denuded with 170-micrometre stripper (cook/ swemed/ MidAtlantic). Oocytes are moved in and out very gradually and repeatedly. The cumulus mass is gently removed till the zona pellucida is clearly seen. These oocytes are then moved through all the bicarbonate media drops to rinse away the Hyaluronidase solution. These are finally collected together in the clean drop of bicarbonate buffered media and incubated till loaded in the injection dish.

Precautions: The oocyte cumulus complexes should be placed into the Hyaluronidase for not more than one minute. Avoid excessive or rough handling of the oocytes during the denuding process. The oocyte is quite fragile and can be susceptible to lyses if aspirated too vigorously.

a) METHOD OF DENUDATION DURING ICSI

1. Allow the single well dish with 400 µl Cooks hyaluronic acid to equilibrate for both pH and temperature in a 37°C, 6.0% CO_2 incubator overnight. The hyaluronic acid of Vitrolife and Medicult companies do not require CO_2 equilibration.
2. Using an appropriate pipette, transfer between 1 and 5 cumulus-oocyte complex (COC) into the Hyaluronidase and aspirate several times until most of the cumulus cells are loosened or removed. Avoid creation of air bubbles as the denuded oocytes may stick to bubbles and get lost. This phenomenon is common we have named it as the parachute effect.
3. Using a series of progressively Narrower Flexipets (170 µm, 140 µm) continue to remove the coronal cells until all zona are free of cells. The Cooks Flexipets are ideal for this, as they are unbreakable polycarbonate and unlikely to harm the oocytes. As each oocyte is denuded it should be moved through the series of wash wells to remove the excess Hyaluronidase. Generally, oocytes are denuded an hour or two after retrieval and ICSI may be conducted immediately after cumulus removal.

V. MANIPULATION OF SPERMATOZOA

1. Load the sperms in the PVP after adequate dilution. Desired concentration is 10 million sperms /ml. A fraction (1 micro L) of the sperm suspension is added to the periphery of the central PVP droplet. This is followed by loading of the denuded oocytes. This helps in the quick manipulation of the sperms and microinjection.
2. It is better to load less sperms than many, as the higher concentration will make the visualization and immobilization of the sperms difficult and cumbersome. If the concentration of the sperms is very low as in case of the surgically retrieved sample, load the sample in the buffered media drop. Sperms could be selected from this media drop and transferred to the PVP drop for micromanipulation.
3. To locate and manipulate spermatozoa in the PVP drop, it is preferable to switch to a 20X objective lens and refocus upon the injection pipette.
4. If the prepared spermatozoa have been added directly to the PVP, then the most motile, and the therefore probably the most viable ones, are found at the periphery of the droplet.
5. The edge of the injection pipette is brought near the edge of the drop. Spermatozoa either swim into the injection pipette

or may be aspirated by creating a very slight negative pressure in the injection pipette by turning the injector screw slightly anticlockwise.

6. To immobilize spermatozoa, localize the sperm near the edge of the PVP drop and bring both the tail of the sperm and the injecting pipette in sharp focus. The sperm is immobilized with a swift moment of the pipette from left to right thus severing the tail of the sperm against the bottom of the dish. This can be easily done by aligning the injection pipette perpendicular to the tail of the sperm. Now the pipette is lowered and the tail is slashed in a quick moment against the bottom of the plate. This should be done quickly and deliberately. If this is repeated again and again the tail becomes sticky and there will be difficulty while aspirating in the injecting pipette. Sperm is immediately immobilized and the kinking or curling of the tail confirms this.
7. It is absolutely vital to damage the plasmalemma of the spermatozoon and to immobilize it before the injection. This method of sperm immobilization is a membrane permeabilization process that may allow the release of a sperm cytosolic factor, which activates the oocyte. This has been shown to markedly enhance fertilization rates following ICSI. Once immobile, the spermatozoon may be aspirated into the injection pipette, tail first, by slowly turning the injector anticlockwise.
8. The sperm's proximal centriole is necessary for the oocyte in bipolar spindle formation for the first mitotic division.
9. After loading the sperm, confirm that the movement of the spermatozoon along the injection pipette is completely under control before proceeding to manipulate the oocyte.

VI. MANIPULATION OF OOCYTES

1. Oocytes must be examined under the stereo zoom microscope before we proceed with the ICSI. Emphasis should be laid on the polar body, ooplasm, zona pellucida and oolemma.
2. Denuded oocyte has an outer zona pellucida, narrow perivitelline space and ooplasm surrounded by the plasma membrane. The oocyte is at Germinal vesicle (GV) stage when a big nucleus is seen in the ooplasm. Oocytes are considered to be at Metaphase 1(MI) stage when GV breaks down and disappears and polar body is not visualized. The appearance of polar body classifies the oocyte at Metaphase II (MII) stage. Only MII oocytes are suitable for microinjection.The immature oocytes (GV &MI) should not be injected, as they will not fertilize.
3. While microinjecting we must position the polar body at 6 or 12'o clock position based on the theory that metaphase spindle is located near the first polar body. It has been documented that nearly 80 % of the MII oocytes have spindle located in the same hemisphere as the first body. Placement of the PB at 6 or 12 o' clock ensures that the injection pipette does not penetrate the oocyte closer to where the spindle is presumed to lie.
4. It is essential to have a heated stage /platform on the micromanipulator while manipulating oocytes. These stages have small holes/apertures through which light can pass. Larger aperture will provide greater the field of view but will cool the plate quickly thus leading to thermal damage of the spindle apparatus of the oocytes. On the other hand, the smaller the aperture in the heated stage, the more efficient the heating of the ICSI dish.
5. Zona pellucida and oolemma is very sensitive to mechanical stress. If an unnecessary negative pressure is applied on the oocyte, the oolemma is sucked towards the holding pipette and deforms. Consequently, there is a risk of damage to the cytoskeleton of the oocyte. Hence, very mild negative pressure on the oocyte is recommended. Oolemma should not be deformed or sucked in the holding pipette during the procedure. On the other hand, the oocyte should not be held so loosely that it becomes detached from the holding pipette while withdrawing the injection pipette from the oocyte. Ideally, the oocyte should be positioned on the holding pipette so that the first PB lies at or near 12'o clock positions.
6. Manipulation of the oocyte in the media droplet to bring polar body at the determined location is an art. Holding pipettes can blow as well suck the oocyte. These movements may be used to rotate the oocyte. Injecting needle can be used to manipulate the oocytes mechanically. X, Y, Z movement of the needle can easily rotate the oocytes in all the directions.
7. Coronal cells attached to the oocyte can be used as a means of attaching the oocyte to the holding pipette. The advantages of using coronal cells for attachment of the oocyte to the holding pipette is that superior and gentle suction may be applied, with less risk of damaging and distorting the oolemma.

VII. MICROINJECTION OF OOCYTES WITH MATURE SPERMATOZOA

1. Place the warm microinjection dish on the pre-warmed stage of the stereo zoom microscope.
2. Load the sperms in the central PVP drop or central PVP line. Move the dish containing denuded oocytes out of the CO_2

incubator. Match the name and identification number of the patient on the culture dish with the injection dish. Transfer the oocytes to the media drops.

3. Immobilized spermatozoon is now aspirated into the injection pipette, and the oocyte is held by the holding pipette with gentle negative suction. Before holding the egg brings the oolemma and the holding pipette tip in focus at one time. It is ensured that the polar body is either at 6 or 12 o'clock position before we proceed further with the injection of the oocyte.
4. Now while keeping the oocyte focused, position the injection pipette tip adjacent to the zona pellucida at the midline of the oocyte and bring the tip in focus keeping the oolemma focused. It is important that both oolemma and injection pipette tip should be in focus concurrently, as it ensures that the injection pipette will initially pierce the oocyte at its midline.
5. Gradually turn the injector syringe clockwise to move the spermatozoon towards the tip of the injection pipette. As the sperm moves towards the pipette tip apply anticlockwise force to stabilize the sperm movement. If this force is not applied correctly, the sperm may be expelled in the injection droplet and the procedure is delayed. With fine control, it is possible to stabilize the movement of the sperm at the tip of the injection pipette.
6. The injection pipette with loaded sperm is advanced cautiously in a straight line towards the oocyte to perforate the zona pellucida. Here, it should be ensured that the injections pipette enters the oocyte at the approximate midline of the geometrical curvature.
7. As the injecting needle perforates the zona pellucida and the oolemma, usually, the oolemma remains intact due to its inherent strength and elasticity. The oolemma remains wrapped around the injecting pipette. Ooplasm may now be aspired into the injection pipette by slowly turning the suction syringe in the anticlockwise direction.
8. To begin with, the aspiration of ooplasm into the injection pipette will be slow as it is impeded by the intact oolemma. With persistent negative suction, the oolemma will rupture and is succeeded by a sudden free flow of ooplasm. At this juncture, it is important to halt the influx of ooplasm into the injection pipette by quickly reversing the negative pressure being created by the injector syringe.
9. Now by slowly turning the injector syringe in a clockwise direction replace the ooplasm that has been aspirated, along with the immobilized sperm, into the oocyte. Quickly withdraw the injection pipette from the oocyte as this will avoid minimal deposition of PVP in the cytoplasm. After the pipette is removed, the breach area is observed, and the order of the opening should maintain a funnel shape with a vertex into the egg. Immediate rupture of the oolemma without any aspiration has been associated with lower oocyte survival rates.
10. If the oolemma fails to rupture despite aspiration, we try to perforate the membrane by employing a very rapid jabbing movement of the injection pipette into the oocyte. While giving a jab, contact with the opposite segment of the oolemma must be avoided. In case we still fail it is better to compromise by depositing the spermatozoon into the perivitelline space rather than subjecting it to more excessive manipulation, which might well result in its demise.
11. A deliberate attempt is made to deposit the spermatozoa towards the centre of the oocyte as this not only confirms the placement but also ensures that proper funnel is created during the placement of the sperm. Now we can gently release the negative suction of the holding pipette and release the egg by clockwise movement of the suction syringe. The same procedure is done for the remaining oocytes.
12. If the number of injectable oocytes is more than 5 these may be injected in the similar fashion, before washing them all through into bicarbonate-buffered medium for culture.
13. Rarely the equipment may develop an error or the pipettes may malfunction and desirable aspiration and injection may not be obtained. In such cases, the procedure is stopped and the oocytes are transferred to the media dish till the problem is resolved. This will avoid unnecessary shock to the oocytes.
14. Fertilization assessment is done as routine.

VIII. CONCLUSION

The intracytoplasmic sperm injection (ICSI) procedure involves the placement of a single spermatozoon directly into the cytoplasm of the oocyte. This technique bypasses the ZP and the oolemma. The ability of ICSI to achieve higher fertilization and pregnancy rates regardless of sperm characteristics makes it the most prevailing micromanipulation procedure with which to treat male factor infertility.

IX. ICSI PROTOCOL

Figure 1: The media required for ICSI.

We use the Vitrolife range of media comprising of G-IVF PLUS, G-MOPS PLUS, OVOIL, ICSI (Polyvinylpyrrolidone - PVP) and Hyase. (hyaluronidase)

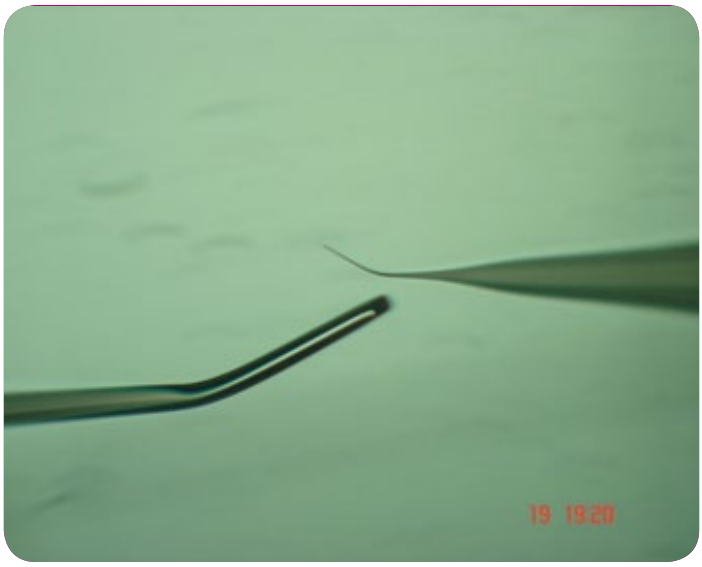


Figure 2: ICSI holding (left) and injecting Pipettes (Right).

Viewed under the inverted microscope, the focal plane of the tip of the injection and holding pipette is higher and lower respectively, indicating that it is not allign correctly in the horizontal plane but is tilted upword

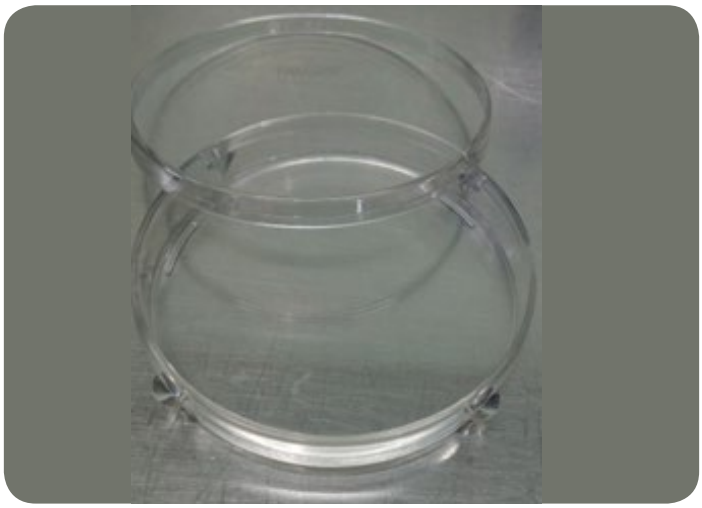


Figure 3: ICSI dish

Falcon Petri Dish (351006) made up of polysterene with dimensions of 50 X 90 mm is used

Figure 4 (a): PVP Plate.

Falcon Petri Dish (351006) is used. The thin central line is made with the PVP. Four 10-µl droplets are made of G - IVF PLUS for oocyte injection. These are then covered with the Oil.

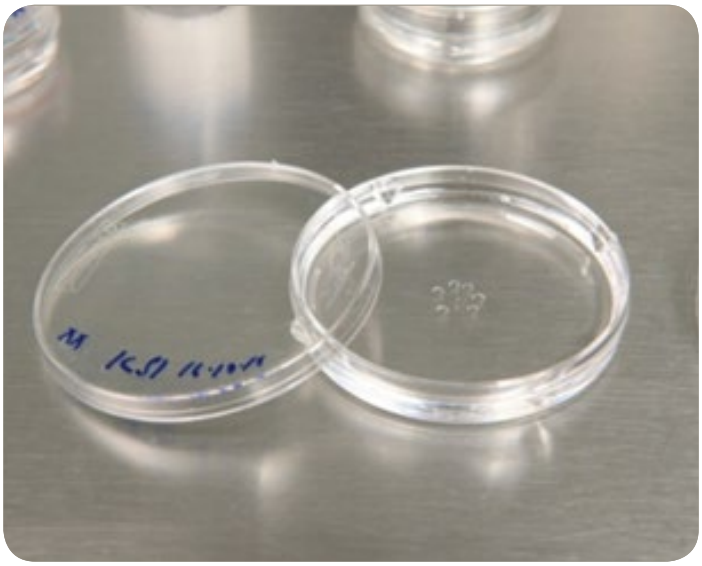


Figure 4 (b): PVP Plate.

A central droplet of PVP surrounded by G-IVF PLUS plus droplets for microinjection. PVP is very viscous and difficult to handle with narrow pipettes. We used the wider end of 140 micrometre or non-toxic sterile pipette tip to make the droplets or central streak of PVP.

Figure 5: HYASE™ (hyaluronidase) is used to facilitate the dispersal of the cumulus mass and corona.

HYASE™ is concentrated 10 times and should be diluted 1:10 with supplemented MOPS™ PLUS.

Exposure to HYASE™ for longer than 30 seconds may damage the oocyte.

Figure 6: Denudation plate.

200-300 µl drop of Hyase at 6 'o clock position is made. Around this drop 4 – 5 drops of G- IVF media are made to wash the oocytes.

Exposure to HYASE™ for too long or rough handling as well as exposure to sub-physiological pH and temperatures may also damage the oocyte.

The diameter of the pipette should be slightly larger than that of the oocyte (approximately 130–175 µm)

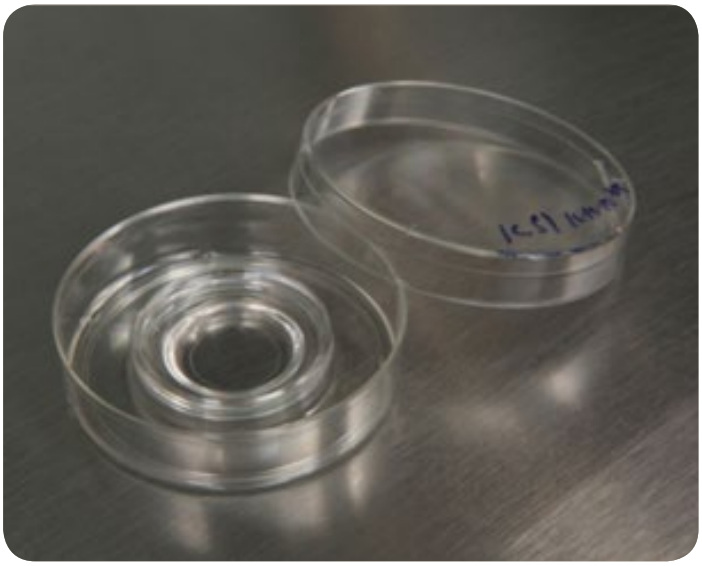


Figure 7: Single well Petri dish.

500 µl of G- IVF Plus media is dispensed and covered with the same amount of oil. Injected oocytes are placed in this dish for further assessment and culture.

A 100 µl droplet is made in the outer well at (11 o' clock) position to rinse the microinjected eggs before placing them into the G- IVF in the inner well.

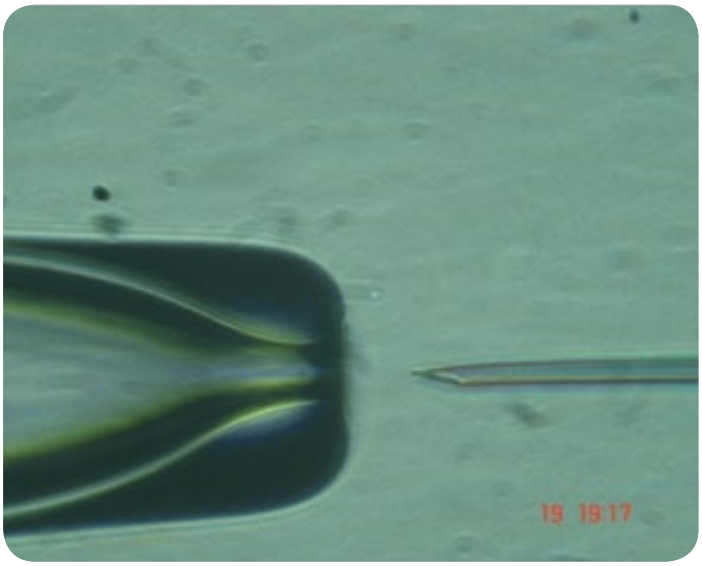


Figure 8: Final alignments of the pipette.

The alignment methodologies of the holding and injection pipettes differ. Holding pipette is aligned without tilt in the horizontal plane, as it has to align flat on the bottom of the dish to aspirate the denuded Oocyte. On the other hand, the injection pipette needs to be tilted downwards towards its tip in the horizontal plane so that the tail of the spermatozoon can be slashed and easily aspirated. Thus the final alignment of the injection pipette is recognized by it touching the bottom of the dish.

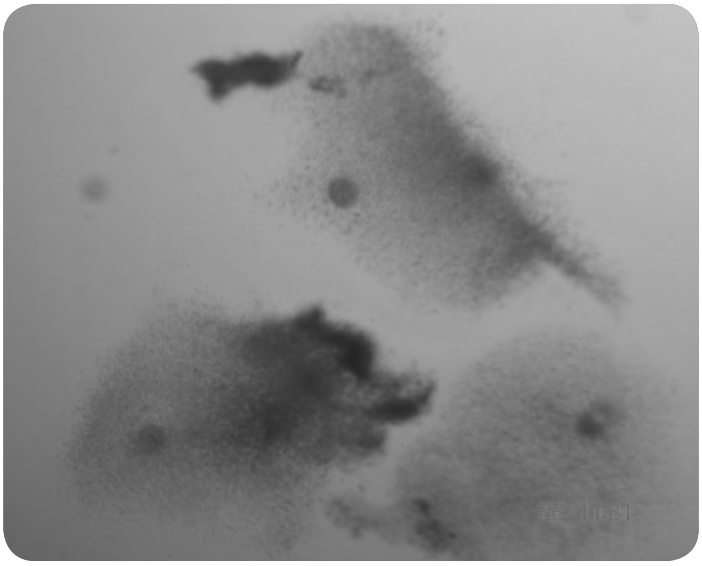


Figure 9: Freshly aspirated oocyte cumulus complex.

We always equilibrate the oocyte cumulus complexes in 4-well dishes with IVF medium in the CO_2 incubator from aspiration until denudation for 2 hrs for ICSI.

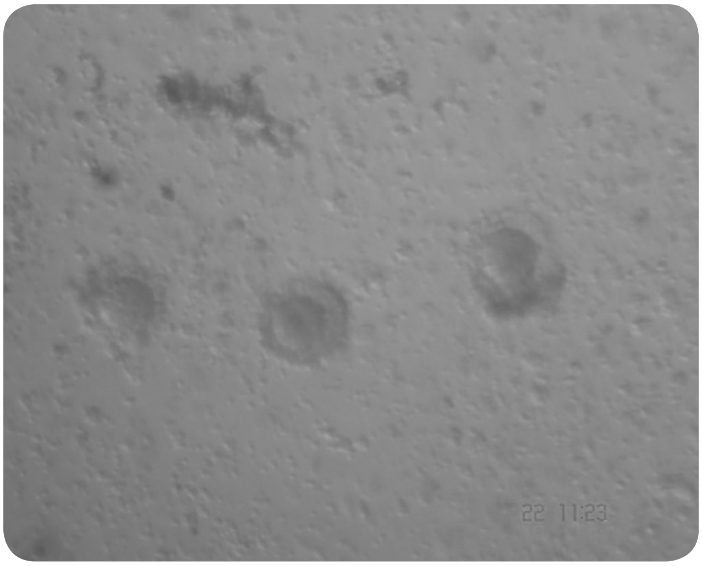


Figure 10: For ICSI the oocytes will need to have their cumulus mass and corona removed.

This process is called denudation. This process may be performed either using the large volume method without oil, using multi-wells and dishes or the droplet method under oil. HYASE™ (hyaluronidase) is used to facilitate the dispersal of the cumulus mass and corona.

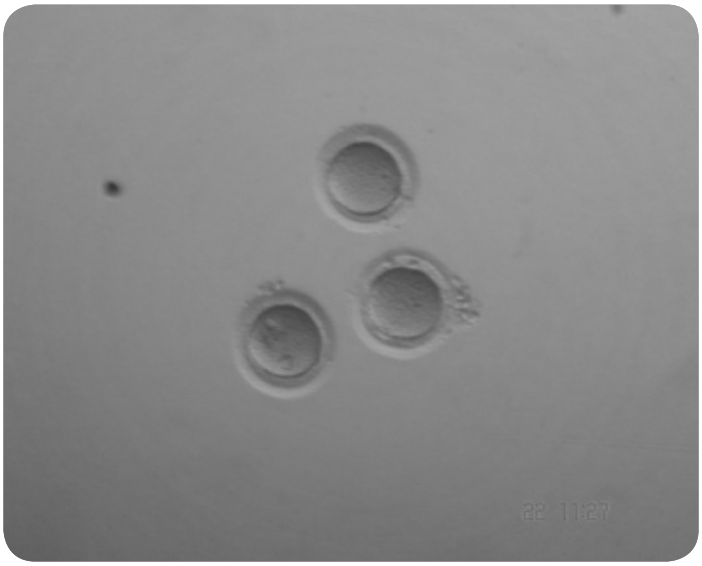


Figure 11: The-denuded oocytes. These may be used for ICSI immediately after the process of denudation.
The oocytes are scored on the inverted microscope and their maturation stages are noted.

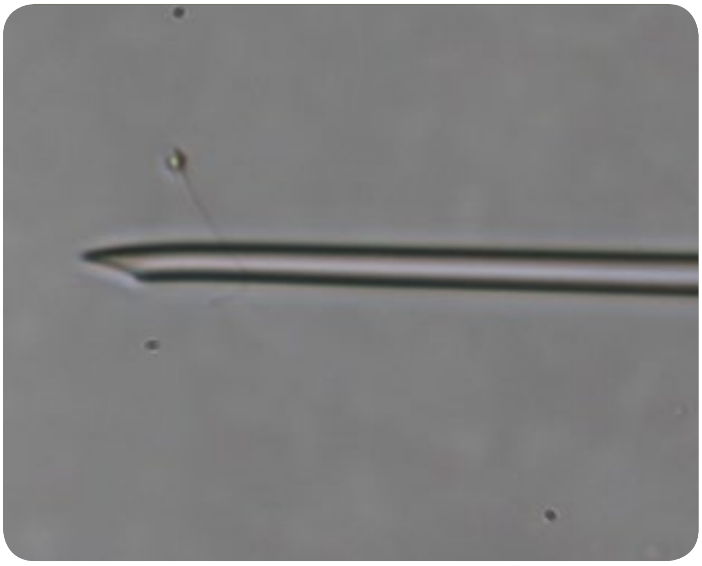


Figure 12: A motile spermatozoon, which is moving slowly along the bottom of the dish, is located.

The injection pipette is moved close to the spermatozoon.
The spermatozoon is immobilized by crushing the tail with the pipette.

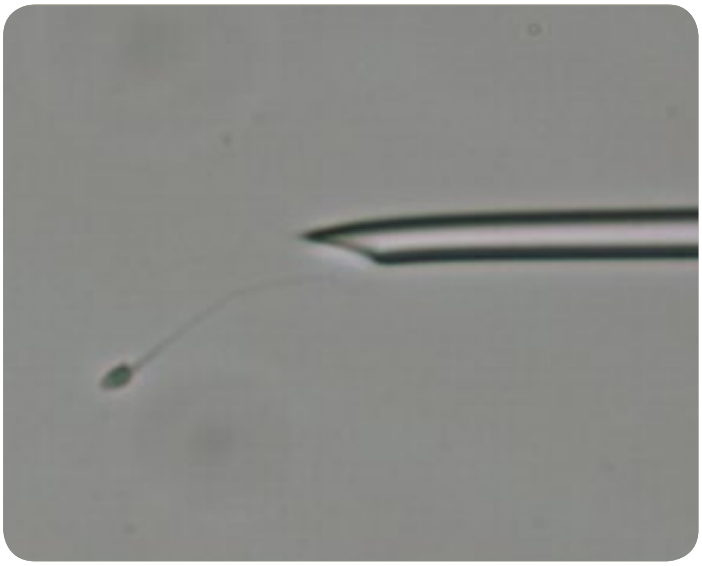


Figure 13: The spermatozoon is aspirated into the injection pipette, tail first.

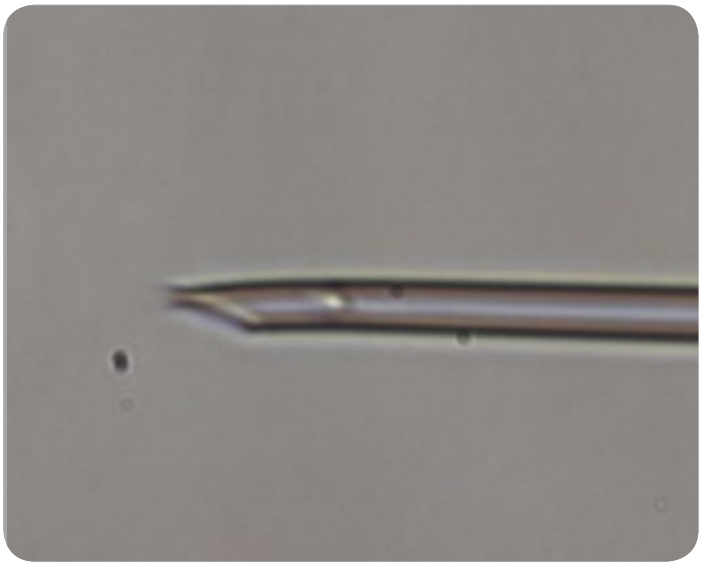


Figure 14: Spermatozoon is moved along the injection pipette and brought to rest at its tip

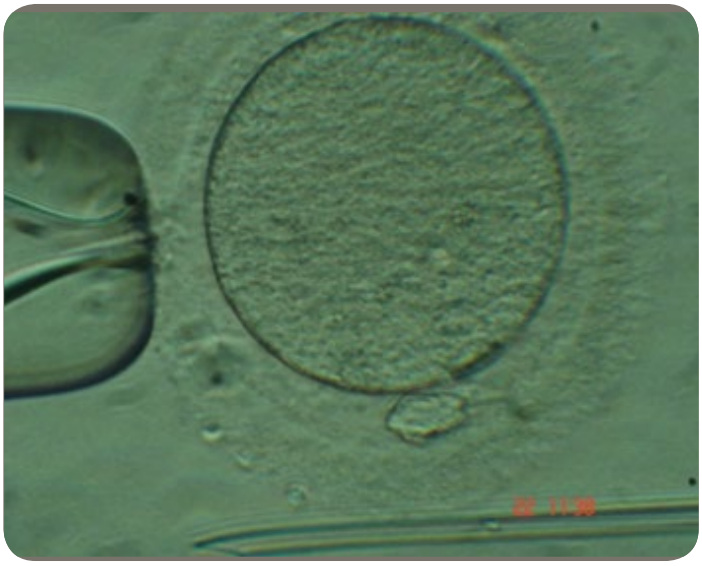


Figure 15: After the spermatozoon has been immobilized and aspirated into the injection pipette, and the oocyte has been attached to the holding pipette with the PB at either 6 or 12 o'clock, injection of the spermatozoon may be done.

The use of slight negative pressure or even just capillary action should be all that is required to attach the oocyte gently but firmly to the holding pipette.

On the other hand, the oocyte should not be held so loosely that it becomes detached from the holding pipette when withdrawing the injection pipette from the oocyte

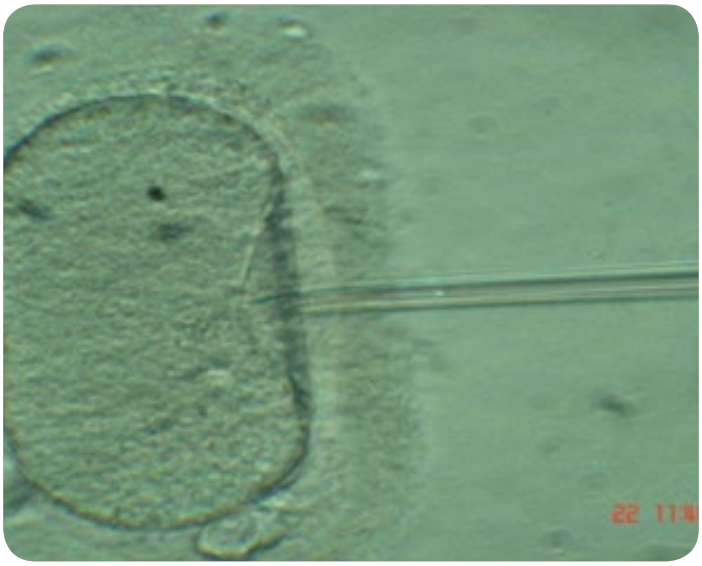


Figure 16: The injection pipette may then be advanced carefully in a straight line towards the oocyte to penetrate the ZP.

Enter the egg from the geometric centre horizontally with a minimum shake.

Observe the pattern of indentation that it creates.

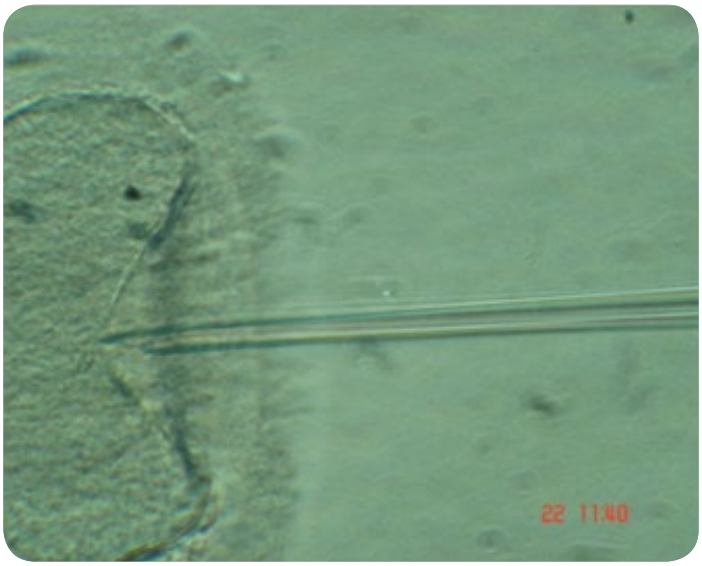


Figure 17: A deep indentation is created in the oolemma with the injecting pipette and acute funnel is obtained.

Please do not push the injection pipette much more than halfway into the oocyte, as to avoid piercing the oolemma on the opposite side. Ooplasm is now aspirated into the injection pipette by slowly turning the injector screw in an anticlockwise direction. Initially, the flow of ooplasm into the injection pipette will be slow as it is impeded by the intact oolemma. Latter with increasing negative suction the oolemma will rupture to allow a sudden free flow of ooplasm in the injecting pipette. Immediately halt the influx of ooplasm into the injection pipette by quickly reversing the negative pressure.

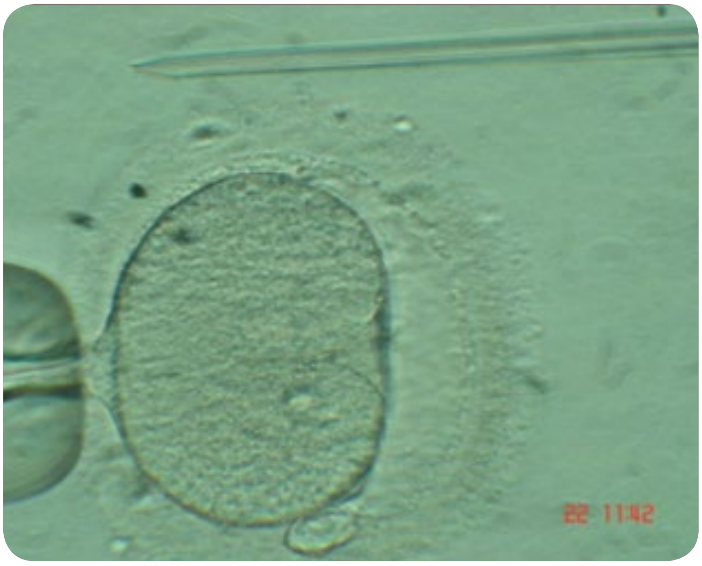


Figure 18: The oolemma funnel will quickly disappear after the withdrawal of the injecting pipette.

Earlier the egg attains the original state healthier it is as funnelling is the indirect evidence of elasticity of the oolemma.

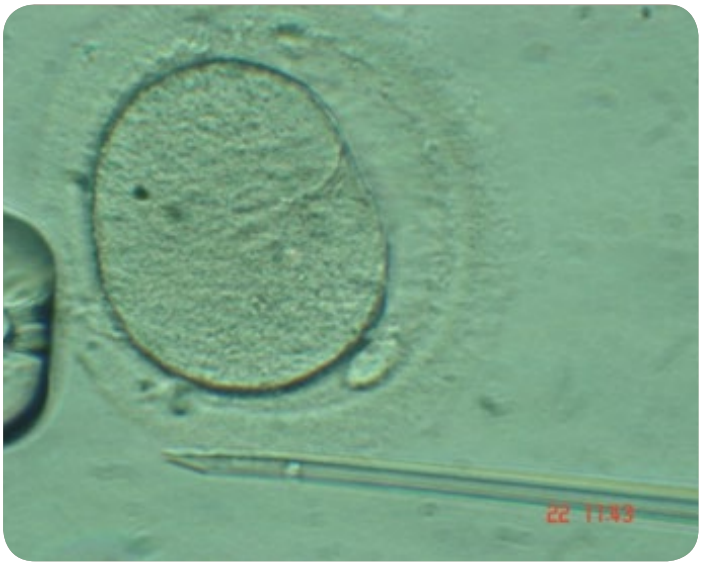


Figure 19: The Oocyte is now released from the holding pipette by turning the injector screw clockwise.

A gentle knock by the injecting pipette may help in the final release of the egg from the holding pipette. The egg is now shifted to the single well dish containing G IVF Plus media.

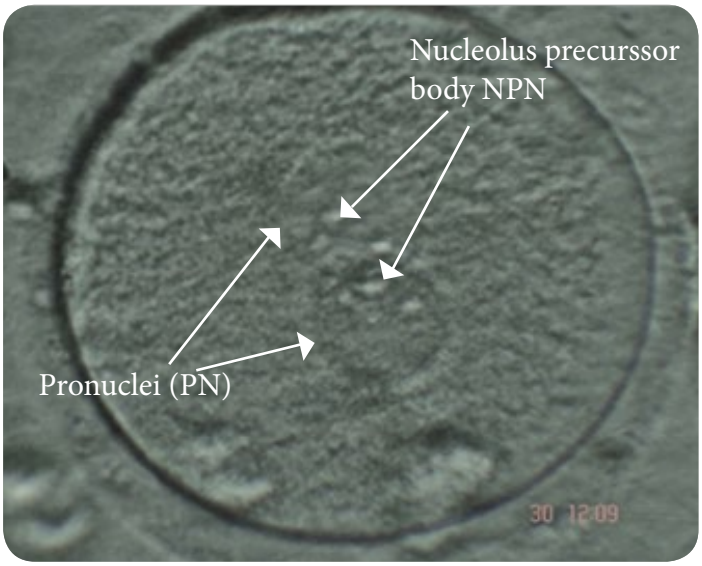


Figure 20: Post ICSI two Pronuclei as observed after 18 hrs.

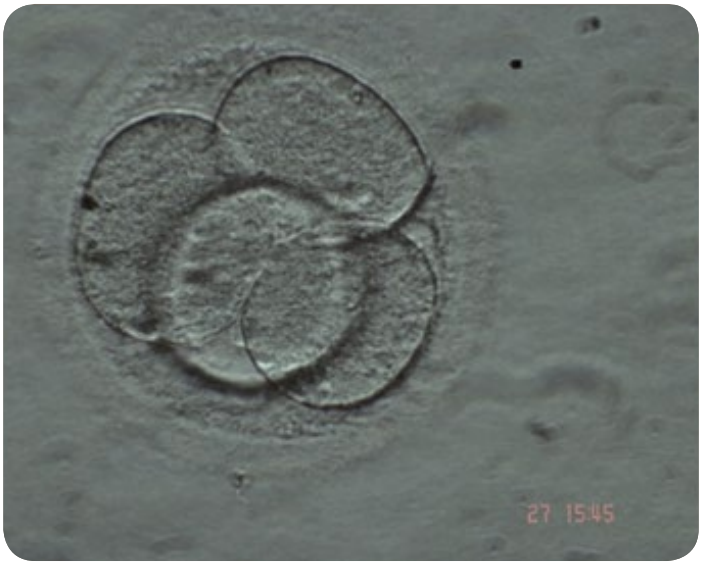


Figure 21: Grade 1, 4 cell embryo as observed after 44 hrs

PART-5

Ready Reckoner

Part 5

I. Intracytoplasmic sperm injection

S. No.	Salient features
1	ICSI bypasses both the ZP barrier and sperm defects in the male gamete that compromises its ability to fertilize.
2	Therapeutic possibilities of ICSI range from cases in which, after sperm selection, the spermatozoa show poor progressive motility, to its application in azoospermic men where spermatozoa are microsurgically retrieved from the epididymis and the testis. Retrieval of a low number of oocytes represents a further indication for this procedure.
3	ICSI has also made the consistent fertilization of cryopreserved oocytes possible since freezing can lead to premature exocytosis of cortical granules and ZP hardening that inhibit natural sperm penetration
4	When preimplantation genetic screening is to be performed on oocytes, the removal of the polar body requires the stripping of cumulus corona cells, thus supporting ICSI as the only insemination method to avoid polyspermy.
5	ICSI is the preferred method of insemination by several groups for HIV-discordant couples because it virtually avoids the interaction of oocytes with semen, thereby reducing the risk of viral exposure
6.	TESA ICSI is also choice of method for men with high sperm DNA fragmentation as DNA damage occurs at post testicular levels.

2. Collection and preparation of oocytes

S. No.	Salient features
1	One day prior to the ovum pick-up (OPU), baseline blood work and pelvic ultrasound are performed on menstrual cycle day 2 for patients treated with gonadotropin-releasing hormone (GnRH) antagonist protocols and on menstrual cycle day 3 for patients treated with the long GnRH agonist protocol
2	Normal baseline parameters include follicle-stimulating hormone (FSH) <12 mIU/mL, estradiol <75 pg/mL, and progesterone <1 ng/mL. Pelvic ultrasound is performed to evaluate endometrial thickness and to assess the antral follicle count and the presence of ovarian cysts.
3	Controlled ovarian super ovulation (COS) is carried out to maximize follicular response while minimizing the risk of ovarian hyperstimulation syndrome. Generally, hCG trigger is given when the two lead follicles attain a mean diameter of 17 mm.
4	Oocyte retrieval is performed under conscious sedation using transvaginal ultrasound guidance approximately 35–36 hours after hCG administration.
5	Under the inverted microscope at 100 X, the cumulus–corona cell complexes are scored as mature, slightly immature, completely immature, or slightly hypermature. Thereafter, the oocytes are incubated for about four hours for micromanipulation.
6	A good and timely cumulus removal is necessary for observation of the oocyte and effective use of the holding and injecting pipette during micromanipulation.
7	Before carrying out micromanipulation, it is important to examine each oocyte under the microscope to assess nuclear maturity and morphology. Metaphase II (MII) is assessed according to the absence of the germinal vesicle and the presence of an extruded polar body.

3. Selection of the spermatozoon and Immobilization

S. No.	Salient features
1	The viscous medium i.e. PVP, decelerates the spermatozoon, allowing the selection of sperm by evaluating its 3-D motion pattern and morphological parameters as well as favouring aspiration into the pipette, which is normally not possible at a magnification of 400 X.
2	Although ICSI does not require any specific spermatozoon pre-treatment, gentle immobilization achieved through mechanical pressure is needed to permeabilize the membrane, which allows the release of a sperm cyto¬solic factor, resulting in oocyte activation and improved fertilization rates.
3	ICSI has also made the consistent fertilization of cryopreserved oocytes possible since freezing can lead to premature exocytosis of cortical granules and ZP hardening that inhibit natural sperm penetration
4	Standard protocol for immobilization is performed by positioning spermatozoon at 90° to the tip of the pipette, which is then lowered gently to compress the sperm flagellum maintaining the shape of its tail. If the latter is damaged then that spermatozoon is discarded and the procedure is repeated with another spermatozoon.
5	Aggressive immobilization, another more effective procedure used for immobilization in which the sperm tail is rolled over the bottom of the Petri dish in a loca¬tion posterior to the mid-piece in order to induce a perma¬nent crimp in the tail section, making it kinked, looped, or convoluted

4. Ooplasmic injection

S. No.	Salient features
1	The oocyte is held in place by the suction applied to the holding pipette.
2	During penetration, as pipette reaches the approximate centre of the oocyte, a break in the membrane should occur. This is reflected by a sudden quivering of the convexities (at the site of invagination) of the oolemma above and below the penetration point, as well as the proximal flow of the cytoplasmic organelles and the spermatozoon moving upward into the pipette
3	To optimize the interaction with the ooplasm, the sperm cell should be ejected past the tip of the pipette to ensure an intimate position among the organelles that will help to maintain the sperm in place while withdrawing the pipette.
4	Once the pipette is removed, a breach area is observed, and the order of the opening should maintain a funnel shape with a vertex into the oocyte and if the border of the oolemma becomes inverted, ooplasmic organelles can leak out
5	The introduction of sequen¬tial media, fashioned by glucose and protein starvation, can result in complications during the execution of ICSI, resulting in increased oocyte damage.
6	To avoid such complications while performing ICSI, it is necessary to have multiple sequential strikes of the sperm flagellum to obtain effective immobilization and consequent membrane permeabilization.

5. Extended sperm search

S. No.	Salient features
1	In cases where no spermatozoa are identified at the initial analysis and after high-speed centrifugation still, no sperm cells are found, an extensive search is performed.
2	A dish is made in the same manner as an injection dish, with PVP solution placed in the central drop. The surrounding droplets of the medium can be replaced with the actual specimen and pentoxifylline is added to each drop to help augment sperm motility. Each drop is browsed and motile sperma tozoa that are identified should be picked up and transferred to the PVP drop.
3	In TESE specimens, sperm may be extremely rare, if not totally absent. In such cases, the extended searches may take greater than three hours to complete, depending on the number of oocytes awaiting injection
4	There is a pronounced decrease in pregnancy outcome as extensive search time increases
5	The search is still an important and valuable tool, as it represents the best opportunity for a male patient with NOA to bear their own biological child.

6. Optional Sperm Selection Techniques

i. Intracytoplasmic morphologically selected sperm injection (IMSI)

Salient features
Intracytoplasmic morphologically selected sperm injection (IMSI) is a procedure where high-power magnification is adopted to morphologically screen for optimal spermatozoa. This technique referred to as motile sperm organelle morphology examination (MSOME), uses an inverted light microscope with high-resolution Nomarski optics followed by computer-assisted magnification up to 6300 X or even higher. • When using MSOME, the criteria for sperm selection include a normal nuclear shape with lengths and widths no greater than two standard deviations away from the average measurements of 4.75 μm by 3.28 μm. • A spermatozoon with normal nuclear content is identified, meaning that no greater than one vacuole (taking up to <4% of the nuclear area) can be present in the nucleus. • Since searching for normal sperm under high magnification and strict criteria takes more time, the sperm sample must be kept at a lower temperature of 21°C to reduce sperm cell metabolism • IMSI is best suited for cases where the selection of morphologically normal spermatozoa is feasible, but it cannot practically be employed in severe oligozoospermic cases such as cryptozoospermia and NOA, where only scarce viable cells are present.

ii. Physiologic ICSI (PICSI)

Salient features
Physiologic ICSI (PICSI) makes use of hyaluronic acid (HA), a substance naturally present in the human body. • HA can be found in the cumulus oophorus around the oocyte and represents a barrier to the immature gametes by only relenting to "mature" spermatozoa. • The "mature" spermatozoa that have undergone the complete process of plasma membrane remodelling, cytoplasmic extrusion, and nuclear maturity will have a significantly higher number of HA receptors and binding sites. **Two methods have been proposed for performing PICSI.** i. In the first method, a special dish is used in which microdots of HA hydrogel have been attached to the bottom of the dish. When spermatozoa are added, this allows examination of bound spermatozoa. At this point, only HA-bound sperm are recovered using a standard ICSI injection pipette. ii. The second method uses a viscous medium composed partially of HA, which would fully replace PVP. • Moreover, spermatozoa capable of HA binding have lower DNA fragmentation rates than simple post-swim up spermatozoa. • The nucleus normalcy rate (according to MSOME criteria) has been shown to be higher in spermatozoa bound to HA as compared with sperm in PVP

7. Safety of ICSI

S. No.	Salient features
1	ICSI's safety has often been criticized because the fertilizing spermatozoon neither binds to the ZP nor fuses with the oolemma
2	The specific concerns in regard to ICSI, whether real or theoretical, involve the use of spermatozoa with genetic or structural defects, and the possible introduction of foreign genes.
3	Several epidemiological studies reported a two-fold increase in infant malformations, a recurrent reduction in birth weight, certain rare syndromes related to imprinting errors, and even a higher frequency of some cancers.
4	However, current evidence do not prove that there is an increased risk of imprinting disorders and even less so childhood cancers in ICSI children.
5	Epigenetic imbalances have been similarly linked to the exposure of the embryos to long-term culture

Take home message

- For ICSI, droplets of the medium should be small as they stick to the bottom of the plate and are therefore more stable
- Not more than five oocytes are injected at one sitting
- The oocyte cumulus complexes should be placed into the Hyaluronidase for not more than half minute
- It is better to load fewer sperms than many, as the higher concentration will make the visualization and immobilization of the sperms difficult
- While immobilizing sperm, both the tail of the sperm and the injecting pipette must be in sharp focus
- Immobilization should be done quickly and deliberately. If this is repeated again and again the tail becomes sticky and there will be difficulty while aspirating in the injecting pipette
- Kinking or curling of the tail of sperm confirms immobilization is successful.
- The immature oocytes (GV &MI) should not be injected, as they will not fertilize.
- Placement of the PB at 6 or 12 o' clock ensures that the injection pipette does not disturb the spindle
- Smaller the aperture in the heated stage of the micromanipulator is preferred as there is more efficient heating of the ICSI dish
- It is important that both oolemma and injection pipette tip should be in focus concurrently, as it ensures that the injection pipette will initially pierce the oocyte at its midline
- It is important to break oolemma by negative suction
- Spermatozoa should be deposited towards the centre of the oocyte as this not only confirms the placement but also ensures that proper funnel is created during the placement of the sperm
- Injection pipette should be quickly withdrawn from the oocyte after the release of sperm to avoid minimal deposition of PVP in the cytoplasm

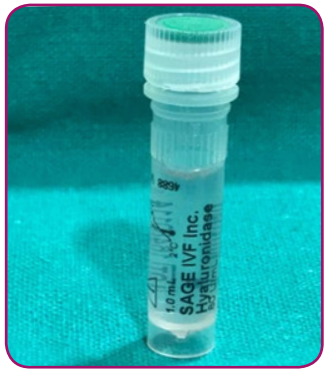
PART-6

Media and Micropipettes

Part 6

Table 1: Preparation and packaging of PVP and HYALURONIDASE medium from different OEMs

S. No.	OEM	Media	Specifications	Preparation	Packaging
1	**SAGE (Cooper surgical company)**	**Polyvinylpyrrolidone 7% solution (PVP)** **(Product Code:** ART- 4005A)  	PVP of average molecular weight 360,000 in HEPES –HTF, 5 mg/ml Human Serum Albumin, filtered through a sterile 0.2 µm filter. It contains 10 mg/l of gentamicin, an aminoglycoside antibiotic. It provides better sperm function and improves visibility to detect sperms. Decreases the motility of spermatozoa and prevents sperm from sticking to the ICSI pipette injection Provides more control over the flow of fluid in the ICSI needle **Ready to use** **Storage**- Stored at 2-8°C **Shelf life**- 6 months from the date of manufacture	Remove PVP Medium and preferred holding medium at 2-8°C Depending on the number of oocytes for injection, pipette a corresponding number of **10 µl droplets of holding medium** onto the bottom of the ICSI dish. In the middle of the same dish place a **5-10 µl droplet of PVP Medium.** Cover with pre-equilibrated oil and place the dish in a 6% CO_2 environment at 37°C prior to use. Introduce **2 µl of prepared** and **washed sperm to the droplet of PVP Medium.** The PVP will reduce the motility of the sperm and facilitate the capture and loading of a single spermatozoon in the injection pipette.	**One unit** of the medium is packaged with **six vials of 0.5 ml** of PVP 7% (w/v) in HEPES-HTF. **Quality Assurance: One-cell MEA tested** and passed with 80% or greater blastocyst. **USP Endotoxin** tested and passed with <1 EU/ ml

S. No.	OEM	Media	Specifications	Preparation	Packaging
		Hyaluronidase, 80U/ml in HEPES-HTF with 5mg/ml HAS **(Product Code:** ART- 4007A) 	Hyaluronidase is an enzyme found in high concentration in the acrosome of most mammalian spermatozoa. It randomly attacks the Hexosaminidic linkage of hyaluronic acid, the Glycosaminoglycan that makes up the extracellular matrix holding cumulus cells together. It contains **10 mg/l of Gentamicin,** an Aminoglycoside antibiotic.	Oocyte-cumulus complexes (OCC) can be placed in a 300µl drop of hyaluronidase medium under oil. After 30 to 45 seconds, the OCCs are pipetted in and out of a fine-bore glass pipette with an inner diameter of 250 to 300 µm to loosen the weakened cumulus mass and release the oocyte with attached corona radiata cells. The oocyte is then transferred to a **100-µL drop of Quinn's** Advantage™ Medium with HEPES containing 5 mg/ml HSA and the corona radiata cells removed by gentle pipetting using a narrow-bore glass pipette with an inside diameter of 135 to 140 µm. The cumulus-free oocytes are further washed through a series of **100 µl drops of Quinn's Advantage™ Medium with HEPES containing 5 mg/ml HSA** to remove excess hyaluronidase and detached cumulus cells The oocytes are now ready for the ICSI procedure.	**One unit** of the medium is packaged with **six vials of 1 ml** of Hyaluronidase, 80U/ml in HEPES-HTF with 5mg/ml HSA **Quality Assurance** **One-cell MEA tested** and passed with 80% or greater blastocyst. **USP Endotoxin tested** and passed with <1 EU/ml**.**

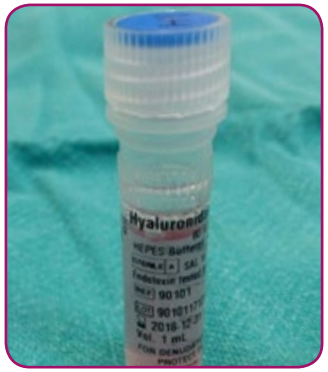
S. No.	OEM	Media	Specifications	Preparation	Packaging
2	Vitrolife	**ICSI™**  	ICSI™ is a viscous sperm handling solution containing **Polyvinylpyrrolidone (PVP) and recombinant human albumin (rHA)** but no antibiotics. Media for immobilization and isolation of sperm prior to intracytoplasmic sperm injection (ICSI). **ICSI™ is antibiotic free.** For use after equilibration at +20 ±5°C and ambient atmosphere **Storage**- Store dark at +2 to +8°C. **Stability**- ICSI™ is stable until the expiry date shown on the container labels and the LOT-specific. Media bottles should not be stored after opening. Discard excess media after completion of the procedure.	Prepare warmed and equilibrated dishes with droplets of handling-medium and a droplet of ICSI™ in the center. Cover with OVOIL™. Place the oocytes into the handling medium droplets, one oocyte per droplet. Place a **small volume (1–2 µl) of prepared sperm suspension** into the centre of the ICSI™-droplet. Incubate the dishes for a few minutes to allow the sperm to migrate to the outer perimeter of the droplet. Prime the injection pipette with ICSI™. Immobilize individual sperm by using the injection pipette to crush the membrane of the sperm tail. Aspirate the immobilized sperm. Move the oocyte droplet into the field of view. Use a holding pipette to secure the oocyte for injection. Inject the sperm with the least amount of ICSI™. Place the injected oocytes in culture medium.	**One unit** of medium is packaged **with five vials of 0.1 ml** of ICSI™ **Quality Assurance:** **Sterile filtered SAL** 10^{-3} **Bacterial Endotoxins** (LAL assay) [EU/ml] < 0.25 **Human sperm motility recovery assay** ≥40% of initial motility **Microscopic evaluation** **Mouse Embryo Assay** (Not performed)
		HYASE™- 10X	**HYASE™ is a physiological salt buffer containing hyaluronidase, human serum albumin and penicillin G as an antibacterial agent.** For use after dilution 1:10 with either of the following two alternatives: 1. **G-MOPS™ PLUS or G-MOPS™** supplemented with G-MM™ or HSA-solution™ and equilibration at +37°C and ambient atmosphere	Dilute HYASE™ either with supplemented G-MOPS™/G-MOPS™ PLUS/G-GAMETE™ to a **ratio of 1:10**, mix well. Prepare and label sterile, non-toxic multiwall dishes and prepare 3 wells of either supplemented G- MOPS™/G-MOPS™ PLUS/ G-GAMETE™ for every well of HYASE™.	**One unit of the medium is packaged with five vials of 0.1 ml of HYASE™- 10X.**

S. No.	OEM	Media	Specifications	Preparation	Packaging
			**2. G-GAMETE™** and equilibration at +37°C and 6 % CO_2 atmosphere. The concentration of hyaluronidase in HYASE™ is **800 IU/ml** before dilution. Medium is used for removal of cumulus cells. **Storage**- Store dark at +2 to +8°C. **Stability**- HYASE™ is stable until the expiry date shown on the container labels and the LOT-specific Media bottles should not be stored after opening. Discard excess media after completion of the procedure.	Pre-warm the multi-well dishes with diluted HYASE™ and supplemented G-MOPS™/ GMOPS ™ PLUS for 15 minutes. If G-GAMETE™ is used as diluting medium; equilibrate the dishes at +37°C and 6 % CO2 for 3 hours. Using a sterile, large bore pipette, place 3–5 oocytes into the HYASE™. Gently pipette the HYASE™ and the oocytes to disperse the cumulus cells. Do not expose oocytes to HYASE™ solution for more than 30 seconds. Use a sterile pipette to transfer oocytes from HYASE™ to the first washing well. Using a fine bore denudation pipette, aspirate each oocyte singly up and down to remove the corona Use the remaining two washing wells to rinse the oocyte. Transfer oocyte to new supplemented G-MOPS™/G-MOPS™PLUS/ G-GAMETE™ or other equivalent droplets for oocyte handling or, if needed, to the culture medium for further incubation and maturation.	**Quality Assurance:** **Sterile filtered SAL** 10^{-3} **Bacterial Endotoxins** (LAL assay) [EU/ml] < 0.5*(*after diluting 1:10 in G-MOPS™ PLUS) Cumulus Cell Removal Assay **Mouse Embryo Assay** (Not performed)

S. No.	OEM	Media	Specifications	Preparation	Packaging
3	**COOK**	**COOK Sydney IVF PVP** 	Sydney IVF PVP is supplemented with **Human Serum Albumin (10 mg/ml) and gentamicin (0.01 mg/ml).** Ready to use after equilibration to 37°C and 6% CO_2 It is intended for use as an aid in the immobilization and isolation of individual sperm cells prior to intracytoplasmic sperm injection (ICSI) procedures. **Storage** - Store at 2-8°C Avoid storing after opening. **Shelf life** - 12 months (at -20°C)	Aseptic technique should be used. Warm the PVP to 37°C and equilibrate in a 6% CO_2 incubator for a minimum of 4 hours prior to use. PVP is suitable for both open and micro-culture. If using micro drops ensure Culture Oil is used to avoid evaporation. PVP consistency can be reduced by addition of Sperm Medium if required. For procedures that will be completed in less than 10 minutes, the injection dish should be prepared with Cleavage Medium warmed to 37°C and equilibrated in a 6% CO_2 incubator for a minimum of 4 hours prior to use. Alternatively, if the procedure will take longer than 10 minutes, the injection dish should be prepared with Gamete Buffer warmed to 37°C in air for a minimum of 4 hours prior to use. Injection dish should be prepared with a concentric pattern of Cleavage Medium droplets. Place a drop of PVP in the centre of the dish.	**One unit** of a medium is packaged **with five vials of 200 µl** of COOK Sydney IVF PVP solution. **Quality Assurance:** **2-cell MEA:** ≥ 80% of control expanded to blastocysts at 72 hours **Endotoxins:** < 0.4 EU/ml **Sterile:** Aseptically filtered **HSA assay:** 5.00-20.00 mg/ml

S. No.	OEM	Media	Specifications	Preparation	Packaging
		COOK Sydney Hyaluronidase 	Hyaluronidase is a **bicarbonate-buffered enzyme preparation containing 80 IU/ml hyaluronidase of ovine origin.** It is supplemented with Human Serum Albumin (5 mg/ml) and gentamicin (0.01 mg/ml). It is intended for use to facilitate removal of the cumulus cells surrounding oocytes in assisted reproductive technology (ART) procedures. **Storage**- Store at 2-8°C Avoid storing after opening. **Stability**- 6 months (-20°C)	Aseptic technique should be used. The oocyte cumulus complexes should be placed into Hyaluronidase for **NO LONGER** than one minute. Warm the Hyaluronidase to 37°C and equilibrate in a 6% CO_2 incubator for a minimum of 4 hours prior to use. Ensure Culture Oil is used to avoid evaporation. Place the Hyaluronidase in one well of a 4-well dish, and aliquots of either Cleavage Medium or Gamete Buffer in the other three wells. Use these three wells for washing the oocytes following the Hyaluronidase treatment.	**One unit** of a medium is packaged with **five vials of 1 ml** of COOK Sydney Hyaluronidase solution. **Quality Assurance:** **2-cell MEA:** ≥ 80% of control expanded to blastocysts at 72 hours **Endotoxins:** < 0.4 EU/ml **Sterile:** Aseptically filtered **HSA assay:** 5.00-20.00 mg/ml

S. No.	OEM	Media	Specifications	Preparation	Packaging
4	Irvine	**7% PVP Solution with HSA** 	**Polyvinylpyrrolidone (PVP) is dissolved in mHTF (an isotonic HEPES buffered medium) to make a 7% (w/v) solution.** It contains 5 mg/mL Human Serum Albumin. 7% PVP Solution with HSA* is intended for use in assisted reproductive procedures which include human gamete and embryo manipulation. These procedures include the use of PVP solution for immobilizing sperm for ICSI procedures. **Storage** - Store the unopened vials refrigerated at 2°C to 8°C. Do not freeze or expose to temperatures greater than 39°C. **Stability** - PVP solutions are stable in unopened vials until the expiration date shown on the kit label when stored as directed.	The 7% PVP Solution is recommended for low motility (or poor quality) semen specimens. Place an aliquot of sperm into liquid PVP solution, 7%. Fill ICSI pipette with PVP solution and then capture single, immobilized sperm according to standard laboratory protocols. Sperm is now ready for ICSI procedure.	**One unit** of a medium is packaged with **five vials of 0.5 ml** of 7% PVP Solution with HSA. **Quality Assurance:** PVP solutions are aseptically processed according to manufacturing procedures which have been validated to meet a **sterility assurance level (SAL) of 10^{-3}**. Each lot of PVP Solution is tested for: **Endotoxin by Limulus Amebocyte Lysate (LAL) methodology** **Sterility by the current USP Sterility Test <71>**
		10% PVP Solution with HAS 	Polyvinylpyrrolidone (PVP) is dissolved in mHTF (an isotonic HEPES buffered medium) to make a 10% (w/v) solution. It contains 5 mg/ml Human Serum Albumin. 10% PVP Solution with HSA* is intended for use in assisted reproductive procedures which include human gamete and embryo manipulation. These procedures include the use of PVP solution for immobilizing sperm for ICSI procedures. **Storage** - Store the unopened vials refrigerated at 2°C to 8°C. Do not freeze or expose to temperatures greater than 39°C. **Stability** - PVP solutions are stable in unopened vials until the expiration date shown on the kit label when stored as directed.	The 10% PVP Solution is recommended for normal and high motility sperm specimens. Place an aliquot of sperm into liquid PVP solution, 10%. Fill ICSI pipette with PVP solution and then capture single, immobilized sperm according to standard laboratory protocols. Sperm is now ready for ICSI procedure.	**One unit** of a medium is packaged with **five vials of 0.5 ml** of 10% PVP Solution with HSA. **Quality Assurance:** PVP solutions are aseptically processed according to manufacturing procedures which have been validated to meet a **sterility assurance level (SAL) of 10-3.** Each lot of PVP Solution is tested for: **Endotoxin by Limulus Amebocyte Lysate (LAL) methodology** **Sterility by the current USP Sterility Test <71>**

S. No.	OEM	Media	Specifications	Preparation	Packaging
		Hyaluronidase solution 	**An enzymatic solution containing 80 IU/ml of bovine-derived hyaluronidase in a HEPES buffered HTF medium supplemented with 5.0 mg/ml (or 0.5 %) therapeutic grade Human Serum Albumin (HSA) and Gentamicin Sulfate (10 µg/ml) as an antibiotic.** Hyaluronidase Solution uses a buffering system composed of a 21 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid) and 4 mM Sodium Bicarbonate combination. This buffering system provides optimum pH maintenance over the physiologic range (7.2 to 7.4) and does not require the use of a CO_2 incubator. It is used for removing cumulus cells surrounding human oocytes (denudation) in preparation for ICSI or other ART procedures. **Storage** - Store the unopened bottles refrigerated at 2-8° C. Do not expose to temperatures greater than 39°C. **Stability** - Hyaluronidase Solution is stable until the expiration date shown on the bottle label when stored as directed.	Pre-equilibrate the Hyaluronidase Solution to 37° C before use. **(NOTE: Hyaluronidase Solution should be tightly capped if warmed in a CO_2 incubator to avoid pH levels of 7.0 or less.)** Dispense contents of the vial into an appropriate culture dish and place the oocytes in the Hyaluronidase Solution for no more than 30 seconds while stripping off the surrounding cumulus cells with continuous pipetting. Immediately remove the oocytes with a minimal volume of solution (leaving behind as much cumulus as possible) and transfer to wash medium (Modified HTF + 5 mg/ml HSA). Aspirate the oocytes with a pipette to remove any remaining cumulus and corona cells. Wash the denuded oocytes 2 more times until completely denuded. Transfer the oocytes to Early Cleavage Medium supplemented with protein and identify which oocytes will be used for ICSI (or other) procedures. Denuded oocytes should be cultured for a minimum of 1 hour before beginning ICSI procedures.	**One unit** of a medium is packaged **with five vials of 1 ml** of Hyaluronidase solution. **Quality Assurance:** Hyaluronidase Solution is **membrane filtered** and aseptically processed according to manufacturing procedures which have been validated to meet a **sterility assurance level (SAL)** of 10^{-3} Each lot of Hyaluronidase Solution is tested for: **Endotoxin by Limulus Amebocyte Lysate (LAL) methodology** **Biocompatibility by Mouse Embryo Assay (one-cell)** **Enzymatic Activity by the current USP Test** **Sterility by the current USP Sterility Test <71>**

S. No.	OEM	Media	Specifications	Preparation	Packaging
5	**Origio (Cooper Surgical Company)**	**SpermSlow™** **(Product Code:** 10944000A)  	SpermSlow™ is a **semi-viscous medium** containing Hyaluronan for slowing down the movement and for the selection of an individual sperm with enhanced genetic and developmental integrity. **It contains Human albumin solution (HAS) Recombinant human insulin Gentamicin sulphate 10 µg/ml** **Key features:** • Replaces PVP in the sperm selection process • Significantly higher embryos development rate with the use of SpermSlow compared to PVP • Better embryo quality • Lower rates of chromosomal aneuploidies in Hyaluronan-selected sperm • Lower rates of DNA fragmentation in Hyaluronan-selected sperm • Lower rates of early miscarriage The products are aseptically processed and supplied sterile. **Storage** - Store in original container at 2-8°C, protected from light. The product is provided in vials intended for single use. **Stability** - When stored as directed by the manufacturer the product is stable until the expiry date shown on the vial label.	Remove SpermSlow™ and preferred holding medium from storage at 2-8°C and leave at room temperature for 10 minutes. Pipette 2 x 10 µl of SpermSlow™ onto the bottom of the ICSI dish, which should be kept at 37°C during the whole procedure (1 drop in centre, 1 drop at the rim of the dish). Depending on the number of oocytes used in the ICSI procedure, pipette a corresponding number of 5-10µl droplets of holding medium. Introduce a small amount prepared and washed sperm close to the SpermSlow™ drop in the centre of the ICSI dish. Use the pipette tip to create a junction between the sperm droplet and the SpermSlow™ drop in the centre. Immediately cover the ICSI dish with pre-equilibrated oil and place it in a 6% CO_2 incubator at 37°C for 15 minutes prior to use. Wash/rinse the injection pipette in pre-equilibrated Sperm Preparation Medium. Aspirate 10-20 mm into the holding pipette and 2-5 mm into the injection pipette After 1 minute expel the Sperm Preparation Medium from the injection pipette into the dish with the Sperm Preparation Medium. The injection pipette is now coated to avoid sperm and SpermSlow™ adhering to the needle. Place an oocyte in each droplet of holding medium. Aspirate 5-6 mm SpermSlow™ from the SpermSlow™ drop at the rim of the ICSI dish. Carefully select a mature spermatozoon from near the junction between the sperm droplet and the SpermSlow™ drop. Mature spermatozoa will be bound to the Hyaluronidase in SpermSlow™. Therefore, look for the spermatozoon with the best morphology, and which has a moving tail but no forward motion (i.e. is 'moving in place'). Spermatozoa moving freely in the SpermSlow™ drop are immature spermatozoa and should not be selected.	**One unit** of a medium is packaged with **four vials of 0.1 ml** of SpermSlow™. **Quality Assurance:** **Sterility tested** **Endotoxin tested** ≤ 0.1 EU/ml **HSA analysis** **Sperm Survival tested** **Performance tested**

S. No.	OEM	Media	Specifications	Preparation	Packaging
		ICSI Cumulase® **(Product Code:** 16125000A) 	ICSI Cumulase® is **HEPES-buffered and pH stable** for use outside the incubator without prior equilibration. ICSI Cumulase® **does not contain bicarbonate and should therefore not be placed in the CO_2 incubator without the cap on.** ICSI Cumulase® is for the removal of the cumulus complex and corona radiata surrounding the oocyte in preparation for ICSI. It contains **Human albumin solution (HAS), Recombinant human hyaluronidase (rHuPH20) 80 U/ml** The products are aseptically processed and supplied sterile. **Storage-** Store in original container at **2-8°C**, protected from light. Do not freeze. Discard excess (unused) media following warming. The product is provided in **vials intended for single use.** **Stability-** When stored as directed by the manufacturer the product is **stable until the expiry date** shown on the label.	Warm ICSI Cumulase® to 37°C for 2 hours with the cap on. Fill one of the wells in a four-well dish with the contents of one vial of ICSI Cumulase® and the other three wells with your preferred, equilibrated culture medium. Place one or several oocyte-cumulus complexes in the well with ICSI Cumulase®. After 60 seconds, gently aspirate the oocyte(s) up and down until the oocyte(s) is denuded. Transfer the oocyte(s) to a well with culture medium. Wash the oocyte(s) thoroughly by transferring it between several wells of culture medium. The oocyte(s) is then transferred to the injection dish and placed in individual micro drops covered with equilibrated Liquid Paraffin.	**One unit** of a medium is packaged with **five vials of 0.5 ml** of ICSI Cumulase®. **Quality Assurance:** **Sterility tested** **Osmolality tested** **pH tested** **Endotoxin tested** ≤ 0.1 EU/ml **HSA analysis** **Mouse Embryo Assay (MEA) tested** **Enzyme activity**

S. No.	OEM	Media	Specifications	Preparation	Packaging
6	**Medicult (Origio)**	**PVP (Product Code: 10890001A)** 	The PVP medium is used for slowing down the movement of the spermatozoa for ICSI. 10% concentration of the PVP medium is sufficient for easy immobilization of the spermatozoa. Contains: Phenol red, Human Serum Albumin (HAS), Recombinant human insulin, Gentamicin sulphate (10µg/ml) **Shelf life** - 8 weeks **Storage** - Store in original container at 2-8°C, protected from light. Do not freeze. Discard excess (unused) media following warming. The product is provided in vials intended for single use. **Stability** - When stored as directed by the manufacturer the product is stable until the expiry date shown on the label. **Validity after opening** - 7 days	Remove PVP Medium and preferred holding medium at 2-8°C and leave at room temperature for 10 minutes. Depending on the number of oocytes for injection, pipette a corresponding number of 10 µl droplets of holding medium onto the bottom of the ICSI dish. In the middle of the same dish place a 5-10 µl droplet of PVP Medium. Cover with pre-equilibrated Liquid Paraffin and place the dish in a 5-6% CO_2 environment at 37°C for 30 minutes prior to use. Introduce 2 µl of prepared and washed sperm to the droplet of PVP Medium. The PVP will reduce the motility of the sperm and facilitate the capture and loading of a single spermatozoon in the injection pipette.	**One unit** of the medium is packed with the **one vial of 1 ml** of PVP medium. **Quality Assurance:** **Sterility tested** **Endotoxin tested** ≤0.2 EU/ml **HSA analysis** **Sperm Survival tested** **Sperm Motility tested**

S. No.	OEM	Media	Specifications	Preparation	Packaging
		SynVitro® Hyadase (Product Code: 15110001A) 	SynVitro® Hyadase is used for the removal of the cumulus complex and corona radiate surrounding the oocyte in preparation for ICSI. **Contains :** Recombinant Human Albumin Pharmaceutical grade hyaluronidase, 80 IU/ml (non-bovine source) **Ready to use** **Storage** - Store in original container at 2-8°C, protected from light. Do not freeze. Discard excess (unused) media following warming. The product is provided in **vials intended for single use.** **Stability** - When stored as directed by the manufacturer the product is **stable until the expiry date** shown on the label.	Pre-Warm SynVitro® Hyadase to 37˚C for 2 hours with cap on. Fill one of the wells in a four-well dish with SynVitro® Hyadase (0.5 ml) and the other three wells with your preferred. Pre-equilibrated culture medium. Place one or several oocyte-cumulus complexes in the well with SynVitro® Hyadase. After approximately 10 seconds, gently aspirate the oocyte up and down in a denudation pipette. Transfer the oocytes to a well with culture medium and remove the remaining cumulus complex by gently aspirating the oocytes up and down in the denudation pipette. Wash the oocytes thoroughly by transferring them between several wells of culture medium. The oocytes are then transferred to the injection dish and placed in individual micro drops covered with pre-equilibrated Liquid Paraffin. **NOTE** - It is recommended to leave the oocytes in culture medium for short period before proceeding with ICSI.	**One unit** of the medium is packed with the **five vials of 1 ml** of SynVitro® Hyadase. **Quality Assurance:** **Sterility tested** **Osmolality tested** **pH tested** **Mouse Embryo Assay (MEA) tested**

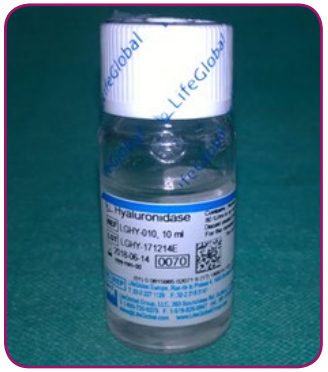
S. No.	OEM	Media	Specifications	Preparation	Packaging
7	Life GLOBAL	**PVP (Product Code: LPVP-502)** 	**Composition** - sodium chloride, Potassium Chloride, Calcium Chloride, Potassium Phosphate, Magnesium Sulfate, Sodium Bicarbonate, Glucose, Sodium Lactate, Sodium Pyruvate Phenol Red, Gentamicin Sulfate, HEPES, Human Serum Albumin **Storage** - Store at 2-8˚C and protected from light **Shelf life** - No more than 9 months from the date of production.	Warm PVP to 37°C. Place a 5–10 µl droplet of PVP in a dish. Place four to five 10 µl droplets of HTF w/HEPES containing 10% HSA in close proximity of the PVP droplet, and cover with LiteOil. Place selected oocytes for ICSI into droplets of HTF w/HEPES. Add a small volume (1–2 µl) of washed sperm into the center of the PVP droplet. Within a few moments, sperm will migrate to the periphery of the droplet. Select and recover the spermatozoa for injection.	**One unit** of medium is packaged with **one vial of 1 ml** of LPVP-001 and **five vials of 0.2 ml** of LPVP-502. **Quality Assurance:** **pH tested** **Viscosity tested** **LAL Endotoxin tested** ≤ 0.1 EU/ml **Mouse Embryo Assay (MEA) tested**
		Hyaluronidase (Product Code: LGHY-010) 	**Composition** - Hyaluronidase (80 IU/ml) in an aqueous solution consists of: Sodium Phosphate, Sodium Bicarbonate, Phenol Red, Potassium Chloride, Glucose, Calcium Chloride, Sodium Lactate, HEPES, Potassium Phosphate, Sodium Pyruvate, Magnesium Sulfate, Human Serum Albumin (5mg/ml), Gentamicin Sulfate (10mg) **Storage** - Store at 2-8˚C and protected from light **Shelf life** - 6 months from the date of manufacture.	Warm Hyaluronidase to 37°C. Prepare a dish containing one 100 µl droplet of Hyaluronidase and three to five 100 µl droplets of HTF w/HEPES for oocyte washing, all under LiteOil. Place up to five oocytes in the Hyaluronidase droplet and begin to pipette up and down repeatedly in a fine bore glass pipette until the oocyte is partial denuded. Using a fine bore glass pipette, transfer the partially denuded oocytes into the first HTF w/HEPES droplet. Remove the corona cells by pipetting the oocytes up and down.	**One unit** of medium is packaged with **one vial of 10 ml** of Hyaluronidase solution. **Quality Assurance:** **pH tested** **Osmolarity tested** **LAL Endotoxin tested** ≤ 0.1 EU/ml **Sterility tested (SAL 10-3)** **Mouse Embryo Assay (MEA) tested**

Table 2. Comparison of Micropipettes from different OEMs

S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
1	**HUMAGEN (now marketed as Origio) (CooperSurgical Company)**	**Injecting pipette**	**Type**- ICSI with Spike **Reference no.**- MIC-SI-XX **Available angle (XX in Ref. no.)**- 0°, 20°, 25°, 30°, 35°, 45° **Distance tip to bend**- 0.5 mm **Bevel**- 30- 35° **Bevel length**- 10- 11 µm **Inner diameter**- 5- 5.7 µm **Others**- Standard Taper	Origio injection pipette is used for gentle injection of spermatozoa into the cytoplasm of an oocyte during ICSI thus, minimizing oocyte damage.	**Advantages:** • Minimizes damage to the oocyte by creating a small entry point • More parallel taper but still rigid when injecting • Excellent control when puncturing the oolemma
		Injecting pipette	**Type- SlimLine** ICSI pipette **Reference no.**- MIC-SLM-XX **Available angle (XX in Ref no.)**- 0°, 15°, 20°, 25°, 30°, 35°, 45° **Distance tip to bend**- 0.5 mm **Bevel length**- 10-12 µm **Inner diameter**- 4.3-4.9 µm **Others**- Narrow taper	They have: • Short taper • Long parallel wall section • Small diameter • Narrow taper design	**Advantages:** • Minimizes damage to the oocyte by creating a small entry point • Maintains a constant narrow diameter while keeping the necessary rigidity to easily penetrate the zona pellucida. • The right choice for loading multiple sperms for quick injections
		Holding pipette	**Type**- Medium Holding **Reference no.**- MPH-MED-XX **Available angle (XX in Ref. no.)**- 0°, 20°, 25°, 30°, 35°, 45° **Distance tip to bend**- 0.5 mm **Inner diameter**- 15-20 µm **Outer diameter**- 95-120 µm	Holding micropipettes are used to hold and immobilize the oocyte or embryo. They have a flat, square opening with a large outer to inner diameter ratio for maximum control. Four standard sizes are offered to suit all micromanipulation techniques.	

S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
	RI (CooperSurgical Company)	**Injecting pipette**	**Type**- ICSI Spiked Injection **Reference no.**- 7-71-T4SIXXL/20 **Available angle (XX in Ref. no.)**- 30°, 35° **Distance tip to bend**- 1 mm **Bevel**-30° **Inner diameter**- 4 µm	RI ICSI injection micropipettes can be used to immobilize, then inject spermatozoa into the cytoplasm of an oocyte during ICSI and intracytoplasmic morphologically-selected sperm injection (IMSI). ICSI injection micropipettes are designed for the **smoothest control** of sperm injection and cytoplasm suction, and to **minimize damage to the oocyte**. Multiple sperms can be loaded and injected. Injection micropipettes from RI are available **with a spike (SI)** or **without a spike (NI)** and in 2 different glass **thicknesses – 1 µm (standard) or 0.9 µm (thin – T code).** For human ART, the **larger 8 µm ID injection micropipette is ideal for collecting sperm from a testicular tissue sample** prior to ICSI and is only available in thin (T code) glass.	Finished product rigorously tested by **1-cell MEA, LAL and HSSA.** Each m spected 5 times during manufacturing. Pipettes are **super sharp, non-sticky,** with **consistent geometries** RI Pipettes have a **shelf life of three years as standard.** **Packed into the high-quality Twista-pak**™ which provides protection from tip damage and operates like a lipstick. The simple twisting action allows easy access and ensures neither end of the micropipette is touched during removal
		Holding pipette	**Type**- Holding **Reference no.**- 7-71-IHXX/20 **Available angle (XX in Ref. no.)**- 0, 20, 25, 30, 35 **Distance tip to bend**- 0.75 mm **Inner diameter**- 15 µm **Outer diameter**- 90-110 µm	These micropipettes are designed for anchoring the oocyte or embryo during ICSI or other micromanipulation procedures. A smooth fire-polished tip cradles the cells gently and securely. 32 µm large holding micropipettes are available with a 30° and 35° bend angle.	

S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
	TPC (CooperSurgical Company)	**Injecting pipette**	**Type**- Standard Injection **Reference no.**- LICR-TA35 **Available angle**- 35° **Distance tip to bend**- 1 mm **Bevel**- 30° **Inner diameter**- 5 µm	**Long, parallel tapers** provide smooth, effortless fluid control during micromanipulation. The **bases of all pipettes are cut square** and are **lightly heat polished** to prevent sharp edges from damaging silicon seals inside microtool holders. **Sharp-Blunt Spike** for easy penetration of the zona pellucida and gentle progression through the oolemma to minimize the risk of tearing. TPC spikes are very consistent and uniform in both shape and length Long bevel	**Advantages:** • Easy immobilization of sperm • Very fine fluid control and suction during use • Easy penetration of the zona pellucida and gentle progression through the oolemma with minimal risk of tearing • Optimal design for loading and injecting multiple sperms
		Holding pipette	**Type**- Standard holding **Reference no.**- LHC-ID30TA35 **Available angle**- 35° **Distance tip to bend**- 1 mm **Inner diameter**- 30 µm **Outer diameter**- 120 µm	Holding pipettes have **a large, square, flat face** and a **proportionately large inner diameter,** which provides better support for the oocyte and embryo and reduces distortion during micromanipulation.	

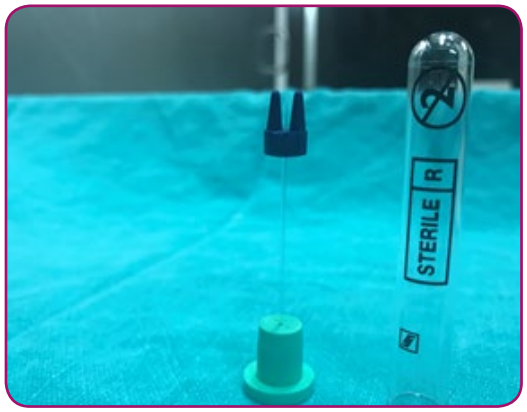
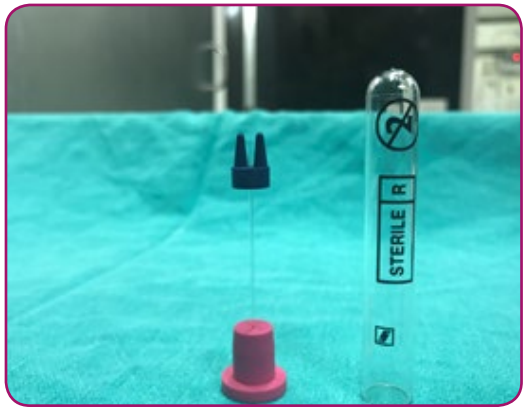
S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
2	**Vitrolife**	**Injecting pipette**	**Type**- A- series **Reference no.**- 15406 **Inner diameter**- 4-5.5 µm **Distance tip to bend**- 500 µm **Available angle**- 0- 35° **Bevel**- Short sharp bevel with a short spike **Others**- Has a unique taper that gets wider later to give less damage to the oocyte	A wide range of pipettes with different characteristics are available: • The **A-series** has short bevel with a short spike. • The **B-series** has short sharp bevel with a longer spike. • The **C-series** has a short bevel and a very short spike. • The **D-series** has parallel walls allowing for greater control of loaded sperms with a small outer diameter for minimally invasive ICSI. Inner diameter optimized to give little space between sperm and the wall, reducing final volume injected. • The **E-series** has a medium bevel and short taper with parallel walls. • ICSI pipettes without spike. All ICSI pipettes are available with various design features including **bend angles from 0° to 35°.**	Micromanipulation Pipettes are **sterile, pyrogen free, disposable, borosilicate glass** lab products designed for manipulation of cells in vitro. **Storage:** Store at room temperature. **One unit** of micropipettes is packaged with **10 pieces** of injecting pipette. All pipettes are **MEA and LAL tested**
		Holding pipette	**Type**- Holding **Reference no**- 15306 **Available angle**- 35° **Distance tip to bend**- 1000 µm **Inner diameter**- 20-30 µm **Outer diameter**- 120 ± 10 µm **Aperture**- 25 ± 5 µm **Others**- Flat surface.	Vitrolife holding pipettes have **an optimal lumen diameter and smooth edges.** This means that oocytes or embryos can be handled with minimal trauma. These pipettes are **available with rounded tip and polished opening including bend angles from 0° to 35°, with and without spike.**	**One unit** of micropipettes is packaged with **10 pieces** of holding pipette. **Storage**- Store at room temperature.

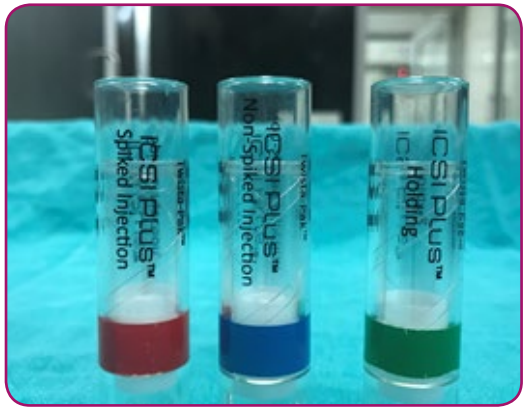
S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
3	**COOK**	**Injecting pipette**	**Reference no.**- K-MPIP-1035 **Inner diameter**- 5.0 µm **Outer diameter**- 7 µm **Distal tip angle**- 35° **Bevel angle**- 30° **Tip to bend length**- 700 µm	The microinjection pipette is used for the intracytoplasmic single sperm injection of oocytes. These pipettes have **well-defined bevels** to aid zona pellucida puncture, and **are supplied in three groups:** • **"10" series** • **"31" series** • **"33" series.** The "10" series has a longer taper-to-tip length than the "33" series. The bend-to-tip length is the same for all three series.	**Sold in boxes of 10**
		Holding pipette	**Reference no.**- K-HPIP-2135 **Inner diameter**- 23 µm **Outer diameter**- 110 µm **Distal tip angle**- 35° **Tip to bend length**- 700 µm	The holding pipette is used to hold an oocyte, embryo, or blastocyst in position with the application of vacuum during intracytoplasmic single sperm injection or assisted hatching/zona drilling. Cook Precision Holding Pipettes are **supplied in three groups:** • The **"10" series** has the smallest inner and outer diameters • The **"21" series** has larger inner and outer diameters, and • The **"33" series** has the largest diameters.	Featuring a **smooth, flame-polished surface**, the holding pipette provides stability and holding power. **Sold in boxes of 10.**

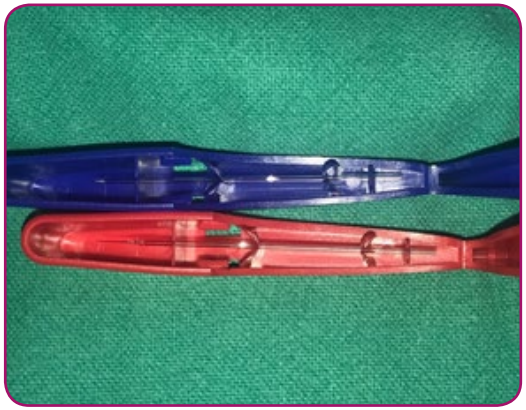
S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
4.	**Sunlight**	**Injecting pipette** SLM ICSI pipettes are used to perform intra-cytoplasmic sperm injection (ICSI). SLM offers **three types of spiked** and **two types of non-spiked** ICSI injection pipettes. SLM **offers three sizes of ICSI injection pipettes.** All injection pipettes are supplied with an **angle of 0°- 45°** and a **tip to bend length of 0.55 mm and 1.0 mm.**	**Tip Type**- V, spiked **Reference no.**- SIC-50V-35 **Inner diameter**- 4.5 - 5.0 µm **Tip angle**- 35° **Tip to bend length**- 0.55 mm	V-type injection pipettes have: • **Short taper** • **Long parallel wall section** • Standard tip to elbow length (0.55mm) **Gamma irradiated** **Mouse embryo assay tested** **Secure packaging** that is **colour coded** for easy identification	**Advantages:** • Smooth control of sperm injection and cytoplasm suction • Less cytoplasm draws back when breaking oocyte membrane • Less damage to oocyte • Better fertilization and embryo development • Multiple sperms can be loaded and injected **Disadvantages:** May not be easy in immobilizing sperm for some embryologists who need press down very hard on sperm tail.
			Tip Type- H, spiked **Reference no.**- SIC-50H-30 **Inner diameter**- 4.5 - 5.0 µm **Tip angle**- 30° **Tip to bend length**- 0.55 mm	H-type injection pipettes have: • Longer taper • Short parallel wall • Longer tip to elbow length (0.75 mm) **Gamma irradiated** **Mouse embryo assay tested** • **Secure packaging** that is **colour coded** for easy identification.	**Advantages:** • Multiple sperms can be loaded and injected • Smooth control of sperm injection • Less damage to oocyte • Better fertilization and embryo development **Disadvantages:** Sperm may turn around when loading and injecting multiple sperms

S. No.	OEM	Type of pipette	Specifications	Characteristics	Other details
			Tip Type- W, spiked **Reference no.**- SIC-50W-35 **Inner diameter**- 4.5 - 5.0 µm **Tip angle**- 35° **Tip to bend length**- 0.55 mm	W-type injection pipettes have: • Short taper • No parallel wall • Long parallel wall section • Standard tip to elbow length (0.55mm) **Gamma irradiated** **Mouse embryo assay tested** • **Secure packaging** that is **colour coded** for easy identification	**Advantages:** • Strong and less flexible tip, so easy in immobilizing sperm. **Disadvantages:** • May suck lots of cytoplasm, especially for people who are not familiar with this type of pipette. • Sperm may turn around when loading and injecting multiple sperms. **Packed and sold in boxes of 10 pipettes.**
		Holding pipette	**Reference no.**- SHP-100-35 **Outer diameter**- 100 µm **Inner diameter**- 15-20 µm **Tip angle**- 35° **Tip to bend length**- 0.65 mm	Holding Pipettes are used to hold an oocyte or embryo in position during intracytoplasmic sperm injection (ICSI) and other micromanipulation procedures There are **three series** and **six sizes** for selection: • The classics series has an ID 15-20 µm and OD in 90, 100, 120, 130 and 150 µm. • The **'S' series** has smaller ID (10-15 µm). • The **'B' series** has an ID 25-30 µm and OD in 120, 130, 150 and 180 µm. The 'B' series may be used for holding an oocyte with cumulus cells attached and for embryo biopsy.	Each micropipette is handmade and examined **Secure packaging** that is **colour coded** for easy identification **Mouse embryo assay tested** **Gamma irradiated** **Packed in a box of 10.** **Storage:** Store at 15-30°C **Shelf Life:** 3 Years

Table 3: Packaging of micropipettes

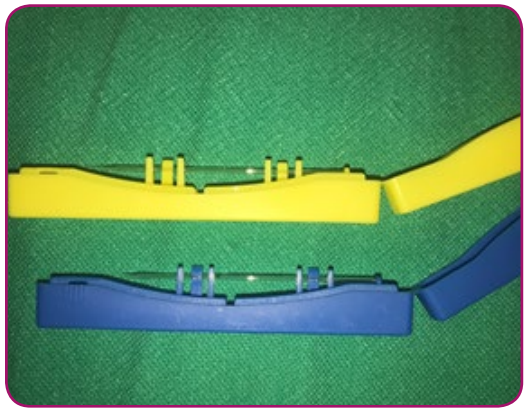
S. No.	OEM	Micropipettes	Figure	Packaging
1	**HUMAGEN (now marketed as Origio) (CooperSurgical Company)**	**Injecting pipette**		Humagen micropipettes are seated in santoprene caps (various colors indicate the type of micropipette), and covered with a polystyrene tube that fits onto the cap and forms a microbial barrier. All micropipettes come together with a removable tip protector which makes it easier to remove the micropipette from the tube without breaking the tip. **Green color** - Injection micropipette **Pink color** - Holding micropipette
		Holding pipette		

S. No.	OEM	Micropipettes	Figure	Packaging
	RI (CooperSurgical Company)	**Injecting pipette**		Micropipettes are **packed into the high quality Twista-Pak™**. Twista-Pak™ provides protection from the tip damage and operates like a lipstick, where the simple twist action allows easy access and ensures neither end of the pipette is touched during removal. RI micropipettes have a **shelf life of three years.**
		Holding pipette		

S. No.	OEM	Micropipettes	Figure	Packaging
	TPC (CooperSurgical Company)	**Injecting pipette**		Pipettes are packaged in protective holders, which are color coded for easy identification of the different types. **Blue color** - Injection micropipette **Red color** - Holding micropipette
		Holding pipette		

S. No.	OEM	Micropipettes	Figure	Packaging
2	**Vitrolife**	**Injecting pipette**		Vitrolife micropipettes are packaged individually in color coded silicon holders for to deliver undamaged product. **Pink color** - Holding pipette **Blue color** - Injecting pipette
		Holding pipette		

S. No.	OEM	Micropipettes	Figure	Packaging
3	COOK	**Injecting pipette**		Micropipettes are packaged individually in containers that are designed to deliver undamaged product. These containers are easy to use and are made of recyclable materials. **Yellow color** - Holding pipette **White color** - Injecting pipette
		Holding pipette		

S. No.	OEM	Micropipettes	Figure	Packaging
4	**Sunlight**	**Injecting pipette**		Micropipettes are packed in convenient color coded pipette safe cases. The case is designed with a green concept by using minimal materials for conservation and minimizing weight and size of the total package in the effort to reduce energy used for transportation. The outer box, including all labels, is made of paper for easy recycling. **Yellow color** - Holding pipette **Blue color** - Injecting pipette Irvine micropipettes have a **shelf life of three years.**
		Holding pipette		

PART-7

Original equipment manufacturer details

Part 7

1. OEM & Vendor Related Contact Information for Micropipettes

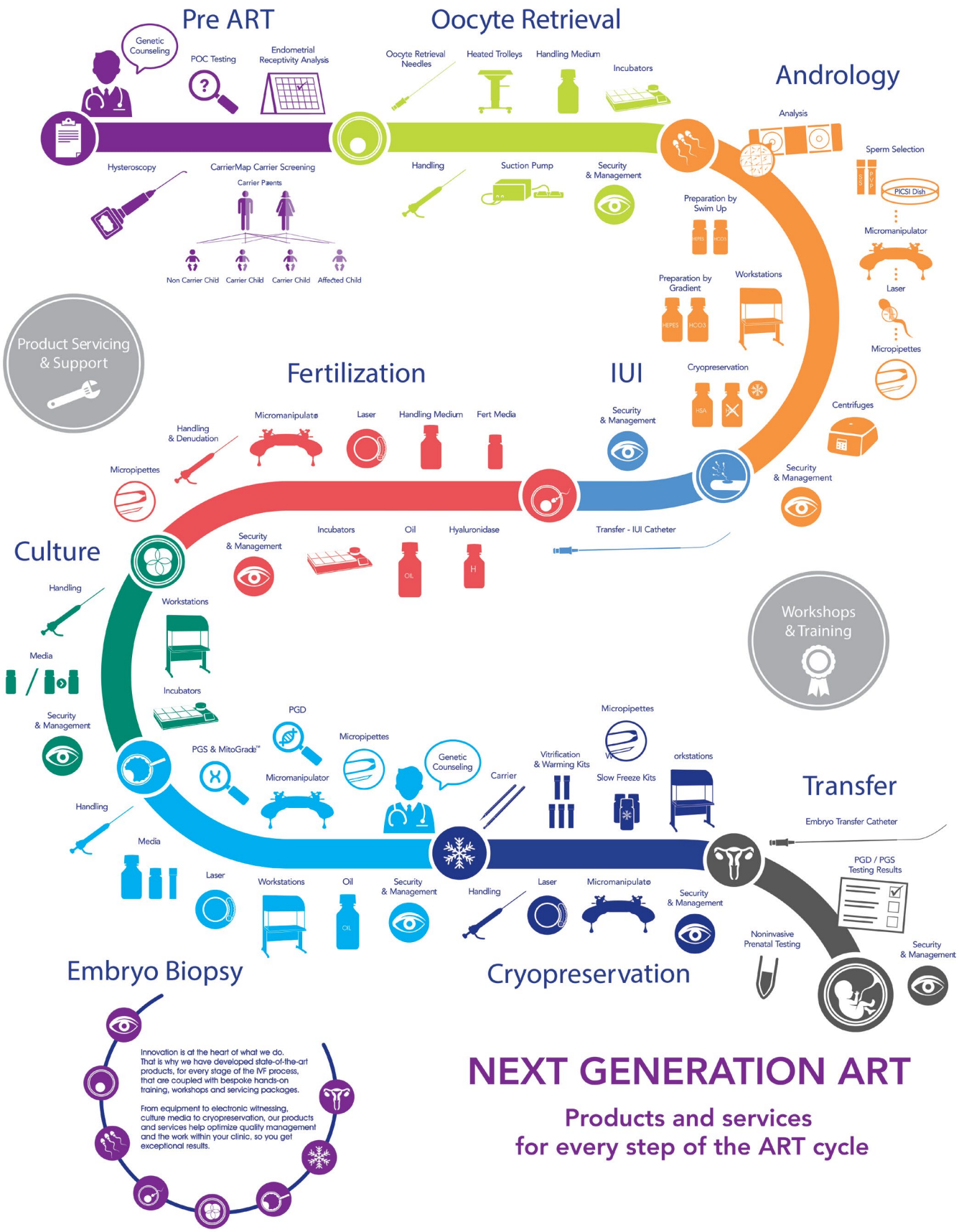
S. No.	ORIGINAL EQUIPMENT MANUFACTURER (OEM)	INDIA DISTRIBUTER	DEVICE BRAND	PRICE PER DEVICE	CONTACT PERSON	PHONE	EMAIL
1	**ORIGIO** a/s Knardrupvej 2 2760 Malov Denmark	Origio India Pvt. Ltd.	**HUMAGEN** micropipettes	Not disclosed	Mr. Piyush Jain	+91 7042396972	pjain@origio.com
2	**ORIGIO** a/s Knardrupvej 2 2760 Malov Denmark	Sar Healthcare Pvt. Ltd.	**TPC** micropipettes	Not disclosed	Mr. Atul Walia	+91 9958029696	info@sarhealth.com
3	**ORIGIO** a/s Knardrupvej 2 2760 Malov Denmark	Shivani Scientific India Pvt. Ltd.	**RI** micropipettes	Not disclosed	Mr. Amol Sharan	+91 9990199933	amols@shivanisciencetific.com amolsharan@gmail.com
4	**Vitrolife Sweden** AB Gustaf Werners gata 2 SE-421 32 Vasta Frolunda Sweden	Vision Diagnostics Pvt. Ltd.	**Vitrolife** micropipettes	Not disclosed	Mr. Punit Khatnani	+91 9910188771	punit@vision-groups.com
5	**CryoBio System** Groupe I.M.V Technologies France	CryoBio System India	**Sunlight** micropipettes	Not disclosed	Mr. Jitender Kumar	+91 9650602424	jitender@cryobiosystemindia.com
6	**COOK Medical Inc.** P.O Box 4195, Bloomington, In 474024195, USA	Intermedics	**COOK** microppettes	Not disclosed	Mr. Gopal	+91 9212798185	projects@intermedics.in

2. OEM & Vendor Related Contact Information for Media

S. No.	ORIGINAL EQUIPMENT MANUFACTURER (OEM)	INDIA DISTRIBUTER	PACK BRAND	PRICE PER DEVICE	CONTACT PERSON	PHONE	EMAIL
1	**ORIGIO** a/s Knardrupvej 2 2760 Malov Denmark	Origio India Pvt. Ltd.	**Origio Medicult SAGE**	Not disclosed	Mr. Piyush Jain	+91 7042396972	pjain@origio.com
2	**Vitrolife Sweden** AB Gustaf Werners gata 2 SE-421 32 Vasta Frolunda Sweden	Vision Diagnostics Pvt. Ltd.	**Vitrolife**	Not disclosed	Mr. Punit Khatnani	+91 9910188771	punit@vision-groups.com
3	**CryoBio System** Groupe I.M.V Technologies France	CryoBio System India	**Irvine**	Not disclosed	Mr. Jitender Kumar	+91 9650602424	jitender@cryobiosystemindia.com
4	**Life Global** LLC 393, Soundview Road, Guilford, CT, 06437, USA	Shivani Scientific India Pvt. Ltd.	**Life Global**	Not disclosed	Mr. Amol Sharan	+91 9990199933	amols@shivaniscientific.com amolsharan@gmail.com
5	**COOK Medical Inc.** P.O Box 4195, Bloomington, In 474024195, USA	Intermedics	**COOK Sydney**	Not disclosed	Mr. Gopal	+91 9212798185	projects@intermedics.in

Notes

Notes



ORIGIO India Private Limited
C - 401, Delphi, Hiranandani Business Park, Powai,
Mumbai - 400 076, Maharashtra, India
Board Line No. : +91 22-49280000 I Fax No. +91 22-49280010

CooperSurgical Companies Product Portfolio

Pre ART

- CarrierMap ™
- Genetic Counseling
- Endosee ® (hysteroscopy)
- ERGrade ™ Endometrial Receptivity Analysis
- POC Testing

Oocyte Retrieval

- Oocyte Retrieval Needles
- Wallace ® Single Lumen Oocyte Recovery Needle*
- Wallace ® Single Lumen Oocyte Recovery Sets
- Wallace ® Dual Lumen Oocyte Recovery Sets*
- ORIGIO Stiff Oocyte Retrieval Needles*
- ORIGIO Regular Wall Oocyte Retrieval Needles*
- ORIGIO Thin Wall Oocyte Retrieval Needles*
- ORIGIO Double Lumen Oocyte Retrieval Needles*
- Suction Pump
- ORIGIO Suction Pump*
- Handling Devices
- The Stripper ® (Standard / BP / CC / PGD)
- RI EZ-Range (RI EZ-Grip ®, RI EZ-Strip ®, RI EZ-Squeeze ™, RI EZ-Tip ®)
- IVF Pasteur Pipets
- The Stripper Tips (Individual or Multi Pack)
- Media
- Quinn's Advantage Medium with HEPES
- Flushing Media
- SynVitro Flush
- Heated Trolley
- K-Systems Trolley with Warmed Table Top*
- K-Systems Warming Plates T45/T47*
- Incubators
- K-Systems G210 InviCell*
- K-Systems G185*
- ORIGIO ® / PLANER Benchtop Incubator BT37
- Security & Management
- RI Witness ™

Andrology

- Analysis
- HBA ® Assay
- Preparation by Swim Up
- ORIGIO Sperm Preparation Media
- Quinn's ™ Sperm Washing Medium
- Preparation by Gradient
- PureCeption ™
- Quinn's ™ Sperm Washing Medium
- SupraSperm ®
- ORIGIO Sperm Preparation Media
- Centrifuges
- Midi/Maxi
- Sperm Selection
- SpermSlow ™
- PICSI ® Dish
- PVP (PVP Medium, PVP Clinical Grade, PVP 7% Solution)
- ORIGIO, TPC and RI Micropipettes
- Cryopreservation
- CryoSperm ™
- Sperm Freezing Medium
- Quinn's Advantage ™ Sperm Freezing Medium
- Micromanipulator
- Integra 3 ™ Micromanipulator
- Laser
- Saturn 5 ™ Laser System
- Workstations – Class I
- K-Systems 100 Series (L124 / L124 ICSI/ L126 MP)*
- ORIGIO Fortuna
- ORIGIO Titan
- Workstations – Class II
- K-Systems L234 CellTouch*
- K-Systems G603 Work Chamber*
- ORIGIO Mars
- Supporting Products
- Warming Blocks
- Glass Hoods
- Gas Line Filters
- Security & Management
- RI Witness ™

IUI

- Handling Devices
- Wallace ® Intrauterine Insemination Catheter
- ORIGIO IUI Cathether*
- IVF Pasteur Pipets
- Security & Management
- RI Witness ™

Fertilization

- Handling and Denudation Devices
- The Stripper ® (Standard / BP / CC / PGD)
- RI EZ-Range (RI EZ-Grip, RI EZ-Strip, RI EZ-Squeeze, RI EZ-Tip)
- IVF Pasteur Pipets
- The Stripper Tips (Individual or Multi Pack)
- Media
- ORIGIO ICSI Cumulase ®
- SAGE Hyaluronidase
- ORIGIO SynVitro ® Hyadase
- Quinn's Advantage ™ Fertilization (HTF) Medium
- Quinn's Advantage ™ Protein Plus Fertilization (HTF) Medium
- ORIGIO Sequential Fert ™
- Oil
- ORIGIO Liquid Paraffin
- SAGE Oil for Tissue Culture
- Incubators
- K-Systems G210 InviCell*
- K-Systems G185*
- ORIGIO ® / PLANER Benchtop Incubator BT37
- Workstations – Class I
- K-Systems 100 Series (L124 / L124 ICSI / L126 MP)*
- ORIGIO Fortuna
- ORIGIO Titan
- Workstations – Class II
- K-Systems L234 CellTouch*
- K-Systems G603 Work Chamber*
- ORIGIO Mars
- Micropipettes
- ORIGIO Holding and Injection Pipettes
- TPC Holding and Injection Pipettes
- RI Holding and Injection Pipettes
- Wallace ® Holding and Injection Pipettes
- Micromanipulator
- Integra 3 ™ Micromanipulator
- Laser
- Saturn 5 ™ Laser System
- Security & Management
- RI Witness ™

*Not available in the USA

Culture

- Media
- Quinn's Advantage ™ Cleavage Medium
- Quinn's Advantage ™ Blastocyst Medium
- Quinn's Advantage ™ Protein Plus Cleavage Medium
- Quinn's Advantage ™ Protein Plus Blastocyst Medium
- ORIGIO Sequential Cleav ™
- ORIGIO Sequential Blast ™
- EmbryoGen ® & BlastGen ™
- SAGE 1-Step ™
- Oil
- ORIGIO Liquid Paraffin
- SAGE Oil for Tissue Culture
- Handling Devices
- The Stripper ® (Standard / BP / CC / PGD)
- RI EZ-Range (RI EZ-Grip, RI EZ-Strip, RI EZ-Squeeze, RI EZ-Tip)
- IVF Pasteur Pipets
- The Stripper Tips (Individual or Multi Pack)
- Incubators
- K-Systems G210 InviCell*
- K-Systems G185*
- ORIGIO ® / PLANER Benchtop Incubator BT37
- Workstations – Class I
- K-Systems 100 Series (L124 / L124 ICSI/ L126 MP)*
- ORIGIO Fortuna
- ORIGIO Titan
- Workstations – Class II
- K-Systems L234 CellTouch*
- K-Systems G603 Work Chamber*
- ORIGIO Mars
- Security & Management
- RI Witness ™

Cryopreservation

- Carrier
- VitriFit ™ *
- CryoLeaf
- VitriGuard (US only)
- **Vitrification & Warming Kits**
- SAGE Vitrification Kit with DMSO
- SAGE Vitrification Warming Kit
- MediCult Vitrification Cooling Kit DMSO free
- MediCult Vitrification Warming Kit
- Slow Freeze & Warming Kits
- CSC Freezing Medium (Oocytes)
- CSC Thawing Medium (Oocytes)
- ORIGIO Oocyte Thaw
- ORIGIO Embryo Freezing Kit
- ORIGIO Embryo Thawing Kit
- ORIGIO BlastFreeze
- ORIGIO BlastThaw
- SAGE Embryo Freeze Kit
- SAGE Blastocyst Freeze Kit
- SAGE Thaw Kit
- Handling Devices
- The Stripper ® (Standard / BP / CC / PGD)
- RI EZ-Range (RI EZ-Grip ®, RI EZ-Strip ®, RI EZ-Squeeze ™, RI EZ-Tip ®)
- IVF Pasteur Pipets
- The Stripper Tips (Individual or Multi Pack)
- Workstations – Class I
- K-Systems 100 Series (L124 / L124 ICSI/ L126 MP)*
- ORIGIO Fortuna
- ORIGIO Titan
- Workstations – Class II
- K-Systems L234 CellTouch*
- K-Systems G603 Work Chamber*
- ORIGIO Mars
- Micromanipulator
- Integra 3 ™ Micromanipulator
- Laser
- Saturn 5 ™ Laser System
- Security & Management
- RI Witness ™

Embryo Biopsy

- Handling Devices
- The Stripper ® (Standard / BP / CC / PGD)
- RI EZ-Range (RI EZ-Grip ®, RI EZ-Strip ®, RI EZ-Squeeze ™, RI EZ-Tip ®)
- IVF Pasteur Pipets
- The Stripper Tips (Individual or Multi Pack)
- Micropipettes
- ORIGIO Biopsy and Holding Pipettes
- TPC Biopsy and Holding Pipettes
- Media
- ORIGIO SynVitro Hyadase
- Biopsy Medium
- Quinn's Advantage ™ medium with HEPES (without Ca/Mg)
- Acidified Tyrodes Solution
- Oil
- ORIGIO Liquid Paraffin
- SAGE Oil
- Micromanipulator
- Integra 3 ™ Micromanipulator
- Laser
- Saturn 5 ™ Laser System
- Workstations – Class I
- K-Systems 100 Series (L124 / L124 ICSI/ L126 MP)*
- ORIGIO Fortuna
- ORIGIO Titan
- Workstations – Class II
- K-Systems L234 CellTouch*
- K-Systems G603 Work Chamber*
- ORIGIO Mars
- Genetic Testing
- PGD
- PGS
- MitoGrade ™
- Genetic Counseling
- Security & Management
- RI Witness ™

Transfer

- Genetic Testing
- PGD / PGS Testing Results
- NIPT
- Transfer Catheters
- Wallace ® Classic Catheter Range
- Wallace ® Sure View ® Range
- Wallace ® Sure-Pro ® Range
- Wallace ® Sure-Pro Ultra ® Range
- ORIGIO Transem Classic Cathether Range *
- ORIGIO EchoGen ™ Ultrasound Range *
- ORIGIO Spherical Tip Catheter *
- ORIGIO Angled Catheter *
- ORIGIO Soft Catheter *
- ORIGIO Supported Catheter *
- Security & Management
- RI Witness ™

Availability of a device for clinical use is dependent on the regulatory approval status within the country the device is intended to be sold into .

Next Generation ART Solutions

origio TPC RI K wallace

CooperSurgical Companies

CooperGenomics™ a CooperSurgical company

Reprogenetics™ Recombine™ Genesis Genetics™

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