Whitepaper

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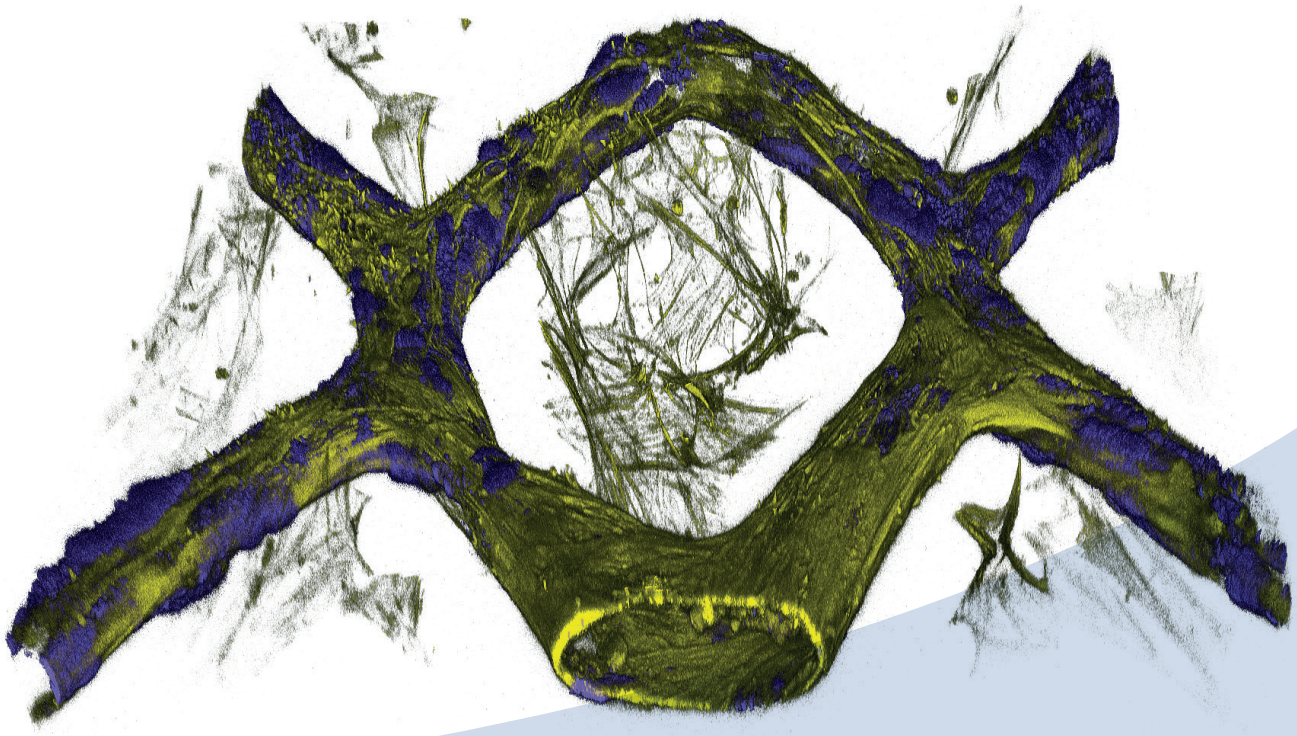
# High-resolution bioprinting

by Simon Sayer and Aleksandr Ovsianikov



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# Glossary

In a 2006 review, summarizing the first international meeting on the topic, Mironov et al. defined **bioprinting** *"as the use of material transfer processes for patterning and assembling biologically relevant materials – molecules, cells, tissues, and biodegradable biomaterials– with a prescribed organization to accomplish one or more biological functions"* [1].

**High-definition (HD) bioprinting,** was defined in 2022 as the capability to consistently produce 3D structures with feature sizes below 50 µm, using materials containing cells. This definition does not only include techniques where the material is deposited line by line or layer by layer, but also modern approaches where 3D scanning is performed within a predeposited volume leading to a localized modification of the material [2].

In their 2016 consensus publication, Groll et al. provided an updated definition of **biofabrication,** as *"the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through Bioprinting or Bioassembly and subsequent tissue maturation processes"* [3].

**Biomaterials** refers to a class of materials that can reside in a biological system without causing adverse effects. Various classes of materials, such as polymers, metals, ceramics, and glasses can fulfill this requirement. Both **bioinks** and **biomaterial inks** are a subset of biomaterials that are processable with biofabrication technologies. The difference is that bioinks generally contain cells, whereas biomaterial inks are used to print 3D structures or scaffolds which can subsequently be populated with cells [4]. Importantly, bioinks should not be considered as something static, as their properties will change with time [5].

**Hydrogels** are composed of hydrophilic polymers that are interconnected via crosslinks and are therefore capable of taking up comparably large amounts of polar liquids, such as water, while retaining their shape. Hydrogels can be of either synthetic or natural origin. The material characteristics are similar to soft tissues and hydrogels are highly permeable to oxygen, nutrients and other water-soluble metabolites, making them an overall highly suitable material for 3D cell cultures [6].

**Tissue engineering** was coined by Langer and Vacanti in 1993, based on the working definition from the first tissue engineering meeting at Lake Tahoe, US in 1988: *"Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function"* [7], [8].

Bhatia and Ingber defined **organs-on-a-chip (OOCs),** also referred to as microphysiological systems, as *"[…] microfluidic devices for culturing living cells in continuously perfused, micrometersized chambers in order to model physiological functions of tissues and organs "* [9]. The goal of such systems is not to build a whole organ, but to create a minimal viable system that recapitulates tissue and organ functions [9].

# Contents



The **cover image** displays a 3D printed microchannel network (50 µm diameter), created with the NanoOne (UpNano) and HYDROBIOINX© U200 (BIO INX), colonized by human endothelial cells and surrounded by human lung fibroblasts in fibrin gel (Purple: endothelial cells, Yellow: F-actin). The image is a courtesy of Federico Cantoni, EMBLA group, Uppsala University.

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UpNano GmbH is a Vienna-based high-tech company specialized in high-resolution 3D printing systems utilizing multiphoton lithography (MPL). Founded in 2018 as a spin-off from TU Wien, UpNano leverages over two decades of research experience from its founding team, Aleksandr Ovsianikov, Peter Gruber and Denise Hirner. The company's pioneering work in the development of biocompatible materials, high-throughput MPL systems and smart software solutions has positioned it as a leader in the field of high-definition bioprinting.

# About the authors

## Prof. Aleksandr Ovsianikov

Prof. Aleksandr Ovsianikov is a head of the research group, 3D Printing and Biofabrication, at the Institute of Material Science and Technology TU Wien (Vienna, Austria). He is a member of the Austrian Cluster for Tissue Regeneration and was recently elected to the board of directors of the international society of biofabrication. His research deals with the use of additive manufacturing technologies and bioprinting for tissue engineering and regeneration. Prof. Ovsianikov has background in laser physics and material processing with femtosecond lasers. A particular focus of his current research is the development of multiphoton lithography technologies for engineering of biomimetic 3D cell culture matrices and realization of novel tissue engineering scaffolds. He was awarded a prestigious Starting Grant in 2012 and a Consolidator Grant in 2017 from the European Research Council (ERC) for projects aimed at these topics.

Since 2004, Dr. Ovsianikov has delivered multiple invited and keynote lectures, and has contributed to over 90 publications. He is co-editor of a living book project, 3D Printing and Biofabrication.

He is also a co-founder and head of research at the TU Wien spin-off, UpNano GmbH (www.upnano.com).

## Simon Sayer

Simon Sayer is a senior research engineer at UpNano GmbH, based in Vienna, Austria. His passion for high definition (HD) bioprinting was sparked during his master's thesis, where he employed multiphoton lithography to alter the chemical composition of hydrogels surrounding cell spheroids. This innovative approach allowed him to investigate the directional sprouting of endothelial cells.

In 2021, Simon joined UpNano GmbH, where he has been actively engaged in multiple research projects, with a strong focus in biofabrication.

Simon is currently pursuing an industrial PhD in collaboration with the 3D Printing and Biofabrication research group at TU Wien, led by Prof. Aleksandr Ovsianikov.

His work has garnered attention at several international conferences, where he has had the opportunity to present his cutting-edge research.

# Abstract

Since the inception of 3D printing in the 1980s, there have been remarkable advancements that have given rise to a wide array of technologies. In the field of bioprinting, many of these technologies are being leveraged to embed living cells into precisely defined 3D structures. Droplet- and extrusion-based bioprinting, two of the earliest techniques, have undergone significant improvements but still face limitations in resolution and versatility. As the role of the cellular microenvironment becomes increasingly recognized, the need for technologies that operate at the microscale has become more critical.

Among high-definition (HD) bioprinting techniques, which achieve resolutions below 50 µm, multiphoton lithography (MPL) stands out for its unparalleled precision and versatility. MPL uses a laser to initiate a chemical reaction within a photosensitive material at the laser's focal point, resulting in highly precise and truly 3D printing capabilities. Recent innovations have focused on improving cytocompatibility and increasing throughput to levels suitable for creating tissue-relevant structures. UpNano leverages over a decade of expertise in photoinitiator and material development, combined with advanced hardware and sophisticated software solutions, to deliver MPL-based bioprinting at the highest level.

In addition to classic photopolymerization, additional processes like photoablation, photocleaving, and photopatterning enable precise temporal and spatial control over cell microenvironments. These photochemical methods hold great promise for enhancing the complexity and functionality of 3D-printed tissue models. As commercial MPL systems become more widespread, a growing number of biofabrication applications are emerging, ranging from organs-on-a-chip, designed to replace animal testing, to tissue engineering, where microscaffolds are used to create millimeter-sized tissue constructs, and vascularization, where MPL's precision allows for the creation of microvessels.

# Controlling the microenvironment – A new frontier in biofabrication

Tissues and organs are characterized by a highly complex architecture with features down to the micrometer scale. Conventional 2D cell culture systems fail to sufficiently replicate the cell's *in vivo* microenvironment in which they interact with other cells, the surrounding matrix and soluble biomolecules. To an extent, the use of spheroids and organoids allows to overcome these limitations. However, the extracellular matrix (ECM), within which the cells are embedded, directly influences cell behavior, as cells sense local mechanical, biochemical, and microarchitectural cues, thereby regulating the structure and function of tissues and organs [10]. Recent studies repeatedly highlighted the importance of ECM composition and microstructure even in the case of organoids, showing that if e.g. intestinal organoids are provided with a spatial template mimicking the target tissue, they more closely resemble *in vivo* conditions [11].

As the importance of the microenvironment is becoming more and more apparent, **bioprinting** technologies, which can manipulate the engineered cell matrix at the micrometer scale, are indispensable. In recent years, significant strides have been made in **HD bioprinting** technologies, which are capable of recapitulating tissue-specific microarchitectures [2]. In some cases, it can be beneficial to dynamically manipulate the microenvironment. **Multiphoton lithography (MPL, Box 1)** stands out as the sole technology offering three key capabilities: 1) printing at subcellular resolution, 2) fully 3D structuring within cell culture plates and microfluidic chips, and 3) mechanical, (bio)chemical and architectural manipulation of the microenvironment throughout the entire cell culture process. MPL bioprinting enables the fabrication of heterogeneous microenvironments containing various cell types, as well as microvessels, semipermeable barriers or tissues scaffolds [12], [13], [14].

BOX 1

## Multiphoton lithography

In multiphoton lithography (MPL), similar to stereolithography (SLA), a laser is scanned through a photosensitive resin, triggering a photochemical reaction. This leads to a localized modification of the material, e.g. crosslinking. Combined with a positioning system that can move vertically and horizontally, a 3D object or pattern is formed.

In SLA, a single photon carries enough energy to excite a molecule to a higher energy state (one-photon absorption, 1PA), which is indicated by the blue arrow in Figure 1a. Once the molecule is in an excited state, it can return to the ground state by emitting fluorescence or by forming radical species (through an intersystem crossing to the triplet state T1), as depicted by the green arrow in Figure 1a. The practical consequences of this principle are shown in Figure 1b, depicting a laser beam focused into an absorbing medium. 1PA is a linear process, where the absorption is proportional to the light intensity (number of photons passing through a unit area per unit time). Therefore, most of the light is absorbed close to the surface and gradually decays as the light is passing deeper into the medium. Consequently, during 3D printing, objects attach to the base of the resin vat and need to be detached after each layer, as indicated in Figure 2a.

In MPL, two or more photons, each carrying a fraction of the required energy, have to be absorbed almost simultaneously, as indicated for two-photon absorption (2PA) by the red arrows in Figure 1a. 2PA probability is very small, requiring a high photon flux. Therefore, MPL utilizes a femtosecond-pulsed laser that is focused through a microscope objective generating an extremely photon-dense regime in the focal spot, while keeping the average power comparably low. Since the rate at which 2PA occurs depends quadratically on the intensity (i.e. doubling the intensity results in four times the fluorescence), the absorption is confined to the focal spot as illustrated in Figure 1c. Besides enabling high-resolution printing, this also permits free-form structuring anywhere in the material, as illustrated in Figure 2b. As a result, there is no attachment between the resin vat and the printed object, allowing to create intricate structures, even within cell culture substrates and microfluidic chips.

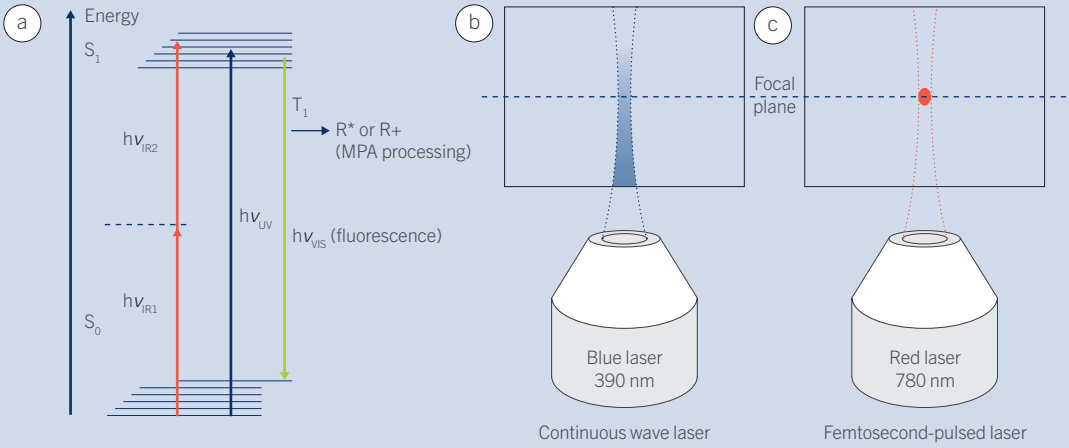



Figure 1: Comparison of one-photon absorption (1PA) and two-photon absorption (2PA). **a,** Jablonski diagram showing the generation of radicals or fluorescence and a schematic illustration showing the absorption pattern for **b,** 1PA and **c,** 2PA.

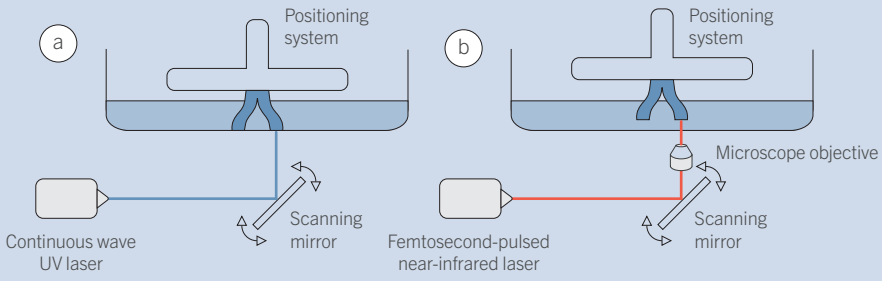
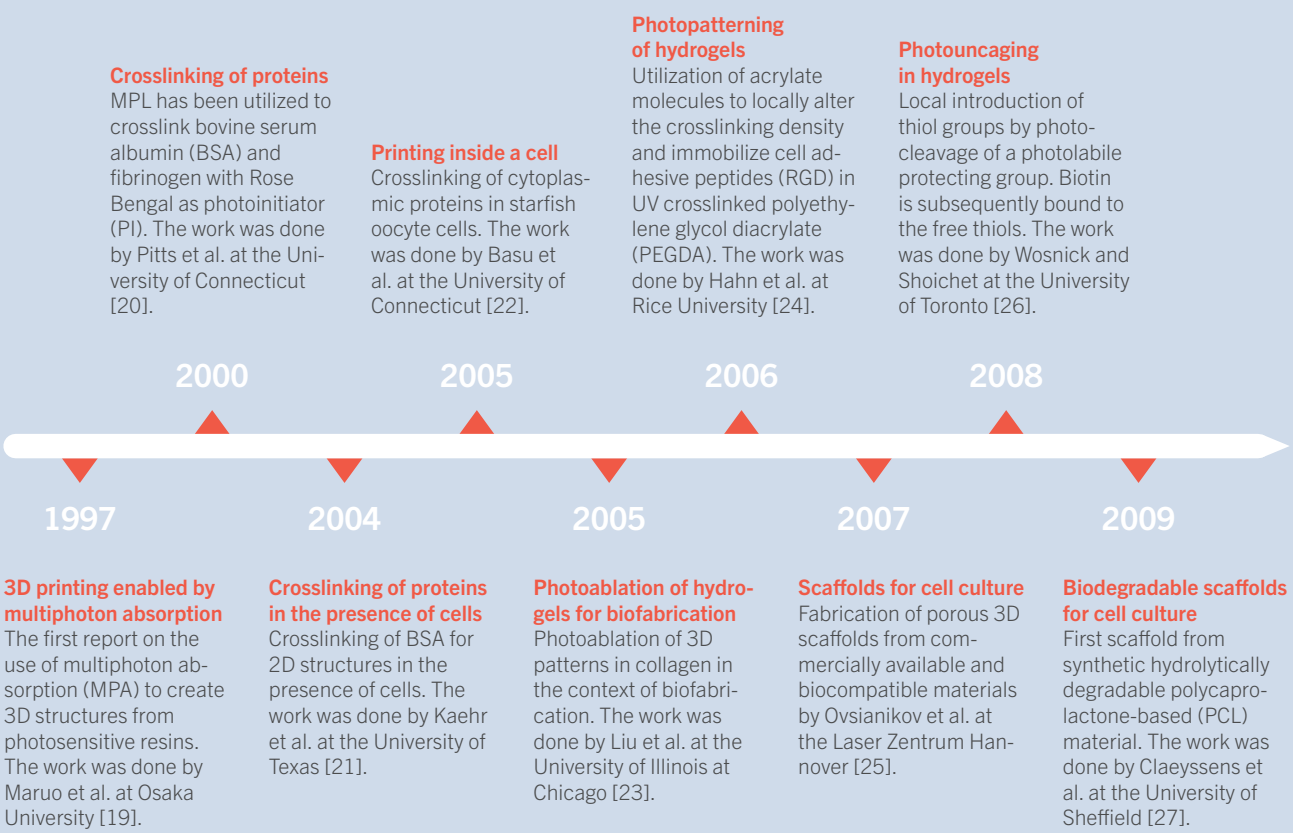



Figure 2: Illustration of the 3D printing process for **a,** stereolithography (SLA) and **b,** multiphoton lithography (MPL). In MPL, the resin is cured exclusively in the focal spot, allowing to avoid any polymerization above and below it, and therefore preventing any bonding between the resin vat and the printed object.

# A historical perspective on bioprinting – Progress and challenges over the years

Let's rewind a bit. Bioprinting is still a relatively young field that gained momentum in the mid-2000s and has experienced steady growth ever since [15]. The first demonstration of bioprinting dates back to 2003, when a commercially available Hewlett-Packard (HP) inkjet printer was modified to deposit simple patterns of mammalian cells onto hydrogel layers [16]. It wasn't until 2004 that 3D bioprinting for the creation of collagen-based constructs with an extrusion-based printer was reported [17]. Since then, more than a dozen bioprinting technologies have been developed, categorized into droplet-based, extrusion-based, and light-based methods [18]. While considerable advancements have been made with both extrusion-based and droplet-based printing, extrusion-based printing typically produces filaments with diameters exceeding 100 µm and droplet-based printing faces challenges in supporting multiple layers due to limitations in the utilized bioinks [2].



# Light-based bioprinting

Light-based approaches are the earliest to have been utilized for 3D printing, with stereolithography (SLA) having been patented in 1984 [28]. Two decades later, in 2004, this technology was utilized to fabricate cell-containing hydrogel structures [29]. Light-based bioprinting was already recognized as a valuable technology during the First International Workshop on Bioprinting and Biopatterning, held in Manchester, UK, in 2004 [1]. All light-based printing systems utilize photosensitive resins, which contain at least two components: (macro)molecules that can bond to each other and a photoinitiator (PI). The molecules form covalent bonds once the PI is excited through light exposure and thereby the resin solidifies locally in the irradiated areas. Standard PIs and their fragments show high levels of cyto- and phototoxicity and developing cell-friendly PIs has therefore been one of the key challenges in advancing light-based bioprinting.

Over the years, a lot of progress has been made in terms of materials, hardware, software and process optimization. Various light-based 3D printing technologies are currently being used for bioprinting, namely digital light processing (DLP), SLA, volumetric 3D printing and MPL [2].

**2009 — Photocleavage of hydrogels**
PEG-based hydrogels containing photocleavable o-nitrobenzyl groups were synthesized. Biocompatibility and the potential to cleave 3D structures upon MPA were demonstrated. The work was done by Kloxin et al. at the University of Colorado Boulder [30].

**2011 — Enzymatically degradable hydrogel**
Gelatin methacryloyl (GelMA) was crosslinked to form scaffolds that were subsequently populated with mesenchymal stem cells. The enzymatic degradability of GelMA was demonstrated. The work was done by Ovsianikov et al. at the Laser Zentrum Hannover [31].

**2012 — Encapsulation of a living organism**
A living organism (C. elegans) was encapsulated in a PEGDA hydrogel by MPA induced crosslinking. The work was done by Torgersen et al. at TU Wien [32].

**2012 — Photografting of hydrogels**
Universal method to locally modify the chemical composition of hydrogels by insertion of aromatic azide molecules into the C-H bonds of the polymer backbone. The work was done by Ovsianikov et al. at TU Wien [33].

**2014 — MPL induced cell encapsulation**
Custom-made MPL-efficient PIs were synthesized and used to encapsulate cells in GelMA containing 80% cell culture medium. This is the first demonstration of MPL-based bioprinting with cells. The work was done by Ovsianikov et al. at TU Wien [34].

**2017 — Biocompatible MPL induced cell encapsulation**
A custom MPL PI bound to the backbone of hyaluronan (HAPI) was synthesized. High viability of MC3T3 cells was observed. The work was done by Tromayer et al. at TU Wien [35].

**2018 — Biocompatible MPL induced cell encapsulation**
Development of a cleavable MPL PI that has a high biocompatibility and a good MPL processing efficiency. ASC/ TERT1 cells were encapsulated in GelMA and a high viability was observed over 5 days. The work was done by Tromayer et al. at TU Wien [36].

**2021 — Launch NanoOne Bio**
The first MPL-based bioprinter was launched by UpNano in 2021. The NanoOne Bio supports direct cell printing, comes with an incubation module (BioUnit), a custom bio software and has an open material platform. Commercial biocompatible materials are supported.

# Multiphoton lithography – Shaping the landscape of HD bioprinting

Given its versatility and unprecedented resolution, MPL is the most promising technology for HD bioprinting [2]. With its origins in fluorescence microscopy, the use of MPL for creating 3D structures was demonstrated for the first time in 1997 by Maruo et al. [19]. Merely three years later, Pitts et al. made the first stride towards processing **biomaterials** by demonstrating the use of MPL to crosslink proteins, namely bovine serum albumin (BSA) and fibrinogen [20].

This step was pivotal, as it showcases the printability of a class of materials called **hydrogels,** which possess several characteristics of the ECM. Materials used for bioprinting should be degradable, and can therefore be remodeled by cells, provide anchoring points for the cells, be permeable, to facilitate nutrient and gas exchange, and should ideally have the appropriate mechanical, chemical and biological characteristics [37].

In 2004, Kaehr et al. crosslinked BSA in the presence of cells using MPL. Although the cells were not embedded in the crosslinked matrix, this work marks a significant milestone on the way to HD bioprinting. It also pointed out one of the major bottlenecks of the technology at the time, namely the throughput, since a scanning speed of a mere 5 µm/s had to be used [22]. In their 2005 study, Basu et al. structured proteins within starfish oocyte cells, offering preliminary evidence that photodamage and phototoxicity are manageable and thereby demonstrating the potential of MPL-based bioprinting [23].

# Overcoming toxicity challenges – Custom synthesized PIs and cell-compatible materials

In their expert review in 2012, Ovsianikov et al. identified the rather low MPL throughput and the limited availability of suitable water-soluble PIs as the main bottlenecks on the way to widespread adoption and medical translation of this technology [38]. To advance MPL bioprinting to its current state, significant improvements were attained with regard to materials, hardware, and software. Between showcasing that proteins can be processed with MPL and being able to print with high shape fidelity, efficiency and under physiological conditions lie decades of research. Many of the advancements in material development were made possible due to the collaboration between TU Wien and Ghent University, spearheaded by Aleksandr Ovsianikov and Sandra Van Vlierberghe. In 2011, they demonstrated for the first time the possibility to create intricate scaffolds by MPL of an enzymatically degradable natural hydrogel. They used gelatin methacryloyl (GelMA) and showed that the resulting scaffolds support the adhesion, proliferation and differentiation of mesenchymal stem cells [31], [39].

**First steps towards MPL bioprinting**

Two years later, the same group reported for the first time the realization of 3D hydrogel structures containing MG63 cell. Here, they used custom synthesized PIs optimized for MPL, which allowed them to produce structures from a GelMA bioink with a water content of up to 80%. While viable cells were successfully trapped within the intricate hydrogel structures, the cells directly exposed to the laser radiation during MPL process were damaged [34]. Given that the PI molecules enter the cytoplasm of cells, the cytotoxic species generated during light-induced radical formation damage the cells, akin to the mechanism of photodynamic therapy (see Box 2). Therefore, to prevent the PI from entering the cells, the same group synthesized a PI that is bound to a macromolecular hyaluronan backbone (HAPI). With this compound they managed to successfully embed viable MC3T3 cells within 3D constructs produced from a GelMA-based bioink [35]. In

BOX 2

## Toxicity challenges in light-based bioprinting

To ensure a high cell viability in bioprinting, all components and processes must be cytocompatible. In its most basic form, a photocrosslinkable hydrogel consists of polymer chains containing reactive functional groups and a photoinitiator (PI), which is typically the primary concern regarding toxicity. PIs can be toxic in the absence of light exposure (cytotoxicity) or upon activation (phototoxicity), with concentration-dependent toxicity levels in both cases. Therefore, finding a balance between efficient polymerization and cytocompatibility is crucial. Phototoxicity can be attributed to the presence of reactive oxygen species (ROS) and radicals that form upon PI activation. Many water-soluble PIs have the ability to enter cells, where these phototoxic species can exert significant damage.

2018, they introduced the first cleavable MPL PI, which also allows direct HD bioprinting by means of MPL [36].

**Advances in photocrosslinkable bioinks**

Further progress on photocrosslinkable hydrogels has been made at the University of Ghent. Most notably, Van Hoorick et al. enhanced GelMA by adding further photocrosslinkable groups, producing GelMA-AEMA. This enhancement leads to a hydrogel with a denser crosslinked network, offering greater stiffness, a lower polymerization threshold, and reduced swelling, which improves shape fidelity. Furthermore, this modification permits the use of lower gelatin concentrations [40].

A year later, in 2018, Van Hoorick et al. modified gelatin with norbornene functionalities (GelNB), which shows even further improved MPL processing capabilities, enabling processing of concentrations as low as 5 w/v% [41]. This material proved to be highly effective for cell encapsulation, with high cell viability and fast processing times [42]. It is also particularly suitable for printing challenging cell types, such as HUVECs, enabling the creation of blood-vessel-on-chip models with vascular structures as small as 10 µm in diameter [12].

In a 2021 study, Van Hoorick et al. demonstrated that modifying the thiolated crosslinker in a GelNB-based formulation could drastically alter its mechanical properties, achievable resolution, processing range, and biological performance. They also showed that MPL could be used to selectively cleave the gelatin backbone, facilitating the generation of softer regions or channels within pre-printed structures. This suggests that there is an optimal laser power window for gelatin-based resins where both resolution and mechanical stiffness are maximized. Therefore, increasing laser power is not always effective for making gelatin-based materials printable, which explains the typical difficulties in achieving acceptable resolutions with GelMA solutions using MPL, especially at low gelatin concentrations [43].

**Translation from academia to industry**

Building on this expertise, the company BIO INX was founded as a spin-off from Ghent University, which offers various MPL-compatible biomaterials. Leveraging expertise in MPL PI synthesis at TU Wien, UpNano developed a proprietary PI that is cytocompatible, highly efficient in MPL, and shows low fluorescence. This PI serves as the basis for the HYDROBIOINX© U200 bioink developed in partnership with BIO INX and was the first commercial resin enabling HD bioprinting of living cells using high scanning speed, and facilitating high reproducibility, using MPL.

Furthermore, there is a synthetic PEG-based hydrogel available, HYDROTECH INX© U200, which can be used for manufacturing large flexible scaffolds as well as for printing in microfluidic chips. Additionally, DEGRADINX© U100, a polyester-based biodegradable resin, which can be applied as a film, enables the production of arbitrary geometries with very high spatial resolution and good mechanical properties. All resins offered by BIO INX are biocompatible according to ISO 10993-5 standards.

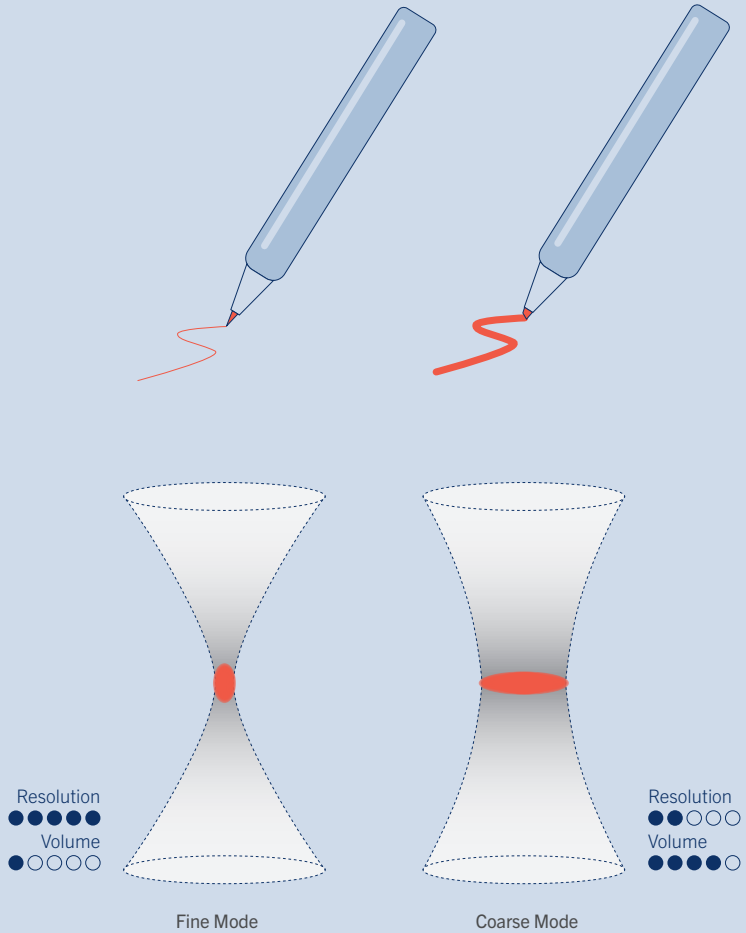
# The frontier of bioprinting using MPL – Cutting-edge hardware and software solutions

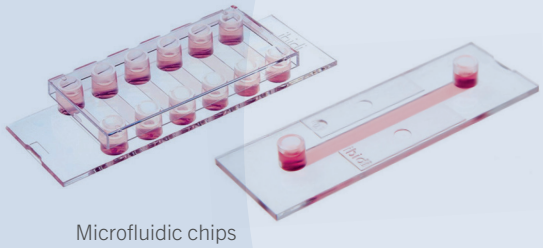
The interplay between hardware and material is crucial. In comparison to other 3D printing technologies, conventional MPL setups are notably slower. To address this limitation and facilitate the fabrication of size-relevant tissue models, UpNano has introduced an innovative technology allowing for the dynamic adjustment of the resolution (Adaptive Resolution, Box 3) during the MPL process, which subsequently increases the throughput. This approach effectively allocates high resolution to relevant areas while rapidly filling in the bulk volume using the Coarse Mode. Additionally, the NanoOne utilizes a powerful laser capable of delivering sufficient energy to the material, even if focused through a low numerical aperture (NA) objective, to trigger the photochemical reaction. This enables throughput rates of 450 $mm^3/h$ and higher, with a scanning speed of up to 1200 mm/s. Furthermore, high laser power enables other useful photoinduced processes, such as photoablation of hydrogels, to be carried out with the NanoOne.

**BOX 3**

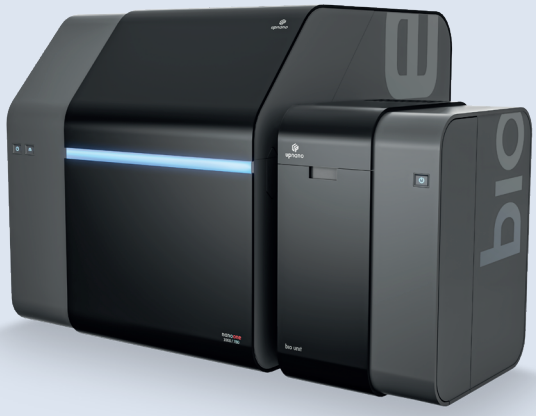
## Adaptive Resolution

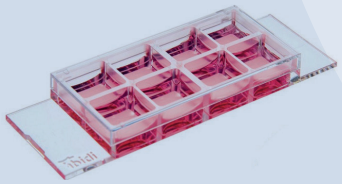
The patented Adaptive Resolution feature enables the real-time dynamic tuning of the volume of the voxel, the region where the photochemical reaction occurs, during a print job. This capability optimizes throughput while ensuring that the resolution is high enough in critical areas. Imagine coloring a picture using pencils with different line widths: Broader strokes fill larger areas quickly (Coarse Mode), while finer pencils are needed for detailed sections (Fine Mode). Adaptive Resolution automatically changes the pencils according to the respective geometry.



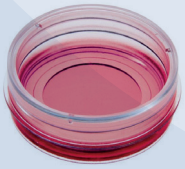


Microfluidic chips
commercial or custom-made

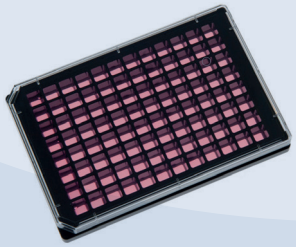




µ-dishes
in different formats



Petri dishes
20–74 mm diameter



Well-plates
with 348, 96, 48, etc. wells

**State-of-the-art hardware for HD bioprinting**

Building on years of expertise in MPL bioprinting, UpNano introduced the NanoOne Bio to the market in 2021. The core of the printer is a femtosecond-pulsed laser with a wavelength of 780 nm. Given the transparency of cells at this wavelength, light-induced cell damage can be avoided. The NanoOne is a desktop system with an integrated HEPA filter and vibration isolation and is therefore perfectly suitable for biological laboratories without the need of special modifications. The NanoOne Bio comes with a modular extension, called the BioUnit. The BioUnit serves as a compact incubation system that can be seamlessly integrated into the build room of the NanoOne Bio. With the BioUnit it is possible to control and monitor environmental parameters, such as temperature, humidity and $CO_2$ concentration, that are critical for the bioprinting process. This level of environmental control is crucial to ensure optimal conditions for cells and hydrogels during the printing process. The introduction of the NanoOne Bio, with its accompanying BioUnit, is a significant leap forward in advancing the reproducibility and stability of the MPL-based bioprinting process.

**Boosting throughput and usability – Interplay between hardware and software**

Given the enhanced throughput enabled by innovations such as Adaptive Resolution or the use of

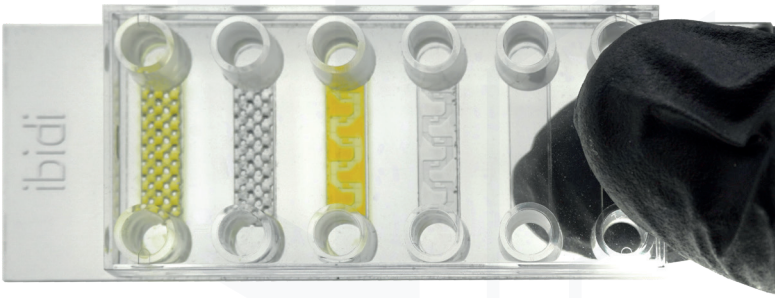



Figure 3: This image depicts a commercial microfluidic chip (µ-Slide VI 0.5, ibidi GmbH) featuring internal structures printed with the NanoOne. The yellow structures were printed using UpPhoto and the transparent structures with UpFlow, a resin with minimal fluorescence and low viscosity tailored for microfluidic applications.

low NA optics, manual handling of the produced structures by the user can be a limiting factor in MPL bioprinting. Hence, the NanoOne Bio is designed to accommodate various commonly used cell culture substrates, providing flexibility with options such as commercially available well-plates or µ-dishes, as well as custom-made microfluidic chips and petri dishes. Alongside the hardware support, the NanoOne Bio comes with a dedicated software, called Plate Dock, embedded within the Think3D user software, which facilitates the creation of a multi-well print job by offering a dynamic user interface and visual feedback, making it easier to design and keep track of the prints. Moreover, individual wells can be linked, which facilitates the easy replication and subsequent parameter change of individual print jobs. On top of that, the Plate Dock feature enables a fully automated process, creating a versatile, fast and user-friendly MPL bioprinting experience.

## Printing inside of microfluidic chips

Printing directly inside of microfluidic chips combines the benefits of high-throughput technologies, like injection molding, with the high resolution of MPL. This process, illustrated in Figure 4, is simple and applicable to any chip with an accessible bottom, though the best results can be achieved with chips having a 170 µm thick glass bottom. Since samples in the NanoOne are positioned over the objective, similar to an inverted microscope, the chip can be inserted into the printer with its top facing up. After printing, unpolymerized resin is removed by flushing the channels with the appropriate solvent, leaving the chip with a functional microstructure. UpNano's UpFlow resin, designed for in-chip printing, is transparent in the visible range, and has low fluorescence and low viscosity, making it ideal for creating intricate structures with small pores, as depicted in Figure 3.

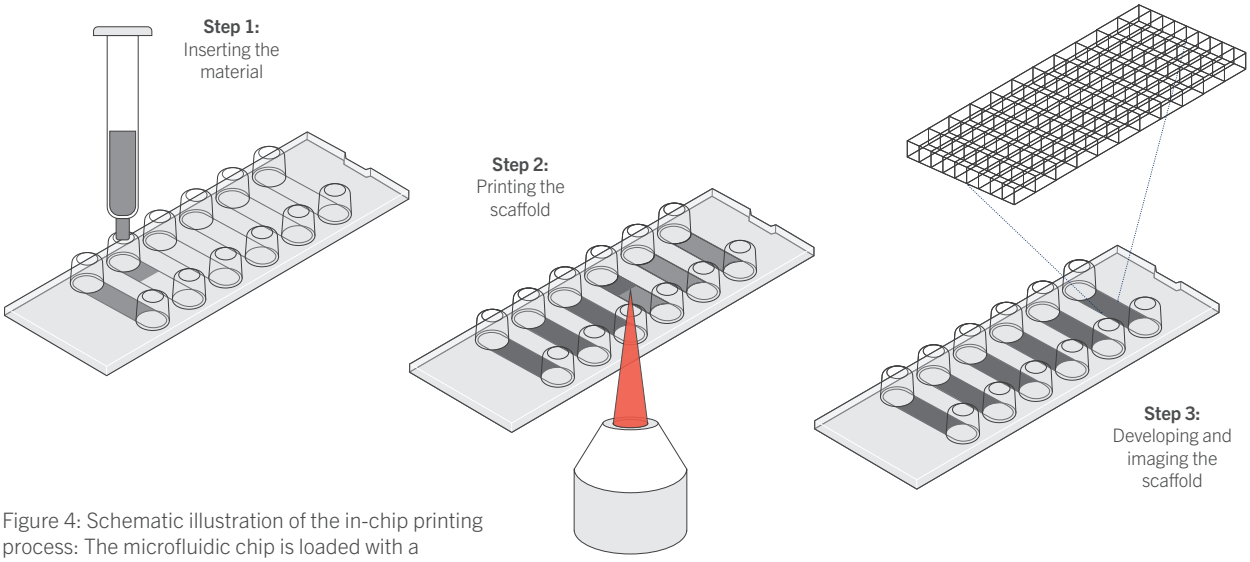



Figure 4: Schematic illustration of the in-chip printing process: The microfluidic chip is loaded with a photosensitive resin and transferred to the NanoOne. The printing starts from the top and moves downward, followed by the removal of the unpolymerized resin.

# Precision engineering at the molecular level – Beyond crosslinking-based bioprinting

It is worth noting that the NanoOne Bio supports crosslinking-based MPL bioprinting as well as additional processes that enable temporal and spatial manipulation of cell microenvironments. These photochemical processes are very useful and have the potential to enhance the significance and physiology of 3D printed tissue models.

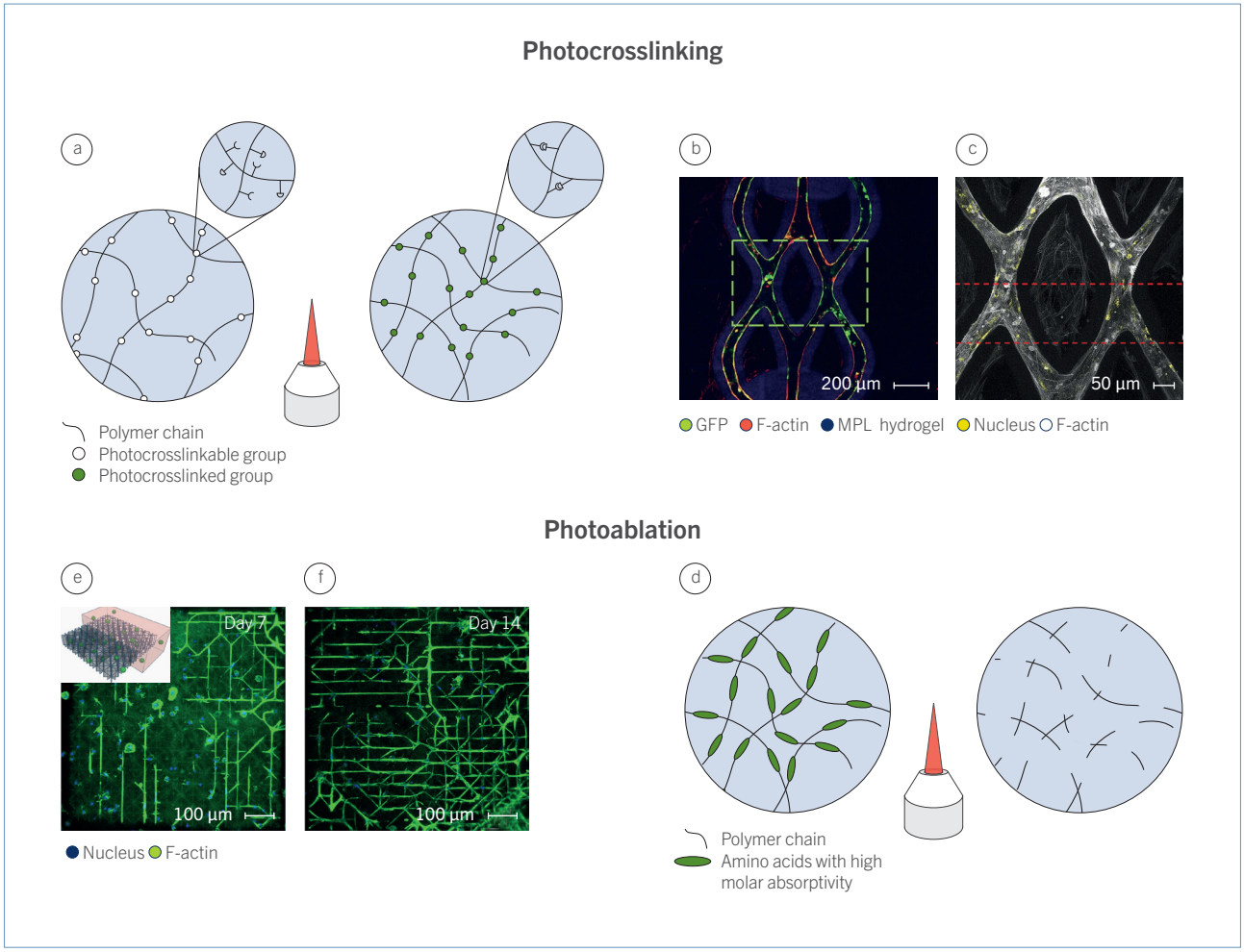



Figure 5: **a,** Schematic illustration of the photocrosslinking process. Functional groups on the polymer chains form covalent bonds upon light irradiation through either chain- or step-growth polymerization. **b,** Overview and **c,** close-up confocal images of a microchannel network (50 µm diameter) structured with MPL (blue), with endothelial cells (GFP labelled) on the inside and fibroblasts in the surrounding fibrin hydrogel. Adapted from [44], CC BY 4.0. **d,** Schematic illustration of the photoablation process. Light-induced chemical denaturation by breakage of hydrogen bonds and disassembly of the protein structure. Confocal images of mesenchymal stem cells on **e,** day 7 and **f,** day 14. The cells are aligning to the photoablated pattern shown in the left corner of image **e.** Adapted from [45], CC BY 4.0.

### Photoablation – A versatile approach for rapid microchannel fabrication

One such approach is MPL photoablation, a subtractive process based for example on the light-induced denaturation of proteins [46]. Given the subtractive nature of photoablation, the processing time for certain structures (e.g. microchannels) is a lot shorter compared to additive approaches. Furthermore, this approach

allows for the manipulation of the structure at any point during the culture process. In addition to complete denaturation, which results in the transition from a solid to a liquid state, it is also possible to locally vary the mechanical properties by controlling the energy dose. This enables the creation of patterns and gradients with different stiffnesses. Another significant advantage of this approach is that the hydrogel does not need to contain specialized functional groups for MPL. This allows for the utilization of well-established biomaterials such as collagen or Matrigel, leveraging existing knowledge bases. However, a relatively high energy input is required to denature proteins. The majority of commercially available MPL systems have been designed for standard microfabrication purposes and therefore do not provide the laser power that is necessary to perform photoablation. In 2005, Liu et al. published the first application of photoablation, showcasing its capabilities to create various patterns within a collagen matrix, which were subsequently seeded with mesenchymal stem cells [23]. Many other applications utilizing photoablation have been published over the years [47], [48], [49], [50], [51], [52]. More recent applications are the creation of perfusable mini-gut tubes [53], organ-specific microvasculature [54] or the creation of an interconnected bone cell network [45].

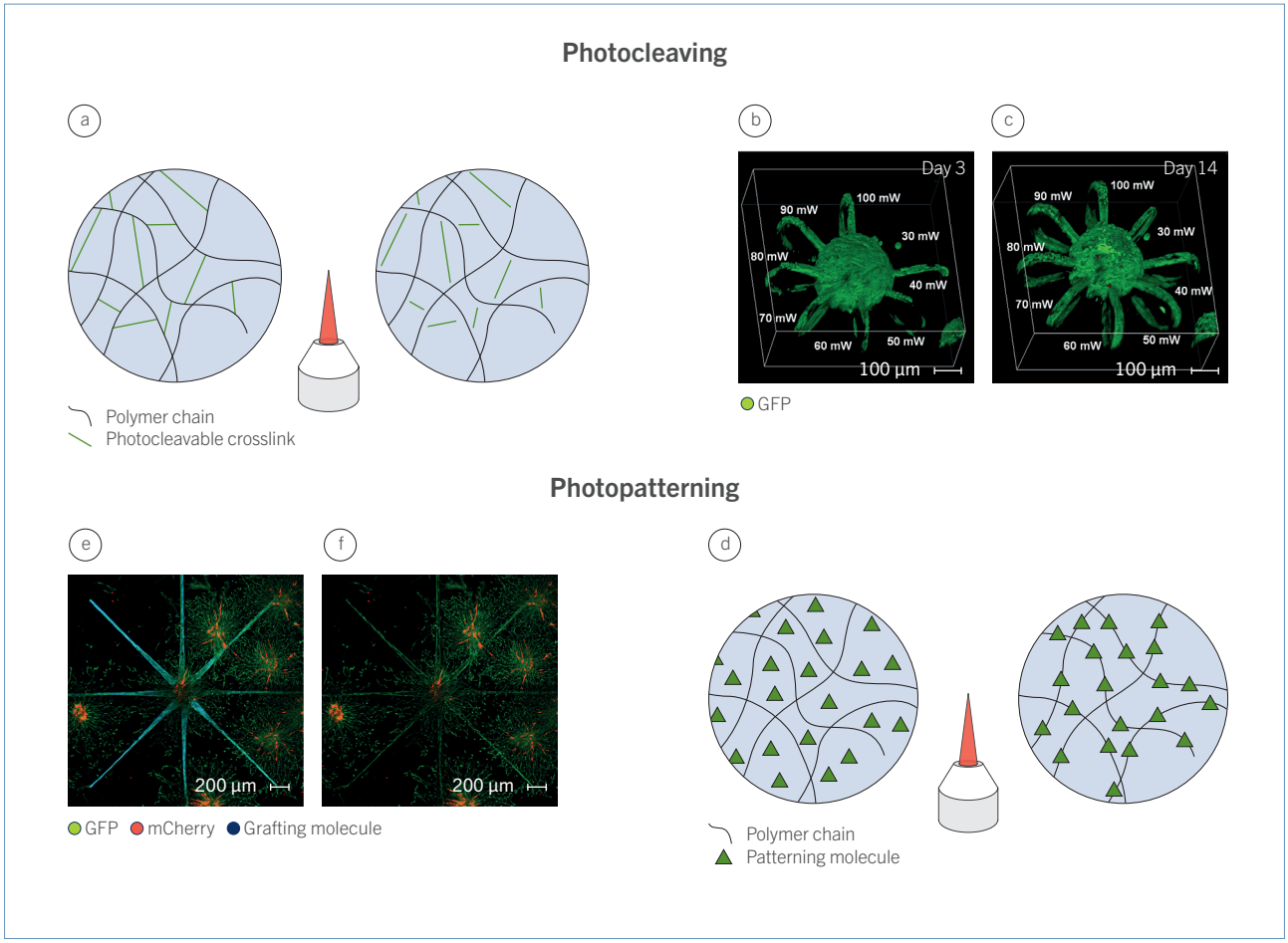



Figure 6: **a,** Schematic illustration of the photocleaving process. Photolabile linkers are cleaved upon light irradiation. Confocal image of adipose-derived stem cells (ASCs) migrating into photocleaved horseshoe-shaped microchannels on **b,** day 3 and **c,** day 14. Adapted from [55], CC BY 4.0. **d,** Schematic illustration of the photopatterning process. Local chemical modification by binding molecules to the polymer chains upon light irradiation. Confocal image of a star shape photopatterned around an ASC and endothelial cell spheroid on day 3 showing **e,** the patterned molecule fluorescing in blue and **f,** GFP-labelled ASCs aligning according to the pattern. Adapted from [56], CC BY 4.0.

**Photocleaving – Harnessing photocleavable groups for biofabrication**

MPL-induced photocleaving, another subtractive approach, utilizes hydrogels with photolabile groups, which are cleaved upon light exposure. This leads to a local change in the cross-linking density and, if enough groups are cleaved, the creation of void regions. The first publication demonstrating the potential of photocleaving with MPL was published in 2009 by Kloxin et al. Here, a PEG-based hydrogel containing o-nitrobenzyl (oNB) groups was used. Upon irradiation, channels were cleaved and the migration of fibrosarcoma cells along these channels was studied [30]. Since then, many other applications of MPL photocleaving have been published [57], [58], [59], [60], [61], [62]. Arakawa et al. were among the first to showcase the potential of MPL photocleaving for the creation of vasculature networks. They used click chemistry to crosslink a tetraalkine-modified PEG with a diazide-modified synthetic peptide containing photocleavable oNB groups. This enabled the creation of microchannels as small as 10 x 10 $\mu m^2$ in the presence of stromal cells. These channels were then successfully lined with endothelial cells, demonstrating the capabilities of creating functional vascular structures in a co-culture system [63]. Notably, the group around A. Ovsianikov at TU Wien was able to enhance the efficiency of the oNB photocleavage reaction by using a MPL photosensitizer and demonstrated the potential to alter a hyaluronic acid matrix in the presence of cells [55].

**Photopatterning – Local chemical modification of predeposited matrices**

In addition to changing the mechanical characteristics of the engineered cell matrix, various methods enabling local chemical modifications have been reported. Photopatterning, for instance, enables the localized introduction of molecules into a hydrogel matrix. This approach was first demonstrated in 2006 by Hahn et al., who tethered cell adhesive peptides to PEG molecules containing crosslinkable acrylate end groups [24]. These macromolecules were then attached to free acrylate groups within the PEG-DA matrix upon laser exposure. In photografting, molecules are covalently bound to the backbone of a polymer. This was initially demonstrated in 2012 by Ovsianikov et al. and recently adapted to showcase the potential for guiding cell migration in hydrogels [33], [56].

**Photouncaging – Light-guided release of molecules**

In another approach, functional groups are already present in the polymer but are exposed only upon laser irradiation. This method, called photouncaging, was first demonstrated by Wosnick and Shoichet in 2008. Here, the researchers utilized a modified agarose that released protected thiol groups upon laser exposure, to which biotin was subsequently bound [26].

# Applications

## Organs-on-chip

Currently, animal models are still considered the gold standard in many of these areas, partially because they are well established and people know how to work with them. Given the ethical concerns, regulators are prompted to advocate for alternatives to animal testing, as evidenced by a resolution passed by the European Parliament in 2021 aimed at phasing out animal test-

ing [64]. While the Food and Drug Administration (FDA) had mandated animal testing in drug development since 1938, the FDA Modernization Act 2.0, signed into law in 2022, rebuked this and rendered animal testing optional [65]. Furthermore, the significant differences between human and animal biology, particularly rodents, often result in promising drugs failing during clinical trials. Contrary to animal models, **organ-on-a-chip (OOC)** systems typically focus on specific aspects of human biology they aim to replicate. Consequently, there is no one-size-fits-all *in vitro* system; instead, each tissue requires a tailored model, with multiple models for each tissue, optimized to emulate specific processes [66]. Considering that these systems enable a better preselection of drug candidates, leading to improved success rates in clinical trials, significant cost reductions can be realized. This in turn accelerates the availability of novel treatments.

Advancements in MPL have positioned it as a key technology for developing physiologically advanced OOC systems, which are currently being adopted by pharmaceutical companies. Mandt et al. utilized MPL to fabricate a placenta-on-a-chip to simulate placental transport across the basal membrane. The researchers have fabricated a hydrogel membrane directly within a microfluidic chip, showcasing one of the major advantages of MPL compared to other methods [13]. Jayne et al. used MPL to construct a heart-on-a-chip, allowing them to study the response of cardiac microtissues derived from human induced pluripotent stem cells (iPSCs) under different mechanical loading conditions. The platform can be used for fundamental studies and drug screening on cardiac microtissue [67].

### Intestine-on-a-chip model enabled by photoablation

A highly promising approach is the integration of organoids and microfabrication. This allowed Nikolaev et al. from the group of Matthias Lutolf to create an intestine-on-a-chip model which showed the formation of crypt- and villus-like domains akin to *in vivo* conditions [53]. They

*"Using NanoOne, we have reduced the production time for a microfluidic chip from many hours on our in-house tool to just minutes. The exceptional combination of high laser power, fine resolution and intuitive software was instrumental in achieving this remarkable speed boost. NanoOne has become an indispensable instrument, playing a pivotal role in numerous research projects at the Roche Institute of Human Biology. It greatly facilitates the prototyping of new microfluidic chips and the bioengineering of next-generation organon-chip models for various R&D projects at Roche. Looking ahead, we're already exploring avenues to further expand our capacity, and we're confident that we have the ideal partner by our side."*


Dr. Mike Nikolaev, Scientist at Roche Institute of Human Biology

loaded a collagen-Matrigel mixture into a microfluidic chip, and subsequently photoablated a perfusable villi and crypt structure that mimics the architecture that is found in the small intestine. Subsequently, mouse intestinal stem cells formed a densely packed epithelium, sustained over several weeks by the continuous removal of dead cells from the growing epithelium through flow, a capability not achievable with conventional organoids. Upon cell differentiation, the spatial distribution of various cell types along the villi and crypt domains closely resembled *in vivo* conditions [53].

Building on the aforementioned model of the small intestine, Lorenzo-Martín et al. from the same group utilized this platform to investigate cancer development [68]. They successfully controlled tumorigenic transformation in a spatiotemporal manner through exposure to blue light, which is sketched in Figure 7a. Unlike animal models, the human mini-colons allowed them to monitor tumors with single-cell resolution over several weeks without disrupting the culture. The study demonstrated the ability to spatially confine tumor formation to either crypt or villi regions, revealing morphological variations in tumors according to their initiation site, as highlighted in the bright-field images in Figure 7b. These tumors exhibited a vast intra- and intertumoral diversity, closely resembling colorectal tumors *in vivo,* as indicated in Figure 7c. Their cancerous nature was validated by comparing the growth of mini-colon-derived to primary colon cancer cells, as shown in Figure 7d and e. Furthermore, the researchers demonstrated the utility of this model in screening for tumorigenic factors [68]. Overall, this study highlights the potential of precisely engineered organoid-on-a-chip systems in advancing cancer research.

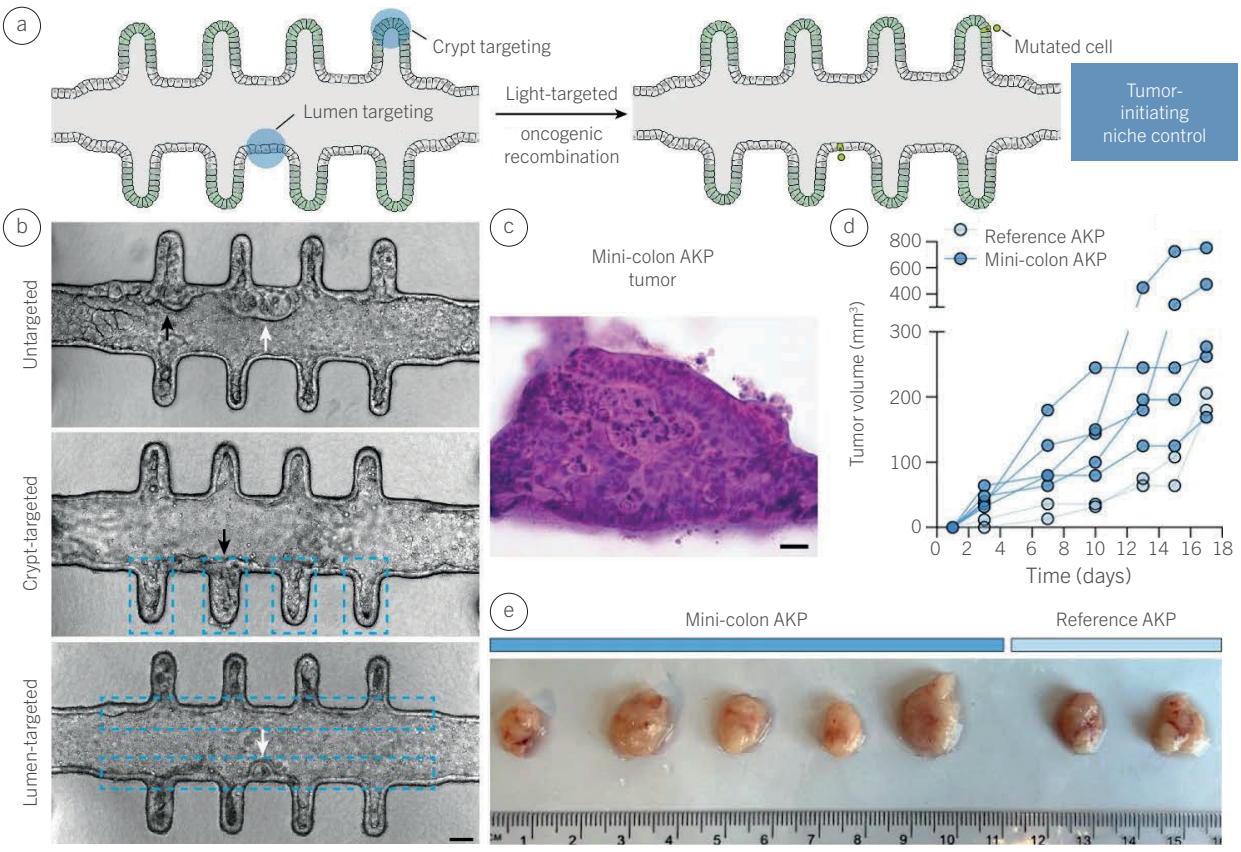



Figure 7: **a,** Workflow schematic of the spatially targeted tumorigenesis. **b,** Bright-field images of the human mini-colons that have undergone targeted and untargeted tumorigenesis. Scale bar is 75 µm. **c,** Hematoxylin and eosin staining of a mini-colon tumor section. Scale bar is 25 µm. **d & e,** Tumor growth comparison between mini-colon derived and primary tumor cells. Adapted from [68], CC BY 4.0.

# Tissue engineering

In the field of **tissue engineering,** multiple techniques are utilized to reconstruct and restore tissues. These include scaffold-free strategies using cells alone, or scaffold-based strategies where the scaffolds are either populated with cells *in vitro,* or *in vivo* after implantation [69]. The scaffold's architecture influences cell behavior, a crucial consideration when engineering the matrix for a specific tissue [70]. MPL facilitates the fabrication of complex scaffolds with intricate microarchitectures. Moreover, MPL can also be used to create microstructures on the surface of the scaffolds, or enable local binding of proteins [71], [72]. Vassey et al. have demonstrated the effect of MPL printed objects with complex microarchitectures on the behavior of cells [73]. Scaffolds printed with MPL have been used to recreate various tissues, such as bone, retinal and neural tissue.

The incidence of bone-related diseases is on the rise, with critical bone defects frequently resulting in amputations. Tissue engineering offers a promising toolbox to tackle these challenges [74]. Timashev et al. conducted a study where they produced a scaffold by means of MPL and seeded it with adipose-derived stem cells (ASCs), which they subsequently differentiated towards the osteogenic lineage. Their results indicated that the scaffold facilitated differentiation and augmented mineralized tissue formation. Furthermore, they implanted scaffolds into mice and showed that bone formation was enhanced [75]. Hauptmann et al. investigated triply periodic minimal surface structures regarding their porosity, flow resistance and stiffness, demonstrating their potential application in bone tissue engineering [76].

The retina, a complex tissue, susceptible to degenerative diseases causing irreversible vision loss, lacks effective therapeutic interventions. Tissue engineering has the potential to bridge this gap [77]. Worthington et al. engineered a 3D structure with horizontal and vertical micropores, mimicking the cell orientation and density of native tissue. Seeding these scaffolds with human iPSC-derived retinal progenitor cells resulted in successful cell attachment and the formation of neuronal processes aligned with the vertical pores [78]. In another study, Thompson et al. used a degradable polymer to fabricate a porous 3D structure. Their findings showcased the successful cultivation of iPSC-derived retinal progenitor cells within the scaffold, with evidence of cell infiltration into the pores and subsequent proliferation. Furthermore, they implanted a scaffold into the sub-retinal space of a pig. Over the course of 30 days, native photoreceptor cells infiltrated the scaffold and no signs of cytotoxicity are visible [14].

**Bioassembly of microscaffolds fabricated with MPL**

Another intriguing approach is the synergy of scaffold-free and scaffold-based tissue engineering approaches in what has been coined the third strategy in tissue engineering. Essentially, cell aggregates are combined with microscaffolds. Utilizing this strategy, one can achieve a high initial cell density, provide mechanical stability, facilitate the self-assembly of tissue and have the possibility to provide biomolecules locally [79].

Researchers from the lab of Aleksandr Ovsianikov have demonstrated in a first study the successful assembly of scaffolded spheroids from human ASCs into millimeter-sized tissue constructs. Moreover, they showed that the microscaffold-based tissue units have higher cell retention and decreased compaction compared to standard cell spheroids [80].

A subsequent study by Kopinski-Grünwald et al. from the same group explored the potential of pre-differentiated scaffolded spheroids to form tissues [81]. They loaded 500 building blocks,

spheroids and scaffolded spheroids respectively, which had been differentiated in chondrogenic medium for three weeks, into an agarose mold and studied the bioassembly after 7 days, as depicted in Figure 8b. Unlike conventional spheroids, which did not form a well-integrated structure as shown in Figure 8d, scaffolded spheroids displayed robust and stable fusiogenicity as highlighted in Figure 8c & e. This advancement enables the generation of cartilage-like tissue through the bioassembly of chondrogenically differentiated scaffolded spheroids, a feat hard to attain with standard spheroids [81].

Furthermore, this work highlights the importance of suitable materials and hardware. The group collaborated with researchers from Ghent University to develop a biodegradable urethane-based resin with high processing efficiency, enabling the fabrication of stable high-resolution structures, as depicted in Figure 8a [82]. Starting from there, BIO INX developed a commercial synthetic biodegradable resin, DEGRAD INX U100. This gives researchers a material with low batch-to-batch variability, important for translational aspects in tissue engineering. Given the number of microscaffolds needed to create size-relevant tissue constructs, the importance of improved hardware that facilitates high-throughput fabrication becomes evident, especially in a translational context [80], [83].

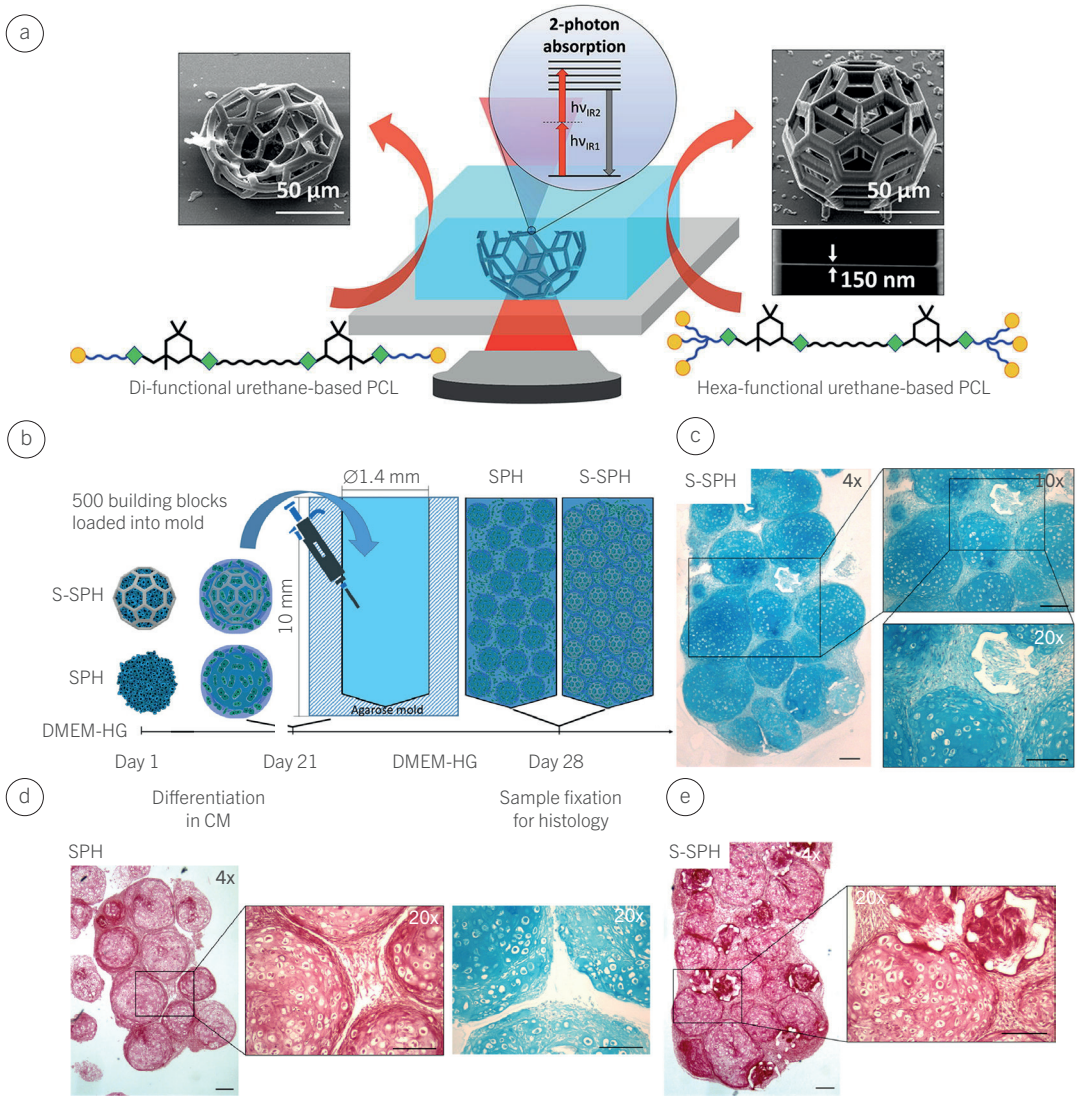



Figure 8: **a,** Schematic of the MPL process. SEM images of microscaffolds printed with di – and hexa-functional UPCL. Adapted from [82], CC BY 4.0. **b,** Schematic of the workflow to create cartilage-like tissue. PicroSirius Red and Alcian Blue histological staining of **c & e,** scaffolded spheroids and **d,** spheroids. Scale bars are 200 µm. Adapted from ref [81], CC BY 4.0.

# Vascularization

Engineered tissue of clinically relevant size usually requires a vasculature to prevent necrosis [84]. Furthermore, *in vitro* models require vessels to accurately mimic physiological conditions. MPL is a promising tool for incorporating microvessels within the tissue.

Dobos et al. have successfully produced channels with diameters ranging between 10 – 30 µm in a hydrogel consisting of thiolated gelatin (GelSH) and gelatin-norbornene (GelNB) inside a microfluidic chip. Both endothelial and supporting cells were directly embedded in the produced hydrogel structure. The functionality of the vessels was validated through immunostaining of vascular endothelial cadherin (VE-cadherin) under static and dynamic conditions [12]. Grebenyuk et al. constructed a 3D vessel network using a custom hydrogel, showcasing the long-term perfusion of neural and liver tissue. Their study illustrated the viability, proliferative capacity and intricate morphogenesis of the tissue during culture, without evidence of hypoxia or necrosis [85]. This is a highly encouraging proof of concept, highlighting MPL's potential to address a significant hurdle in tissue engineering. Rayner et al. used photoablation to create microchannel networks in collagen hydrogels. They demonstrated the fabrication of organ-specific capillary networks based on *in vivo* data, the successful endothelialization and perfusion with blood [54]. This is a big step towards the incorporation of vessels for tissue engineering and organ-on-a-chip models.

**Perfusable microvessels facilitated by MPL**

In their recent work, Cantoni et al. introduced a custom microfluidic chip to create perfusable microvessels through a two-step method. First, they printed microchannels using HYDROBIO INX U200, then injected a fibrin hydrogel containing human lung fibroblasts into the surroundings. This approach not only provides a cell-type specific microenvironment but also enables the rapid creation of 10 µm channels, as shown in Figure 9a & b, significantly faster than printing the entire construct. Confocal images showcase the formation of a human umbilical vein endothelial cell (HUVEC) monolayer alongside the microchannels, as shown in Figure 9c & d [44]. Overall, this approach exemplifies how smart fabrication techniques can boost MPL capabilities for creating microphysiological models.

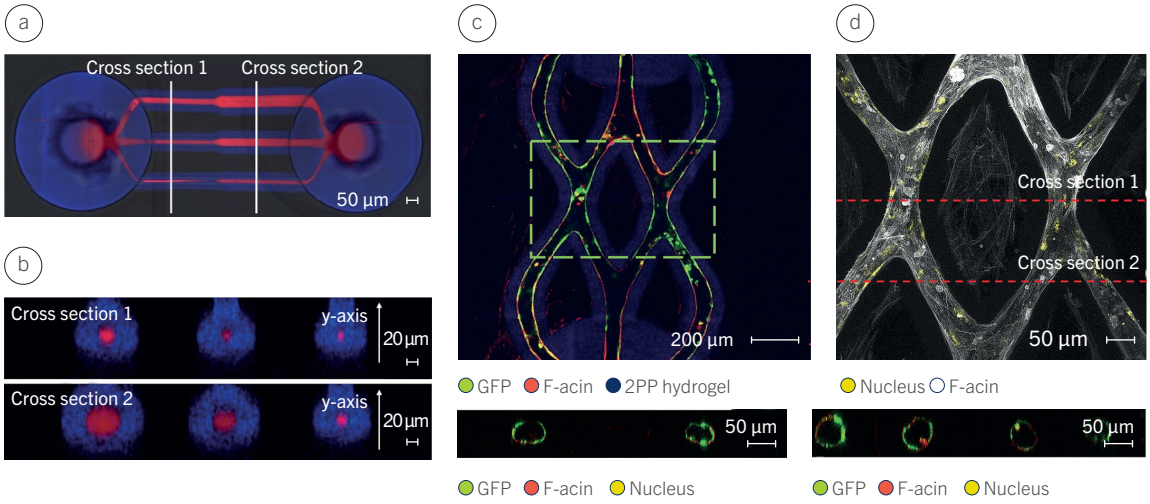



Figure 9: **a,** Top view and **b,** cross section of a MPL fabricated channel structure after 10 days, with diameters of 30, 20 and 10 µm for cross section 1 and 60, 40 and 20 µm for cross section 2. MPL printed structure in blue and Antonia Red-dextran in red. **c,** Overview, **d,** zoomed-in and respective cross-section image of human umbilical vein endothelial cells (HUVECs) and fibroblasts lining the MPL printed microchannels. Adapted from [44], CC BY 4.0.

# Overview research projects

UpNano is involved in several high-profile research projects that deal with various aspects of MPL for biomedical applications.

The EU Horizon 2020 project **Immune Niches for Cancer ImmunoTherapy Enhancement (INCITE)** aims to improve current cancer immunotherapies by culturing T memory stem cells ($T_{SCM}$) that have a high efficacy against tumors in an artificial immune niche. The immune niche consists of a scaffold that is printed inside a microfluidic chip using MPL. First results indicate that the artificial immune niche enhances the population of $T_{SCM}$.

At present, most bioinks originate from non-human sources. There is a huge need for human-based alternatives to resemble processes and conditions in human tissue more closely. Therefore, UpNano has partnered with biomaterials experts in the IraSME research project **Human bioinks for 3D printing (HU3DINKS)** to develop human placenta-based bioinks for MPL. UpNano provides their expertise in the development of PIs for MPL and process establishment.

The scope of the FFG-funded project **Optiflow3D** is to develop a novel two-stage rotodynamic blood pump for pediatric patients. UpNano develops a process to produce polymeric micro- and nanostructures that are subsequently transferred to ceramics using nanoimprint lithography (NIL). Moreover, similar structures will be produced inside a microfluidic chip, with the aim of developing a thrombus-on-a-chip model.

**ELEVATE,** another IraSME-funded project, deals with the development of an Electronic-Tumor Impedance Readout platform. This platform will enable the non-invasive electronic detection of cancer cells, three-dimensionally printed tumor spheroids and patient-derived microtumors. UpNano is printing 3D structures that can hold cell spheroids in place in specific locations on microelectrode arrays (MEAs).

# Outlook

Over the past decade, significant advancements have propelled the field of HD bioprinting. Researchers have undertaken considerable efforts to advance MPL-based bioprinting, resulting in numerous promising applications across various domains, including drug testing, tissue engineering and the development of organs-on-a-chip systems. One of the key achievements in MPL bioprinting is the creation of intricate and functional tissue constructs with exceptional resolution. MPL enables the realization of complex 3D structures that closely mimic the architecture of native tissues.

Despite its numerous benefits, MPL has long been known as a comparatively slow 3D printing technology. Increasing the throughput is crucial and requires advancements across hardware, software and materials. By leveraging the Adaptive Resolution in combination with state-of-the-art cytocompatible photochemistry, the NanoOne Bio can process hundreds of cubic millimeters of cell-containing hydrogel within an hour. The NanoOne Bio is compatible with a wide range of substrates, and its software includes a Plate Dock feature, enabling automatic printing of separate jobs at precise locations across the substrates.

Another significant development in MPL bioprinting is the increasing diversity of materials that can be processed. Researchers are exploring a wide range of biomaterials, including natural and synthetic polymers, and bioactive compounds, to expand the capabilities of MPL bioprinting and create more versatile and functional tissue constructs.

The advancements in MPL have propelled the development of microphysiological systems. These microengineered models, which replicate the structure and function of human tissue and organs on a miniature scale, offer a powerful platform for studying disease mechanisms, evaluating drug efficacy and toxicity, and advancing personalized medicine.

In conclusion, MPL bioprinting has made remarkable progress in recent years, with promising applications emerging in diverse biomedical fields, such as tissue engineering and organs-on-a-chip. While challenges remain, ongoing research and innovation are driving the field forward, paving the way for the development of advanced biomedical solutions and personalized therapies. The availability of the NanoOne and NanoOne Bio system, along with the portfolio of suitable materials, provide a powerful tool for research and development teams to be able to focus on advancing applications and their translation.

# Acknowledgements

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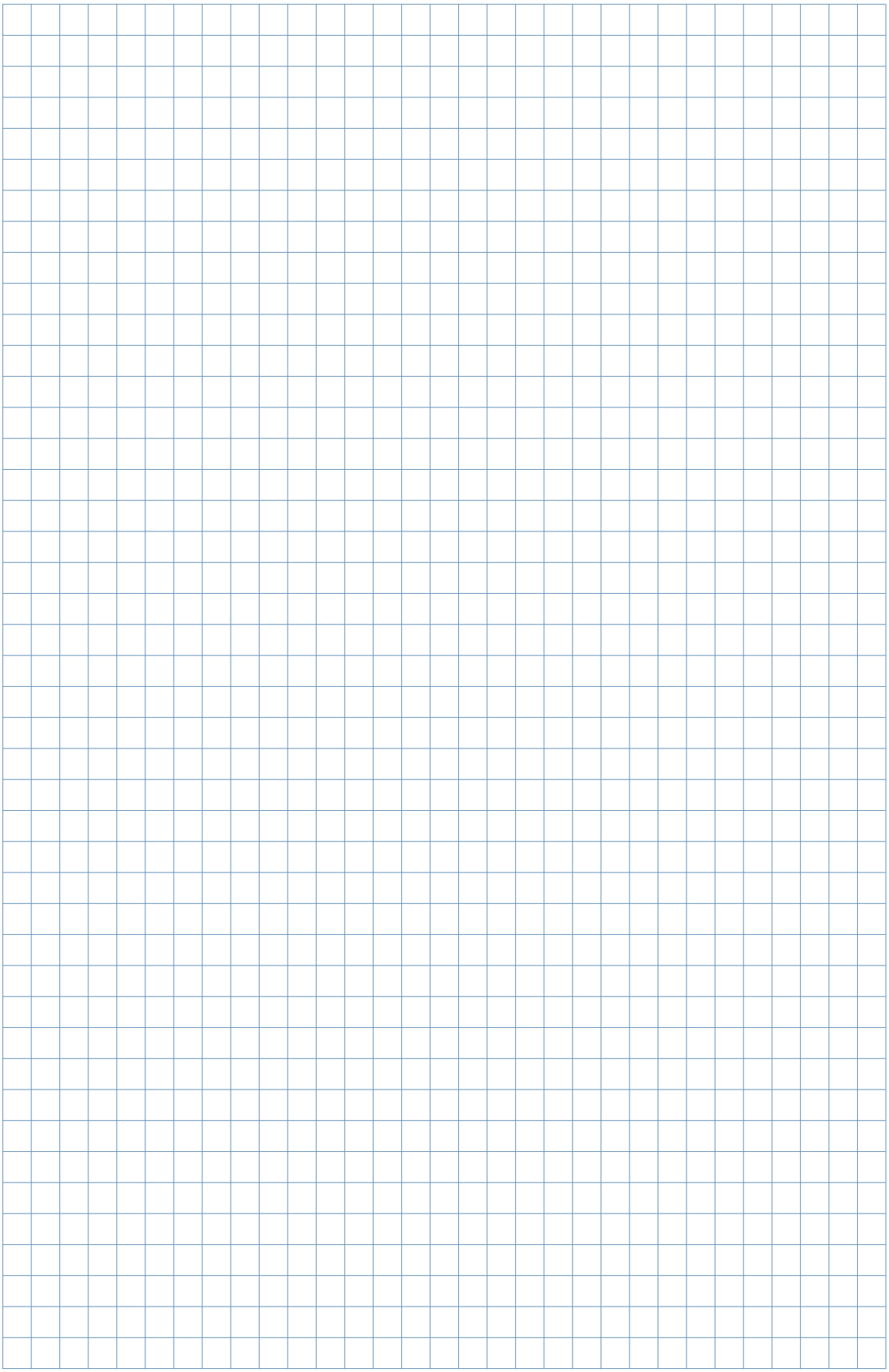
# Notes



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