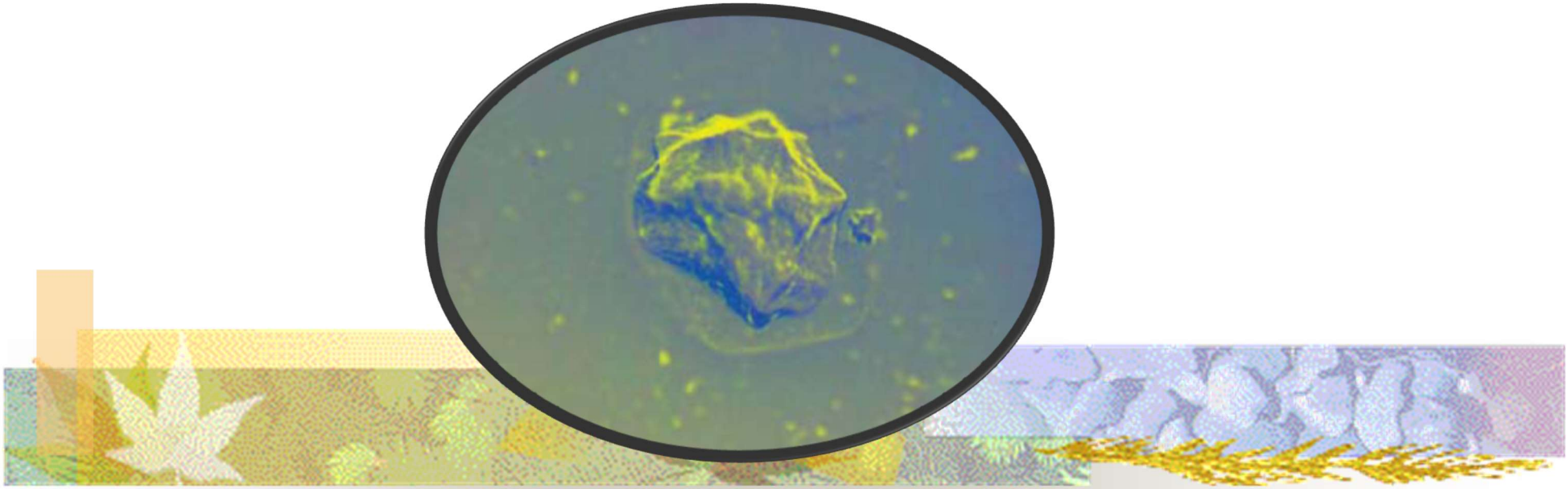
Vitrification in ART



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Two techniques were developed

1 Cryopreservation

(Controlled, slow freezing *(Whittingham et al., 1972)*

2 Vitrification

Ultra-rapid freezing procedure *(Rall & Fahy, 1985)*

- The first successful pregnancy resulting from the transfer of a human embryo that had been cryopreserved by slow cooling was reported in 1983 (Trounson and Mohr, 1983).

- Today, IVF programmes routinely use embryo vitrification to augment cumulative pregnancy rates from a single oocyte retrieval, which rapidly becoming the method of choice (Vajta and Kuwayama, 2006).

cryopreservation

Steps of Cryopreservation

- Equilibration in the cryoprotectant
- Freezing process
- Storage in LN_2
- Thawing (warming) process
- Removal of the cryoprotectant
- Culture in the physiological medium

Factors influencing the success of cryopreservation

1. Possible temperature shocks (+ 15°C or -5°C)
2. Possible changes in the plasma membrane
3. Selection of the right cryoprotectant
4. Dehydration: intensity and time
5. Critical cell volume
6. Solute concentration
7. Cooling rate
8. Thawing rate

Temperature shock

This happened if the cells cooled too fast (also without ice crystalization)

- This shock starts at the plasma membrane due to:
  - * Shrinkage of the different parts of the membrane
  - * Mechanical effect
  - * Reduction of the volume

Characteristics of cryoprotectants

- High soluble in water
- Relative low molecular weight
- Fast cell permeability
- Conjunction with water to built stable H_2 bridges
- With high concentration should be non-toxic
- Reducing the freezing point of the extracellular fluid
- Low influx of the intracellular water to avoid the sudden shrinkage of the cell.

Cryoprotectants

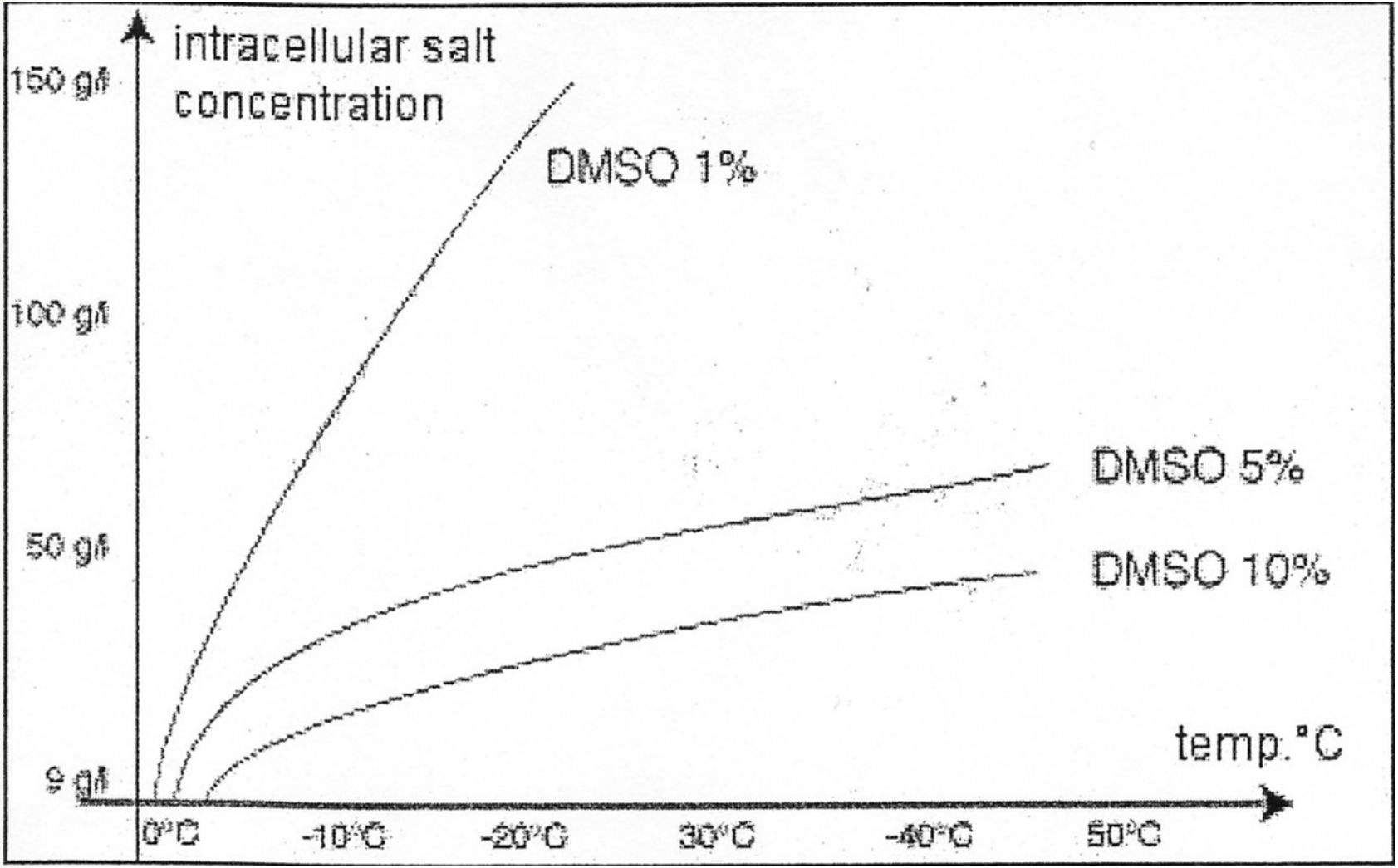
Permeable	Non permeable
Methanol CH_3OH	Polyethylenglycol
Ethanol C_2H_5OH	Polyvinyl pyrrolidone
Ethylenglycol $C_2H_4(OH)_2$	Ficoll
1-2 Isopropanol $C_3H_6(OH)_2$	Sucrose
Glycerol $C_3H_5(OH)_2$	
DMSO (CH_3) SO	

Cryprotectant concentration and solute concentration during freezing (exp. DMSO)

- Isotonic saline solution (9g NaCl/Liter)

replaced by	replaced by	replaced by
1% DMSO	*5% DMSO*	*10%DMSO*
↓	↓	↓
It will reach a concentration of 50g/Liter by		
-5°C	*-20°C*	*-50°C*

DMSO concentration and solute concentration during freezing



Action of the cryoprotectant

Penetration of cryoprotectant in the cell and partially replacing the

↓

intracellular water

↓

dehydration of the cell

Cooling rate

- Avoiding temperature shock
- Avoiding damage effect of the cell during dehydration
- Avoiding damage of the colloidal milieu of the cell

Cooling rate

Optimal cooling rate,

if the cell gives the maximum

amount of the intracellular water to avoid the

intracellular ice crystal formation

Cooling rate

- *Optimal cooling rate is dependent on the critical volume of the cell which can be defined as:*

- The permeability of the cell membrane to the water
- Large membrane surface
- The relation between cell surface to the cell volume according to these phenomenon each cell has its cool rate

Thawing rate

- *The thawing rate is closely related to the cooling rate in general: the fast thawing is preferable*
- Thawing rate has no influence to the slow freezing

The most important principle of the cryopreservation of the oocytes and embryos is:

The formation of ice crystals which should be avoided during the process of freezing of the cells and tissues

vitrification in ART

Physical definition:

Vitrification is solidification of a solution to be similar to the state of the glass

- Vitrification is a process in which liquids solidify without crystallization (Luyet, 1937).

- Compared with the slow cooling procedures, vitrification methods are very rapid.

- Three key factors influence the probability of successful vitrification (Rios et al., 2010):

* Cooling and warming rates
* Composition of the cryoprotectant solution which is reflected in the viscosity of the sample
* Sample volume.

- Increasing the cooling/warming rate (Vajta and Kuwayama, 2006), raising the cryoprotectant content or decreasing the sample volume will each increase the probability of vitrification (Arav, 1992).

- In the past, vitrification was achieved by simply plunging the sample into liquid nitrogen (LN) at -196°C (Rall and Fahy, 1985).

- During this process, heat transfer from the sample into the LN leads to the evaporation of LN around the sample, resulting in the formation of a nitrogen gas layer, which acts as an insulator.

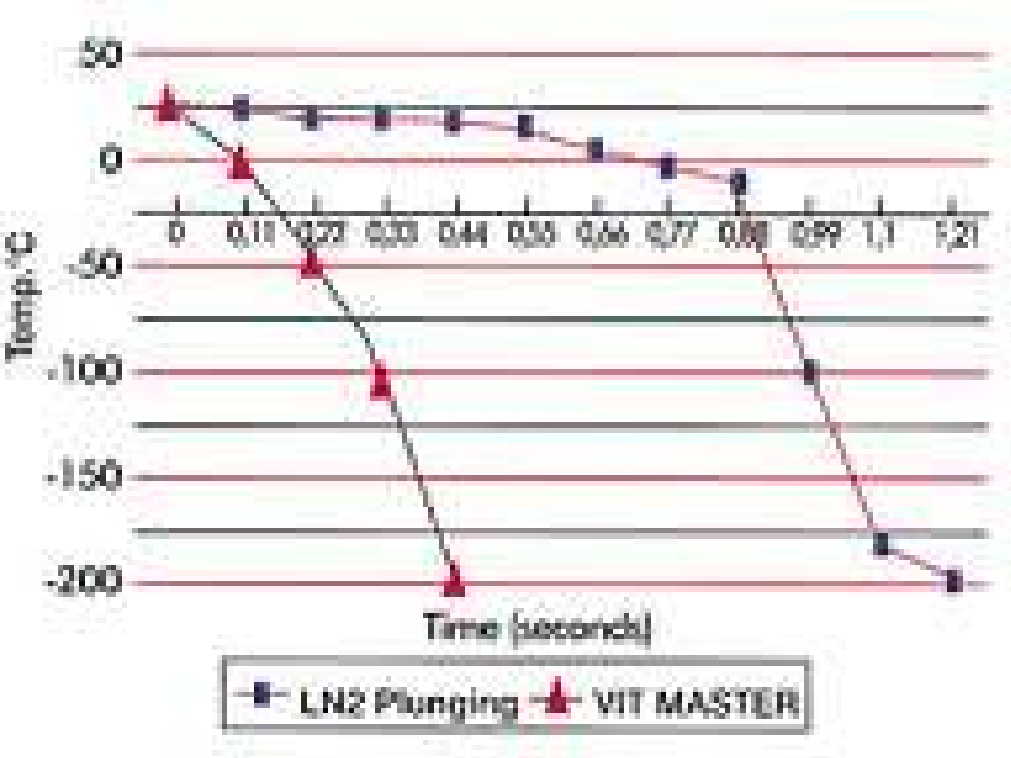
Main principles of the vitrification in ART:

- Guarantee of fertilization (oocyte)
- High survival rate after warming
- Increasing the success rate through a significant
- High cumulative pregnancy rate

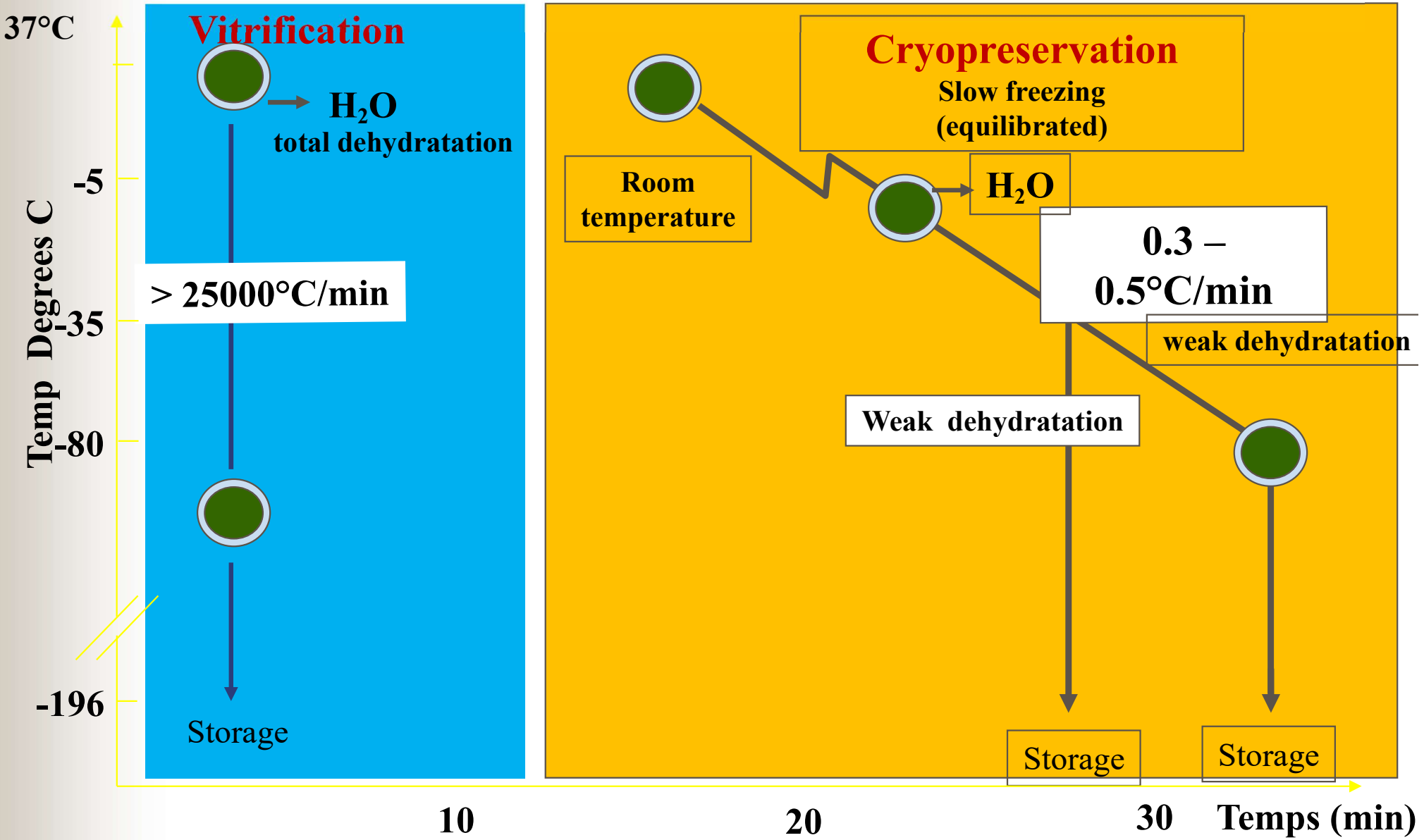
Freezing in the liquid nitrogen LN_2 (Vitrification)

Physical definition:

solidification of a solution to be similar to the state of the glass



Vitrification vs Cryopreservation



Cryopreservation vs. Vitrification

	Cryopreservation	Vitrification
CPA-Concentration	1.5 M	3.0- 5.0 M
Volume	0.3-1.0 ml	< 1 µl
Contact with N_2 & the cell	no	yes
Cooling rate	~ 0.5°C /min	15.000- 50.000 °C/min
Freezing	slow	ultrarapid
Thawing/ Warming	slow	rapid
Time consuming	≥ 180 min.	2 sec.
Dehydration	not controlled	controlled

Terminology

- Instead of Freezing → Vitrification
- Instead of Thawing → Warming

Why we prefer the vitrification-procedure now ?

- There is no mechanical injury (extracellular crystal formation
- Less osmotic stress for the cell
- No intracellular crystal formation
- Less labor in the laboratory daily work
- Simple protocol
- It is useful for cells like oocytes and blastocyst which have less success with slow freezing
- No need for expensive device

Cooling rate and Vitrification (importance)

- High cooling rate needs high concentration of cryoprotectant
- There is a practical limit to achieve high cooling rate which correlates with biological limit of the cryoprotectant of cells during vitrification
- For this reason it is important to find a balance between a maximum cooling rate and a minimum concentration of cryoprotectant.

Example for cooling rates

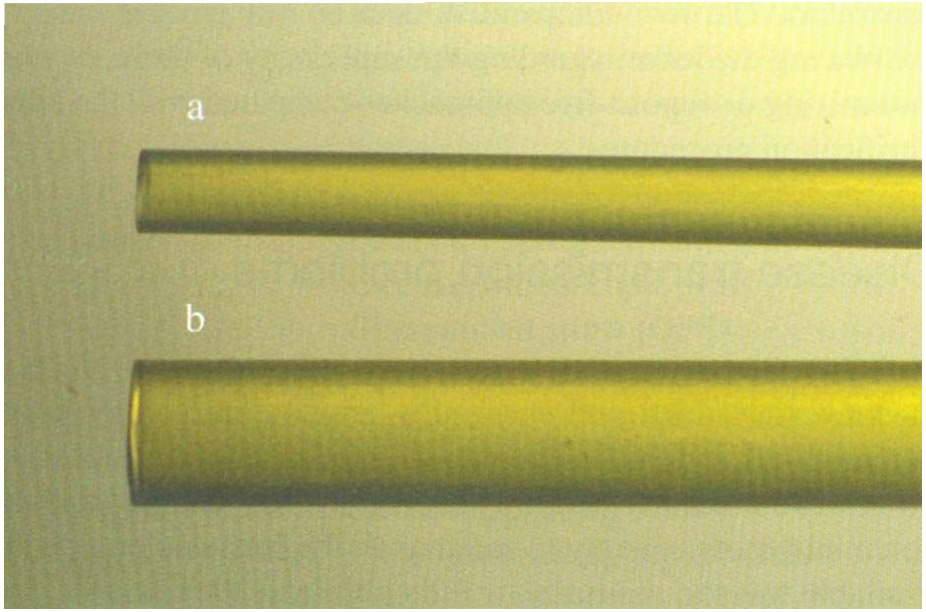
- ~ 2500°C/ min by using 0.25 ml straws. Thick straw and large volume of medium do not allow a high cooling rate and thawing rate

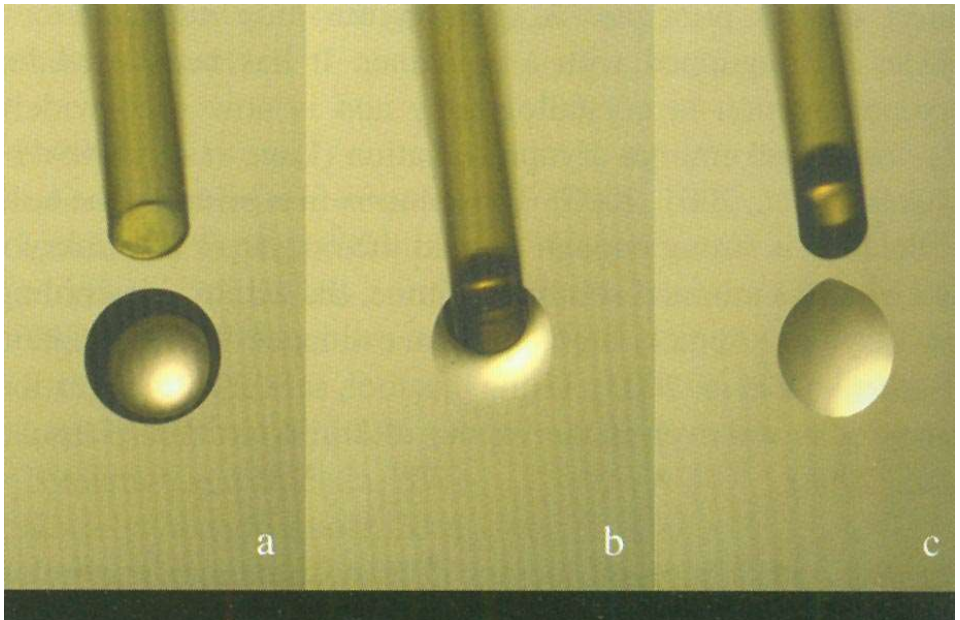
- ~20000-25000°C/min by using a carrier which allows a very small volume which can get a direct contact with LN_2

Cell carrier systems

- Open- pulled straws (OPS) *Kuleshova et al. 1999; Chem et al. 2000*
- Electron microscope copper grid *Hong et al. 1999, Park et al. 2000*
- French ministraws *Vanderzwalmen et al. 2002*
- Flexipet-denuding pipette (FDP) *Liebermann et al. 2002*
- Cryo loop (CL) *Reed et al. 2002*
- Hemi-straw system (HSS) or Cryotop also *Stehlik et al. 2003*

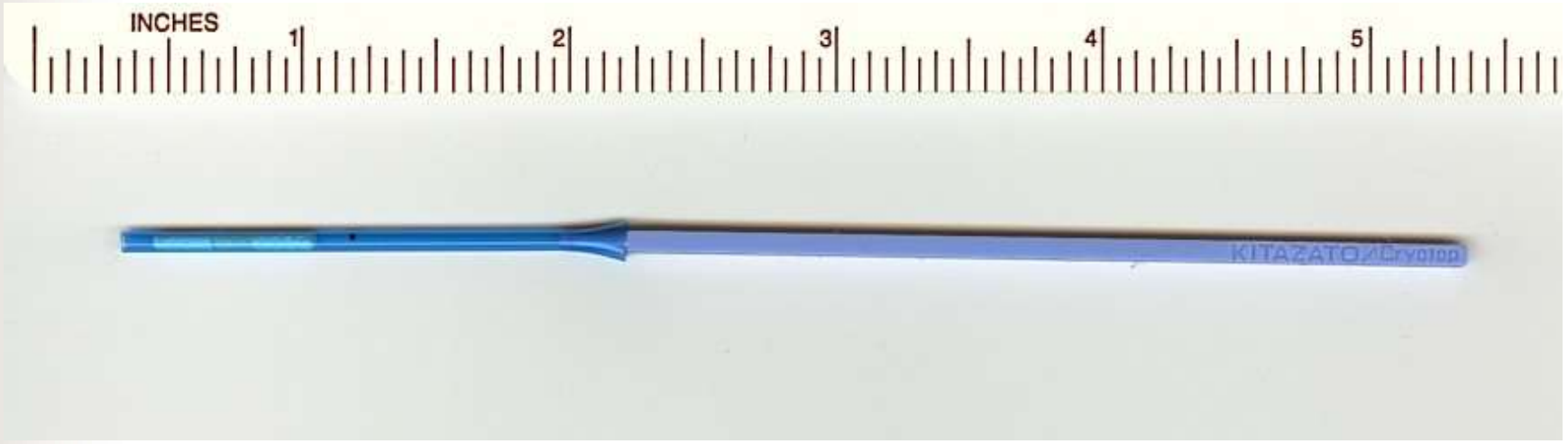
Open- Pulled straws (OPS)

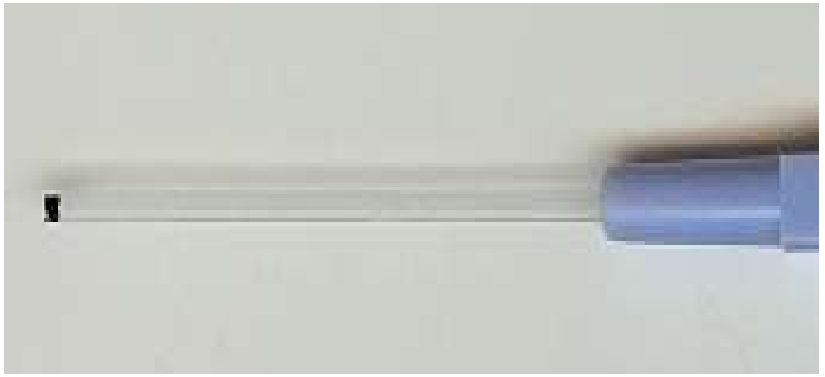




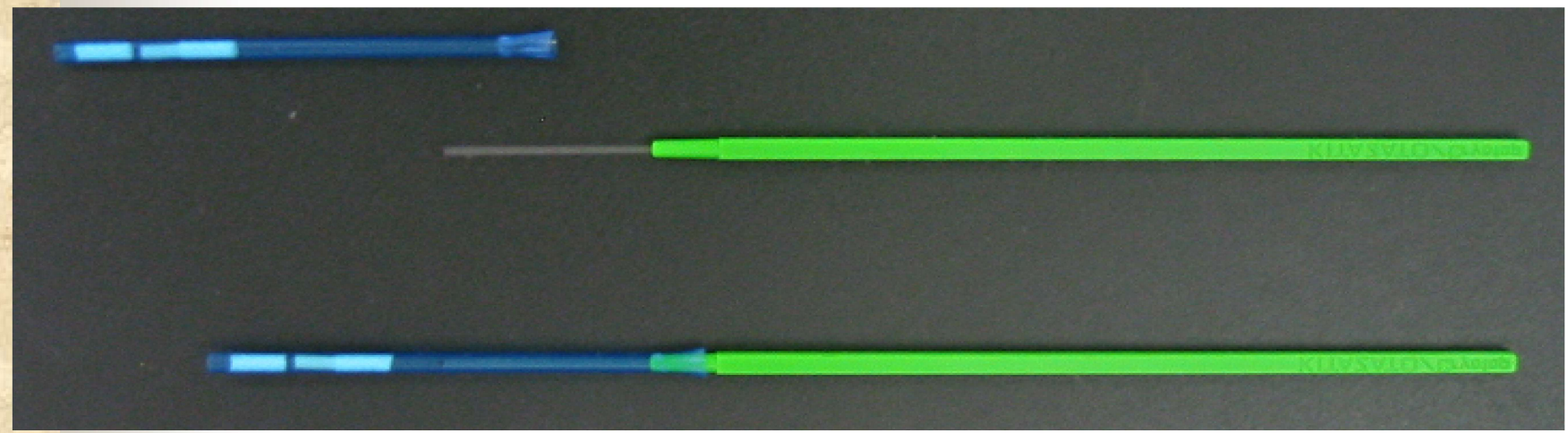
Cryoloop

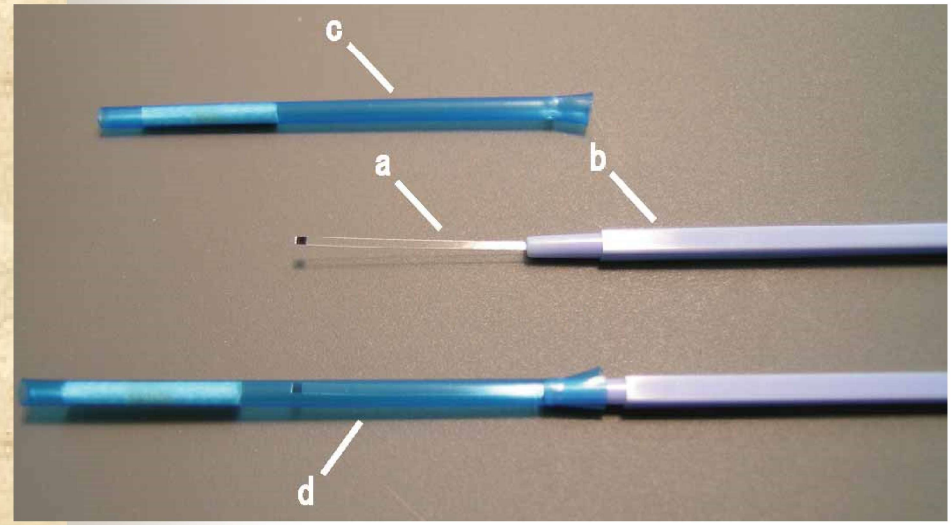
Cryotop





Ultra-rapid Vitrification container: Cryotop





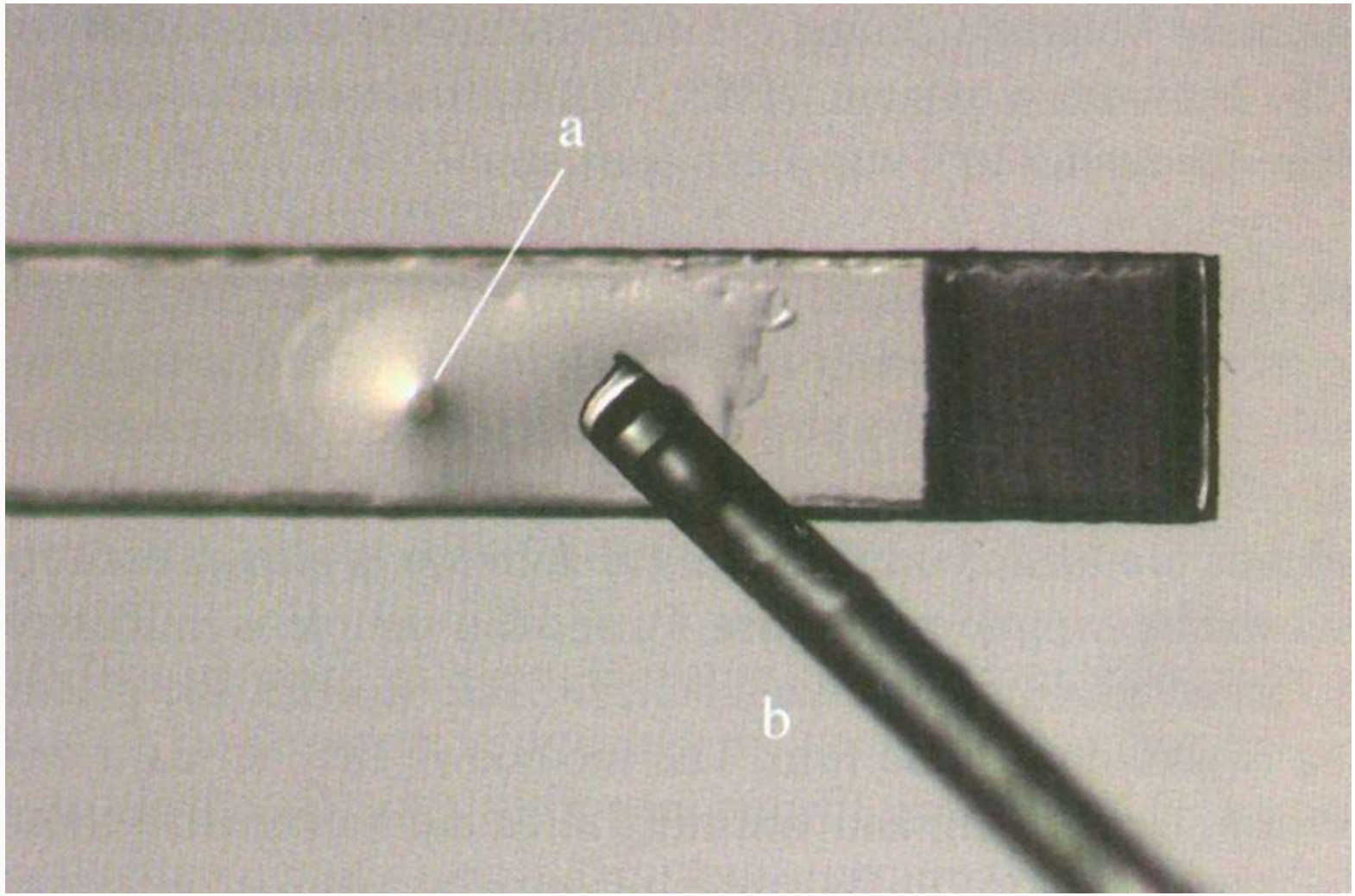
a: Transparent fine Polypropylene sheet (0.8mm x 2cm)

b: Plastic handle

c: Cover cap

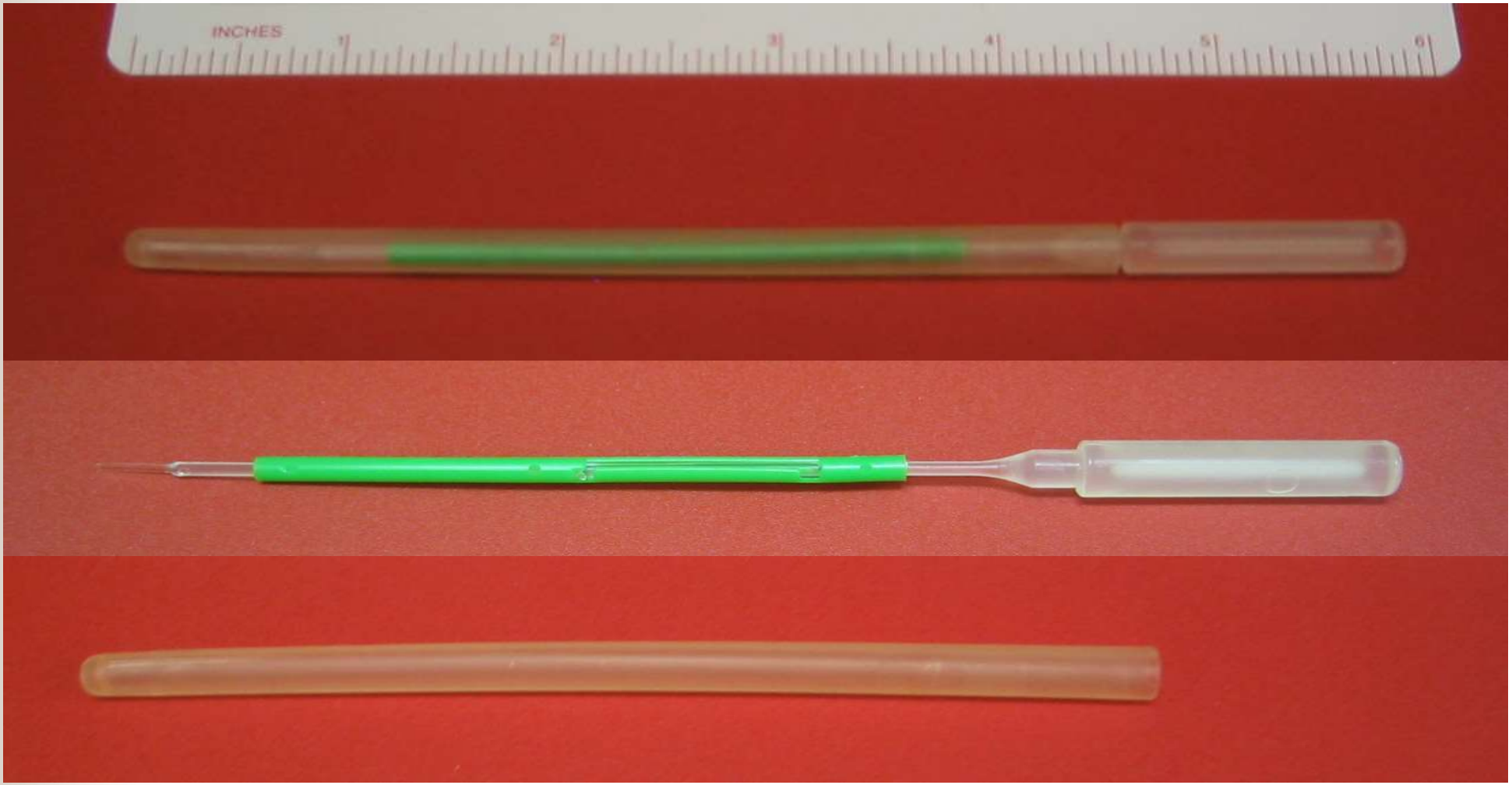
d: Cover Top part during storage in LN2

Cryotop



Cryotip

Cryoleaf (McGill)



Critical side of Vitrification

- Very high cooling rate

- Protection against potentially toxic chemicals

(Vitrification needs high concentration of permeable cryoprotectant to induce a intracellular vitrification)

(which is sometimes biologically and technically difficult and problematic)

- There is a direct contact between the vitrification medium and LN_2 which is eventually a source of contamination.

Solution for Vitrification

- To reach a high cooling rate, special container or carrier should be used
- To minimize the toxicity of the cryoprotectant, at least two different types of cryoprotectant should be used in a stepwise incubation and two different concentration *(lower-strength and full-strength)*
- LN_2 as a source of contamination ?

Contamination during LN_2 storage

- Virus and bacteria can survive the LN_2 at -196°C like Hepatitis- and HIV-virus ?
- Extra tank for vitrified material
- <u>Infected material should not be frozen</u>

Yavin etal 2009

Yavin etal 2009 describe an innovative method; ‘sealed pulled straws’ (SPS), which is designed to reduce the potential risk of contamination during vitrification while maintaining a high cooling rate in LN slush at -210°C.

This method protects the biological sample from direct contact with the LN during the vitrification process and subsequent storage.

What are the different solutions for vitrification ?

- Permeable cryoprotectant for the cell membranes
  (Glycerol, Ethylenglycol, DMSO)

- Non- permeable cryoprotectant
  (Sugar, Proteines, Polymere)

What are the characteristics of the vitrification solutions

- Essential substances: permeable cryoprotectant

- ✓ The solution should have the power for dehydration
- ✓ The possibility to reduce the freezing point
- ✓ These cryoprotectants should be non-toxic

- Non-permeable substances (Sucrose, Trehalose)

- ✓ Dehydration of the cell through osmosis
- ✓ Reduction of the swelling shock during rewarming
- ✓ Support the process of dehydration through reduction of the incubation time
- ✓ The sucrose leads to increase of the viscosity of the solution

Equilibration in the protective substance

A) Time

- The incubation should be short as possible
- Two-step equilibration is important to reduce the toxicity

B) Temperature

- The fast entrance and the degree of toxicity of the cryoprotectant can be influenced by temperature
- Equiliberation at 37°C avoid the re-expansion of the cell especially the first step of warming
- It is advisable to equilibrate between 22 and 25°C

Is the technique of vitrification standarized to be adopted in IVF-centers?

All the developmental stages are now vitrified sucessfully

These are some technical difficulties…….

a- Type and concentration of the cryoprotectant

b- Variability in the volume of the media or the carrier

c- Temperature of the solution during equilibration

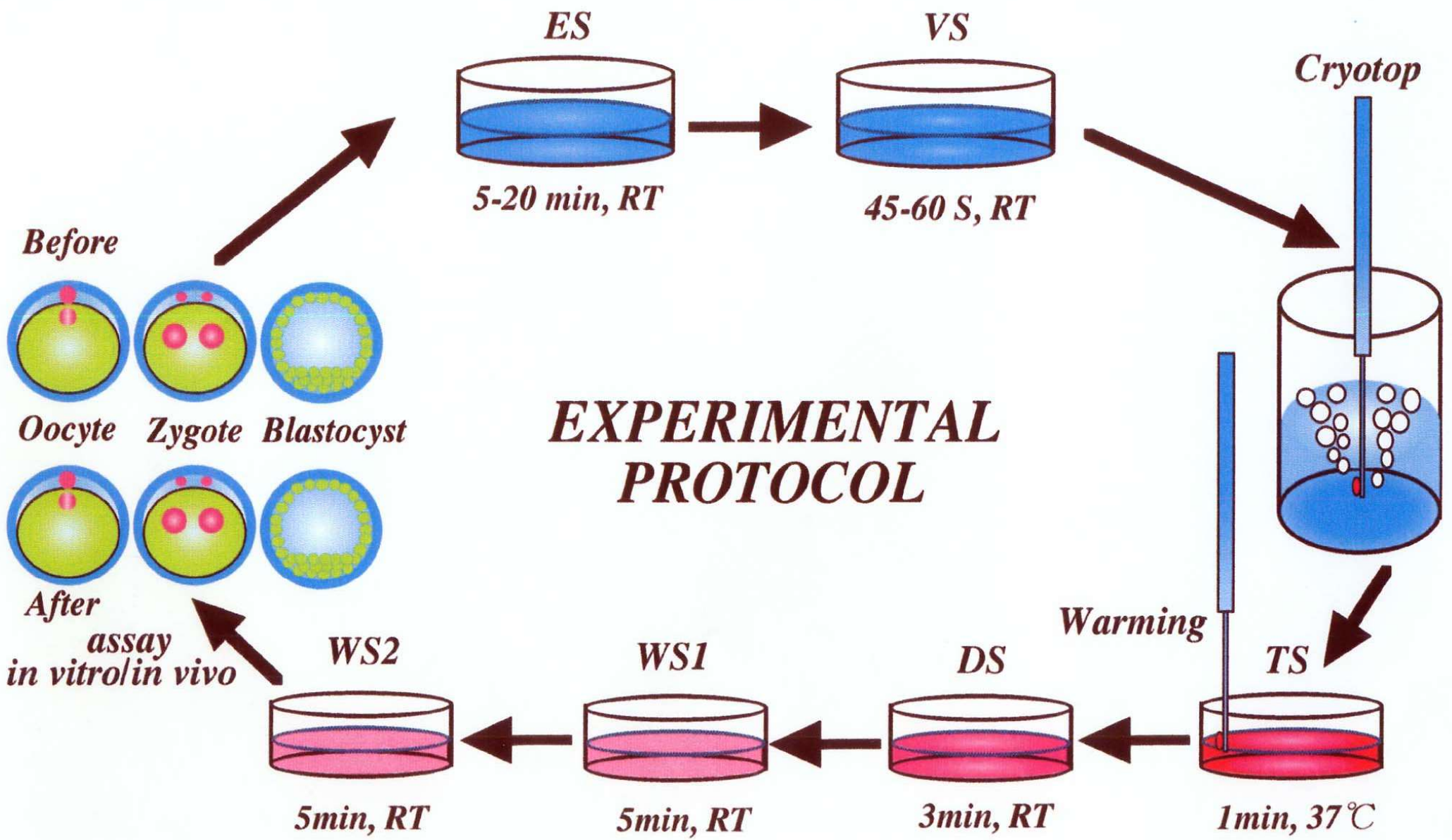
d- Type of vitrification container

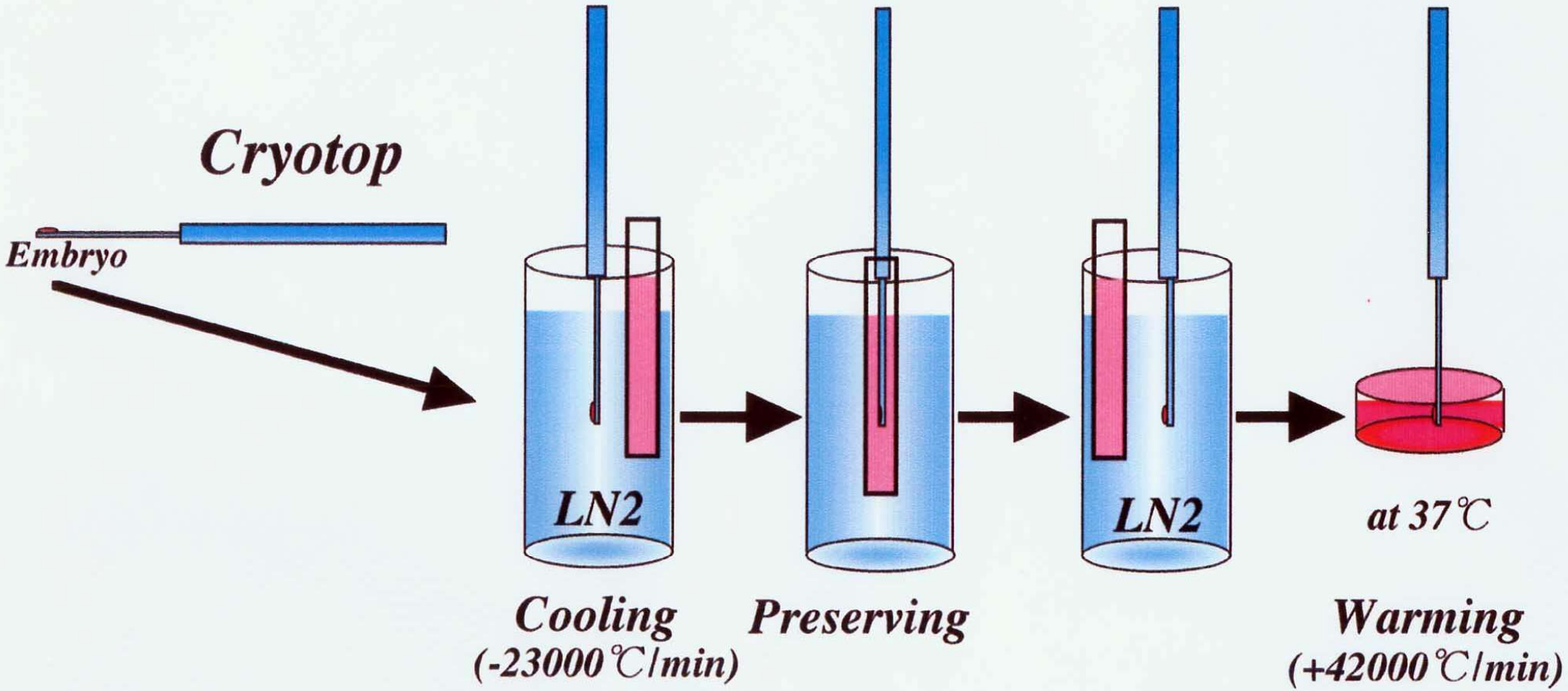
e- Skillness of the embryologist

Successful vitrification

- High cooling rate (>25.000 °C)
- Fast cooling period (< 2 sec.)
- Low volume (< 1µl)

This will lead to avoid crystal formation

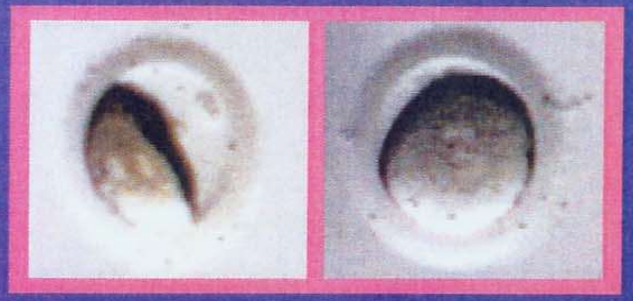

ES
VS
Cryotop
5-20 min, RT
45-60 S, RT
Before
Oocyte
Zygote
Blastocyst
EXPERIMENTAL PROTOCOL
After
assay
in vitro/in vivo
WS2
WS1
DS
Warming
TS
5min, RT
5min, RT
3min, RT
1min, 37℃



How to cool & warm the specimens by MVC method

Vitrification Protocol

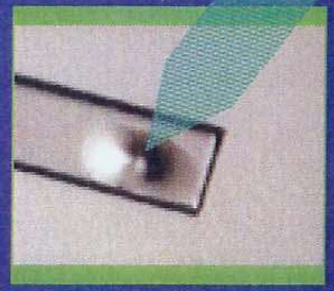
Zygote



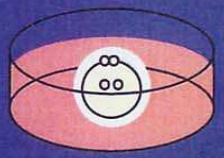
(Shrink)(Recovery)(Shrink)

Blastocyst

Embryo

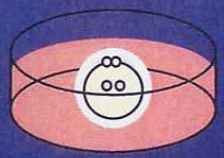


Top of Cryotop



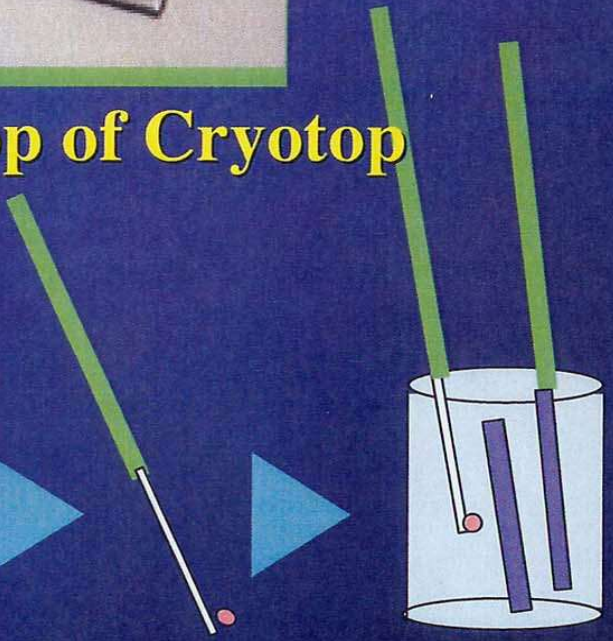
ES

7.5% EG, 7.5% DMSO
5-15min, RT



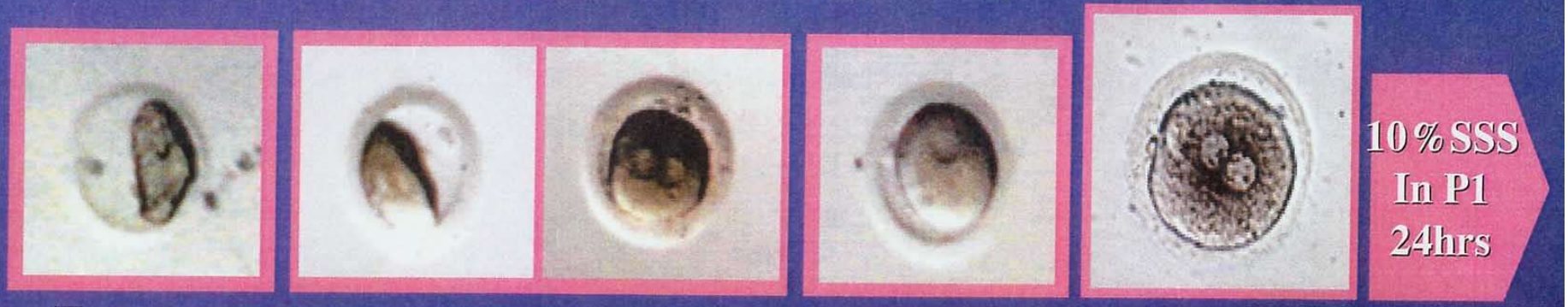
VS

15% EG, 15% DMSO,
0.5MS
45-60s, RT

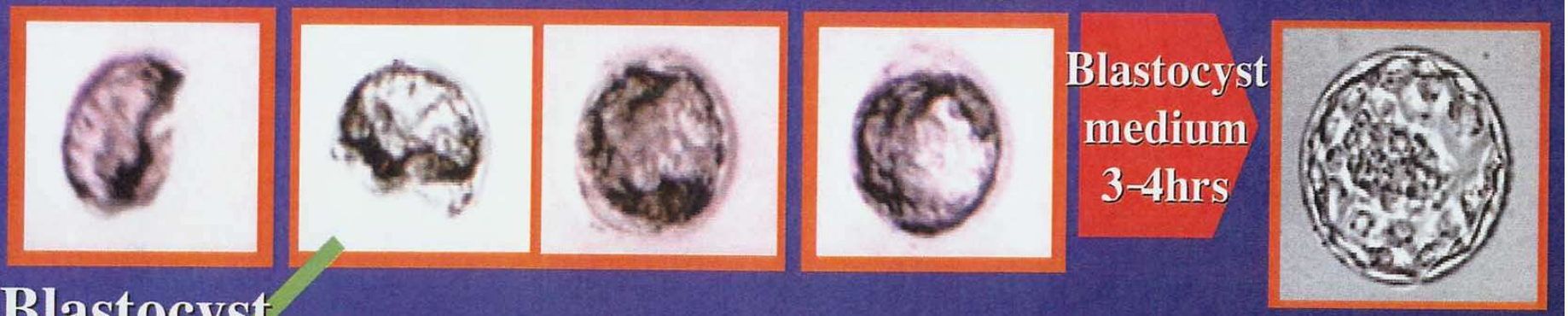


Cryotop LN_2

Thawing Protocol



Zygote (Shrink～Expand～Shrink) (Recovery)



Blastocyst

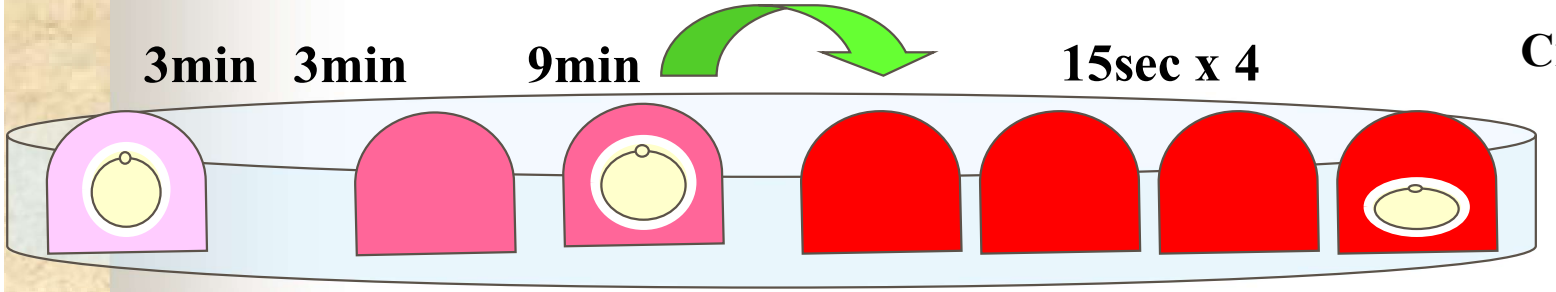
LN2 TS DS WS1,2 Culture ET

1MS
1min, 37℃

0.5MS
3min, RT

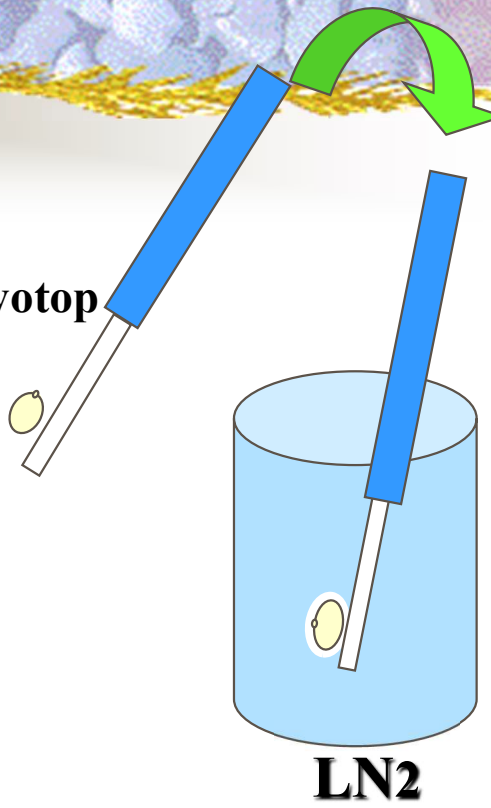
5min × 2, RT

Vitrification

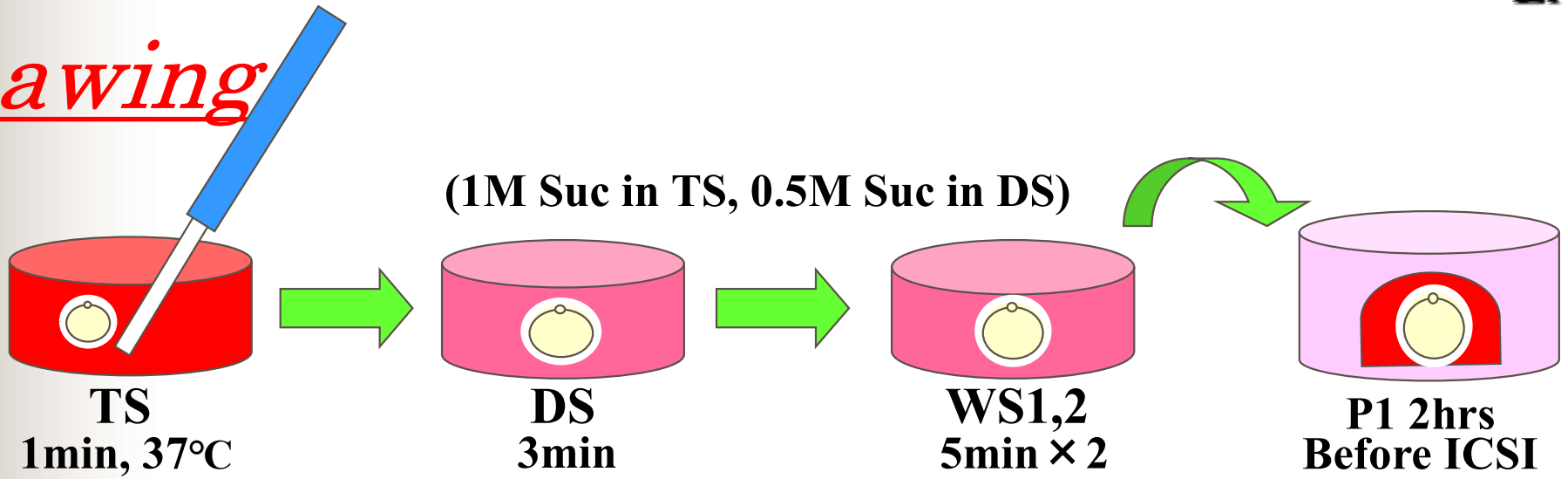


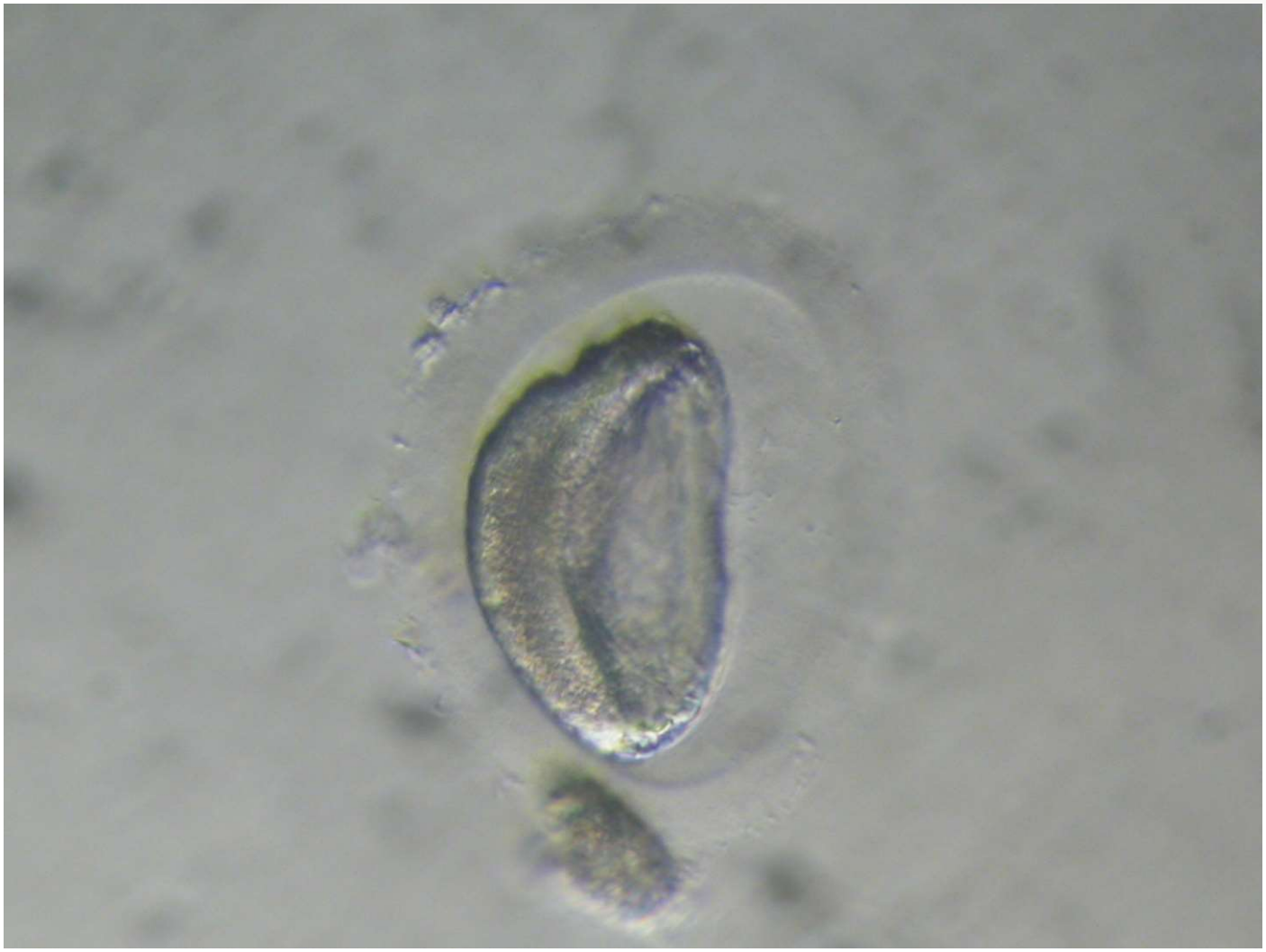
(20μl for each drop, at R.T.)

(7.5%EG,7.5%DMSO in ES)
(15%EG,15%DMSO,0.5M Suc in VS)



Thawing





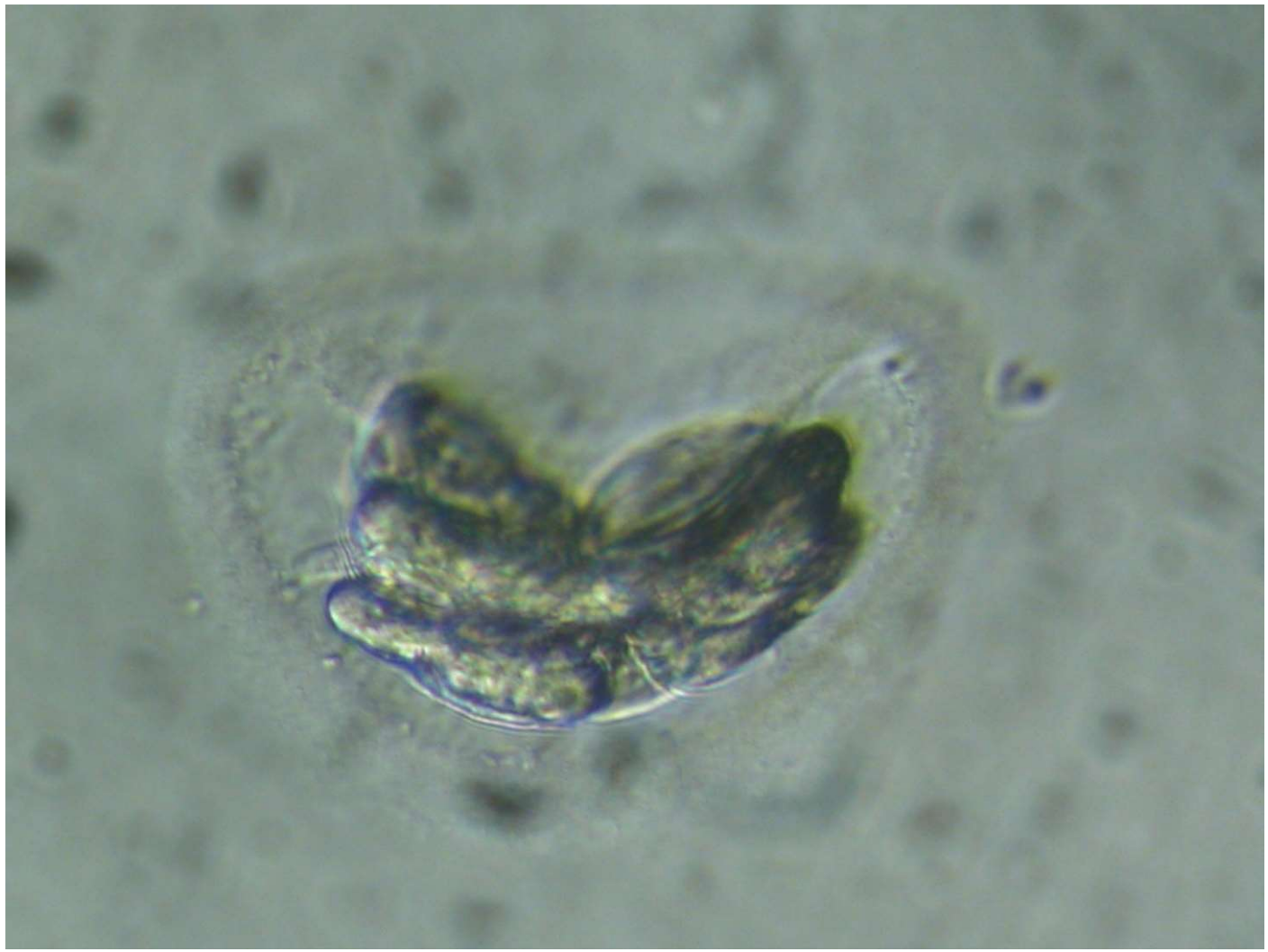


Table 1. Survival rates of MII oocytes after cryopreservation by vitrification or conventional slow freezing methods.

VS	Cumulus Layer	No.(%) Oocytes		(%)			
		Vitrified	Recovered	Surviving	2PN	4cell	Blast.
Vitrification[a]	+	64	64 (100)	91	90	81	33
Slow Freezing Method[b]	−	9	9 (100)	22	50	0	0

a: Vitrification Kit (Vitification Media and Thawing Media)
b: Conventional slow freezing method using 1.5M propanediol

Towako Kato Ladies' Clinic

Kuwayama et al. 2005, RBM online, 11, 300-308

No. of oocytes	No. (%) of survived	No. (%) of fertilization	No. (%) of blastocyst	No. of embryos transferred	No. of pregn.	No. of birth	No. of ongoing
64	58 (91)	52 (89.6)	32 (50)	29 (22)	12	7	3

Results of vitrified Human Oocytes

No. Of Cycles		120
No. Of Occytes vitrified		463
No. Of Oocytes warmed	330	
No. & % survived		328 (99.4)
No. & % cleaved		295 (96.7)
Implantation rate		13.2%
No. & % clinical preg.		39 (32.5)
No. & % abortions		8 (20.5)
No. Of deliveries		3

M. Antinori et al. 2007

RBM online 1, 14

Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates Dr. Raju (2005)

	Vitrifi.	slow-freezing
No. of embryos	436	420
No. of thawed	127	120
No. & % survived	121 (95.3)	72 (60)
No. & % pregnant	14 (35)	4 (17.4)

40% EG + 0.6 mol sucrose, nylon loop

Statement: EG is a good croyprotectant to preserve 8-cell embryos because of its low toxicity as shown by the high survival rate, and vitrification is a promising alternate to the conventional slow-freezing method.

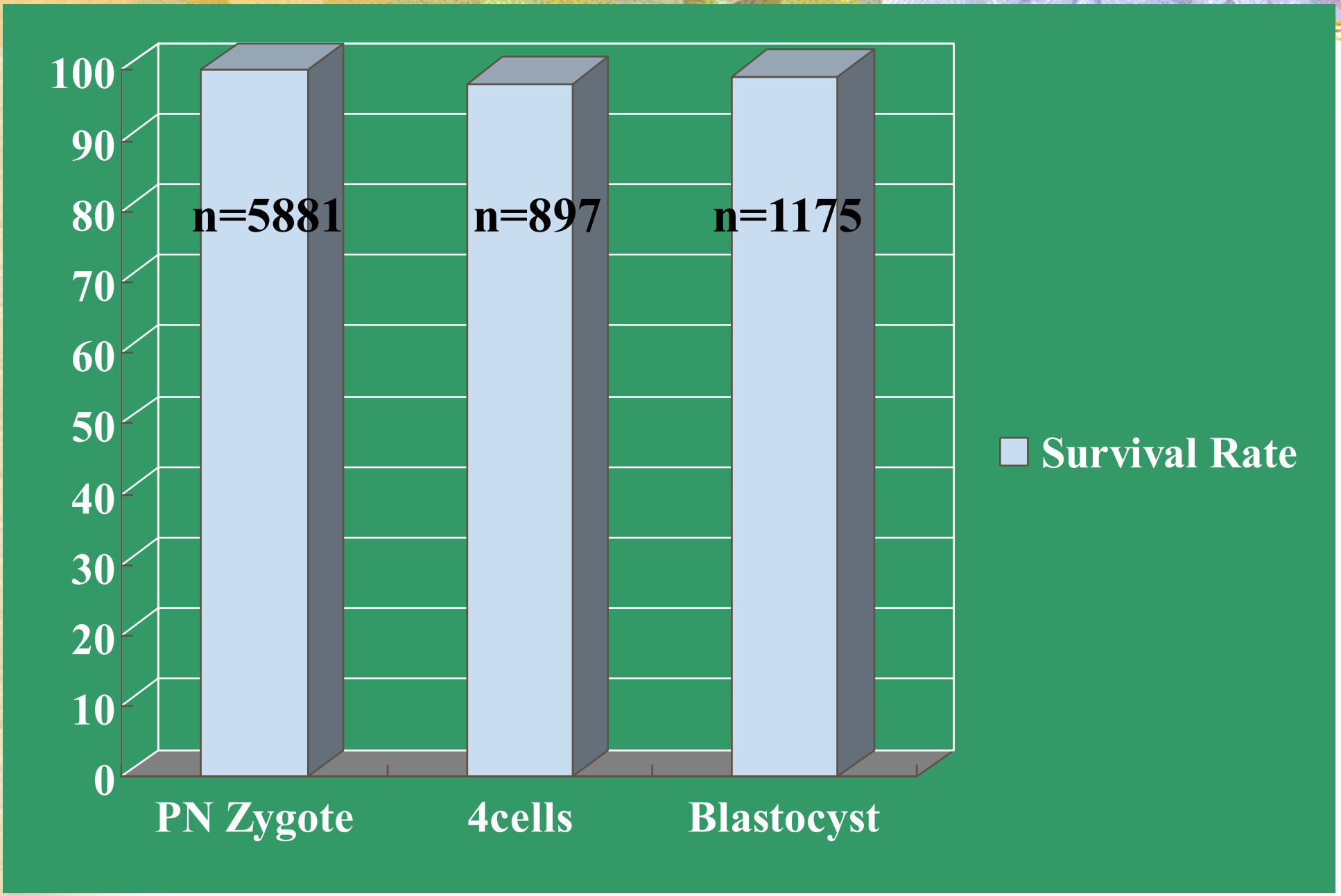
RBM online, 11, 434-437

Vitrification demonstrates significant improvement versus slow-freezing of human blastocysts Stehlik et al. (2005)

	Day 5		Day 6	
	slow	Vitr.	slow	Vitr.
No. of embryos transferrerd	24	20	27	15
% of survival	83	100	89.5	100
% pregnant	16.7	50	18.5	33.3

Survival & pregnancy rates of day 5 Vit. Blastocysts have significantly increased over day 5 of slow-frozen blastocysts. A similar trend was observed with day 6 blastocysts.

RBM online, 11, 53-57



Post-thaw survival rates of human PN zygotes, 4-cells stage embryos and blastocysts after vitrification

Escriba et al., 2009 Fertil Steril

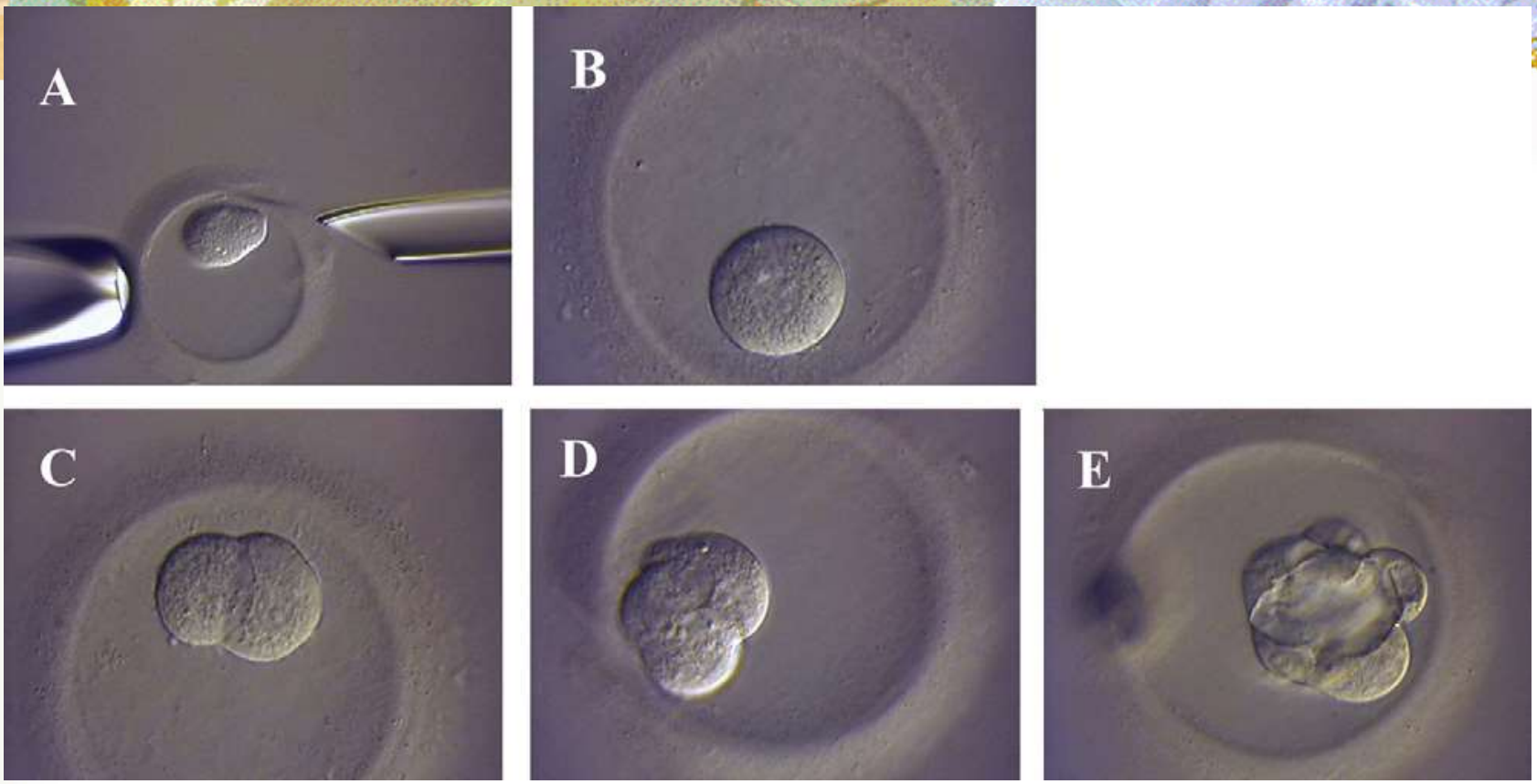
Describe a new methodology for preserving and banking isolated human blastomeres

75.7% After warming, of blastomeres survived and developed at a rate comparable to that in noncryopreserved blastomeres

62.5% Cleavage

26.6% Compaction

20.3% Cavitation



A) An emptied zona pellucida packed with a single blastomere
B) Using a biopsy instrument (tool on the right) and a holding pipette (left tool). Blastomere detail at 30 minutes after manipulation
C) During in vitro culture, some blastomeres cleaved
D) Compacted
E) Cavitated (E).

Vitrification of spermatozoa

Spermatozoa cryopreservation empirical methods developed in the 1949 are still used today.

The motility of cryopreserved/thawed spermatozoa normally falls to about 50% of the motility before freezing, wherein inter-individual fluctuation can be considerable.

Despite routine application, the problem of toxicity due to osmotic stress during saturation and dilution of the cryoprotectant as well as the possible negative influence on the genetic material is as yet unresolved .

Classical vitrification requires a high percentage of permeable cryoprotectants in medium (30-50% compared to 5-7% with slow-freezing) and is unsuitable for the vitrification of spermatozoa due to the lethal osmotic effect.

Shape and size of the sperm head could be factors, which define the cryosensitivity of the cell.

New Method for Vitrification of Small Volume of Normal Human Sperms: Use of Open Pulled Straw Carrier Saki etal 2009

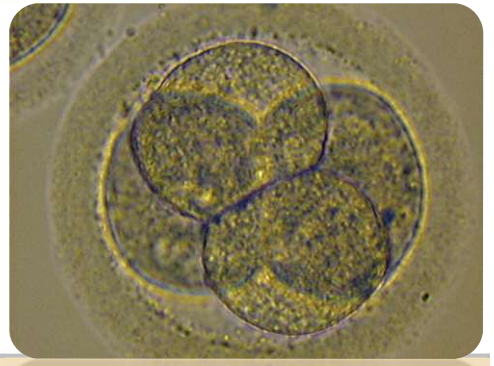
Progress motility of sperm in fresh group was evaluated as 59.2±7.6 comparing to

37.5±8.2 Vitrification method

26.3±6.4 Cryopreservation

Lamis Infertillity Centre preliminary results 2008-2010

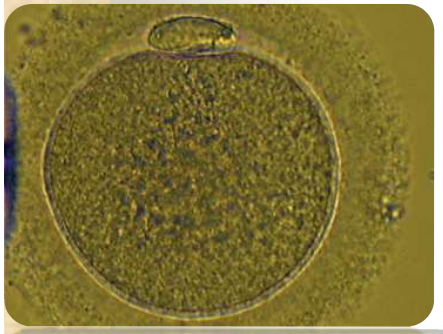
Embryo Vitrification



No. of Cycles	No. of embryo vitrified	No. Of embryo Thawed (cycles)	No. & % Embryo survived	No. & % Continous development
173	865	282(79)	282(100%)	271 (96%)

- Four deliveries, 12 biochemical pregnancy

Lamis Infertillity Centre preliminary results 2008-2010



Oocyte Vitrification

No. of Cycles	No. of Oocytes vitrified	No. Of Oocytes Thawed (cycles)	No. & % Oocytes survived	No. & % of 2PN and cleaved
22	138	32 (6)	32(100%)	26 (81%)

Open Questions??????????

1-Toxicity of the Cryoprotactants

2-Type of the Carrier

3-LN2 Contamnination

Future Aspects

- In case of OHSS and PCO
- All 2PN cryopreservation

Quality is evolution
Vitrification is a revolution

Prof. van der Elst

19.1.2007

Summary

- The vitrification procedure is easy to be done
- Much less costs
- It is the procedure of the first choice in the future
- The survival rate is very high with all different stages of development
- It may be need to be standardized
- It needs skillness of the embryologist

Thank you !