Optimising Vitrification

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CooperSurgical
Fertility and Genomic Solutions

Aims & Objectives

- introduction to cryobiology and use of CPAs
- highlight key areas of concern
- detail stage-by-stage considerations
- discussion

Cryopreservation

- the main aim of any cryopreservation programme is to ensure a high percentage of survival and viability after thawing

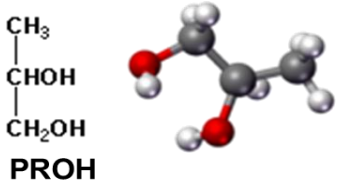
- six steps:
  - equilibration with cryoprotectant agents to induce dehydration
  - reduction of the temperature to a cooling point
  - storage
  - warming
  - rehydration
  - resumption of cellular functions

Cryoprotectants

- agents which influence the transition from water to ice
- stabilise bio-molecules as water substitutes
- reduce ice nucleation and growth during thawing
- intracellular or permeating
- extracellular or non-permeating

Cryoprotectants

- intracellular or permeating



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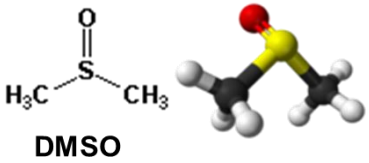
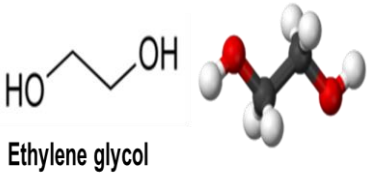
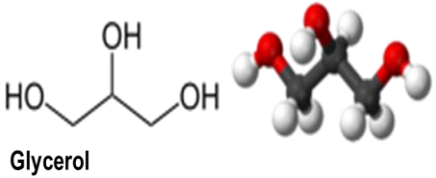


DMSO

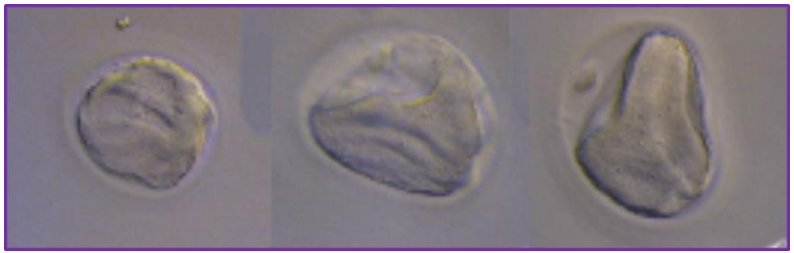


Ethylene glycol



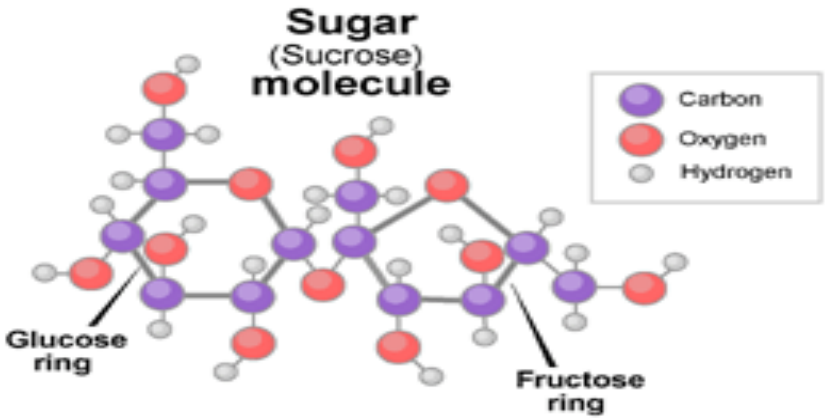
Glycerol

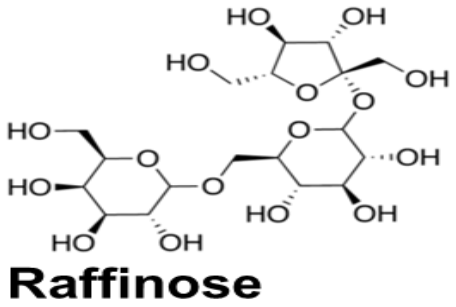
  - **enter cells and replace water**
  - **as they enter cells more slowly than water exits, there is shrinking and re-expansion**



Cryoprotectants

- extracellular or non-permeating





Warming/thawing

- after thawing, need progressive dilution of CPA
- form osmotic gradient by creating an extracellular environment with lower osmolarity
- this results in the gradual movement of water in to the cell = rehydration
- associated diffusion of intracellular CPA to the extracellular environment

Optimising vitrification: what is important?

Recurring questions…

- what should be vitrified?
- which media/CPA is best?
- how and when must we control temperature?
- are volumes of media important?
- how long must we equilibrate?
- must we collapse blastocysts and, if so, how is equilibration affected?
- choice of carrier: open or closed systems?
- how do we optimise warming procedures?

Embryo grading: what to vitrify?

- cleavage stage
  - appropriate cell number (by age hpi)
  - <20% blast diameter difference; <20% fragmentation

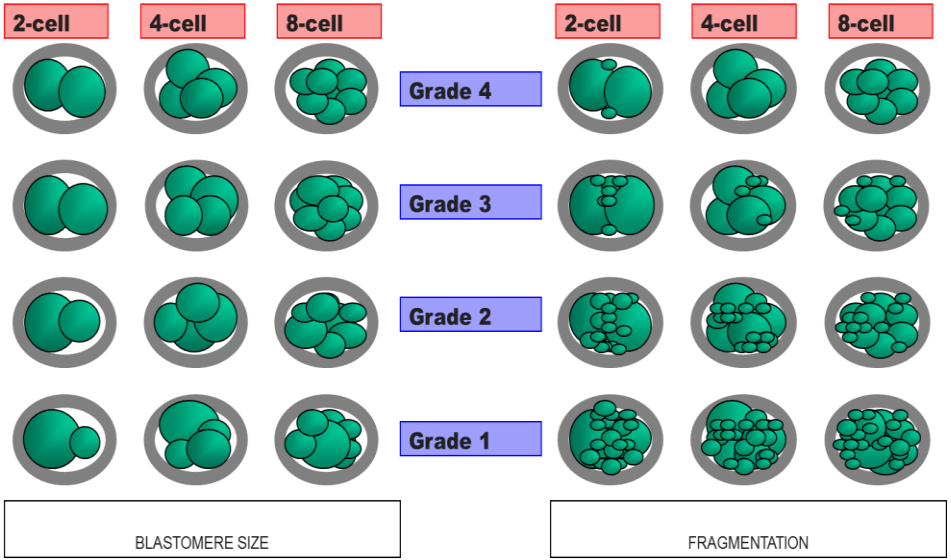
- blastocyst stage
  - full blastocyst
    - compacted ICM, loosely adhered, some individual cells
    - confluent TE layer, fewer and larger cells
    - leave early blasts to D6 but FBT as a day 5

Embryo grading

Blastomere number

Blastomere size
4 = regular, even division
3 = <20% difference (blast diam)
2 = 20-50% difference
1 = >50% difference *after Hardarson et al 2001[33]*

Fragmentation
4 = <10% frags by volume
3 = 10-20%
2 = 20-50%
1 = >50% *after van Royen et al 2003[35]*



Elective Single Embryo Transfer: Guidelines for Practice British Fertility Society and Association of Clinical Embryologists

RACHEL CUTTING[1], DAVE MORROLL[2], STEPHEN A. ROBERTS[3], SUSAN PICKERING[4], & ANTHONY RUTHERFORD[2] (ON BEHALF OF THE BFS AND ACE)

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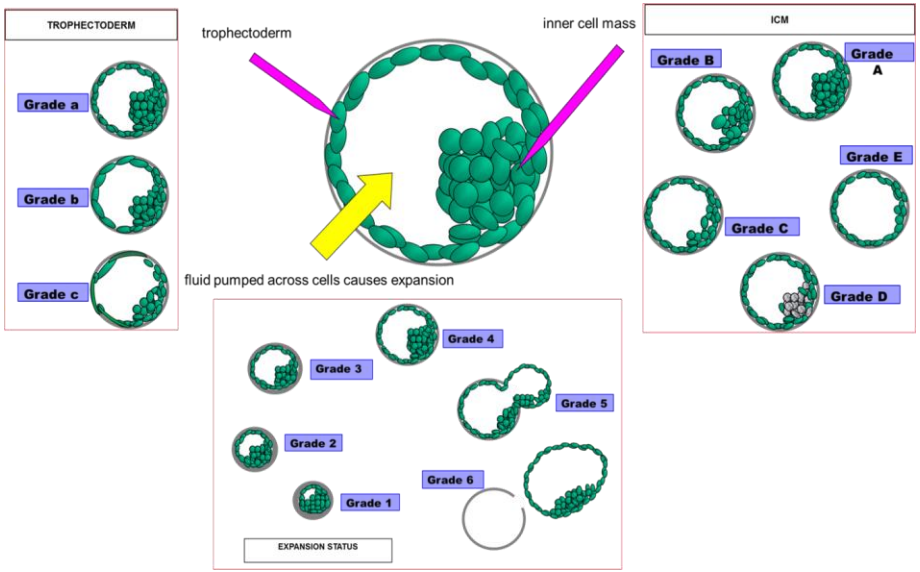
Blastocyst grading

Expansion Status	1 = Early blastocyst; blastocoel less than half the volume of the embryo, little or no expansion in overall size, zona pellucida (ZP) still thick
	2 = Blastocyst; blastocoel more than half the volume of the embryo, some expansion in overall size, ZP beginning to thin
	3 = Full blastocyst; blastcoel completely fills the embryo.
	4 = Expanded blastocyst: blastocoel volume now larger than that of the early embryo. ZP very thin
	5= Hatching blastocyst; trophectoderm has started to herniated through the ZP
	6 = Hatched blastocyst; the blastocyst has evacuated the ZP
ICM grading	A = ICM prominent, easily discernible and consisting of many cells, cells compacted and tightly adhered together
	B= Cells less compacted so larger in size, cells loosely adhered together, some individual cells may be visible
	C = Very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophectoderm
	D = Cells of the ICM appear degenerate or necrotic
	E = No ICM cells discernible in any focal plane
Trophectoderm	a = Many small identical cells forming a continuous trophectoderm layer
	b = Fewer, larger cells, may not form a completely continuous layer
	c= Sparse cells, may be very large, very flat or appear degenerate



Which cryoprotectant?

- no clear evidence to favour any one system
- optimise system within each laboratory
- anecdotally, DMSO favoured for oocytes?

How and when to control temperature?

- optimised at RT°C (constant in all seasons)
- permeability of CPAs into cells is temperature-dependent
- rate of ingress of CPA can impact on outcomes

- warm solutions to RT °C at least 30 minutes before use
- switch off heated stages or use ambient plate
- do not leave over light source

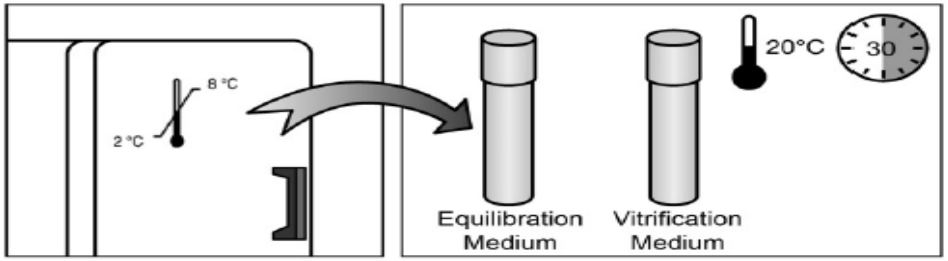
Are media volumes important?

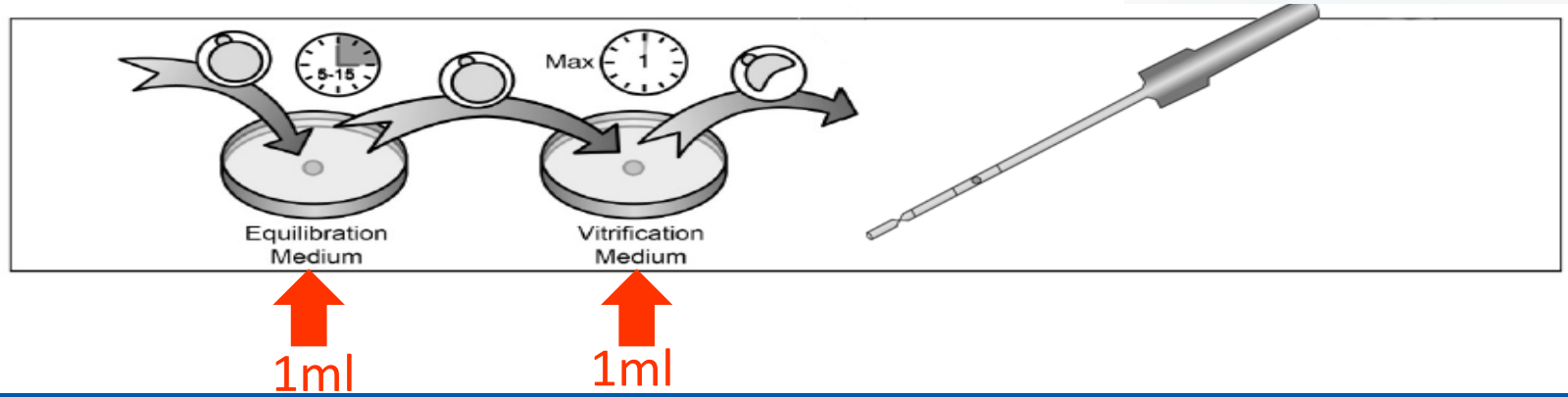
- yes … and no!

- people use small volumes successfully but…
- control of osmolarity changes due to evaporation
  - dispense only immediately before use
  - be aware of effects of airflow in workstation
- pipetting
- number of oocytes/embryos handled at one time

*** most new users improved outcomes using larger volumes ***

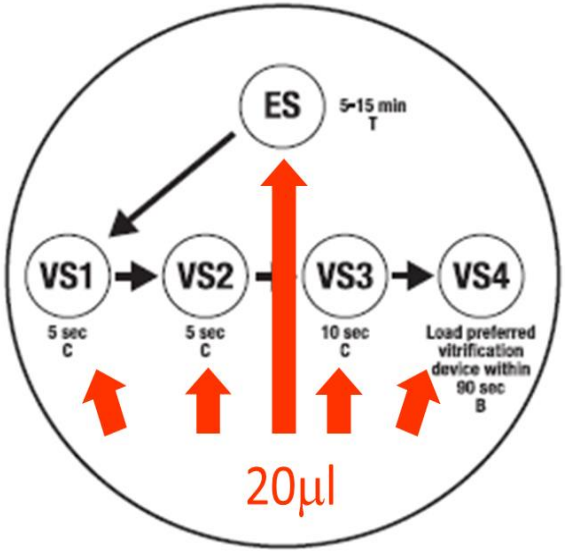
Vitrification Cooling Protocol: ORIGIO





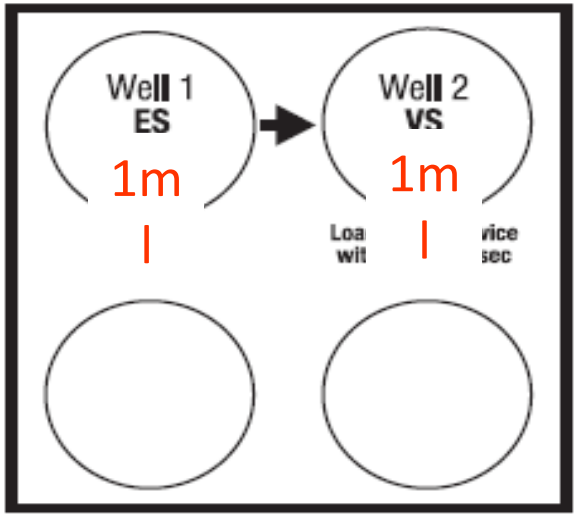
Vitrification Cooling Protocol: SAGE

Fig. 1



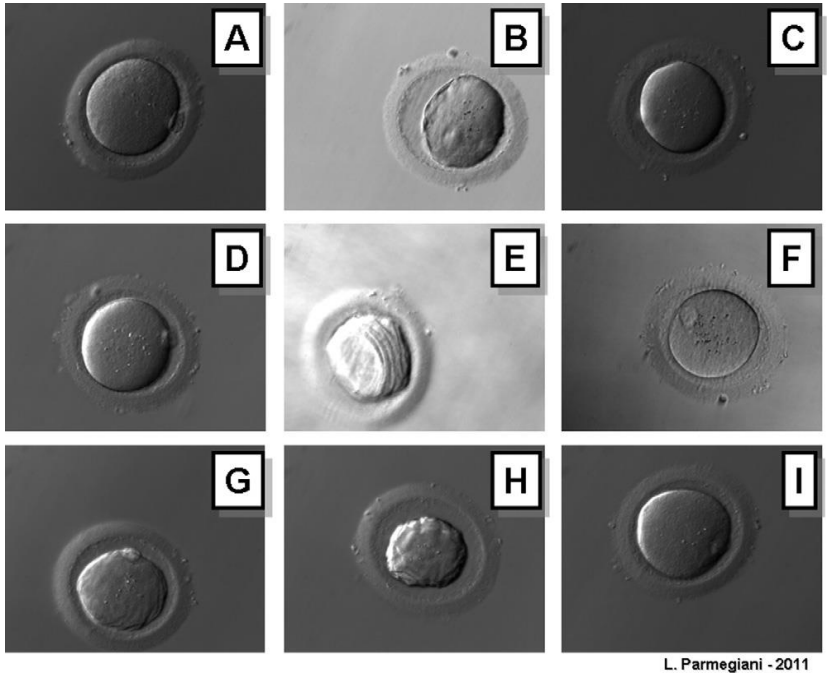
KEY:
ES = Equilibration Solution
VS = Vitrification Solution
➡ = transfer embryo to next drop
T = Top of drop
C = Center of drop
B = Bottom of drop

Fig. 2



KEY:
ES = Equilibration Solution
VS = Vitrification Solution
➡ = transfer embryo to next well

How long should we equilibrate?



- stage-dependent
- 5 – 15 minutes
- options
  - observe and wait to see full (90%) re-expansion
  - establish median (fixed) time for your laboratory
- blastocysts – effect of collapse
  - collapsed: use 5 minutes
  - non-collapsed: check for re-expansion of cells NOT blastocoel

Should we collapse blastocysts?

- some clinics doing well without collapsing
- preferred options
  - puncture with ICSI needle
  - laser
  - small tip (micropipetting)
- generally advised
- wait for 50% shrinkage and then move straight to VM

Reproductive Biology and Endocrinology 2009, **7**:99 http://www.rbej.com/content/7/1/99

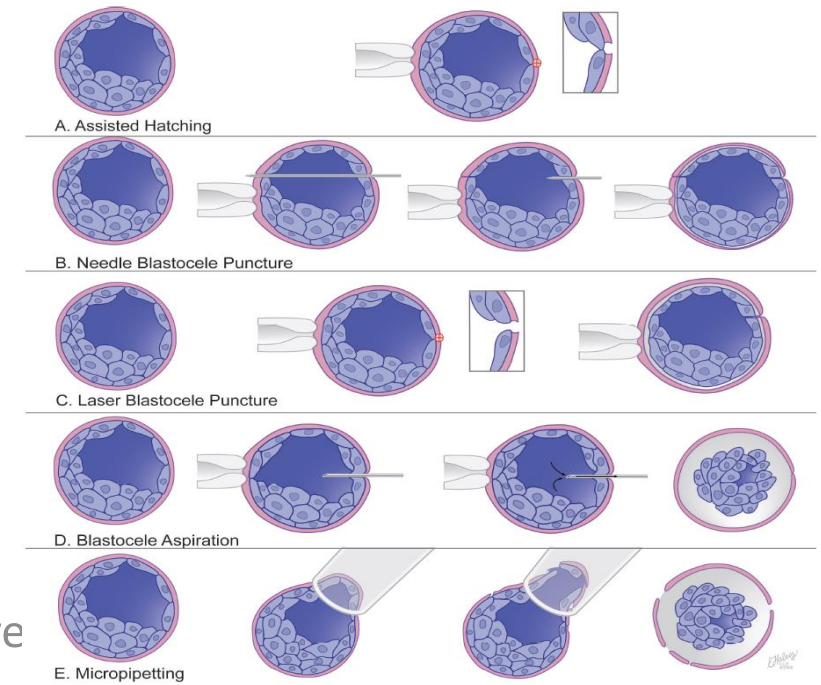
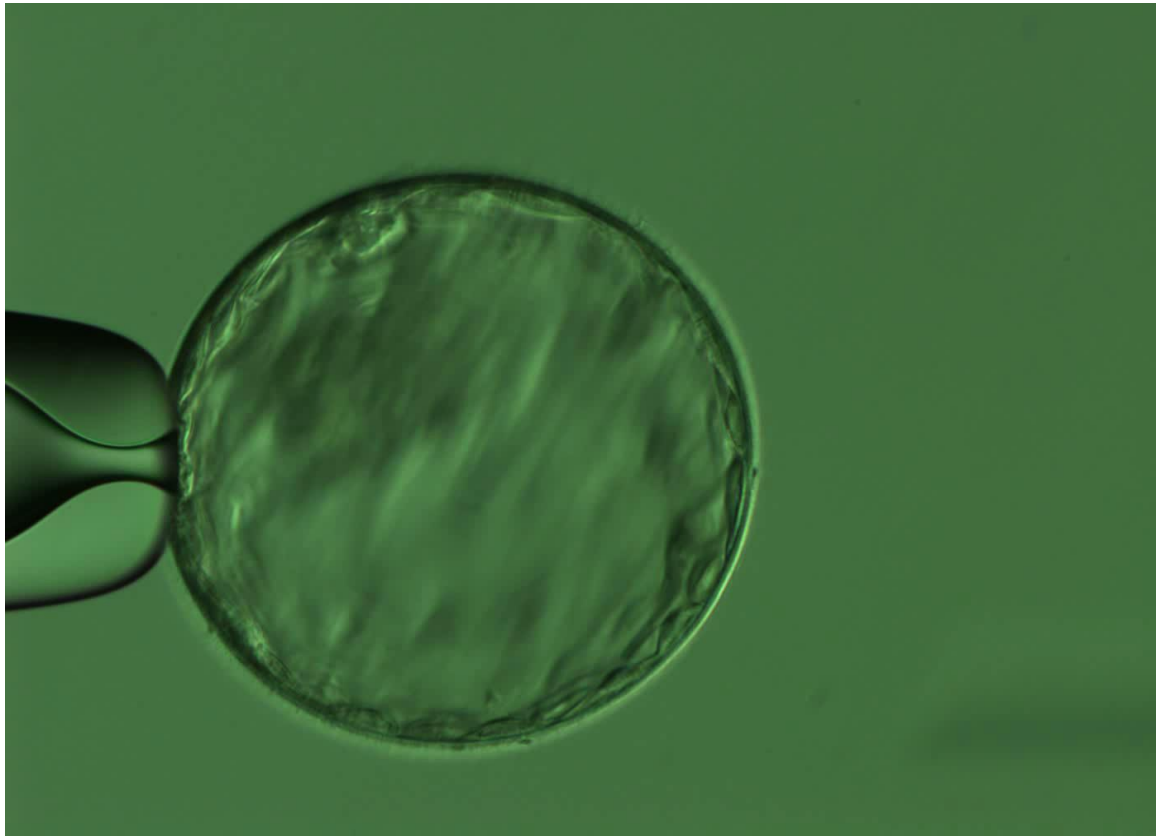


Figure 1
Different pre-vitrification interventions for blastocysts. A. Assisted hatching: An opening is created in the zona using laser pulse B. Needle blastocoele puncture: A needle is passed through the zona and blastocoele and retracted allowing the blastocelic fluid to freely leak. C. Laser blastocoele puncture: laser pulse creates an opening in the zona and a small defect in the trophectoderm causing the blastocoele to leak. D. Blastocoele aspiration: An injection needle is introduced into the blastocoele and blastocoelic volume is sucked out. E. Micropipetting: Passing the blastocysts through a narrow pipette would crack the zona and compress the blastocoele to leak through the cracked zona.

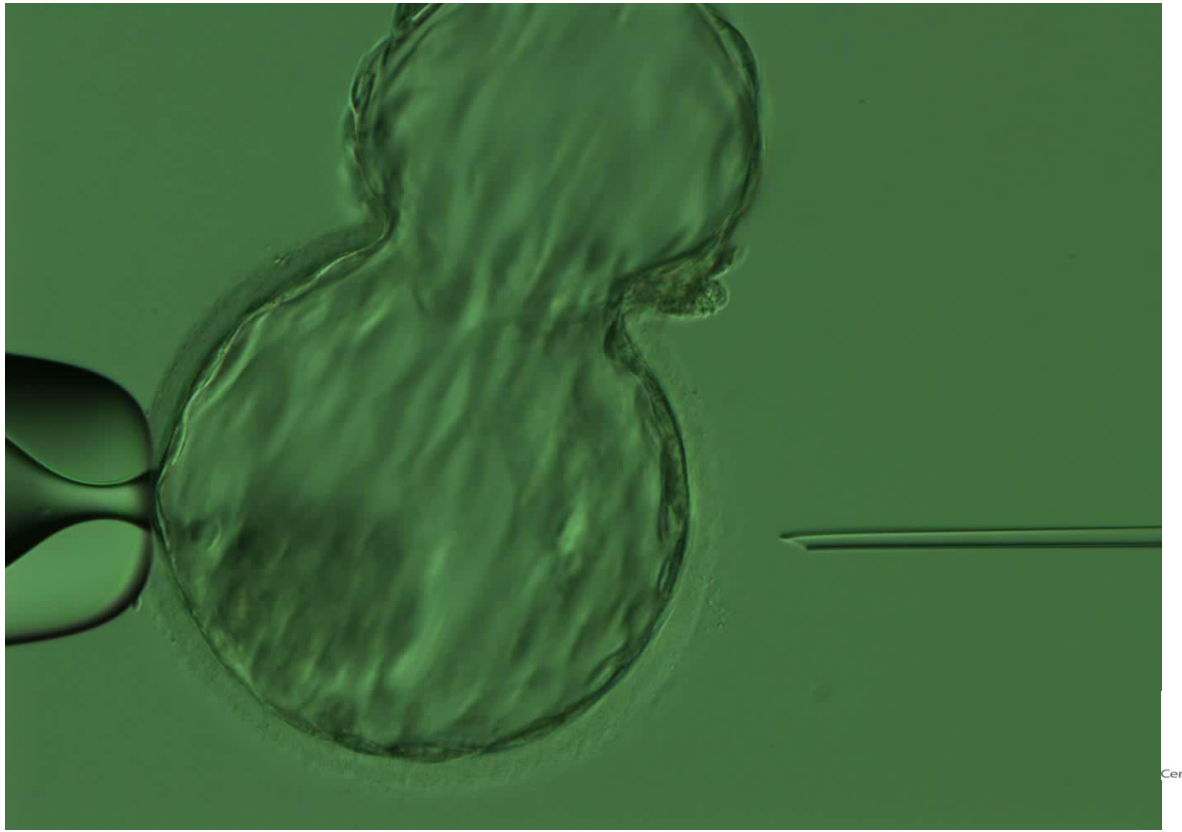
Should we collapse blastocysts?



video courtesy of
Lyndon Miles, Cardiff

CRGW
Centre for Reproduction and Gynaecology Wales

Should we collapse blastocysts?



video courtesy of
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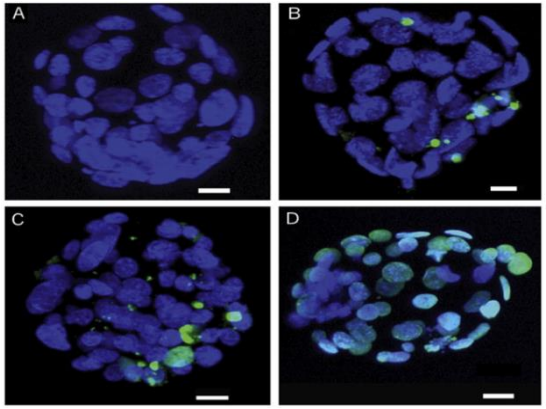
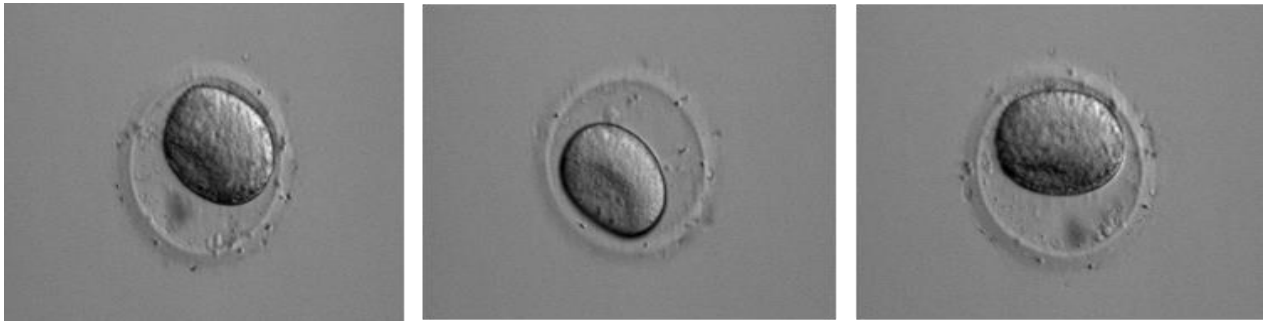
Should we collapse blastocysts?

Table 2. Effect of blastocele aspiration and spontaneous hatching on expanded blastocysts postwarming DNA integrity.

	Expanded blastocysts			
	Fresh	Vitrified		
Parameter	Control (n = 14)	No intervention (n = 22)	Blastocele aspirated (n = 22)	Spontaneously hatched (n = 12)
Total blastomeres mean ± SD	48.00 ± 15.66	46.91 ± 15.05	39.95 ± 12.84	62.00 ± 13.10
Survival	100%	90.9%	100%	100%
DNA integrity index mean ± SD	95.47 ± 4.25	77.61 ± 16.65 (P<.001)[a]	90.08 ± 5.59 (P=.003)[a] (P=.001)[b]	88.45 ± 4.35 (P=.001)[a] (P<.012)[c] (P=.35)[d]



Vitrification step

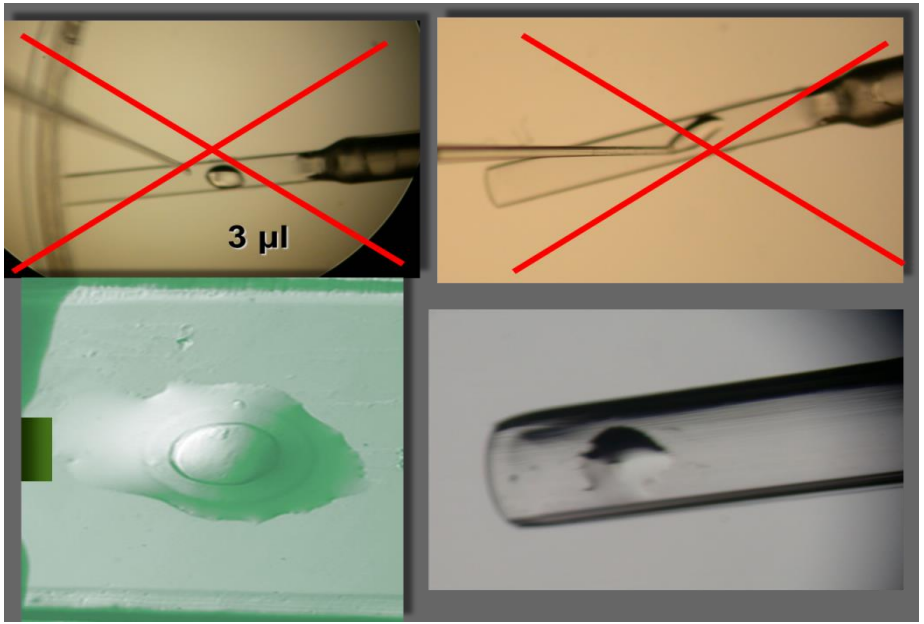


5 secs | 30 secs | 60 secs

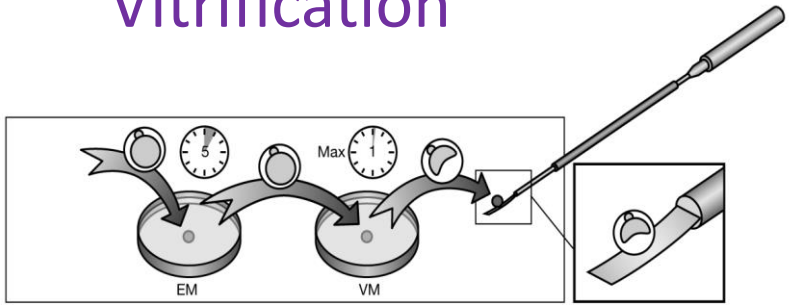
- do not pre-load pipette with VM/VS
- ensure complete mixing
- care with timing before loading

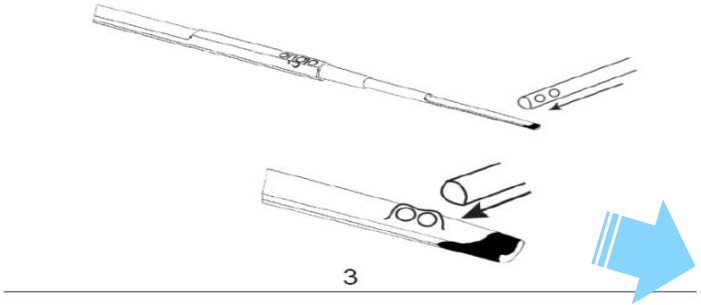
Choice of carrier: open or closed?

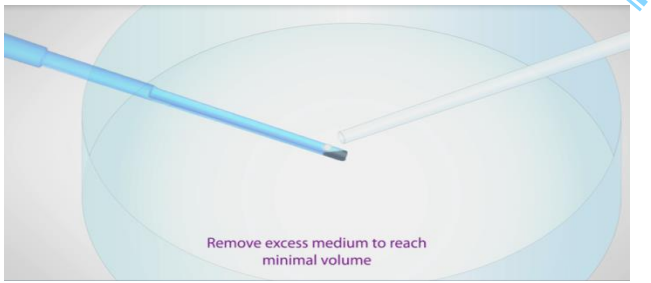
- loading
  - use a holder
  - minimal volume
  - numbers
  - maximal cooling
  - timing (not too soon so evaporation significant)

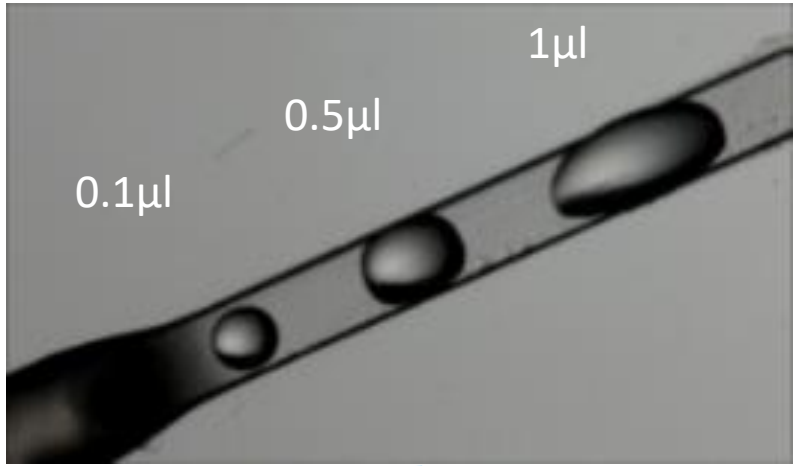


Vitrification



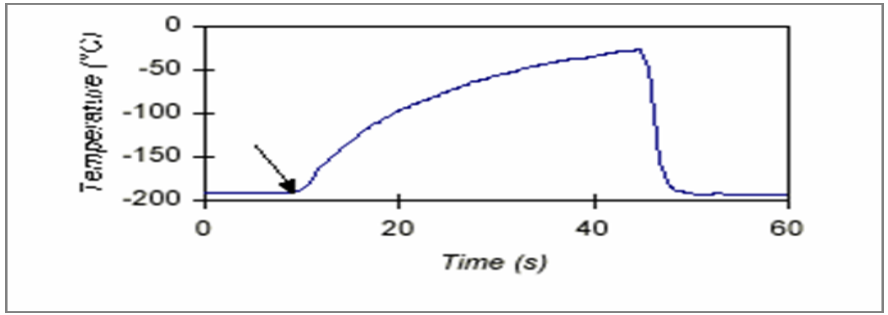




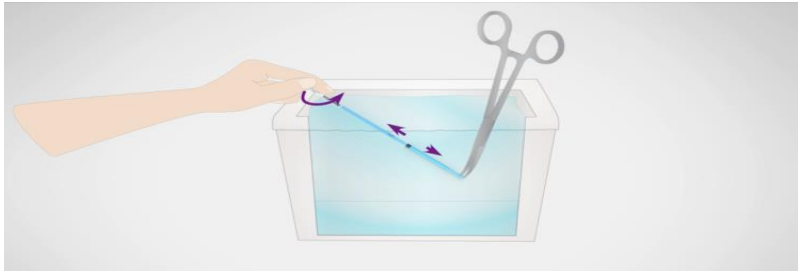


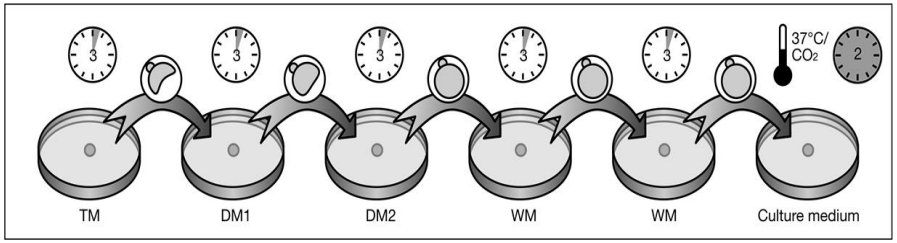
Storage conditions

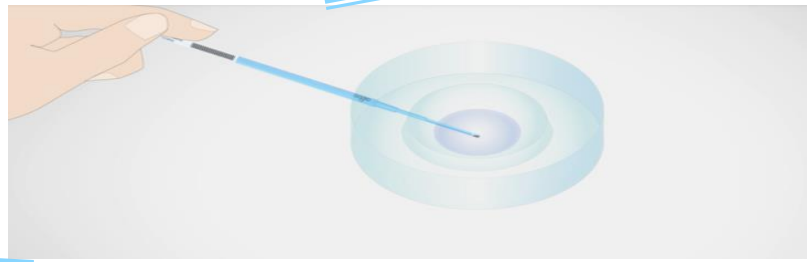
- liquid or vapour phase
- care with handling
  - movement to small vessels prior to warming
  - audits
- even brief exposure to temperature above glass transition temperature could be disastrous

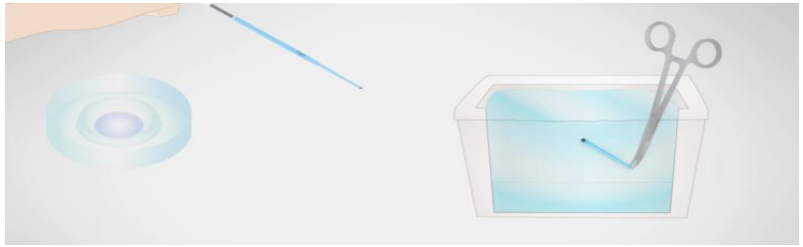


Warming

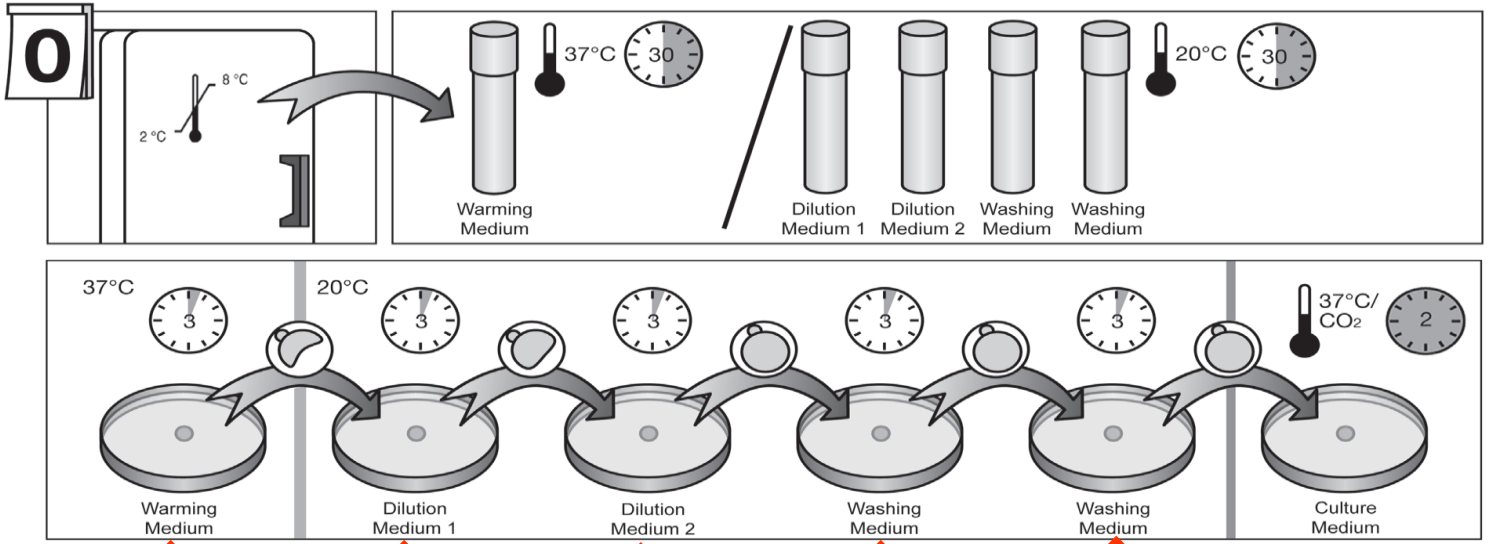








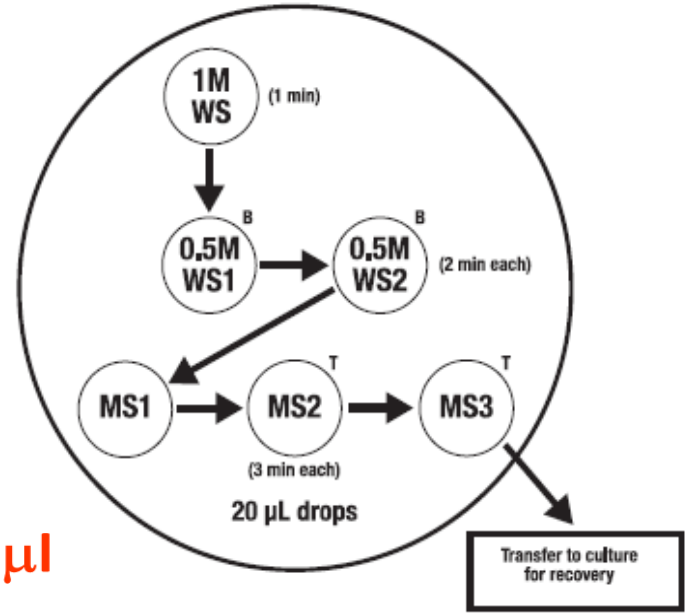
Vitrification Warming Protocol: Origio



warming step critical – care with volumes; otherwise use smaller drops (± oil)

Vitrification Warming Protocol: Sage

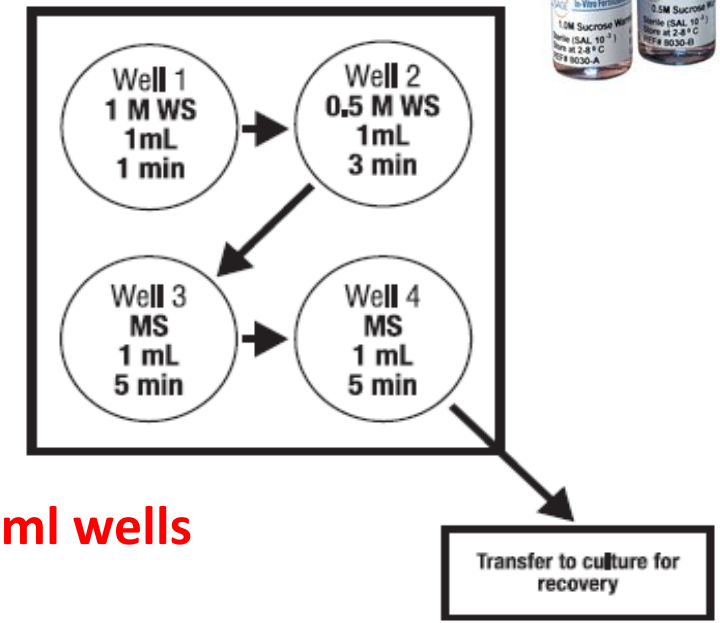
Fig. 1



20µl

KEY:
1M WS = 1 M Sucrose Warming Solution
0.5M WS = 0.5 M Sucrose Warming Solution
MS = MOPS Solution
➡ = transfer embryo to next drop
T = Top of drop
B = Bottom of drop

Fig. 2



1ml wells

KEY:
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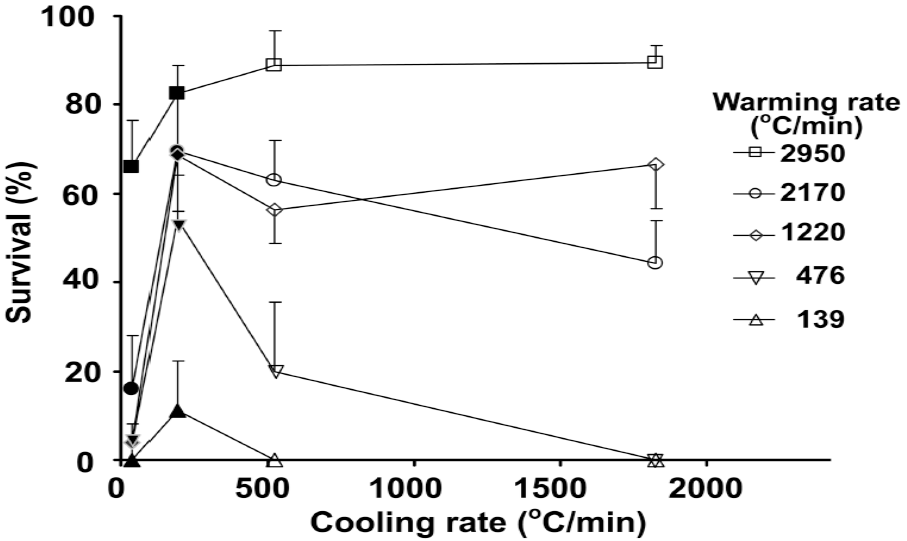
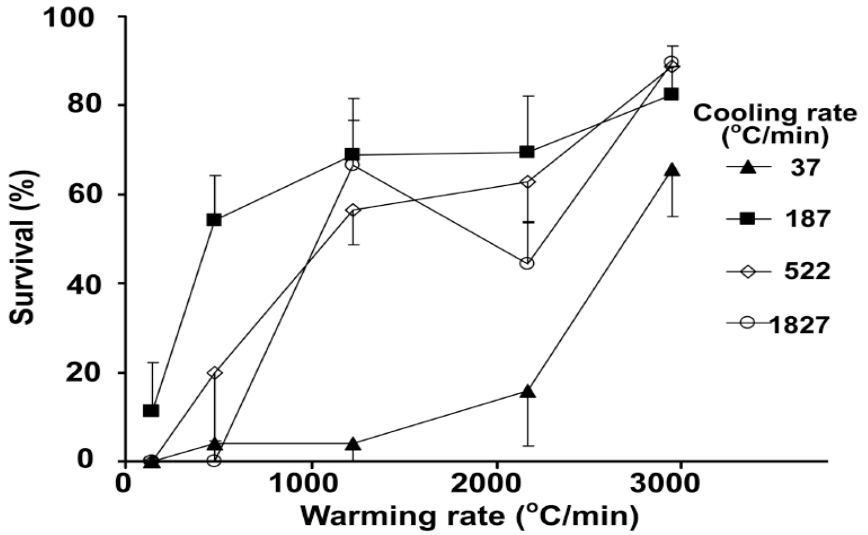
Vitrification Warming Protocol





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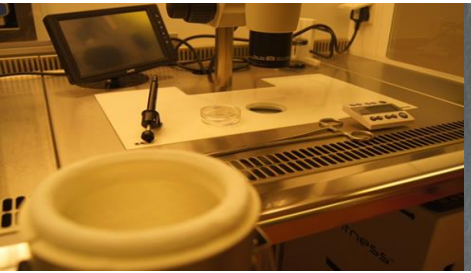
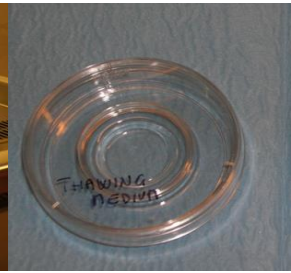
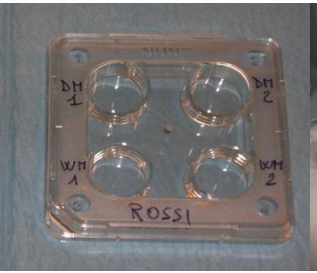
The Dominance of Warming Rate Over Cooling Rate in the Survival of Mouse Oocytes Subjected to a Vitrification Procedure*

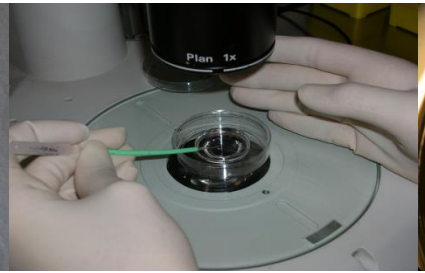
Shinsuke Seki and **Peter Mazur***
Fundamental and Applied Cryobiology Group, Department of Biochemistry and Cellular and Molecular Biology, The University of Tennessee, Knoxville, Tennessee 37932-2575

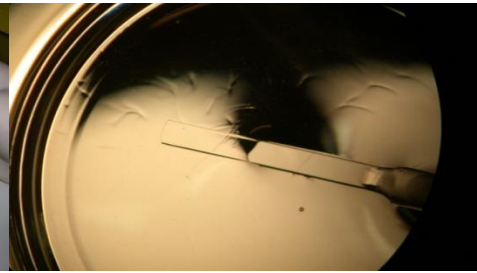
Vitrification Warming Procedure

- prepare in advance
- first step must be at 37°C
  - keep warming medium at 37°C (volume; oil/water in outer well)
  - shortest possible time from liq N2 to WM







- remaining steps at RT°C or 35-37°C depending on kit used

Comparison of Slow Freezing & Vitrification

Table 1 Oocyte key performance indicator values.

KPI			*Competence*	*Benchmark*
O1	Morphological survival	Freezing	≥50%	75%
		Vitrification	70%	85% (95% for donors <30 years)
O2	Fertilization rate	No more than 10% (absolute; i.e. 10 percentage points) lower than that for the comparable population of fresh oocytes at the centre		
O3	Embryo development rate	Freezing	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre	The same as for the comparable population of fresh embryos at the centre
		Vitrification	The same as for the comparable population of fresh embryos at the centre	
O4	Implantation rate	No more than 10–30% (relative) lower than that for the comparable population of fresh embryos at the centre		

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Comparison of Slow Freezing & Vitrification

Table 3 Embryo key performance indicator values.

KPI			*Competence*	*Benchmark*
E1	Morphological survival: fully intact	Freezing	40%	55%
		Vitrification	70%	85%
E2	Morphological survival: ≥50% intact	Freezing	60%	85%
		Vitrification	85%	95%
E3	Post-thaw development (including implantation rate) for fully intact embryos		≤10% (relative) lower than that for the comparable population of fresh embryos at the centre	The same as for the comparable population of fresh embryos at the centre

The KPI values are calculated as the proportion of all thawed/warmed embryos.

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Comparison of Slow Freezing & Vitrification

Table 4 Blastocyst key performance indicator values.

KPI			*Competence*	*Benchmark*
B1	Survival rate	Freezing	70%	85%
		Vitrification	80%	95%
B2	Transfer rate	Freezing	70%	85%
		Vitrification	80%	95%
B3	Implantation rate		≤10% (relative) lower than that for the comparable population of fresh embryos at the centre	The same as for the comparable population of fresh embryos at the centre

The KPI values are calculated as the proportion of all thawed/warmed blastocysts.

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How to start vitrification successfully (or optimise your results)

- select only good quality gametes and embryos
- be fastidious with control of timings, temperature, osmolarity and pipetting
- evaluate time for shrinkage and re-expansion in EM (use median time as fixed time)
- load in very small volumes
- take care with handling and storage
- remember that warming is as critical as vitrification steps


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