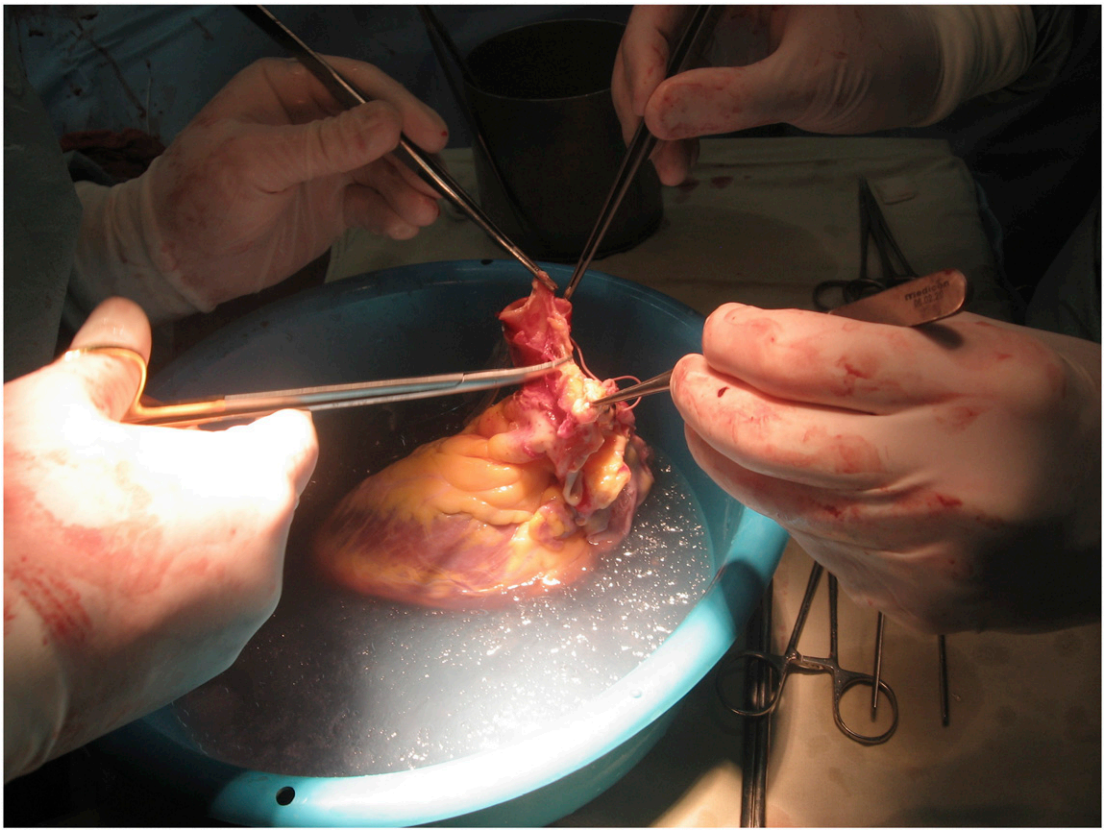
Cryopreservation aims to engineer novel ways to freeze, store, and thaw organs

Megan Scudellari, *Science Writer*

Five years ago, even top cryobiologists doubted that a human organ would ever be successfully frozen and thawed. It felt like "a long, long scientific step" from the freezing of cells or millimeter-thick tissues to the freezing of whole organs, says Janet Elliott, Canada Research Chair in Thermodynamics at the University of Alberta. "My opinion has changed," Elliot professed this past August after an organ-banking conference at Harvard Medical School hosted by the nonprofit Organ Preservation Alliance (OPA).

OPA and the US Department of Defense (DoD), which is seeking better ways to treat combat trauma, have spent the past five years attempting to unite researchers and to encourage new participation in the field of cryopreservation. The results are now manifest: What started as a long-shot effort to preserve a rabbit kidney is now a collaborative, government-funded endeavor. The practitioners of cryopreservation are experimenting with new engineering approaches for preserving larger and larger tissues, from pig heart tissue to whole rat limbs.



Cryopreservation methods have the potential to revive damaged organs, improve the quality of tissue models for drug discovery, and extend the amount of time a transplant organ can be preserved. Image courtesy of Shutterstock/kalewa.

www.pnas.org/cgi/doi/10.1073/pnas.1717588114

Although whole-organ banking remains years if not decades away, cryopreservation tools are already being applied to help revive damaged organs, improve the quality of human tissue models for drug discovery, and stretch the amount of time a transplant organ can be preserved—when even a few extra hours can save a life.

Outrunning Ice

Long-term cold banking of organs, or cryopreservation, has been pursued since at least the 1950s, when researchers attempted (with limited success) to cool golden hamsters to below 0 °C and rewarm them. In 1970 surgeon Thomas Starzl, who performed the first human liver transplant, wrote that there was "no way" that widespread organ transplants would be possible without major developments to preserve organs for weeks or months (1). Yet in the decades since, that feat has not been achieved.

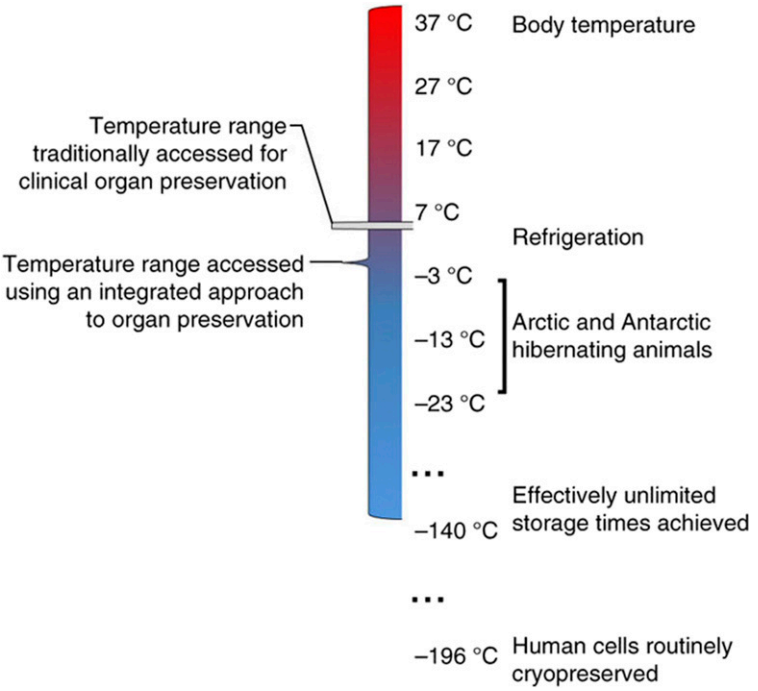
Vitrification—a form of cryopreservation that transforms a substance into a noncrystalline solid that's essentially a type of glass—was first proposed in the 1930s but wouldn't actually be demonstrated for another 50 years. That's when, in 1985, Greg Fahy and Bill Rall at the American Red Cross developed a method to vitrify mouse embryos (2). The process was rapidly adopted to preserve sperm, oocytes, and embryos, but only a few have attempted to apply it to the preservation of larger tissues and organs.

That's largely because vitrification—as well as freezing—wreaks havoc on biological tissues. Living things are filled with water, and the formation of ice crystals during freezing causes massive physical damage: tissues are distorted and may crack apart on the macroscale; cells shrink and collapse or form lethal intracellular ice on the microscale. To avoid ice crystals, vitrification relies on high levels of cryoprotectants, such as glycerol, to supplant water and maintain tissue structure. Yet most cryoprotectants are toxic to human tissues at the levels needed.

And then there's the trouble with warming the organs. In one illustrative attempt in 2002, Fahy vitrified a rabbit kidney at −130 °C for a short time and then rewarmed it using a special conductive warming technique combined with perfusion. He then transplanted the kidney into a recipient rabbit that lived for 48 days with a working kidney before being killed for research purposes (3). Fahy used a novel cryoprotectant solution called M22 to preserve the kidneys, and he has been tweaking the formula and delivery method ever since.

Although the rabbit kidneys avoided ice formation during cooling, says Fahy, it has been more difficult to prevent ice formation during warming. Glassy organs are inherently brittle and can crack when warmed too rapidly or unevenly. Slow cooling and slow warming can prevent cracking, notes Fahy, yet slow warming may allow more ice nuclei to form and grow. If a tissue is not thawed quickly enough, ice crystals can form during the process, disrupting the vasculature or forming intracellular ice.

Complicating matters, the requirements for cryopreservation vary from tissue to tissue. Human sperm, for example, are some of the easiest cells to freeze, whereas pig sperm or endothelial cells are more difficult. It hasn't helped that the fields of cryobiology and reproductive medicine have remained relatively isolated from one another. Technologies developed by early cryobiologists—including Fahy and Amir Arav, a cryobiologist based in Ness Ziona, Israel, and founder of several companies—became widely used to vitrify human eggs and ovaries. Yet there is little overlap, Arav laments, among scientists in each field, even though they're often striving to reach similar goals.



Colder temperatures allow for different preservation conditions and strategies. Reprinted from ref. 7 with permission from Macmillan Publishers Ltd: *Nature Biotechnology*, copyright 2017.

Recent efforts tackle both the challenges of ice and lack of academic teamwork in the field: collaborations among multidisciplinary specialists—including biologists, engineers, and physicists—have sought to use novel engineering solutions to circumvent the damaging mechanisms of ice. "In the last five years, we've seen new formulations, different cryoprotective agents . . . and clever approaches based on rational design at the molecular level," says Luis Alvarez, former cofounder and deputy director of DoD's Tissue Injury and Regenerative Medicine Program and now director of organ manufacturing at United Therapeutics Corporation. "There's not a physics limitation—it's an engineering limitation. And in that case, it's surmountable."

Since 2014, under Alvarez's guidance DoD has invested in five organ-banking grant programs, seeding an estimated $15 million into collaborations among 35 groups. "It has made a difference," says Shannon Tessier, a postdoc in Mehmet Toner's lab at Massachusetts General Hospital, site of one of the DoD-funded projects. "Not only because of the money but because it has made cryobiologists and others in this area have hope. It's created momentum."

Out on a Limb

At Harvard, Toner, Tessier, and colleagues recently began exploring the temperatures between hypothermic storage—about 4 °C, a common temperature used to store tissues and cells for shipping—and vitrification. Their new "partial freezing" method, presented at the recent OPA conference and inspired by wood frogs that freeze solid in frigid temperatures, cools tissues far enough to trap water in the vasculature as ice but not the water inside cells. The technique requires only low concentrations of cryoprotectants, protecting tissues from toxicity. The lab is currently partially freezing rat livers and cartilage to −6 °C with the hopes of getting temperatures down to −10 °C to −20 °C, says Tessier. She expects the approach will enable organs to be preserved for perhaps five to seven days and up to a month if combined with molecular metabolic inhibitors.

> "If you can bring an organ down to −10 °C and buy yourself 48 hours, that would be amazing. And we're not very far from that."
>
> —Luis Alvarez

Looking to nanotechnology for answers, cryobiologist John Bischof at the University of Minnesota infused human fibroblasts, pig arteries, and pig heart tissue with iron oxide nanoparticles, vitrified the tissues, and then activated the nanoparticles via radiofrequency. The activated nanoparticles uniformly and rapidly warmed tissues by about 130 °C per minute in volumes of 50 mL (4). The technology has since been scaled up to 80-mL volumes, says Bischof—large enough to try a rabbit kidney in collaboration with Fahy—with the intention of achieving volumes of 1 L in the future. "Hopefully we'll be able to show some viability and that this can actually work for kidneys, and then other organs," says Bischof.

The iron oxide nanoparticle project focuses on thawing large pieces of tissue. On the microscale, the team developed a method to revive vitrified fish embryos, which are more difficult to freeze than human embryos. The team microinjected a cryoprotectant and gold nanorods into the 800-μm embryos and then used a laser pulse to warm the tissues at an ultrafast 1.4×10^7 °C per minute, outrunning any ice formation (5). The technology is also being applied with collaborators at the Smithsonian to cryopreserve coral species on the brink of extinction.

And in Israel, Arav, along with Nir Shani, head of the laboratory of microsurgery and plastic surgery at Tel-Aviv Sourasky Medical Center, recently used a method called directional freezing to freeze a whole rat limb, thaw and reattach it, and watch the "life come back to the organ," reports Shani (6). The reattached limbs successfully pumped blood for three days before the animals were killed, and the team is now planning long-term survival studies to see if the leg nerves can regrow.

Directional freezing involves passing a tissue in solution slowly though a temperature gradient. As the sample cools, ice crystals grow in the opposite direction of the sample's movement, enabling precise control of ice crystal formation and reducing tissue damage. The technique has also been used to cryopreserve a human ovary, rat and pig livers, and cartilage tissue, but the rat hindlimb was the most ambitious attempt to date. "Most organs are a homogenous type of tissue, but limbs have everything there: skin, muscle, bone and cartilage, fascia, arteries and veins," says Arav. "It was really surprising to see that everything survived. It was very good news."

Partial Victories

Some, though, suggest that long-term organ cryopreservation is a solution in search of a problem. That was the criticism levied by surgical participants at the OPA organ-banking conference at Harvard in August. Right now, there aren't even enough organs available for the people who want them, so there is no surplus of organs waiting to be frozen.

But cryobiologists and OPA organizers contend that technologies created in the field will have immediate impacts on the economics and logistics of today's organ transplants. Reperfusion technologies perfected for cryopreservation to restore the flow of blood to thawed organs can also be used to repair damaged organs for transplant, for example. And techniques such as partial freezing may extend the time transplant organs can be maintained before surgery, even by just a few hours. "If you can bring an organ down to −10 °C and buy yourself 48 hours, that would be amazing," says Alvarez. "And we're not very far from that."

Long-term organ banking remains a long-term goal, researchers agree. If routine bioengineering of synthetic organs comes to pass, a reliable means of organ banking would be quite valuable. At the moment, however, that's not really the point, says Bischof. "It's not about hitting the home run," he says. "It's about getting on base, and coming up with something new that keeps the game going."

1 Starzl TE (1970) A look ahead at transplantation. *J Surg Res* 10:291–297.
2 Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature* 313:573–575.
3 Fahy GM, et al. (2009) Physical and biological aspects of renal vitrification. *Organogenesis* 5:167–175.
4 Manuchehrabadi N, et al. (2017) Improved tissue cryopreservation using inductive heating of magnetic nanoparticles. *Sci Transl Med* 9:eaah4586.
5 Khosla K, Wang Y, Hagedorn M, Qin Z, Bischof J (2017) Gold nanorod induced warming of embryos from the cryogenic state enhances viability. *ACS Nano* 11:7869–7878.
6 Arav A, Friedman O, Natan Y, Gur E, Shani N (2017) Rat hindlimb cryopreservation and transplantation: A step toward "organ banking". *Am J Transplant* 10.1111/ajt.14320.
7 Giwa S, et al. (2017) The promise of organ and tissue preservation to transform medicine. *Nat. Biotechnol* 35:530–542.