Cell presses

The holy grail of printed human organs remains a long way off, but commercial efforts to print simple structures and tissues are forging ahead. Gunjan Sinha reports.

These days it seems there is no end to the remarkable ways in which three-dimensional (3D) printers can revolutionize medicine—from printing tracheal splints that have helped infants breathe to printing prosthetic noses, ears and even a beak for an injured zoo penguin. Galvanized by the potential of 3D printing to radically change the way products are designed and manufactured, startups have sprung up aiming to apply the technology to the printing of cells and tissues—so-called bioprinting. As yet, the technology is capable of creating only rudimentary human tissues not much larger than a postage stamp. What's more, the resulting manufactured tissues often lack the functionality of their counterparts in the body. As a result, commercial bioprinting applications are focusing on drug toxicity testing or disease modeling rather than creating fully functional organs for use in transplants.

Printing options

Several technologies for 3D bioprinting exist, each with advantages depending on the application (**Box 1**). What they have in common is the input and mechanical process. All bioprinters start with a 3D image of the desired object and create 3D tissues by laying cells down layer by layer. Some printing technologies print cells within a support structure such as collagen or hydrogel—usually made out of a polymer, such as polyglycolic acid or alginate, a cross-linking compound and water—that not only provides support for the cells during printing, but can also serve as removable filler to create channels or voids in growing tissues through which oxygen and other nutrients can be later delivered. Printing can create different architectures simply by layering cells in varying concentrations in geographic space or by supplying growth factors and other signaling molecules in a gradient fashion.

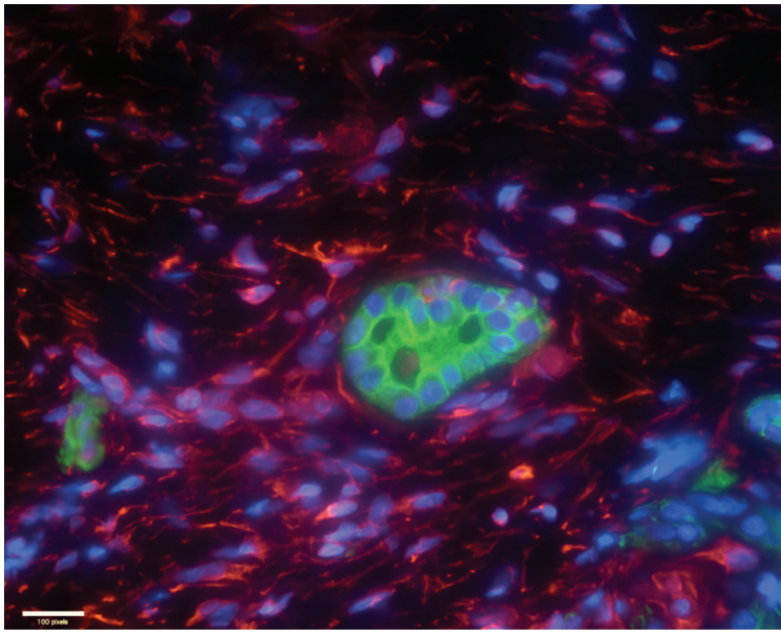
3D printers have already become important bioengineering tools as demonstrated by the myriad inventive ways researchers have been applying them. Early last year, a team of doctors and scientists at the University of Michigan in Ann Arbor used 3D bioprinting to fabricate a customized tracheal splint out of biodegradable polycaprolactone that was wrapped around a two-month-old infant's underdeveloped bronchus, part of the trachea. After a week, the child was gradually weaned off of a ventilator and breathed on his own[1]. In February, researchers at Cornell University demonstrated that it is possible to use a 3D printer to fabricate a custom-fitted human ear prosthetic within a few hours. The researchers used a 3D digitized image of a human ear to print a mold. They injected the mold with collagen derived from rat tails, then added cartilage cells taken from the ears of cows and implanted the mold under the skin of rats. Over three months, the cartilage cells grew to form the shape of a human ear[2].

But although 3D printing technology may suffice to create small pieces of tissue, cartilage and bone, some are skeptical that it will be able to print much more. "I think a lot of the talk is aspirational," says Brian Derby, professor of materials science at the University of Manchester School of Materials. In fact, it isn't even clear that what current technology fabricates can be called tissues, he says. "The definition of what you call these things is very broad." Startup companies aim to achieve something within a time frame that will draw investment, he points out.

The 3D clusters of cells created by bioprinting are a few hundred micrometers across in size—nutrients will readily diffuse across them and consequently the cells are easily cultured into small pieces of tissue. But going from there to larger structures is more difficult, says Derby. Although some tissues, such as cartilage, are easy to print because they don't require extensive vasculature, cells in most organs must be within 150 to 200 micrometers—the width of a few human hairs—of the nearest capillary to survive. "You are moving from something that you are culturing as an isolated blob to something with a structure. At that point you need to put in vascularization or a network of prevascularized channels to get nutrients through. We are still waiting for a breakthrough at that level," Derby says.

Indeed, it isn't any particular technological advance driving the rise in media attention, says Dietmar Hutmacher, professor of biomedical engineering at the Queensland University of Technology in Brisbane, Australia. It is rather a combination of other factors at play. The plummeting cost of 3D printers and the growing availability of open source software have brought 3D printing into the mainstream. The popularity of the printers, especially with young people who have been tinkering with them to print everything from food to toys, and the 'cool' factor of adapting them to print human tissues has had a spillover effect on research. "Ten years ago, it would have been difficult to entice a software engineer to work on bioprinting with me," says Hutmacher. Today, because of the prevalence of the technology, students know that they have an opportunity to work on something that could potentially get published in a high impact journal, he explains.

Researchers began experimenting with 3D bioprinters over a decade ago as a way to scale up production of biomaterials. At the time, most tissue engineers were painstakingly handcrafting scaffolds into desired shapes out



Pancreatic tumor constructed using a 3D printer with dissociated tumor cells (right) resembles the real thing (far right). Source: Reproduced with permission from Brittany Allen-Peterson, Oregon Health & Science University.

of biocompatible and biodegradable polymers. 3D printing offered a way to speed up the process and also make it reproducible. The idea isn't new. The automotive industry, for example, has been using computer-assisted design and 3D printing to make parts for decades, so it was relatively easy to adapt the software and hardware to print scaffolds.

The first medical application of 3D printers was, in fact, to print scaffolds. Hutmacher, for example, helped found Singapore-based Osteopore nine years ago—a spinoff from the National University of Singapore, which specializes in making biodegradable scaffolds. Scaffolds are made out of polymers and composites, such as polycaprolactone-tricalcium phosphate, that can be seeded with mesenchymal stem cells and growth factors to improve healing of human bone and cartilage[3].

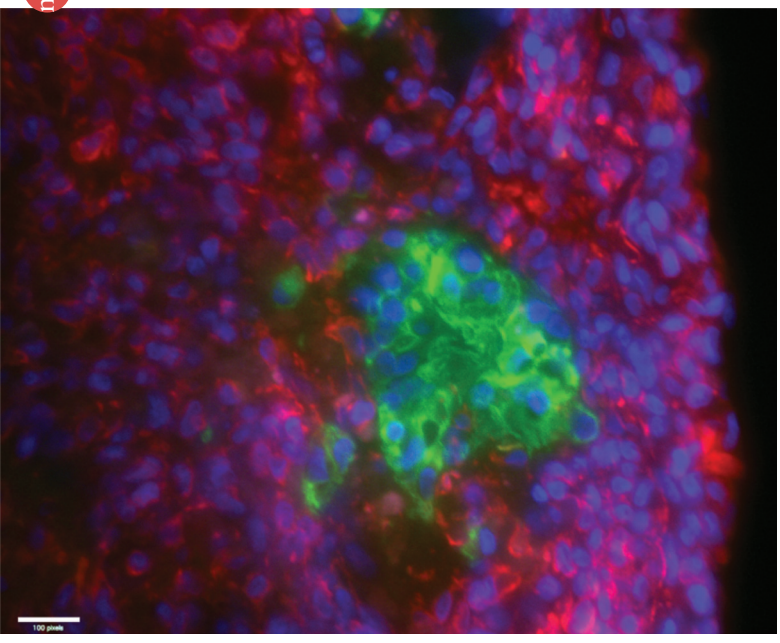
The same technology could be adapted to print scaffolds to reconstruct breast tissue from breast cancer patients' own stem cells, says Hutmacher, who is also a senior fellow at the Institute for Advanced Study, Technical University of Munich. The scaffolds would be custom made from 3D scans of an individual patient's breasts, and the cells would come from liposuctioned fat tissue. Once the adipose tissue is injected into the scaffold, the scaffold would be implanted into patients where the aspirated adipose tissue would remodel itself into mature fat tissue and the scaffold would degrade over time. The technology could be in the clinic within three years, he ventures.

Although Hutmacher's laboratory is also exploring technology to print cells rather than just scaffolds, "such technology is far away from [being] in the clinic," he says. Cells require nutrients, oxygen, and the appropriate temperature and moisture conditions. Whereas engineers have solved many of the mechanical issues, "the big question is biology," says Derby. "There is optimism coming from the engineers who say 'we can hack it', but this reflects a lack of understanding of the biological complexity," Derby adds. "I don't see much of a coherent approach where both the engineers and the cell biologists understand the problems from each other's point of view." Hutmacher agrees: "After printing, we need to make sure that the cells form enough of a natural tissue-like architecture and composition. That's where the biology kicks in and we still need to learn a lot more about how we can direct the biology."

Fabricating startups

The lack of knowledge hasn't deterred startups, however. In fact, it's almost unimportant. "We do leverage developmental biology," says Michael Renard, Organovo's executive vice president of commercial operations. San Diego–based Organovo, an early entry into the commercial bioprinting sector, was formed in 2007 with a proprietary bioprinting platform, NovoGen, pioneered by and licensed from Gabor Forgacs at the University of Missouri[4]. The company entered the public markets in 2012 by means of a reverse merger process, raising $15.2 million in financing.

According to Renard, the company's technical forte is in choosing and acquiring the appropriate cells and preparing them for printing. Once the cell aggregates are assembled into "the right architecture," he says, nature takes its course. "Inherent in our technology is that when we put these cells together in a precise fashion, they organize and start to operate as a system," he adds.



To fabricate liver tissue, for example, the company uses three primary cell types: hepatocytes, fibroblasts and endothelial cells in different stages of differentiation[5]. The result is a piece of liver tissue that can be either matured in a bioreactor or *in vivo*. The company has also printed cardiac muscle, nerve tissue grafts, lung tissue and bone, says Renard. "What we have at the end of our process is a 'native-looking' tissue that acts like real human tissue, biochemically and histologically," he says.

Renard admits that the technology cannot yet produce a functional organ. But long-range, the company aims to understand all the major cell types needed to fabricate an organ and how to nudge them down a particular developmental path. In the meantime, Organovo plans to fabricate tissues that function like native tissues for use in medical research to meet the company's short-term needs. Organovo has announced several partnerships with groups interested in drug testing: Oregon Health and Science University in Portland, which will use Organovo's technology to fabricate cancer tissues for testing the efficacy of drug cocktails, and the US National Center for Advancing Translational Sciences (NCATS), which is bringing the technology in house. According to Sitta Sittampalam at the Strategic Alliances office at NCATS, the center plans to use Organovo's technology to bioprint skin tissues for toxicity screens, the results of which will be compared to traditional toxicity assays. Additionally, a pilot program at the US National Eye Institute, aims to use Organovo's technology in collaboration with NCATS to fabricate retinal tissue using induced pluripotent stem cells and use the tissue in chemical toxicity testing as part of the federal program "Toxicology in the 21st Century."

Employing a similar technology is Tokyo-based Cyfuse Biomedical. The company was co-founded in 2010 by Koichi Nakayama at Saga University with a JPY340 ($4.2)-million grant, administered jointly from the Japan New Energy and Industrial Technology Development Organization (Tokyo) and Kyushu University. The company has since raised an additional JPY572 ($6.3) million in venture capital funding.

Cyfuse's patented technology, however, does not employ a bioprinter. It rather relies on manual culture of cell aggregates in microwell plates and then uses a custom-built robot that places each sphere-like cell aggregate onto plates fitted with needle-like projections that secure each aggregate in place by literally skewering the clusters. In this way, cell aggregates can be organized to form desired geometric shapes. The cell aggregates are allowed to mature for a few days after which the "skewers" are removed and the tissue is allowed to further mature. Although not printed *per se*, "our input data is 3D and our output is a 3D object," says Nakayama. "Our technology does fall under the umbrella term of 3D printing."

Cyfuse has three ongoing projects all in preclinical stages. The first is to use autologous mesenchymal stem cells to create a stem cell

Box 1 Printing options

Several types of 3D bioprinting technology are under development. Among the most widely used to print cells are: inkjet printing, laser forward transfer and filament extrusion. Ink-jet printers generally print cells in suspension or media, laser printers lock cells into a gel, and filament microextrusion deposits cells in a continuous thread of material in a process akin to squeezing toothpaste from a tube. Each technology has advantages depending on the application, says Brian Derby. Ink-jet printing tends to be cell dense and can produce larger cell aggregates than the other technologies, whereas laser approaches print cells almost at the level of individual cells; microextrusion prints cells somewhere in between. There are two schools of thought: one posits that it's better to create a cell-dense structure and let biology take over to mature the cells; the other posits that if one can get the cells in the approximate positions in the first place they are more likely to do what one wants, Derby explains.

Organovo's 3D bioprinting process favors the former approach. The process begins by using computer-assisted design software to create a form that reproduces key architectural and compositional elements of a given tissue. The printer simultaneously prints cells and hydrogel components that temporarily act as a scaffold to support the cells as layers are built up vertically, or that function as filler to create channels or voids to mimic features of natural tissue. The cell aggregates are then cultured in media where they form their own extracellular matrix and mature to become tissue.

Scaffolds are an important feature of tissue engineering in that they replace the extracellular matrix in providing initial mechanical strength and stiffness on which cells can grow. Scaffolds typically consist of a material with interconnected pores that enables cellular attachment, migration, proliferation and differentiation. They also enable the flow of nutrients and metabolic waste. Hydrogels have an advantage over other scaffolding material in that they are similarly hydrated to biological tissues. However, alone they likely cannot provide enough mechanical strength to engineer large pieces of tissue or organs. Several research groups are working on different scaffolding materials and composites of hydrogels and polymers to make up for the mechanical deficiencies of hydrogels.

tissue plug that when implanted *in vivo*, can regenerate joint cartilage and subchondral bone. The second is to fabricate blood vessels, which are easier to engineer because they don't require vascularization, and the third is a hepatocyte project aimed at fabricating liver tissues for toxicity testing or grafting.

Another newcomer, TeVido BioDevices was founded in 2012 with a combination of private funding and US National Science Foundation grants, says Scott Collins, chief technology officer. The Austin, Texas–based company, which was co-founded by Thomas Boland of Clemson University in South Carolina, aims to create custom grafts for breast cancer reconstruction. Using preadipocytes as starting material, they are constructing tissues to improve nipple reconstruction and fill lumpectomies. As yet, the research remains in preclinical stages. The company's original goal was to bring a tissue-engineered product to market, says Collins. But now with that goal years, if not decades away, the goal has morphed into asking what products can be marketed that are simpler from a biological and regulatory perspective.

Cells rather than organs

The shift by companies to short-term tangible goals is no surprise to veterans of the tissue-engineering field like Anthony Atala, director of the Wake Forest Institute for Regenerative Medicine in Winston-Salem, North Carolina. "I recall someone declaring at a tissue engineering meeting about 18 years ago that it would be possible to fabricate an entire human heart in 10 years," he says. It is not just that 3D printing technology is not yet at a stage where it can deliver fully functional and sustainably viable tissues for transplant today, it is also that daunting regulatory hurdles remain for this technology. Atala implanted the first engineered bladders, grown from patients' own bladder tissue, over ten years ago. That technology is still in phase 2 clinical trials and is being taken forward by Tengion, also in Winston-Salem. "The leap into humans is critical," Atala says. Any bioprinted tissue implant would likely fall under regulatory guidelines for biological drugs and would also be required to adhere to stringent manufacturing regulations. "From trial to use, there is at least a ten-year road map of regulatory hurdles," Derby agrees.

As addressing these questions and navigating the regulatory process to reach the clinic is likely to be a lengthy and risky process, 3D bioprinting companies are looking to research applications for near-term commercial opportunities. One such application is the use of printed cells in drug toxicity testing assays.

Traditional two-dimensional, mammalian cell culture systems provide only a rough proxy for measuring the toxic effects of drug and chemicals on the body. For example, primary hepatocytes become undifferentiated and die within a few days if cultured in a monolayer[6]. Moreover, many of the most important drug-metabolizing enzymes, like cytochrome P450s, essential for toxicity testing in drug research, are among the first functions to be lost on cell propagation. Organovo claims that its 3D liver tissue produces liver-specific proteins, such as transferrin, cholesterol and albumin, at levels five to nine times greater per cell than matched 2D controls; the company also states its liver tissue can survive for weeks, data that were presented during a poster session at the American Society of Cell Biology meeting in December 2013.

Capitalizing on printing

A second avenue for commercialization is manufacturing of the printers themselves. Whereas off-the-shelf 3D printers can and are being used in some medical settings where the materials are consistent and reproducible, bioprinting with cells or other biological materials requires some adaptations. A small cottage industry is forming around companies looking to turn a profit from adapting or inventing bioprinters that satisfy the needs of researchers. Formlabs of Somerville, Massachusetts, an MIT spin-out, for example, is one such company. It is raising funds through Kickstarter to support the development of a low-cost 3D printer.

However, much of the technology development is still being done by researchers, as academics continue to tinker with cell printing hardware. In fact, most 3D bioprinters in use have been custom-engineered for specific purposes, as researchers continue to push the limits of what they can do. At the Laser Center in Hannover, Germany, for example, Lothar Koch has been fine-tuning his laser bioprinter to print multicellular 3D tissue constructs. Using fibroblasts and keratinocytes, he showed that laser printing can create skin in a layered configuration consisting of dermis and epidermis that mimics the structure of natural skin[7]. At the Cardiovascular Innovation Institute in Louisville, Kentucky, James B. Hoying is working with a local startup, Advanced Solutions Life Sciences, to develop a six-axis (rather than the typical three-axis) printer, that can move nearly 360 degrees.

At the Engineering and Physical Sciences Research Council Centre for Innovative Manufacturing in Regenerative Medicine at the University of Nottingham, UK, Kevin Shakesheff's laboratory is trying to exploit the phase transitions of different materials to create new inks—inks that remain liquid at certain temperatures, for example, but then solidify or gel at 37 °C to become a support structure for cells. His laboratory is primarily working on bone tissue, as going from being a printable liquid to solidifying into bone requires "an extreme change in material properties," he says.

Still others working at the crossroads of materials science and bioengineering are studying better scaffolding materials. Although hydrogels are often used to fix cells into place, they can hinder cell migration and proliferation and the formation of an extracellular matrix. Most hyrodgels currently used in bioprinting have been developed for other purposes, says Hutmacher, and have not yet been tailored specifically for the purposes of printing cells. Scaffolding materials are a major research focus of Hutmacher's laboratory.

But the path to biological materials poses challenges. The material, bioink, is not firm like plastic or rubber, and with soft materials, the printed product doesn't retain its shape; the force used in printing needs to be adjusted to the viscosity of the material. Academics like Hoying are starting to get a handle on this. Advanced Solutions Life Sciences is developing a database of material of different viscosities, which they will incorporate into their computer-aided design. "I don't need to understand the material properties or be a material scientist. I can just design my structure and let the printing system take care of the rest," he says. Hoying sees ways to approach the difficult task of incorporating enervation, with stem cells (nerve cells don't grow well) or using wires made from conducting biomaterials.

Toward the grail

For die-hard tissue engineers, "the ultimate goal that many of us have is to create the initial conditions for an organ to self-form," says TeVido's Collins. At the moment, this likely lies decades away, although 3D printed materials (without living cells) are already making an impact in medicine. Pre-operative surgical devices for repairing congenital heart defects in infants or colon fistulas are created routinely using off-the-shelf 3D printers.

If not for 3D printing, a 27-year-old from Nottingham, England, would be permanently deformed. Earlier this year, he fell from a fifth-floor balcony, landing face down and shattering his teeth, breaking all of his facial bones and limbs. Surgeons at the maxillofacial department of Queen's Medical Centre in Nottingham used a computed tomography scan of his damaged face to print a 3D gypsum model of his skull. Using a bit of creative license, they cut up the model and put it back together to match the average proportions of how facial bones should fit together. This enabled them to prebend the metal plates that would hold his broken facial bones together into custom proportions. They then rebroke most of his facial bones and reconnected them with the prebent plates using the model as a guide.

3D printing has certainly come a long way in being able to position cells at a resolution that one would need to print organs, adds Kevin Shakesheff. Indeed, Atala famously wowed the audience at a 2011 TED conference by printing a human kidney in just a few hours. It was pink, fleshy and bean shaped, just like the real deal, but tragically wasn't anywhere near functional—it lacked the necessary vasculature.

Apart from how to vascularize a printed organ, another big question is, what happens on a subcellular level? Cells communicate with their neighbors and the extracellular matrix, says Shakesheff. "The history of how a tissue developed is not printed with it. In terms of the final maturation of an organ, I'm not sure we will be able to achieve that with existing technology." But, there may be room for optimism. According to Hoying, controlling all aspects is not as critical as once thought. "We just need to create permissive/instructive conditions and rely on the self-assembly and adaptation intrinsic to nearly all cell systems to do the rest," he says.

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