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(56) Documents Cited
WO 96/24018 A1 WO 96/02801 A1 WO 91/01635 A
US 4799358 A

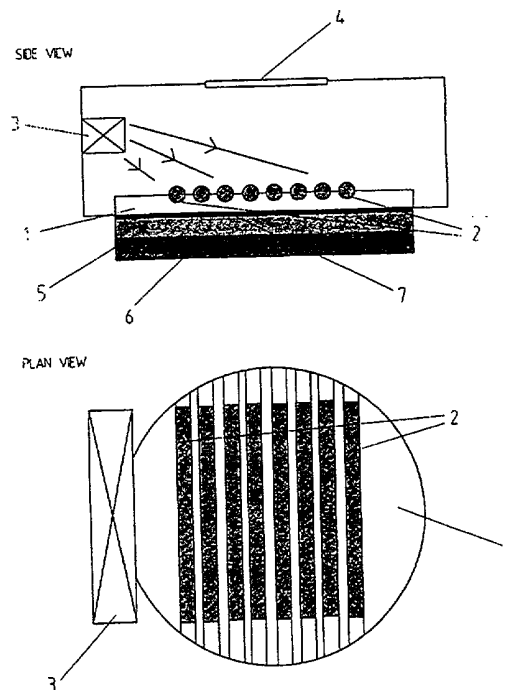
(58) Field of Search
UK CL (Edition P) **A2D DEF DX2 DX3**
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(54) Abstract Title

Cryopreservation of cell suspensions

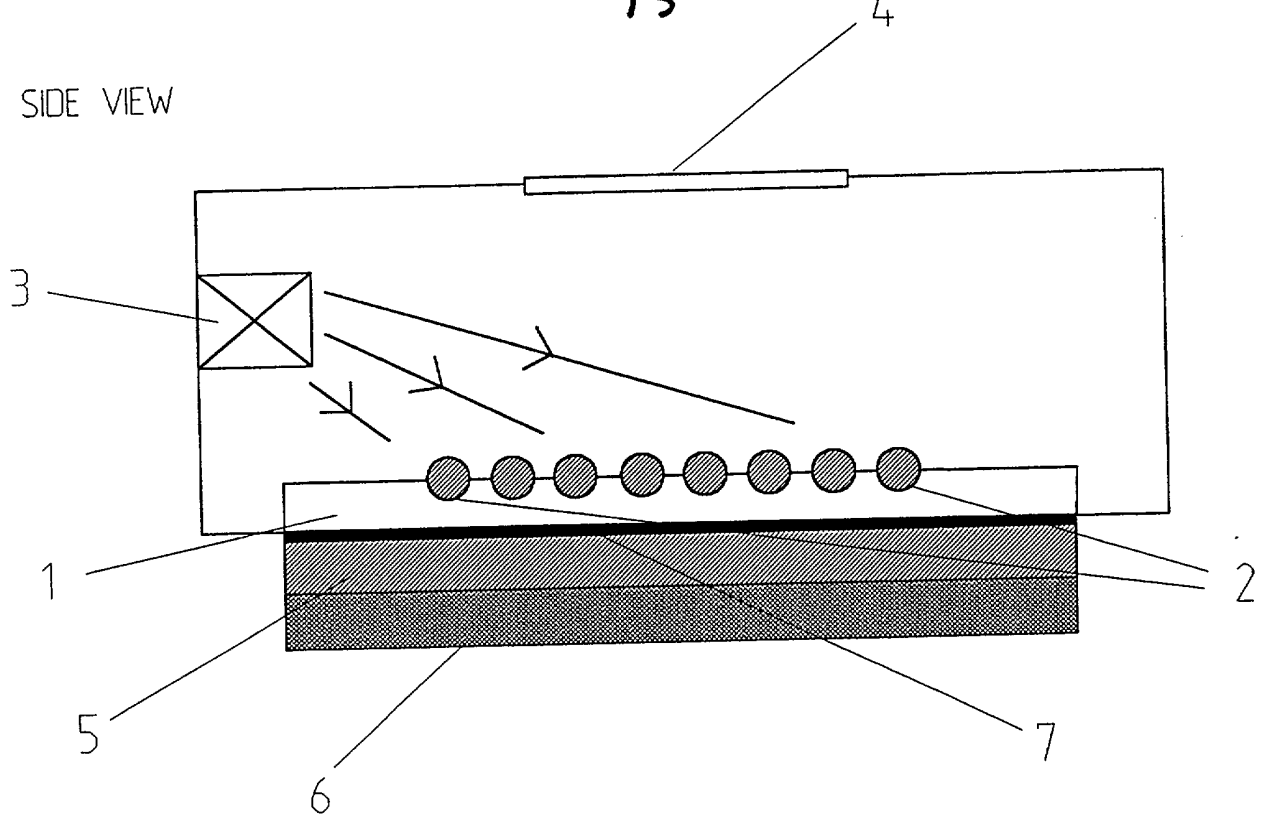
(57) The invention provides a method for the cryopreservation of cell suspensions comprising the steps of determining the osmolality of the suspending solution, nucleating undercooled samples close to the melting point and then controlling the temperature profile during solidification wherein the rate of temperature reduction is non-linear and the time rate of change of the solute concentration of the unfrozen fraction is decreasing over more than 80% of the elapsed time of solidification.

Apparatus for carrying out the method comprises samples 2 cooled by heat sink 6. The ice crystal growth can be monitored by illuminating samples 2 with light source 3 without removing the samples from the apparatus.

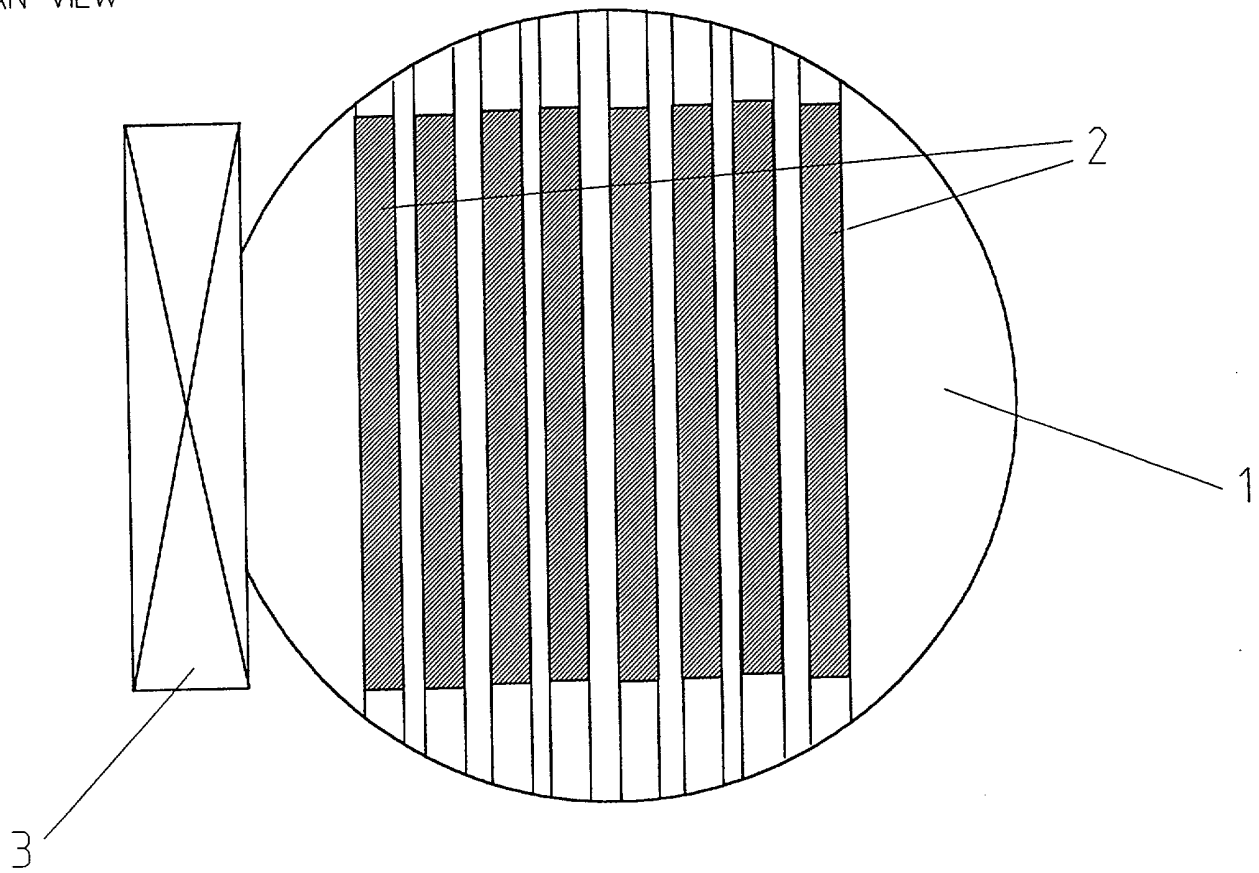


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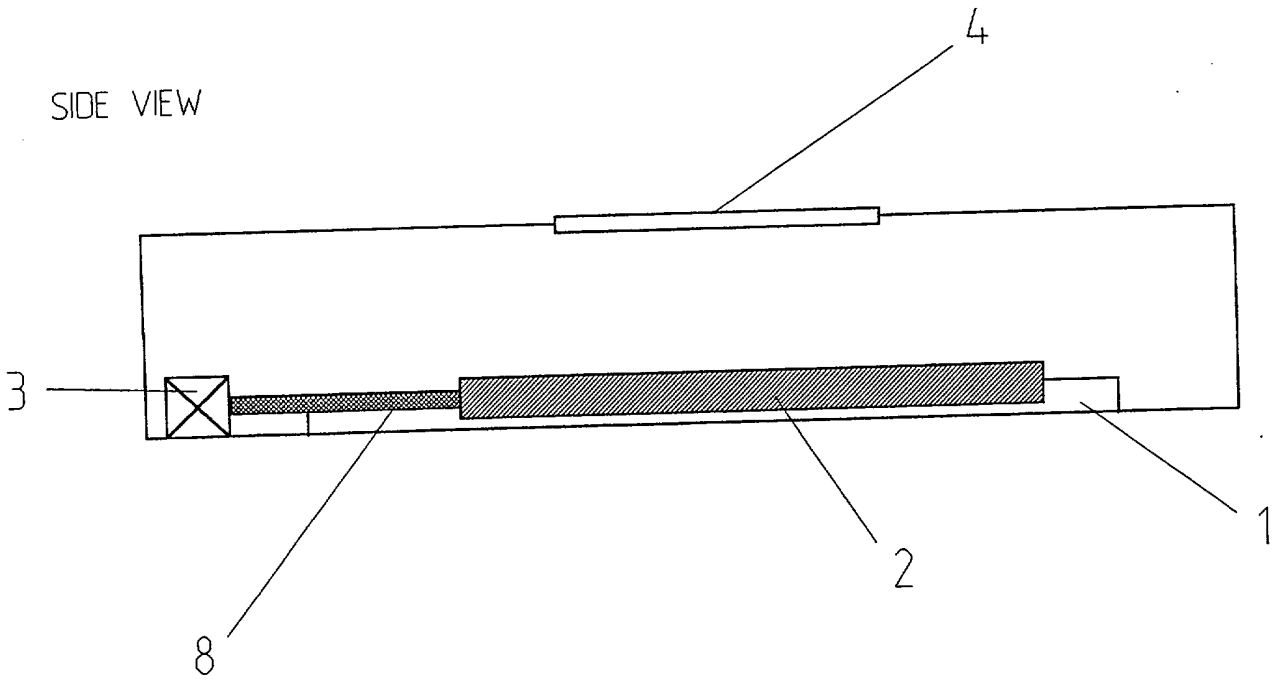
SIDE VIEW



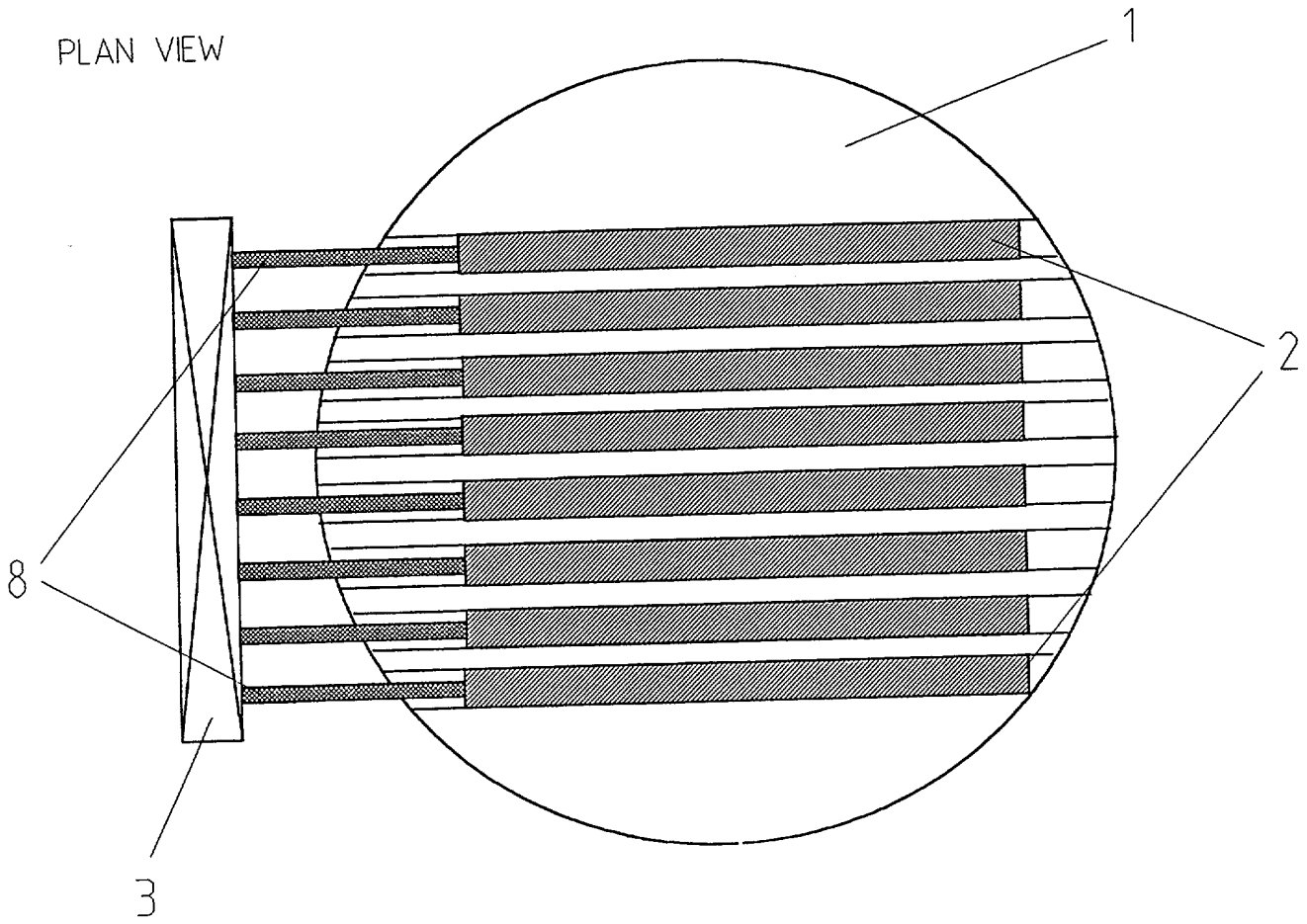
PLAN VIEW



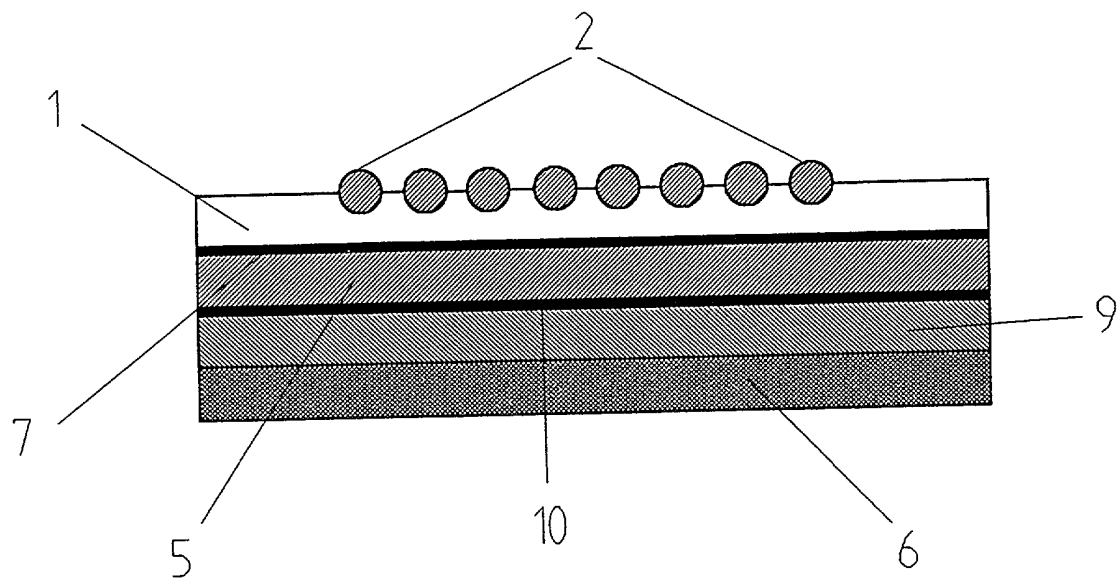
SIDE VIEW



PLAN VIEW

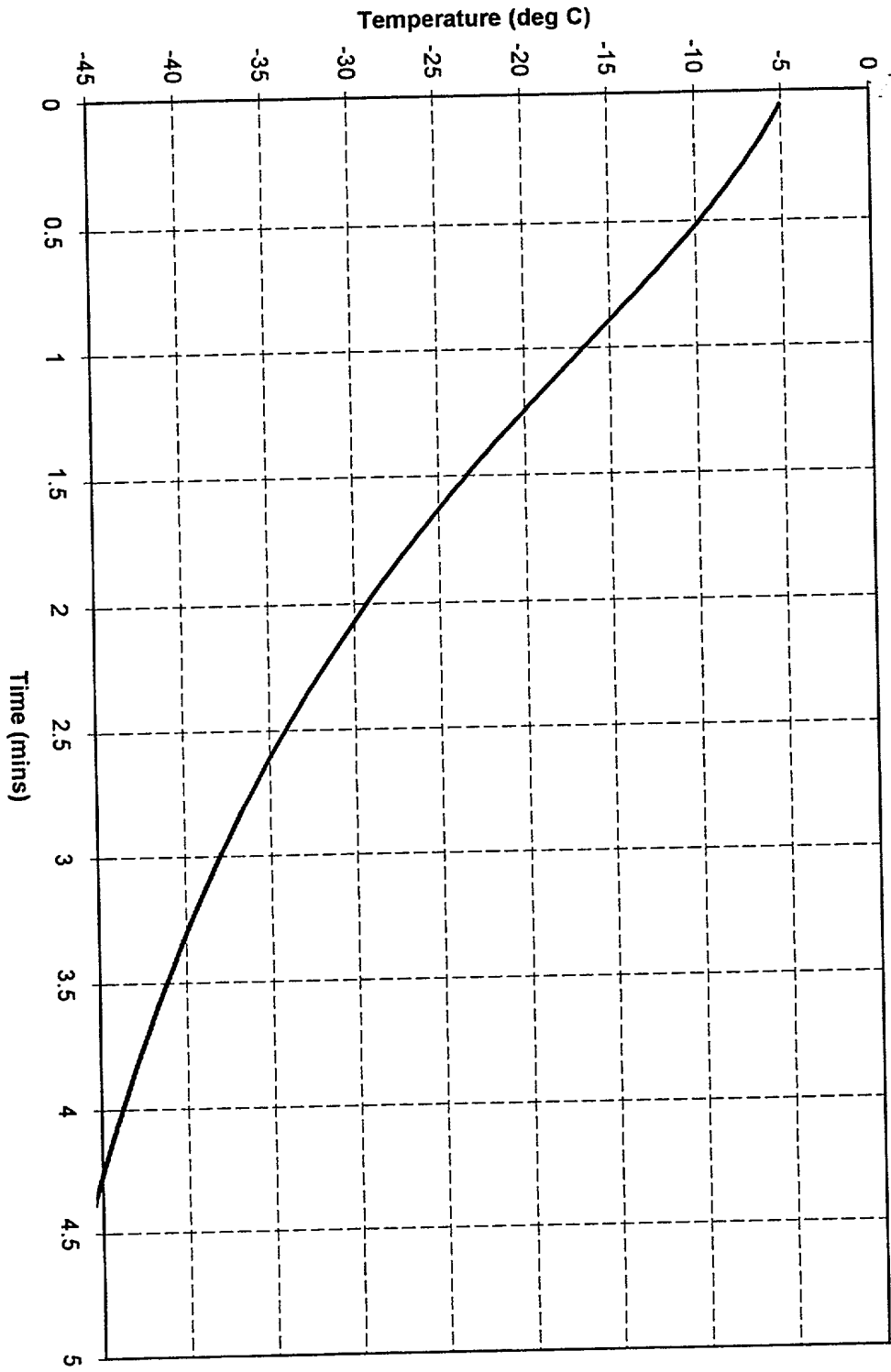


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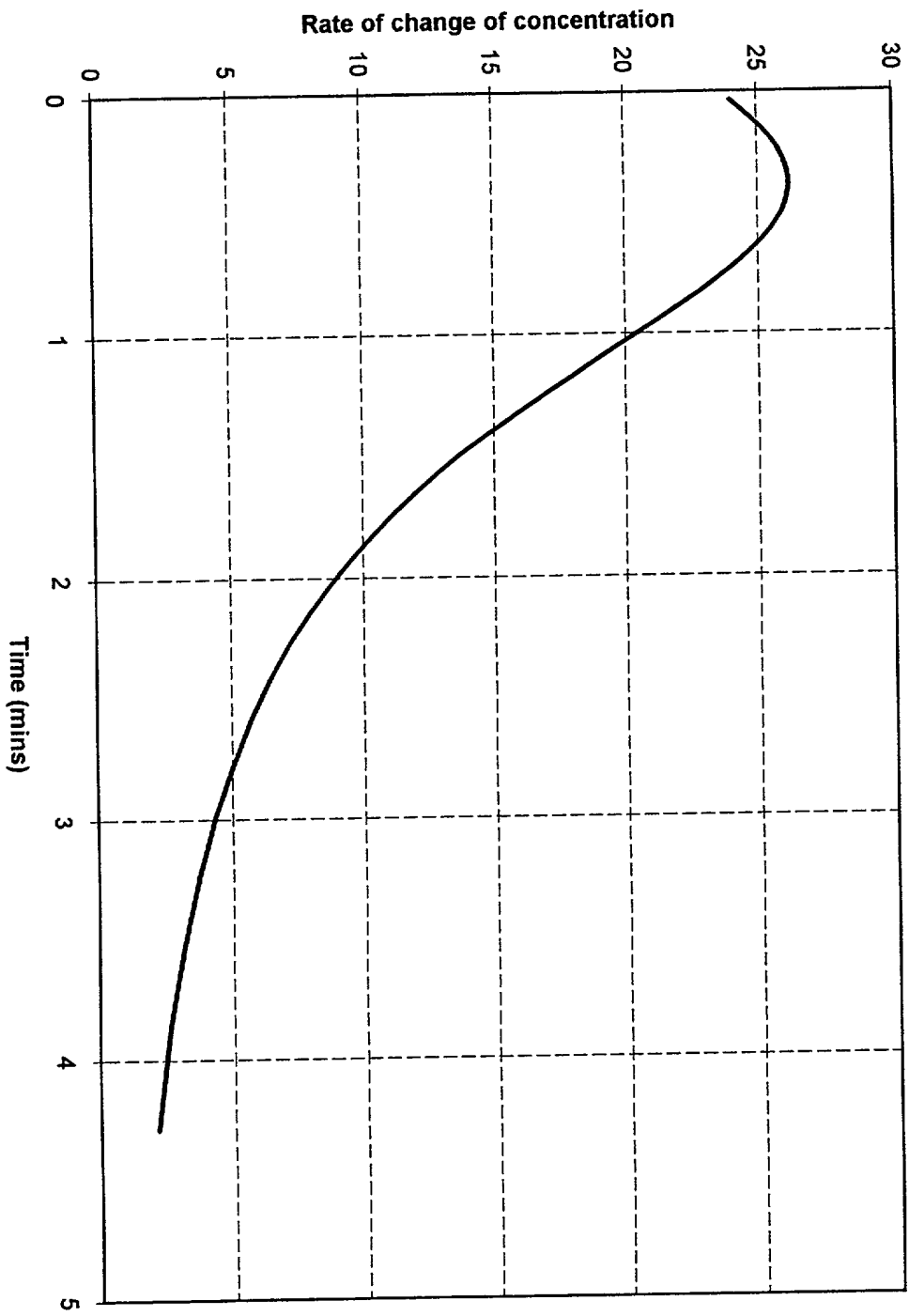
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Temperature-time graph



S/S

Time rate of change of unfrozen liquid concentration



Improved Method and Equipment for Cryopreservation

This invention relates to an improved method and equipment for the cryopreservation of cell suspensions.

Field of the Invention

Cryopreservation is a process in which biological samples are stored at low temperatures. Of particular interest to this application are cells in suspension including embryos and gametes (spermatozoa, oocytes), cell lines, bone marrow, blood stem cells etc. for use in medicine, agriculture, aquaculture, biotechnology etc. These materials are generally frozen in flexible bags, glass or plastic ampoules, or transparent plastic tubes known as straws.

It is well known that cell survival on thawing is dependent both on the addition of suitable cryoprotective additives and by control of cooling rate. Conventionally, samples are cooled at linear rates in freezers of various degrees of sophistication.

The Problem

It is a disadvantage with known methods of freezing that cellular injury may occur upon thawing. Whilst cryopreservation is commonly employed for a wide range of biological materials the technique is not applicable to some cell-types where the recovery upon thawing may either be low or very variable, for example sub-fertile human spermatozoa, human testicular spermatozoa, pig spermatozoa etc., or in other cases where survival is non-existent for example mammalian oocytes, fish eggs and fish embryos.

The critical physical steps in the cryopreservation process are:

- 1) Accurate nucleation of ice within the samples.
- 2) Controlled temperature reduction from nucleation to the temperature of solidification.

Ice Nucleation. It is known to be problematic that when ice nucleation is not externally controlled, it occurs individual samples occurs a wide range of temperatures below the melting point of the samples. While some samples nucleate at or just below the melting point, others do not nucleate until the temperature has reached up to 20°C below the melting point.

With cell suspensions contained in straws or ampoules the range of temperatures is particularly wide both because the volume of sample, and hence the number of available

heteronuclei, is small, and also because the containers are generally sealed before freezing, thus removing the possibility of seeding by airborne ice nuclei in the freezer. Even when cooling at rates apparently optimised for survival, nucleation at temperatures significantly away from the melting point results in transient temperature changes and cell death. (This is further discussed in Whittingham D.G. In The Freezing of Mammalian Embryos. Ciba Foundation Symposium 52 pp 98-102, 1977).

In order to nucleate samples in a reproducible manner it is common practice firstly to cool the samples to a temperature below the melting point of the cryoprotectant solution employed, then after a short period of thermal equilibration, to nucleate ice in the supercooled liquid. This is performed either manually by the application of cold forceps to the outside of the sample, by cold wires, by devices employing 'reverse Peltier effects' and by the application of physical disturbances. Following nucleation and initial crystal growth, cooling of the samples is then resumed. However a major problem exists in conventional controlled rate freezing equipment because following the nucleation step it is not possible to monitor subsequent crystal growth without removing the straws from the apparatus for visual inspection. A high risk of melting of the sample is associated with such removal. At present no method is available in such equipment to monitor, in a non-invasive manner, the success of the nucleation procedure.

Temperature Reduction following Ice Nucleation. It is conventional practice that during cryopreservation, materials are cooled with, as far as possible a linear reduction in temperature with time. All commercially available cryopreservation equipment is designed to control the sample environment in a linear manner, and depending on the heat transfer characteristics of the equipment, the samples cool in a more or less linear manner. Whilst this approach is convenient for simple design of equipment and has produced acceptable results with a range of cell types, with many other cellular materials it has proved unsatisfactory.

We have now devised methods and equipment for the freezing of biological materials in suspension, whereby the problems associated with cellular freezing injury are mitigated or overcome.

Statement of Invention

According to the first aspect of this invention there is provided a method for the cryopreservation of biological materials suspended in a medium containing a suitable concentration of a cryoprotectant, comprising the steps of:

- 1) Determine the osmolality of the suspending solution.
- 2) Determine the melting point of the liquid ($T_{m.p.}$ °C) from the phase diagram for the liquid.

- 3) Decide upon the 'holding temperature' of the liquid ($T_{\text{hold}} \text{ }^{\circ}\text{C} < T_{\text{m.p.}}$) at which ice will be nucleated in the undercooled liquid. Cool the material to the holding temperature, and hold.
- 4) Nucleate ice in the undercooled sample and allow to re-equilibrate at T_{hold} . From the phase diagram determine the new concentration, A_0 , of the unfrozen solute after nucleation at T_{hold} .
- 5) Reduce the temperature of the sample from T_{hold} to $T_{\text{solidification}}$. This temperature reduction should take place in a non-linear manner such that the time rate of change of the solute concentration of the unfrozen fraction is decreasing for more than 80% of the elapsed time. The corresponding temperature - time relationship can be determined from the phase diagram.

The time rate of change of the solute concentration of the unfrozen fraction in a preferred embodiment is decreasing for more than 90% of the elapsed time of the solidification process.

In a preferred embodiment the change in solute concentration, A (% by weight), of the unfrozen liquid during solidification from T_{hold} to $T_{\text{solidification}}$ to take an inverse tangent form closely approximated by:

$$A = 26.9 + 30.6 \tan^{-1}\{(t + t_0 - 0.385)/1.17\},$$

where t is the time (minutes) from the start of this step 5, and t_0 is determined from the concentration A_0 according to the relation:

$$t_0 = 0.385 + 1.17 \tan\{0.0327(A_0 - 26.9)\}.$$

The initial osmolality of the suspending medium may be measured by any convenient method, including freezing point osmometry or may be calculated from the composition of the medium. The equilibrium phase diagram during solidification of this solution is then constructed by any appropriate method, including experimental measurement, by reference to standard text (for example the CRC Handbook of Chemistry and Physics), or scientific publication (for example Rasmussen & McKenzie, *Nature* 220, 1315-1317, 1968, Shepard *et al.*, *Cryobiology* 13, 9-23, 1976, Lane, *Ind. Engr. Chem.* 17, 924, 1925). For solutions with unpublished or incomplete phase diagrams, and for complex mixed solutions the phase diagram may be approximated by any well defined system, for example glycerol and water (Lane, *Ind. Engr. Chem.* 17, 924, 1925).

The holding temperature ($T_{\text{hold}} \text{ }^{\circ}\text{C}$) to allow ice nucleation within the sample should be chosen to be near to the melting point ($T_{\text{m.p.}} \text{ }^{\circ}\text{C}$). In a preferred embodiment the amount of undercooling ($T_{\text{m.p.}} - T_{\text{hold}}$) should be less than 2°C and preferably less than 1°C . Nucleation of ice in the undercooled sample is achieved by any convenient method.

The elapsed time from T_{hold} to of the system is chosen with respect to the cell type being frozen and the type and concentration of the cryoprotective additive employed. This elapsed time is generally similar to that determined from the 'optimal' linear rate of cooling, for example $10^{\circ}\text{C min}^{-1}$ for many types of mammalian spermatozoa and $0.3^{\circ}\text{C min}^{-1}$ for mammalian embryos between the temperatures T_{hold} and $T_{\text{solidification}}$. The 'optimum' linear rate of cooling may be estimated from a knowledge of the basic biophysical parameters which determine osmotic behavior during freezing. Alternatively the elapsed time during cooling by the non-linear protocol described here may be calculated by modelling the osmotic behavior during solidification. The elapsed time is selected to be the shortest time which provides sufficient water loss to avoid any intracellular ice formation within the cell, either within the initial freezing step or during subsequent transfer to the storage temperature. $T_{\text{solidification}}$, the temperature at which all the liquid phase solidifies or vitrifies is determined from the phase diagram or may be measured, for example by differential scanning calorimetry. For most simple systems $T_{\text{solidification}}$ this is the eutectic temperature of the suspending medium.

In a further embodiment of the invention, for example where large volumes of cell suspensions are being frozen in flexible bags, the re-equilibration component of step 4 above is omitted. Step 5 is then modified so that the temperature of the sample is reduced from $T_{\text{m.p}}$ to $T_{\text{solidification}}$ in the manner described.

According to the second aspect of this invention there is provided apparatus for carrying out the method of invention for samples within straws, comprising a sample holder with means of temperature control and of sample nucleation, means of illuminating the sample within the apparatus, and means of viewing the illuminated sample

In an embodiment of the equipment the samples are fitted into machined grooves in a horizontal sample holder made from a good thermal conductor and light is shone at a suitable angle onto the straws from a longitudinal light source orientated parallel to the straws and positioned above and to one side of the sample holder.

In a further embodiment the light is introduced through an optical fibre terminating in a modified plug in the end of each straw. The presence of ice is indicated by sideways scattering from the resulting axial light beam.

In an embodiment the sample holder has a surface finish/colour to further enhance the optical contrast between unfrozen and nucleated samples for the cryoprotectant solution being employed. Indicators may also added to the cryoprotectant solution to detect changes in the properties of solutions which occur when ice is formed. For example, it is well known that the apparent pH of a solution may change during freezing and the simple incorporation of pH indicator may provide a easily detectable colour change.

In embodiments of the invention means of viewing the illuminated sample are provided. The sample chamber may have an optically clear lid of a suitable insulating material, such

as perspex which allows the samples to be viewed directly during freezing. This may be further covered with a insulating lid which may be removed to permit sample examination.

If samples are detected not to have nucleated following the nucleation step, then appropriate action may be taken by the operator. The nucleation step may be repeated with the sample(s) which have failed to nucleate; it is not expected that this will compromise other samples which have nucleated successfully within the apparatus. In all cases, successful detection of ice nucleation following the nucleation step will provide a key parameter for the quality control of the cryopreservation protocol.

In a further embodiment of the invention cell suspensions contained in plastic ampoules or glass vials are processed in equipment similar to that described for straws, except that the sample holder has vertical holes to accommodate these containers. To simplify the detection of ice following nucleation within plastic ampoules optically clear tops are fitted to the ampoules.

In order that the equipment be more fully understood, embodiments thereof will be described, by way of example only, with reference to the accompanying drawings, wherein:

Figure 1 is a schematic of a horizontal sample holder (1) in which the samples (2) are illuminated by a light source (3). This light source is positioned so as to enable ice within the straw to be visualised through a suitable viewing window (4). Heat is extracted from the sample holder through an insulated plate (5) to a cold heat sink (6). The temperature of the sample holder is measured by any suitable technique for example a thermocouple, and a thin film heater (7) is then used to control the sample holder temperature above that of the cold heat sink as required. Temperature control during solidification is achieved using any suitable controller which may be programmed to achieve non-linear rates of temperature change.

Figure 2 is a schematic of a horizontal sample holder (1) in which the samples (2) are illuminated axially through light guides (8) in the end of each straw. A suitable light source (3) provides light to these guides.

Figure 3 is a schematic of a passive cooler. The temperature of the sample holder (1) up to ice nucleation is controlled by the upper thin-film heater (7) counter-acting the cooling to the cold heat sink (6). The insulated plate is split into two pieces (5 & 9) with a second thin film heater between them. This heater (10) is used to maintain the temperature at the bottom of the upper insulated plate (5) at a pre-determined temperature (T_1). After nucleation the upper heater (7) is switched off and the sample holder then cools passively to temperature T_1 . After a pre-determined time the second heater is switched off and the sample holder cools to the temperature of the cold heat sink

Example 1 Cryopreservation of Human Spermatozoa.

Normal human sperm was diluted into a cryoprotectant solution (Richardson 1976) containing glycerol, fructose and sodium citrate prior to freezing. The osmolality of the cell suspension was measured to be $1430 \text{ mOsm Kg}^{-1}$ by freezing point osmometry equivalent to be an aqueous glycerol solution of 13.5 % w/v. The temperature-time curve to achieve a change in the solute concentration of the unfrozen fraction that was decreasing for >90% of the elapsed time was then determined and is shown as Figure 4 together with the calculated concentration-time curve. The elapsed time from -5°C (the nucleation temperature) to -45°C (close to the eutectic temperature for glycerol/water) was 4 minutes.

Straws (0.25ml IMV, L'Aigle, France) were filled with the cell suspension and sealed with PVA powder. The straws were placed into equipment as described in Figure 1 above and cooled at a rate of $2^{\circ}\text{C min}^{-1}$ from 20°C to a holding temperature at -5°C . At -5°C the straws were nucleated within the equipment by touching the straw at the position of the free surface of the liquid column with a nucleating tool which had been cooled by immersion into liquid nitrogen. Confirmation that ice had formed in all straws was achieved by direct observation of illuminated straws on the cooling plate. The temperature of the straws was then reduced to -45°C following the temperature-time curve described above. Straws were then further cooled to -100°C at a rate of $10^{\circ}\text{C min}^{-1}$ and then transferred to liquid nitrogen for storage. When required straws were thawed at room temperature and the motility was assessed according to the methods described by the WHO (WHO, 1993).

With all sperm samples examined, sperm frozen by the method of the invention had a significantly higher recovery than sperm frozen by a passive vapour phase cooling protocol (Richardson 1976) or by linear cooling at a rate of $10^{\circ}\text{C min}^{-1}$ within a conventional controlled rate freezer. For example, with samples from a single ejaculate, vapour phase cooling yielded 60% motile sperm on thawing (relative to unfrozen sperm) whilst linear cooling within a controlled rate freezer yielded 66%, and samples frozen by the method of invention had a recovery on thawing of 88%.

Claims

1. A method of cryopreservation of biological material in suspension, which comprises the steps of :

- i) Determining the melting point ($T_{m.p.}^{\circ C}$) of the suspending solution from its measured osmolality or otherwise.
- ii) Determining a holding temperature ($T_{hold}^{\circ C} < T_{m.p.}$) to allow ice nucleation, and cooling the material to the holding temperature.
- iii) Nucleating ice in undercooled samples and allowing them to re-equilibrate at $T_{hold}^{\circ C}$. From the phase diagram determine the new concentration, A_0 , of the unfrozen solute.
- iv) Reducing the temperature of the sample from T_{hold} to $T_{solidification}$. This temperature reduction should take place in a non-linear manner such that the time rate of change of the solute concentration is decreasing for more than 80% of the elapsed time of cooling between T_{hold} to $T_{solidification}$.

2. A method as claimed in claim 1 in which the time rate of change of the solute concentration of the unfrozen fraction is decreasing for more than 90% of the elapsed time.

3. A method as claimed in claim 1 in which by controlling the temperature profile during cooling the time rate change in solute concentration, A (% by weight), of the unfrozen liquid to take an inverse tangent form closely approximated (within 5 %) by:

$$A = 26.9 + 30.6 \tan^{-1}\{(t + t_0 - 0.385)/1.17\},$$

where t is the time (minutes) from the start of this step iv, and t_0 is determined from the concentration A_0 according to the relation:

$$t_0 = 0.385 + 1.17 \tan\{0.0327(A_0 - 26.9)\}.$$

4. A method as claimed in claims 1 to 3, in which the thermal re-equilibration after the ice nucleation step is omitted.

5. Apparatus carrying out the method of invention which apparatus comprises:

- (a) a sample holder with means of temperature control and of sample nucleation, and
- (b) means of controlling the rate of temperature reduction in an appropriate non-linear manner

6. Apparatus carrying out the method of invention for cell suspensions contained in straws which apparatus comprises:
 - (a) A sample holder with means of temperature control and of sample nucleation, and
 - (b) means of illuminating the sample within the apparatus, and
 - (c) means of viewing the illuminated sample.
 - (d) means of controlling the rate of temperature reduction in an appropriate non-linear manner
7. Apparatus as claimed in claim 6 in which straws on a horizontal sample holder are illuminated by a light from above.
8. Apparatus as claimed in claim 6 in which the samples are illuminated axially through a transparent plug.
9. Apparatus as claimed in claim 6 in which the surface colour and finish of the sample holder is modified to enhance the optical contrast occurring following ice nucleation.
10. Apparatus as claimed in claims 5 and 6 in which the control of temperature reduction is achieved via a feedback loop incorporating a means of temperature measurement, a controller and a thin-film heater.
11. Apparatus as claimed in claims 5 and 6 in which appropriate temperature reduction is achieved passively.
12. Apparatus as claimed in claims 6 to 11 in which the rate of temperature reduction is in a linear manner.



Application No: GB 9722156.8
Claims searched: 1-12

Examiner: Keith Kennett
Date of search: 5 February 1998

Patents Act 1977 Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.P): A2D (DEF, DX2, DX3)

Int Cl (Ed.6): A01N 1/02

Other:

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	WO 91/01635 A3 (CELL SYSTEMS) see page 9 lines 11-23 and Figures 2a & 3	1,5
A	WO 96/24018 A1 (ORGANO-GENESIS) see claims 1 & 3	1,5
A	WO 96/02801 A1 (SECRETARY OF STATE) see Figure 6	1,5
A	US 4799358 (KNOFF) see column 7 lines 15-26	1,5

X Document indicating lack of novelty or inventive step
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P Document published on or after the declared priority date but before the filing date of this invention.
E Patent document published on or after, but with priority date earlier than, the filing date of this application.