Cryobiology

- The word cryobiology is derived from the Greek words "cryo" = cold, "bios" = life, and "logos" = science.
- Cryobiology is the branch of biology that studies the effect of low temperatures on living things.

- Cryobiology is the study of biological material or systems at temperatures below normal.
- Materials or systems studied may include proteins, cells, tissues, organs or whole organisms.

- Temperatures may range from moderately **hypothermic** (the artificial reduction of body temperature to slow metabolic processes) conditions to **cryogenic** (the study of the production and behaviour of materials at very low temperatures) conditions.--

- Cryopreservation (preservation in the frozen state) is based on the reduction and subsequent arrest of metabolic function of biological material by imposition of ultra-low temperature

- At the temperature of liquid nitrogen(-196^{o}C) almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods.

- However, only few biological materials, in their natural state, can be frozen to sub-freezing temperatures without adversely affecting the cell viability.

- The main objective of developing cryopreservation technology is preservation of valuable genetic resources, especially of vegetatively propagated and also of those species which have short lived seeds.

- It is imperative that use of cryopreservation technology required efficient regeneration protocols through tissue culture of the species.

- **Procedure of Cryoprotection and Pretreatment:**
- Some form of cryoprotection is necessary for cryopreservation of plant materials unless they are naturally dehydrated, as in the case of dormant vegetative buds in the winter, or artificially cold acclimated.

- Several chemicals such as Dimethyl sulphoxide (DMSO), Glycerol, various sugars and sugar alcohols protect living cells against damage during freezing and **thawing** (to become unfrozen or warm after preservation at ultra low temperatures).

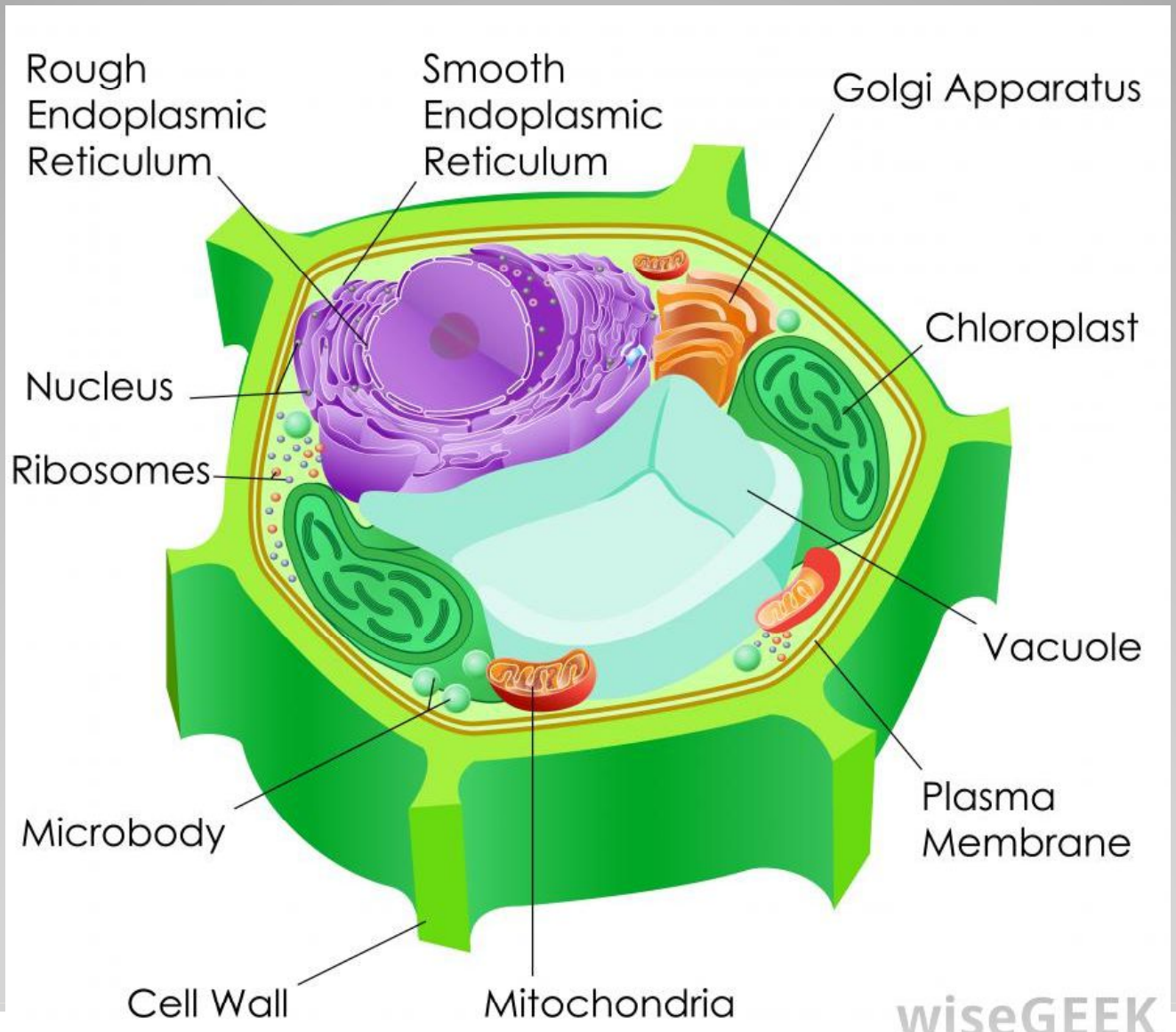
- **High solubility in aqueous** phase and **low toxicity to the cells** are the two essential characteristics for cryoprotectants.=

- Cryoprotectants are categorized as
- 1.Permeating, e.g: DMSO, methanol and glycerol
- 2.Non permeating, e.g: sugars, sugar alcohols, high molecular weight polymers as dextran, polyvinyl pyrrolidone, hydroxy ethyl starch.

- DMSO enters more rapidly than glycerol and therefore require shorter period for treatment.

- Addition of osmotically active compounds in the culture medium such as mannitol, sorbitol, sucrose and proline increase the freezing resistance (the ability to withstand many cycles of freezing and thawing without disintegrating) of the cells.

- **Methods of Cryopreservation**
- **1. Slow freezing:** When plant cells are cooled progressively, ice crystal formation is usually inhibited extra-cellularly (outside the cells proper).
- It is presumed that plasma membrane acts as a barrier that prevents the ice crystal formation in the cytoplasm.


Rough Endoplasmic Reticulum
Smooth Endoplasmic Reticulum
Golgi Apparatus
Chloroplast
Nucleus
Ribosomes
Vacuole
Plasma Membrane
Microbody
Cell Wall
Mitochondria
wiseGEEK

- In absence of ice crystals, cytoplasm remains super cooled. On further lowering of temperature, the concentration of extra cellular liquid increases as more water is converted to ice.

- The slow freezing prevents the intra-cellular ice formation and consequently freezing injury is prevented. It is believed that slow freezing increase the concentration of cytoplasm and increased dehydration increases the survival of the cells.

- The development of efficient slow freezing method depends upon several factors like cooling rates, pretreatment and cryoprotection, type and physiological state of the material, and the temperature prior to immersion in liquid nitrogen.

- The most commonly used methods for the cryopreservation of plant cells generally involves regulated slow cooling at a constant rate of 0.5 to 2°C/min. to terminal temperature between -30°C to -40°C followed by storage in liquid nitrogen.

- **2. Rapid Freezing:** Rapid freezing is employed to cryopreserve shoot tips. It has been used for preservation of shoot tips of potato, strawberry and several others and somatic embryos of oil palms.

- It is accompanied by direct immersion of the cryoprotectant-treated specimens in liquid nitrogen.
- The cooling rate in this method is very high, usually several hundred degrees per minute.

- At such high cooling rates, the intracellular fluids do not have sufficient time to equilibrate with the external ice. It results in the possibility of intracellular ice formation that is lethal for cells.

- **3. Droplet** (a very small drop of a liquid)**Freezing:** In this method the cryoprotectant treated meristems are dispensed in droplets of 2-3µl on an aluminium foil in a petriplate.

- The specimens are frozen by slow cooling (0.5°C/min) to subzero temperature between -20 to -40°C prior to immersion in liquid nitrogen.-

- **4. Storage, thawing and regrowth:** Materials can be stored in liquid nitrogen (-196°C) or in its vapour (-150°).

- Thawing is carried out by removing the sample from liquid nitrogen storage and transferring to a water bath (34-40^{o}C) for about 1-2 min or until material is warmed up

- The growth and regeneration of the cryopreserved cells after thawing will be like normal cells.
- However, care should be taken in handling, plating and subculture of such cells.

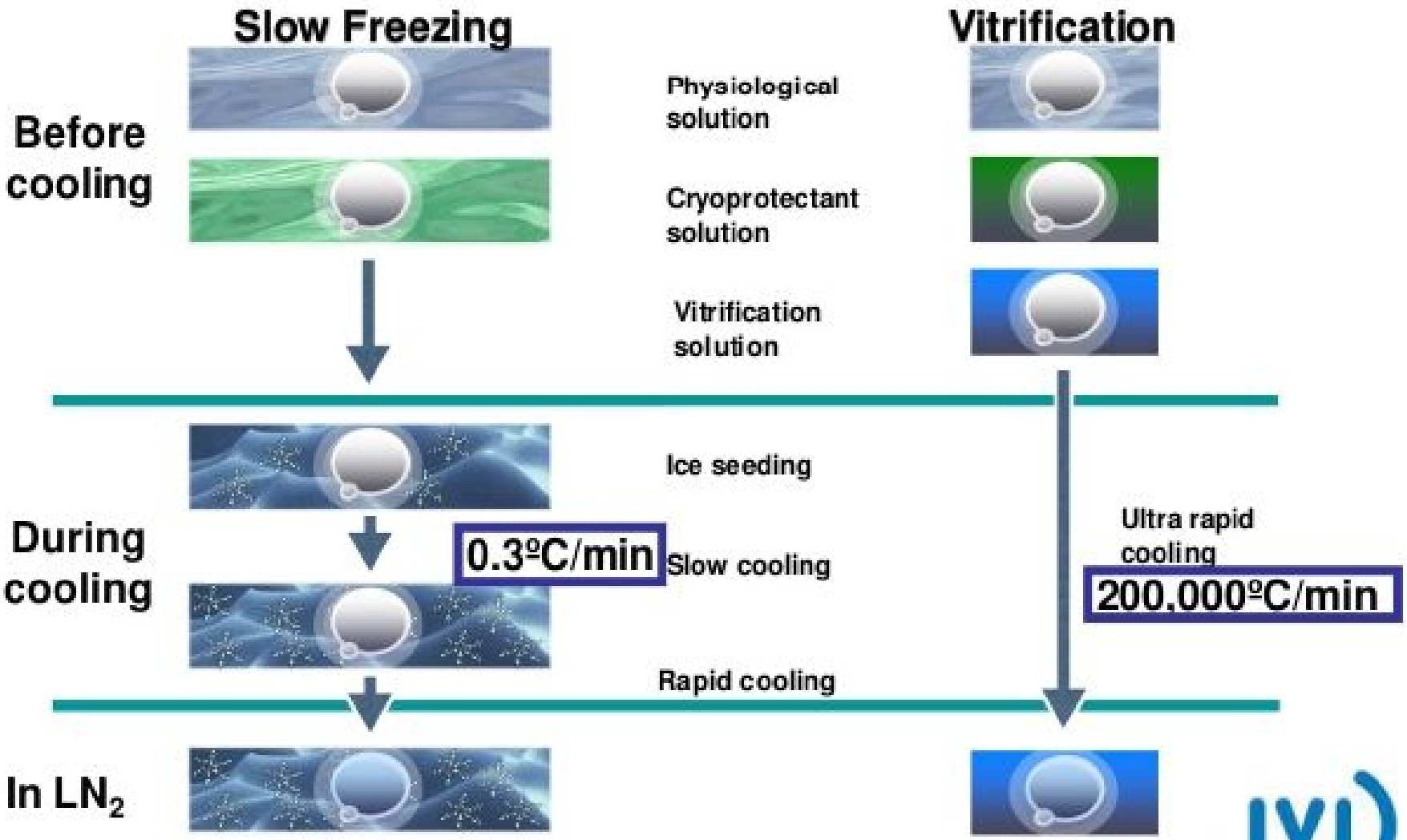
- **Vitrification:**
- Vitrification is a modern cryopreservation technique based on sudden cooling of reproductive cells to a temperature of -196°C in seconds.
- It uses a medium containing high doses of cryoprotectants, protecting cells against damage.

- It is a method in which not only cells but also the whole solution is solidified without the crystallization of ice

- This is based on the ability of highly concentrated solutions of cryoprotectants to supercool to very low temperatures to become viscous at sufficiently low temperatures, and solidify without the formation of ice.

- If the cells are capable of tolerating this severe sense of osmotic stress to allow glass transition to occur, they should be able to survive the freezing process.

Cryopreservation Techniques



- **6.Encapsulation:** This technique is used in synthetic seed technology in which somatic embryos are coated in sodium alginate beads.
- For cryopreservation, shoot tips or embryos are coated in sodium alginate and dehydrated by placing in medium containing high concentration of sucrose (0.3-0.7M.).

- In this way encapsulated material lose water by exosmosis. (The passage of a fluid through a semi permeable membrane toward a solution of lower concentration, especially the passage of water through a cell membrane into the surrounding medium).=

- Further, drying is carried out in Laminar air flow for 2-6 hours and then transferred to liquid nitrogen.

- **Factors affecting Cryopreservation**. First, in addition to the cell cryoinjury caused by the intracellular ice formation (IIF) and "solution effects" (caused by concentration of **solutes** (the minor component in a solution, dissolved in the solvent) in non-frozen solution during freezing as solutes are excluded from the crystal structure of the ice. High concentrations can be very damaging), there are several problems associated with organ cryopreservation:

- (a)Vascular damage/rupture caused by ice formation. To minimize this cryo-destructive effect, increased understanding and prediction of the fundamental mechanisms of ice formation and cell dehydration in tissues/organs are required.
- (b) Fracture of frozen tissue/organ caused by the thermal stress during the warming process. Thermal stress is one type of mechanical stress caused by non-uniform warming in a solid/frozen body (e.g., the fracture of glass when a surface is rapidly heated or cooled). To reduce the thermal stress, uniform heating is needed.

- Recently scientists have developed a slow-cooling technique for relatively uniformly warming the tissues. However, generally speaking, the slow warming may cause intracellular ice re-crystallization killing cells. A rapid and uniform heating technology is desired to prevent both potential lethal ice re-crystallization and thermal-stress-induced fracture and it has been developed.

- (c)Problems associated with vitrification: a dramatically different approach to cryopreservation is to use either high concentrations of certain CPAs (Cryoprotective Agent) or ultra-rapid cooling rates (>10^6°C/min) to induce the cell cytoplasm to form a glass (i.e., to vitrify cells/tissues) rather than to crystallize. Indeed, vitrification is an ideal approach for organ cryopreservation. However, ultra-rapid cooling rates are technically difficult to achieve for tissues/organs. Several of the CPAs that are effective in ameliorating slow freeze injury also act to promote glass formation, but the required concentrations are so high that they can be very toxic to the cells/tissues. In addition, a vitrified organ is very brittle and can be easily fractured by thermal stress if not uniformly heated.

- (d) The lack of a single optimal cryoperservtion condition for all cell types in a tissue/organ. As we know, the optimal cryopreservation condition (e.g., cooling rate) is cell-type dependent due to the cell-type dependence of membrane permeability to water and CPAs, intracellular ice formation, osmotic tolerance limits, cryo-sensitivity and other physical/biological factors for cell cryoinjury.
- Because of many different cell types in a tissue or organ, it is difficult to define one single condition which is optimal for cryopreservation of all cell types.

- The difficulties are *(i)* high specific feature of plant cells, such as their large size, strong vacuolization and abundance of water, *(ii)* cell damage during freezing and subsequent thawing caused by ice crystals formed inside the cells and by cell dehydration, and *(iii)*gradual formation of large crystals of more than 0.1μm whose facets rupture many cell membranes. However, in the presence of cryoprotectants (the chemicals decreasing cryodestruction) and reduced temperature, free water has enough time to leave the cells. Therefore, it can freeze on the crystal surface in the solution. This results in marked dehydration and protoplast shrinkage. Excessive time and degree of plasmolysis (contraction of the protoplast of a plant cell as a result of loss of water from the cell) are the reasons of cell destruction during slow freezing, since they cause irreversible contraction of the plasmalemma.