Lab. Medical Oncology

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Cryogenic tissue fixation for molecular medicine

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Patients

In the clinic we are faced with patients with a large variety of malignancies.

For the best possible treatment many details about their disease should be known. This implies that data from the disease should be collected for diagnosis, but also for research purposes.

A number of technological tools are available to the physician to obtain this information:

- Imaging techniques, like CT, MRI, or PET scanning
- Analyzing body fluids like blood, urine, liquor, CSF
- Obtaining biopsies from e.g. skin, tumor, inflammation sites, etc.

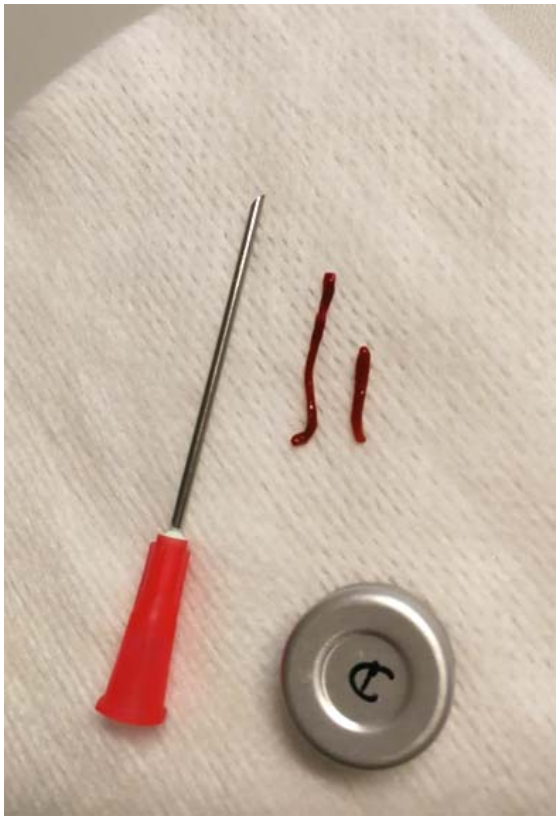
Biopsies

Biopsies in daily clinical practice are often obtained with the aid of a biopsy (hollow) needle, core biopsies.

After obtaining the biopsy, it should be preserved to keep the properties of the tissue as similar as possible to the in patient situation. Ongoing biochemical reactions and decay are unwanted processes which will alter the biopsy properties.

Most common fixation methods of tissue are:

- formalin (= formaldehyde solution)
- alcohols, like methanol or ethanol
- freezing



core biopsy next to hypodermic needle

Fixation of tissue

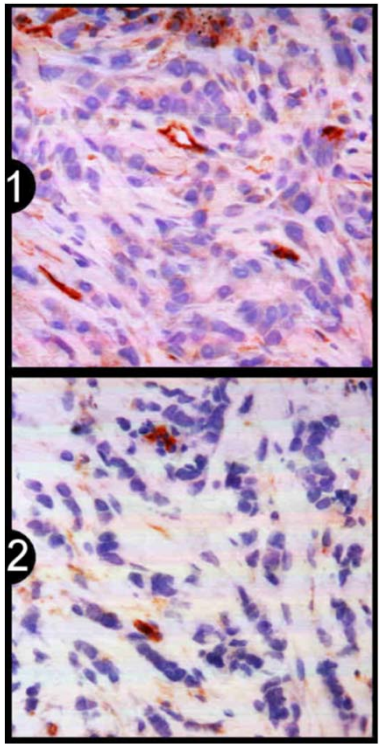
- **Formalin fixation is routinely used**

The protein crosslinking properties are ideal for structure preservation, after imbedding in paraffin the biopsies can be cut in thin slices and the tissue can be studied.

But the crosslinking of the proteins makes this method less well suited when one wants to study the loose proteins themselves. Also the quality of RNA and DNA is detrimental effected by this fixative.

- **Freezing the tissue**

By freezing the tissue the proteins are preserved well, and also the RNA and DNA can be isolated in good quality. So this is the preferred method for e.g. mass spectrometry analysis of proteins.



HE staining of tissue sections

Freezing of tissue

- **standard freezer** **-20 °C** **slow freezing**
- **ultra low freezer** **-80 °C** **slow freezing**
- **Dry ice** **-78 °C** **medium freezing**
- **Liquid nitrogen** **-196 °C** **fast freezing**

- Both dry ice and liquid nitrogen have the problem that they evaporate, so there should be a regular supply. Not always and everywhere available.
- Safety measures when working with liquid nitrogen are to be considered. Not easy in use for an operation-room.

CryoOn project: Cryogenics meets Oncology

The aim of this project is to design a freezing system that:

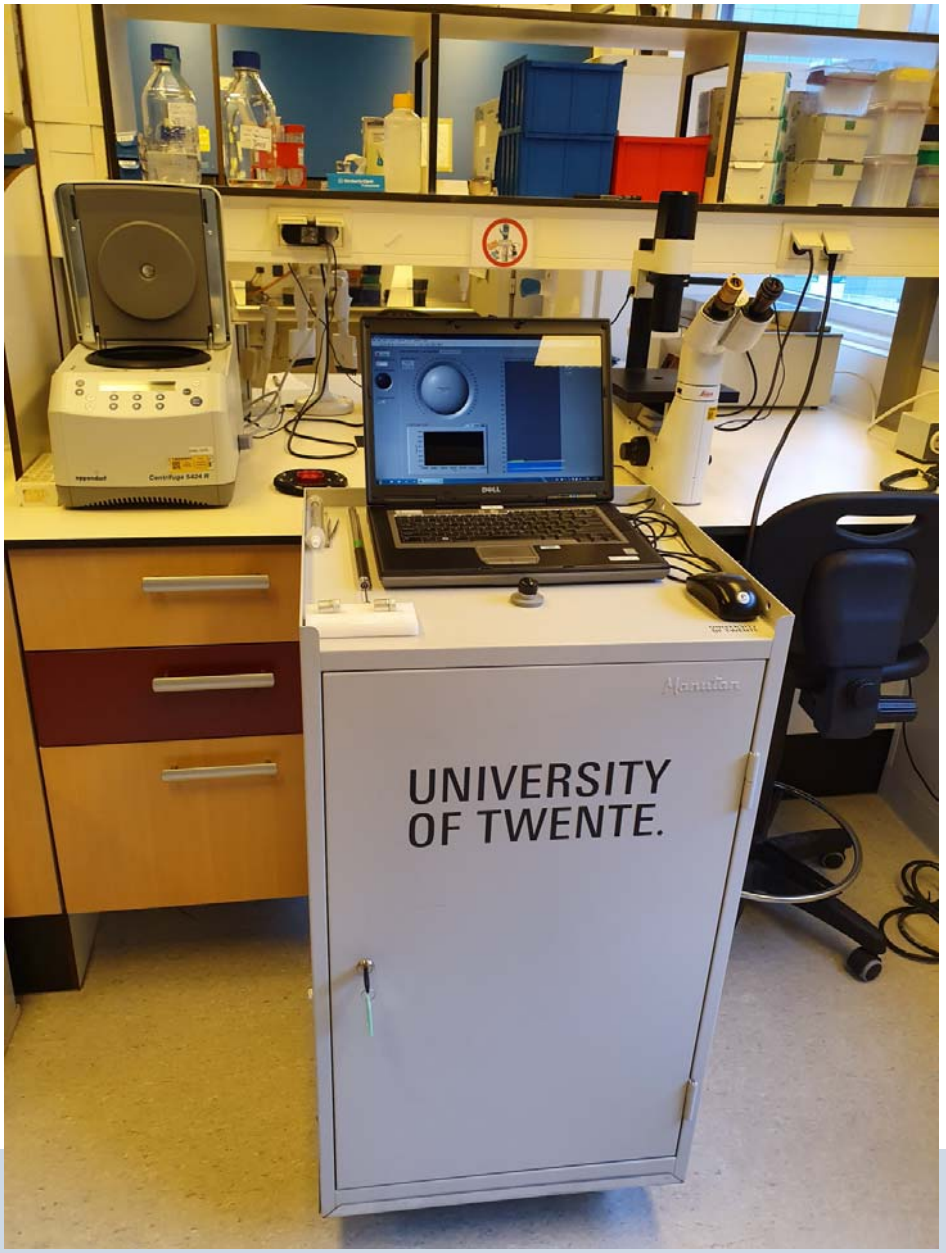
- **quickly freezes a sample**
- **does not need dry ice or liquid nitrogen**
- **can be used in a surgery or biopsy room**
- **can be transported easily**
- **is easy to use**

Snapfreezing device

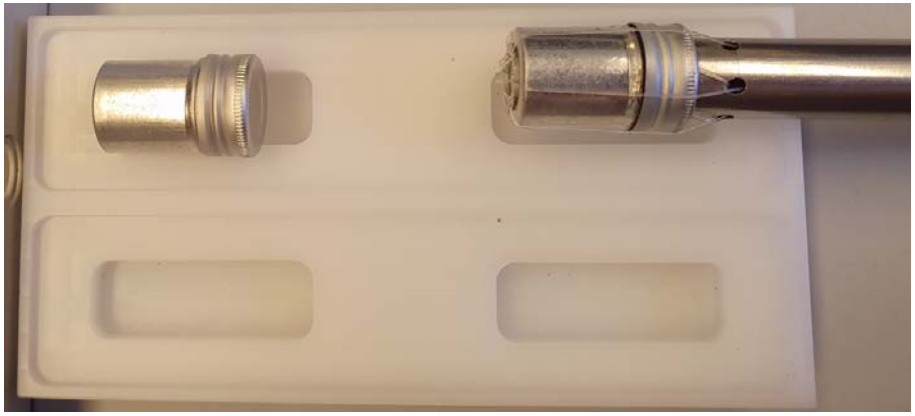
A prototype has been designed, and was build by the University of Twente.

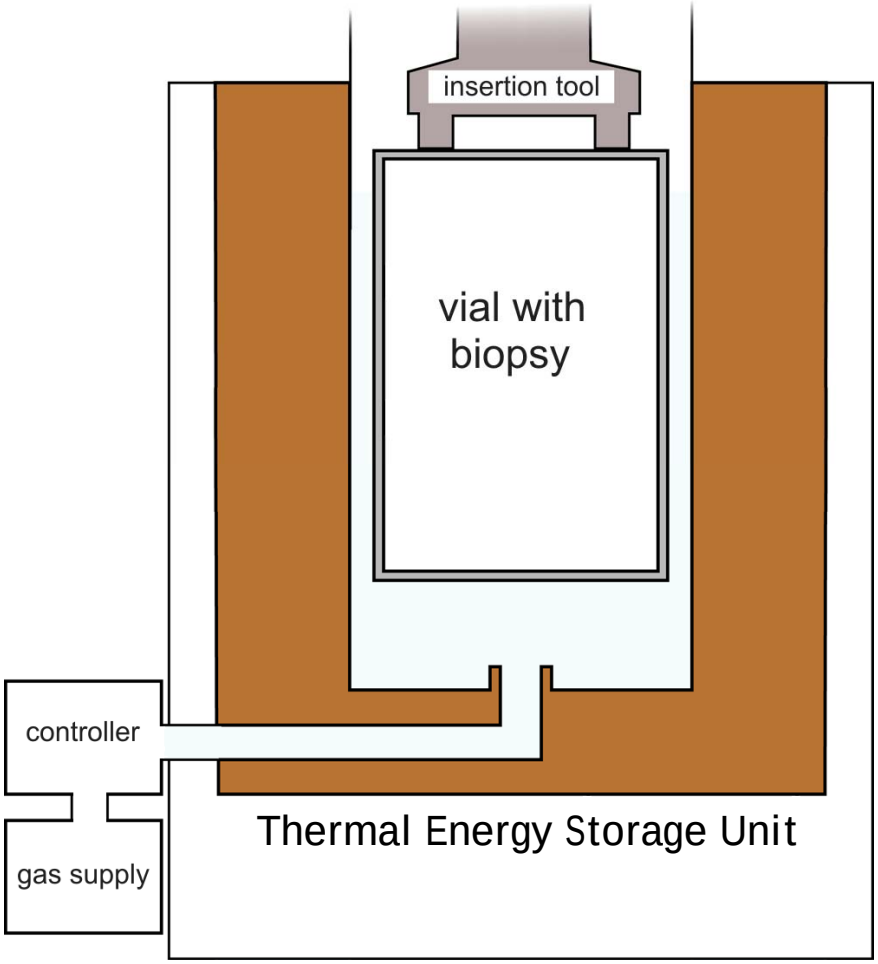
Details of the design can be found in this publication:

M.A.J. van Limbeek, S. Jagga, H. Holland, K. Ledeboer, M. ter Brake & S. Vanapalli, 2019. *Scientific Reports* **9: 3510. doi:10.1038/s41598-019-40115-6**



Snapfreezing device





Snapfreezing device

Needs a power plug

Is pc controlled

Can be set at every wanted temperature, e.g. -80 °C (193 K) or -196 °C (77 K)

Samples can reach very fast the set temperature (circa 15 s to 77 K),

similar, or even faster than in liquid nitrogen (Leidenfrost effect)

Testing samples in snapfreezer

At the Amsterdam UMC, location VUmc, several biological samples were tested.

Freezing conditions tested are liquid nitrogen versus snapfreezer.

Performance is evaluated from quality of:

- **phospho-proteomics data by LCMS**

  Phosphorylated proteins are involved in signaling in the cell, these levels can be easily influenced by stress (lack of oxygen, nutrients, etc.).

- **RNA**

  RNA is very sensitive to Rnases, which degrade the RNA. Rnases are present e.g. in lysosomes, which may be damaged during freezing.

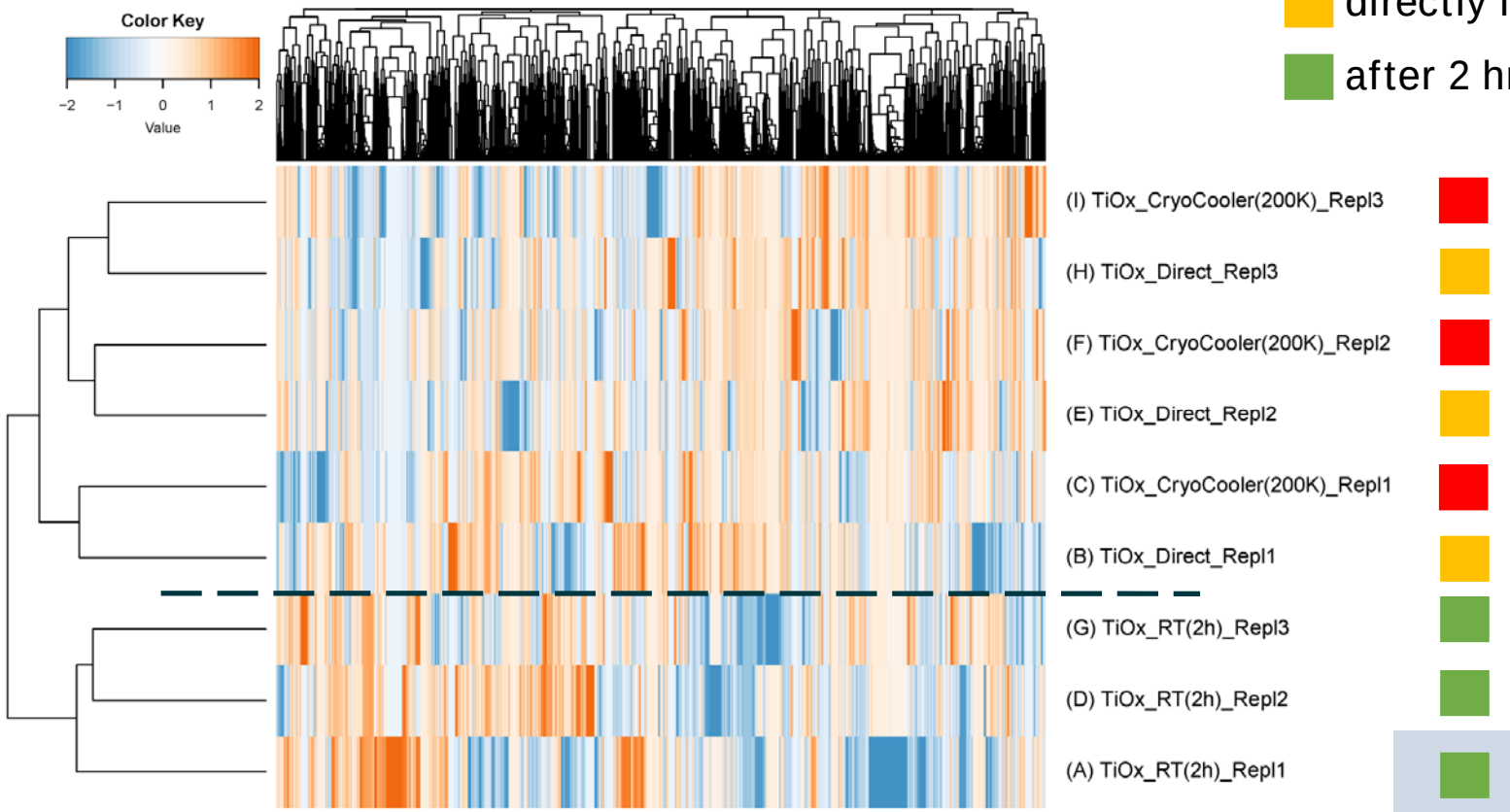
Freezing cells and tissue

- K-562 suspension cells [chronic myelogenous leukemia]
- Liver biopsies from patients taken directly after liver resection in operating room

Snap freezer (-78 C) VS Directly in liquid nitrogen (-196 °C) VS 2 hrs at room temp. before liquid nitrogen

Phospho-site unsupervised cluster analysis

K-562 cells



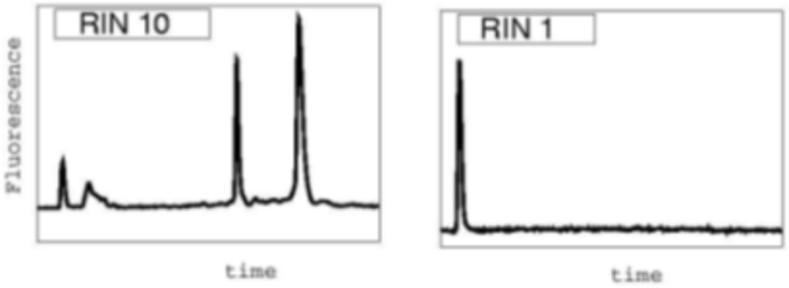
snapfreezer

directly in N2

after 2 hr in N2

RNA integrity

- RNA Integrity Number (**RIN**): calculated value resulting from an algorithm that describes RNA integrity. Used as a standardized procedure of RNA quality control.
- Decreasing RIN value = increasing amount of RNA breakdown, by Rnase activity.
- RNA integrity is visualized by an electropherogram. RNA molecules are separated according to molecular weight.



Results – RNA integrity 1

Cell line K-562

The first results show that the RIN values from cells directly frozen in liquid nitrogen, frozen in the snapfreezer and cells first left for 2 hrs at rom temperature before freezing in liquid nitrogen, are all perfect (>9).

Results – RNA integrity 2

Liver tissue, biopsies taken at the operation room directly after resection

The first preliminary results show that the RIN values from liver tissue directly frozen in liquid nitrogen, frozen in the snapfreezer, or after left for 2 hrs at room temperature before freezing in liquid nitrogen, are all good (>6).

Conclusions

The prototype of the snapfreezing system behaves as anticipated:

- The performance of very fast freezing of biological samples is met
- The quality of the phosphor-proteome and RNA are good
- The newly designed snapfreezer is easy to use
- A good alternative to liquid nitrogen or dry ice

Discussion

After the samples are frozen in the snapfreezer, they are usually stored before being processed for longer time in a ultra low freezer at -80 °C.

A cooled transport system between the snapfreezer and the storage location is needed, this system is being developed by the University of Twente.