

In-lab three-dimensional printing

An inexpensive tool for experimentation and visualization for the field of organogenesis

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The development of the microscope in 1590 by Zacharias Janssenby and Hans Lippershey gave the world a new way of visualizing details of morphogenesis and development. More recent improvements in this technology including confocal microscopy, scanning electron microscopy (SEM) and optical projection tomography (OPT) have enhanced the quality of the resultant image. These technologies also allow a representation to be made of a developing tissue's three-dimensional (3-D) form. With all these techniques however, the image is delivered on a flat two-dimensional (2-D) screen. 3-D printing represents an exciting potential to reproduce the image not simply on a flat screen, but in a physical, palpable three-dimensional structure. Here we explore the scope that this holds for exploring and interacting with the structure of a developing organ in an entirely novel way. As well as being useful for visualization, 3-D printers are capable of rapidly and cost-effectively producing custom-made structures for use within the laboratory. We here describe the advantages of producing hardware for a tissue culture system using an inexpensive in-lab printer.

Introduction

The invention of mechanical printing by Johannes Gutenberg in the 1430s proved to be one of the most significant transformative technologies of the world. In promoting the dissemination of knowledge and opinion it arguably laid the foundations for the reformation, the renaissance and the age of enlightenment from which modern science emerged. Depending initially on contact between paper and metal letters placed manually in a frame, printing has been developed to include methods that now require no physical model page. Instead they rely on the application of either mechanical pressure, an ink spray, an electric field, heat or light, delivered by a print head under computer control. These technologies continue to evolve and are critical to many aspects of science, including the production of the paper version of this journal, but they have one thing in common: they result in the development of a two-dimensional (2-D) pattern.

In 1986 Charles Hull added another dimension to the process of “printing,” with his patent “an Apparatus for Production of Three-Dimensional (3-D) Objects by Stereolithography” (US Patent number 4575330). Coining the term “stereolithography,” he described a process of additive manufacturing using a focused beam of light to turn a liquid polymer into solid form. By focusing the UV beam on the surface of the fluid, a single layer of solid polymer is produced. Once this cross-section has set, the laser is focused on the layer above and the process is repeated until the full 3-D shape has been constructed from a series of sequential cross-sections.

Over the past three decades there have been significant advances in refining this process and applying the principle of additive layering in different ways. In selective laser sintering (SLS), the raw material comes in the form of a powder rather than a liquid and particles are fused together using a focused laser. A range of materials can be used as the powder, including plastic, glass, ceramics, stainless steel, cobalt chromium and titanium. If metals are used, the process is referred to as direct metal laser sintering (DMLS). Alternatively, a liquid “glue” can be sprayed on to the area where powder is to be fused, using an adapted version of an ink-jet printer head.

Fused filament fabrication (FFF) is a form of additive manufacture in which a thermoplastic is melted and extruded from a print head, laying down thin strips of plastic to construct a single cross-sectional slice of the object. The print head is then raised and lays down the next layer on top of this. This is currently the most simple and cost effective means of 3-D printing and is used in a number of commercially available printers. The price of these has reduced dramatically over the last 18 months, to the extent that many (for example, those from BitsfromBytes and from Makerbot) cost less than \$3,000.

The resolution and speed achievable using all of these techniques are constantly improving. Jan Torgersen and Peter Gruber in Vienna have recently printed detailed structures as small as 285 micron wide and at a rate of 5 m/sec; unpublished data supporting this can be found at www.phocam.eu. At the other end of the cost-spectrum, the RepRap project, started in 2005 by Adrian Bowyer at the University of Bath, UK, offers an open-source printer design. Full details of how to assemble this printer are provided under the GNU General Public License software scheme (and can be downloaded from

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www.reprap.org/wiki/RepRap). Once built, the printer is capable of printing almost all of its own components, making some progress toward the creation of a self-replicating machine.

The standard tessellation language (.stl) format is widely used in the preparation of files to print. Stl files describe structures as surfaces defined by a series of triangles, each triangle being specified by its normal and the three points of its vertices. Most printers come with software that converts .stl files into a printer-specific format, describing a series of layers that are printed in order to re-produce the solid shape. Stl files may be generated in a number of ways, as discussed below.

3-D printing is being hailed as a transformative technology that may have far-reaching implications in all spheres of life. It effectively removes the long-held dogma of economy of scale of production and has been described as a potential democratizing force equivalent in impact to Gutenberg's printing press.

3-D Printing as an Aid to Visualization and Explanation

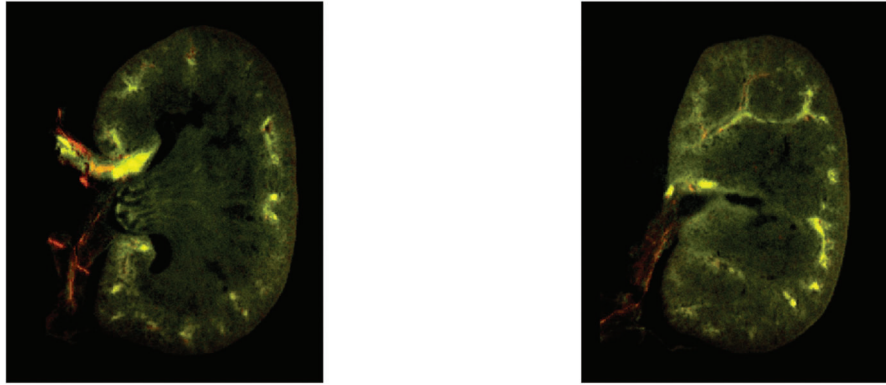
Most mammalian organs are distinctly 3-D structures that consist of several tissue layers. Some are spatially uncomplicated; the smooth, hollow spheres of the capsule of the kidney and the tunica albuginea of the testis show such simplicity, at least until fine-spatial resolutions are considered. Some tissues, on the other hand, have complicated 3-D shapes that are critical to their physiological function. The ramifications of nerves, the networks of blood vessels and the branched trees of glandular epithelia are examples. Errors in making these shapes, for example by faulty axonal path-finding, aberrant angiogenesis and epithelial cystogenesis, result in disease.^{1,2}

Gaining a clear and accurate understanding of these complicated internal anatomies has always been problematic and has been addressed by many techniques, most of which are laborious. The oldest technique, that of careful manual dissection, is suitable for revealing 3-D structures at the gross scale, although preservation of spatial relationships is a problem for any tissue that normally relies on surrounding stroma to prevent collapse. This issue can be addressed by perfusion, or filling of certain structures such as blood vessels, with a resin that hardens, and then dissolving away the remaining tissue to reveal a cast. For microscopic structures, more relevant to most problems in organogenesis research, the oldest technique for visualization is reconstruction from serial sections. This technique was pioneered in the 1870s by Gustav Born, an anatomical assistant at Breslau. To make an accurate model of a developing amphibian skull, Born made serial sections of the embryo and also prepared many wax plates. He then traced the magnified outline of the skull shown on each section on to one wax plate, cut away the wax outside the outline drawn, and stacked the wax plates on one another in order. The result was an accurate, magnified, 3-D model of the embryonic skull. This technique went on to be used by the great model makers of the nineteenth century, including Friedrich Ziegler, a 19th century German model maker who ran a small factory devoted to the task and who supplied most of the great universities of the world.³ Although wax, and later plastic, models are now used mainly for teaching, when they were first made they

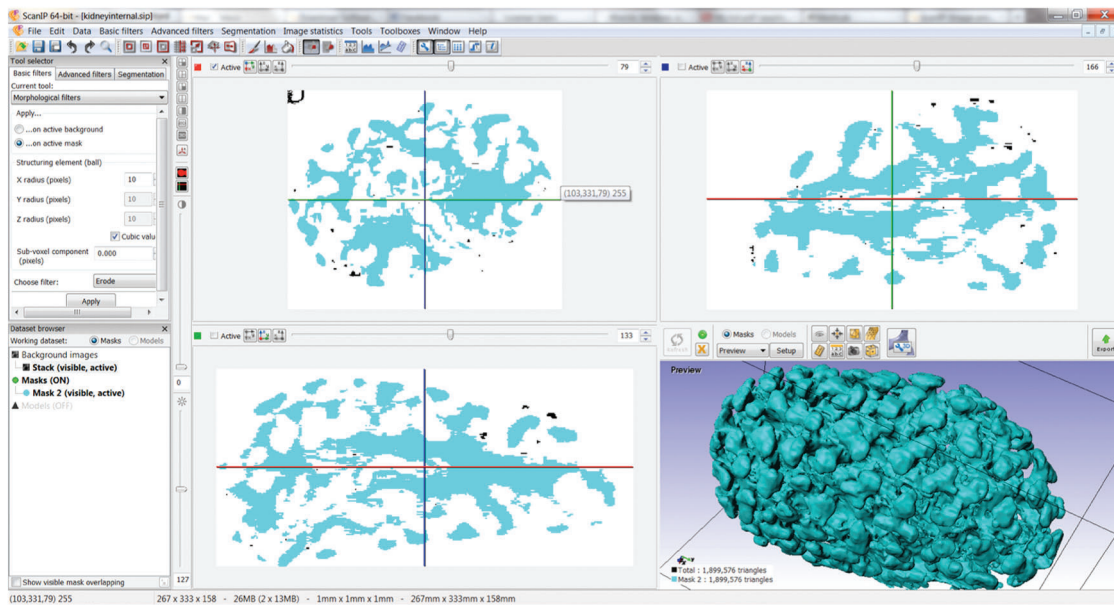
were very much for the purposes of research. In particular, they were used to provide hard data for the debates surrounding Haeckel's biogenetic law. Although old-fashioned and labor intensive, reconstruction of large 3-D structures from serial sections is still used for major projects, although the output tends to be stored digitally in a computer rather than rendered in wax. Examples include the Visible Human⁴ and the Edinburgh Mouse Atlas Project (EMAP).^{5,6} Where the application does not need very high spatial resolution compared with the size of the object, modern 3-D imaging technologies, such as magnetic resonance imaging (MRI), optical projection tomography (OPT) and confocal microscopy, can be used to generate the data sets with much less time and effort.⁷⁻¹¹ The data are still held digitally, and this creates the problem of visualization because the most common digital output device is a computer monitor screen. Many applications exist that allow a projection of a 3-D data set on to a computer screen, the user being able to rotate the object in any plane and so gain an impression of the original 3-D structure. This may be assisted by projection of two adjacent or superimposed images, adjusted for the perspectives of two separate eyes and viewed through 3-D glasses of the type used in cinemas. This system works to some extent but there is a strong base of research literature that demonstrates that correctly appreciating the form of a 3-D shape presented only via a 2-D display is difficult and depends on the viewer having strong visuospatial skills, perhaps enhanced by specific training in mental rotation.¹²⁻¹⁸ Perception is much easier when a physical 3-D model is available to be manipulated directly, a fact reflected by the much better performance of anatomy students trained using 3-D models rather than 2-D representations of 3-D structures.¹⁹

3-D printing of a data set, on the other hand, combines the modern advantages of digital capture and storage with the advantages of perception provided by a truly 3-D, magnified model that can be held in the hands and manipulated in the way that humans evolved to comprehend the world. We have applied this technology to the problem of visualizing the 3-D structure of the developing collecting duct system of the kidney. We started with an OPT data set from kidney rudiments stained with the Troma-I antibody, which recognizes a cytokeratin antigen expressed by cells of the ureteric tree (this data set was originally obtained for our published study of the regulation of kidney development by the Cerberus protein²⁰). We cleaned the data of extraneous noise (see "limitations" below) and converted the image to Standard Tessellation Language (.stl) format by the process illustrated in **Figure 1**. We then printed it in acrylonitrile butadiene styrene (ABS) plastic, using a BitsfromBytes 3-D Touch Triple Head printer. Because the process of printing proceeds layer-by-layer from the bottom up, and some branches of the collecting duct project from the center of the kidney downwards but do not reach the very lowest point of the model, it would be impossible to print these branches without the use of a support matrix (because it is impossible to print an object floating free in space and have it stay there). The Axon2 software that ships with the 3-D Touch printer recognizes this during the conversion from .stl to print-file. The program then calculates the shape and extent of support structure required to facilitate

Image acquisition: Optical Projection Tomography



Segmentation and conversion of 2D images to 3D model mesh: Simpleware



Mesh cleaning and repair: meshlab

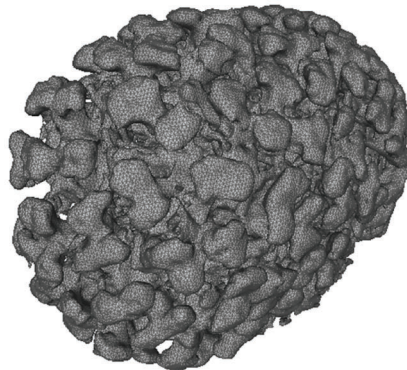


Figure 1. Embryonic mouse kidney imaged using OPT. The image stack was then imported to a commercially available image segmentation package (ScanIP). Threshold and segmentation techniques were then applied to reconstruct a surface mesh of the ureteric tree. Smoothing and island removal tools were applied to the mesh to reduce artifacts at the transition between image slices, and to remove unconnected regions in the model that may pose problems when printing. The final mesh of the embryonic kidney was then saved and exported in the .stl format. Further repairs and clean up/simplification of the kidney surface as required were performed using freely available open source packages such as MeshLab and netfabb. The final .stl file containing the embryonic kidney geometry was then imported into the companion software package of the 3-D printer (Axon2, BitsfromBytes).



Figure 2. Branching ureteric tree from an E15.5 mouse kidney printed in red ABS plastic, demonstrating the extensive white PLA support scaffold required.

printing. As printing proceeds, a second print head extrudes the support material, which can be a different type of plastic to that of the model. In our system this is the starch-based thermoplastic Polylactic acid (PLA). In the case of a branching ureteric tree, the support scaffold required is extensive (Fig. 2). When printing is complete, the PLA matrix was dissolved by incubation in water at 80°C.

The result of this printing is illustrated in Figure 3. In this large 3-D model, the features of ureteric bud development, such as the frequent occurrence of three-way branch events, are immediately obvious. Whether three-way branching events are common or rare has been the subject of contention by people examining sections of fixed material.²¹⁻²⁴ It was settled, on the side of the events being quite common, only comparatively recently by time-lapse recordings of developing GFP-expressing



Figure 3. Red ABS printed branching ureteric tree from an E15.5 mouse kidney with support structure removed. The branching pattern is clearly seen and can be explored in three dimensions.

ureteric buds.²⁵ Had a facility for 3-D printing been available many years ago, the controversy would probably never have arisen. The ability to make 3-D models from OPT and similar data sets, of both wild-type and mutant embryos, may therefore lead to much more rapid insight than emerges from interacting with data sets on a 2-D computer screen. As well as providing a means of making models from real data sets, 3-D printing could also be used to visualize the output of computer models of development or of congenital disease.

3-D Printing as a Means of Producing Custom Hardware for Organ Culture

There is great scope for 3-D printers to produce laboratory hardware rapidly and cost-effectively. A number of open source and simple to use programs are available to generate .stl files, many of which require little or no previous computer aided design (CAD) experience; examples can be downloaded from www.tinkercad.com, www.sketchup.google.com, www.blender.org and www.123dapp.com. Most of these programs allow structures of up to at least 20 × 20 cm to be printed. The print material costs approximately \$60 USD per kg (the culture grids described below weigh approximately 2 g each).

We have deployed this technique in our lab to produce a custom-made structure to support filters on which embryonic mouse intestine is cultured. Custom grids were needed because the size of the gut cultures was greater than the mesh size of metal Trowell screens common in organ culture, and this meant that the metal grid obstructed photography. A range of plastic grids were designed and printed on the 3-D printer. As is often the case with design and manufacture, the first few designs were found to be promising but imperfect and new, desirable features became apparent only when each structure was tested on real cultures. The great advantage of 3-D printing for such applications is that the design can be revised immediately, and the updated product produced within minutes. If this were to have involved re-machining a plastic or metal object in a separate department, that process would have taken much longer and been significantly more expensive.

We have successfully grown embryonic mouse intestine of different gestational ages on these grids. The growth rate of the tissue has been found to be equivalent to that on the previously used metal support grids. The ABS plastic grids withstand sterilization at 120°C and have been shown to be suitable for re-use. Figures 4 and 5 demonstrate the process of generating the print file and the end product in use.

Limitations

One clear limitation of the current generation of inexpensive 3-D printers is their spatial resolution. The maximum resolution of the 3-D Touch printer used for the examples described here is a layer thickness of 125 μm; in the context of making magnified anatomical models this is very good, but it is a limitation for producing culture hardware, where resolution in the micron range would be very useful. As the spatial resolution of 2-D printers for

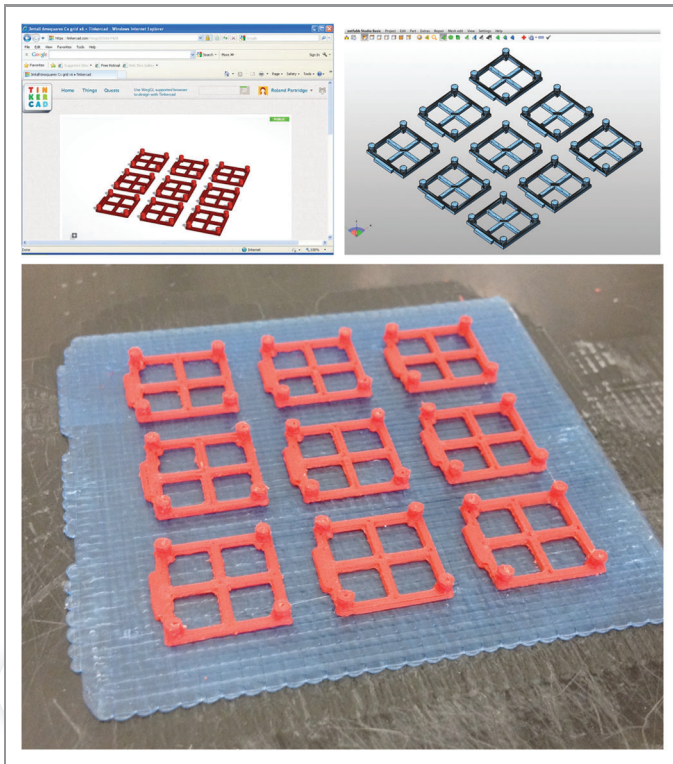


Figure 4. Tinkercad (top left) and netfabb (top right) screen shots showing .stl file generation and “clean-up” process respectively. Bottom photo: the ABS culture support grids printed from this file.

home computers has improved rapidly over the last four decades, so the resolution of their 3-D counterparts can be expected to increase. Torgersen and Gruber have recently demonstrated that resolution at the low micron scale is possible using experimental equipment; a demonstration can be found at www.phocam.eu.

Printing of models from real data sets introduces limitations connected with the quality of the original data. The first is that of the signal-to-noise ratio. The boundaries of an anatomical object to be modeled need to be defined clearly. In an ideal situation, the difference between a confocal, OPT or MRI signal from within the structure, and the signal from without, will be so great that taking all values greater than some threshold as meaning “part of the structure” will define that structure perfectly. In reality, problems of light penetration and antibody diffusion mean that this is seldom the case. Another type of noise, occasional bright speckles, or signals from a few detached cells, can create problems because of the software conventions that are followed by current inexpensive 3-D printers. Because the development of these machines has been done mainly in the context of computer-aided design and manufacturing, and because of the mechanical advantage obtained from printing a continuous stream of plastic rather than isolated dots, the file formats used place an emphasis on definition of surfaces. Automatic conversion of volume-based (voxel) data to surface-based (triangle vertex) data can be affected very badly by outlying bright points. Typically, conversion software will try to include an outlying point by connecting it by a thin line to the nearest point on the main surface, and what looks like a good data set to the eye will be turned into a model like a pin-cushion. Where conversion to surface-based data are required, it is essential that these outlying points be eliminated either by a spatial filter program (if they are small compared with the desired resolution of printing) or manually.

At present, inexpensive 3-D printers print only in a range of thermoplastics. At the other end of the cost-spectrum, experimental printers have been built that can print cells suspended in biological matrix compounds to engineer a printed “tissue.”^{26,27} It is to be hoped that increased uptake of current-generation technology in laboratories interested in development will persuade

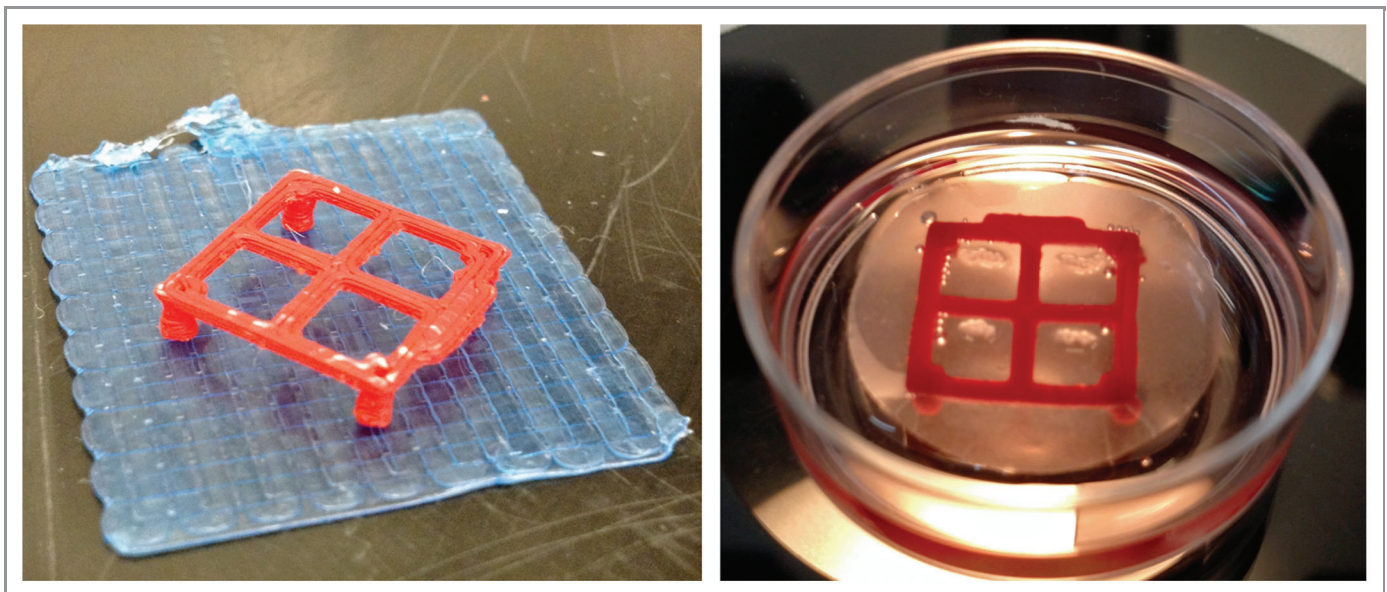


Figure 5. Close-up of tissue culture support grid (left). Grid in use supporting four E11.5 mouse small bowels in culture (right).

manufacturers to produce a machine that can offer biological printing at reasonable cost.

Conclusion

The transition of new techniques from an expensive experimental phase to being available to any laboratory has frequently driven step-changes in the progress of science. By allowing the production of custom hardware for culture and accurate physical models for visualization, 3-D printing has the potential to

facilitate organogenesis research in two distinct, but complementary ways. Its use is likely to become very common, very quickly.

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