C-1

The Use of Engineering Modeling in Designing Cryopreservation Protocols for Articular Cartilage. J. A. W. ELLIOTT[1], A. Abazari[2], N. Shardt[2], L. McGann[1], and N. Jomha[3]. [1]University of Alberta, 7th Floor ECERF, Room 7-026A, 9107 - 116 Street, Edmonton, AB T6G 2V4, CANADA; [2]Chemical and Materials Engineering, University of Alberta, Edmonton, AB T6G 2V4, CANADA; and [3]Surgery, University of Alberta, Edmonton, AB T6G 2B7, CANADA. Email: Janet.Elliott@ualberta.ca

The transplantation of bone-bearing living articular cartilage is an effective treatment for joint injury and disease. Widespread access to this treatment, however, will require the ability to cryopreserve and bank this tissue. The cryopreservation of articular cartilage has been studied for more than 50 years, with early unsuccessful attempts at freezing of cartilage slices [R.C. Curran, T. Gibson, Proc. Royal Soc. London B 144 (1956) 572] and the first successful cryopreservation of isolated chondrocytes reported in 1965 [A.U. Smith, Nature 205 (1965) 782]. Recently, our group developed a protocol to cryopreserve human full-thickness articular cartilage attached to bone resulting in chondrocyte membrane integrity of 75.4 +/– 12.1% with functionality of chondrocytes confirmed by a metabolic assay and the ability of extracted, pellet-cultured chondrocytes to produce of collagen II and sulfated glycosaminoglycans at levels similar to healthy controls [N.M. Jomha et al., Biomaterials, 33 (2012) 6061.] This protocol, aimed at vitrifying the tissue, requires the addition of four different mixtures of four different cryoprotectants added at different temperatures for specific periods of time. To develop this protocol, we relied heavily on engineering modeling. This presentation will review our research over the past 10 years on modeling of various key phenomena relevant to the cryopreservation of articular cartilage including solution thermodynamics; water transport; and the transport, toxicity, and vitrifiability of cryoprotectants. The research being reviewed was funded primarily by the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council of Canada, Alberta Innovates, and the Edmonton Orthopaedic Research Committee. J. A. W. Elliott holds a Canada Research Chair in Thermodynamics.

C-2

Kinetic Vitrification: Basic Thermodynamics, Methods and Devices. IGOR I. KATKOV. CELLTRONIX, San Diego, CA 92126. Email: prodvincell@hotmail.com

Cryopreservation (CP) of cells by ultra-fast (tens of thousands C/min) kinetic vitrification (K-VF) of small samples without additional thickeners and ice-blocking vitrification agents (VFAs) had been the first method of cryopreservation back in the 1940s. The mainstream of CP later shifted towards slow freezing (SF). Equilibrium vitrification (E-VF) with very high concentrations of VFAs and relatively moderate rates of cooling and warming (reported in 1980s) turned attention back to VF due it seemingly simple methodology. It however soon became clear that E-VF had had its own substantial limitations due to very high toxicity and osmotic damage produced by VFAs *per se*. As we pointed out in 2012, the wave has been turning back toward K-VF, especially in the fields of CP of reproductive and germ cells. In this presentation, we touch the basic distinctions between the 3 major ways of CP, with specific emphasis to the means, methods and equipment, as well as pitfalls of the current approaches to K-VF, such as Leidenfrost effect (LFE) and restrictions of the sample size. A short video that demonstrates an entirely new platform for hyper-fast cooling (hundreds of thousands C/min) KrioBlast™ developed by CELLTRONIX, which allows to vitrify relatively large samples (up to 4,000 mcl at the day of reporting, can be further scaled up) practically without a need for potentially damaging vitrificants such as DMSO or propylene glycol, traditionally used nowadays for VF. The system completely eliminates LFE and a need of potentially toxic and osmotically damaging permeating vitrificants such as DMSO or ethylene glycol used in the current methods of VF. We have been able to vitrify12% and in som case as low as 7% glycerol solutions as a cooling rate markers, which theoretically corresponds to the critical cooling rate up to 1,600,000 C/min or higher. This can be considered as a step toward the "Unified Cryopreservation Protocol". preliminary results that demonstrate excellent of survival of human embryonic stem cells and sperm vitrified with KrioBlast™ are also discussed.

Disclosure: Author was or is employed by CELLTRONIX.

C-4

Modulation of Heat and Mass Transfer During Freezing/Thawing of Cell Suspensions. GANG ZHAO[1], Xiaoming Zhou[2], and Dayong Gao[3]. [1]Department of Electronic Science and Technology, University of Science and Technology of China, Hefei 230027, CHINA; [2]School of Mechanical, Electronic and Industrial Engineering, University of Electronic Science and Technology of China, Chengdu 611731, CHINA; and [3]Department of Mechanical Engineering, University of Washington, Seattle, WA 98195. Email: ZhaoG@ustc.edu.cn

From the angel of the discipline of engineering thermophysics, cryopreservation involves typical heat and mass transfer, and thermodynamic processes, e.g., i) freezing and thawing of both extra and intracellular solutions, ii) transport of water and cryoprotective agents across cell membrane, and iii) ice nucleation and the growth of ice crystals in these solutions. To achieve the highest cell survival rate after a freeze-thaw cycle, efforts on optimization of these processes are indispensable. Recent studies have revealed that the introduction of nanoparticles into cell suspensions may affect the thermal

properties of both the extra and intracellular solutions, the nucleation and the growth of ice crystals in them, and the cell membrane permeability, and thus it may be used to change the heat and mass transfer, and the thermodynamic processes. Besides, the superparamagnetic nanoparticles could be used to further enhance the rewarming efficiency of frozen cell suspensions in the external electromagnetic field usually excited in a single mode cavity. This presentation will highlight recent work on the mode of action by nanoparticles influencing heat transfer of the extra and intracellular solutions, mass transfer across cell membrane, and ice nucleation and the growth of ice crystals during freezing/thawing.

C-5

A Versatile and Robust MEMS-based Microdevice for High Accuracy Thermal Conductivity Measurements of Semi-rigid Biomaterials and Solutions. XIN M. LIANG[1, 2], Praveen K. Sekar[2], Xiaoming Zhou[3], Gang Zhao[1], Weiping Ding[1], Jingru Yi[1], and Dayong Gao[2]. [1]Centre for Biomedical Engineering, Department of Electronic Science and Technology, University of Science and Technology of China, Hefei, Anhui 230027, CHINA; [2]Department of Mechanical Engineering, University of Washington, Seattle, WA 98195; and [3]School of Mechanical, Electronic, and Industrial Engineering, University of Electronic Science and Technology of China, Chengdu, Sichuan 611731, CHINA. Email: liangxin@ustc.edu.cn, dayong@uw.edu

Accurate thermal property measurements of biological materials and various solutions (i.e. CPAs with different concentrations) are critical in the development of cryopreservation- and cryotherapy-related applications. Although a number of approaches for obtaining the thermal conductivity of semi-rigid materials and solutions have been developed and characterized, they all exhibit deficiencies in terms of accuracy, data repeatability, device production quality, and sensor configuration flexibility. Here we introduce a MEMS-based micro-thermal conductivity sensor in both single probe and multi-probe forms. The presented sensor along with the standardized measuring procedure has been successfully utilized to study the thermal properties of commonly used CPA (pure and mixtures with different concentrations) and multiple biomaterials under various temperatures. Overall, the MEMS enabled micro-thermal conductivity sensor is a miniaturized robust biomedical microdevice with superior capabilities to overcome the inherent limitations of existing macroscopic counterparts to deliver high accuracy thermal conductivity data for penetrable biomaterials and liquids.

C-6

Clinical Impact of the Cell Death Continuum in Hypothermic Organ Systems Manifest as Ischemia/Peperfusion Injury. MICHAEL J. TAYLOR. Cell and Tissue Systems, N. Charleston, SC and Dept. Mechanical Engineering, Carnegie Mellon University, Pittsburgh, PA. Email: mtaylor@ celltissuesystems.com

The application of hypothermia for *in vivo* and *ex vivo* organ preservation is based upon the protective effect of reduced temperatures against the deleterious effects of ischemia and hypoxia that ensues from blood loss, or organ procurement for transplantation. However, low temperatures have a multitude of effects, not all of which are beneficial. Special conditions have been developed to harness the protective properties of cold for effective organ preservation and the development of specialized solutions is the primary factor in the evolution of hypothermic organ preservation technology. Even in pre-clinical research models, extreme hemodilution by blood replacement with synthetic, acellular crystalloid/colloid solutions has proved efficacious for avoiding ischemia/reperfusion *in vivo* after several hours of circulatory arrest under ultraprofound hypothermia (<10°C). The modern era of organ transplantation, which increasingly uses organs from marginal donors including those compromised by prior warm ischemia, is reliant on preservation technology to stabilize and even resuscitate the donor organ. Ischemic insults (warm and cold) are incurred during each stage of the transplantation process from procurement, through preservation for storage and shipping, to re-implantation and re-perfusion. Each stage demands optimized conditions for the best outcome and hypothermia enhanced by appropriately designed preservation solutions is not only the basis for current standard practices, but offers the path towards improved techniques in the future. The most promising approach for further improvement of hypothermic preservation technology is the selection of cytoprotective and resuscitative agents as supplements to the baseline perfusates to counteract ischemia/reperfusion injury. Specific targets include reactive oxygen species, pro-inflammatory cytokines and other cell stress mediators that lead to death by apoptosis or necrosis. This approach has recently been applied successfully to hypothermic perfusion preservation of clinical livers.

Disclosure: Author was or is employed by Cell & Tissue Systems, Inc.

C-7

The Impact of the Molecular-based Cell Death Continuum on Cryoablation and Biopreservation Outcome. JOHN M. BAUST. CPSI Biotech, 2 Court St., Owego, NY 13827. Email: jmbaust@cpsibiotech.com

The discipline of hypothermic medicine (Cryo) covers a wide diversity of application areas ranging from organ transplantation to biopreservation of cells and tissues to cryoablation. With established techniques Cryo is often viewed as an “old school” discipline yet modern Cryo is in the mist of another scientific and technology development growth

phase. In this regard, today's Cryo finds itself at the forefront of research in the areas of molecular biology and engineering. While covering a wide range of temperatures (~4°C to -196°C, nominally) and exposure times (minutes to years), each Cryo application utilize low temperature to depress or halt biological activity on a transient or permanent basis. While the ultimate outcome desired may vary significantly (preservation vs. ablation) the overall response of a biologic to low temperature exposure share a number of commonalities, as well as differences, at the molecular level. Recent studies have shown that the control and direction of these molecular responses significantly impacts final outcome. This presentation will provide an overview of our current understanding of the molecular stress response of cells to low temperature exposure (ranging from ~4°C to -196°C), the interrelated role of the apoptotic and necrotic cell death continuum and how this impacts outcome in a diversity of clinical and research settings including preservation and ablation. Further, discussion will include studies focused on the targeted modulation of common and/or cell specific responses to cold and freezing temperatures as a potential path to tailoring outcomes, from improved cell function to enhanced cancer ablation. This line of investigation has provided a new direction and molecular-based foundation guiding new research, technology development and procedures. As the use of and the knowledge base surrounding Cryo continues to expand this new path will continue to provide for improvements in overall Cryo efficacy and outcome.

Disclosure: Author was or is employed by CPSI Biotech.

C-8

Molecular Mechanisms of Cell Death Following Cryosurgery and the Impact of Super Critical Nitrogen. ANTHONY ROBILOTTO[1,2], John M. Baust[1,2], Robert G. Van Buskirk[1,2], Andrew A. Gage[2,3], and John G. Baust[1]. [1]Institute of Biomedical Technology, Binghamton University, Binghamton, NY 13902; [2]CPSI Biotech, 2 Court St, Owego, NY 13827; and [3]Department of Surgery, SUNY Buffalo, Buffalo, NY 14260. Email: arobilotto@cpsibiotech.com

The lesion formed during cryosurgery is a highly dynamic environment composed of gradients in ice composition, osmolarity, pH, and temperature that vary both spatially and temporally. Investigations into the mechanisms of cell death within this complex cryolesion have led to the classical view that cell death is transitional with freeze rupture nearest the cryoprobe, necrosis throughout the lesion, and a delayed apoptosis confined primarily to the periphery. More recent studies, however, have shown cell death to be more fluid. Apoptotic cell death has been observed not only in the periphery, but also at colder isotherms deep within a lesion, and studies with caspase inhibitors have shown the ability of cells to transition between apoptosis and necrosis. As studies continue to show the innate, multi-faceted nature of cryosurgery, a window has opened for the development of new strategies and technologies to more effectively ablate targeted tissues. The identification of the combination of physical and molecular cell death throughout the lesion has begun to drive a series of technological advancements in cryosurgical devices. This has led, in part, to the development of a novel cryosurgical device utilizing super critical nitrogen (SCN) as the cryogen. Capable of generating colder temperatures more rapidly and confined to a smaller overall volume, the SCN system represents the next generation of cryosurgical devices. This presentation will focus on the recent findings of rapid-onset cell death and the growing understanding of the cell death continuum, and how the integration of the molecular response with the technological advances of SCN are providing a next generation approach to cryosurgery. As the field of cryosurgery continues to evolve, it is critical that the impact on the biology of cells, tissues, and organs be understood through both device development and laboratory/clinical research. This is particular significant as new technologies begin to offer clinically effective, cryoablative options for disease states beyond the reach of current cryosurgical devices.

C-9

Monitoring the Effects of Dual Thermal Ablation on Pancreatic Cancer Cell Line PANC-1. K. W. BAUMANN[1], K. Snyder[2], J. Baust[2], R. Van Buskirk[1], and J. Baust[1]. [1]Binghamton University, Science 3, Room 210, PO Box 6000, Binghamton, NY 13902-6000 and [2]CPSI Biotech, 2 Court St, Owego, NY 13827. Email: kbauman1@binghamton.edu

Pancreatic cancer (PCC) is the 4th leading cause of cancer deaths in the US and the 8th worldwide. Difficulty in diagnosing PCC and typical poor prognosis from current therapies contribute to low patient 5-year survival (<5%). Cryoablation is a minimally invasive technique using low temperature to treat many solid mass cancers. Radiofrequency ablation (RFA) exposes the tumor mass to high temperatures (>37°C) to induce cell death. This study investigated the effects of a tandem heat and cryotherapy model on PCC. This combination, a dual thermal ablation (DTA) approach, was compared to the results of heat or cryoablation exposure alone on the PCC line PANC-1. DTA samples underwent 5 min heating exposure at temperatures of 45 to 60°C in 5°C intervals with 1 min recovery at RT followed by freezing exposure at temperatures of -10 to -25°C for 5 mins. After freezing, samples were passively thawed at RT for 10 mins. Single thermal exposed samples underwent 5 mins of freezing/10 mins of recovery or 5 mins of heating/1 min of recovery. Fluorescence microscopy (Calcein-AM /PI) and flow cytometry (YO-PRO-1/PI) assessed levels of viability, apoptosis and necrosis during the 24hr recovery period. AlamarBlue metabolic activity assay was performed for 1 week on days 1, 3, 5, and 7, normalized to untreated controls.

Results indicate a single thermal ablation was less lethal than DTA exposure at the same conditions. Following 1 day of recovery, exposure at 50°C (65.2%) or -15°C (58.9%) resulted in higher sample viability compared to DTA (4.7%). Live cell populations measured using flow cytometry 24hrs after exposure to -15°C (48.1%) or 50°C (57.8%) were higher compared to DTA (4.5%). Fluorescence microscopy corroborated these results with observed increases in necrotic cell populations from 1 to 24hrs. Exposure below -25°C or above 60°C, resulted in complete ablation of PANC-1 cells. DTA treatment (50°C/-20°C) also achieved complete cell loss. These results support the utilization of DTA for the treatment of PCC.

C-10

Development of Advanced Technology and Methods for Cryopreservation: Seattle Experience. DAYONG GAO. Department of Mechanical Engineering, Department of Bioengineering, and Research Center for Cryobiology and Cryo-Biomedical Engineering, University of Washington, Seattle, WA 98195. Email: dayong@u.washington.edu

This presentation reports recent activities and results in cryobiology and cryopreservation research with a focus on the new engineering methods, instruments, and advanced technology developed at the Research Center for Cryobiology and Cryo-Biomedical Engineering at University of Washington (UW) collaborating with the UW Medical Center, Fred Hutchinson Cancer Research Center, and Puget Sound Blood Center in Seattle, Washington.

C-11

High-speed Video Imaging of Ice Crystal Formation in Cells, Tissues and Droplets. JENS O. M. KARLSSON. Department of Mechanical Engineering, and Cellular & Molecular Bioengineering Research Group, Villanova University, Villanova, PA 19085. Email: jens.karlsson@ villanova.edu

Attempts to elucidate the causes of cryoinjury have benefited from nearly two centuries of microscopic observation of cell and tissue response during exposure to cold temperatures. However, the mechanisms of intracellular ice formation, a phenomenon that is strongly associated with irreversible cell damage, remains poorly understood: even in suspended, isolated cells, it has not been possible to obtain experimental evidence that conclusively supports or contradicts any of the competing hypotheses of intracellular ice formation (viz., the surface-catalyzed nucleation model, pore theory, or membrane rupture hypothesis). In part, this lack of progress has been due to the limited temporal resolution of conventional cryomicroscopy technology. Until recently, image acquisition rates in video cryomicroscopy studies have typically not exceeded 30 frames per second (fps), yielding a temporal resolution of 33 ms. Because the transformation of cell water to ice is completed in approximately 1 ms or less during rapid-cooling experiments, intracellular ice formation events cannot usually be detected using conventional video-micrography. As a result, cryomicroscopists have relied on the observation of cytoplasmic opacity changes as a proxy for intracellular freezing, an approach that can cause significant errors in estimates of intracellular ice formation kinetics. To detect the initial appearance and growth of ice crystals inside supercooled cells, and to quantify the phase transformation kinetics, it is necessary to use high-speed video cryomicroscopy to acquire micrographs at sub-millisecond resolution. In particular, to accurately measure intracellular ice formation kinetics in rapidly cooled somatic cells, image acquisition rates in the range 10^3-10^4 fps are required, whereas shutter speeds should be faster than ~0.1 ms to prevent motion blur. We have implemented such a high-speed imaging cryomicroscopy system, and have successfully used it to visualize non-equilibrium ice crystal formation and growth inside cells, tissue constructs, and microscale water droplets.

C-12

Microfluidic Processing of Cryopreserved Blood. ADAM Z. HIGGINS and Ratih E. Lusianti. School of Chemical, Biological and Environmental Engineering, Oregon State University, 102 Gleeson Hall, Corvallis, OR 97331-2702. Email: adam.higgins@oregonstate.edu

Cryopreservation of human red blood cells (RBCs) in the presence of 40% glycerol allows a shelf-life of 10 years, as opposed to only 6 weeks for refrigerated RBCs. Nonetheless, cryopreserved blood is rarely used in clinical therapy, in part because of the requirement for a time-consuming (~1 h) post-thaw wash process to remove glycerol before the product can be used for transfusion. The current deglycerolization process involves a series of saline washes in an automated centrifuge, which gradually removes glycerol from the cells in order to prevent osmotic damage. We recently demonstrated that glycerol can be extracted in as little as 3 min without excessive osmotic damage if the composition of the extracellular solution is precisely controlled. Here, we explore the potential for carrying out rapid glycerol extraction using a membrane-based microfluidic device, with the ultimate goal of enabling inline washing of cryopreserved blood. To assist in experimental design and device optimization, we developed a mass transfer model that allows prediction of glycerol removal, as well as the resulting cell volume changes. Experimental measurements of solution composition and hemolysis at the device outlet are in reasonable agreement with model predictions, and our results demonstrate that it is possible to reduce the glycerol concentration by more than 50% in a single device without excessive hemolysis. Based on these promising results, we present a design for a multistage process that is predicted to safely remove glycerol from cryopreserved blood in less than 3 min.

C-13

MEMS Coulter Counters for Dynamic Impedance Measurement of Time Sensitive Cells. JAMES BENSON[1], Yifan Wu[2], and Mahmoud Almasri[3]. [1]Department of Mathematical Sciences, Northern Illinois University, DeKalb, IL 60115; [2]Micron Technology, Boise, ID 83716; and [3]Electrical and Computer Engineering, University of Missouri, 233 Engineering Building West, Columbia, MO 65211. Email: benson@math.niu.edu

There is a long history of cryobiologists adapting a Coulter counter to measure cellular biophysical parameters including establishing that cells are ideal osmometers, estimating water and solute permeabilities and their activation energies, and measuring isosmotic volume. These techniques have been used in many cell types to facilitate prediction of optimal protocols for CPA addition and removal as well as optimal cooling and warming protocols. While it has advantages over other techniques in some aspects, the Coulter counter technique is also challenging in several scenarios. First, the technique requires large sample volumes and cell counts, and introduces a several second time lag between exposure to anisosmotic media and cell volume detection. In many cell types and at low temperatures, this lag may not critical, but for cells with very high surface area to volume ratios, or cells with very high water permeability, this lag makes accurate biophysical measurement nearly impossible. Moreover, it is experimentally challenging to perform measurements at temperatures other than ambient. Finally, volume measurements are taken from individual cells only once as they pass through an aperture. This yields a "population" permeability estimate, and requires considerable data reduction and advanced parameter estimation techniques to obtain mean biophysical parameter values. Here we describe the design, fabrication and testing of a MEMS Coulter counter specifically designed for dynamic biophysical measurements. In particular, as opposed to several existing MEMS coulter counters that size static cells, ours has an ultrarapid mixing region in which individual cells are rapidly mixed with desired reagents (e.g. anisosmotic media) and their volumes are measured at ten specific time points. Our new approach allows initial measurement of cell responses in less than 100 ms, provides a much improved estimate of the population variance of water and solute permeability, and facilitates very simple temperature control of the system. We present initial data and analysis of system output.

C-14

Application of Hydrogel in Cryopreservation as a Novel Cryoprotectant, Encapsulation and Single-cell-based Functional Sensing Material. ZHIQUAN SHU[1], Wanyu Chen[1,2], Amy Q. Shen[1], and Dayong Gao[1]. [1]Department of Mechanical Engineering, University of Washington, Seattle, WA, 98195 and [2]School of Materials Science and Engineering, Wuhan University of Technology, Wuhan, 430070, CHINA. Email: zqshu@u.washington.edu, dayong@uw.edu

Some challenges still remain in Cryopreservation and cytotherapy, including (1) cryoinjury due to intracellular ice formation may cause cell loss during cryopreservation. Non-freezing preservation may be achieved by vitrification with ultra-high cooling rate or cryoprotectant concentration; however, this is unfeasible or cytotoxic. (2) Immune rejection response may happen to the transplanted cell products. Microencapsulation of the transplanted cells has been used to immobilize xenograft cells in the last few decades. However, the progress in this field has not met the expectations yet. The major problems include biocompatibility, possible function loss during encapsulation, mechanical and chemical stability of the encapsulation membranes, generation reproducibility of the uniform capsules, suitable membrane permeability that protects the implants from immune response of the host and simultaneously permits passage of nutrients and secretions. (3) A fast, simple and reliable quality control assay for the cells along the whole process is desirable. Especially, single-cell-based quality assessment has many advantages. In this work, application of hydrogel (application for rat islet as the example) was studied to address the three challenges listed above. A microfluidic device was applied to generate individual-cell based microencapsulation with uniformity and reproducibility. Meanwhile, biosensing agent was embedded in the hydrogel layer for single-cell-based biosensing, which can act as a simple quality control assay in cryopreservation and cytotherapy. It was found that glass transition of hydrogel may be achieved by tuning the crosslinking network density and water content. This implies that hydrogel can be potentially a novel cryoprotectant for non-freezing preservation purposes. In conclusion, hydrogel can serve as a cryoprotectant, immune barrier, and biosensing agent carrier. Further investigations are needed, especially the properties of hydrogel as cryoprotectant, which may lead to many striking and interesting applications in cryobiology.

C-15

Comparative Cryobiology of Germplasms in Non-traditional Species. PIERRE COMIZZOLI. Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC. Email: comizzolip@si.edu

One of the greatest challenges to retaining viability of frozen gametes and reproductive tissues is understanding and overcoming species-specificities. This also is important because there is significant diversity in the cryobiological properties and requirements among cell types and tissues themselves. Systematic studies can lead to successful post-thaw recovery, especially after determining (1) membrane permeability to water and cryoprotectant, (2) cryoprotectant toxicity, (3) tolerance to osmotic changes and (4) resistance to

cooling and freezing temperatures. While species-dependency ultimately dictates the ability of specific cells and tissues to survive freeze-thawing, there are commonalities between taxa that allow a protocol developed for one species to provide useful information for another. This is the primary reason for performing comparative cryopreservation studies among diverse species. Our laboratory has compared cellular cryotolerance, especially in spermatozoa, in a diversity of animals - from coral to elephants - for more than 30 years. Characterizing the biophysical traits of gametes and tissues is the most efficient way to develop successful storage and recovery protocols. Such data are only available for a few laboratory, livestock and fish species, with virtually all others (wild mammals, birds, reptiles and amphibians) having gone unstudied in this arena. Nonetheless, when a rare animal unexpectedly dies, there is no time to understand the fundamentals of biophysics. In these emergencies, it is necessary to rely on experience and the best data from taxonomically-related species. Fortunately, there are some general similarities among most species, which, for example, allow adequate post-thaw viability. Regardless, there is a priority for more information on biophysical traits and freezing tolerance of distinctive biomaterials, especially for oocytes and gonadal tissues, and even for common, domesticated animals.

C-16

Selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species. UTKAN DEMIRCI. Bio-Acoustic-MEMS in Medicine (BAMM) Laboratory, Canary Center, Department of Radiology, Stanford School of Medicine, Palo Alto, CA. Email: utkan@ stanford.edu

Fertilization and reproduction are central to the survival and propagation of a species. Couples who cannot reproduce naturally have to undergo *in vitro* clinical procedures. An integral part of these clinical procedures includes isolation of healthy sperm from raw semen. Existing sperm sorting methods are not efficient and isolate sperm having high DNA fragmentation and reactive oxygen species, and suffer from multiple manual steps and variations between embryologists. Inspired by *in vivo* natural sperm sorting mechanisms where vaginal mucus becomes less viscous to form microchannels to guide sperm towards egg, we present a chip that efficiently sorts healthy, motile and morphologically normal sperm without centrifugation. Higher percentage of sorted sperm show significantly lesser reactive oxygen species and DNA fragmentation than the conventional swim-up method. The presented chip is an easy-to-use high throughput sperm sorter that provides standardized sperm sorting assay with less reliance on embryologist's skills, facilitating reliable operational steps.

C-17

Human Oocyte Cryopreservation: Current Status and Outlook. ALI EROGLU. Medical College of Georgia/Georgia Regents University, 1120 15th Street, CA-2004 Augusta, GA. Email: aeroglu@gru.edu

Human oocyte cryopreservation is desired to preserve future fertility of young cancer patients, delay childbearing years, and avoid legal and ethical issues associated with embryo freezing. Unlike embryo freezing, oocyte cryopreservation has proved to be challenging due to susceptibility of oocytes to various cryoinjuries such as intracellular ice formation, disruption of cytoskeleton and spindle microtubules, premature exocytosis of cortical granules and zona hardening, parthenogenetic activation, and polyploidy. Although the first successful cryopreservation of human oocytes was reported in 1986, it took a decade of research and the use intracytoplasmic sperm injection to partially address some of the cryoinjuries and reproduce the initial success of human oocyte cryopreservation. Even then, the overall success rates were low and usually around 1-5%. These initial studies used slow cooling protocols. The first successful vitrification of human oocytes was achieved in 1999 with an overall success rate of ~6%. Since then, considerably better results have been reported using both slow cooling and vitrification protocols. While the overall success rate after slow cooling still remains significantly lower than that of unfrozen controls, some fertility centers reported fertilization and pregnancy rates comparable to in vitro fertilization cycles using fresh oocytes after a vitrification approach that uses a minimal sample volume (<1µL) and requires direct contact with liquid nitrogen to achieve extremely fast cooling/warming rates. Based on these encouraging results, the Practice Committee of the American Society for Reproductive Medicine removed the "experimental" label of human oocyte cryopreservation in 2012. Yet currently used cryopreservation techniques still suffer from several shortcomings including biosafety issues and devitrification risks associated with vitrification techniques, substantial difficulties in transportation of samples, and reliability issues. Further research is needed to address these shortcomings.

C-19

Zebrafish Cryobiology: Sperm, Oocytes, Embryos and Stem Cells. MARY HAGEDORN. Smithsonian Conservation Biology Institute, Washington, D.C. 20008. Email: hagedornm@si.edu

Cryopreservation is a proven method for long-term maintenance of genetic material, nevertheless current protocols for fish are not standardized and yield inconsistent results, threatening the efficacy of large-scale genetic screening and stock centers. Previous reports have dealt with sperm cryopreservation in >200 fish species from marine and

freshwater species with the most common observation from these publications being the inconsistency in the post-thaw results. Consortia are working to identify processes in zebrafish protocols to try and improve post-thaw variability. Although a great deal of work has been done examining the cryobiology of zebrafish oocytes at various stages of maturation, the successful cryopreservation, maturation and subsequent fertilization of these oocytes has not been proven. Similarly, zebrafish embryo cryopreservation has been extensively examined for the past 25 years, and several aspects of standard cryopreservation methods have made it a challenging system to develop successful protocols. These problems include: 1) a large overall size resulting in a low surface to volume ratio that can slow water cryoprotectant efflux and influx; 2) the presence of compartments, such as the blastoderm and yolk; with different permeability properties, 3) susceptibility to chilling injury; and 4) extreme chilling sensitivity at early relatively undifferentiated developmental stages necessitating vitrification. New laser-warming protocols may help change how we understand this complex suite of challenges in zebrafish embryo cryopreservation, especially the relative need for cryoprotectant and the potential for prevention of damaging intracellular ice crystal formation. To diversify and expand our resource collections, however, cryopreserved zebrafish spermatogonial stem cells may provide one of the most important strategies for resource centers. Researchers have successfully developed simple effective cryopreservation methods for zebrafish testes, allowing the rapid and effective preservation of mutant and transgenic lines.

C-20

Dry Preservation of Decellularized Heart Valve Tissues. WILLEM F. WOLKERS. Leibniz University Hannover, GERMANY. Email: wolkers@imp.uni-hannover.de

Decellularized heart valve tissues are receiving increased attention as matrix implants. Storage of these biological matrices in a dry state has clear economic and practical advantages compared to frozen storage. Long-term storage becomes available at room temperature without the need of liquid nitrogen or expensive freezer systems. We have investigated the effects of drying on heart valve scaffolds using sucrose as protectant. First, diffusion kinetics of sucrose in the scaffolds was studied using attenuated total reflection infrared spectroscopy. These studies indicated that the scaffolds can be homogeneously loaded with sucrose within several hours at 37 degrees C. After loading with sucrose, the scaffolds were either freeze- or vacuum-dried, and the structure was evaluated by histology staining. Freeze-drying in the absence of sucrose caused an overall disintegrated appearance of the histological architecture. Sucrose (5% w/v) protects during drying, but freeze-dried scaffolds were found to have a more porous structure, likely due to ice crystal formation. Rapid freezing, which reduces ice crystal size, was found to reduce the pore size. No pores were observed when samples were incubated in high concentrations of sucrose (80% w/v) and the overall architecture closely resembled that of fresh tissue. Vacuum drying, which lacks a freezing step, has the advantage that drying times are much shorter. Vacuum-dried tissue displayed an intact histological architecture similar to freeze-dried tissue. In conclusion, both freeze-drying and vacuum-drying hold promise for preservation of decellularized heart valve tissues and could possibly replace vitrification or cryopreservation approaches in the near future.

C-21

Tissue Vitrification. KELVIN GM BROCKBANK. Cell & Tissue Systems, Inc., 2231 Technical Parkway, Suite A, North Charleston, SC 29406. Email: kgbrockbankassoc@ aol.com

Application of the original tissue vitrification method developed by Fahy and used by my research group for rabbit blood vessels and articular cartilage has evolved over time. The formulation was 2.21 M propylene glycol, 3.10 M formamide and 3.10 M dimethylsulfoxide in EuroCollins solution and it was employed with multiple addition/removal steps to minimize osmotic damage to the cells. Ice-free cryopreservation using VS55, as originally described, is limited to small or thin samples where relatively rapid cooling and warming rates are possible. VS55 cryopreservation typically provides excellent extracellular matrix preservation with approximately 80% cell viability and tissue function compared with fresh untreated tissues. In contrast, ice-free cryopreservation using higher concentrations of the same cryoprotectants in EuroCollins solution has several advantages. High viability can be achieved for human and porcine articular cartilage employing an 83% formulation, VS83, in combination with multiple addition/removal steps. Lower cryoprotectant concentrations are not effective for large mammal articular cartilage. VS83 can also be used for tissue matrix preservation with retention of material properties at the expense of cell viability. Addition and removal of the cryoprotectants can be done in a single step followed by washing to minimize residual chemical concentrations. This protocol minimizes tissue cell viability and also reduces tissue immunogenicity suggesting that this vitrification strategy may be an alternative to the decellularization methods being employed for engineering tissues for medical implants. The evolution of these vitrification protocols will be reviewed drawing upon research employing natural and engineered tissues.

Disclosure: Author was or is employed by Cell & Tissue Systems, Inc.

C-22

Clinical Banking of Amniotic Membrane Allografts. JELENA L. HOLOVATI[1,2], Natasha MJ Perepelkin[1], Haiyan Jiao[2], Tumelo Mokoena[2], Teresa Fernandez[2], Mike Bentley[2], Mrinal

Dasgupta[1,2], and L. U. Ross-Rodriguez. [1]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, CANADA and [2]Comprehensive Tissue Centre, Alberta Health Services, Edmonton, AB, CANADA. Email: jelena.holovati@ualberta.ca

Amniotic membrane (AM) is currently collected from caesarean section placenta, processed into allograft pieces and cryopreserved by the Comprehensive Tissue Centre (CTC) in Edmonton, Alberta, Canada for regional and national ophthalmological and dermatological transplant applications. Anti-inflammatory, anti-immunogenic, and anti-fibrosis properties of AM allografts are important for post-transplant epithelialization. The purpose of this study was to evaluate quality of cryopreserved AM allografts. AM allograft sections were cultured for bacterial and fungal growth after recovery from donors and after antibiotic decontamination. Non-linear controlled-rate cooling was used to cryopreserve AM allografts (sizes: 3 cm^2, 5 cm^2, 10 cm^2). Post-thaw AM viability was assessed by a fluorescent membrane integrity assay (ethidium bromide/Syto® 13) and confocal microscopy. Cell Tissue Organ Surveillance System (CTOSS) forms provided information on AM quality from surgical end-users. While 40.9% of AMs showed positive bacterial culture results upon recovery, with 55.6% of positive culture due to *Propionibacterium* sp, 100% of cultures were negative after the antibiotic decontamination (n=22). CTOSS feedback indicated quality of 16.3% AM allografts was excellent, 61.2% satisfactory and 2.0% unsatisfactory (n=49).Percent viabilities for cryopreserved AM allografts, including 3 cm^2 (n=8), 5 cm^2 (n=7) and 10 cm^2 (n=6) were 57.8±11.0%, 42.7±8.5%, and 47.7±8.2%, respectively, with no statistically significant difference among the allograft sizes (p=0.54). There was statistical significant difference between percent viability of "fresh" allografts (47.7±13.6%) when compared to cryopreserved samples (p=0.78). The results of this study provide important information on quality of AM allografts for transplant applications. The CTC takes great pride in providing safe allografts of high quality, through excellence in practice and leadership in research.

Disclosure: Author was or is employed by University of Alberta.

C-23

The Next Frontier: Vascularized Tissues. G. FAHY. 21st Century Medicine, Inc., 14960 Hilton Drive, Fontana, CA 92336. Email: gfahy@21cm.com

Cryopreservation of thin tissues such as small animal ovaries, organ slices, skin, and blood vessels has been studied for many years. As long ago as 1980, three dozen organized tissues could be listed that had been successfully cryopreserved by freezing, including partial successes with some organs. But the cryopreservation of vascularized tissues adds another level of complexity to tissue preservation, and requires consideration of vascular dynamic issues that must be added to other common factors in tissue cryopreservation. Most early studies illustrate well the pitfalls of delivering and removing cryoprotectants by perfusion, but fortunately, much has been learned since then, and it now appears that the preservation of even whole organs will be possible by vitrification in the foreseeable future. Extension of current work on kidneys to hearts and other organs and limbs could become and remain a new frontier for decades to come. In the meantime, there are at least four lessons learned that may be of assistance for the preservation of non-vascularized tissues as well. First, models of optimized tissue treatment with cryoprotectants developed for organs may be more applicable to non-vascularized tissues than to organs. Second, whole organ research has highlighted the importance of, and possible remedies for, chilling injury which is a factor that is rarely considerd in conventional tissue freezing or vitirfication protocols. Chilling injury may depend on cell volume and hence on levels of and gradients in tissue CPA concentration. Third, conventional tissue freezing usually ignores the possibility of cryoprotectant toxicity in the frozen state, a problem that may be reduced by using low-toxicity cryoprotectant mixtures of the kind necessary for vitrifying kidneys. Recent studies indicate that even polymeric agents appear to permeate well enough into unperfused tissues to be beneficial. Finally, use of synthetic ice blockers also appears applicable to non-perfused tissues, and may have benefits even during freezing for the prevention of damaging recrystallization.

C-24

Ice-binding Proteins and Their Interactions with Ice Crystals. IDO BRASLAVSKY[1,2], Ran Drori[1], Yeliz Celik[2], Maya Bar Dolev[1], and Peter L. Davies[3]. [1]The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, 7610001, ISRAEL; [2]Department of Physics and Astronomy, Ohio University, Athens, OH 45701; and [3]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON K7L 3N6, CANADA. Email: ido. braslavsky@mail.huji.ac.il

Ice-binding proteins (IBPs) depress the freezing point of the body fluids below the melting point, resulting in a thermal hysteresis (TH) that prevents freezing of the organism in supercooled conditions and inhibits ice re-crystallization in frozen tissues. The potential of these proteins in the medical sector, in cryopreservation, in the frozen food industry, and in agriculture is enormous. The mechanisms by which IBPs interact with ice surfaces are still not completely understood and the potential of IBPs as cryoprotecting agents has not yet been realized. We are investigating the interactions of IBP with ice surfaces. In particular we are interested in the difference between hyperactive antifreeze proteins and moderately active ones, and the dynamic nature of the protein:ice interaction. We have developed novel methods to study these issues, including

fluorescence microscopy techniques combined with temperature-controlled microfluidic devices (Celik *et. al.* PNAS 2013). These techniques enable the replacement of the IBP solution surrounding an IBP-bound ice crystal by buffer, without disturbing the bound IBP. These results show that the binding of IBP to ice is irreversible. We also investigated the dynamic nature of the interactions between IBP and ice. We found that the TH-gap is sensitive to the time allowed for the proteins to accumulate on ice surfaces. This sensitivity changes dramatically between different types of IBPs. In a study of ice shaping during growth and melting we have demonstrated a correlation between ice crystal shapes, the shaping process, and the affinity of IBPs for the basal plane (Bar-Dolev *et al*, R. Soc. Interface 2012). Our results point to a connection between the dynamics and level of activity of different types of IBP to their ability to bind to particular ice orientations. These results contribute to an understanding of the mechanisms by which various IBPs act that will be critical for the successful use of IBP in cryobiological applications.
Disclosure: Supported by ERC, NSF, ISF, and CIHR.

C-25
The Enigmatic Role of Intercellular Junction Structures in Tissue Freezing. JENS O. M. KARLSSON. Department of Mechanical Engineering, and Cellular & Molecular Bioengineering Research Group, Villanova University, Villanova, PA 19085. Email: jens.karlsson@villanova.edu

The increased risk of intracellular ice formation during freezing of intact multicellular tissue, as compared to the intracellular crystallization probability in isolated cells, is commonly attributed to the effects of junctional protein assemblies at the interface between two neighboring cells. In particular, for the past two decades, gap junctions have been suspected to play a major role in accelerating the intracellular freezing kinetics in tissue, ostensibly by making possible crystal propagation via the channels formed by pairs of mating connexons. However, recent findings indicate that the presence or absence of gap junctions does not always correlate with an increase or decrease, respectively, of the intercellular ice propagation rate. Moreover, thermodynamic and kinetic analyses of freezing phenomena in genetically modified cell strains point to a critical role for tight junctions in the response of tissue during freezing. This presentation will review the evidence for and against the gap junction hypothesis of intercellular ice propagation, and enumerate the experimental observations and biophysical models that support a competing view, in which tight junctions and adherens junctions affect the correlation of intracellular ice formation events in neighboring cells. The accumulated evidence raises new questions about the complex interactions between junctional proteins and ice during freezing of multicellular tissue. (Support: National Science Foundation Award No. CBET-0954587)

C-26
Biological Cells, Ice and Bubbles. JOHN MCGRATH. 251 Foust, Central Michigan University, Mt. Pleasant, MI 48859. Email: mcgra2jj@cmich.edu

Cryomicroscopic observations have revealed significant adhesive interaction between extracellular ice and cell-sized liposomes [Cryoletters, 8, 322, 1987]. The energy of adhesion was quantified. Comparable significant adhesion between murine oocytes and extracellular ice was not apparent [Cryoletters, 8, 334, 1987]. Cryomicroscopy has revealed liposome vesiculation, fusion, loss of membrane material and membrane "popping" as a result of freezing and thawing [Cryobiology, 21, 81, 1984]. Cryomicroscopy also demonstrated intracellular bubble formation and apparent cell damage linked to extracellular freezing and thawing of Spirogyra [Cryoletters, 2, 341, 1981]. Finally, freezing-induced alterations to murine osteoblast cytoskeletal components, focal adhesions, and gap junctions are reviewed [IEEE-EMBC05, 27th Annual International Conference IEEE, Engineering in Medicine and Biology, Shanghai, China, Sept 1-4, 2005; Acta Biochimica et Biophysica Sinica, 37, 814, 2005].

C-27
Evaluation of Closed System Medical Device for Low Volume Storage for Clinical Studies Involving Regulatory T Cells. EVONNE R. FEARNOT[1], Henrieta Fazekasova[2], Sarah Thirkell[3], Katie Lowe[2], Andrew Bushell[3], and Giovanna Lombardi[2]. [1]Cook General BioTechnology, LLC, Indianapolis, IN; [2]Immunoregulation Laboratory, Kings College London, London, England, UK; and [3]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, England, UK. Email: Evonne.Fearnot@ CookGBT.com

Clinical use of low volume cellular therapeutics requires closed system medical devices for storage, transport, and delivery. The regulatory T cells have been considered as key regulators in various immunological processes and there has been a great interest in using them as immunotherapy. The objective of the current study was to evaluate the use of CellSeal® Closed-System Cryogenic Vial (Cook Medical, IN, USA) for the application of short-term low volume storage of separated and expanded regulatory T cells and preparation for clinical studies. The CellSeal cryogenic vial is a newly CE Marked medical device intended for the freezing and storage of tissue, cells, blood and blood components for the purpose of introduction into the body by means of infusion or other means of administration. The CellSeal cryogenic vial is made of materials that are stable and durable during freezing and low temperature storage. The design of the CellSeal cryogenic vial has an loading port for ease of loading, a microbial barrier vent to prevent pressure from building up in the vial during loading and to allow air back into the vial during retrieval, tubing that can be sealed to create a functionally closed system, and a

retrieval port allowing maximum recovery of the stored volume. The retrieval port on CellSeal offers seamless transfer at the point of care to standard delivery needles or infusion sets for delivery to patients. The current study provides supportive data for use of a single dose of autologous regulatory T cells cryopreserved using CellSeal vials to be injected *in vivo* to induce prolongation of organ allograft survival. For cryopreservation, separated and expanded regulatory T cells were counted and resuspended in Cryostor CS10 (BioLife Solutions Inc, WA, USA). The cell concentration per single dose was calculated so the volume of the final dose ($3x10^6$/kg of patient's body weight) was frozen and stored in 2 mL CellSeal cryogenic vials using a controlled rate freezer and stored in vapor phase of liquid nitrogen. After cryopreservation, the cells were thawed, resuspended in 5% human serum albumin, and evaluated for viability, phenotype, potency and recovery. After 12 weeks of storage, the phenotype and the potency of the cells remained stable. The viability of the cells was complied with the release criteria of the study (>70%). The cells also had a high degree of recovery. The current results indicate that CellSeal cryogenic vials met expectations in preparation for two clinical trials using regulatory T cells. We conclude that the CellSeal cryogenic vials can be highly suitable for low volume storage in clinical trial settings in cellular therapy.

C-28

Frozen Storage and Transport of Cell Therapies: Developing a Solution to a Common Challenge. DOMINIC CLARKE. Charter Medical, Ltd. Email: DClarke@Lydall.com

Production of cell therapies whether for clinical development or large scale commercial production is often times stored frozen. Frozen storage provides manufacturing process flexibility while enabling long-term product stability. Products are frozen and stored using a variety of technologies from standard cryovials to disposable cryobags. While commonly used for storage and transport, the products are not without limitations for closed-system processing and overall robustness. New technologies are needed to support the growing demand for closed-system processing, transport and delivery of cell therapies. Understanding industry requirements is essential for providing these products and services. Intermediate and final fill containers used for cell therapies typically consist of either single-use cryovials or cryobags. Freezing containers used for cell therapy products should provide a sterile environment, accommodate aseptic filling and removal and protect the integrity of the product during storage and transport. Cryovials are commonly used for ultra-low (cryogenic) storage of cell-based products. They accommodate small volumes, can be filled easily and are generally robust, but the major drawback for standard cryovials is that this represents an open step in the process. Cryobags offer larger volume options and closed-system filling, but concerns with robustness and post-thaw access remain. As the field of cellular therapies continues to advance, the need for robust frozen storage options and cold chain management will be imperative. Single-use containers are available, but a number of aspects should be considered to ensure the proper container is used to support the cold chain requirements of the product. The objective of this presentation is to provide an overview of basic cell therapy manufacturing processes, highlight where single-use freezing containers play a pivotal role and offer alternative solutions for storage and delivery of cell therapy products.

C-30

ISBER Best Practices in Action at CDC's CASPIR Biorepository. JUDITH GIRI. Centers for Disease Control and Prevention, Atlanta, GA 30907. Email: JGiri@cdc.gov

The International Society for Biological and Environmental Repositories (ISBER), has developed a set of best practice guidelines for repositories, reflecting the collective experience of its members and repository professionals. The enterprise has been driven by the need for standardized, evidence based methods for collection, long-term storage, retrieval and distribution of specimens, to ensure availability of high quality biological and environmental specimens for research use. The ISBER Best Practices document represents effective strategies for the management of specimen collections and repositories, and is regularly revised to incorporate new advances. Biorepostories represent the infrastructure that includes both the physical facilities and the full range of activities associated with the repository. The Best Practices address all aspects of a repository required to ensure the quality of both specimens and associated data. A self-assessment tool is available from ISBER to assist repositories gage how well they adhere to the Best Practices. The CASPIR (CDC and ATSDR Specimen Packaging, Inventory, and Repository) at the Centers for Disease Control and Prevention (CDC), is an example of a large, long term biorepository that utilizes the ISBER Best Practices framework to ensure consistent management of specimens and associated data. The CDC accrues millions of irreplaceable specimens in the course of Public Health investigations, including a variety of human, microbial and environmental samples. CASPIR was established as a centralized facility to preserve the integrity of CDC's unique and valuable specimens for future research in a secure facility, with continuously maintained uniform conditions and standard operating procedures and quality measures.

C-32

The Pan-Smithsonian Cryo-initiative-freezing for the Future. PIERRE COMIZZOLI. Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC. Email: comizzolip@si.edu

Among the largest non-human biobanking efforts, the Smithsonian Institution curates and manages frozen biomaterials, including samples of DNA, somatic cells, tissues, blood products, germplasm (sperm and eggs), embryos, as well as other animal, plant, and soil products. Today, this biological collection comprises at least 1 million samples from more than 18,000 species. In addition to being large, it also is a fast growing scientific collection. The value of the systematic and organized biorepository extends far beyond ensuring evidence of the world's unique animal and plant species is protected. Interestingly, many samples in the Smithsonian frozen collection are unique treasures such as ancient DNA or frozen sperm cells from rare and endangered species. Biobanking efforts are fully integrated to conservation efforts (preservation and restoration of black-footed ferret populations for instance) and support a vast array of projects and disciplines. Given the importance of the Smithsonian frozen collections and growing need to preserve them using the best methods available, a multi-step initiative – the Pan-Smithsonian Cryo-Initiative - was created with the goal to preserve, inventory, and enable easy access to the cryo-specimens. As observed in other institutions worldwide, immediate actions are necessary to bring cataloging, management, equipment, and staffing requirements to an appropriate level across the different units. The highest priorities includes standardizing database, barcoding of each individual sample, organizing freezers and liquid nitrogen tanks to optimize their storage capacity, and recording location metadata in a database (location in laboratory freezers, sample type, date and locality of collection, collector, voucher information, link to relevant documentation, DNA sequences, Geographical Information System references). This includes a basic understanding of how laboratories and departments are managing their frozen collections, the current status of each unit, and improvements for archival use and long-term storage.

C-33

Best Practices for Archiving Marine Samples for the National Institute of Standards and Technology's Marine Environmental Specimen Bank (Marine ESB). REBECCA S. PUGH, Amanda J. Moors, Jennifer Ness, Jody Rhoderick, Debra Ellisor, Melannie Bachman, and Paul R. Becker. National Institute of Standards and Technology, Chemical Sciences Division, Hollings Marine Laboratory, 331 Ft. Johnson Rd., Charleston, SC 29412. Email: rebecca.pugh@noaa.gov

Formal environmental specimen banking has been recognized internationally as an important component of long-term environmental research and monitoring programs. The National Institute of Standards and Technology (NIST) has been involved in environmental specimen banking for over 30 years through collaborations primarily with other U.S. government agencies as well as with universities and non-profit organizations. Biological (i.e. marine mammal tissue, seabird egg contents, sea turtle tissue, fish tissue, coral, etc.) and environmental (i.e marine sediment, macroalgae, etc.) specimen collections from individual projects are maintained at the Marine Environmental Specimen Bank (Marine ESB), Hollings Marine Laboratory, Charleston, SC. Carefully designed and standardized protocols for sample collection, processing, and banking have been developed primarily to conduct research on environmental contaminant measurements but specimens have been and could be used for additional purposes, such as genetic studies, biotoxin exposure, and infectious disease research. The carefully designed protocols and procedures for collecting these specimens vary depending on a number of factors, such as specimen type, availability of the specimen, and the collection location, but a best practice guideline is to strictly adhere to these protocols in order to ensure the quality of the sample is not compromised for its intended purpose. Standard Reference Materials (SRMs) and Control Materials (CMs) intended for use in method development and validation as well as for quality assurance and for use in assigning values for selected environmental contaminants are also produced by NIST and provide a resource to ensure sample stability of specimens archived at the Marine ESB.

C-34

Coping With Water Loss: Protecting Your Proteome Under Stress. KEVIN STRANGE, MDI Biological Laboratory, 159 Old Bar Harbor Road, Salisbury Cove, ME 04672. Email: kstrange@mdibl.org

The nematode *C. elegans* is a powerful model system for defining the cellular and molecular mechanisms by which animal cells cope with water loss. We have demonstrated using genome wide RNAi screening that highly conserved genes required for proteostasis are also critical components of a cellular osmosensing and osmoprotective network. Proteostasis is the maintenance of protein function and is mediated by the tightly integrated activities of gene transcription, RNA metabolism and protein synthesis, folding, assembly, trafficking, disassembly and degradation. Hypertonic stress induced water loss causes widespread and striking protein aggregation and misfolding in *C. elegans*. Survival in desiccating environments requires the function of gene products that detect and destroy those damaged proteins. Synthesis of new proteins is rapidly inhibited in response to cellular water loss via activation of the GCN-2 kinase signaling cascade and phosphorylation of the translation initiation factor eIF2a. Reduction of translation rate minimizes hypertonic stress induced protein damage. Inhibition of protein synthesis by hypertonic stress, knockdown of genes required for translation, or protein synthesis inhibitors also functions as a signal that activates osmoprotective gene transcription via the WNK and GCK-VI subfamily kinases WNK-1 and GCK-3.

WNK and GCK-VI kinases are highly conserved and play central roles in cellular and systemic osmoregulation in humans. Taken together, these findings provide the foundation for ongoing development of an integrated understanding of the genetic, molecular and cellular pathways by which animal cells detect osmotic perturbations and cope with osmotic stress induced cellular and molecular damage.

C-35

Lyoprotection During Bovine Sperm Desiccation and Osmotic Damage Model. SANKHA BHOWMICK, Ranjan Sitauala, and Jorge Jimenez-Rois. Department of Mechanical Engineering, Bioengineering and Biotechnology Program, University of Massachusetts, Dartmouth, N. Dartmouth, MA 02747. Email: sbhowmick@umassd.edu

Designing excipients for stable desiccation preservation protocols of bovine sperm at low moisture content and ambient storage conditions for the animal husbandry industry requires subsequent retention of motility. We shall discuss the role of various excipients such as the lyoprotectant trehalose and osmotolerant sorbitol plays in bovine sperm. Since it is hypothesized that much of the damage during drying is related to the osmotic stress encountered due to increased osmolarity of the extracellular environment, we wanted to develop a predictive tool that captured the osmotic damage of sperm during drying. Our approach was to subject bovine sperm to anisosmotic environments for different times and then extract parameters for motility loss and subsequently predict desiccation experiments. We saw a two-step motility response; a rapid drop that can be considered instantaneous and a slower one. We developed two models a) First order rate injury model (Fast or Slow) and b) Multi-modal (MM) injury model. For desiccation, the MM model was able to closely bracket motility loss as an osmotic change event with a time independent and a time dependent component. While the mechanistic basis of osmotic damage requires further exploration, the model can serve as a bracketing tool for predicting motility loss during desiccation based on excipients designed to minimize osmotic damage.

C-36

Origins of Cryoprotectant Toxicity. G. FAHY. 21st Century Medicine, Inc., 14960 Hilton Drive, Fontana, CA 92336. Email: gfahy@21cm.com

The nature of cryoprotectant toxicity has received little attention. Literature studies are mostly confined to in vivo toxicity data that have little to no relevance to events at low temperatures. Cryoprotectant toxicity in the frozen state suggests a rapid mechanism for toxicity that overwhelms the generally protective effects of lower temperatures. Previous evidence argued against generalized protein denaturation as a mechanism of toxicity because a) toxicity is observed at lower concentrations than those needed to denature proteins, b) protein destabilization tendency was associated with less toxicity, not more, and c) preferential exclusion of cryoprotectants from the protein surface implied protein stabilization at lower temperatures. Newer data, however, have reopened the possibility that toxicity may be related to the denaturation of specific proteins. A microarray study showed that vitrification solution exposure increased heat shock protein gene expression, increased transcription of genes in the ERK and JNK pathways, and induced 8 genes associated with ribosome biogenesis, all suggesting a reaction to protein denaturation. The correlation between toxicity in organized tissue and qv* is also compatible with protein denaturation as the primary mechanism of "non-specific" toxicity. Although individual cryoprotectants may be used at sub-denaturing concentrations in vitrification solutions, their cumulative effect could become denaturing, especially if cryoprotectant-mediated destabilization summates with cold destabilization. Pertinent to this possibility, an additional microarray study suggests that chilling injury may be largely related to protein denaturation as well, which raises the question as to whether chilling injury in systems prepared for vitrification may in large part be induced by the presence of the vitrification solution. If so, chilling injury may be considered a toxic effect of cryoprotectants at deep subzero temperatures, consistent with the manifestation of such toxic effects in frozen-thawed systems.

C-37

Kinetic Modeling of CPA Toxicity and Implications for Design of Cryopreservation Procedures. ADAM Z. HIGGINS. School of Chemical, Biological and Environmental Engineering, Oregon State University, 102 Gleeson Hall, Corvallis, OR 97331-2702. Email: adam.higgins@oregonstate.edu

The protection conferred by penetrating cryoprotectants (CPAs) such as glycerol and dimethyl sulfoxide has made it possible to successfully cryopreserve a variety of cell types. However, it is well known that CPAs are not universally beneficial and exposure to CPAs can cause significant damage as a result of both cytotoxicity and osmotically-induced cell volume changes. Consequently, the design of cryopreservation procedures involves a balance between achieving sufficient protection and avoiding the damaging effects of CPAs. This is particularly true for vitrification methods, which involve the use of high CPA concentrations to completely prevent formation of ice. Recently, it has become increasingly clear that optimally balancing the beneficial and damaging effects of CPAs will require an improved understanding of the kinetics of CPA-induced cytotoxicity. For example, selection of the optimal temperature for CPA loading is not trivial and depends on the relative rates of CPA-induced damage and CPA transport, both of which are temperature dependent. In

addition, the optimal method for equilibrating cells with CPA depends on how the kinetics of CPA-induced damage varies with concentration. This presentation will review work by us and others on the kinetics of CPA toxicity, including the effects of factors such as temperature, concentration and CPA type. The implications of these results for design of cryopreservation procedures will also be discussed.

C-38

Preservation of Mammalian Sperm by Freeze-drying. TAKEHITO KANEKO. Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, JAPAN. Email: tkaneko@anim.med.kyoto-u.ac.jp

Stable conservation of genetic resources is important in various research fields. Sperm preservation is a valuable method for bio-banking of mammalian genetic resources. Freeze-drying is a useful technique for storing biological materials as samples can be preserved for a long-term in a refrigerator (4°C). Freeze-drying sperm have been studied as new simple preservation method in various mammals. Effective method of freeze-drying sperm have been reported that offspring could be obtained from mouse and rat sperm preserved long term at 4°C after freeze-drying using a simple solution containing 10 mM Tris and 1 mM EDTA adjusted pH to 8.0 (TE buffer) (Kaneko T., Cryobiology 64, 211-214, 2012; Kaneko T., PLoS One 7, e35043, 2012). Freeze-drying sperm results in lower costs and safety, as specialized cryoprotectants and a constant supply of liquid nitrogen is not required. A further advantage is that freeze-dried sperm can be temporarily stored at room temperature. Freeze-drying is possible to transport sperm oversea at ambient temperature that requires neither liquid nitrogen nor dry ice. Furthermore, freeze-dried valuable samples can be also protected safety even at ambient temperature in the event of unexpected power failure and disaster such as earthquakes and typhoons. Present, freeze-drying sperm has been applied as “Freeze-drying Zoo” for conservation of wild endangered animals (http://www.anim.med.kyoto-u.ac.jp/reproduction/home.aspx).

C-41

In-situ Determination of the Relaxation Characteristics of Sugar Glasses Embedded in Microfiber Substrates. LINDONG WENG and Gloria D. Elliott. Department of Mechanical Engineering and Engineering Sciences, University of North Carolina at Charlotte, Charlotte, NC 28223. Email: lweng@uncc.edu

Recently there has been considerable interest in developing anhydrous preservation strategies that enable storage of biologics without refrigeration. Anhydrous preservation strategies typically employ trehalose as a protectant, as it can provide specific molecular protection, while also forming a glassy matrix that suppresses adverse chemical reactions. Glass microfiber filter paper is a good substrate for drying biologics, providing an even distribution of samples and an enhanced surface area for drying, but the opaqueness prevents macroscopic observation of the sample and can introduce complexities that impede physical characterization. Because drying kinetics and processing conditions can impact the relaxation dynamics, which can influence the efficacy of the glass as a stabilizer, methods are needed that can enable an in-situ determination of relaxation phenomena of sugar glasses within such complex environments. In this study we present a method which provides verification of the absence of crystallinity following drying trehalose/water and sucrose/water mixtures on filter paper and also enables the determination of relaxation characteristics of amorphous sugar compositions embedded within these substrates. Using stainless steel material pockets to contain the sugar glass-embedded microfiber paper, the α-relaxation temperature, T_α, was determined as a function of the water content in the sugar samples using Dynamic Mechanical Analysis (DMA). Results were verified by comparison with previous calorimetric and spectroscopic studies. The data demonstrated the plasticizing effects of water, as T_α was shown to correlate with water content via a Gordon-Taylor equation. This plasticization effect was also mirrored in the increase in the magnitude of the change of storage and loss moduli. Our findings validate a new approach for determining the relaxation characteristics of microfiber embedded sugar glasses, and offer new insights into the relaxation characteristics of glasses prepared by microwave-assisted drying on filter papers.

C-42

Measurement of Intracellular Water and Trehalose in Desiccated Cells. ALIREZA ABAZARI[1], Steven Hand[2], Alptekin Aksan[3], and Mehmet Toner[1]. [1]The Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School, and Shriners Hospital for Children, Boston, MA; [2]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA; and [3]Department of Mechanical Engineering, University of Minnesota, Minneapolis, MN. Email: abazari.alireza@mgh.harvard.edu, mtoner@hms. harvard.edu

Stability of intracellular glass, a requirement for long-term storage in the dry state, largely depends on the residual water content and the heterogeneity in trehalose and water distributions. In light of studies on the slowed rate of desiccation in sessile droplets due to glassy skin formation at the air-liquid interface, we investigated the potential heterogeneity in water and trehalose distributions at the single-cell level in spin-dried samples. We utilized label-free detection capabilities of Raman microspectroscopy for quantitative analysis and measurement of water and trehalose concentrations in the intra- and extracellular milieu. The results

confirmed the previous measurements of water content in trehalose thin film (~0.1 g water/g trehalose). Intracellular trehalose resulted in a marginally higher water content compared to non-loaded cells. Nonetheless, we were able to show that the retained water was bound, and we observed no free intracellular water in the absence or presence of intracellular trehalose. Also, cross-sectional analysis of the desiccated cells showed that the concentrations of intracellular trehalose and water were the highest at the cell membrane adjacent to the extracellular milieu, and both followed a decreasing trend toward the middle of the cell. This was the first study to probe into desiccated cells at the single-cell level and to quantify intracellular water and trehalose contents and distributions, which are essential information for studies on storage stability of desiccated cells. The results suggest that, for optimum stabilization of trehalose-loaded cells, further desiccation for increasing the Tg of the desiccated cells and the trehalose layer is required, or otherwise, storage at below-ambient temperature is recommended. Also, our results warrant further studies on the observed uneven intracellular distribution of trehalose and investigation of its intracellular glassy/crystalline state, which may adversely impact the biostabilization properties of trehalose.

C-43

Biopreservation Considerations for GMP Bioprocessing, Clinical Development, and Delivery. ABY J. MATHEW. BioLife Solutions, Inc., 3303 Monte Villa Pkwy, Bothell, WA 98021. Email: amathew@biolifesolutions.com

Often in cell and tissue processing, there may exist a gap between biopreservation method optimization from a cryobiology perspective and the process development that results in the cryopreserved or non-frozen cell/tissue product. This presentation will discuss Best Practices recommendations for integrating biopreservation methods within Good Manufacturing Practices (GMP), share lessons learned from cell therapy manufacturing – including some positive and negative examples of biopreservation issues, and suggestions for integration of biopreservation methods within biobanking and Regenerative Medicine with consideration to the Quality and Regulatory footprint.

Disclosure: Author was or is employed by BioLife Solutions, Inc.

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The Impact of Cryopreservation on Fitness of Cell Therapy Products in Human Clinical Trials - the MSC Paradigm. JACQUES GALIPEAU. Department of Hematology and Medical Oncology & Pediatrics, Emory University, Atlanta, GA 30322. Email: jgalipe@emory.edu

Marrow-derived mesenchymal stromal cells (MSCs) from either autologous or from random donor origin are being investigated in a large number of clinical trials as a cellular pharmaceutical for treatment of immune disorders and in regenerative medicine applications. Most typically, a polyclonal pool of culture expanded cells is cryopreserved after serial passaging and banked for later use. Human subjects subsequently receive an intravenous transfusion of MSCs which were retrieved from cryostorage no more than a few hours beforehand. It is assumed that viable, immediate post-thaw MSCs deploy the same biochemical, homing and immune modulatory features as their pre-freeze counterparts. We here propose that this assumption may be erroneous and may provide, in part, an explanation for discrepancy between pre-clinical models of MSC effectiveness and negative outcome of a large prospective randomized Phase III clinical trial (NCT00366145) of random donor MSCs for treatment of steroid-resistant acute GvHD. We show that MSC functional fitness as a transfusional product can be significantly altered due to post thaw heat shock response and cytoskeletal disruption.

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Cryobiology: Changing the Face of Regenerative Medicine. COLIN D. WHITE, Kate Benson, Pratima Cherukuri, and Timothy Beals. Parcell Laboratories, Inc., 21 Strathmore Road, Natick, MA 01760. Email: white@parcelllabs.com

Cryobiology, or the study of how low temperatures affect the living cell, has been a field of active investigation for several decades. Notwithstanding the progress made to date, many manufacturers, distributors, and/or end users of cell-based therapies still rely on less than optimal biopreservation, storage, and shipping modalities for their final cellular product(s). The current paradigm is somewhat conflicted, with those in industry trying to educate end users on the biological principles justifying cryopreservation. On the flip side, end users often find themselves ill equipped and without enough experience to handle receipt of cellular products that have been cryopreserved and shipped in a fashion designed to maintain optimal viability and shelf life. As the regenerative medicine field continues to advance, addressing this paradigm will become of significant importance. Parcell Laboratories develops and manufactures a variety of cellular products and therapies. The Company was founded around the discovery of a novel type of adult stem cell, the Early Lineage Adult (ELA) stem cell. Immunophenotype analyses have demonstrated that this cell is distinct from other stem cells currently reported in the literature, and genetic and functional assays are ongoing to ascertain the potential of the cell to be used as a treatment for various disease states. Regardless of the indication, all cellular products manufactured by the Company are control rate frozen in CryoStor CS10 (BioLife Solutions), and stored in vapor-phase liquid nitrogen post-processing. Products may be shipped on either dry ice or in cryogenic storage, depending on the preference of the distributor and/or end user. This

presentation will summarize our experience in processing, storing and shipping cellular products, as well as our perception as to how the cryobiology field will advance in the future.
Disclosure: Author was or is employed by Parcell Laboratories.

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Pre-cryopreservation Processing and Storage: A Biologist's Perspective on the Balance Between Manufacturing Efficiency and Post-Thaw Quality. JASON P. ACKER. Canadian Blood Services, 8249-114 Street, Edmonton, CANADA AB T6G 2R8. Email: jason.acker@blood.ca

The *ex vivo* storage of cells at low temperatures for extended periods of time has been one of the most important advances that led to the creation of biobanks and the widespread therapeutic use of cellular products. Most cellular products used today spend a large portion of their product lifecycle at hypothermic temperatures or cryopreserved and stored frozen in freezers. As it is generally understood in the biobanking industry that the length and temperature of pre-cryopreservation storage can influence the post-thaw recovery of cells, biobankers have empirically established thresholds for the "optimal" conditions for the: shipment of raw material to centralized manufacturing sites, cell selection, donor testing and processing. Unfortunately, there is a general lack of appreciation of the impact that pre-cryopreservation processing and storage has on critical cell properties that are crucial to post-thaw recovery of viable cells. This presentation will provide a biologist's perspective on the impact that cell manufacturing conditions can have on the quality of cellular therapeutics. Emerging data on the hypothermic storage lesion and cryoinjury of red blood cells and hematopoietic progenitor cells will be discussed with a focus on changes to cell metabolism, oxidative injury and membrane remodeling. The impact of the biological changes on the ability for cells to respond to the physical and chemical stresses encountered during cryopreservation will be introduced. Current quality control methods and international regulations will be briefly described. Recent clinical data on the effects of hypothermic storage and cryopreservation on recipient outcomes will be reviewed. While there is a practical / commercial need to optimize the pre-cryopreservation manufacturing efficiency of cellular products, consideration must be given to the impact and dependency that each manufacturing step has on the quality and potency of the final product.