A CRITICAL ASSESSMENT OF CURRENT VITRIFICATION METHODS

Steven F. Mullen Ph.D.
Scientific Director
The World Egg Bank

THE WORLD EGG BANK®

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Scientific Director
The World Egg Bank

Disclosure

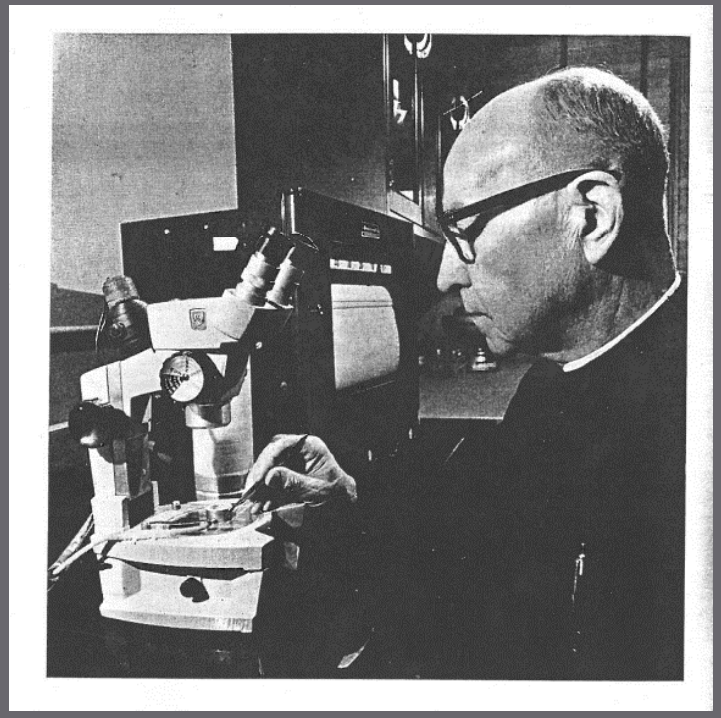
Scientific Director for The World Egg Bank
A For-Profit Company providing Oocyte Donors and Cryopreserved Donor oocytes to Fertility Clinics and Patients in the US and Worldwide

No financial interests in any products being Marketed to IVF Clinics other than TWEB Products and Services

Goals:

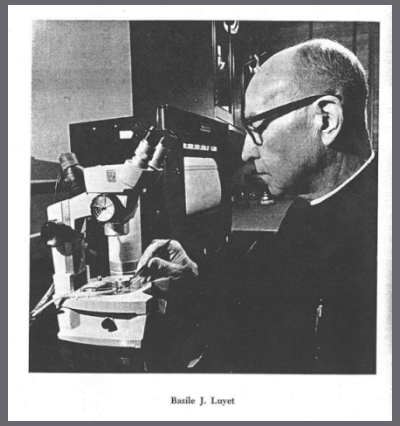
- Learn about the historical developments of Ultra-Rapid Vitrification
- Understand the sensitivities of current vitrification methods
- Explain what we should know, and yet do not
- Interpret the sensitivities and use that information as a guide to day-to-day practices
- Be able to examine a vitrification protocol and determine if the method is likely to be robust/repeatable

History of vitrification by "ultra-rapid" cooling



Basile J. Luyet (1897 – 1974)

Photo from: Meryman HT. Basile J. Luyet: In Memoriam. Cryobiology 1975;12:283-92.


Basile J. Luyet

Luyet. 1940

Life and Death at Low Temperatures

"One will obtain rapid elimination of heat by reducing the material to sheets with the smallest possible thickness and the largest possible area". P. 208

"In order to reduce the heat capacity of the preparation, one must use very thin
supports." P. 218

"Instead of thin supports, we sometimes used, with advantage, a ring of about two millimeters in diameter, made of as thin a metal wire as possible and fastened to a light, rigid rod. One simply dips this loop into the culture and thus obtains, in the thin film within the ring, quite a considerable number of organisms." P. 218

Photo from: Meryman HT. Basile J. Luyet: In Memoriam. Cryobiology 1975;12:283-92.
Text from Luyet BJ, Gehenio PM. Life and death at low temperatures. Normandy, Missouri: Biodynamica, 1940;341.

representing a $^1\Sigma - {}^1\Sigma$ transition, gave $B_e' = 0{\cdot}5747$ and $B_e' = 0{\cdot}5797$ cm.$^{-1}$. The agreement is good.

Our analysis shows that the red system represents a $^1\Sigma - {}^1\Pi$ transition. A more detailed paper on the red bands will appear in *Arkiv för Fysik*.

ALBIN LAGERQVIST
ULLA UHLER

Physics Department,
University of Stockholm,
April 23.

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Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures

THE effect on spermatozoa of vitrification at temperatures of −79° C. and below has been studied by several authors. Human spermatozoa appear to be the most resistant; a substantial proportion may show good motility on thawing after even prolonged vitrification. Revival is far better when semen is frozen in bulk than when minimal amounts in capillary tubes are used[1]. No explanation of this result is yet forthcoming, but it would appear that rapidity of freezing is less important than the avoidance of surface effects.

Positive results have also been obtained with frog and fowl spermatozoa[2,3], though in both these cases

ing 40 per cent glycerol, the spermatozoa resume full motility on thawing. So far as retention of motility is concerned, the specimen is indistinguishable from its unvitrified control; it shows even the wave motion characteristic of fowl semen. Decreasing the final concentration of glycerol below 10 per cent decreases the protection against vitrification. Increasing it above 20 per cent results in progressive immobilization of the spermatozoa, which cannot altogether be reversed by further dilution with Ringer's solution; but with these higher concentrations, no additional loss of motility is caused by vitrification. Specimens of spermatozoa have been found to resume motility completely after long periods (up to ten weeks) of vitrification. Other experiments showed that both propylene glycol and ethylene glycol were more toxic than glycerol, and in relation to their toxicity less protective against vitrification.

The fact that spermatozoa resumed full motility after vitrification under the conditions described above made it possible to investigate the effects of freeze-drying. 1 c.c. of fowl semen was diluted with 1 c.c. of 20 per cent glycerol in Ringer's solution, and vitrified as a thin layer in a 100-c.c. distilling flask at −79° C. The temperature was then allowed to rise to −25° C. and the flask connected to a high-vacuum distillation system of which the condenser unit contained liquid air. After 3 hr., when the distillation was stopped, the semen had the appearance of being dry, and 1·7 c.c. of water was thawed from the condenser. While still cold, the dehydrated semen was

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Steponkus PL, Myers SP, Lynch DV, Gardner L, Bronshteyn V, Leibo SP, Rall WF, Pitt RE, Lin TT, MacIntyre RJ. Cryopreservation of Drosophila melanogaster embryos. Nature 1990;345:170-2.

Bovine Blastocyst Development

Control = 40%

5 seconds at 0 °C ~= 20%

Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996;54:1059-69.

Human Oocyte Cryopreservation Outcomes

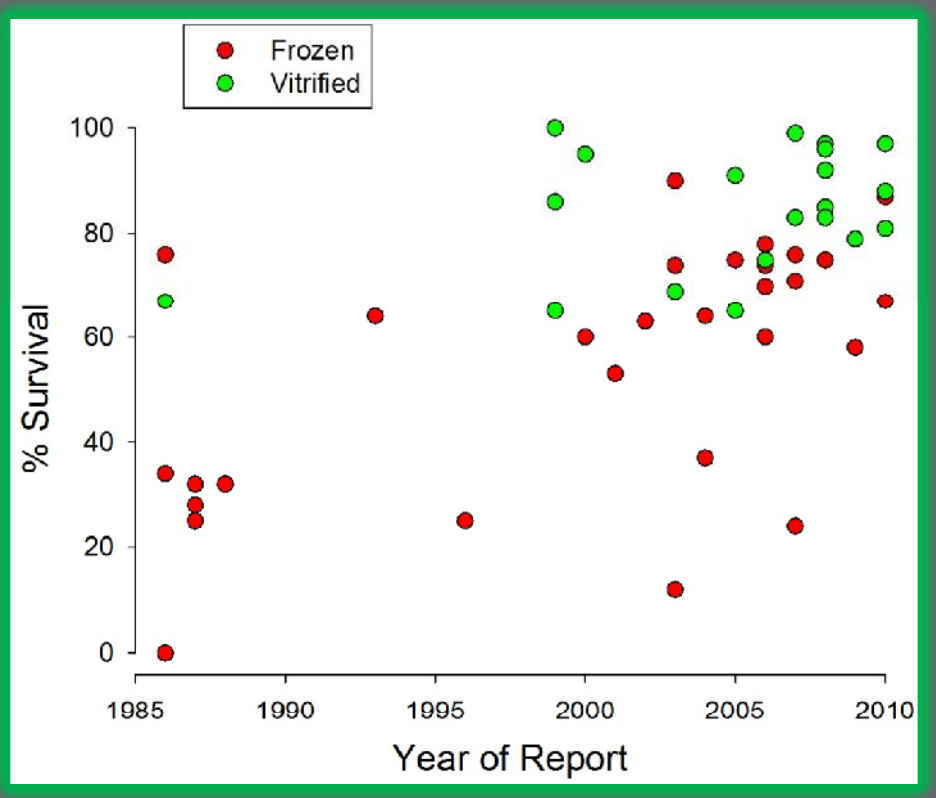


Figure from Steven Mullen, The World Egg Bank, Phoenix AZ USA. See Reference List

Today

Image from Irvine Scientific, Santa Ana, CA USA

Image from Kitazato, Shizuoka Japan

CURRENT VITRIFICATION METHODS

The Good, The Bad, and The Ugly

Technically Demanding

For skill embryologists/technicians:

1.Observation during the first week of training

2.Start training with spare material (IVM or unfertilized oocytes), under the guidance of the supervisor. Signed informed consent (the material will be used for teaching purposes).

3.Daily training during approximately 2 months (Estimated, depending on the skills and material available).

4.Start loading 1 oocyte per device and increase the number sequentially to be able to load 4 oocytes per device, in strict compliance with the protocol directions.

5.Over 80% SV in approximately 200-250 oocytes.

6.Beginers are not allow to perform entire cases.

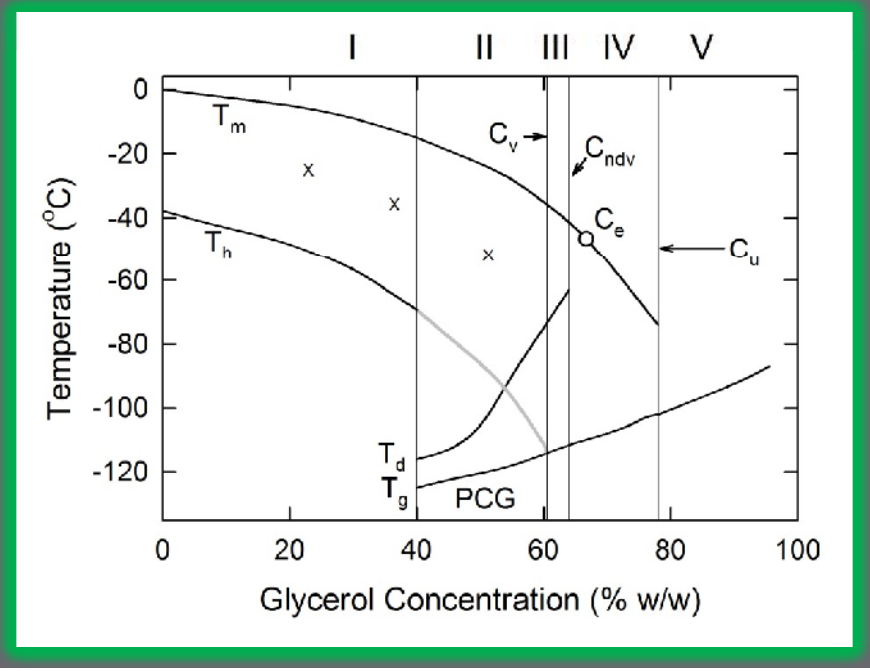
The challenge of vitrifying oocytes

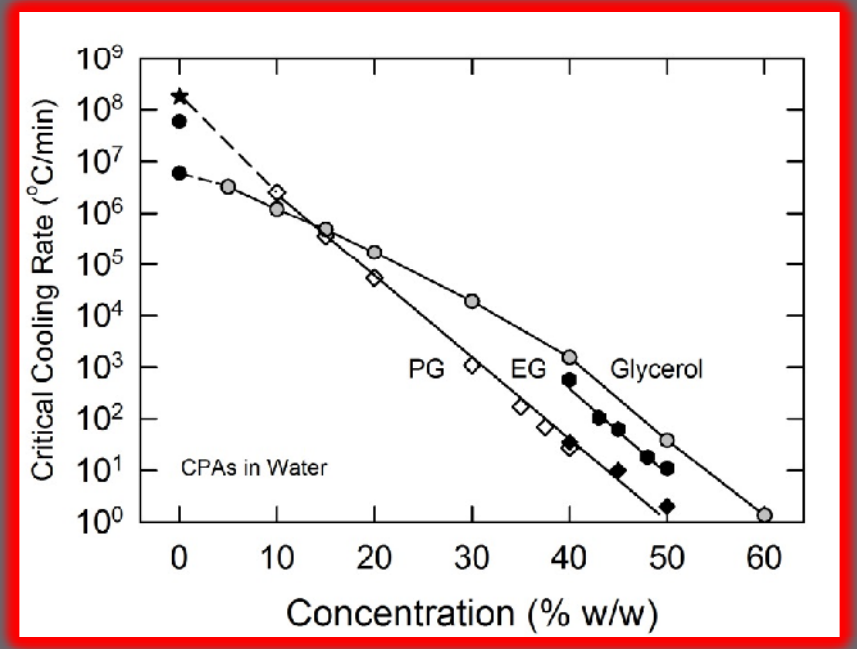
Image from Kitazato, Shizuoka Japan

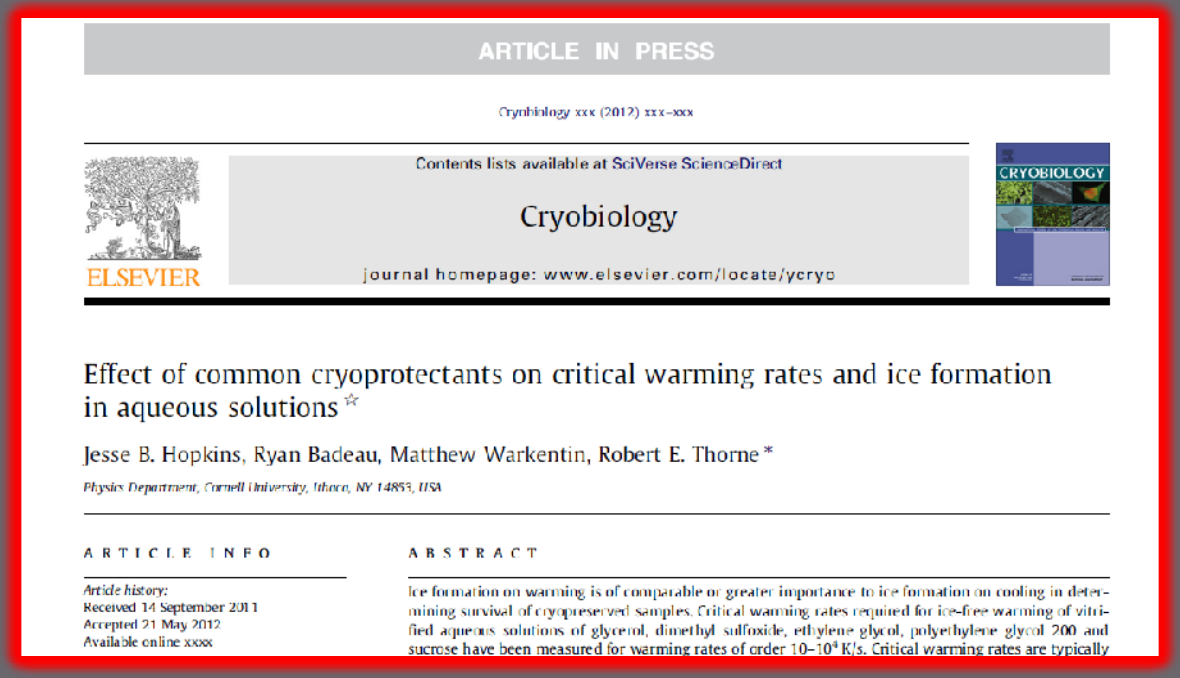
Are We Really Vitrifying??

"…survival alone is not unequivocal proof of vitrification."

Mullen SF, Fahy GM. Fundamental Aspects of Vitrification as a Method of Reproductive Cell, Tissue, and Organ Cryopreservation. In: Donnez J,Kim SS (eds), Principles and Practice of Fertility Preservation. Cambridge: Cambridge University Press, 2011;145-63.







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CRYOBIOLOGY

Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions ☆

Jesse B. Hopkins, Ryan Badeau, Matthew Warkentin, Robert E. Thorne *

Physics Department, Cornell University, Ithaca, NY 14853, USA

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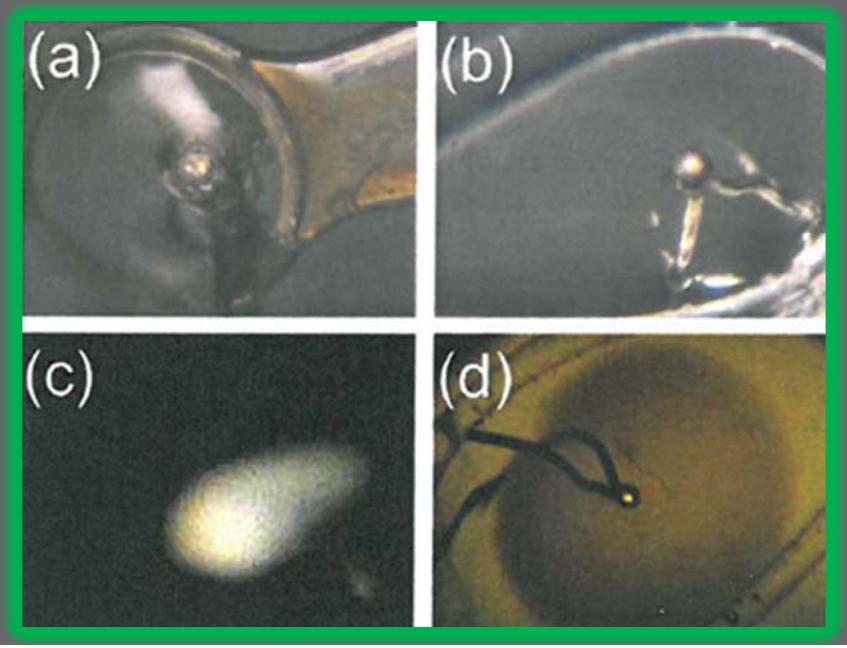
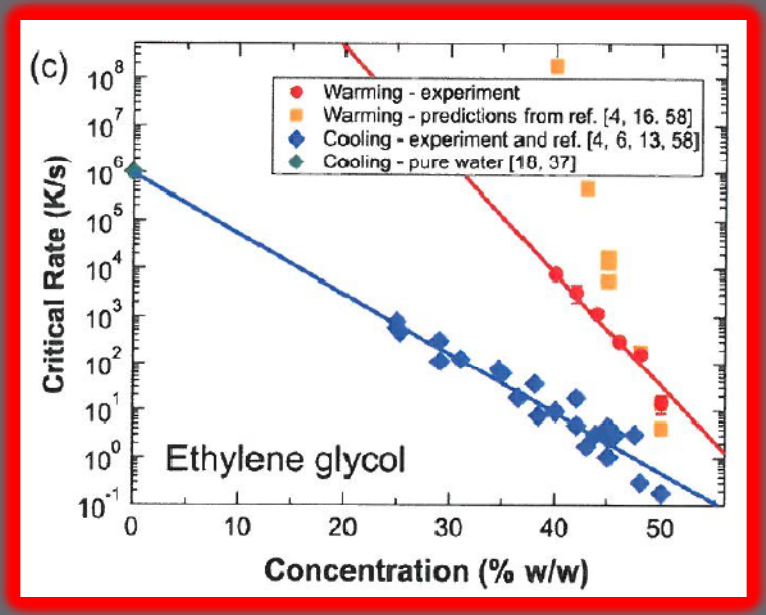
ABSTRACT

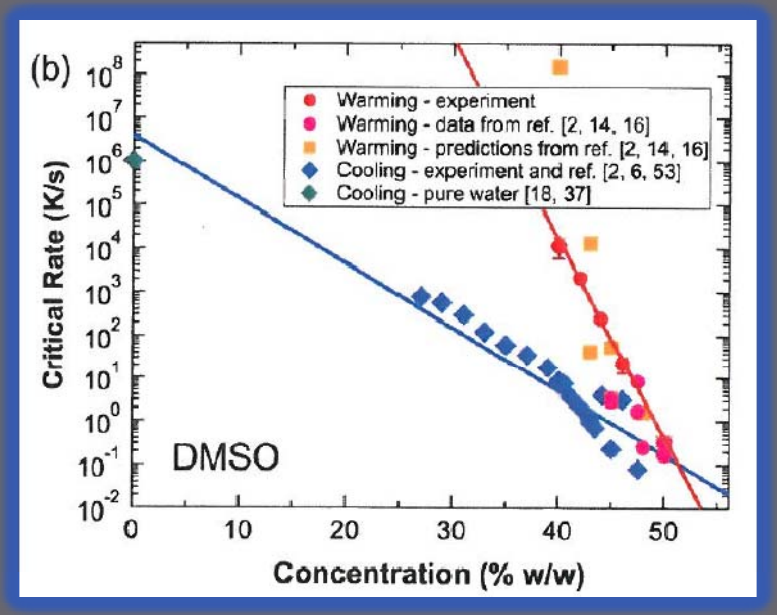
Ice formation on warming is of comparable or greater importance to ice formation on cooling in determining survival of cryopreserved samples. Critical warming rates required for ice-free warming of vitrified aqueous solutions of glycerol, dimethyl sulfoxide, ethylene glycol, polyethylene glycol 200 and sucrose have been measured for warming rates of order $10–10^4$ K/s. Critical warming rates are typically

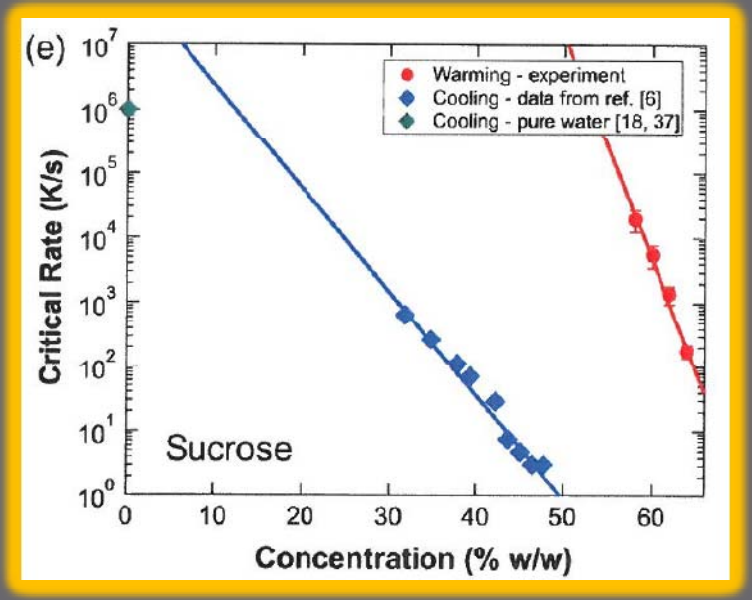
Finally!!



Hopkins JB, Badeau R, Warkentin M, Thorne RE. Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions. Cryobiology 2012;65:169-78.

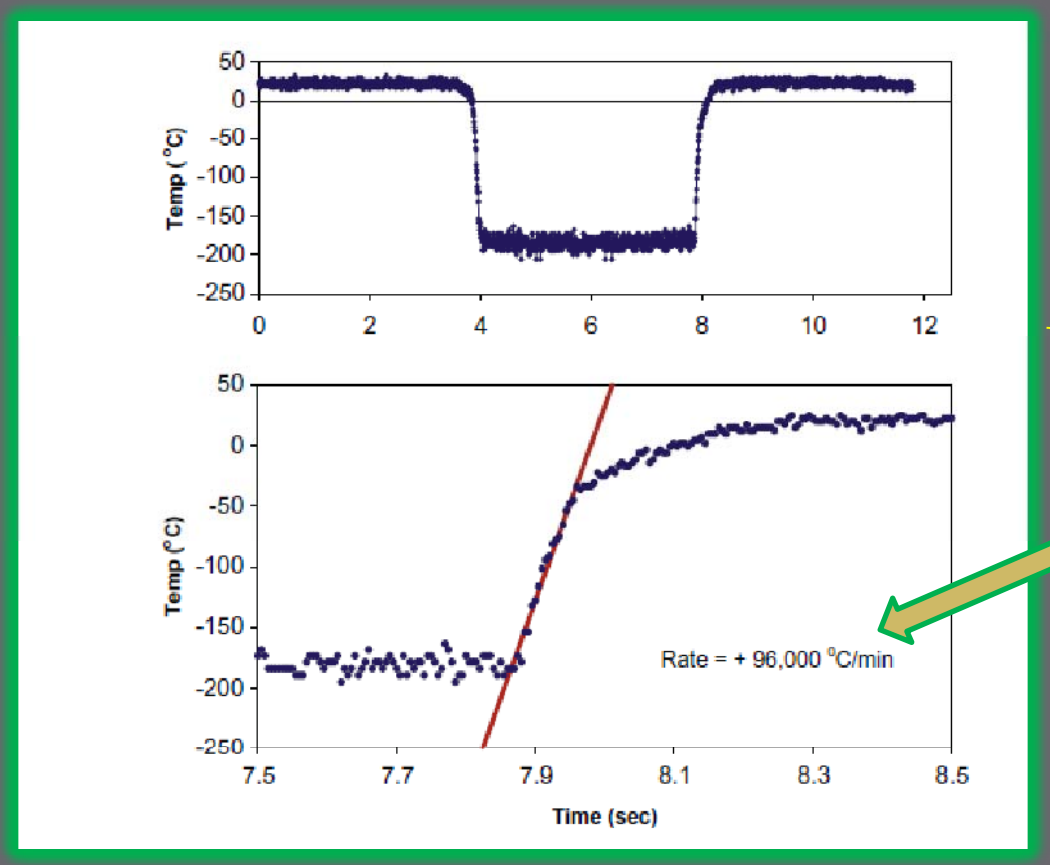






Relevant Critical Warming Rates

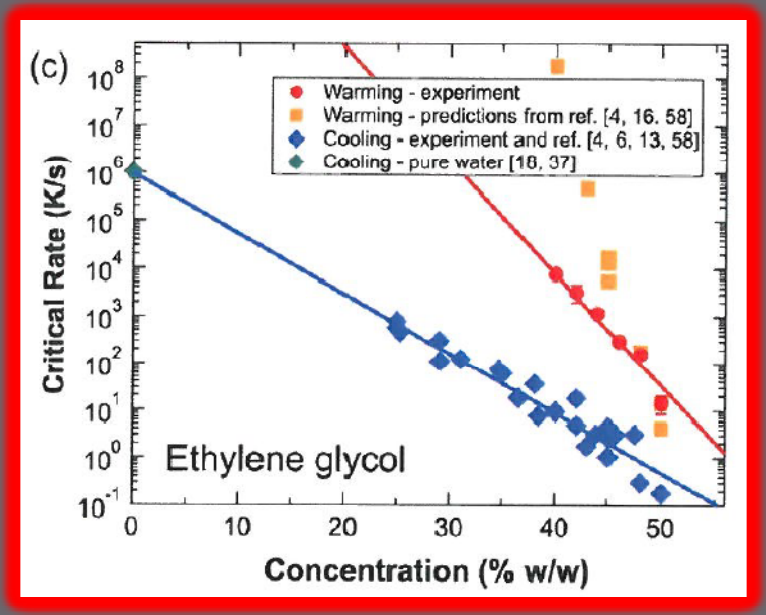
Hopkins JB, Badeau R, Warkentin M, Thorne RE. Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions. Cryobiology 2012;65:169-78.

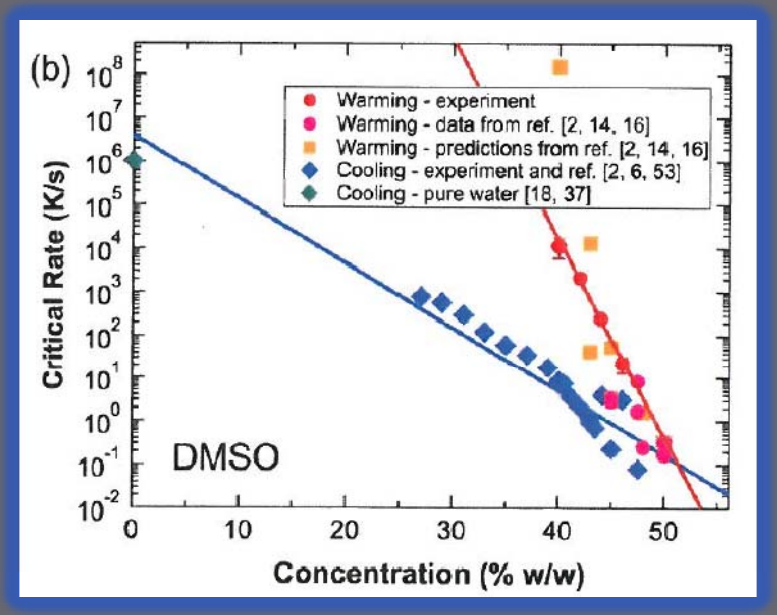


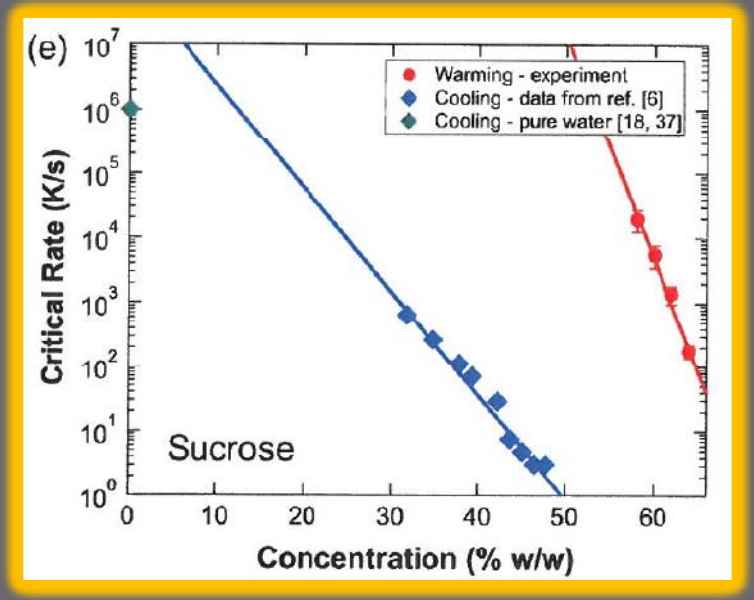
Warming Rate 96,000 K/min
(= 1.6 x 10^3 K/sec)

Kleinhans FW, Seki S, Mazur P. Simple, inexpensive attainment and measurement of very high cooling and warming rates. Cryobiology 2010;61:231-3.

Image from Kitazato, Shizuoka Japan







What about 15% DMSO 15% EG 0.5 mol/L Sucrose, Etc ???? Current Warming for Cryotop about 10^3 K/s

"Our preliminary attempts to measure critical cooling and warming rates in aqueous solutions containing up to 50% w/w lysozyme suggest that soluble proteins are very poor cryoprotectants, as has been found in studies of ice formation in hydrated protein powders ."

Hopkins JB, Badeau R, Warkentin M, Thorne RE. Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions. Cryobiology 2012;65:169-78.

Image from Kitazato, Shizuoka Japan

Big Unknown

Image from Kitazato, Shizuoka Japan

What is the vitrification solution composition in this scenario?

Shouldn't we Know??

Moving On…

Photo by S. F. Mullen

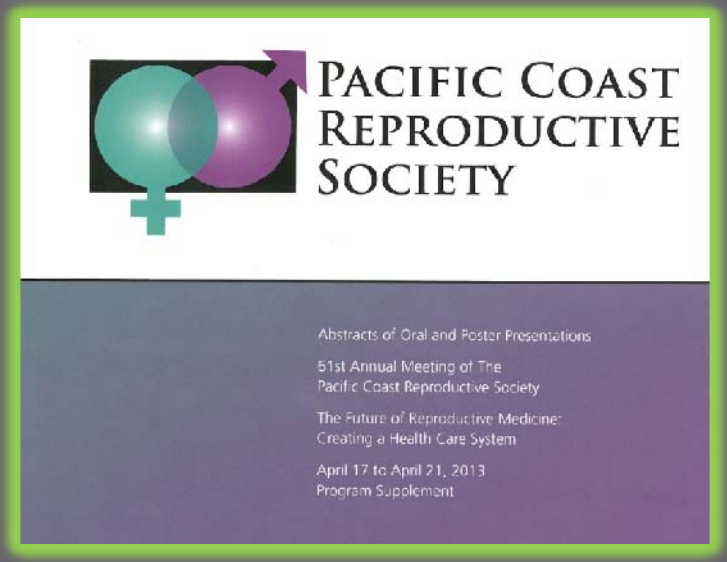
Thermal Sensitivity…

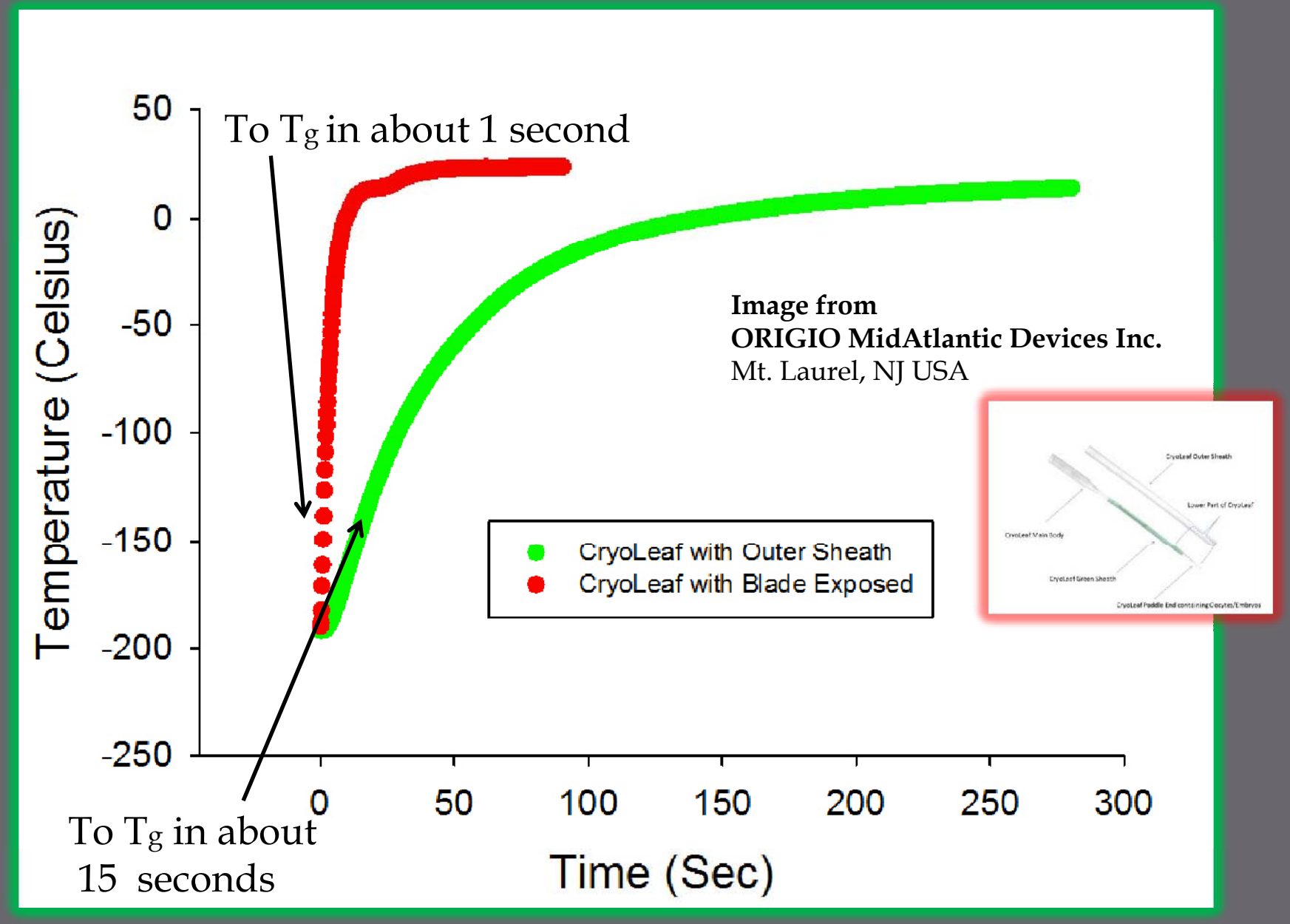
Seconds of Exposure to Room Temperature	Percent of Viable Embryos 5 hours post-warming
1	100
5	80
10	0
30	0
60	0

Image from Kitazato, Shizuoka Japan

P-40

Vitrified Embryos Are Vulnerable to Temperature Change. Charity Reeves, B.Sc., T.S.,[a] Marlena Duke, M.S.,[a] Lawrence Grunfeld, M.D.,[a,b] Benjamin Sandler, M.D.,[a,b] Tanmoy Mukherjee, M.D.,[a,b] Alan B. Copperman, M.D.[a,b] [a]Reproductive Medicine Associates of New York, 635 Madison Avenue, 10th Floor, New York, NY 10022; [b]Department of Obstetrics, Gynecology and Reproductive Science, Mount Sinai School of Medicine, Klingenstein Pavilion, 1176 Fifth Avenue, 9th Floor, New York, NY 10029.





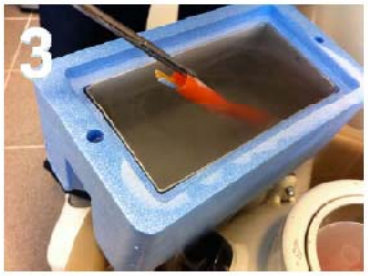
Data from Steven Mullen, The World Egg Bank, Phoenix AZ USA

So…what does this mean in practice ?

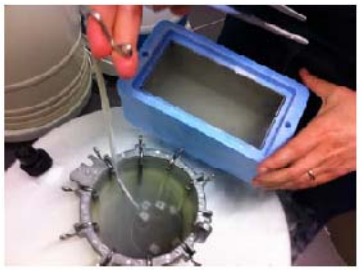
Sample reception at the destination

Fill the Dry Shipper with LN

Bring a recipient with LN



Take the gobelet to the container quickly



4 Storage

Temperature

The challenge of vitrifying cocytes

Moving On…

Mechanical Sensitivity…

Massive Fracture Event in Vitrified Solution in a Straw

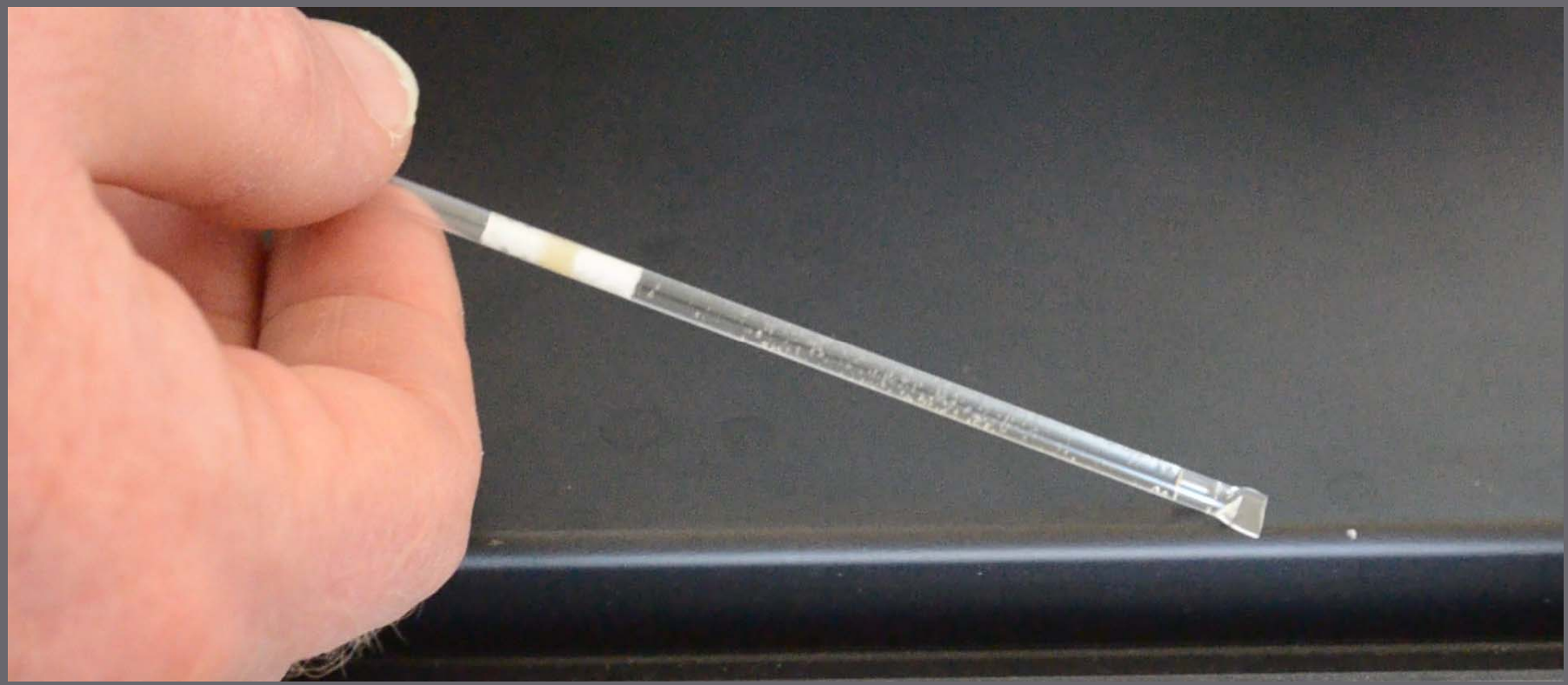
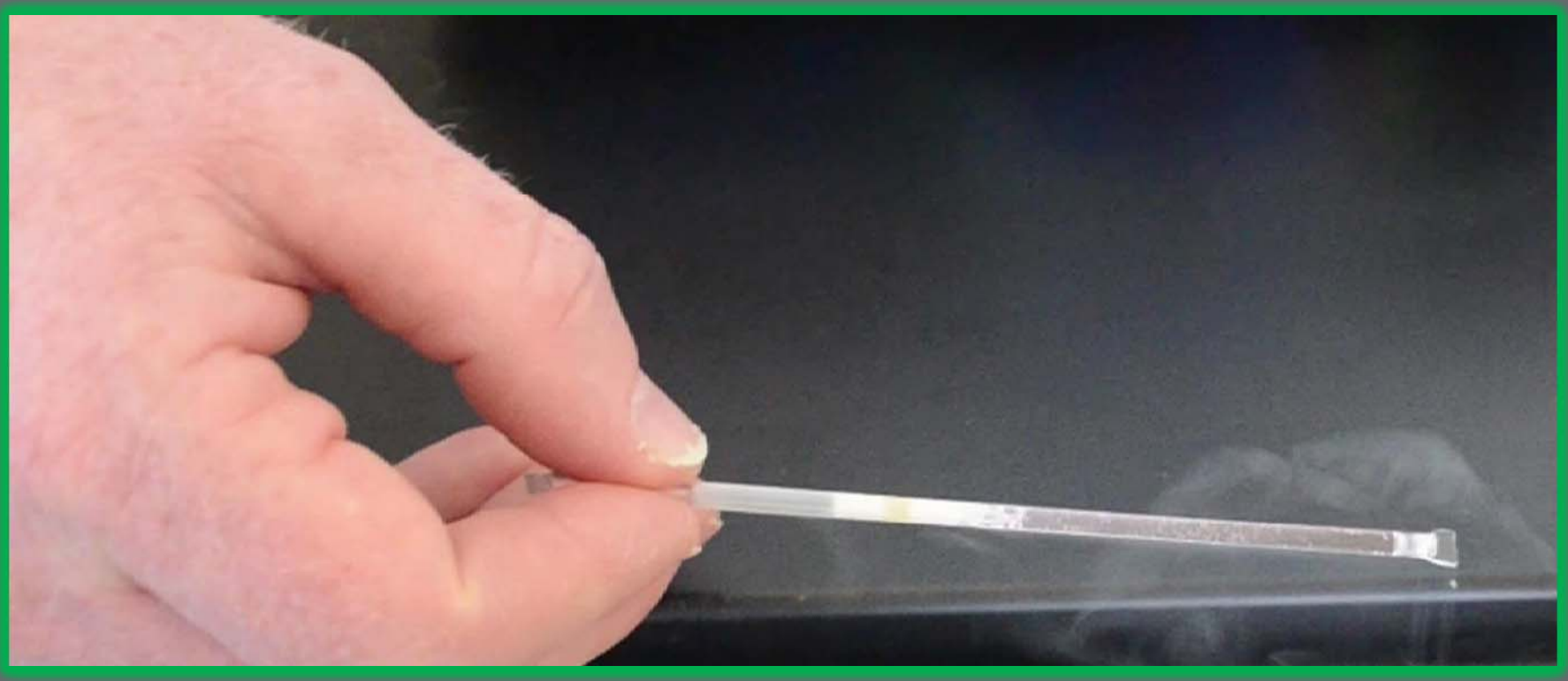


Figure from Steven Mullen, The World Egg Bank,
Phoenix AZ USA



Movie from Rebecca De La Cruz and Steven Mullen, The World Egg Bank, Phoenix AZ USA

What to Take Away…

- Vitrification research has a long history, and we are probably re-visiting many issues already investigated

- Vitrification, as is currently practiced, is technically demanding and and requires a lot of practice and training to become proficient

- There are a lot of details that are simply unknown, and can lead to increased variability and difficulties in troubleshooting problems

  These include:
  - Whether vitrification is actually occurring
  - To what solution composition the cells are being exposed
  - How sensitive the samples are to accidental warming
  - How sensitive the samples are to thermomechanical stress

- Do we really need to go to these extremes?

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A CRITICAL ASSESSMENT OF CURRENT VITRIFICATION METHODS

THANK YOU FOR YOUR ATTENTION!