A tissue snap-freezing apparatus without sacrificial cryogens

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A tissue snap-freezing apparatus without sacrificial cryogens

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Abstract. Molecular technologies in cancer diagnosis require a fresh and frozen tissue, which is obtained by means of snap-freezing. Currently, coolants such as solid carbon dioxide and liquid nitrogen are used to preserve good morphology of the tissue. Using these coolants, snap freezing of tissues for diagnostic and research purposes is often time consuming, laborious, even hazardous and not user friendly. For that reason snap-freezing is not routinely applied at the location of biopsy acquisition. Furthermore, the influence of optimal cooling rate and cold sink temperature on the viability of the cells is not well known. In this paper, a snap-freezing apparatus powered by a small cryocooler is presented that will allow bio-medical research of tissue freezing methods and is safe to use in a hospital. To benchmark this apparatus, cooldown of a standard aluminum cryo-vial in liquid nitrogen is measured and the cooling rate is about -25 K/s between 295 K and 120 K. Sufficient cooling rate is obtained by a forced convective helium gas flow through a gap formed between the cryo-vial and a cold surface and is therefore chosen as the preferred cooling method. A conceptual design of the snap-apparatus with forced flow is discussed in this paper.

1. Introduction

Worldwide cancer is the third most common cause of death, one of eight deaths is cancer related [1]. Biopsy of suspected cancerous tissue is a crucial procedure to guide cancer diagnosis and treatment. Obtained tissue specimens are examined to determine the presence of malignant, invading cells, to identify markers indicative of the primary tumor. Acquisition of a tissue and subsequent processing are usually performed by different experts, usually at separate locations.

The most common and widely adopted method to prepare a tissue sample for sectioning and diagnosis is formalin and paraffin embedded (FFPE) process [3]. The FFPE procedure takes time for fixation, therefore for a rapid intra-operative diagnosis tissue samples are generally frozen in a cryogenic coolant such as solid carbon dioxide, liquid nitrogen or liquid isopentance cooled with liquid nitrogen [2]. It is suggested that FFPE tissue is not suitable for emerging molecular diagnostic procedures among others genomic, transcriptomic and proteomic profiling. Therefore in research laboratories, additional tissue samples are frozen for molecular diagnostics and research purposes.

Current practise in a hospital where the biopsy is performed does not include a simple and reliable solution to obtain fresh and frozen tissue. Variation between the used freezing procedures results in different freezing rates [4]. Differences may occur when the same type of tissue is frozen by different freezing procedures because a lot is still unknown about the ideal freezing rate for certain types of tissues. The ideal cooling rate depends on the cells critical volume (surface

area of he membrane, surface to volume ratio, permeability of the plasma membrane). More standardized and fixed freezing procedures per type of tissue is likely to improve the tissue quality and will minimize the variables between the same types of a tissue.

Commercial snap-freezing devices use coolants such as dry ice or liquid nitrogen, and a heater to control the cooling rate. The cooling rate of Mr. FrostyTm freezing container is maintained at -1 K/min to a temperature of 193 K [5]. CoolRack uses dry ice and can cool the vials to 193 K in 1-2 minutes [6] . The HistoChill uses 3M Novec 7000 as a liquid coolant bath cooled with a mechanical refrigerator [7]. Higher freezing rates compared to CoolRack is reported. Stand-alone Gentle Jane is a portable device that uses liquid nitrogen with a controllable freezing rate [8]. All these commercial devices use sacrificial cryogens and cannot be used in the room where the biopsy procedure is performed.

In this paper, the design of a tissue snap-freezing apparatus is presented that will allow tissue processing at the location where the biopsy is performed. In addition, the apparatus has additional features to facilitate research on the impact of freezing characteristics on the viability of the tissue. The main requirements of the apparatus are elaborated below:

- Adjustable cold sink temperature between 80-200 K.
- Cooling rate may be varied with a minimum value lower than the liquid nitrogen quenching rate
- No use of dangerous substances and limited venting of gases in line with the safety norms of the operation theatre.
- Standard cryo-vials should be used.

In order to quantify and benchmark the apparatus to established cooling protocols, experiments are performed to determine the cooling rate of a standard aluminum cryo-vial in liquid nitrogen. The experimental data of cooling of a beef specimen in a cryo-vial is presented in the next section followed by the systematic design of the snap-freezing apparatus.

2. Quenching of a cryo-vial in liquid nitrogen

Quantitative information on the freezing rate and the duration of quenching is not well documented in the literature. TuBaFrost (European Human Frozen Tumor Tissue Bank) recommends quenching in liquid nitrogen for atleast 5 seconds [9]. In practise the hospitals whom the authors consulted quench the cryo-vials with the tissue in it for about 10 seconds [10]. Quenching experiments in liquid nitrogen are performed with commercially used cryo-vials. To quantify the cooling rate a standard cryo-vial made of aluminum of weight 1.4 g, an outer diameter of 14.5 mm and a height of 24.2 mm is used.

A type-E thermocouple is attached to the inner wall of the cryo-vial through a hole drilled on the side wall, which is later closed with a glue. The accuracy of the thermocouple is $\pm$ 3 K. A special cryo-vial holder is designed to reduce the thermal mass around the cryo-vial. Quenching experiments in liquid nitrogen are performed with an empty cryo-vial (Exp #1) and with a sample of beef acquired using a 14G needle (Exp #2). The beef specimen weights about 15 mg. In the case of beef experiments, an additional thermocouple is inserted in the beef specimen.

Figure 1 shows the inner wall temperature of an empty cryo-vial (Exp #1) during cooldown. Two distinct cooling regions are visible in the plot, corresponding to the pool boiling characteristics, namely film and nucleate boiling regime. Initially the temperature difference of the cryo-vial surface and the liquid nitrogen saturation temperature $\approx$ 77 K is large causing a thin vapor layer of nitrogen to form at the interface (film boiling regime). When the cryo-vial surface temperature decreases below $< \approx$112 K, the vapor layer collapses, initiating liquid-solid contact and transforms to a columns of bubbles (nucleate boiling regime). Optical observations with a high speed camera reveal the two boiling regimes and support the above arguments.

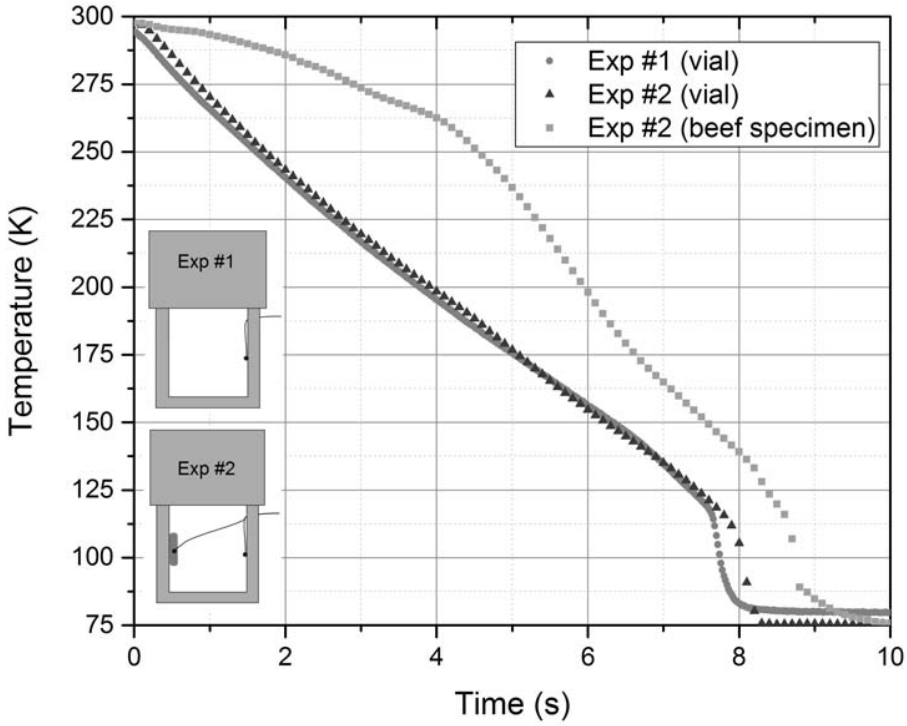

Figure 1. Temperature measurement of the cryo-vial inner wall while cooling in liquid nitrogen, with out (Exp #1) and with a beef sample (Exp #2).

In Exp #2 with a beef specimen, the cryo-vial inner wall temperature shows similar characteristics as in Exp #1. The beef specimen temperature shows a discontinuity and a rather gradual slope around 273 K, which is due to the latent heat content of water. The difference in slope of the temperature plot above and below the liquid-solid transition is due to the differences in specific heat capacity of liquid and solid water. The large temperature difference between the beef specimen and cryo-vial inner wall can only be explained by a large thermal contact resistance between them as the heat capacity of the beef specimen is much lower than the cryo-vial.

For freezing tissues, the cooling rate between 300 - 150 K is of interest, as below 150 K the cell morphology in the tissue do not change. The experiments discussed above, shows that in the temperature range of 300-150 K, a cooling rate of about -25 K/s is achieved. Therefore the goal is to obtain a cooling rate below this value in the snap-freezing apparatus.

3. Assessment of various cooling principles

In this section, a brief assessment of the various cooling principles is elaborated followed by experimental verification of the chosen cooling principle.

3.1. Quenching in a pool of liquid coolant
This is a popular method used to snap-freeze biological materials. Some of the coolants used are liquid nitrogen, isopentane or ethanol cooled in liquid nitrogen. Due to safety concerns with the use of liquid nitrogen in a operation theatre, the tissue is usually frozen in a pathology laboratory. Since molecular changes in the cells can occur within minutes after excision from the body, this cooling approach requires dedicated expedited transport and the presence of trained personnel. In-situ production of a small quantity of liquid nitrogen using a cryocooler and spray cooling the cryo-vial is a possible alternative. However, with liquid cryogens it is not easy to adjust the cold sink temperature.

3.2. Solid contact
In this procedure, a vial is directly brought in contact with a cold solid surface, greased with a thin layer of isopentane to bridge the surface roughness. A 10 micron thick layer of isopentane provides a heat transfer rate of about 1 $W.cm^{-2}K^{-1}$. The disadvantages of this approach are: the cooling rate cannot be altered; the cylindrical side wall of the cryo-vial require a robotic arm in cryogenic environment to make physical contact with the heat sink.

3.3. Forced convective flow
A pre-cooled non-condensible gas is force flowed through a gap formed by a cryo-vial outer wall and a cold surface of the thermal storage container. When a gap size of sub-mm is used the flow can be considered to be in the laminar flow regime, resulting in a heat transfer coefficient that is proportional to the ratio of the gas thermal conductivity and the gap size. The enthalpy carried by the gas flow is proportional to the mass flow rate allowing control of the cooling rate of the cryo-vial. The advantage of this approach is the possibility of controlling both the cold heat sink temperature and the cooling rate.

Experimental verification of forced convective flow cooling:
A vial-container is designed and a test rig is constructed as shown in Figure 2 to verify the performance of the forced flow technique. In these experiments helium gas flow is allowed to flow in a coiled stainless steel heat exchanger with liquid nitrogen as the cooling medium. The vial-container is immersed in a pool of liquid nitrogen. The polypropylene vial-container is additive manufactured with inlet and outlet ports for the gas flow. The gap between the cryo-vial and the vial-container is 1.4 mm. A type-E thermocouple is inserted in the cryo-vial similar to experiments discussed above. The cold helium gas enters the vial-container from the bottom side and flows out from the top side.

The measured temperature of the inner wall of the cryo-vial is shown in Figure 3. Quenching data of the cryo-vial in liquid nitrogen is also shown in the same figure for comparison purposes. As expected with increase in mass flow of helium gas the cryo-vial cooling rate also increases, mainly due to the increase in flow heat capacity. A helium gas flow rate of about 40 $mg.s^{-1}$ provides a cooling rate of -25 $K.s^{-1}$ between 300 K to about 230 K. At higher flow rates above 100 $mg.s^{-1}$ the cooling rate is larger than that of cooling in liquid nitrogen, in the temperature range of interest 300 K to 150 K.

Although at first sight, the flow rate seem to be rather high to realize a compact snap-freezing apparatus, it must be noted that the heat transfer coefficient scales with inverse of the gap size. In these experiments, a wider gap of 1.4 mm is used due to the ease of additive manufacturing and quick tests. A gap size of 0.2 mm is within the reach of conventional fine machining methods. It must also be noted that polypropylene is used for producing the vial-container. Using the properties of polypropylene at room temperature (α, thermal diffusivity), the thermal diffusion depth ($\approx \sqrt{\alpha t}$) is about 1 mm when a diffusion time (t) of 10 s is considered. To reduce this

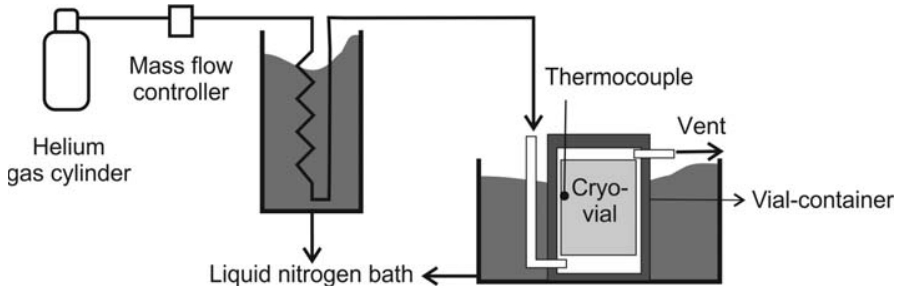

Figure 2. Test rig to characterize the cooling of a cryo-vial with forced flow of helium gas.

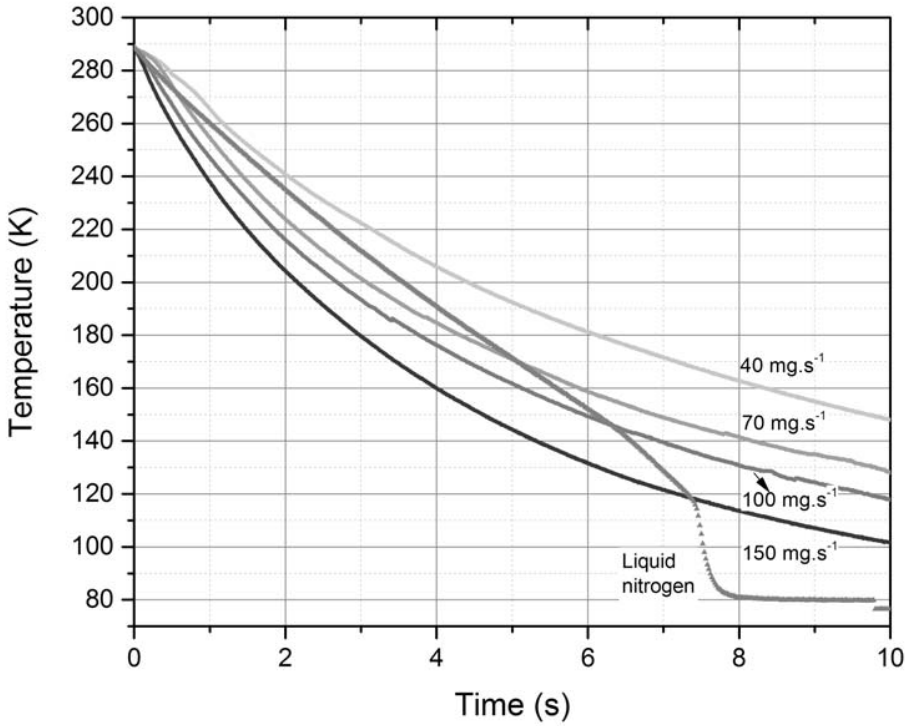

Figure 3. Temperature measurement of the cryo-vial inner wall for several helium gas flow rates.

thermal skin effect, a material with higher thermal diffusivity such as aluminum or copper is recommended. This measure reduces the requirement of the helium gas flow rate.

4. Conceptual design of the snap-freezing apparatus

Following the assessment of the cooling principles in the previous section, the forced convective flow principle satisfy all the apparatus requirements detailed in section 1. The main objectives of the design process is compactness (size) and energy efficiency. From the usage perspective,

Table 1. Main components of the snap-freezing apparatus and a description of their function.

Component	Function
Cryocooler	Generates cooling
Thermal Energy Storage Unit (TESU)	A cold buffer
Gas-flow circulator	Controls helium gas-flow in the apparatus
Counter Flow Heat Exchanger (CFHX)	Pre-cool warm gas flow
Cold Heat Exchanger (cold-HX)	Cool the gas flow to a set low temperature of the TESU
Vial Heat Exchanger (vial-HX)	Cool the cryo-vial
Insulation	Reduce parasitic heat leak to the cold environment

Table 2. Material properties of the biopsy tissue sample, the cryo-vial and TESU

	Biological tissue [11]	Cryo-vial (aluminum)	TESU (copper)
Density (ρ)	1 g/cm^3	2.7 g/cm^3	8.96 g/cm^3
Heat capacity (c_p)	4 J/g·K (T $>$ 273 K) 334 J/g (T = 273 K) 2 J/g·K (T $<$ 273 K)	0.91 J/g·K (T = 300 K)	0.39 J/g·K (T = 300 K)

the apparatus should be able to cool, six cryo-vials sequentially in one batch. In this section, a conceptual design of the apparatus and the various functional components are discussed.

Figure 4 shows the schematic of the apparatus depicting the main components and Table 1 gives a functional description of each component. The apparatus should be prepared for snap-freezing; this is done by cooling the TESU with a cryocooler to a set cold temperature and circulating a low flow of helium gas, about 4 mg.s^{-1}. The flow of helium gas cools the counter flow heat exchanger and brings the system to a steady cold state. While a low flow is maintained, the lid of the apparatus is opened and a cryo-vial is inserted. The low flow prevents moisture deposition in the apparatus. Once the vial is inserted, the flow is increased to the set value depending on the desired cooling rate. After the specified cool down time, the circulating helium gas flow rate is reduced and the cryo-vial is removed from the apparatus. During the insertion and removal of the cryo-vials some amount of helium gas escapes to the ambient which is replenished by the gas-handling system. For usage in an operation theatre, instead of pure helium gas medically approved mixture of helium and oxygen may be used in the system.

Thermal energy storage unit (TESU) is essentially a large heat capacity used to bridge the gap between cold energy demand and cold supply with a cryocooler. Without a TESU, a large power cyrocooler is required increasing the overall size of the apparatus. An estimation of the required cooling power without a TESU clarifies this hypothesis. The cooling energy required is equal to the sum of the energy content in the tissue and the cryovial between 310 K to 77 K. The tissue may be assumed to be made of only water with a latent heat of liquid to ice transformation at 273 K. The thermo-physical properties of the tissue and the cryo-vial are shown in table 2. The energy content of the tissue is about 13.0 J and that of the cryo-vial is about 238.4 J. Therefore an average cooling power of 25.1 W in the cooling time of 10 seconds

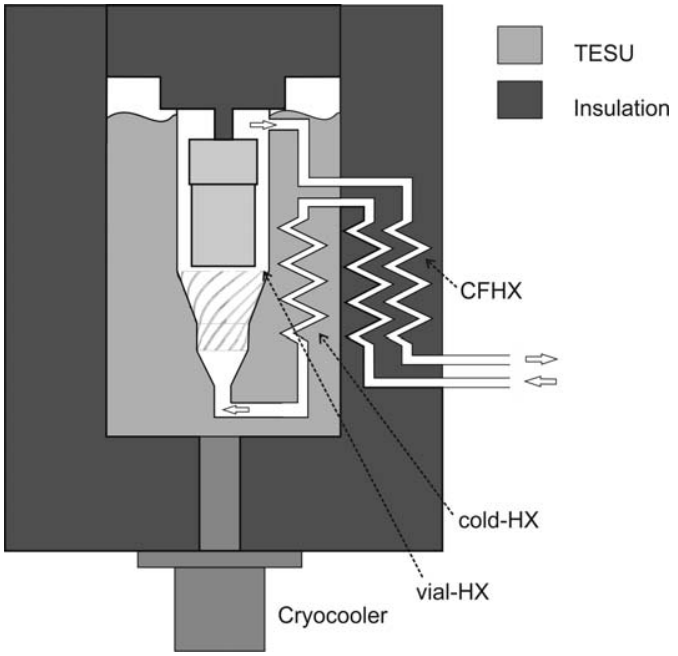

Figure 4. Conceptual design of the apparatus showing the main functional components.

is required to cool only the tissue and the cryo-vial, without considering the parasitic heat leaks to the environment. A commercial cryocooler that satisfies this requirement weighs 5.6 kg and occupies 0.25 m^3 [12]. A thermal buffer mitigates the cold energy supply and demand gap, and reduces the size of the cryocooler. Considering design margins and a temperature tolerance of 2 K, a copper mass of 1 kg with an energy content of 391 J, is sufficient to provide the desired cooling capacity. By using a TESU, a much smaller power cryocooler may be used to prepare the apparatus for snap-freezing.

The reason for using a Counter Flow Heat Exchanger (CFHX) in the apparatus is two fold; first it improves the energy efficiency of the apparatus, and second the outgoing gas from the TESU should be heated to the ambient temperature as the flow instrumentation, and the circulator are specified to operate at room temperature. The thermal energy content of a 40 $mg.s^{-1}$ flow of helium gas between 300 K and 77 K is about 45 W. Without a CFHX all this energy should be absorbed by the TESU. A counter flow heat exchanger with an efficiency of about 95 % will deposit about 2.25 W reducing the required size of the TESU.

The gas circulator consists of a membrane pump to circulate the helium gas. A small bottle of helium gas is connected to the circulator to fill the apparatus with working gas. The membrane pump speed is varied to control the mass flow rate to the apparatus.

To summarize, the apparatus described in this paper: allows adjustment of cold heat sink temperature during snap-freezing of a tissue; the cooling rate to a pre-set cold temperature can be varied, venting of low quantity of helium gas or a mixture of medical approved helium, oxygen mixture may be used; standard aluminum cryo-vial may be used. The current status of the development is sizing and production of the components.

From the operational point of view the following phases are recognized. The preparation phase in which the cryocooler is turned on to cool the TESU and later a small flow of helium gas is circulated to cool the counter flow heat exchanger. This phase takes about 2-3 hours. The cooling phase where a cryo-vial is inserted in the apparatus for snap-freezing, which takes

less than a minute. The regeneration phase, in which the cryocooler cools the TESU to the original setting, which takes about 2 minutes when the apparatus is once again ready for the next cryo-vial insertion.

5. Conclusions

A conceptual design of a tissue snap-freezing apparatus without sacrifical cryogen is being developed that has a unique capability of adjusting cold sink temperature and the freezing rate. A cooling rate of about -25 K/s from 300 K to 150 K is measured for an aluminum cryo-vial while quenching in liquid nitrogen. It is observed that the thermal contact resistance between the biological specimen and the cryo-vial is large and does not influence the cooling characteristics of the cryo-vial. Among the various cooling principles, forced convection of helium gas in a gap between the cryo-vial and cold surface is chosen because of the simplicity. Experiments performed with an additive manufactured prototype showed that cooling rates of a cryo-vial faster than quenching in liquid nitrogen is feasible. In the final design, a pulse tube cryocooler rated at 4 W at 80 K will provide the cooling to a Thermal Energy Storage Unit (TESU). To reduce the size of the TESU, a counter flow heat exchanger is included to pre-cool the warm helium gas flow to the apparatus. We believe that this apparatus will allow unprecedented bio-medical research of tissue freezing and will enable molecular diagnosis.

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