

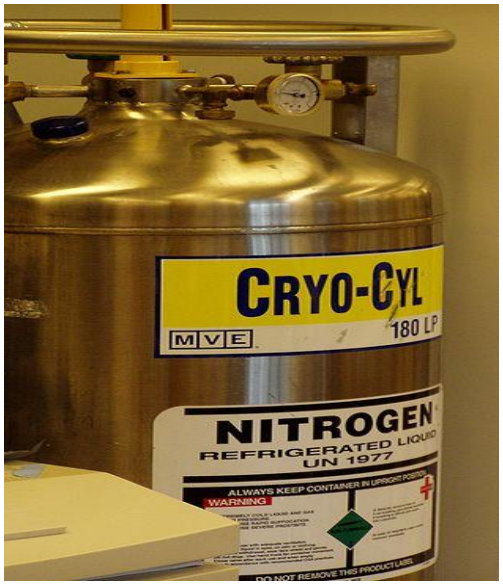
Cryopreservation

cell freezing and thawing

Science of Freezing

Cryogenics: The study and use of low temperature phenomena in physics and chemistry.

Cryopreservation: The storage of living organisms at ultra-low-temperatures such that it can be revived at a later period of time.



WHY CRYOPRESERVATION?

Every cell line is unique! It may be impossible to replace; at best, replacement would be expensive and time consuming.

Therefore, it is essential to protect this considerable investment by preserving the cell line.

WHY CRYOPRESERVATION?

Artifacts of Culture:

- Genotypic drift due to genetic instability
- Senescence and the resultant extinction of the cell line
- Transformation of growth characteristics and acquisition of malignancy-associated properties
- Phenotypic instability due to selection

Handling mishaps:

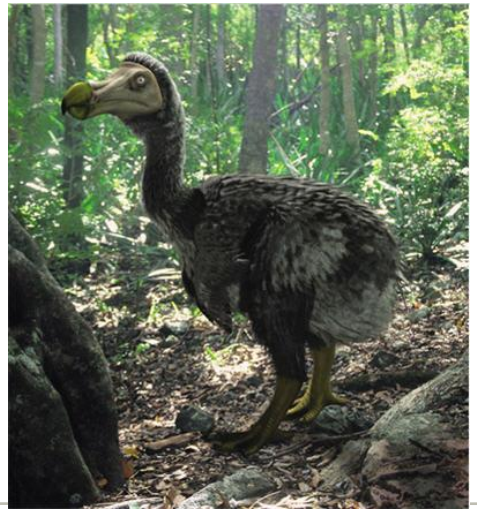
- Contamination by microorganisms
- Cross-contamination by other cell lines
- Misidentification due to careless handling
- Incubator failure

Planning and Needs

- Saving time and materials
- Need for distribution to other users

ADDITIONAL APPLICATIONS

- Preservation of blood and gametes (sperm, eggs) in medicine
- Maintaining germplasm for important or endangered species
- Preservation of plant seeds
- Preservation of stem cells for clinical and research use



Challenges of Cryopreservation

Why don't we use cryogenics to freeze and revive people or animals? (cryonics)

Ice Crystals



Challenges of Cryopreservation

Causes of cellular damage during cryopreservation:

1. Direct effects from freezing (and thawing)
   - Mechanical damage from extracellular ice
   - Intracellular crystallization



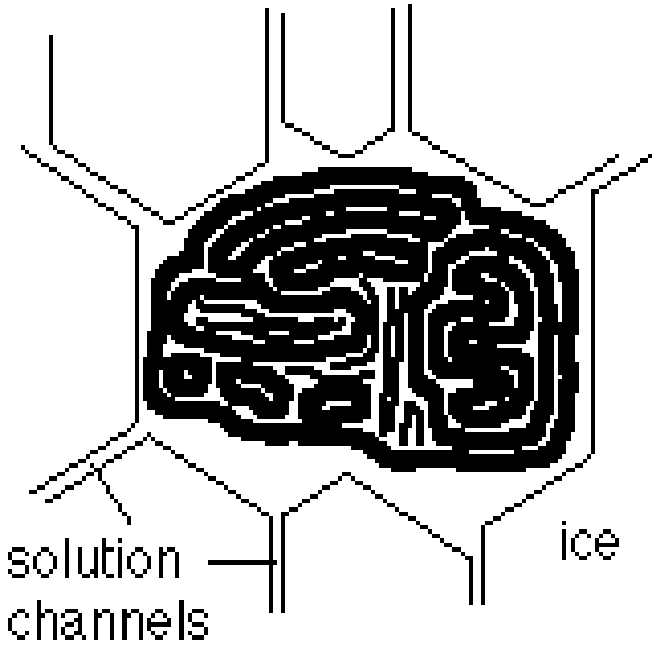
1. Nucleation

2. Crystal growth

Challenges of Cryopreservation

2. Indirect effects from freezing (and thawing)
   - Severe dehydration and increased ion concentrations, leading to altered electrical interactions and instability of proteins (enzymes, cell membrane)





"Cryobiology and anhydrobiology of cells" Joe Wolfe, Gary Bryant

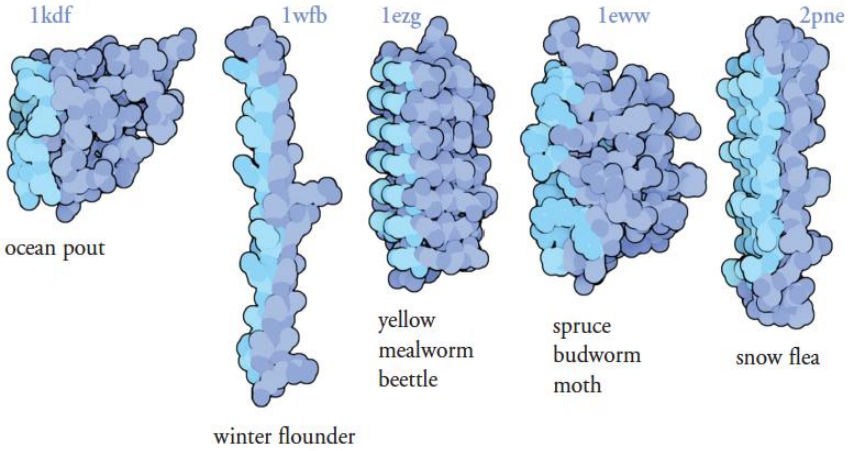
Goal of Cryopreservation

Achieve intracellular **vitrification** (*form an amorphous, glass phase*) while avoiding intracellular ice formation and membrane damage.

AVOIDING INTRACELLULAR ICE

1. **Supercooling** – taking a liquid below its equilibrium freezing point, without freezing





AVOIDING INTRACELLULAR ICE

2. **Freezing point depression** – accumulation of soluble molecules in the intracellular and extracellular environment to lower the equilibrium freezing temperature. Also makes cells more resistant to dehydration due to osmotic shifts.



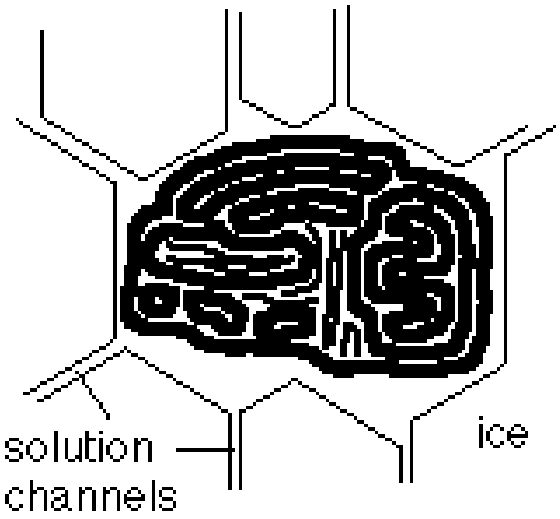
Wood frog (Rana sylvatica) able to survive up to 65% of total body water freezing during winter through the accumulation of urea and glucose in the blood (cryoprotectants)

AVOIDING INTRACELLULAR ICE

3. **Dehydration** – Moderate dehydration increases the osmotic pressure of the cytoplasm which depresses the cells freezing point and promotes vitrification – preventing intracellular crystallization. Severe dehydration can cause cell death.

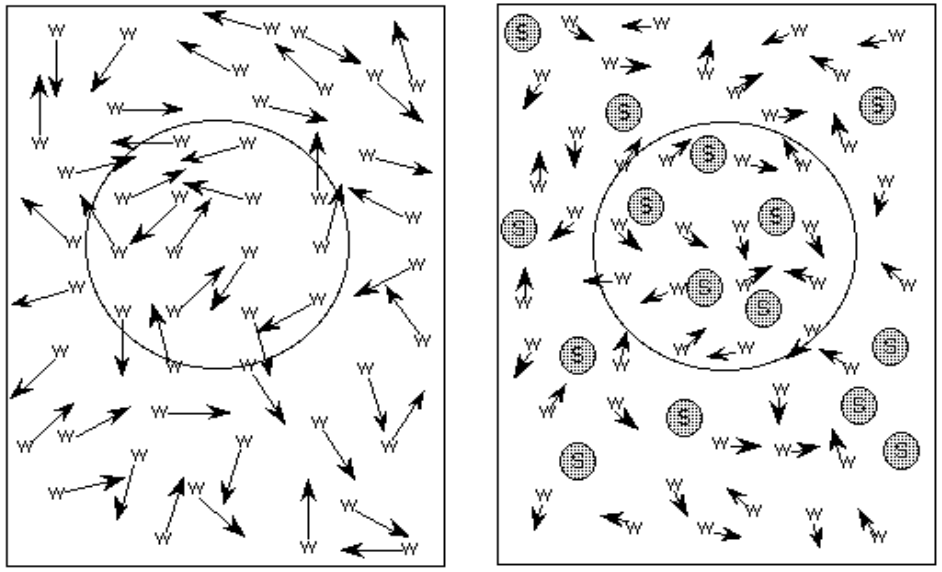


unfrozen suspending medium



AVOIDING INTRACELLULAR ICE

4. **Intracellular Vitrification** – rapid cooling and increased solution concentration increase the viscosity of a solution, thereby decreasing the likelihood of crystal nucleation and leading to vitrification.



a – water **b – water + solute**

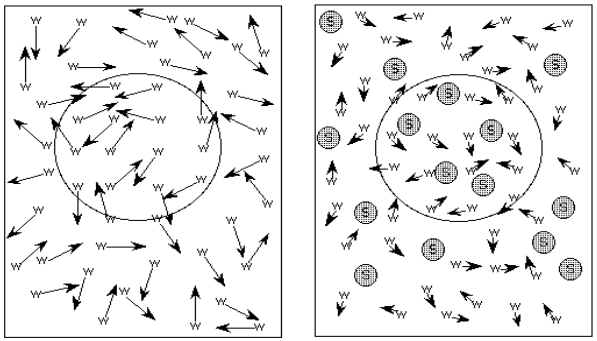
"Cryobiology and anhydrobiology of cells" Joe Wolfe, Gary Bryant

CRYOPROTECTANTS

• Protect cells by increasing intracellular solution concentration (freezing point depression, promotes vitrification)

• Properties:

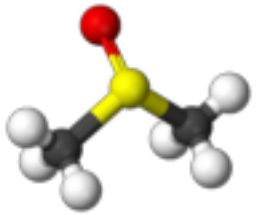
1. Highly soluble in water (even at low temperatures)
2. Penetrates easily into cells, freely diffusable
3. Low toxicity

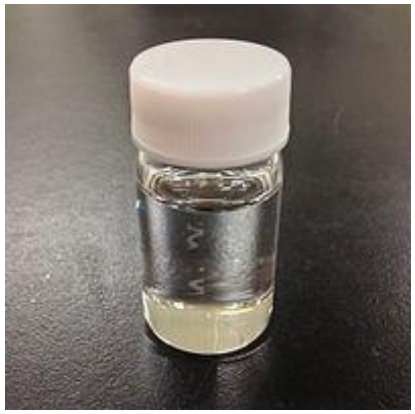


DMSO (DIMETHYL SULFOXIDE)

- Polar, aprotic compound that readily dissolves both polar and non-polar compounds (i.e. good solvent for salt solutions, readily form hydrogen bonds)
- Diffuses readily into cells

- Minimally toxic compared to like compounds, but not without toxicity both to cells and to RESEARCHERS
    - alters cell permeability and tight junction adhesions
    - readily absorbed through skin and can have detrimental health effects (irritation, mutagenic, organ damage)





ΔTEMPERATURE: COOLING & WARMING

How can we incubate before vitrification?

- ***"Slow"* cooling -** toxic effects of DMSO on cells, profound dehydration

- ***"Fast"* cooling -** allows equilibration of DMSO across cell membrane, minimize dehydration prior to intracellular vitrification

To Achieve Vitrification

- ***"Rapid/Instant"* cooling -** results in vitrification of intracellular *and* extracellular solution due to lack of crystal nucleation (also called flash freezing, requires high cryoprotectant concentrations)

Thawing

- **Warming** → always rapid to minimize recrystallization of vitrified solutions

STORAGE TEMPERATURE

- Short Term Preservation (weeks to months):
  -80°C freezer

- Long Term Preservation (years):
  liquid nitrogen (-200°C)



WHY?

Cells stored at -80°C maintain low levels of enzyme activity which may cause damage to cellular structures. Cells stored in liquid nitrogen have enzyme inactivation.

ORGAN AND ORGANISM CRYOPRESERVATION?

• Extracellular crystallization disrupts important extracellular structures (rupture of blood vessels, muscle bundles, basement membrane attachments)

• Multicellular systems also contain heterogenous cells which likely have very different optimum requirements for preservation

• Tissue vitrification through rapid freezing (and warming!) and concentrated solutions aims to entirely avoid crystallization. However, rapid temperature changes are difficult in bulky organ systems and this procedure generally requires toxic levels of cryoprotectants.

• Some success with Drosophila embryos, human skin, small elastic arteries and articular cartilage using vitrification, antifreeze proteins, and gradual cyropreservative additive.



LAB #4: CRYOPRESERVATION EXPERIMENTAL DESIGN

- Hypothesis
  - Establish your hypothesis with your group. It's ok for group members to have different hypotheses
- Experimental protocol
  - This weeks protocol is like having a lot of pokers in the fire. Read it over ahead of time and develop your plan before stepping into the culture room.
- Assign jobs and discuss effective use of time
  - 30 minutes allotted per group for freeze work in hood
  - 30 minutes allotted per group for thaw work in hood

Experimental Plan

Table: Cryo-Conditions Label		
COOLING ➡ MEDIA ⬇	Slow Cool Kept at 4°C	Fast Cool Kept at -20°C
D15	**Group Name** **Cell Line** **D15-SC**	**Group Name** **Cell Line** **D15-FC**
Freezing Media (FM)	**Group Name** **Cell Line** **FM-SC**	**Group Name** **Cell Line** **FM-FC**