Cryobiology for Clinical Embryologists

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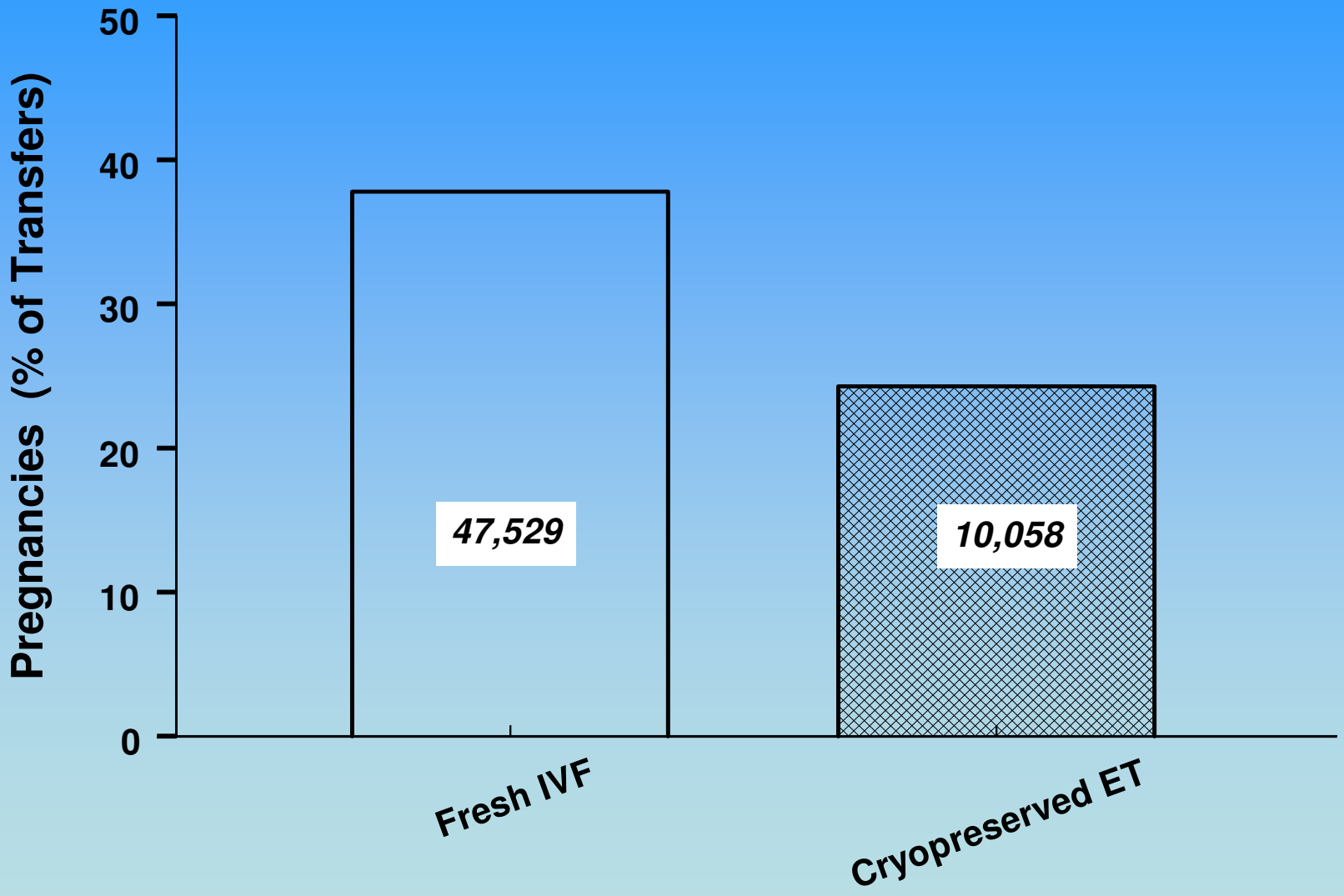
During the past 37 years, cryopreserved oocytes and embryos used to produce …

- ***Millions of cattle and other domestic animals***
- ***Hundreds of thousands of lab animals***
- ***Tens of thousands of children***

1998 ART Results in the U.S.



ASRM/SART Registry 2002 Fert Steril 77:18

Domestic Offspring from ET with Cryopreserved Embryos

Antelope	*Horse*
Cat	*Mouse*
Cattle	*Pig*
Gerbil	*Rat*
Goat	*Sheep*
Hamster	*Rabbit*

Non-Domestic Offspring from ET with Cryopreserved Embryos

Baboon	***Marmoset***
Eland	***Red deer***
Fallow deer	***Wapiti***
Cynomolgus	***Rhesus***

African Wildcat

Cryopreservation Methods

Category	CPA Concn.	Cooling Rate (°C/min)
Equilibrium Cooling	10% ≈ 1.5 M	~0.5
Non-Equilibrium Cooling	>40% ≈ 6 M	>>1,000

Generalized Approach to Cryopreservation

- Suspend cells in cryoprotectant (CPA)
- Cool cells to subzero temperatures,

  i.e. below -130°C
- Use conditions to dehydrate cells,

  i.e. minimize intracellular ice formation
- Store in LN_2 at -196°C
- Warm so as to avoid recrystallization

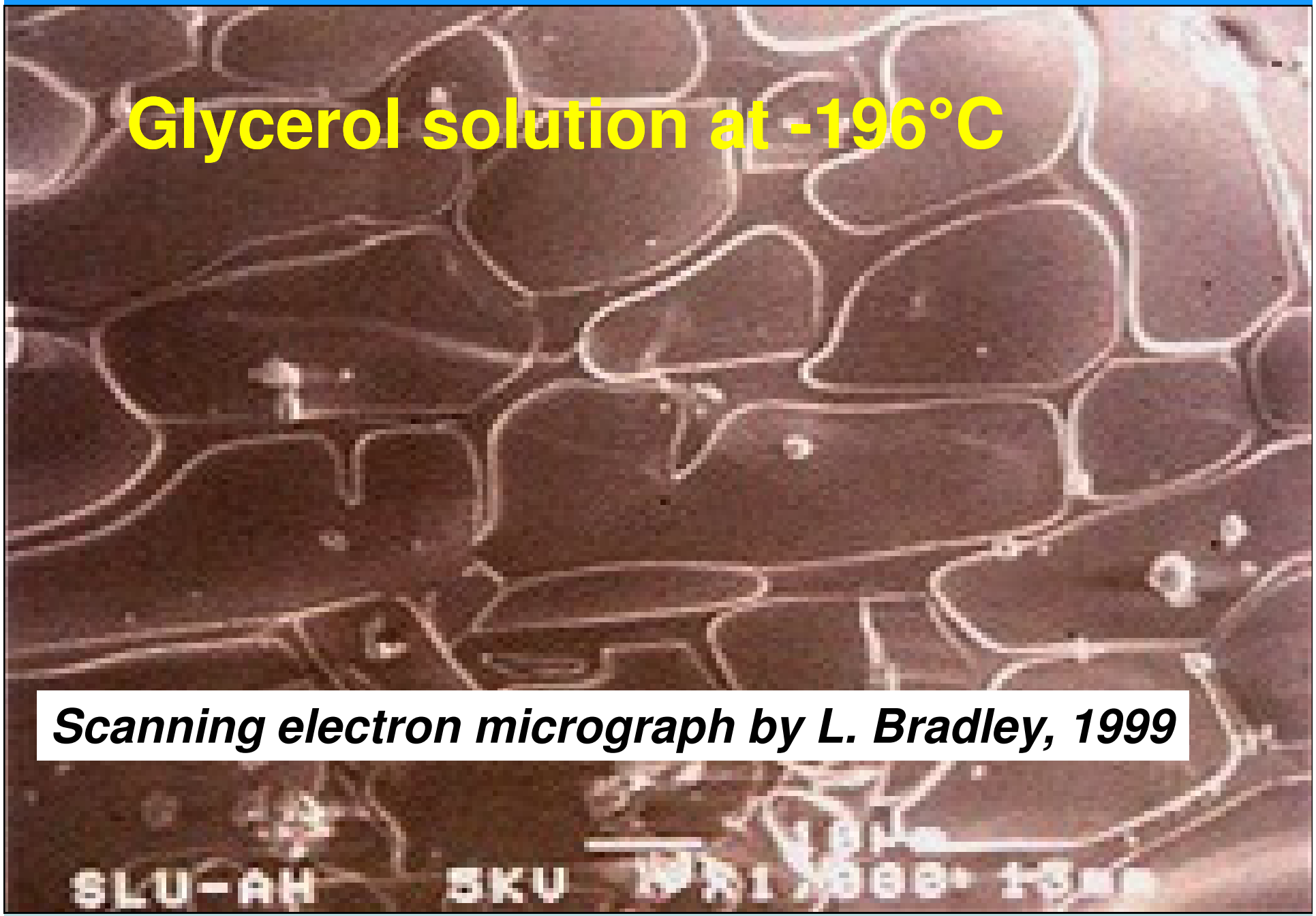
Basic Cryobiology

Equilibrium Cooling "Slow Cooling"	
10 to 15% CPA Cool at ~1°C/minute Cells dehydrate during freezing Little or no Intracellular Ice	

Fundamentals of Cryobiology

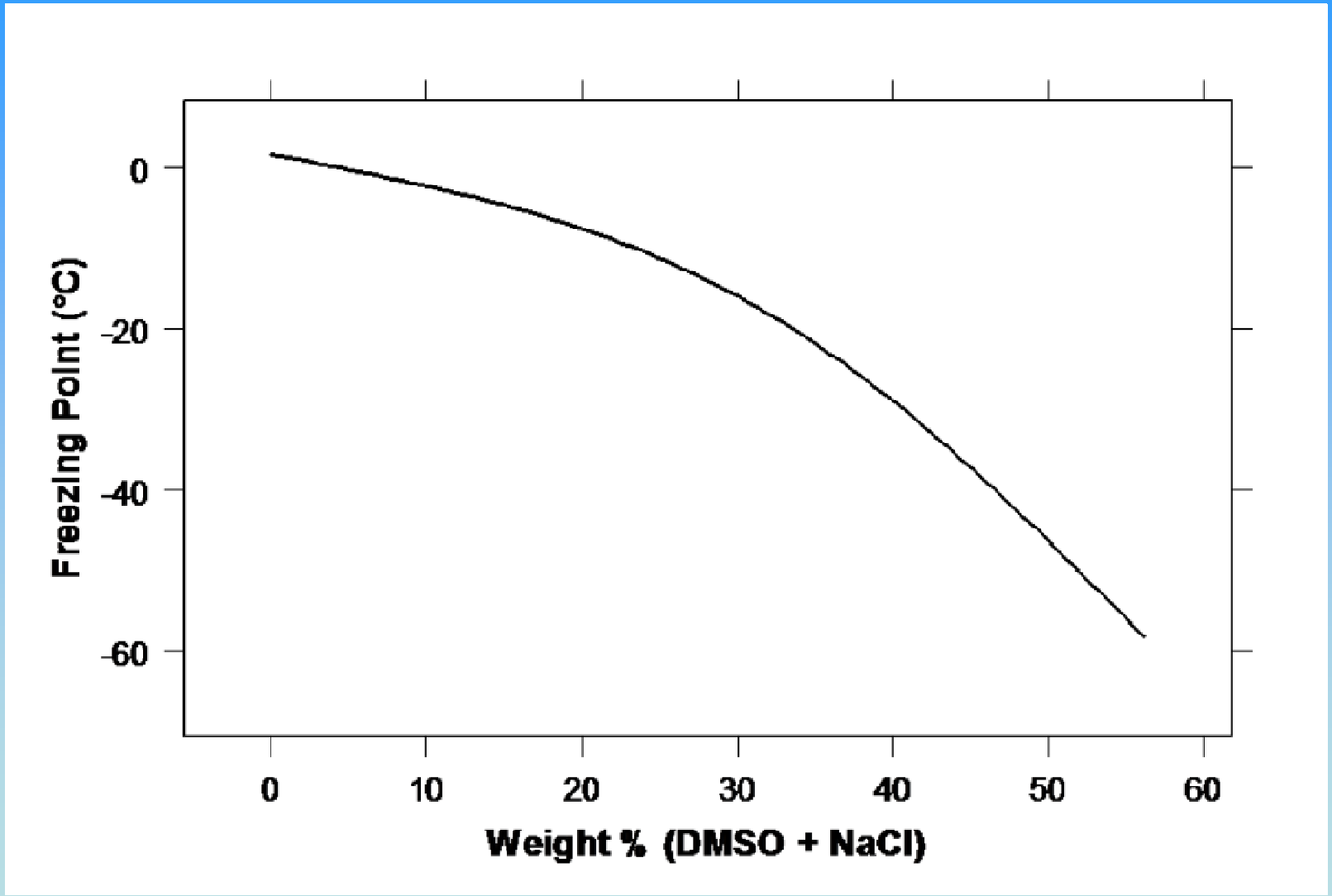
- **What happens to solutions cooled to subzero temperatures?**
- **What happens to cells exposed to such solutions?**

Glycerol solution at -196°C



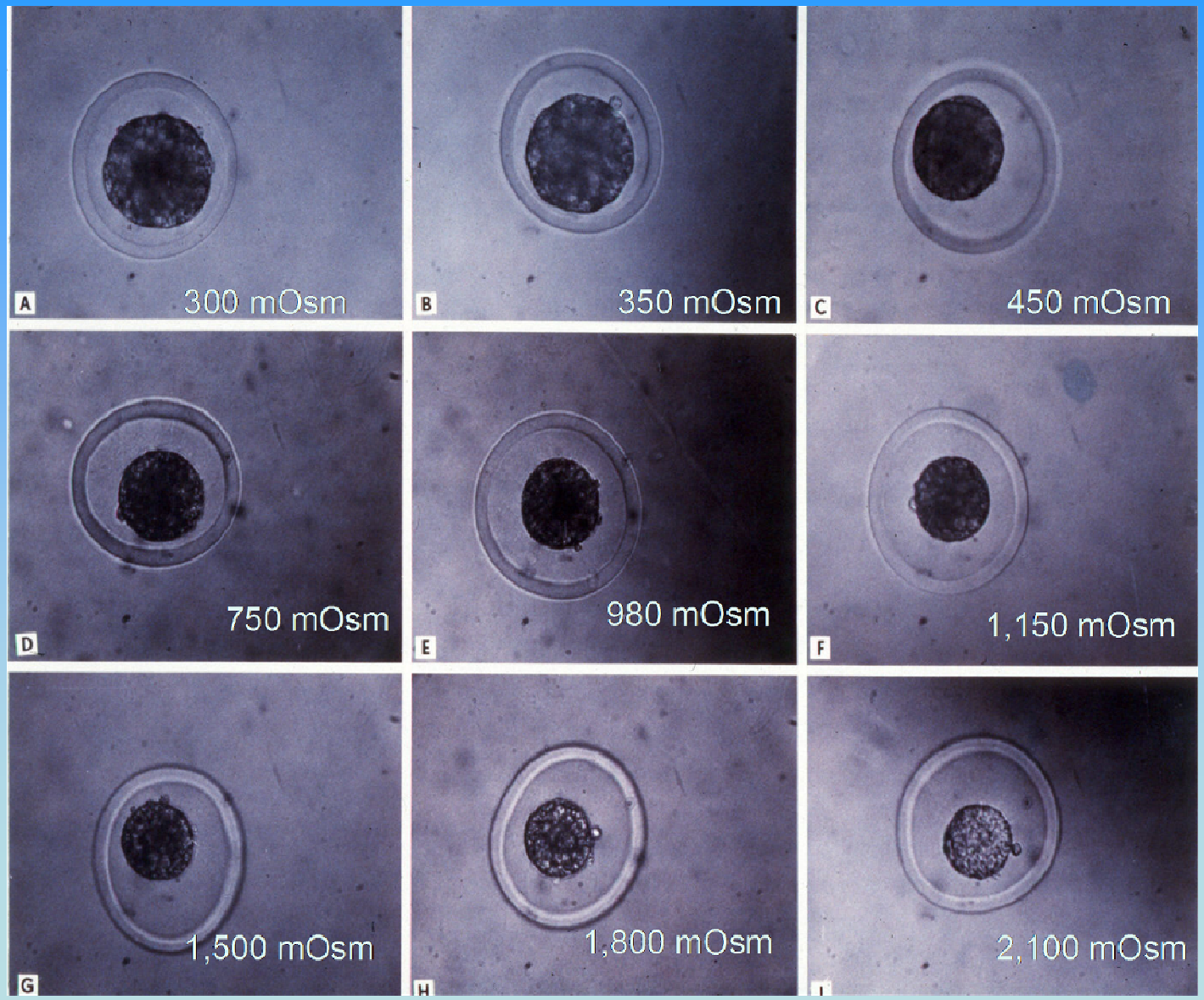
Scanning electron micrograph by L. Bradley, 1999

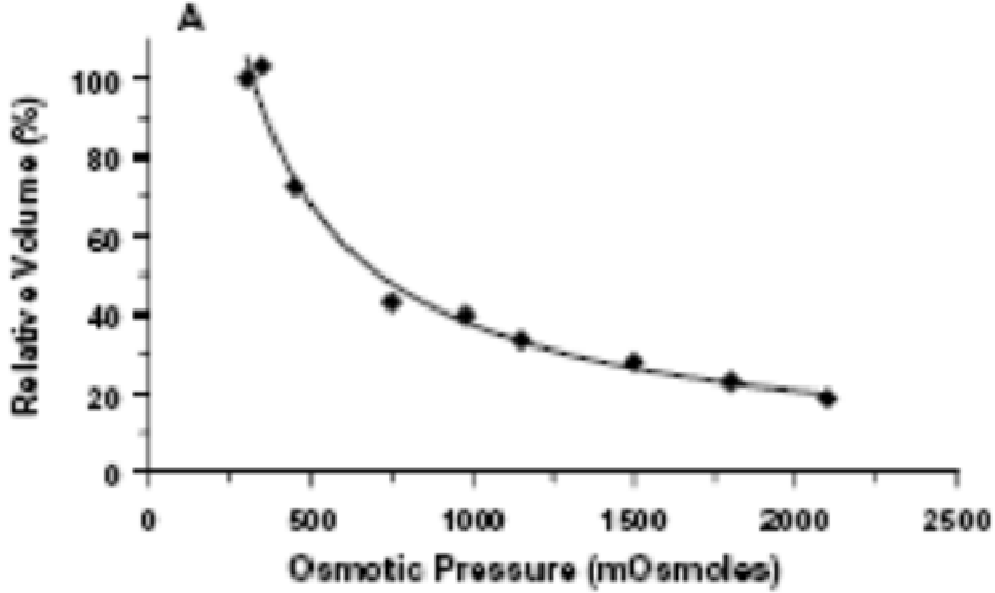
Phase Diagram of Dimethyl Sulfoxide

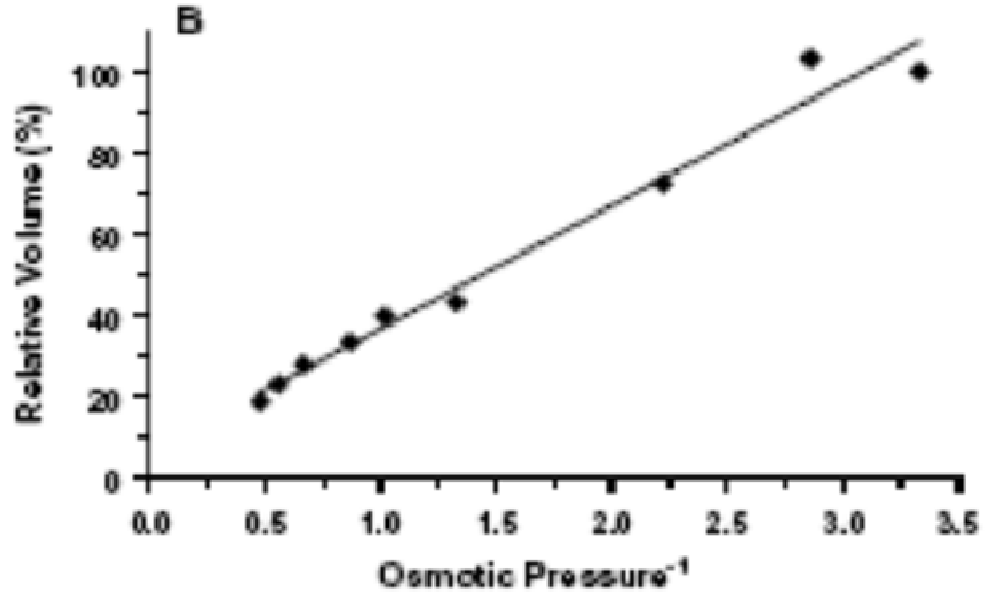


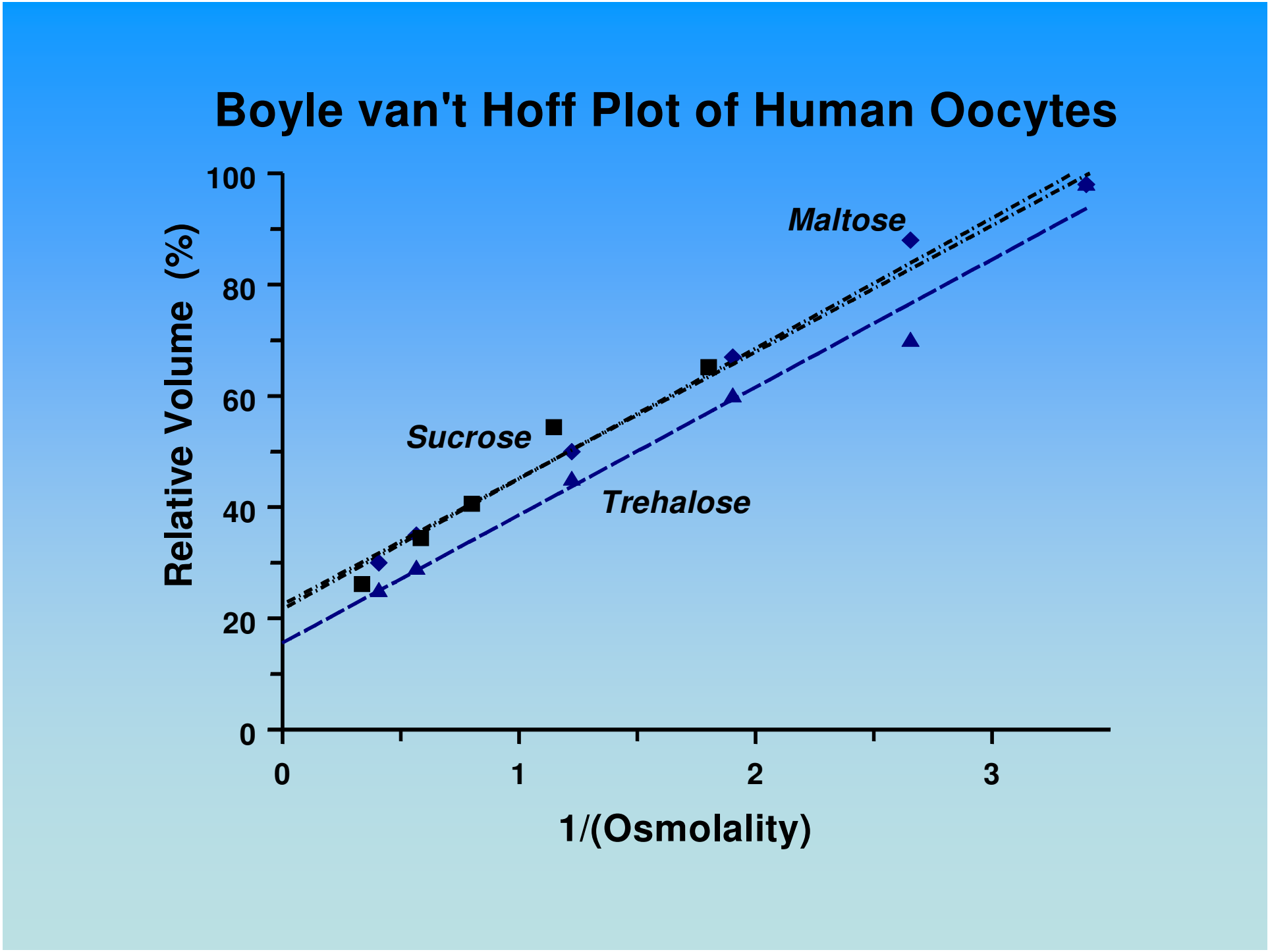
data of Kleinhans & Mazur, 2007

Bovine morula suspended in PBS or sucrose solutions




A
Relative Volume (%)
0
20
40
60
80
100
0
500
1000
1500
2000
2500
Osmotic Pressure (mOsmoles)


B
Relative Volume (%)
0
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Osmotic Pressure^{-1}


Boyle van't Hoff Plot of Human Oocytes
Relative Volume (%)
100
80
60
40
20
0
0
1
2
3
1/(Osmolality)
Maltose
Sucrose
Trehalose

Reprinted from *The Journal of General Physiology*, November 1963
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Printed in United States of America

Kinetics of Water Loss from Cells at Subzero Temperatures and the Likelihood of Intracellular Freezing

PETER MAZUR

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Mazur's Equations of Theoretical Cryobiology

$$L_p = L_p^* \exp(-E / R[1/T - 1/T^*])$$

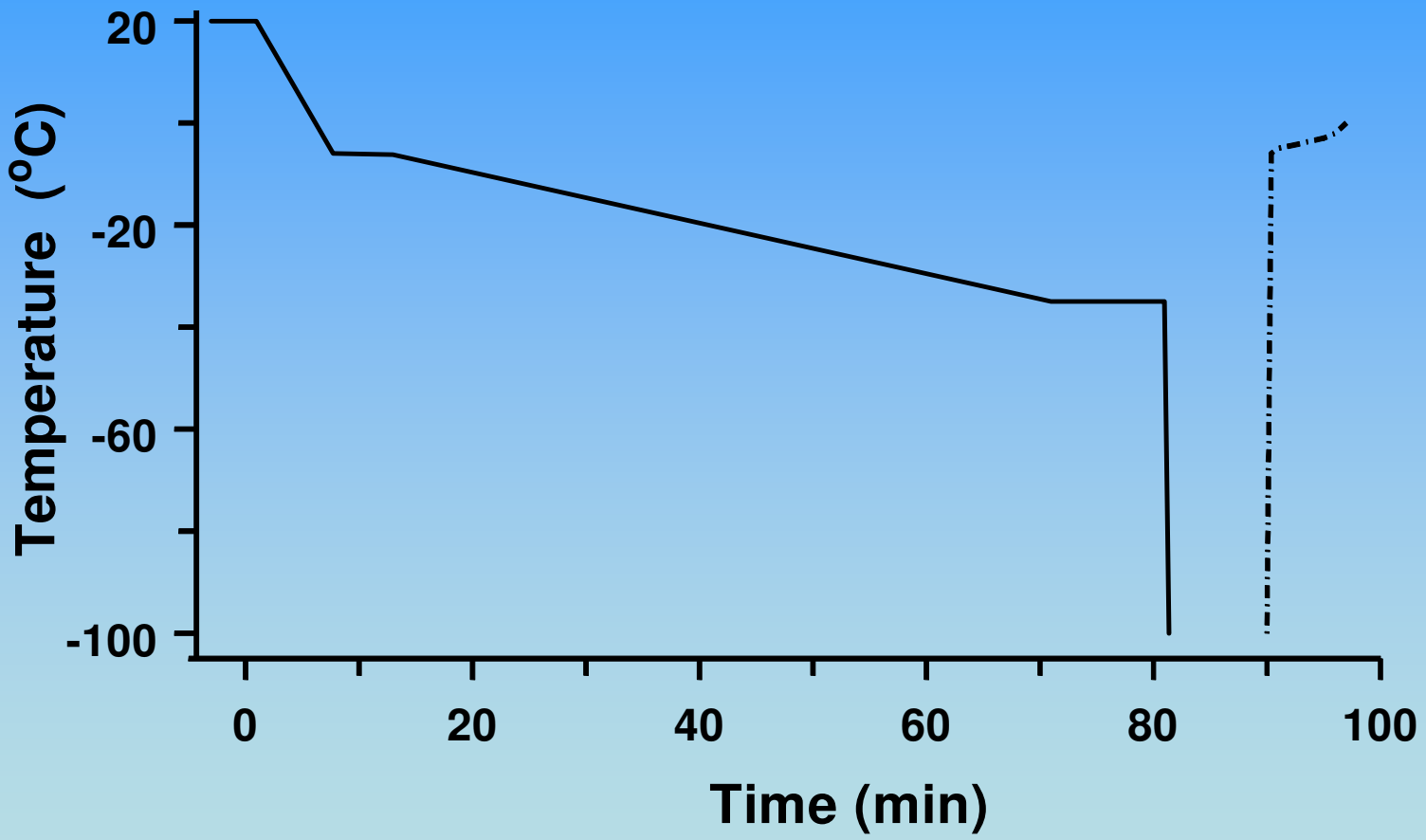
- ***Temperature affects cell's water permeability***

Mazur's Equations of Theoretical Cryobiology

$$dV / dt = \{L_p A R T \ln P_e / P_i \} / \upsilon^o$$

- ***When cell exposed to hypertonic solution, cell loses water as a function of time***

Diagram of "Standard" Cooling/Warming Curve

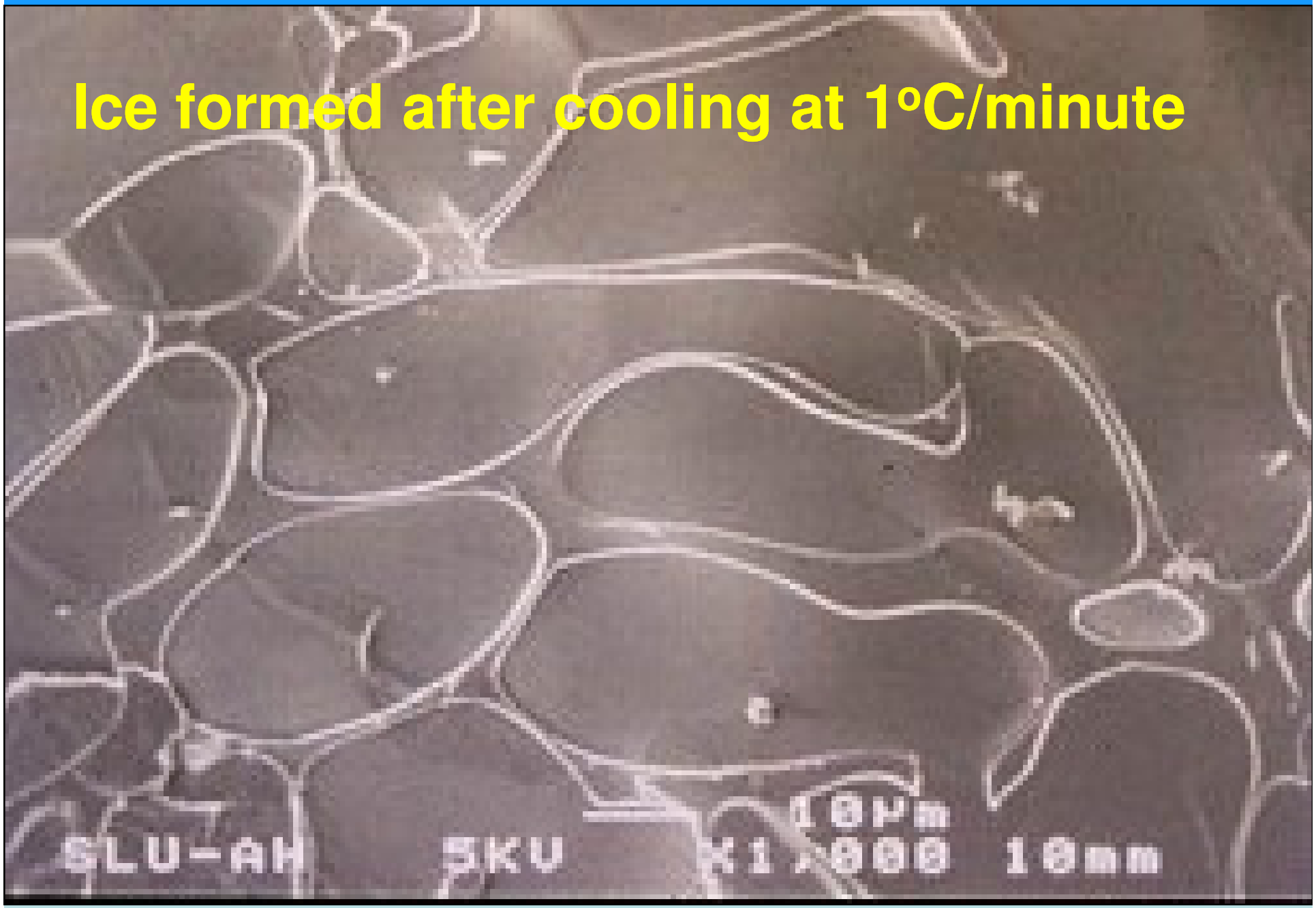


Mazur's Equations of Theoretical Cryobiology

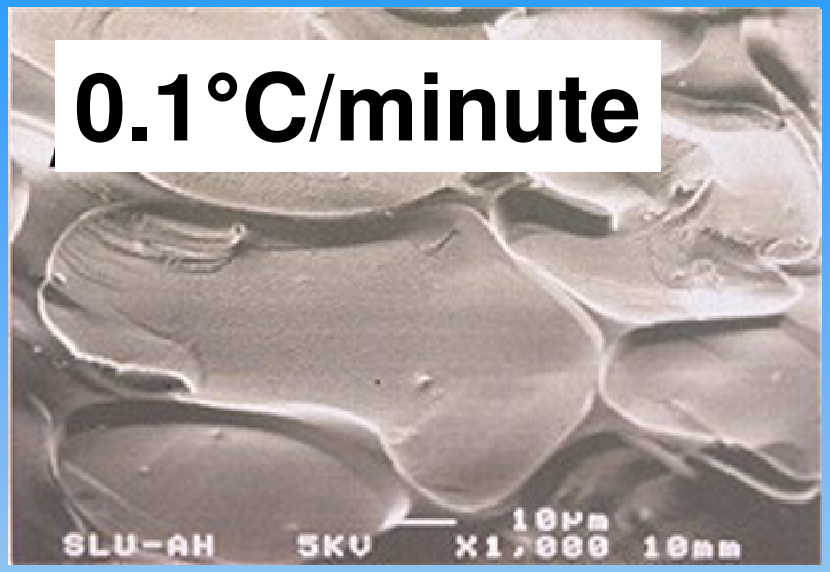
$$dT / dt = B$$

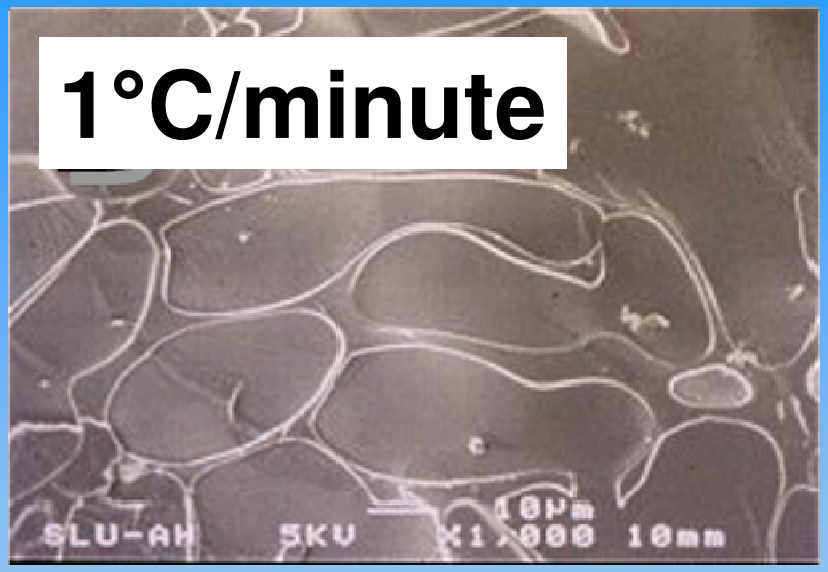
- ***Solutions may be cooled at various rates***

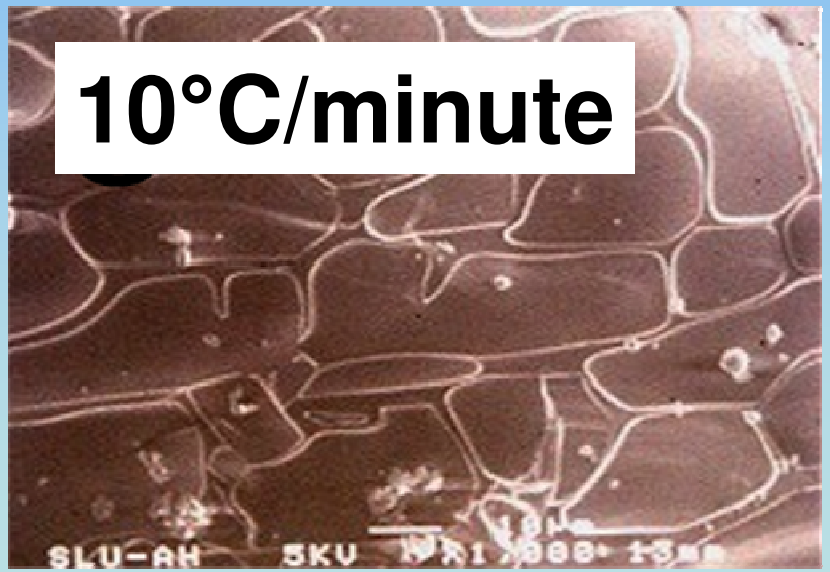
Ice formed after cooling at 1°C/minute

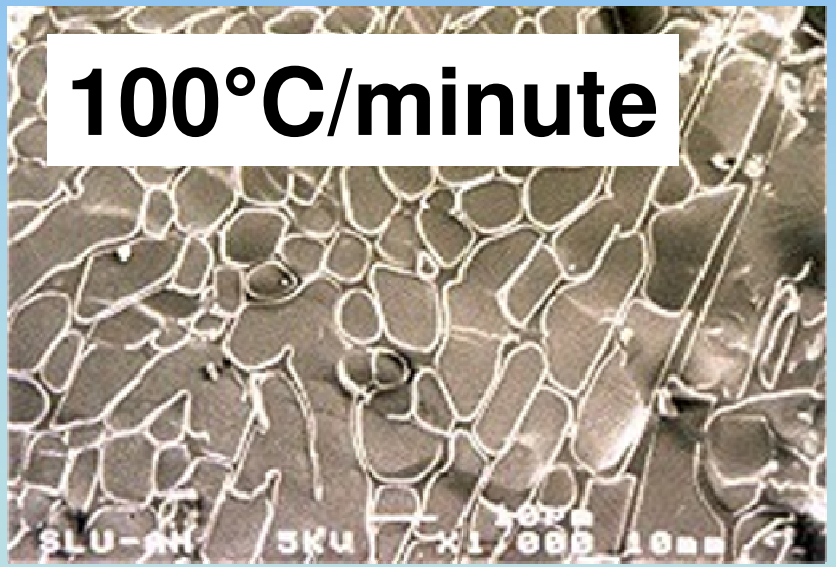


Frozen solutions of glycerol





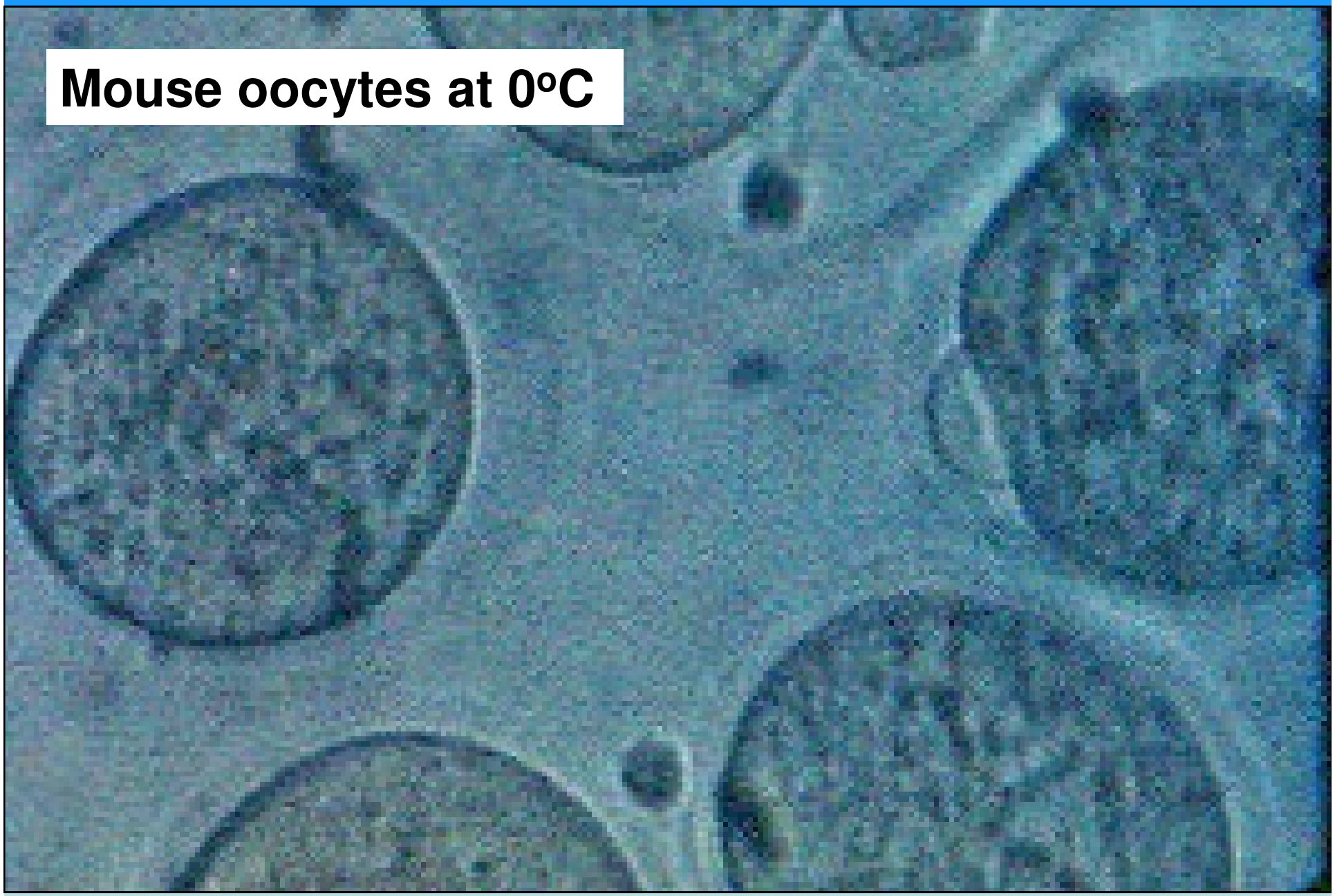


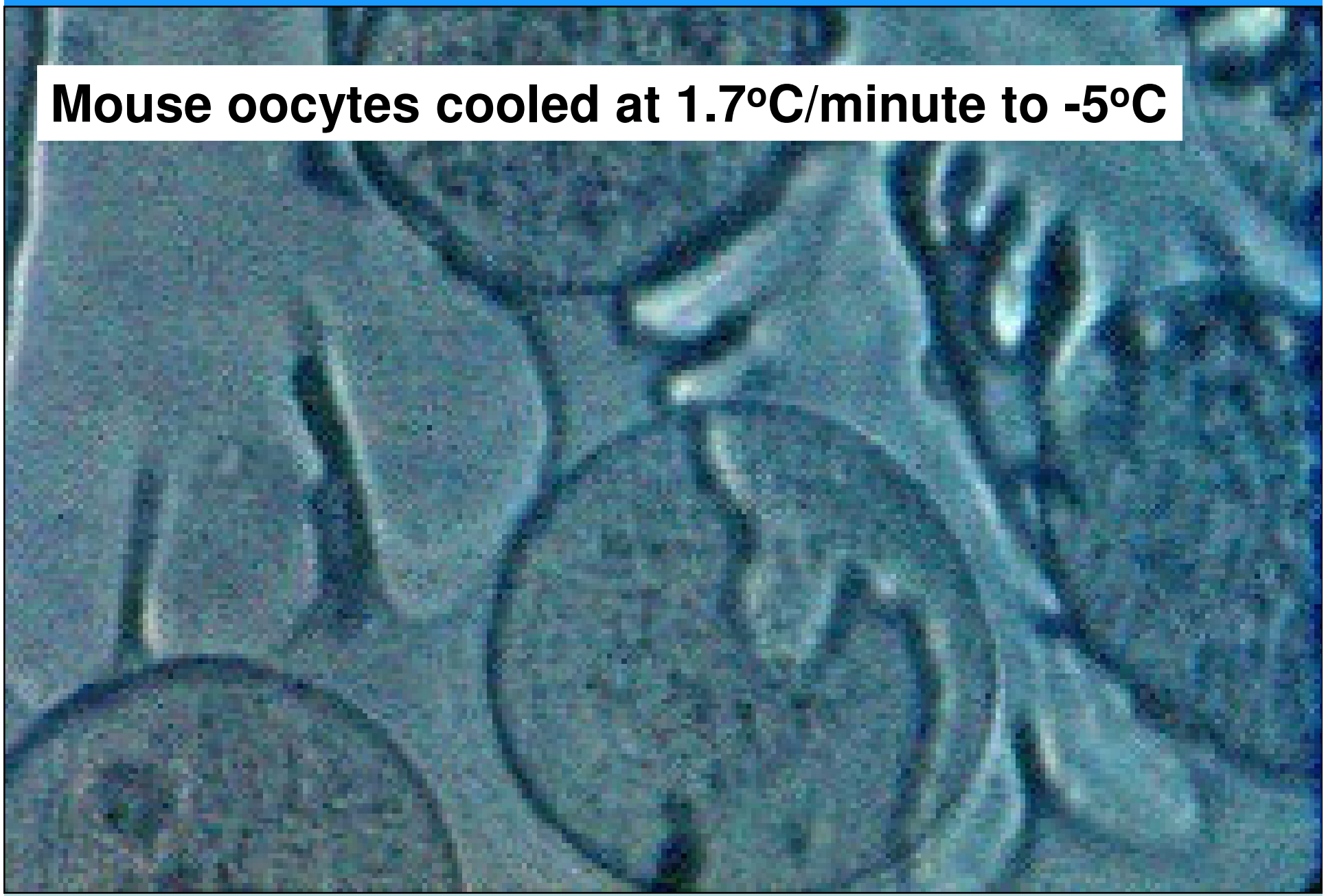


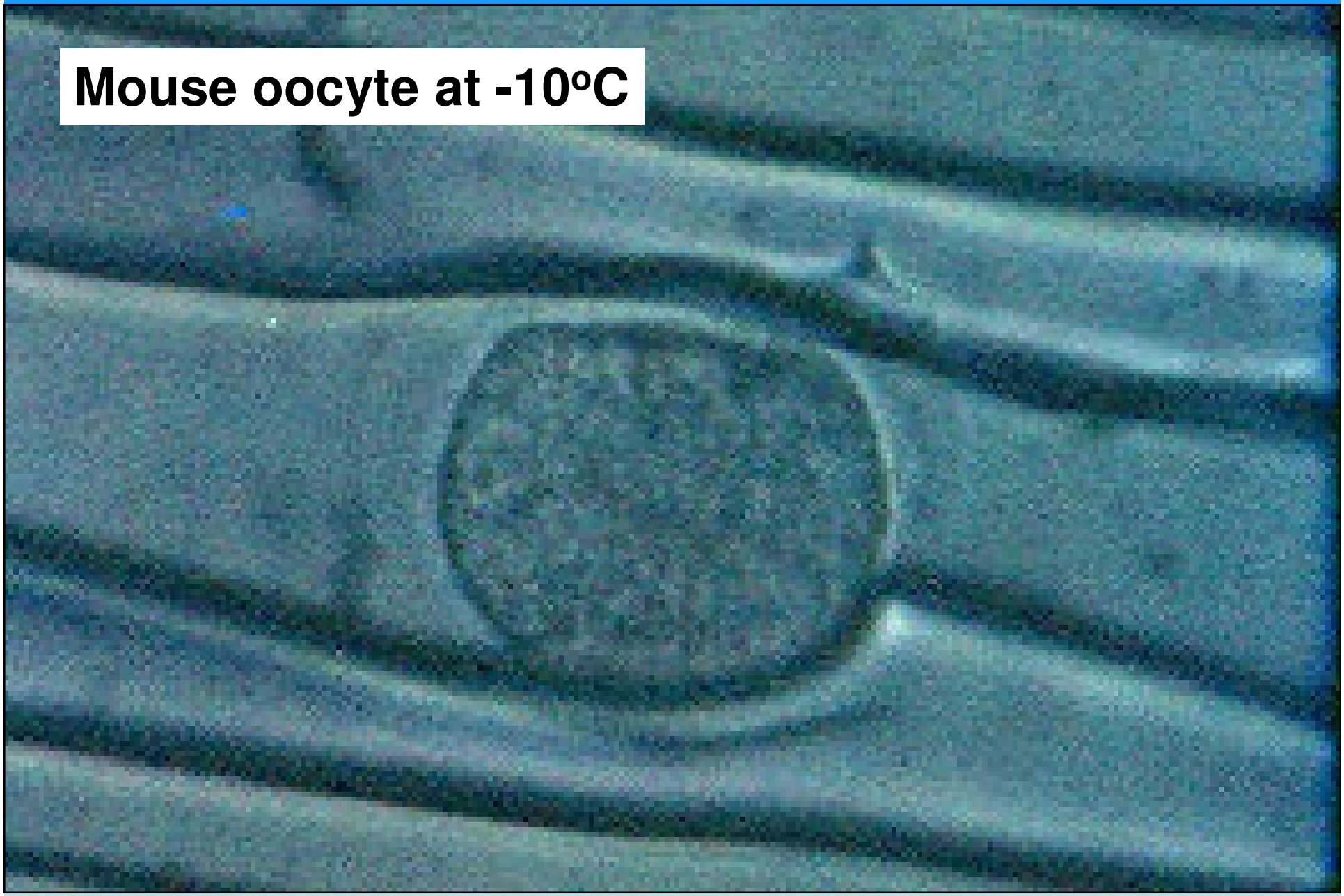
Mazur's Equations of Theoretical Cryobiology

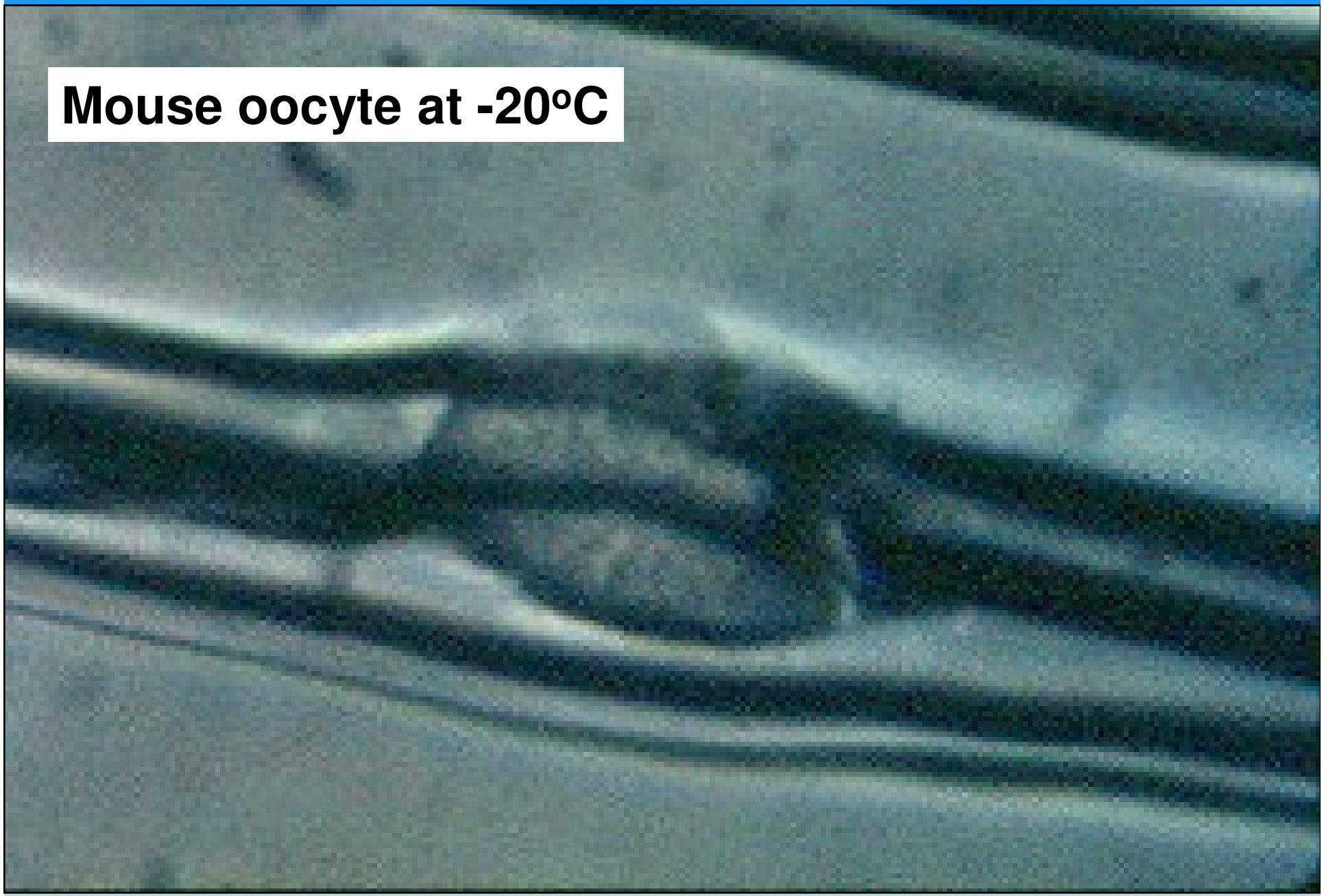
$$\ln [V / V + N\upsilon^{\circ}] = (L_f / R) (1/273 - 1/T)$$

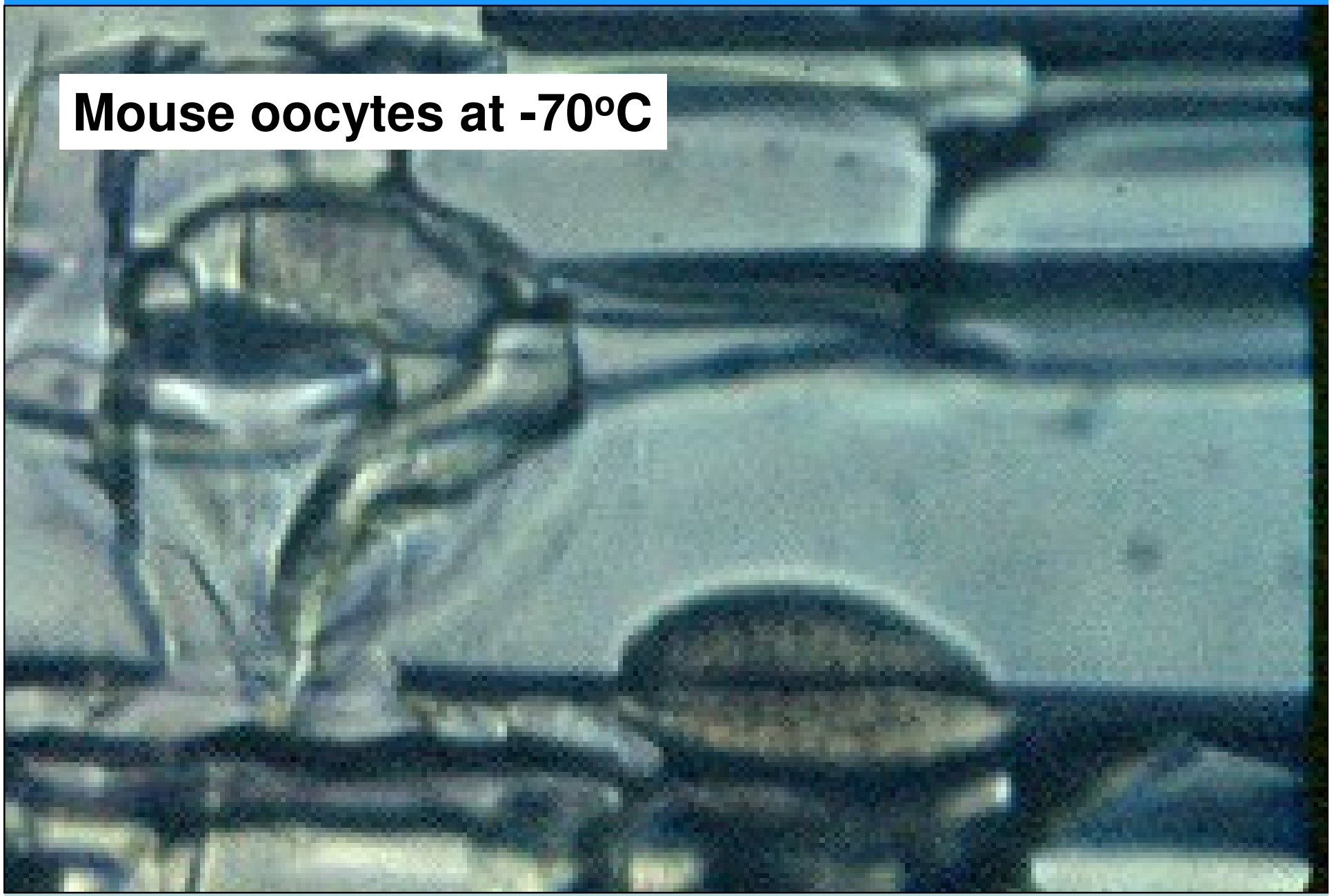
- ***If cell is cooled infinitely slowly, cell water volume will remain in equilibrium with extracellular solution***


Mouse oocytes at 0ºC

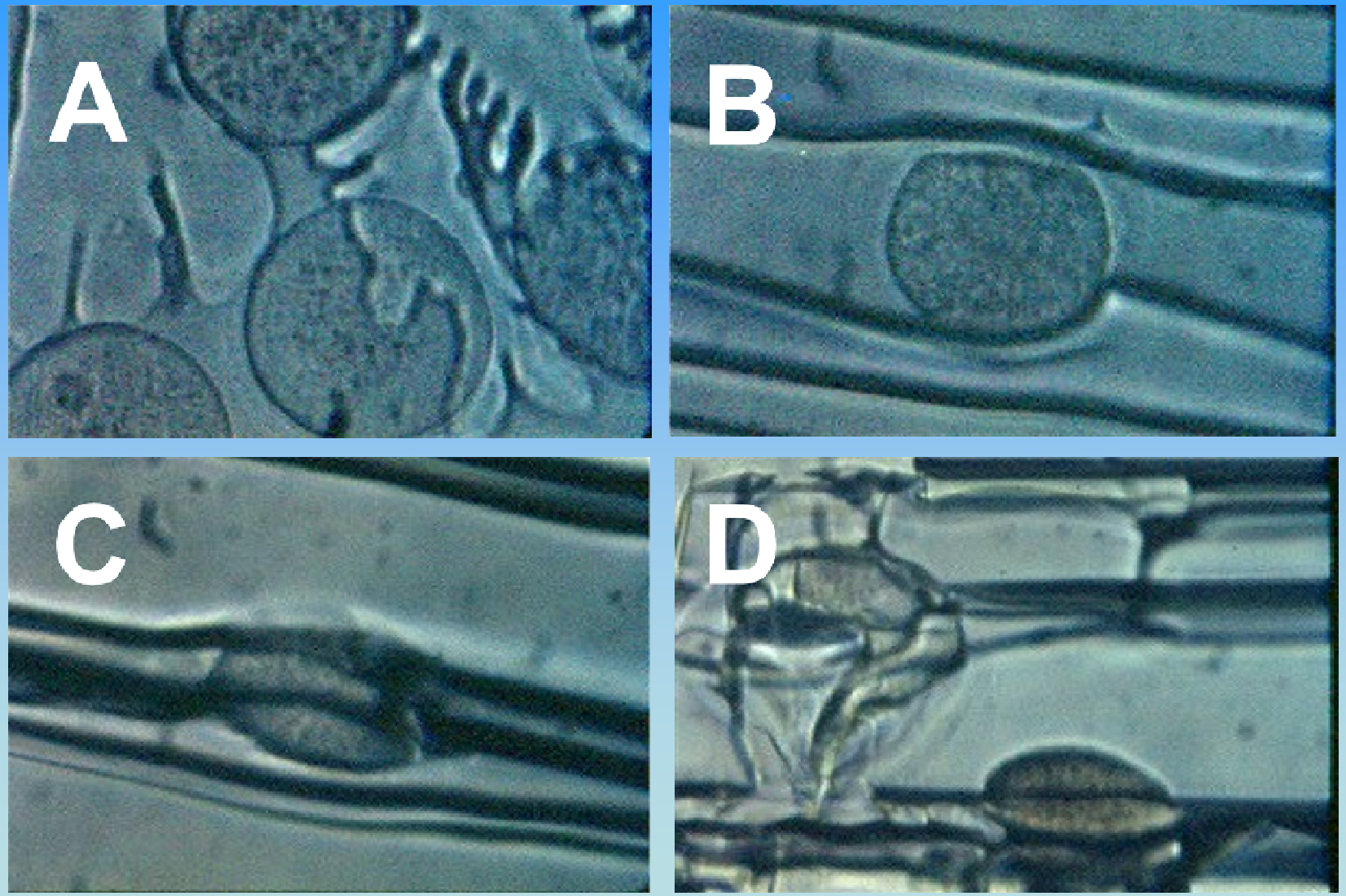

Mouse oocytes cooled at 1.7°C/minute to -5°C


Mouse oocyte at -10°C

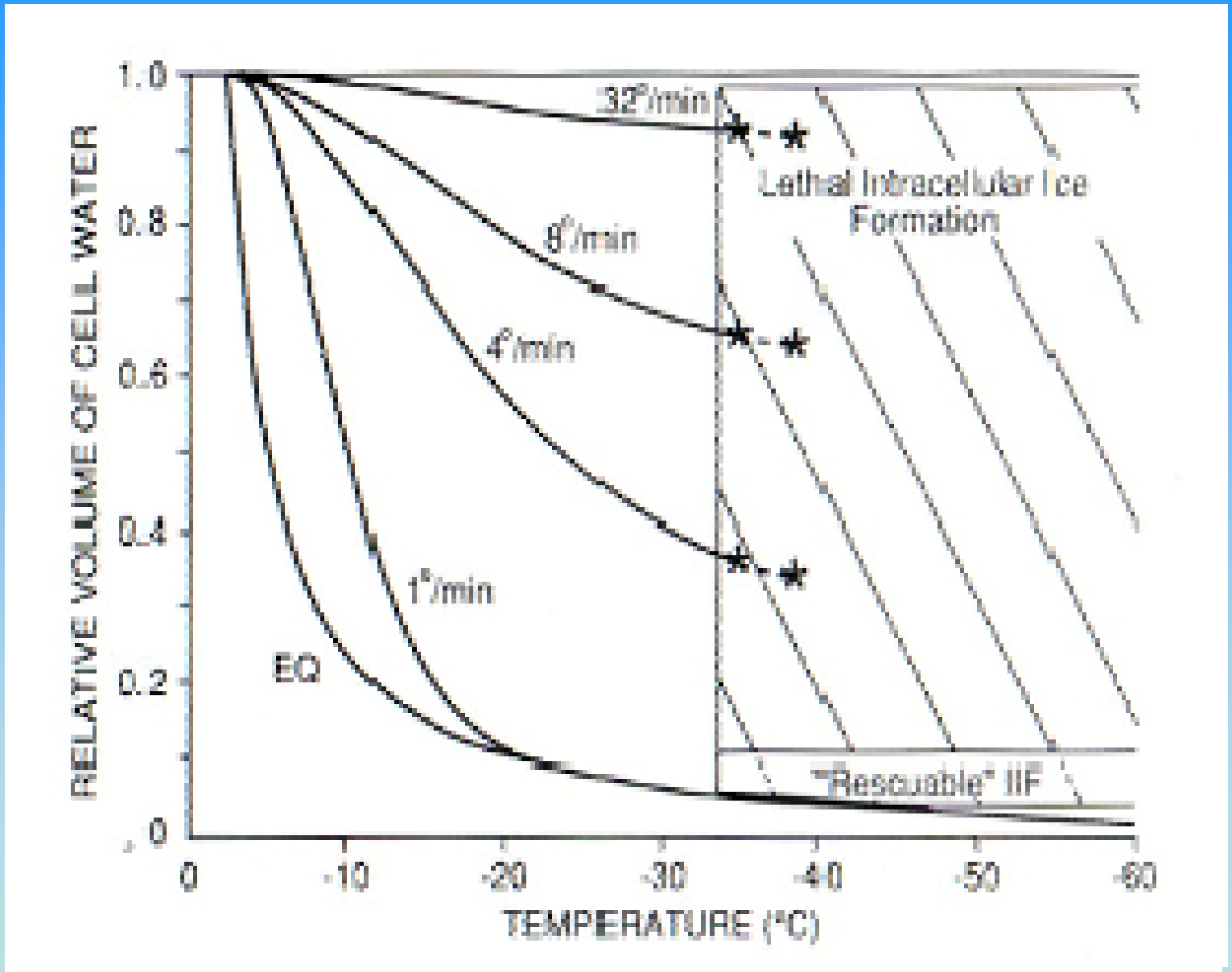

Mouse oocyte at -20°C


Mouse oocytes at -70°C

Mouse oocytes cooled at 1.7ºC/minute



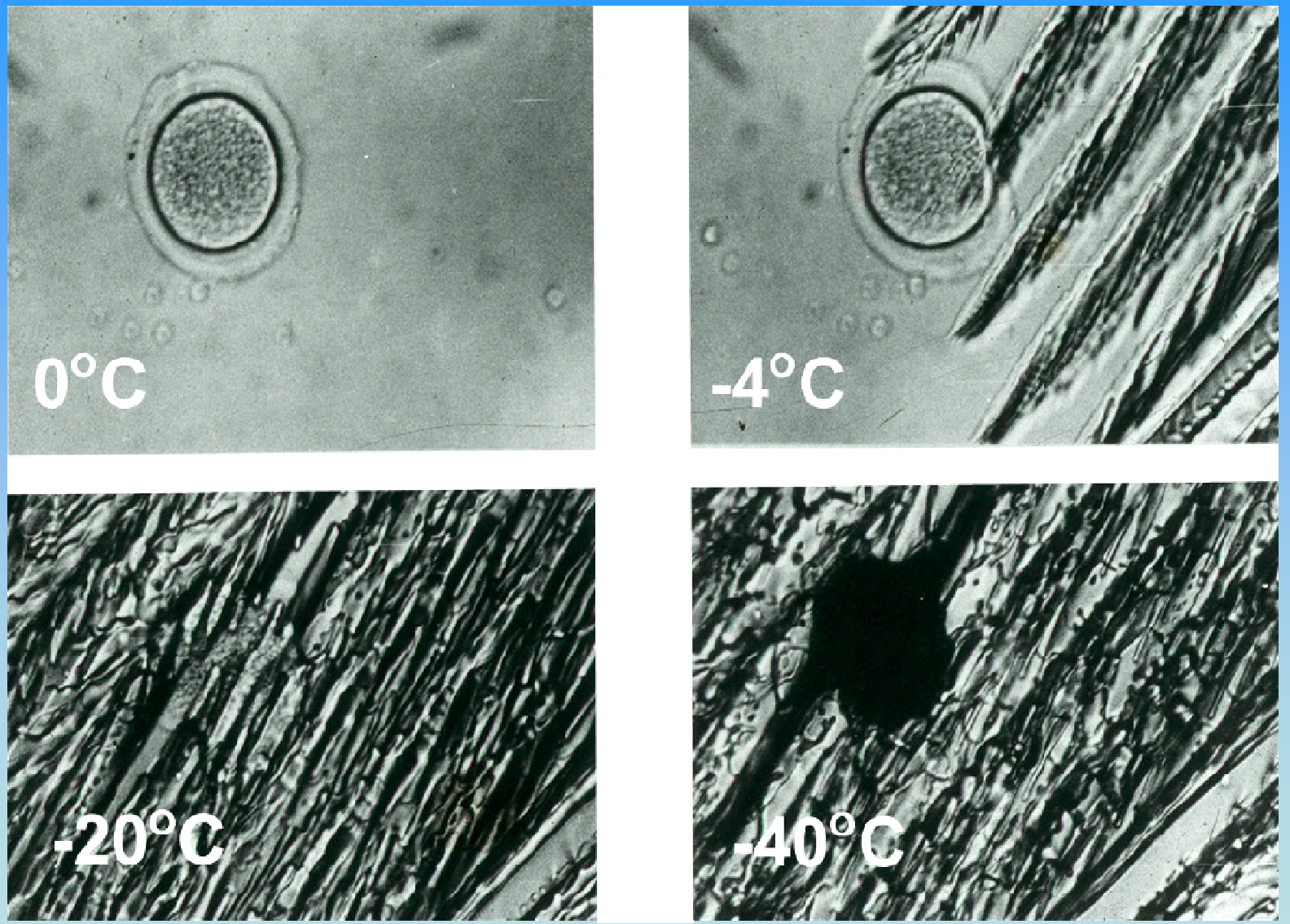
Theoretical response of oocytes cooled at various rates



Theoretical analysis shows that …

- ***Cells cooled slowly lose water at high subzero temperatures.***
- ***Cells cooled rapidly do not dehydrate.***
- ***Therefore, they freeze intracellularly!***

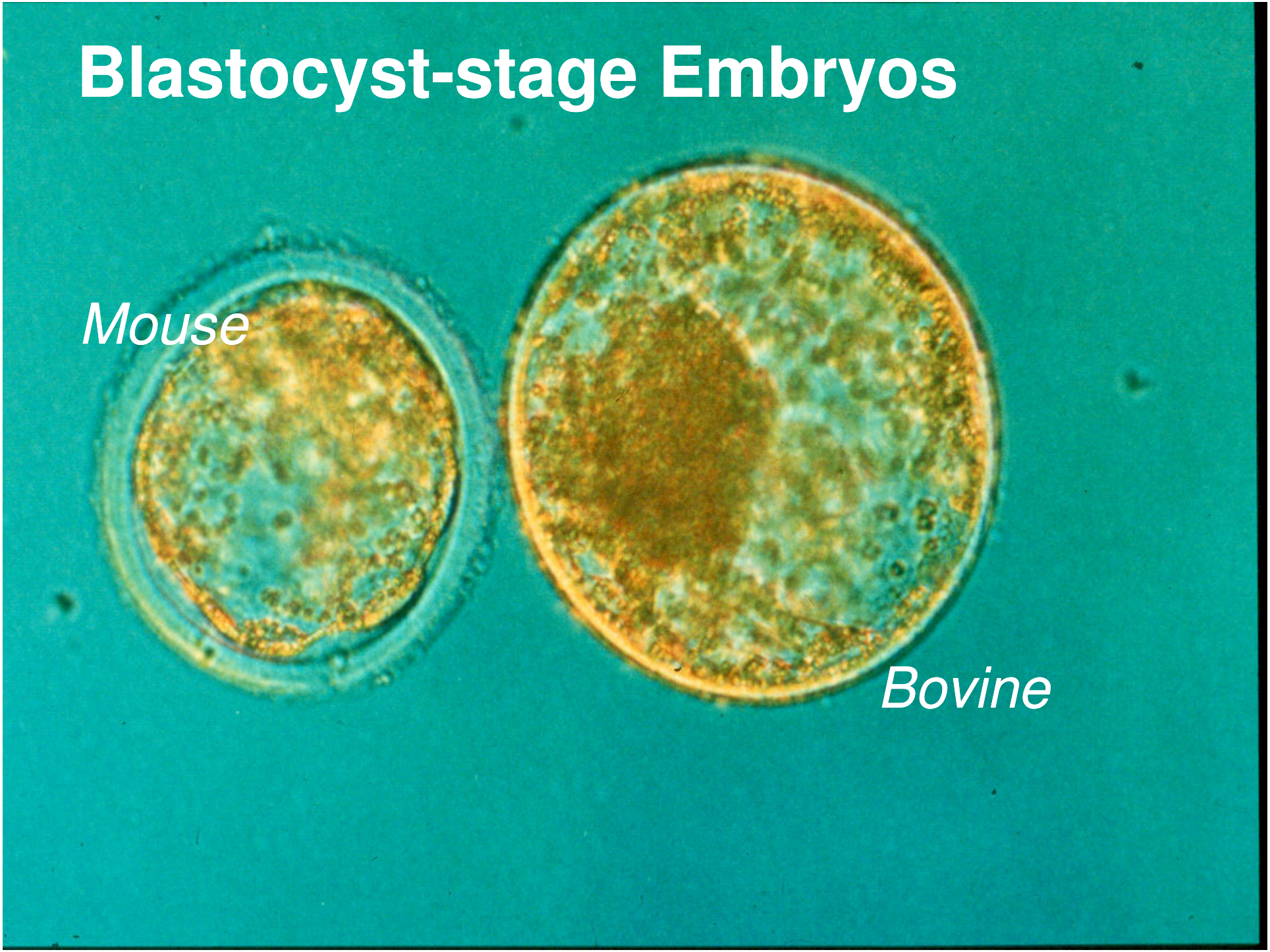
Oocyte cooled at 32ºC/minute



Theory dictates that optimum cooling rate determined by …

- ***Surface area to volume ratio***
- ***Water permeability (Lp)***

Blastocyst-stage Embryos



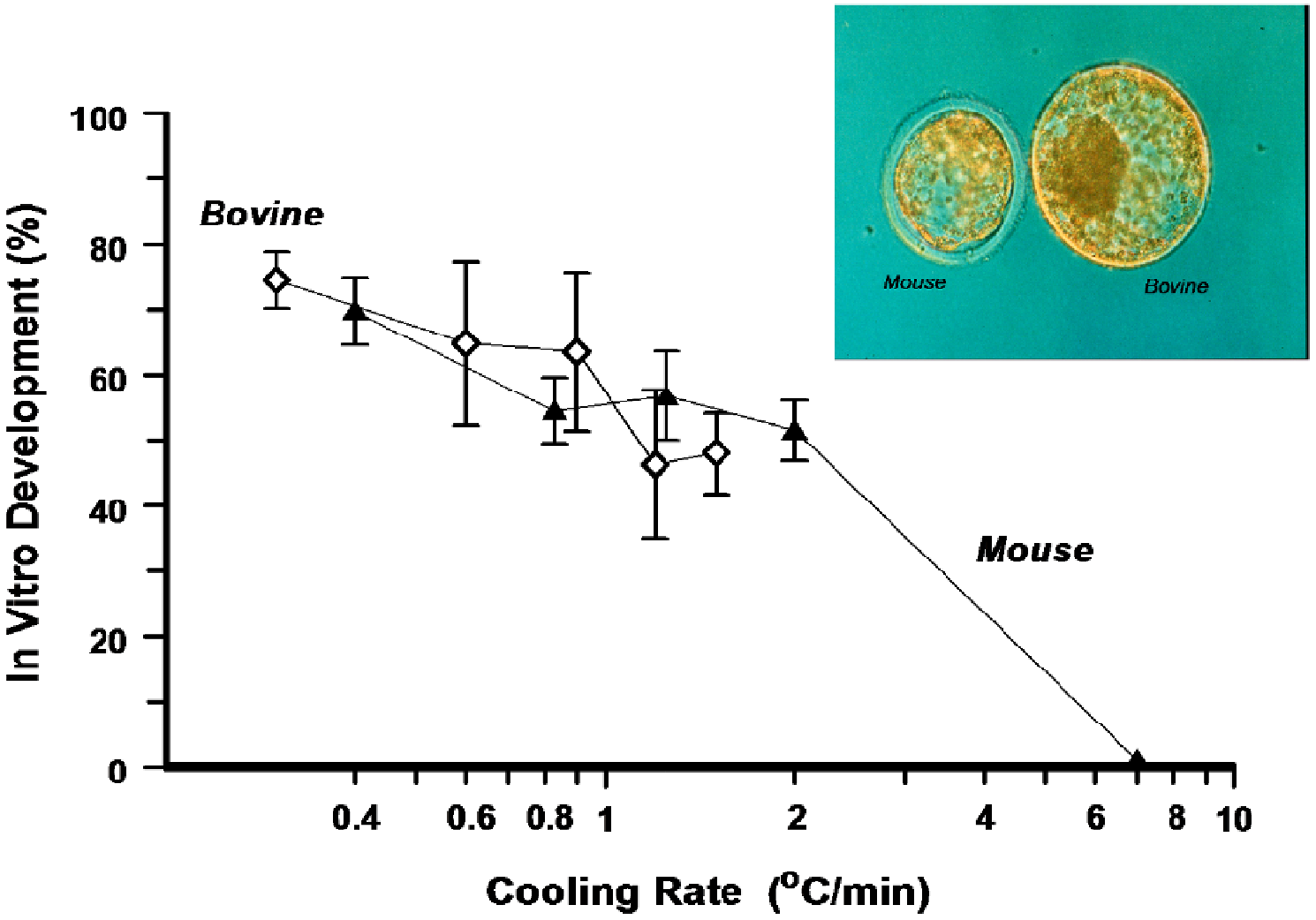
Embryo Characteristics

Species	Water Permeability (µm/min-atm)	Reference
Mouse	0.48	Hunter ’92
Bovine	0.84	Agca ‘93

Embryo Characteristics

Species	Surface Area $(\mu m)^2$	Volume $(\mu m)^3$	SA / V
Mouse	1.7×10^4	2.2×10^5	0.08
Bovine	7.1×10^4	17.7×10^5	0.04

Comparison of mouse and bovine embryos



Water permeability coefficients of oocytes of various species

Species	Water Permeability
Human	0.40
Hamster	0.80
Monkey	0.23
Goat	0.97

Water permeability coefficients of individual human oocytes at 22°C

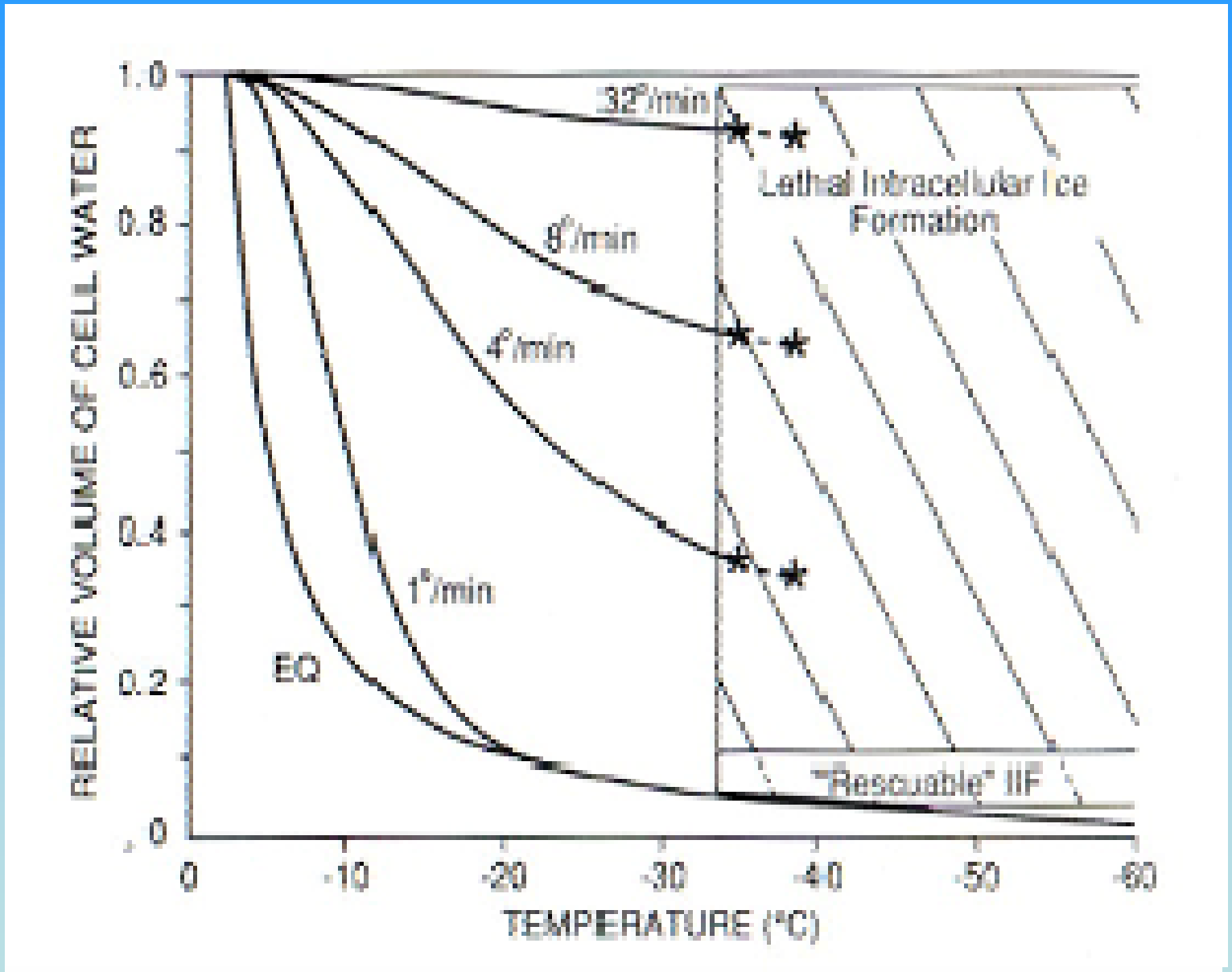
(data of Van den Abbeel 2007)

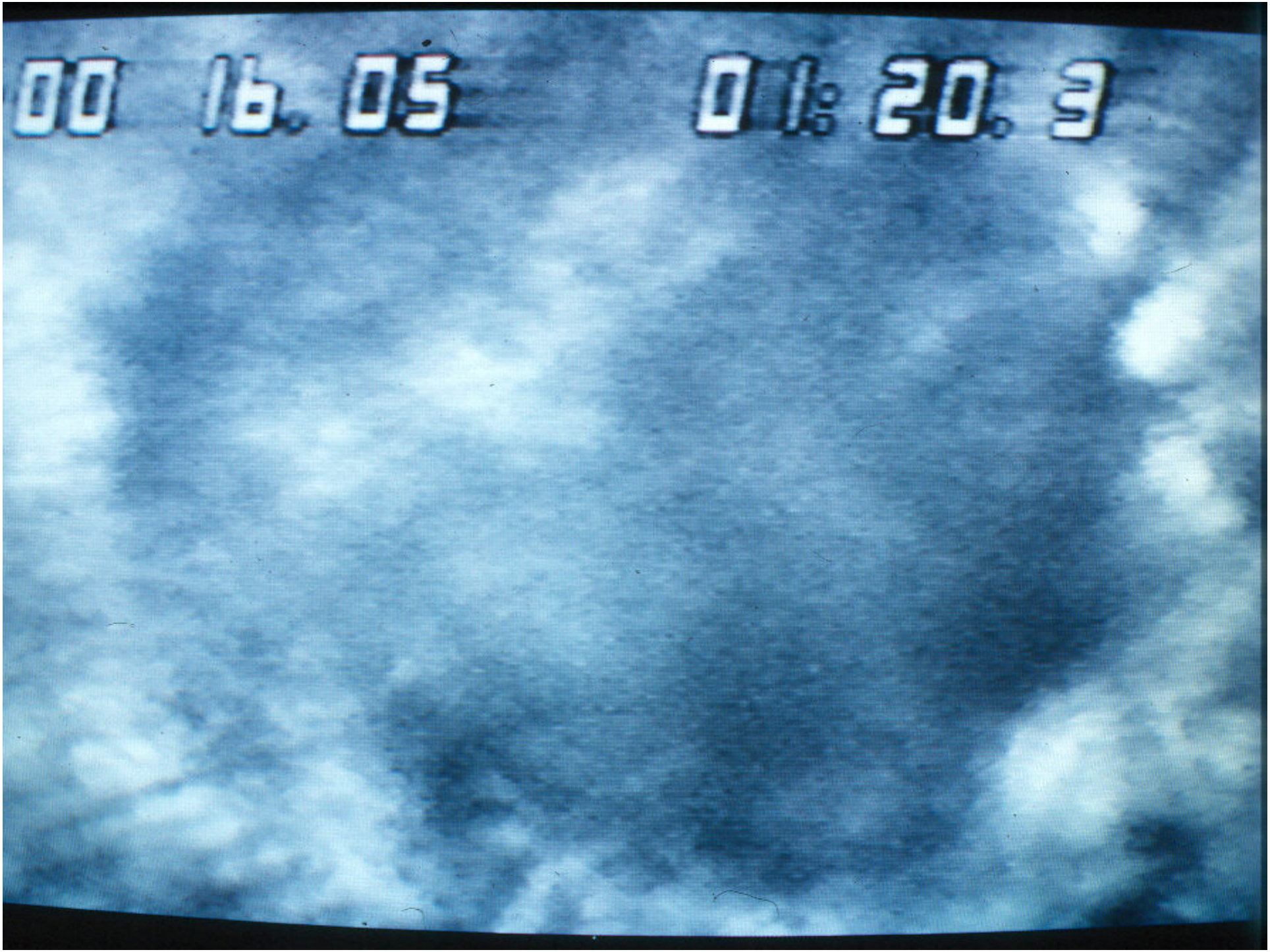
in DMSO	In Propylene Glycol
0.24	0.68
0.52	0.71
0.56	0.75
0.63	0.78
0.65	0.86
0.66	0.92
0.69	1.14
0.74	1.18
0.77	2.15
0.87	2.18

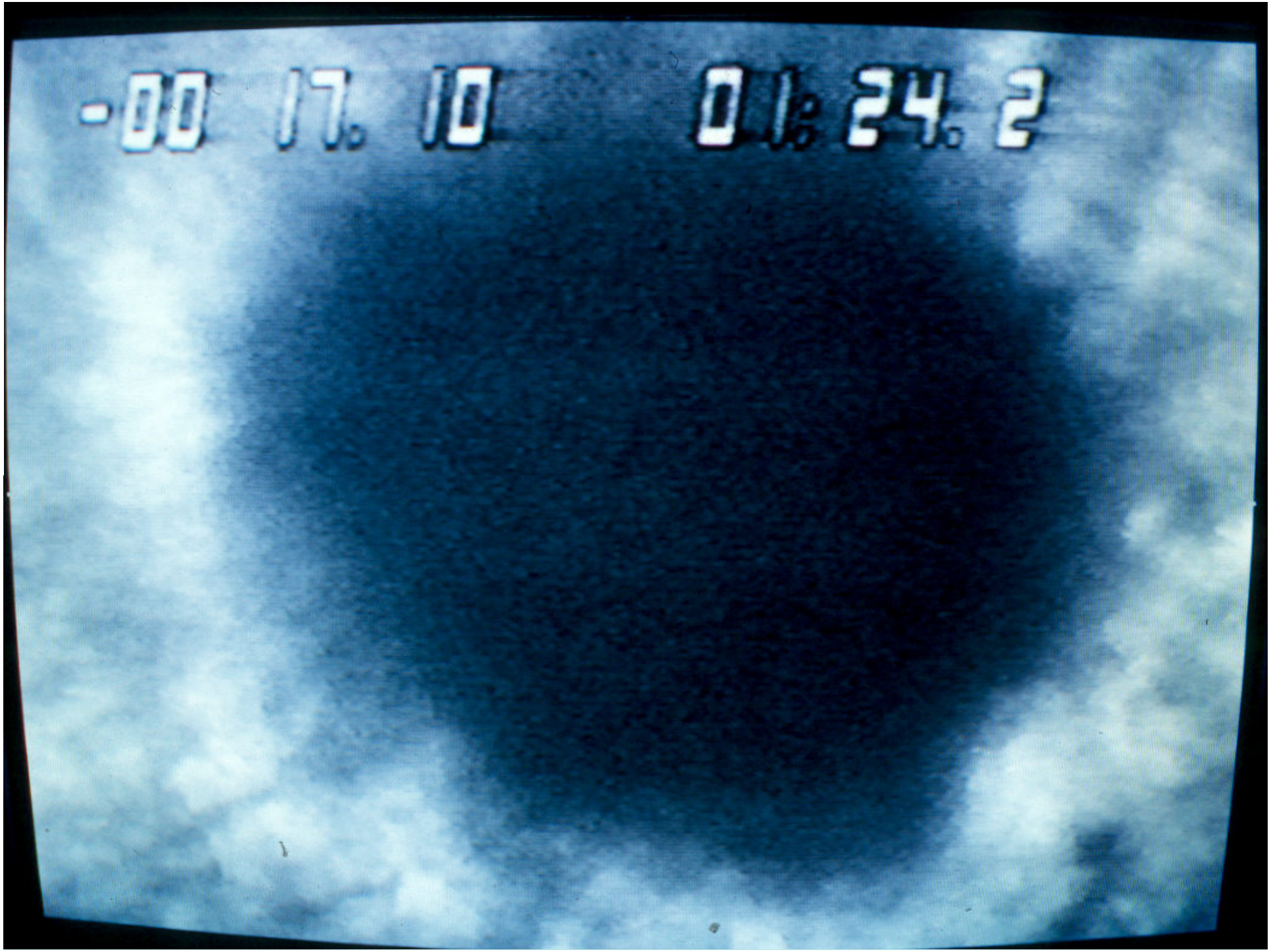
Theory dictates that likelihood of intracellular freezing determined by …

- ***Constant nucleation temperature***

Theoretical response of oocytes cooled at various rates

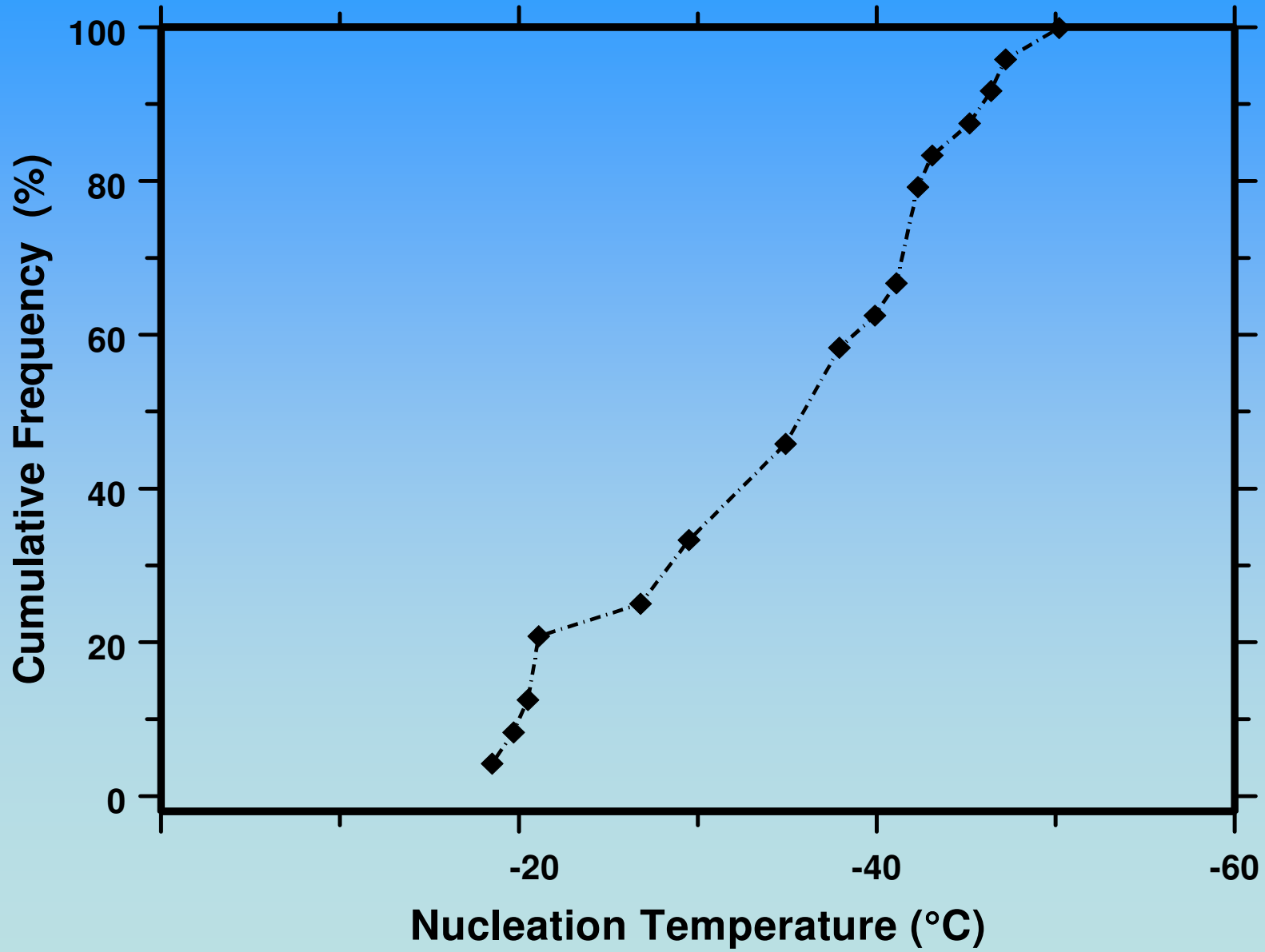



00 16. 05
01: 20. 3

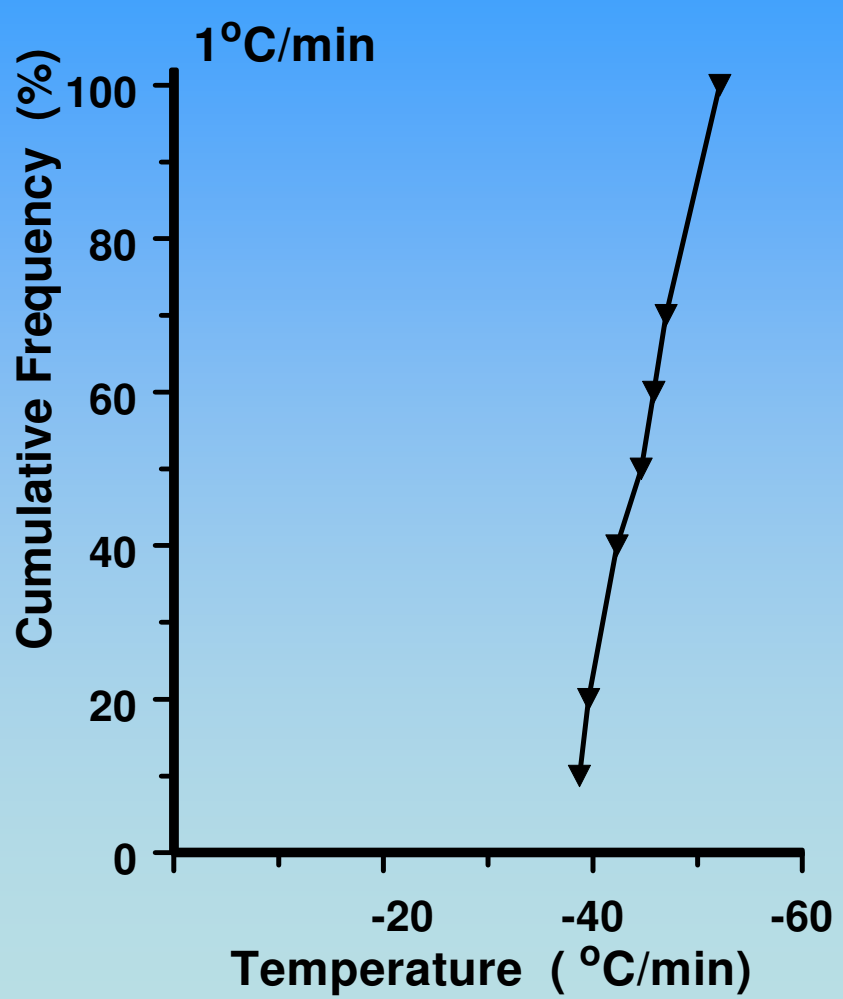

-00 17. 10
01:24. 2

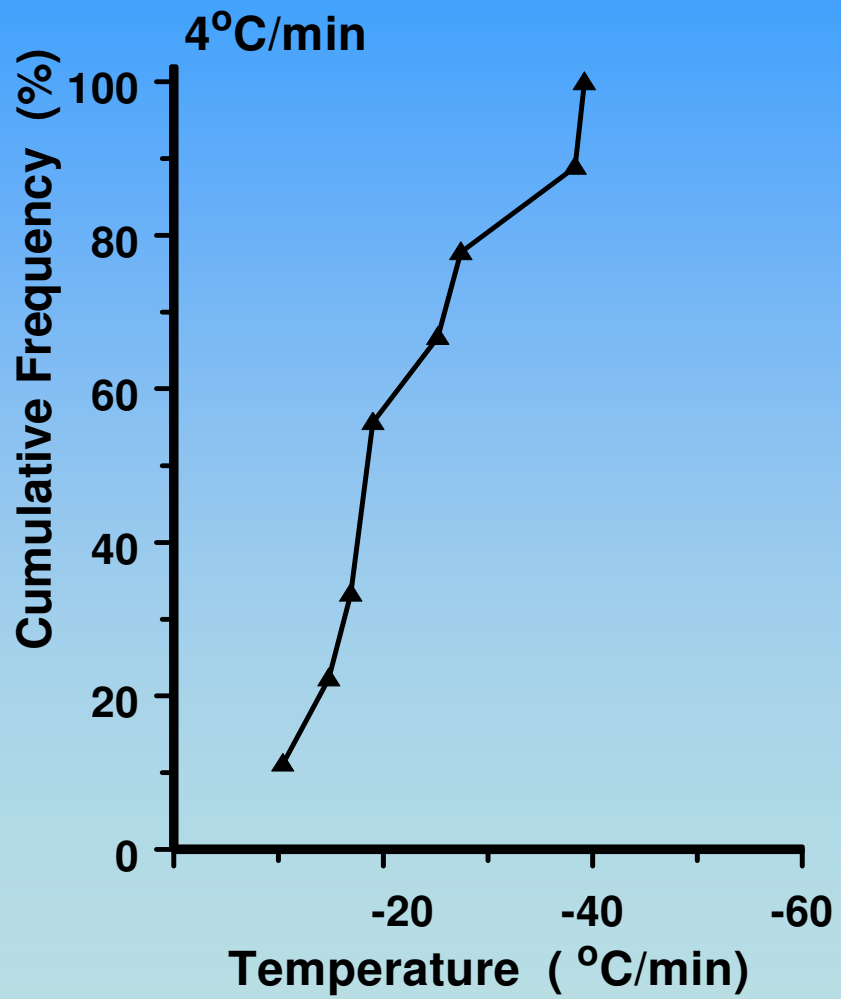
Intracellular Ice Formation in Bovine Embryos

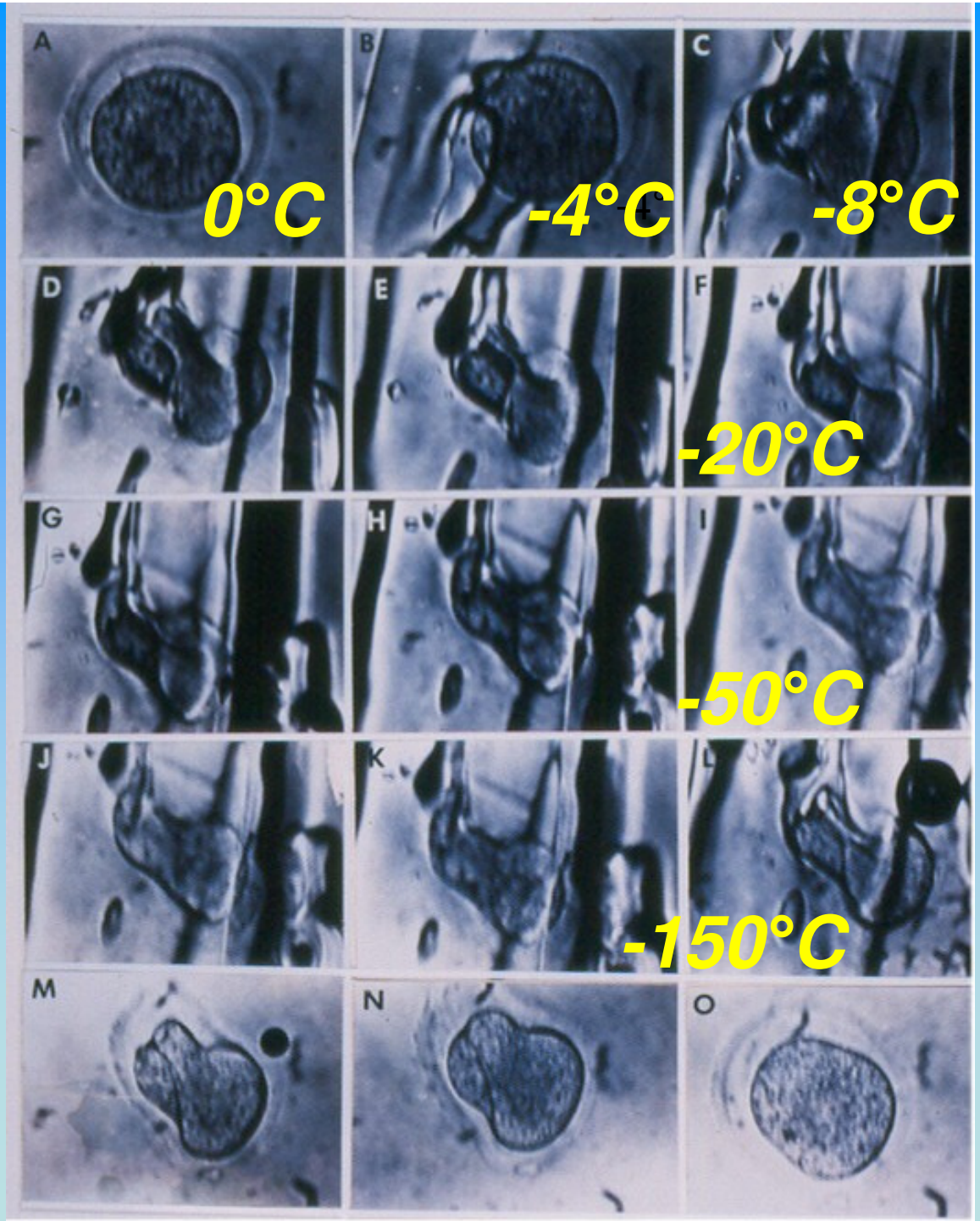
Each point shows nucleation of one embryo



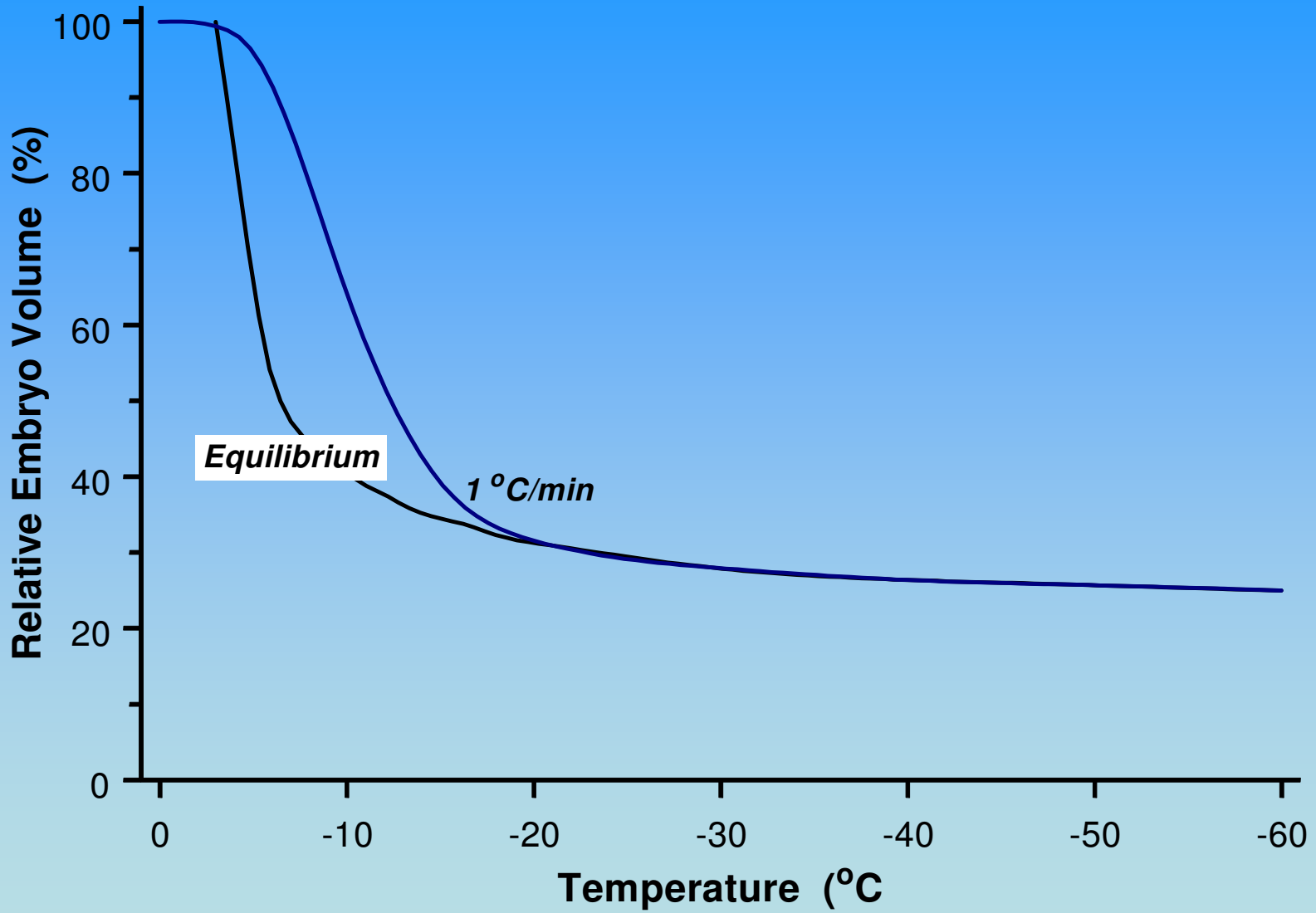
Nucleation of Intracellular Ice in Bovine Embryos






A
0°C
B
-4°C
C
-8°C
D
E
F
-20°C
G
H
I
-50°C
J
K
L
-150°C
M
N
O

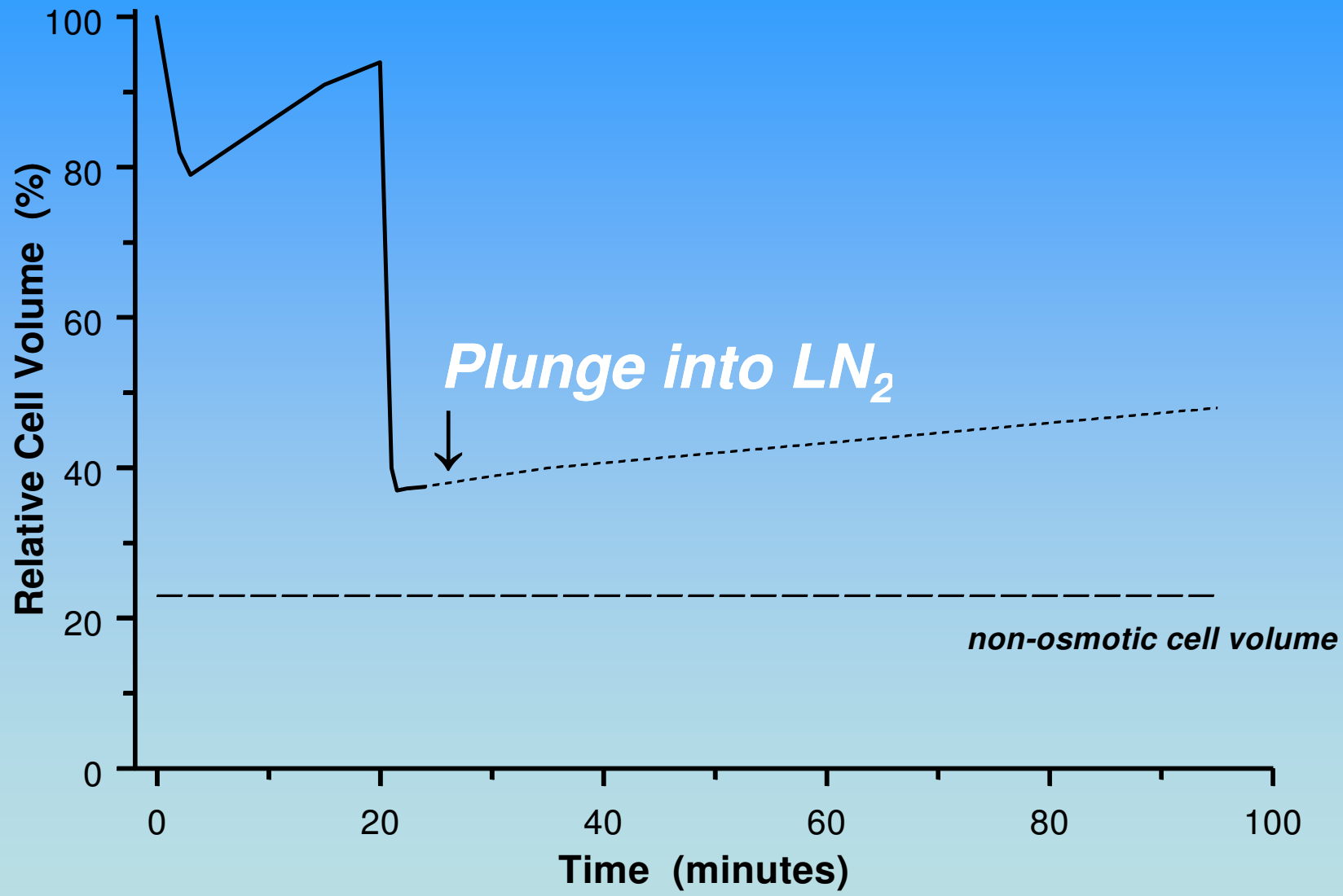
Calculated Volume of Embryo during Cooling



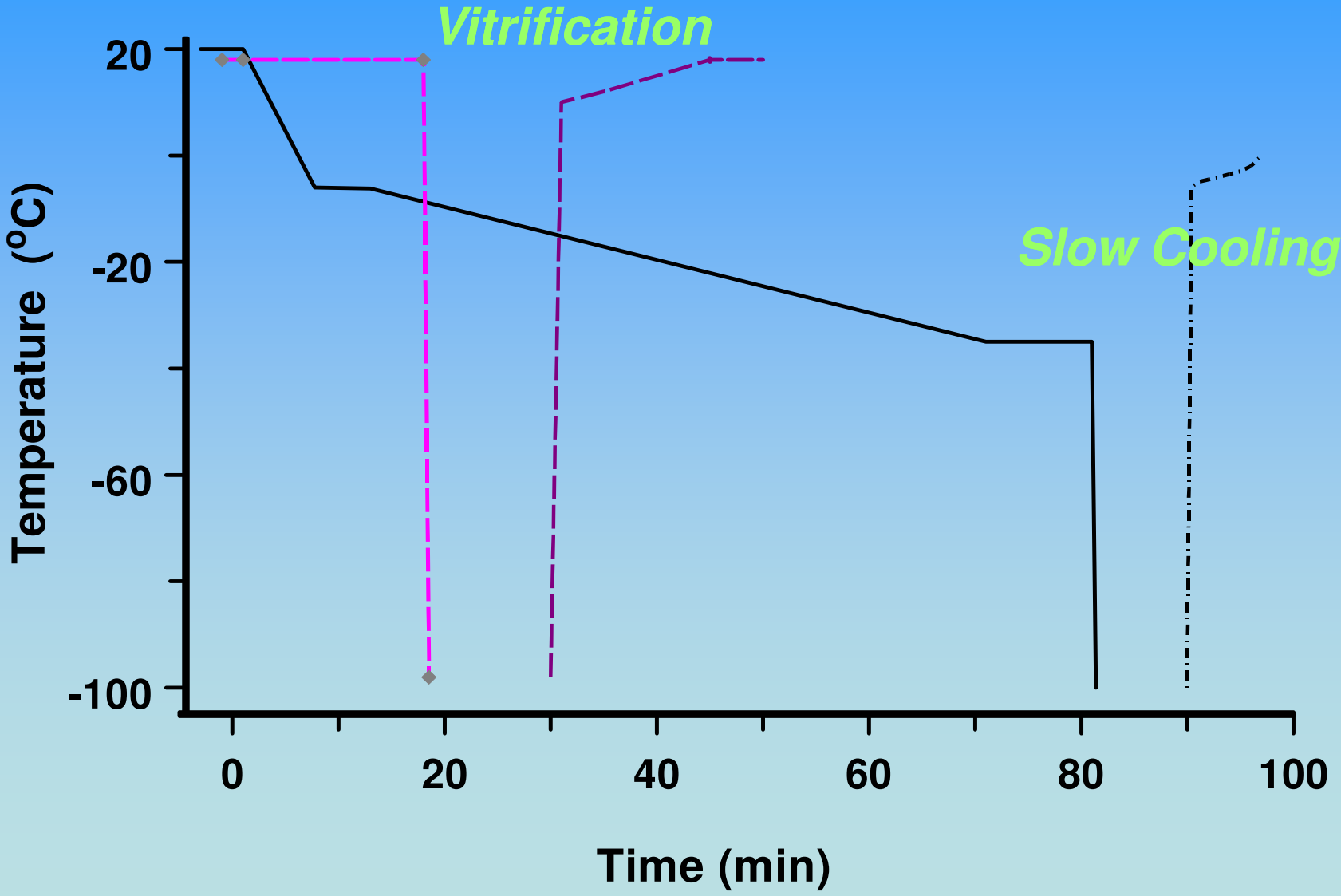
Basic Cryobiology

Equilibrium Cooling "Slow Cooling"	Non-equilibrium Cooling "Vitrification"
10 to 15% CPA	40 to 60% CPA
Cool at ~1°C/minute	Cool at >1000°C/minute
Cells dehydrate during freezing	Cells dehydrate ***before*** cooling
Little or no Intracellular Ice	Some intracellular ice

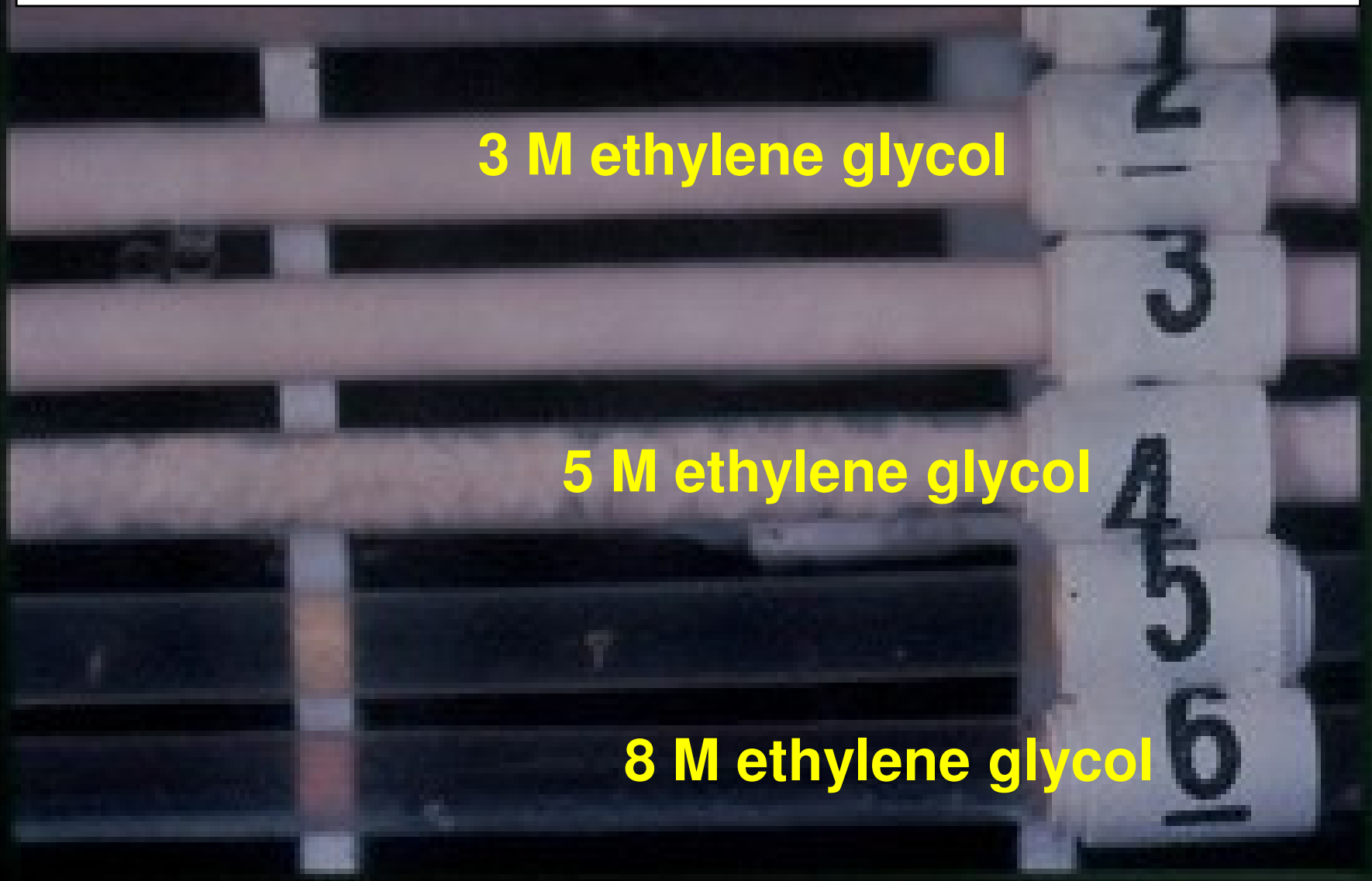
Calculated Volume of Embryo in Two CPAs



Comparison of "Slow Cooling" and "Vitrification"



Straws submerged in LN_2 at -196°C



Conclusion

- ***Mazur's theoretical analysis (and derivative theories of others) describes generalized response of cells when frozen.***

Limitations of Theoretical Analysis

- *Water permeability of different oocytes & embryos vary*
- *Intracellular nucleation temperatures vary among oocytes & embryos*
- *Non-equilibrium cooling may avoid these differences*

Final Conclusion

- ***Empirical analysis***
- ***Imagination***
- ***Creativity***

can contribute to optimization of
Cryopreservation of Oocytes and Embryos