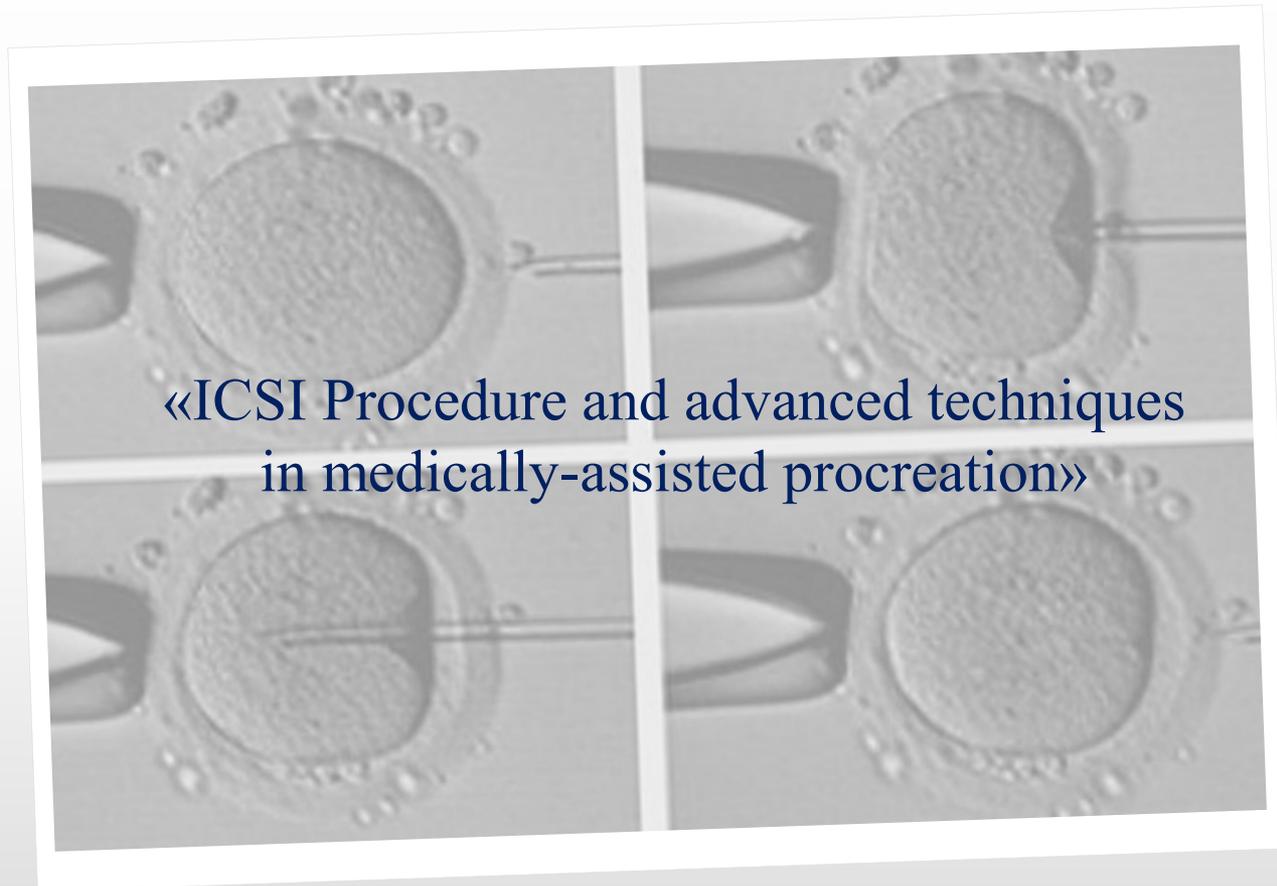


Second-Cycle Degree Course in “REPRODUCTIVE BIOTECHNOLOGIES”



A.Y. 2024 - 2025

Ilaria Listorti
Head of Villa MafaldaART lab
ilistorti@unite.it

MAIN TOPICS

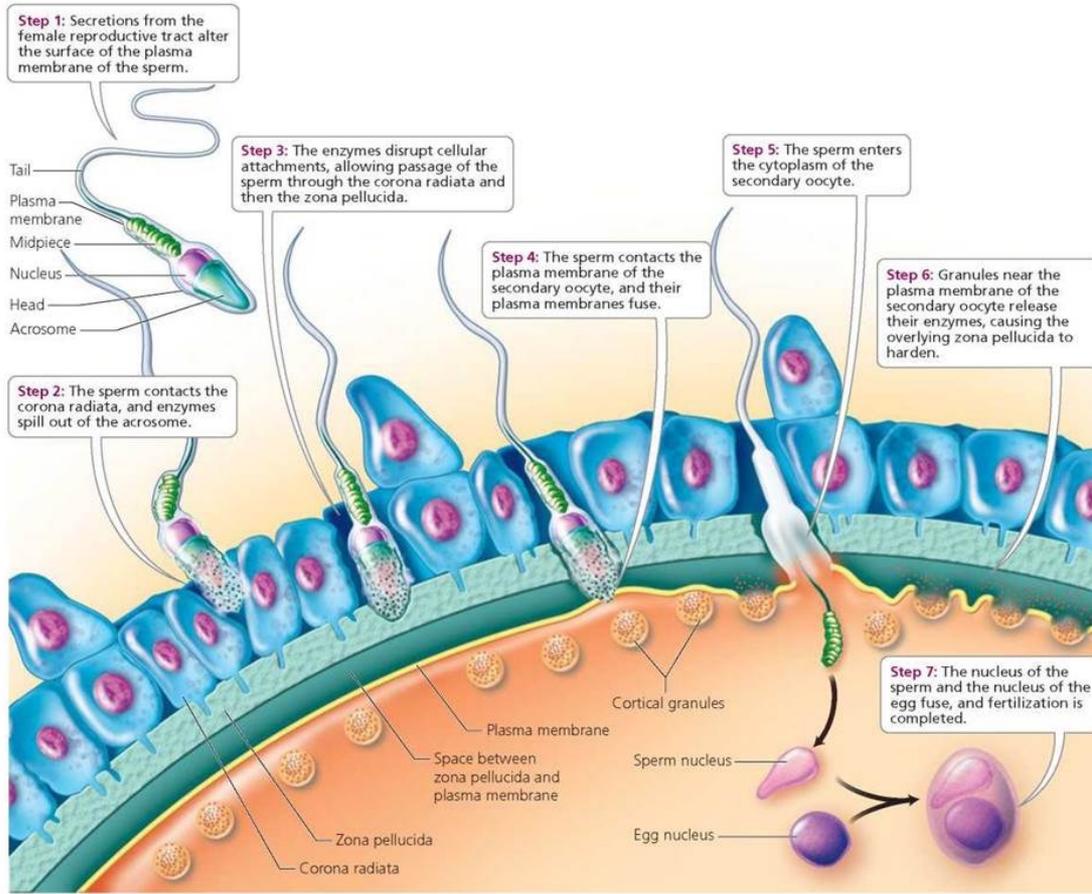
- ASSISTED REPRODUCTIVE TECHNIQUES

- The oocyte retrieval (*Pick-Up*);
- In-vitro* insemination;
- Intracytoplasmic Sperm Injection (*ICSI*);
- Assisted zona hatching**;
- Blastocyst biopsy;
- Pre-implantation genetic diagnosis and embryo screening;
- Vitrification of oocytes and embryos;

THEROICAL LESSON

ASSISTED ZONA HATCHING

ZONA PELLUCIDA: acellular matrix composed of sulfated glycoproteins (ZP1, ZP2, ZP3)



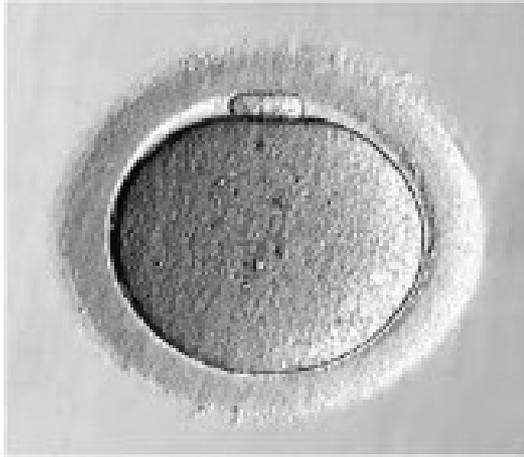
BINDING OF REACTED SPZ
DURING FERTILIZATION

PREVENTION OF
POLYSPERMIC FERTILIZATION

PROTECTION OF THE
EMBRYO
AND INTEGRITY
MAINTENANCE

PREVENTION OF
ECTOPIC PREGNANCY

ZP SIZE AND SHAPE



MII oocyte normal in shape



MII oocyte with a thick and dense ZP



Oocyte with an abnormally shaped ZP and with what appears to be a duplication of the ZP. The oocyte has a regular shape.

ASSISTED ZONA HATCHING

In-vivo

EMBRYO HATCHING: a spontaneous rupture of the ZP that allow the embryo to interact with the endometrial layer of the uterus. *In-vivo*, hatching occurs at blastocyst stage and is due to:

- ❑ CHEMICAL DIGESTION OF ZP (principal hypothesis): lysins from the embryo and/or the uterus are involved;
- ❑ MECHANICAL LYSIS OF ZP (secondary hypothesis): due to contraction and expansion cycles.



In-vitro

IN-VITRO GENERATED EMBRYOS
DEVELOP MORE SLOWLY THAN THE
IN-VIVO ONES;

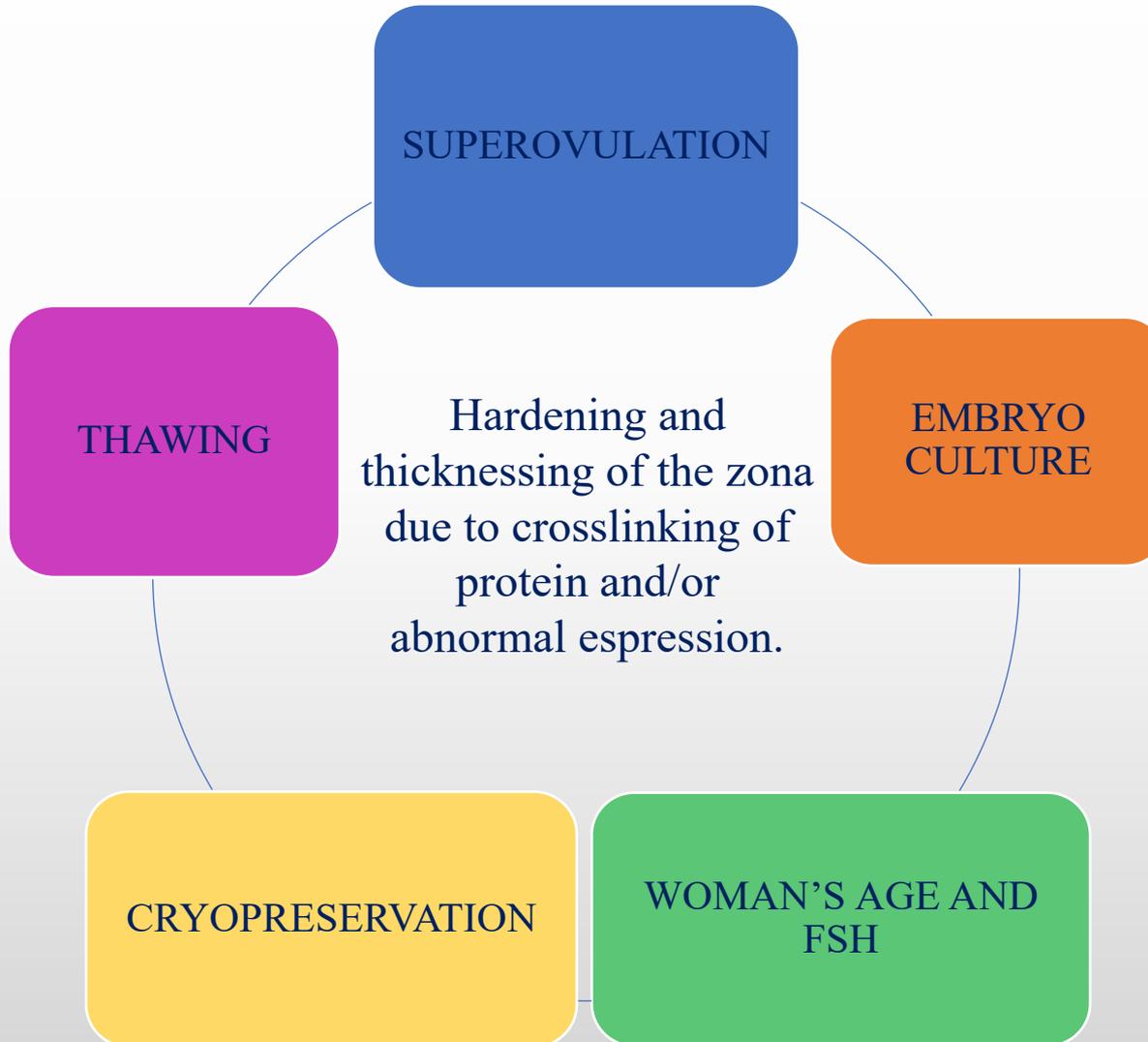
MANIFEST A RELATIVE HIGH
DEGREE OF GENETIC
ABNORMALITIES;

UNDERGO CELL FRAGMENTATION;

HATCH AND IMPLANT AT A LOWER
RATE THAN NATURAL.



ASSISTED ZONA HATCHING

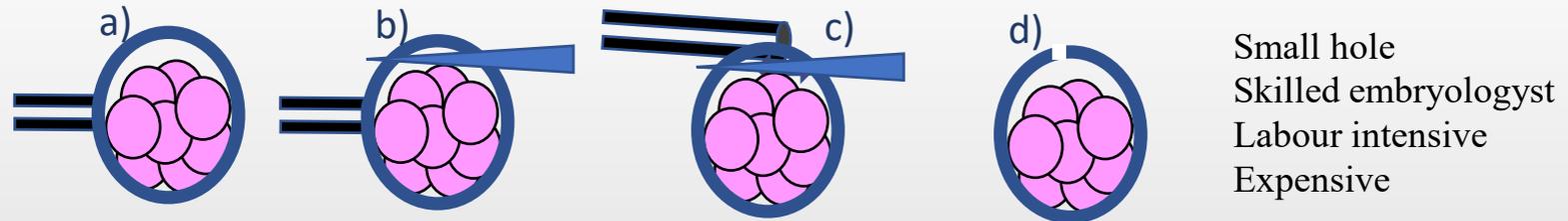


ASSISTED ZONA HATCHING

In order to help the embryo to implant:

1) MECHANICAL PARTIAL “ZONA DISSECTION” a) the embryo is held with a holding pipette and b) the ZP is tangentially pierced with a needle (from 1 to 11 o’clock position). c) the embryo is released from the holding pipette and the part of the ZP between the two points is rubbed against the holding pipette d) until a slit is made in the ZP.

(Cohen et al., 1990)



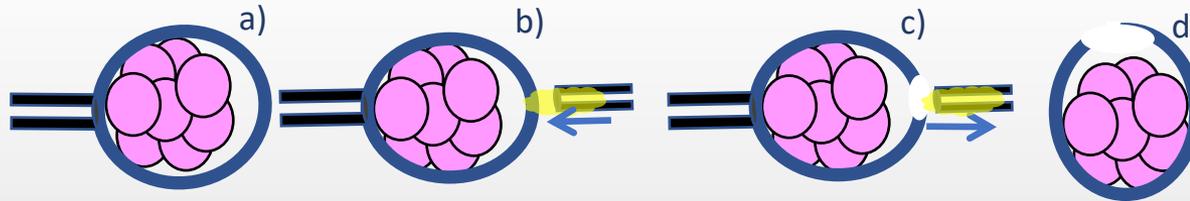
We use this technique for day 3 or day 4 embryos to avoid osmolarity imbalance caused by larger hole

ASSISTED ZONA HATCHING

In order to help the embryo to implant:

2) CHEMICAL “ZONA DRILLING” a) the embryo is held with a holding pipette and b) an acid solution (Tyrode’s pH 2.2 – 2.6) is gently delivered over a small area of the ZP c) as soon as a hole in the ZP is created c) a suction is applied to avoid damage arisen from toxic solution.

(Cohen *et al.*, 1992)



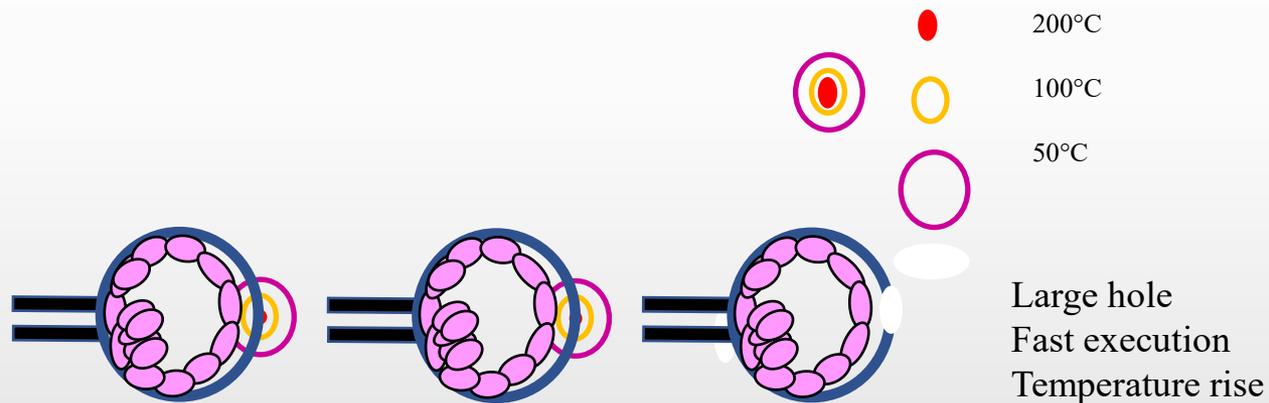
Large hole
Skilled embryologist
Labour intensive
Expensive
Toxic effect

We never use this technique due to low pH solution toxicity to the embryos

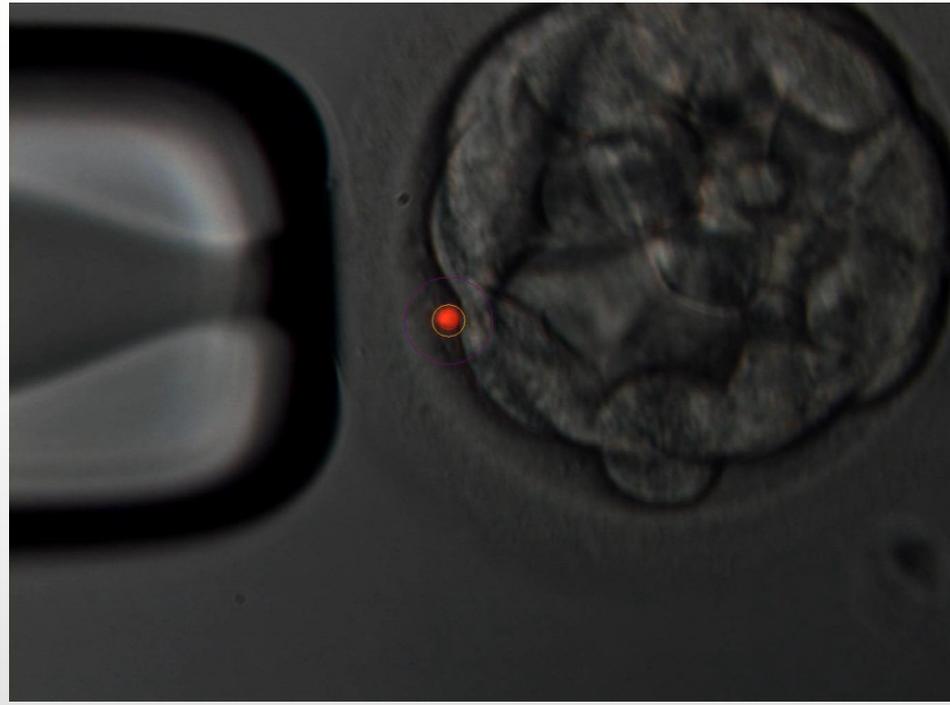
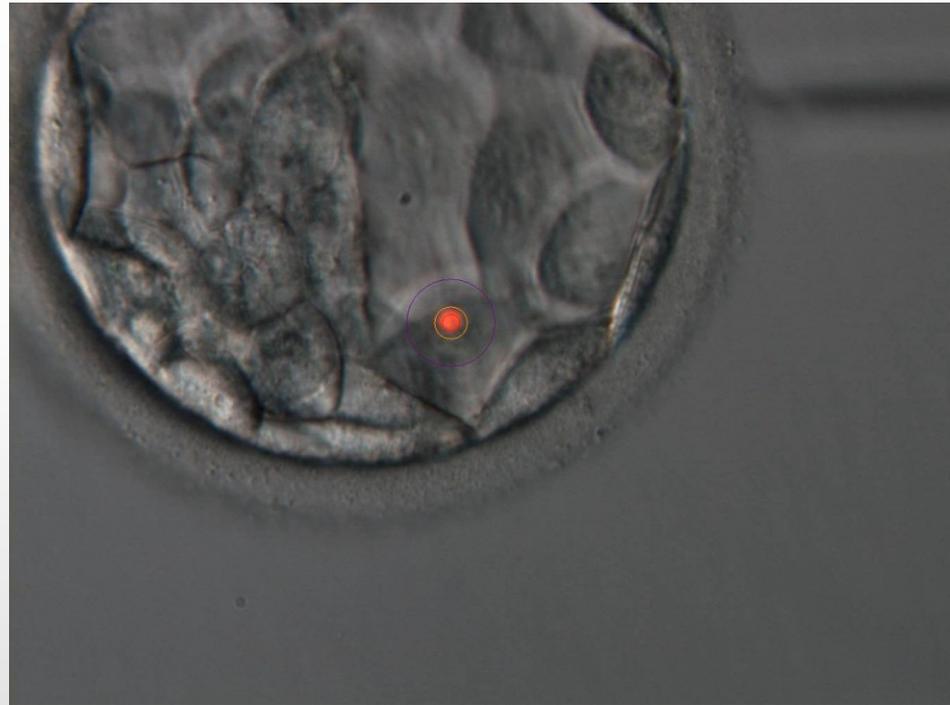
ASSISTED ZONA HATCHING

In order to help the embryo to implant:

3) “LASER ASSISTED” HATCHING a) the embryo is held with a holding pipette and b) four 200 – 450 μ s impulses are applied until a hole in the ZP is made. (*Obruca et al., 1994*)



LASER ASSISTED HATCHING



MAIN TOPICS

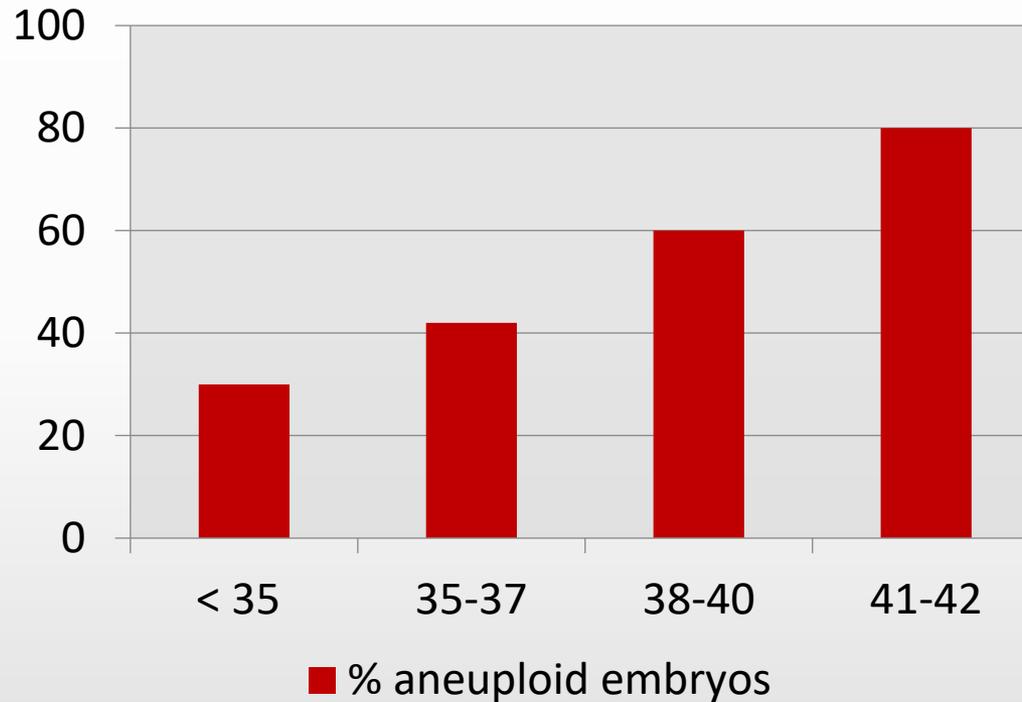
- ASSISTED REPRODUCTIVE TECHNIQUES

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- Pre-implantation genetic diagnosis and embryo screening;
- Vitrification of oocytes and embryos;

THEROICAL LESSON

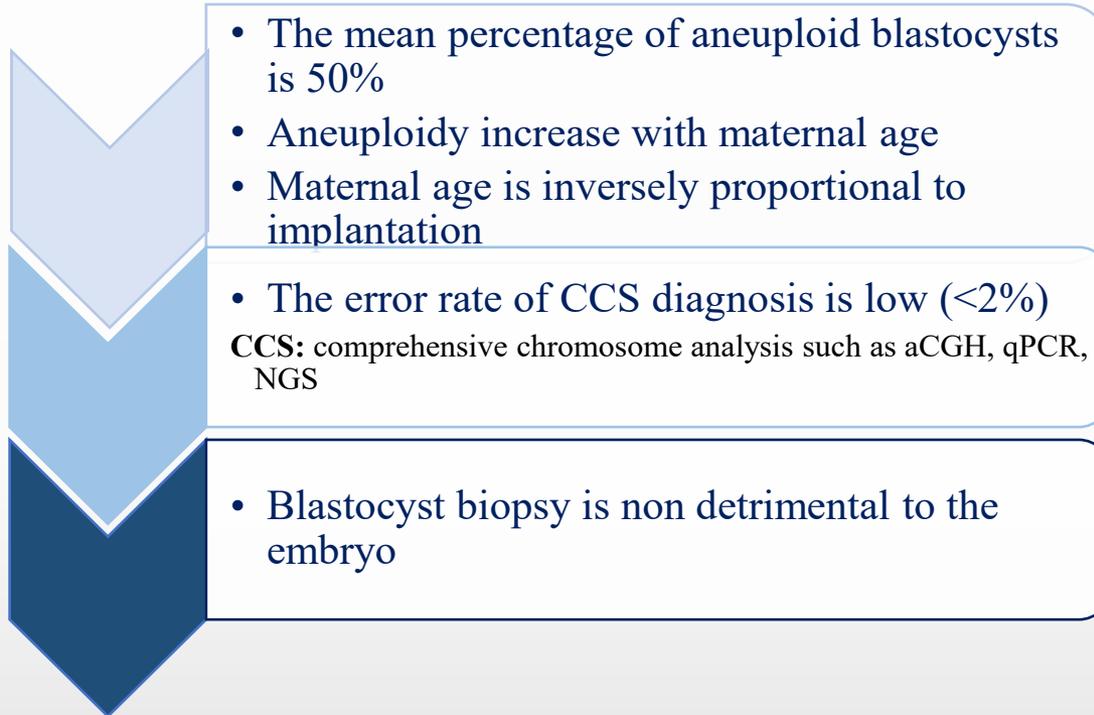
BLASTOCYST BIOPSY

MOST LOSS OF IMPLANTATION IS CAUSED BY CHROMOSOME ABNORMALITIES



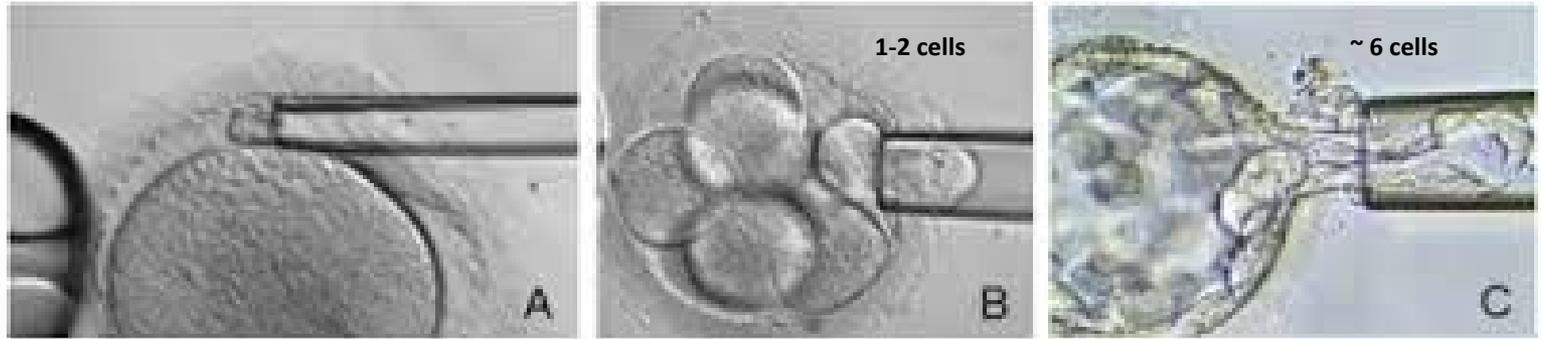
2014. Data from Reprogenetics, our partner in PGS/PGD >19000 blastocysts analyzed.

BLASTOCYST BIOPSY



Genetic diagnosis in blastocyst embryo improve implantation rates and eliminate maternal age effect on implantation (miscarriage; birth defects)

WHEN TO BIOPSY



EFFECT OF DAY 3 AND BLASTOCYST BIOPSY

Implantation rate	<i>cleavage stage</i>		<i>blastocyst stage</i>		
	biopsy	not	biopsy	not	
	31%	53%	54%	41%	

BLASTOCYST BIOPSY



- Not all embryos reach the blast stage and not all the same day
- 4,5% monozygotic twins after hatching

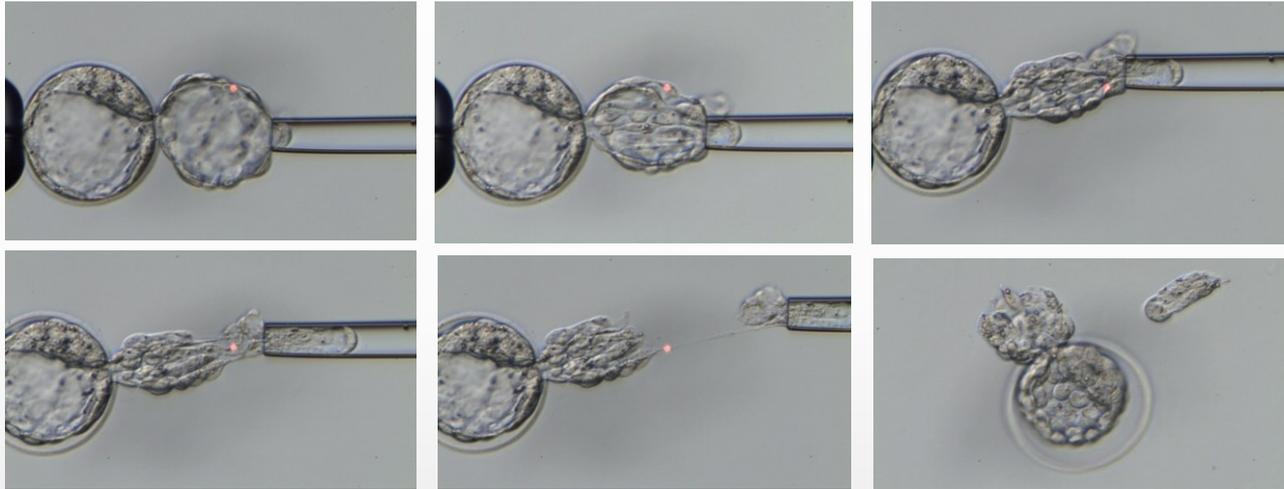
DISADVANTAGES:

ADVANTAGES:

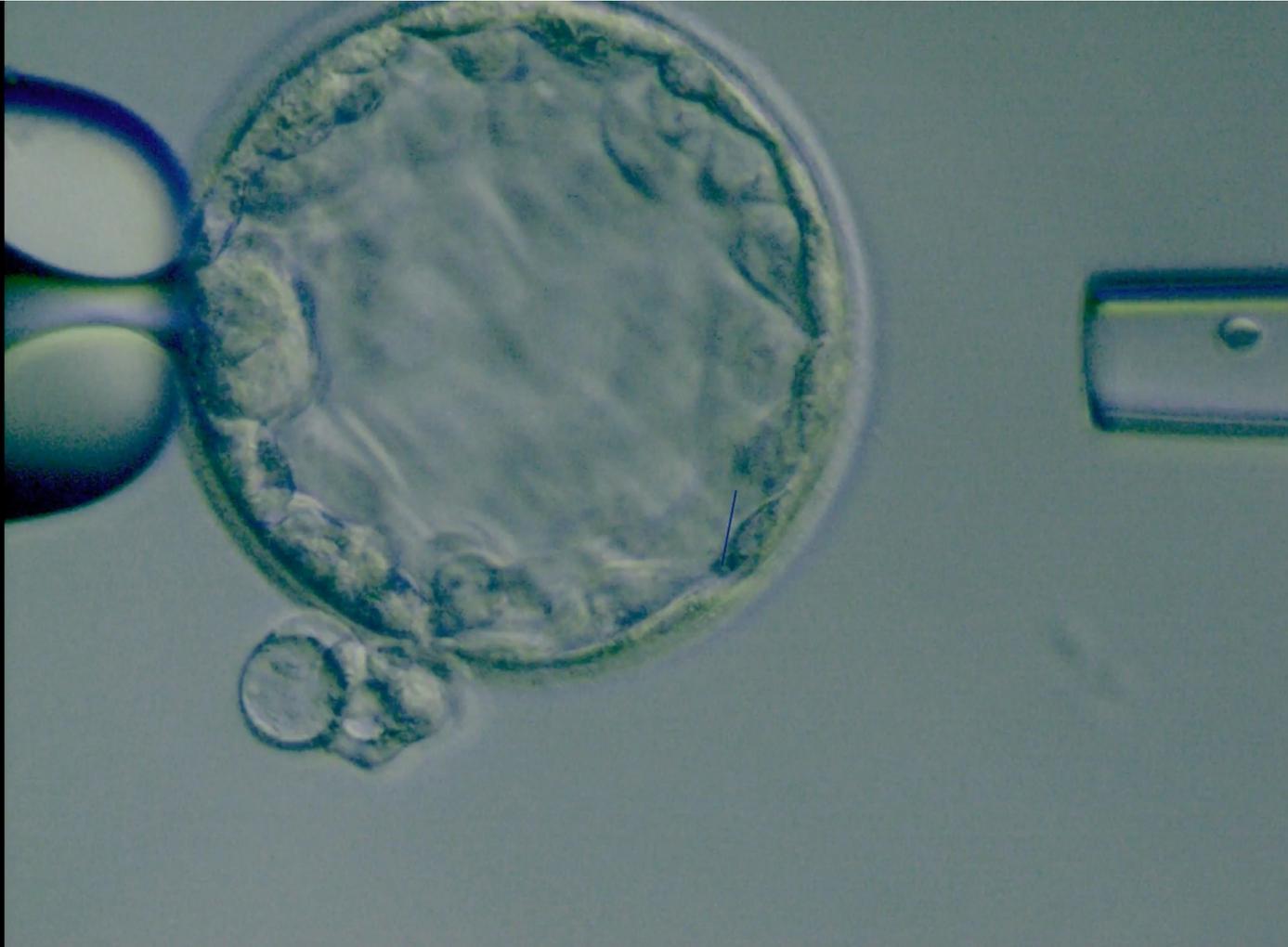
- More DNA: less NO results
- Less mosaicisms = low error rate
- Reduced impact of embryo biopsy
- Less embryos to process (only blasts!)
- Uterine environment optimized after thaw
- The trophoectoderm is representative of ICM (97%)

BLASTOCYST BIOPSY

The “PULLING” method: ideal for hatching blastocyst

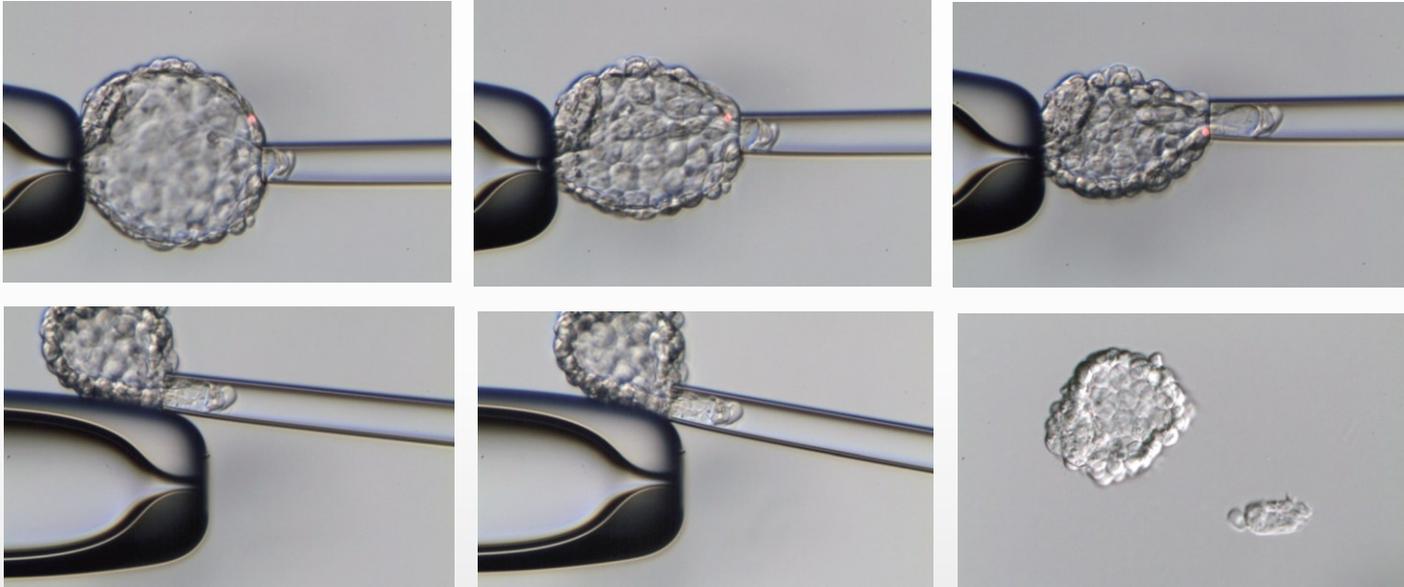


BLASTOCYST BIOPSY



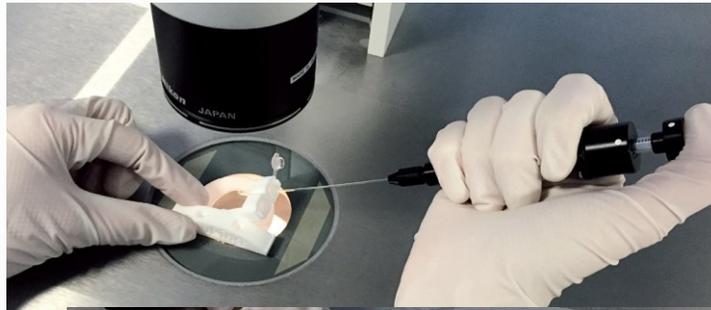
BLASTOCYST BIOPSY

The “FLICKING” method: ideal for fully hatched blastocyst



<https://www.youtube.com/watch?v=K2AX3zIYpuE>

BLASTOCYST BIOPSY



TUBING



<https://www.youtube.com/watch?v=zhBxyEVEkIs>

050- 2.34

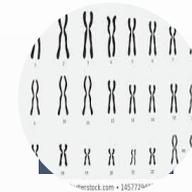
MAIN TOPICS

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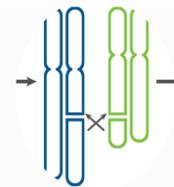
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THEROICAL LESSON

PREIMPLANTATION GENETIC TESTING



PGT-A



PGT-SR



PGT-M

PGT-A

Preimplantation Genetic Testing for Aneuploidy

Simultaneous testing of the entire set of chromosomes of an embryo on the day 5 of development by *aCGH* (*array Comparative Genomic Hybridization*) on microchips or by *NGS method* (*Next generation sequencing*) before the embryo is transferred into the uterus.

Indications:

- Repeated failed IVF cycles;
- Pregnancy occurs, but then repeatedly interrupted or stopped (Repeated miscarriages);
- Age after 40 years (Advanced maternal age).

In such cases, embryos with an altered chromosome set often develop, which are not able to be implanted into the uterus or stop in their development in the early stages of pregnancy.

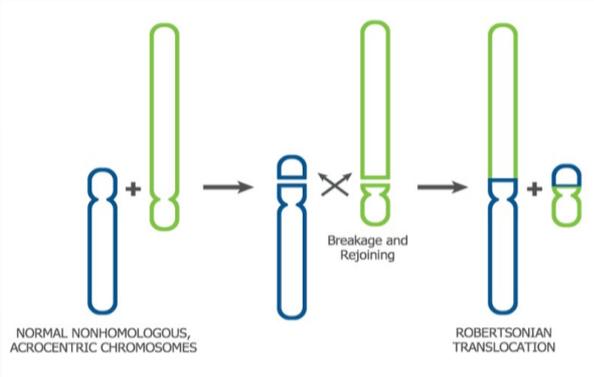
PGT-A is carried out:

to screen the entire set of chromosomes for abnormalities;
to improve IVF results;
without personalized test preparation.

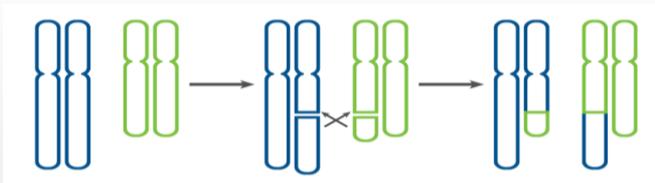
PGT- SR

Preimplantation Genetic Testing for chromosomal Structural Rearrangements

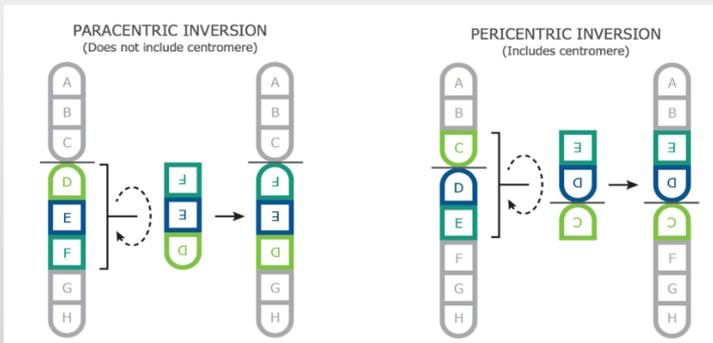
Robertsonian translocations



Reciprocal translocations



Inversion



Chromosomal rearrangements are changes from the normal size or arrangement of chromosomes.

Indications:

- Changes in the parental karyotype
- Child or pregnancy with a chromosome rearrangement

The majority of rearrangement cases:

Require no extra test preparation

Require no extra family member testing

PGT-M

Preimplantation genetic testing for monogenic/single gene diseases

The examination of monogenic hereditary diseases (which appeared because of changes in a single gene).

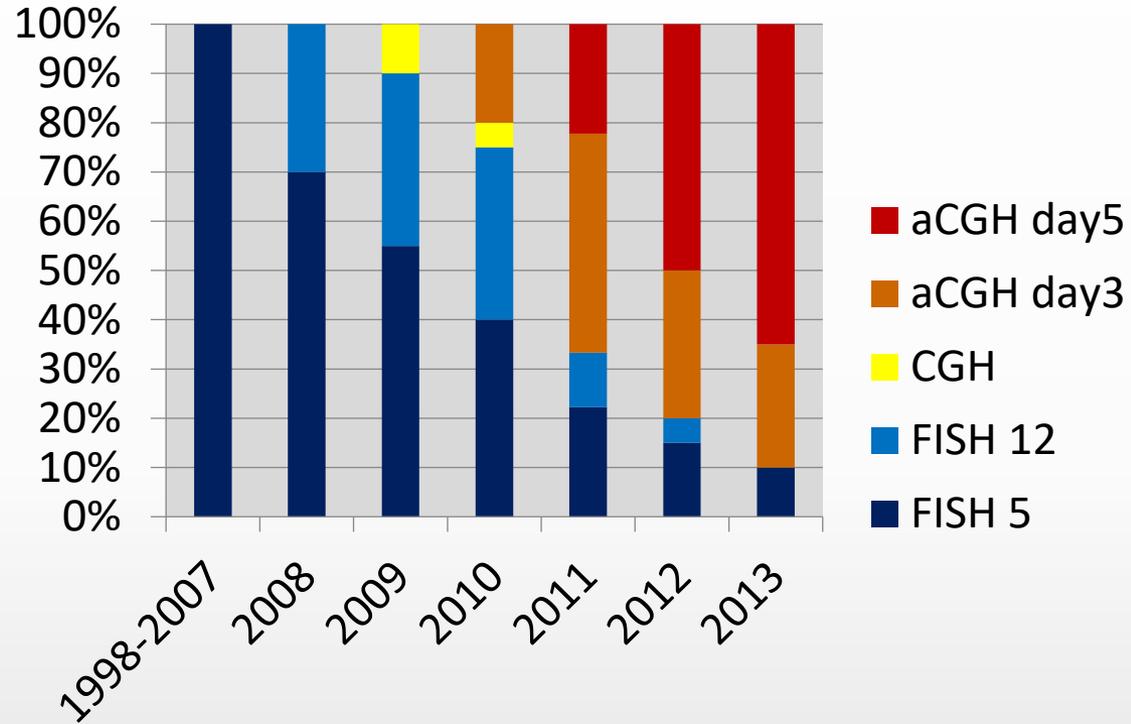
Indications:

- Confirmed monogenic hereditary diseases, which are transmitted from generation to generation;
- A monogenic disease has already been detected in one of family children

(PGT-M) is carried out:

- to reduce genetic disorder risk;
- to check for monogenic hereditary diseases;
- with personalized test design & preparation.

PGT EVOLUTION



2014. Data from Reprogenetics, our partner in PGS/PGD >19000 blastocysts analyzed.

TABLE 1
A comparison of current preimplantation genetic screening platforms for comprehensive chromosomal screening.

Characteristics	qPCR	aCGH	SNP array	High resolution NGS
Total independent data signals ^a (reads per sample)	96	2,700	32,000	700,000
Resolution in million megabytes	20	6	6	3
Misdiagnosis of aneuploidies (4, 9, 12, 13, 15)	1%	2%	2%	0
Unbalanced translocations (16)	No	Yes	Yes	Yes
Partial aneuploidies	No	Yes	Yes	Yes
Polyploidy	No	No	Yes	Yes
Percent mosaicism detectable (17, 18, 19)	No	40%–60%	No	20%–80%

Note: aCGH = array comparative genomic hybridization; NGS = next generation sequencing; qPCR = quantitative polymerase chain reaction; SNP = single nucleotide polymorphism.

^a Number of reads per run × number of samples per run × percent of reads lost = number of reads per sample.

Friedenthal. NGS increases ongoing PRs. Fertil Steril 2017.

Next generation sequencing is the newest platform for PGT, which performs high throughput and high resolution sequencing by synthesis.

It can assess: aneuploidy of full chromosomes with low error rates, unbalanced translocations, segmental aneuploidies, some triploidies(20), and lower levels of mosaicism

THE MOSAIC EMBRYO

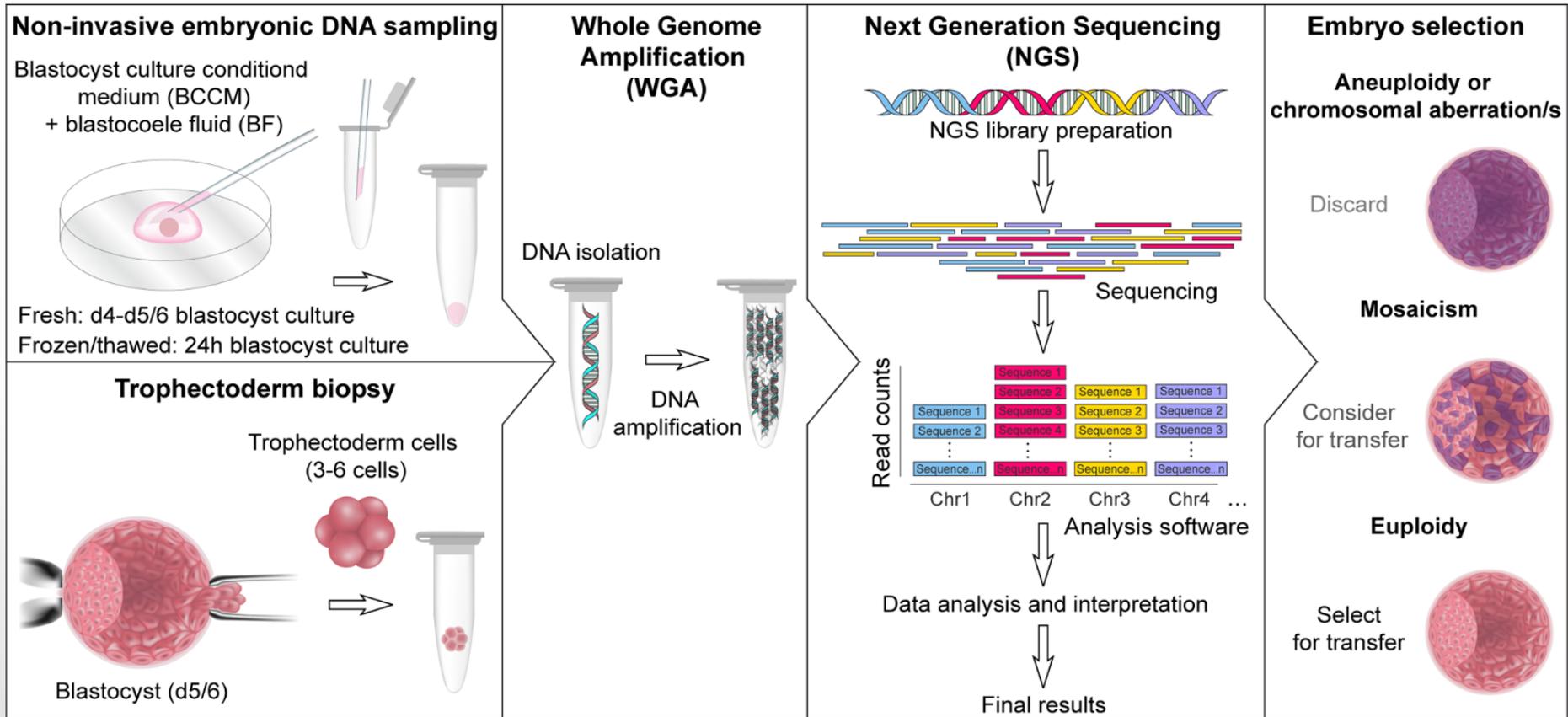
Mosaicism is defined as the presence of two or more populations of cells, each with different genotypes, within the same embryo and results from mitotic errors occurring after fertilization

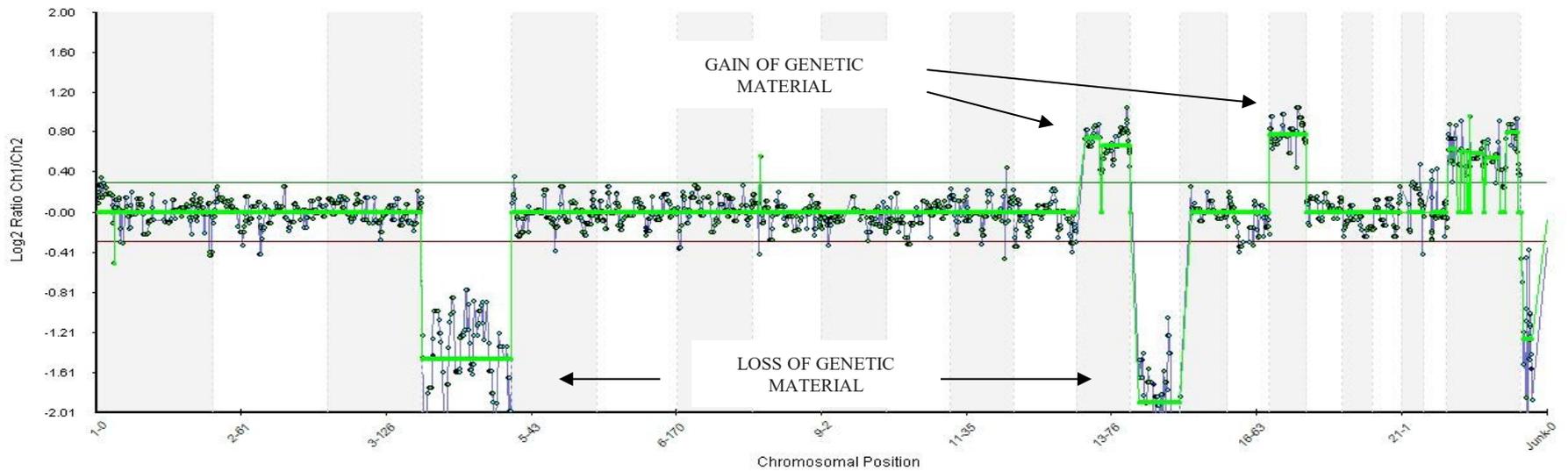
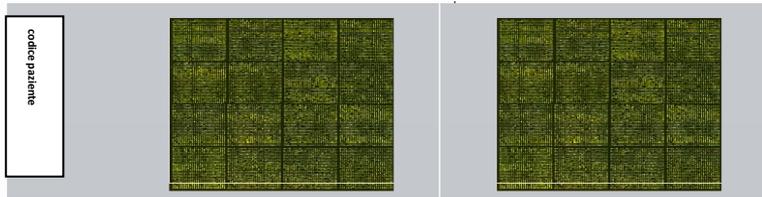


**PGT-A AND
MOSAICISM - THE
GOOD, THE BAD AND
THE UGLY**

<https://blog.vitrolife.com/togetheralltheway/pgt-a-and-mosaicism-the-good-the-bad-and-the-ugly>

TIMING OF THE TECHNIQUE





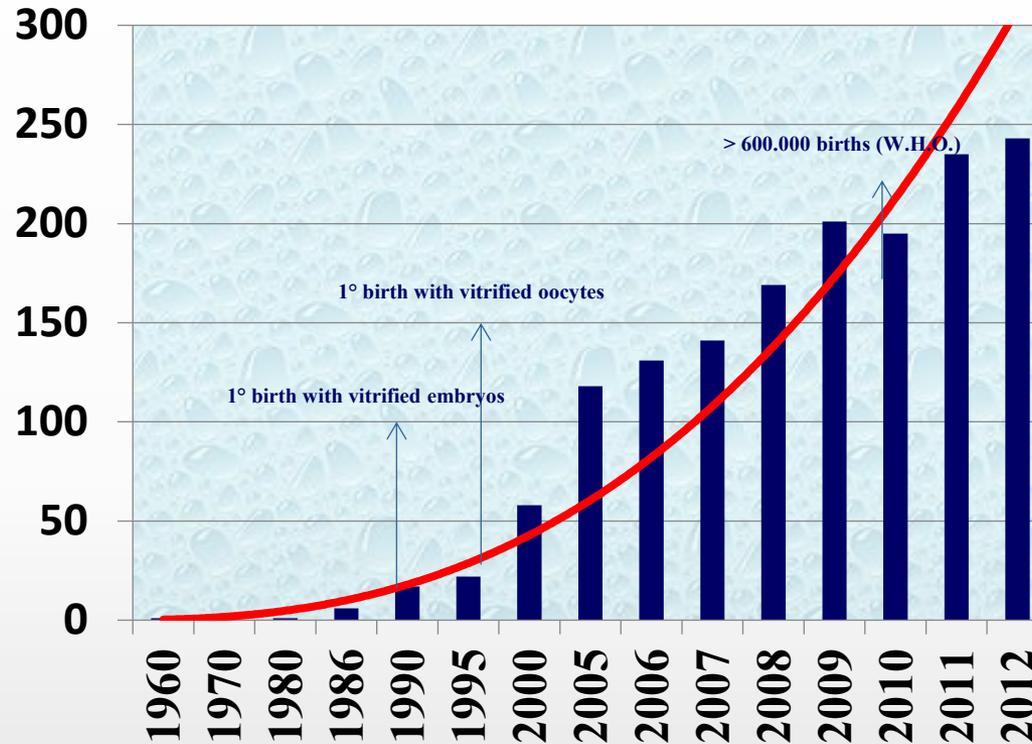
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THEROICAL LESSON

VITRIFICATION



[http://www.ncbi.nlm.nih.gov/pubmed/
?term\(KeyWords\)=oocyte+embryo+vitrification.](http://www.ncbi.nlm.nih.gov/pubmed/?term(KeyWords)=oocyte+embryo+vitrification)

VITRIFICATION

“supercooling”

37 °C Aqueous solution at liquid stage

~~0 °C Aqueous solution at crystalline stage~~

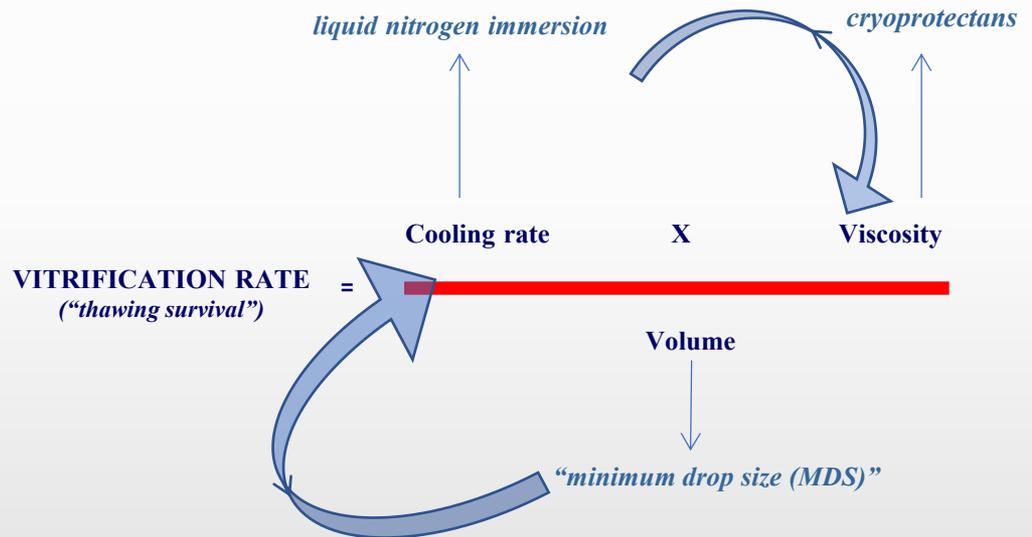
↓ -196 °C Aqueous solution at amorphous stage

“Glass”: liquid with very high viscosity

VITRIFICATION

The successful of vitrification requires a balance of three major properties:

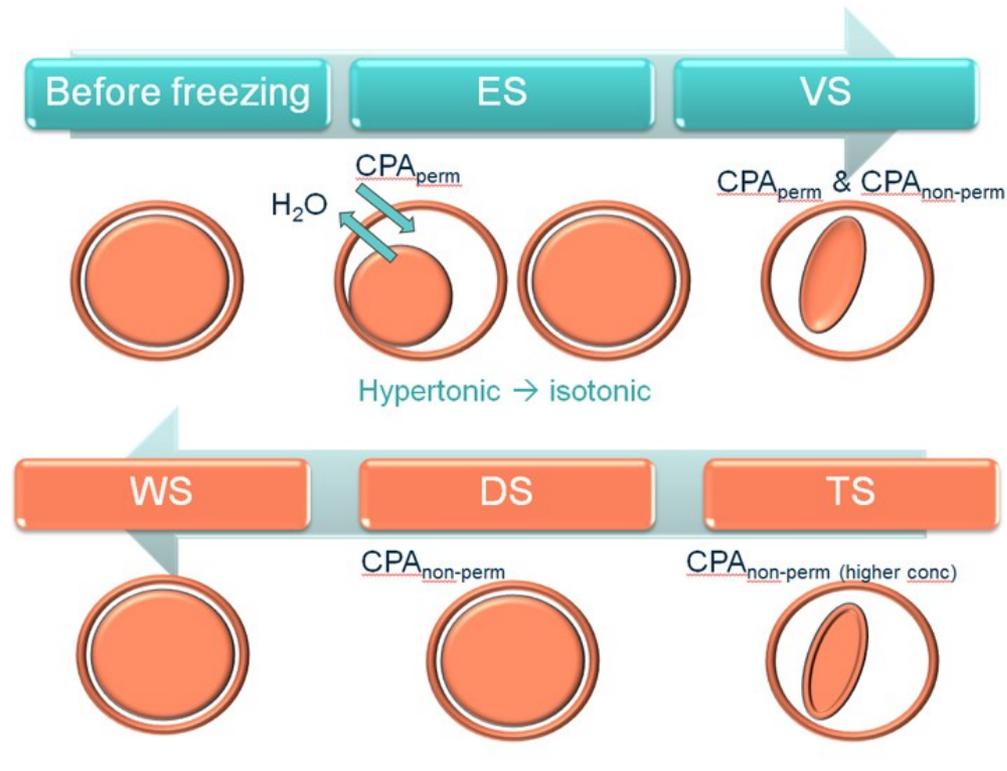
- 1) rapid cooling and warming;
- 2) CPA concentration (to increase viscosity and lower the freezing point of solutions);
- 3) limited media volume on cryodevice.



These three factors together prevent the intracellular crystallization of water!!

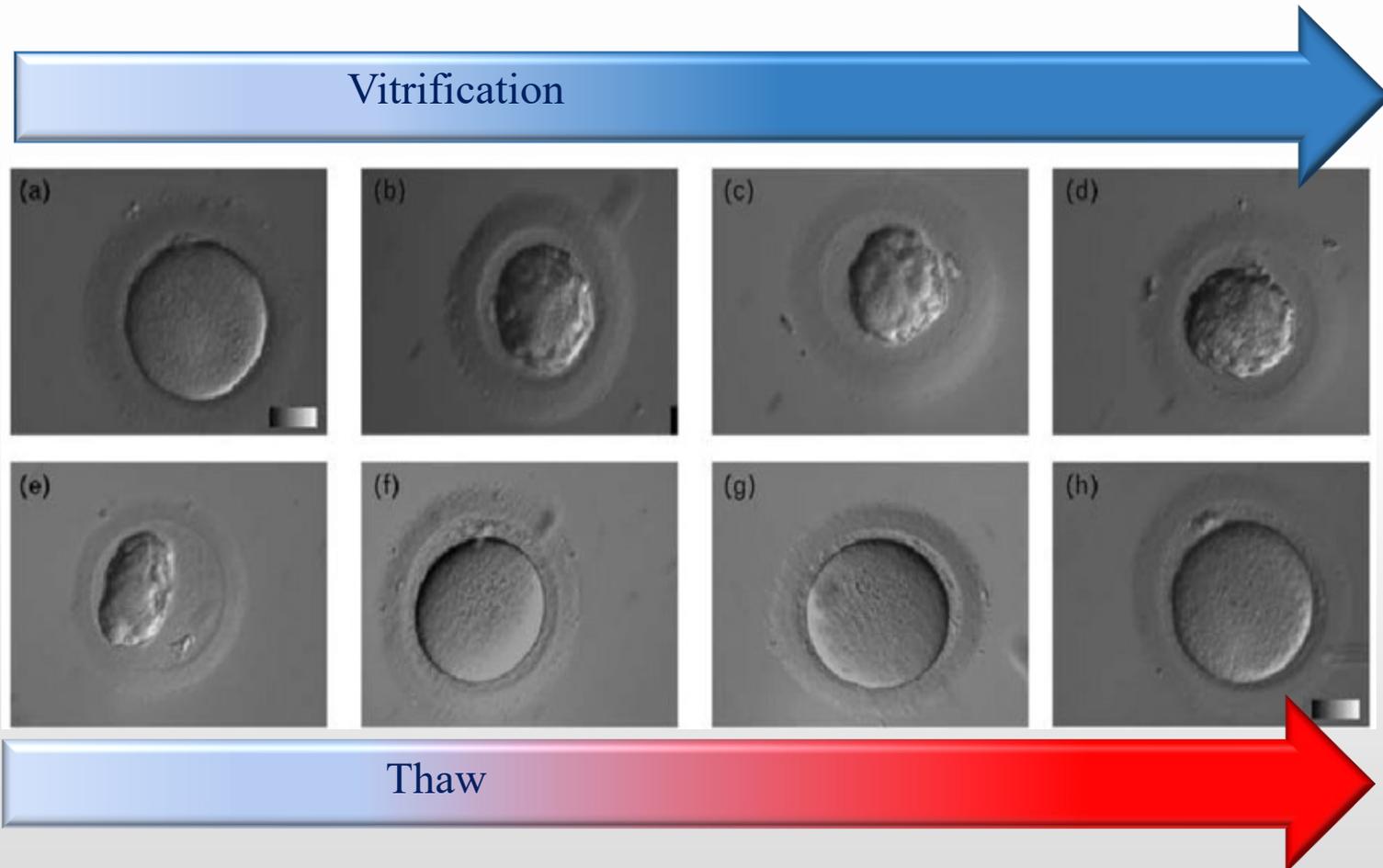
VITRIFICATION AND THAW

During vitrification step, an embryo is exposed to Equilibration Solution (ES) containing CPA_{perm} from 6 to 10 min and undergoes osmotic change (hypertonic \rightarrow isotonic state). When osmotic pressure reaches equilibrium, the embryo is placed in Vitrification Solution (VS) containing higher concentrations of CPA_{perm} and 0.5M sucrose ($CPA_{non-perm}$) for 30 seconds to dehydrate, loaded onto a cryodevice with a very small volume of vitrification medium, and plunged into liquid nitrogen within 80 seconds.



In the warming steps, the embryo is rapidly warmed in Thawing Solution (TS) for 1 minute at $37^{\circ}C$ and undergo further dehydration. Stepwise reduction of sucrose concentrations from 1M in TS to 0.5M in Dilution Solution (DS) induces rehydration of the cells while providing an osmotic buffer. In Wash Solution, the embryo is pre-equilibrated before transferring to culture medium

OOCYTE VOLUME CHANGES DURING VITRIFICATION AND THAW.



(a) De-cumulated oocyte before cryopreservation. (b)-(e) Oocyte undergoing vitrification. (f)-(g) Oocyte during warming phase of vitrification protocol. (h) Oocyte after cryopreservation. Images courtesy of Herrero et al., 2011.

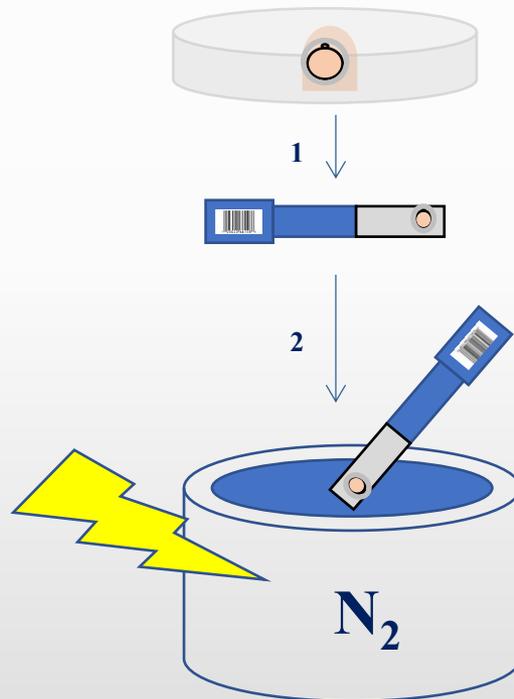
VITRIFICATION & THAWING MEDIA COMPOSITION

		CPA _{perm}		CPA _{non-perm}
Solution		DMSO (v/v)	EG (v/v)	Sucrose
Vitrification Kit	Equilibration solution (ES)	7.5 %	7.5 %	0
	Vitrification Solution (VS)	15 %	15 %	0.5M
Thawing Kit	Thawing Solution (TS)	0 %	0 %	1.0M
	Dilution Solution (DS)	0 %	0 %	0.5M
	Washing Solution (WS)	0 %	0 %	0

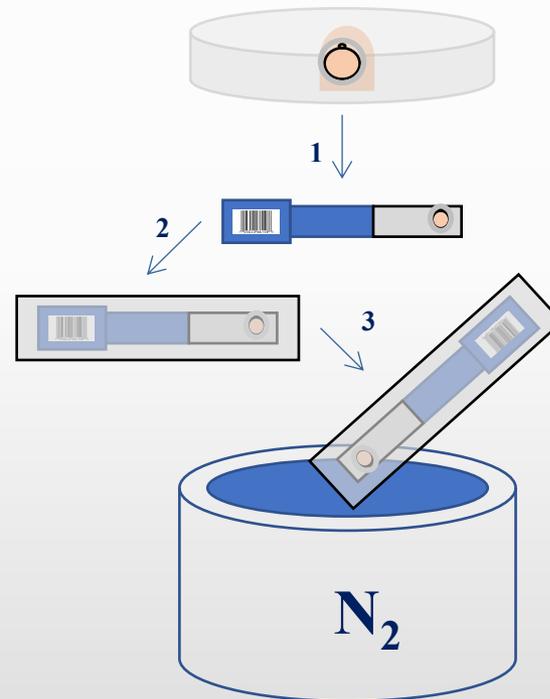
Irvine Scientific Vit Kit® – Freeze and –Thaw are supplemented with 20% DSS and 35 µg/mL gentamicin.

VITRIFICATION DEVICES

A) OPEN SYSTEM

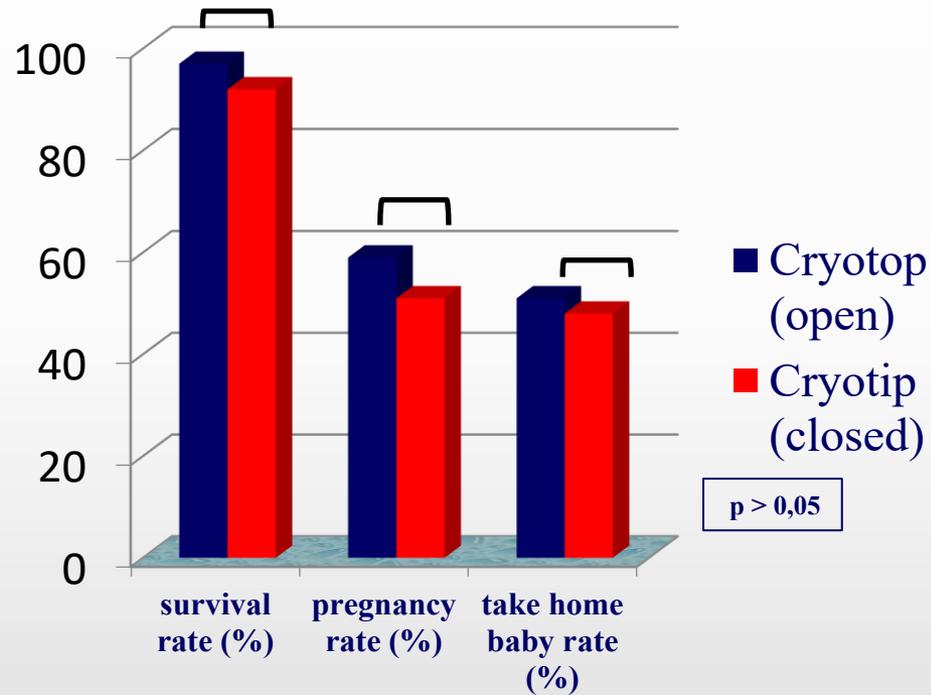


B) CLOSED SYSTEM



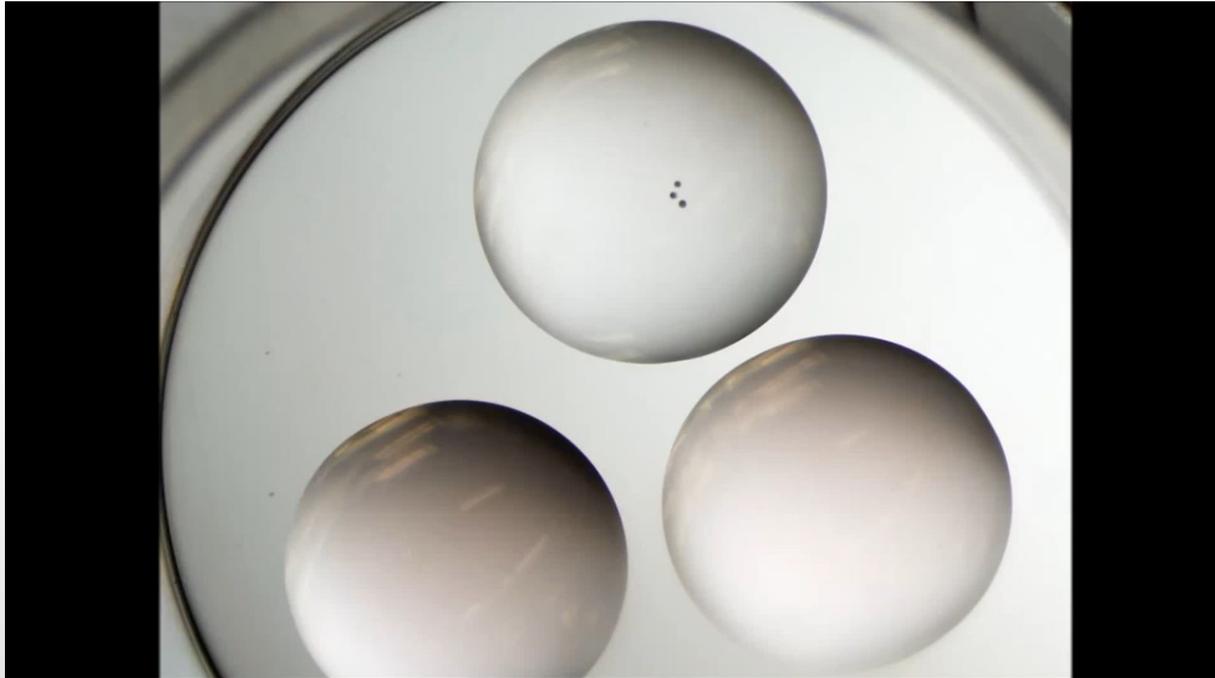
VITRIFICATION

OPEN SYSTEM VS CLOSED SYSTEM



Kuwayama et al. "Comparison of open and closed methods for vitrification of human embryos and the elimination of Potential contamination". 2005. *RBM Online*, Vol.11, N.5 pp. 608-614.

VITRIFICATION PROTOCOL



VITRIFICATION & THAWING PROTOCOL

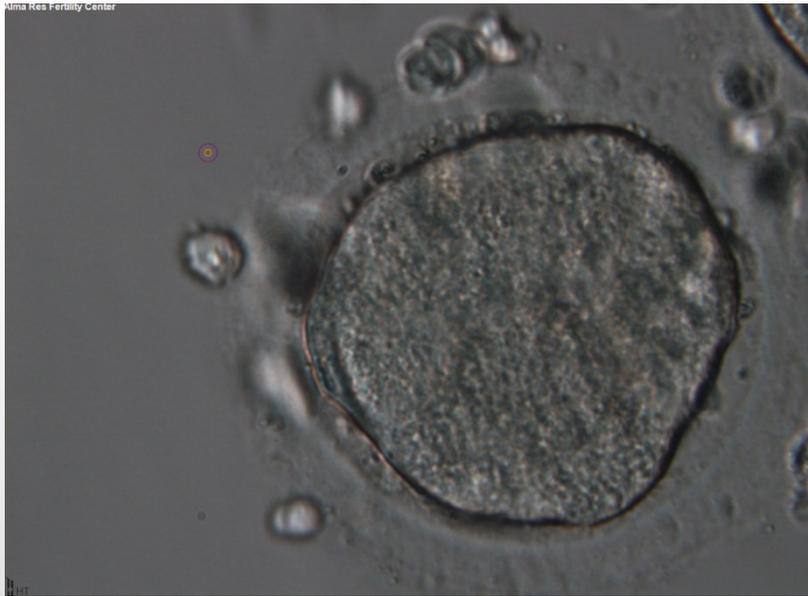
Kitazato Cryotop® Oocyte Vitrification+Thawing - Open System

<https://www.youtube.com/watch?v=TUPfZhP64IA>

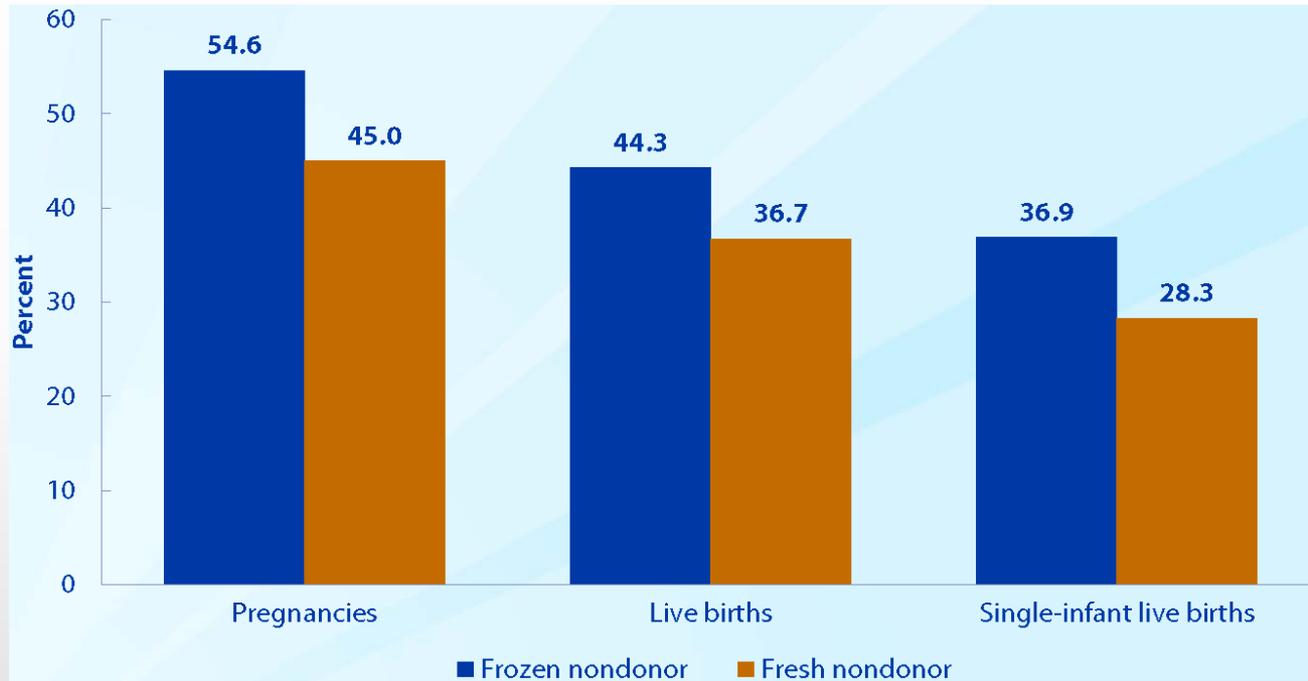
Kitazato Cryotop® Embryo Vitrification+Thawing - Open System

<https://www.youtube.com/watch?v=g0m3xK-Zvaw>

VITRIFICATION: When?



FROZEN VS FRESH EMBRYO TRANSFER



VITRIFICATION

FROZEN EMBRYO TRANSFER (FET)

Ovarian hyperstimulation negatively affect the endometrial receptivity during ART treatments.

High levels of E_2 e P_4 induce biochemical and morphologic alterations to the uterus.
(*Simon et al., 1999*)

Oocytes or embryos from the same cohort give better results in recipient patients than in donor patients
(*Simon et al., 1999; Shapiro et al., 2009*)

Vitrified embryos give high percentage regardless embryo morphology rate.
(*D'Angelo et al., 2010; Griesinger et al., 2011*)

