



Use of X-Ray Computed Tomography for Monitoring Tissue Permeation Processes

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Abstract

Cryoprotectants are essential to prevent ice formation during tissue cryopreservation procedures. However, the control of their concentration and spatial distribution in the tissue is necessary to avoid toxicity and other damages associated with the cryopreservation procedures, especially for bulky samples such as tissues and organs. X-ray computed tomography measures the attenuation of an X-ray beam when it passes through a substance, depending on the material properties of the samples. The high electronic density of the sulfur atom of the dimethyl sulfoxide makes it an excellent cryoprotectant to be assessed by X-ray CT, and its concentration is proportional to the X-ray attenuation either at room or cryogenic temperatures. In addition, this imaging technique also allows to detect the formation of ice and eventual fractures within tissues during the cooling and warming processes. Therefore, X-ray CT technology is an excellent tool to assess and develop new cryopreservation procedures for tissues and organs.

Key words Tissue permeation, X-ray computed tomography, Tissue and organ cryopreservation, Cryoprotectant concentration assessment, Ice formation, Tissue fractures formation

1 Introduction

Whereas a wide variety of cell types can be cryopreserved, either by the traditional slow freezing protocol or by vitrification, the creation of a biobank for tissues is still a challenge. The larger volume and complexity of tissues and organs imply heat and mass limitations during cryopreservation procedures making them more likely to be damaged by ice, cryoprotectant toxicity, and fractures during the cooling and warming processes. Therefore, cryopreservation of large samples requires accurate control of temperature and cryoprotective agent (CPA) concentration. We have established the use of X-ray computed tomography technology to noninvasively monitor cryopreservation protocols and assess CPA concentration for the particular case of dimethyl sulfoxide at lower acceleration voltage (65–75 kV) [1].

X-ray CT devices measure the attenuation of an X-ray beam when it penetrates a substance. The beam intensity is reduced according to the attenuation coefficient and the mass density of the material [2]. This attenuation can be attributed to several physical processes, whose predominance depends on the energy used in the CT devices and therefore on the X-ray acceleration voltage. Compton scattering is predominant in conventional CTs, in which case the attenuation is proportional to the density matter. However, at lower energies, the photoelectric effect governs the attenuation process, depending mainly on the atomic number [3]. Whereas most cryoprotectants are alcohols, composed of carbon, hydrogen, and oxygen, the higher atomic number of the sulfur atom in dimethyl sulfoxide (Me_2SO) makes the X-ray attenuation proportional to the concentration for this particular CPA. This makes Me_2SO more suitable for CT imaging of CPA permeation processes in tissues compared to other CPAs.

X-ray CT has already been used to map crystalline and amorphous phases in frozen samples [4] with applications to cryosurgery and cryopreservation, although a higher acceleration voltage was used (420 kV).

We have applied this CT imaging technique to assess cryopreservation procedures of several tissues. We visualized the formation of ice crystals (up to 2 μL) inside vitrified kidneys [5]. We have also used X-ray computed tomography for ovarian tissue cryopreservation: we analyzed and optimized freezing procedures for bovine [6] and human ovarian tissue [7]. We developed a different procedure for ovarian tissue cryopreservation based on a slow vitrification procedure [8], consisting of a gradual increase of the vitrification solution concentration while decreasing the temperature. Finally, we have characterized the cooling process for a Me_2SO solution in order to minimize the formation of fractures by studying the influence of some parameters: cooling rates, type of vials and insulating containers, degasification, and orientation of the container [9, 10].

To summarize, this patented X-ray imaging technique [11] is an excellent tool to monitor any cryopreservation procedure and to obtain 3D images of tissues and organs with a spatial resolution of up to 50 μm .

2 Materials

2.1 CT Imaging

1. A NanoCT device (Bioscan NanoCT[®], USA; currently Mediso, Hungary), with an acceleration voltage range of 45–75 kV (*see Note 1*).
2. A cylindrical carbon fiber CT bed with dimensions according to the CT device.

3. Software for image acquisition: NuLine (Mediso, Hungary).
4. Software for 3D image reconstruction: IVS image processing (Invicro, USA).
5. Software for image analysis: PMOD 3.7 (PMOD Technologies LLC, Switzerland).

2.2 Cooling Equipment for CT Measurement of Cryopreserved Samples

1. A liquid nitrogen dewar (about 2 L of volume) containing a copper coil of about 10 mm inner diameter and the maximum length and width possible to fit inside the dewar (*see Note 2*).
2. A compressed nitrogen gas tank with a pressure-reducing valve to control the nitrogen gas flow.
3. A polypropylene tube of 20 mm inner diameter from the compressed nitrogen gas outlet to the copper coil described above (room temperature gas) and a polystyrene tube of 20 mm inner diameter and 20 mm of thickness from the copper coil to the insulating container described below (cooled nitrogen gas), with dimensions according to the carbon fiber CT bed (*see Note 3*).
4. A cryochamber for holding the sample to image, made of a cylindrical insulating container of polystyrene (60 mm outer diameter maximum), with several tubes of 1.5 mm inner diameter connected at the end of the tube system described above, through which the cooled nitrogen gas passes (*see Figs. 1 and 2*).
5. A type K thermocouple, located inside the insulating container described above and connected to a data logger (TC-08 Temperature Data Logger, Picolog).
6. A small computer (e.g., notebook) to install the Picolog software to monitor the temperature during the CT measurements.

2.3 Solutions and Containers

1. Isotonic phosphate-buffered solution (PBS) as solvent, prepared from deionized water (NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.44 g/L, KH₂PO₄ 0.24 g/L) and adjusted to pH 7.4.
2. Dimethyl sulfoxide as cryoprotective agent in concentrations ranging from 5% to 70% (v/v).
3. There are two conditions that are going to influence the selection of the material and the dimensions of the sample containers. The material used for containers has to be transparent to X-rays, in order to avoid interferences in the images (*see Note 4*). The dimensions of the containers are limited by the size of the CT bed, and also depend on the size of the samples to be analyzed (*see Note 5*).

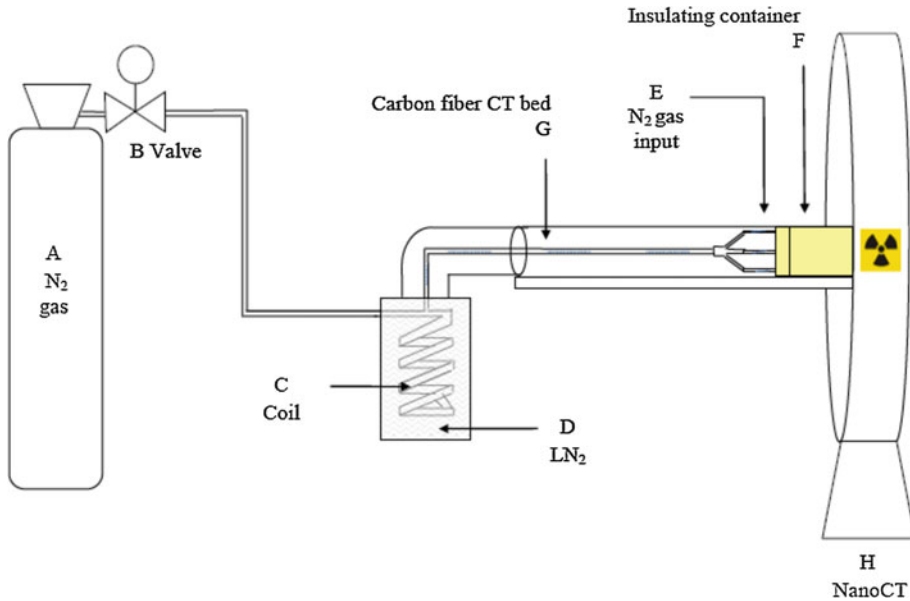


Fig. 1 Scheme of the cooling system for CT measurements of cryopreserved samples. The flow of the nitrogen gas (A) is controlled through a pressure-reducing valve (B) to the copper coil (C), which is immersed in liquid nitrogen (D), for the heat exchange. Then, the cooled nitrogen gas enters through some tubes (E) inside the insulating container (cryochamber) (F), where the sample is placed once the temperature is low enough. The insulating container is placed inside the carbon fiber bed (G) of the NanoCT device (H). (Figure adapted from [1])

3 Methods

3.1 CT Calibration at Room Temperature

1. Prepare the different Me₂SO solutions in PBS or other solvent, in several concentrations within the range of concentration you expect to obtain in your sample, and add a sample of each concentration in a different container, including a sample of the solvent with no CPA and a sample with Milli-Q water. Try to prevent air bubbles when filling the containers. The containers should be the same to the one you are going to use for analyzing your biological sample. Other CPAs could be used, depending on the characteristics of the CT device used, but the difference of attenuation for each concentration will not probably be enough to use it as quantification, as can be seen in Fig. 3.
2. Place the cooling system on the CT device, as can be seen in Fig. 1. Even though CT measurements are made at room temperature (RT), the conditions for the CT imaging should be the same ones.
3. Place the first sample inside the insulating container, and use a sample holder in order to have all the samples in the same position (*see Note 6* and Fig. 2).

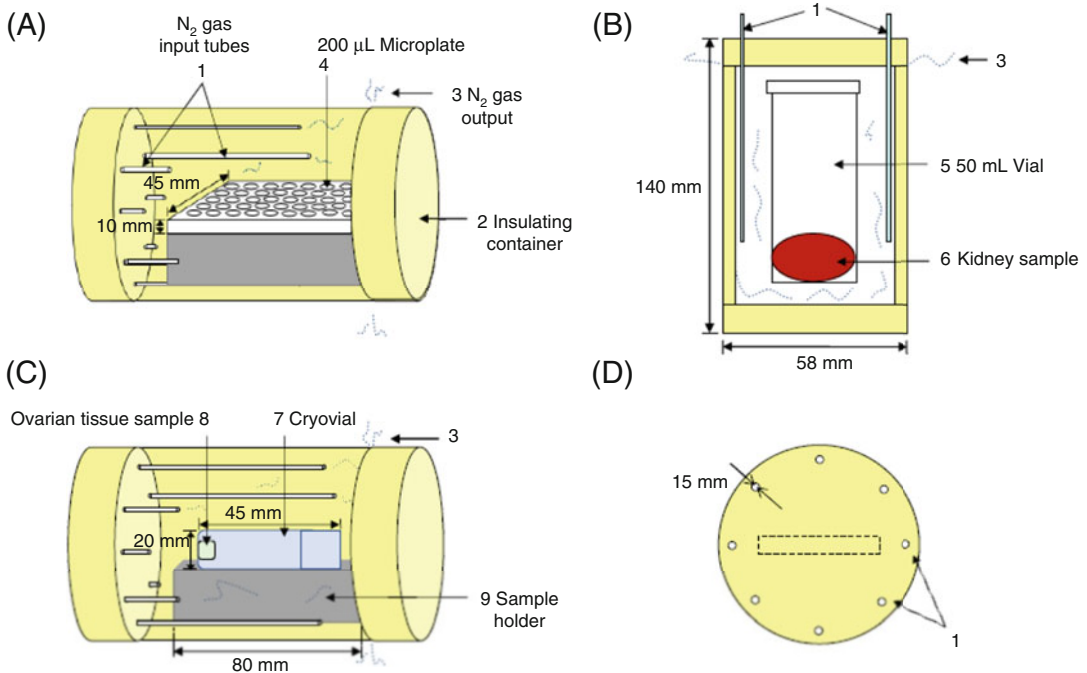


Fig. 2 Design of the holding cryochamber for cryopreserved samples to be imaged. In order to keep the samples below the glass transition temperature during the CT measurements, this specific chamber was built, consisted mainly of a cylindrical insulating container made of polystyrene. **(a)** 3D view and details of the cryochamber. The cooled nitrogen gas enters through eight 1.5 mm inner diameter tubes (1) into the insulating container (2). The nitrogen gas leaves the container through the cap (3). The dimensions of one of the containers used, the 200 µL well microplate (4), are also shown (mm). **(b)** Front view of the system and dimensions of the insulating container (mm), showing a 50 mL vial (5) used for imaging a rabbit kidney sample (6). **(c)** 3D view and details of the cryochamber, showing in this case another vial typically used for imaging, a cryovial (7), for ovarian tissue samples (8). The cryovial is laid on a polystyrene holder (9), to hold the vial at the center of the image. **(d)** View and dimensions (mm) of the nitrogen gas input tubes (1) inserted in the cap of the insulating container. (Figure adapted from [1, 8])

4. Open the CT acquisition software, create a new patient, and identify each sample with an ID number, the name of the solution, and date. Make a topogram, and select the region of interest to image, where your sample is located. Try to select the shortest possible length in order to get a single rotation of the CT gantry; this way the acquisition time will be lower (*see Note 7*). Make sure the thermocouple is out of the area to image.
5. Select the following acquisition parameters: 360 projections, pitch 1, ultra-fine frame resolution, 500 ms of exposure time, and 75 kV of the X-ray tube voltage. The total time of the image acquisition will depend on these parameters and on the length of the sample to image (*see Note 8*).

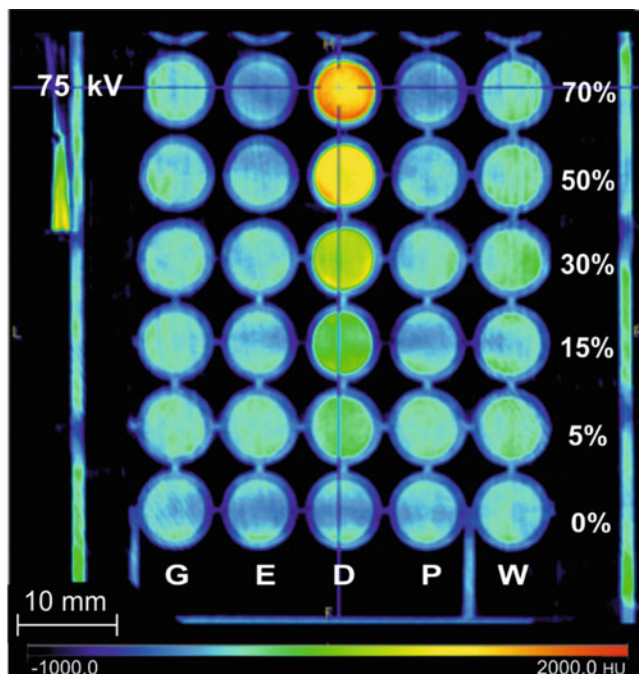


Fig. 3 CT image of different CPAs at different concentrations. The image shows the X-ray attenuation for different CPAs (*G* glycerol, *E* ethylene glycol, *D* dimethyl sulfoxide, *P* propanodiol) at different concentrations in PBS (0–70% v/v) and water (*W*), placed in a 200 μ L well microplate. It was acquired with a NanoSPECT/CT device, at a voltage of 75 kV and spatial resolution of 200 μ m. The color scale goes from a dark blue color for the lowest attenuation (–1000.0 HU) to an intense red for the highest one (2000.0 HU), showing that the attenuation increases with the concentration only in the case of the dimethyl sulfoxide (*D*). (Figure adapted from [1])

6. Open the reconstruction software and select the CT image, send it to batch CT, and reconstruct it to a spatial resolution of minimum 0.2 mm (*see Note 9*), using the filtered back-projection (FBP) algorithm.
7. Repeat the **steps 3–6** for all the samples (water, solvent, and each different Me₂SO concentration).
8. Open the analysis software, and select first the water image. Select the image scale that allows you to visualize better the different concentrations (*see Note 10*). Create a volume of interest (VOI) with the size and shape according to the biological sample you are going to analyze and at the same location where your sample is going to be placed (*see Note 11*). Use the statistic tool of the analysis software to get the average CT values of the VOI and the error, expressed as the standard deviation of all the CT values of each vowel contained within the VOI. Write down the average CT values of the sample of water.

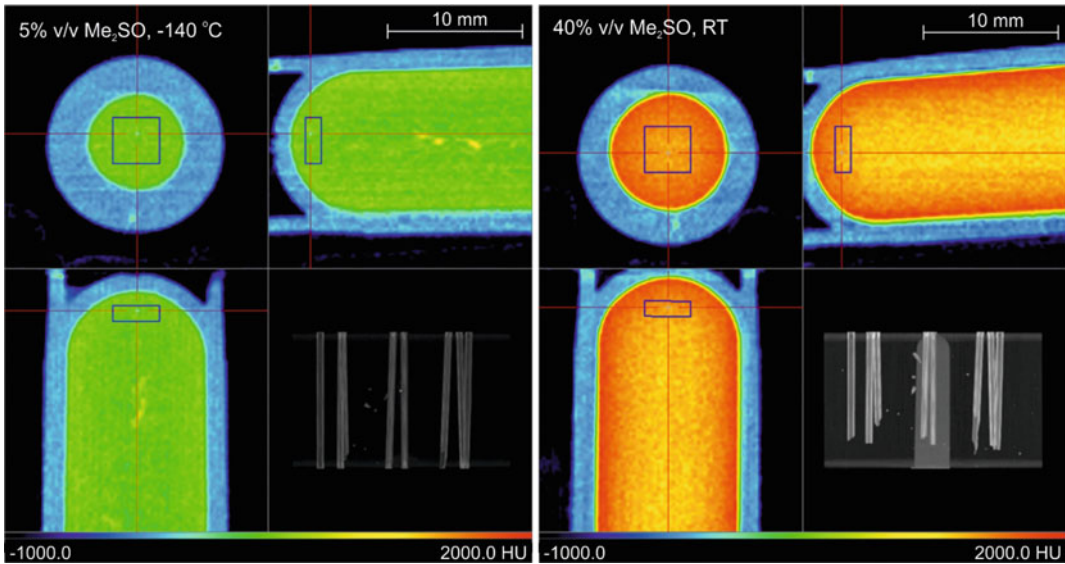


Fig. 4 Example of VOIs used in CT images for calibration. The figure shows the volumetric VOIs in blue, of dimensions $3 \times 3 \times 1 \text{ mm}^3$, for two different samples: a cryovial with a solution of 5% v/v Me_2SO in PBS, imaged at $-140 \text{ }^\circ\text{C}$ (left image), and a cryovial with a solution of 40% v/v Me_2SO in PBS, at RT (right image). The calibration curves were obtained for analyzing pieces of bovine ovarian tissue of $5 \times 5 \times 1 \text{ mm}^3$ dimensions and located at the bottom of the cryovial, where the VOIs were created. The acquisition of images was made at 75 kV, and images were reconstructed to a spatial resolution of 0.2 mm

9. Open the first sample of the Me_2SO solutions and select the color scale. Use the specific tool of the analysis to transform the CT values to Hounsfield Units (HU), the standard units for CT output, according to the following equation:

$$\text{HU} = 1000 \times \frac{\text{CT}_{\text{sample}} - \text{CT}_{\text{water}}}{\text{CT}_{\text{water}} - \text{CT}_{\text{air}}}$$

where HU is the X-ray attenuation in HU and CT in the raw units of the CT device (CT values). Use the CT values of water obtained at the **step 8** for (CT_{water}), and use 0 for the CT value of air (CT_{air}). Save the image in HU units.

10. Create a VOI with the same dimensions and at the same location to the one used for the sample of water (*see* Fig. 4). An easier option is to open the VOI created for water, and replace it in this sample. Save the VOI and get the average HU and SD of the VOI.
11. Repeat the **steps 8–10** for each sample of the Me_2SO solutions.
12. In a spreadsheet program (e.g., Excel), create a listing with the average attenuation (HU) for each Me_2SO concentration (% v/v), and perform the linear regression analysis to obtain the following calibration curve:

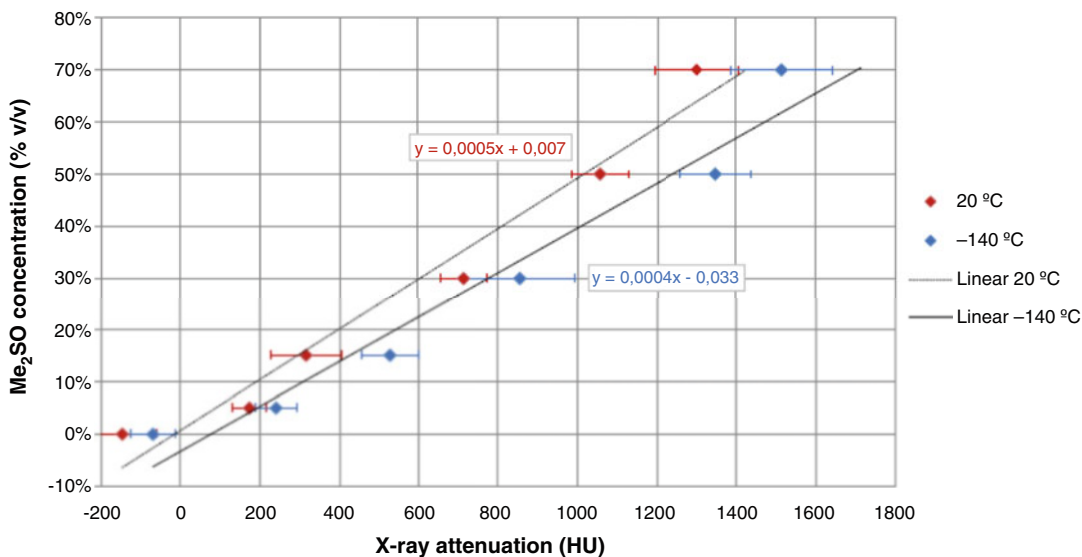


Fig. 5 Example of calibration curves at room temperature and $-140\text{ }^{\circ}\text{C}$: X-ray attenuation versus Me_2SO concentration. The graph shows the linear dependence between the Me_2SO concentration and the X-ray attenuation, both at room temperature and $-140\text{ }^{\circ}\text{C}$. These calibration curves will allow us to calculate the sample concentration from their CT images, through the linear equations $y(\%v/v \text{ Me}_2\text{SO}) = a * x(\text{HU}) + b$

$$[\text{Me}_2\text{SO}] = a * \text{HU} + b$$

being $[\text{Me}_2\text{SO}]$ the Me_2SO concentration, in % v/v, HU the average attenuation in HU, and a and b the slope and the y -intercept of the curve, respectively. *See* an example of this calibration curve at room temperature in Fig. 5.

3.2 CT Calibration for Samples Imaged Below $-140\text{ }^{\circ}\text{C}$

1. Place the cooling system on the CT device, as can be seen in Fig. 1.
2. Start the Picolog software to monitor the temperature inside the insulating container.
3. Open the pressure-reducing valve to the optimized outlet pressure of the nitrogen gas that allows to cool the system down fast enough to $-140\text{ }^{\circ}\text{C}$ and keep the temperature constant during the CT measurement (*see* **Note 12**).
4. Add liquid nitrogen inside the dewar until the copper coil is totally immersed in liquid nitrogen. It is recommended to use a cap of insulating material (not pressurized) to avoid much loss of liquid nitrogen by vapors.
5. Observe that the temperature is cooling down, and make sure that the nitrogen gas pressure keeps constant. Fill the dewar with liquid nitrogen whenever is necessary.
6. When the temperature has cooled down to $-140\text{ }^{\circ}\text{C}$, place the first of the samples prepared in **step 1** of Subheading 3.1 inside

the insulating containers (*see* Figs. 1 and 2), and wait for about 10 min to cool it down, depending on the size of the sample (*see* **Note 13**).

7. Check that the nitrogen gas pressure is the required one and the temperature is down to $-140\text{ }^{\circ}\text{C}$, and refill the liquid nitrogen of the dewar.
8. Repeat the **steps 4–7** of Subheading 3.1. Before the CT acquisition of each sample, wait until the temperature is cooled down to $-140\text{ }^{\circ}\text{C}$, wait for 10 min to cool it down, check the pressure of the nitrogen gas, and refill the liquid nitrogen of the dewar.
9. Once all the samples have been imaged, close the valve of nitrogen gas, and then turn off the Picolog software.
10. Repeat the **steps 8–12** of Subheading 3.1, and obtain the calibration curve for the samples imaged below $-140\text{ }^{\circ}\text{C}$ (*see* Fig. 5 as an example).

3.3 CT Acquisition of the Biological Samples After Cryopreservation

1. Proceed with your routine or any other cryopreservation protocol of the samples you wish to analyze under the CT. Store the cryopreserved samples in liquid nitrogen or any other usual storage.
2. Place the cooling system on the CT device, as can be seen in Fig. 1. Check that the insulating container and the holder you use are suitable for the container of your samples (*see* Fig. 2).
3. Repeat the **steps 2–5** of Subheading 3.2.
4. When the temperature has cooled down to $-140\text{ }^{\circ}\text{C}$, place the first sample to image inside the insulating container. Make sure you do this operation very quickly in order to avoid any devitrification of the sample.
5. Check that the nitrogen gas pressure is the required one and the temperature is down to $-140\text{ }^{\circ}\text{C}$, and refill the liquid nitrogen of the dewar.
6. Repeat the **steps 4–7** of Subheading 3.1. Before the CT acquisition of each sample, wait until the temperature is cooled down to $-140\text{ }^{\circ}\text{C}$, check the pressure of the nitrogen gas, and refill the liquid nitrogen of the dewar.
7. Once all the samples have been imaged, close the valve of nitrogen gas, and turn off the Picolog software.

3.4 CT Acquisition of the Biological Samples After Rewarming

1. Place the cooling system on the CT device, as can be seen in Fig. 1.
2. Proceed with your routine or any other warming protocol of the cryopreserved samples you wish to analyze under the

CT. For a better accurate washing analysis, it is recommended to consider the time of the image acquisition in the last washing step.

3. Repeat the **steps 3–7** of Subheading **3.1**.

3.5 Quantification of Me_2SO Concentration in the Biological Samples

1. Open the analysis software, and select first CT image of the sample to analyze. Select the color scale that allows you to visualize better the different concentrations (*see Note 10*). Use the specific tool of the analysis to transform the CT values to the standard units Hounsfield units (HU), according to the following equation:

$$\text{HU} = 1000 \times \frac{\text{CT}_{\text{sample}} - \text{CT}_{\text{water}}}{\text{CT}_{\text{water}} - \text{CT}_{\text{air}}}$$

where HU is the X-ray attenuation in HU and CT in the raw units of the CT device (CT values). Insert the average CT values obtained for water at the **step 8** of Subheading **3.1** for CT_{water} , and use 0 for the CT value of air (CT_{air}). Save the image in Hounsfield units.

2. The color scale in the image indicates the areas of minimum and maximum Me_2SO concentration and, therefore, the areas where ice is likely to have formed (*see Fig. 6*). Select a volume of interest (VOI) with the size and shape according to the biological sample you are going to analyze and at the location where your sample was placed (*see Note 11*) or any other area you are interested to analyze (*see Fig. 6*). Use the statistic tool of the analysis software to get the average HU values of the VOI and the error, expressed as the standard deviation (SD) of all the CT values of each voxel contained within the VOI. Save the VOI and copy the average HU and SD of the VOI in a

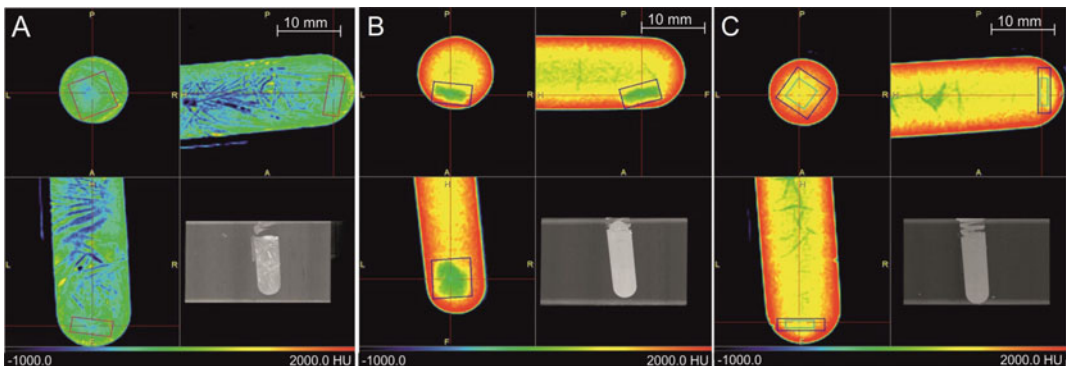


Fig. 6 CT image of cryopreserved ovarian tissue. The figure shows the CT images of $5 \times 5 \times 1 \text{ mm}^3$ pieces of bovine ovarian tissue cryopreserved by different protocols: (a) slow freezing, with a $5 \times 5 \times 1 \text{ mm}^3$ pink VOI for analysis; (b, c) stepped vitrification, with a $5 \times 5 \times 1 \text{ mm}^3$ blue VOI in (b) and a $5 \times 5 \times 1 \text{ mm}^3$ cyan VOI in (c). (Figure adapted from [6, 8])

spreadsheet (e.g., Excel). If you are also interested in the Me₂SO concentration range within the sample, you can also select and copy the minimum and maximum attenuation within the VOI.

3. Repeat the **steps 1–2** for each sample. Remember the option of opening the first VOI created, and replace it in the new sample. It is recommended to use the same size and shape of VOIs for similar samples.
4. To calculate the Me₂SO concentration (% v/v), use the calibration curve obtained in Subheading 3.1 at RT, in the case of rewarmed samples, or the one obtained in Subheading 3.2 at –140 °C, for the cryopreserved samples. Introduce the average attenuation (HU) of the VOI in the equation of the calibration curve to obtain the Me₂SO concentration:

$$[\text{Me}_2\text{SO}] = a * \text{HU} + b$$

Do the same to calculate the minimum and maximum Me₂SO concentration of the samples. *See* an example of Me₂SO ovarian tissue sample quantification, after cryopreservation and rewarming, in Table 1.

Table 1
Example of minimum, maximum, and average Me₂SO concentration within several ovarian tissue samples

ID number	VOIs dimensions (mm ³)	Maximum concentration (% v/v Me ₂ SO)	Minimum concentration (% v/v Me ₂ SO)	Average concentration (% v/v Me ₂ SO)
ID 2241 Control (10% Me ₂ SO)	5 × 5 × 2.5	39.0%	0.0%	8.0%
ID 2242 Control (10% Me ₂ SO)	5 × 5 × 2.2	27.0%	0.0%	8.4%
ID 2245 Group 5 (5% Me ₂ SO)	5 × 5 × 2.4	54.0%	0.0%	3.0%
ID 2246 Group 5 (5% Me ₂ SO)	5 × 5 × 2.5	54.0%	0.0%	3.0%
ID 2247 Group 6 (20% Me ₂ SO)	5 × 5 × 2.0	43.0%	3.0%	22.0%
ID 2248 Group 6 (20% Me ₂ SO)	5 × 5 × 2.1	44.7%	7.9%	19.2%

The samples were imaged at –140 °C, and the Me₂SO concentration was calculated from the attenuation values of the VOIs created within the tissues and through the calibration curve previously calculated

4 Notes

1. The CT device should have the option of using lower acceleration voltage than conventional ones (about 70 vs 120 kV), in order to obtain a higher predominance of the photoelectric effect and a higher dependence on the atomic number.
2. It is recommended the maximum width of the coil possible and the maximum number of turns according to the dimensions of the dewar, in order to ensure a better heat exchange between the liquid nitrogen and the nitrogen gas.
3. The dimensions of the polystyrene tube must be the maximum allowed to fit inside the CT bed; in our case the outer diameter must be lower than 60 mm. The total length of the polystyrene tube is the length needed from the copper coil to the cryochamber described below, in our case, about 700 mm. It is also necessary the use of connectors for the joining of different tubes (e.g., the copper coil and the polystyrene tube, which have a different diameter).
4. It is recommended to test containers of different materials in order to find out the interaction in the images. We selected the following materials after our tests: polypropylene (PP), polyethylene (PE), and polystyrene (PS).
5. We used these following materials depending on the sample to analyze: 2 mL cryovials (for ovarian tissues), 200 μ L microplates (for solutions of different CPA concentrations), and 20 and 50 mL vials (for bigger samples, e.g., rabbit kidneys).
6. In order to get a centered image and the same position for all the samples to image, it is recommended to use a holder for the sample. Remember the holder must be made of an X-ray transparent material (e.g., polystyrene) and must fit inside the cryochamber (*see* Fig. 2).
7. According to our NanoCT device, the maximum length to get a single rotation of the CT gantry is 37 mm (*z*-axis); so depending on the sample, you will need more than one rotation.
8. With these parameters and considering a selected area to image shorter than 37 mm (*z*-axis), the total time acquisition is 3 min. This time is relevant when imaging cryopreserved samples, since the temperature must be kept below the glass transition temperature for the entire duration of the measurement. Nevertheless, other parameters could be used according to the desired spatial resolution.
9. Our NanoCT device achieves a spatial resolution up to 50 μ m, although the higher the spatial resolution, the greater the noise. It is important to keep in mind the signal-to-noise ratio (SNR), which is relevant in imaging techniques.

10. In PMOD software for image analysis, there are many options of image visualization scale, from grayscale to different colors scales. In our analyses, we normally selected the “color scale,” which goes from a dark blue color for the lowest attenuation to an intense red color for the highest one, passing through green, yellow, and orange colors for intermediate attenuation in increasing order (*see* Figs. 3, 4, and 6).
11. An example of samples we have thoroughly analyzed is ovarian tissue (bovine and human). We normally prepare ovarian tissue samples in strips of about $5 \times 5 \times 1 \text{ mm}^3$ dimensions, which are placed at the bottom of the cryovial where they are going to be cryopreserved. To analyze the samples and quantify the X-ray attenuation and therefore the Me_2SO concentration inside the samples, we created VOIs of dimensions slightly shorter than the ovarian tissue samples, to make sure the VOIs contain only tissue. For example, a typical VOI dimension we use for these samples is $3 \times 3 \times 1 \text{ mm}^3$ (*see* Fig. 4).
12. For our CT measurements at temperatures below -140°C , we used an outlet nitrogen gas of about 0.4 bars. To optimize this pressure, previous tests should be performed to fix this parameter.
13. In the case of ovarian tissue samples, we use 1.8 mL cryovials which need around 10 min to cool at that temperature and with that nitrogen gas pressure. Previous tests are necessary for different vials, depending on the volume. To monitor the time needed to reach the required temperature, connect a thermocouple to the Picolog data logger and place the hot junction inside the container, immersed in the solution.

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