MSLASpheroidStamp: 3d cell spheroids for everyone

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Abstract

3D cell cultures, such as cell spheroids, are actively used in biology for modeling biological processes, studying cell-cell interactions and screening various drugs and are becoming indispensable objects in cell culture laboratories. There are many methods for producing spheroids, varying in cost and convenience. One of the most handy and affordable is the use of agarose microwells. We have developed approaches to fabricate agarose microwells in standard culture plastic with the assistance of a hobby-grade MSLA 3D printer. The use of 3D printing allows you to customize microwells in a wide range of shapes and sizes, and scale the production process from a few spheroids to tens of thousands. We demonstrated the ability to cultivate spheroids in a glass bottom dish and dynamically observe their formation, perform in situ optical clearing, and demonstrated the ability to study the cytotoxicity of various substances and nanoparticles in 96-well plates.

And finally, in this article we describe the difficulties and limitations of our approach and suggest ways for solving them, allowing you not only to reproduce it, but also to adapt it to the specific needs of your laboratory, using 3D models and our instructions.

Introduction

Recently, three-dimensional cell cultures like spheroids and organoids have gained equal importance in laboratory research compared to traditional suspension or adherent cell cultures. The behavior of cells in 3D culture is similar to their interactions and growth in the animal body, and data obtained from experiments on 3D cultures can be more valuable for drug development [https://doi.org/10.1186/s12951-021-01213-8] and research in developmental biology [https://doi.org/10.1002/adhm.202000608] or the study of tumor metastasis [https://doi.org/10.1016/j.critrevonc.2021.103511]. Despite the numerous benefits, 3D culture demands increased effort and expenses in production. In this study, we suggest employing a do-it-yourself (DIY) method to lower costs and enable the affordable production of cell spheroids in any biological laboratory.

There are many ways to make cell spheroids: using nonadherent cultural plastic, hanging drop technique, agarose 3D microwell technique [https://doi.org/10.1177/0963689720937292] or using specialized viscous media such as Matrigel [https://doi.org/ 10.1054/bjoc.2001.1967].
Matrigel and other analogs are an almost mandatory matrix for culturing organoids, but its use is expensive and not always suitable for high-throughput screening of drugs.
Nonadherent petri dishes do not provide control over the spheroid's size, which negatively affects the reproducibility of the experiment [https://doi.org/10.1002/biot.201700417].
Hanging drop technique is a rather labor-intensive and poorly reproducible procedure.
Agarose 3D microwell is a simple, accessible and reproducible technique that does not require large expenses, which is why we chose this method. There are two approaches: first - the use of stamps that make an impression - microwell in agarose or agar poured into a container (e.q. dish or well of a culture plate), the second - the use of a silicone mold with micropins into which agarose is poured and, after hardening, agarose mold removed and transferred to the container. We decided to replicate both approaches and study how and where they are applicable. At the same time, our task was not just to implement such a project, which has already been partially done, but also to make it accessible for reproduction by the general public, make it DIY.

In recent years, the term DIY involves the use of a 3D printer as a device that allows the reliable and reproducible production of complex objects. A 3D printer in a modern scientific laboratory, including a biological one, is a relevant device that allows you to save money and time by manufacturing objects directly in the laboratory [https://doi.org/10.1002/advs.202202610]. In the problem that is solved in our article, 3D printing is not something new. For example, a 3D printed (using an FDM printer) system for producing spheroids using the hanging drop method is described in https://doi.org/10.1038/s41598-019-56241-0.

However, FDM printing is a relatively rough technology for producing such miniature objects as microwells for cell spheroids. Hence, the more preferable method is photoplastic 3D printing of various types. In this area, authors rarely delve into the details of the printing process itself, which makes these approaches difficult to reproduce. For example, the use of a 3D printed stamp for the production of microwells in the cells of a 96-well culture plate is described [10.1177/2472630318756058], but the authors do not describe the 3D printing process in detail, limiting themselves to only mentioning the company that made the stamp. From the above illustrations, it can be assumed that the technology used was laser stereolithography (SLA), which is an expensive method, compared to MSLA, which has now become cheaper. In another work, a professional MSLA 3D printer was used to make stamps, the cost of which exceeds 150,000 USD, which is also a significant amount for many laboratories [10.1089/3dp.2019.0129]. At the same time, the features of the printing process are not reflected in this work. An interesting approach is that the authors use a stamp with a hole through which agarose is poured (instead of first pouring angarose and then installing the stamp). A slightly different technique was used by the authors of the article, who used a 3D printing form (made using AutoDesk Ember STL, the price of which is approximately 6000 USD) [10.1088/1758-5090/ab30b4] to produce cells in a polyacrylamdine gel applied to a glass substrate in which they were cultured spheroids. In a recent paper [10.32607/actanaturae.11603], the authors used a 3D printing stamp to make depressions in agarose, however, with the exception of the printer model (far from the cheapest FormLabs Form3, USA) they did not provide any details about the printing process or the design features of their stamps, which limits the ability to reproduce this approach.

In this article, we set out to study the limitations and possibilities of low-cost MSLA 3D printing technology and develop a set of approaches to affordable and customizable production of cell spheroids. In this article, we emphasize the importance of DIY, not only showcasing what we have done but also providing detailed instructions on how to replicate and adapt it to your specific needs, highlighting potential pitfalls along the way. The article and supporting materials show the designs of stamps for the production of microwells in a glass bottom dish, in 96 well plates, as well as molds for mass production of spheroids in 6 well plates and in culture flasks. STL files and editable 3D models of all objects described in the article are given in supplemental materials.

Materials and Methods

3d printing

The models were designed in the open source software FreeCAD (0.21.0), and the Chitubox (1.9.4) slicer was used for printing.

The MSLA 3D printer Anycubic Photon Mono (Anycubic, China) was used in the work (pixel size 50x50 microns, resolution 1620x2560, print area (130x165x82 mm). It is possible to use any other MSLA 3D printer available on the market. Anycubic Basic Translucent Green resin (Anycubic, China) was used for printing. Translucent resin was chosen because it allows objects to be studied using optical transmission and LCSM microscopes. Specific printing settings were selected by printing test objects (“Cones of Calibration” test was used) and are given in the supplemental materials (S1). The authors note that the settings may vary depending on the printer model and resin type, and must be selected for specific tasks and conditions.

After printing, the parts were separated from the stage and washed with isopropyl alcohol. Initially, in the first portion of alcohol in a plastic container, then in a cleaner one in the Cure and Wash Station (Anycubic, China) for 15 minutes and finally completely clean using a rinse bottle. Alcohol was removed from the printed products using compressed air from compressed air dusters, after which the products were cured, also using a Cure and Wash Station. Finished products were stored in plastic zip-lock bags. Stamps for 96 well plates were stored in empty boxes of 10-200 µl tips (S2).

Silicone molds

For the manufacture of secondary molds, two-component cast silicone based on tin Alcorsil 315 (China) was used. The silicone base was mixed with the hardener using a wooden spatula in a ratio of 100:2 base to hardener for 4-5 minutes. A dye was added to visually assess the quality of mixing. In addition, the dye allows the use of color coding to separate finished molds by well size (it is impossible to sign silicone products with a sharpie).

Next, the silicone was poured into a printing form, evenly distributed with a wooden spatula and vacuumed for 5-7 minutes in a vacuum chamber, pressure 6 kPa. Vacuuming removes small air bubbles that spoil the finished product. During vacuuming, some of the silicone may be lost, and to compensate for the losses, silicone with a hardener was added

to the mold. In this case, you can use silicone with the addition of another dye, which increases the color palette available for marking finished silicone molds. The cast silicone used in the work thickens within 20–40 minutes, depending on the temperature, and acquires final strength in 24 hours. After this, the stamps were washed with isopropyl alcohol and stored in ziploc bags to protect from dust.

Optical and confocal microscopy

Macro photos were taken using the macro camera of the Redmi Note 10 Pro mobile phone (MIUI Global 14.0.2).

For microscopy of test objects, stamps, wells, spheroids and cell cultures, an Olympus IX-71 (Olympus, Japan) inverted optical microscope with a Toupcam (ToupTek Photonics, China) UCOMS03100KPA digital camera with ToupView (4.11.19782.20211022) software was used.

A laser scanning confocal microscope LSM-710 (Carl Zeiss, Germany) was also used. The use of confocal microscopy allows analyze the objects in three dimensions, which is critical for microscopy of large objects such as printed mold parts and spheroids. Z-stacks of 3D printed objects were observed at an excitation wavelength of 405 nm and emission detection in the range of 410-550 nm, using an EC Plan-Neofluar 10x/0.30 M27 objective. During microscopy, the brightness correction function was used to correct the height of the subject to acquire the optimal image.

To observe the formation of a spheroid in dynamics, cells were cultured in an Incubator PM S1 (Carl Zeiss, Germany) at 37C.

Cell culture

Rhabdomyosarcoma cells (RD), Human Skin Fibroblasts (HSF) were obtained from Cell Bank of Institute of Cytology of the Russian Academy of Sciences (Russia), hTERT MSC ASC52telo cells obtained from Cell Bank of MSU (Russia). Cells were cultured T25 flasks (JetBiofill, China) or 6 well plates (JetBiofill, China) in DMEM (BioInnLabs, Russia) containing 10% fetal bovine serum (Intl Kang, China). Cell cultures were maintained in an incubator at a temperature of 37°C in a humidified atmosphere with 5% CO2. Cells were passaged after 90% confluency of the monolayer, every three days for RD culture and every five days for hTERT and HSF cultures. Cell detachment was performed using 0.25% trypsin-Versen solution (BioInnLabs, Russia). Cells were routinely checked for mycoplasma.

Agarose molds productions

We should start with a small but important comment. It is a big mistake to think that culture plastic (cups, plates, everything else) is standardized. We should start with a small but important comment. It is a big mistake to think that culture plastic (cups, plates, everything else) is standardized. In the course of this work, we have seen that almost all dimensions can vary markedly (down to the millimeter) from one manufacturer to another. The only truly reproducible value is the distance between wells in culture plates. The only really stable value is the distance between wells in culture plates. Accordingly, all stamp sizes, agarose volumes, and other things may vary slightly when using plastic other than ours. In the supporting materials for the article and in the project's git repository

(https://github.com/arteys/MSLASpheroidStamp), .stl objects of stamps and molds are given, as well as .fcstd files that can be edited to the size of the plastic used, which requires a certain amount of trial and error adjustment.

For the production of molds in glass bottom dishes and in 6 well plates, 2% agarose (Panreac Applichem, Spain) was used, for the production of molds in 96 well plates - 2.5%, both prepared in phosphate-buffered saline (Bioinnlabs, Russia). To prepare, 600 (750) mg of agarose was weighed into a 50 ml test tube, then 30 ml of PbS (pH 7.4) was added. After shaking, the agarose was gently heated in the microwave until melted (90-95C).

2% agarose (Panreac Applichem, Spain) was used for making molds in glass bottom dishes and 6-well plates, and 2.5% agarose was used for making molds in 96-well plates. For its preparation 600 (750) mg of agarose was taken into a 50 ml tube, then 30 ml of phosphate buffer was added. After shaking, the agarose was gently heated in a microwave until melted (90-95C).

Two approaches were used to construct the agarose molds. First, agarose was poured into a culture plastic (dish, culture plate), and then a MSLA printer-printed or silicone stamp was placed there. About 1.5 ml of agarose should be poured into glass bottomi dishes (here it is convenient to use a disposable Pasteur pipette). Agarose was dispensed into 96-well plates using a multi-channel pipette (65 µl each), drawn from a standard polypropilene reagent reservoir (JetBiofill, China). The volume of agarose must be sufficient: in a small volume, microwells are not formed; in a large volume, the adhesion of agarose to the stamp is too strong and the agarose mold can tear when stamp removed.

After agarose solidification (about 5-7 minutes) the stamp was carefully removed. This approach was used to make molds in glass bottom dishes to visualize spheroids using CLSM and in 96-well plates for cytotoxicity tests.

In another option, molten agarose was poured into a silicone mold. To avoid the formation of small air bubbles, the silicone mold under the agarose layer was smoothed with a 200 µL tip. After cooling, the agarose mold was carefully separated and transferred to a culture container and filled with culture medium for 30 minutes.. This was used to make spheroids on a large scale in six-well plates or T-125 flasks. When using molds with microwells smaller than 400 µm, due to surface tension the liquid did not fill all the microwells and bubbles were formed (S3). In this case, the agarose mold was briefly washed with isopropyl alcohol to reduce surface tension and then washed three times with DMEM.

Commercial silicone molds (Microtissues, Inc., USA) were used in the same way according to the manufacturer's recommendations.

Spheroid fabrication

Cells were grown to a monolayer in 6-well culture plates (approximately 3.1E+05 cells per well), then detached using trypsin solution, transferred to tubes and centrifuged (750 RPM, 5 min, 4C) after which trypsin was carefully removed as excess trypsin can impair spheroid formation. Cells were resuspended and the suspension was transferred to agarose mold at the rate of 1000 cells per microwell (or another number of cells if necessary, with dilution of the cell suspension with culture medium).

Spheroids staining

Spheroids were fixed with 4% formaldehyde for 1 hour. Before and after fixation, spheroids were washed twice with a phosphate-buffered saline (PBS). The fixed spheroids were stained with Hoechst 33342 (Bio-Rad Laboratoties, USA) (10 µl of the dye (1.1 mg/ml) were dissolved into 190 µl of PBS, adding 200 µl to the well for 1 hour).

Optical clearing of spheroids

Spheroids were cleared using several post-staining purification approaches.

Incubation in 88% glycerol solution [10.3389/fmolb.2020.00020]

A 99.5% solution of glycerol (Reachem, Russia) was used for the preparation of a saline solution. The agarose mold containing spheroids was filled with 2 ml 88% of the glycerol solution and was incubated in the dark at room temperature. The clearing solution was changed twice for every day of incubation. Clearing takes at least 48 hours.

ClearT method [10.1016/j.optlastec.2018.04.002]

Spheroids were incubated sequentially in 20, 40, 80, and 95% (v/v in PBS) formamide solutions (Fluka Analytical, Sweden) for 5 min each. Finally, incubate in a fresh 95% solution, then leave in the dark at room temperature for 15 minutes.

ClearT2 method [10.1117/1.JBO.23.5.055003]

The ClearT2 №1 clearing solution with a volume of 13 ml, contained 25% (v/v) of formamide and 10% (w/v) of PEG 8000 (Panreac, Spain). Clearing solution ClearT2 №2 with the same volume contained 50% formamide (v/v) and 20% (w/v) PEG 8000. Both solutions were carefully mixed until the PEG precipitate dissolved.

Spheroids were incubated for 10 minutes in solution №1, 5 minutes in clearing solution №2, twice. The spheroids were incubated in the dark at room temperature for one hour.

ScaleA2 method [10.1117/1.JBO.21.8.081203]

To prepare a 13 ml ScaleA2 clearing solution, 3.12 g of urea (Dia-M, Russia), 26 µs of Triton X-100 (Loba Chemie, Austria) and 1 ml of glycerol were mixed with distilled water. Thus, a solution with a concentration of urea 0.24 g/ml, glycerol - 0.1 ml/ml, Triton X-100 - 0.002 ml/ml was obtained. The solution was heated to 40°C in a water bath and thoroughly mixed. Spheroids were incubated in the clearing solution in the dark for 72 hours, changing the solution every day.

ScaleS4 method

To prepare ScaleS4 clearing solution in 13 ml, 5.2 g sorbitol-D (Labochem international, Germany), 3.12 g of urea, 26 µl Triton X-100, 1 ml of glycerol and 1.95 ml of DMSO were taken. Total, a solution with a concentration of urea of 0.24 g/ml, glycerol - 0.1 ml/ml, Triton X-100 - 0.002 ml/ml, DMSO - 0.15 ml/ml, sorbitol-D - 0.4 g/ml was obtained. The mixture was also heated to 40°C in a water bath and thoroughly mixed. It took 72 hours to incubate the spheroids in the clearing solution, changing the solution every day.

MTT assay of spheroids

Agarose molds with 17 cone-shaped microwells were created using 3D printing and silicone stamps within the wells of a 96-well plate. hTERT culture cells were added to each well (500 cells per microwell) using a multichannel pipette. After 15 minutes, the top of the

wells was washed with a dispenser to remove excess cells, and the plates were placed in an incubator to form spheroids for 24 hours.

To study the cytotoxic effect on spheroids, we used DMSO (PanReac Applichem, Spain), camptothecin (Thermo Fisher Scientific, USA) and suspensions of Fe@C-NH2 and Fe@C-COOH magnetic nanoparticles. DMSO was diluted with the culture medium, 10 mM camptothecin was diluted with phosphate buffer to the required concentration. Magnetic nanoparticles were synthesized and modified in the Laboratory of Applied Magnetism, Ural Branch of the Russian Academy of Sciences, Institute of Physics and Mathematics, the synthesis was carried out using the gas-phase method [https://doi.org/10.1016/j.carbon.2014.03.034], surface modification using aryl-diazonium derivatives [https:/ /doi.org/10.1016/j.colsurfb.2019.01.009]. The nanoparticle suspension was filtered before adding to the cells through 0.22 μm PTFE syringe filters (JetBiofill, China) and diluted with nutrient medium to the desired concentration.

To obtain the blank data, the optical density of the wells with spheroids was measured using a plate reader (Elx808, Bio-Tek Instruments, USA) at a wavelength of 570 nm (optical density from the plate itself, agarose and spheroids).

One day after incubation, the culture medium was replaced with an MTT (Dia-M, Russia) solution at a concentration of 1 mg/ml for 4 hours. After incubation, the nutrient medium was removed with a multichannel dispenser and 100 μl of DMSO was poured into the wells of the plate, extracting the colored reaction product from the spheroids. With DMSO, the spheroids were incubated for 30 minutes in an incubator at 37 degrees, after which the optical density was recorded using a plate reader.

Image processing

Image processing

Automatic image processing of stamps and cell spheroids was performed using CellProfiler (4.2.5) and Ilastik software. As a rule, CellProfiler was used, but in cases when satisfactory segmentation could not be achieved, Ilastik (1.4.0) was used. Manual image processing was performed using FIJI (1.54f).

3d model processing

In order to clarify some peculiarities of MSLA printing, we present images comparing simultaneously the model created in CAD, the model obtained after the slicer and the real appearance of the object obtained with the help of CLSM. Obtaining these images and corresponding videos turned out to be a relatively non-trivial task, so we decided to describe our pipeline in a bit more detail.

To illustrate the features of MSLA printing, the work contains images that simultaneously compare the model created in CAD, the model obtained after the slicer, and the real appearance of the object obtained using CLSM. One of the approaches we used was to export a .stl file from the CAD and then use the stl-to-voxel (0.9.3) library for Python to convert it into a layered set of .tiff images (with resolution=512). Using a slicer, the same stl model was converted into a .pwmo file, which was again converted into a .tiff set using UVTools (4.3.2). After collecting images with a confocal microscope, a multilayer .tiff file was also obtained. In the ImageJ program, the images were manually aligned relative to each other and transferred to napari (0.4.19), where 3D images and animations were already

produced. The attenuated_mip rendering mode was used for CAD and slicer models, and mip for images from a confocal microscope.

Another approach involves using Blender (4.1) with the tif2blender (0.1.1) plugin. This plugin made it possible to import both data obtained from a confocal microscope and a 3D model converted to tiff using UVTools, as in the previous method. In Blender, the 3D printed object model was converted into a mesh, and the microscopic data was visualized using the emission volume shader.

Results and discussion

Principles of MSLA 3d printing

MSLA (masked stereolithography) printer is based on stereolithography to produce three-dimensional models. The process is carried out through the polymerization of photosensitive compounds under the influence of light, and the mask controls which part of the composition will be illuminated.

There are several main components in a typical MSLA 3D printer. The liquid photopolymer is poured into a vat, the bottom of which is made of a transparent film made from fluorinated polymers (FEP film), which ensures minimal adhesion. The bath is installed in the printer on top of an LCD screen, under which an array of LEDs (with a wavelength of 405 nm) is installed.

While the printer is in use, a platform is automatically lowered into the resin-filled vat. Light from photodiodes is then projected through an LCD screen onto the platform. The screen's individual pixels control the passage of light, functioning as a mask (“M” - in “MSLA”) in the printing process.

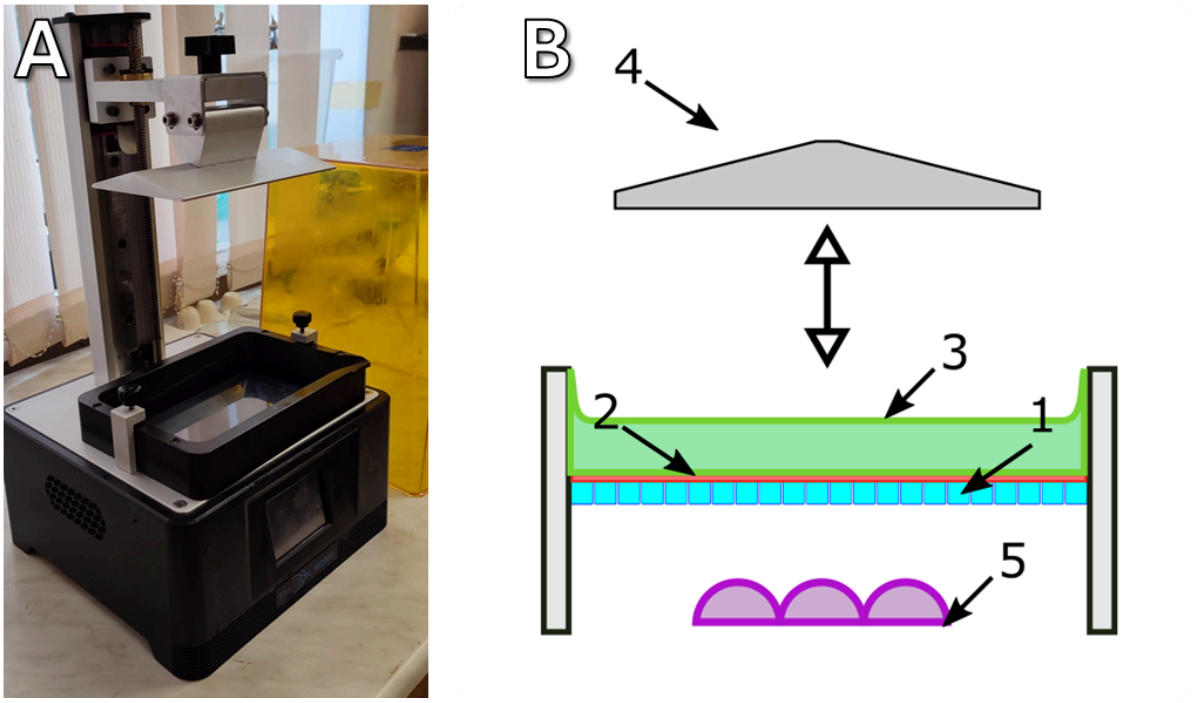

Figure 1. Anycubic Photon Mono 3d printer (A) with the protective cap removed and an MSLA diagram of the printer showing the main construction components (B).

At the first printing cycle the stage is lowered almost close to the FEP-film, at a distance of one layer (usually in 3d printers of hobby class it is 50-25 μm), after which the irradiation is switched on. The layer of photopolymer between the film and the stage polymerizes and adheres to the stage, and the process is selective, only in those areas where the light passes through the LCD screen.

When one layer of the part is illuminated and stuck to the stage, the stage is raised to a certain height, necessary for fresh photopolymer to flow under it, then lowered back down, with the new distance from the FEP to the stage being greater by the height of one layer, after which a new layer is illuminated (polymerized), sticking this time to the already solid first layer. The process is repeated the necessary number of times to print the entire part.

Capabilities and limitations of MSLA-3d printing

Angle of printing

In this whole process of MSLA 3d printing, there are a number of non-obvious factors that need to be considered for successful printing of various objects with small surface features [https://doi.org/10.1002/bit.28031].

- FEP film has minimal adhesion to the photopolymer, but not zero, and, in addition, over time the film degrades and the printed objects begin to stick to it more and more, which eventually leads to the object coming off the bed. The adhesion is also aggravated by the hydrodynamic pressure generated by the upward movement of the stage.
- If the area of the object that is adhered to the bed is too small, it can also tear off.

A common way to eliminate such problems in stereolithographic printing (not only MSLA, but other types as well) is to place objects at an angle (Figure 2), which minimizes the contact area with the film. At the same time, to increase adhesion of the object to the stage, supports are added to the model, which ensure successful printing and are separated from the object after printing.

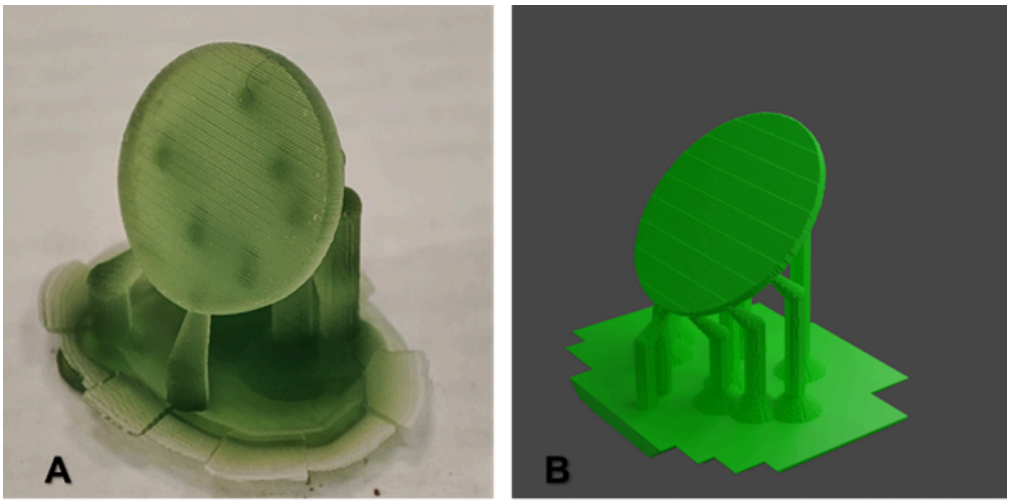

Figure 2. Macrophoto (A) and (B) 3d rendering of a test object printed at a 60 degree angle to a table with supports

However, the above-mentioned ways to achieve successful printing have their significant disadvantages. The fact is that the MSLA 3d printer has limited resolution in all three axes. On the xy axis the resolution is limited by the pixel size of the LCD screen (approximately 40x40 μm, slightly different for different printer models available on the market), and on the z axis the resolution is limited by the layer height (25-50 μm, depending on the settings).

This leads to the fact that the object printed by the printer is not a perfect reproduction of the digital model, the software (slicer) used for printing approximates this model with voxels, which leads to various artifacts of this transformation, as shown in the image (figure 3) below.

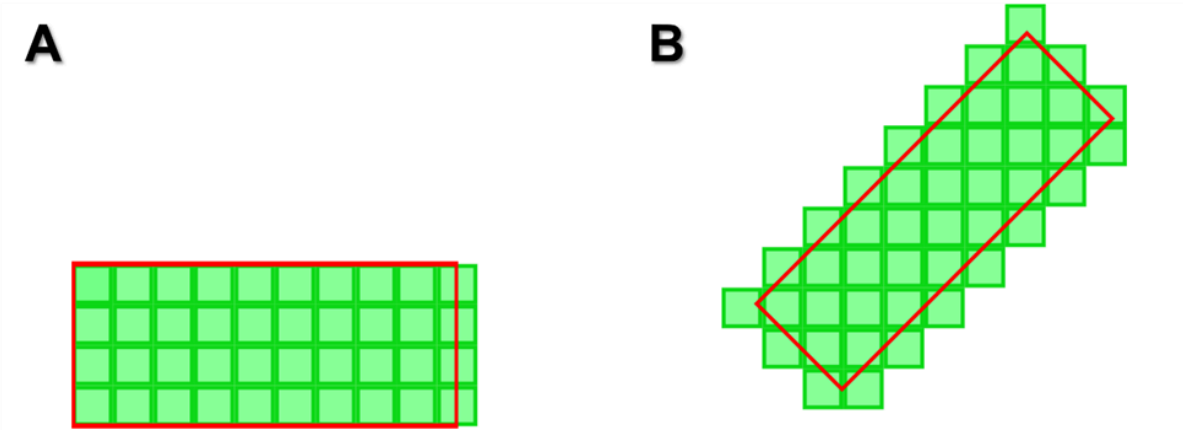

Figure 3. Illustration of the process of approximation of a flat model by voxels

The scheme is presented in 2d to simplify perception. Red denotes the original model embedded in the slicer, green - the resulting model, approximated by voxels (in case of 2d illustration - pixels). Object A is located parallel to the printing plane, object B is located at an angle of 45 degrees (the diagram does not indicate the support necessary for printing in this orientation).

It is shown how the conversion to voxels performed by the slicer can distort the geometric dimensions of the object if it is slightly smaller or larger than an integer number of pixels - this can be seen on the right side of the object A (Figure 4).

The example of object B demonstrates how flat faces are distorted, in case of printing at an angle - the slicer approximates them with steps, which leads to a significantly inhomogeneous surface of the plane.

Below are the images of the test object in the 3d software and how it will look when printing. Also, a clarification must be made that this is still an idealistic digital representation of the object. In reality, to the already distorted model will be added various mechanical and optical artifacts of the printing itself, which further affects its geometry (S4).

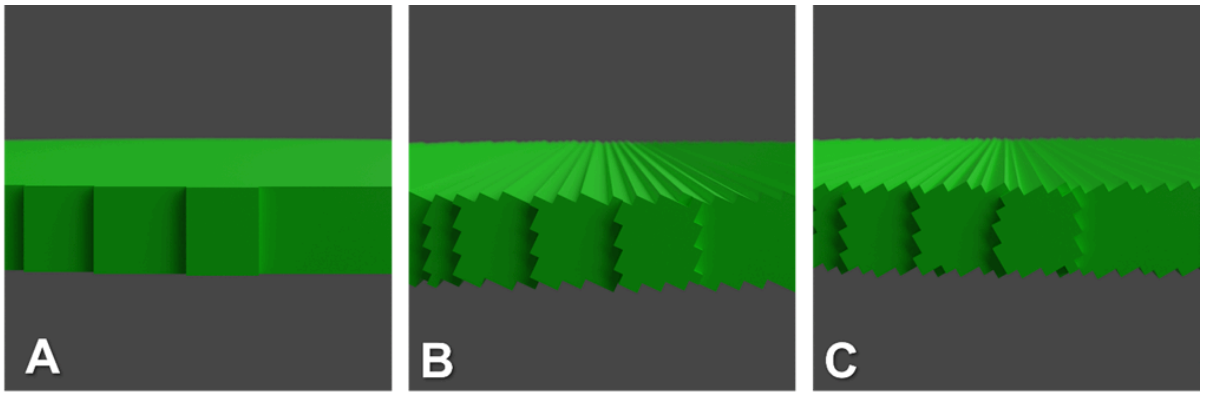

Figure 4. 3d render test objects, profile view. A - the object is oriented in the print plane, B - at an angle of 25 degrees, C - at an angle of 60 degrees

Diameters, depth and height capabilities

The mechanism of 3d model approximation by voxels discussed above also contributes to the macrorelief structure by adding its corresponding distortions. The process of formation of such distortions is schematically depicted in the figure below. Model A is a macro-relief model oriented in the printing plane, model B, respectively, at an angle of 45 degrees to the plane. Both models show a noticeable significant deviation of the final shape from what was intended (Figure 5). It must be said, however, that everything in this illustration is significantly exaggerated.

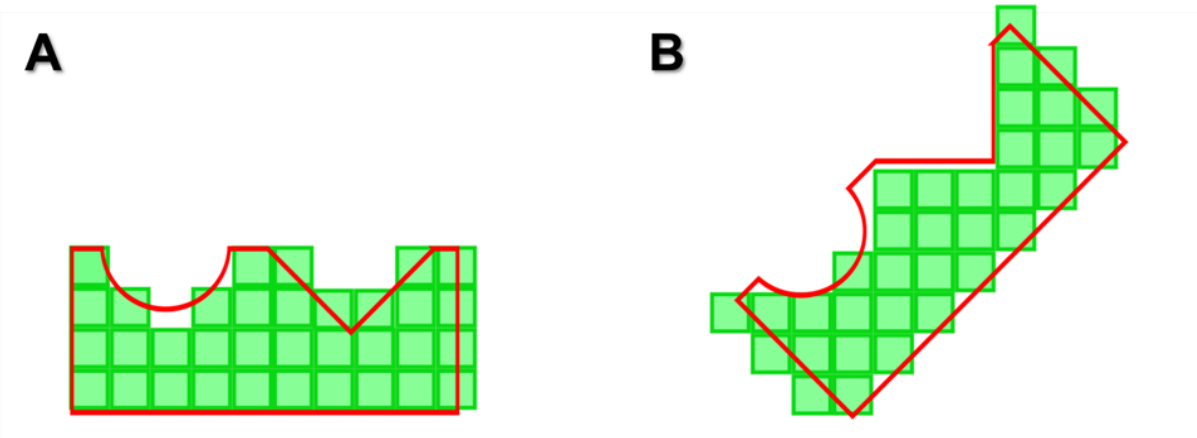

Figure 5. Illustration of the process of approximation of a model with macro-relief by voxels. A - printing in the plane of the table, B - printing at an angle of 45 degrees

In order to determine the limits of limitations of the used technology already in terms of macro relief, a test object was printed, which contains many depressions and elevations of different sizes and shapes: cones, pyramids, cylinders (Figure 6). Detailed description of geometric dimensions and features it contains is given in the auxiliary information (S5). All

features have been studied with CLSM, which proved to be a very convenient tool for studying such objects.

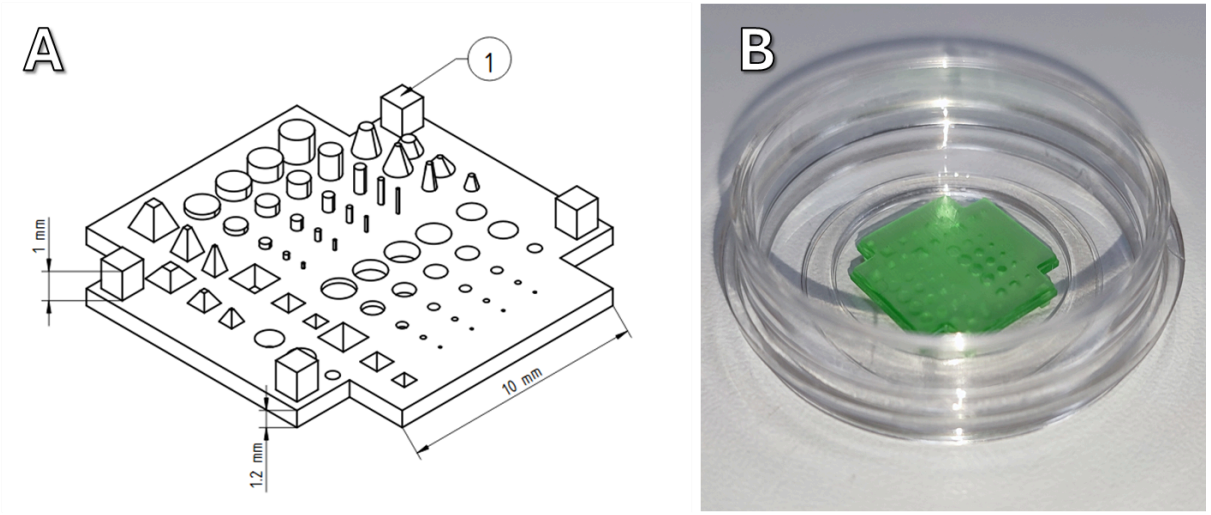

Figure 6. A Sketch of the test object. The cubes (1) at the corners are necessary to serve as supports, because the test object is installed with the relief downwards and supports are needed to make it stand flat. Detailed dimensions of each feature are given in the supplementary materials. B - photo of the test object located in the glass bottom dish

The smallest objects in the test object used are cylinders with diameter 100 μm and height 200 μm and corresponding recesses. Neither of them could be printed. The cylinders are most likely not printable due to their brittleness and simply collapse when torn from the film. The recesses have another problem - high viscosity of the resin leads to the fact that it does not flow out of the holes and is illuminated there by parasitic light from neighboring pixels of the LCD screen.

Larger sized figures are printed and as expected, the shape of the printed objects is significantly different from what was conceived and designed in CAD. Some examples are shown in the figure, others are included in the supporting materials. Cones (figure A, B and C) become pyramids of rather complex shape, with many irregular steps, and pyramids with smooth sides become stepped. Cylinders and cylindrical recesses are distorted the least (Figure D, E, F), merely acquiring a characteristic pixelized profile (Figure 7). Animations generated with napari that allow the reader to experience this in 3d are presented in the

supplementary materials (Supplementary video 1).

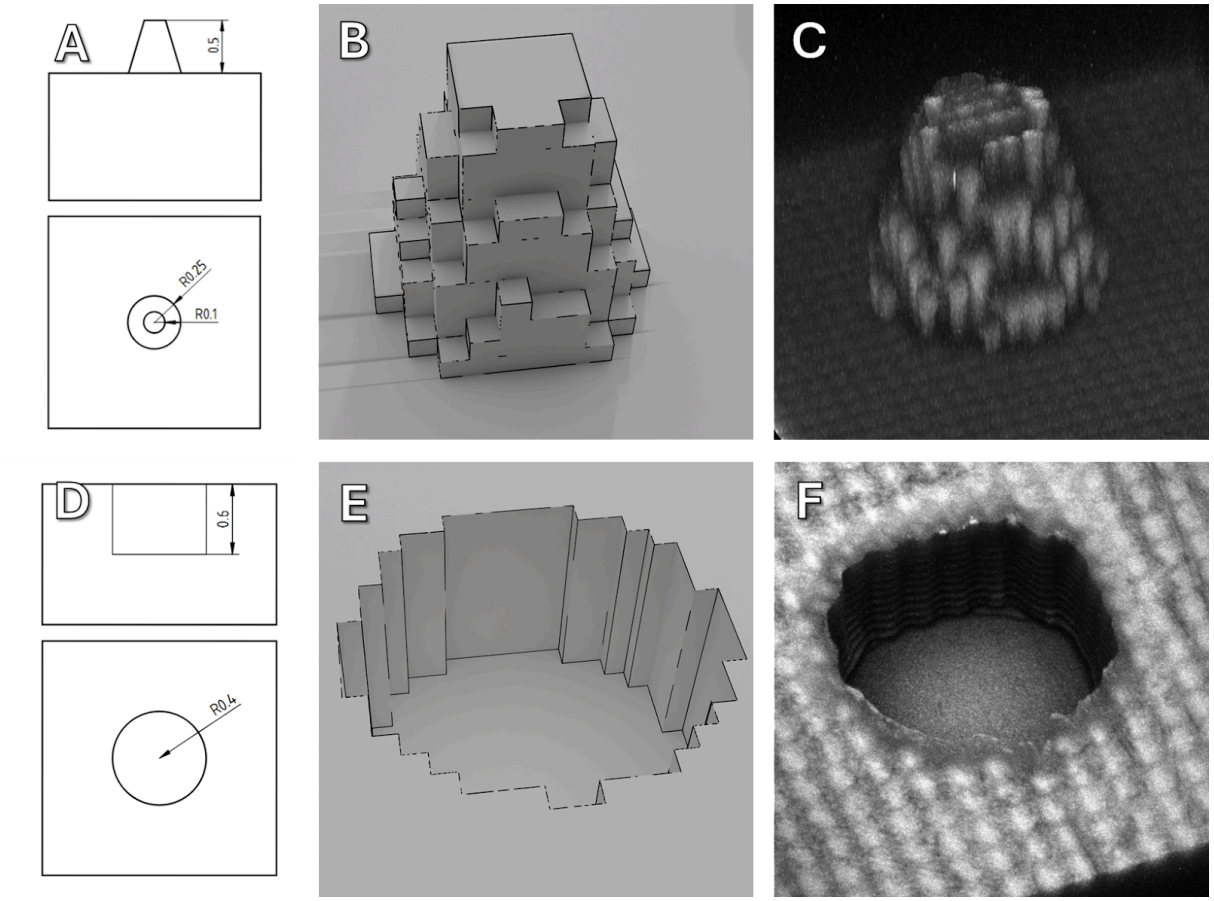

Figure 7. Sketch with dimensions, 3d rendering of the model formed by the slicer and 3d reconstruction obtained by confocal microscopy for objects of different geometry.

However, the fact that a printer can print single objects, even if it distorts their geometry, does not mean that it will do so reliably. Quite quickly it was discovered that when tens or hundreds of 200 μm diameter cylinders were printed, a noticeable fraction of them would end up damaged and broken when printed, as shown in Figure 8A. Another limitation is the distance between cylinders, if it is too small, bridges of cured polymer form between them during printing (Figure 8B). As a result, it is optimal to print cylinders with diameters of 300 μm or more at a distance of about 300-400 μm from each other, as shown in Figure 8 (C and D).

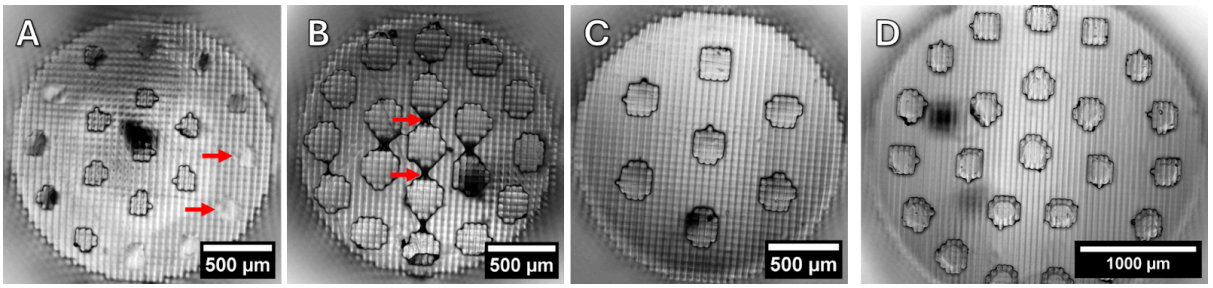

Figure 8. Stamps for making microwells in a 96-well plate. A - 200 μm cylinders, arrows indicate cylinders that broke off during printing; B - 300 μm cylinders with bridges

between them; C - 400 µm cylinders at a greater distance from each other and D - 300 µm cylinders on a larger diameter stamp.

Similar difficulties were encountered when making silicone molds. Despite the fact that in the 3d printing mold the 200 µm diameter recesses are obtained successfully when making a silicone impression from this mold, some of the cylinders are damaged. Below is an image of a silicone mold with cylinders of different sizes, including the defective 200 µm (Figure 9).

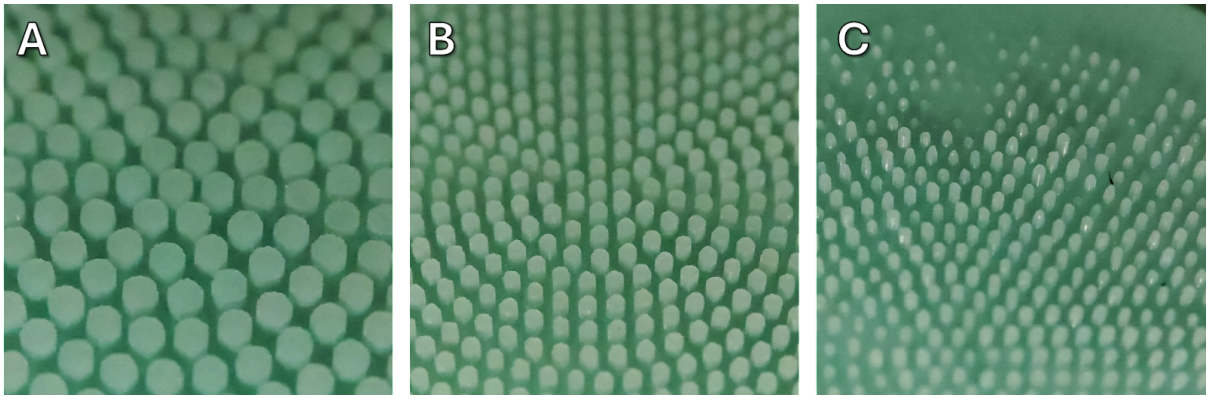

Figure 9. Macrophoto of silicone molds with cylinders of different diameters. A - 600 µm, B - 300 µm, C - 200 µm.

It has already been said that the CAD model is approximated during printing by voxels, which, accordingly, distort the geometry of the object. However, the question was how strong these distortions are. To check this, test objects with 100 cylinders of different diameters were printed and photographed with a microscope (Figure 10).

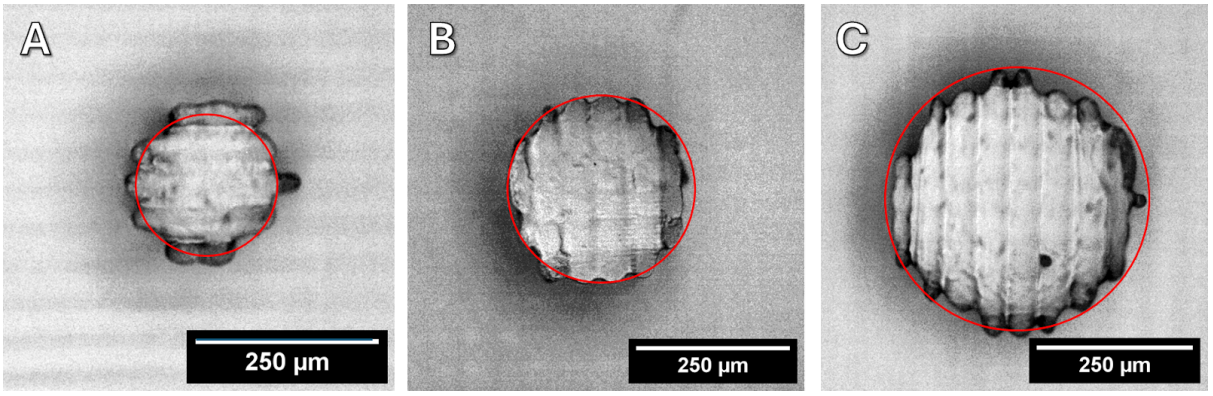

Figure 10. Cylinders with different diameters. A - 200 µm, B - 300 µm, C - 400 µm. The circle of the corresponding diameter is marked in red.

The microphotographs were further segmented using ilastik and further the geometric dimensions of the objects were measured in the CellProfiler program. The results are summarized in table 1 and plot (Figure 11) below.

Table 1. Planned and actual sizes of micropins in cylindrical stamp

Diameter in CAD model, μm	Measured equivalent diameter, μm
200	223 ± 11
300	318 ± 10
400	446 ± 17

The presented data show that the obtained diameters are relatively reproducible, the intragroup difference is less than 10%. At the same time, the real diameter of the cylinder slightly differs from the model, but this difference is not so significant if we compare the areas of the objects.

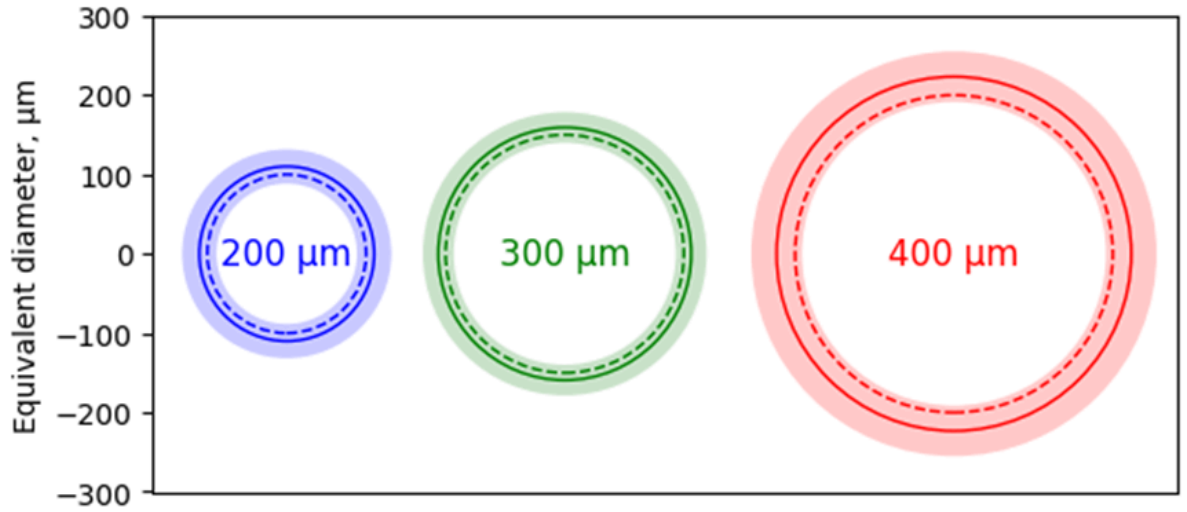

Figure 11. Graph showing the measured dimensions of the test cylinders. The dashed line is a circle of a given diameter, the solid line is the measured circle of equivalent diameter, the translucent bar is the standard deviation of the measured equivalent diameter.

In general, we have seen that on the MSLA 3d printer it is possible to reliably print even quite small objects, which can then be used directly as stamps for creating agarose molds or for making silicone molds, in which agarose molds will already be made.

Stamp and mold design

The design of molds and stamps was carried out by iterative prototyping. Here the final designs for stamps and silicone molds are given and described.

Stamp for glass bottom dishes

For confocal microscopy, specialized glass-bottomed petri dishes are often used to minimize the distance from the objective lens to the sample. Accordingly, the agarose layer should also be minimized so as not to increase the distance even further. Agarose of such thickness becomes surprisingly delicate and can be damaged at any careless movement, so the variant with the use of silicone molds for making such constructions was discarded at once and a stamp was designed, allowing to make a mold directly in a glass bottom dish or in a usual petri dish, at appropriate change of the stamp depth. After a number of iterations (examples are shown in the supplementary materials), a stamp of the following design was developed (Figure).

The stamp was designed to be printed flat on the printer's table, without supports and easily detachable after printing. On the upper part of the stamp (in working position) there is a plane (1) on which an inscription can be embedded in the design. In the main part of the stamp (2) there are holes (3), by which the stamp can be conveniently held and, importantly, removed from the solidified agarose (Figure 12A). The holes (4) lie on the edges of the petri dish (Figure 12B) and allow centering the stamp on it, while the part (5) is immersed in the agarose and on it are located the actual micropins (6), which leave an impression in the agarose (Figure 12C) after removing the stamp.

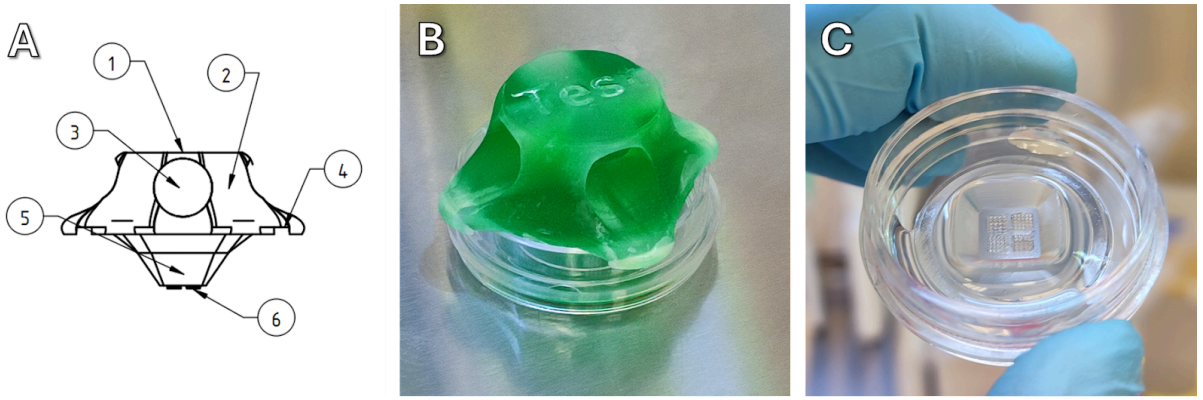

Figure 12. A - sketch of a stamp for a dish. 1 - flat part for marking, 2 - holes for easy grip, 3 - recesses for centering the stamp on the walls of the dish, 4 - part to be immersed in the well, 5 - working part for creating microwells. B - photo of the stamp installed in a glass bottom dish with poured agarose. C - microwells created in agarose

Above we have already shown in what limits it is possible to vary the geometry of wells and now it is time to check how spheroids are formed in microwells of different geometry. It is possible to check all possible shapes and sizes, but we stopped at a few of the most representative ones. Stamps for a glass bottom dishes (Figure 13) containing micropins of different shapes were designed: cylinders with a diameter of 300 μm (1), truncated pyramids with an upper square edge of 600 μm and a lower one of 100 μm (2), truncated cones with an upper diameter of 500 μm and a lower one of 200 μm, cylinders with a diameter of 300 μm (3), truncated pyramids with an upper square edge of 600 μm and a lower one of 300 μm (4).

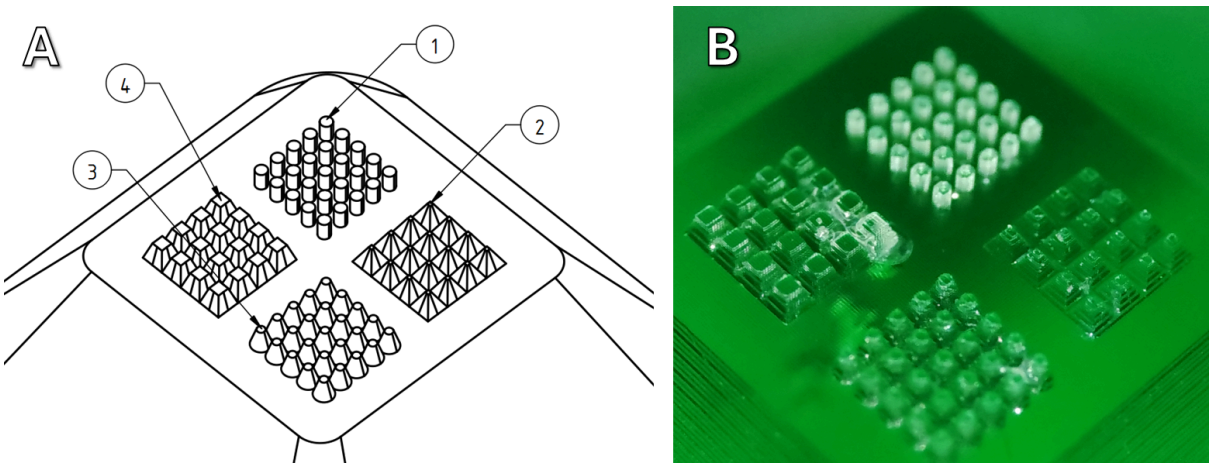

Figure 13. Schematic diagram of the working part of the stamp (A) for testing micropins of different shapes and its macrophoto (B). Explanations are given in the text.

As discussed above, when printing on an MSLA printer, the CAD program shapes are approximated by voxels, which leads to a noticeable distortion of the shape. It is the formation of "steps" on walls other than vertical that is most sensitive. These steps cause cells added to the microwells to get stuck.

With the help of a stamp with different shapes of micropins, the corresponding agarose molds were made in a glass bottom dish. Here it is necessary to make an important statement - agarose should be poured into the cup in a relatively little volume, for our cups it is about 1 ml. If the agarose is more - when solidifying it sticks too firmly to the stamp and when removing it, the agarose starts to tear or the mold is removed from the cup entirely.

hTERT culture cells with nuclei labelled with the fluorescent dye Hoechst 33258 were added to the prepared agarose molds, and images were then taken using a confocal microscope in z-stack mode, which allows a 3D image to be obtained.

We were unable to obtain a high-quality confocal 3D image of the agarose mold itself, so the illustration below shows a 3d rendering of the microwells of the corresponding shapes overlaid with confocal microscope images. The figure shows the cells immediately after addition to the wells after the procedure of washing off excess cells and spheroids formed in the same wells after a day (Figure 14).

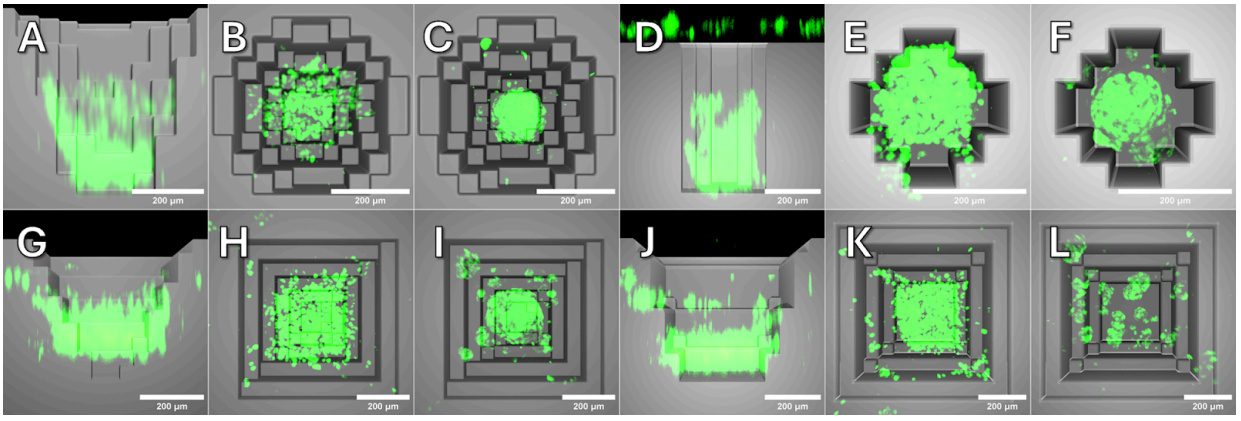

Figure 14. Combination of confocal microscopy (hTERT cells, cell nuclei stained with Hoechst 33258) and 3d rendering of differently shaped microwells. The scale bar is 200 µm. All wells are 500 µm deep. Side and top views are provided In the case of the side view, half of the microwell model is cropped, for illustrative purposes. A, B and C are truncated cones with upper diameter 500 µm and lower 200 µm, D,E,F are cylinders with diameter 300 µm,

G, H, I are truncated pyramids with upper square edge 600 μm and lower 100 μm, J, K, L are truncated pyramids with upper square edge 600 μm and lower 300 μm. A, D, G, J - side view of cells in a microwell shortly after seeding. B, E, H, K - top view of cells in a microwell shortly after seeding. C, F, I, L - top view of spheroids formed a day after seeding.

In cone-shaped microwells, the cells are partly stuck on the steps, but in a day all the cells gather at the bottom forming one spheroid. In cylindrical wells the picture is similar, although it should be noted that some cells remain on the surface formed by the flat part of the stamp. In pyramidal wells the picture is different, quite a lot of cells get stuck on the steps and not all of them fall to the bottom. In a pyramid with a bottom square edge of 100 μm, besides one large spheroid, several small spheroids were formed and some cells did not become part of the spheroid at all. In the pyramid with the bottom cube edge of 300 μm several small spheroids were formed.

For reliability and reproducibility of biological studies, it is optimal to have one spheroid of known size per well. Accordingly, we subsequently used cone- and cylinder-shaped microwells.

The main advantage of forming spheroids directly in the dish is the possibility to observe the process of their formation, which may also be important in some studies. The corresponding experiment was performed, we planted hTERT culture cells in microwells made in the glass bottom dish and observed spheroid formation for several hours (figure 15), the corresponding video is given in the supplementary materials (Supplementary video 2).

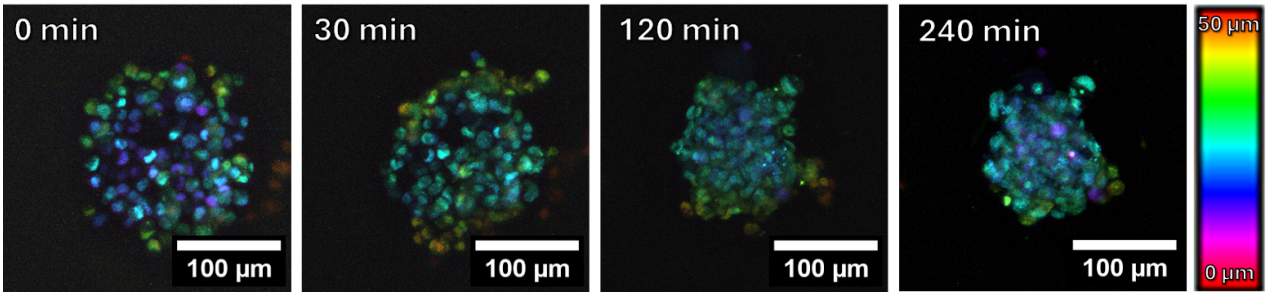

Figure 15. Spheroid formation in a microwell, z-stack. The color-bar on the right allows one to get an idea of the height distribution of cell nuclei.

Spheroids optical clearing

The main problem with studying spheroids using optical microscopy is their optical properties: they are not very transparent. One way to overcome this limitation is optical clearing - a process in which an object is treated with different substances to remove the coloured substances and to make its RI (refractive index) equal to the RI of the medium that is optimal for the optical system.

Of course, many such methods have been developed, but we want to simplify and speed up high-throughput analysis on spheroids. We therefore decided to test the possibility of clearing spheroids directly in the agarose molds in which they were formed.

We tested 5 different optical clearing methods on spheroids of human skin fibroblast and rhabdomyosarcoma cell cultures (Figure 16). In both cases, there were control groups of spheroids that were stained but not fixed. We evaluated not only how the optical properties of the spheroids changed, but also how the clearing process affected their size, but these results are too extensive and have been moved to the supplementary materials (S6).

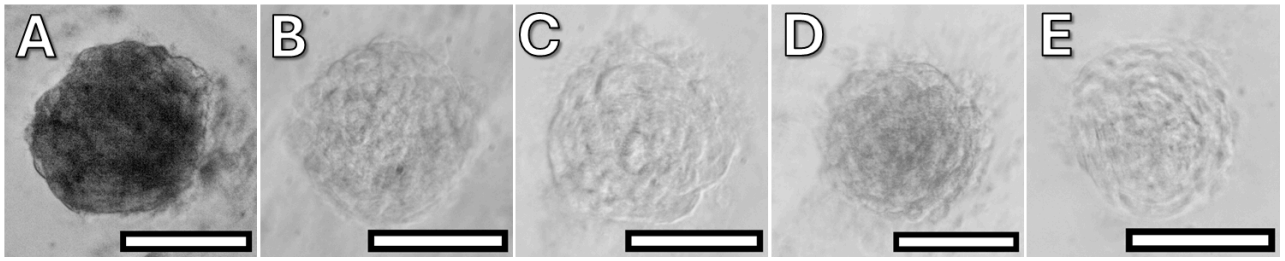

Figure 16. Spheroids of human skin fibroblasts cleared by various methods: A - Control uncleared spheroid, B - Spheroid after clearing with glycerol, C - Spheroid after ClearT, D - Spheroid after ClearT2, E - Spheroid after ScaleA2, F - Spheroid after ScaleS4. Scale bar - 200 μm

The CLSM images of selected spheroids were also further analysed. It can be noticed that a spheroid with a diameter of more than 200 μm forms a central dark region from which it is impossible to detect the dye fluorescence signal, which could theoretically lead to incorrect conclusions about the processes occurring in-side the spheroid (Figure 17, A). In spheroids treated with clearing solutions, internal optical sections are uniformly visible (Figure 17, B–F).

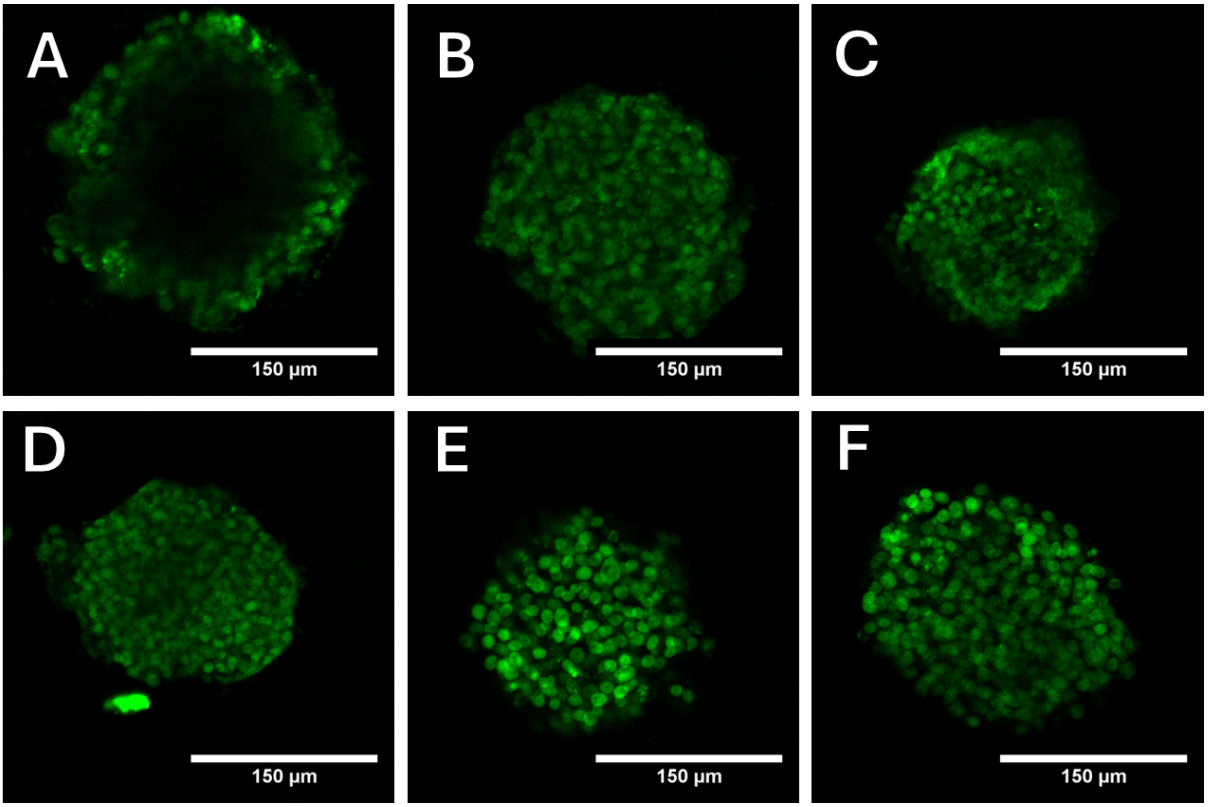

Figure 17. Fluorescent visualization of spheroids of rhabdomyosarcoma of dif-ferent optical cleared groups: A - control, B - glycerol, C - ClearT, D - ClearT2, E - ScaleA2, F - ScaleS4.

A similar pattern is observed in spheroids of human fibroblasts (S6). Additionally, it is noted that the spheroids from ScaleA2 and ScaleS4 exhibit more distinctly outlined nuclei that

stand out against the background. It is suggested that this may allow counting them even in dense fibroblast spheroids.

A characteristic directly reflecting the efficiency of optical clearing of biological tissue is the thickness of the outer layer that can be cleared (clearing depth). Using ScaleA2 and ScaleS4 techniques, it was possible to increase the depth of the fluorescent dye signal by 85 μm. Overall, the average clearing depth of rhabdomyosarcoma spheroids was 57 μm. With each of the five techniques used, it is possible to significantly increase the thickness of the spheroid's outer layer on which the dye fluorescence can be detected.

It was previously reported that optical clearing can cause a significant reduction in the fluorescence intensity of stained tissues, which can lead to incorrect results, for example, when using fluorescent proteins. Thus, formamide in ClearT destabilizes protein conformation. Displacement of water during clearing may also negatively affect fluorescence [10.1117/1.JBO.23.5.055003]. Polyethylene glycol in ClearT2 should preserve and increase fluorescence intensity by stabilizing protein structures [10.1242/dev.091844].

Overall, we have shown that it is possible to optically illuminate spheroids directly in agarose molds, thus speeding up and simplifying their study.

Molds for mass production of spheroids

Petri dishes, including glass bottom ones, are well suited for observing and experimenting on a relatively small number of spheroids. For some studies, however, it is necessary to work up a large number of spheroids and accordingly a substantially larger vessel area is needed. In this case, we are not limited by the optical features of confocal microscopy and, consequently, the thickness of the agarose mold can be noticeably larger, so we used an approach with silicone molds into which agarose is poured and then transferred to a culture vessel for further manipulation. Molds were constructed for 6 well plates and for T-125 culture vials.

The MSLA 3d printer is used to print a master model (Figure 18A) where a set of microwells of the required size and number are arranged in concentric circles, two-component injection molding silicone is poured into it and vacuumed so that the silicone completely fills all the holes and form, respectively, the silicone micropins. Than melted agarose is poured into the prepared silicone molds (Figure 18B) under sterile conditions; after solidification, the agarose molds (with many microwells) are removed (Figure 18C) and transferred into the wells of a 6-well plate (S7).

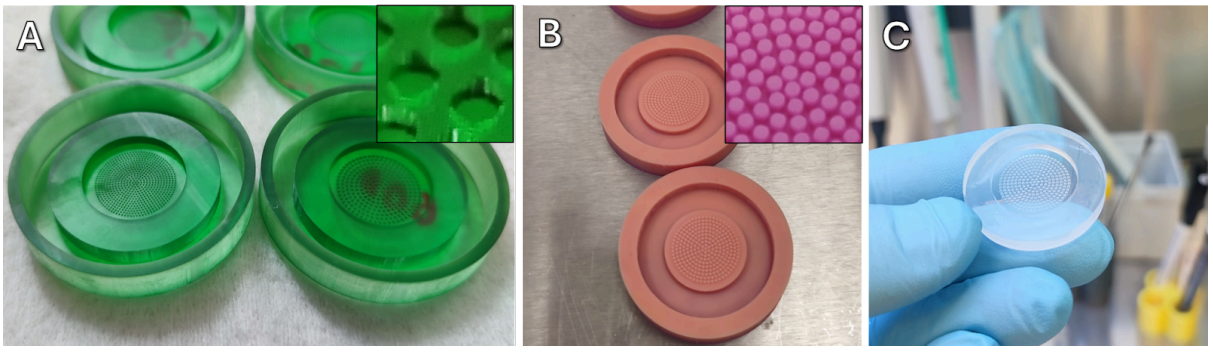

Figure 18. A - 3d-printed primary molds with different size and number of microwells (on the inset macrophotograph of microwells), B - silicone molds (on the inset macrophoto of mold's micropins), C - agarose mold made with the help of these molds.

To demonstrate the ease and convenience of customization of microwells using our approach, we produced primary molds with different diameters of cylindrical wells: 200 μm, 300 μm and 600 μm (Figure 19).

As mentioned above, the printing of recesses with diameters smaller than 300 μm on MSLA printer is not reliable, respectively, silicone molds using 200 μm were also obtained with many defective micropins, but we still conducted experiments with them, although the number of obtained spheroids was less than it could be.

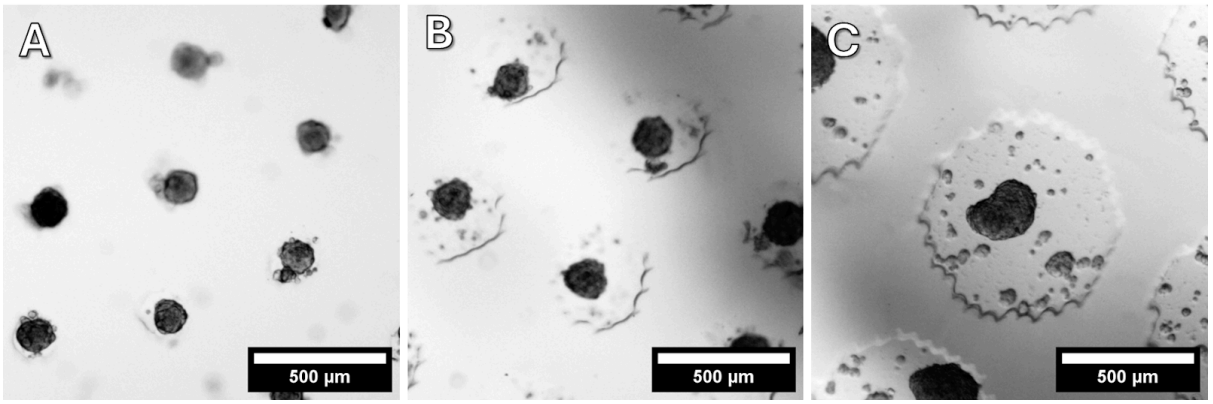

Figure 19. Spheroids in cylindrical wells of different sizes: A - 200 μm, B - 300 μm, C - 600 μm.

Spheroids from hTERT cells were obtained in these agarose molds and photographed using an inverted optical microscope.

Using a combination of Ilastik and CellProfiler software, the size of spheroids and their roundness were determined (form factor - equal to 1 for a perfectly round spheroid), which is a screening method for assessing their viability proposed in [https://doi.org/10.3389/fmolb.2021.784962]. The results are presented in the figure below (Figure 20).

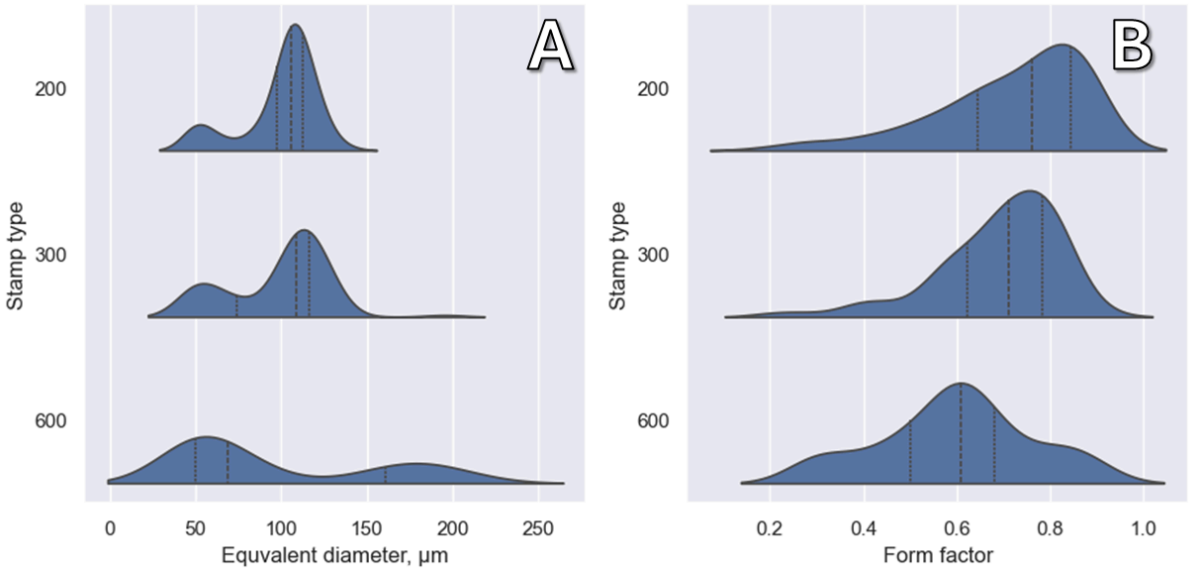

Figure 20. KDE-plots showing the distribution of spheroid sizes (A) and form factors (B) of spheroids in wells made with different stamps.

The size of the spheroids is always smaller than the well diameter, which was also seen in the images; at the same time, the size distribution is bimodal, with some smaller spheroids present. This is especially pronounced in large (600 μm) microwells, where there are many small spheroids and fewer large ones. In general, large wells are not very suitable for spheroids fabrication, which can be seen from the shape factor distributions, it can be seen that a noticeable number of spheroids have an irregular shape, most likely this is because in the wells several small spheroids merge into one large one.

Another approach to adjusting the number of resulting spheroids is to vary the number of cells placed in the wells of a given area. To demonstrate this, we planted different quantities of cells in a same microwells with a diameter of 600 μm (Figure 21).

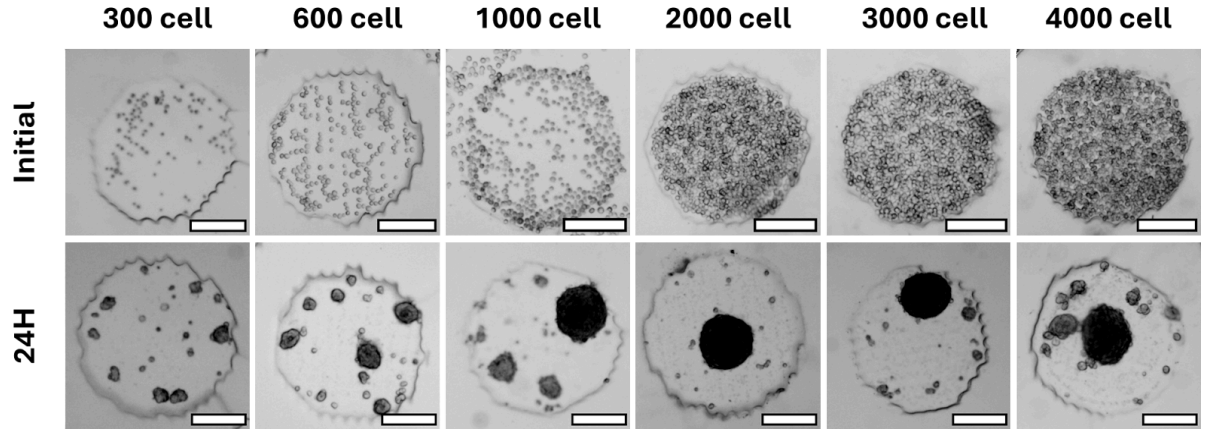

Figure 21. Different numbers of cells in a 600 μm diameter microwell planted in a microwell and the spheroids formed from these wells after 24 hours. Scale bar 200 μm

When cells are few, they are unable to form large spheroids and form individual small spheroids consisting of dozens of cells. When the number of cells in a microwell becomes excessive, several spheroids are formed, and also irregularly shaped spheroids can be seen, arising from the fusion of several smaller ones. And finally there is a “goldilocks zone”, about 1000-3000 cells per well of such area, when only one large spheroid is formed in a well.

We also compared our system to a commercial system made by MicroTissue. These silicone molds are made using much more precise equipment than the MSLA printer and the shape of the holes is much smoother (Figure 22). However, is this a critical flaw in our approach?

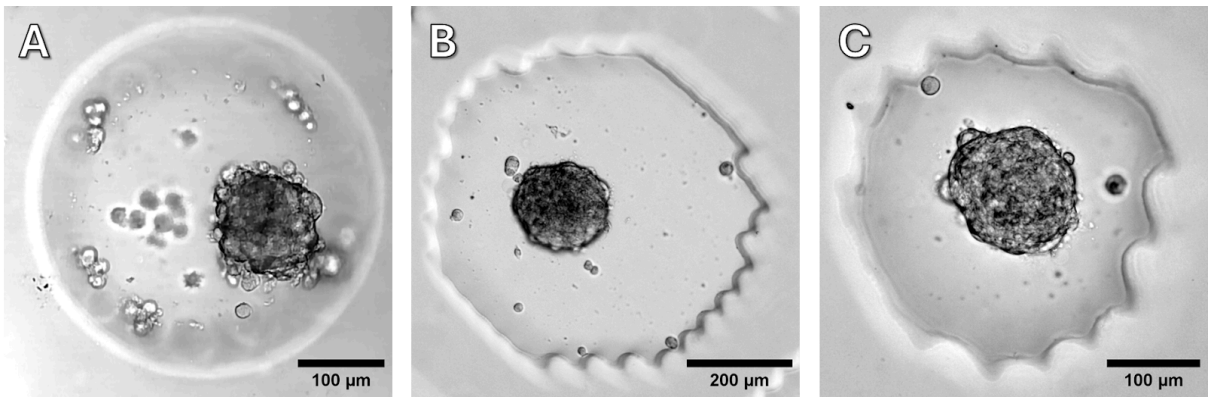

Figure 22. Spheroid formed in MicroTissue molds (A) with a bottom diameter of 300 µm, and in molds described in the article with diameters of 600 um (B) and 300 µm

Despite the fact that visually the cells in molds made with MicroTissue look perfectly even, the shape and size of spheroids are not significantly affected. Moreover, in commercial molds they are even less uniform and less rounded than in our molds (Figure 23).

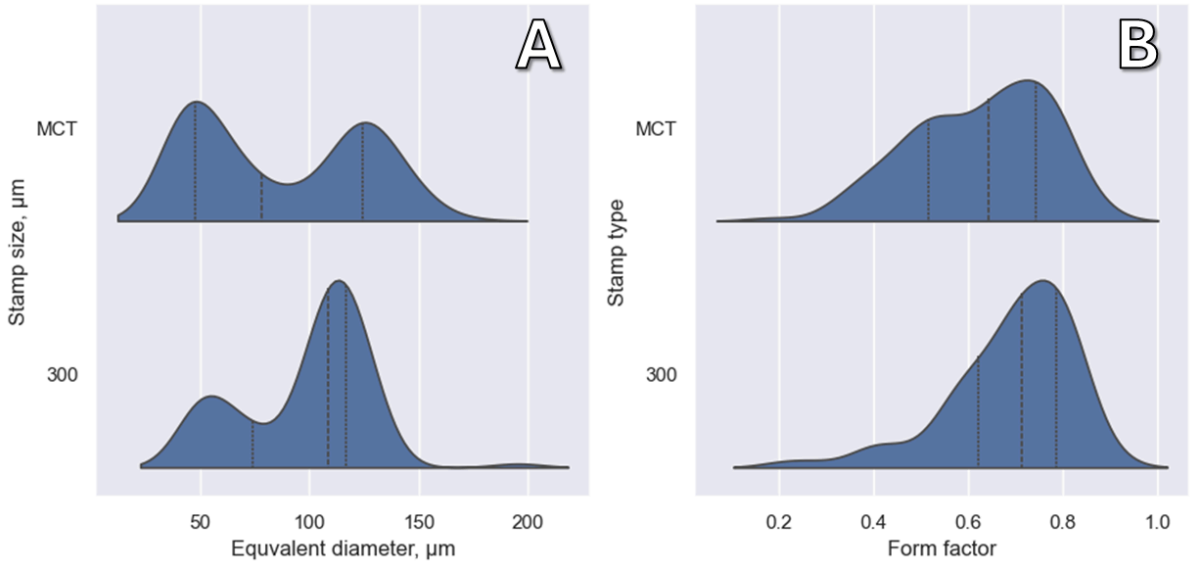

Figure 23. KDE-plots showing the distribution of spheroid sizes (A) and form factors (B) of spheroids in wells made with MicroTissue system (denoted as MCT) and approximately similar mold of our construction with 300 µm microwells.

Some studies may require even larger numbers of cell spheroids. To achieve this, spheroids can be grown in culture vials instead of cups or tablets (the development of molds for the bioreactor is definitely beyond the scope of this study). For this purpose we chose vials with a closed lid on the top. It is rather inconvenient to insert a stamp there due to the complex shape of the lid, besides the printing of such a large object is also not a very easy task. It was much more reliable for us to print a mold, make a silicone cast of it and use it to make agarose molds with 14282 wells inserted in the flask in two pieces, allowing 28564 spheroids to be formed in the flask. Such a mold is shown in the picture below (Figure 24). The obvious difficulty when working with such a mold is the necessary number of cells to form spheroids in each well. With an optimal number of 1000 cells per well, 2.8E+07 cells are needed to fill these microwells, which requires the use of two or three (or more, depending on the density of cells in the monolayer) T-225 flasks.

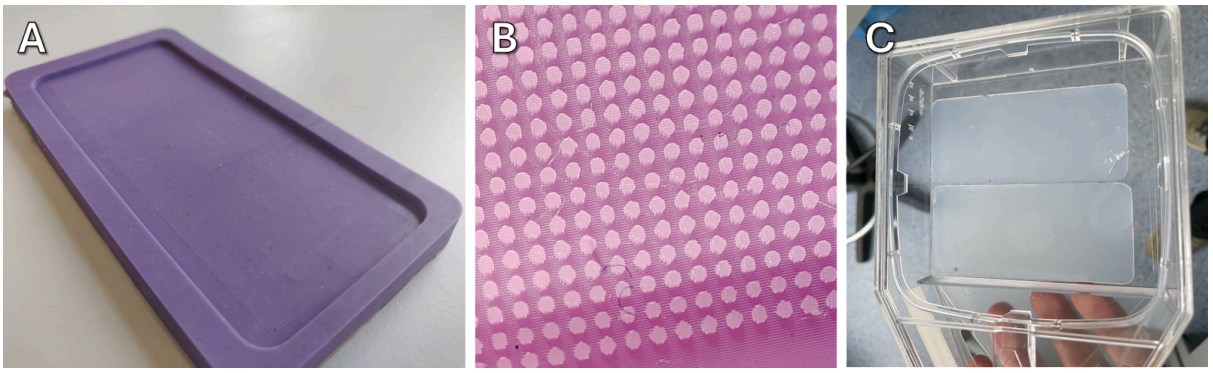

Figure 24. Photograph (A) and macrophotograph (B) of a silicone mold with 14282 cylindrical micropins and agarose molds made with it placed in Tissue Culture Flask with re-closable Lid (TPP).

Stamp for 96-well plates and cytotoxicity study

The 96-well plate is the workhorse of cell biology, where a wide variety of tests and assays can be performed on cell cultures, while remaining relatively affordable and not requiring the use of robots, as is sometimes necessary for plates with a large number of wells. We have tried to show that it is quite easy to adapt the wealth of available screening assays to spheroids as well.

Agarose thickness is not a problem here as with dish stamp, but the problem is quantity. It would not be so difficult to make a silicone mold in which agarose would be poured and after solidification would be transferred to the tablet, but to do it 96 times for each tablet would be quite time-consuming and simply tedious procedure. Consequently, we went back to the stamp approach. At the same time, we tried to make the process as fast and convenient as possible, since we are talking about screening studies, which are often quite large and small time costs start to add up. In addition, the whole approach is designed for the use of a multichannel pipette, which allows efficient dosing including molten agarose.

Making a stamp for a 96-well plate also took several iterations and we finally settled on the following design (Figure 25). Like the dish stamp, this one is printed directly on the printer's bed, without supports. In the upper part there is a flat plane for labels (1), in the main part (2) there are holes for better handling (3). Centering rings (4) allow to locate the stamp in the wells of the plate when the rods (5) are immersed in agarose and on its end there are micropins (6) forming microwells. In addition to printed stamps, we decided to try stamps made of silicone to compare the usability (Figure 25B), which are completely identical in the lower part of the design, but due to the difficulties of working with silicone do not have a handle on top.

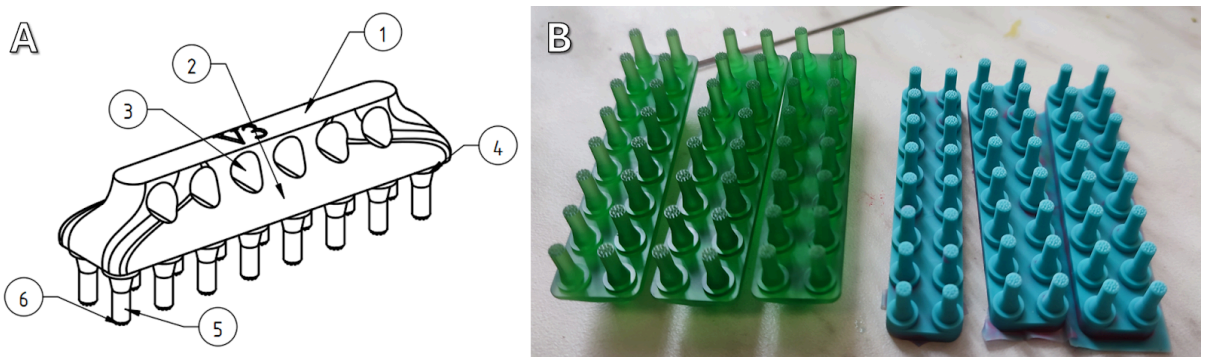

Figure 25. A - sketch of a stamp for a 96-well plate. 1 - flat part for labels, 2 - holes for easy grip, 3 - centering ring, 4 - pin immersed in the well, 5 - working part for creating microwells. B - photos of stamps printed on MSLA printer (left) and silicone stamps similar to them in design of the working part (right).

What you should pay attention to at once - each stamp is designed for two columns - 16 wells of a 96-well plate. Larger stamps are rather inconvenient both for printing and for handling, as well as they are extremely difficult to remove due to adhesion of the stamp in agarose in each well.

Accordingly, agarose is poured into two columns with a pipette, after which the stamp is placed there (Figure 26). As we mentioned above, the most homogeneous distribution of spheroids was obtained in cone-shaped and cylindrical microwells, but after a number of pilot experiments with 96-well plates, we noticed that cylinders are much more likely to cause damage to the agarose mold, probably due to the larger surface and, as a consequence, greater adhesion to agarose, respectively, we finally settled on the cone shape of microwells (B), which allows to achieve the formation of 1 spheroid per microwell (Figure 26C).

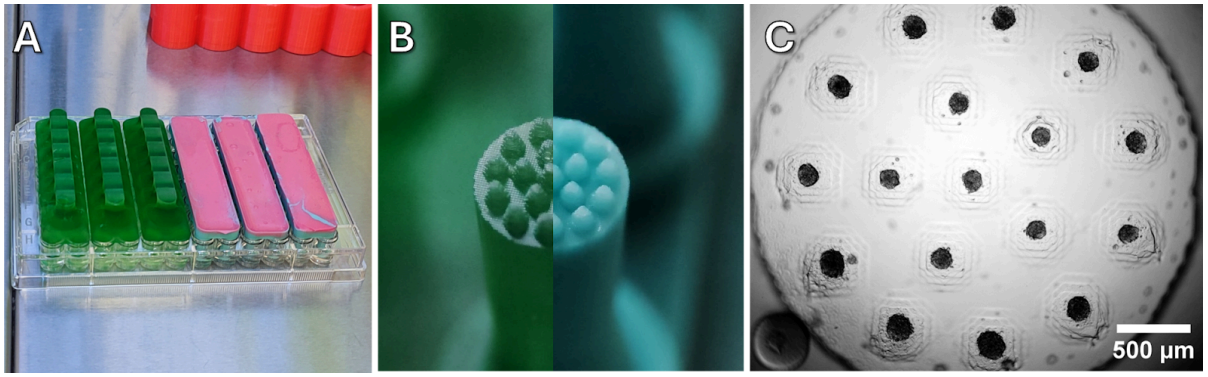

Figure 26. A - printed (left) and silicone (right) stamps installed in the plate. B - working part of the printed and silicone (left and right, respectively) dies for 96-well plates for the production of cone microwells. C - spheroids formed in microwells

Out of several variants of configuration of microwells arrangement in the mold we decided on 17 pieces located in three concentric circles. The area of the mold itself is limited by the size of the field of view in the microscope (Figure 26C) - we wanted the whole mold to fit into it, this greatly simplifies screening observation, and the location and number of pins is somewhat arbitrary. Microwells should not be placed too close (as mentioned in the section "Diameters, depth and height capabilities"), and otherwise there can be many variants of their configuration, which we have not found significant pros and cons.

As in the case of glass bottom dishes, the volume of agarose in the well is important here. For our stamps and the plates used in this work, the optimal volume was 60±10 µl. If there is too much agarose, it will adhere more strongly to the stamp and when the stamp is removed, the microwell will tear or be completely removed from the plate. If there is too little agarose, then, logically, the stamp does not reach it at all. However, even with the optimal amount of agarose, defects may occur during the production of microwells, the most common ones are shown in the figure below. Sometimes an air bubble is trapped under the agarose and does not come out of it (Figure 27 A) - such defects do not affect the formation of spheroids, but can complicate computer quantification. If the bubble gets trapped in the gap between the agarose and the stamp (Figure 27 B and C) - some of the wells may be damaged, resulting in spheroids not forming there.

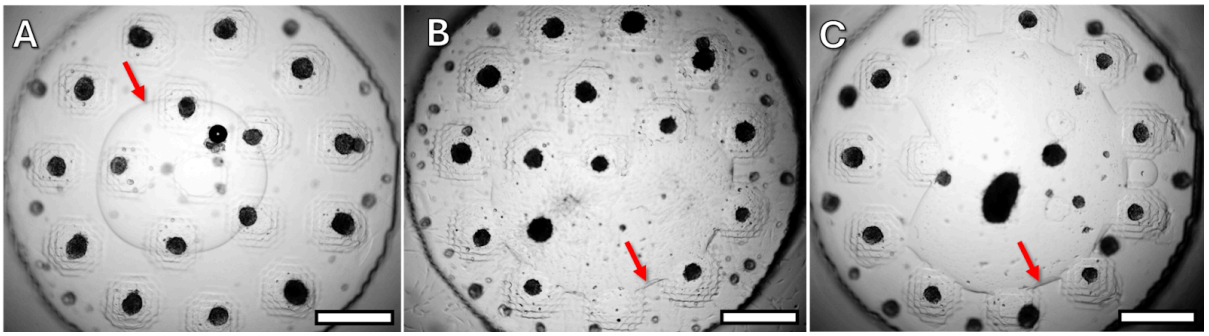

Figure 27. Various defects formed during the manufacture of stamps. A - bubble located under the agarose mold. B - absence of cells in the mold. C - bubble in the area of the mold where microwells are located. The arrows indicate bubbles in different parts of the mold. Scale bar - 300 µm.

Stamps printed on MSLA printer and stamps made of silicone with identical shape were made. Immediately at microscopic inspection it was found out that the defects described above occur in molds made with silicone stamps very often, somewhere in one third of wells. Probably the adhesion of agarose to silicone is higher than to photopolymer. Nevertheless, the spheroids were photographed and their size and shape were measured (Figure 28).

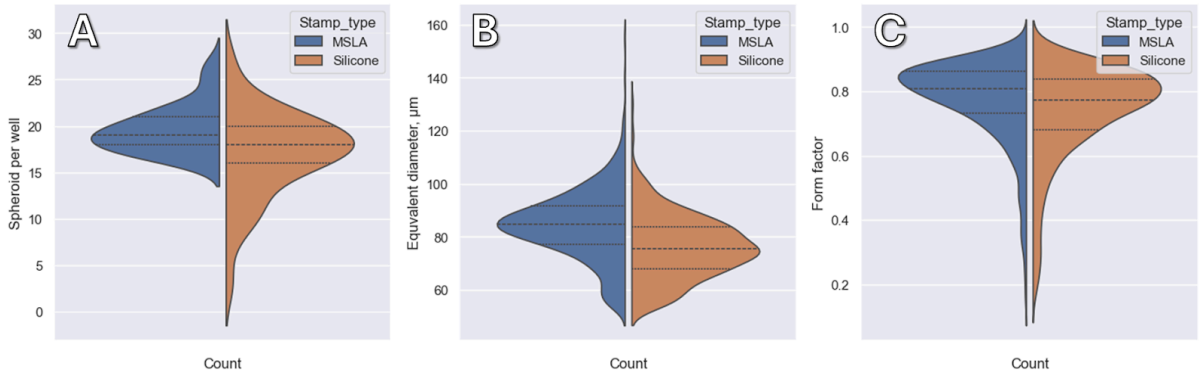

Figure 28. Violin-plot showing the number of spheroids per cell (A), size distribution (B) and form factors (C) of spheroids in microwells made with printing dies and their silicone counterparts .

In spite of the fact that 17 wells of spheroids are created in each microwell, more or less spheroids can be formed in the end. On the average in molds made with printing dies there were 19±2 spheroids, mainly due to the fact that in the wells sometimes two spheroids are formed instead of one. In silicone molds the scatter is noticeably higher, on the average there are 17±4 spheroids in a well, but as can be seen from the graph there are many wells

with noticeably less number, mainly these are wells containing various kinds of defects in the relief of agarose.

The average size of the MSLA molds was 84±12 μm and in the ones made with silicone stamps 76±12 μm, the average form factor was 0.77 and 0.76, respectively. Overall, when looking only at the numerical values MSLA and silicone stamps are very close to each other. Although if we evaluate the distribution (figure), it is obvious that the number of spheroids in molds made with silicone stamps is much less reliable. In addition, making silicone stamps is a noticeably more tedious process, hence in the future we will use exclusively printed ones.

To demonstrate the applicability of agarose microwell plates made by our method, we chose the MTT assay, a well-known and incredibly popular technique for screening the effect of various substances on cell viability. This method is not without drawbacks, but it is amazingly cheap, simple, and relatively reliable when performed correctly [https://doi.org/10.1038/s41420-022-01207-x].

Of course, we are not the first to propose to study cell spheroids in tablets, it is a rather obvious idea for someone who works with cell cultures, but it is often quite inconvenient. For example, in a paper the authors noted the non-homogeneity of spheroid sizes in low-adhesion coated plates, which can affect the subsequent results of the study [https://doi.org/10.1177/1087057116651867]. Also MTT-test on such spheroids requires some tricks, for example, in [https://doi.org/10.1038/s41598-022-05426-1] authors dispersed spheroids mechanically before MTT assay.

The use of our approach allows us to create microwells of the necessary shape and size for a particular task in the wells of a 96-well plate, in which the necessary number of spheroids will be obtained.

Speaking about the MTT-test it is necessary to make a disclaimer at once. In the classical formulation of the test, the optical density of the reaction product is linearly related to the number of living cells (although it actually reflects the activity of mitochondria, but this usually remains behind the scenes). In our case, however, it is rather difficult to establish this relationship, since the viability (and activity) of cells in one spheroid can vary greatly. Accordingly, in the following we simply give the optical density value or the ratio of the OD of the experiment to the OD of the control.

The pre-test was simple (blank experiment), spheroids were formed in wells of a 96-well plate and the MTT assay was performed. After subtracting the blanks, the standard deviation of the optical density was of the order of 11% of the mean, which we found good enough for subsequent experiments.

To demonstrate the possibility of studying cytotoxicity by this method, several different substances that can have a cytotoxic effect were taken. Namely, a solution of DMSO, the topoisomerase inhibitor camptothecin, and magnetic nanoparticles were taken. It should be noted that a noticeable part of the well volume is occupied by agarose gel, the water from which dilutes the solution of the substance introduced into the well.

DMSO and camptothecin are often used in biology as positive controls of various toxic effects. Magnetic nanoparticles are a popular tool for application in biology for magnetogenetic studies, magnetic hyperthermia, development of promising MRI contrasts and so on and since studies on spheroids provide closer to the living organism data screening of nanoparticles on spheroids is quite a challenge.

DMSO and camptothecin were added to already prepared spheroids growing in microwells in a 96-well plate as solutions in nutrient medium. Nanoparticles of iron core-carbon shell composition (Fe@C) modified with carboxyl (-COOH) and amino (-NH2) groups were used.

These nanoparticles are able to penetrate and accumulate in cells [https://doi.org/10.1134/S0031918X19130027], but the uptake of nanoparticles by spheroids is extremely inefficient. Therefore, we took a different route. Cells were incubated with nanoparticles in a monolayer, after which spheroids were formed from these cells, where each cell knowingly contained nanoparticles (Figure 29A).

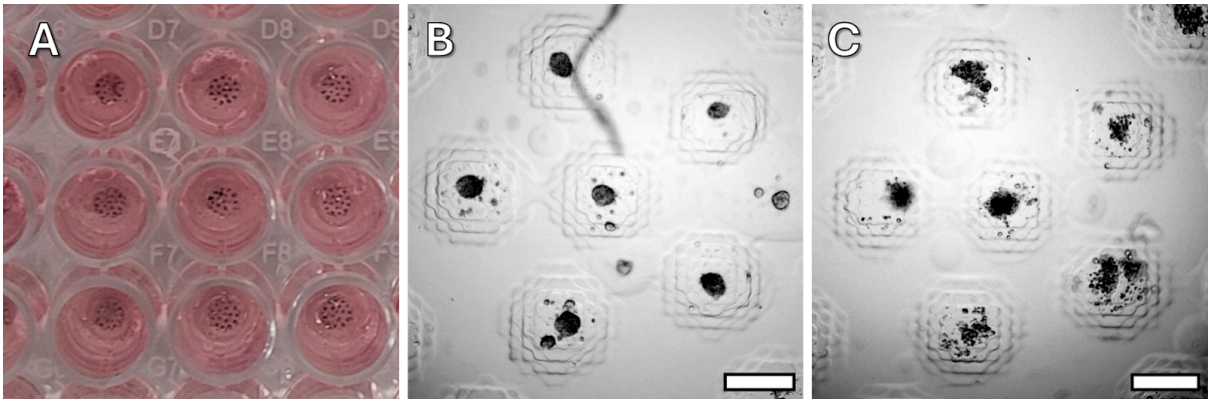

Figure 29. A - photo of spheroids saturated with magnetic nanoparticles visible to the naked eye in the wells of the plate. B - intact spheroids, C - spheroids incubated in a medium containing 20% DMSO.

The effect of DMSO on cell spheroids is noticeable even without the MTT assay (Figure 29B and C): spheroids incubated in a high concentration of DMSO disaggregate into individual cells. In the MTT assay, the effect is also quite clear (Figure 30A). The effect of camptothecin is less pronounced and only appears at very high concentration (Figure 30B), which is expected for cell spheroids (http://dx.doi.org/10.4172/2155-9872.100024). Magnetic nanoparticles did not show any noticeable toxicity on cell spheroids (Figure 30C), nor did they show it on monolayer culture S8.

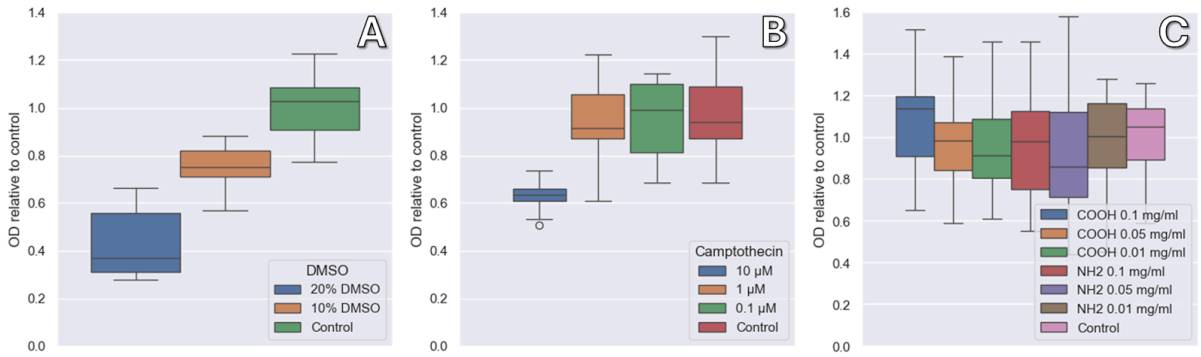

Figure 30. Evaluation of the cytotoxic effect of DMSO, camptothecin and magnetic nanoparticles on spheroids formed from hTERT culture cells.

As noted above and demonstrated in Figure A, cells saturated with nanoparticles are optically dense by themselves (S9), so are spheroids made of them. To obtain the results, the optical density of spheroids with nanoparticles was taken before the MTT assay in order to subtract it and obtain reliable data.

Pricing

Speaking about the possibility of adapting our approach, it is impossible not to mention its cost. In our opinion, it turned out to be quite affordable by the standards of any scientific organization.

The prices given below (table) are somewhat conditional, as the cost of different items may vary significantly depending on regional exchange rates, taxes and duties. Also, the prices do not take into account the cost of electricity and other household needs, as well as payment to the printer operator. However, we hope that this will give an idea at least to an order of magnitude. The main thing you need to have (in addition to standard equipment and supplies for working with cell cultures) is a 3d printer, photopolymer resin and two-component silicone. For making silicone molds it is also necessary to have a vacuum chamber with an appropriate vacuum pump.

Table 2. Equipment prices

Equipment and Materials	Quantity	Price, USD
MSLA 3d printer	1 pcs	400
Vacuum chamber with vacuum pump	1 pcs	450
MSLA resin	1 liter	50
Two component silicone	1 liter	50

If only direct costs are taken into account, the cost of stamps and molds is determined by their volume. The die designs presented in this article were partially optimized in terms of reducing the cost of photopolymer and silicone, but this was not the main goal; the price, however, was already relatively low (Table).

Table 3. Mold and stamp prices

Pices	MSLA resin volume/ Silicone volume, ml	Cost, USD
Petri dish stamp	7/none	0.4
6 well mold	6/5	0.3/0.3
T-150 flask mold	44/39	2.5/2.1
96 well stamp	10/none	0.6

As you can see above, the cost of one mold is quite low, especially when compared to the commercial options (a set of Microtissue molds costs a couple hundred dollars) described above in the article. At the same time, printed stamps and silicone molds can be used many times. The master molds from which silicone replicas are made, however, gradually degrade and you can make 4-5 casts from one before the quality starts to drop and the micropins are destroyed when removing the silicone mold from the mold.

In addition to the relatively low price, the DIY approach allows you to customize stamps and molds to suit your specific needs. It takes two to three hours to print several stamps (depending on the specific printer), it takes about a day to make a silicone mold from a printed stamp (time for final polymerization of silicone), hence per working one can conduct two or three experiments to cultivate spheroids in new microwells.

Conclusion

In order to democratize, simplify and make accessible to every laboratory, we have developed approaches to reliable and reproducible agarose microwell fabrication, with rich customization possibilities. Using MSLA 3d printing, we have produced a series of dies as well as master molds for making silicone molds that allow us to make agarose microwells at a wide variety of scales. From a few cell spheroids in a well of a 96-well plate to tens of thousands in T-150 culture vials.

We have shown how the fundamental limitations of this 3d printing method determine the size and shape of microwells and, using confocal microscopy, demonstrated how this affects the process of spheroid formation. We compared our approach to the mass production of spheroids with its commercial counterpart and showed that, despite the limitations of our technique, spheroids are generally similar. Finally, we have shown the prospects of growing spheroids in 96-well plates, which opens up the possibility of high throughput screening of various substances on spheroids, using standard approaches used in the cell culture laboratory, by modifying the routine MTT test on cellular spheroids with a number of model objects.

CRediT author statement

Minin, A.: Conceptualization, Methodology, Software, Validation, Investigation, Writing - Original Draft, Visualization. Semerikova, T.: Investigation, Resources. Karavashkova, O.: Investigation; Pozdina, V.: Investigation. Tomilina M.: Investigation, Zubarev, I Supervision, Funding acquisition.

Conflict of interest statement

There is no conflict of interest to declare

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