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Cryopreservation of mammalian oocytes: Slow cooling and vitrification as successful methods for cryogenic storage

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Summary

Two basic methods for the laboratory-focused cryopreservation of mammalian oocytes are described, based on work with murine oocytes. One method uses a relatively low concentration of the cryoprotectant propanediol plus sucrose and requires controlled rate cooling equipment to achieve a slow cooling rate. This method has also produced live births from cryopreserved human oocytes. The second method, which is described here, employs a high concentration of the cryoprotectant dimethyl sulfoxide plus a low concentration of polyethylene glycol. This is a vitrification method, which involves ultra-rapid cooling by plunging standard straws into liquid nitrogen vapour, hence avoiding the need for specialised equipment, but requires technical ability to manipulate the oocytes quickly in the highly concentrated cryoprotectant solutions. Murine oocytes that have been vitrified using this technique have resulted in live births. Vitrification using other cryoprotectant mixtures is now a popular clinically accepted method for cryobanking of human oocytes.

Key words: oocyte, slow-cooling, vitrification, murine, human

1. Introduction

Successful cryopreservation of mature oocytes has been one area of reproductive cryobiology, which has challenged our understanding of cryobiology over the past 30 years, in ways unforeseen at the beginning of the studies. Much had to be learnt about the specific ultrastructural and physiological characteristics of these large unique cells, in order to be able to recover functional oocytes from cryogenic storage, and these details have been published in many previous reviews **[1—5]**. With improved outcomes, in the past decade, there was a renaissance of interest in applying oocyte cryopreservation **[6]** and the technology has moved from a largely research-focused activity into the main stream of clinical reproductive medicine, resulting in the recent designation from the ASRM as a justified therapy **[7]**.

Storage of unfertilised oocytes has numerous applications. Originally conceived as a potential option for women undergoing anti-cancer treatment which may render the women infertile **[8]**, it may now have a wider remit, including application in banking of donated oocytes, premature ovarian failure, ethical objections to embryo cryopreservation, or in women seeking to defer their reproductive potential until later in life but who do not have a current partner **[4]**. The method can also be used to cryo-bank excess oocytes produced within a particular cycle during in vitro fertilization.

Applications in animal management include the preservation of important genetic lineage; the ability to restock following outbreaks of diseases such as foot and mouth; the preservation of genetically modified strains, thereby reducing the cost of continuous breeding and avoiding problems of genetic drift; and the preservation of endangered species.

Oocyte cryopreservation has also developed alongside a greater understanding of what is needed for successful recovery of function after cryogenic storage. The need for optimized cryogenic dehydration of the cells, coupled with introduction of specific non-toxic stabilizing solutes called cryoprotectants (CPA) is a central tenet of cryobiology **[2,9,10]**. The term cryopreservation itself has come to mean different things in applied cryobiology. For true long-term cryogenic preservation, cells and their supporting medium need to be stored in this optimally cryo-dehydrated state below the temperature range at which any residual water molecules undergo a 'glassy transition' **[2,11]**. However, there are two different approaches to achieve this optimally dehydrated state which can be termed controlled rate slow cooling (CRSC), or vitrification (VS - literally changing into a glassy state). In CRSC CPAs are essential to support cell survival but the dehydration is produced by the extracellular water within the sample being changed into ever-increasing ice fraction as temperatures progress slowly (at rates < −1°C/min) down from 0°C to -60°C and beyond. In VS CPAs are equally essential but they are applied in very high concentrations to produce optimal cryo-dehydration and suppress ice crystal formation *before* cooling begins **[2−4,11]**. Because these high CPA concentrations are potentially toxic, the samples must be cooled very rapidly (at rates >200°C/min) to below the glassy transition range (about −120°C, depending on the CPA mixture selected). VS as this is widely used can only be practically achieved using small volume samples (usually <10 μL) in special containers to achieve these high cooling rates. Lastly, for both CRSC and VS, the rate of rewarming back from cryogenic storage is important to control, and should be fast, to avoid the propensity for free water molecules to form ice crystals as the temperature profile climbs (the so-called event of freezing during warming) **[12]**.

Oocyte cryopreservation has been at the heart of this debate within applied cryobiology. Originally, the early reports of oocyte cryopreservation in both human and animal oocytes were made using CRSC **[13−20]**. Since then pregnancies and live births resulting from

cryopreserved oocytes have been reported in a number of species **[21−24]** and human **[25]**. Clinical programmes were developed in the early 2000's using CRSC with optimized CPA mixtures **[26,27]**. At the same time VS techniques were becoming optimized **[4,28]**. It became apparent that experienced practitioners could recover higher percentages of oocyte from cryogenic storage using VS methods **[29−31]**.

Historically, oocytes have been considered to be difficult cells to cryopreserve for a number of reasons. The oocyte is a large single cell with relatively low permeability to water and cryoprotective agents (CPA). This means that oocytes have a tendency to retain water as ice starts to form in the suspending medium during cooling and, if this leads to formation of intracellular ice as cooling proceeds, damage to the cell results. Permeability of oocytes varies between species, strains and maturational status of the oocyte. The mature oocyte is also a short-lived cell that must undergo fertilization in order for it to continue to survive and develop. For fertilization to occur naturally, the oocyte must retain integrity of a number of its' unique structural features. These include the surrounding zona pellucida, the cortical granules and the microtubular spindle on which the chromosomes are organized. The zona pellucida is a glycoprotein coat surrounding the oocyte. Changes to this layer, triggered by the action of a single sperm binding to its' receptor, induce the cortical granules to release their contents. The enzymes released from the cortical granules act to crosslink the glycoproteins of the zona pellucida, thus rendering it impenetrable to further sperm **[32]**. Cryopreservation has been shown to result in the premature release of the contents of cortical granules, thus creating a block to sperm penetration **[33]**. CPAs themselves can cause a transient increase in intracellular calcium similar to the initial increase caused by sperm entry and subsequent induction of zona hardening **[34]**. Conversely cryopreservation can lead to physical damage to the zona pellucida resulting in multiple sperm entry. Both of these problems are overcome by application of the intracytoplasmic sperm injection (ICSI) technique, whereby a single sperm is injected into the

oocyte to achieve fertilization. The microtubular spindle is the structure upon which the condensed chromosomes are aligned in mature oocytes and is responsible for the movement of chromosomes during cell division. Damage to this structure can lead to aneuploidy after fertilization. During cooling the microtubular spindle has been shown to disassemble, although there is growing evidence that the spindle is capable of repair on warming and post-thaw culture **[35−37]**. One way of avoiding the potential for damage to the microtubular spindle is to cryopreserve the immature oocyte before the spindle is formed, when the chromosomes are contained within the germinal vesicle and the oocyte is termed immature **[38]**. However, success following cryopreservation of immature oocytes is less than that following cryopreservation of mature oocytes **[39]**. If protocols for hormonal stimulation of ovaries to induce release of numerous mature oocytes are not available or not easily applicable, for example in patients with polycystic ovary syndrome or in cancer patients, when harvesting of ovarian tissue is the only option for fertility preservation, then only immature oocytes, contained within the ovaries, may be available for storage. The major problem with cryopreserving immature oocytes is that they must be matured in vitro in order to become fertilisable. This maturation involves communication with the cumulus cells that surround the oocyte **[40]**. The cumulus cells are much smaller than the oocyte and are connected with it by numerous gap junctions. Development of cryopreservation protocols that allow survival of the oocyte, the cumulus cells and connections between the two is difficult **[41]**. However it was shown that preservation of this cell-to-cell connection can be achieved even within such a complex ovarian tissue when the method of VS was applied **[42]**. Also, techniques for maturation in vitro require further refinement in most species. In vitro maturation of oocytes prior to cryopreservation may produce poor quality oocytes that are more prone to damage during cryopreservation **[43]**. As well as these general problems, the oocytes of some species have further characteristics that make them still more difficult to freeze; for example, porcine

oocytes have a high lipid content and are sensitive to chilling **[44]**.

The protocols used for the cryopreservation of unfertilised oocytes have largely been adopted from embryo cryopreservation techniques. As with embryo cryopreservation both CRSC and VS techniques have been successfully applied. Murine oocytes have been successfully cryopreserved using a range of CPA such as glycerol or dimethyl sulfoxide, whilst the technique most commonly applied to the controlled-rate cryopreservation of human oocytes is that of slow cooling in the presence of propanediol and sucrose. This technique, combined with ICSI, first yielded a live birth from cryopreserved human oocytes in 1997 **[25]**. Since then, the number of human live births has been increasing steadily, with live births escalating into hundreds **[36,45]**. VS has also produced accumulating numbers of human live births **[46,47]**, with more than 250 recorded by 2009 **[48]** and coming to more than thousands babies born till 2016−2017 **[49−52].** Following significant improvement of oocyte VS by the Cryotop method **[53,54]**. Combination of the (i) stepwise equilibration at the room temperature in increasing concentrations of permeating CPA with addition of non-permeating agents to the VS solution (minimising the toxic effect by using a mixture of CPAs and equilibration at room temperature); (ii) placing an oocyte on the surface of the fine propylene strip attached to a plastic handle in the minimum (<1 μL) volume of VS solution (reaching a high cooling rate to minimise the toxic effect of the CPAs and associated osmotic injury, also preventing any accidental ice formation, when a lower concentration of CPA is used, and avoiding any fracture of the vitrified oocyte or surrounding solution); (iii) direct plunging into liquid nitrogen in an open system thus reaching a high cooling rate (if required, to avoid contamination of the sample, liquid nitrogen can be sterilised by filtration or exposure to UV); (iv) rapid warming at 37°C with stepwise equilibration of oocytes in decreasing concentration of sucrose to prevent the effects of de-vitrification and osmotic shock resulted in good morphological survival of 95% of vitrified oocytes, high rates of fertilization (91%), in vitro blastocyst formation (50%)

and subsequent delivery of healthy babies (*see* **Figure 1**). The Cryotop method using different mixtures of CPAs is the most commonly used worldwide method for oocyte VS.

As both CRSC and VS have been more commonly used around the world, the debate continues about which (or, if either) technique, is superior for oocyte cryopreservation **[55−58]**. Recent systematic reviews in human oocyte cryopreservation comparing either slow cooling methods or VS techniques found some evidence that oocyte recoveries, fertilization and embryo development were higher in the VS groups **[31,51,59−61]**. Due to different requirements in operator skills, process times, equipment availability and species-specific factors in oocyte biology, no single cryopreservation method is likely to be suitable in all situations, for all species or for all developmental stages of oocytes. Many ancillary factors such as oocyte quality and selection, hormonal stimulation regime, and in vitro manipulation conditions can impact on cryopreservation outcomes **[50,62,63]**. Even if VS is superior for oocyte cryopreservation further studies on the fundamental biophysics of cryogenic exposure could optimise both CRSC and VS protocols. As far as can be assessed from current information, oocyte cryopreservation does not impact in a negative way on birth abnormalities **[48,50]**. Oocyte cryopreservation has moved away from assignment as an experimental procedure, towards recognition of the true value of this powerful technique with far-reaching potential in reproductive technologies. As we approach the 2020's, there is a highly significant switch to VS for those centres performing oocyte cryopreservation, such that, for example, in the UK and Scandinavia none are now using CRSC (Fuller, Keros personal communication 2019). Therefore in this chapter, we shall concentrate on providing details of the VS method. However still some scientists are interested in details and principles of slow cooling, especially for animal oocyte cryo-banking. Thus we also present a robust method and protocol details for murine oocyte CRSC **[2]**. Detailed explanation of the Cryotop® – Open System method (Kitazato Biopharma, Shizuoka, Japan) successfully applied worldwide for human oocyte VS

can be found on the Kitazato company's homepage (https://www.kitazato-dibimed.com/wp-content/uploads/CRYOTOP_SAFETYKIT_A5_2019_LOW.pdf)

Two methods of oocyte cryopreservation are described below, with the necessary technical details to introduce these into a standard laboratory setting and which have been developed over several years for murine oocytes. One method is for slow controlled rate cooling and the other is a VS method. They can yield reliable, good outcomes, but (as discussed above) there may still be opportunities for further optimization in the various steps, should investigators be interested to pursue those.

2. Materials

2.1 Slow controlled rate cooling

1. A controlled rate freezing machine (available from several sources such as Planer plc, Middlesex, UK or Asymptote, Cambridge, UK) set to hold at 20 ºC, then cool at –2ºC/min to –7ºC, allow for seeding (*see* Note 1), hold at –7ºC for 10 min after seeding, then cool at 0.3ºC/min to –30ºC, then at –50ºC to –150ºC and finally hold at –150ºC for 10 min.
2. Plastic straws (IMV, L'Aigle, France).
3. Plugs for straws (IMV, L'Aigle, France), sealing powder or heat sealer (*see* **Note 2**).
4. Pulled glass pipettes, denudation glass or plastic pipettes.
5. Tissue culture dishes (Falcon, Becton and Dickinson Co., USA).
6. Dissecting microscope.
7. Hotplate set at 37ºC.
8. Forceps cooled in liquid nitrogen (optional - *see* **Note 1**).
9. Liquid nitrogen dewars, preferably at least two storage dewars (*see* **Note 3**) and one for

transporting samples to storage dewar and cooling forceps, if used.

10. Scalpel.
11. Syringes and needle (optional - *see* **Notes 4 and 5**).
12. Heated water bath set at 30ºC.
13. Safety equipment e.g. cryo-gloves, face shield, oxygen depletion monitor.
14. Heated gassed incubator.
15. Oocyte culture medium e.g. for human oocytes Fertilisation medium (Cook IVF, Brisbane, Australia), for murine oocytes Tyrode's medium (Invitrogen, Paisley, UK).
16. Dulbecco's phosphate-buffered solution (PBS; Invitrogen, Paisley, UK) for human oocytes supplemented with 30% protein supplement e.g. plasma protein supplement (Baxter AG, Vienna, Austria) or serum protein supplement (Pacific Andrology, CGA/Diasint, Florence, Italy) for mouse oocytes supplemented with 5% heat-inactivated foetal bovine serum (Invitrogen, Paisley, UK).
17. Freezing solutions: 1.5 M 1,2-propanediol (PrOH) and 1.5 M PrOH plus 0.2 M sucrose, both made up in PBS with protein supplement.
18. Thawing and dilution solutions: 1.0 M PrOH plus 0.2 M sucrose; 0.5 M PrOH plus 0.2 M sucrose; 0.2 M sucrose, all made up in PBS with protein supplement.
19. Hyaluronidase (optional - *see* **Note 6**).

2.2 Vitrification

1. Plastic straws (IMV, L'Aigle, France) (*see* **Note 7**).
2. Plugs for straws (IMV, L'Aigle, France) or sealing powder or heat sealer (*see* **Note 2**).
3. Pulled glass pipettes, denudation glass or plastic pipettes.
4. Tissue culture dishes (Falcon, Becton and Dickinson Co., USA).
5. Dissecting microscope.

6. Thermocouple.
7. Device to hold straw horizontally above liquid nitrogen vapor without covering area of straw containing oocytes (*see* **Note 8**).
8. Hotplate set at 37ºC.
9. Liquid nitrogen dewars, preferably at least two storage dewars (*see* **Note 3**), plus a container capable of holding liquid nitrogen and accommodating a straw held horizontally.
10. Scalpel.
11. 3× 1 mL syringe and 2× needle.
12. Heated water bath set at 20ºC.
13. Safety equipment e.g. cryo-gloves, face shield, oxygen depletion monitor.
14. Heated gassed incubator.
15. Oocyte culture medium e.g. for human oocytes Fertilisation medium (Cook IVF, Brisbane, Australia), for murine oocytes Tyrode's medium (Invitrogen, Paisley, UK).
16. Dulbecco's phosphate-buffered solution (PBS; Invitrogen, Paisley, UK) for human oocytes supplemented with 30% protein supplement e.g. plasma protein supplement (Baxter AG, Vienna, Austria) or serum protein supplement (Pacific Andrology, CGA/Diasint, Florence, Italy) for mouse oocytes supplemented with 5% heat-inactivated foetal bovine serum (Invitrogen, Paisley, UK).
17. VS solutions: 6 M dimethyl sulfoxide (Me_2SO) plus 1 mg/mL polyethylene glycol (PEG; MW 8000) added to 4× strength PBS medium without $CaCl_2$ (*see* **Note 9**). The solution (for a volume of 10 mL) should be made up by adding 1 mL of distilled water to 2.5 mL of 4× PBS prior to adding the Me_2SO (4.69 mL) and PEG (1 mL of 10 mg/mL aqueous stock). Then add protein supplement (e.g. 0.5 mL FBS) and make up almost to volume with water prior to adding $CaCl_2$ (100 μL of aqueous stock solution of 15.9 g/L). Finally, add distilled water to make up to the final volume (10 mL in this case). Keep at room temperature until

required. This solution will be referred to as VSDP (*see* **Note 10**). Make up dilutions of 25% and 65% VSDP using PBS containing protein supplement as required.

18. Dilution solution consisting of 1 M sucrose made up in PBS containing protein supplement.
19. Hyaluronidase (optional - *see* **Note 6**).

3. Methods

3.1 Slow controlled rate cooling

The slow controlled rate cooling method described uses a mixture of the permeating cryoprotectant PrOH and non-permeating sucrose. It has been shown that 0.3 M sucrose yields better survival than 0.2 M sucrose in human oocytes **[64,65]**. This is thought to be largely due to greater dehydration of the cells prior to freezing. Optimal exposure time to PrOH plus 0.2 M sucrose is 5–10 min whereas optimal exposure time to PrOH plus 0.3 M sucrose is yet to be determined. However, an exposure time of 2 min will give a level of dehydration equivalent to that achieved with 5 min exposure to PrOH plus 0.2 M sucrose **[45]**. A further potential modification to the method described below is the choice of media in which to dilute the CPAs. Studies have also shown a medium in which some of the sodium has been replaced by choline to be preferable to PBS **[37,66,67]**.

1. Straws should be loaded with a column of 1.5 M PrOH plus 0.2 M sucrose and left at room temperature (20±2ºC) until required (*see* **Note 4**).
2. Place one 0.5 mL droplet each of PBS plus protein supplement, 1.5 M PrOH and 1.5 M PrOH plus 0.2 M sucrose in a tissue culture dish. Label each droplet.
3. No more than five oocytes (*see* **Note 11**) should be placed in the droplet of PBS (cumulus cells may be removed, *see* **Note 6**) and then placed in the droplet of 1.5 M PrOH for 10

min at room temperature. The oocytes are then moved to the droplet of 1.5 M PrOH plus 0.2 M sucrose for 5 min at room temperature.

4. The oocytes are then loaded into the prepared straws within the column of 1.5 M PrOH plus 0.2 M sucrose and the straw sealed (*see* **Note 2**).
5. The straws should be placed within a freezing machine set at 20ºC. The machine should be cooled to –7°C at –2°C/min. Having reached this temperature ice nucleation should then be initiated in the solution containing the oocytes (*see* **Note 1**). The straws are then replaced in the cooling machine and the cooling regime resumed with the temperature being held at –7°C for 10 min to allow dissipation of the heat of crystallisation. Once the cooling protocol has been completed and the samples are being held at –150°C, wearing appropriate protective equipment, the straws are placed in a liquid nitrogen storage dewar (*see* **Note 12**).
6. Prior to warming the straws, prepare dishes containing a 0.5–1 mL droplet each of 1.0 M PrOH plus 0.2 M sucrose, 0.5 M PrOH plus 0.2 M sucrose, 0.2 M sucrose and PBS with protein supplement. Label each droplet.
7. Wearing suitable protective equipment, warm the straws by holding them in air for 30 s, hold either end of the straw not the area containing the oocytes. Then place the straws in the water bath set at 30°C for 30 s or until the ice has just melted.
8. Wipe the straw dry. The plug, if used, should be removed from the straw or the ends of the straw cut with a scalpel and the contents then expelled (*see* **Note 5**) into the dish containing 1 M PrOH plus 0.2 M sucrose.
9. The oocytes should remain in this solution for 5 min at room temperature before being moved into the droplet of 0.5 M PrOH plus 0.2 M sucrose again for 5 min at room temperature.
10. The oocytes are moved into 0.2 M sucrose for 10 min at room temperature.

11. The oocytes are then placed in the droplet of PBS for 20 min, 10 min at room temperature and 10 min at 37°C on a hotplate.
12. The oocytes should then be cultured (2–3 h for human, 30 min for mouse) within an incubator in a suitable culture medium to allow recovery prior to attempted fertilisation (*see* **Note 13**).

3.2 Vitrification

Vitrification is advantageous over slow controlled rate cooling in that neither expensive cooling machines nor special laboratory environment are required. However, the need for high concentrations of CPA means that the times stated for CPA exposure must be strictly adhered to and manipulation of oocytes in these highly viscous solutions is technically demanding. Different CPA mixtures and minimum volume VS have been proven to be effective for oocyte VS. Toxicity of VS solution can be reduced by combination of decreased concentration of permeating CPA, which can be balanced by increasing concentrations of non-permeating agents like polymers and sugars **[53,68−70]**. Accurately performed VS (*see* **Figure 2**) can result in oocytes with similar to unfrozen oocytes post-warming morphological recovery, embryo development and life birth in human. The VS method described using VSDP has resulted in high blastocyst formation **[71]** and live births **[72]** in mice, although the technique is prone to variability **[48,68]**. The CPA mixture and cryopreservation vessel could be modified but the basic techniques described are applicable to all VS protocols.

1. Straws should be prepared by pushing the cotton plug approximately one third of the way down the straw. Using a needle and syringe, inject a column of sucrose solution, via what was the open end, so that the plug is wetted. Take care not to wet the sides of the straw with sucrose (*see* **Note 14**). The column of sucrose should fill approximately one third of the straw. Using a separate needle and syringe, inject a small ~0.5 cm column of 100% VSDP

leaving at least 1 cm between the sucrose and the VS solution. Leave prepared straw at room temperature until required.

2. Place one 50 µL droplet each of 25%, 65% and 100% VSDP in a tissue culture dish. Label each droplet.
3. Place liquid nitrogen, to a level equivalent to at least the length of the straw, into the vessel capable of accommodating the straw horizontally. Use the thermocouple to determine the point above the liquid nitrogen at which the temperature is -140°C and temporarily affix the thermocouple at this level.
4. Pipette a maximum of 5 oocytes (*see* **Note 11**) into 25% VSDP droplet and leave at room temperature for 3−5 min.
5. Transferring as little of the solution as possible (*see* **Note 15**), pipette the oocytes from the 25% VSDP into 65% VSDP droplet.
6. As quickly as possible, move oocytes into droplet of 100% VSDP.
7. Immediately draw up a small amount of 100% VSDP into a glass pipette and collect the oocytes. Transfer the oocytes into the column of 100% VSDP contained within the prepared straw.
8. Seal the open end of the straw (*see* **Note 2**). Hold straw using holder and, wearing suitable protective equipment, position the straw horizontally at the position above the liquid nitrogen at which the temperature is –140°C (*see* **Note 16**). Keep the straw in this position for 3 min and then plunge the straw into liquid nitrogen.
9. Transfer the straw to a liquid nitrogen storage vessel (*see* **Note 12**).
10. Prior to warming the straw, place 1 mL of 1 M sucrose solution in a culture dish, half fill a 1 mL syringe with 1 M sucrose and place 2× 50 µL droplets of sucrose solution and 2× 50 µL droplets of PBS with protein supplement in a culture dish. Label the droplets.
11. Wearing suitable protective equipment, remove the straw from liquid nitrogen storage.

Hold the straw in air for 10 s, hold either end of the straw not the area containing the oocytes, then plunge the straw into the water bath at 20°C for 10 s (*see* **Note 16**).

12. Wipe the straw dry and cut through the straw using a scalpel in the area containing the sucrose. Remove the plug from the other end, if used, or cut with a scalpel. Attach the syringe containing sucrose to one end of the straw and hold the other end over the dish containing 1 mL 1 M sucrose solution. Flush the contents of the straw and syringe into the dish and ensure good mixing.
13. Immediately begin to look for the oocytes using the dissecting microscope (*see* **Note 17**). As soon as the oocytes are identified place them in one of the droplets of 1 M sucrose solution.
14. Immediately move the oocytes into the second droplet of 1 M sucrose solution. When the oocytes have been in contact with 1 M sucrose for a total of 5 min, transfer the oocytes into a droplet of PBS and leave for 10 min at room temperature. Move the oocytes into the second droplet of PBS and leave them for 10 min on the hotplate.
15. The oocytes should then be placed in oocyte culture medium, within an incubator, (2−3 h for human, 30 min for mouse) prior to addition of sperm (*see* **Note 13**).

4. Notes

1. Ice nucleation can be initiated automatically by some freezing machines or can be initiated manually by removing the straws from the machine and touching the solution with forceps or cotton tipped applicators/cotton swabs that have been pre-cooled in liquid nitrogen or by insertion of an ice crystal from a pipette tip.
2. Straws can be sealed by heating of the ends (and is required for clinical samples), although care must be taken not to heat the solution containing the oocytes, or by inserting plugs,

which should be wet in order to give a good seal, or with sealing powder.

3. Ideally, oocytes should be stored individually within straws and the straws kept in at least two different locations so that should an accident occur with one straw then all is not lost. Accurate records should be kept of their location within the storage vessel.
4. The straw can be filled using a small syringe and needle. Pushing the cotton plug along the straw will reduce the capacity of the straw. The plug should be wetted as this will help prevent liquid nitrogen entry. If the plug is wetted by insertion of a sizeable column of the primary thawing/dilution solution (in this case a 4 cm column of 1 M sucrose solution), then expulsion of this solution along with the oocytes following thawing will aid dilution of the CPA. An air gap (e.g. a 0.5 cm column) should be left between this section (1 M sucrose solution) and the area to contain the oocytes - a 1 cm column of CPA. Straws should be clearly labelled, with an appropriate marker.
5. The straw contents can be expelled upon thawing by inserting a 1ml syringe containing at least 0.5 mL of primary thawing/dilution solution and expelling the contents of both the straw and syringe into a dish.
6. Studies have been performed with oocytes that have been denuded of cumulus cells (by means of treatment with hyaluronidase and/or gentle pipetting) prior to freezing or frozen with the cumulus intact. No clear advantage either way is evident. Human oocytes ought to be denudated within 37−40 hours post -hCG administration for ovulation induction and to be vitrified directly after denudation. Cryopreservation of denuded oocytes is routine method, however it has been shown that mouse oocytes with remaining intact cumulus cells successfully survive VS in calcium-free medium with ethylene glycol as CPA **[73]**.
7. Faster cooling/warming rates can be achieved and the risk of ice crystal formation reduced, by using straws that have been pulled to reduce the wall thickness, or by use of such devices as nylon loops or microscope grids. However, such devices are even more susceptible to

temperature change and some of these systems are ‘open’ i.e. the sample is in direct contact with liquid nitrogen (*see* **Note 12**).

8. A piece of plastic that can be inserted into a straw and is approximately twice the length of a straw is ideal. The plastic is inserted into the end of the straw and then bent through 90º. The straw can then be placed close to the nitrogen liquid surface in the horizontal position and the holder can be held by hand in the cold nitrogen vapour at a safe distance from the liquid nitrogen. A floating straws carrying device (CryoFloater, Nidacon, Sweden), that provides a constant distance between the straws and the nitrogen surface, thereby allowing freezing in the cold nitrogen vapour, can be also the option.
9. Addition of the high concentrations of CPA used in VS solutions directly to the PBS medium will result in reduction of the salt concentrations within that medium. Using a concentrated PBS medium allows the CPA to be added prior to the addition of water to give the required volume of single strength PBS medium with appropriate isotonic salt balance.
10. The VS solution described here could be replaced with other combinations of CPA, for example, ethylene glycol together with sucrose, or ethylene glycol plus propanediol plus sucrose often in combination with a macromolecule such as Ficoll or trehalose, which has been used successfully for the preservation of bovine and human oocytes, or in combination with newly applied cryoprotective agent carboxylated ε-poly-L-lysine resulted in production of live offspring in mice **[74]**. Exposure to this CPA mixture can be performed at 37°C thereby avoiding any cooling-related damage.
11. In order to adhere to the timing of each step of the procedure oocytes should be processed in small batches.
12. It should be noted that straws allow rapid heat transfer and hence are susceptible to temperature change during handling, for example on transfer from the cooling machine to

liquid nitrogen for storage and during handling for identification purposes prior to thawing. Straws can be stored in the liquid or vapour phase of liquid nitrogen. If stored within the liquid phase care should be taken to prevent leakage of liquid nitrogen into the straw as this will expand on warming and may cause the straw to explode or crack. Also, the liquid nitrogen may be contaminated with viruses, which have been shown to survive such temperatures. Straws can also be placed within a second vessel, for example a second larger straw (straw in straw method), to reduce liquid nitrogen entry. One end of the larger straw should be pre-sealed and plunged into liquid nitrogen, then VS straw containing oocytes placed inside of it and another end of the covering straw accurately sealed avoiding warming of the sample. However, debate continues about the impact on cooling and warming rates which enclosure in a second straw produce, and whether this has an impact on oocyte survival **[6]**. If storing in the vapour phase is chosen, the level of liquid nitrogen in the storage dewar should be carefully monitored to ensure stability of storage temperature. Alarms and automatic filling systems are available, but it is recommended that the level of liquid nitrogen still be checked manually at regular intervals.

13. There is evidence to suggest that the microtubular spindle is capable of repair during a period of culture post thaw.
14. The presence of the sucrose solution on the internal sides of the straw would dilute the concentration of CPA in the VSDP column and, more importantly, may allow the propagation of ice crystals from the sucrose solution into the area within which the oocytes are contained.
15. Care must be taken not to dilute the CPA concentration in these small droplets, the use of small droplets aids location of the oocytes in these viscous solutions.
16. The straw is held at –140°C (below the glass transition temperature range for aqueous CPS mixtures), rather than plunging directly into liquid nitrogen in order to reduce the

occurrence of cracks in the vitrified glass which, on warming, may form sites of ice nucleation. It is important to measure the temperature as extreme fluctuations in temperature are present across a few centimetres in liquid nitrogen vapour. Higher cooling/warming rates are recommended when lower CPA concentrations are used and oocyte is vitrified in the low - less than 1 μL volume - of the VS solution. Immediate warming in a large volume of warming solution at 37°C gives better survival if oocytes are vitrified in a closed system **[75]**.

17. Oocytes are particularly difficult to locate in this solution and operator practice is important. The oocytes must spend no longer than 5 min in 1 M sucrose solution at this point and this time can pass quickly. Other groups have developed post-vitrification dilution steps using 0.5 M sucrose solutions **[74]**, which could be applied here, although we have not direct evidence about their use with VSDP protocols.

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Figure captions

Fig. 1. A human embryo undergoing development from oocyte vitrified using Kitazato Cryotop® - Open System method and fertilized by intra cytoplasmic sperm injection. (A) Matured MII oocyte, recovered from a vitrification after warming and 3 hours incubation in the IVF medium (Vitrolife) at 5% O_2 and 6% CO_2. After ICSI the oocyte retains good refractive cytoplasm with intact plasma membrane, typical 1st polar body and intact, homogenous zona pellucida. (B) Zygote 16 hours after insemination. (C) Two-cell embryo 27 hours after insemination. (D) Four-cell embryo 44 hours after insemination. (E) Morula 94 hours after insemination. (F) Blastocyst 114 hours after insemination.

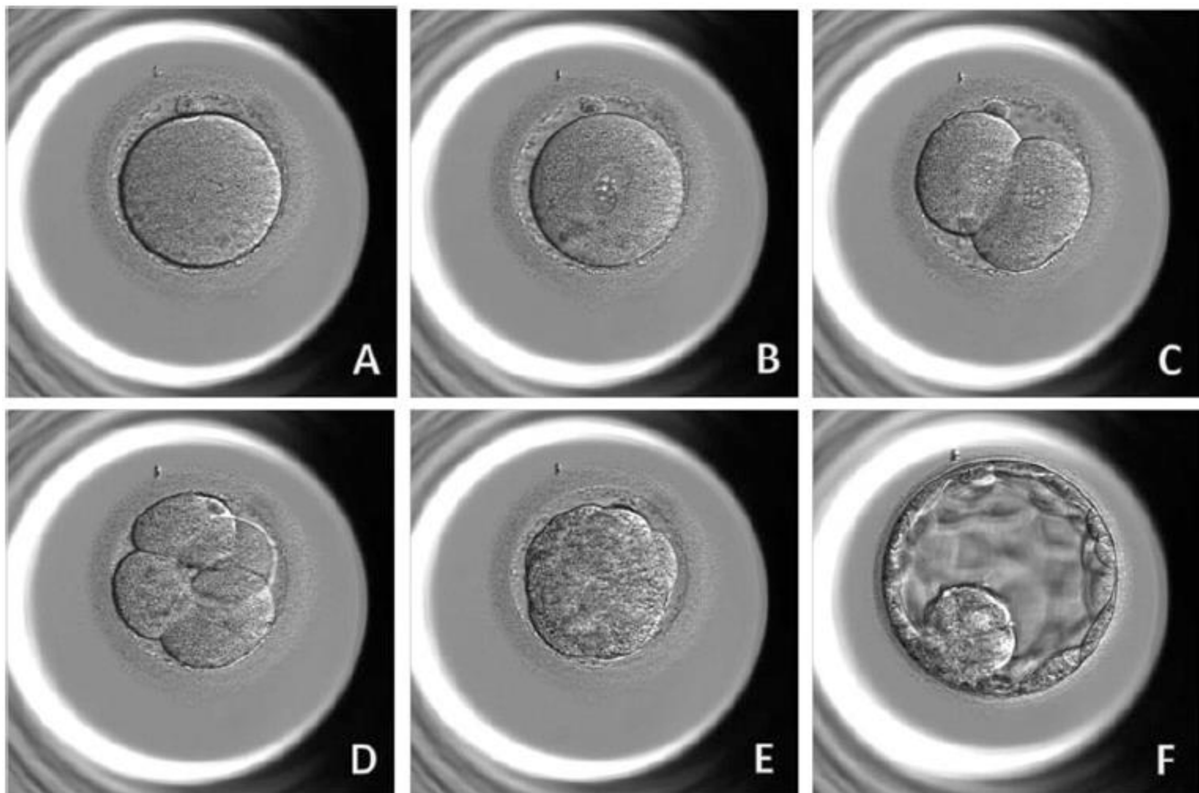

Fig. 2. Dynamical changes in the shape of mature human oocyte during equilibration in CPA solutions according to the Kitazato Cryotop® - Open System vitrification protocol. (A) M2 oocyte in the basic solution (BS) before addition of equilibration solution (ES). (B) Reduced volume of oolemma and concurrent increased perivitelline space in the shrunken M2 oocyte after edition of ES containing CPA. (C) Expanding M2 oocyte immersed in ES. (D) Completely expanded oocyte after immersing in ES showing width of perivitelline space equal to non-immersed oocyte. (E) Dehydrated shrunken oocyte in the vitrification solution just before transferring to the Cryotop and plunging into liquid nitrogen.