«ICSI Procedure and advanced techniques in medically-assisted procreation»

Second-Cycle Degree Course in "REPRODUCTIVE BIOTECHNOLOGIES"

A.Y. 2021 - 2022

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MAIN TOPICS

- **ASSISTED REPRODUCTIVE TECHNIQUES**

•Basic semen analisys;
•Selection of spermatozoa for *ICSI;*
•The oocyte retrieval (*Pick-Up*);
•*In-vitro* insemination;
•Intracytoplasmatic Sperm Injection (*ICSI*);
•Assisted zona hatching;
•Blastocyst biopsy;
•Pre-implantation genetic diagnosis and embryo screening;
•Vitrification of oocytes and embryos.

1° THEROICAL LESSON

-HUMAN EMBRYO CULTURE: TIPS AND TRICKS

- QUALITY CONTROL INSIDE A.R.T. LABORATORY

2° THEROICAL LESSON

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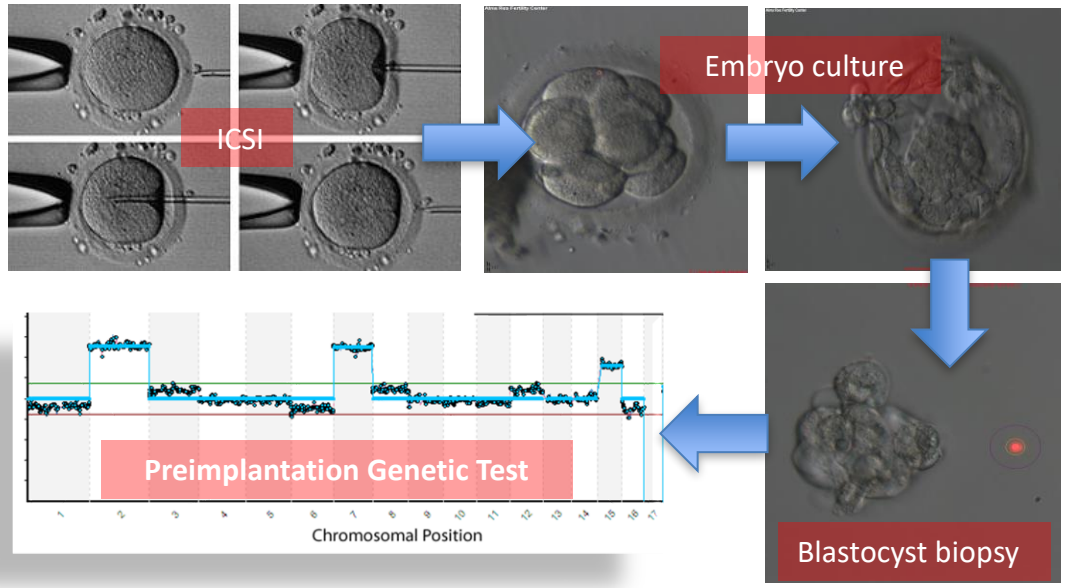
MAIN TOPICS

- “ATLAS OF HUMAN EMBRYOS”

……YOU HAVE TO CHOOSE!!!.....

3° INTERACTIVE LESSON



4° PRATICAL LESSON

Recommended books:

- **Pratical Manual of in Vitro Fertilization**. Advanced Methods and Novel Devices
Editors: **Nagy**, Zsolt Peter, **Varghese**, Alex C., **Agarwal**, Ashok

- **In Vitro Fertilization. A Practical Approach**
Editor: **Gardner** David K.

- **Eshre Atlas of Human Embriology**

http://atlas.eshre.eu/

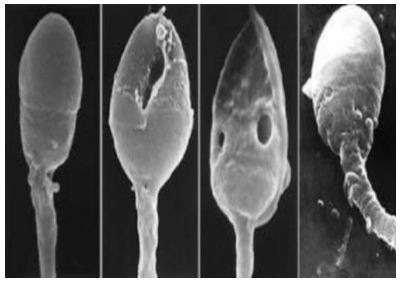
MAIN TOPICS

- **BASIC SEMEN ANALISYS**
- **SELECTION OF SPERMATOZOA FOR ICSI**

THEROICAL LESSON

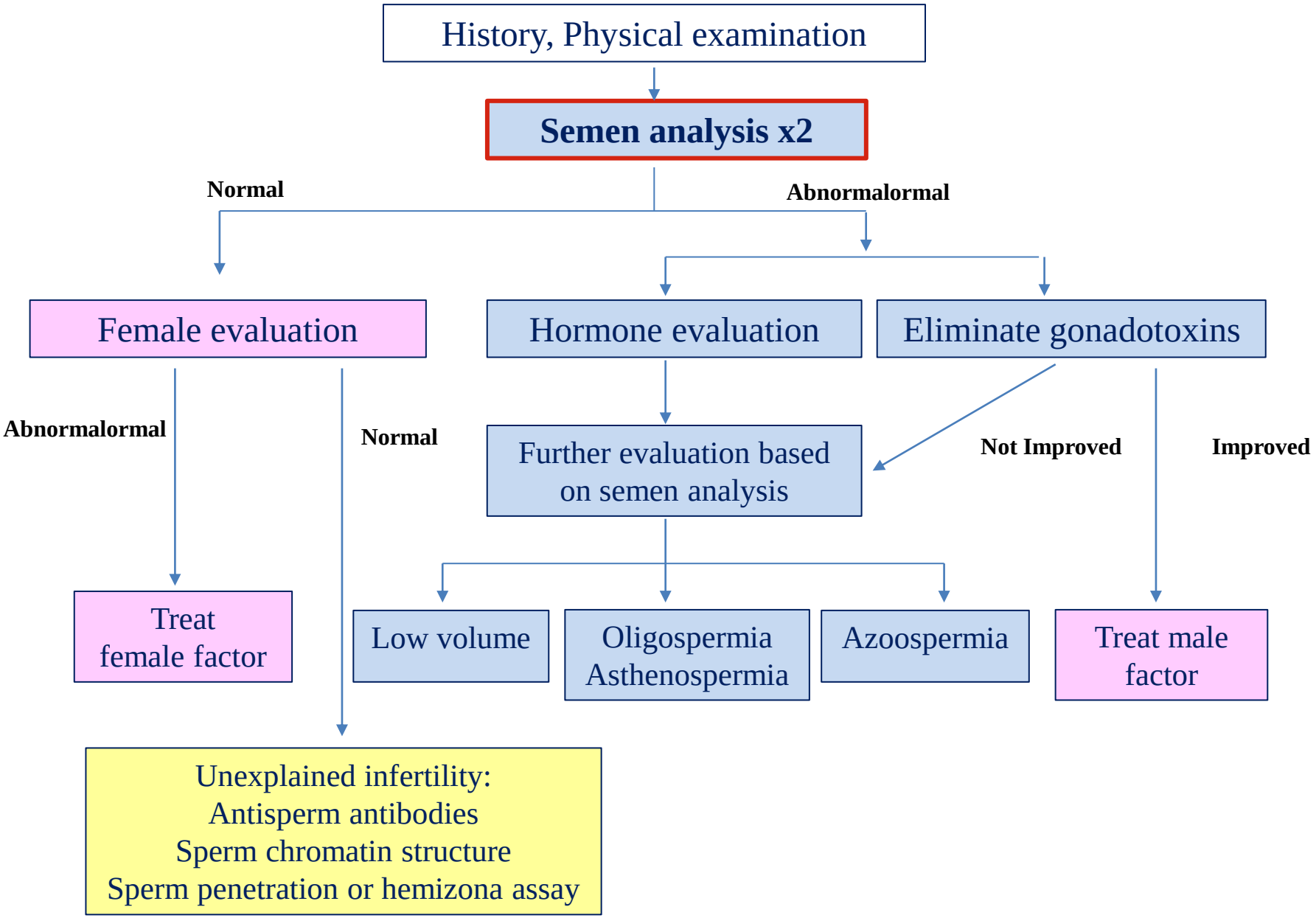
- **“ATLAS OF HUMAN SPERMATOZOA”**

……YOU HAVE TO CHOOSE!!!.....



INTERACTIVE LESSON

INFERTILITY WORK - UP



INFERTILITY WORK - UP

When the diagnosis is completed, the individual or couple treatment must to be based on three principal options:

1. Medical treatment in order to restore the fertility. (hormonal therapy; infections therapy).

2. Surgical treatment in order to restore the fertility. (tubal function restoring; myoma or fibroid removal; varicoceles).

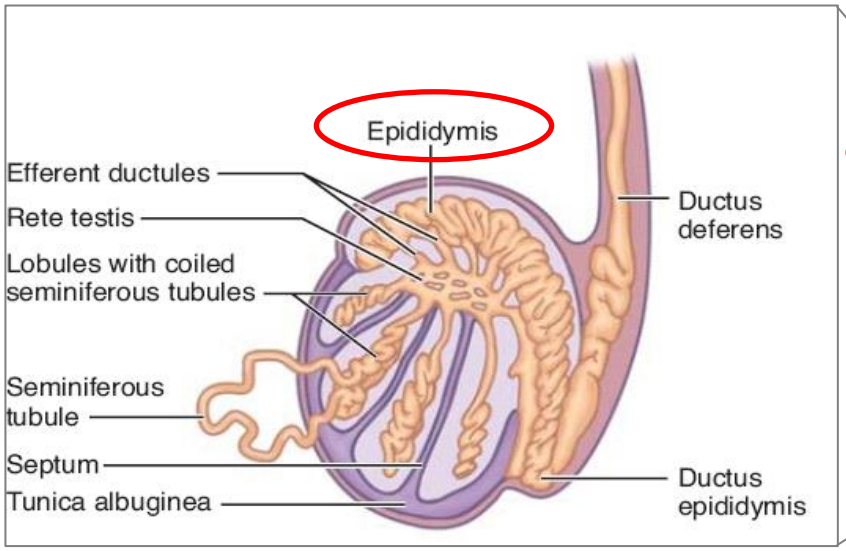
3. Access to the medically-assisted techniques.

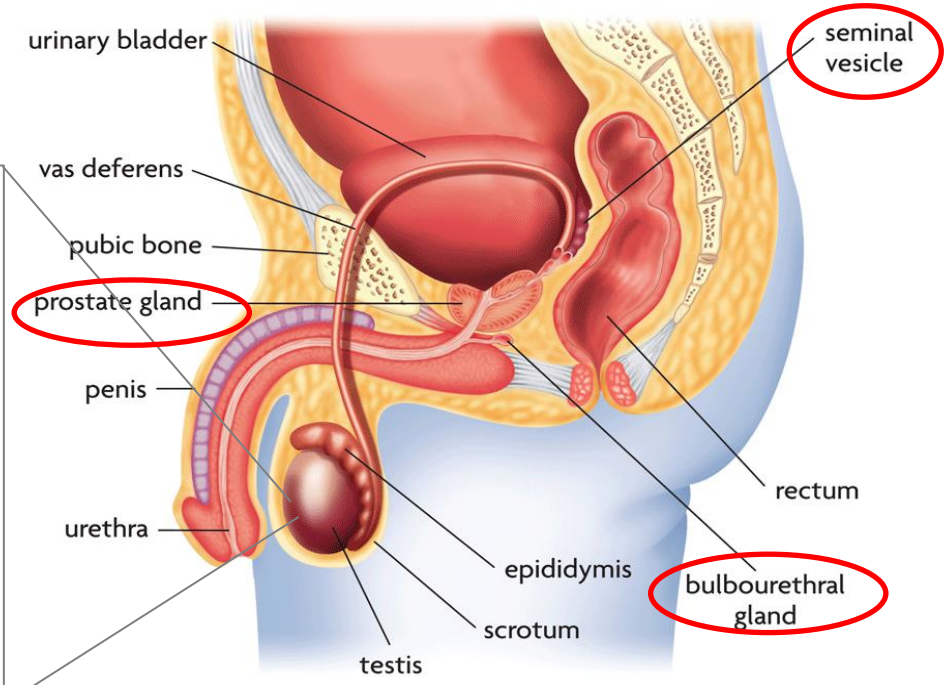
THE MODERN ANDROLOGY

- In 1902, *Edward Martin*, the founder of the modern andrology, proposed that an analysis of a semen sample, should be incorporated into all infertility assessments.

- In 1956, *John MacLeod* advanced the basic semen analysis from beyond a *mere* observation and introduced the importance of certain sperm parameters such as morphology and motility.

MALE REPRODUCTIVE SYSTEM





During ejaculation, semen is produced from a concentrated suspension of spermatozoa, stored in the paired epididymides, mixed with, and diluted by, fluid secretions from the accessory sex organs. About 90% of semen volume is made up of secretions from the accessory organs (Weiske,1994), mainly the prostate and seminal vesicles, with minor contributions from the bulbourethral (Cowper's) glands and epididymides.

SEMEN ATTRIBUTES

- **The total number of spermatozoa**: this reflects sperm production by the testes and the patency of the post-testicular duct system;

- **The total fluid volume** contributed by the various accessory glands: this reflects the secretory activity of the glands.

The nature of the spermatozoa (their vitality, motility and morphology) and the composition of seminal fluid are also important for sperm function.

LABORATORY MEASUREMENTS

The results of laboratory measurements of semen quality will depend on:

1. Whether a complete sample is collected. During ejaculation the first semen fractions voided are mainly sperm-rich prostatic fluids, whereas later fractions are dominated by seminal vesicular fluid .

Björndahl & Kvist, 2003

2. The activity of the accessory sex glands, the fluids of which dilute the concentrated epididymal spermatozoa at ejaculation .

Eliasson, 2003

3. The time since the last sexual activity. In the absence of ejaculation, spermatozoa accumulate in the epididymides, then overflow into the urethra and are flushed out in urine. Sperm vitality and chromatin are unaffected by increased length of abstinence unless epididymal function is disturbed.

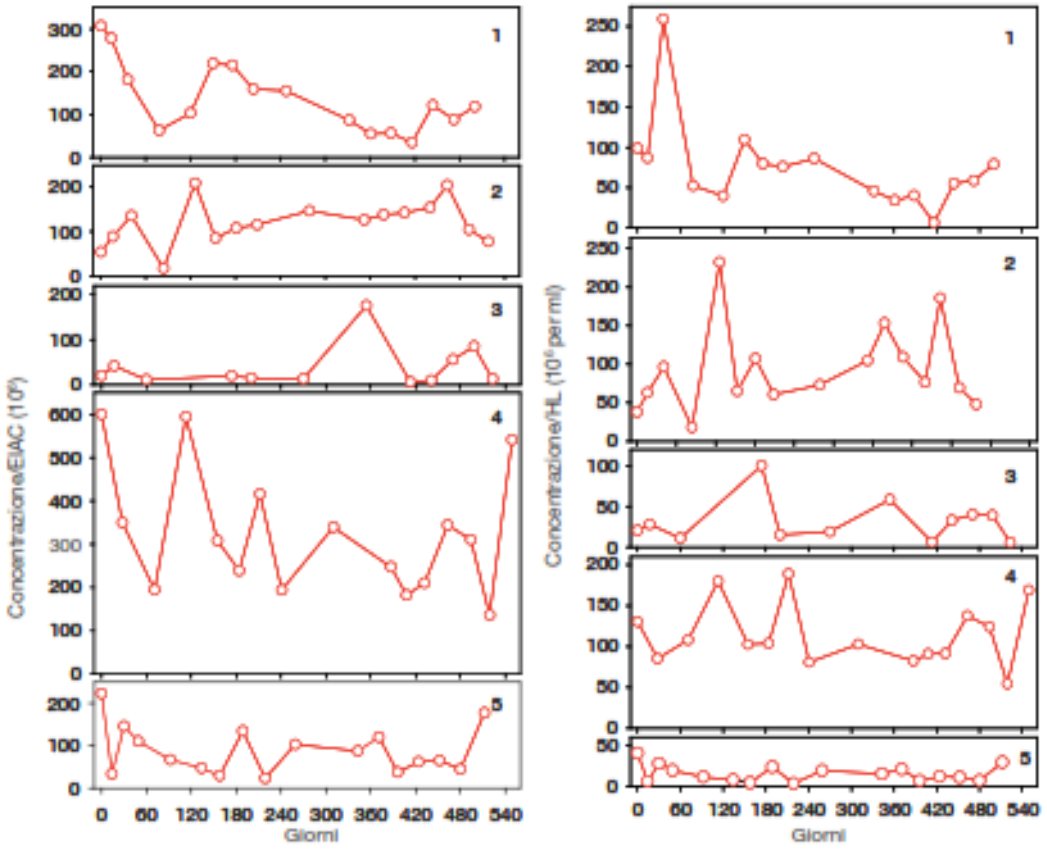
Cooper et al., 1993; De Jonge et al., 2004; Tyler et al.,1982b; De Jonge et al., 2004; Correa- Perez et al., 2004

4. The size of the testis, which influences the total number of spermatozoa per ejaculate.

Handelsman et al., 1984; WHO, 1987; Behre et al., 2000; Andersen et al., 2000

INTRA- INDIVIDUAL VARIATION IN SEMEN COMPOSITION

Fig. 2.1 Variazione nella concentrazione di spermatozoi per eiaculato e per ml in un periodo di oltre un anno e mezzo



Dati gentilmente concessi da Schering Plough e Bayer Schering Pharma AG.

It is impossible to characterize a man's semen quality from evaluation of a single semen sample.

It is helpful to examine two or three samples to obtain baseline data.

Poland et al., 1985; Berman et al., 1996; Carlsen et al., 2004; Castilla et al., 2006; Keel, 2006.

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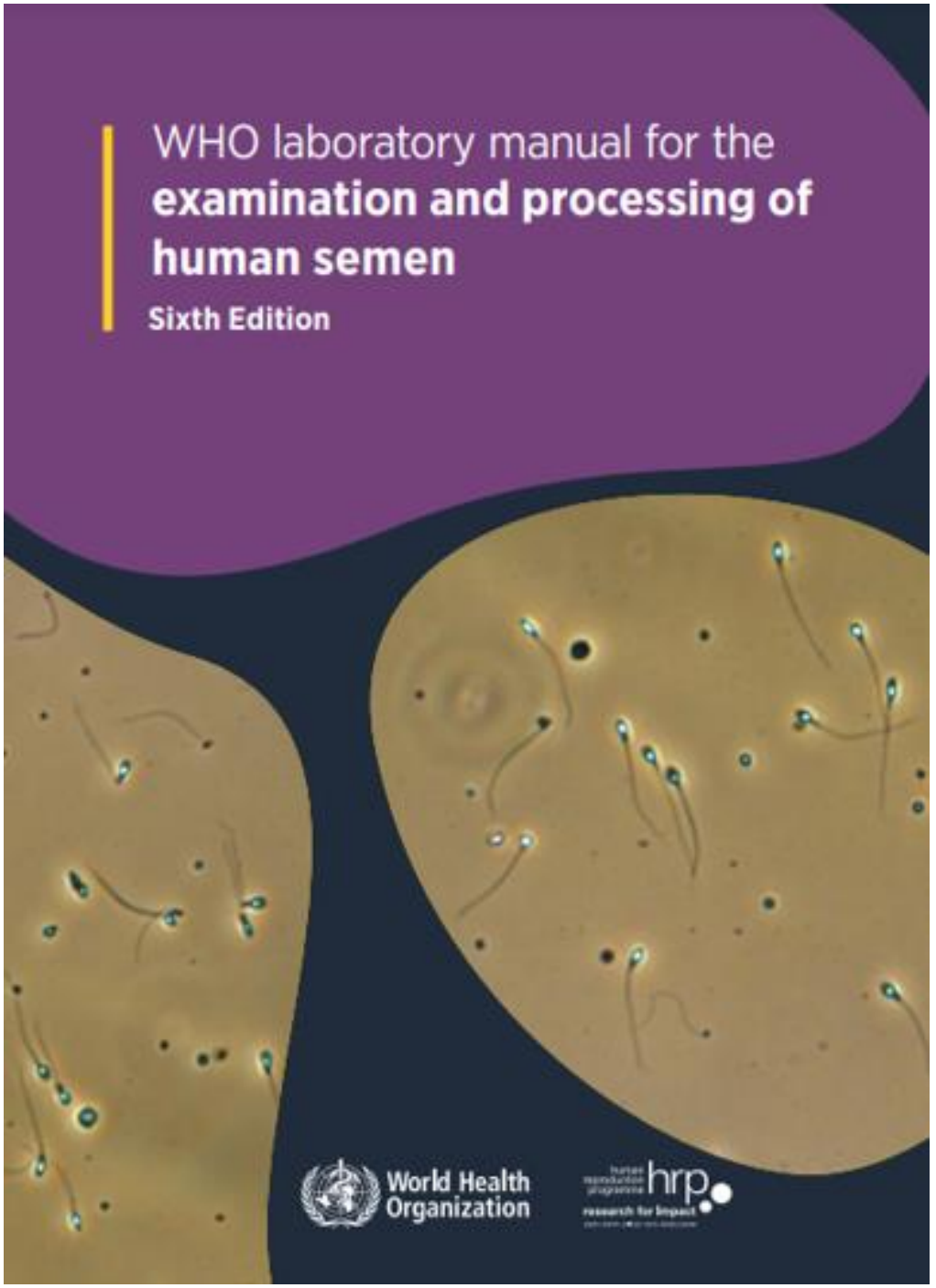
In response to a growing need for the standardization of procedures for the examination of human semen, this manual is offered as a resource for scientists, technicians and managers undertaking semen analysis in clinical and research laboratories.



Pre-examination procedures

1. 

patient information

2. 

sample collection

3. 

sample reception

4. 

initial sample evaluation

SAMPLE COLLECTION

1. The sample should be collected in a private room near the laboratory, in order to limit the exposure of the semen to fluctuations in temperature and to control the time between collection and analysis.

2. The sample should be collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence.

3. The man should be given clear written and spoken instructions concerning the collection of the semen sample. These should emphasize that the semen sample needs to be complete and that the man should report any loss of any fraction of the sample.

4. The sample should be obtained by masturbation and ejaculated into a clean,wide-mouthed container. The specimen container should be kept at ambient temperature, between 20 °C and 37 °C.

SEMEN EXAMINATION

MACROSCOPIC EXAMINATION	✓ Liquefaction ✓ Viscosity ✓ Volume ✓ pH
MICROSCOPIC INVESTIGATION	✓ spermatozoa ▪ concentration ▪ motility ▪ morphology ▪ sperm aggregation or agglutination ✓ epithelial cells ✓ Leukocytes ✓ germ cells ✓ isolated sperm heads or tails

MACROSCOPIC EXAMINATION

Immediately after ejaculation into the collection vessel, semen is typically a semi-solid coagulated mass. Within a few minutes at room temperature, the semen usually begins to liquefy. The complete sample usually liquefies within 15 minutes at room temperature, although rarely it may take up to 60 minutes or more.

Occasionally samples may not liquefy, making semen evaluation difficult.

Semen analysis should begin with a simple inspection soon after liquefaction, preferably at 30 minutes, but no longer than 1 hour after ejaculation, to prevent dehydration or changes in temperature from affecting semen quality.

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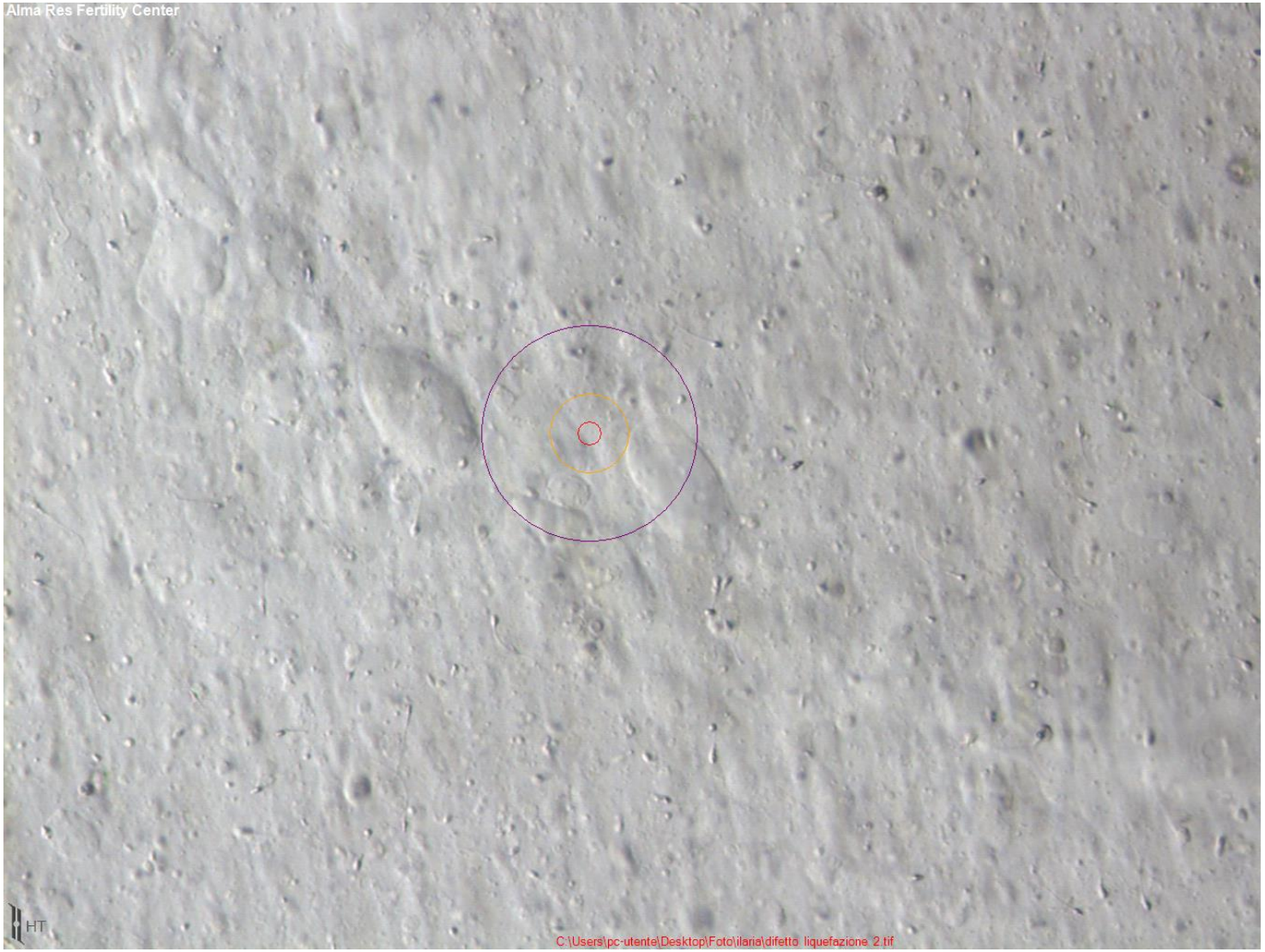

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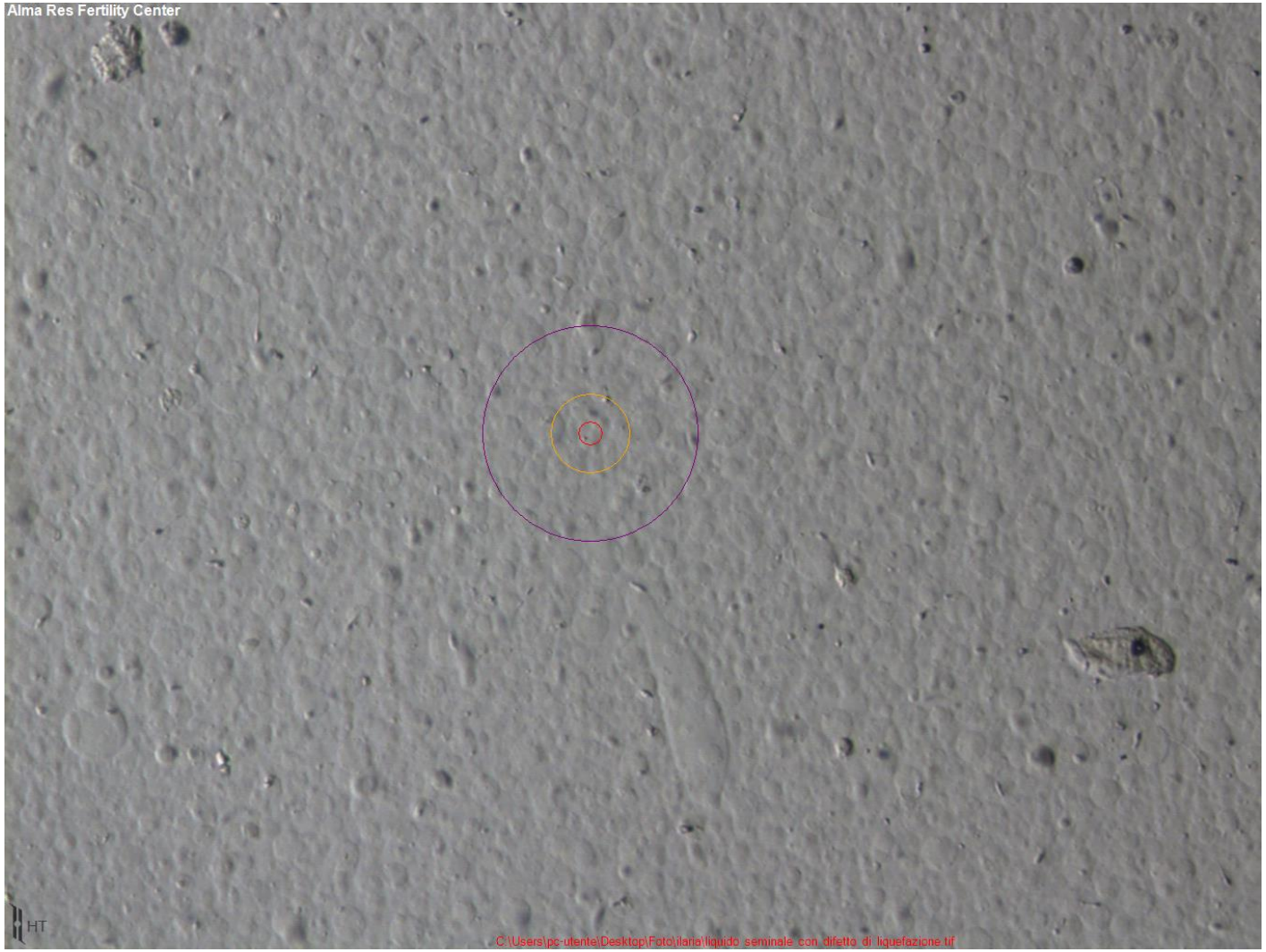

APPEARANCE OF THE EJACULATE

A normal liquefied semen sample has a homogeneous, grey-opalescent appearance.

Less opaque	If the sperm concentration is very low
Red-brown	when red blood cells are present (haemospermia)
Yellow	in a man with jaundice or taking certain vitamins or drugs

SEMEN VISCOSITY

After liquefaction, the viscosity of the sample can be estimated by gently aspirating it into a wide-bore (approximately 1.5 mm diameter) plastic pipette, allowing the semen to drop by gravity and observing the length of any thread. A normal sample leaves the pipette in small discrete drops. If viscosity is abnormal, the drop will form a thread more than 2 cm long.

High viscosity can interfere with determination of sperm motility and sperm concentration.

SEMEN VOLUME

The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymides. Precise measurement of volume is essential, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated.

reference limit for semen volume ≤ 1.4 ml

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VOLUME

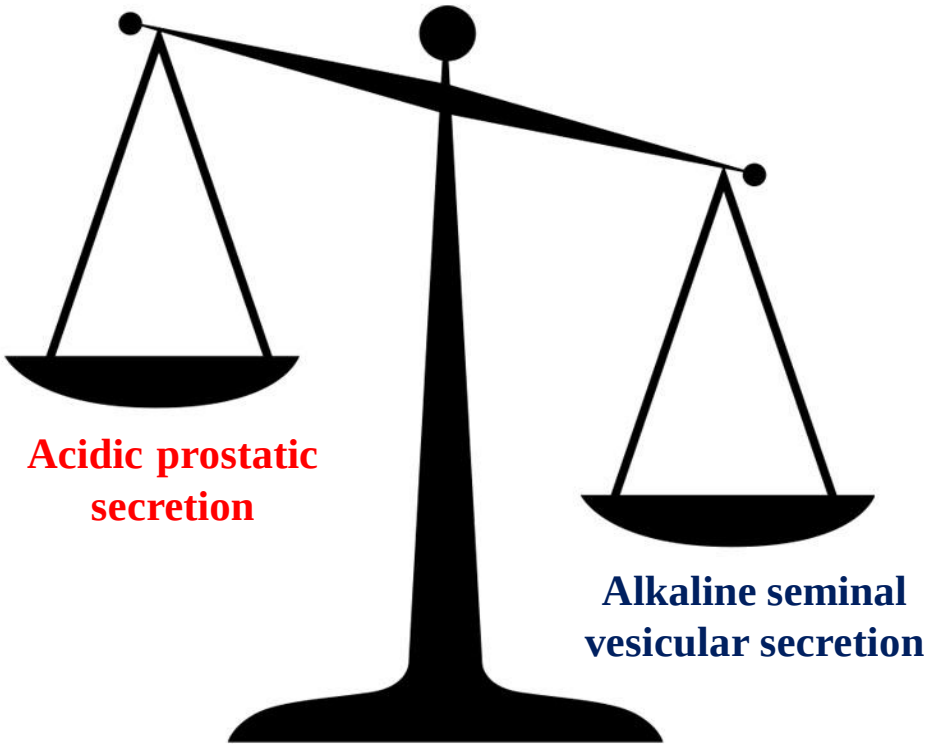
Low semen volume is characteristic of obstruction of the ejaculatory duct or congenital bilateral absence of the vas deferens (CBAVD)

de la Taille et al.; 1998; Daudin et al., 2000; von Eckardstein et al., 2000; Weiske et al., 2000

Low semen volume can also be the result of collection problems (loss of a fraction of the ejaculate), partial retrograde ejaculation or androgen deficiency.

High semen volume (> di 6 ml) may reflect active exudation in cases of active inflammation of the accessory organs.

SEMEN pH



Reference limit for semen pH are 7.2 – 8.0

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The pH should be measured after liquefaction, at a uniform time, preferably after 30 minutes, because it is influenced by the loss of CO_2 that occurs after production. So, semen pH increases with time.

SEMEN pH

A pH value under 7.2 may be indicative of a lack of alkaline seminal vesicular fluid. It can also be due to urine contamination

If the pH is less than 7.0 in a semen sample with low volume and low sperm numbers, there may be ejaculatory duct obstruction or congenital bilateral absence of the vas deferens

de la Taille et al.; 1998; Daudin et al., 2000; von Eckardstein et al., 2000; Weiske et al., 2000

SEMEN EXAMINATION

MACROSCOPIC EXAMINATION	✓ Liquefaction ✓ Viscosity ✓ Volume ✓ pH
MICROSCOPIC INVESTIGATION	✓ spermatozoa ▪ concentration ▪ Motility ▪ morphology ▪ sperm aggregation or agglutination ✓ epithelial cells ✓ Leukocytes ✓ germ cells ✓ isolated sperm heads or tails

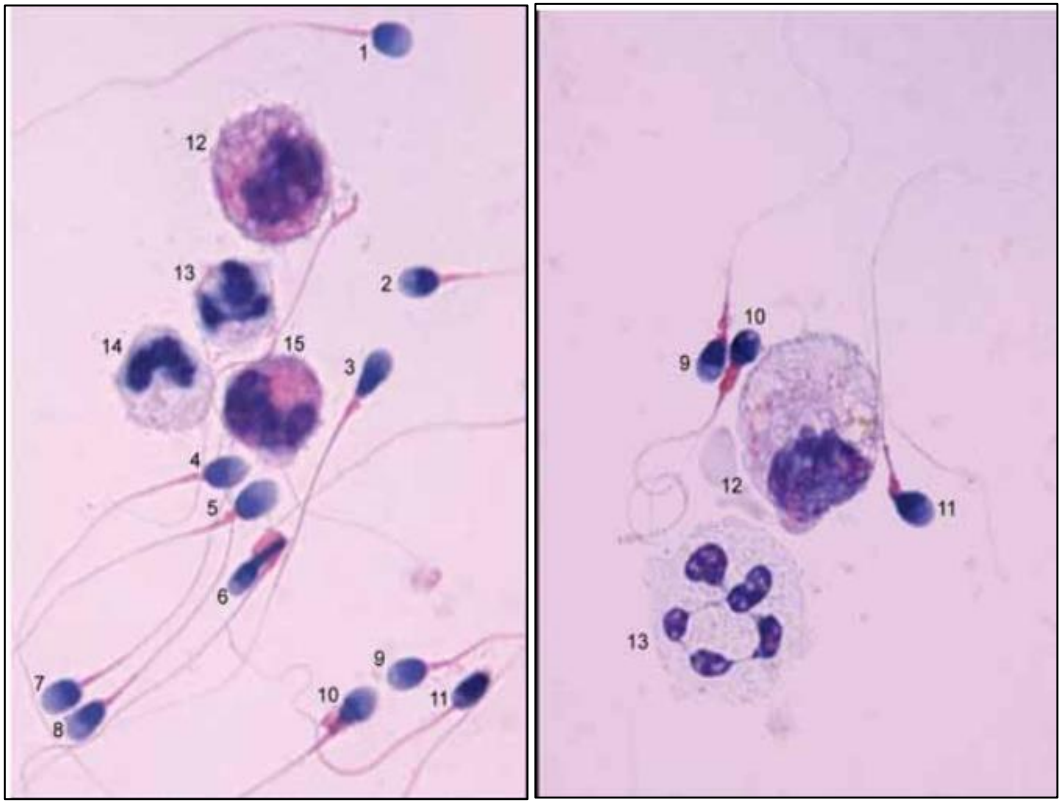
MICROSCOPIC INVESTIGATION

Microscopic investigation provides an overview of the sample, to reveal:

- mucus strand formation;
- sperm aggregation or agglutination;
- the presence of cells other than spermatozoa, e.g. epithelial cells, “round cells” (leukocytes and immature germ cells) and isolated sperm heads or tails;
- assessment of sperm number;
- assessment of sperm motility;
- assessment of sperm morphology.

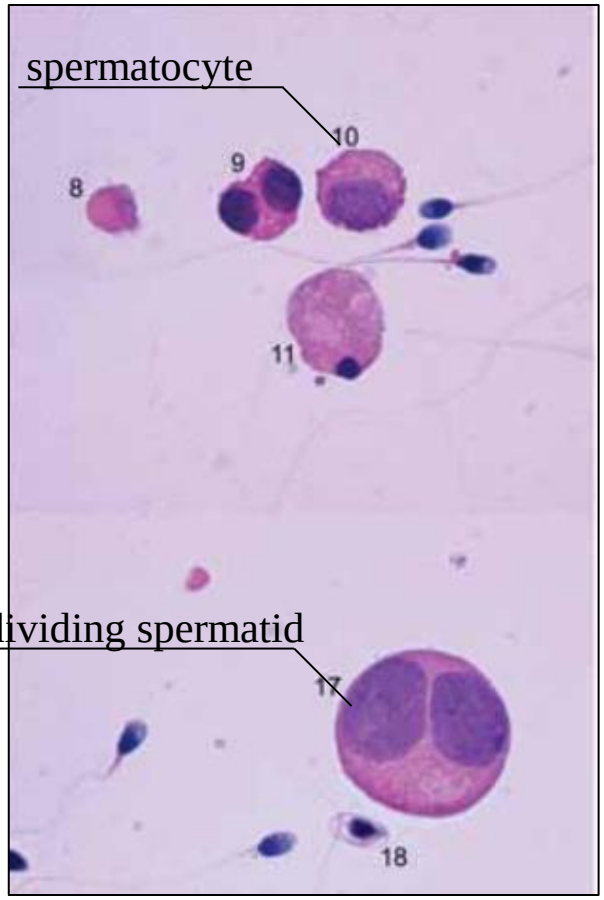
NON- SPERM CELLULAR ELEMENTS

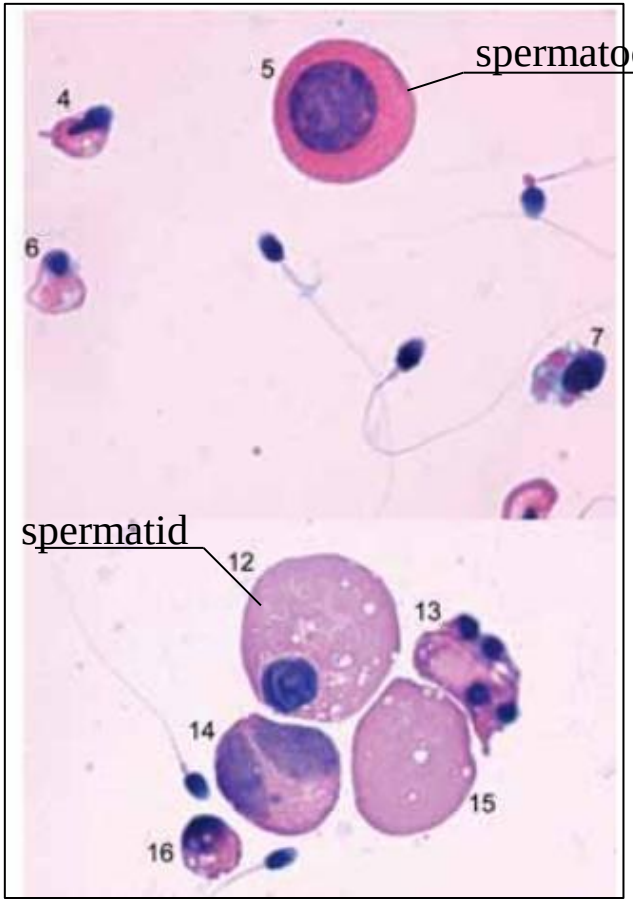
Leukocytes are the most commonly observed cells in a semen sample besides spermatozoa.

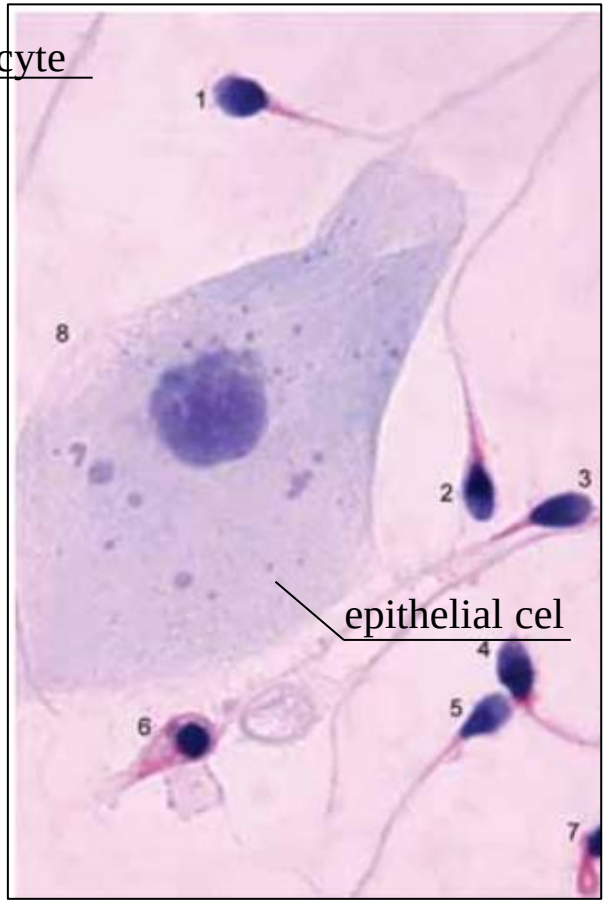


The presence of leukocytes that exceed the **WHO refernce value of more than 10^6/ mL** is indicative of a genital tract infection.

NON- SPERM CELLULAR ELEMENTS







SPERM NUMBERS

Sperm concentration refers to the *number of spermatozoa per unit volume* of semen and is a function of the number of spermatozoa emitted and the volume of fluid diluting them.

The lower reference limit ≤16Milioni/mL $_{\text{spermatozoa per ml}}$

Total sperm number refers *to the total number of spermatozoa in the entire ejaculate* and is obtained by multiplying the sperm con- centration by the semen volume.

The lower reference limit is 39×10^6 $_{\text{spermatozoa per ejaculate}}$

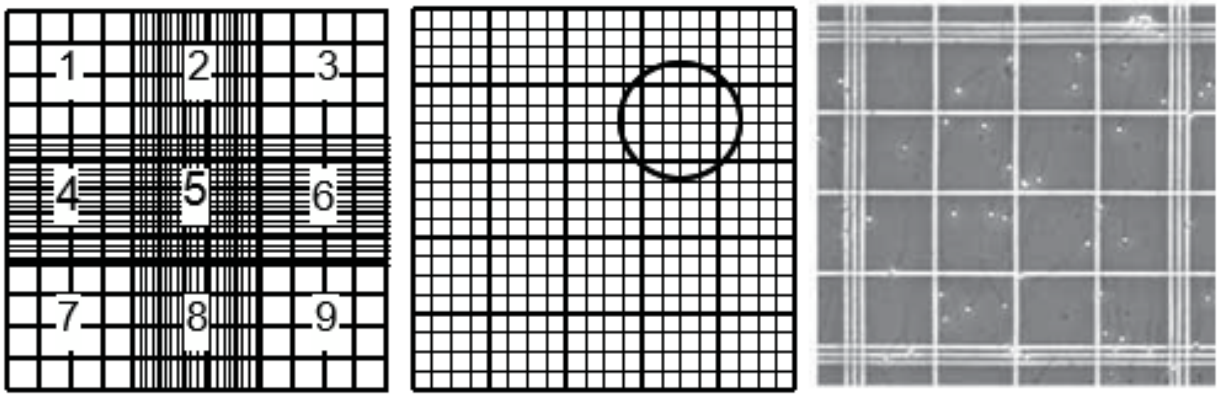
The total number of spermatozoa per ejaculate and the sperm concentration are related to both time to pregnancy and pregnancy rate and are predictors of conception.

Slama et al., 2002; WHO,1996; Zinaman et al., 2000; Bonde et al., 1998; Larsen et al., 2000.

NEUBAUER HAEMOCYTOMETER CHAMBERS

The use of 100-µm-deep improved Neubauer haemocytometer chambers is recommended to evaluate the sperm number.

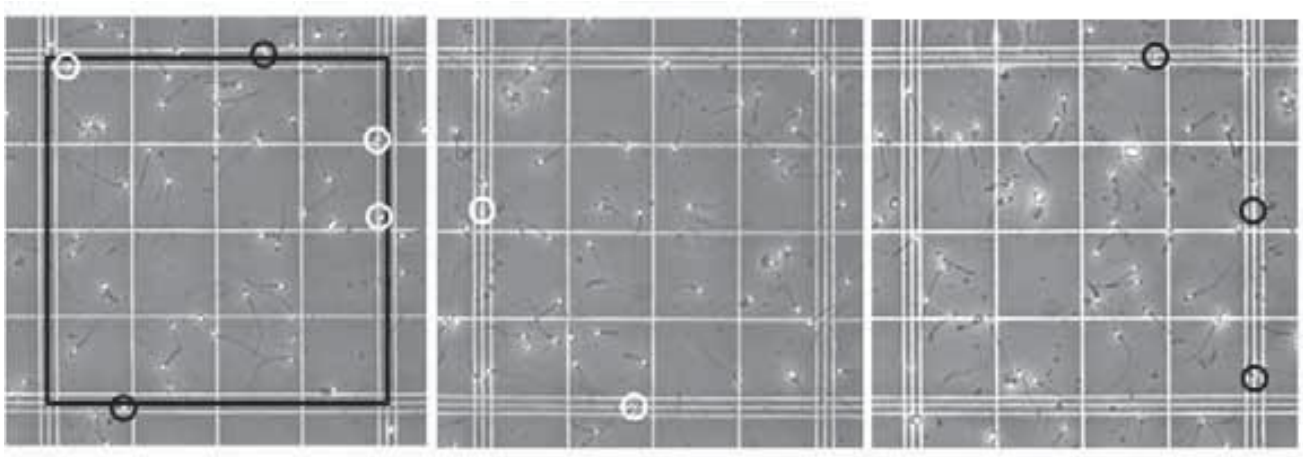
The chamber has two separate counting chambers, each of which has a microscopic 3 mm × 3 mm pattern of gridlines etched on the glass surface. It is used with a special thick coverslip (thickness number 4, 0.44 mm), which lies over the grids and is supported by glass pillars 0.1 mm above the chamber floor.



Micrograph courtesy of C Brazil.

Depending on the dilution and the number of spermatozoa counted, different areas of the chamber are used for determining sperm concentration.

NEUBAUER HAEMOCYTOMETER CHAMBERS



The middle of the three lines defines the square's boundary (black line, *left panel*). All spermatozoa within the central square are counted, as well as those with their heads between the two inner lines (white circles), but not those whose heads lie between the outer two lines (black circles). A spermatozoon with most of its head lying on the central line is counted only if that line is the lower or left-hand line of the square (white circles, *middle panel*) but not if it is the upper or right hand line of the square (black circles, *right panel*).

COMPUTER ASSISTED SEMEN ANALYSIS

Computer Assisted Semen Analysis (CASA) are automatic or semi-automatic semen analysis techniques based on image analysis.

The use of CASA techniques allows the accurate, repetitive and automatic assessment of sperm motility, concentration and morphology.

However the manual analysis of semen parameters is still an extremely effective indicator of the quality and composition of semen.

SPERM MOTILITY

The motility of each spermatozoon is graded as follows:

- **Progressive motility (PR):** spermatozoa moving actively, either linearly or in a large circle, regardless of speed.
- **Non-progressive motility (NP):** all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed.
- **Immotility (IM):** no movement.

SPERM MOTILITY

Reference value:

1. Total motility (PR+ NP, %)≥42 %
2. Progressive motility ≥30%

The extent of progressive sperm motility is related to pregnancy rate.

Jouannet et al., 1988; Larsen et al., 2000; Zinaman et al., 2000

Sperm vitality

Sperm vitality, as estimated by assessing the membrane integrity of the cells, is not necessary when at least 40% of spermatozoa are motile. But in samples with poor motility**, the vitality test is important to discriminate between immotile dead sperm and immotile live sperm**.

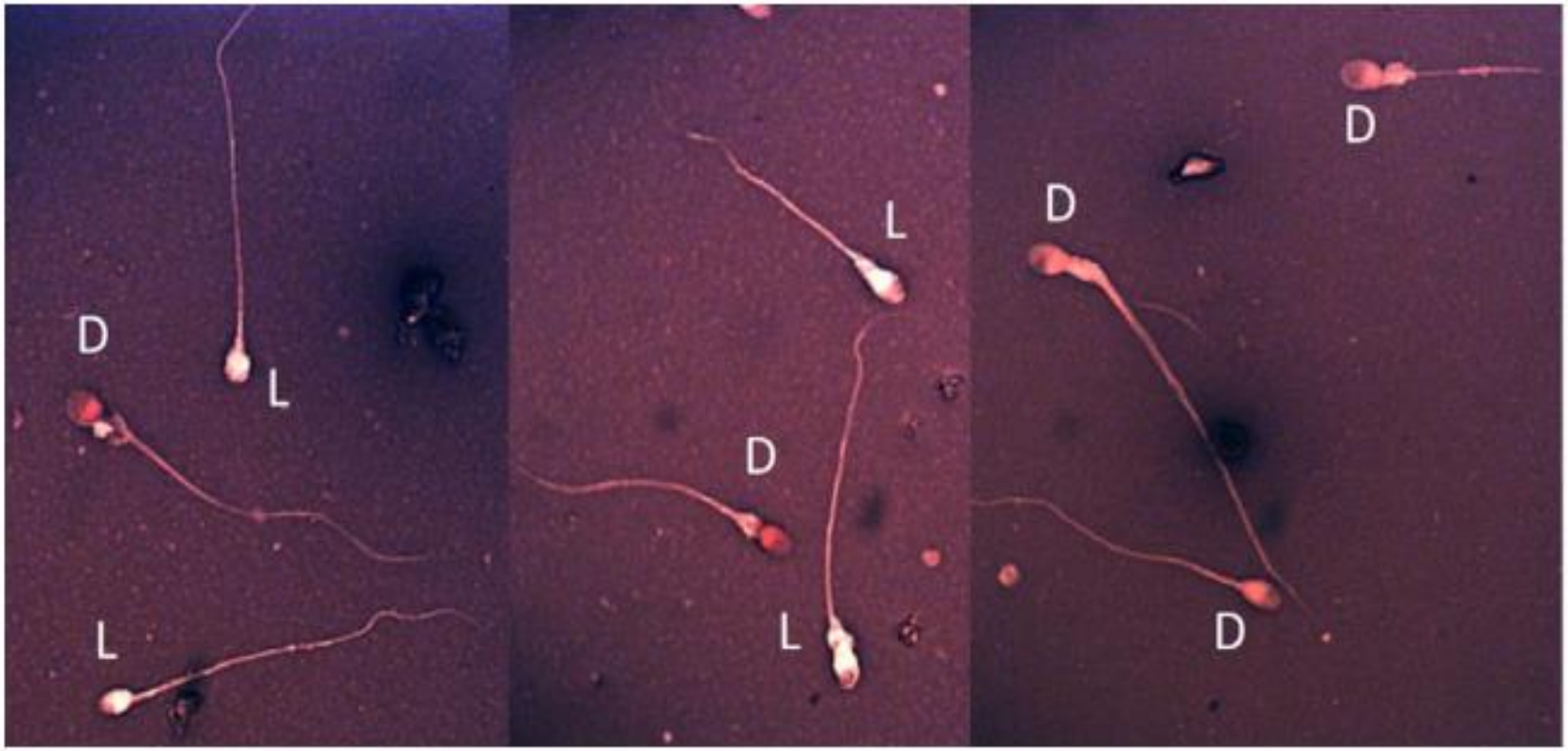
The presence of a large proportion of live but immotile cells may be indicative of structural defects in the flagellum; a high percentage of immotile and dead cells may indicate epididymal pathology or an immunological reaction due to an infection.

The percentage of live spermatozoa is assessed by identifying those with an intact cell membrane, by **dye exclusion** (dead cells have damaged plasma membranes that allow entry of membrane-impermeant stains) or by **hypotonic swelling.**

Sperm vitality should be assessed as soon as possible after liquefaction of the semen sample, preferably at 30 minutes, but in any case, within 1 hour of ejaculation, to limit deleterious effects of dehydration or changes in temperature on vitality

The eosin–nigrosin test.

The recommended test for diagnostic use of vitality is the eosin–nigrosin test. Spermatozoa with red or dark pink heads are considered dead (D), whereas spermatozoa with white heads (L) are considered alive

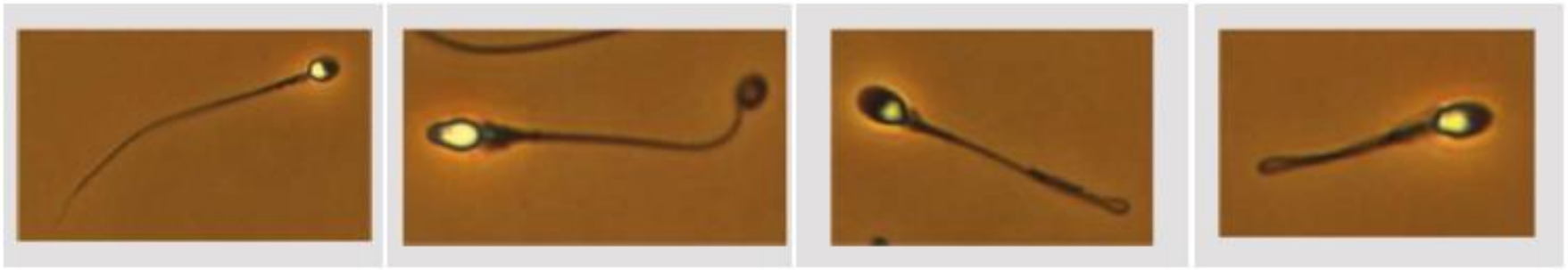


Micrograph courtesy of L. Björndahl.

Hypo-osmotic swelling test

An alternative to dye exclusion, the hypo-osmotic swelling test may be used to assess vitality. *This is useful when staining of spermatozoa must be avoided*, e.g. when choosing spermatozoa for intracytoplasmic sperm injection (ICSI). The hypo-osmotic swelling test presumes that only cells with intact membranes (live cells) can swell in hypotonic solutions.

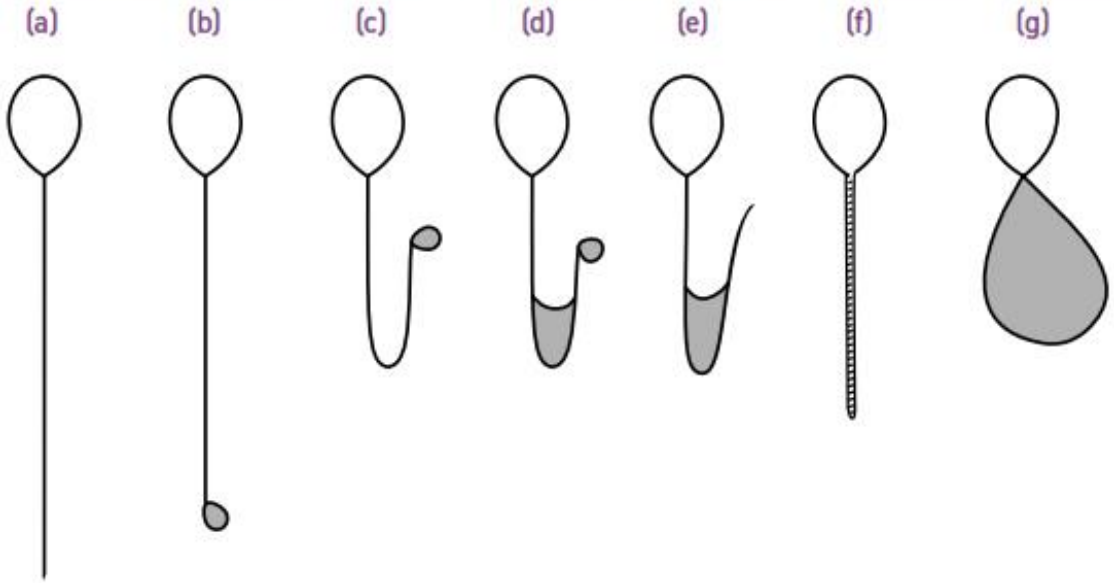
Fig. 2.18 Photo micrographs under phase contrast microscope of spermatozoa subjected to hypo-osmotic stress



Courtesy of E. Holmes *(147)*.

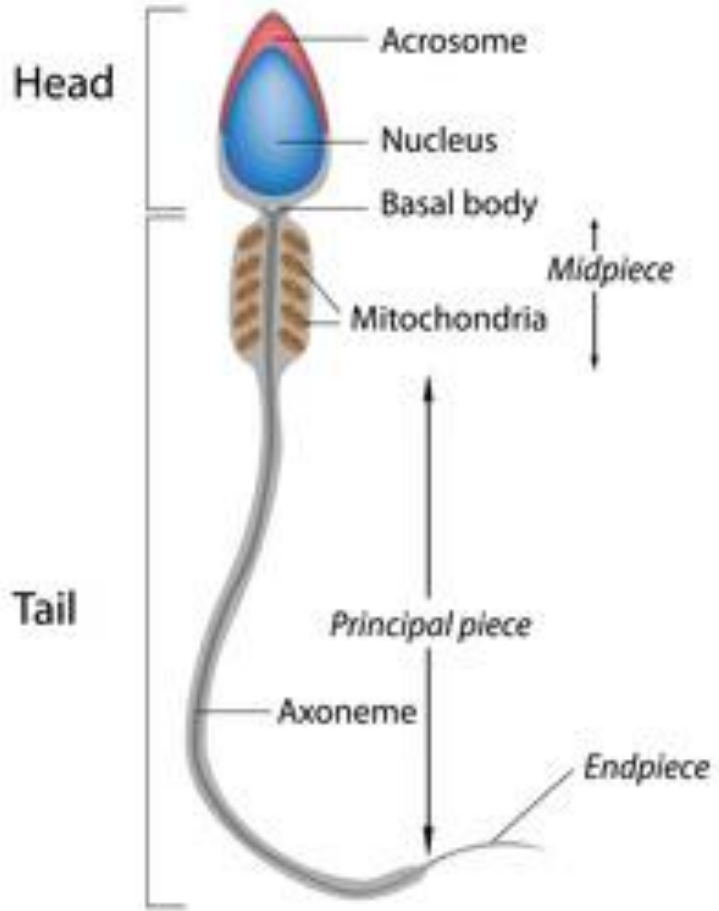
Hypo-osmotic swelling test

Fig. 2.17 Schematic representation of typical morphological changes in human spermatozoa subjected to hypo-osmotic stress



(a) = no change; (b)–(g) = various types of tail changes. Swelling in tail is indicated by the hatched area.

SPERM MORFOLOGY

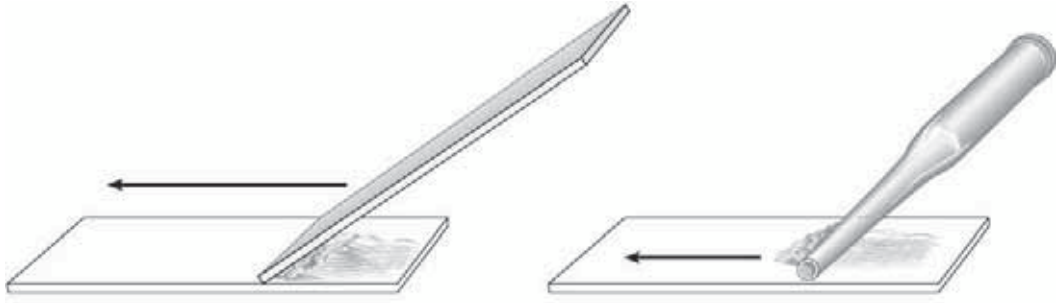


- The head should be smooth, and oval in shape. There should be a well-defined acrosomal region comprising 40-70% of the head area (Menkveld et al., 2001).
- The acrosomal region should contain no large vacuoles, and not more than two small vacuoles, which should not occupy more than 20% of the sperm head.
- The post-acrosomal region should not contain any vacuoles.
- The midpiece should be slender, regular and about the same length as the sperm head. The major axis of the midpiece should be aligned with the major axis of the sperm head.
- The principal piece should have a uniform calibre along its length, be thinner than the midpiece, and be approximately 45 µm long

DETERMINATION OF SPERM MORPHOLOGY

Determination of sperm morphology comprises the following steps:

- Preparing a smear of semen on a slide
- Air-drying, fixing and staining the slide.



- Examining the slide with brightfield optics at ×1000 magnification with oil immersion.
- Assessing approximately 200 spermatozoa per replicate for the percentage of normal forms or of normal and abnormal forms

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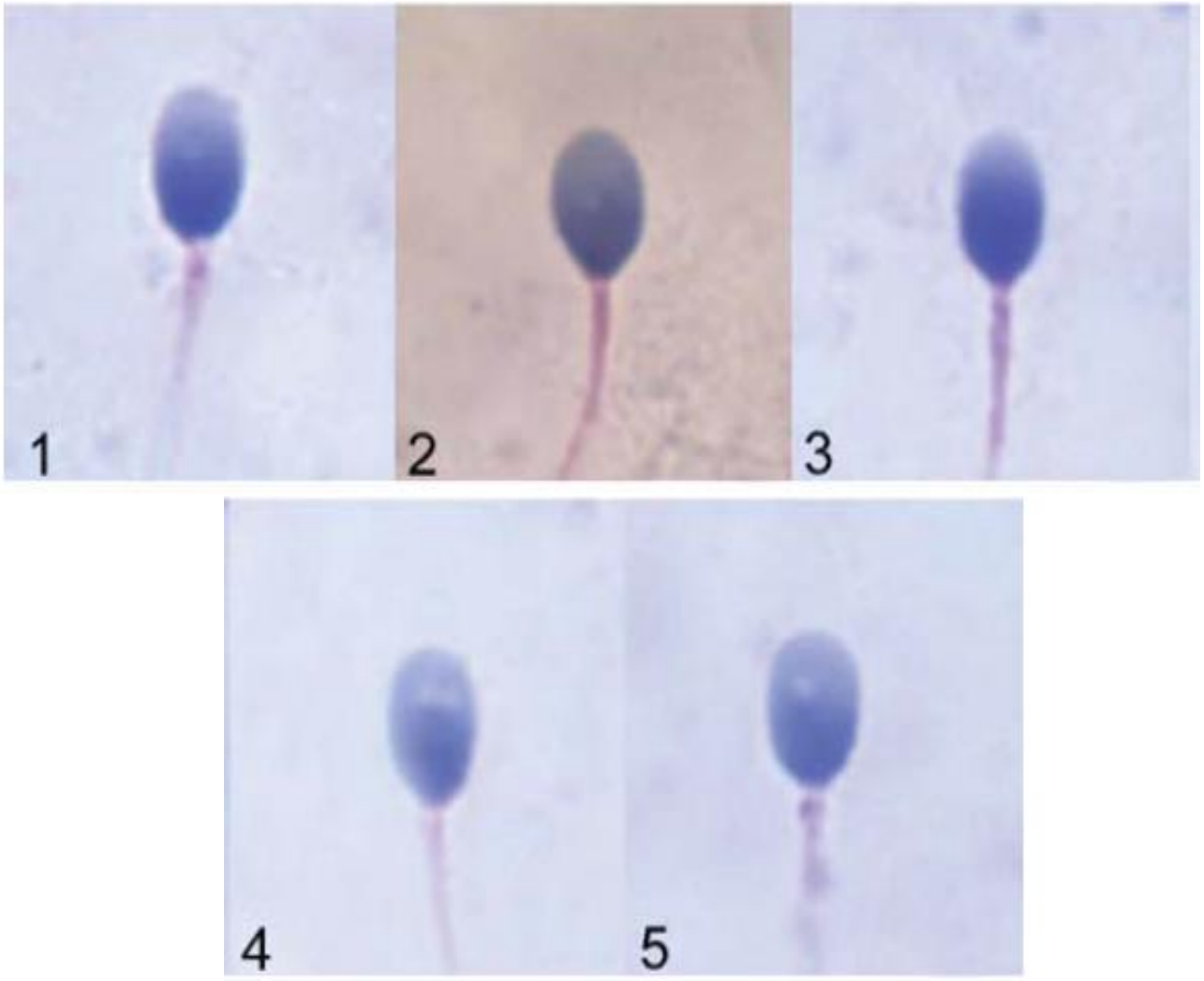
SPERM MORFOLOGY



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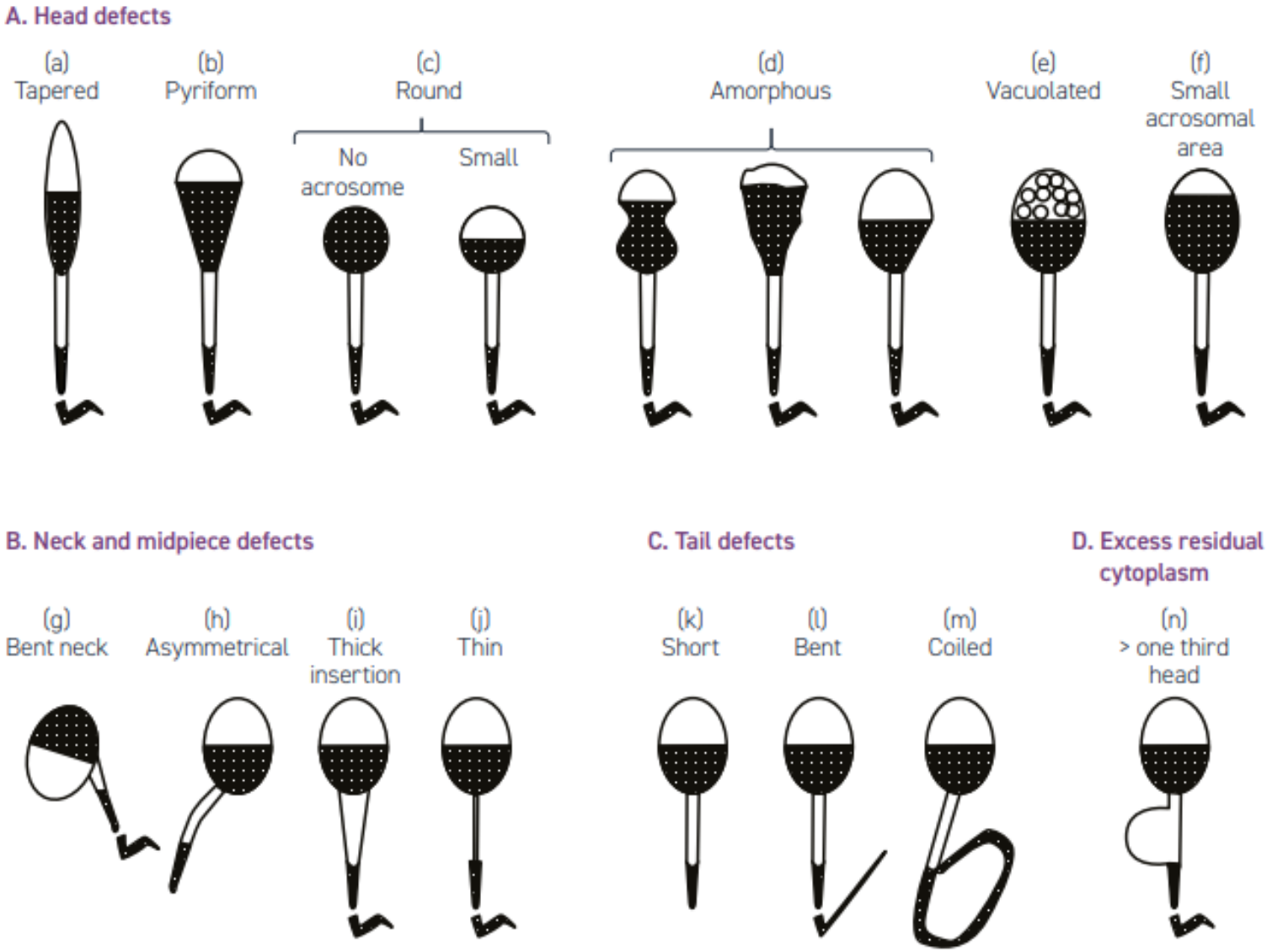
SPERM MORFOLOGY



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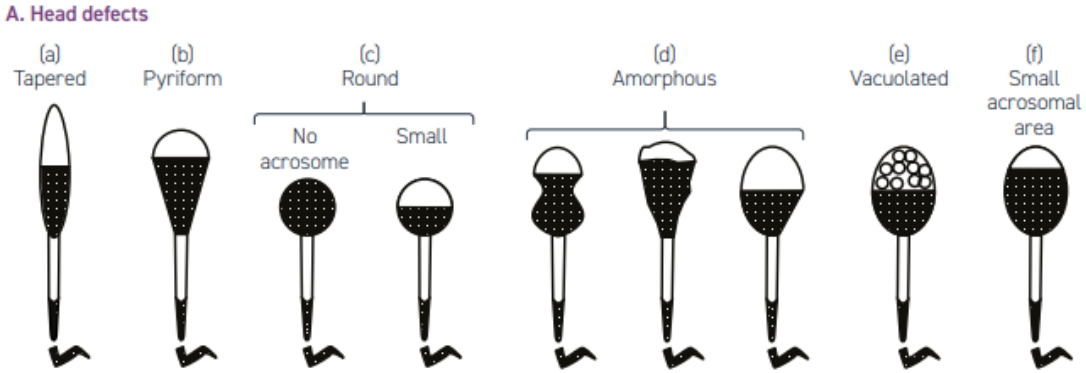
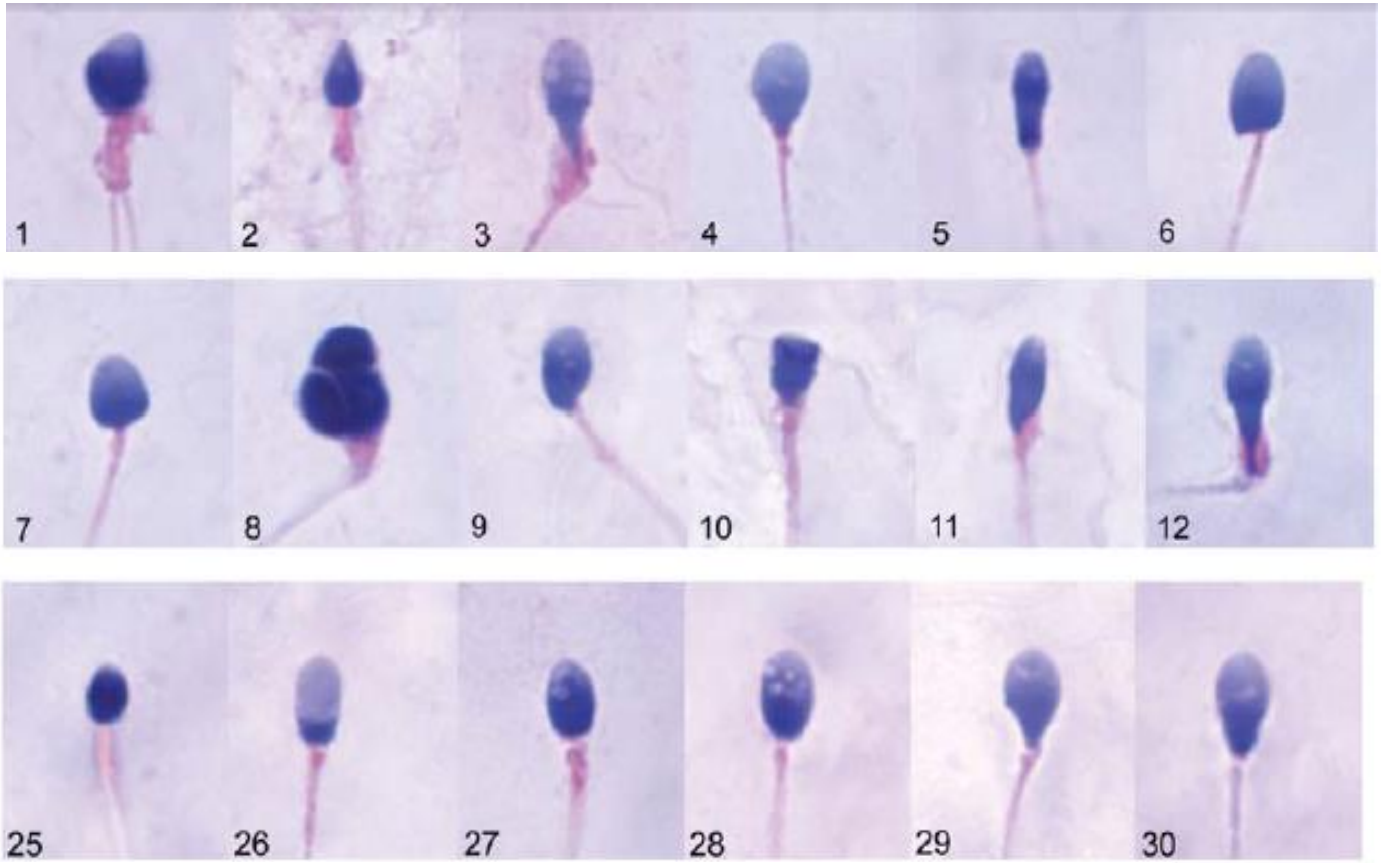
HEAD DEFECTS





Microfotografie gentilmente concesse da C Brazil.

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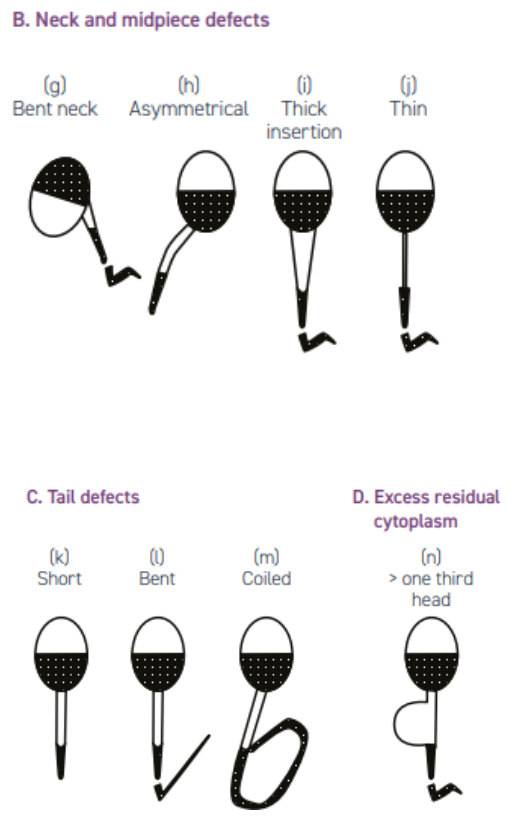
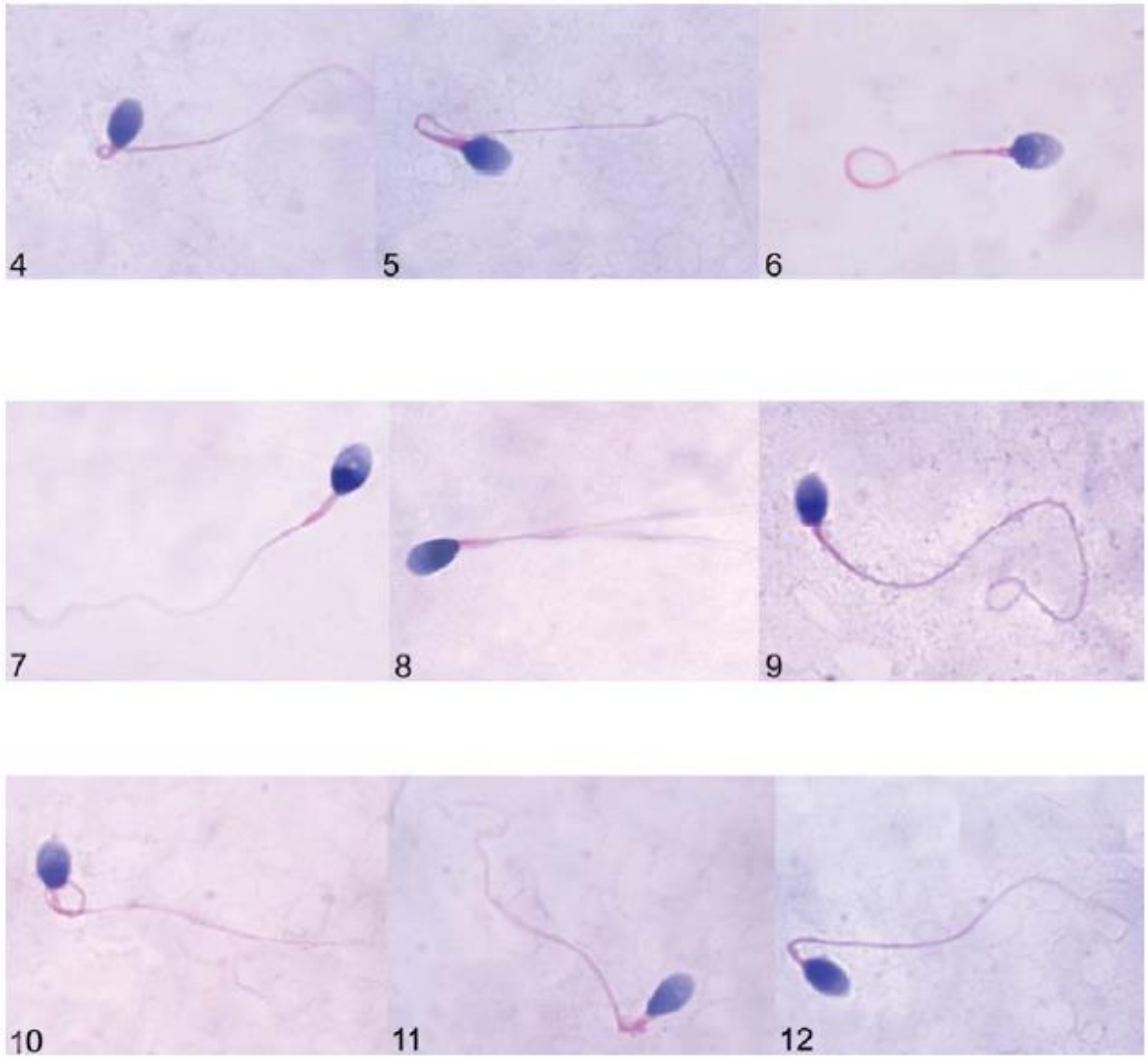
NECK AND TAIL DEFECTS





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SPERM MORFOLOGY

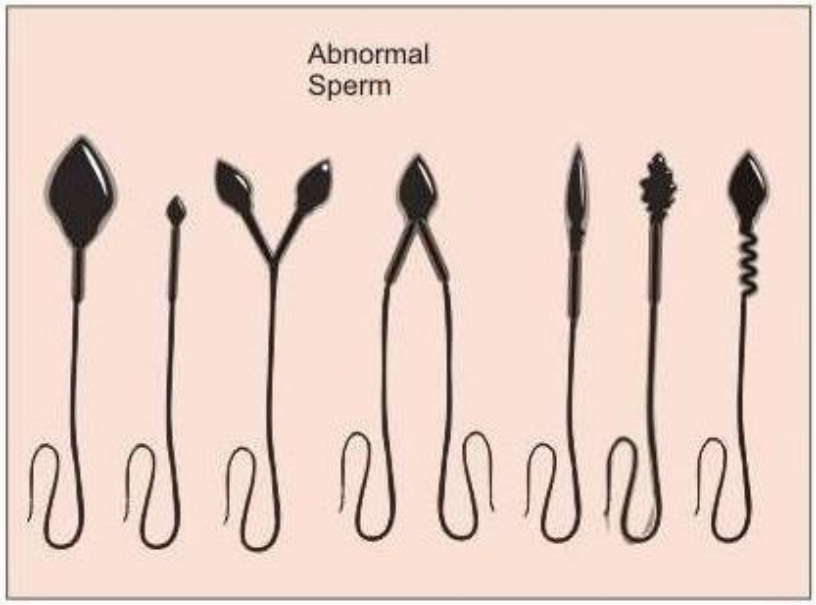
Reference limits: Percentage of normal forms ≥ 4%

Relationships between the percentage of normal forms and various fertility endpoints (time-to-pregnancy (TTP), pregnancy rate in vivo and in vitro) have been established and may be useful for the prognosis of fertility.

Eggert-Kruse et al., 1996; Jouannet et al., 1988; Toner et al., 1995; Coetzee et al., 1998; Menkveld et al., 2001; Van Waart et al., 2001; Garrett et al., 2003; Liu et al., 2003

Manuale WHO 2021

SPERM MORFOLOGY



Abnormal spermatozoa generally have a lower fertilizing potential, depending on the types of anomalies, and may also have abnormal DNA.

Morphological defects have been associated with:

- increased DNA fragmentation (Gandini et al., 2000);
- increased incidence of structural chromosomal aberrations (Lee et al., 1996);
- immature chromatin (Dadoune et al., 1988);
- aneuploidy (Devillard et al., 2002; Martin et al., 2003).

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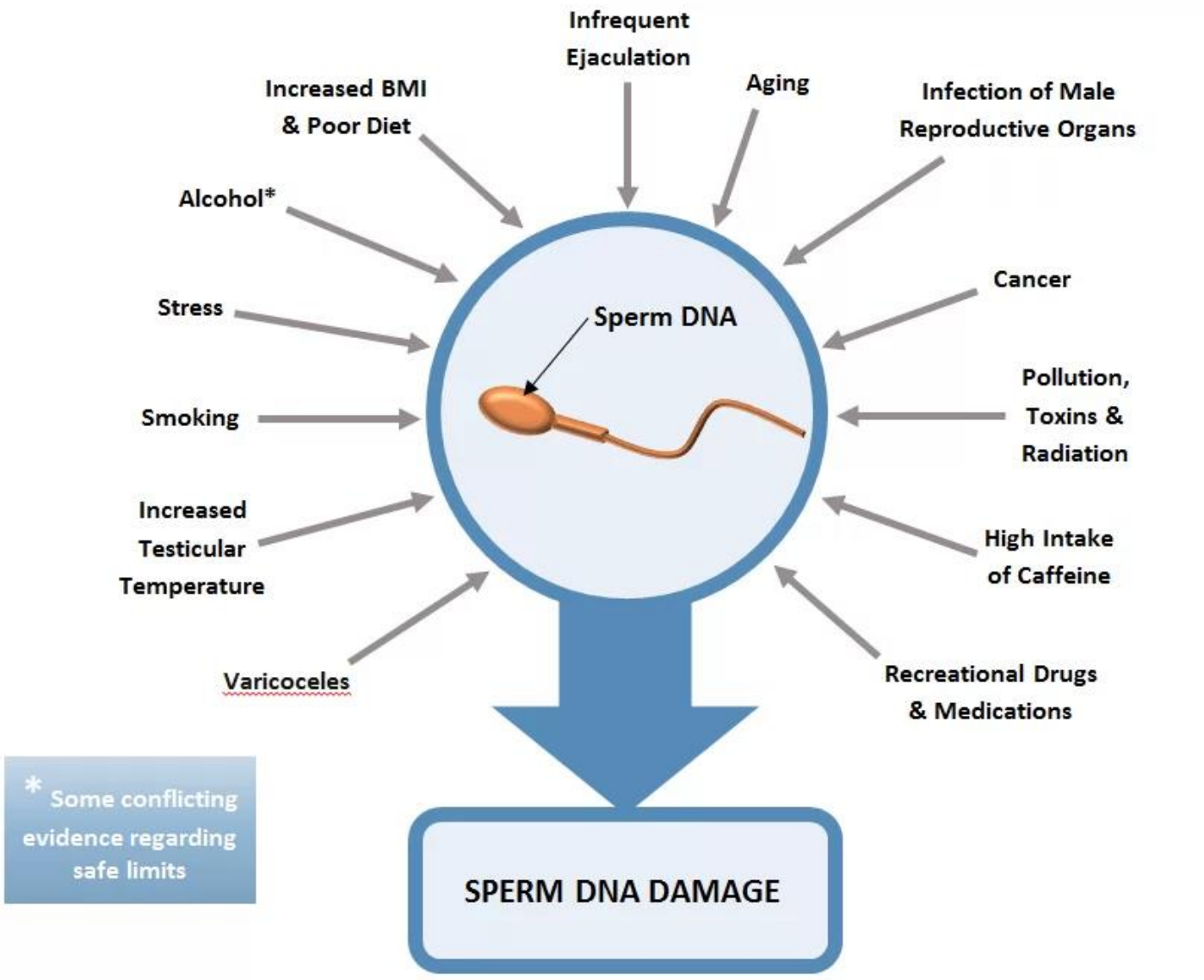
Sperm DNA fragmentation

Sperm DNA damage can be defined as any chemical change in the normal structure of the DNA. Among these changes, sperm DNA fragmentation (sDF) is one of the most common disturbances affecting the genetic material in the form of single or double strand breaks.
sDF may be triggered by different processes, including the defective packaging of the DNA during spermatogenesis, and processes of cell death and oxidative stress which may be associated with several pathological and environmental conditions

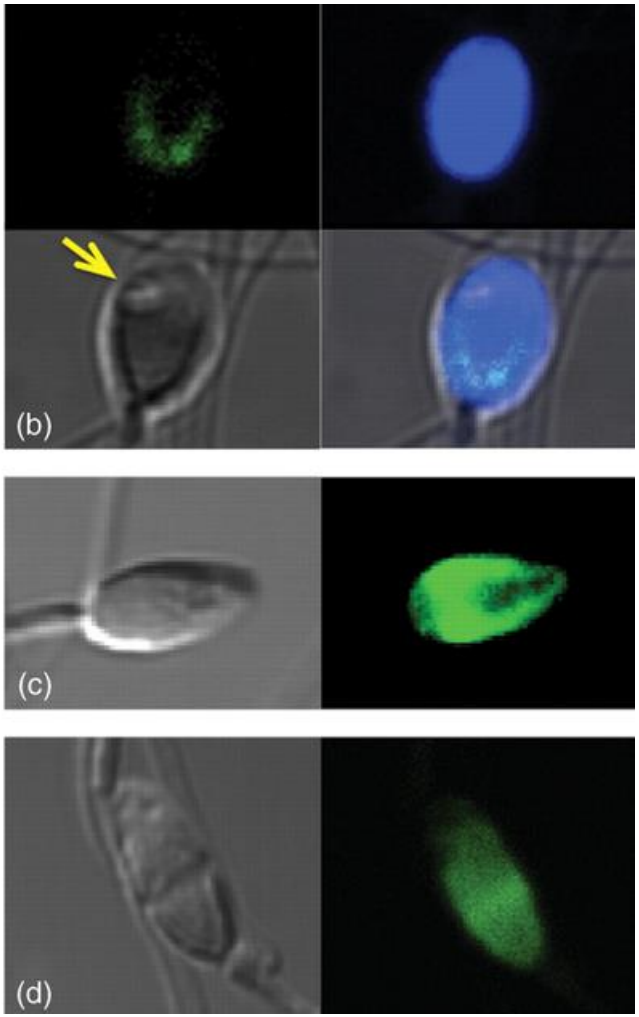

Infrequent Ejaculation
Increased BMI & Poor Diet
Aging
Infection of Male Reproductive Organs
Alcohol*
Cancer
Stress
Sperm DNA
Pollution, Toxins & Radiation
Smoking
Increased Testicular Temperature
High Intake of Caffeine
Varicoceles
Recreational Drugs & Medications
* Some conflicting evidence regarding safe limits
SPERM DNA DAMAGE

Sperm DNA fragmentation

sDF may affect embryo development, implantation, and pregnancies in both natural and assisted reproduction. It is also known that sDF is prevalent among men with abnormal ejaculate parameters, and it has been proposed to be related to cases of infertility in normozoospermic individuals. Since sDF is only partially related to semen quality, it could represent an important addition in the work-up of male infertility.

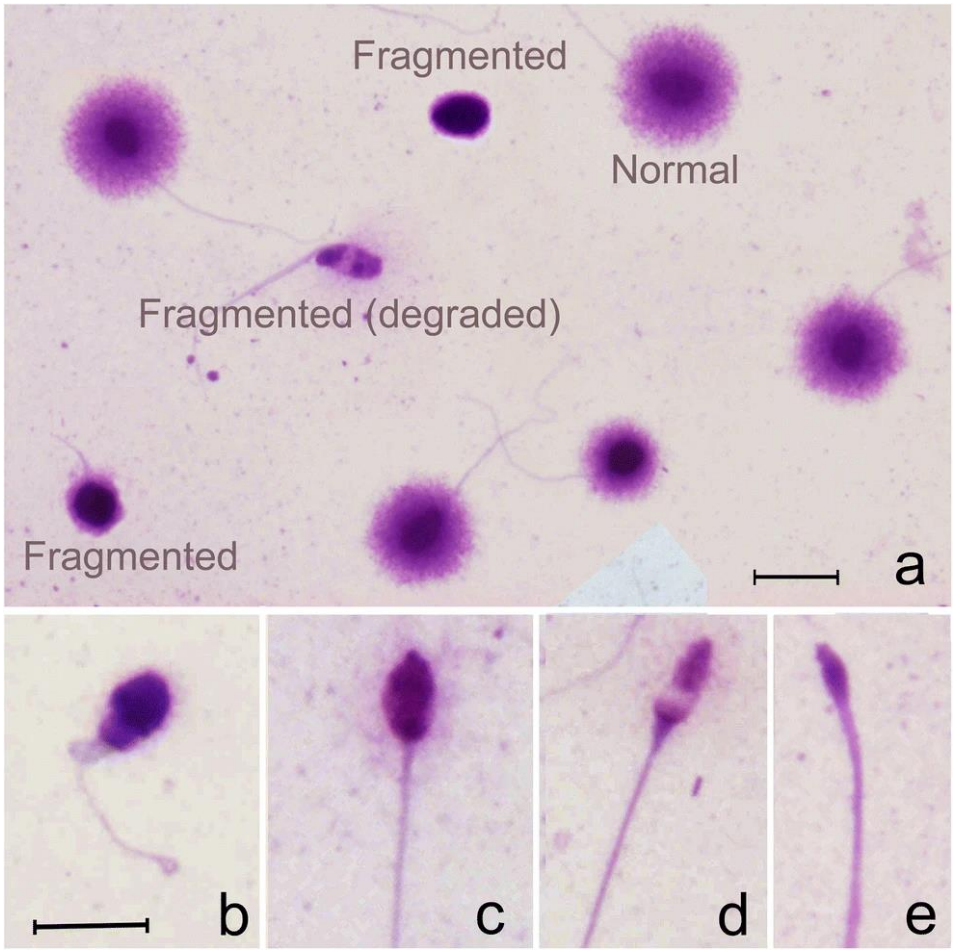
Terminal deoxynucleotidyl transferase (dUTP) nick end labelling (TUNEL)

Directly assess the presence of single and/or double strand breaks in the DNA



(b) Normal-shaped sperm with a large vacuole (an arrow) was not fluorescent. A sperm head where the posterior area emitted a positive fluorescent signal. (c). Fluorescence was relatively intense, suggesting that sperm DNA was seriously damaged by the chemical. (d) An example of a tapered sperm head where intensive fluorescence was detected.

Sperm chromatin dispersion test



a Sperm chromatin dispersion test resulting in the differentiation of three main sperm nuclear morphotypes: (1) normal sperm free of sperm DNA fragmentation and displaying large or moderate sized haloes around a compact core; (2) fragmented sperm displaying small or absence of haloes and (3) fragmented (degraded) sperm showing varying size of the faintly stained nuclear core (**b**–**e**).

The sperm chromatin dispersion (SCD) test is a light microscopy method to evaluate the susceptibility of sperm DNA to acid denaturation. SCD is based on the principle that intact DNA loops expand following denaturation and extraction of nuclear proteins, whereas when DNA is fragmented, dispersion does not develop or is minimal.

SEMEN ANALYSIS

Semen analysis involves the following steps :

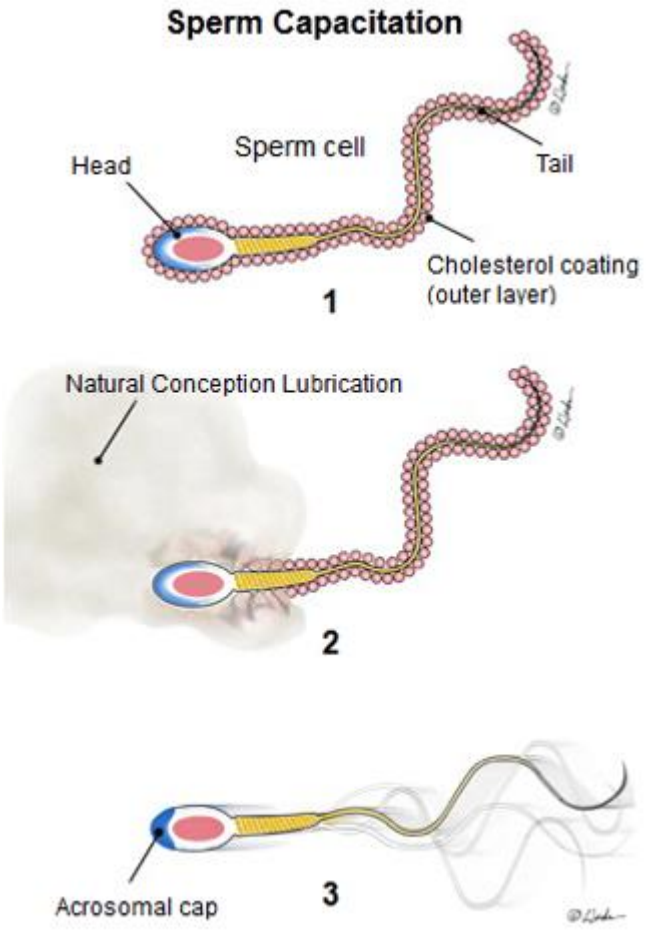
- Placing the specimen container on the bench or in an incubator (37 °C) for liquefaction.
- Assessing liquefaction and appearance of the semen.
- Measuring semen volume.
- Measuring semen pH (if required).
- Preparing a wet preparation for assessing microscopic appearance, sperm motility and the dilution required for assessing sperm number.
  - Mix the semen sample well
  - Remove an aliquot of semen immediately after mixing, approximately 20 µm deep. Wait for the sample to stop drifting (within 60 seconds).
  - Examine the slide with phase-contrast optics at ×200 or ×400 magnification.
- Calculating the concentration in spermatozoa per ml and the total number of spermatozoa per ejaculate with appropriate chambers.

THE (-*IN VIVO*) CAPACITATION

In vivo, separation of motile sperm most capable of fertilizing oocytes from immotile sperm, seminal plasma or leukocytes is taking place in the female genital tract by active migration through the cervical mucus.

This process also prepares male germ cells for fertilization process by means of fundamental changes called **capacitation**.

The capacitation involevs changes in the motility pattern, metabolism and the removal of cholesterol from the plasma membrane leadind changed fluidity of the sperm plasam membrane, enabling the sperm to undergo acrosome reaction.



SPERM PREPARATION TECHNIQUES

In order to select functional sperm or preserve sperm functions, *criteria* for a good sperm selection are as follows:

- ✓ Elemination of seminal plasma and debrids
- ✓ Elimination/ reduction of dysfunctional and ROS-producing sperm
- ✓ Elimination/ reduction of leukocytes
- ✓ Elimination/ reduction of bacteria
- ✓ Enrichment of functional sperm in term of motility, DNA integrity, acrosome reaction and normal sperm morphology
- ✓ Easy and quick to perform
- ✓ Cost- effectiveness

SPERM PREPARATION TECHNIQUES

The choice of sperm preparation technique is dictated by the nature of the semen sample:

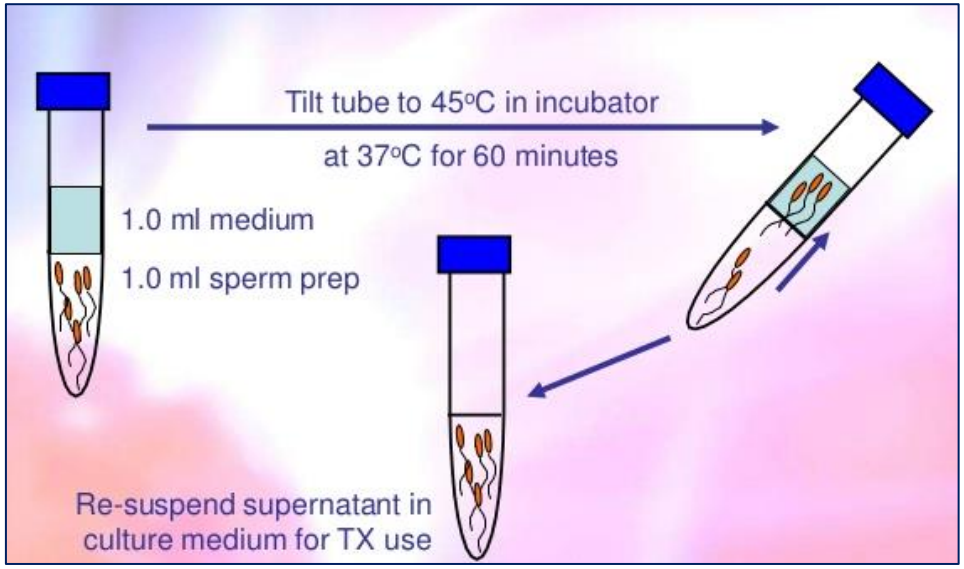
If we have a sample with normal count, motility and morphology of sperms (normozoospermia) we choose a **sperm washing or a swim up method**.

By contrast, with a suboptimal quality sample (severe oligozoospermia, teratozoospermia or asthenozoospermia) we usually prefer a **density gradient centrifugation**.

SWIM UP METHOD

By this technique, the sperms are selected on their motility and the capability to swim out of the seminal plasma.

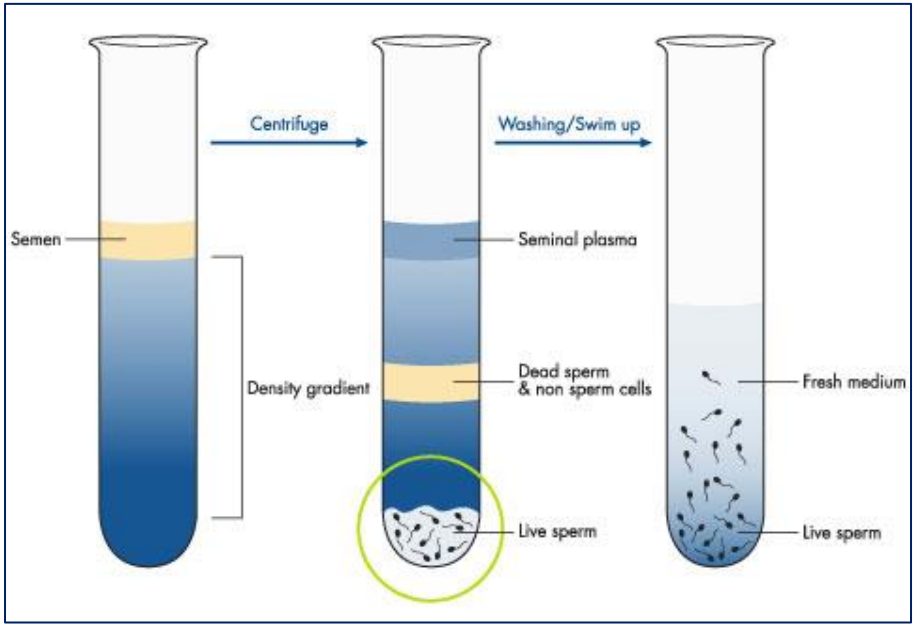
Is performed with the centrifugation of the semen followed by the stratification of the medium over the resuspended pellet.
The liquefied semen is divided in fractions of 1 ml into each tubes, the medium is added (1:1) and after the centrifugation the supernatant is gently removed. Over the resuspended pellet, 0,2- 1 ml of medium is replaced with caution and the tubes is put into the incubator from 30 to 60 min at 37° C (inclined at 45°); after the migration of the sperms, the volume of the semen to perform the IVF tecnique is removed.



DENSITY DISCONTINUOUS GRADIENT METHOD

Cells with different density and motility can be selected during the centrifugation by the colloidal silica coated with silane of the gradient; The most applied discontinuous density-gradient is a two layers density-gradient, formed by a top layer of 45% (v/v) and a lower layer of 90% (v/v).

After the fluidification, 1 ml of the semen is layered over the upper layer (45%) and centrifuged.
At the end of the centrifugation, the supernatant is removed and 1 ml of new medium are added. The centrifugation is repeated again in order.
0.2/ 1 ml of medium is replaced with caution on the final pellet and the tubes is put into the incubator from 30 to 60 min at 37° C (inclined at 45°).



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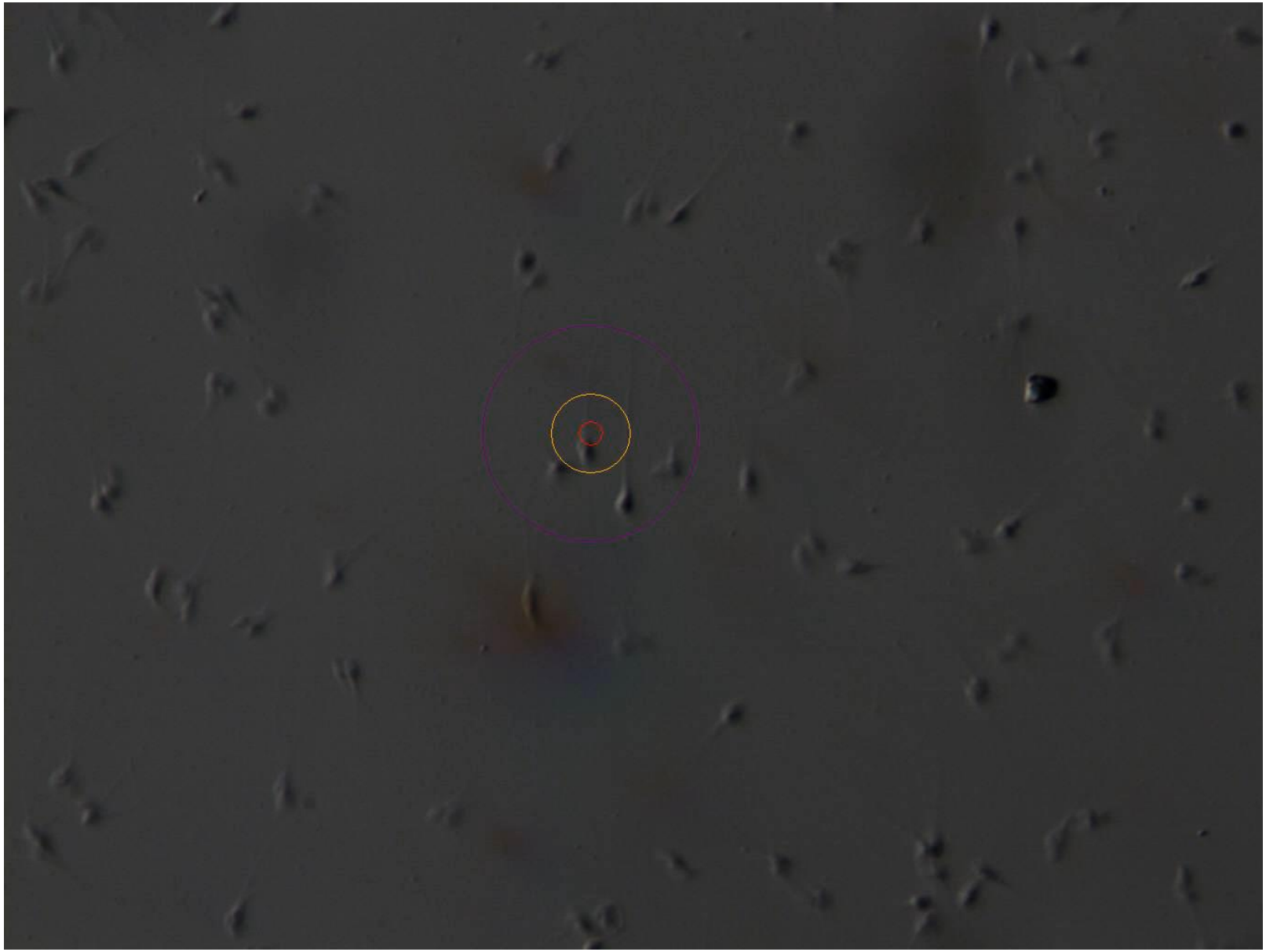
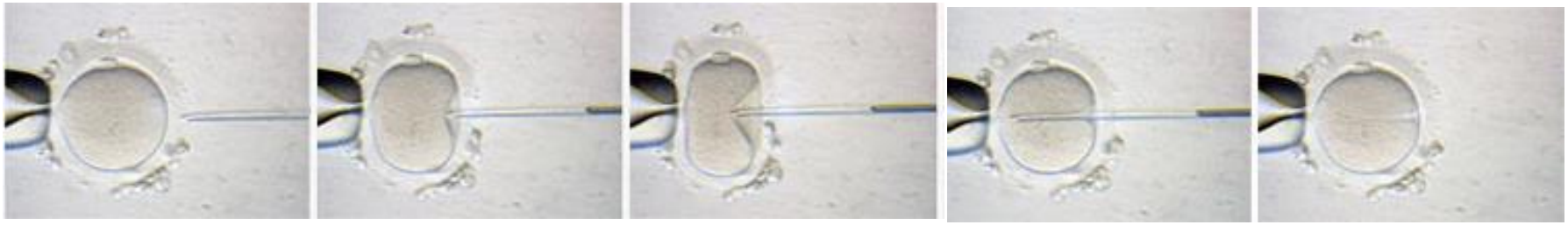


ART MECHANISM

Assisted reproductive technology increases the pregnancy by a double mechanism:

1. It facilitates the interaction between spermatozoa and oocytes;

2. it bypasses seminal abnormalities, such as a reduced number, motility or increased morphological defects of spermatozoa.



INDICATIONS FOR INTRACYTOPLASMIC SPERM INJECTION (ICSI)

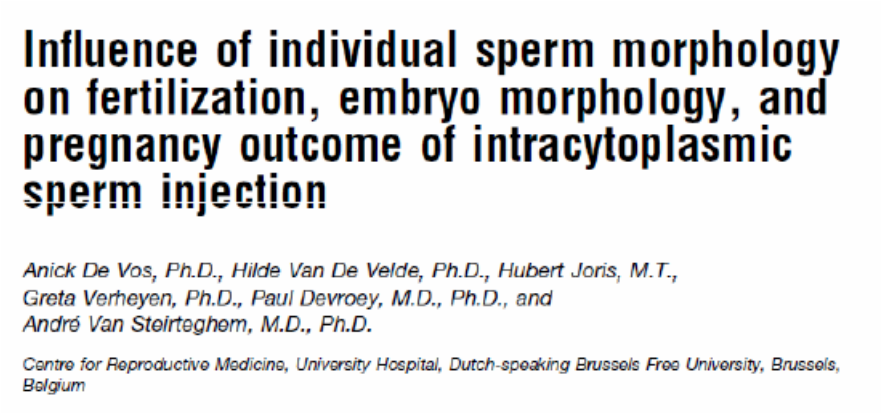
- Oligo-astheno-teratozoosermia
- No or poor fertilization in previous attempts of IVF
- Frozen-thawed spermatozoa with poor survival
- Epididymal/testicular spermatozoa

The microinjection techniques allow to obtain an "in vitro" fertilization also in the presence of severe male factor infertility.

THE ROLE OF SPERMATOZOA IN FERTILIZATION AND EMBRYO DEVELOPMENT

The role of spermatozoa in fertilization and embryo development was minimized to being a carrier that transports DNA to the oocyte.

It is now proved that human spermatozoa play an extensive role that extends even beyond the early stages of fertilization to include abnormal embryogenesis leading to implantation failure (Barroso *et al.*, 2009).



Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection

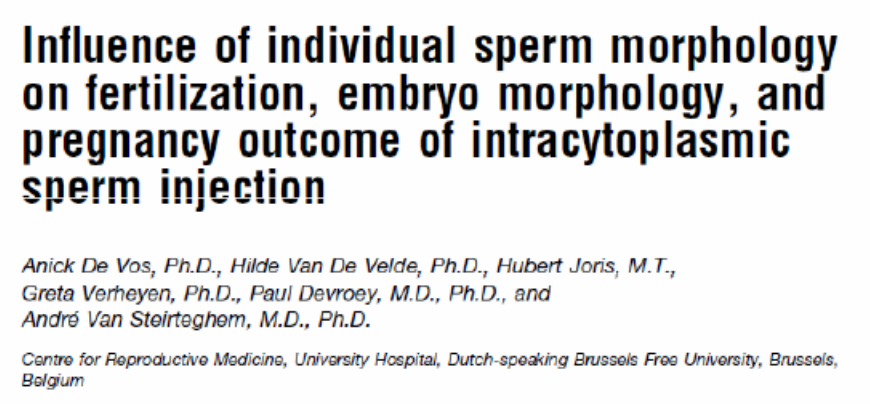
Anick De Vos, Ph.D., Hilde Van De Velde, Ph.D., Hubert Joris, M.T., Greta Verheyen, Ph.D., Paul Devroey, M.D., Ph.D., and André Van Steirteghem, M.D., Ph.D.

Centre for Reproductive Medicine, University Hospital, Dutch-speaking Brussels Free University, Brussels, Belgium

Several studies have demonstrated a positive correlation between sperm morphology and positive ICSI outcomes.

INFLUENCE OF SPERM MORPHOLOGY

Sperm morphology was suggested to play an important role in determining fertility and was proven to be closely related to fertilization rate, embryo morphology and pregnancy rate



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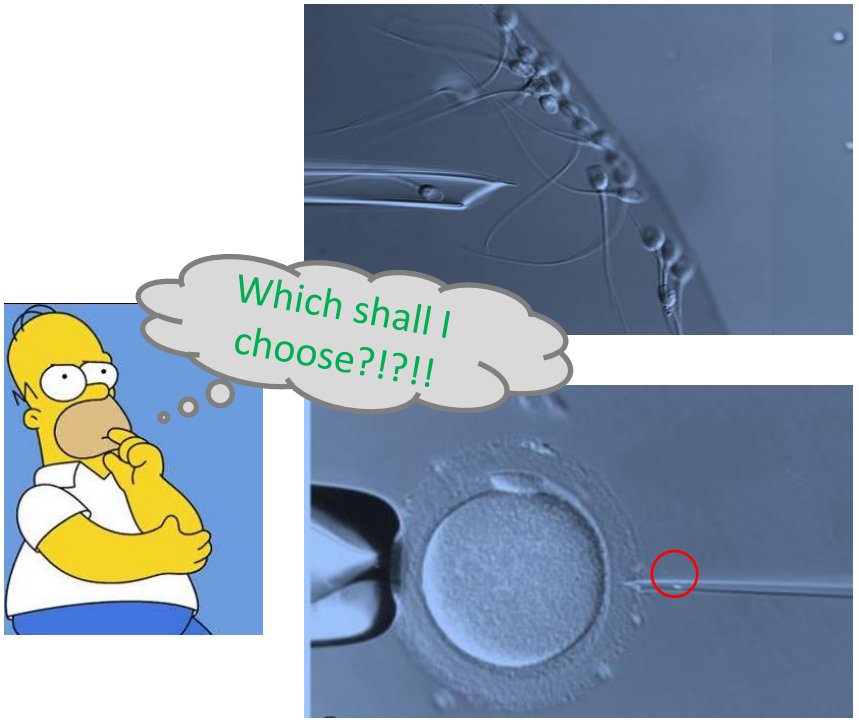
IMPORTANCE OF SPERM SELECTION

Routine sperm preparation techniques such as density gradient centrifugation and swim-up are currently used as main components of ART procedures. These routine techniques appear to be equally effective in selecting motile, morphologically normal sperm (Le Lannou and Blanchard, 1988).

However, other sperm characteristics such as apoptosis and apoptosis-like manifestations, DNA integrity, membrane maturation and ultrastructure are not directly targeted by routine sperm preparation techniques. These characteristics could be influenced by sperm selection and concomitantly be important determinants of fertility.



NEW SPERM SELECTION METHODS

Several advanced sperm selection methods have been developed with the objective of improving sperm preparation protocols used during ART.

These methods aim at isolating mature, structurally intact and non-apoptotic spermatozoa with high DNA integrity.

While ICSI has revolutionized ART and offered an effective treatment option for severe male factor infertility, its application, if using spermatozoa with defective DNA, may result in serious consequences for the offspring.

(Ji *et al.*, 1997; Aitken *et al.*, 2003; Aitken and De Iuliis, 2007).

SPERM SELECTION

- **Transmission electron microscopy and scanning electron microscopy**: ultramorphological analysis of subcellular organelles
- ***SCSA, Tunel assay, SCD test:*** Nuclear integrity analysis
- ***FISH test:*** chromosomal integrity

These techniques **do not allow a real-time analysis of the sample**. Therefore they are to be considered only for screening.

SPERM SELECTION

- Selection based on sperm **birefringence**
- Selection based on sperm surface charge : ***Microflow® CS- 10, potenziale elettrico.***
- Non–apoptotic sperm selection: ***Magnetic Activated Cell Sorting (*MACS*).***
- Selection based on sperm membrane maturity: *Physiological ICSI* (**PICSI**).
- Selection based on sperm ultramorphology: *Intracytoplasmatic Morphologically Selected Sperm Injection* (**IMSI**).

These techniques provide a real-time selection of spermatozoa for ICSI

SPERM SELECTION BASED ON BIREFRINGENCE

The presence of birefringence is the expression of an organized and very compact texture that characterizes normal sperm nuclei, acrosomes, and motile tails.

On the basis of these considerations, the application of polarization microscopy to the ICSI technique has been proposed as a novel tool for sperm selection, based on the properties of birefringence that human spermatozoa naturally possess.

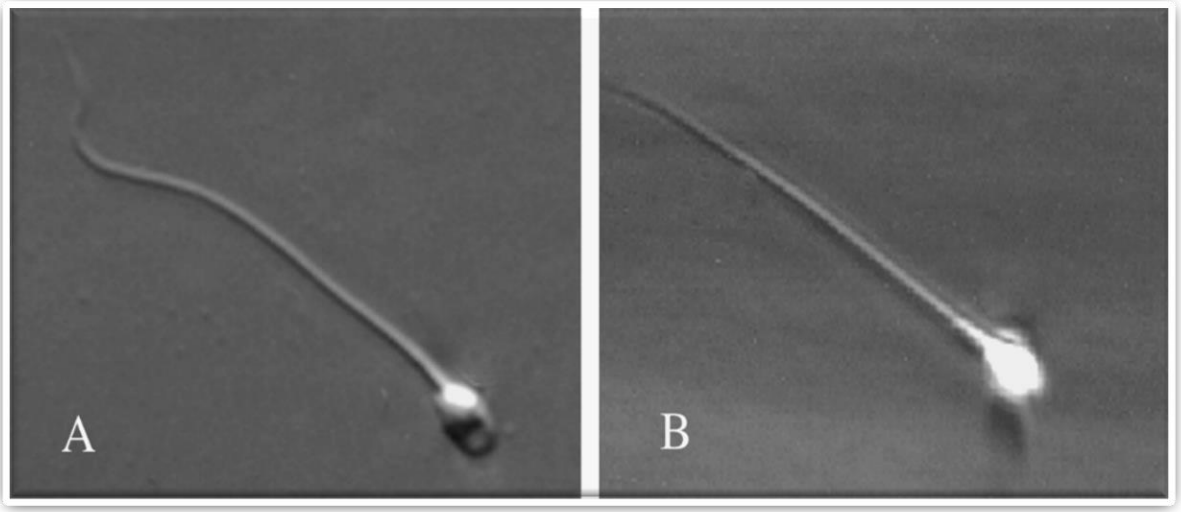
Fertil Steril. 2010 Feb;93(3):807-13. doi: 10.1016/j.fertnstert.2008.10.024. Epub 2008 Dec 6.

Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection.

Gianaroli L[1], Magli MC, Ferraretti AP, Crippa A, Lappi M, Capitani S, Baccetti B.

SPERM SELECTION BASED ON BIREFRINGENCE

Gianaroli. Selection of reacted spermatozoa for ICSI. Fertil Steril 2010



(A) The localization of birefringence in the postacrosomal region indicates that the acrosome reaction has already occurred. (**B**) The presence of birefringence in both compartments of the head, acrosome, and nucleus, identifies an intact acrosome in a nonreacted spermatozoo.

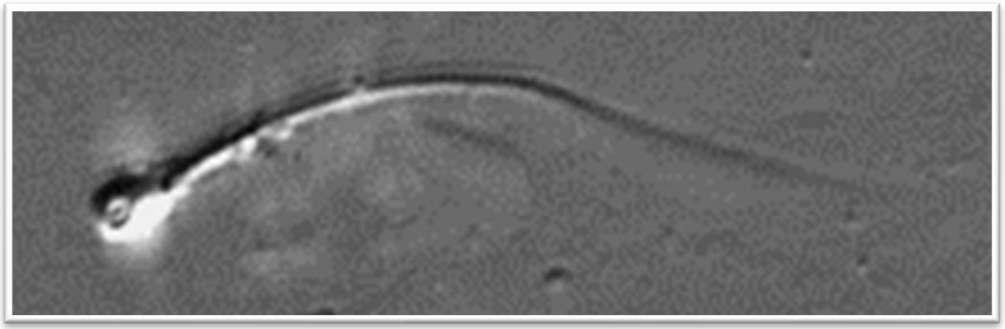
It is well known that the acrosome reaction includes a series of events that sperm cells need to undergo to naturally achieve fertilization of the oocyte.

SPERM SELECTION BASED ON BIREFRINGENCE

Recent studies document an association between sperm head birefringence and DNA integrity:

- Damage in DNA structure could negatively affect the sperm capacity to undergo the acrosome reaction and the consequent steps following its entry into the oocyte.

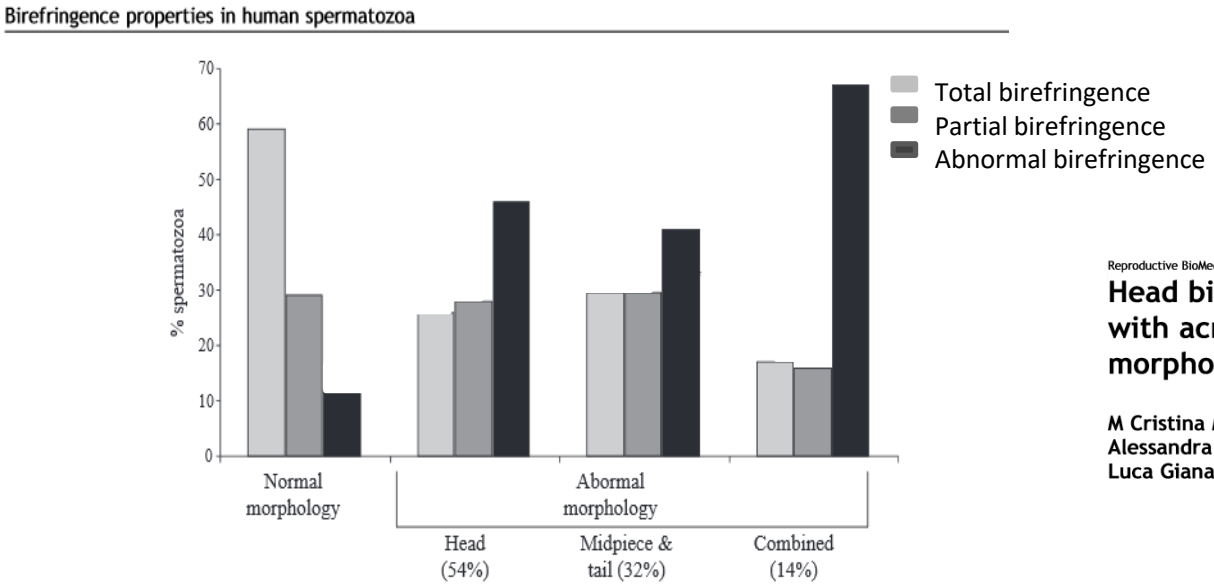
  Petersen et al., 2011



- The polarized light also permitted easily visualization of irregularities such as vacuoles in the sperm head.

  Magli et al., 2012

SPERM SELECTION BASED ON BIREFRINGENCE



Reproductive BioMedicine Online (2012) 24, 352–359

Head birefringence properties are associated with acrosome reaction, sperm motility and morphology

M Cristina Magli [a], Andor Crippa [a], Luigi Muzii [a], Erbeha Boudjema [a], Alessandra Capoti [a], Giulia Scaravelli [b], Anna P Ferraretti [a], Luca Gianaroli [a,*]

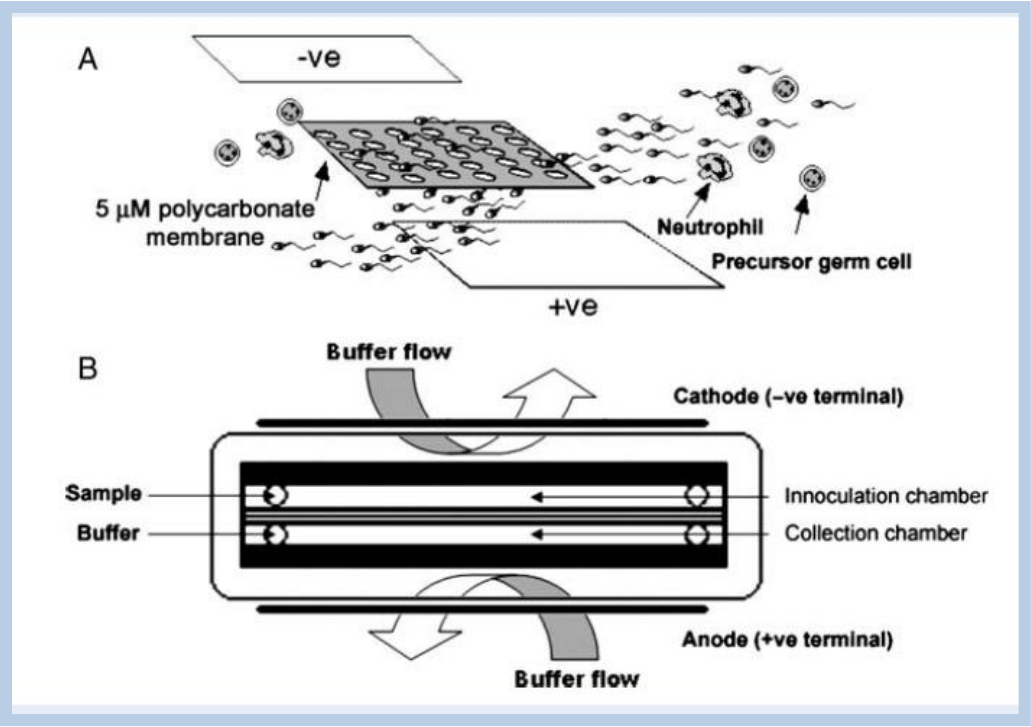
Analysis of birefringence type was used to distinguish spermatozoa on the basis of their acrosome integrity, to evaluate whether some differences could derive from the injection of either reacted or nonreacted sperm cells. Apparently there was no effect on the fertilizing capacity of either type of sperm, nor on the initial cleavage divisions of the fertilized oocytes and consequent embryo quality. Nevertheless, the viability of the generated embryos was significantly different, suggesting that spermatozoa that have undergone the acrosome reaction seem to be more prone to supporting the development of viable embryos.

SELECTION BASED ON SPERM SURFACE CHARGE

***Microflow*® Cell Sorter –** 10 *An electrophoresis-based technology has been developed to separate spermatozoa based on size and electronegative charge.*

The size criterion ensures that only spermatozoa are included, while leukocytes and immature germ cells are excluded.

An electronegative surface charge indicates that the sperm is normally differentiated and has CD52 on its surface: CD52 expression was found to be correlated with normal sperm morphology and capacitation (Giuliani et al., 2004).

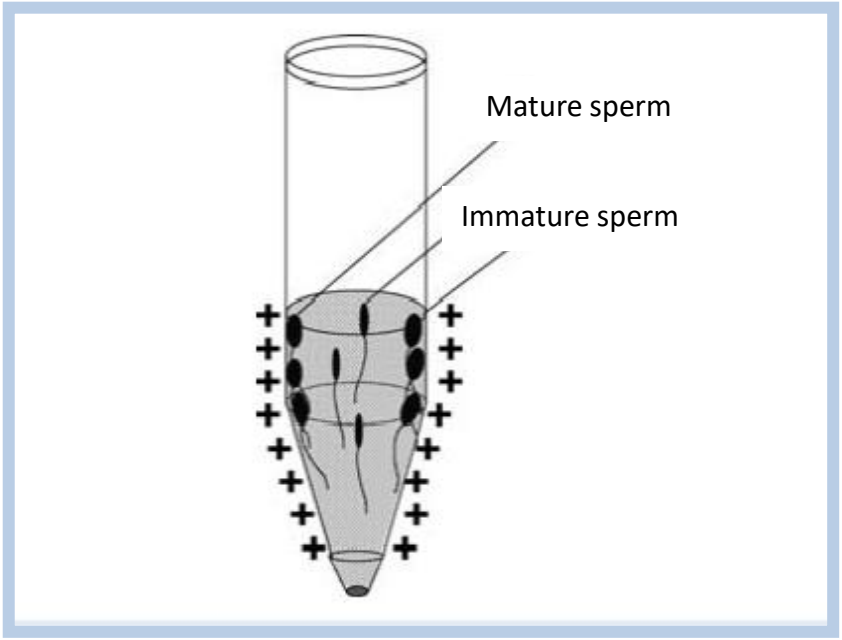


Ainsworth et al., 2005

SELECTION BASED ON SPERM SURFACE CHARGE

Potenziale *ζ electric potential between the sperm membrane and its surroundings measuring - 16 to -20 mV in mature sperm.*

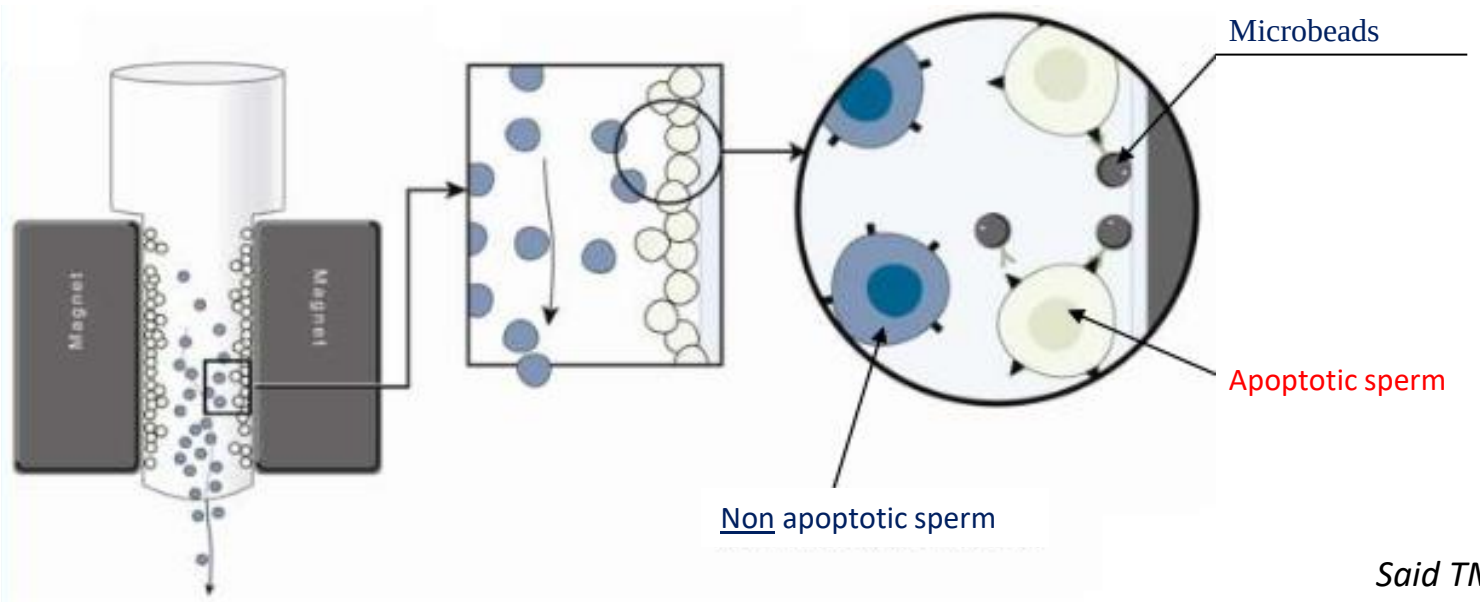
The zeta potential further decreases with capacitation (**Della Giovampaola et al., 2001**). The method entails pipetting washed sperm into a positively charged centrifuge tubes. Thereafter, adhering (negatively charged, mature) sperm can be retrieved by rinsing the tube with serum-supplemented media.



Chan et al., 2006

NON-APOPTOTIC SPERM SELECTION

The externalization of phosphatidylserine (PS) to the outer surface of the sperm membrane, a feature of early apoptosis, has been used as a basis for selection of non-apoptotic spermatozoa.



Said TM et al., 2008

A heterogeneous sperm cell suspension is incubated with Annexin-V-conjugated microbeads, which bind to only apoptotic sperm with externalized PS. Thereafter, the bead/sperm mixture is allowed to run through the MACS column, which is placed inside a magnet. The magnetic force will cause the retention of the cells labeled with microbeads inside the column, while the nonlabeled cells will freely flow (Manz et al., 1995).

EFFECTS ON SPERM QUALITY

Non-apoptotic sperm selection is simple, fast, inexpensive and highly specific.

However, the combination of DGC and MACS will **involve repeated steps of centrifugation and re-suspension**, which might be detrimental when applied to semen samples characterized by limited sperm counts, as low sperm recovery may be expected.

In addition, the technique still requires special laboratory equipment, which may not be feasible or available in all settings.

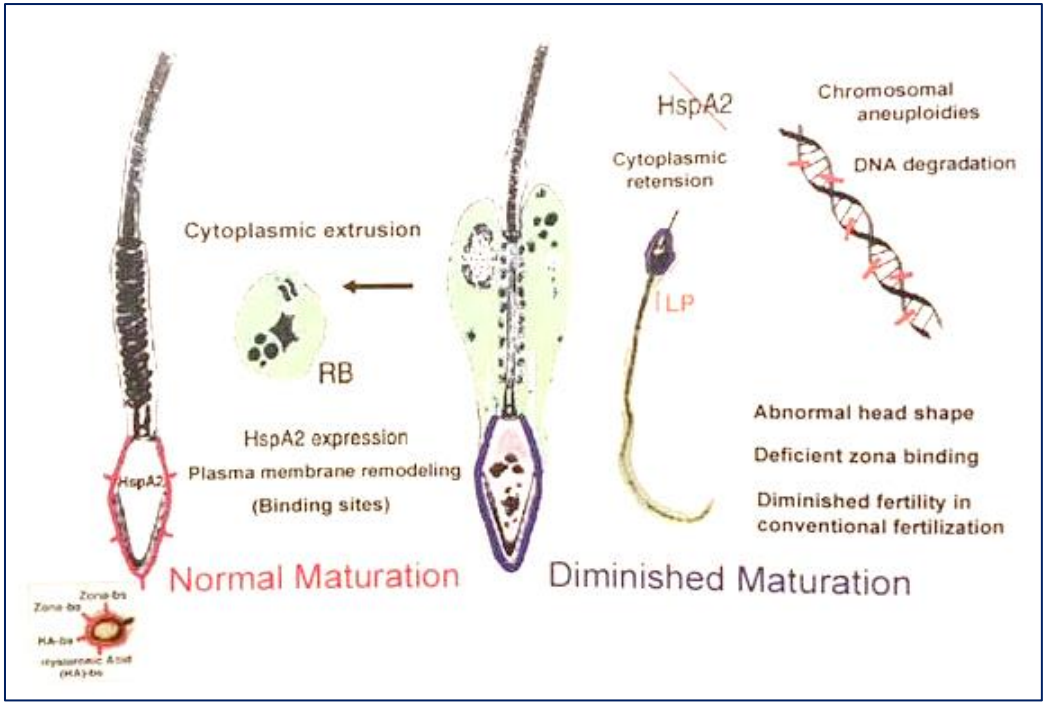
Said et al., 2008

SELECTION BASED ON SPERM MEMBRANE MATURITY

The formation of hyaluronic-acid (HA)-binding sites on the sperm plasma membrane is one of the signs of sperm maturity.

Huszar *et al.*, 1997

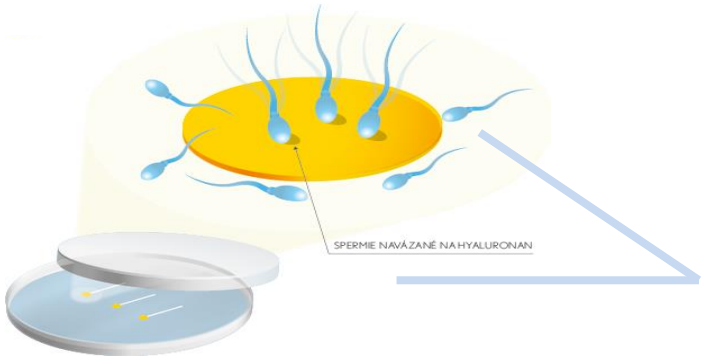
There is a relationship between the levels of hyaluronic-acid (HA)-binding sites in elongated spermatids and the related spermiogenetic events, such as cytoplasmatic extrusion, formation of the normal sperm shape or the sperm plasma membrane remodeling.

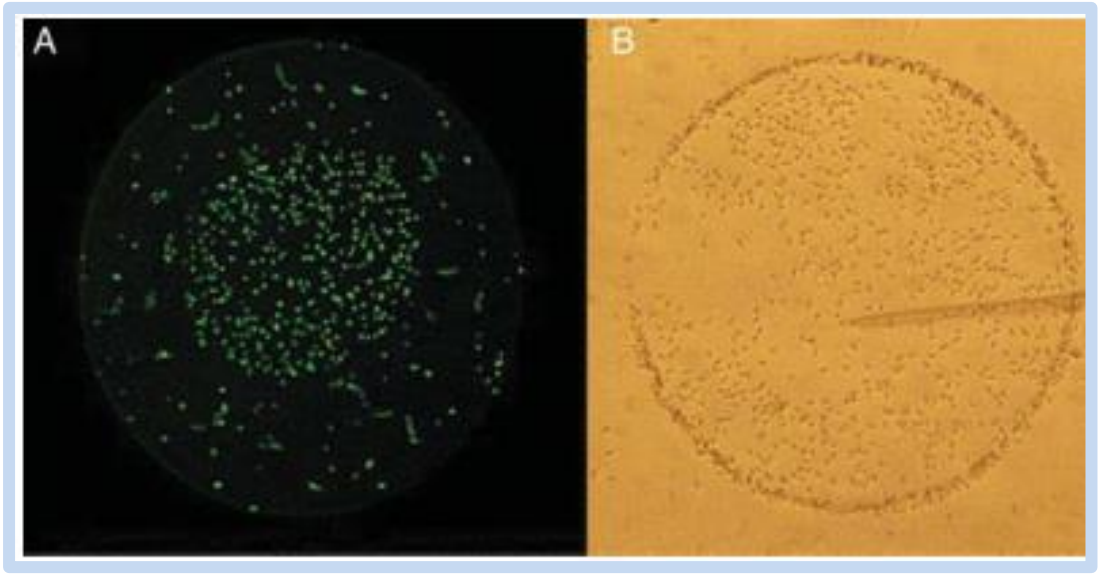


SELECTION BASED ON SPERM MEMBRANE MATURITY (PICSI)

The formation of hyaluronic-acid (HA)-binding sites on the sperm plasma membrane has been used as a basis for sperm selection.

Huszar et al., 1997





(**A**) A sperm drop is placed at the periphery of a HA drop, mature sperm binds to the HA-spot, while immature sperm moves freely. (**B**) Bound sperm could be picked up with the ICSI pipette.

Jakab et al., 2005

EFFECTS ON SPERM QUALITY

When compared with spermatozoa prepared by DGC, HA-bound spermatozoa have displayed:

1. a greater degree of maturity
2. a positive correlation with motility
3. less DNA fragmentation

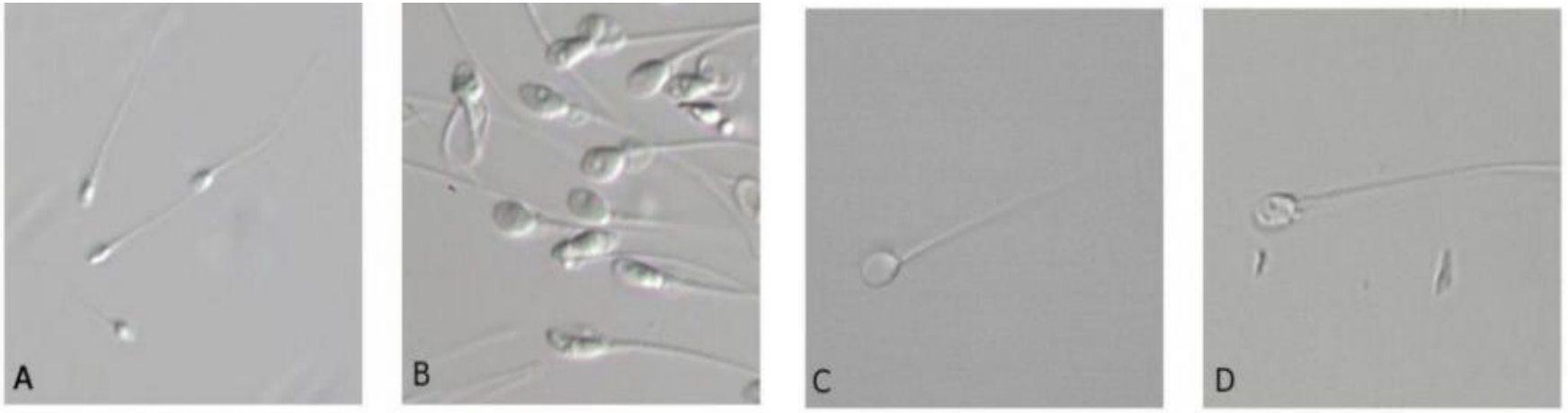
But it does not provide any additional information about the sperm morphology.

Tamer et al., 2011

ULTRA-HIGH MAGNIFICATION (IMSI) SPERM SELECTION

Sperm morphology has been described as one of the major determinants of male in vivo and in vitro fertility.
A new sperm selection method has been developed based on the inclusion of only normal sperm assessed using real-time motile sperm organelle morphology examination (**MSOME**) at a magnification of ×6300.

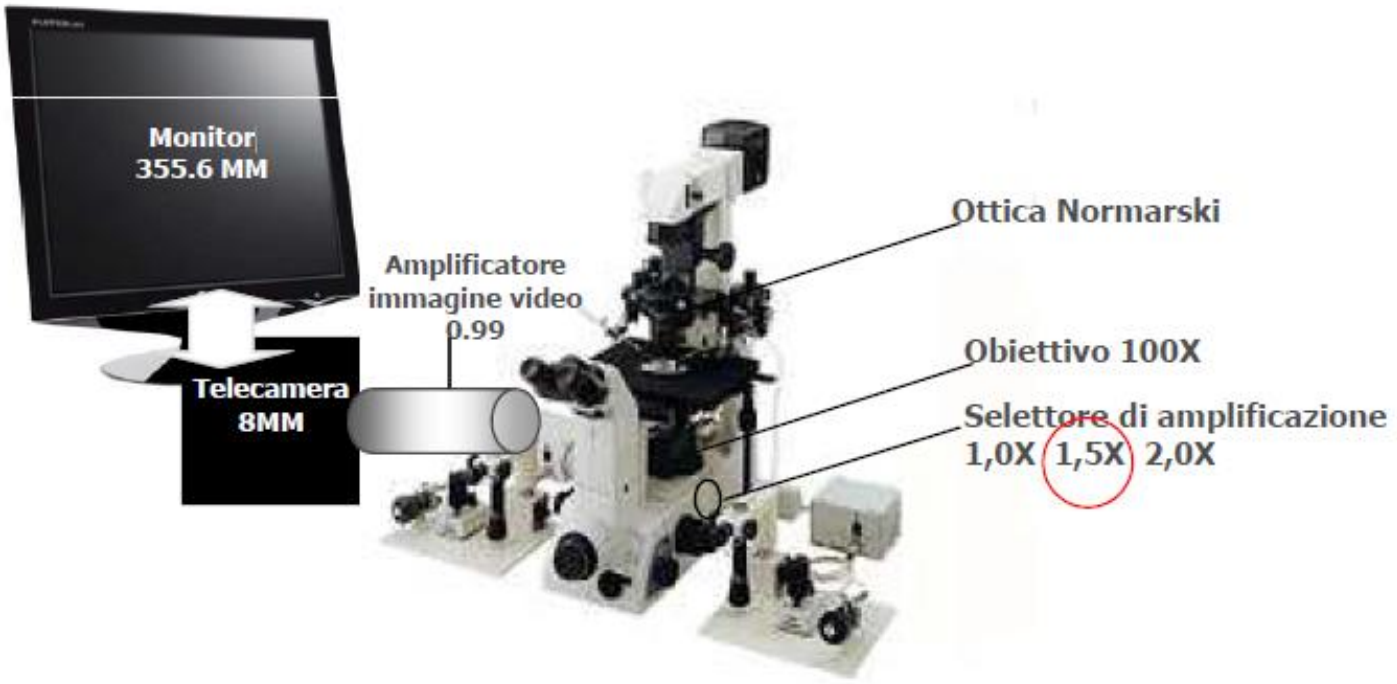
Bartoov et al., 2002



(A) Semen observed by conventional magnification (400X); (B. C. D) Semen observed by MSOME (x 6300).

ULTRA-HIGH MAGNIFICATION (IMSI) SPERM SELECTION

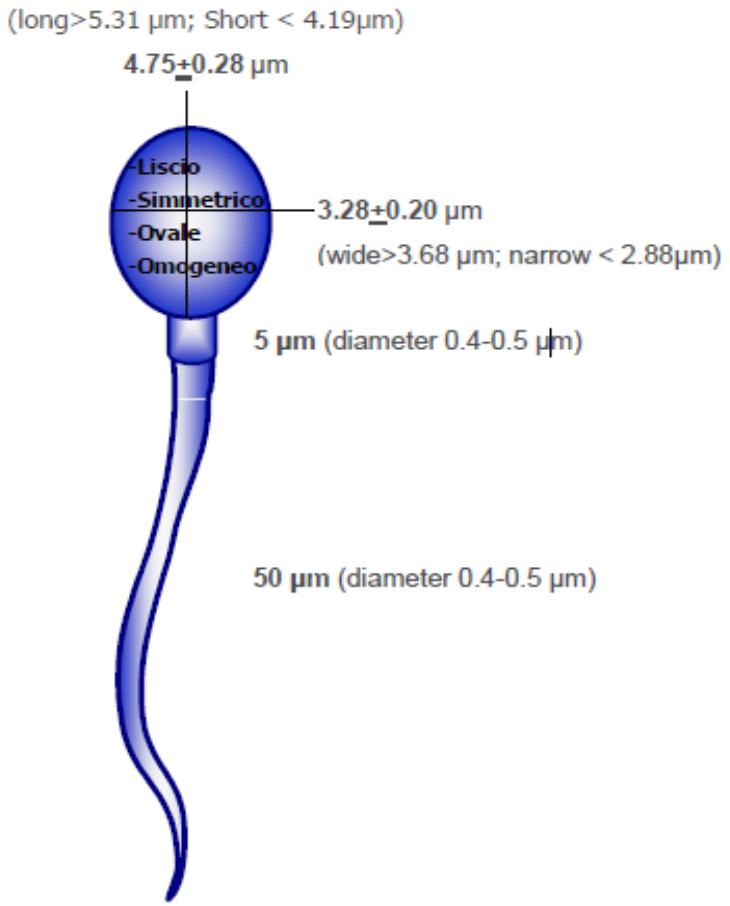
During MSOME, a micro-droplet of motile sperm suspension prepared by a routine sperm preparation technique is examined under oil immersion, with an inverted light microscope fitted with high power Nomarski optics (differential interference contrast optics) with digital enhancement. Multiplying the lens magnification, with that of selector, camera and monitor, the final magnification will be equal to 6600 X.



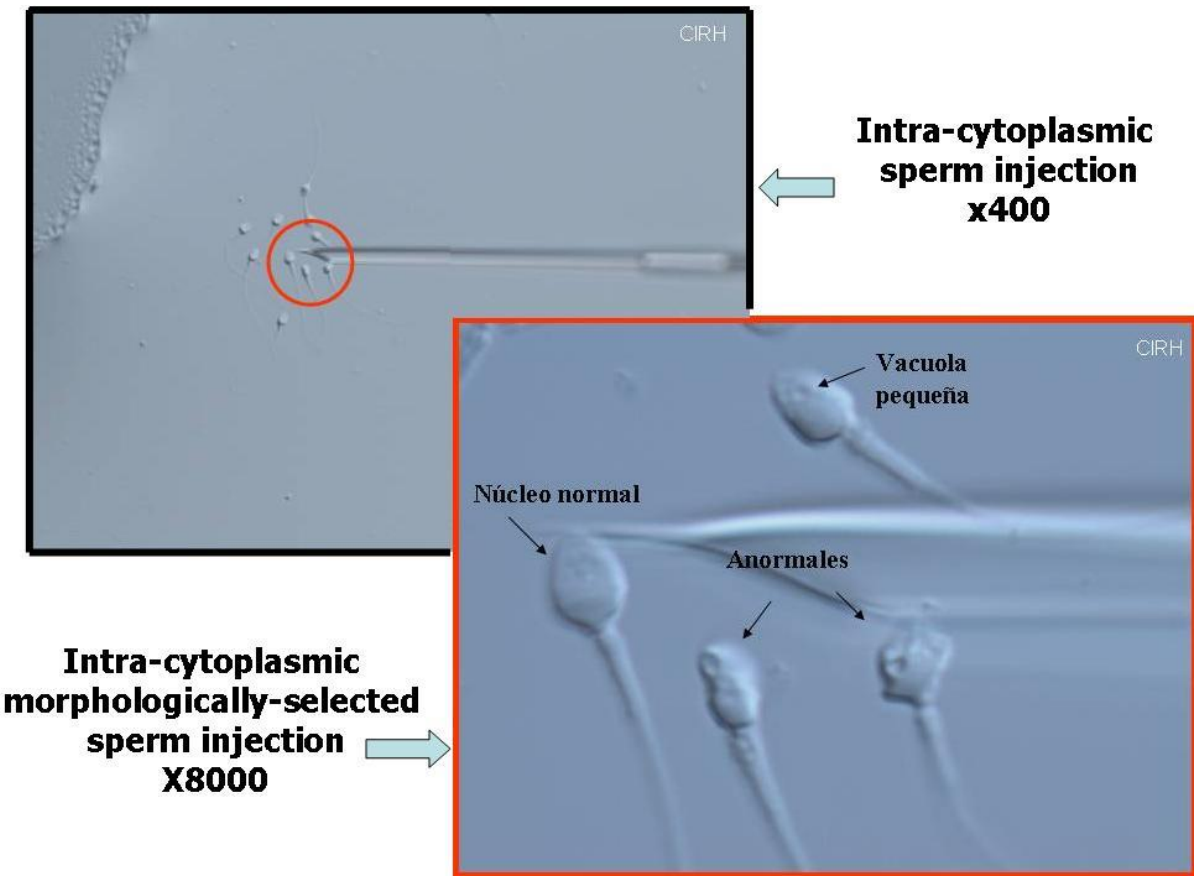
ULTRA-HIGH MAGNIFICATION (IMSI) SPERM SELECTION

MSOME assesses five sperm organelles (acrosome, postacrosomal lamina, neck, tail and mitochondria) that can be classified as either normal or abnormal. The sixth organelle (the nucleus) is evaluated for both shape and chromatin content (vacuolar area).

- **Head**: the shape has to be smooth, symmetric and oval. The chromatin mass has to be homogeneous and contain no extrusion or invaginations with a maximum of one vacuole involving less than 4% of the nuclear area.
- **Acrosome and post acrosomal lamina**: they were considere abnormal if absent, partial or vesiculated.
- **neck**: an abaxial neck with disorderes or cytoplasmatic droplets was considered abnormal.
- **Tail**: was considered abnormal if broken, short or double.



IMSI Bartoov et al., 2003



This approach is of particular benefit when used in situations where identification of specific sperm organelles is required, such as the acrosomal components in cases of globozoospermia . Check et al., 2007

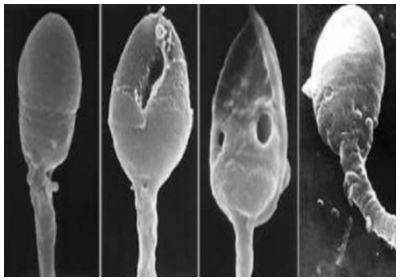
MAIN TOPICS

- **BASIC SEMEN ANALISYS**
- **SELECTION OF SPERMATOZOA FOR ICSI**

THEROICAL LESSON

- ***"ATLAS OF HUMAN SEMEN"***

……YOU HAVE TO CHOOSE!!!.....



INTERACTIVE LESSON

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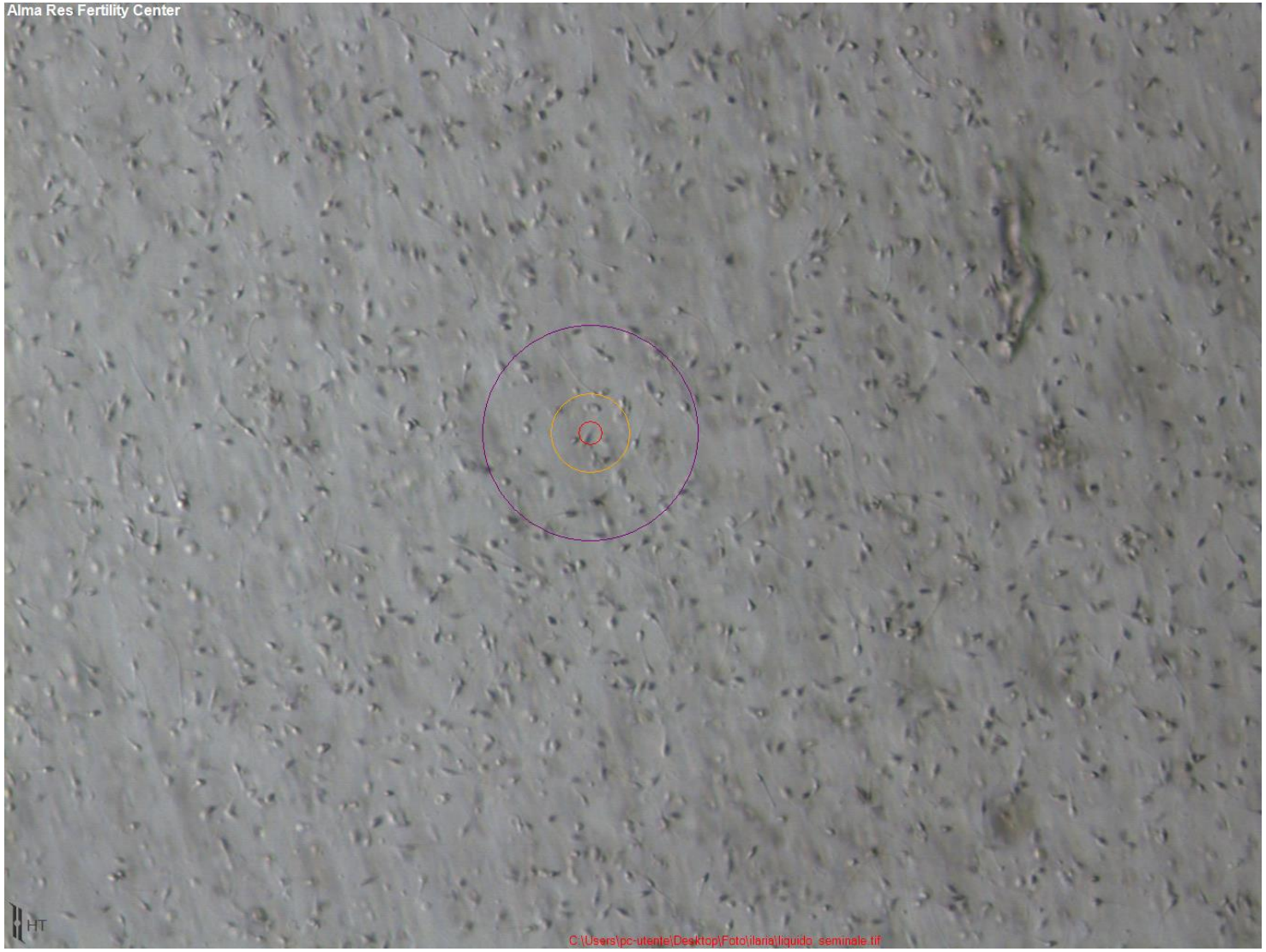
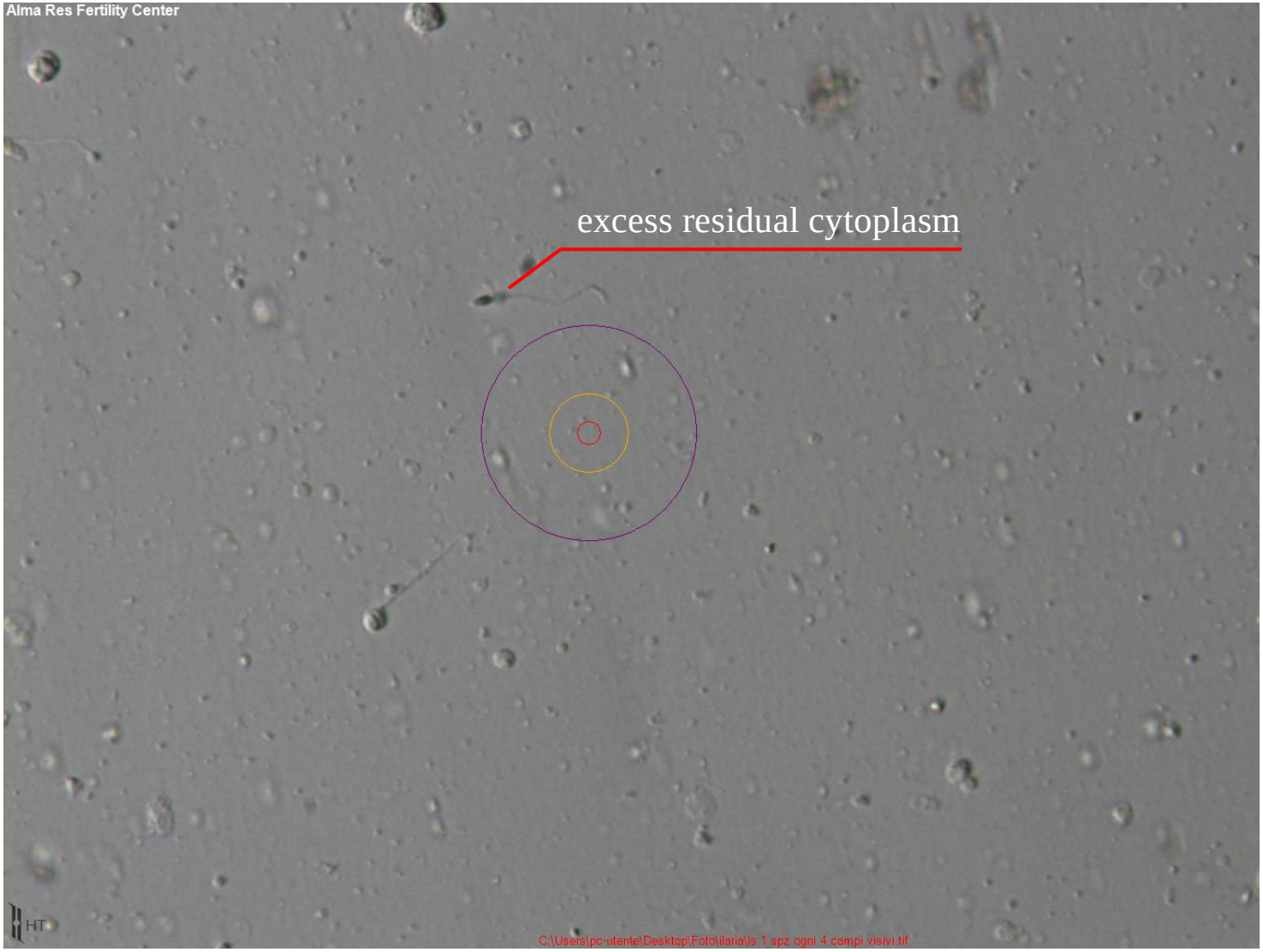
Normo-zoospermia



Oligoasthenoteratozoospermia


Alma Res Fertility Center
excess residual cytoplasm
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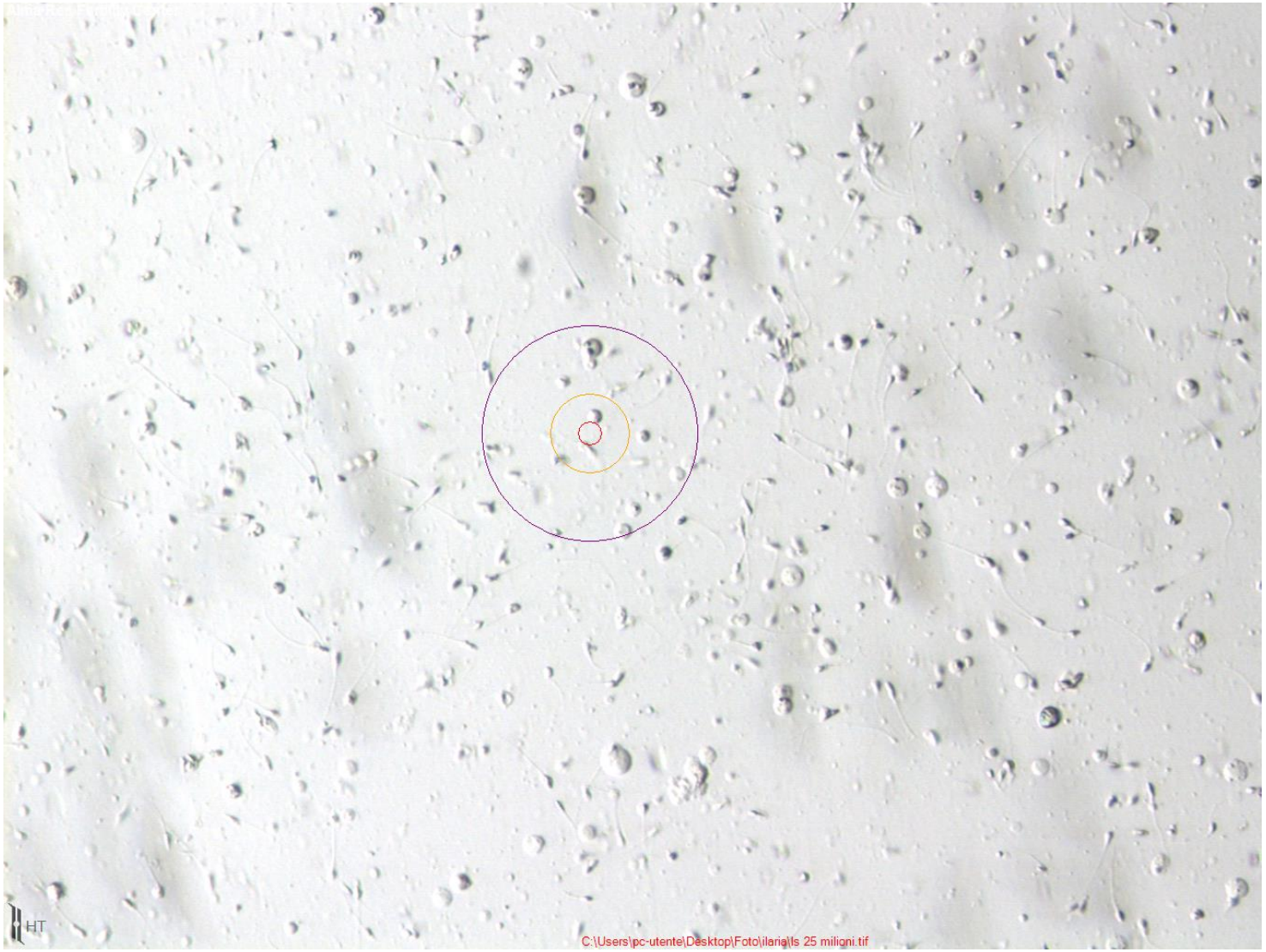
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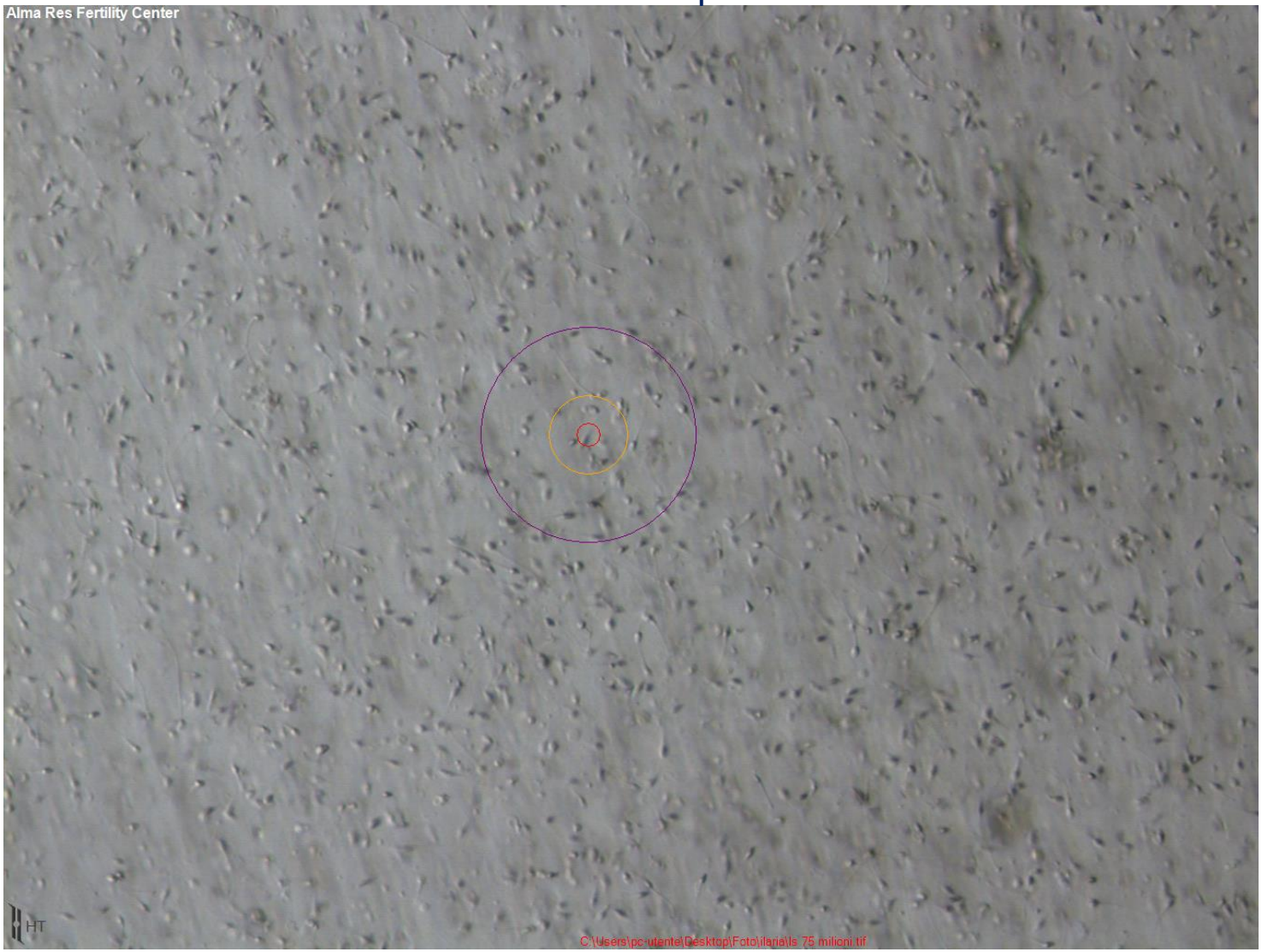
Normo-zoospermia



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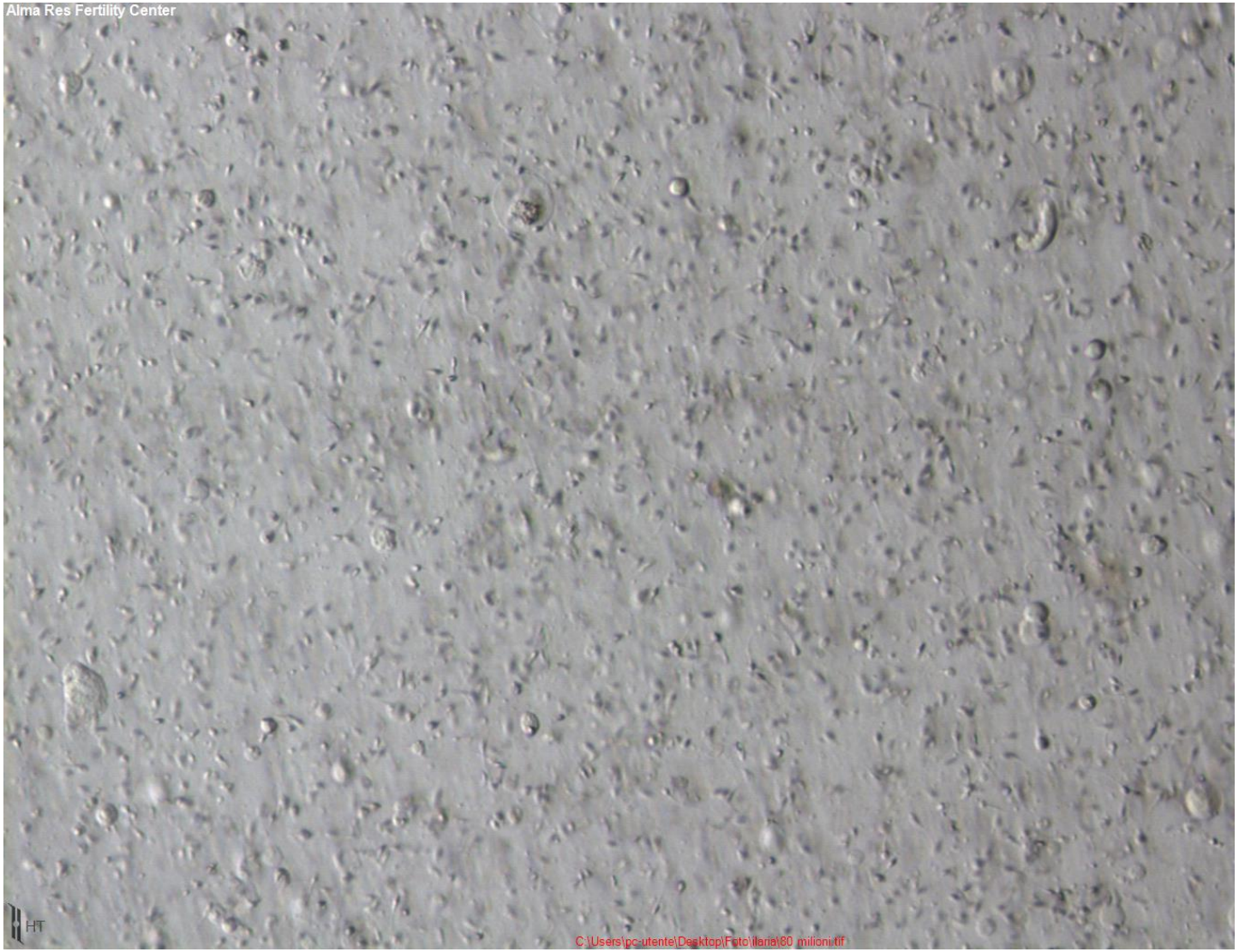

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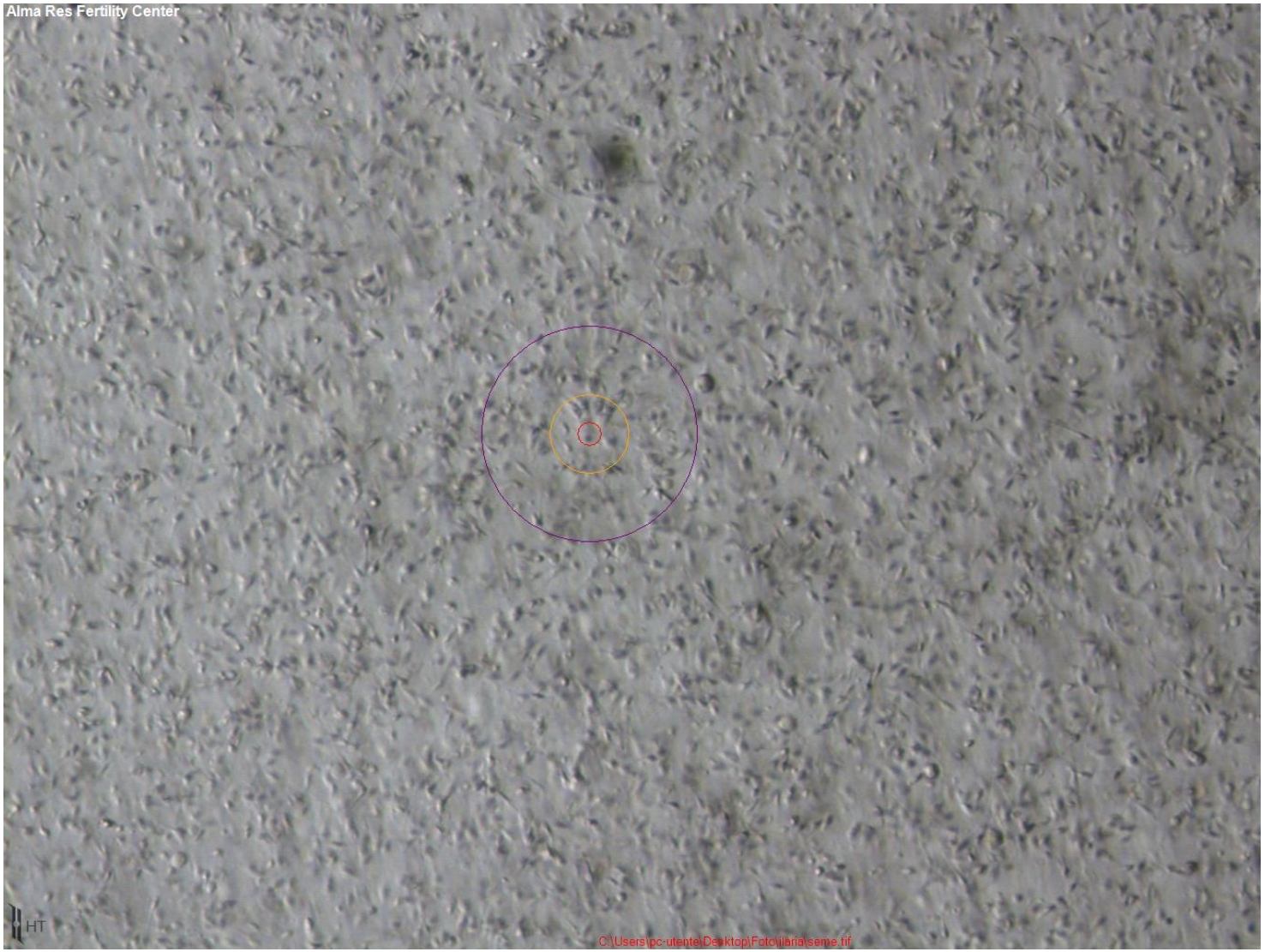

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UNITE

Sperm agglutination

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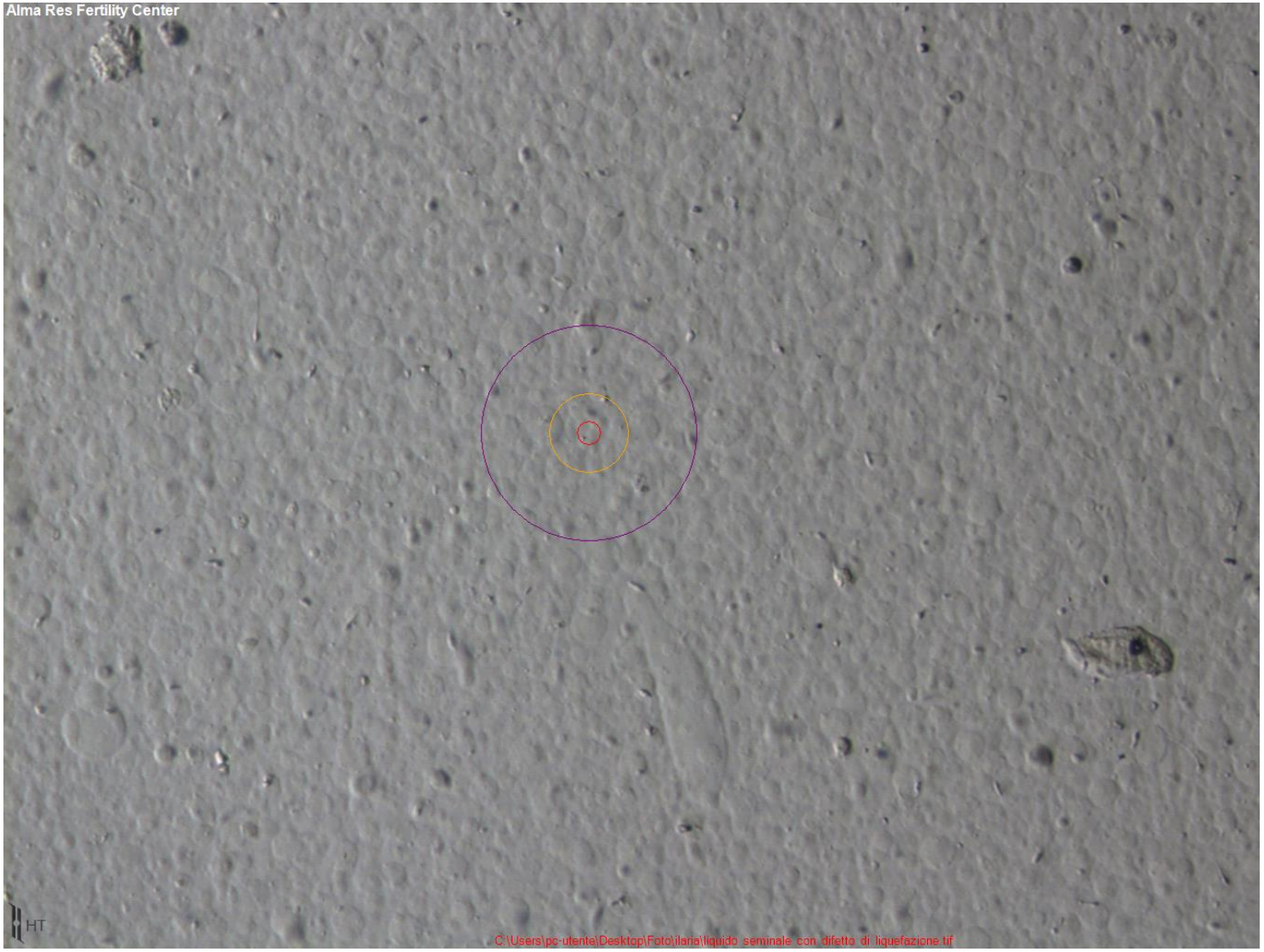
Sample not liquefy



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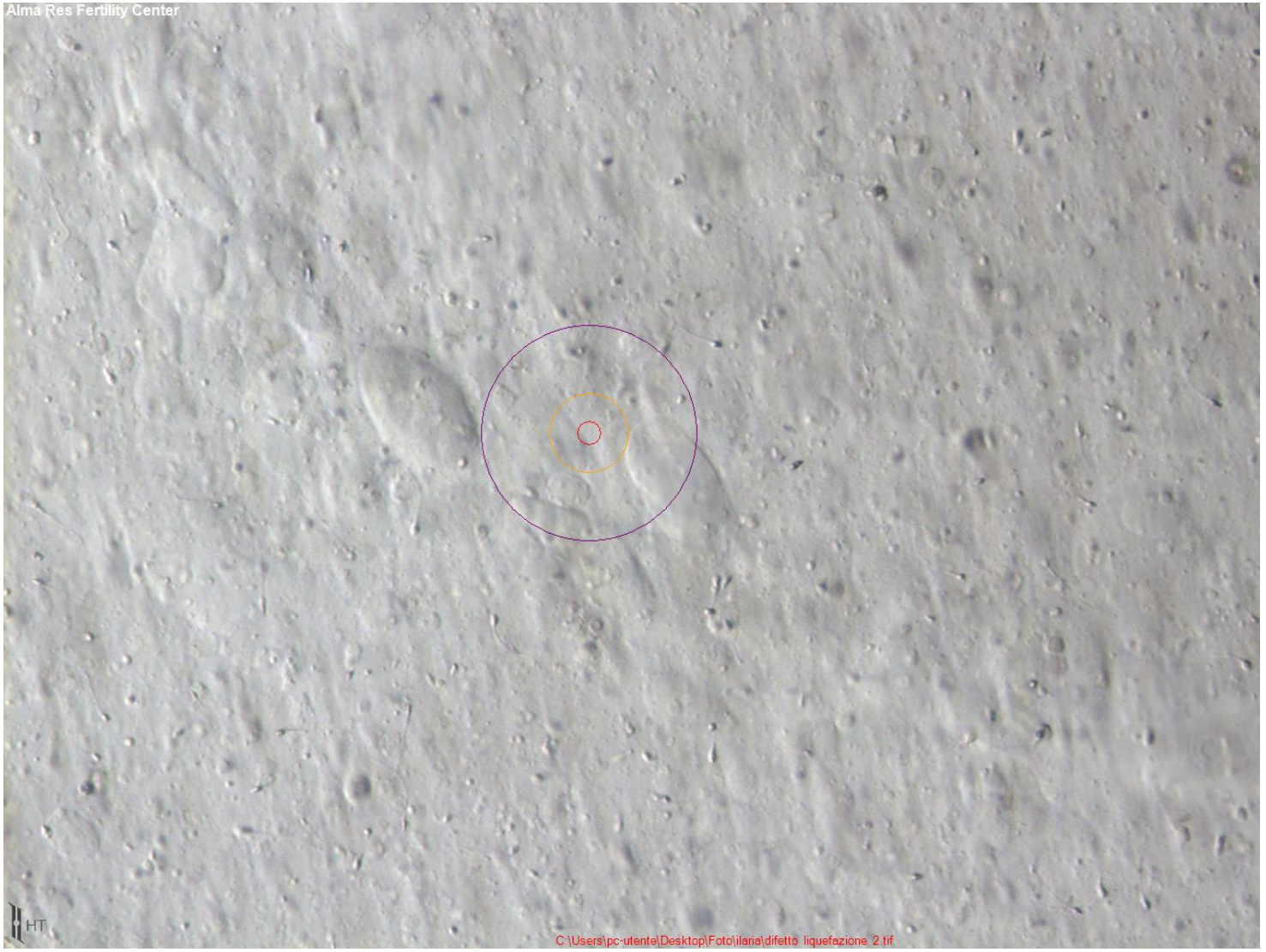
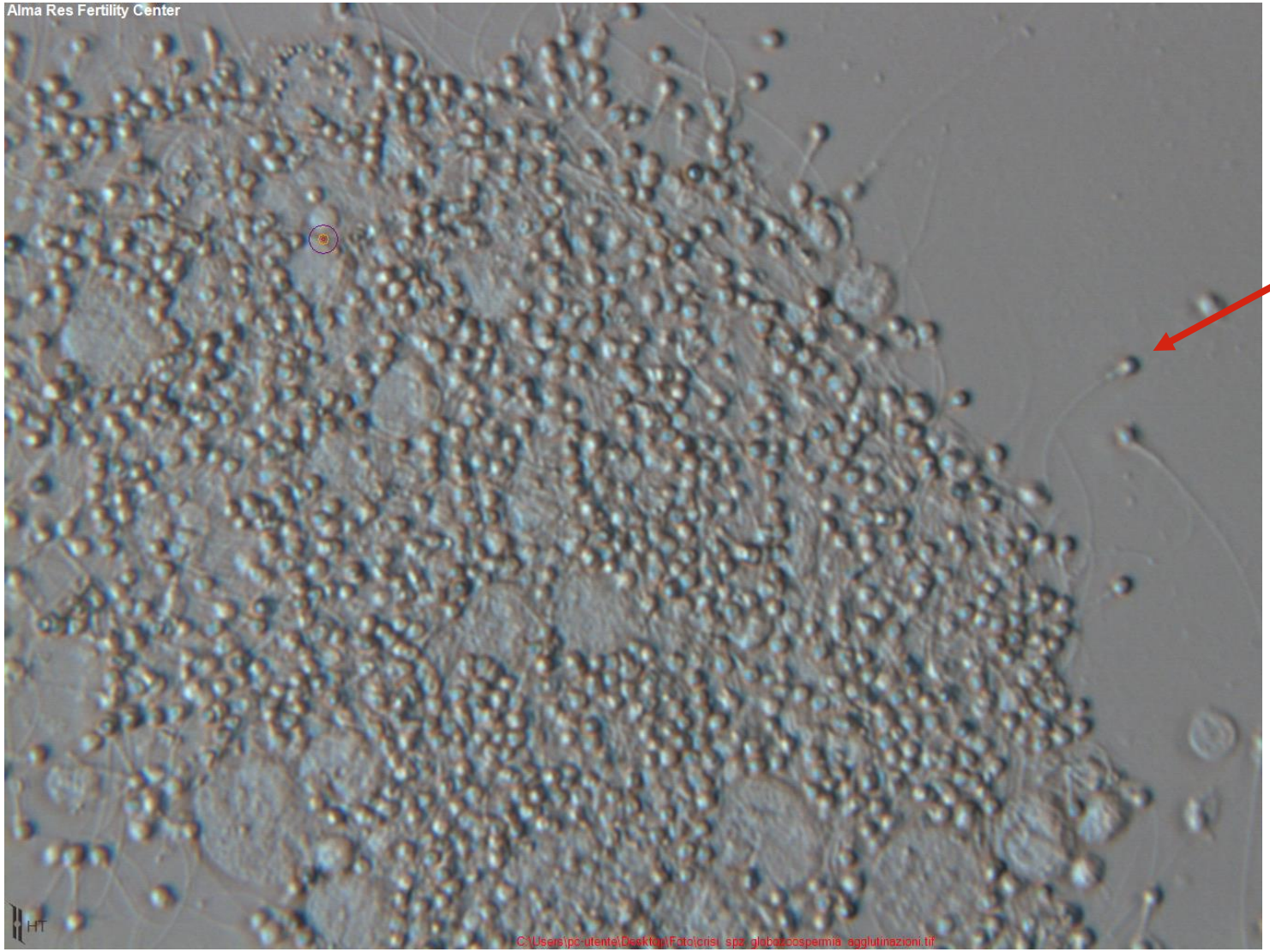
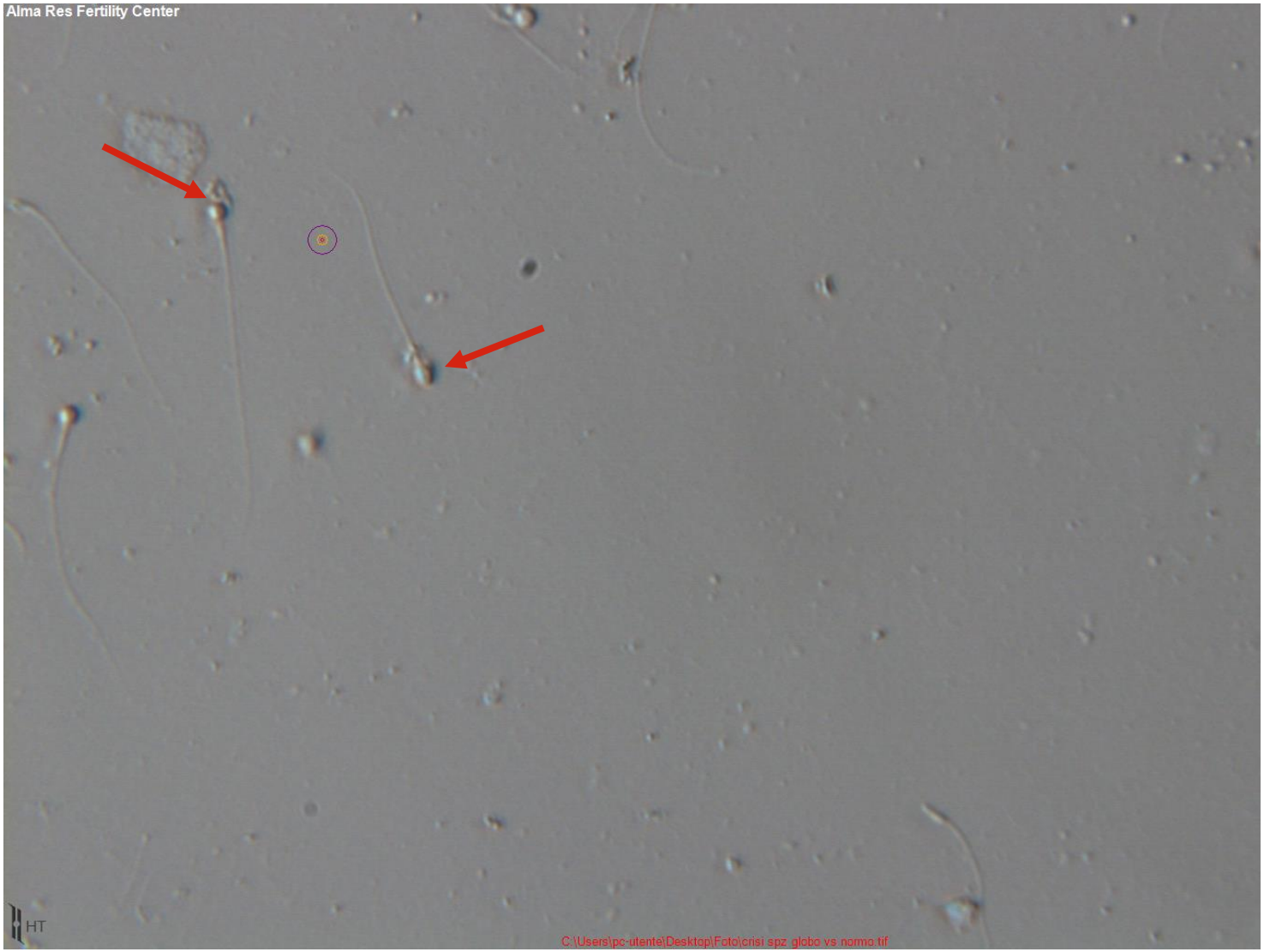
Sample not liquefy



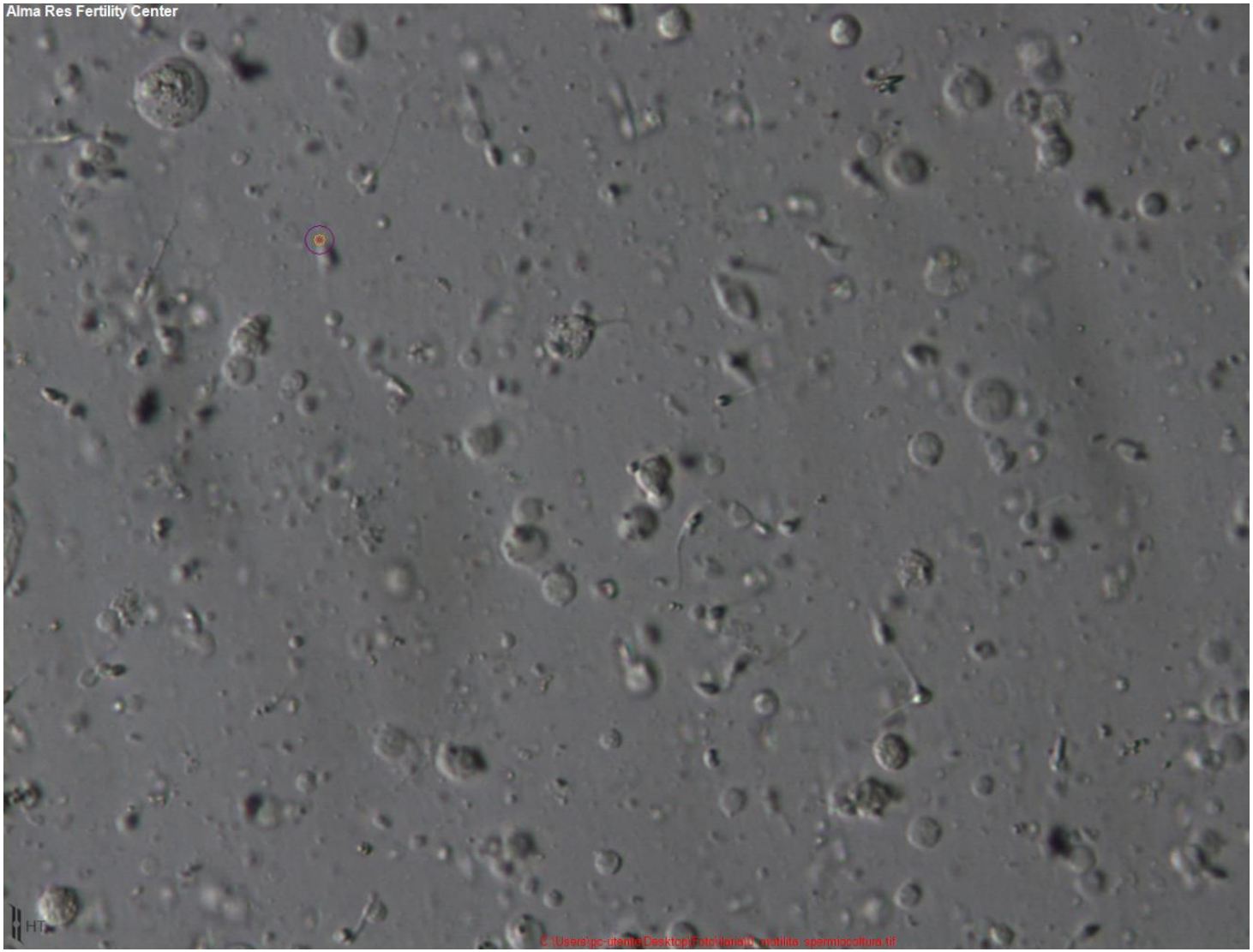
Globozoospermia

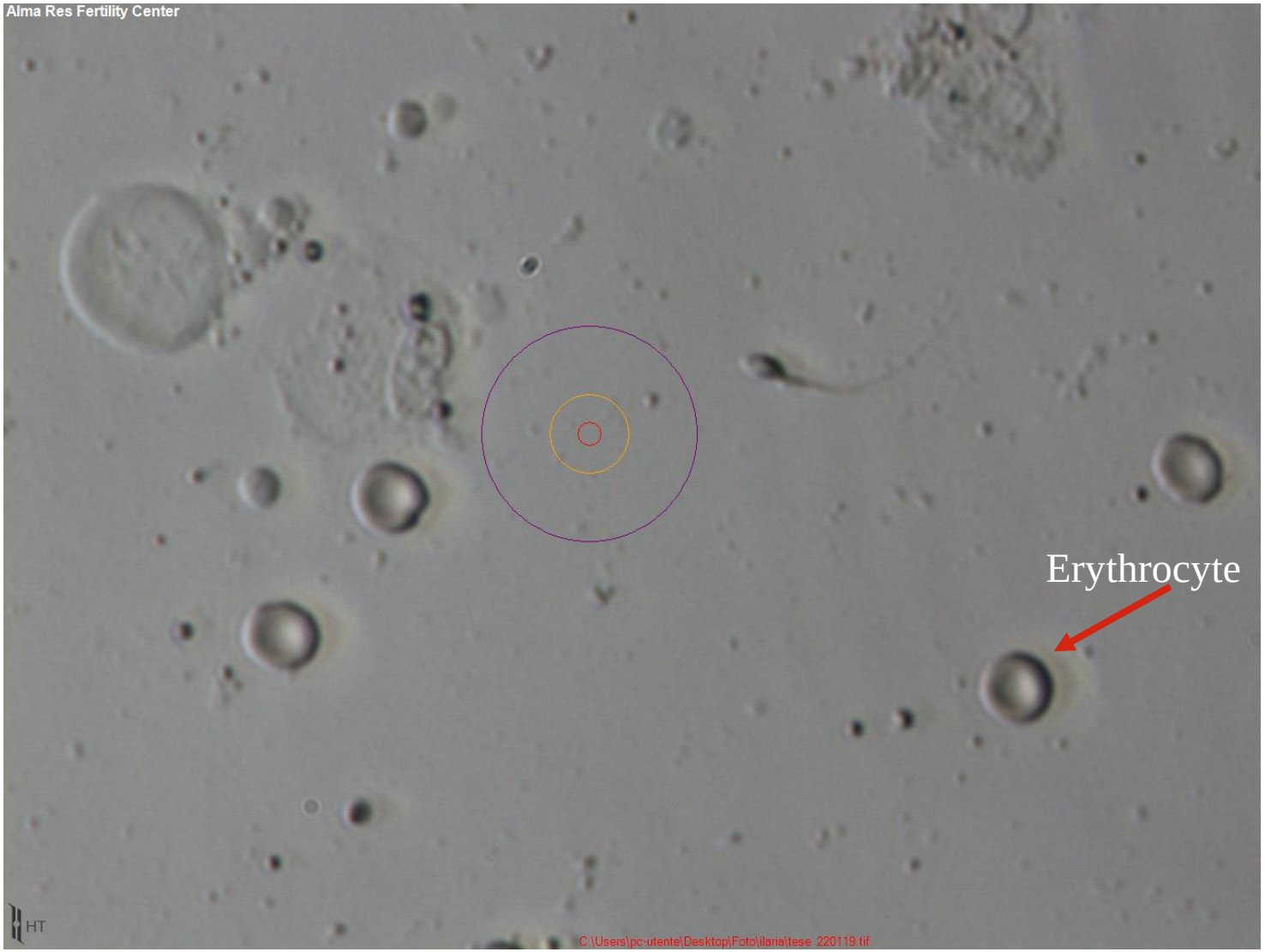


Globozoospermia



Round cells

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Erythrocyte
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