Medical Biostasis Protocol (V 1.0)

By Aschwin de Wolf[1], Dr. Ville Salmensuu, M.D.[2]

Medical biostasis is an experimental procedure that induces metabolic arrest at cryogenic temperatures to allow terminally ill patients to benefit from future medical advances and restore them to good health. Practiced as a hospital-based, elective medical procedure, medical biostasis consists of three distinct procedures: induction of hypothermic circulatory arrest, cryoprotection, and long-term care at intermediate temperatures (between -120℃ and -130℃). This document sets out a detailed protocol for medical biostasis, outlines a variation of this protocol for out-of-hospital emergency cases, and outlines research directions to further optimize this protocol.

Introduction

Medical biostasis is an experimental medical procedure to stabilize terminally ill patients in a state of low-temperature biostasis in order to transport them to a time where their condition can be treated and any adverse effects of the biostasis procedure itself can be reversed. The concept of medical biostasis is an extension of existing mainstream medical procedures in which the temperature of a patient is lowered sufficiently to stop the heart to conduct advanced surgical procedures on the brain (i.e., deep hypothermic circulatory arrest). The premise of medical biostasis is that modern vitrification technologies can lower the temperature of the patient sufficiently enough to induce complete metabolic arrest. Placing a patient in medical biostasis prevents any kind of critical condition from advancing and allows science and medicine to catch up to the point where matter can be manipulated at the molecular level and restoration of the patient to good health is feasible.[3]

Eligibility for Medical Biostasis

As an experimental life-saving procedure, medical biostasis seeks to preserve life. There are two basic conditions in which a choice to induce medical biostasis should be considered. The most common situation is one in which a patient is diagnosed with a condition for which no contemporary medical treatment is available. Examples of these conditions include advanced forms of cancer that are resistant to further treatment, untreatable bacterial or viral infections, and severe cases of trauma. A second category of cases are those in which cardiovascular

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[3] Robert A. Freitas Jr., "Chapter 23. Comprehensive Nanorobotic Control of Human Morbidity and Aging," in Gregory M. Fahy, Michael D. West, L. Stephen Coles, and Steven B. Harris, eds, The Future of Aging: Pathways to Human Life Extension, Springer, New York, 2010, pp. 685-805

recovery is possible but progressive and irreversible loss of identity-critical brain function is expected. Examples of this category of cases include prolonged cases of cardiac arrest, untreated global cerebral ischemia, and forms of dementia for which no contemporary cure is available. In such cases medical biostasis takes advantage of the fact that the fine structure of the mammalian brain persists longer than the duration of the insult that is compatible with functional brain recovery.[4] In cases of prolonged cardiac arrest or stroke, the initial stages of medical biostasis will bear close resemblance to out-of-hospital treatment of cardiac arrest and severe stroke victims.

Induction of Deep Hypothermic Circulatory Arrest

The initial step in a medical biostasis protocol is to lower the temperature of the patient to the point of electrocerebral silence where blood can be replaced with a universal hypothermic organ preservation solution. This organ preservation solution is designed to provide metabolic support at low temperatures and prepare the patient for cryoprotectant perfusion. Induction of deep hypothermic circulatory arrest consists of five distinct procedures: (1) pre-operative procedures, (2) induction of general anesthesia, (3) surgery for cardiopulmonary bypass, (4) vascular cooling, and (5) blood substitution.

Pre-operative procedure

To protect the patient during deep circulatory arrest, a detailed medical assessment should be made to determine whether any recent and existing medication regimes contra-indicate exposure to deep hypothermic circulatory arrest (DHCA). Administration of heparin or specific anti-thrombin inhibitors prevent hypothermia-induced coagulation abnormalities. Anti-inflammatory drugs such as corticosteroids mitigate the inflammatory response to induction of hypothermia. The patient should also be evaluated for any medical conditions that complicate blood sugar stabilization, pH management, and cannulation of the vessels. Local anesthetics, anxiolytics, and narcotics are administered to facilitate arterial line placement and monitoring devices.

Induction of general anesthesia

Induction of general anesthesia is practiced in line with institutional practice and typically includes intravenous administration of agent to induce analgesia and muscle relaxation and propofol and/or thiopental. A continuous infusion of these agents is given to achieve a normal surgical plane of anesthesia. Maintenance dosage of general anesthetics should be adjusted to reflect temperature measurements.

[4] Aschwin de Wolf, Chana Phaedra, R.M.Perry, and Michael Maire, "Ultrastructural Characterization of Prolonged Normothermic and Cold Cerebral Ischemia in the Adult Rat", *Rejuvenation Research*, June 2020

Surgery for cardiopulmonary bypass

A median sternotomy is performed to gain access to the heart vessels, followed by placing an arterial cannula in the aorta and a venous cannula in the right atrium for venous return. If the medical condition of the patient or the condition of the heart vessels preclude cannulation of the heart, femoral cannulation can be considered as an alternative.

Vascular cooling

Upon completion of cannulation, the tubing is connected to a roller pump and extracorporeal perfusion is initiated. The hemodiluted blood of the patient is run through an oxygenator to provide metabolic support and a heat exchanger to lower core body temperature. This procedure is continued until the EEG monitor indicates electrocerebral silence, which occurs around a mean temperature of 18 °C, at which point the blood of the patient will be replaced with a universal organ preservation solution.

Blood substitution

The blood substitution procedure consists of two distinct procedures: an initial open-circuit flush solution to remove the blood and reverse any residual (micro)clotting, followed by circulation of the organ preservation solution to a temperature of 0 °C to prepare the patient for cryoprotectant perfusion. The organ preservation solution of choice is B1, which is a modification of the carrier solution of the M22 vitrification solution that includes calcium chloride, magnesium chloride, and hydroxyethyl starch. During the initial blood removal flush, 250,000 units of streptokinase are added to the washout solution. Blood washout is continued until the effluent on the venous side of the patient is clear, after which the circuit is closed, and cooling continues. Oxygenation of the perfusate prevents energy depletion of the brain, loss of ion hemostasis, and the development of cerebral edema. Perfusion pressures should not exceed 100 mmHg as measured in the arterial line.

When the patient reaches a tympanic temperature of 3.5°C, incisions are made in the scalp by a neurosurgeon to expose and open the dura to create two bi-lateral holes in preparation of cryoprotectant perfusion. To prevent excessive heating to the tissue and brain tissue, chilled saline is sprayed on the surgical drill and the parts of the skull subject to the burr hole procedure. After completion of the burr hole procedure, acoustic fracturing recording probes and brain volume measuring probes are placed in contact with the patient’s brain.

To maintain low temperatures and further lower the patient's temperature during cryoprotection, a preferred version of this protocol employs a patient enclosure with (software) controlled periodic injections of nitrogen-vapor.

Composition of B1 washout solution

Chemical	Concentration
Glucose	90 mM
Mannitol	45 mM
Lactose	45 mM
Potassium Chloride	28.2 mM
Potassium phosphate	7.2 mM
Gluthathione (reduced)	5 mM
Adenine	1 mM
Sodium Bicarbonate	10 mM
Calcium Chloride	1.0 mM

Magnesium Chloride	2.0 mM
Hydroxyethyl Starch	5%
Streptokinase (initial flush)	250,000 IU

Cryoprotection

The goal of cryoprotection is to replace the blood and liquid parts of the cell with a cryoprotectant to suppress ice formation when the patient is cooled below the freezing point of water. Modern vitrification solutions use high concentrations of cryoprotectants to inhibit ice formation altogether. M22 is a published, peer-reviewed, vitrification solution that has been successfully used in organ preservation to preserve cellular viability after introduction and removal from kidneys and hippocampal brain slices[5]. M22 is delivered in a hypertonic carrier solution named LM5 to support tissues during cryoprotectant exposure and to eliminate chilling injury. M22 must be introduced in gradual fashion to prevent osmotic injury.

Current medical biostasis protocol is to gradually introduce the vitrification solution at +3.5°C, followed by lowering the temperature of the patient to -3.5°C, followed by continued perfusion until a concentration of 100% M22 venous concentration has been achieved.

M22 Vitrification Solution

Dimethyl sulfoxide	2.855M
Formamide	2.855M
Ethylene glycol	2.713M
N-methylformamide	0.508M

[5] Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E, in: Cryobiology (2004 vol. 48), "Cryopreservation of organs by vitrification: perspectives and recent advances", pg. 157-78.

3-methoxy-1,2-propanediol	0.377M
Polyvinyl pyrrolidone K12	2.8%
X-1000 ice blocker polyvinyl alcohol (PVA)	1%
Z-1000 ice blocker polyglycerol (PGL)	2%

M22 has a total concentration of 9.345 M. M22 should also include an oncotic agent to mitigate cryoprotectant-induced edema in the lower body of the patient.

LM5 carrier solution

Chemical	**Concentration**
Glucose	90 mM
Mannitol	45 mM
Lactose	45 mM
Potassium Chloride	28.2 mM
Potassium phosphate	7.2 mM
Gluthathione (reduced)	5 mM
Adenine	1 mM
Sodium Bicarbonate	10 mM

Operating Room Requirements

Medical biostasis protocol requires an operating room of at least 70 square meters. During the whole procedure the patient is placed inside a temperature-controlled enclosure which is also used for the sub-zero rapid cooling to cryogenic temperatures. To allow real-time whole-body CT scanning during cryoprotectant perfusion, the room should have wide doors, and walls and/or other appliances compatible with the radiation protection requirements of the installed CT scanner as determined by a medical or health physicist. Ample room should remain available for laboratory equipment and supporting systems such as mobile liquid ventilation units.

Patient enclosure

A human-sized patient enclosure is used for the majority of medical biostasis procedures and needs to satisfy the following criteria:

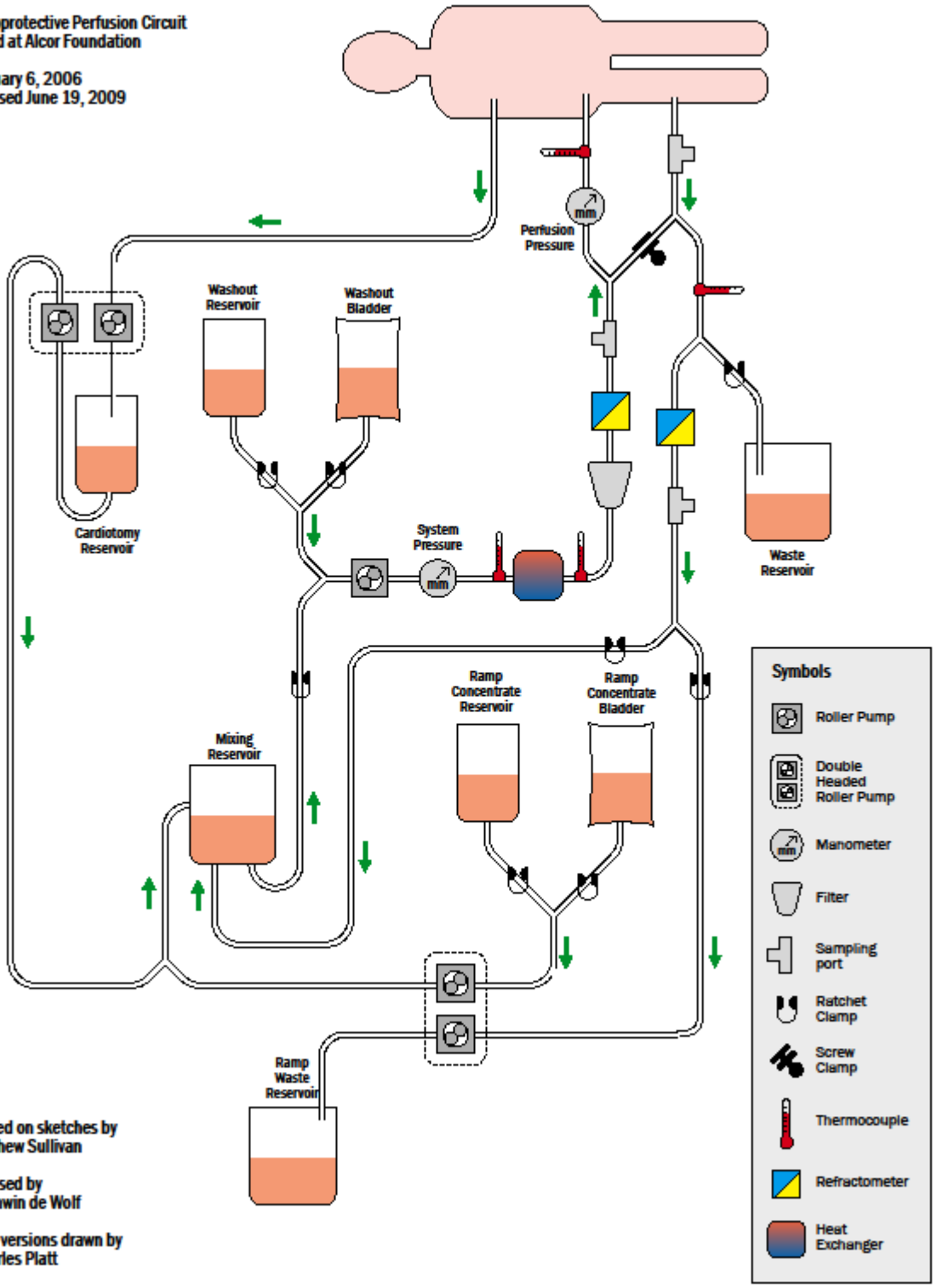
- Can fit within the bore of a mobile CT scanner
- Permits anesthetic and surgical procedures
- Materials and design tolerate injections of nitrogen vapor
- Real-time measurements of body weight (that don't interfere with CT scanning)

Cryoprotection circuit design

The cryoprotectant circuit is an enhanced version of a standard extracorporeal circuit (including components such as a bubble trap and heat exchanger) with the most notable added component being a mixing reservoir which allows gradual delivery of the vitrification solution to the patient. The tubing assembly allows for protocol in which the blood of the patient or cryoprotectant is allowed to be discarded on the venous side or recirculated to achieve a specific temperature or perfusate concentration. To prevent contamination of the circuit, the initial washout circuit is replaced with the cryoprotectant circuit prior to starting cryoprotectant perfusion. An example of an advanced recirculating cryoprotection perfusion circuit is shown below.

Cryoprotective Perfusion Circuit
Used at Alcor Foundation

January 6, 2006
Revised June 19, 2009



Based on sketches by
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PDF versions drawn by
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Computer control of perfusion

There are three basic possibilities for control and data collection during cryoprotectant perfusion, and various combinations thereof:

Manual data collection and manual cryoprotection control
Automated data collection and manual cryoprotection control
Automated data collection and automated cryoprotection control

Circulation of the vitrification solution and temperature control informed by real-time measurement of cryoprotectant concentration favors the substitution of manual control of perfusion by software-controlled perfusion. A computer-controlled perfusion circuit relies on sensors converting physical phenomena (such as pressure, concentration, and flow rate) into electrical signals that can be used to control and log perfusion operations.

A computer-controlled medical biostasis vitrification system should have at least the following features:

Control of the cryoprotectant and patient enclosure temperature based on measured cryoprotectant concentration.

Control of flow rate to stabilize a specific arterial pressure.

Monitor and display real-time in-line cryoprotectant concentration (refractive index).

Control the gradual increase of cryoprotectant concentration by separate volumetric addition of CPA concentrate and base perfusate.

Monitoring of the mixing reservoir level.

Air bubble and perfusate reservoir level alarms.

To prevent interruption of cryoprotection and cooling during a software failure, the perfusion system should have a failure mode that allows (temporary) manual control of all aspects of perfusion.

Conduct of cryoprotectant perfusion

Upon completion of washout, the software control system is activated to start cryoprotectant perfusion. The procedure starts at 3.5°C with the withdrawal of the carrier solution from the mixing reservoir while a 105% solution of M22 (the concentrate) is gradually delivered to the mixing reservoir to increase the concentration. The mixing reservoir delivery- and withdrawal pumps are independently controlled by in-line refractive index readings to ensure the concentration conforms to a gentle, osmotically-safe, profile chosen in the software. If the concentration on the venous side increases too rapidly or slowly the mixing reservoir delivery pump will respectively decrease or increase its flow rate. Level sensors in the concentrate reservoir and mixing reservoir protect levels from running too low. The perfusate in the arterial line runs through a 40-micron filter and heat exchanger to remove particulates and cool the vitrification solution. A small portion of the perfusate on the arterial and venous side is redirected to a separate temperature-stabilized circuit where in-line refractive index measurements are taken, which are used to control cryoprotectant concentrate add- and withdrawal rates and temperature settings.

When the arterial concentration of M22 approaches 50% of CNV (**C**oncentration **N**ecessary to **V**itrify) cryoprotectant concentrate addition is reduced until a stable 50% reading is obtained. When this condition is met, nitrogen gas is injected in the patient enclosure until the core temperature of the patient measures -3.5°C. At this temperature, cryoprotectant concentration should be ramped as quickly as possible to 100% CNV. Cryoprotectant perfusion is complete when manual benchtop refractometer readings of the venous effluent concentration reach 100% of target and there little fluctuation in the refractive index is observed - or when no notable gains in M22 equilibration are observed. In case CTs scan of the brain indicate uneven equilibration of the cryoprotectant, perfusion can be continued for these areas to catch up, provided the time limit for good ultrastructural preservation is not exceeded. Arterial perfusion pressure is not to exceed 100 mmHg.

Monitoring of cryoprotectant perfusion

Cryoprotectant perfusion data are automatically logged by the software and should include:

- Start time of blood washout
- Start time of cryoprotectant perfusion
- Arterial and venous line pressures
- Reservoir levels
- Flow rates
- Temperature data
- Patient weight
- In-line refractive index measurements
- Completion time of cryoprotectant perfusion.

Cryoprotection data that require scribing and manual entry include benchtop refractometer data, blood sample analysis, tissue lab results, and unusual events. For quality control purposes, global and close-up video recordings should be made of all pertinent procedures, which should be stored in a secure, encrypted, database. These recordings can be used for protocol review, training, and education purposes.

Patient weight

Patient weight change during cryoprotection is a function of cryoprotectant composition, cryoprotectant concentration, and carrier solution tonicity. Perfusate composition is a strong predictor of weight gain and vitrification solutions with high toxicity tend to produce more (abdominal) weight gain. Conversely, vitrification solutions with toxicity-neutralizing components and weak glass formers (such as M22) tend to produce weight loss

In non-ischemic conditions, the high tonicity and osmolality of the whole-body M22 formulation typically produces weight loss. Integration of a scale in the patient enclosure can track weight changes in real time. To prevent inaccurate weight readings during perfusion, the patient weight with inserted cannulae and tubing should be used as a baseline.

If the patient suffered extensive periods of ischemia, or suffered from a condition that caused vascular leaking or damage to the lining of the abdomen, (extensive) weight gain is a typical response to cryoprotection, including swelling of the face and the brain.

Visual brain monitoring

The condition of the brain is visually monitored through two burr holes made using a 14 mm Codman perforator. This procedure permits observation of the osmotic response of the brain to cryoprotectant exposure and the development of cerebral edema. In ischemically compromised patients, brain swelling is routinely observed, with parts of the brain extruding from burr holes in the skull in extreme cases.

After completion of cryoprotection temperature probes and bilateral placement of acoustic fracturing monitoring sensors are placed on the surface of the brain.

Refractive index monitoring

Accurate determination of the concentration of cryoprotectant solutions and estimation of the concentration of cryoprotectant in the tissue is necessary to deliver cryoprotectant in a safe and gradual manner and to protect the tissues against ice formation. The antifreeze nature of

cryoprotectants precludes freezing point depression osmometry. An alternative is to measure the refractive index of different cryoprotectant concentrations and determine concentration through refractometry. Refractometry can be done in-line (to establish trends) or through high standard benchtop refractometry (to make decisions). In software-controlled cryoprotection, inline refractometers are integrated on the arterial and venous side of the circuit. A preferred option is to measure a small portion of the vitrification solution in a temperature-stabilized environment to obtain stable readings. Prior to use, the in-line refractometers need to be calibrated for the specific vitrification solution. Manual arterial samples are used to determine final concentration. When the refractive index on the venous side fails to increase for a prolonged period, and issues such as cannula displacement have been ruled out, a decision should be made to stop cryoprotection. Satisfactory refractive index readings on the venous side can co-exist with poor regional perfusion. The preferred protocol is to decide to stop cryoprotection based on both refractive index readings and CT scan images.

Blood gas and electrolyte analysis

Intermittent samples of the venous effluent can be run through benchtop or hand-held blood gas analysis devices to determine the concentration of electrolytes and pH. This data can be used to adjust perfusate composition in real-time in case of extreme values (like a low pH) or used for subsequent scientific study to optimize cryoprotection protocols for histological preservation and viability.

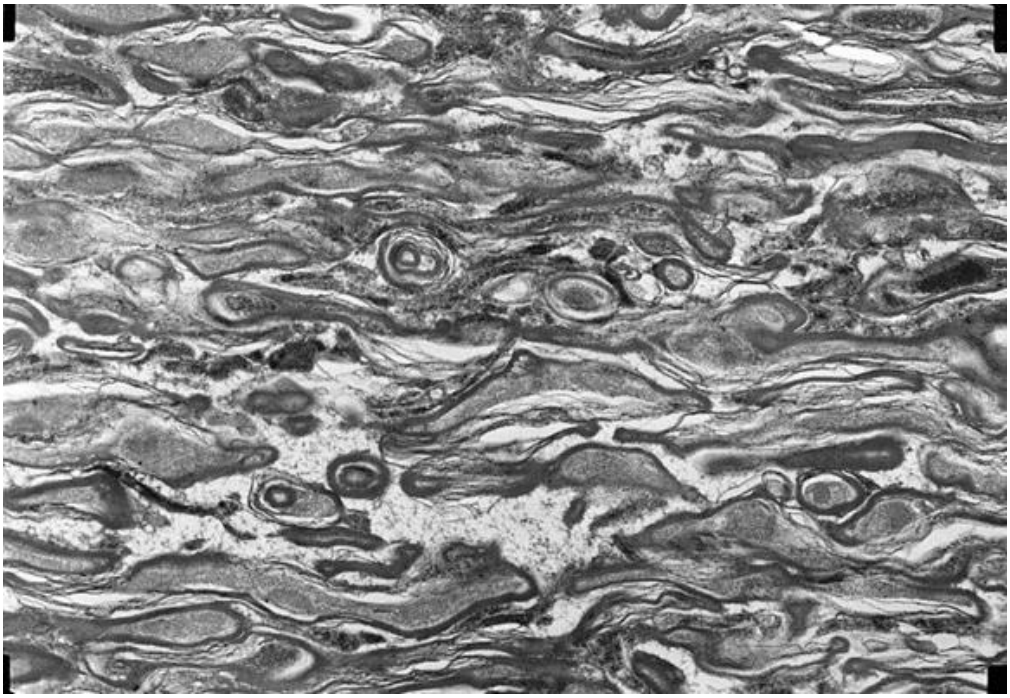
Ultrastructural and viability tests of the cryoprotected brain

The placement of bilateral burr holes permits the taking of microliter brain samples. These biopsies should preferably be taken from an area that is not associated with identity-critical information and under guidance of a CT scan. Brain tissue can be subjected to two distinct procedures: cell viability testing and electron microscopy. Brain biopsies can be taken during at any point during cryoprotectant perfusion. A preferred protocol is to take brain samples at the completion of cryoprotectant perfusion. It is important to know the concentration of the cryoprotectant in the sample to prevent processing the sample in a solution with vastly different osmotic properties. To understand the relationship between human biostasis protocol, brain ultrastructure, and cellular viability one sample can be prepared for electron microscopy and the other sample can be prepared for viability testing.

Fully equilibrated cryoprotected tissue cannot be processed for either electron microscopy or viability assays in its initial condition and needs to be stabilized in a mixture that contains the same concentration of cryoprotectant as the tissue. For electron microscopy this means a mixture of the full concentration of the cryoprotectant and glutaraldehyde (or a mixture of formaldehyde and glutaraldehyde), after which the cryoprotectant is gradually unloaded from the tissue, and the

tissue is washed and prepared for electron microscopy. Compatibility of the carrier solution, cryoprotectant, and the fixative needs to be considered. High molecular weight polymers or incompatible buffers (such as THAM) should be omitted from these solutions.

Interpretation of electron micrographs of cryoprotected brain tissue needs to be informed by detailed knowledge of the conditions under which the patient has been cryopreserved. Cryoprotection with contemporary vitrification solutions produces severe cryoprotectant-induced volume reduction of the brain caused by water osmotically leaving tissue faster than cryoprotectants penetrate the blood brain barrier, up to a 50% reduction of brain volume. The in-procedure or post-procedure CT scan of the patient will provide information about the degree (or absence) of cryoprotectant-induced volume reduction. In patients with significant cryoprotectant-induced cerebral volume reduction, electron micrographs will look distinctly different from normal controls and will show evidence of shrunken myelinated axons with reduced synaptic detail. The M22 image below is a good example of the histological appearance of cryoprotectant-induced brain shrinking.



Suprahippocampal white matter after perfusion with M22 for 60 min at -3°C, cooling to below the glass transition temperature, rewarming, and perfusion fixation (courtesy of 21st Century Medicine)

Ischemia-induced damage to the blood brain barrier reduces, or completely eliminates, this kind of cryoprotectant-induced volume reduction and electron micrographs will have a more regular appearance. Under such conditions cell damage, regional perfusion discrepancies or suboptimal

cryoprotectant equilibration will produce cells with poor ground substance, low electron-density, damage to the cell membranes, and pooling of fluid around capillary cells.

Brain samples should also be subjected to a cell viability assays to determine the response of cell metabolism to cryoprotectant exposure. The following three viability assays can be used for evaluating to viability of cryoprotected tissue samples:

Potassium / Sodium assay

The potassium/sodium assay (K^+/Na^+ assay) is a popular assay in cryobiology research. It is one of the most sensitive assays to measure viability in brain slices because ion homeostasis strongly correlates with both intact cell membranes and the ability to do metabolic work. After slicing, unloading, and incubation of the sample in oxygenated aCSF, extracellular ions are washed from the sample and a strong acid is used to release the intracellular ions, which can be measured in a flame photometer. Some flame photometers also measure calcium, which constitutes an additional viability assay because intracellular calcium levels are good indicators of cell injury. Under optimal loading and unloading conditions, hippocampal brain slices can recover between 80% and 90% viability.

A complication of doing K+/Na+ assays on cryoprotected brain tissue is that it is not possible to take a true control sample from the same patient, which makes calculating the ratio between control and experimental brains problematic. One solution is to take an additional brain biopsy after blood washout but prior to cryoprotection. Another alternative is to use the average control values in the literature, or other human cases, to calculate viability.

MTT assay

The MTT assay is a colorimetric assay that assesses cell metabolic activity by converting a tetrazolium dye to an insoluble compound with a dark purple color. In areas with good viability the slice will turn a dark purple and the dye can be released into the medium to be read in a spectrophotometer.

The MTT assay is not as sensitive as the K^+/Na^+ assay or an LDH assay but can be used to measure differences between controls and extreme conditions such as freezing.

LDH assay

The lactate dehydrogenase (LDH) enzyme is acutely released during cell injury. The main difference from the K^+/Na^+ and MTT assays is that instead of measuring the intracellular contents of the slice, one measures the medium in which the brain slices are incubated. If the cells are injured, the slices will release LDH into the artificial cerebral spinal fluid (aCSF) which can be measured with a spectrophotometer.

This assay is moderately sensitive to viability differences and is able to distinguish between control, the same cryoprotectant at different concentrations, and the addition of cryopreservation (cooling to -130° C).

The LDH and the K^+/Na^+ assays can be conducted on the same tissue sample because the former requires the measurement of the aCSF medium and the latter requires release of intracellular ions from the sample. In a preferred version of this protocol, K^+/Na^+ measurements are taken after LDH measurements. These measurements can be combined to create a multi-assay viability score that can be correlated to different solution composition and cryoprotection protocols.

Artifical Cerebrospinal Fluid sample incubation medium

NaCl	127 mM
KCl	1.0 mM
KH2PO4	1.2 mM
NaHCO3	26 mM
Glucose	10 mM
CaCl	2.4 mM
MgCl	1.3 mM
pH and oyxgen levels are maintained by bubbling 95% oxygen and 5% carbon dioxide	

Since samples for histology and viability are taken after completion of cryoprotection, the results cannot be used for real-time protocol changes. In conjunction with other automatically collected data, those can be used to specifically design bio-medical experiments. It is also an option to change the timing of sampling. If cell viability after cryoprotection is close to zero, future measurements can be taken at earlier points during cryoprotection to understand the decline of viability as a function of time, temperature, and concentration. Sample taking is not confined to the brain and additional samples should be taken from other organs. Endothelial samples should be taken to understand the effects of the washout solution and cryoprotectant on the vessels.

CT scanning

CT scans of the whole body or head can be conducted in the vitrified state to understand the relationship between cryoprotectant concentration and ice formation. In more advanced human biostasis protocols mobile CT scanners are used to monitor and guide cryoprotection. Real-time

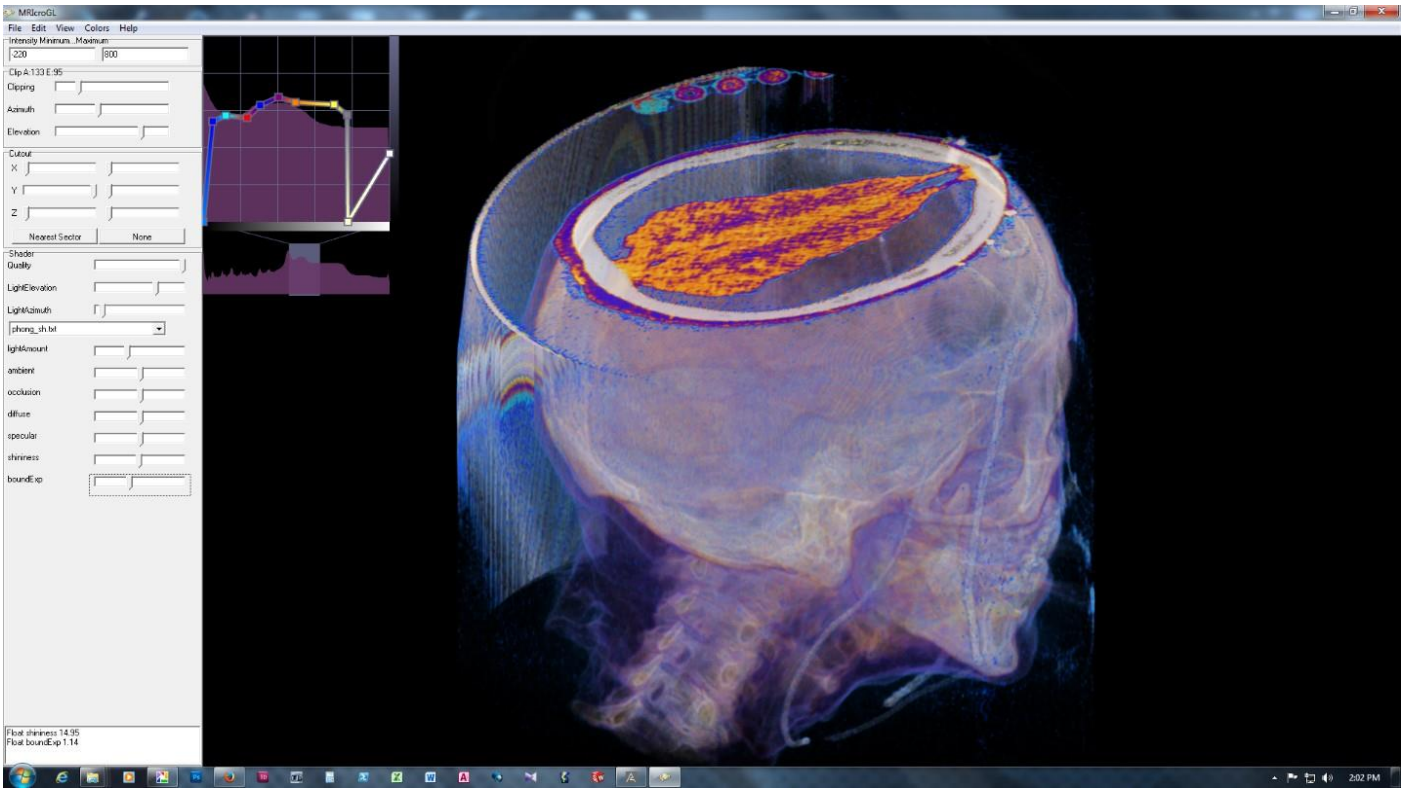
CT scans can reveal regional cryoprotectant distribution / concentration and cryoprotectant-induced dehydration.

Conducting CT scans requires radiation protection measures determined by a medical or health physicist, depending on the model of CT scanner and other considerations.CT scanning requires a patient enclosure that can fit in the bore of the CT scanner. For whole body patients, an enclosure that allows for a narrower width at the head of the patient to fit in the CT scanner might permit scanning of the head only.

Differences between different concentrations of vitrification solutions can be meaningfully distinguished in CT scans. DMSO-containing vitrification solutions (like M22) further favor meaningful CT scan interpretation due to the better photoelectric absorption of x-rays of its sulfur content. Meaningful CT scanning of cryoprotection procedures requires detailed calibration of different concentrations of a solution to Hounsfield Units (HU), also called "CT numbers." Different vitrification solutions (or variations of the same solution) require new calibration and color scales. Because physical density, and presence or absence of ice in sub-vitrifiable concentrations, also depends on temperature, calibration depends on the temperature at which measurements are being made. Calibration for tissue is in general expected to be different from calibration for solutions in absence of tissue, even if the solution contains the same concentration of cryoprotectants as the tissue. However according to published literature, the difference tends to be small, especially at high concentrations.

CT scans can be used to monitor cryoprotectant equilibration in real-time and identify regional variation in cryoprotectant distribution. If venous refractive index readings indicate 100% equilibration but CT scans indicate inadequate distribution, cryoprotect perfusion can be continued. Real-time CT scanning also allows a detailed understanding of the response of the brain (and rest of the body) to different pressures, flow rates, concentrations, and tonicities. CT scans are of special interest in case the patient is ischemically compromised and a determination needs to be made whether continued cryoprotection produces meaningful results. CT scans of patients with extensive periods of cerebral ischemia typically lack the features of cryoprotectant-induced dehydration.

After completion of cryoprotection, CT scans can be used for guiding brain sample biopsies and correct placement of probes for acoustic detection of fracturing events during cryogenic cooling. If CT scanning is not available during the procedure, whole body and head-only scans can still be performed when the patient is immersed in liquid nitrogen. CT scans at these temperatures will also reveal signature of ice formation if freezing occurred.



CT scan of M22-induced cerebral dehydration (courtesy of the Alcor Life Extension Foundation)

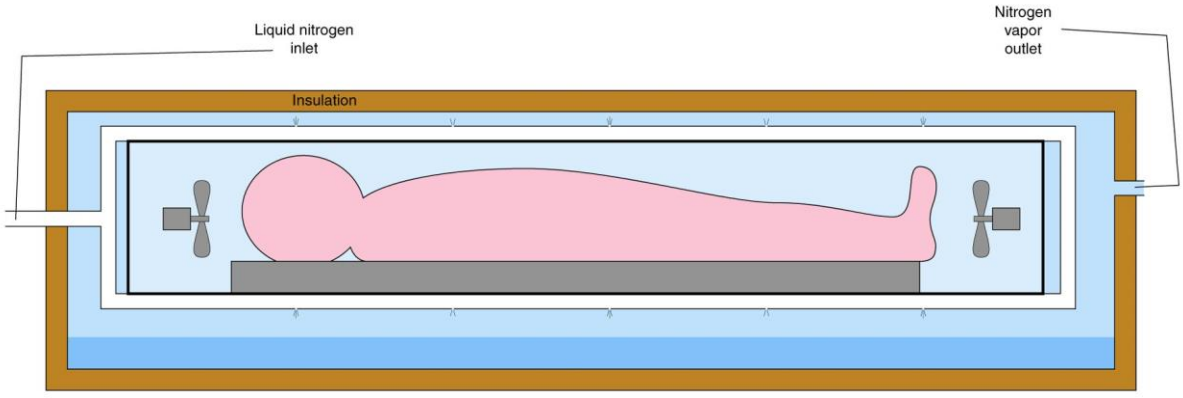
Cooling to the Glass Transition Temperature

After completion of cryoprotection, the body should be cooled as quickly as possible to deep subzero temperatures to minimize exposure to the highest concentration of the vitrification agent and to prevent ice formation. To reduce thermomechanical stress and later risk of fracturing, conventional practice is to cool quickly to between -80 degC and -110 degC and then more slowly through the glass transition point (Tg) of the vitrification solution, which is -123°C for M22. As the glass transition temperature is approached, the vitrification solution will sharply increase in viscosity and will undergo a phase transition to a glassy state. After reaching Tg the heterogeneity of the tissue will produce increased stress on the tissue, culminating in fractures. To minimize thermal stress and to avoid lowering the temperature too far below Tg, cooling between -110 °C and -130 °C is conducted especially slowly. At -110 °C the patient is held at a plateau for 12 hours to allow annealing and the temperature is then dropped more slowly over 100 hours to a temperature slightly below Tg (~ -130°C for M22 cryoprotected patients). After reaching the desired long-term maintenance temperature, the patient is transferred to a whole body intermediate temperature storage unit.

In a preferred embodiment of the human medical biostasis protocol the patient enclosure doubles as a cryogenic cooling box. This dual-purpose enclosure requires a choice of material and insulation that will not interfere with CT scanning and be prone to significant heat leak at lower temperatures. Cooling of the patient is conducted by injecting liquid nitrogen vapor. One or several fans are used to accelerate the cooling rate and avoid hot spots. Cooling of the patient, during its initial rapid phase and the slower phase close to Tg, is controlled by software. The software controls the cooling rate by opening or closing cryogenic-temperature compatible solenoid valves. During the initial rapid cooling phase the valves are almost constantly open, during the annealing phase valves are closed or only intermittently opened, and during the slow descent below Tg the valves are opened intermittently to conform to a specified cooling rate. The software allows the operating a cooling protocol that is optimized for a specific patient. For example:

(1) cool to −110ºC in 8 hours
(2) hold at −110ºC for 12 hours
(3) cool to −130ºC in 100 hours

Software control of cooling requires the placement of one or more controlling temperature probes in the tissue of the patient. The placement of additional controlling temperature probes allows the software to distinguish between exterior and interior temperature of the patient, which is not uniform in external nitrogen vapor-controlled cooling.



Simplified image of a cryogenic cooling enclosure (Courtesy of Charles Platt)

In cases where only partial vitrification, or cryopreservation without cryoprotection, is expected there will be a phase transition from water to ice at higher temperatures. In such circumstances thermal stress and fracturing will occur at higher temperatures and a different, slower, cooling protocol should be chosen in the software.

The patient should be monitored for fracturing during cooldown. One technology uses probes to record and amplify acoustic phenomena associated with fracturing to infer fracturing. An

alternative approach is to install a cryogenic camera in the cooling box to directly observe external freezing and fracturing events.

Long Term Maintenance

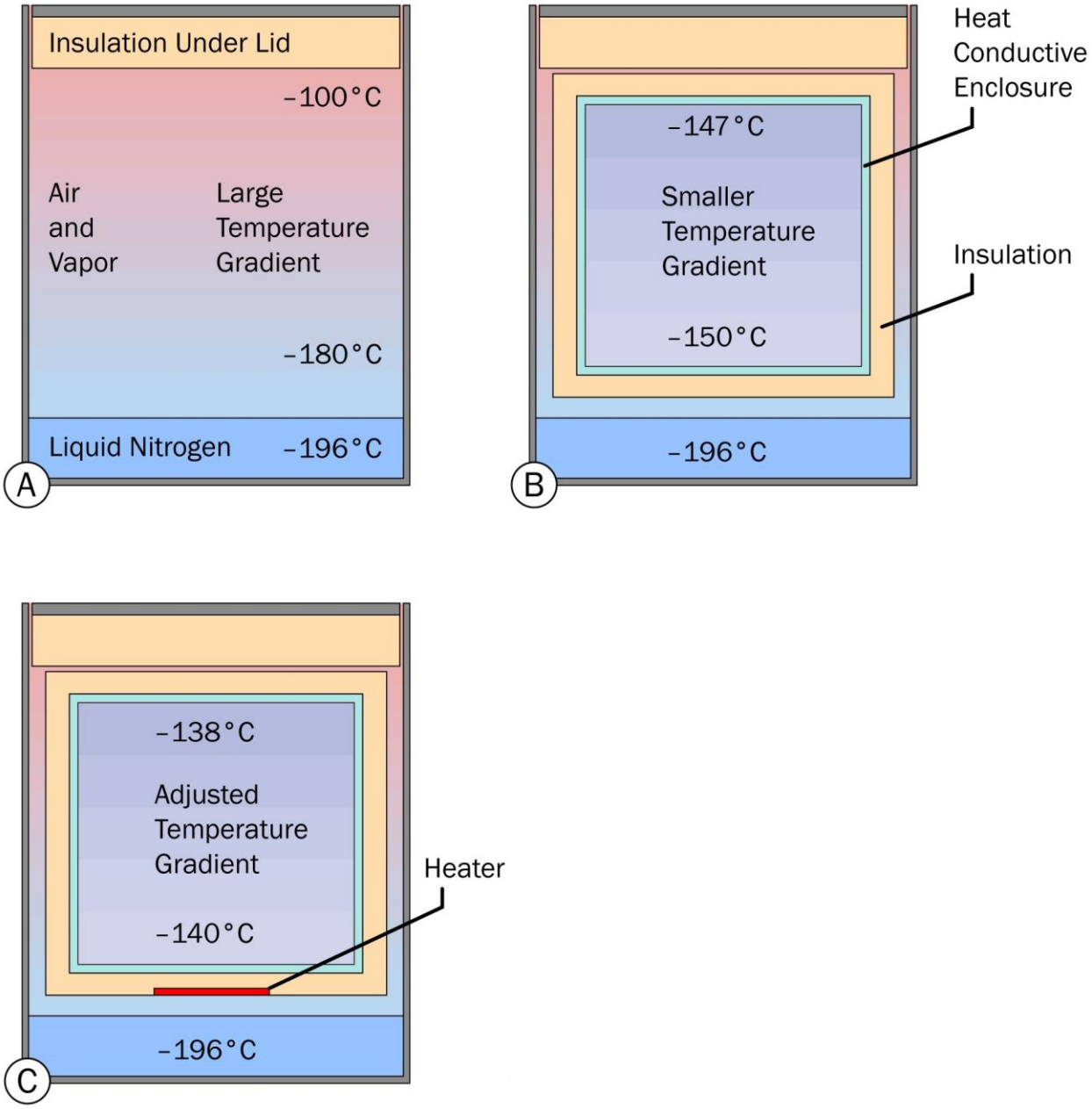
Long-term maintenance of patients needs to conform to the following requirements: complete inhibition of metabolism, avoidance of thermal stress-induced fracturing, long term safety, and electricity-independency. The traditional solution for long-term cell and tissue preservation is liquid nitrogen. At -196°C all diffusion-based chemistry is inhibited and no chemically meaningful chemistry occurs for thousands of years, which should be sufficient for the purposes of medical biostasis. Liquid nitrogen storage is silent and odorless, relatively non-reactive (allowing for direct patient contact) and does not require power for temperature maintenance. The fundamental drawback of long-term maintenance of human medical biostasis patients at this temperature is significantly lower than the glass transition temperature of common vitrification solutions and fracturing of tissues (including the brain) will likely occur. Intermediate temperature storage (ITS) is a technology that allows for safe storage of cryopreserved patients at temperatures where fracturing is minimized, or even avoided.[6]

Intermediate temperatures can be maintained in electronic cryogenic freezers. Because this technology is dependent on an uninterrupted power supply, needs to be filled with (dry) ice for thermal ballast, and ultra-low temperature refrigeration produces significant amount of heat, the preferred option for human biostasis is to place patients in specifically designed intermediate temperature storage units. ITS units take advantage of the fact that the nitrogen vapor above a pool of liquid nitrogen has a higher temperature than the liquid nitrogen itself. The temperature variation in the vapor phase can be narrowed by placing a heat-conductive enclosure in the ITS unit surrounded by insulation. Temperature variations within that unit are further reduced to just several degrees by adding a heating element at the bottom of the unit. While the addition of an electronically controlled heater introduces some degree of electricity reliance, the failure mode of this system is such that the temperature *decreases* instead of increases in case of a power or equipment failure. The temperature in the unit should be continuously monitored and failure of the system to keep a specified temperature should trigger an alarm system that will alert staff and outside stakeholders.

Choice of the ideal intermediate temperature storage temperature is a function of the glass transition temperature of the vitrification solution. Lowering temperatures far below Tg increase the probability of fracturing while higher temperatures risk storing patients at a temperature where ice crystal nucleation is at its fastest. Since nucleation at this temperature does not translate into ice formation-induced damage, but fracturing does produce actual damage, a

[6] Brian Wowk, "Systems for Intermediate Temperature Storage for Fracture Reduction and Avoidance", *Cryonics*, 3rd Quarter 2011

temperature close to Tg is advised. Since different vitrification solutions have different glass transition temperatures, patients cryopreserved with significantly different vitrification solutions cannot be stored in a single unit. The drawings below illustrate the principle behind ITS.



Achieving a controlled intermediate temperature environment for medical biostasis patients (images courtesy of Charles Platt)

Out-Of-Hospital Cases

Medical biostasis is an elective medical procedure but some circumstances dictate that the initial stabilizing procedures are conducted in an out-of-hospital setting. Examples of such cases are patient with prolonged circulatory arrest or extensive, non-recoverable, trauma. If there is prior patient consent for medical biostasis procedures, a modified protocol needs to be followed in such cases. Here we set forth a general out-of-hospital protocol for patients that are determined to be outside the conventional realm of resuscitation and require emergency preparation for cryopreservation.

Medical biostasis out-of-hospital protocol consists of three distinct interventions:

1. Cardiopulmonary support
2. Induction of rapid hypothermia
3. Administration of stabilization medications

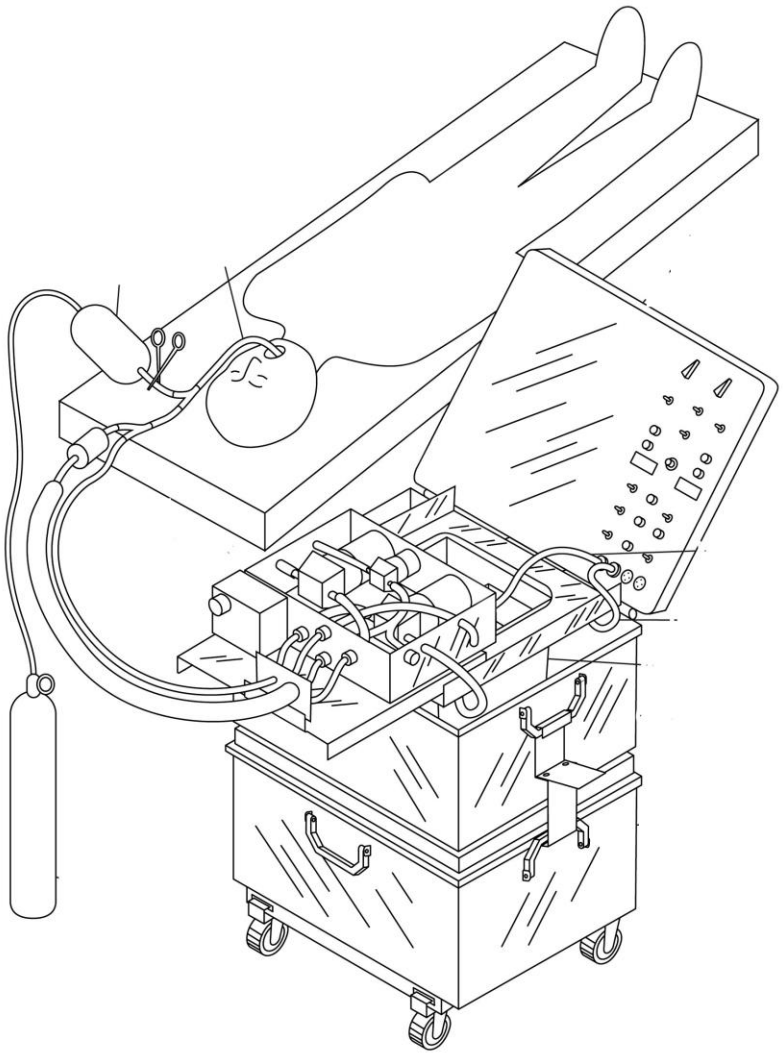
Cardiopulmonary support

When a determination is made that the patient's health prospects are best served by placing him in biostasis, the patient's circulation needs to be restored to meet the metabolic demands of the brain, enhance cooling, and circulate medications. Since the aim of restoring circulation and oxygenating the brain is to stabilize the patient's condition in anticipation of cryopreservation, the name for this procedure is cardiopulmonary *support* (CPS). Circulation is restored with a mechanical chest compression device (such as the Michigan "Thumper" or LUCAS) that can deliver continued high-impulse, active-compression decompression chest compressions. Utilization of a mechanical chest compression devices facilitates uninterrupted circulation in situations where logistical concerns would dictate pausing them (i.e. transport from the ambulance to a hospital room). Mechanical chest compression devices also prevent fatigue from medical professionals. Oxygen to the brain is delivered through a mechanical ventilator which also supports liquid ventilation.

Induction of rapid hypothermia

Induction of rapid hypothermia is achieved through two procedures: external ice bath cooling and liquid ventilation. After a determination is made to stabilize a patient for medical biostasis the patient should be immediately moved to a mobile, portable, ice bath which also provides a stable environment for mechanical CPS. The cooling rate of ice bath immersion is optimized when an additional submersible pump actively moves the ice-water and sprays it over the patient.

The most rapid cooling rates are achieved by complementing ice bath cooling with liquid ventilation. Liquid ventilation can be used to rapidly induce cooling by cyclic delivery of cold perfluorocarbons (which are inert and have higher oxygen solubility than water) to the lungs of the patient. Since all of the patient's blood flows through the lungs, the lungs are essentially used as an endogenous heat exchanger. Unlike emergency cardiopulmonary bypass, which is an invasive and technically complex procedure, liquid ventilation only requires the placement of an endotracheal tube for delivery and removal of the oxygenated perfluorocarbons. The liquid ventilation system consists of a reservoir that chills the perfluorocarbons prior to delivery through an insulated line to the patient. A software-controlled pump regulates volume, delivery, and withdrawal of the perfluorocarbons. Heat exchange is substantially improved by only partially filling the lungs and delivering oxygen independently from the perfluorocarbons to create "small-scale mixing" of gas and liquid. In ideal conditions, cooling rates of approximately 1 °C per minute can be achieved, which, in conjunction with CPS and administration of neuroprotectants should be sufficient to mitigate cerebral ischemia.



Liquid ventilation diagram

Administration of stabilization medications

To stabilize the patient's condition and mitigate events that follow circulatory arrest and insufficient blood flow, a number of medications should be administered to prevent blood coagulation, restore blood volume, increase blood pressure, protect the brain, prevent cerebral edema, and neutralize stomach acids. All medications and fluids should be administered according the body weight and conventional medical practice (if applicable).

Sodium Citrate
Citrate prevents the formation of blood clots that interfere with blood circulation and cryoprotectant perfusion. Unlike heparin, its anti-coagulation properties are not pH-sensitive. Citrate also works as a neuroprotectant by chelating calcium.

Heparin
Heparin is another anticoagulant that prevents the formation of blood clots and works in synergy with sodium citrate.

Epinephrine
Epinephrine is a vasopressor that is used to increase blood pressure during cardiopulmonary support. Vasopressin can be used as an alternative if epinephrine administration is ineffective.

Magnesium
Magnesium sulfide is a safe, inexpensive, natural cation with multi-factorial neuroprotective properties. Magnesium's neuroprotective mechanisms include inhibition of glutamate receptor-mediated excitotoxicity, inhibition of intracellular calcium overload, protection of membrane integrity, and decreased cerebral metabolism at higher dosages.

Minocycline
Minocycline is a broad spectrum bacteriostatic antibiotic and free radical scavenger with good tissue and brain penetration that possesses a broad variety of neuroprotective properties including inhibition of -metalloproteinases, -iNOS, PARP, mitochondrial cytochrome c release and, apoptosis.

Decaglycerol/THAM

Decaglycerol is a glycerol polymer used to osmotically inhibit cerebral edema similar to mannitol. THAM is a buffer that is used to mitigate acidosis. Decaglycerol/THAM is administered as a custom formulation of 20% w/v decaglycerol and 4.5% w/v THAM in water.

Dextran 40

The synthetic colloid Dextran 40 restores blood pressure in hypovolemic patients, improves cerebral blood flow during reperfusion by decreasing viscosity and ischemia induced leukocyte-endothelial adhesiveness, and also has a mitigating effect on blood coagulation and hypothermia-induced cold agglutination and rouleaux formation. Administration of Dextran 40 is optional and contraindicated in patients with volume overload.

Aluminum Hydroxide & Magnesium Hydroxide

Maalox is an antacid used to stabilize the pH of stomach contents to prevent erosion of the stomach wall by hydrochloric acid at low temperatures. Failure to prevent this ischemia- and hypothermia-induced phenomenon can lead to contamination of the circulatory system with stomach contents and abdominal swelling during cryoprotection. Maalox is administered through a gastric tube, preferably prior to endotracheal intubation for liquid ventilation.

Monitoring of out-of-hospital stabilization procedures

The two most important things to monitor during out-of-hospital medical biostasis stabilization procedures are temperature and metabolic support of the brain. Since CPS cannot generate adequate cerebral blood flow on a consistent basis, it is crucial to determine whether metabolic demand is reduced through rapid induction of hypothermia and administration of cerebro-protective drugs.

At least two temperature probes should be placed to obtain reliable core temperature data. A preferred protocol is to place a nasal probe and a rectal probe. It is crucial to validate that the temperature probes measure core temperature by touching tissue instead of the ice water. Slow cooling rates indicate a failure of effective ice water circulation, circulation of blood, or failure of the liquid ventilation equipment.

One of the most reliable indicators of adequate artificial circulation is the measurement of expired CO2 (ETCO2). Since liquid ventilation excludes this mode of monitoring it needs to be replaced by a technology such as cerebral oximetry. Cerebral oximeters measure transcutaneous oxygenation of the frontal cerebral cortex by exploiting the property of hemoglobin to absorb light. The frontal cerebral cortex has high metabolic demands and limited oxygen reserve, so oxygen deficiency in this area can indicate poor delivery of oxygen to the brain as a whole.

Cerebral oximetry equipment can operate down to 16°C which allows collecting cerebral oxygenation data during stabilization and subsequent in-hospital procedures.

The efficacy of medications to suppress cerebral metabolism can be validated by obtaining a Bispectral Index (BIS). BIS is an emerging technology that aims to measure the depth of anesthesia by calculating a single number from electroencephalographic measurements. The BIS index ranges from 0 (equivalent to electrocerebral silence) to 100 (equivalent to fully awake and alert). The BIS sensors are simple and can be non-invasively applied to the forehead of the patient.

Human Biostasis Research

This human biostasis protocol reflects contemporary knowledge from disciplines such as anesthesiology, neuroscience, emergency medicine, extracorporeal perfusion, cryobiology, and cryogenic engineering in conjunction with reasonable extrapolations from these fields into the novel field of human cryopreservation. To further refine and improve this protocol a number of topics require detailed investigation. It is beyond the scope of this document to provide a comprehensive list of topics, but we will describe the most important areas of investigation.

- ✓ In patients without cerebral ischemia, current cryoprotection protocol induces severe cerebral dehydration. It is currently unknown whether the a-typical ultrastructural effects can be consistently reversed after unloading the cryoprotectant. Development and validation of a safe unloading protocol can contribute to answering these questions.

- ✓ Addition of low concentrations of detergents like sodium dodecylbenzenesulfonate or sodium deoxycholate to the carrier solution of the vitrification solution can open the blood-brain-barrier and eliminate cryoprotectant-induced dehydration. Experimental research needs to be conducted to understand how the addition of such blood-brain-barrier modifiers affect ultrastructure and brain slice viability.

- ✓ The effects of different carrier solution composition, pressure regimes, and temperature protocols require additional investigation. In particular, the ultrastructural effects of the current protocol should be compared to protocols in which perfusion is completely conducted above 0 °C and protocols in which the highest concentration of the vitrification solution is perfused at -20 °C.

- ✓ Potential benefits may be obtained from using pulsatile flow and oxygenation of the perfusate during cryoprotectant perfusion. Research needs to be conducted to understand the effects of such additions.

- ✓ An alternative to external cryogenic is to perfuse the circulatory system with a cold gas. This technique could potentially improve post-perfusion cooling rates and eliminate thermal stress-induced fracturing.

- ✓ Optimization of long-term maintenance temperature requires better understanding of the (long-term) behavior of bulk vitrification solutions and cryoprotected tissue just below the glass transition temperature.

- ✓ The effect of stabilization medications in out-of-hospital medical biostasis protocols need more detailed investigation. The effects of neuroprotectants, metabolic inhibitors, anti-thrombotic drugs, and protocols to prevent and reverse cerebral edema are of specific interest.

This project is sponsored by the Aeracura Stichting in The Netherlands

Selected Journal Articles Supporting Human Cryopreservation:

First paper showing recovery of brain electrical activity after freezing to -20°C. Suda I, Kito K, Adachi C, in: Nature (1966, vol. 212), "Viability of long term frozen cat brain in vitro", pg. 268-270.

First paper to propose cryonics by neuropreservation: Martin G, in: Perspectives in Biology and Medicine (1971, vol. 14), "Brief proposal on immortality: an interim solution", pg. 339.

First paper showing recovery of a mammalian organ after cooling to -196°C (liquid nitrogen temperature) and subsequent transplantation: Hamilton R, Holst HI, Lehr HB, in: Journal of Surgical Research (1973, vol 14), "Successful preservation of canine small intestine by freezing", pg. 527-531.

First paper showing partial recovery of brain electrical activity after 7 years of frozen storage: Suda I, Kito K, Adachi C, in: Brain Research (1974, vol. 70), "Bioelectric discharges of isolated cat brain after revival from years of frozen storage", pg. 527-531.

First paper suggesting that nanotechnology could reverse freezing injury: Drexler KE, in: Proceedings of the National Academy of Sciences (1981, vol. 78), "Molecular engineering: An approach to the development of general capabilities for molecular manipulation", pg. 5275-5278.

First paper showing that large organs can be cryopreserved without structural damage from ice: Fahy GM, MacFarlane DR, Angell CA, Meryman HT, in: Cryobiology (1984, vol. 21), "Vitrification as an approach to cryopreservation", pg. 407-426.

First paper showing that large mammals can be recovered after three hours of total circulatory arrest ("clinical death") at +3°C (37°F). This supports the reversibility of the hypothermic phase of cryonics: Haneda K, Thomas R, Sands MP, Breazeale DG, Dillard DH, in: Cryobiology (1986, vol. 23), "Whole body protection during three hours of total circulatory arrest: an experimental study", pg. 483-494.

First detailed discussion of the application of nanotechnology to reverse human cryopreservation: Merkle RC, in: Medical Hypotheses (1992, vol. 39), "The technical feasibility of cryonics", pg. 6-16.

First successful application of vitrification to a relatively large tissue of medical interest: Song YC, Khirabadi BS, Lightfoot F, Brockbank KG, Taylor MJ, in: Nature Biotechnology (2000, vol. 18), "Vitreous cryopreservation maintains the function of vascular grafts", pg. 296-299.

First report of the consistent survival of transplanted kidneys after cooling to and rewarming from -45°C: Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E, in: Cryobiology (2004 vol. 48), "Cryopreservation of organs by vitrification: perspectives and recent advances", pg. 157-78.

First paper showing ice-free vitrification of whole brains, the reversibility of prolonged warm ischemic injury without subsequent neurological deficits, and setting forth the present scientific evidence in support of cryonics: Lemler J, Harris SB, Platt C, Huffman T, in: Annals of the New York Academy of Sciences, (2004 vol. 1019), "The Arrest of Biological Time as a Bridge to Engineered Negligible Senescence", pg. 559-563.

First discussion of cryonics in a major medical journal: Whetstine L, Streat S, Darwin M, Crippen D, in: *Critical Care*, (2005, vol. 9), "Pro/con ethics debate: When is dead really dead?", pg. 538-542.

First demonstration that both the viability and structure of complex neural networks can be well preserved by vitrification: Pichugin Y, Fahy GM, Morin R, in: *Cryobiology*, (2006, vol. 52), "Cryopreservation of rat hippocampal slices by vitrification", pg. 228-240.

Rigorous demonstration of memory retention after cooling to +10°C (59°F). Alam HB, Bowyer MW, Koustova E, Gushchin V, Anderson D, Stanton K, Kreishman P, Cryer CM, Hancock T, Rhee P, in: Surgery (2002, vol. 132), "Learning and memory is preserved after induced asanguineous hyperkalemic hypothermic arrest in a swine model of traumatic exsanguination", pg. 278-88.

Review of scientific justifications of cryonics: Best BP, in: Rejuvenation Research (2008, vol. 11), "Scientific justification of cryonics practice", pg. 493-503.

First successful vitrification, transplantation, and long-term survival of a vital mammalian organ: Fahy GM, Wowk B, Pagotan R, Chang A, Phan J, Thomson B, Phan L, in: Organogensis (2009, vol. 5), "Physical and biological aspects of renal vitrification" pg. 167-175.

First demonstration of memory retention in a cryopreserved and revived animal: Vita-More N, Barranco D, in: *Rejuvenation Research*, (2015, vol. 18), "Persistence of Long-Term Memory in Vitrified and Revived Caenorhabditis elegans", pg. 458-463.

First demonstration of whole brain vitrification with perfect preservation of neural connectivity ("connectome") throughout the entire brain: McIntyre RM, Fahy GM, in: *Cryobiology*, (2015, vol. 71), "Aldehyde-stabilized cryopreservation", pg. 448-458.