Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Vitrification in « closed » carrier device.

A way towards « aseptic » vitrification.

Vanderzwalmen P, Ectors F, Prapas Y,

Panagiotidis Y, Papatheodorou A, Lejeune B, Vanderzwalmen S, Zech N

IVF Centers Prof. Zech, Bregenz, Austria

Iakentro IVF Centre, Thessaloniki, Greece

Centre Hospitalier Inter Régional Cavell (CHIREC), Braine l'Alleud – Bruxelles, Belgium

Outline of the presentation

- How to obtain a succesful vitrified state ?
- Evolution of the vitrification technique:
  - Towards vitrification in „closed“ carrier devices
  - How to vitrify in reduced cooling conditions ?
- Clinical application of vitrification in „closed“ carrier for:
  - Oocytes
  - Embryos
- How safe is the vitrification procedure? A cytoskeletal analysis approach

The intracellular control of foci of nucleation and ice crystal formation are key factors that determine the viability of oocytes and embryos following the cooling and warming process.

Vitrification: definition

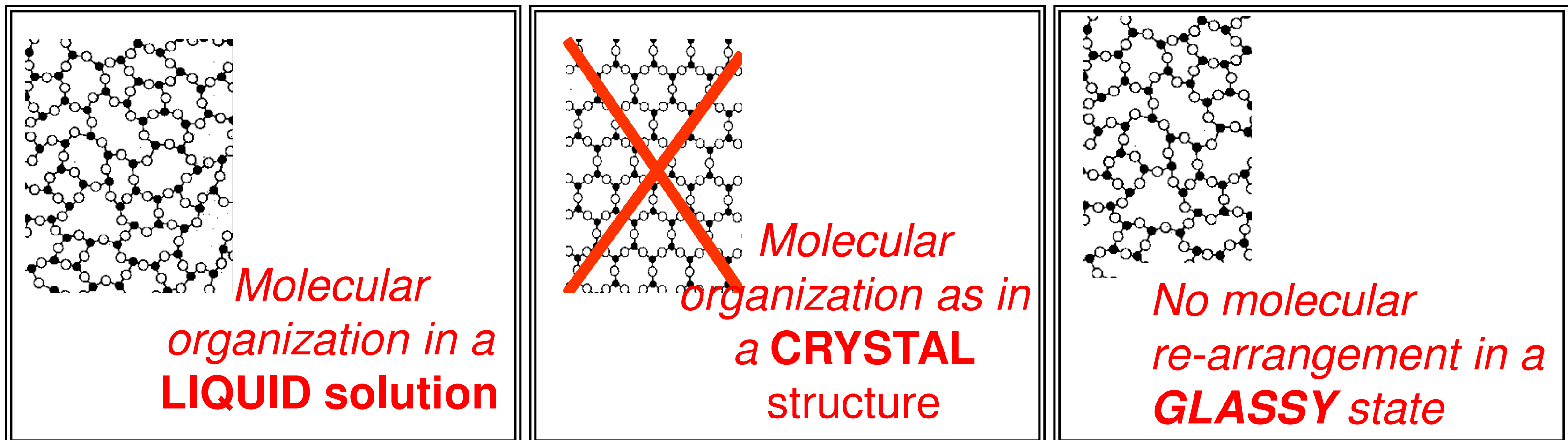
Vitrification consists in the solidification of a solution without ice crystal formation.

Vitrification involves :

an extreme elevation of viscosity before and during cooling,

resulting ultimately in a liquid that has the same lack of internal motions as a crystalline solid, and thus has no capacity for change over time,

yet lacks the molecular rearrangements of crystallization that do so much damage.



Institute für
Reproduktionsmedizin
und Endokrinologie

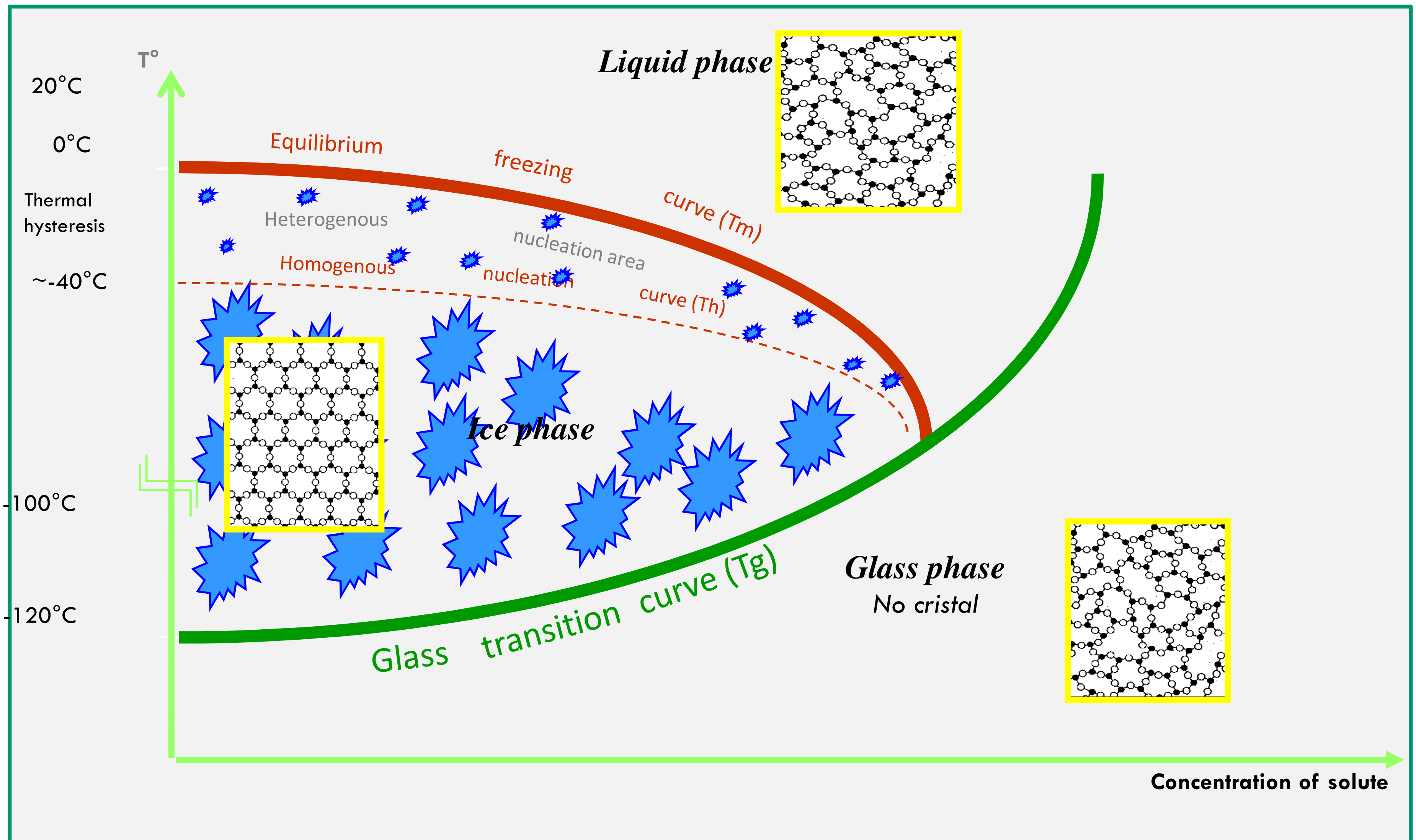
GIGA

How to reduce the likelihood of lethal ice crystal formation during the transit through the crystalline phase?

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Binary Phase diagram

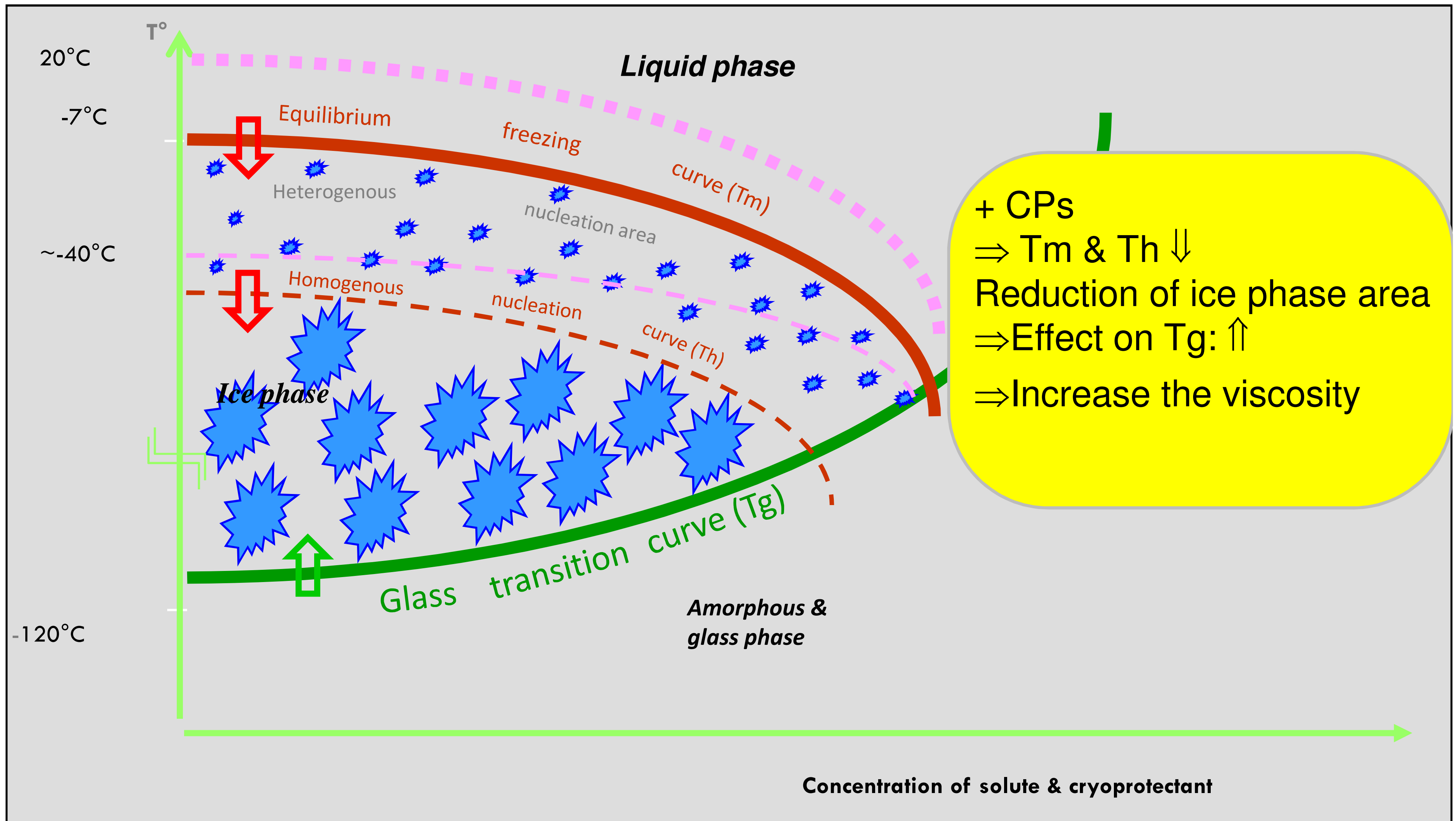


Institute für Reproduktionsmedizin und Endokrinologie

GIGA

How to reduce the likelihood of lethal ice crystal formation during the transit through the crystalline phase?

1 the use of concentrated solutions of CP

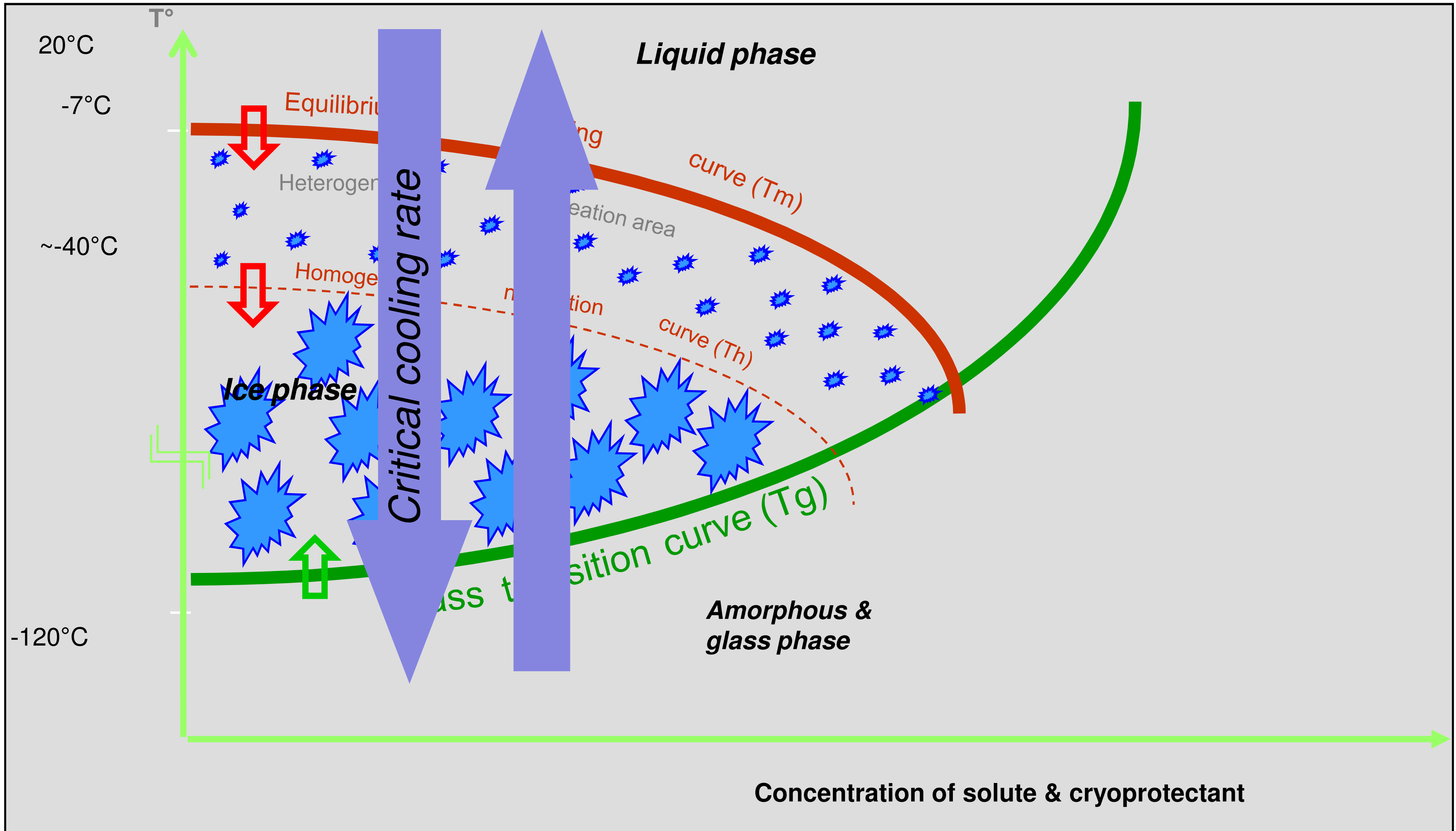


Institute für Reproduktionsmedizin und Endokrinologie

How to reduce the likelihood of lethal ice crystal formation during the transit through the crystalline phase?

GIGA

***2** with rapid cooling and warming*



Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Which factors influence the formation of a glass-like state?

Cooling – warming rates × Viscosity

Volume of the sample

Types of cells

The higher the cooling rate, the lower the required cryoprotectant concentration is, and *vice versa*.

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Vitrification : different steps

- **Exposure of biological material to CP solutions (viscosity)**
- Loading on carrier devices - (cooling warming rate)
- Storage
- Warming
- Removal of the CP solutions

Vitrification: Basic principle

<u>Before plunging the biological material in LN2:</u>

cells are exposed 2 different solutions of CP

<u>not vitrified</u> solutions (permable CP)

- *Prepare the intracellular part to an optimal concentration of CP*

  Dehydration and permeation of CP

  (Cooling-warming rate,time, concentration of CP, type of cells)

<u>vitrified</u> solutions (permeable and not permeable CP)

- *Create an intra and extracellular vitrified state*

  Dehydration and concentration of CP

Create an intracellular environment that will remain vitrified for a defined cooling-warming rate

Institute für Reproduktionsmedizin und Endokrinologie

G I G A

EVOLUTION OF THE VITRIFICATION TECHNIQUES

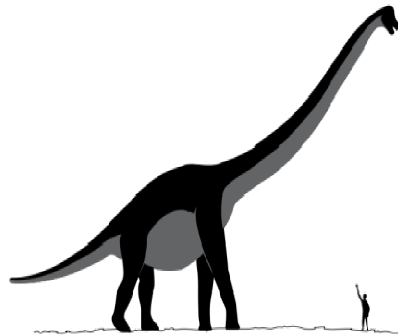
EVOLUTION OF THE TECHNIQUE (1985 – ……)

Vitrification in straw

« closed » carrier

Cooling < 2.000℃/ min
Warming ~500℃/ min

Mice, bovine, human



Human blastocysts	
% survival	**45% - 70%**
Birth/ vitri cycles	**15% - 25%**

(Rall and Fahy 1985, **Massip**, Scheffen, Vanderzwalmen 1986, Kasai 1990, Mukaida 1998, Vanderzwalmen 1997,2000, Chen 2000 Yokota 2000, 2001)

EVOLUTION OF THE TECHNIQUE (2000 – 20??)

Institute für Reproduktionsmedizin und Endokrinologie

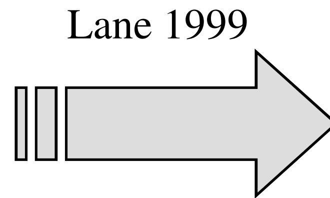
G I G A

Vitrification in straw

« closed » carrier

Cooling < 2.000 °C/ min
Warming ~500 °C/ min

Lane 1999



Vitrification in

« OPEN » carrier

Cooling > 20.000 °C/ min
Warming > 20.000 °C/ min

Current vitrification procedure

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

ULTRA – RAPID VITRIFICATION

cooling / warming rates: >20.000°C/min

7.5%-7.5% 10%-10%

DMSO-EG

PROH-EG

15 %-15% 20%-20%

2 to 4 min (8 cell- Morula -Blast)

7 - 10 min (Oocyte zygote)

30 – 90 sec

Advantage:

- cells are exposed in 2 steps to increased concentrations of CP,
- however only for a <u>short period of time</u>,
- long enough to permit the extraction of the intracellular water while <u>limiting the amount of CP permeating into cells.</u>

Reduced: osmotic stress, solute effects

Current vitrification procedure

ULTRA – RAPID VITRIFICATION

DMSO-EG

PROH-EG

7.5%-7.5%   10%-10%

15 %-15%   20%-20%

2 to 4 min* *(8 cell- Morula -Blast)

7 - 10 min (Oocyte zygote)

30 – 90 sec

Load on an "open" carrier device

Plunge in LN2

Institute für Reproduktionsmedizin und Endokrinologie

Current vitrification procedure

GIGA

ULTRA – RAPID VITRIFICATION

cooling / warming rates: >20.000°C/min

Small straws with thin walls

(1) Open-pulled straws *(OPS)*

(2) Open-pulled straws – quartz micro-capillary

Direct contact between a small volume of vitrification solution and LN2.

(1) Electron microscope copper grid (EM grids)

(2) Cryoloop

(3) Hemi-straw system

(4) Cryotop

(5) Cryoleaf

ULTRA – RAPID VITRIFICATION: **DISADVANTAGE**

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

ULTRA – RAPID VITRIFICATION : **DISADVANTAGE**

Embryos are

Directly in contact with liquid nitrogen

during cooling and/or storage.

Liquid nitrogen can be a **source of contamination** by bacteria, fungi, virus, heavy metals

(Tedder 1995, Fountain 1997, Bielanski 2000, Fountain 1997, Bielanski 2003)

Institute für Reproduktionsmedizin und Endokrinologie

ULTRA – RAPID VITRIFICATION : **DISADVANTAGE**

GIGA

European Union directive

concerning the packaging of cells and tissues

„Following procurement, all recovered ***tissues and cells must be packaged in a manner which minimises the risk of contamination*** *and must be stored at temperatures that preserve the required characteristics and biological function of the cells/tissues"*

ULTRA – RAPID VITRIFICATION : **DISADVANTAGE**

European Union directive

concerning the packaging of cells and tissues

*„Following procurement, all recovered **tissues and cells must be packaged in a manner which minimises the risk of contamination** and must be stored at temperatures that preserve the required characteristics and biological function of the cells/tissues"*

Vitrification usi

« **OPEN** »
carrier

Institute für Reproduktionsmedizin und Endokrinologie

Solutions to avoid contact with LN2 ?

GIGA

« open » carrier devices:

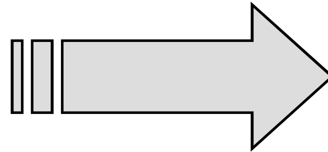
- Sterile LN2
- Vapor

EVOLUTION OF THE TECHNIQUE (2006 – 20??)

Vitrification in straw

« closed » carrier

Cooling < 2.000°C/ min
Warming ~500°C/ min



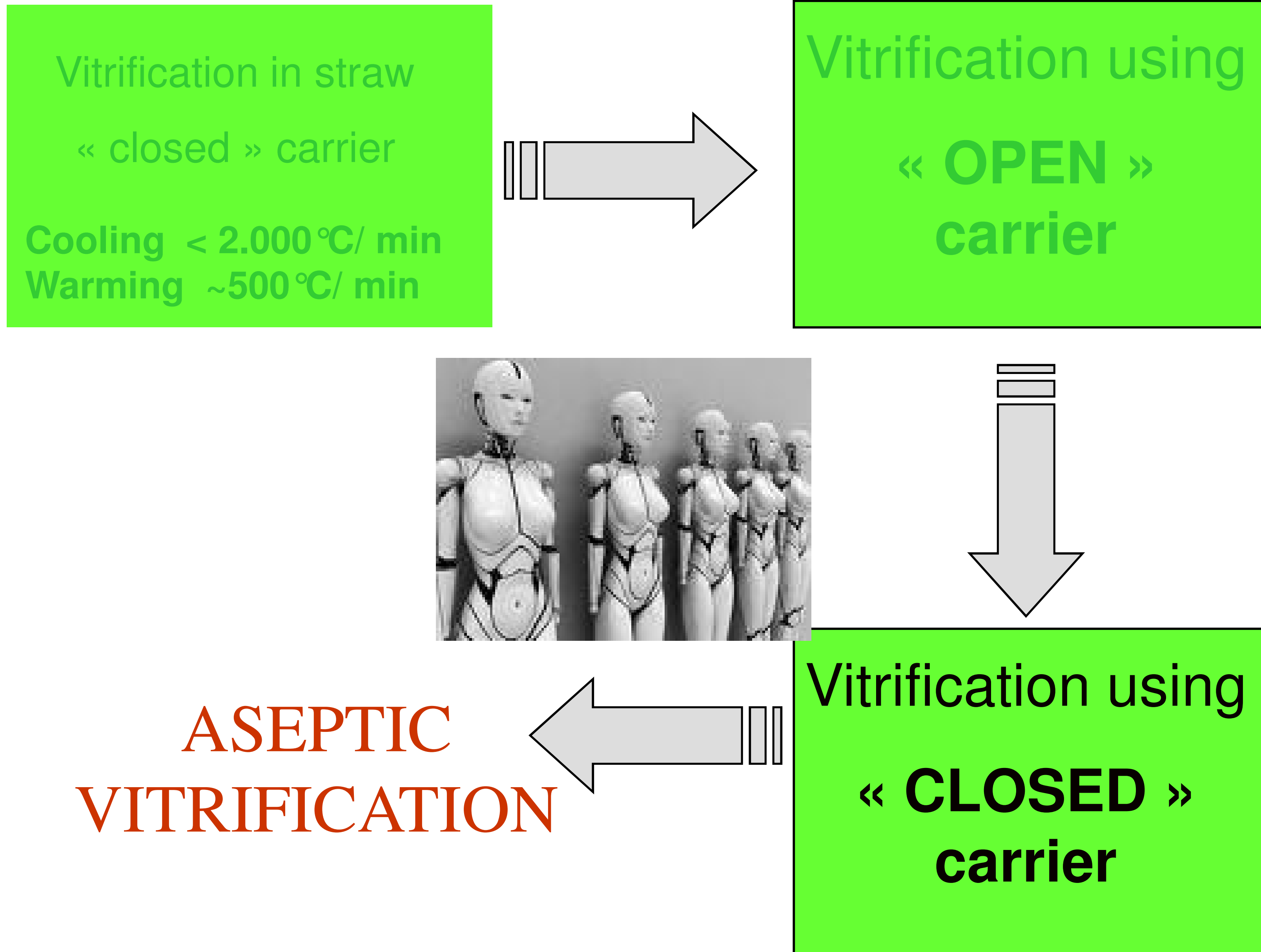
Vitrification using

« OPEN » carrier

Institute für Reproduktionsmedizin und Endokrinologie

EVOLUTION OF THE TECHNIQUE (2006 – 20??)

GIGA



Institute für Reproduktionsmedizin und Endokrinologie

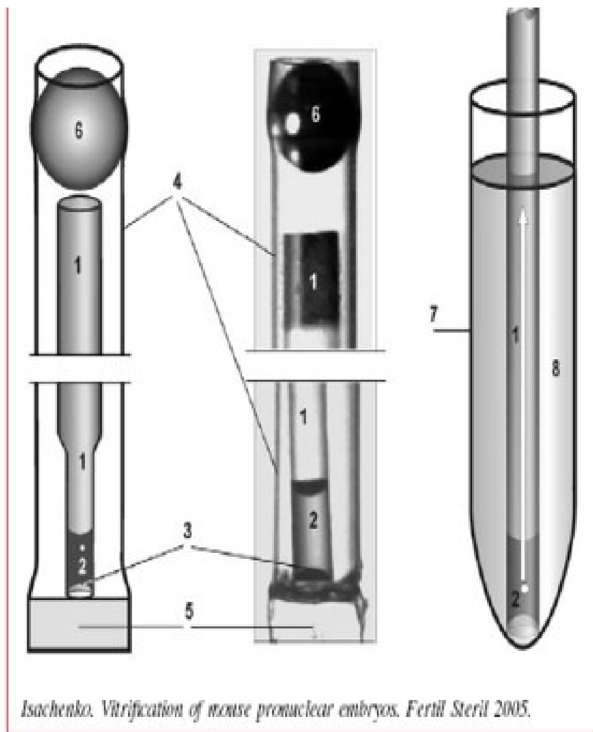
G I G A

rification of mouse pronuclear embryos after polar body biopsy without direct contact with liquid nitrogen *FS 2005*

Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws *Hum Reprod 2005*

("straw in straw" vitrification)

Isachenko V



Isachenko. Vitrification of mouse pronuclear embryos. Fertil Steril 2005.

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

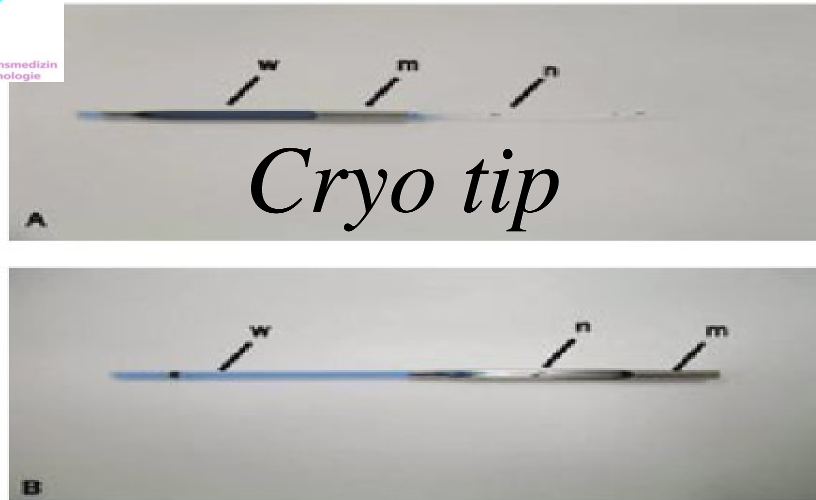
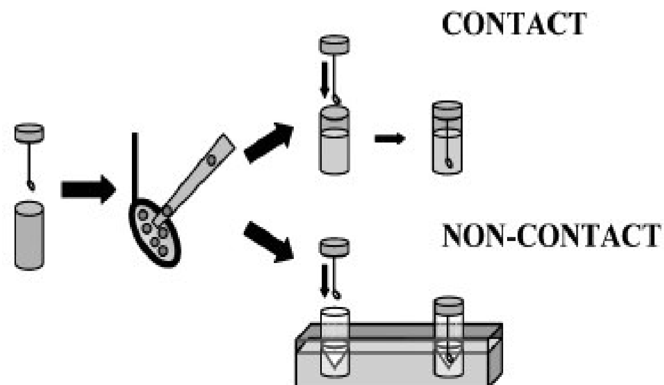


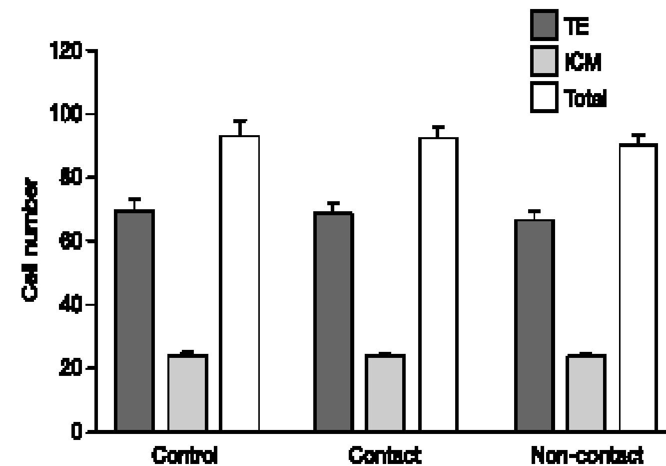
Table 4. Survival, pregnancy and delivery rates after single embryo transfer of human blastocysts vitrified with either the Cryotop or the CryoTip method.

	Cryotop	CryoTip
Survived/vitrified rate (%)	221/227 (97)	82/88 (93)
Pregnancy/transfer rate (%)	131/221 (59)	42/82 (51)
Delivery/transfer rate (%)	113/221 (51)	39/82 (48)

No significant differences between corresponding values were found.

Comparison of open and closed methods for vitrication of human embryos and the elimination of potential contamination Kuwayama 2005





Vitrication of mouse pronuclear oocytes with no direct liquid nitrogen contact Larman 2006

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

VITRIFICATION

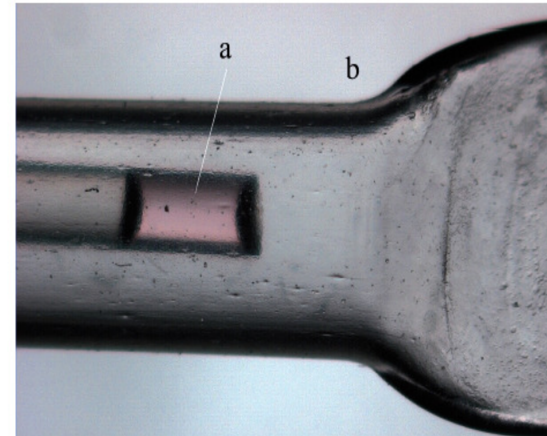
ULTRA – RAPID
„open" carrier

Cooling rate
> 20.000 °C/min

Warming rate
> 20.000 °C/min

EM grids
Cryoloop
Hemi-Straw
Cryotop

„CLOSED" carrier



Isolation of the sample from LN2

create a Thermo-insulating barrier

Decrease in the cooling

< 2.000 °C/min

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Ultra-rapid vitrification « open » device

> 20.000 °C/min

Successful results

short exposure to CP

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Ultra-rapid vitrification « open » device

> 20.000 °C/min

→ Successful results

short exposure to CP

?

« CLOSED » device

Reduced cooling and / or warming rates < 2.000 °C/min

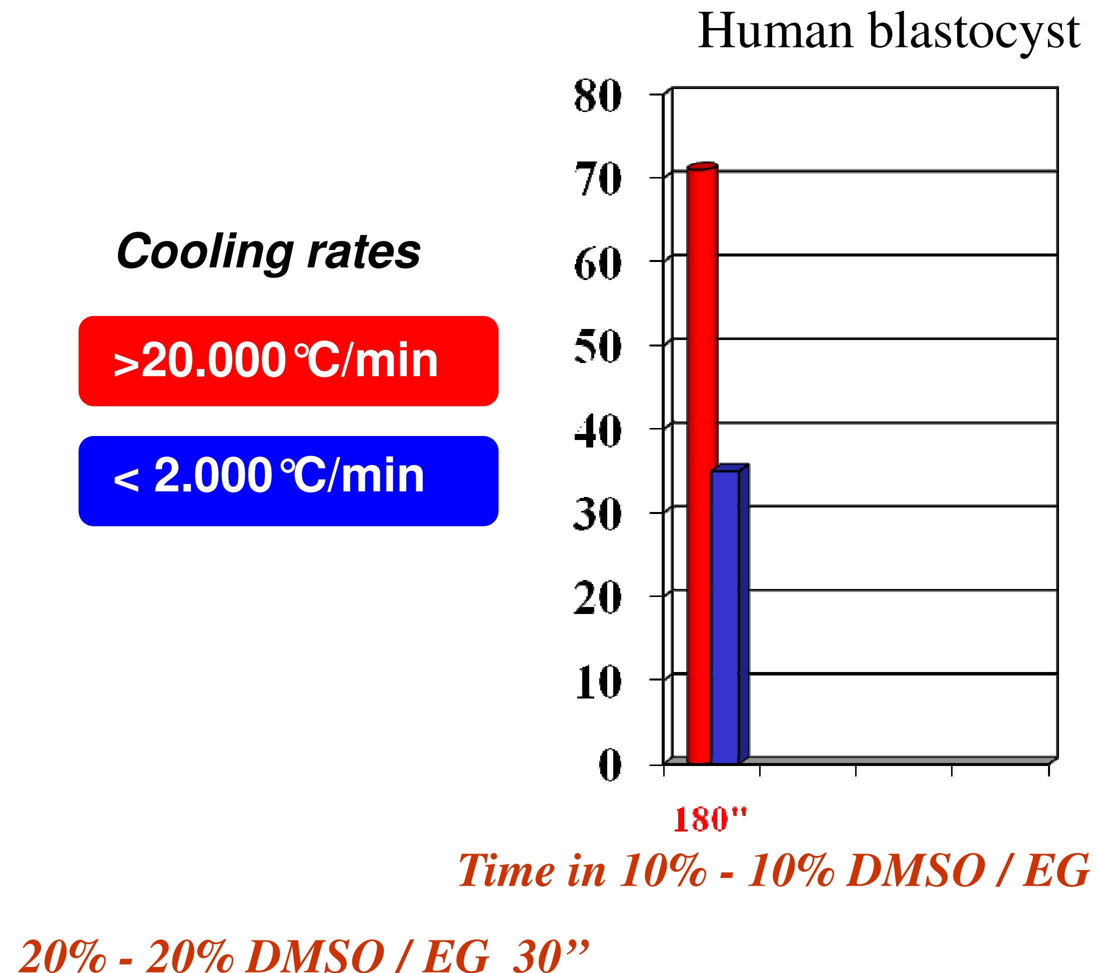
→ Do we have to modify the procedure ?

EFFECT OF A REDUCTION IN THE COOLING RATE

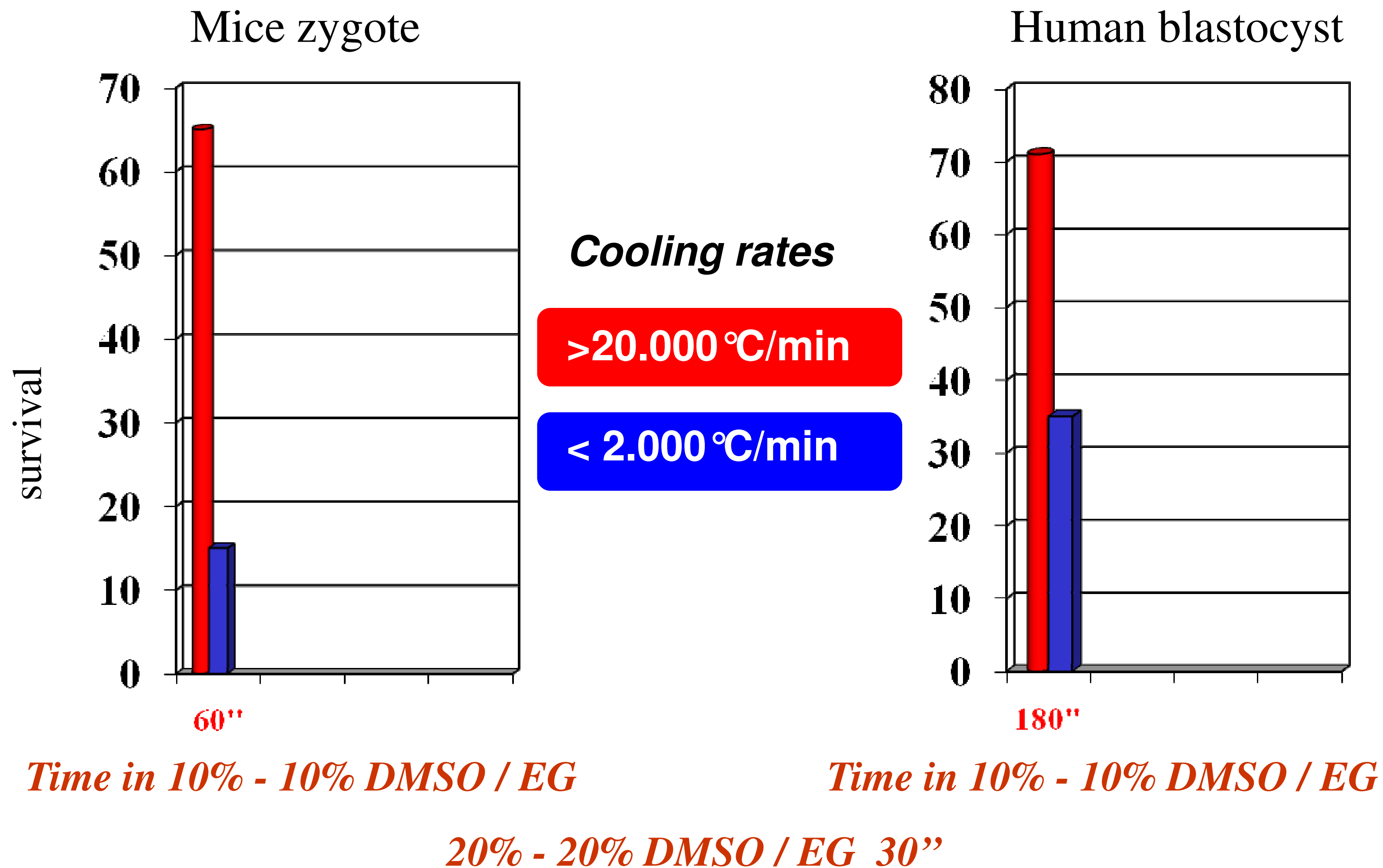
Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Effect of short time exposure to 10%-10% DMSO-EG before vitrification in « open » and « closed » carrier devices



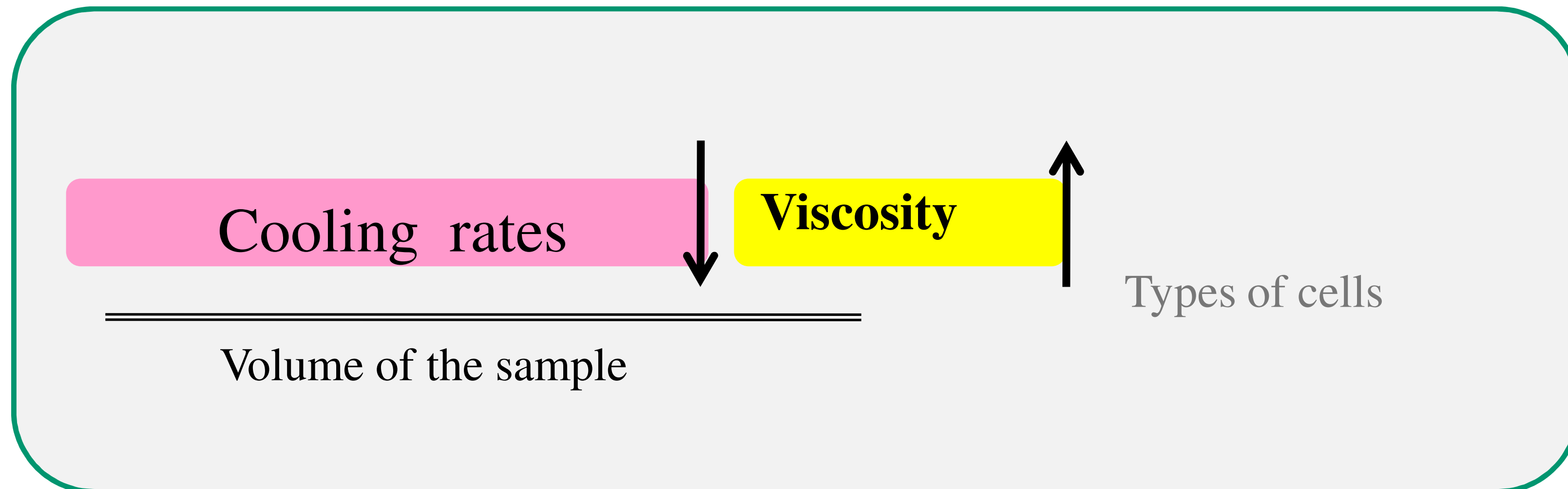
Effect of short time exposure to 10%-10% DMSO-EG before vitrification in « open » and « closed » carrier devices



20% - 20% DMSO / EG 30”

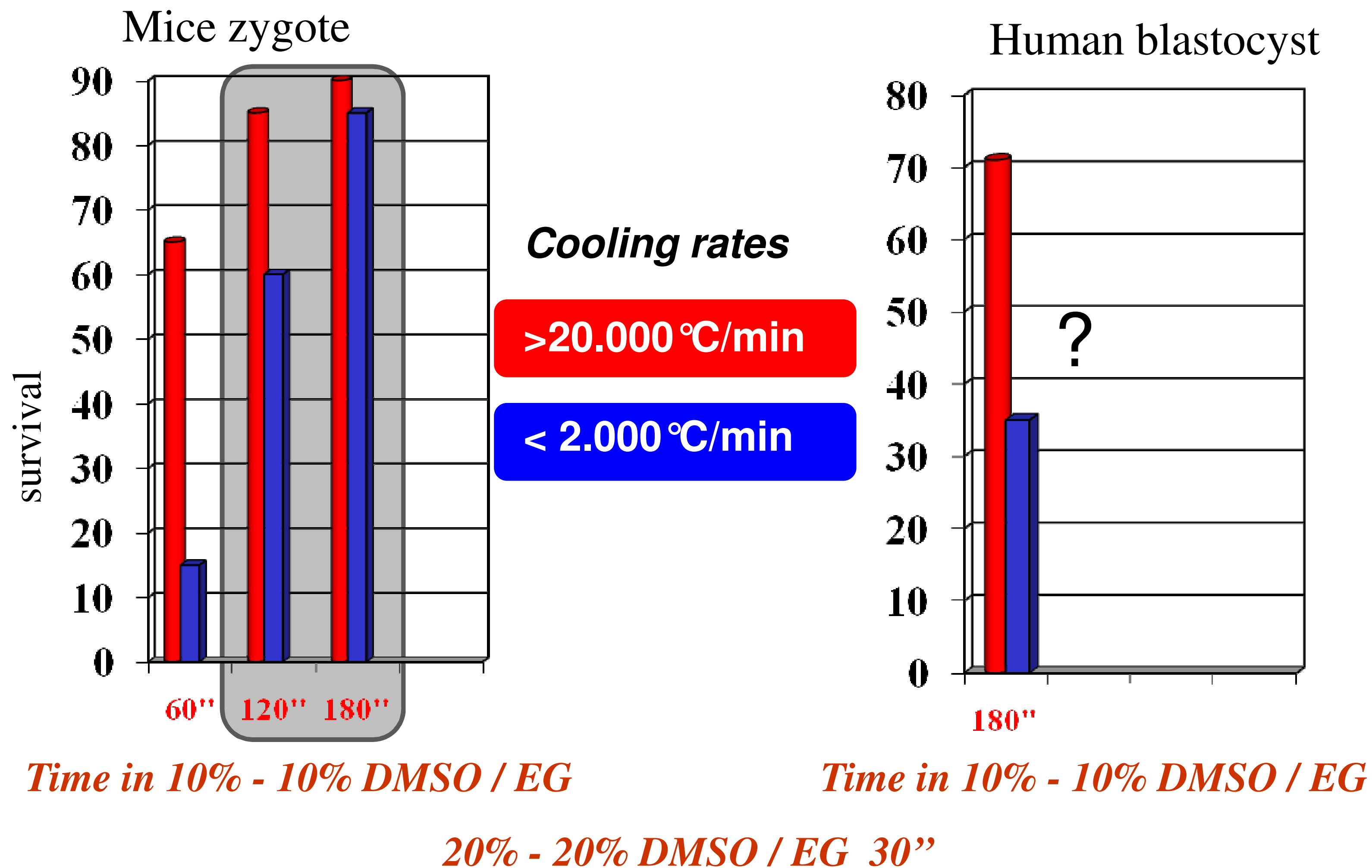
Institute für Reproduktionsmedizin und Endokrinologie

GIGA



INCREASE THE EXPOSURE TIME TO THE CPs

Effect of increasing the time of exposure to 10%-10% DMSO-EG before vitrification



Institute für
Reproduktionsmedizin
und Endokrinologie

G I G A

DEVELOPEMNT OF A VITRIFICATION TECHNIQUE IN A « CLOSED »CARRIER

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

1 DEVELOPMENT OF AN ASEPTIC DEVICE

2 DEVELOPMENT OF A PROTOCOL IN REDUCED COOLING CONDITIONS

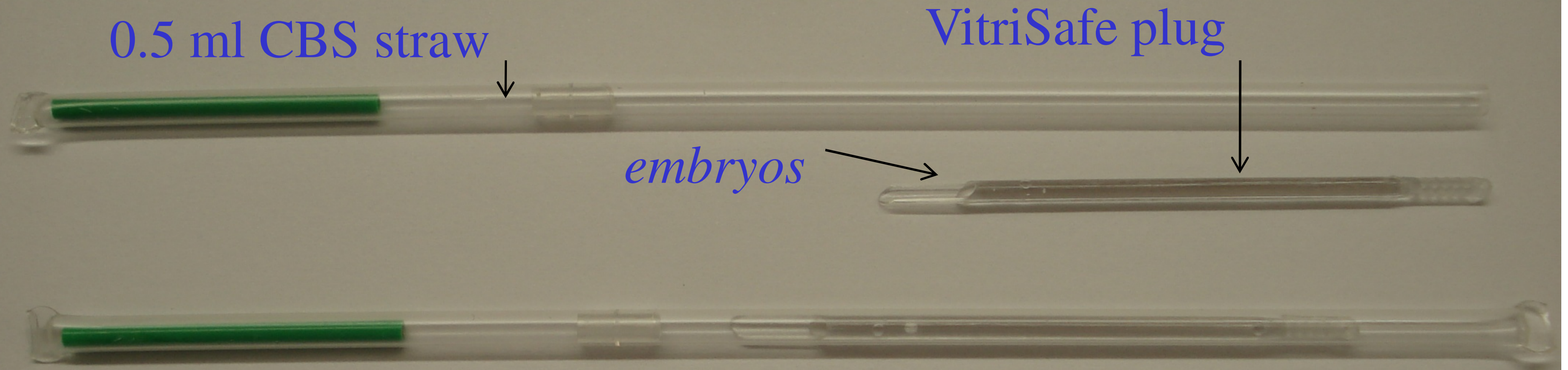
Institute für Reproduktionsmedizin und Endokrinologie

GIGA

1 DEVELOPMENT OF AN ASEPTIC DEVICE

- easy to handle
- allow complete isolation of embryos from LN2 cooling and storage
- Ultra-fast warming rate
  - *It is more difficult to prevent ice formation during rewarming than during cooling so that we need more CP for the warming step*

Aseptic vitrification kit "VitriSafe"

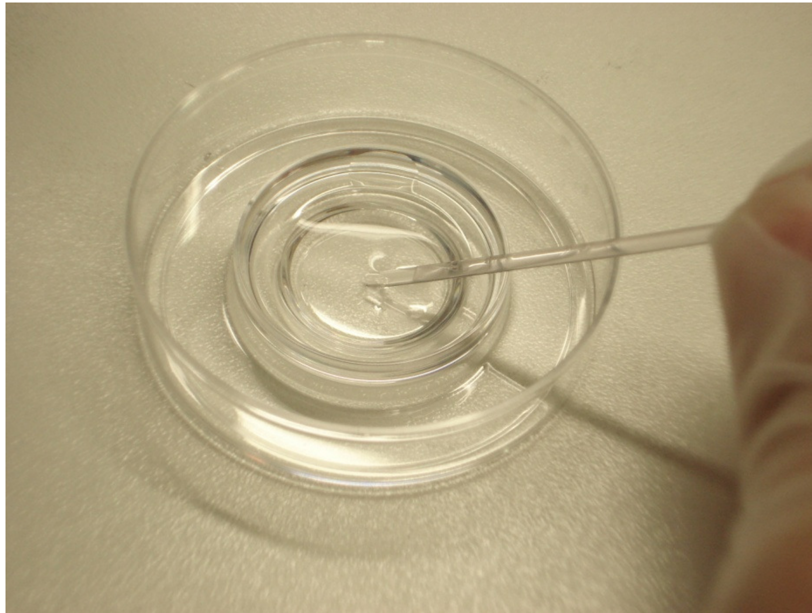


Vitrisafe inserted in 0.5 ml straw
Heat sealed
Ready to plunged in LN2

Cooling ~ 1.300°C / min
Warming > 20.000°C / min

ULTRA-RAPID WARMING

> 20.000 °C/min



Plunge the tip of the VitriSafe in 1M sucrose

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

2 DEVELOPMENT OF A PROTOCOL IN REDUCED COOLING CONDITIONS

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

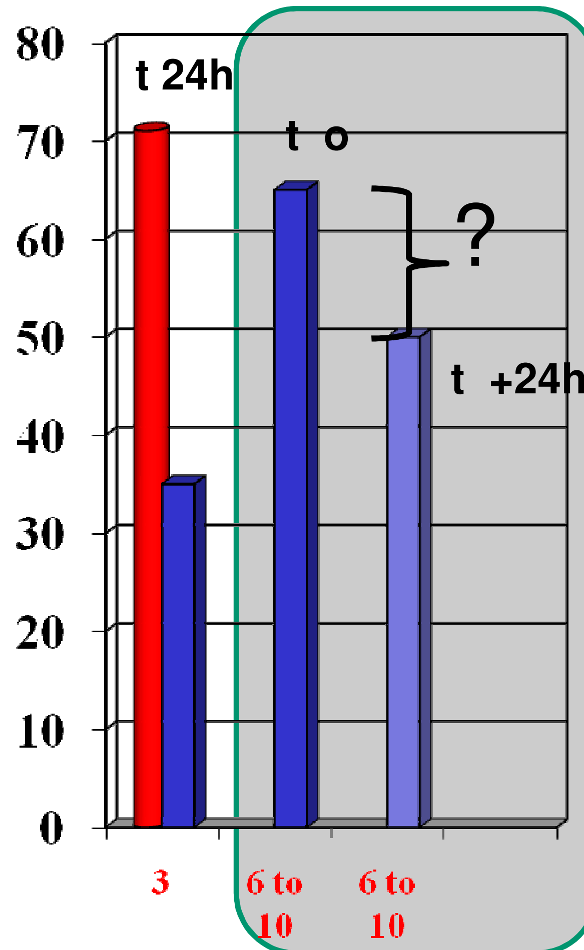
Effect of increasing the time of exposure to 10%-10% DMSO-EG before vitrification

Human blastocyst

Cooling rates

>20.000°C/min

< 2.000°C/min



Time in 10% - 10% DMSO / EG

20% - 20% DMSO / EG 30"

decrease in the re-expansion rate following 24h culture

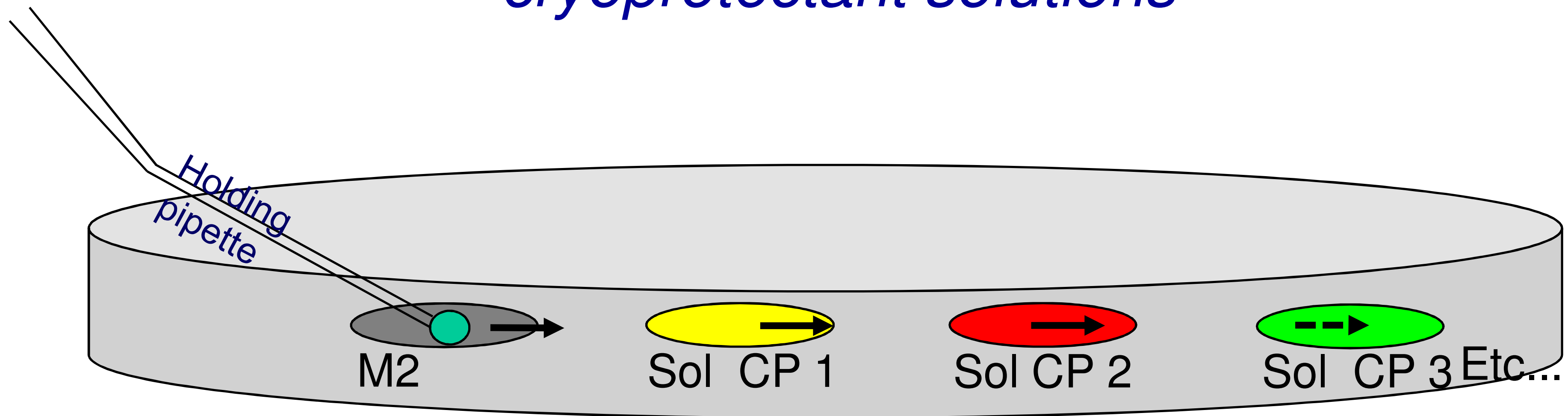
- Toxicity effect due to:
  - Exposure in one step to the 10% 10%?
    - Shrinkage / swelling stress?
  - To long period of exposure to CP?
    - Toxicity effect?

How to bring an answer?

How to understand how cells react in presence of CP?

microscopic cinematographic evaluation: time lapse evaluation

Volume analysis in relation with the exposure to cryoprotectant solutions



Zygote is maintained with a holding pipette
One picture every 5 -10 sec ***(FVB/N zygotes)***

Ectors F. & Vanderzwalmen P. GIGA – ULg
Transgenic unit and mice facilities

Institute für Reproduktionsmedizin und Endokrinologie

G I G A

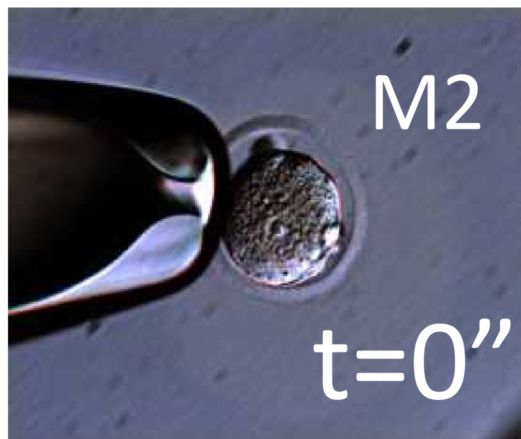
Objectives of Cinematographic analysis

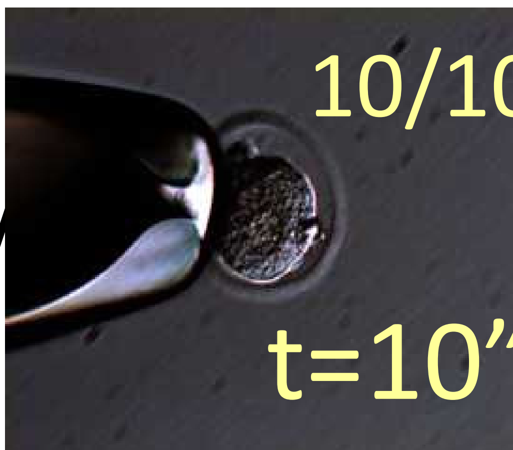
First objective:

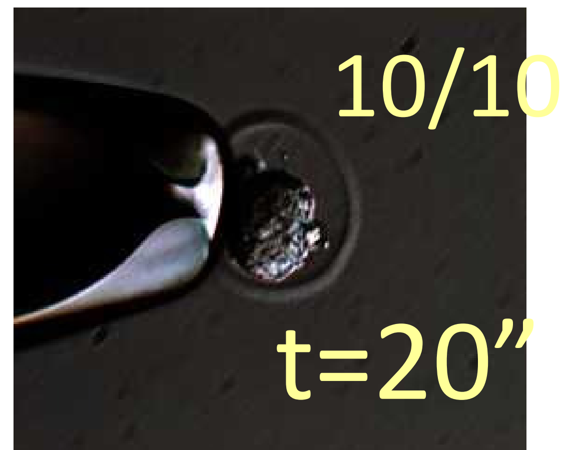
1 Understand how cells react to different CPs?
2 Which phase of the CP exposure is important to analyse:
dehydration
entrance of CP

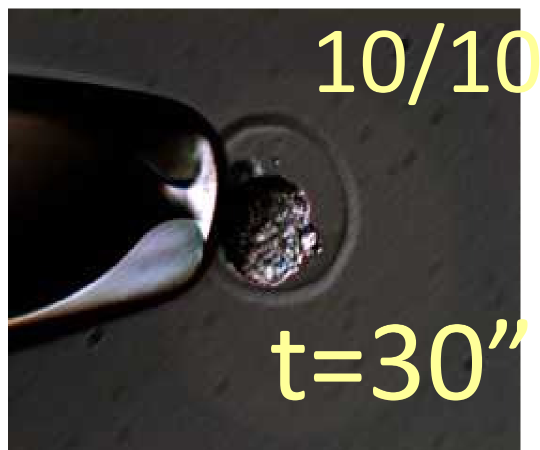
Final objective:

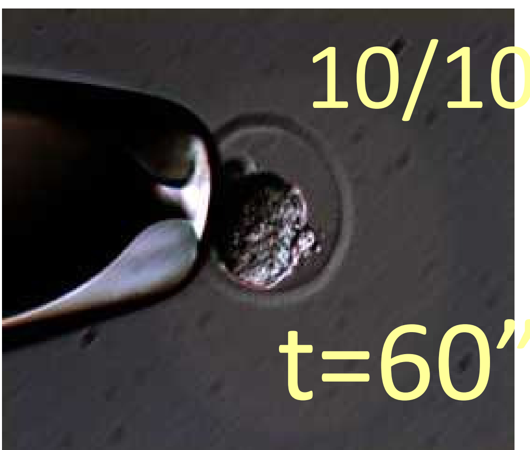
Analyse the shrinkage/ swelling process in order to stay between acceptable limits of volume variation.

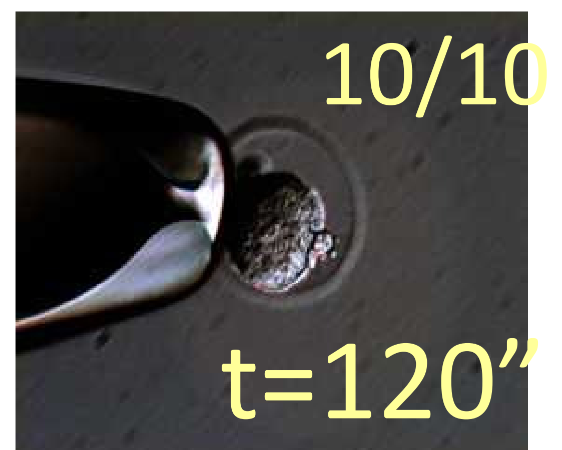

M2
t=0”

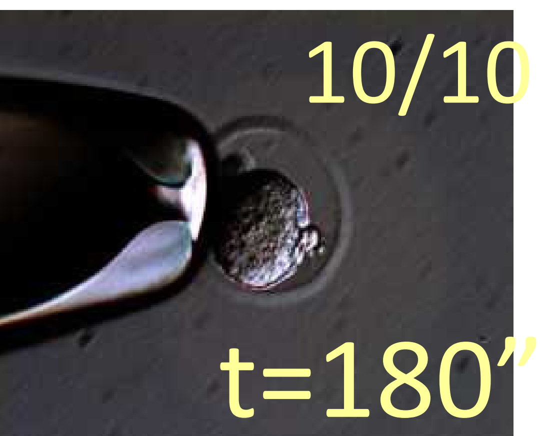

10/10
t=10”


10/10
t=20”

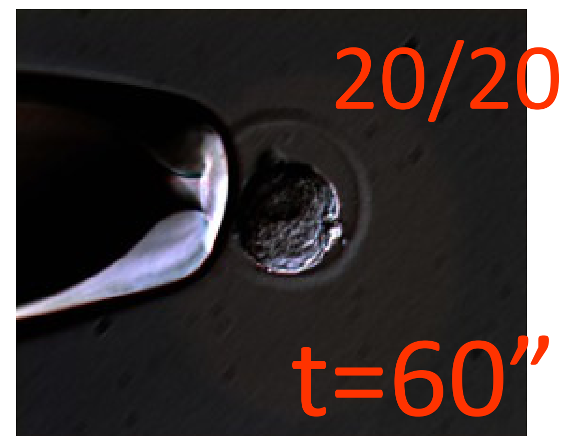

10/10
t=30”


10/10
t=60”

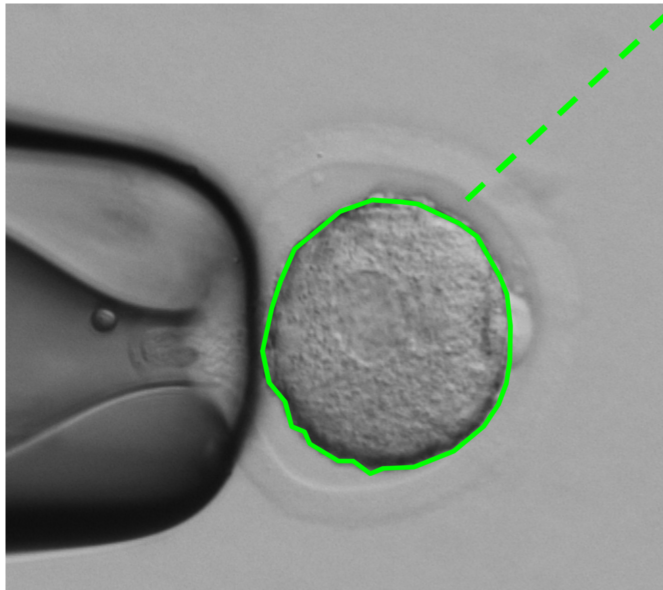

10/10
t=120”


10/10
t=180”

20/20
t=10”


20/20
t=60”

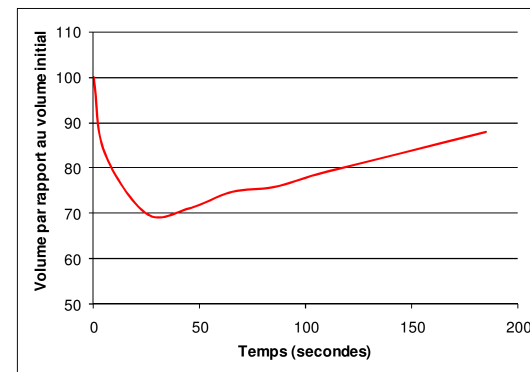
Acquisition of results:



Perimeter and volume

T0 to Tn

Ratio T0 to Tn / T0 (%)



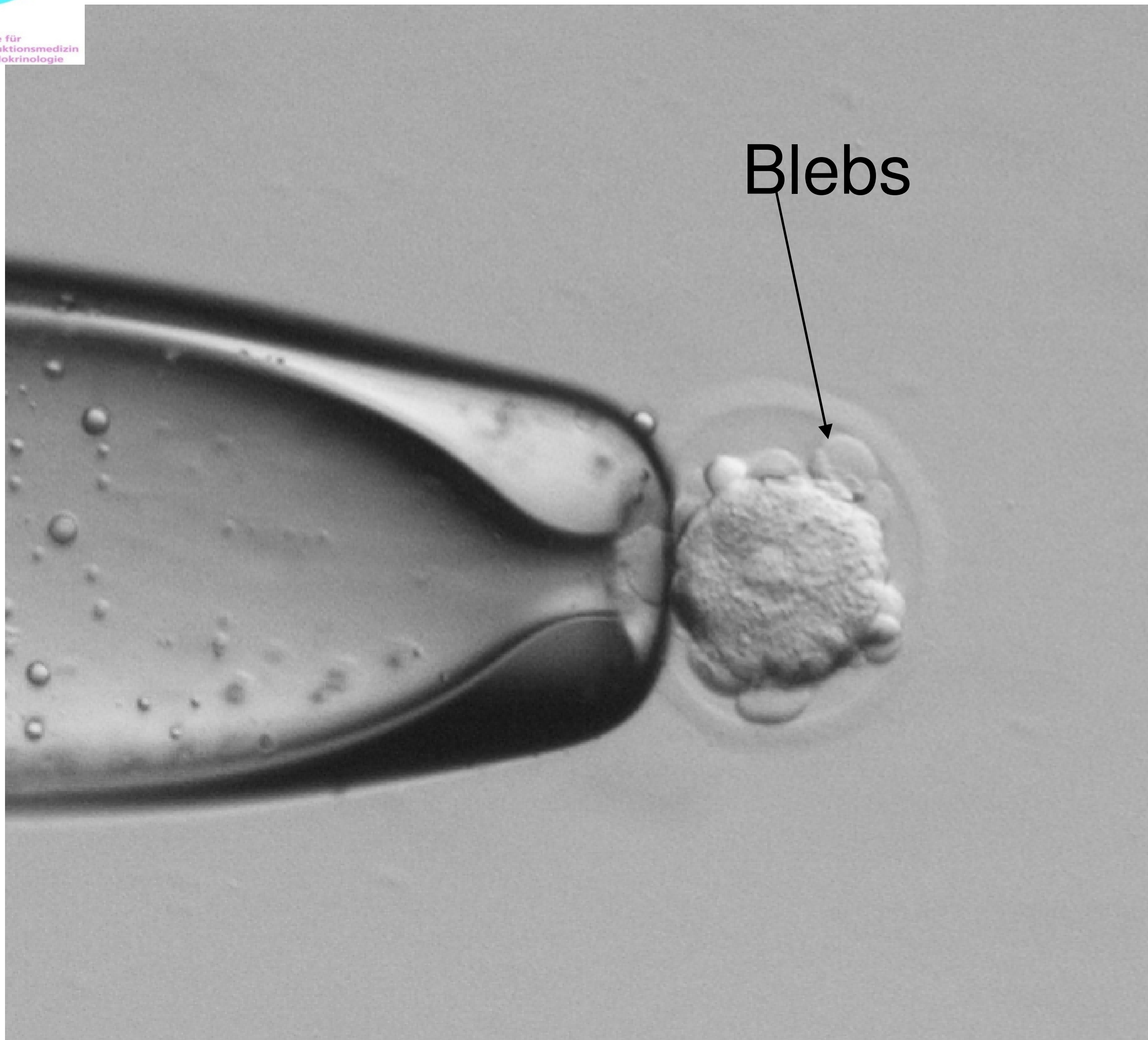
T0 Tn

Analyse the response of cells during the dehydration and entrance of CP

- After increasing the concentration of CP
- After direct or gradual addition of CP

- According to the type of CP
- In combination with solution of different CP

GIGA



Very high outflow of H_2O and fast entry of CP

Analyse the response of cells during the dehydration and entrance of CP

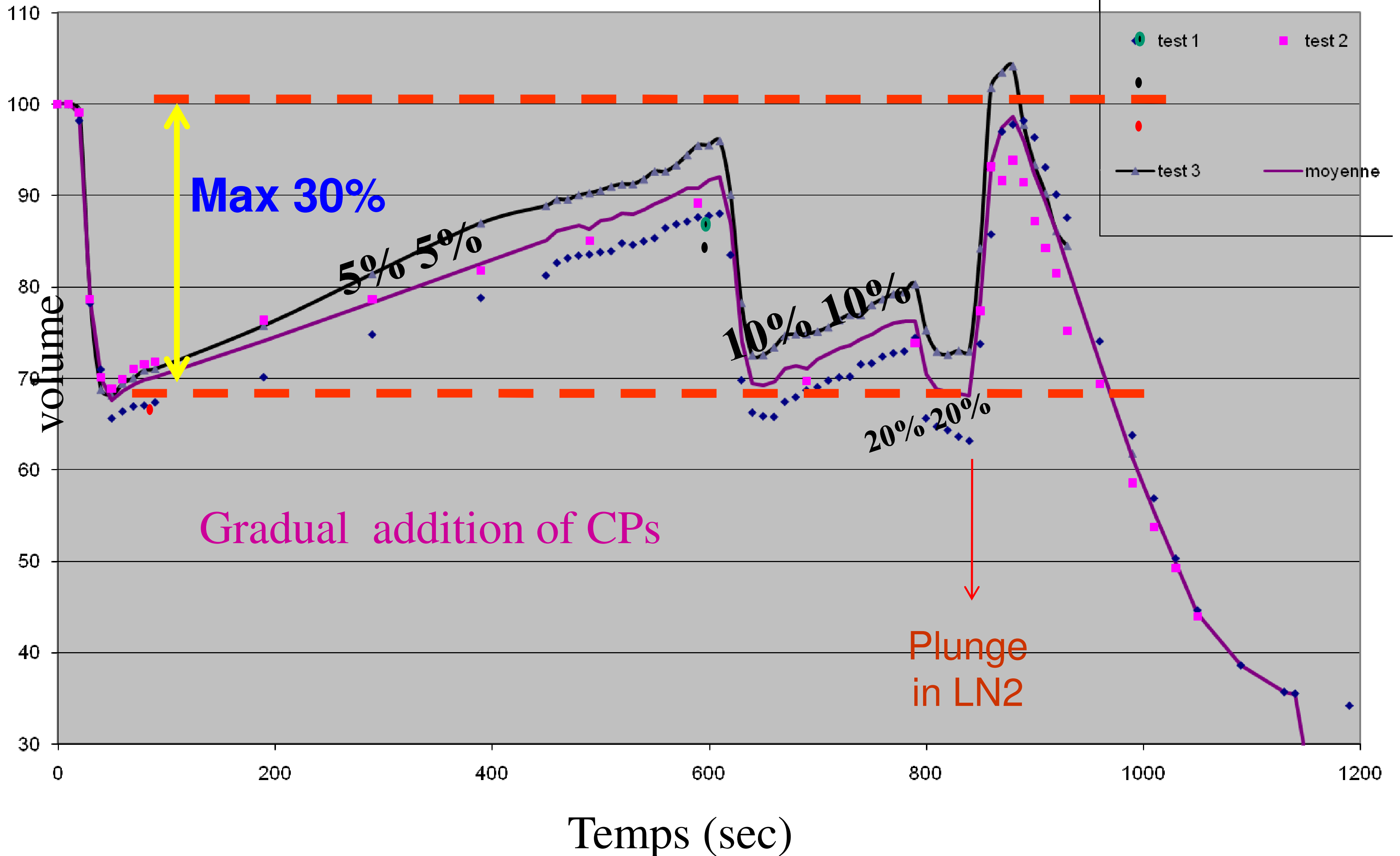
- After increasing the concentration of CP
- After direct or gradual addition of CP

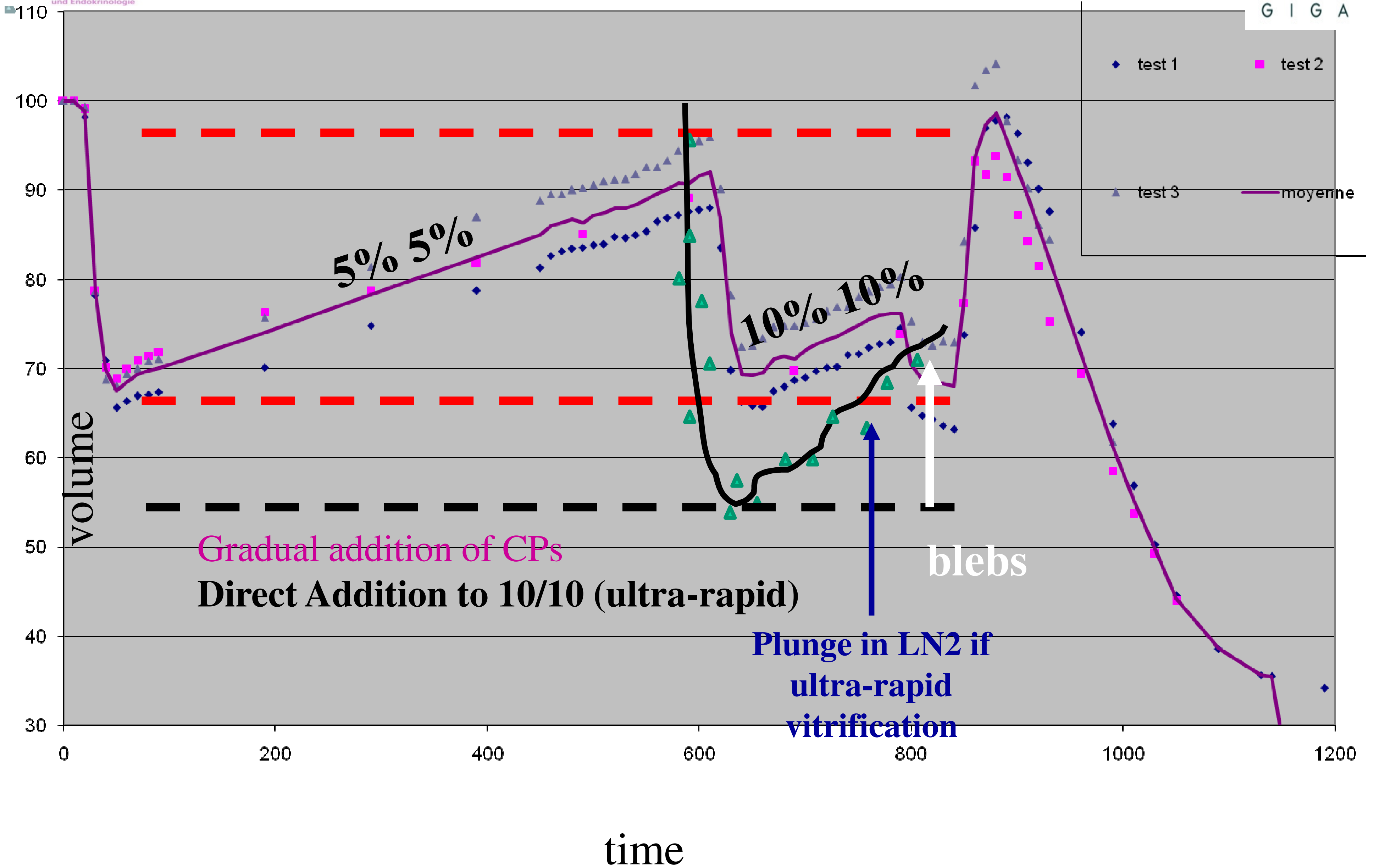
- According to the type of CP
- In combination with solution of different CP

Institute für Reproduktionsmedizin und Endokrinologie

Variation du volume en relation avec différentes concentrations en DMSO/EG solutions

G I G A




Institute für Reproduktionsmedizin und Endokrinologie
GIGA
test 1
test 2
test 3
moyenne
5% 5%
10% 10%
Gradual addition of CPs
Direct Addition to 10/10 (ultra-rapid)
blebs
Plunge in LN2 if ultra-rapid vitrification
volume
time
110
100
90
80
70
60
50
40
30
0
200
400
600
800
1000
1200

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Conclusions from cinematographic analysis

- One step addition of the CP:
  - ⇨ Shrinkage of more than 50% of the initial volume during the dehydration step
  - ⇨ Faster entrance of CP
  - ⇨ Higher frequency of apparition of blebs (faster with PROH)
- Several steps:
  - ⇨ No excessive dehydration
  - ⇨ Lower rates of blebs (more frequent with PROH)
- Only a dehydration step in the vitrification solution (20% - 20%)

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Exposure time and concentration of CPs for blastocysts

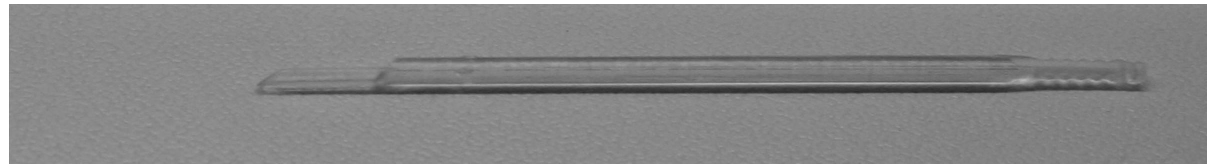
Nb blastocysts	DMSO - EG 2.5%/2.5 %	5%/5%	10%/10%	20%/20%	Survival rates t= 0h	t= 24h
10			2'-4'	40''- 60''	20%	20%
41			6'-10'	40''- 60''	68%	51%
100		**5 '– 10 '**	**3'-4'**	**40''- 60''**	**96%**	**87%**
21	5'	10'	3'-4'	40''- 60''	95%	95%

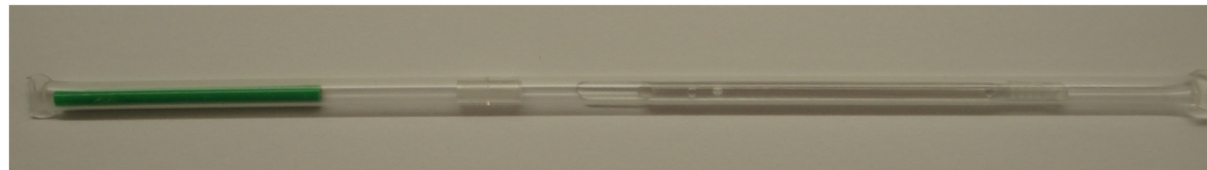
EVOLUTION OF THE VITRIFICATION TECHNIQUE IN „CLOSED" CARRIER

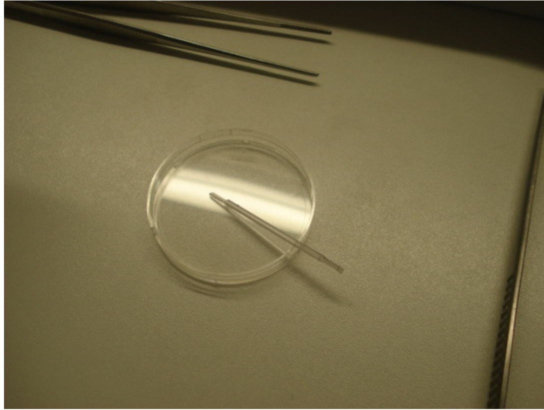
Institute für
Reproduktionsmedizin
und Endokrinologie

GIGA

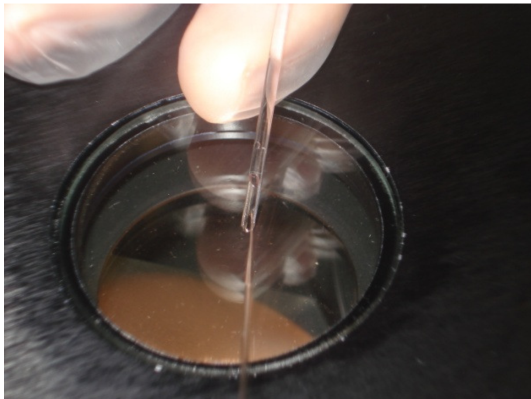
CLINICAL APPLICATION OF ASEPTIC VITRIFICATION USING THE « VITRISAFE » AS CARRIER

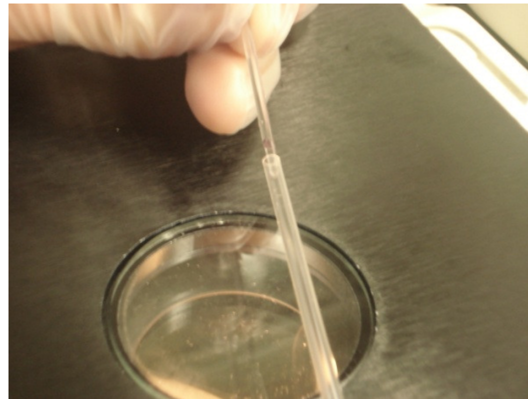


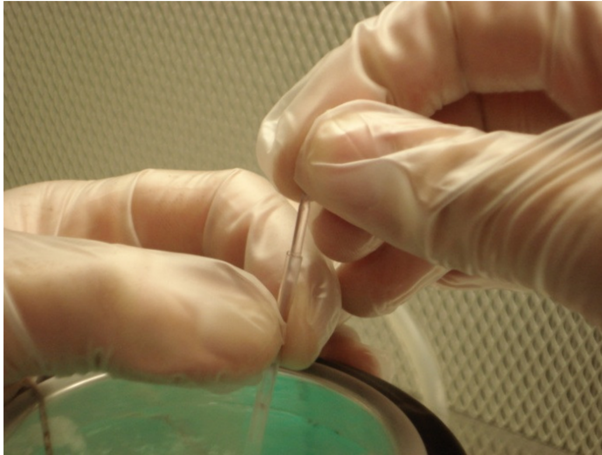


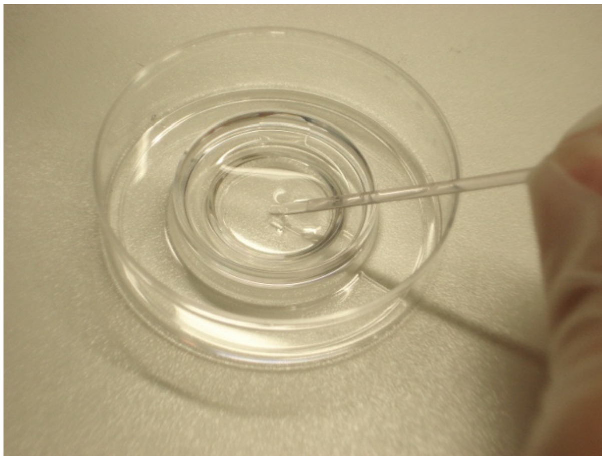


Loading of oocytes or embryos on the "VitriSafe" carrier









Warming and dilution

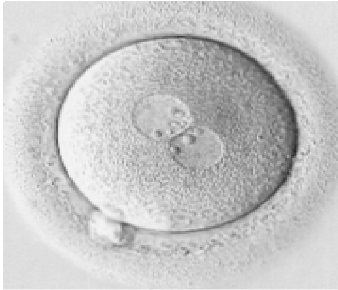
ZYGOTES

DMSO – EG

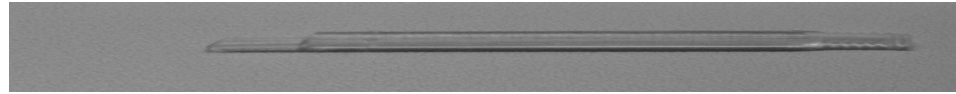
5%-5% (5 - 10 min) 10% - 10% (4 min) 20% - 20% (45 – 60 sec)

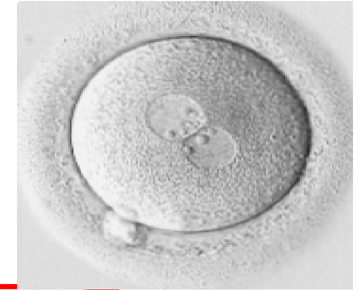
WARMING: Suc 1M 1'Suc 0.75M 1'30'' Suc 0.5M 2'

Suc 0.25M 2-3' Suc 0.125M 2-3'



ASEPTIC Vitrification of ZYGOTES (OHSS)

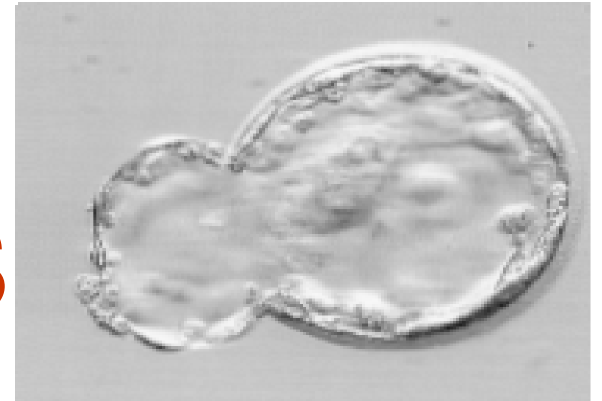


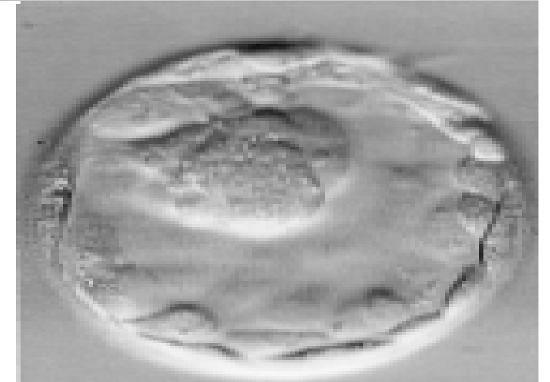


# Vitrification – warming cycles	26 (102 x 2PN)
Survival after warming (%)	96% (98)
Day 5 culture	
% blastocysts	52%
# Ongoing pregnancies (%)/ V-W cycle	10 (38%)
Implantation rates (%)	27%

** revitrification of 8 blastocysts

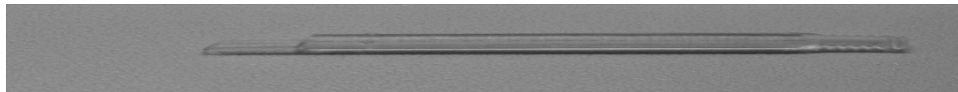
BLASTOCYSTS

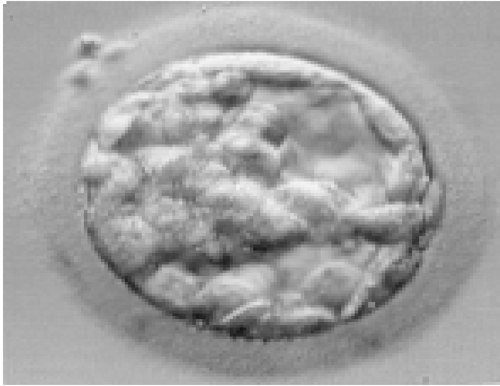




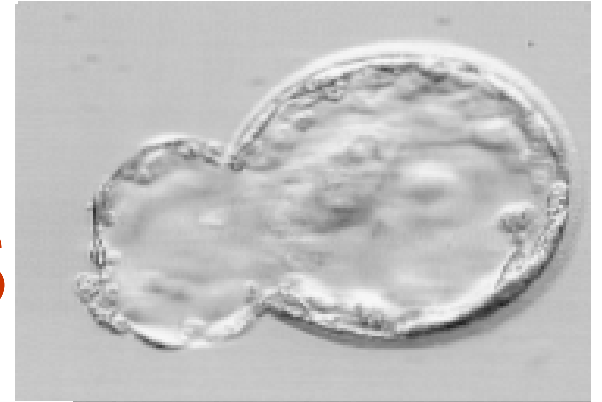
"Development of an aseptic vitrification technique: application to blastocysts originating from infertile patients, egg donors and after in vitro maturation"

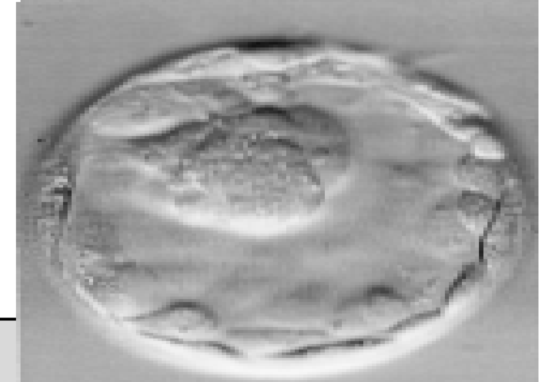
Vanderzwalmen 2009 RBMonline in Press November





BLASTOCYSTS





DMSO – EG

5%-5% (5 - 10min) 10% - 10% (4 min) 20% - 20% (45 – 60 sec)

<u>WARMING</u>: Suc 1M 1’ Suc 0.75M 1’30” Suc 0.5M 2’

Suc 0.25M 2-3’ Suc 0.125M 2-3’

NOT NEED TO PERFORM ARTIFICIAL SHRINKAGE OF THE BLASTOCOELE

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Application of aseptic vitrification of blastocysts in a group of male and female infertiles patients

« closed » Embryo carriers:	Vitrisafe
Nº vitrification - warming cycles	153
Survival before embryo transfer after 24h culture	77%
Ongoing PR / vitrification - warming cycles	66 (43%)
N° FHB - % Implantation	78 (32 %)

Institute für Reproduktionsmedizin und Endokrinologie

Clinical outcomes of aseptic vitrification of blastocysts generated from **EGG DONORS**

GIGA

« closed » embryo carriers:

Nº vitrification - warming cycles	*172*
Survival before embryo transfer 24 H	*86%*
Ongoing PR / vitrification - warming cycles	*80 (47%)*
IR	*35%*

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Clinical outcomes *of **IVM embryos***

after FRESH or VITRIFIED embryo transfer

	Fresh	*« closed » vitrification*
Nº IVM cycles	*34*	*49*
Survival before embryo transfer 24 H		*69%*
Ongoing PR / vitrification - warming cycles	*7 (21%)*	*18 (44%)*
IR	*10%*	*30%*

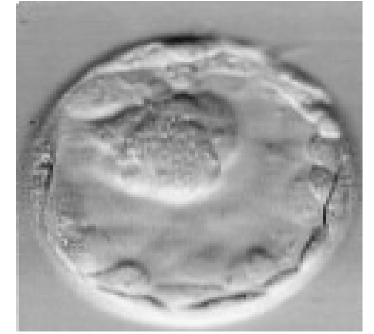
Institute für
Reproduktionsmedizin
und Endokrinologie

GIGA

ASEPTIC VITRIFICATION OF BLASTOCYSTS WITH

GLYCEROL IN „CLOSED“ CARRIER

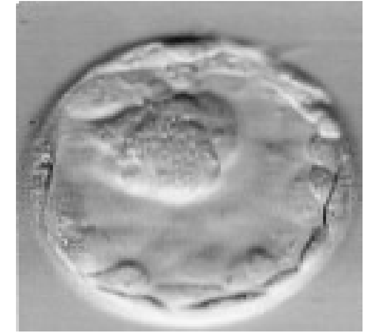
ASEPTIC Vitrification of BLASTOCYSTS



Glycerol - PROH

# Vitrification – warming cycles	*21*
Blastocysts after 24 h (%)	*83*
# Ongoing pregnancies	*6*
vitrification cycles (%)	*29 %*
Implantation rates (%)	*23*

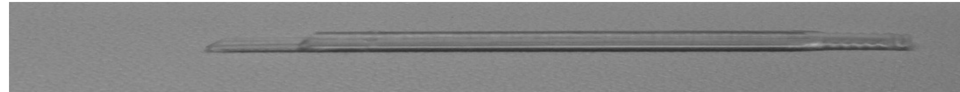
ASEPTIC Vitrification of BLASTOCYSTS



Glycerol – EG (ADDITION IN 4 STEPS)

# Vitrification – warming cycles	12
Survival after warming (%)	98
Blastocysts after 24 h (%)	92
# Ongoing pregnancies vitrification cycles (%)	6 (50%)
Implantation rates (%)	42%

OOCYTES



5 steps dilution before vitrification

6 steps dilution after vitrification

	Carrier device	
	“open”	*“closed”*
Nb vitrificatin cycles	19	15
Nb vitrified oocytes	127 (6.7)	96 (6.4)
Survival rates	94.4% (120)	92.7% (89)
2 PN	73.8% (88)	86.5% (77)
Ongoing pregnancy / vitrified cycle	21.1% (4)	40.0% (6)
Implantation rate		
nb transferred embryos	9.6%	14.3%
nb vitrified oocytes	4.0%	7.3%

Iakentro - Prapas

Institute für Reproduktionsmedizin und Endokrinologie

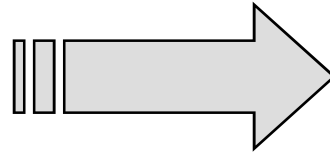
EVOLUTION OF THE TECHNIQUE (2008 – 20??)

GIGA

Vitrification in straw

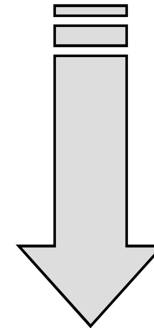
« closed » carrier

Cooling < 2.000°C/ min
Warming ~500°C/ min

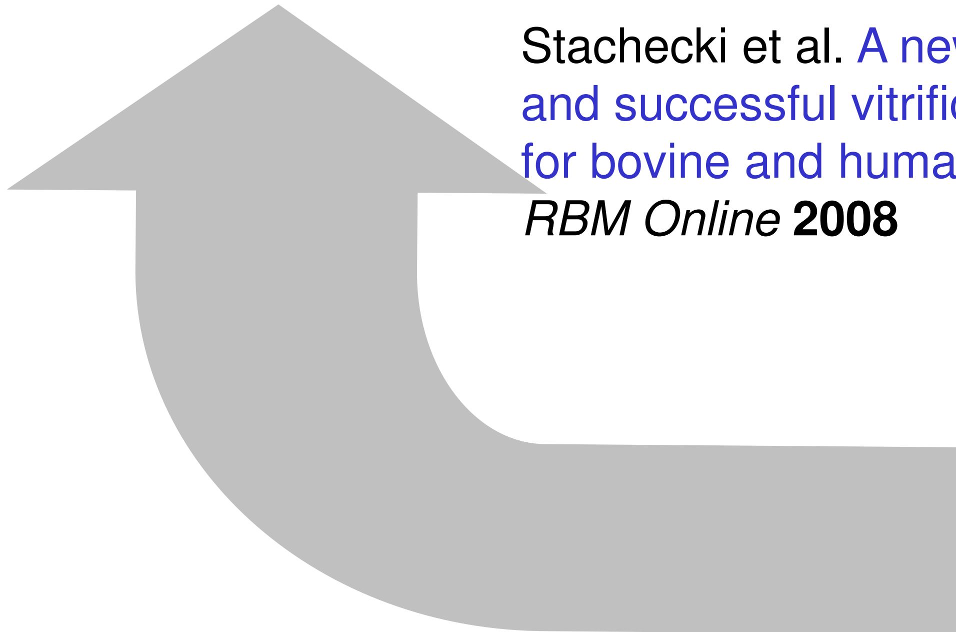


Vitrification using

« OPEN » carrier



Stachecki et al. A new safe, simple and successful vitrification method for bovine and human blastocysts. *RBM Online* **2008**



Vitrification using

« CLOSED » carrier

Institute für Reproduktionsmedizin und Endokrinologie

GIGA

Nb thawed blastocysts	884
Intact	89%
Nb Transfers	358
Nb blast transferred	768
Pregnancy/transfer	56.4 %
FHB	42.8%

Clinical results after vitrification of blastocysts in **0.25 ml straw** and exposed to S^3 CP solution

Stachecki et al. RBM online 2008

Conclusions (I)

- *Vitrification has suffered from two drawbacks:*

  the non-aseptic cooling/storage conditions and

  the use of DMSO/EG as CP solutions.

- *Vitrification in reduced cooling rates ("closed carrier") is proven to be effective for MII oocytes and all stage of embryo development.*

- *The balance required in vitrification is between*

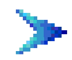
  (i) establishment of a safe system for maximal, reliable and optimal cooling and warming rates while avoiding consequent damage of the cells, and

  (ii) to find, the minimal and optimal conditions of exposure (reduce shrinkage – swelling stress) to high cryoprotectant concentrations (time and T°) needed to obtain and maintain an intracellular non-toxic vitrified environment.

Conclusions (II)

- *Addition of CPs as the removal in several steps reduce the shrinkage/swelling stress*
- *No need to perform artificial shrinkage of the blastocoel cavity.*
- *Replacement of DMSO/EG by Glycerol/PROH or Glycerol/EG is effective for blastocysts.*
- *The »closed » carrier (VitriSafe – HVS) are efficient devices:*
  - *easy to handle*
  - *Permit very high warming up to 20.000°C/min (eliminate de risk of devitrification)*
  - *Easy to store in the LN2 tank*

Conclusions (III)



Ultra-rapid vitrification is also safe because preliminary results show no increase of major and minor birth defects . (Takahashi 2005)