Cryopreservation of hematopoietic stem cells: how did we get here and where are we going?

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Clinical context for HSC cryopreservation

Cryopreservation is typically used for

- Umbilical cord blood
- Autologous HSC transplantation

(Short term liquid storage is used for other applications)

Cryopreservation permits

- Coordination with patent care regimes
- Completion of typing and safety testing
- Genetic diversity of cells available

Standard protocol #1

- Cryoprotectant: 10% DMSO
- Cooling rate: 1 C/min
- Controlled rate freezing
- Storage on LN_2

(Areman, Bone Marrow Transplantation, 1990, Rowley,J. Clin. Apheresis, 1992)

Standard protocol #2

- Cryoprotectant: 5% DMSO+6% HES.
- Cooling rate: approximately 1 C/min
- Cooling in a mechanical freezing
- Storage: LN_2 or -80 C

(Stiff, Blood, 1987, Rowley, BMT, 2003)

Components of a Cryopreservation Protocol

Liquid storage/
prefreeze processing

- Introduction of solution
- Cooling protocol
- Storage conditions
- Warming protocol
- Post thaw assessment

Additional factors:
- Cell density
- Cell source

Formulation and introduction of a cryopreservation solution

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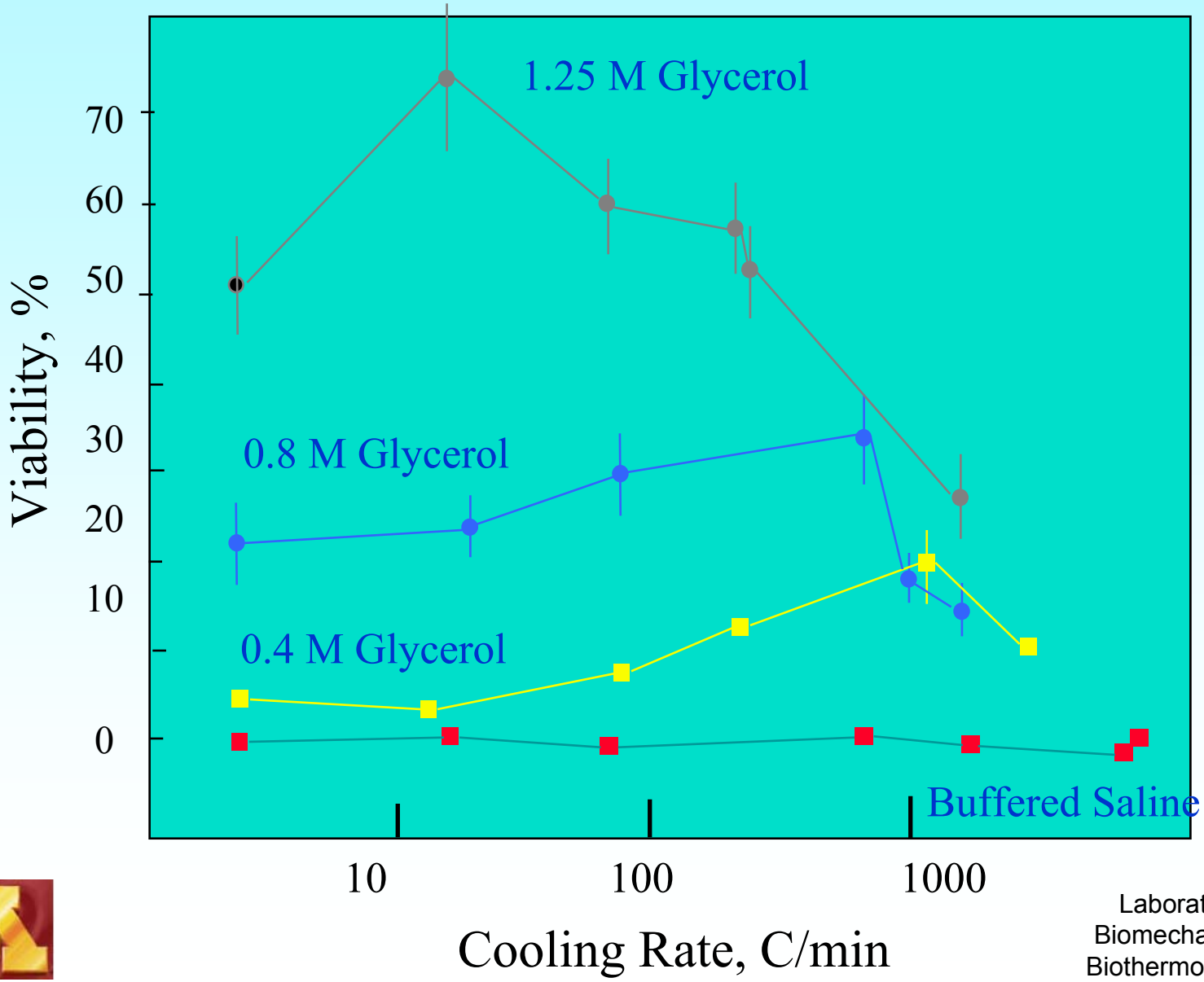
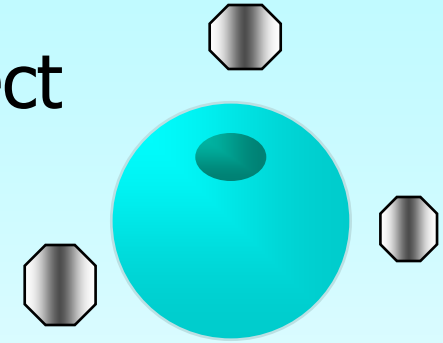
CPAs influence post thaw viability



Mechanisms of action for CPAs

Colligative Effect



Stabilization of Cell Membrane

Change in crystal growth and structure
-vitrification
-growth inhibitors

These changes can occur where the protective agent is found (intracellular and/or extracellular space)

Partitioning of CPAs

High Molecular Weight Agents

- Polymers (HES, PVP)
- Sugars (trehalose, raffinose)
- Antifreeze Proteins

How did we get here: DMSO is a low molecular weight CPA that penetrates the cell membrane. This property is largely why we use it.

Introduction of CPA solutions

Method #1: Combining equal volumes of cell suspension and 20% v/v DMSO solution.

Method #2: Using a syringe pump to infuse slowly high concentrations of DMSO.

DMSO and cells are typically cooled on ice prior to addition of the CPA solution.

Introduction of CPA solutions, cont

Cryopreservation solutions are not physiological.

Mechanisms of Damage

- Osmotic Stresses
  - Volumetric excursions resulting from efflux of water followed by influx of CPA.
- Biochemical Toxicity
  - cytoskeletal reorganization
  - suppression of normal metabolism
  - shift in membrane composition

Strategies to reduce cell losses from introduction

Protocol Modifications

- Step-wise or gradual introduction.
- Introduction at reduced temperatures.
  - Acts on both mechanisms of toxicity.

Evolutionary changes

Base of cryopreservation solution

Then: tissue culture medium

Now: licensed electrolyte solutions (Normosol R, Plasmalyte A)

Protein additive

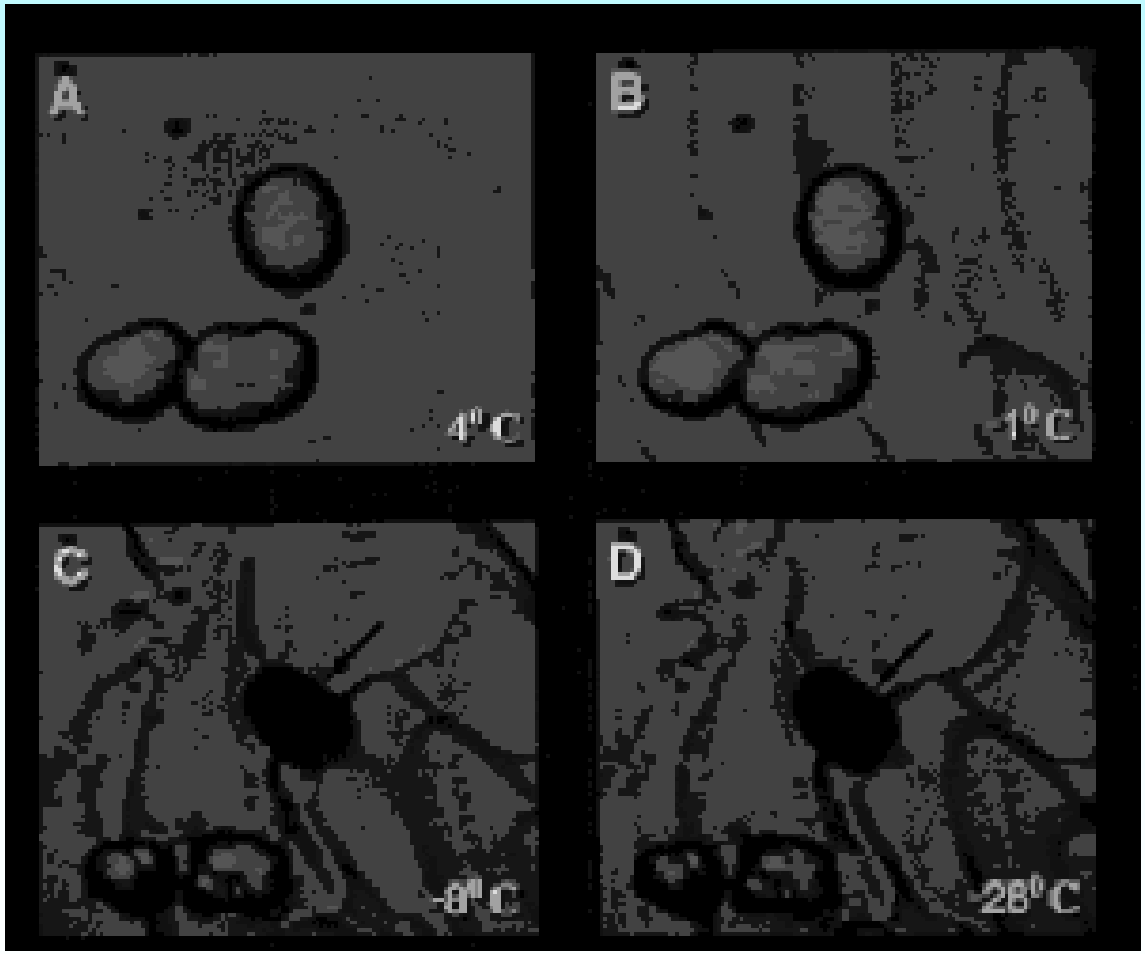
Then: fetal calf serum

Now: human serum albumin, polymers

These changes have improved the safety of the product without damaging viability or transplantation potential.

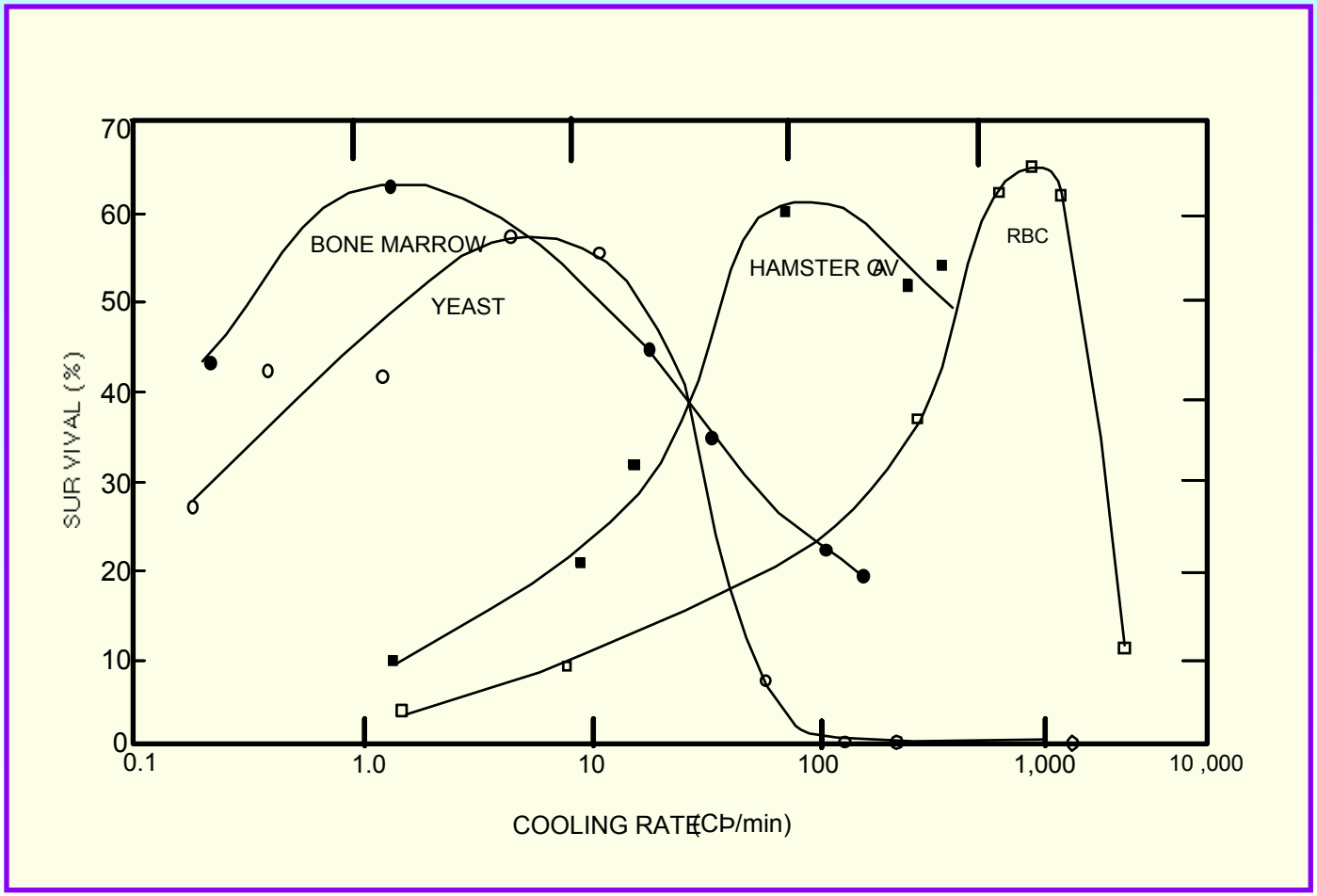
Importance of cooling rate and its selection

Cooling rate and freezing response: cryomicroscopy of hepatocytes



- Isolated rat hepatocytes were frozen at 100 C/min in isotonic saline solution.
- Dehydration of intracellular ice formation were observed.

Survival as a Function of Cooling Rate



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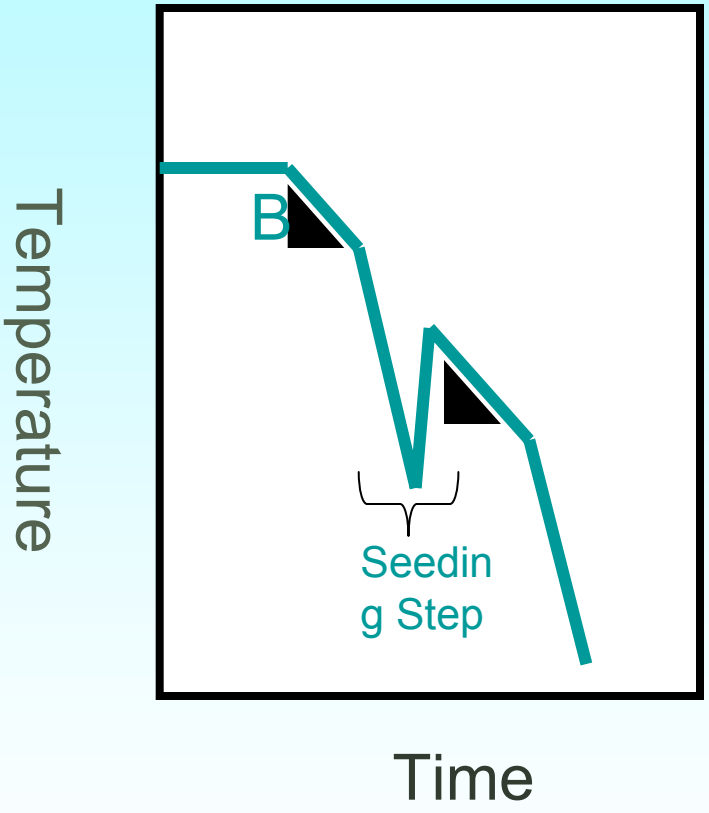
Cooling protocol

Protocol elements

- Initial equilibration phase
- Initial cooling
- Seeding Step
- Secondary cooling
- Cooling to final temperature

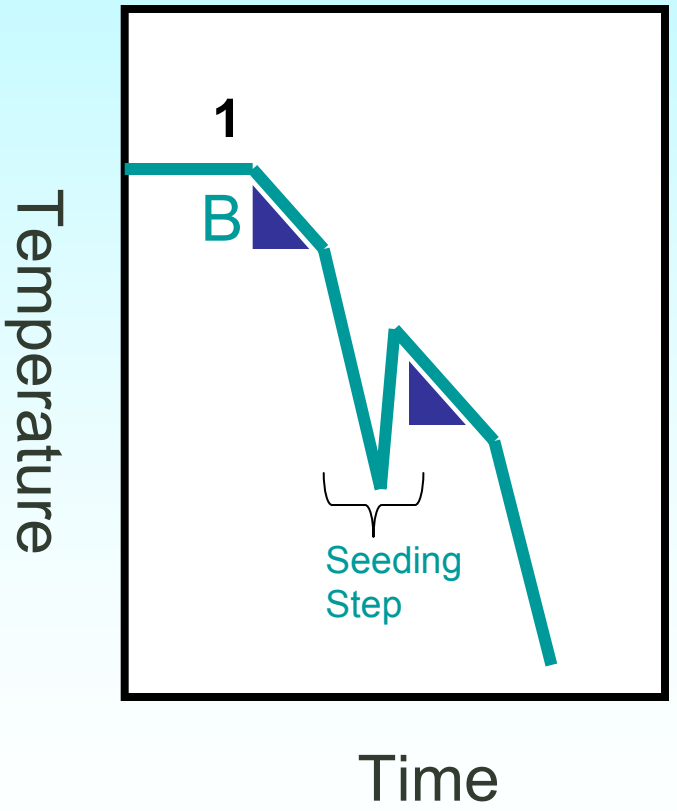


Optimal cooling protocols are influenced strongly by solution composition

Segments of cooling protocol

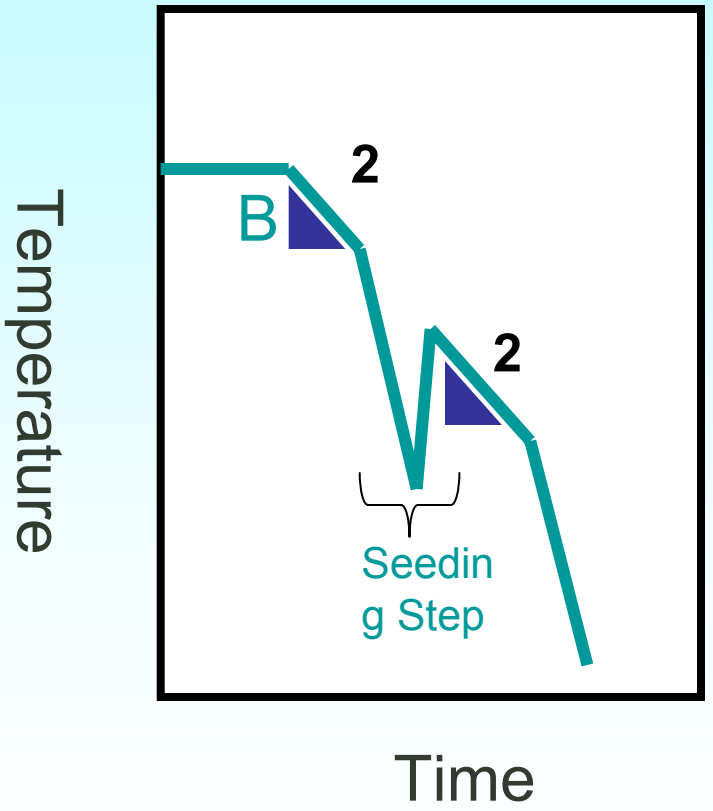
Segment One:

- Typically the chamber is pre-cooled. This segment permits the sample to achieve a uniform initial temperature.
- Insufficient time for equilibration in harmful.



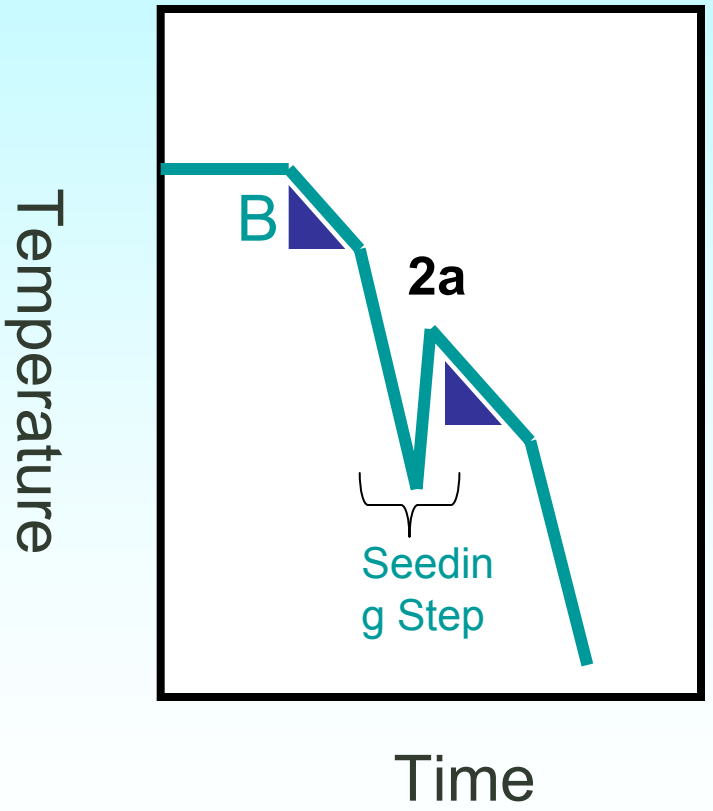
Cooling protocol, cont.

- Segment two:
  - The cooling rate for high subzero temperatures.
  - When a protocol specifies a cooling rate (i.e. 1 C/min),that means the cooling rate at segment two.



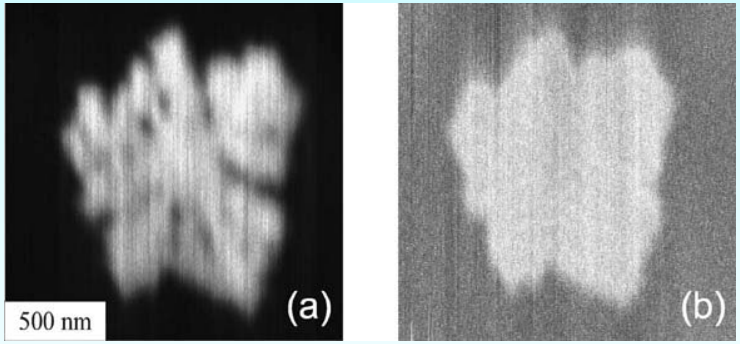
Cooling Protocol, cont.

- Segment 2a:
  - Also known as the seeding step.
  - Sharp drop in temperature followed by the increase is intended to force nucleation of the extracellular solution



Methods of seeding sample

- "Automatic seeding"
  - Clinical protocols.
- Manual seeding
  - IVF protocols.
- Uncontrolled seeding.
  - Both clinical and laboratory settings.



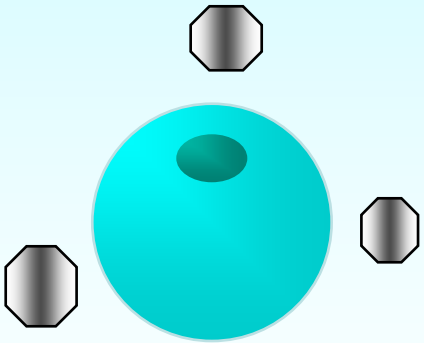
“Delayed” Latent heat

Nucleation and growth of ice releases the latent heat of fusion.

The temperature at which this occurs, T_{nuc} is important.

<u>Material Science</u>

- Concentration of extracellular solution
- Ice crystal growth characteristics



<u>Biology:</u>

- Permeability of cell to water
- Membrane phase characteristics

Relationship between T_{nuc} and IIF

- IIF is an accepted mechanism of damage.
- Cooling rate, T_{nuc} and cell type influence fraction of cells with IIF.

Low temperature storage

- Convention:
  - LN_2
- Emerging interest in the use of -80 C freezers for storage of products.

Relevant biophysics during storage

- Material science
  - Extracellular solution is only partially frozen until the eutectic temperature.
  - T_{eut} for 10% DMSO solution ~ -70 C.
- Cell biology
  - Complete suppression of biochemical reactions < -150 C.
  - Storage at -80 C: biochemical function still present.

Storage comparison

- LN2 storage
  - Requires capital equipment and access to LN2
  - Stability of products: 10,000-30,000 years.
- Mechanical freezer
  - Reduced capital expenditures
  - Need for back up power supply
  - Stability of products:
    - Increasing the temperature of storage decreases the shelf life

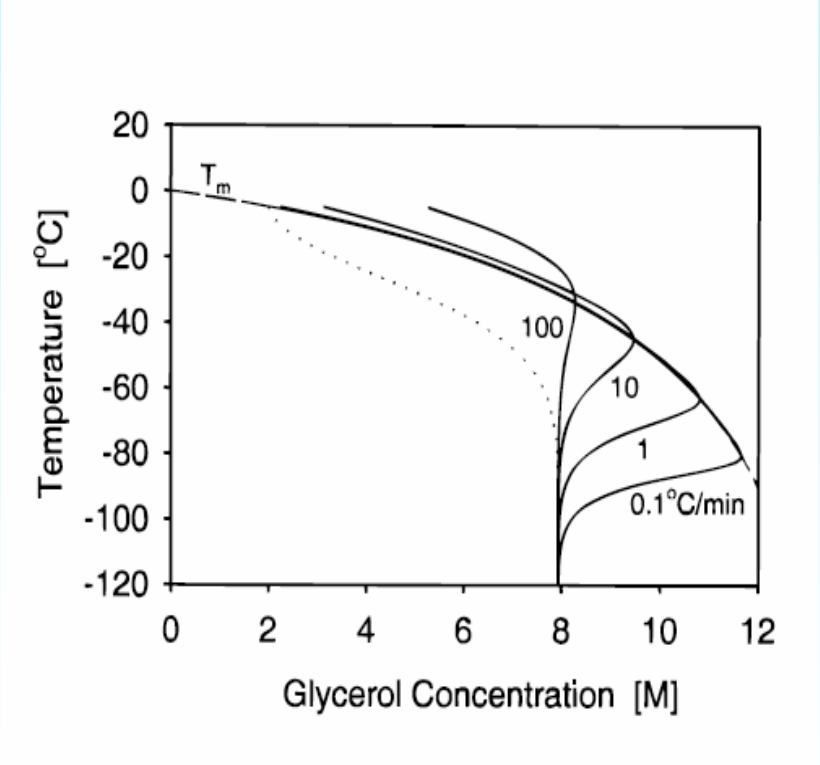
Product is not replaceable. Improper storage can result in product loss.

Warming Protocol

- The same temperature range must be traversed during during warming.
  - Recrystallization damage
  - Long-term exposure to high concentrations can also be damaging
- Cooling protocol influences warming response.

Cooling and warming interaction

- Dotted line: intracellular thermodynamic state upon cooling.
- Solid lines: intracellular thermodynamic state during warming.
- Different cooling protocol will shift dotted line and influence intracellular states upon warming.



Karlsson, Cryobiology, 2001.

Thawing protocols

- Conventional freezing: rapid thawing.
- Typically, thawing is performed in a warm water bath.
- Hints:
  - Swirling sample in the bath increases heat transfer and increases warming rate.
  - Increasing bath temperature increases warming rate.

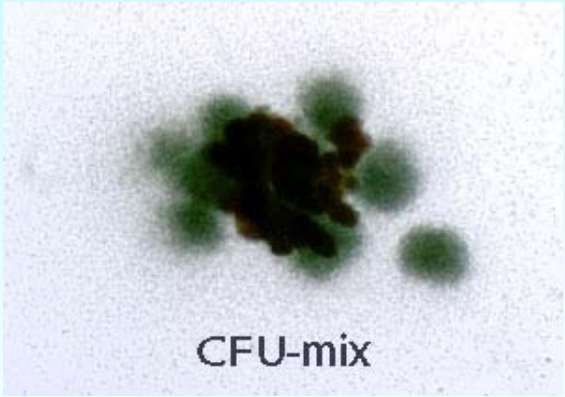
Thawing baths, cont.

- Bath temperature:
  - Convention: 37 C.
  - Higher bath temperatures can be used.
    - 42 C is the lower threshold for hyperthermic damage
    - >45 C is the upper threshold for hyperthermic damage. You will experience cell losses from high temperatures
    - Between the two thresholds, there are specific kinetics of damage.

Post thaw assessment: HSC

Convention:

- TNC, CD34+, ‘viability’, CFU
- Assessment is performed prefreeze but not always post thaw.
- Material to be assayed: integral segments, vials, bag, etc.



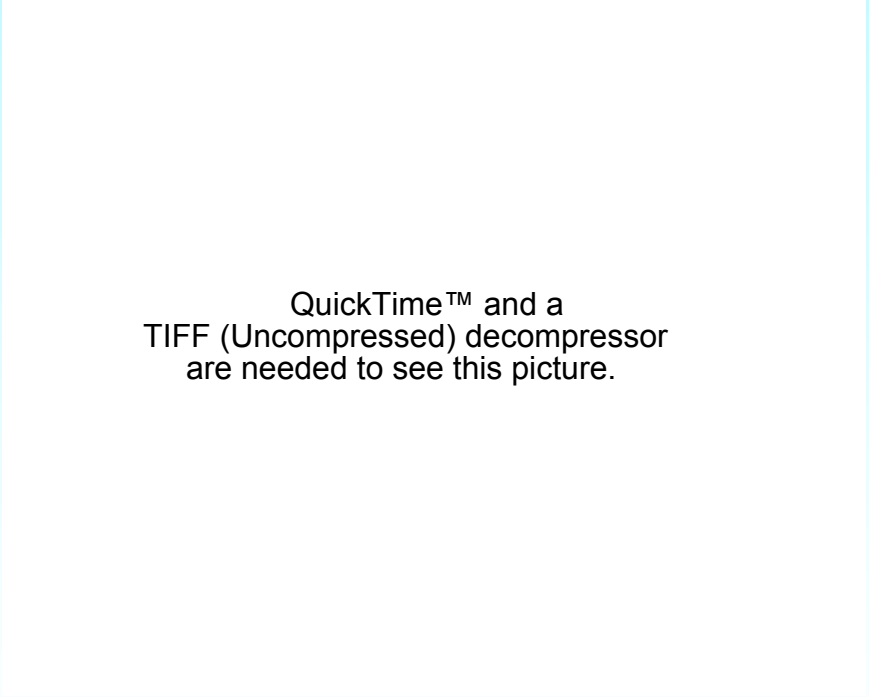
Post thaw assessment, cont.

Complexity intrinsic to cryopreservation:

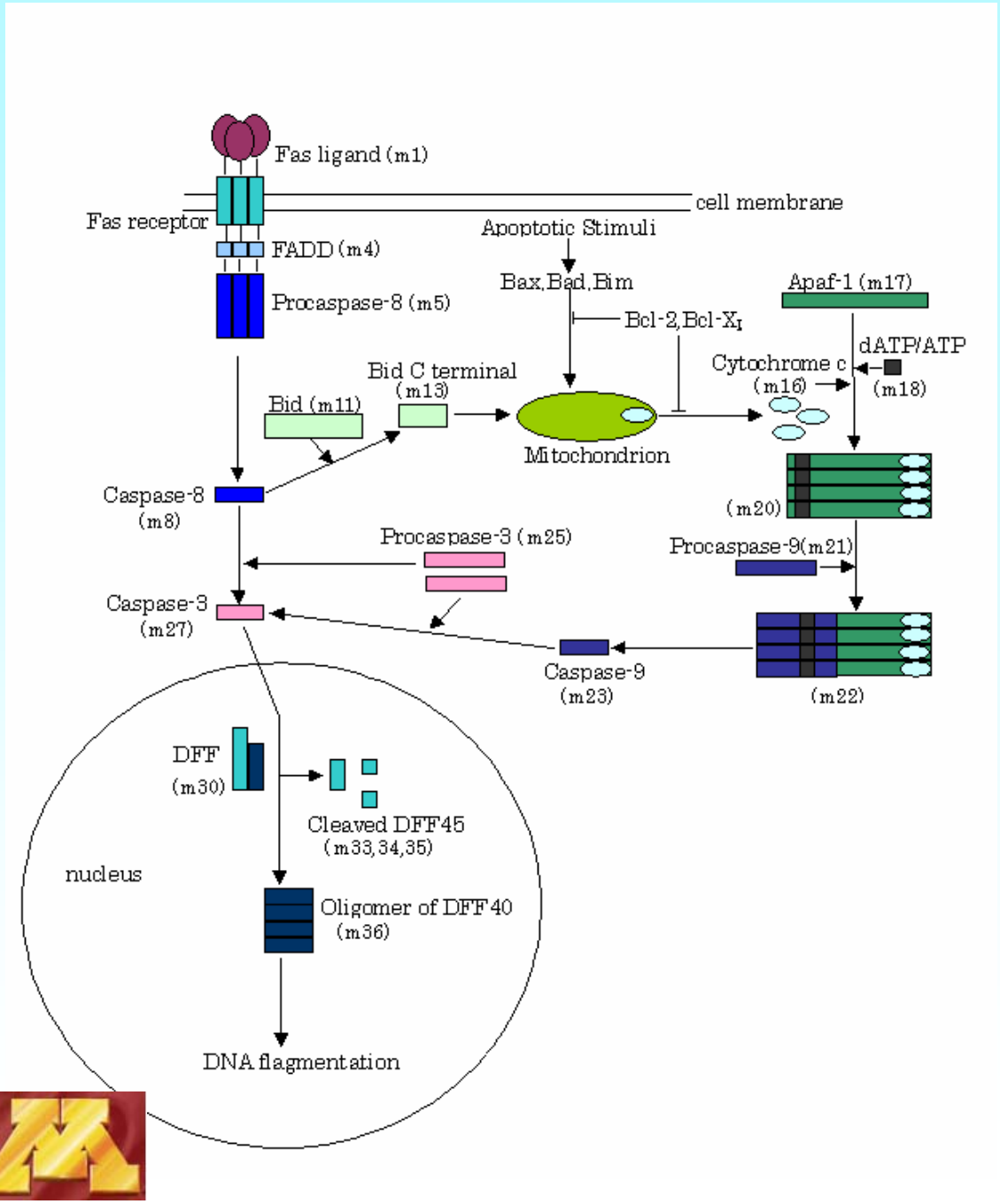
- DMSO can cleave surface receptors
- Residual DMSO can influence colony formation.
- Freezing environment changes with volume frozen.
- Membrane integrity assays are not reliable.

Post thaw viability assessment

- Lymphocytes were frozen using conventional methods.
- Cell counts and viability were performed at different times post thaw.



Post thaw apoptosis



- Post thaw cell death
  - Measured starting in the 1990s
  - Observed in various cell types
- Mediated by the mitochondria and activation of Caspase-3

  (Stroh, 2002)
- Caspase inhibition has been shown to reduce post thaw apoptosis losses.

Caspase Inhibition

- Commercial solutions exist which contain caspase inhibitors (cryopreservation and hypothermic storage).
- Exposure to caspase inhibitors can result in reduced/delayed cell proliferation.
- Further work is needed to understand and optimize use of caspase inhibitors in preservation solutions.

Issues unique to HSCs

<u>CD34+ cell/colony forming cell</u>

- Finding a needle-in-a-haystack.
  - Measurement variances are high and vary with operator.
- Surface marker/cell biology paradox
  - Ex vivo culture
  - Cryopreservation?

Emerging science and technology

New sources of HSCs

- Fetal liver-based hematopoietic progenitor cells
  - Gestational age influences post thaw recovery.
  - Osmotic characteristics have been determined.
- Blood cells from hESC.

Freezing response for different cell types may suggest that different freezing strategies may be needed.

Alternatives to DMSO as a CPA

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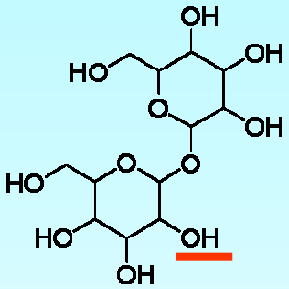
Osmolyte system (occurrences)	Principal osmolytes
A. Polyhydric alcohols-polyols	
Cyanobacteria	
Synechococcus sp.	Glucosylglycerol
Fungi	
Saccharomyces rouxii	Arabitol
Asteromyces cruciatus	Arabitol, glycerol, mannitol
Lichens	
Lichina pygmeae	Mannosidomannitol
Unicellular algae	
Dunaliella spp.	Glycerol
Chlorella pyrenoidosa	Sucrose
Ochromonas malhamensis	Isofloridoside
Multicellular algae	
Fucus spp.	Mannitol
Vascular plants	
Gossypium hirsutum L.	Glucose, fructose, sucrose
Insects (freeze-tolerant or -resistant)	
Eurosta solidaginis (Diptera)	Glycerol, sorbitol
Bracon cephi (Hymenoptera)	Glycerol
Crustaceans	
Artemia salina (emerging larvae)	Glycerol, trehalose
Vertebrates	
Hyla versicola	Glycerol
B. Amino acids and amino acid derivatives	
Eubacteria	
Klebsiella aerogenes	Glutamic acid, proline
Salmonella oranienburg	Glutamic acid, proline
Streptococcus faecalis	γ-Aminobutyric acid, proline
Protozoa	
Miemiensis avidus	Glycine, alanine, proline
Vascular plants	
Spartina townsendii	Betaine
Atriplex spongiosa	Betaine
Aster tripolium	Proline
Mesembryanthemum nodiflorum	Proline
Invertebrates	
All phyla of marine invertebrates (see examples in Table 1)	
Cyclostomes	
Myxine glutinosa (hagfish)	Various amino acids
Amphibia	
Bufo marinus	Various amino acids
C. Urea and methylamines	
Cartilaginous fishes (elasmobranchs and holocephalans: marine and estuarine): see examples in Table 1	
Coelacanth (*Latimeria chalumnae*)	
D. Urea: estivating forms	
Mollusks	
Bulimulus dealbatus	
Lungfishes: African and South American	
Amphibians	
Scaphiopus couchi (spadefoot toad)	
E. Inorganic ions	
Archaebacteria	
Halobacterium spp.	K^+

Yancey, P.H., et al., 1982, Science, 217, p1214

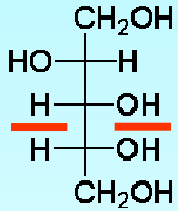
Preservation in nature

What's the common theme?

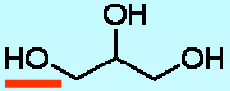
Trehalose



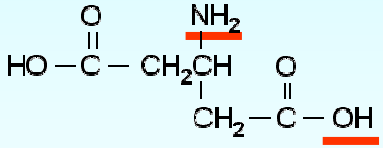
Arabitol



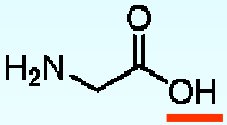
Glycerol



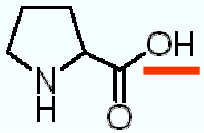
Glutamic Acid



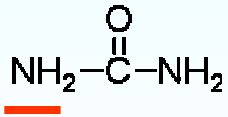
Glycine



Proline



Urea



"Natural osmolytes affect the structure of water"

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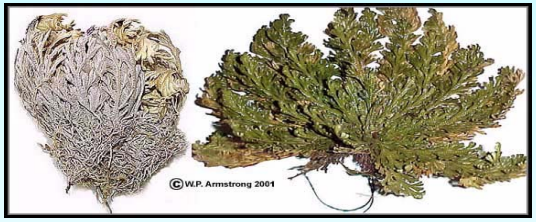
Sugars for HSC preservation

- Mammalian cells are stabilized by sugars/polyols
  - Glucose/sucrose
  - Trehalose
  - 3MOG
  - Sugars act synergistically with glycerol



- Trehalose added to conventional freezing protocols improves post thaw recovery

Sugars, cont.

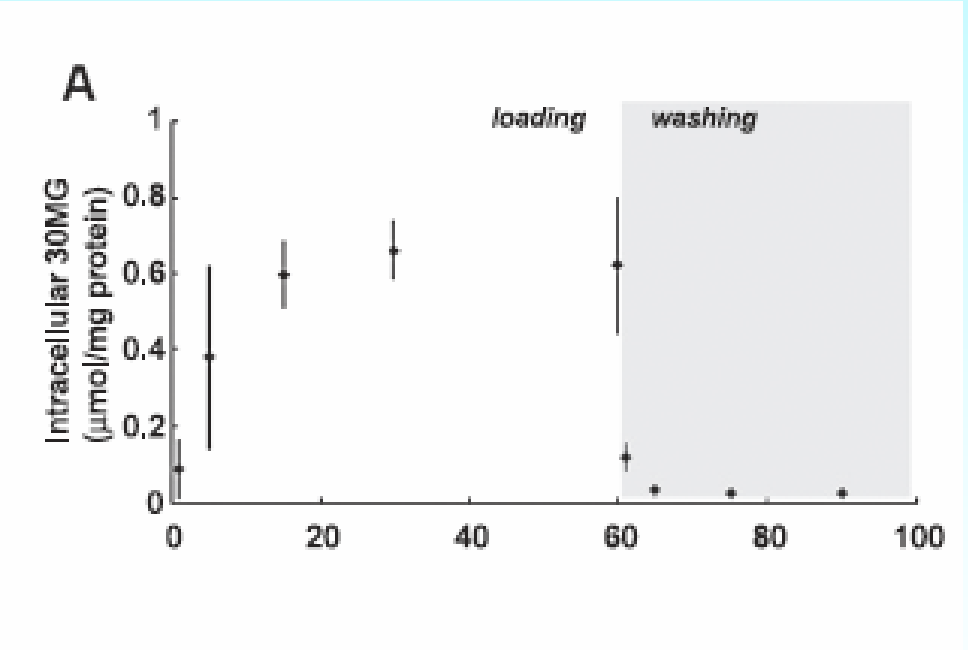
- Cells have been frozen with low levels of trehalose (200-300 mM) with high levels of recovery.
- Principal challenge: ‘delivery’
- Secondary challenge: selecting sugars.

Methods of introducing sugars

- Many methods have been used.
- Very few would be appropriate for the clinic
  - Cell numbers involved
  - Regulatory concerns

Sugar uptake: Glut receptor

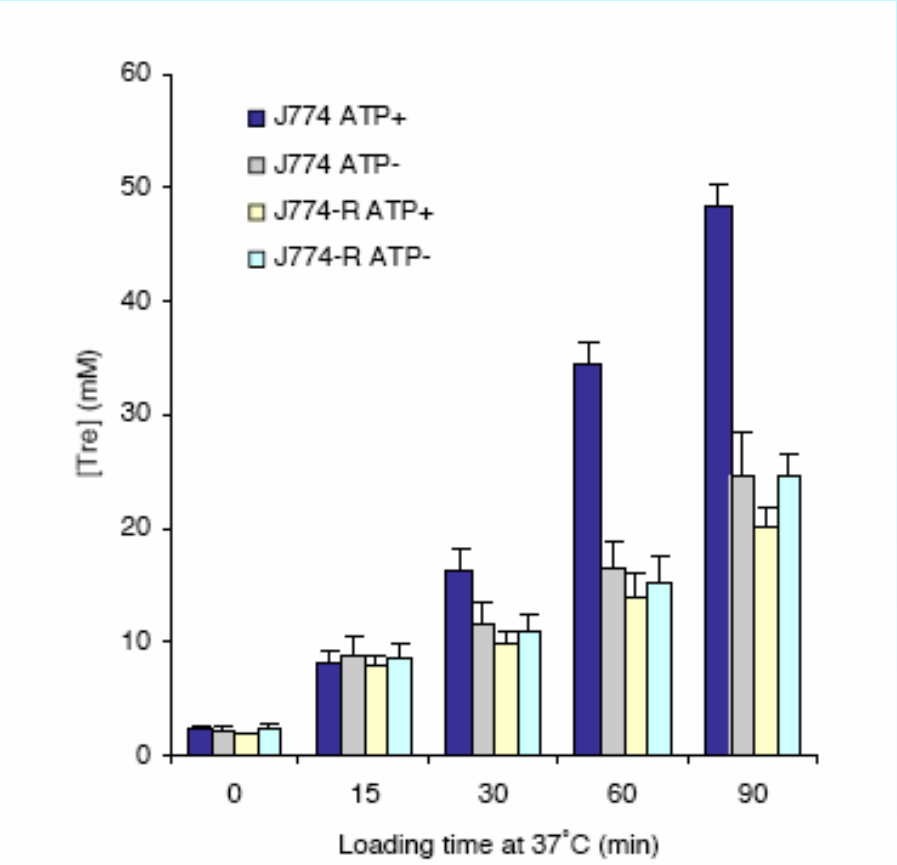
- Hepatocytes were incubated with 3-O-methyl-glucose.
- Sugar uptake via Glut- II receptor.



Sugimachi, Tissue Engineering, 2006

Sugar uptake: purinergic receptor

- Mouse macrophage cells were incubated with trehalose solutions containing ATP.
- Uptake via purinergic receptor was determined.



Elliot, Cryobiology, 2006

Cytoprotective agents

Observations:

- A significant number of cells are lost from post thaw apoptosis.
- Oxygen free-radical generation is observed during freezing.

Outcome

- Supplement media with cytoprotective additives to mitigate these effects.
  - Catalase, α-tocopherol
  - Caspase inhibitors

Post thaw processing

PBSC or Bone Marrow

- Thaw at bedside
- Immediate infusion

Umbilical cord blood

- Wash with abumin/ dextran solution
- Reinfuse

Issues in post thaw processing

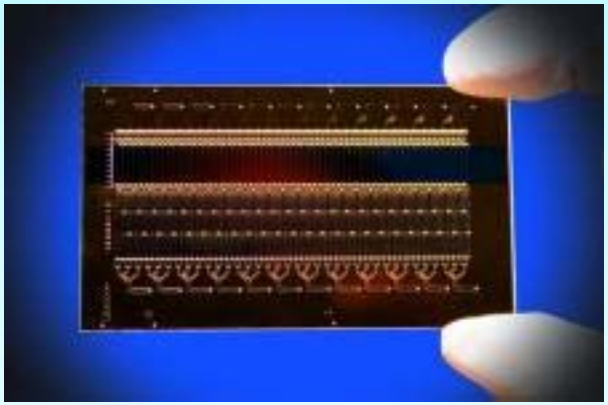
- Frozen and thawed HSCs are reinfused directly into adult patients.
  - DMSO present in the cell suspension is responsible for adverse reactions.
- Umbilical cord blood is routinely washed and cell losses >25%.

Therein lies the conundrum: direct infusion of cryopreserved hematopoietic stem cells results in significant adverse reactions but washing cells to remove DMSO results in significant cell losses that, in turn, adversely affect transplant outcome

Microfluidics

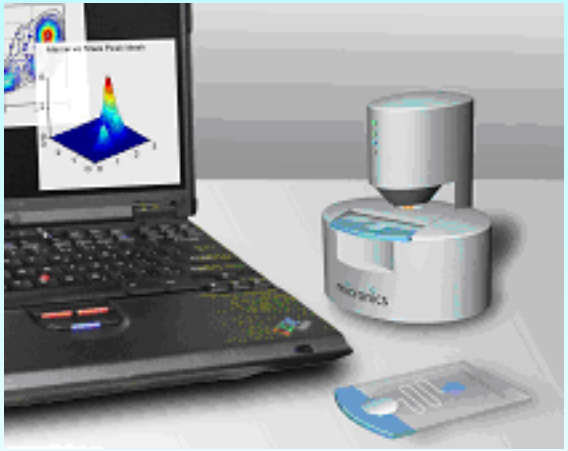
- Microfluidics: flow of fluids in a microscale channel (laminar flow).
- Advantages: low power requirements, manufacturability, control over cell motion.
- Microfluidics will grow to play an increasing role in the clinical laboratory.

Microfluidics, cont.



Cytonome

Cell sorting of 10^9 cells/hr



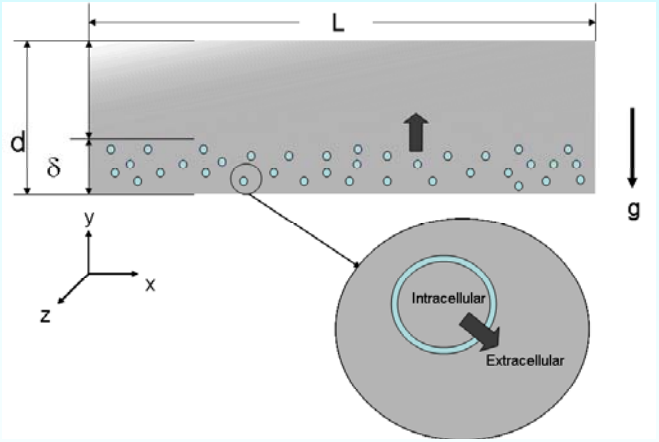
Micronics

CBC and Differential

This technology will permeate the clinical lab/doctor's office in the next 5 years.
More clinical laboratory services will move from the laboratory to the point of use.

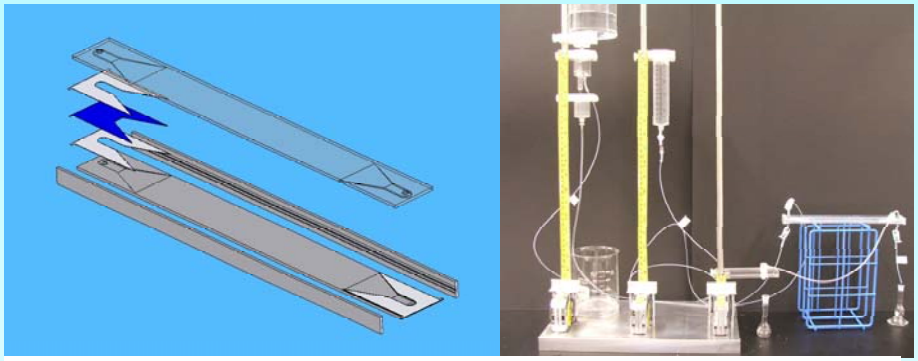
Microfluidic processing of cells

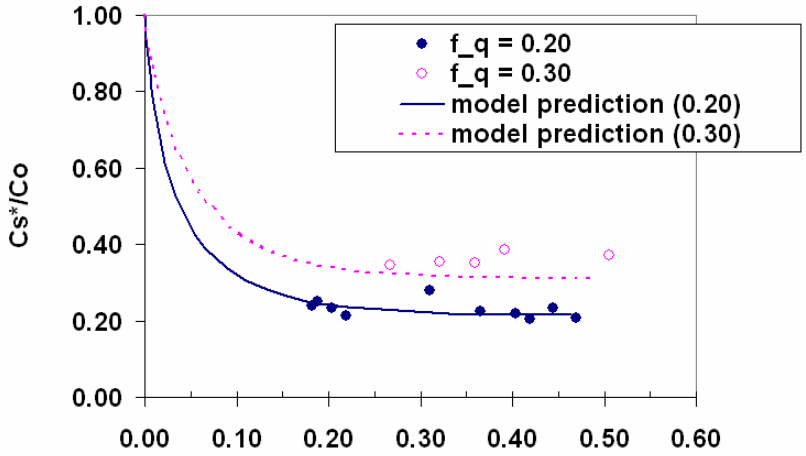
- Remove DMSO from frozen and thawed cell suspension using a microscale device.
- Volume throughput of 2-3 ml/min.
- Removal of 95% DMSO concentration (1.4M to 0.07 M).
- Device size should permit bench top or bedside processing.



Device prototype

- Prototype was developed and performance measured.
- Excellent correlation between theory and experiment.
- Cell recovery > 90%.





HSC preservation: the future

- Cell processing for freezing
  - Closed microfluidic devices (no centrifugation).
  - Improvement in bags and devices.
- Non-toxic protective agents for cell preservation
  - Low level (~300 mM concentraitons).
  - No or low adverse effects.

Interested in learning more?

- Professional short course

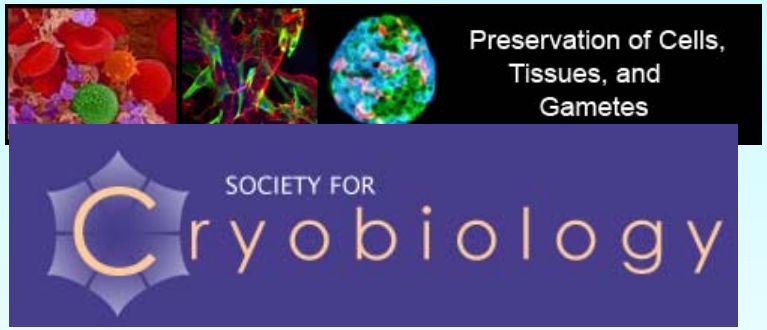
http://www.me.umn.edu/education/shortcourses/preservation/index.htm

- Society for Cryobiology

http://www.societyforcryobiology.org/

- International society for cell therapy

http://celltherapysociety.org/



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- Funding
  - 3M Foundation Fellowship
  - University of Minnesota Grant-in-aid Program
  - National Blood Foundation
  - National Institutes of Health

Evolution cont: cell concentration

- Freezing at high concentration reduces
  - Total dosage of DMSO infused.
  - Storage space required.
- 'Normal' concentration: 30-50 x10^6 cells/ml.
- "High" concentration: ~200x10^6 cells/ml.
- Increasing cell concentration beyond approximately 20% cytocrit, results in reduced post thaw recovery.

Cell concentration, cont.

Relationship between cell density and cytocrit:

$$C = \frac{V_{totalcell}}{V_{total}}$$

Sample calculation: Estimate the maximum cell concentration for a cell with diameter of 20 μm

$$V_{cell} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi(10x10^{-6}m)^3 = 4.2x10^{-15}m^3 = 4.2x10^{-9}ml$$

$$0.2 = CV_{cell} \qquad C = 48x10^6 cells/ml$$

Freezing response is altered when the water content of the cells becomes a significant fraction of the water present.