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Overview/Abstract*

An initial patterning specification covering the generation of a spatially and temporally modulated laser source and the scanning methodology used to address three-dimensional locations in the goal volume is presented. Additional information concerning the relationship and requirements on the laser source with respect to the polymerisation of the hydrogel are also introduced. Risk analysis for the presented areas is also included.

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Led by

Name	Gustaf Mårtensson	**Partner**	MYCRONIC	**Date**	**22/11/2019**
Name	Robert Eklund	**Partner**	MYCRONIC	**Date**	**22/11/2019**

Reviewed by

Name	Francesco Pampaloni	**Partner**	GUF	**Date**	**04/12/2019**
Name	Elena Martínez	**Partner**	IBEC	**Date**	**10/12/2019**
Name	Núria Torras	**Partner**	IBEC	**Date**	**10/12/2019**
Name	Javier Selva	**Partner**	IBEC	**Date**	**18/12/2019**

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1. Background

To unleash the full potential 3D cell culture models, at practice advanced imaging techniques that allow extraction of relevant information are needed. In this context, one scientific breakthrough has been the development of Digital Scanned Light-Sheet Microscopy (DSLM).8,9 In DSLM, illumination is provided through a light-sheet, generated by rapidly scanning a µm-thin laser beam vertically through the specimen. Light-sheet based imaging makes high resolution compatible with fast acquisition, being then ideal for long-term life imaging of biological processes. This project proposes to exploit these unique characteristics of light-sheet illumination in the bioprinting field.

The electronic industry has reached impressive breakthroughs in realizing laser mask writers that allow writing complex patterns in light sensitive media at impressive speed and precision. This sophisticated technology is used for the production the high-resolution display in our every day's electronic gadgets, but it has been never exploited to achieve a substantial leap forward in bio-printing.

By gathering these key advances in different scientific domains, BRIGHTER will develop a new generation of bioprinting technologies able to produce tissue surrogates in an ultrafast, highly precise and cost-efficient manner. Light-sheet illumination from two directions combined with high-resolution digital photomasks will be used to produce the localized crosslinking of cell-laden materials, creating 3D structures in a top-down lithography process (Fig. 1). Our novel approach will make for the first-time high resolution fully compatible with high speed in a bioprinting process. Ultimately, this will lead to constructs that will faithfully mimic the heterogeneous architecture, biochemical and mechanical properties of cell microenvironment within tissues without compromising cell viability due to long bioprinting periods. We envision that the unique tailoring capabilities of our technology will be key in providing cells with instructive environments that guide their self- organizing abilities to form functional tissues.

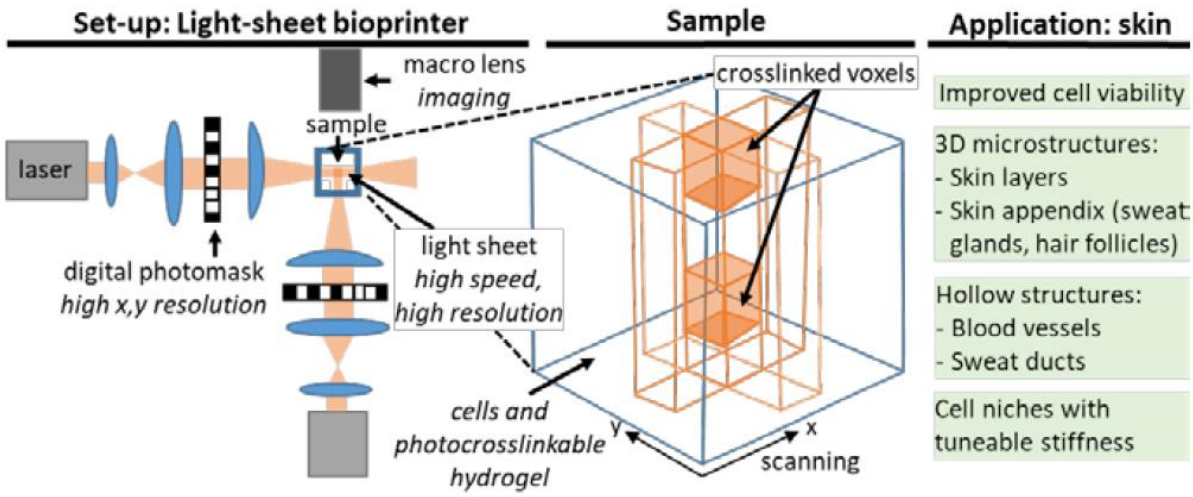


Figure 1: Conceptual vision of the BRIGHTER setup.

The proposed optical setup is based on a two-stage process where 1) Mycronic AB (MYC) will provide a temporally and spatially modulated light source using a special version of their standard patterning setup utilising an acoustic optical modulator and an acoustic optical deflector into 2) the light scanning set from Göthe-Universität Frankfurt (GUF). A detailed description of the respective setups is presented below. A SWOT analysis is presented to clarify the strengths, weaknesses, opportunities and threats to the proposed patterning design.

2. Technical design

T1.1. Generation of the light-sheet (GUF, MYCRONIC). Light-sheet will be generated by a pair of galvanometer scanning mirrors coupled with an F-Theta lens (scanning lens) (Fig. 2a, b). A collimated Gaussian laser beam will be scanned. A homogeneous DSLM illumination profile equivalent to a light-sheet in SPIM, is obtained by applying a constant scan speed and a constant laser power during sample illumination. The illumination optics also include a tube lens and a low N.A. and low magnification illumination objective lens (2x-10x and N.A =0.1-0.3). It is expected with this set-up to have a height of light-sheet of ~ 4 mm using a 5x objective lens. Light-sheet illumination will be spatially and temporally structured, as the laser intensity can be digitally modulated during the scan process.

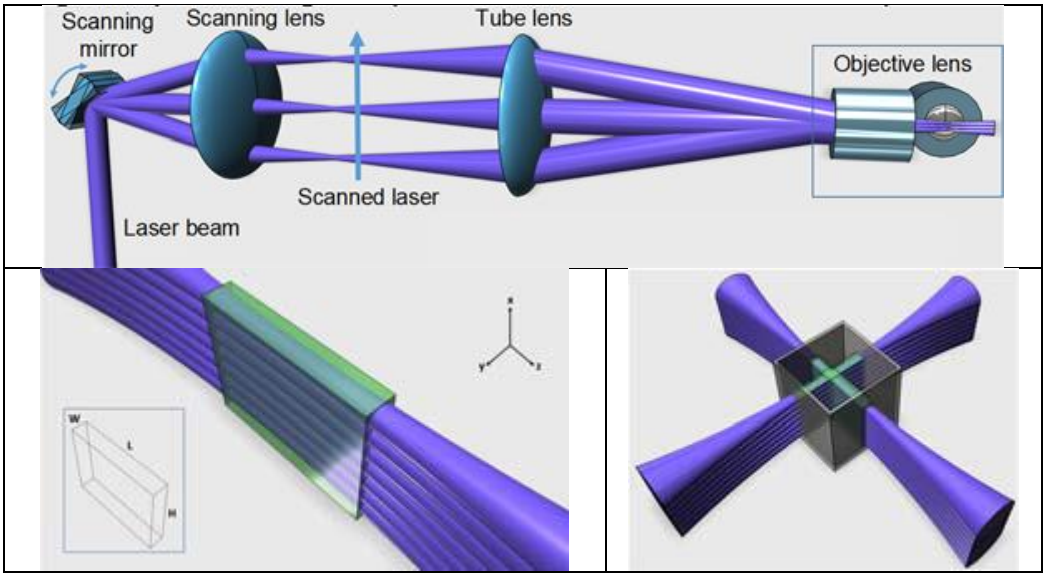


Figure 2. (a) Scheme of the light-sheet generation, (b) light-sheet size parameters and (c) 90º crossed set-up.

T1.2. Development of a system including two light-sheet illumination sources (**GUF**). Two light-sheets will be combined (Fig. 2c) to increase both the illumination energy required for the hydrogel polymerization and the spatial resolution. In this configuration, the hydrogel polymerization occurs at the cross-point of the two light-sheets.

T1.3. Coupling light-sheet with a pattern generator (**MYCRONIC**, GUF). A high-resolution digital photomask along with the required relay optics will be placed before the scanning mirror. An acousto-optic modulator (AOM), which provides high resolution at high speed, would be adapted to light-sheet at λ = 404 nm. The spatial light modulator will be equipped with dedicated control software to bring dynamic resolution capabilities.

T1.4. Development of the imaging system (GUF). The bioprinting system will be equipped with modern objective lenses (e.g. Nikon CFI75 LWD 16xW), which offer high N.A., long working distance, low magnification, and large field of view, and are ideal to image large specimen with light-sheet-based microscopy.

T1.5. Development of the bioprinting chamber (**GUF**, CED, IBEC, TECHNION). Dedicated cuvettes (Fig. 4c) will be produced to contain the bioinks to be printed. A fluorocarbon polymer (FEP) will be tried as a first option, as it provides excellent mechanical and optical properties (the refractive index is 1.34, very close to water, so it will avoid aberrations and impairments of the optical resolution). Compatibility of cuvettes with bioprinting materials and procedure and cell biocompatibility will be also performed in this task.

2.1 MYCRONIC

The pattern generation system that is utilised in the commercial systems from MYC is a raster scan system based on acousto-optic deflection and modulation of the exposure beam [2], see Figure 3. A key benefit with the system architecture is that the acousto-optic components allow extensive calibration of the beam, which improves the CD control. Both the positional linearity and the dose for the sweep of the laser beam are calibrated. The sweep calibration simplifies the optical design since there is no need for the f-theta optics required in the case of deflection using a rotating polygon. Because a single optical component, the acousto-optic deflector (AOD), is used to generate all sweeps the same well-controlled sweep is used throughout the exposure, which improves the CD uniformity. The beam control allows a single pass writing strategy since there is less reason to reduce errors by averaging using multiple passes.

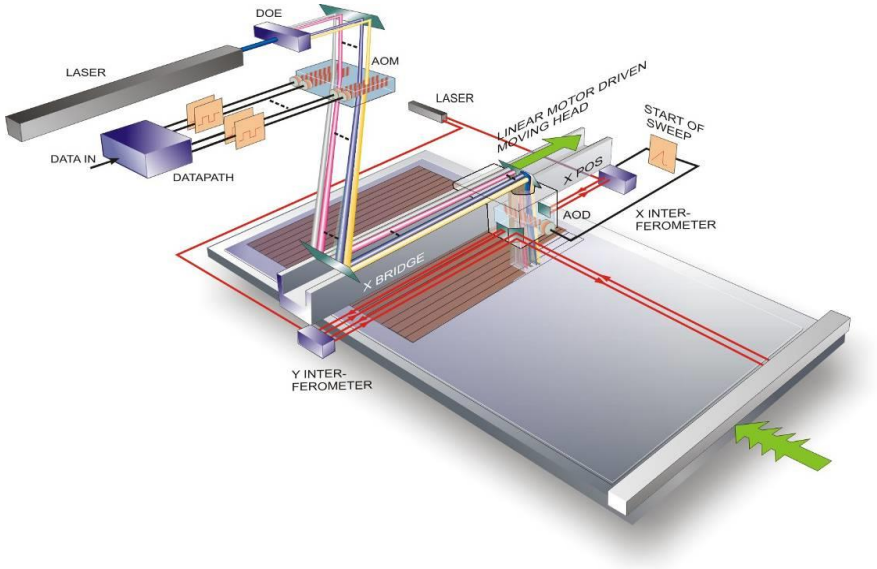


Figure 3: The pattern generation design is a raster scan system with acousto-optic deflection and modulation of the exposure laser.

2.1.1 Original data

The three-dimensional pattern that is to be realised in the hydrogel is initially created in a digital format that supplies information about every voxel (elemental spatial volume) in the sample. The necessary information is

a) voxel index
b) spatial information (x, y, z)
c) dose intensity

This list may be supplemented with additional fields, as they are found to be necessary.
The goal voxel size for the patterning example proposed is 10 x 10 x 10 μm^3.

2.1.2 Data path

The original data described in 2.1.1 is the result of the manipulation of the laser beam over time. Therefore, a complementary input dataset is necessary to control the modulation and deflection of the continuous laser light that is fed into the system. This dataset will be created using a conversion software that synchronises the acousto-optic components with respect to a given signal that is used as a reference. This signal is called the Start-of-Sweep and is denoted SOS. It is proposed that the Galvano mirror is used to generate the SOS signal due to the inertia of the mechanical system that is assumed to possibly introduce effects that are easier to follow and compensate for using the optical system (Figure 4).

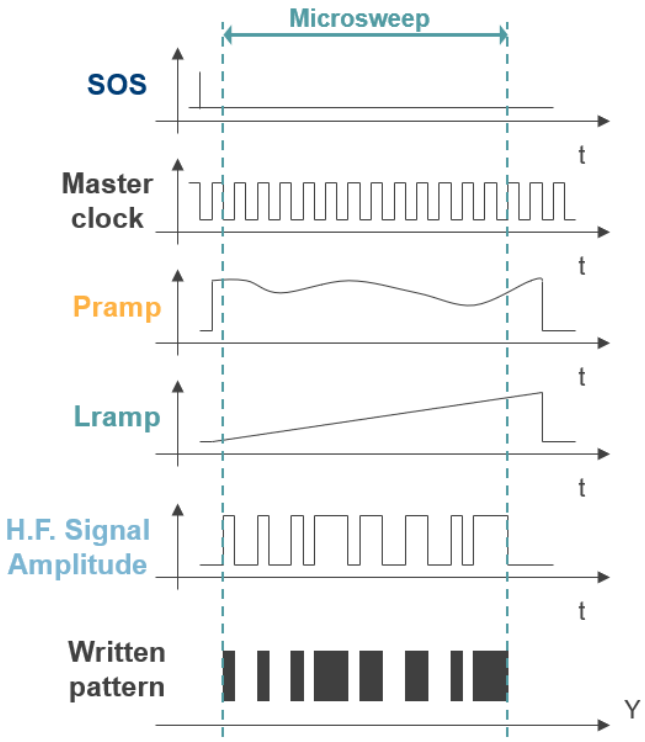


Figure 4: The synchronisation of an external event is used to localise the subsequent patterning.

The data path is shown in Figure 5, which also shows the optical assembly from MYC and the scanning assembly from GUF. It is generated in the related computer array, triggered by the SOS and delivers signals to control the acousto-optic modulator and acousto-optic deflector. The deflected and modulated laser beam is then delivered to the galvano mirror. The beam is further split and scans through the patterning sample.

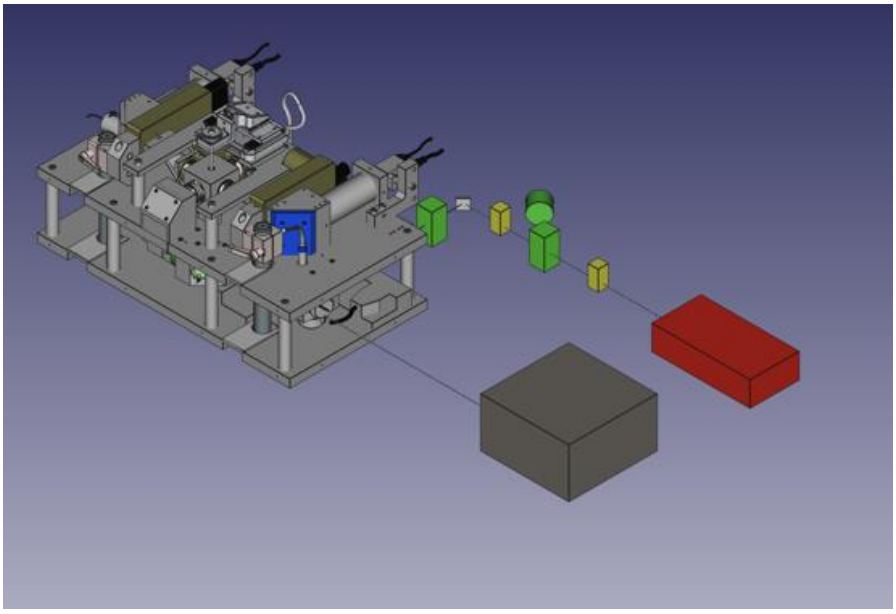


Figure 5: Fundamental setup of the BRIGHTER project.

2.1.3 Acousto-optic modulator (AOM)

The MYC setup involves both the temporal and spatial control of the beam. The temporal control is made possible by the acousto-optic modulator (AOM). The principle of the AOM is the utilisation of a crystal that is actuated using a piezoelectric material in order to create sound waves changes which will

impose a change in the index of refraction. The resulting periodic index modulation will scatter the incoming light. The angle of the output beam is proportional to the sound frequency, which allows manipulation of the beam to either pass through an aperture and subsequently pattern in the sample volume, or be collected in a beam dump and thus not pattern in the sample volume.

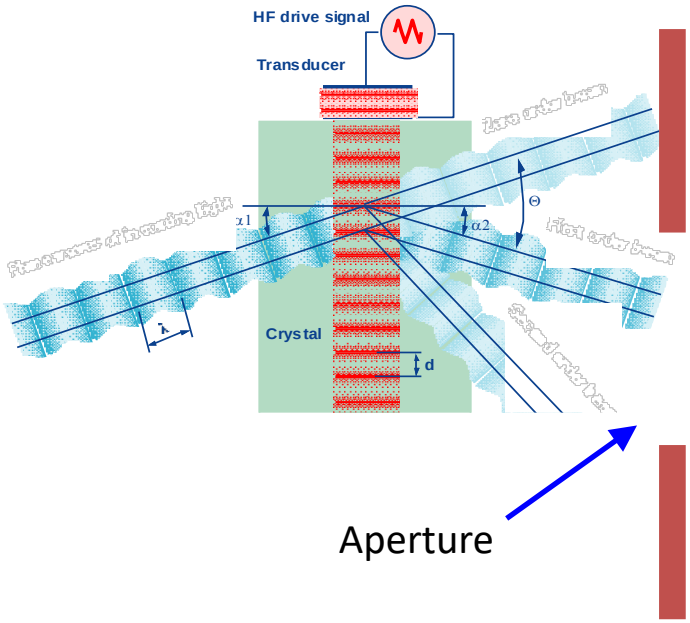


Figure 6: Schematic of a acousto-optic modulator.

An example of an AOM can be seen in Figure 6. The AOM is sensitive to the wavelength of the laser light, so a change of light wavelength will introduce changes in the optical assembly that are not trivial.

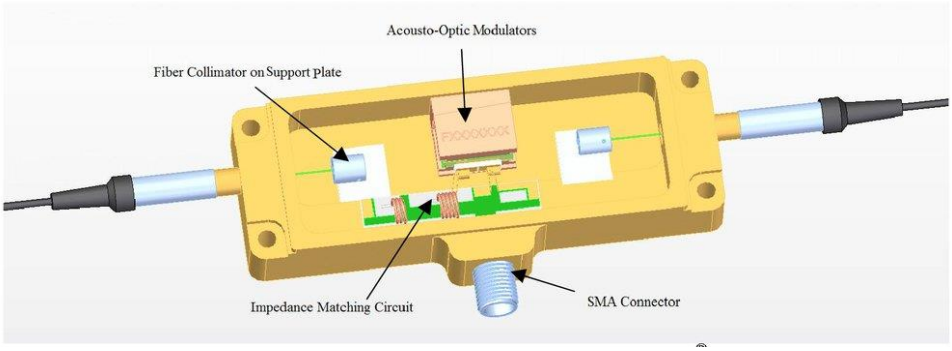


Figure 7: Example of the commercial acousto-optic modulator.

The light intensity in the first-order beam is proportional to the frequency of the sound introduced into the crystal and we can, therefore, manipulate the amplitude of the beam through the adjustment of the beam. The intensity of the first-order beam is modulated by changing the amplitude of the AOM drive signal. Only the first-order beam is used (Figure 7).

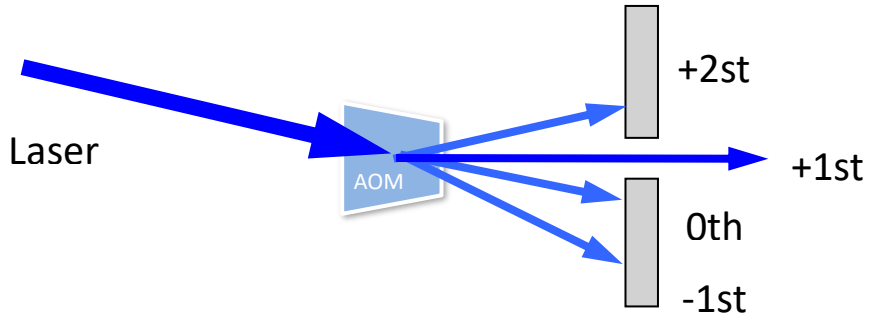


Figure 8: Amplification of the first order beam in the AOM.

2.1.4 Acousto-optic deflector (AOD)

The acousto-optic deflector utilises the same physical principle of the AOM, in order to introduce a sweep of the light beam or beams. In Figure 8, the case of a multiple beam configuration is shown which starts with a diffraction optical element (DOE) that splits the beam into multiple beams and controls their position in the AOM, which controls the amplitude and converts the pattern data to exposure light.

The AOD is a single-channel device that generates a sweep for all beams and controls the power and linearity over the sweep. In the BRIGHTER project, a single beam will be deflected and swept over the Galvano mirror which is the entry point into the GUF setup.

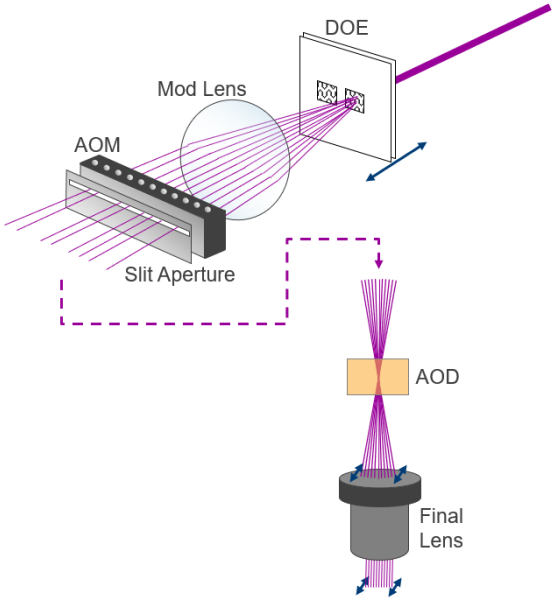


Figure 9: Schematic of the situation of the AOD in connection to the DOE and AOM.

2.1.5 Relay optics

Relay optics is necessary to provide the beam with adequate spot size and divergence. Relay optics is needed before the modulator, before the deflector and after the deflector, as well.

2.1.6 Wavelength

The proposed patterning system will utilise a λ = 405 nm laser source. There are a number of available manufacturers of systems that can provide λ = 405 nm, such as Toptica and its product Toptica iChrome CLE, https://www.toptica.com/products/multi-laser-engines/ichrome-cle/. An advantage of this type of product is that it can provide multiple laser wavelengths.

The choice of laser wavelength is connected both to the sensitivity of the hydrogel and its polymerisation, but also to the optical setup, specifically the acousto-optic modulator and deflector.

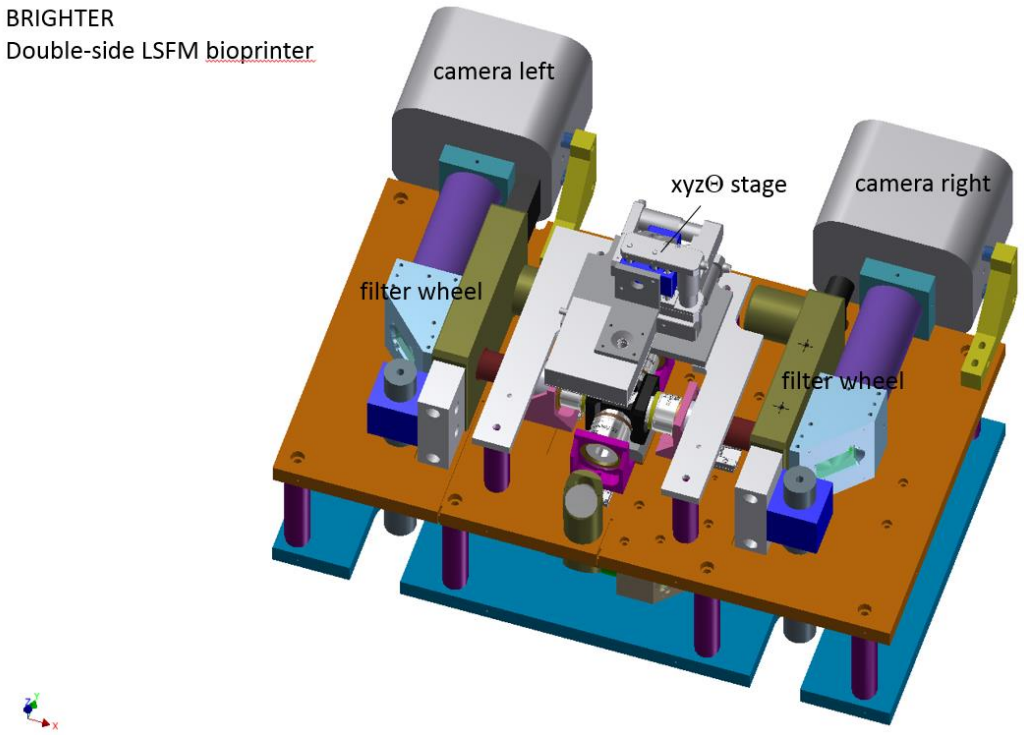
2.1.7 Number of beams

It is possible to generate a multiplicity of beams and control them individually in the optical assembly. In the commercial pattern generators offered by MYC, a set of fifteen (15) beams are controlled individually. In the proposed patterning design, a single beam will be implemented, but consideration will be taken to the necessary design changes that are connected with the addition of additional beams to the setup if one would like to enable a throughput increase.

2.2 GUF

2.2.1 Basic design principles

We pursue the design and building of the **Light Sheet Bioprinter** (LSFM-Bioprinter) through a series of iterations involving multiple working set-ups. The goal is achieving a throughout understanding of the photochemical and photophysical parameters required for a rapid (in the timescale of seconds) and high-resolution (≤10 μm) polymerization of the hydrogel in a volume up to 10^3 mm^3 (=1 ml of hydrogel/cell mixture). Specifically, the required laser power (at a wavelength of 405nm) and the scanning rate of the pattern are identified in the process. The first iteration working set-up implemented for the BRIGHTER project is shown in Figure 10.



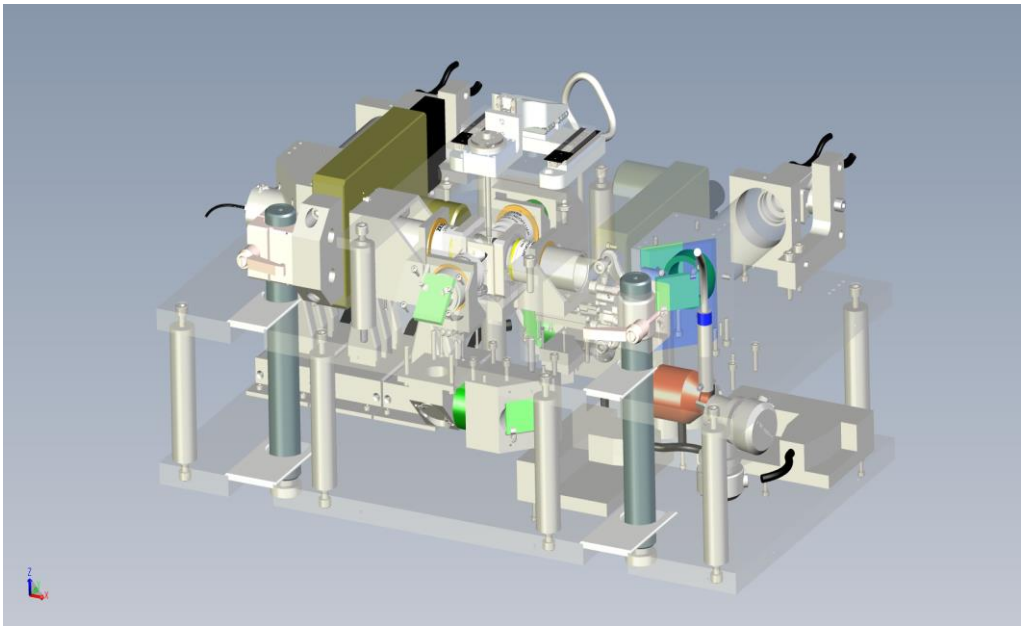


Figure 10: Overview of the first-iteration double-side LSFM Bioprinter realized for BRIGHTER.

The system features two-side light-sheet illumination and two-side fluorescence detection. The main component of the bioprinter is listed in **Table 1**.

Table 1 – Main components of the LSFM bioprinter

Component type	**Component features**	**Company**
Multi-laser engine iChrome CLE	Output wavelengths λ = 405, 488, 561, 640 nm. Output power for each line = 20 mW, max modulation speed 1 MHz.	Toptica Photonics
dynAXIS S	2x galvanometric mirrors scanners, Step response time 1% of full scale = 0.25 msec.	ScanLab
CMOS camera DMK 23UX174	CMOS, USB 3.0 monochrome industrial camera • $^{1}/_{1.2}$ inch Sony CMOS Pregius sensor (IMX174LLJ) • 1,920×1,200 (2.3 MP), up to 54 fps • Global shutter • Trigger and I/O inputs	ImagingSource
Illumination objective lenses 2.5x	Air objective lens EC Epiplan-Neofluar 2,5x/0,06 M27 (a=15,1mm)	Carl Zeiss
Detection objective lenses, 10x, 20x, 40x	Water dipping objective lens W N-Achroplan 10x/0,3 M27 (a=2,6mm)	Carl Zeiss
XYZΘ-piezo stage assembly	Linear piezo stick-slip stage with nanometric range resolution and long travel. SLC series.	SmarAct

The optical set-up of the LSFM bioprinter is shown in Figure 11. The optical fibre from the multi-line laser engine directs the laser beam to the galvanometric mirror scanners. One of the two scanners generates the light sheet, while the second one directs the light sheet to the illumination objective lenses. Two-side illumination or single-side illumination is possible. After passing the scan lens, the laser beam is reflected by the mirror surface of the prism and by the two mirrors M1 and M2. The next optical element is the tube lens, which injects the collimated beam in the back pupil of the illumination

objective lens. The illumination lens is used for both polymerizing and for fluorescence imaging. The detection objective lenses collect the fluorescence signal. Then, the emitted light passes through the emission filters in the filter wheel and it is imaged by the CMOS cameras.
The LSFM bioprinter is operated with a software interface written in C++, which allows controlling or the relevant parameters, including laser power, exposure time, positioning of the container ("FEP cuvette", see below) containing the liquid hydrogel solution, and patterning.

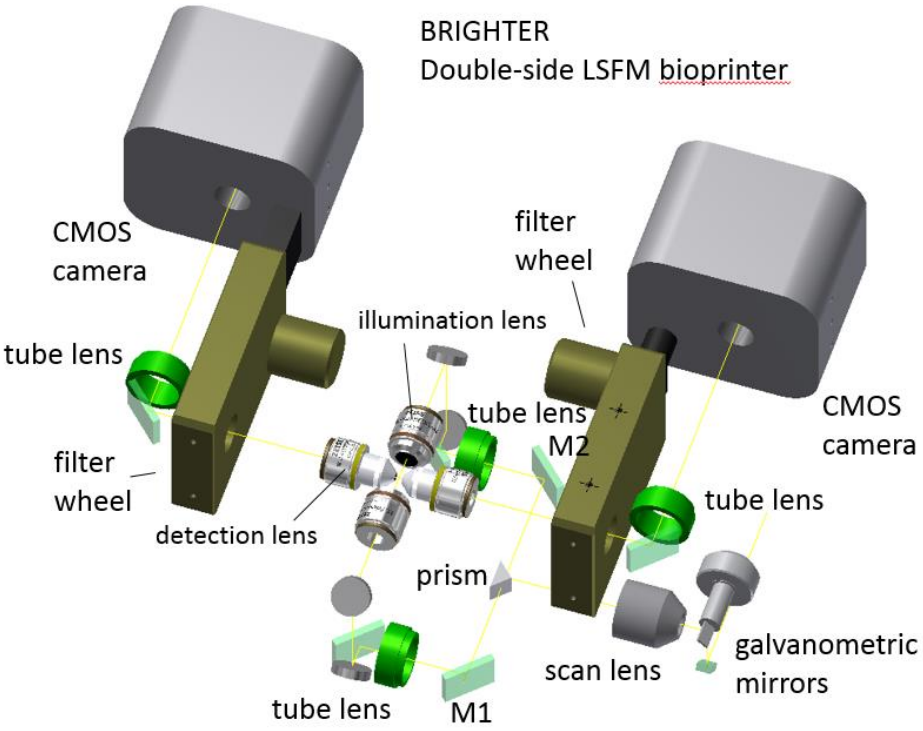


Figure 11: Optical set-up of the LSFM Bioprinter realized for BRIGHTER.

For photo-polymerization, the hydrogel solution is pipetted in a specially designed and custom produced fluoro-ethylene polymer (FEP) cuvette, which ensures nearly 100% transparency at all the relevant wavelengths, features a wall thickness ≤50 μm, a refractive index n = 1.34 (close to the one of water, 1.33) and it is both chemically inert and biocompatible. The cuvettes are produced by vacuum-forming of the raw FEP foil. The cuvette has a square cross-section and has a size of the of 2mm x 2mm at the base and a height of 4mm (Figure 12). This size has been determined for the preliminary and optimization experiments. Accordingly, to the project's requirements, the final size of the cuvette will be 5mm x 5mm x 5mm (minimum) or 10mm x 10mm x 10mm (preferred).
During operation with the LSFM bioprinter, the cuvette filled with the hydrogel solution is connected to the XYZΘ stage and positioned for polymerization at the selected starting spatial coordinates. During photo-polymerization, the stage translates the cuvette accordingly to a pre-determined pattern set with the software interface.

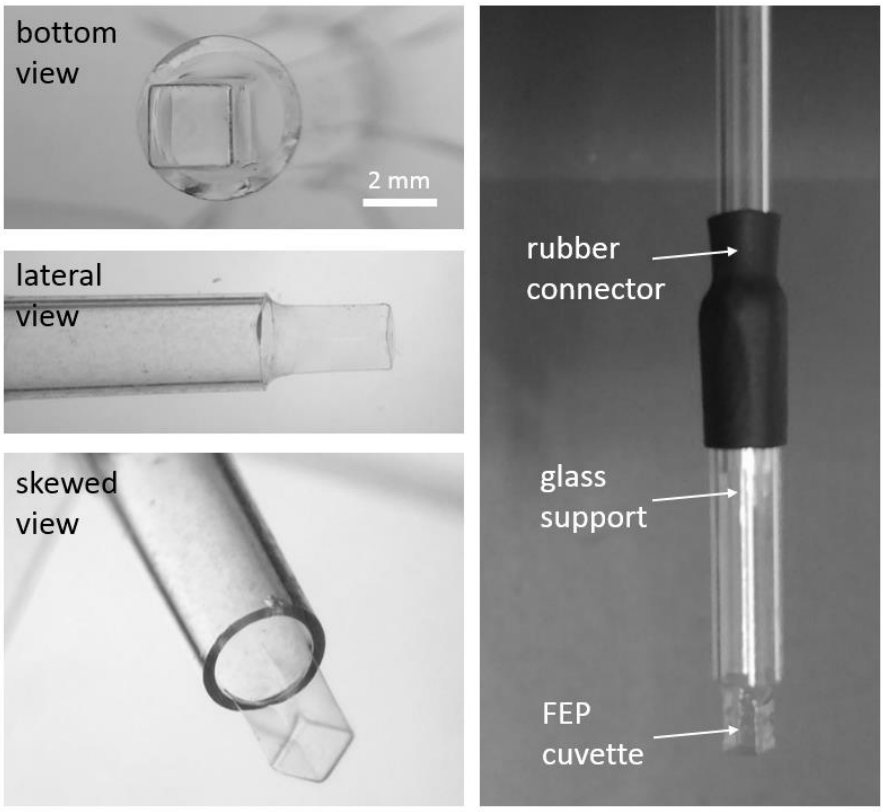


Figure 12: Optical set-up of the LSFM Bioprinter realized for BRIGHTER.

The FEP-cuvette allows for a straightforward preparation for printing of the hydrogel solutions. The production approach by using vacuum forming allows great flexibility in determining the cuvette size and shape. Thus, other cross-sectional shapes than a square can be easily realized (e.g. octagonal or cylindrical).

2.2.2 Focus strategy

The focusing strategy is flexible and includes multiple units that can work independently. The optimum focusing strategy, resulting in a combination of the individual focusing/positioning units is going to be identified further during the project. The available focusing options are summarized in Table 2. Figure 13 shows the reference coordinate system of the bioprinter.

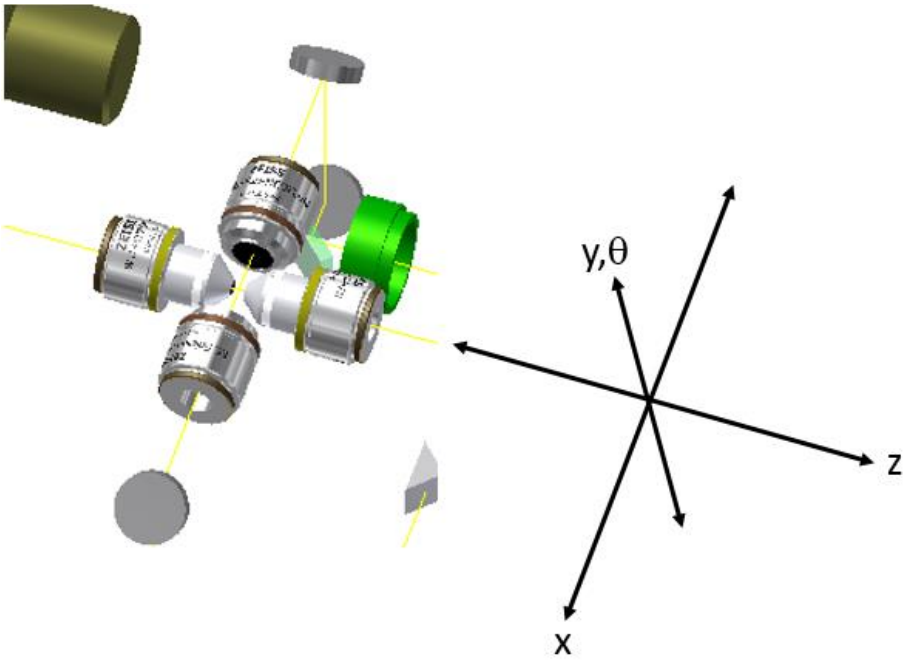


Figure 13: Reference coordinate system of the SLFM bioprinter.

Table 2 – Available focusing options

Unit	**Action**
XYZΘ-stage	Translation along the Z-axis.
XYZΘ-stage	Rotation of an angle Θ along the Y-axis.
XYZΘ-stage	Translation along the XY axes.
Galvanometric scanner	Pattern scanning on the XY-plane.
Galvanometric scanner	Light-sheet translation along Z.
Galvanometric scanner	Skewing of the light sheet on the XY-plane.
A piezoelectric slider at the illumination objective lenses	Light-sheet focus translation along the X-axis.

3. Hydrogel

Testing and fine-tuning of the laser power and exposure time required for the hydrogel photopolymerization are ongoing. So far, the following hydrogel formulations have been preliminary tested (Table 3):

Table 3 – Tested hydrogel formulations (λ=405 nm)

Hydrogel #1	GelMA-PEGDA-LAP (IBEC)
Hydrogel #2	PN-PEGLink-LAP (Cellendes)

The results indicate that the polymerization at λ=405 nm is possible for both hydrogels. Since both hydrogels are highly transparent, and their optical properties do not change upon photopolymerization, we have devised a method for the imaging of the polymerized shape by the same light sheet system used for printing. Accordingly to this method, high molecular weight dextran, labelled with the fluorescent dye fixed is pipetted in the FEP-foil cuvette after polymerization. The FITC-dextran can only diffuse in the non-polymerized regions of the hydrogel solution. Consequently, the polymerized

shape is imaged as a negative (black) 3D region inside a bright background produced by the FITC fluorescence (Figure 14).

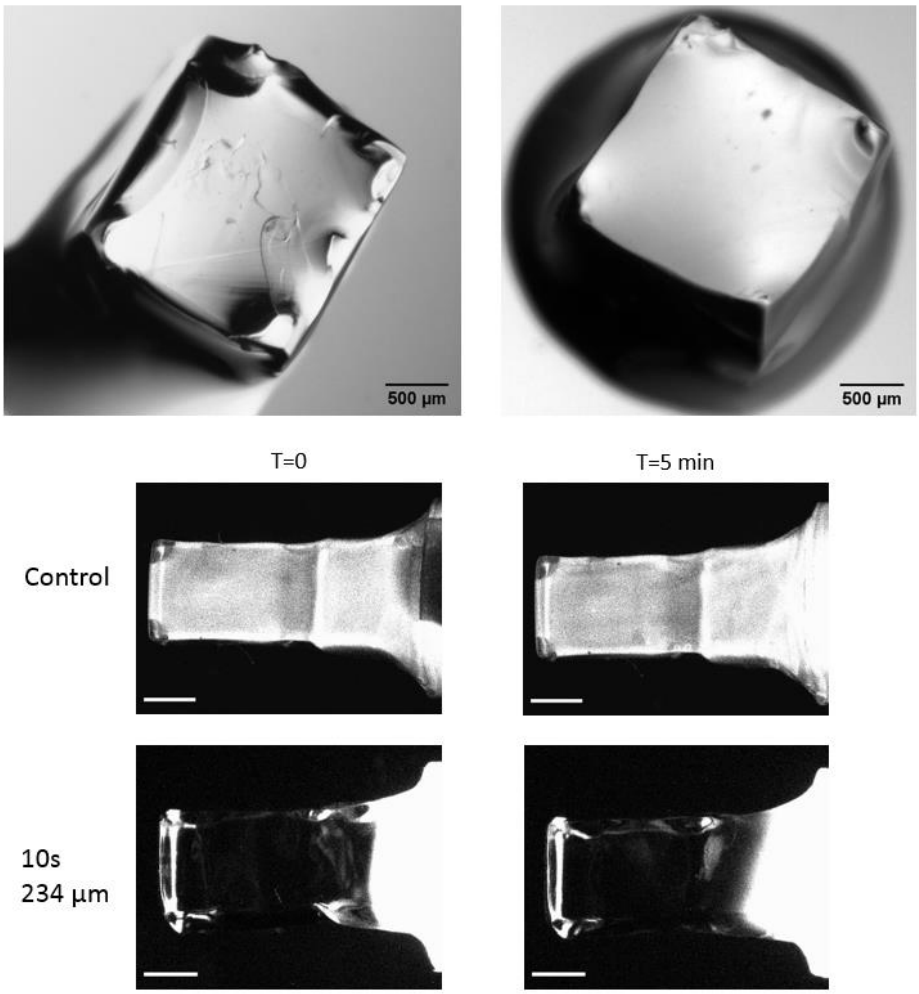


Figure 14: Top: examples pf polymerized cubes of GelMa-PEGDA (left) and PN-PEGLink (right). Bottom: method to determine the 3D shape of the polymerized hydrogel regions by dissolving FITC-dextran in the FEP-cuvette after polymerization.

4. Risk Analysis

4.1 Gap Map

A technical gap analysis has been initiated and is continuously updated during the development work performed in the project. The technical gap analysis has focused on the patterning aspect of the project but could be extended to include the more biological aspects of the project (Figure 15).

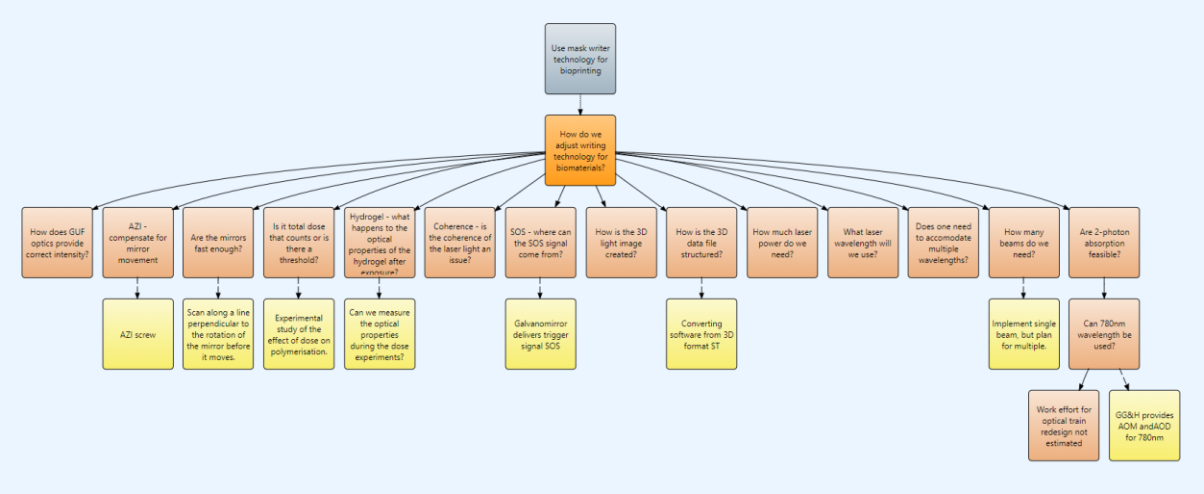


Figure 15: Technical gap analysis of the patterning design to be utilised in the BRIGHTER project.

There are a number of questions concerning technical solutions, where both primary and secondary solutions have been formulated. The primary questions that have been identified as requiring a special degree of attention are

1. Implementation of the positioning of focus within the sample
2. Implementation of the extent of focus within the sample
3. Degree of the sensitivity of polymerisation to radiation dose
4. Degree of the sensitivity of polymerisation to the radiation intensity
5. Resolution of polymerisation in the sample

Alternative optical solutions are being considered as an alternative to patterning with a movement of the focus position of the patterned light surface within the sample. An alternative could be to use a tomographic alternative where a rotation of the sample is utilised.

4.2 SWOT

An attempt has been made to analyse the patterning principle from a SWOT (Strength – Weakness – Opportunity - Threat) perspective. The SWOT analysis is shown in Table 4.

Table 4: SWOT analysis of patterning principle.

SWOT ANALYSIS OF PATTERNING SOLUTION	
STRENGTHS	**WEAKNESSES**
- The rapid rate of patterning - High resolution - The flexibility of choice of wavelength	- The threshold of sensitivity for polymerisation - Focusing on the laser in the sample - Expansion of patterning volume -
OPPORTUNITIES	**THREATS**
- Possibility of pattern and image at the same time - Higher patterning rates - Differential patterning using multiple wavelengths -	- unsure of polymerisation effect of radiation - -

1. References

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