Cell Culture Techniques

Course 4: Cryopreservation

Assoc. Prof. Dr. Emrah Şefik Abamor

What is Cryopreservation?

Cryo (Greek) = *krayos*. Meaning- frost.

Literally cryopreservation is preservation in "frozen state".

Cryopreservation is the long-term preservation of the biological materials at sub-zero temperature where all the metabolic activities are arrested keeping its viability intact, to be used in future.

Cryopreservation

- **The aim of cryopreservation** is to enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times.
- It is invaluable when dealing with cells of limited life span.

Cryopreservation

- This practice is crucial for biomedical research, clinical medicine, zoology, botany, and biotechnology.
- When frozen and kept properly, specimens may **remain in a state of suspended cellular metabolism indefinitely and can be thawed as needed.**

WHY SHOULD YOU FREEZE YOUR CELLS?

- Store your cells for future study.
- Cell storage acts as an insurance policy in case of contamination, failure, or cell shortage.
- Actively growing your cells over a long period of time may alter gene expression and differentiate cells. Continued growth and passage of cells may cause them to lose their original features. This endangers reliable research results!
- Freezing and storage of cells is a crucial step to ensure long-term cell use and reproducible results.
- Save time and money. Obtaining new cells is costly and time-consuming!

Basic Principles for Cryopreservation

1.Cultures should be healthy with a viability of >90% and no signs of microbial contamination.

2.Cultures should be in log phase of growth (this can be achieved by using pre-confluent cultures i.e. cultures that are below their maximum cell density and by changing the culture medium 24 hours before freezing).

Basic Principles for Cryopreservation

3.A high concentration of serum/protein (>20%) should be used. In many cases serum is used at 90%.

4.Use a cryoprotectant such as **dimethyl sulphoxide (DMSO) or glycerol** to help protect the cells from rupture by the formation of ice crystals.

5.The most commonly used cryoprotectant is DMSO at a final concentration of 10%, however, this is not appropriate for all cell lines e.g. where DMSO is used to induce differentiation. In such cases an alternative such as glycerol should be used

- In practice, the frozen cells are stored at a temperature of -196°C in liquid nitrogen (N_2).

- At low temperatures, any enzymatic or chemical activities which might cause damage to the biological material in question is effectively stopped.

- Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice during freezing.

- Traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed as Cryoprotectants, which protects the biological materials from freezing damage.

- Today, the most common procedures are cryopreservation of embryos, spermatozoa and some somatic cells and tissue.

Cryopreservation of Cell Lines

- Reduced risk of microbial contamination
- Reduced risk of cross contamination with other cell lines
- Reduced risk of genetic drift and morphological changes
- Work conducted using cells at a consistent passage number
- Reduced costs (consumables and staff time)

Why Liquid Nitrogen?

- In simple words , we use liquid nitrogen to store animal cells **because nitrogen in this liquid state has very low temp. so all the metabolic activities of a cell pauses and the cell enters in the stable state**

Why Liquid Nitrogen?

- They can remain for thousand of years in this stable state, further **when we remove liquid nitrogen the cell will again start the metabolism and other normal activities.**
- **So it is the best method to store animal cells.**

TYPES OF CRYOPRESERVATION:

- Cryopreservation at -196˚C.
- Cryopreservation at above -196˚C.
- Freeze Drying.
- Vitrification.

Cryopreservation at -196°C

- The biological materials are stored at -196°C (liquid nitrogen temperature).

- The viability of storage by this process is independent of the period of storage.

- The stresses associated with this cryopreservation are not mutagenic.

Cryopreservation at above -196°C:

- Storing biological materials in a conventional freezer is more convenient than liquid nitrogen.

- Freezers are available which maintain temperatures down to -130°C.

- However, unless biological material is stored at temperatures below -135°C the viability decreases during long-term storage.

- This may be appropriate with microbial suspensions or with mammalian tissue cultures where large numbers of cells are frozen and some loss of viability may not cause practical problems, storage at these temperatures would not be acceptable with embryos, oocytes etc.

STORAGE OF CELLS AT ULTRA LOW TEMPERATURES

-80^{0}C

-196^{0}C

- Storage in liquid phase nitrogen allows the lowest possible storage temperature to be maintained with absolute consistency, but requires the use of large volumes (depth) of liquid nitrogen which is a potential hazard.
- There have also been documented cases of cross contamination by virus pathogens via the liquid nitrogen medium. For these reasons ultra-low temperature storage is most commonly in vapour phase nitrogen.

Freeze Drying:

- Freeze Drying is the process of preserving the biological materials by rapidly freezing it and then subjecting it to high vacuum which removes ice by sublimation.

- It is convenient as the cost and maintenance associated with frozen storage can be avoided.

- However, evidently, the freeze drying is **highly mutagenic** and that **viability of the cells** would be expected to decrease with storage time.

- These two factors precludes the use of freeze dried spermatozoa in IVF.

Vitrification:

- Vitrifications is the process which, by combining the use of concentrated solutions with rapid cooling, avoids the formation of ice.

- Samples reach low temperatures in a glassy state, which has the molecular structure of a viscous liquid and is not crystalline.

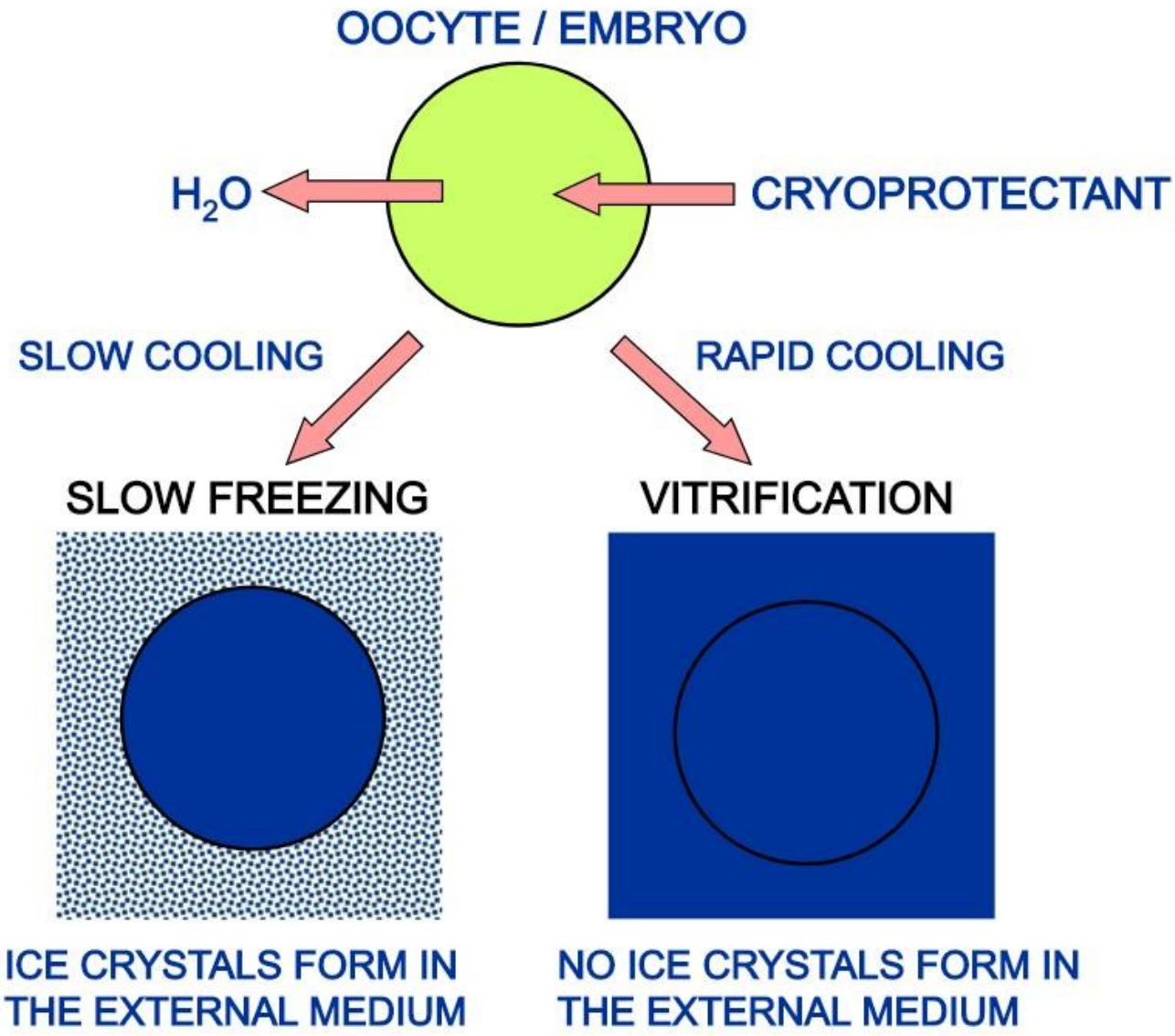
- This method has the potential advantages of being rapid to carry out and does not require controlled rate cooling apparatus.

- Although good survival has been demonstrated, however is still experimental, as the additives used are potentially cytotoxic and tendency of vitrified solutions to devitrify during storage and thawing leads to lose the viability.

Nature of water

- The temperature below 0ºC will introduce formation of water ice-crystal;
- Below -130ºC is the glass transition temperature of water;

Slow freezing versus vitrification



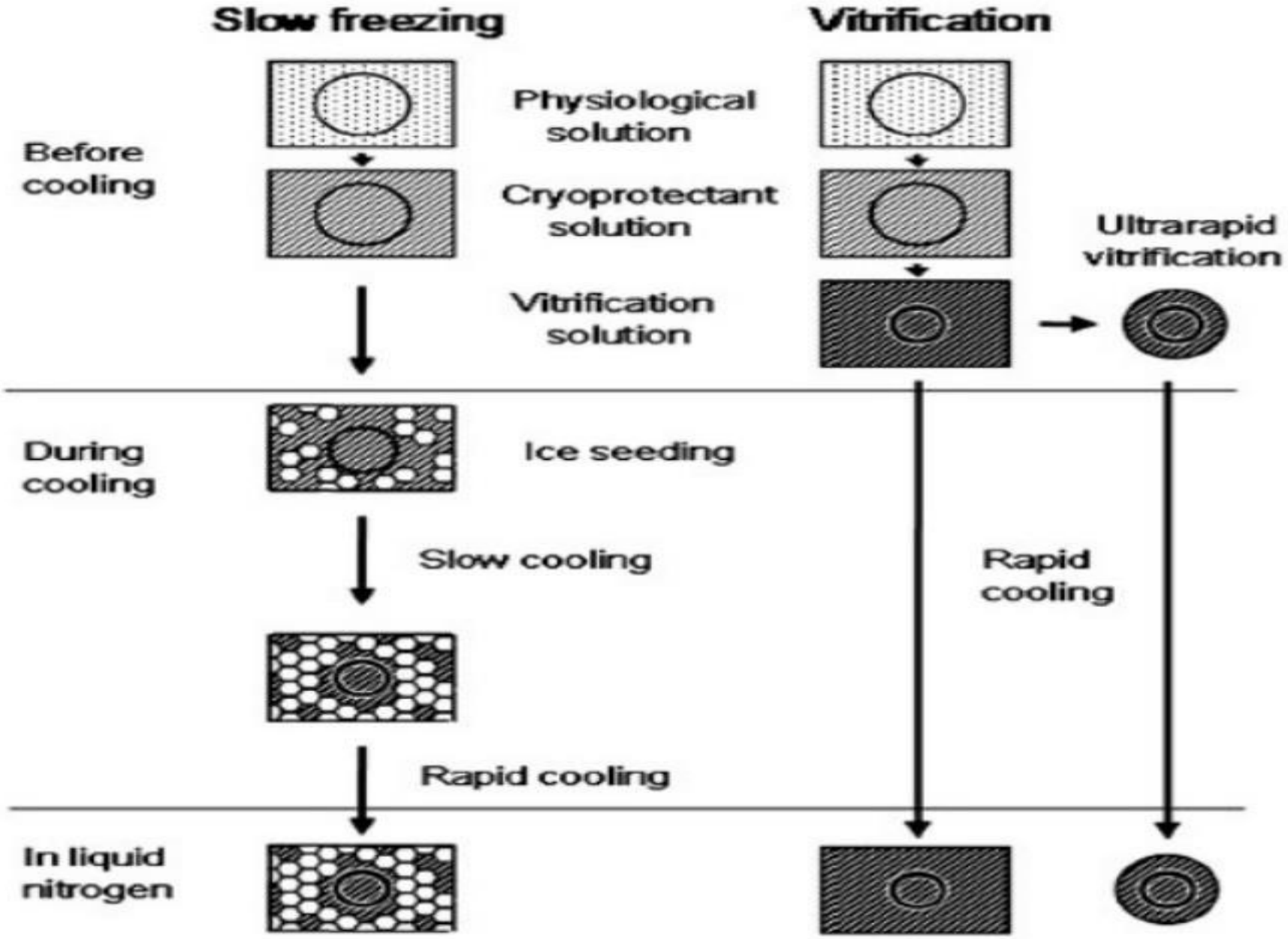


Figure 1. Schematic presentation of an embryo (circle) before cooling, during cooling and in liquid nitrogen in slow freezing, conventional straw vitrification, and ultrarapid vitrification. White hexagons represent ice crystals.

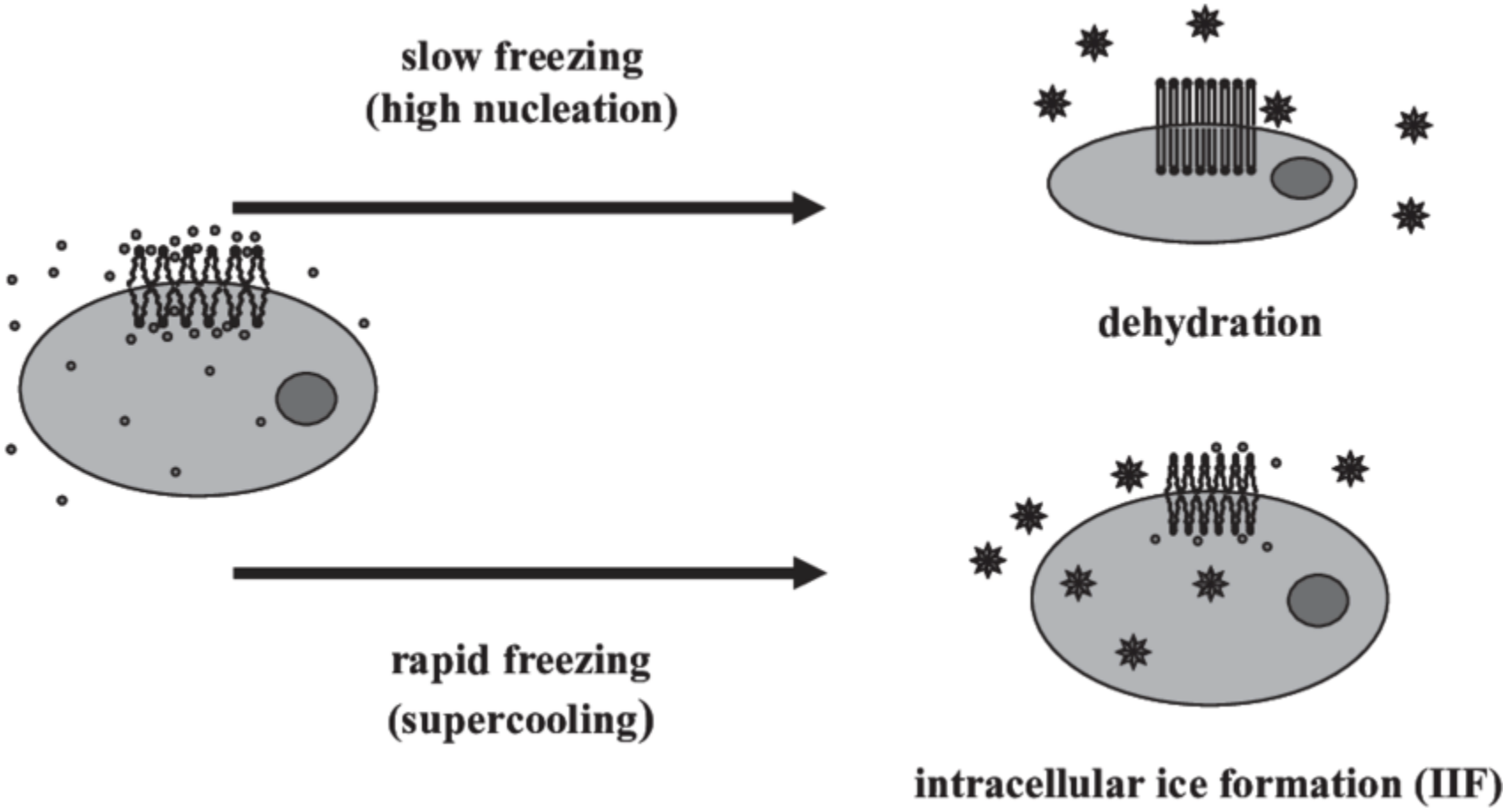
Slow freezing versus vitrification

Slow-freezing

- low levels of cryoprotectants
- slow controlled rates of cooling (0.1-0.3°C/min)
- slow dehydration of cells to minimize ice crystal formation and damage
- freezing machine required (calibration, expenses)
- *takes hours*

Vitrification

- high levels of cryoprotectants
- extremely fast rates of cooling (>20,000°C/min)
- no ice crystal formation or damage; straight to a glass-like structure
- no freezing machine required
- *takes seconds*


slow freezing
(high nucleation)
dehydration
rapid freezing
(supercooling)
intracellular ice formation (IIF)

EFFECT OF FREEZING ON CELLS:

- During freezing, it is essential for cells remove a particular amount of water from cells osmotically to avoid intracellular freezing.

- At slow rates of cooling, cells try remain in equilibrium with external solution because of the protective effect of cell membrane, thus exosmosis takes place and outside of the cell becomes hypertonic, resulting in equilibrium disturbance.

- As cooling rate is increased, water gets less time to move out of the cell, becomes increasingly supercooled, and intracellular ice formation occurs that is lethal to the cells.

- So optimum rate of cooling, results from the balance of these two phenomena.

Risks associated with cryopreservation

- Damage to cells during cryopreservation occur during the freezing stage, and include,
  1)solution effects,
  2) extracellular ice formation,
  3)dehydration
  4)intracellular ice formation.
- These effects can be reduced by cryoprotectants.
- Once frozen stage is reached, it is safe from further damage.
- It has a maximum storage period of 1000 years.

Solution effects

- As ice crystals grow in freezing, water solutes are excluded, causing them to become concentrated in the remaining liquid water.

- High concentrations of some solutes can be very damaging

Extracellular ice formation

- When tissues are cooled slowly, water migrates out of cells and ice forms in the extracellular space.

- Too much extracellular ice can cause mechanical damage to the cell membrane due to crushing.

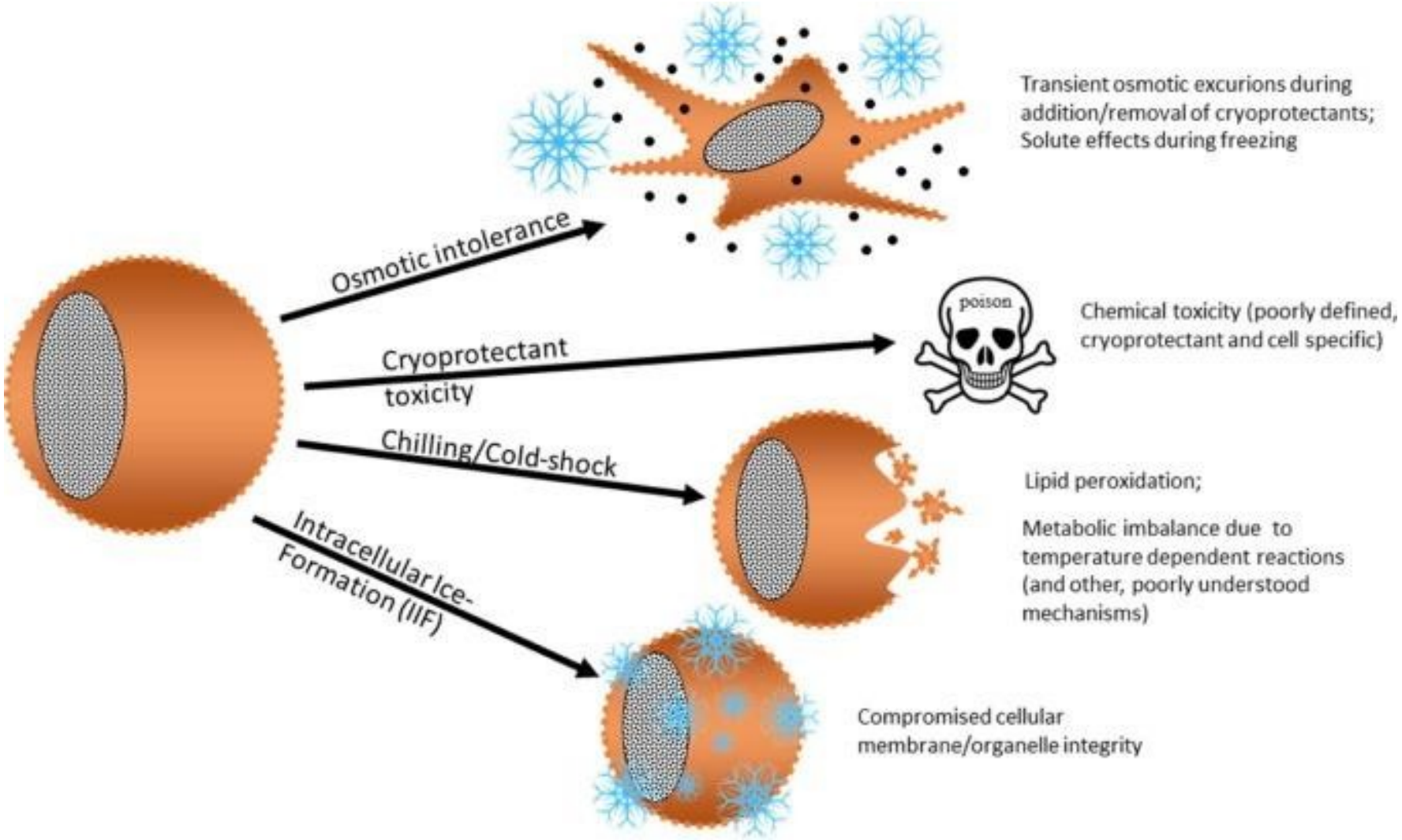
Dehydration

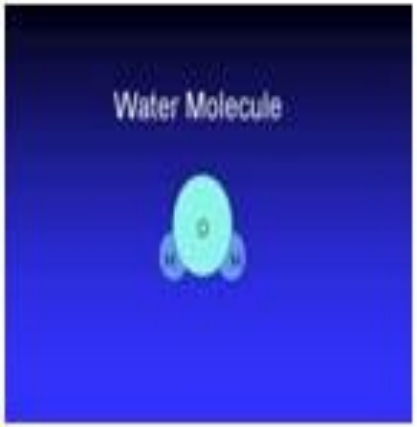
- The migration of water causing extracellular ice formation can also cause cellular dehydration.

- The associated stresses on the cell can cause damage directly.

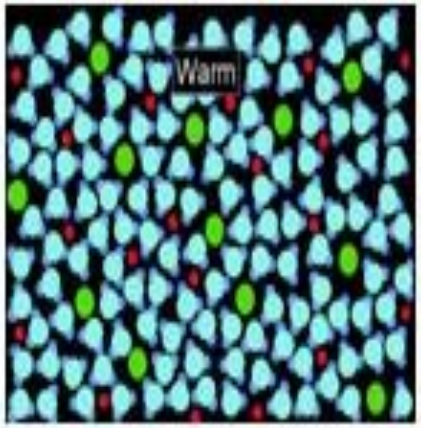
Intracellular ice formation

- Some organisms and tissues can tolerate some extracellular ice, but any appreciable intracellular ice is almost always fatal to cells.


Osmotic intolerance
Transient osmotic excurions during addition/removal of cryoprotectants; Solute effects during freezing
Cryoprotectant toxicity
poison
Chemical toxicity (poorly defined, cryoprotectant and cell specific)
Chilling/Cold-shock
Lipid peroxidation;
Metabolic imbalance due to temperature dependent reactions (and other, poorly understood mechanisms)
Intracellular Ice-Formation (IIF)
Compromised cellular membrane/organelle integrity


Water Molecule

(a)


Warm

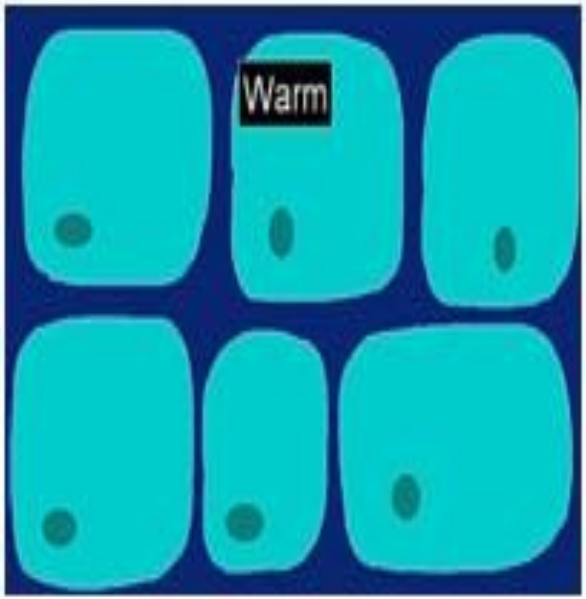
(b)


Cold (Freezing)

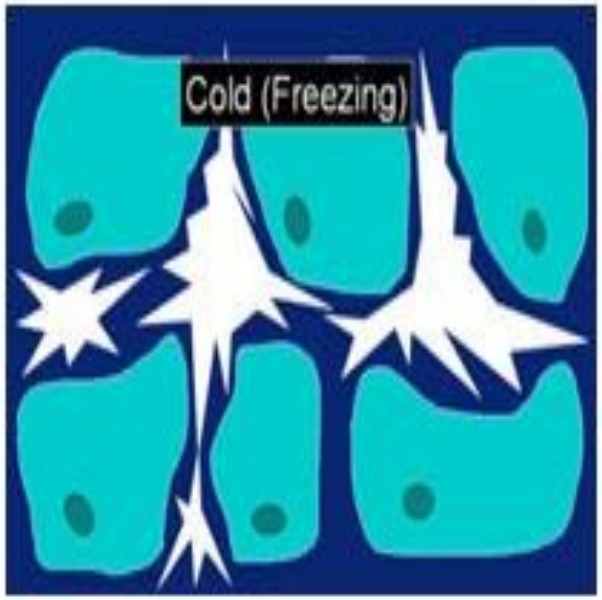
(c)


Very Cold (Frozen)

(d)


Warm

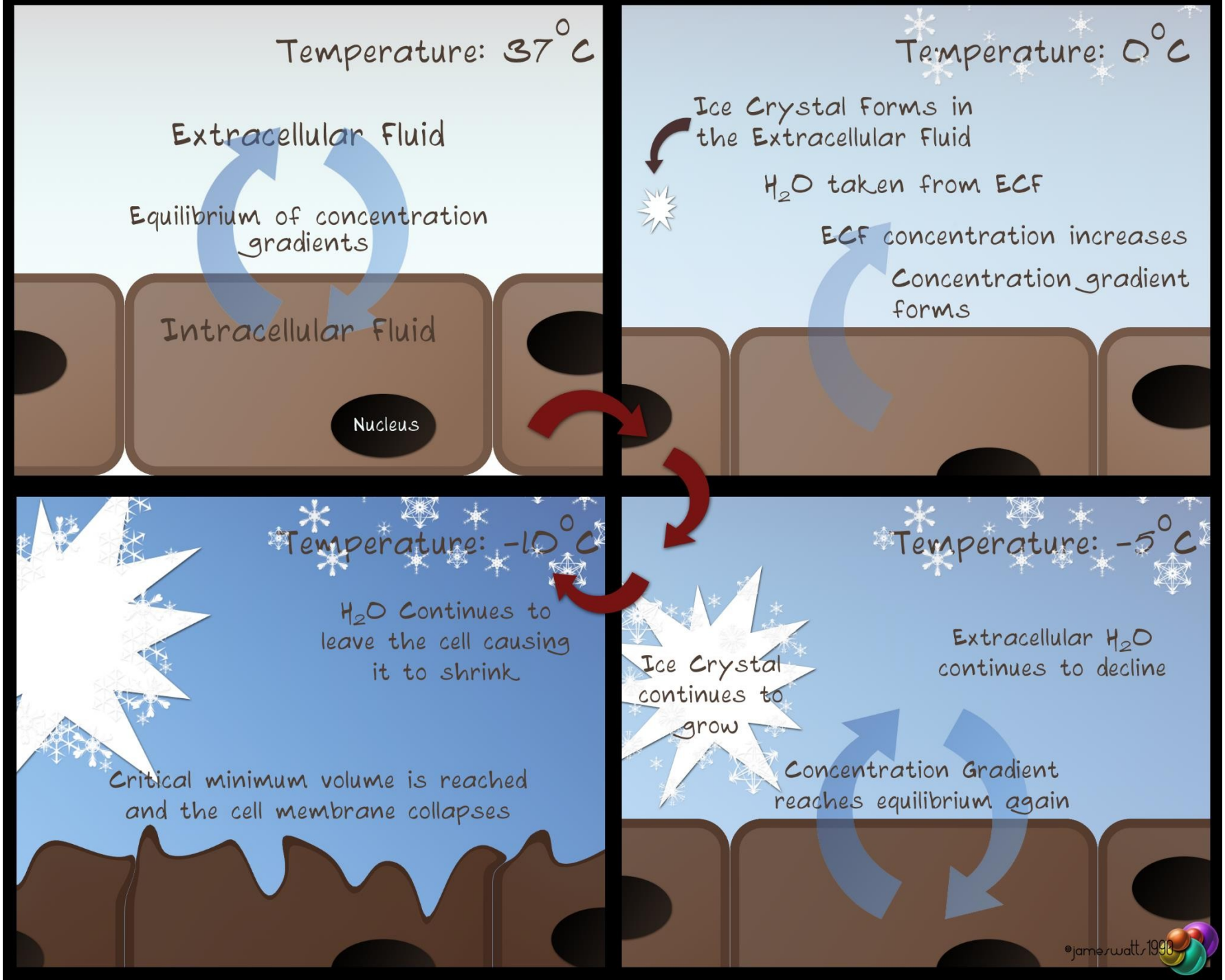
(e)


Cold (Freezing)

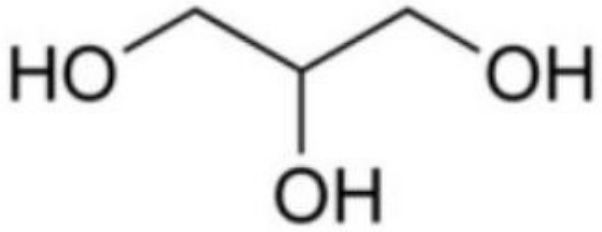
(f)


Very Cold (Frozen)

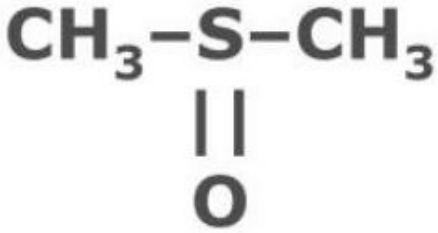
(g)


Temperature: 37°C
Extracellular Fluid
Equilibrium of concentration gradients
Intracellular Fluid
Nucleus
Temperature: 0°C
Ice Crystal Forms in the Extracellular Fluid
H_2O taken from ECF
ECF concentration increases
Concentration gradient forms
Temperature: -10°C
H_2O Continues to leave the cell causing it to shrink
Critical minimum volume is reached and the cell membrane collapses
Temperature: -5°C
Ice Crystal continues to grow
Extracellular H_2O continues to decline
Concentration Gradient reaches equilibrium again
©jameswatt/1990

Cryoprotectants

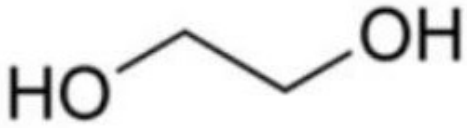


Glycerol



Dimethylsulphoxide
(DMSO)

Propylene glycerol
(PROH)



Ethylene glycol
(EG)

Types of cryoprotectants

Based on their ability to diffuse across cell membranes, two types of cryoprotectants are there :

- Penetrating cryoprotectants
- Non-penetrating cryoprotectants

Penetrating cryoprotectants

- Penetrating cryoprotectants are so called because they penetrate the cell membrane and enter the cytosol.
- They are exclusively small molecules.
- They form hydrogen bonds with water to prevent ice crystallisation.
- They act by replacing water and therefore controlling cell size changes as well as preventing intracellular ice formation and prevent excessive dehydration during cell cryopreservation.
- Common penetrating cryoprotectants are DMSO (Dimethyl sulfoxide), glycerol, ethylene glycol.

Non-penetrating cryoprotectants

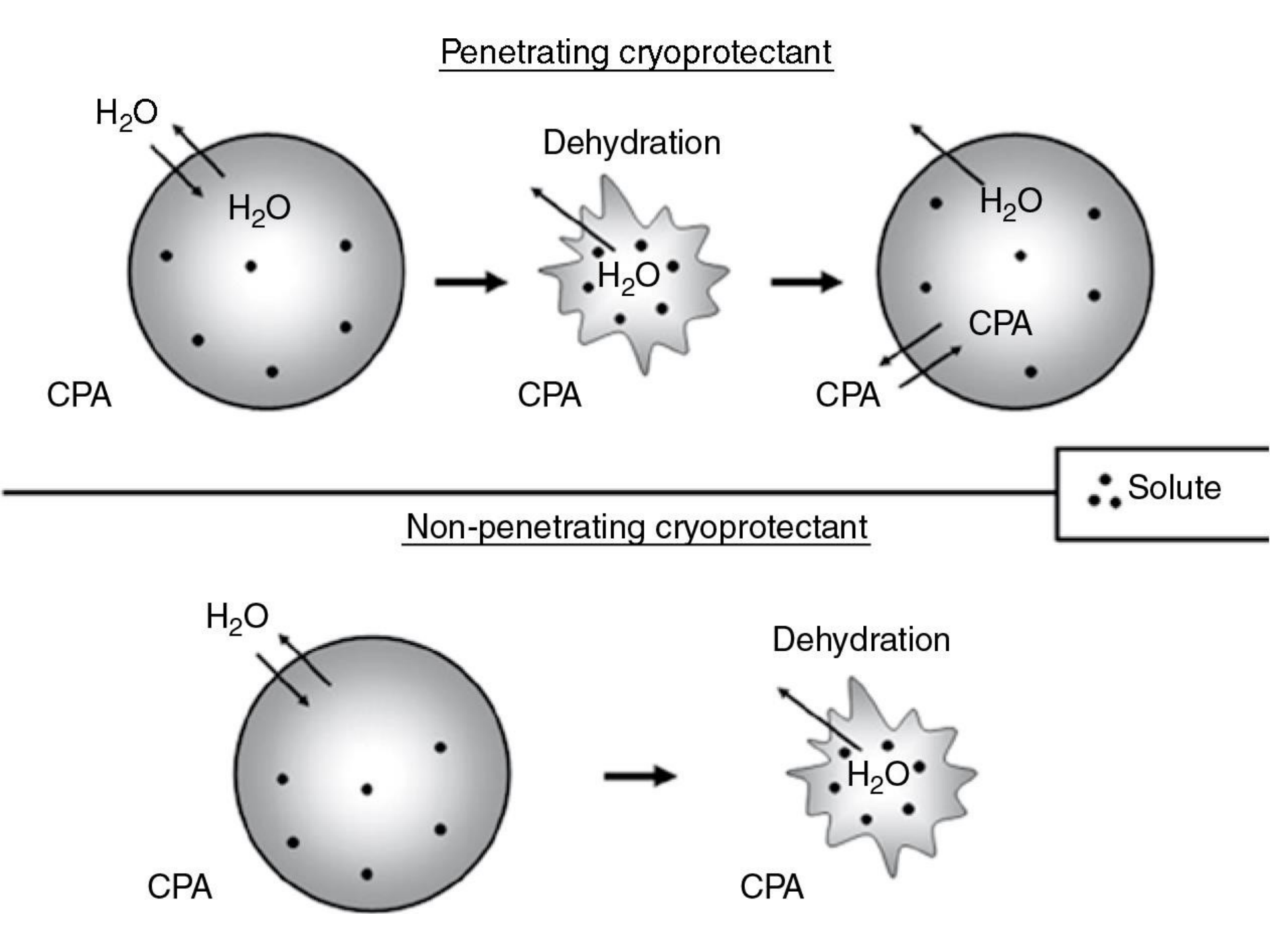
- This type of cryoprotectants do not penetrate the cell membrane.
- They are larger molecules, usually polymers such as polyethylene glycol or saccharides such as sucrose.
- Non-penetrating cryoprotectants are thought to act by dehydrating the cell before freezing, thereby reducing the amount of water that the cell needs to lose to remain close to osmotic equilibrium during freezing.
- They inhibit ice growth by the same mechanism as penetrating cryoprotectants, but they do not enter cells.
- They help to dehydrate the cell prior to cryopreservation by altering the osmotic balance and also help to prevent damage to cells during recovery from cryopreservation by preventing solutes, particularly larger protoplasmic elements, from escaping the cell too rapidly.

Characteristics of cryoprotectants

- High soluble in water
- Relative low molecular weight
- Fast cell permeability
- Conjunction with water to built stable H_2 bridges
- With high concentration should be non-toxic
- Reducing the freezing point of the extracellular fluid
- Low influx of the intracellular water to avoid the sudden shrinkage of the cell.

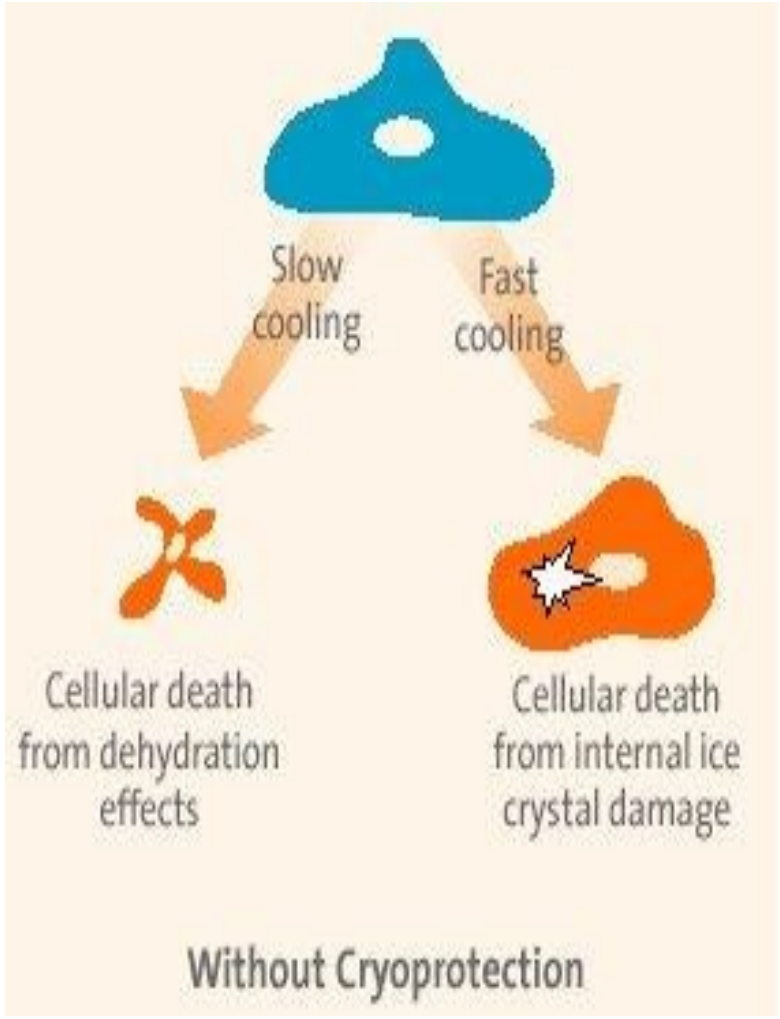
Cryoprotectants

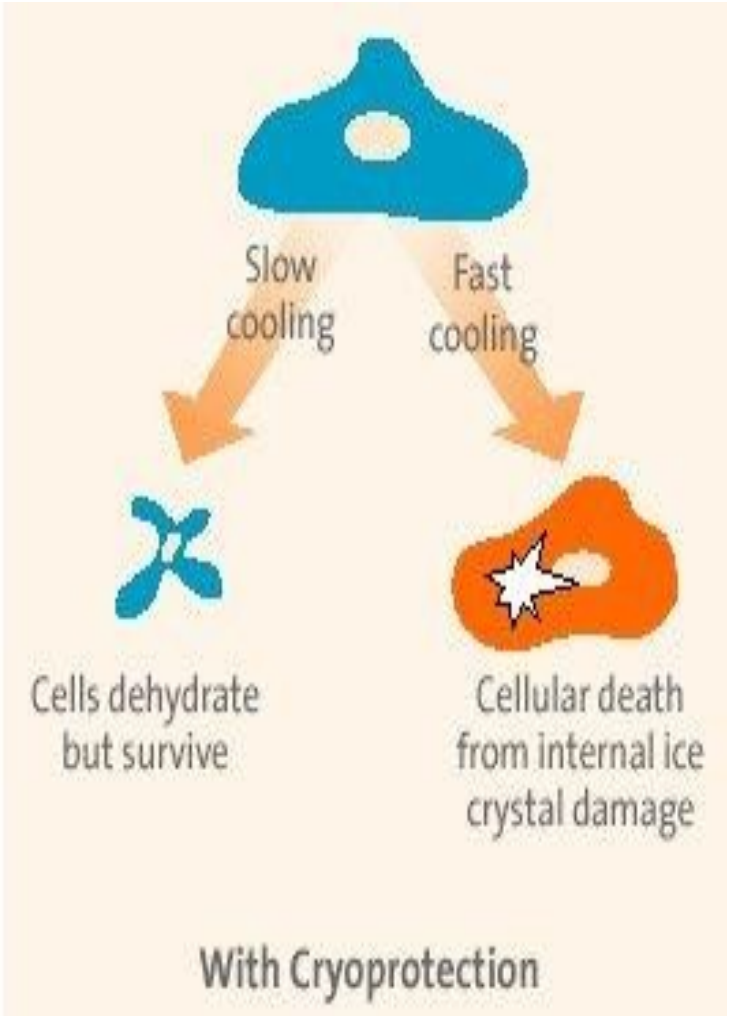
Permeable	Non permeable
Methanol CH_3OH	Polyethylenglycol
Ethanol C_2H_5OH	Polyvinyl pyrrolidone
Ethylenglycol $C_2H_4(OH)_2$	Ficoll
1-2 Isopropanol $C_3H_6(OH)_2$	Sucrose
Glycerol $C_3H_5(OH)_2$	
DMSO (CH_3) SO	


Penetrating cryoprotectant
H_2O
H_2O
CPA
Dehydration
H_2O
CPA
H_2O
CPA
CPA
Solute
Non-penetrating cryoprotectant
H_2O
CPA
Dehydration
H_2O
CPA

Principles of cryopreservation

- **Water in cell:** Around 90% of water is free (water) while the remaining 10 % bounds to other molecular components of the cell (proteins, lipids, nucleic acids and other solutes). This water does not freeze and called hydrated water
  - Removal of water is necessary during freezing to avoid ice crystal formation, dehydration is limited to the free water
  - Removal of hydrated water could have adverse effect on the cell viability and the molecular function (freezing injuries)


Slow cooling
Fast cooling
Cellular death from dehydration effects
Cellular death from internal ice crystal damage
Without Cryoprotection


Slow cooling
Fast cooling
Cells dehydrate but survive
Cellular death from internal ice crystal damage
With Cryoprotection

Cryopreserving cultured cells

1.Prepare freezing medium and **store at 2-8°C until use.** Note that the appropriate freezing medium depends on the cell line.

2.For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Resuspend cells in the complete medium required for that cell type.

Cryopreserving cultured cells

3.Determine the total number of cells and percent viability using a hemocytometer, cell counter, and trypan blue exclusion, According to the desired viable cell density, calculate the required volume of freezing medium.

4. Centrifuge the cell suspension at approximately 100–200 × g for 5–10 minutes. Aseptically decant supernatant without disturbing the cell pellet.

Cryopreserving cultured cells

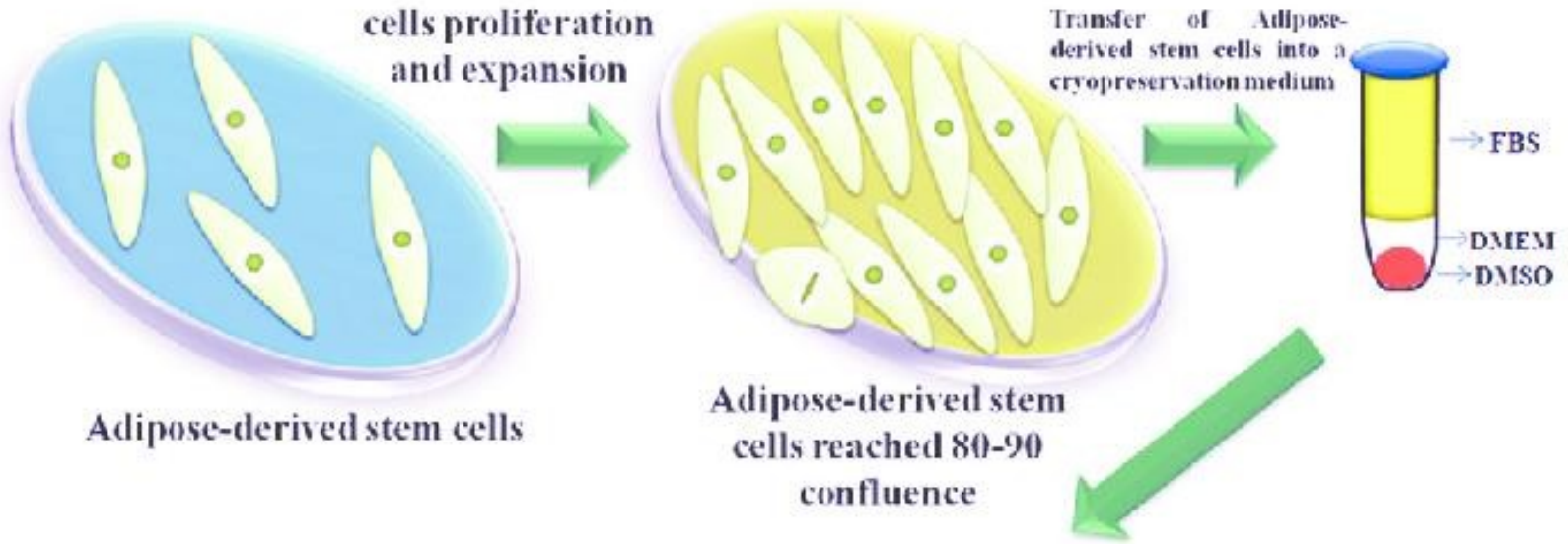
5.Resuspend the cell pellet in cold freezing medium at the recommended viable cell density for the specific cell type.

6.Dispense aliquots of the cell suspension into cryogenic storage vials. As you aliquot them, frequently and gently mix the cells to maintain a homogeneous cell suspension.

Cryopreserving cultured cells

7.Freeze the cells in a controlled-rate freezing apparatus, decreasing the temperature approximately 1°C per minute and store them at –80°C overnight.

8.Transfer frozen cells to liquid nitrogen, and store them in the gas phase above the liquid nitrogen.


cells proliferation and expansion
Transfer of Adipose-derived stem cells into a cryopreservation medium
FBS
DMEM
DMSO
Adipose-derived stem cells
Adipose-derived stem cells reached 80-90 confluence

Thawing frozen cells

1.Remove the cryovial containing the frozen cells from liquid nitrogen storage and immediately place it into a 37°C water bath.

2.Quickly thaw the cells (< 1 minutes) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.

3.Transfer the vial into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.

4.Transfer the thawed cells dropwise into the centrifuge tube containing the desired amount of prewarmed complete growth medium appropriate for your cell line.

Thawing frozen cells

5.Centrifuge the cell suspension at approximately 200 × g for 5–10 minutes. The actual centrifugation speed and duration varies depending on the cell type.

6.After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.

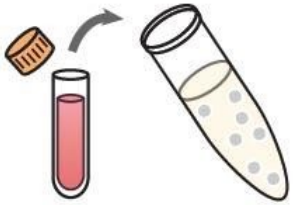
7.Gently resuspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment.

1. abm's Cell Culture Thawing Procedure

1

Place cryovial into a 37°C waterbath

2



Transfer vial contents to a 15ml conical tube with pre-warmed medium

3

Centrifuge at 200 x g for 3 mins to pellet the cells

4

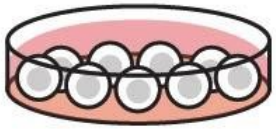
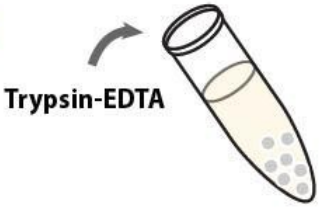


Plate cells once resuspended with culture medium into culture vessel

2. abm's Cell Culture Subculture Procedure

5



Add equal volume of complete medium to neutralize trypsin. Centrifuge at 200 x g for 3 mins

6

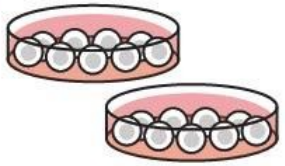
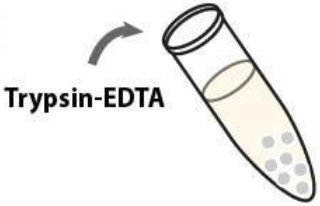


Plate cells once resuspended with culture medium into culture vessels

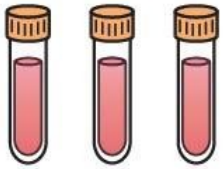
3. abm's Cell Culture Freezing Procedure

7



Add equal volume of complete medium to neutralize trypsin. Centrifuge at 200 x g for 3 mins

8



Cryopreserve the cells using abm's cryopreservation medium