Advances in three-dimensional rapid prototyping of microfluidic devices for biological applications

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Abstract

The capability of 3D printing technologies for direct production of complex 3D structures in a single step has recently attracted an ever increasing interest within the field of microfluidics. Recently, ultrafast lasers have also allowed developing new methods for production of internal microfluidic channels within the bulk of glass and polymer materials by direct internal 3D laser writing. This review critically summarizes the latest advances in the production of microfluidic 3D structures by using 3D printing technologies and direct internal 3D laser writing fabrication methods. Current applications of these rapid prototyped microfluidic platforms in biology will be also discussed. These include imaging of cells and living organisms, electrochemical detection of viruses and neurotransmitters, and studies in drug transport and induced-release of adenosine triphosphate from erythrocytes.

I. Introduction

During the past three decades, microfluidic systems have evolved from relatively simple single-function devices to multiple-function analytical systems[1] used for a wide range of biological applications in clinical and forensic analysis,[2] proteomics and metabolomics,[1] immunoassays,[3] cell analysis,[1] point-of-care (POC) diagnostics,[1] drug discovery,[4] genetic analysis,[5] and organs-on-chip.[6–9] These micro scale systems have a number of advantages over traditional macro scale methods used in biological and biomedical research, including the capability of (i) streamlining complex assay protocols, (ii) providing investigators with accurate manipulation of the cell microenvironment, and (iii) reducing the sample and reagent volume maximising the information obtained from precious samples and reducing costs. However, most of the publications in the field of microfluidics are still appearing in engineering journals (85% in 2010) compared to biology and medical journals.[10] This

indicates that there is still a lot of scope for further bio-focused research in microfluidics as well as for the emergence of new bio application domains in the coming years.

In fact, though microfluidics is widely considered to be a key component in both development of laboratory-based, high-throughput analytical methods and POC diagnostics, a "killer application" for microfluidics is still anticipated.[11–13] Such an application is one that will generate large revenue in a relatively short period of time and will strongly promote the microfluidic industry as a whole. In a recent series of articles by Becker it is suggested that the diagnostic market is one such area where microfluidics will find its niche.[12,13] However, before this is realised a significant amount of work still needs to be carried out in the area of design, manufacture, and integration of microfluidic components (e.g. valves, micropumps, and separation columns) within a single microfluidic platform.[14,15] Thus, recent advances in rapid prototyping (RP) techniques, such as the availability of 3D RP equipment with much higher resolution, have the ability to propel the field of microfluidics forward towards finding such a "killer application" by easing the fabrication of complex designs as well as speeding up the fabrication process allowing for mass production.

3D printing[16] and direct internal 3D laser writing[17] methods allow for direct fabrication of 3D microstructures in one single step. In comparison, standard replication methods for fabrication of microfluidic devices such as injection moulding, hot embossing and casting (i.e. soft lithography[18]) can be termed multistep manufacturing methods as they require the creation of a replication master before casting or moulding of the final device. These replication methods, while particularly useful for industrial scale manufacture in the case of injection moulding, are often expensive and time-consuming on a smaller scale owing to the

need for fabrication of the replication master. Alternatively, direct microfabrication methods such as computer-numerical-control (CNC) milling and 2D laser surface ablation allow for direct fabrication of microchannels on a variety of substrates, including glass and polymers. However, all these standard microfabrication methods typically lead to the production of open channels on the surface of a substrate, which then need to be sealed by an additional layer creating an enclosed microfluidic channel. Thus, standard microfabrication methods employed in microfluidics will generally involve a multistep procedure as compared to 3D printing and direct internal 3D laser writing methods. In this paper, these direct 3D fabrication methods are presented and their capabilities for fabrication of microfluidic devices for biological applications are reviewed.

II. 3D printing fabrication methods

The term 3D printing covers a wide range of techniques, some of which are already well established and widely used in industry. That is the case of stereolithography (SL), also sometimes referred to as micro-stereolithography (μSL), which is one of the most important rapid prototyping processes in industry today.[19] SL allows for the automated production of complex 3D shapes in polymeric materials at low to medium volume throughputs.[20] SL presents an inherent advantage over other lithographic methods (i.e. photolithography and soft lithography) in that no alignment or bonding is necessary to produce 3D structures. SL, developed by Hull in 1986,[21] involves curing a photocurable liquid polymer layer by layer using a UV light to build up a solid 3D object.

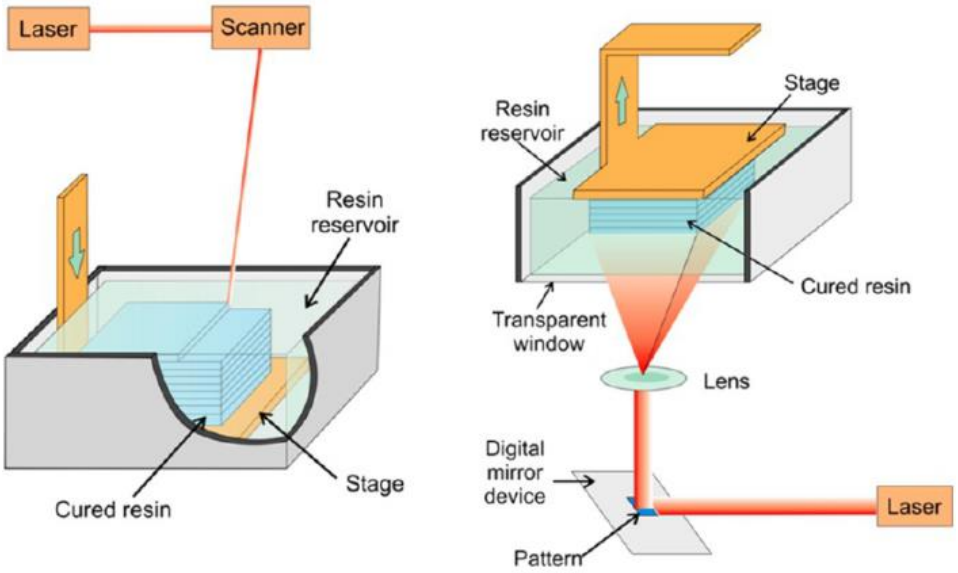
A list of popular commercially available high resolution SL systems and their corresponding attributes is given in Table I. These systems range from the Form 1+ at the lower end of the price scale to DigitalWax and EnvisionTEC systems at the higher end. This large discrepancy in price could be attributed to three main factors: resolution, build area, and build speed.

Table I. Commercially available SL systems, quoted specifications, and approximate prices.

Company	Model	Resolution (μm)		Available Resins		Price
		XY	Z	Transparent	Biocompatible	$
Formlabs	Form 1+	300	25	Y	N	1,088
MiiCraft	MiiCraft	56	50	Y	N	2,299
Smart3D	MiiCraft HR	Not stated	25	Y	N	6,674
Asiga	Freeform PRO	50 - 75	25	Y	Y (coming soon)	24,990
	Freeform Pico	27 - 39	25	Y	Y (coming soon)	6,990 – 8,990
3D Systems	Projet 1200	56	30	N	N	4,900
Solidator	Solidator	270	30	N	N	4,950
FSL3D	Pegasus Touch	80	25	Y	N	2,000
Old World Laboratories	OWL Nano	Not stated	0.1	Y	N	2,000-5,000
LightForge	LightForge	150	25	Y	N	2,000-5,000
B9 Creator	v1.2	50	6.35	N	N	2,400
MakeX	M-One	140	20	N	N	2,000
mUVe	mUVe 1 3D	100	Not Stated	N	N	599 – 1,699
Kudo 3D	Titan 1 HD	37	1	N	N	2,000
DigitalWax Systems	Dental range	Not stated	10	Y	Y	20,000-125,000
EnvisionTEC	Perfactory 3	16	15	Y	Y	100,000

Curing time and resolution are defined by these parameters as well as the chosen material and curing method used. A variety of curing methods have been developed for use within commercially available SL instruments, these include laser raster scanning, laser vector scanning, and digital light processing (DLP) methods. In the first SL systems commercially introduced by 3D Systems in 1988, a low-power, highly focused UV laser was raster scanned according to the area to be cured. The introduction of galvanometer-based vector scanning regimes for the laser allowed for faster curing of each 2D layer and reduced production times. Projection systems have recently been introduced which allow for a complete 2D layer to be cured in one step. These systems employ a data projector type method of photon exposure often termed Digital Mirror Display (DMD).[19] Choice of curing method can be seen to

depend on the specific application with vector scanning typically providing a larger build volume (e.g. Form 1+) compared to DLP methods which provide increased productivity (e.g. EnvisionTEC Perfactory 3). Most commercial systems sold today offer the ability to adjust the resolution to speed up productivity (lower resolution resulting in faster build speeds), while in some cases the lens can be changed to improve resolution further, sacrificing build area as a result (e.g. EnvisionTEC's Perfactory 3 Mini Multi Lens). Figure 1 shows a schematic representation of these two different curing approaches, i.e. direct laser writing (Figure 1(a)) and DMD-based writing (Figure 1(b)).[22] In addition, two different configurations are also possible depending on the orientation of the light source: the bath configuration (Figure 1(a)) and the layer configuration (Figure 1(b)). In the bath configuration the part is built from the bottom up, with the z-stage moving down into the resin vat after each layer has been cured to start building the next layer. The layer configuration uses a light source situated beneath the z-stage which cures the part through an optically transparent window beneath the resin vat. After each layer has been cured, the z-stage moves upwards producing a part that is "upside down" when finished. Of these two configurations, the layer configuration is the most popular as the part height is not restricted by the size of the vat, there is less resin waste, and the layer thickness can be more readily controlled by the z-stage positioning as opposed to controlling the laser depth of focus.[22]



(a) (b)

FIG. 1. (a) Schematic of a SL bath configuration with direct laser writing and (b) a SL layer configuration with DMD-based writing. Reprinted with permission from Gross et al., Anal. Chem. 86(7), 3240 (2014). Copyright 2014 American Chemical Society

Folch et al. recently investigated the capabilities of SL methods for fabrication of microfluidic systems [20]. They found that the main limiting factors were the effective drainage of the uncured liquid resin, optical clarity, and z-height resolution. Current research and development into photocurable and SL systems aims to overcome these limitations focusing toward the implementation of new advanced resins and improvement in the xy- and z- system resolutions. Table I presents an overview of a broad range of currently available SL systems, and their capabilities and prices as provided by the manufacturers. New resins exhibiting improved optical transparency and biocompatibility are also continuously being introduced to the market, which no doubt will favour further applications of 3D printing in microfluidic-based biological assays/platforms with optical detection/imaging. For example, biostable resins based on polyester/polyether oligomers with acrylate or methacrylate functions, as well

as biodegradable composites of methacrylate-functionalised polyesters have been developed in recent years by DSM.[23] These biocompatible resins have been used in SL equipment to produce implants meeting Intracutaneous Test standard ISO 10993-10. Another commonly used material is the Eshell supplied by EnvisionTEC which is classified as Class-IIa biocompatible. Improved biocompatibility of SL produced components could be also achieved via surface treatments (e.g. plasma or laser irradiation) to improve wettability or surface functionalisation with –OH or NH_2 groups to allow biomolecule attachment.[24] However, for the most part, current SL resins are limited to non-biocompatible, translucent and opaque materials (commonly used in jewellery and structural modelling), with a limited range of suppliers offering a biocompatible and transparent resin option (see Table 1). In the case of the Asiga SL 3D printer, it is suggested by the supplier that the printed resin requires the application of a lacquer in a post-processing step to make it transparent. Such methods for achieving transparency may be difficult to apply within long narrow internal microstructures. Other resin properties to consider when fabricating microfluidic platforms by SL are permeability to gases, degree of hydrophobicity, and chemical stability in the presence of solvents.

Other 3D printing methods of note are two-photon polymerisation (TPP), fused deposition modelling (FDM), and 3D inkjet printing.[19,22] TPP is based on the polymerisation of a photocurable resin via two-photon absorption upon illumination with a femtosecond pulsed near-infrared laser. Compared to conventional SL, the TPP process is not hindered by the diffraction limit of the light source leading to much better structural resolution.[25] Thus, resolutions in the order of 100 nm are feasible for TPP instruments (e.g. Nanoscribe GmbH systems). Honegger et al. studied different photoresists, including SU- 8, AZ1512-HS, polyethylene glycol (PEG), Photomer 3015, and 4-hydroxybutyl acrylate (4-HBA), for production of 3D structures within microfluidic channels by TPP.[26] Results showed that PEG

and 4-HBA were suitable materials for production of arm structures within channels with submicrometer resolution.

FDM is based on the extrusion of melted bulk material through a heated nozzle.[27] As with the other 3D printing technologies, each 2D layer is traced out with subsequent layers being added to build up the required 3D design. Common materials used in the FDM process include acrylonitrile butadiene styrene (ABS), polylactic acid (PLA), and nylon. FDM printers can write in many colours/materials without the need for changing the filament between colours/materials by usage of multiple extrusion nozzles. Due to the nature of the printing process, the resolution achievable is limited by the xy-plotter (two stepper motors), the z-stepper motor, the thickness of the filament, and the extrusion nozzle diameter. FDM printers are widely available from companies such as RepRap, Ultimaker, MakerBot, and 3D Systems. Finally, 3D inkjet printing involves applying droplets of bonding resin according to a prescribed 2D design onto powder to bond each 2D layer. The 2D layer of bound and unbound powder provides support for the subsequent layer. In an alternate 3D inkjet printing process, a low viscosity photocurable resin is printed alongside a support material such as wax onto the build platform. The wax support material acts as a mould, constraining the liquid resin until it is hardened during the curing stage. As with other 3D printing methods, this process is repeated, layer by layer, until the part is finished. A post-processing step is then required to remove the unbound powder or wax support material.

III. Direct internal 3D laser writing fabrication methods

Direct internal 3D laser writing methods have been recently employed for fabrication of channels and other micro features (e.g. optical components) in microfluidic devices. These methods consist in the internal processing of in-bulk materials by laser ablation using ultrashort-pulsed lasers with low pulse energy.[17] These ultrafast lasers, having pulse widths in

the pico- to femtosecond range, can produce high quality microstructures within glass and polymer materials owing to a significant reduction in the heat-affected zone (HAZ) surrounding the ablation focal position with decreased pulse width. Femtosecond lasers (e.g. 800 nm Ti:sapphire), in particular, can modify materials at wavelengths for which they are normally transparent. This occurs by depositing energy through high-order non-linear absorption processes inducing optical breakdown, which makes these lasers very useful tools for micromachining.[28] Femtosecond lasers also offer the possibility to produce sub-wavelength features as these non-linear absorption processes are not limited by optical diffraction.[29] A major disadvantage of these ultrafast laser systems to date has been their cost, with femtosecond lasers typically being three to six times more expensive than standard nanosecond CO_2, excimer or Nd:YAG systems of similar power.

In 1996, Davis *et al.* showed that it was possible to write 3D structures for fabrication of optical waveguides in different bulk glasses, including silica and soda-lime, via multiphoton interactions with femtosecond laser radiation.[30] A procedure for fabrication of 3D interconnected channels as narrow as 10 μm inside silica was then presented in 2001.[31] It consisted in optical damaging of bulk silica by a 795 nm femtosecond laser, followed by selective etching of the written structures with hydrofluoric acid (HF) solutions. Photosensitive glasses such as Foturan haven been also employed for production of 3D U-shaped microchannels following infrared femtosecond exposure.[32] Femtosecond irradiation induces a local phase change in this phosensitive glass, from amorphous to crystalline. This process was then completed by heating and subsequent etching of the crystalline areas with 10% HF solution. Recently, internal processing of polymethyl methacrylate (PMMA), polydimethylsiloxane (PDMS), polystyrene (PS), and polyvinyl alcohol (PVA) polymers has also been investigated.[33] A 800 nm Ti:sapphire laser with a maximum pulse energy of 1 mJ was used in this work.

IV. Applications in biology

Microfluidic systems are very valuable tools for fundamental studies of complex biological systems since they provide precise control of small volumes of fluids over very short distances. Flow cytometry analysis,[34] cell-based assays (such as cytotoxicity[35] or induced cellular stress assays[36]), sorting, manipulation and imaging of single-cells,[37] and cell/tissue engineering,[38,39] are just some of the current applications of microfluidics in biology. Microfluidics also offer the means to create and maintain environments that closely resemble those encountered *in vivo*.[40,41] This is essential in ensuring that the experimental results are not biased with artefacts caused by, for example, early triggered apoptosis and, therefore, creating the right environment has important implications in cell analysis. Moreover, current microfabrication techniques allow the production of large arrays of microwells that can entrap single/multiple cells to perform molecular analysis,[42] or study cell response to chemical and physical stimuli following exposure to different environments;[43] the major advantage of this approach being the ability to perform parallel screening of a large number of cells.

In addition, one of the main advantages of 3D printing over more conventional techniques typically employed for production of biomicrofluidic devices is the simplification of the process which does not require the fabrication of a replication master nor extensive labour.[19,20,22,44] A 3D microvascular network enabling chaotic mixing was one of the earliest microfluidic devices produced by 3D printing more than 10 yr ago.[45] The microfluidic mixer integrating cylindrical channels with diameters between 10 μm and 300 μm was fabricated by direct-write assembly of a fugitive organic ink. Sixteen-layer scaffolds were first produced by robotic deposition of a paraffin-based organic ink, followed by infiltration with an epoxy resin, and subsequent curing of the resin at 22 °C and ink removal at 60 °C. A photocurable resin was then infiltrated in the resulting microchannels and polymerised through a photo-

mask for production of the final interconnected network. The efficiency of the 3D mixer was then tested by mixing two fluorescent dyes (red and green) as a function of varying Reynolds numbers (Re). Alternatively, direct printing of fugitive ink filaments within a photocurable gel reservoir recently allowed the fabrication of a 3D microvascular network for potential applications in 3D cell culture, tissue engineering, and drug delivery. Subsequent photopolymerisation of the gel (Pluronic F127 diacrylate) and removal of the fugitive ink led to the generation of the microvascular network within the hydrogel matrix.[46] Inkjet printing has also been used for generation of 3D hydrogel scaffolds with embedded microchannels for adequate supply of nutrients and oxygen to cells in tissue engineering applications.[47] An in-house 3D printer capable of dispensing a chemically crosslinkable collagen hydrogel precursor, a heated gelatin solution (used as the sacrificial element for channel fabrication) and cell suspensions, allowed the generation of a 3D collagen scaffold with microfluidic channels capable of performing adequate perfusion of cells printed inside the scaffold.[47]

In comparison to the above multi-step 3D printing methods, a one-step procedure was recently applied to the fabrication of optically transparent microfluidic devices using stereolithography.[20,44] These optically transparent chips were successfully employed in imaging of Chinese hamster ovary (CHO) cells previously seeded within the microchannels.[20] Albeit a certain degree of autofluorescence was exhibited by the biocompatible resin employed in the chip fabrication, discrete cells were clearly observed under fluorescence as well as phase-contrast modes. The capability of stereolithography for direct integration of standard connectors to the macro-world within the final device was also demonstrated (e.g. female Luer connectors[20] and 10-32 threads[44]). Breadmore *et al.* showed that it was possible to produce a 40 mm × 25 mm × 17 mm micromixer chip (including connectors) in less than an hour using a rather cheap 3D printer (approx. $2,300) and incurring a material expenditure of only $2 (Figure 2).[44] Gradient generators, droplet extractors and

isotachophoresis chips were also successfully generated with the same 3D printer confirming the feasibility of this approach for cost-effective, rapid prototyping of microfluidic devices, which could open the door to many future low-cost analytical applications.

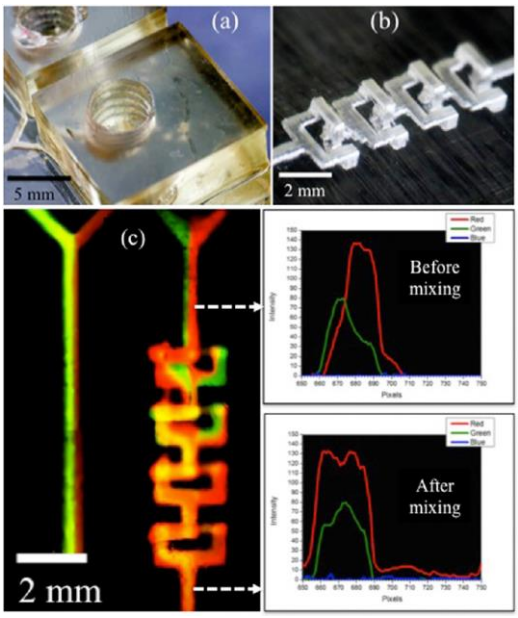


FIG. 2. Optically transparent microfluidic mixer chip integrating 10-32 threads. Reprinted with permission from Shallan et al., Anal. Chem. 86(6), 3124 (2014). Copyright 2014 American Chemical Society

Integration of commercially available polycarbonate membrane inserts into a reusable microfluidic chip containing eight parallel channels was also demonstrated recently (Figure 3).[48] The chip, also integrated with standard threaded connectors, was manufactured with a 3D printer by inkjet deposition of a biocompatible photocurable resin within approximately 4 h. This chip was successfully employed in studying the transport of drugs (levofloxacin and linezolid) across a polycarbonate membrane in view of its future applications in pharmacokinetic profiling of cultured cells. Cell viability studies were also performed with

this platform via exposure of bovine pulmonary artery endothelial cells to a detergent (saponin) which was pumped into the channels inducing cell death.

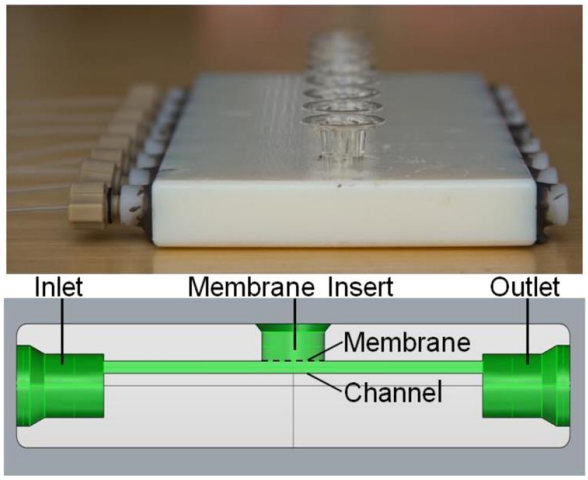


FIG. 3. Microfluidic chip integrating membrane inserts. Reprinted with permission from Anderson et al., Anal. Chem. 85(12), 5622 (2013). Copyright 2013 American Chemical Society

Stereolithography was previously implemented for production of a two-electrode electrochemical flow cell with channel dimensions of 3.5 mm × 3 mm × 192/250 μm.[49] Inkjet-based 3D printing technology has also been used for the fabrication of transparent microfluidic devices integrating interchangeable electrodes of different materials (glassy carbon, platinum, gold, and silver) and sizes (from 250 μm to 2 mm diameter) for electrochemical detection.[50] The resulting two-electrode microfluidic cells presented a straight channel and threaded receiving ports for integration of the electrodes as well as the standard connectors to the syringe pump (see Figure 4). The removable working and pseudo-reference electrodes were prepared by insertion into PEEK fitting nuts in a serial configuration. This approach prevented the need for careful alignment of the electrodes with the microchannel each time the electrodes were interchanged. These microfluidic devices were successfully employed in the detection of dopamine (neurotransmitter) and nitric oxide,

as well as the collection of adenosine triphosphate (ATP) released from red blood cells flowing through the channels while simultaneously measuring oxygen concentration (release stimulus). For collection of ATP, polyester membrane inserts were fitted into a couple of well ports integrated in the device. Collected ATP was then analysed by chemiluminescence detection.

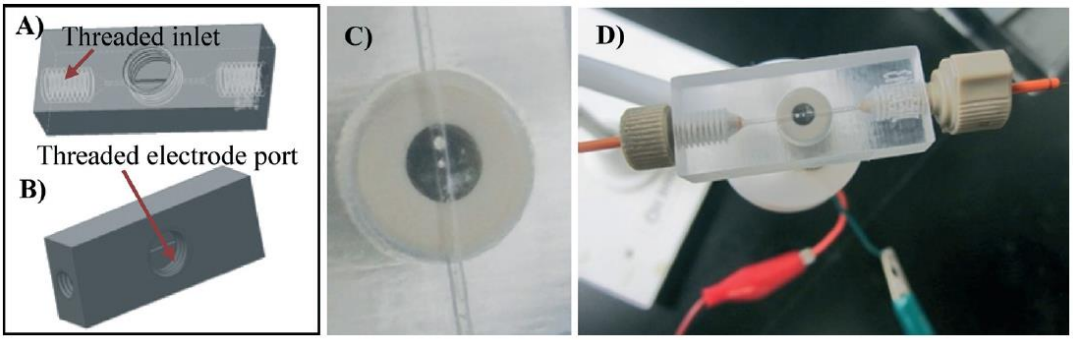


FIG. 4. Microfluidic chip for electrochemical detection: a–b) schematics of the chip showing the threaded ports; c) picture showing alignment of both working and pseudo-reference electrodes with the channel; d) picture showing the chip connected to the syringe pump. Reprinted with permission from Erkal et al., Lab Chip 14(12), 2023 (2014). Copyright 2014 Royal Society of Chemistry

A microfluidic chip integrating ports for a three-electrode system was recently produced from PLA by FDM, and was used for specific electrochemical detection of influenza virus.[51] Influenza hemagglutinin labelled with CdS quantum dots was first isolated within the reaction chamber by glycan-modified paramagnetic beads via hemagglutinin-glycan interaction. Electrochemical quantification of cadmium(II) ions by differential pulse voltammetry was then carried out to determine the presence of the virus.

FDM has also been investigated for fabrication of capillary valves in centrifugal microfluidic discs.[52] Results showed that 3D printing can be considered a viable alternative to other fabrication techniques typically employed for the fabrication of microfluidic discs (e.g. CNC milling and soft lithography) in view of their application in the development of biochemical assays.[53] Although channels produced in ABS possessed ridged or ‘‘scalloped’’ patterns, structures containing predictably-operable valves were obtained. Valve structures comprising channels with widths of 254 and 508 μm, and heights between 254 and 1016 μm were successfully fabricated.

There are few examples in the literature where direct internal 3D laser writing has been used for production of microfluidic platforms for biological applications. This might be due to the fact that ultrafast lasers have been quite expensive for most research labs until very recently. In addition these systems require highly skilled personnel operating such lasers. A 1045 nm femtosecond laser was used to fabricate a microfluidic platform for investigation of the factors that induce cyanobacteria Phormidium to glide toward a seedling root which could be used for promoting accelerated growth of vegetables.[54] Internal microfluidic channels were produced in photostructurable glass (Foturan) followed by annealing and successive wet etching in dilute hydrofluoric acid solution. Optical waveguides and lenses used for imaging of Phormidium were then also created with the femtosecond laser in a single step process. Results showed that CO_2 secreted from the seedling root was the most likely cause for the Phormidium gliding (see Figure 5). A similar fabrication method was previously developed by the same group using a 775 nm femtosecond laser.[55] In this work, the biophotonic microchip was used to inspect the movement of living Euglena’s flagellum within the channels. This new microchip design allowed for wider field of view and greater depth of focus to readily image living microorganisms in a manner which could not be performed by conventional optical observation in Petri dishes using high-speed camera capture.

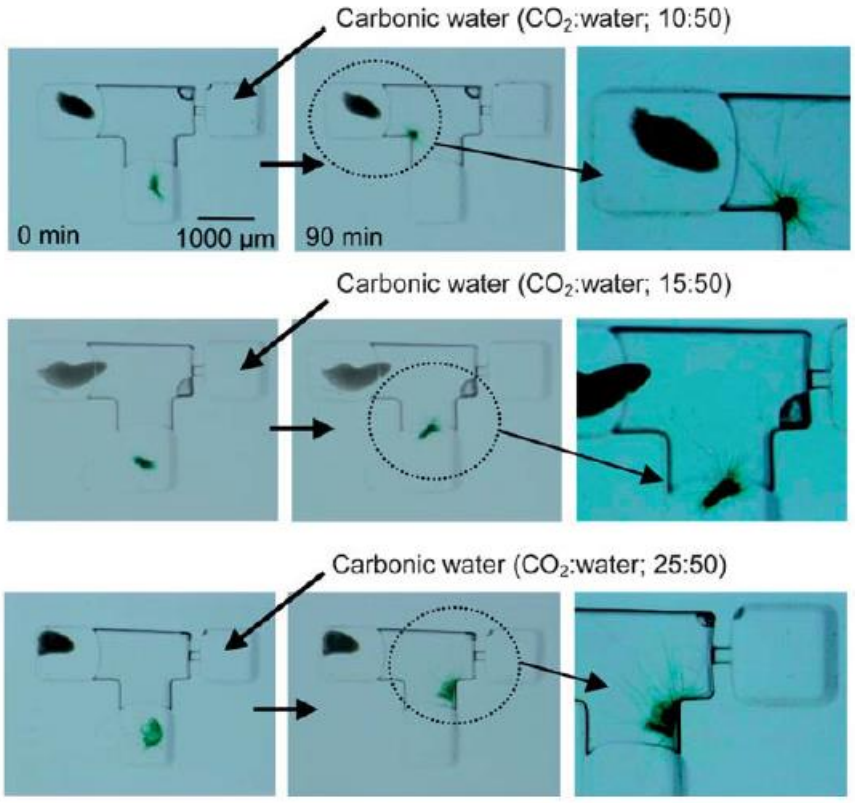


FIG. 5. Investigation of the attractant inducing Phormidium gliding within the microfluidic channels. Adapted with permission from Hanada et al., Lab Chip 11(12), 2109 (2011). Copyright 2011 Royal Society of Chemistry

V. Concluding remarks

3D printing fabrication methods have to date not been widely utilised within the field of microfluidics. This can be attributed to the poor resolution of cheap equipment, the prohibitively high cost of high resolution equipment, and the lack of availability of suitable materials from 3D printing technologies. However, the expiration of key 3D printing patents in recent years has brought many 3D printing technologies to the consumer market, making them more affordable and spurring competition between the newly formed companies

supplying into this area. The rapid increase in the capabilities and availability of these rapid prototyping technologies, and at much reduced cost, has open the door to the exploration of 3D printing as an alternative to more conventional 2D microfabrication methods typically employed in the fabrication of microfluidics platforms. Recent studies have actually shown that 3D printing methods can effectively be used for producing micrometer scale internal channels within bulk biocompatible and transparent materials for a cost as low as $2 per chip,[44] confirming the feasibility of this approach for rapid prototyping of cost-effective microfluidic devices in a single step. Continuous improvements in resolution are expected, even below the currently available TPP devices. However, it is clear that further research is needed in two main areas: removal of support material from complex geometries and development of new resins for SL.

Direct internal 3D laser writing of microchannels presents another promising option for fast accurate production of complicated 3D microfluidic systems. Using a high frequency pulsed femtosecond laser it is possible to create internal channels in a range of glass and polymer materials. Internal optics and actuating elements can also be fabricated using such laser writing techniques. However, the equipment complexity and operator's skill level is still relatively high for this fabrication route in comparison to 3D printing methods.

The benefit of these direct 3D fabrication technologies can be seen in the biological applications for which they have already been used. Resin biocompatibility and optical transparency as well as high accuracy of SL 3D printing have allowed fabrication of microfluidics platforms for applications in cell culture and cell imaging. New microfluidic designs integrating electrodes and membrane inserts were successfully employed in the

electrochemical detection of neurotransmitters and viruses, collection of biologically relevant analytes (e.g. ATP), and drug transport studies. Direct internal 3D laser writing also facilitated the production of suitable microfluidic-based photonic platforms for observation of living organisms. Based on all the above, it is fair to expect that direct 3D fabrication methods will play an important role in biomicrofluidics in the near future, with possible new applications in POC diagnostics, cell culture, drug discovery, organs-on-chip, or even forensic analysis, to name just a few.

Acknowledgments

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