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The Grand Challenges of Organ Banking: Proceedings from the first global summit on complex tissue cryopreservation

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ABSTRACT

The first Organ Banking Summit was convened from Feb. 27 - March 1, 2015 in Palo Alto, CA, with events at Stanford University, NASA Research Park, and Lawrence Berkeley National Labs. Experts at the summit outlined the potential public health impact of organ banking, discussed the major remaining scientific challenges that need to be overcome in order to bank organs, and identified key opportunities to accelerate progress toward this goal. Many areas of public health could be revolutionized by the banking of organs and other complex tissues, including transplantation, oncofertility, tissue engineering, trauma medicine and emergency preparedness, basic biomedical research and drug discovery – and even space travel. Key remaining scientific sub-challenges were discussed including ice nucleation and growth, cryoprotectant and osmotic toxicities, chilling injury, thermo-mechanical stress, the need for rapid and uniform rewarming, and ischemia/reperfusion injury. A variety of opportunities to overcome these challenge areas were discussed, i.e. preconditioning for enhanced stress tolerance, nanoparticle rewarming, cyroprotectant screening strategies, and the use of cryoprotectant cocktails including ice binding agents.

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1. A first global summit on the Grand Challenges in Organ Banking

1.1. Sebastian Giwa, Jedediah Lewis, Alessandro Tocchio, Erik J. Woods, Jason P. Acker

Key governmental bodies charged with spurring American innovation, including the White House and the Department of Defense, have begun to take note of the immense potential of organ and tissue banking to change the landscape of transplant, trauma and regenerative medicine and save millions of lives. The Department of Defense has led the way, recently funding the first ever grant programs targeted toward long-term preservation of organs and complex tissues. Subsequently a number of agencies are exploring the potential of organ and complex tissue banking, including several of the National Institutes of Health (NIH), the White House Office of Science and Technology Policy (OSTP of EOP), the Biomedical Advanced Research and Development Authority (BARDA) and the Defense Advanced Research Projects Agency (DARPA).

Many experts within the medical and scientific communities have called for a coordinated national effort to address the multifaceted challenge of organ banking. Government and academia have not yet made the banking of organs and other complex tissues a national priority, and many have cited institutional causes. A wealth of cross-disciplinary knowledge is needed to achieve the ambitious and complex goal of long-term storage of complex tissues. Yet research surrounding tissue preservation has historically been fragmented, and relatively few incentives and little support currently exist to make progress.

The key feature that had made complex tissue banking vulnerable to stagnation in the current scientific ecosystem is also what gives it the potential to be transformative to science and medicine: its benefits are widespread and diffuse, enabling advances in many areas of public health spanning cancer treatment, organ transplantation, trauma care, disaster preparedness, tissue engineering, drug screening and disease modeling, regenerative medicine, and more. Long-term storage of healthy tissue is a foundational technology that could become a cornerstone of biomedicine. Yet its role as an enabler of many other scientific and medical efforts, rather than as a central player in any, has left organ and complex tissue banking without a champion.

To address these issues, the first global Summit on the Grand Challenges in Organ Banking (Organ Banking Summit 2015) was convened at Stanford University and in Palo Alto, CA, with events at Lawrence Berkley National Lab as well as Singularity University Labs at NASA Research Park. The Summit brought together representatives from venture capital, the biotech industry, and organ procurement organizations with top scientists in the fields of transplantation, tissue preservation, cryobiology, regenerative medicine, and related fields. These participants combined their expertise in nanotechnology, organic chemistry, molecular biology, physics, thermodynamics, biomedical engineering, metabolism, public health, finance, and many other subjects to chart a scientific path toward banking of organs and complex tissues for transplantation.

In his talk at the Summit, Dr. Robbie Barbero of the White House Office of Science and Technology Policy called on the scientific community to articulate a Grand Challenge of organ banking. The experts presenting at the Summit responded enthusiastically, presenting a vision for focused progress in the organ banking field that can reduce organ transplant waitlists and greatly impact human health, give birth to new industries and create jobs, help alleviate the economic burdens of the national healthcare system, lessen global and national health disparities, and expand the frontiers of basic scientific knowledge.

Moreover, the Summit participants have discussed how this Grand Challenge can be divided into tractable sub-challenges that, if properly supported, the scientific community is well equipped to tackle. We have outlined surrounding scientific fields, technologies and tools, many of which advanced rapidly in recent years, that can greatly accelerate progress on these organ banking challenges. We have highlighted model organisms used to study principles of tissue stasis that can be applied to organ banking; nature has already solved many organ banking problems, and a large body of basic research has revealed biological mechanisms that may enable organ banking. We have identified a vast array of ideas that have seen early experimental success and, if adequately resourced, could lead to breakthroughs in tissue preservation. And we have discussed recent “proof of concept” breakthroughs that suggest that organ banking is possible.

These proceedings from the first global Summit on the Grand Challenges in Organ Banking, as well as the published abstracts from the Summit [48], reflect over 50 lectures from distinguished scientists, doctors, surgeons, investors, philanthropists, and government officials. They outline the huge need for and value of banking of organs and other complex tissues, the promising advances in the field over the last decades, the challenges (both scientific and institutional) remaining, and strategies for leveraging the immense knowledge and expertise available in the broader scientific community in order to overcome these challenges.

2. The need and value of organ banking[1]

2.1. Jedediah Lewis, Alessandro Tocchio, Sebastian Giwa

In the decades since its development, organ transplantation has saved hundreds of thousands of lives and granted over 2 million years of aggregate life to patients with otherwise incurable diseases in the U.S. alone [55]. With incredible medical, surgical, and scientific advances in this field, the large and growing shortage of donor organs has become a central problem limiting the effectiveness of transplantation, alongside transplant rejection and the need for immunosuppression. Despite longstanding efforts to increase organ donation waitlists have grown swiftly and steadily [49]. Officially at least 1 in 5 patients on transplant waitlists die waiting for an organ that they never receive [49]. Currently over 120,000 patients are currently on organ transplant waitlists in the U.S. [49], although the waitlist captures only a small fraction of patients who would benefit from an organ transplant if supply constraints were removed.

End-stage organ disease accounts for over 700,000 deaths per year in the U.S., dwarfing the number of patients added to transplant waitlists [51], and according to some estimates over 30–35% of all U.S. deaths, over 900,000 deaths per year, could be prevented or delayed by an organ transplant and broader tissue engineering [22,36,37]. In the U.S. organ impairment leads to more deaths than cancer, and chronic organ impairment makes up a substantial

[1] Session Participants and Speakers: **Dr. Robbie Barbero**, Assistant Director for Biological Innovation, White House Office of Science and Technology Policy (OSTP); **Dr Abbas Ardehali**, UCLA School of Medicine, Chief Cardiac Surgery, Director Heart and Heart-Lung Transplant Program, Professor Cardiothoracic Surgery; **Lt Col. Luis Alvarez, PhD**, Director of the DoD's three new organ and tissue banking grant programs, former co-founding Deputy Director of the DoD's Tissue Injury and Regenerative Medicine Program that also oversees the AFIRM; **Dr. John Scandling**, Medical Director of Kidney and Pancreas Transplantation and Professor of Medicine, Stanford University **and Dr. Gabor Forgacs**, Scientific Founder of Organovo, Authority in biomechanics, tissue engineering and pioneer in 3D bio-printing. Session Chairs: **Robin Farmanfarmaian**, Summit Executive Director and **Dr. Sebastian Giwa.**

fraction of these deaths [12]. The organ shortage is more severe for ethnic minorities, who face a lower likelihood of finding a matching organ [56]. The shortage is even more severe worldwide: the World Health Organization conservatively estimates that organ transplantation meets less than 10% of the global demand [32], and many countries perform less than 1% of the transplants per capita carried out in the U.S. and Western Europe [27].

Yet a large fraction of organs that could potentially be used for donation are discarded, in large part due to their short safe preservation times – only 4–12 h for most vital organs [46]. Although each cadaveric donor can provide up to eight lifesaving vital organs, on average only 3–4 per donor are transplanted [39]. Currently almost two thirds of potential donor hearts and lungs are discarded [6,35], along with nearly 20% of kidneys [59]. A large fraction of healthy donor kidneys are discarded simply because they exceed their maximum preservation times [59]. Likewise, the inability to adequately preserve transplant organs is a decisive factor in the heart discard rate as "borderline cases" are discarded due to the anticipating compounding of ischemic damage during transport. The Organ Preservation Alliance has estimated that if only half the discarded hearts and lungs were utilized for transplantation, the waitlists for these organs could be extinguished in 2–3 years.

While roughly 8000 people per year become cadaveric organ donors, it has been estimated that at least 8000–10,000 additional patients who die from traumatic brain injuries could become donors if organ assessment and preservation barriers were overcome (accounting for medical suitability and logistics) [51], more than doubling the donor pool. Reducing or eliminating damage from prolonged cold ischemia during transplantation could reduce the need for stringent donor selection and allow time for evaluation and rehabilitation of organs from extended criteria donors (ECDs) [51]. Meanwhile, reduced ischemic injury would mean that organs from standard criteria donors would be healthier at the time of implantation.

The short preservation times for vital organs severely limits the lifesaving potential of transplantation in many other ways. Longer windows for transplantation would improve matching between donors and recipients, both by allowing more and better matching assays [31] and by expanding the geographic areas across which transplantation could occur. Better matching could in turn reduce the incidence of graft rejection, increase graft lifespan, and reduce the need and the cost of immunosuppressants. Short preservation times currently limit many screening procedures that could prevent transmission of infectious diseases between donors and recipients. Organ banking could also revolutionize the field of immune tolerance induction - the "Holy Grail" of transplantation, potentially enabling donor-specific tolerance induction protocols to be initiated before implantation of cadaveric donor organs and increasing applicability and effectiveness of current immune tolerance protocols [61].

The benefits of complex tissue preservation would extend far beyond organ transplantation. The fertility of young cancer patients could be protected by removal of the ovaries or testes before chemotherapy and subsequent re-implantation. In the U.S. alone there are an estimated 380,000 survivors of childhood cancer [12] and 630,000 survivors of cancer contracted as young adults (ages 21–40) [4], indicating that in each 1–2 generations there are over 1 million cancer survivors in the U.S. alone who could potentially benefit from protective storage and re-implantation of their reproductive organs. Similarly, in many cases amputated limbs could be preserved and reattached. This would allow wounded service members to receive advanced care for battlefield extremity injuries [3] and would have dramatic public health benefits for the population at large: in the U.S. over 2 million people are living with limb loss [76], and 185,000 amputations are currently performed per year [50]. Meanwhile skin, blood vessels, and bone marrow could be banked for natural disasters, military conflicts, terrorist attacks and other mass casualty events.

Moreover, as tissue engineering matures, complex tissue preservation will increasingly become a bottleneck limiting the entire field [34]. The storage, transport and quality control of engineered tissues and organs are a vital and required part of the tissue engineering supply chain. Tissue storage has already become a major barrier for tissue engineering companies working to manufacture skin graft substitutes, blood vessels, and other engineered products [3]. If measures are not taken now to give complex engineered tissues and eventually organs a shelf-life, tissue storage constraints are likely to plague regenerative medicine just as the organ shortage limits transplantation today.

Not only does organ and tissue banking hold the promise of dramatically improving health, but it can alleviate the large and growing economic burdens associated with organ impairment. When alternatives to organ replacement exist, the costs are often substantial. The global economic cost of treating end-stage renal disease has been estimated at over $1 trillion over the course of a decade [40]. $34.3 billion of the U.S. Medicare budget was spent on dialysis in 2010 alone, over 6% of the total Medicare budget [13]. In addition to the financial burdens, dialysis patients typically experience a poor quality of life compared with transplantation [14]. Organ banking can help to bring down the costs of transplantation, averaging almost $1 million for a single heart transplant, $600,000 for a liver transplant and over $250,000 for a kidney transplant [7], by eliminating logistical burdens such as the need to fly donor organs to their recipients. By making transplantation safer and more effective, and by reducing complications, rejection, and the need for re-transplantation, the need for costly treatments can in turn be reduced.

Alongside its cost-saving benefits, organ banking would give rise to new industries and economic opportunities, increasing national wealth and competiveness for countries that lead organ banking efforts. Combined with tissue engineering, the ability to bank organs could enable up to hundreds of thousands of additional organ transplants per year [22,51] in the U.S. alone. Although organ banking is likely to decrease the overall cost of transplantation, even at low price points where the banking procedure costs $30–50,000 per organ we estimate that a multi-billion dollar annual industry will materialize. The potential market for limb replacement is even larger: given that 185,000 amputations occur each year just in the US, if 20% of amputations instead result in limb transplantation then the market will be larger than the current market for vital organ transplants. Combining all of this with a larger need to preserve tissue engineering and xeno tissues and organs and taking into account global growth the Organ Preservation Alliance presented preliminary analysis that total industry market capitalization could reach north of a trillion dollars.

These opportunities can encourage substantial private investment, leading to new collaborations between the public and private sectors and making government and philanthropic investments highly leveraged. Once a critical mass of infrastructure and basic research knowledge is reached, organ preservation research is likely to give rise to the birth of a vital and self-sustaining, self-reinforcing organ banking industry and private research enterprise. Complex tissue banking capabilities would further the missions of myriad federal agencies and potentially hundreds of private non-profits. The Department of Defense has already signaled its interest in the banking of organs and other complex tissues for trauma care and regenerative medicine, launching three calls for grant applications targeted toward tissue cryopreservation. Also tied to national defense needs, as well as public health more broadly, is the stockpiling of skin, blood vessels, bone marrow and other tissues

for trauma care and mass casualty events. Such an effort is closely tied to the aims of Project Bioshield, the federal program to develop medical countermeasures against chemical, biological, radiological and nuclear attacks, as well as other efforts by the Biomedical Advanced Research and Development Authority (BARDA) to address natural and intentional threats to public health [67]. Likewise NASA's Technology Roadmap for Human Health, Life Support and Habitation Systems, is closely tied to the preservation of tissues, as are the overall missions of the heart, liver, kidney, lung, veterans, cancer societies and associations and numerous other non-profit organizations.

The ability to bank organs and complex tissues impacts the missions of organizations spanning the National Institutes of Health. Among these are the National Heart, Lung, and Blood Institute (NHLBI), charged with supporting research to promote the treatment of heart, lung, and blood diseases [68], the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), with the mission of combatting diseases of the liver and kidney, as well as Diabetes [69], the most common cause of kidney failure [70], and the National Institute of Biomedical Imaging and Bioengineering (NIBIB), committed to "leading the development and accelerating the application of biomedical technologies" [71], as well as the National Cancer Institute, the National Institute of Allergy and Infectious Diseases (Transplant Group), the National Institute of General Medical Sciences, the National Institute on Minority Health and Health Disparities, and the National Center for Advancing Translational Sciences.

Despite the great promise of complex tissue banking, it has historically received little funding or attention [3]. Long-term storage of biological materials has been characterized by many researchers as an under-resourced, neglected, or even orphaned field. Until the recent Department of Defense calls for grant applications [3], the federal government has historically not provided any targeted funding specifically toward research on banking of organs large tissues. Government agencies and academic research institutes have yet to adopt complex tissue banking as a priority.

Achieving the long-term storage of organs and other complex tissues is an exceptionally interdisciplinary undertaking, and often even incremental progress requires combining knowledge from cell biology, mechanical engineering, physical chemistry, structural biology, thermodynamics, materials science, and many other fields. This makes complex tissue banking especially vulnerable to stagnation when supported on an ad hoc, grant-by-grant basis and when focused only on immediate research applications. Research fields surrounding organ banking would benefit enormously from a cohesive institutional infrastructure that can combine and coordinate expertise on a longer-term large scale.

Ironically, another major hurdle to organ banking research so far may have been exactly what makes it so attractive: its widespread and diffuse benefits, which do not fall within the purview of one funding agency or organization but are spread across many. This makes organ banking ideally suited for a coordinated, inter-agency research effort, one that is likely to find many allies within the private and non-profit sectors. If properly resourced, banking of organs and other complex tissues has the potential to address a huge breadth of public health needs, and impact economic vitality, health disparities, national security, and fundamental scientific understanding.

3. The promise of organ banking to transform medicine is now within reach[2]

3.1. *Jedediah Lewis, Alessandro Tocchio, Erik J. Woods, Jason P. Acker, Sebastian Giwa*

A growing body of scientific evidence suggests that the long-term banking of organs and large tissues is not only achievable but now within reach. Storage under extreme low-temperature conditions can be used to initiate metabolic arrest and prevent ischemic damage that would otherwise be deleterious to transplantable tissues, greatly extending preservation times. Recent breakthroughs in the low-temperature storage of large tissues and organs overcome many of the challenges of this approach and provided proof-of-concept. Experimental successes in recent years include the successful re-implantation of rodent hearts [5] porcine and rodent livers [25], and sheep ovaries [25] after low-temperature storage. One group has reproducibly achieved success storing rabbit kidneys at −45 °C, which function and support life after transplantation [21], as well as successful re-implantation of a functioning rabbit kidney after vitrification and long-term storage below −120 °C in the glass state [23]. And multiple advances have recently been made by Chinese labs, including the successful cryopreservation and reattachment of functioning rat hind limbs [73] and human digits [72].

In addition to the successful organ cryopreservation pilot experiments that have drawn heavily on engineering innovations, Nature has already provided many of the solutions to organ banking challenges. Many studies have shown that a variety of vertebrates undergo sustained metabolic arrest under extreme low-temperature conditions, emerging with no long-term tissue damage. For instance the wood frog *Rana Sylvatica* can tolerate temperatures close to −20 °C during periods of hibernation, with as much as 70% of its body water as extracellular ice [15]. In nature these freeze-tolerant hypometabolic states can last for weeks; with simple therapeutic interventions researchers have extended *Rana Sylvatica's* periods of cryotolerance as long as 218 days [38]. As Prof. Kenneth Storey outlines in his talks, a variety of regulatory events involving epigenetic modifications, miRNA suppression of translation, and post-translational modifications can condition cells, tissues, and entire organisms to tolerate extreme low temperature conditions in a hypometabolic state. Many of these mechanisms are conserved in mammals and even primates [17,24,43,74].

The organ banking effort also stands to benefit from surrounding fields and technologies that have seen enormous advances in the last decade, providing tools that have yet to be brought to bear on cryobiology problems. For example, cyoprotective agents have historically been discovered either fortuitously or through the study of cryotolerant organisms. Over the last 50 years, the discovery of dozens of cryoprotectants with diverse chemical structures has suggested that many effective cryoprotectants have yet to be discovered or designed, yet rational design high throughput chemical screening has yet to be used at scale for this purpose.

Other untapped opportunities to apply state-of-the-art technologies abound. As Prof. John Bischof explains in a later section of these proceedings, nanotechnology has already been utilized for rapid, uniform tissue rewarming; nanoparticles hold enormous potential for tissue cryobiology, where spatial and temporal aspects must be tightly controlled. Genomics, proteomics transcriptomics,

[2] Session Participants and Speakers: **Dr. Mehmet Toner**, Professor at Harvard, Mass Gen Hospital and Co-founder of the Center for Engineering in Medicine; **Dr. Kenneth Storey**, Canada Research Chair in Molecular Physiology; **Dr. Boris Rubinsky**, Professor at UC Berkeley; **Dr. Janet Elliott**, Canada Research Chair in Thermodynamics and Professor at University of Alberta; **Dr. Erik Woods**, President of the Inter-national Society for Cryobiology, Senior Vice President and Lead Scientist, Cook Regentec; **Dr. Robert Ben**, Canada Research Chair in Medicinal Chemistry, Professor Bioorganic Chemistry at the University of Ottawa; **Dr Greg Fahy**, Chief Science Officer at 21st Century Medicine; **Dr. Utkan Demirci**, Host Professor, Leading Cryobiologist and Director of Stanford's Bio-Acoustic MEMS in Medicine Labs; **Dr. John Bischof**, Director of Bioheat and Mass Transfer Lab at the University of Minnesota. Sessions Chairs: **Dr. Alessandro Tocchio** and **Dr. Sebstian Giwa.**

metabolomics, and other systems biology approaches can be applied to uncover mechanisms of cryoinjury and cryotolerance in complex tissues. 3-D engineered tissue models can be developed to study cryopreservation in tissue systems, an unprecedented opportunity in a field where 3-dimensional tissue organization presents unique challenges. Computational modeling can be used to overcome many organ preservation challenges, from understanding the dynamics ice crystal growth to simulating cryoprotectant diffusion.

Organ and complex tissue banking efforts also stand to benefit from a vast array of basic research knowledge from related fields that has yet to be explored in the context of complex tissue storage. Research on cellular injury, stress responses, and cell death present many untapped opportunities to uncover and therapeutically target mechanisms that harm tissues during extreme temperature transitions. Likewise studies of tissue organization, metabolism, enzymology, and even thermodynamics have yet to be tightly integrated with research on tissue stasis and cryogenic storage. And many experimental approaches, such as gas persufflation to facilitate heat transfer, manipulation of tissue volume and ambient pressure to minimize ice crystal formation during cooling, and temporal control of cryoprotectant delivery, have shown experimental promise and have the potential to yield breakthroughs quickly if adequately supported.

In the sections of the proceedings that follow, top researchers in fields related to complex tissue preservation discuss the major challenges in organ banking and outline the most promising advances, proofs-of-concept, and untapped resources that may be leveraged to overcome them. The sessions of the summit follow each of the six remaining organ banking sub-challenges: controlling ice formation, reducing cryoprotectant toxicity, preventing thermal and mechanical stress, minimizing ischemic injury, limiting chilling injury, and developing protocols for revival, rehabilitation, and functional assessment of organs. A session was also held on rapid and uniform tissue rewarming, because of important recent advances in this field and their potential to help overcome some of the key challenges related to ice formation, mechanical and thermal stress, and cryoprotectant toxicity. Each of these challenges presents exciting opportunities for high-impact research. Taken together, they chart a scientific path toward the banking of organs and many other tissues, providing the first outline of organ banking as an ambitious but achievable scientific goal.

4. Scientific and technical obstacles that need to be overcome[3]

4.1. Jason P. Acker and Sebastian Giwa

The successful preservation of human organs presents a number of technical and practical challenges that at the surface would seem to be insurmountable based on the current state of low temperature science. While advances in many interdisciplinary fields has allowed for routine preservation of many cell types, the complexity of tissues and organs introduces many new problems that have required innovative strategies and technologies to be explored.

While composed of individual cells, which in themselves may be readily cryopreserved, the unique physical properties of tissues and organs have been shown to significantly affect the biological response to freezing and thawing (reviewed in [2,34]). The diversity of cell types and cell densities as well as the morphological differences between constituent cells significantly affects the osmotic and thermal state of tissues and organs. This has dramatic implications on the cooling and thawing rates that can be attained and hence the response of the tissue and organs to freezing and thawing. In addition, the requisite cell–cell and cell–matrix interactions in a tissue have been implicated in the poor survival of tissues following freezing. In tissue and organ systems, ice crystallization becomes much more complicated and difficult to balance through the use of conventional cryoprotectants and cooling/thawing rates due to thermal and mass transfer limitations creating damaged cellular zones within the tissue or organ. What has emerged from the 60 years of research into the cryobiological response of tissues and organs is that there is an interconnectiveness between the biophysical, thermal, mechanical and biological behavior of the system which must be considered when developing strategies for low temperature preservation.

Through systematic studies examining the mechanisms of damage to tissues and organs and the current state of advances in the preservation sciences, it is possible to identify specific areas where interdisciplinary, collaborative research could be focused in order to achieve measureable advances in the state of organ preservation [26]. Six focus areas emerge as key areas where scientific and/or technical advances would have a major impact on the overall goal of achieving successful, long-term organ preservation. Each of these sub-challenges have seen major advances over the last decade and present many more opportunities for both basic and translational research. The key scientific and technical obstacles detailed by the speakers throughout the Organ Banking Summit and discussed in these proceedings include: ice crystal formation, cryoprotectant and osmotic toxicity, chilling injury, control of heat and mass transfer profiles, thermo-mechanical stress, ischemic injury and tissue/organ revival and repair.

What emerged through discussions at the Organ Banking Summit was a general sense that due to the interconnectiveness of the scientific and technical challenges, progress in any one area of focus could have rippling effects on the other 6 areas. This could significantly reduce the need to achieve breakthrough advances in all areas of focus. For example, developing less toxic cryoprotectants could allow for higher concentrations to be tolerated by the tissues or organs, thereby reducing (or eliminating) the amount of ice formed at any one temperature and altering the requirements for rapid cooling or warming. As the accumulated stress during cryopreservation needs only to be kept under threshold levels to allow for survival and healthy function post-thaw, none of the six sub-challenges represents an absolute barrier to organ cryopreservation. The problems facing organ cryopreservation could therefore be restated in terms of the following challenges:

1 Control excessive ice formation
2 Hold cryoprotectant toxicity within acceptable levels
3 Limit disproportionate mechanical/thermodynamic stress
4 Control excessive chilling injury
5 Avoid unacceptable levels of ischemic injury
6 Ensure acceptable repair and revival protocols

While to a large extent universal in nature, the targets for each of these challenges will mean something different depending on the tissue or organ being considered and it will be important for the community to develop common understanding as to what “excessive” or “acceptable” means. Developing standard biological

[3] Session Participants and Speakers: **Dr. Mike Taylor**, Chief Science Officer, Sylvatica Biotech, and Adjunct Professor at Carnegie Mellon; **Dr. Ido Braslavsky**, Director of the Food-Biophysics and Cryobiology Laboratory and Professor at The Hebrew University of Jerusalem; **Dr. Greg Fahy**, Chief Science Officer at 21st Century Medicine; **Dr. Yoed Rabin**, Director of the Biothermal Technology Laboratory and Professor at Carnegie Mellon University; **Dr. Erik Woods**, President of the International Society for Cryobiology and CEO of Cook General BioTechnology and Genesis Bank. Sessions Chairs: **Dr. Jason Acker**, President-Elect Society, Cryobiology and Professor at the University of Alberta and **Dr. Sebastian Giwa.**

model systems to help define and characterize these tolerable limits for ice formation, toxicity, thermo-mechanical stress, chilling injury and ischemia is key.

In defining areas where advances in our scientific understanding and/or technical achievement are needed, we have leveraged the outstanding work of the pioneering cryobiologists in the field. As we look forward to the challenges that are before the field, it is clear that continued investment into interdisciplinary research and transdisciplinary collaborations will be absolutely key to achieving success. As never before, valuable understanding and tools in other - often radically accelerating - domains are empowering cryobiologists who can, via interdisciplinary collaborations, help with each of the six sub-challenges.

To advance the science of tissue and organ cryopreservation will require a concerted effort by a broad interdisciplinary field of scientists, engineers, and clinicians working to develop new strategies and tools. The ideas presented at this first Organ Preservation Summit will hopefully stimulate not only the search for improved techniques for the cryopreservation of tissues and organs, but more importantly, a desire to clearly understand the response of biological systems to low temperatures.

5. Controlling excessive ice formation[4]

5.1. Kelvin Brockbank and Ido Braslavsky

This session focused on the fundamental issue of ice formation for development of organ cryopreservation integrating concepts from multiple disciplines including biology, chemistry, physics, engineering disciplines and current practices in tissue banking. Ice formation separates water from the biological tissue resulting in damage by various mechanisms [42]. The control or avoidance of ice in cryopreservation procedures is crucial for successful preservation.

Several methods and approaches were discussed in the session. Mike Taylor described avoidance of ice by vitrification using high concentration of solutes which is an approach that is used successfully in cryopreservation of cells and simple tissue [9,54,65]. How to use it in organs is an open question. The notion that ice by itself within cells is necessarily lethal was challenged in a presentation by Jason Acker. Damage to membranes might be a key for the understanding of the destructive power of ice [1]. John Morris discussed the importance of diffusion limited ice formation, and the interplay between nucleation, crystal growth that depends on concentration, and the solute concentration that depends on the amount of ice in the system. In some cases loss of viability is clearly due to osmotic imbalance, not intracellular ice formation [44]. Controlling ice by high pressure offers a method to control ice growth and nucleation that was presented by of Boris Rubinsky and Nicholas Greer. The use of closed containers that can tolerate high pressures can result in methods in which ice grows in part of the container, leaving other parts without ice, avoiding ice in critical parts of the sample [29,58]. Controlling ice recrystallization and growth and improving cell cryopreservation is being attempted by Robert Ben's group by synthesis of molecules of interest [10]. Ido Braslavsky described Ice binding molecules from nature such as antifreeze proteins and ice recrystallization inhibitors [16,18,19,52]. These proteins are emerging as promising candidates to control ice in cryopreservation procedures, potentially facilitating ice control in both vitrification and slow cooling freezing methods [8,30,66,57]. Brian Wowk described a unique cryoprotectant formulation that facilitates vitrification at slow cooling rates. This formulation include a non-protein ice active molecule, namely Polyvinyl Alcohol [75].

Thermodynamics describes the driving force for crystallization and osmotic pressure and thus provides a framework for the understanding of cryobiology and the role of ice in cryopreservation [42,20]. Janet Elliott emphasized that it is more than heat transfer during cooling and warming. Thermodynamics describes the freezing point of intra and extracellular solutions, how much ice is formed and the fluxes of water and cryoprotectants into and out of biological materials. Small specimens in the scale range of cell-suspensions and cell clusters to small, organized tissues ranging from volumes in the microliter to a few milliliter have been successfully cryopreserved using freezing and ice-free vitrification techniques [60]. However, in complex biological materials ice formation damages both the cells and extracellular matrices during cryopreservation by freezing, usually compromising the materials' function in vivo as a transplant [62]. Methods for avoidance of ice by vitrification in medically important tissues that include pieces of muscle, short lengths of blood vessels, both natural and engineered, and cartilage discs were presented [9]. However, application of these methods in large tissues and organs is limited by cryoprotectant diffusion, risk of cryoprotectant cytotoxicity and, last but by no means least, ice formation, because we can't currently cool and warm fast enough to prevent ice nucleation.

The big issue identified and discussed throughout this organ cryopreservation summit, was how do we go from the single rabbit kidney successfully cryopreserved and transplanted [21] to reproducible procedures that can be used for human organs ranging from internal organs to hands and faces? Several presentations emphasized the development of ice-free vitrification methods that avoid both ice formation and cryoprotectant cytotoxicity. We were reminded that pressure is an important variable for avoidance of ice that may combine the benefits of less ice with use of less cytotoxic lower concentration cryoprotectant formulations. Clearly, better control of ice was identified as a significant issue. However, based on studies of freeze tolerant organisms and mammalian cell and tissue models, complete avoidance of ice may not be a necessity. Investigation of the impact of location of ice within biological structures and the form of the ice that can be tolerated without loss of function are important issues that need further work.

Further presentations and discussion focused on natural and synthetic ice control compounds that might prevent or modify ice. Ido Braslavsky made it clear that the terminology for ice control compounds in this field is too complex ranging from synthetic ice blockers and ice modulators to ice active compounds, ice crystallization inhibitors, ice nucleators, ice-active agents, ice binding agents, and antifreeze compounds. Ice-active agents include ice nucleating agents, ice nucleation inhibitors, some of which specifically inhibit certain types of ice nucleators, and ice growth inhibitors, that target specific ice crystal planes. Ice-growth inhibitors are also ice recrystallization inhibitors, but not all ice recrystallization inhibitors are effective ice-growth inhibitors. Research on antifreeze proteins and ice recrystallization proteins found in nature revealed that they bind to ice. Thus the term ice binding proteins was suggested as a united term to describe the different functionalities of these proteins. Ice-binding proteins depress the

[4] Session Participants and Speakers: **Dr. Mike Taylor**, Chief Science Officer, Sylvatica Biotech, and Adjunct Professor at Carnegie Mellon; **Dr. Robert Ben**, Canada Research Chair in Medicinal Chemistry, Professor Organic and Bioorganic Chemistry at the University of Ottawa; **Dr. Boris Rubinsky**, Professor at UC Berkeley; **Dr. Janet Elliott**, Canada Research Chair in Thermodynamics and Professor at University of Alberta; **Dr. Brian Wowk**, Cryobiologist and Senior Physicist at 21st Century Medicine; **Nickolas Greer**, Chief Science Officer, Rissali - Technology and Devices for Freezing and Thawing Under Pressure and **Dr. Jason Acker**, President-Elect of the Society for Cryobiology and Professor at the University of Alberta. Sessions Chairs: **Dr. Kelvin Brockbank**, President and Chief Science Officer of T3 - Tissue Testing Technologies and **Dr. Ido Braslavsky**, Director of the Food- Biophysics and Cryobiology Laboratory and Professor at The Hebrew University of Jerusalem.

freezing point of fluids resulting in a thermal hysteresis that prevents freezing in supercooled conditions, inhibiting ice recrystallization. Ice binding proteins are found in many freeze avoiding and freeze tolerant organisms and these proteins vary extremely in their ice binding activity. Antifreeze proteins that allow for several degrees of supercooling at low micro molar concentration levels are termed hyperactive antifreeze proteins [28,66]. These have found to be able to bind to the basal plane of ice in contrast to moderated antifreeze proteins [19,52,63]. The binding differences influence the shape of the ice crystals and may influence their role as cryoprotectants in cryopreservation procedures. In addition to the ice binding proteins there are interesting low molecular weight ice nucleation inhibitors such as a polyglycerol polymer that is effective against bacterial ice-nucleating proteins. Ice active agents tend to be effective at very low concentrations. Clearly this terminology requires integration. Combination of ice binding proteins and other ice active agents with several cryopreservation methods is being investigated. For example Boris Rubinsky observed that when ice binding proteins are used in an isochoric system there is a further 4–5 fold depression of the freezing temperature [53].

It was exciting to see the impact of Robert Ben's low molecular weight synthetic ice crystallization inhibitors, such as para-bromophenyl glucoside, modeled on naturally occurring antifreeze compounds, glycoproteins, found in deep sea Teleost fish that inhabit sub-zero environments, on ice formation in the presence and absence of red blood cells during repetitive cooling and warming cycles [11]. The best of these compounds may protect against transient warming injury, which may be a problem in clinical settings. The limiting factor in this research was not the production of compound libraries of potential ice crystallization inhibitors, rather it was screening of the compounds for ice activity and protection of biological materials during cooling and warming. Most of the presentations emphasized freeze tolerant organisms. Kelvin Brockbank concluded with discussion of work from Jack Duman's group on the larval form of *Cucujus clavipes*, an insect, which actually vitrifies and survives temperatures as low as −100° centigrade [64]. It has a complex survival strategy that includes seasonal behavior, dehydration, cryoprotectants, antifreeze proteins, antifreeze glycolipids and clearance of ice nucleators from their digestive tract. These are freeze-avoiding creatures that vitrify. How can we translate freeze avoiding and freeze tolerant survival lessons from Nature into cryopreservation methods for organs? The development of new microscopy methods, computed tomography, magnetic resonance imaging and microfluidics-based tools will help us understand the mechanisms by which diverse natural and synthetic ice binding inhibitory compounds act and guide their successful utilization in ice-free, or near ice-free, organ cryopreservation protocols.

Where do we go from here? We need a better understanding of molecular structure and ice activity mechanisms, leading to rational design of ice active agents. We need potent, stable, small, and affordable ice growth and recrystallization inhibitors, especially for vitrification. Do these compounds need to be inside the cell? Can ice be tolerated in some organ tissues or spaces as observed in freeze tolerant frogs? The answers to these and other questions will result in the development of the critical knowledge, tools and methods for a holistic approach to the challenge of scale–up from cell and tissue models to organ cryopreservation.

6. Holding cryoprotectant toxicity and chilling injury within acceptable Levels[5]

6.1. Greg Fahy

Cryoprotectant toxicity and chilling injury are the most fundamental obstacles to cryopreserving living systems in general and whole organs in particular. In principle, toxicity can be overcome by using different cryoprotectant solutions or by using different methods of cryoprotectant treatment, and both chilling injury and toxicity can be overcome ultimately by better understanding and blocking their mechanisms.

Finding better cryoprotectant cocktails would benefit from better search methods, but this involves searching an n-dimensional space in which each variable interacts with every other. Variables include not only the choice of cryoprotectants, but also the total concentration of the solution (i.e., the stability of the amorphous state of the solution), the carrier solution in which the cryoprotectants are delivered and removed, the temperature(s) at which cryoprotectants are administered, the times of exposure at each step or stage of addition and washout, the speed of addition and washout, and size of the concentration steps if steps are employed, and how osmotic buffering is done. To cut through some of the complexity of finding the peak of viability in this multi-dimensional landscape, both brute-force methods (high throughput screening) and conceptual simplifications and predictive heuristics would be of great value. Beyond this, the field might be benefitted immeasurably by understanding not just how injury relates to the above variables, but how it relates to the internal biochemical state of the cell, i.e., how the observed injury arises from cellular changes at the molecular level. Even more powerful would be the ability to discover cells that are naturally resistant to cryoprotectant toxicity and chilling injury and learn how to emulate them.

Utkan Demirci led off the session by speaking about a microfluidics system that might in the future be used to enable high-throughput screening of new cryoprotectant candidates. However, it will be important to take into account that different cryoprotectants have different vitrification tendencies, and so screening methods must also be done that compare different cocktails at the same vitrification tendencies, a significant challenge for the future.

Gloria Elliott spoke about how optimizing interactions between different molecular species, such as magnesium and glycerol or glycerol and trehalose, can drastically and beneficially alter the glass transition temperature and the “fragility” of the amorphous state in non-aqueous systems, but acknowledged that it will be important to understand how such effects work in the context of water. Gloria Elliott spoke about how optimizing interactions between different molecular species, such as magnesium and glycerol or glycerol and trehalose, can drastically and beneficially alter the glass transition temperature and the “fragility” of the amorphous state in non-aqueous systems. Nevertheless, her experience supported the current idea of using complicated cocktails of cryoprotectants rather than single or few agents to adjust the stability of the amorphous state.

Janet Elliott noted that in large living systems, cryoprotectants can kill cells by indirect effects such as by osmotically-induced long-range mechanical stress, adding yet another dimension to

[5] Session Participants and Speakers: **Dr. Kenneth Storey**, Canada Research Chair in Molecular Physiology and Professor in Biochemistry at Carleton University; **Dr. Utkan Demirci**, Director of Bio-Acoustic MEMS in Medicine Labs at Stanford University; **Dr. Gloria Elliott**, Director of the Biostability Lab and Professor at University of North Carolina–Charlotte; **Dr. Janet Elliott**, Canada Research Chair in Thermodynamics and Professor at University of Alberta; **Dr. Adam Higgins**, Director of the Bio-transport and Biomedical Process Engineering Lab and Professor at Oregon State University**; Dr. John Morris**, Founder and CEO of Asymptote; **Dr. Joao Pedro Magalhaes**, Leader of the Integrative Genomics of Aging Group at the Institute of Integrative Biology, University of Liverpool, UK and **Dr. Tom Johnson**, Professor at the Institute for Behavioral Genetics, University of Colorado; Professor of Integrative Physiology and Fellow of Biofrontiers Program. Sessions Chair: **Dr. Greg Fahy**, Chief Science Officer at 21st Century Medicine.

the problem of controlling injury from cryoprotectant exposure. With respect to the issue of predicting the toxicity of cryoprotectant mixtures, she noted that rules could be developed that parse out the effects of two-, three-, and four-cryoprotectant interactions, but that rules developed for one set of circumstances did not apply when applied to different circumstances, such as different temperatures, isolated cells rather than intact tissue, or the same intact tissue from different species, although the evidence did, again, favor mixtures rather than single agents for intact tissues.

Based on modeling tools and experimental results, Adam Higgins described a method for greatly reducing the relevance of all of the numerous complexities underlying cryoprotectant toxicity by enabling the exposure time to peak CPA levels to be greatly reduced, thus lessening toxicity regardless of its mechanism. This can be accomplished by adding the absolute mass of intracellular cryoprotectant needed while a cell is in a swollen state, after which restoring the cell to its normal, or to a slightly subnormal, volume immediately elevates intracellular concentrations even to vitrifiable levels. The method works well on endothelial cell monolayers simulating the vascular bed. Extending it to whole organs will add much more complexity, but results indicate that viable methods can be reduced to narrow classes that should be relatively easy to test even on a complex whole organ.

John Morris then reviewed what is known about lipid phase changes as a mechanism of chilling injury. As he pointed out, little work has been done on this since the original idea was introduced that membrane phase separation creates packing defects in the cell membrane that cause a damaging increase in cell permeability, but a number of investigators have shown improved resistance to chilling injury by modifying cooling rate or membrane composition in ways that reduce the tendency for phase separation. Accomplishing this in whole organs, however, remains a formidable challenge, although it may be possible to adjust membrane fluidity in a favorable direction. Thermoelastic stress to the plasma membrane has also been proposed as a mechanism of chilling injury by McGrath, and may be more tractable.

Ken Storey then pointed out that tackling the final problem, the elucidation of biochemical mechanisms of cryoprotectant toxicity, is not as hard as it used to be, thanks to all the "'omics" technologies now available, including transcriptomics, proteomics, metabolomics, and other global methods for assessing the state of cells on a comprehensive basis.

The next speaker, Joao Pedro de Megalhaes, described a study that did just this, assessing mechanisms of the toxicity of 60% v/v ethylene glycol using transcriptomics. Treating human endothelial cells, used as a model of the vascular system in perfused organs, with this more-than-vitrifiable concentration of ethylene glycol resulted in little cell death or loss of proliferative ability compared to cold control cells, but in many ways specifically affected membrane functions, in some cases resulting in changes that did not entirely revert back to normal even 72 h after CPA exposure [41]. This study raises the basically new possibility that the problem with CPA toxicity in whole organs might be less about cell death than the induction of long term functional changes in cells that might affect the viability of the organ as a whole. Selective changes in vascular cells, for example, might make normal function of the rest of the organ unsustainable.

But what if we could discover ways to make cells more resistant to such changes? Tom Johnson described methods of preparing populations of mutagenized embryonic stem cells that enable the populations to be screened for resistance to CPA exposure. Preliminary studies indicate that some mutations can be found that do confer protection, which should provide much more direct insights into mechanisms of protection from toxicity than can be obtained with 'omics approaches. Further, by identifying the specific mutations, he has found in similar studies that it was possible to identify an already FDA-approved drug whose mechanism of action mimics the effect of the mutation. Such a drug might have great value in whole organ studies if it can block susceptibility to cryoprotectant toxicity in vivo or in vitro, and one of Johnson's mutants has been found to both block the toxicity of dimethylsulfoxide and freezing injury using this agent as the cryoprotectant.

In further support of such possibilities, the last talk, which was impromptu, by Robert Shmookler Reis, confirmed that not just cells but even whole organisms can be made more resistant to cryobiological stresses using not just genetic modification but activation of genetic pathways of stress resistance. By starving C. elegans or subjecting them to heat stress, their freeze-thaw survival could be improved, and by using both stresses together, survival was improved many fold.

What are the biochemical mechanisms of chilling injury and the toxicity of vitrifiable concentrations of cryoprotectant? How can cells be made more immune to these mechanisms? Can whole organs be perfused under hypotonic conditions and if so, what are the best cryoprotectant choices and how will these vary from organ to organ? Is high throughput screening for new cryoprotectants really possible and if it is, are there enough new candidates to screen? Is the biggest problem cell death, or cell dysfunction? And which cells are most at risk? For the first time, we are finally beginning to be able to not only ask such questions, but to answer them. The prospects for harnessing natural protective pathways using drugs or drug-like interventions (miRNAs, CRISPR/cas9, etc.) and to add in better perfusion methods, apoptosis blockers, etc., are truly promising.

7. Minimizing thermo-mechanical stress in order to preserve structural integrity and functionality in cryopreservation[6]

7.1. Yoed Rabin

Key to biobanking success is the preservation of the tissue's structural integrity. Structural changes in cryopreservation may come from two primary sources: irreversible changes at the cellular level induced by exposure to extreme temperatures and cryopreservation solutions, and mechanical stresses induced by the tendency of all materials to change volume with temperature. The physical property that describes this effect is known as the *thermal expansion coefficient* and the mechanical response of the tissue to those volume changes is known as the *thermo-mechanical stress*. When the thermo-mechanical stress exceeds the strength of the material, structural damage follows, with fracture formation as its most dramatic outcome.

To a large extent, irreversible structural changes at the cellular level are the reflection of insult on the biological material by a host of physical phenomena, conveniently classified as resulting from tissue handling, exposure to low temperatures, cryoprotective agents (CPAs) flow, CPA toxicity, kinetics of ice crystallization, and degradation of biological material in a suspended state. The extent

[6] Session Participants and Speakers: **Dr. Barry Fuller**, Lead Global Professor at the UNESCO Chair in Cryobiology and Professor at UCL Medical School/Royal Free Hospital, **Dr. Yoed Rabin**, Director of the Biothermal Technology Laboratory and Professor at Carnegie Mellon University; **Dr. James Benson**, Biomathematician predominantly focusing on cryobiology and Assistant Professor at Northern Illinois University; **Dr. John Bischof**, Director of Bioheat and Mass Transfer Lab at the University of Minnesota; **Dr. Ramon Risco**, Professor of Engineering, University of Seville, CEO of SafePreservation, Senior Physicist at National Accelerator Centre (Spain) and **Dr. Brian Wowk**, Cryobiologist and Senior Physicist at 21st Century Medicine. Sessions Chair: **Dr. Yoed Rabin**, Director of the Biothermal Technology Laboratory and Professor at Carnegie Mellon University.

of structural changes at the cellular level can be quantified by comparing the mechanical properties of the tissue before and after cryogenic storage, in both cases at normothermic conditions. This comparison highlights the accumulated structural changes over the entire cryoprotocol, where relevant mechanical properties include strength, elasticity, and stiffness. While methods to measure mechanical properties are well established in the engineering literature, a major challenge in the context of cryobiology is to reduce those methods to practice at the cellular level. Another key challenge is in correlating the accumulated structural changes with specific events occurring during the process.

At the tissue level, thermo-mechanical stress is dominated by tissue inhomogeneity and anisotropy, temperature non-uniformity, phase change effects, and tissue interaction with its holding container. Furthermore, the development of thermo-mechanical stress during cryopreservation is a path-dependent effect, which means that the level of stress at any given instant is not merely dependent upon the temperature or any other thermodynamic property, but it is in fact affected by the history of events that the tissue has experienced up to the specific point in of interest. It follows that one can design different pathways from normothermic conditions to cryogenic storage and back to tissue recovery, which would result in different mechanical stresses along the way. Representative results demonstrated how some of the protocols might lead to structural damage while others may avoid it. Ongoing discussions stressed the need to develop basic knowledge, computation tools, and new CPA solutions that will create favorable conditions to preserve structural integrity.

Developing the ability to plan and optimize cryopreservation protocols in order to reduce and possibly circumvent structural damage is paramount. This ability becomes even more critical with ongoing efforts to preserve larger tissues and organs, where thermo-mechanical stresses might only intensify with the increasing size. While engineering methods to study thermo-mechanical effects are well established, their application for the benefits of cryopreservation represents a relatively uncharted territory. Due to the complexity and path-dependency nature of cryopreservation processes, it is argued that the only practical approach to study thermo-mechanical stress in large-size samples is to develop testing devices that can mimic the cryopreservation protocol while simultaneously measuring the mechanical response. Another unmet need in this area of research is the development of computation tools that can make use of the measured data in order to predict the outcome of cryopreservation. This investigation process of measuring physical effects, describing them mathematically (i.e., modeling), and consequently creating computation tools that can predict the outcome of potential cryopreservation protocols, essentially underlies a mechanistic approach to the study of cryopreservation. This approach stands in contrast to the phenomenological approach that has dominated the field since the dawn of history of cryopreservation.

While the investigation of ways to preserve structural integrity represents a relatively uncharted territory, session discussions have focused on developing research areas at the forefront of cryopreservation research, namely: mechanical behavior of cryopreserved materials, cryogenic protocols and solutions to avoid fractures, cell-based modeling of mechanical and chemical stress, challenges in thermo-mechanical stress modeling for large-size tissues and organs, computed tomography as a means to observe physical effects that dominate cryopreservation success, and neutron scattering as a means to interrogate molecular changes during rewarming.

The mechanical behavior of biomaterials varies tremendously along the cryogenic protocol. During vitrification for example, the viscosity changes by twelve orders of magnitude, from a fluid-like material at room temperature to a solid-like material at the storage temperature (below glass transition). As a result, different devices and measurement techniques are needed in order to characterize the viscosity at different temperatures. Viscosity measurement is only one example, where other physical properties call for a similar strategy. A selection of recently developed measurement devices has been presented and representative results have been discussed, obtained with the CPA cocktails VS55, DP6, and in combination with synthetic ice modulators (SIMs)—a cutting-edge technology in vitrification. VS55 and DP6 have drawn significant attention in recent years, where the base of knowledge on their mechanical behavior is the most extensive at the current stage. Nonetheless, additional efforts are required in order map the behavior of new and promising cryopreservation solutions and biological materials.

Thermo-mechanical stress is driven by differential thermal expansion, which may be induced by the container walls, variation in material properties between different locations and in different directions, temperature non-uniformity, and crystallization, where water exhibits dramatic expansion upon solidification. The extent of temperature non-uniformity and the likelihood of partial crystallization increases with the specimen size, which serves as a driving force in the development of new CPAs. An advanced vitrification cocktail has been discussed, M22, which is characterized by extraordinary low critical cooling and rewarming rates. M22 is a complex cocktail containing eight CPAs, two of which are also known as synthetic ice blockers (SIBs)—a subset of SIMs. Promising experimental results obtained with M22 suggest that its application may prevent gross fracture formation. However, it has also been noted that thermo-mechanical stress was a suspected cause of more subtle, but still-significant damage after transplantation of vitrified rabbit kidneys. Possibly, this is the results of some plasticity effects. With M22 as an example, the need to develop superior vitrification solutions to alleviate the stress on the tissue is an ongoing effort.

Thermo-mechanical stress and chemical stress modeling are essential to explain observed phenomena and develop predicting tools, in order to design the next generation of cryopreservation materials and protocols. Computation tools are needed to simulate cryopreservation process according to those models. Cryopreservation presents multiscale and multiphysics challenges for process simulations, which come with high computation costs. A cost-effective approach is to separately simulate cellular-level and tissue-level effects. At the cellular level, recent advances display more realistic cell-based and CPA-based modeling. This enhances the knowledge about cell interactions with their environment, mass transport, cell growth, and cell death. Furthermore, it is now feasible to model cellular-level tissue damage due to osmotic pressures, cell-to-cell ice propagation, and the impacts of accumulated damage due to the cryopreservation process. Following these expanding computation capabilities, an unmet need to measure cell-specific physical properties has arisen. As the cryopreserved material solidifies, cellular-level effects become less prominent, while tissue-level effects take center stage. This is evident by the nature of gross fracture surfaces, where they appear to be oblivious to the cellular detail. Classical models of thermo-mechanical stress may not serve as good representations under such conditions, and an unmet need to develop material-specific mechanical models at cryogenic temperatures has concurrently developed. This also necessitates additional efforts to widen the database on material properties.

Nondestructive imaging modalities are essential for the study of structural integrity. Imagining modalities can further provide feedback on mass transport and crystallization events. Three alternatives are readily available: computed tomography (CT), neutron scattering (NS), and cryomacroscopy. It has been

demonstrated that CT can be used to demonstrate fractures, ice formation, and monitor CPA concentration within the specimen. CT has the ability to render three-dimensional effects. NS has been demonstrated on selected CPAs in cryogenic temperatures. Clear evidence of ice recrystallization has been demonstrated, as the vitrified material rewarms from cryogenic storage. Verification of crystallization at the same temperature and subject to a similar cryogenic protocol has been obtained by means of differential scanning calorimetry. CT and NS technologies provide exceptional tools for studying the structure and phase change effects at the molecular level, which can be instrumental for the development of new cryopreservation protocols and verification of mathematical models. The cryomacroscope is an optical device to visualize physical events *in situ*, in large-size samples. While the cryomacroscope capabilities have been demonstrated in selected cases throughout the summit, polarized light capabilities have been further demonstrated as a nondestructive measurement technique of mechanical strain, using the principles of photoelasticity. It is proposed to further develop and expand CT, NS, and cryomacroscopy capabilities as complementary tools for the study of large-size cryopreservation.

In summary, thermo-mechanical stress and preservation of structural integrity is perhaps the least populated area of research within the field of cryobiology. The unmet need for advanced knowledge in this area is evident; this unmet need only intensifies with concurrent trends to increase the size of the cryopreserved material. Given the state of knowledge in the field, research efforts are expected to concurrently expand in several thrust areas: developing of new mechanical experimentation tools that can simultaneously mimic routine cryopreservation protocols, establishing material properties databases, formulating models for the behavior of materials, and developing rapid simulation technique and optimization methods to help the cryobiologists in the design of new CPAs and cryoprotocols.

8. Optimal rewarming modalities[7]

8.1. John Bischof, Brian Wowk

Cryopreservation by vitrification is generally believed to be the most promising approach for complex tissue and organ banking because it nominally avoids damaging ice formation. Instead of freezing, tissues are transformed into a solid water/cryoprotectant glass. The concentration of cryoprotectant agents (CPA) necessary for vitrification, and associated toxicity, can be minimized by cooling and warming at the fastest rates possible. Rapid warming is especially important because the minimum warming rate necessary to avoid ice crystallization at a given CPA concentration is typically an order magnitude greater than the minimum cooling rate necessary. An additional consideration is that warming should be uniform to reduce thermo mechanical stress. The conflicting requirements of rapid warming and uniform warming can be met by warming tissue from within instead of by surface conduction. In this session Dr. Brian Wowk from 21st Century Medicine, Inc., and Dr. John Bischof from the University of Minnesota Bioheat and Mass Transfer Laboratory respectively presented work on electromagnetic warming and magnetic nanoparticle warming for faster and more uniform heating of tissue during recovery from the vitrified state.

There has been limited study of electromagnetic warming for cryopreserved organs and tissues since the 1970s. In electromagnetic warming, tissue is warmed by a combination of dielectric and ohmic heating from an applied radiofrequency or microwave electric field. The frequency must be low enough to have a large skin depth and no resonant effects on the spatial scale of the specimen being rewarmed. The frequency must be high enough to couple efficiently with dipolar molecules in the vitrification solution within the temperature range of interest. For the M22 solution that has been used for kidney vitrification, a frequency in the range of 10 MHz−100 MHz is preferred. The specimen must be placed in an applicator consisting of opposed capacitor plates to deliver an electric field of this frequency range. Experimentally, 200 W of 27 MHz illumination achieved a peak warming rate of 160 °C/min at −55 °C during rewarming of vitrified 20 mL volume of M22. (Although M22 has a critical warming rate to avoid ice crystallization of only 1 °C/min, such faster warming rates are important because the CPA concentration in some parts of organs doesn't reach the full concentration of the perfused solution during a tolerable time course of perfusion.) This peak warming rate was advantageously near the temperature of maximum ice growth rate in slightly-diluted M22. A vitrified rabbit kidney warmed slower and more heterogeneously than the pure M22 solution, with the pelvic temperature lagging the cortical temperature by 15 °C at the end of warming.

Magnetic nanoparticle warming ("nanowarming") adds superparamagnetic nanoparticles to CPA solutions that are either perfused into an organ (perfusion loading) or into solutions that surround a specimen (boundary loading). A radiofrequency magnetic field applied with a helical coil then warms the nanoparticles and surrounding solution. This is new approach to heating tissue in cryobiology, motivated by the increasing experimental use of nanoparticles in medicine for drug delivery and magnetic hyperthermia. 1 mL samples of VS55 vitrification solution loaded with 10 mg Fe/mL ~10 nm Ferrotec EMG-308 nanoparticles in a 20 kA/m magnetic field at 360 kHz warmed at ~200 °C/min below the glass transition temperature (−120 °C) and ~100 °C/min above it. Nanoparticle warming is advantageous because such low frequencies of magnetic fields and associated electric fields interact negligibly with tissue compared to magnetic nanoparticles, making heating entirely dependent upon nanoparticle distribution rather than temperature-dependent tissue properties. This also allows nanoparticle warming to begin in the solid state at very low temperatures, a regime in which classical electromagnetic heating is comparatively ineffective. The distribution and density of nanoparticles can also be imaged by SWIFT MRI to predict warming uniformity and associated heating rate limitations in different organs.

Nanowarming can rewarm vitrified tissue at an approximately steady rate proportional to applied power beginning at very low temperatures. The heating rate of electromagnetic warming changes continuously with temperature, but this can be used to advantage to prevent "thermal runaway" if the frequency and system tuning are chosen so that the dielectric warming rate drops off as a target temperature is approached. Both nanowarming and electromagnetic warming are vulnerable to problems of inhomogeneous warming rate. During nanowarming heat is delivered via nanoparticles inside blood vessels, limiting the heating rate to that which does not overheat large vessel walls and surroundings. During electromagnetic warming, heat is delivered most effectively to tissue rich in polar liquids, limiting the heating rate to that which does not cause overheating as heat conducts away from faster-warming regions to slower-warming regions. The continued development of nanowarming and electromagnetic warming requires the construction of larger systems so that these issues can be

[7] Session Participants and Speakers: **Dr. John Bischof**, Director of Bioheat and Mass Transfer Lab at the University of Minnesota and **Dr. Brian Wowk**, Cryobiologist and Senior Physicist at 21st Century Medicine. Sessions Chair: **Dr. John Bischof**, Director of Bioheat and Mass Transfer Lab at the University of Minnesota.

studied in larger tissues and organs.

Future research/development directions: During nanowarming and electromagnetic warming, future developments will require efforts on the part of nanoparticle design, applied electromagnetic field, imaging, and biological impact. For nanowarming, the nanoparticle design will require the highest heating, biocompatible magnetic (i.e. iron oxide) nanoparticle that can be batch produced for vitrification solutions. The applied fields for both nanowarming and electromagnetic warming will require scaled up electromagnetic field (i.e. RF or microwave) power supplies in the 10 or more kWs. For electromagnetic warming, computer management of tuning and matching will be important and frequency sweeping to follow peak absorption frequencies as viscosity decreases during warming between Tg and 0 °C will be important. Similarly, for nanowarming, the magnetic properties of the nanoparticles will typically rise at lower temperatures giving a higher heating near the glass transition temperature that will need to be studied for optimal re-warming protocols. Finally, imaging will be important to both assess the vitrified state by computed tomography and the nanoparticle distribution by both MRI (i.e. SWIFT pulse sequencing – Michael Garwood, University of Minnesota). Finally, assessing both nanowarming and electromagnetic warming approaches in biological systems from cells, tissues to whole organs will be important in fully translating this technology.

9. Enhance cellular and tissue repair and avoid excessive ischemia reperfusion injury[8]

9.1. Barry J. Fuller and Erik J. Woods

Recent years have seen many advances in our understanding of the physiology of ischemia/reperfusion (I/R) injury and contributions to tissue injury more generally. These create promising possibilities for reducing sources of injury during organ preservation and transplantation, as well as conditioning tissues to tolerate I/R injury and directly repairing injured tissues. Cells and tissues can be critically injured in response to both ischemia and, paradoxically, restoration of blood flow. This ischemia/reperfusion (I/R) injury is especially applicable to vascularized tissues or organs which can be considered complex tissue allografts (CTA). Ischemia as a term to denote deficient blood supply to tissues was first used in the early nineteenth century. Since then researchers have endeavored to understand the underlying mechanisms of ischemia related damage to find methods to limit the health/medical burden associated with disorders affecting organ-specific blood flow, including those associated with solid organ transplant [33].

Over the past 30 years, many new discoveries have been made that are particularly impressive, vastly increasing our understanding of the molecular, cellular, tissue-specific, as well as systemic events that occur during ischemia *per se*. Evidence supporting the concept that reperfusion could paradoxically induce and exacerbate tissue injury and necrosis was also discovered early in this period and provided a major drive for research because this component of tissue injury has the potential to be alleviated through therapeutic intervention. I/R has been linked to a number of different effects, including oxidative stress, signaling of cellular damage pathways, and recruitment of inflammatory responses. For vascularized DCT, there are additional consequences of I/R which may impact the host immune system and injure distant organ systems. A number of pharmacological agents and therapeutic strategies have been shown to ameliorate different aspects of I/R, however a holistic approach can be complex and challenging. Indeed, despite years of intensive investigation, we are still far away from thoroughly understanding the underlying mechanisms of I/R [47].

9.1.1. Learning from animals (Dr. Kenneth Storey)

Selected amphibian and reptile species living in seasonally cold climates have developed the capacity to survive long term freezing with about two-thirds of their body water locked up in extracellular ice. Long term survival in the frozen state requires regulated responses by cells and organs to sustain homeostasis and viability upon thawing. This includes a diversion of energy expenditures into a minimum of actions to sustain viability and protection of macromolecules beyond just metabolite cryoprotectants (e.g. enhancement of chaperones and antioxidant defenses). In this way freeze tolerance shares components of responses to two other widely encountered environmental stresses (oxygen limitation and dehydration). New and ongoing studies are documenting freeze/thaw-responsive control of gene expression not only via traditional methods such as signaling pathways that impact transcription factors but also via posttranscriptional regulation of mRNA transcripts by microRNAs, small non-coding RNA species that influence the fate of mRNA. Transcriptional regulation via epigenetic controls on gene expression also occurs, creating unique patterns of freeze-responsive gene and protein expression. A thorough understanding of freeze/thaw and other natural mechanisms of animal cold hardiness is not only key to understanding the principles and mechanisms by which organisms survive thawing after subzero temperatures, it may also enable breakthroughs in the banking of human organs and other complex tissues. Importantly, many of these mechanisms are conserved in hibernating mammals and primates, with some evidence that analogous mechanisms are found even in humans.

9.1.2. Novel trophic factors (Dr. A Yu Petrenko)

Recently, novel sources of trophic factors to assist in organ repair/recovery following I/R injury have been investigated. Ongoing work by Petrenko focusing on fetal liver stem cells (both hepatic and hematopoietic) has yielded trophic factor “cocktails” which can be utilized as cell free extracts. Petrenko has previously shown significant therapeutic effects of these trophic factors (TF) on models of acute toxic hepatitis, experimental cirrhosis, chronic alcohol poisoning, solid tumor progression and wound healing. Most recently, utilizing a non-hepatic source of mesenchymal stromal cell derived cell-free fetal-specific factors; Petrenko was able to demonstrate that supplementation of preservation solutions with such factors modulated redox-dependent processes and led to strengthening of cell adaptive responses to stress against I/R injury. These results indicate the potential for TF derived from adult stem/progenitor cells and or conditioned culture media may be useful in reducing organ I/R injury.

9.1.3. Ex vivo perfusion (Dr. Pablo Sanchez)

Machine perfusion is an area undergoing rapid advances; one example is the use of ex vivo organ perfusion, in particular for

[8] Session Participants and Speakers: **Dr. Robert Shmookler-Reis**, Professor of geriatric medicine, molecular biology, and pharmacology at the University of Arkansas; **Dr. Kenneth Storey**, Canada Research Chair in Molecular Physiology and Profes-sor in Biochemistry at Carleton University; **Dr. John Baust, Sr**, UNESCO Professor, Chief Scientific Adviser at CPSI Biotech, Director of the Institute of Biomedical Technology at the State University of New York, Binghamton; **Dr. Barry Fuller**, Lead Global Professor at the UNESCO Chair in Cryobiology and Professor at UCL Medical School/Royal Free Hospital; **Dr. Alexander Petrenko**, Head of the Biochemistry Department and Professor at the Institute for Problems of Cryobiology and Cryomedicine Ukraine (IPC&C) and Kharkov University; **Dr. Pablo Sanchez**, Lead Scientist for XVIVO Perfusion, Inc and Assistant Professor of Surgery, University of Maryland; **Dr. Klearchos Papas**, Scientific Director of the Institute of Cellular Transplantation and Professor of Surgery, University of Arizona. Sessions Chair: **Dr. Erik Woods**, President of the International Society for Cryobiology, Senior Vice President and Lead Scientist, Cook Regentec.

lungs, which holds the potential to increase the number of transplantable organs. Current limits for human or porcine lung perfusion totals about 12 h while maintaining minimal edema formation, which allows time for lung reconditioning. Current available therapies could be rapidly translated to lung repair before transplantation. Fluid clearance can be stimulated by intratracheal instillation of terbutaline. Vascular permeability and epithelial fluid transport can be restored with allogeneic mesenchymal stem cells (MSC). Other MSC properties, such as the antibacterial secretion of indoleamine 2,3-dioxygenase, LL-37 peptide, the immunomudulatory effects of IL-10 and IL-4 secretion, the inhibition of INF-γ and TNF-α, and the positive effects described on ischemia-reperfusion injury in the liver and kidney could translate cell administration during ex vivo perfusion in a therapy tailored to modulate a wide range of conditions affecting lung quality.

Gene therapy has also been proposed as an option to recondition donor lungs ex vivo. Human lungs that received IL-10 transfection exhibited improved functional quality, increased oxygenation, decreased vascular resistance, improved cell–cell interaction, and presented a positive shift from a pro-inflammatory to an anti-inflammatory cytokine release environment. Finally, the idea of growing immunocompatible lungs "on demand" was recently proposed. Cadaveric rat lungs that were de-cellularized, re-cellularized and transplanted demonstrated good function up to 7 days.

9.1.4. Persufflation in organ preservation (Dr. Klearchos K. Papas)

Enhanced oxygenation during organ preservation for kidney, heart, pancreas and liver results in improved organ viability. Persufflation (PSF), or the flow of hypothermic humidified oxygen gas through the vasculature of organs has been shown to be effective in oxygenating and preserving as well as recondition a variety of organs including kidneys, livers, hearts, and pancreata from expanded criteria donors in a variety of small and large animal models with excellent outcomes. Persufflation is currently in a clinical trial for reconditioning (after hypothermic static storage) and transplanting livers that would have otherwise been discarded in Europe.

PSF has also extended significantly the allowable widow for organ preservation (14 h instead of 4–6 for heart and 24 instead of 8–12 h for the pancreas) without compromising outcomes. PSF has been primarily limited to reconditioning organs after prolonged cold ischemia times, but ongoing work by Papas has been evaluating systems which would allow PSF to be used in a portable manner, which would potentially allow humidified oxygen gas to be delivered to the vasculature of organs during transport.

9.1.5. Preconditioning and other strategies (Dr. Erik J. Woods)

Most if not all tissues can withstand short periods of ischemia that do not produce detectable functional injury. However, once a critical duration of ischemia is reached, cell injury and/or death results. In 1986, Murry et al. made the discovery that prior exposure of the heart (or other tissues) to short bouts of ischemia and reperfusion (ischemic preconditioning) prior to prolonged reductions in coronary blood flow exerted powerful infarct-sparing effects [45]. This seminal finding created an explosion of interest with regard to identification of therapeutic strategies that might prove effective in reducing the risk for and/or outcome of adverse cardiovascular events. In addition, the discovery of ischemic preconditioning indicates that the response to ischemia is bimodal, with longer periods of ischemia inducing cell dysfunction and/or death that is exacerbated by reperfusion, while short cycles of conditioning ischemia can actually be protective, rendering tissues resistant to the deleterious effects of prolonged ischemia followed by reperfusion via activation of intrinsic cell-survival programs.

In recent years there has been an explosion in various emerging approaches to assess and manipulate genetic integrity of cells and tissues at the phenotypic, cytologic, biochemical, and molecular level. These approaches can now be exploited with respect to organ preservation. A better understanding of the implications of epigenetics, combined with renewed interest in cell differentiation and de-differentiation has opened new philosophies of biological mechanisms that can be developed to the advantage of future cryobiologists as they become better understood. For instance, new understanding of induction of pluripotency in somatic cells (e.g. iPS cells) has led to a better understanding of some fundamental mechanisms of cellular repair and propagation. A deeper understanding of the epigenetic influences on cell phenotype also shows great promise to help yield improved cryopreservation outcomes of cells, tissues and organs. Cellular reprogramming through molecular or environmental cues may be able to modulate cell pathways, inducing a rejuvenated state capable of restoring primary energy metabolism while avoiding free radical production post thaw. Better understanding of how to manipulate these mechanisms could potentially allow and induction of cryotolerance in difficult to freeze models to avoid quiescence or apoptosis post thaw. By combining these tools with novel chemistry and physics, systems can be developed which will allow for more stable preservation through "classic" cryopreservation (e.g. slow cooling approaches), vitrification and potentially lyophilization.

10. Outcomes from the Summit

The leading scientists, surgeons and stakeholder at the first global Organ Banking Summit have identified a strong and widespread need for capabilities to bank organs and complex tissues. Promising approaches to achieving organ banking through focused research efforts has been outlined, breaking the needed research into a few discrete remaining set of sub-challenges that can be overcome in parallel. Importantly, these sub-challenges are interrelated and benefit from substantial spillover effects, and none represent an absolute barrier to successful organ banking.

A plethora of scientific opportunities have been identified for each of these sub-challenges, which have resulted from promising recent breakthroughs, historically underexplored research leads that have the potential to be high impact, and the need to integrate complementary fields and approaches into the organ banking research efforts that have not yet been brought to bear. Critically, pursuing these opportunities depends on appropriate allocation of resources to organ banking research and in turn on prioritization by major funding sources. If organ banking is made a research priority, the field is poised for rapid progress in the coming years.

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Note: co-authors are only responsible for their respective sections.

Conflict of interests

IB, JPA and BJF have no conflicts of interest to declare.
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