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Paris, 3 July 2014

Press file

3D Laser printing of living things: what advances can be expected?

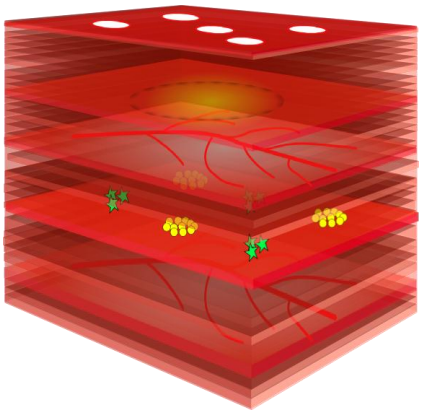
From 3D printing to bioprinting

- 3D printer + Biology: Bioprinting on the rise

In recent years, 3D printing has developed in the area of health. Custom medical devices and prostheses were the first applications for this new technology. In 2011, for example, the first prosthesis (a titanium jaw) made with the help of 3D printing was implanted. In May 2013, a tracheal prosthesis was made for a newborn infant. The same year, a 3D printed cranium (the largest ever placed) was implanted into a 22-year-old woman in the Netherlands.

In addition to these medical devices made of inert materials, researchers took on a challenge of an entirely different nature:

→ **Bioprinting** involves printing **living** cellular materials.



Schema of different layers of biological tissues

© F. Guillemot/Inserm

Bioprinting was developed in response to the challenges of tissue engineering, which is aimed at stimulating the regeneration of missing tissue or creating substitutes for restoring, maintaining or improving tissue function. Until now, treatments based on tissue engineering techniques have not been able to employ manufactured cellular structures in a clinical setting. **In the laboratory, bioprinting employs the principles of 3D printing to assemble the components of biological tissues** (such as the cells and the extracellular matrix) **layer by layer** in digitally designed predefined patterns.

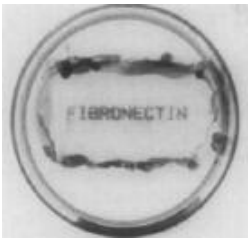
Researchers define bioprinting as:

"The use of digital manufacturing processes enabling the 3D patterning and assembly of biological tissue components in order to produce grafts for regenerative medicine or physiological models for biological research."

F. Guillemot, V. Mironov & M. Nakamura, Biofabrication (2010)

- The concept of bioprinting: a long story

1984: Development of the first 3D printer capable of printing with inert material.



1988: Robert J. Klebe at the University of Texas describes in an article published in *Experimental Cell Research* a technique *"that could help to produce artificial tissues resembling natural tissues and organs."* Using an office inkjet printer, he succeeds in printing the word "Fibronectin" in fibronectin, an extracellular matrix protein. He carries out the 1st 3D assembly of 2 layers of collagen.

1994: Several years later, he succeeds in controlling the micro-positioning of cells using a cell-sorting device coupled to a computer. This technique would enable the preparation of structures resembling tissues composed of several cell types.

2003: Several projects using inkjet bioprinting emerge around the world (Thomas Boland – Clemson University US/Makoto Nakamura – Tokyo University). For the first time, the journal *Science* mentions this new technology.

2005-2007: Development of laser-assisted bioprinting (LAB) in the Inserm "Tissue Bioengineering" laboratory, which prints living cellular structures from 2006.

2007 – Creation of the first bioprinting company, "Organovo," based in San Diego, USA. This company uses the technique of bioextrusion, developed several years earlier at the University of Missouri by Gabor Forgacs (see Fig.2, p5).

2010 – Demonstration of the feasibility of printing *in vivo*, directly in a mouse, by the Inserm "Tissue Bioengineering" laboratory in Bordeaux.

2013 – Bioprinting of human embryonic stem cells by researchers at Heriot Watt University in Edinburgh.

2014 – Creation of a human liver tissue model by Organovo.

Despite advances in research, it is not presently possible to print functioning organs, contrary to what was suggested by the TED (Technology Entertainment Design) conference given in March 2011 by Antony Atala entitled "Printing a human kidney."

- Principle of 3D bioprinting and opportunities for application

The manufacture of a biological tissue by 3D bioprinting can be broken down into several steps (see Figure 1):

- **computer-aided design** of the architecture of the biological tissue, during which the spatial patterning of the tissue components is defined (based, for example, on the architecture of organs and tissues observed by medical imaging or cellular microscopy)
- **programming** of the printing parameters for the inks (containing the cells), enabling the resolution, in particular, to be defined
- **printing** of the biological tissues layer by layer with the help of robots that reproduce the computer-designed patterns by depositing microdrops of biological inks (bioinks),
- **maturation** of the printed tissue, which allows the cells to self-assemble (4D), ultimately giving rise to specific biological functions.

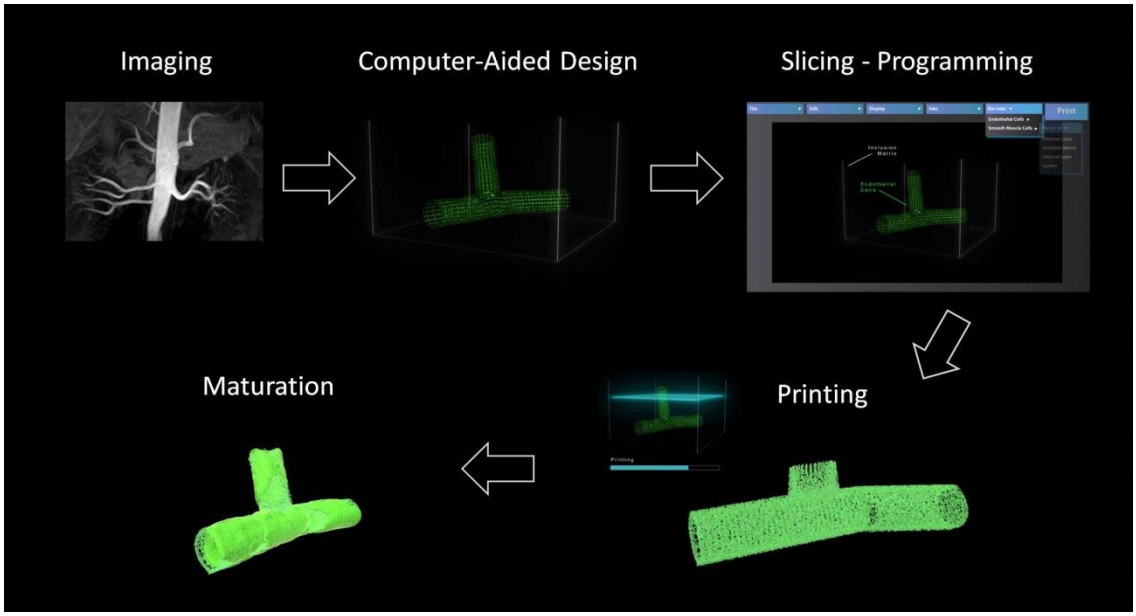


Figure 1. Sequence for the manufacture of biological tissues by bioprinting

When compared with traditional methods of cell culture and tissue engineering, bioprinting introduces **new technical possibilities** leading to new opportunities for manufacturing biological tissues:

New technical possibilities	New application opportunities
Tissues are manufactured by computer-controlled robots	Production of reproducible tissues on demand
Spatial patterning of tissue components is controlled at cellular level	Manufacture of complex 3D tissues, reproducing defined microarchitecture
Each tissue is associated with a digital file	Custom design and manufacture of personalised tissues (clinical, pretreatment screening). Sharing or outsourcing of design.

- Printing techniques developed around the world

Different bioprinting processes are being developed around the world:

- Inkjet printing
- Bioextrusion
- Laser bioprinting

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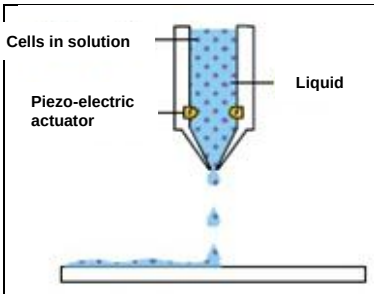
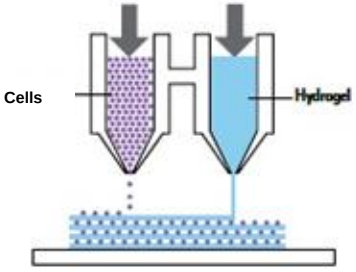
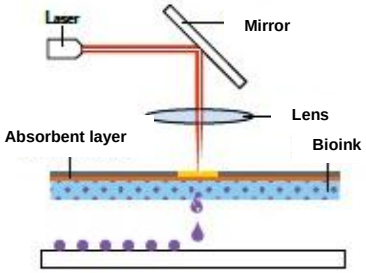
<td> 

Inkjet</td>
<td> 

Bioextrusion</td>
<td> 

Laser bioprinting</td>
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<td>The print head projects microdrops of a liquid containing the cells. Ejection of the drop is induced by a thermal or piezo-electric process.

Advantage: Low cost of instruments (office printers)

Disadvantages: Technique limited to inks that contain a low concentration of cells (to avoid clogging the print heads). Considerable shearing constraints imposed on cells as they pass through the orifice.</td>
<td>The tissue components are mechanically pushed through a microsyringe.

Advantages: Simplicity. First commercialised technique.

Disadvantages: High cost of instrument. Low resolution.</td>
<td>Each laser pulse generates a microdrop of liquid containing cells. The optical scanner allows the formation of complex patterns.

Advantages: High resolution. Orifice-free technique, which ensures excellent cell viability.

Disadvantages: Only one instrument commercialised. Robustness has yet to be demonstrated.</td>
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<td colspan="3">Figure 2. Description of the main bioprinting technologies</td>
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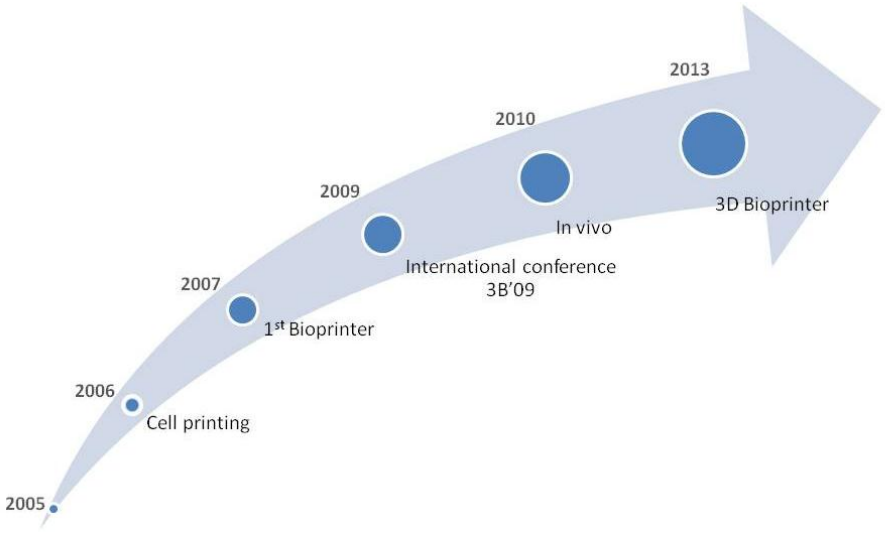
Laser bioprinting in Bordeaux, an innovative approach

- The innovative "Tissue Bioengineering" laboratory (Inserm Unit 1026)

The laboratory was established in 1981 by Charles Baquey. Biologists, physicians (ophthalmology, dental surgery), physicists and chemists work there (approximately 30 people), since 2007 under the direction of Joëlle Amédée, Inserm Research Director.

One of the goals of the laboratory is to develop laser and microfabrication technologies with the aim of printing tissues *in vitro* and *in vivo*. The researchers in the laboratory were pioneers in Europe, developing laser-assisted bioprinting from 2005. This Inserm/University of Bordeaux joint research unit is one of a very few worldwide to use this process.

The main phases of the laboratory:



- The laser bioprinting approach: how does it work?

Work carried out by Fabien Guillemot, Inserm Research Fellow, and his team since 2005 has led to the development of innovative devices and methods for laser-assisted bioprinting (see Figure 3).

Their bioprinting approach involves taking the complexity of the tissue into account in order to:
- control the 3D distribution of the cells by using laser bioprinting
- lead to cellular self-assembly (4D) to produce functional tissues

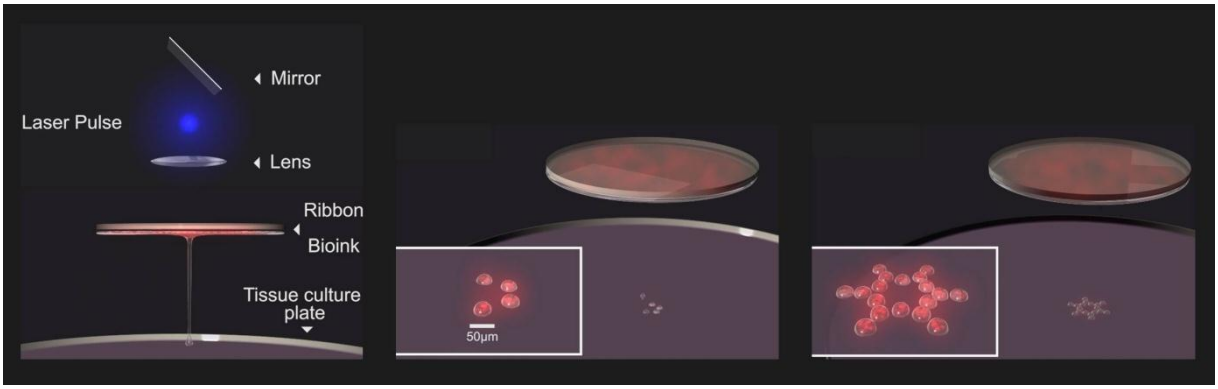


Figure 3. Principle of laser-assisted bioprinting.

The laser pulse (in blue) directed at a ribbon (comprising a film of ink spread over a glass plate) causes the projection of a jet of ink towards a substrate on which the microdrops of cells are collected. By controlling the physical conditions of ejection (energy etc.), the volume of the droplets can be precisely controlled (~ picolitre scale). The cell patterns are obtained by rapid scanning of the ribbon by the laser, which effects the formation of 10,000 droplets per second.

The laser pulse

The team led by Fabien Guillemot showed[1] that the volumes deposited depend on the energy of the laser pulse, viscosity of the bioink, surface tension, and the thickness of film on the ribbon. Controlling its parameters enabled the definition of an optimal "ejection regime."

Energy below the threshold: no ejection

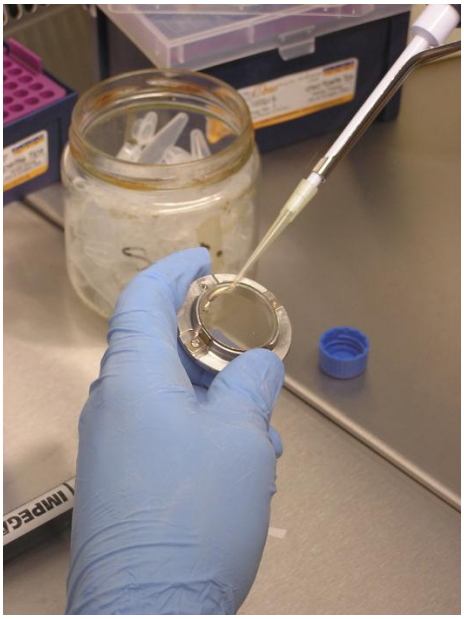
Optimal regime, favouring precise application of the bioink

Energy above the threshold: deterioration of the jet, uncontrolled ejection

Figure 4. Ejection regimes

[1] Guillemot et al. Nanomedicine (2010)

Preparation of the bioink



© Ludovic Lescieux Alphanov/Fabien Guillemot Inserm.

Bioinks consist of a suspension of cells in a specific medium. Before they are suspended, the cells are cultured (multiplication/differentiation) under traditional cell culture conditions. They are then placed in a specially defined medium to ensure that the cells' integrity is maintained during printing.

- Laser-assisted printers: technological developments

1st laser bioprinter

In 2007, Fabien Guillemot's team develops a first bioprinter in collaboration with NovaLase S.A (Canéjean, France). This printer allows 2D computer modelling of the structure to be printed, and is coupled to an imaging system. Printing several layers (up to 5 bioinks may be used) generates a complex 3D tissue structure.

© UMR Inserm 1026 – Tissue Bioengineering

2nd laser bioprinter

In 2013, the team designs a 2nd printer in collaboration with Alphanov (Talence, France).

As an integral part of a completely sterile environment, it facilitates handling of biological materials and allows direct 3D modelling from the computer. It can accommodate up to 7 different cell inks.
Several patent applications are currently being made for this printer with SATT (Society for Accelerated Technology Transfer) Aquitaine Science Transfert.

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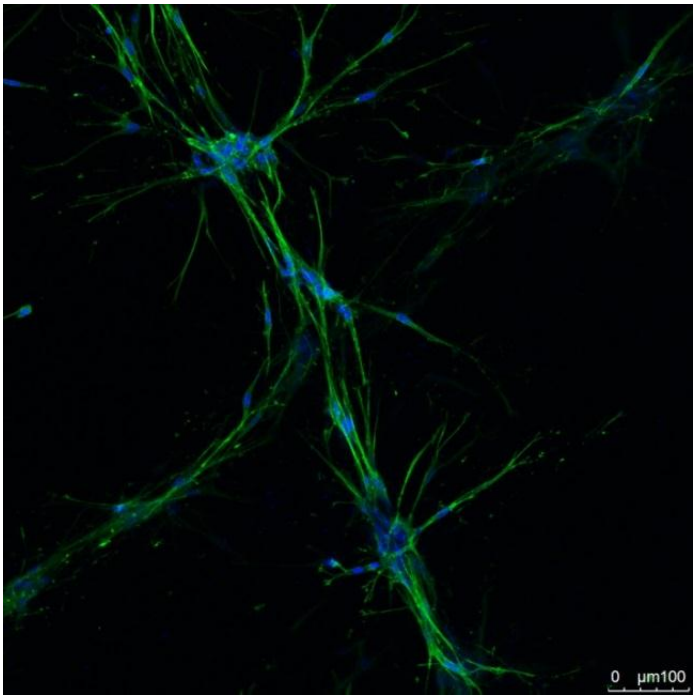
What are the results using the laser approach?

- Structures printed using computer-assisted laser

Laser-assisted bioprinting enables the manufacture of complex tissues with the help of printing using bioinks with cell concentrations close to physiological conditions, with a high degree of resolution (micron scale, pL volume) and a high operating speed (.> 10,000 droplets per second).

"In the area of bioprinting, laser technology offers the highest resolution," explains Fabien Guillemot, Inserm Research Fellow**.**

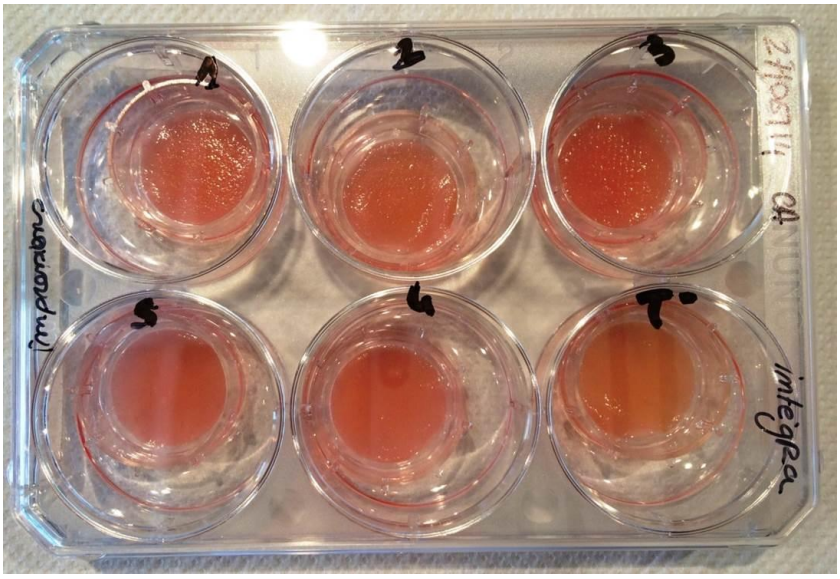
Since 2005, the research team has succeeded in printing different structures and cell types with multiple layers of keratinocytes (cells of the superficial layer of the skin and appendages—nails and body and head hair) and collagen. At the moment, researchers are working on printing corneal and skin tissues in order to meet the needs of regenerative medicine, pharmacology, cosmetics, etc.



Printing human corneal fibroblasts
© Ludovic Lescieux Alphanov/Fabien Guillemot Inserm

Printed cells are viable (97% viability after 6 h), and the researchers confirmed that bioprinting did not affect cell differentiation in the case of human adult stem cells.

Printing of several cell types has been carried out with the help of the printer, which can accommodate several cell inks, enabling the researchers to print skin last June.



Key publications by the Inserm team

Methods Cell Biol. 2014;119:159-74. doi: 10.1016/B978-0-12-416742-1.00009-3.
Cell patterning by Laser-assisted bioprinting.
Devillard R, Pagès E, Correa MM, Kériquel V, Rémy M, Kalisky J, Ali M, Guillotin B, Guillemot F.

Biomaterials. 2010 Oct;31(28):7250-6. doi: 10.1016/j.biomaterials.2010.05.055. Epub 2010 Jul 2.
Laser assisted bioprinting of engineered tissue with high cell density and microscale organization.
Guillotin B[1], Souquet A, Catros S, Duocastella M, Pippenger B, Bellance S, Bareille R, Rémy M, Bordenave L, Amédée J, Guillemot F.

Acta Biomater. 2010 Jul;6(7):2494-500. doi: 10.1016/j.actbio.2009.09.029. Epub 2009 Oct 9.
High-throughput Laser printing of cells and biomaterials for tissue engineering.
Guillemot F[1], Souquet A, Catros S, Guillotin B, Lopez J, Faucon M, Pippenger B, Bareille R, Rémy M, Bellance S, Chabassier P, Fricain JC, Amédée J.

Biofabrication. 2010 Mar;2(1):014101. doi: 10.1088/1758-5082/2/1/014101. Epub 2010 Mar 10.
In vivo bioprinting for computer- and robotic-assisted medical intervention: preliminary study in mice.
Keriquel V[1], Guillemot F, Arnault I, Guillotin B, Miraux S, Amédée J, Fricain JC, Catros S.

Patents

"*Bioprinting station, assembly comprising such Bioprinting station and Bioprinting method*."
F Guillemot, V Keriquel, S Catros, JC Fricain.
European patent EP10305224.7 filed on 4 March 2010

Several patents are currently being filed in relation to the second machine.

- The challenge of 4D and *in vivo* bioprinting

In contrast to traditional 3D printing, bioprinting involves consideration of a 4th dimension: the time dimension, in which the printed cells assemble themselves, migrate and differentiate autonomously to form functional tissues. Bioprinting is therefore aimed at guiding cell self-assembly processes that occur naturally during embryogenesis, development and even during tissue remodelling.

The objective of Fabien Guillemot's team is therefore not only to position cells in 3D, but to define and model the self-assembly dynamic of the printed cells.

At the same time, the research team also conducts *in vivo* experiments in mice. In 2010 it succeeded in printing mesenchymal stem cells in the bone of live mice. The next step will involve testing computer-aided surgery which would allow *in vivo* printing of tissues directly where required.

What is the outlook for tomorrow?

- The challenge of bioprinting

The challenge of bioprinting remains the production of functional tissues with the aim of creating:

Here and now....

> **Predictive models** that reproduce the physiology of healthy human tissues or diseased tissues, enabling the more predictive testing of drugs, components and candidate drugs. These physiological models will be used in the pharmaceutical field. (For cosmetic applications, the overall market for alternative methods has been estimated at €1 billion in 2015 (Source: Transparency Market Research), with an annual growth of 13.1%.)

In the next 3-5 years...

> **Individualised tissues**, made using patient cells, that allow ***in vitro* selection of treatment *based on these tissues***, and development of **personalised treatment solutions**. Fabien Guillemot's team hopes to include bioprinting in the developments of the new Cancer Plan concerning individualised medicine.

In the next 7-10 years...

> **Implantable tissues for regenerative medicine.** The development and manufacture of biological tissues represent major socioeconomic challenges. The market for tissue engineering was valued at $15 billion in 2014, and should double by 2018 (source: MedMarket Diligence, LLC.). Moreover, because of the increase in life expectancy, and in the incidence of major diseases such as cancer and diabetes, the number of people waiting for an organ transplant is constantly increasing (51,000 people in Europe in 2013).

- Ethical questions

3D bioprinting is a technology that raises many expectations.

"This characteristic ***stems from the digital-physical (or biological) duality of bioprinting, which paves the way for on-demand manufacture of complex and potentially customised biological tissues,"*** says Fabien Guillemot, a research fellow at Inserm.

Bioprinting technology will raise ethical questions stemming from its own particular developments.

"Indeed, once researchers are able to create functional tissues, they will then be able to modify these tissues to improve them," points out Fabien Guillemot. *Ethical debate will be needed to determine the extent to which tissue modification is possible, and for what purposes," emphasises the researcher.*

- Development of the start-up company Poietis

Encouraged by the results obtained in the laboratory, Fabien Guillemot's team is presently creating a start-up company, "Poietis."

In June, the latter was awarded the "coup de cœur" (high recommendation) by the Aquitaine Region and Bordeaux Unitec, and has just won an award from the National Competition to support the creation of innovative technology companies, organised by the French Ministry of Research.

Initially, this structure will allow printed tissues to be supplied to laboratories all over the world for research purposes.

In Europe, academic laboratories are working on bioprinting in Edinburgh, Manchester (UK), Hanover (Germany), Utrecht (Netherlands) and Zurich (Switzerland).

The following companies are seeking to commercialise biological tissues manufactured by bioprinting:
- Organovo, San Diego, USA (created in 2007; extrusion bioprinting)
- TeVido Biodevices, Austin, USA (created in; inkjet bioprinting)
- Cyfuse Biomedical, Tokyo, Japan (created in 2010)
- Regenovo Biotechnology Co. Ltd, Hangzhou, China (created in 2013; extrusion bioprinting)

The following companies market bioprinters (mainly to academic players):
- Fraunhofer Institute for Laser Technology (ILT), Aachen, Germany (laser bioprinter)
- EnvisionTEC GmbH, Gladbeck, Germany (extrusion bioprinter)
- RegenHU, Villaz-St-Pierre, Switzerland (extrusion bioprinters)

Photos:

Photographs are available on the Inserm image gallery:
http://www.serimedis.inserm.fr/fr/feature/1016/biotis/page/1/nobc/1

Investigator contact

Fabien Guillemot
Inserm Research Fellow
Unit 1026 Tissue Bio-engineering (BIOTIS) (Inserm/University of Bordeaux)
Tissue Engineering Assisted by Laser (TEAL) Group
+33 (0)5 57 57 14 95 / (0)6 83 54 47 99
Fabien.guillemot@inserm.fr

Press contact

Juliette Hardy
+33 (0)1 44 23 60 98 / (0)6 19 36 46 45
presse@inserm.fr