Spatially organized and reproducible spheroid formation using PRIMO contactless photopatterning system

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Abstract

With the increasing use of spheroids in cancer research and drug screening, different techniques have been developed to produce these three-dimensional (3D) tumor cell aggregates. However, besides the fact that they are relatively labor-intensive and have limited throughput, they often lack reproducibility in the shape and size of the spheroids they form. In this application note, we show two ways of using PRIMO contactless photopatterning system to make hundreds of very reproducible 3D cell aggregates. A first method consists in growing cells in 3D cuvettes made of non-adherent hydrogel, while the other allows their growth in 3D from a two-dimensional (2D) micropattern. Another limitation of current methods is the difficulty to monitor spheroid evolution or to image them because of their difficult handling. We also show by making a well-organized 3D tumor invasion assay that our techniques allow a perfect organization of experiments in space, thus allowing easier subsequent imaging and data analysis. Altogether, this application note shows that PRIMO can be a powerful tool to produce reproducible 3D cell aggregates, control their shape, and organize them in space for a precise automated data analysis.

Introduction

The physiological relevance and advantages of three-dimensional tumor cell culture models over conventional two-dimensional culture models in cancer biology and drug discovery have been widely acknowledged[(1)]. Indeed, 3D tumors in vivo display several structural, functional and physiological features that are not replicated in 2D cultures. Spheroids are in vitro 3D aggregates of tumor cells that better mimic the physiology of in vivo tumors. Indeed, they have complex cell-cell and cell-to-matrix interactions, and they develop chemical gradients of oxygen, nutrients and catabolites resulting in a similar organization as in-vivo tumors with an outer zone of proliferating cells, followed by an inner hypoxic area with quiescent cells, which contains a necrotic core **(Fig. 1)**. All these characteristics make spheroids one of the best 3D model of cancer[(2)], not only for cancer biology understanding but also for drug discovery.

Up to now, different techniques have been developed for spheroid assembly, including the hanging drop method, the liquid overlay technique, the use of spinner flask, or more recently, the use of low-attachment and round bottom wells[(3)].

The hanging drop method consists in seeding dissociated cells in small droplets of media that hang on the upper lid of a culture dish lid, allowing them to sediment and eventually aggregate. It is really easy to implement so it became one of the most used technique, together with the liquid overlay technique which principle is to seed single cells on non-adherent surfaces and let them aggregate. However, while these two techniques do not require specialized equipment or expensive consumables, they come with a major drawback: a low reproducibility in the control of shape and size of the spheroids. Besides, they are not adapted for subsequent analysis like imaging since you cannot follow every spheroid and/or maintain it.

Some more recent devices have been developed to overcome these issues. Indeed, people use more and more some 96 well-plates made of low-attachment U-bottom wells that allow the

formation of one spheroid per well. In this case the reproducibility is good and it is adapted for subsequent analysis but it only allows to work with 96 spheroids per plate, which is a low output regarding their substantial price.

Altogether, this shows that the most cost-effective techniques often lack reproducibility and/or ease of use for monitoring during experiments.

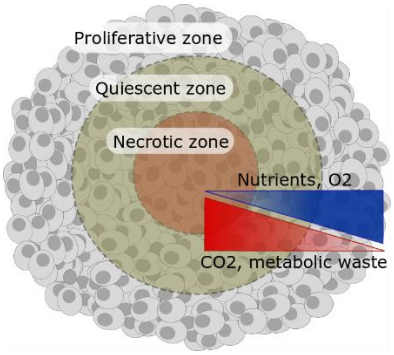

***Figure 1**: The structure of spheroids resembles the in-vivo tumors*

In this application note, we show that it is possible to use PRIMO contactless photopatterning technique to produce spheroids in a very controlled and reproducible manner and to spatially organize the experiment to make it more compatible with imaging analysis. We show that this can be done either by creating non-adherent hydrogel cuvettes or by making spheroids grow from 2D micropatterned cells and that these two techniques have their own pros and cons. We also show an example of application with a very common experiment: the 3D cancer invasion assay.

Experiments and results

Setup description

PRIMO is docked on an inverted microscope **(Fig. 2)** and allows for a wide-field spatially modulated UV-light illumination thanks to an array of micromirrors. The UV-light (375 nm) is modulated according to an image previously designed and loaded into Leonardo software. The corresponding UV pattern is then reflected on a UV dichroic mirror placed in the filter turret of the microscope and passes through a 4X S-Fluor objective before reaching the sample **(Fig. 2)**. The images used as micropatterns can be drawn using Inkscape or Fiji software; any grayscale pdf or tif file can be loaded into Leonardo software. The gray levels of the image are computed into relative UV doses allowing a local control of the UV dose on a single image.

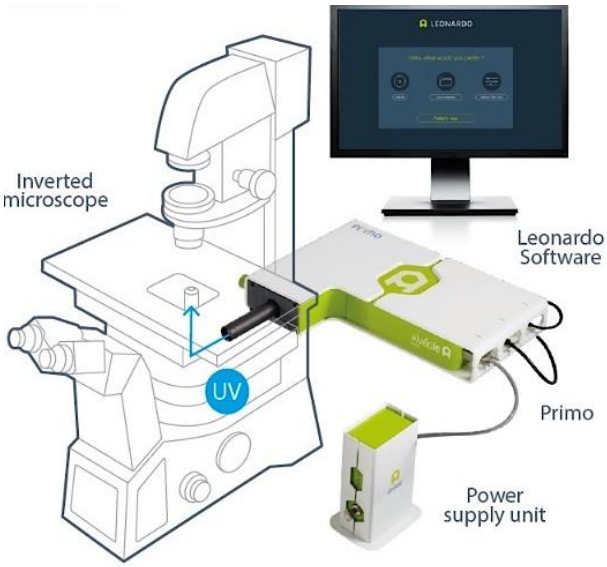

***Figure 2**: Docking of the PRIMO on an inverted microscope*

Spheroids made in hydrogel cuvettes

To create non-adhesive hydrogel cuvettes, we used the capacity of PRIMO to locally control the UV power exposure, which is mandatory to control hydrogel structures' height. The principle is to take advantage of the oxygen polymerization inhibition to control the height of hydrogel structure[4]. A porous PDMS ceiling is used to allow the oxygen diffusion at the top of the illuminated area which creates an "inhibition zone" that stops the polymerization at a given height. Increasing the UV power increases the oxygen consumption rate leading to a taller structure **(Fig. 3B)**.

To locally control the UV power, we use grayscale images that are interpreted by PRIMO as different local photon flows leading to a topographical structure.

To create the hydrogel cuvettes, we proceeded as follow:

1) Sample preparation

The microreactors were prepared as shown in the **Figure 3A**: two PDMS stencils (Alvéole) were stacked onto a 22x22 mm 170 µm thick glass coverslip (Schott Nexterion, Schott Jena, Germany) to create a PDMS chamber.

Some 4-Arm-PEG-Acrylate (MW 10K, Laysan Bio, Arab, USA) was diluted at 50 mg/mL in PLPP photo-initiator (Alvéole) then pipetted into the PDMS chamber.

2) Hydrogel polymerization with PRIMO

The sample was positioned on the inverted microscope equipped with PRIMO system. A grayscale 8-bit image was loaded into the Leonardo software then projected through the sample during 30 seconds at 130 mW/mm^2 for polymerization. Once structured, the hydrogel was washed with PBS to remove the solution containing the remaining non-

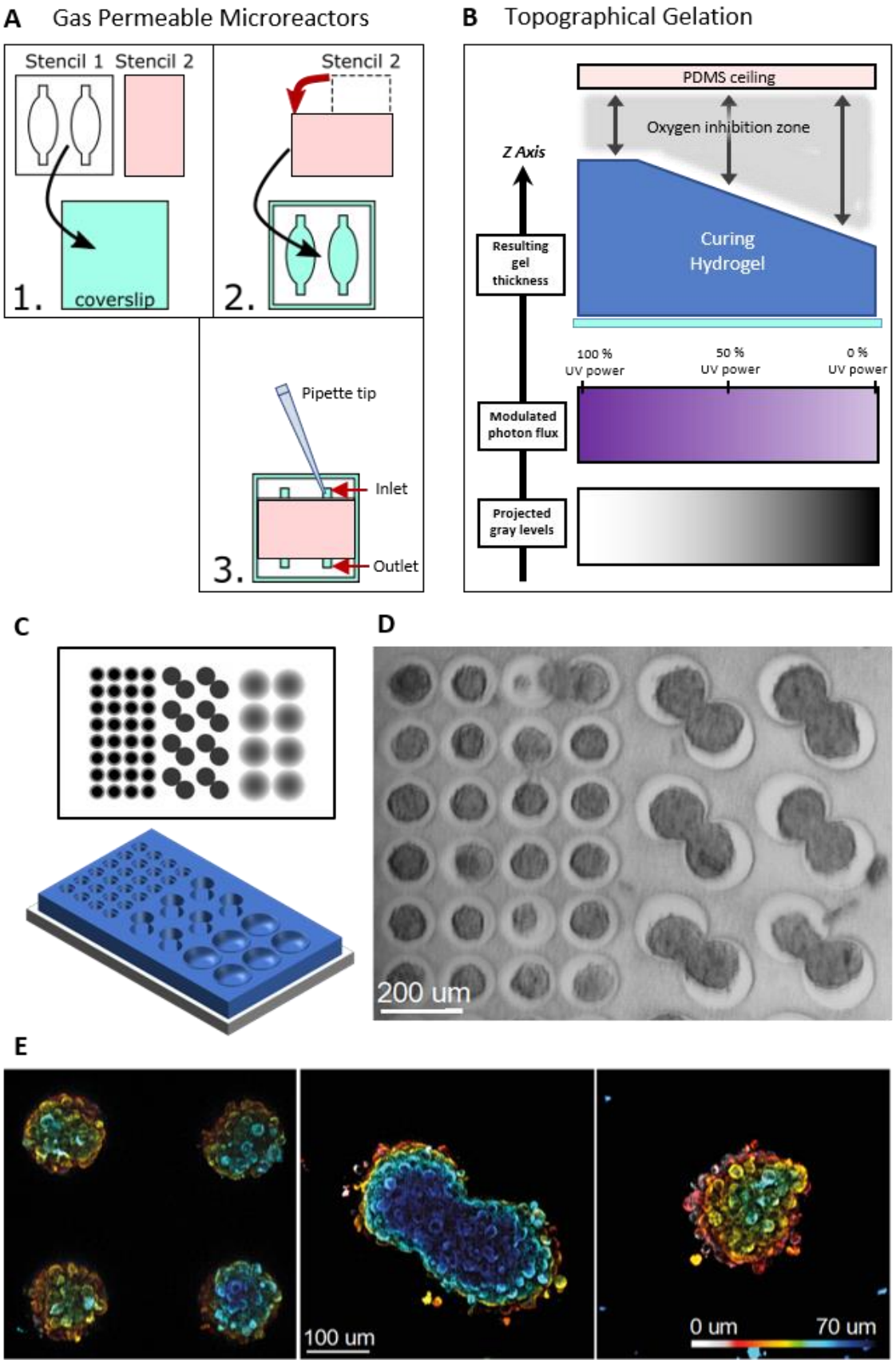

Figure 3: Production of spheroids using hydrogel cuvettes. (A) Build-up of the gas permeable microreactor using PDMS stencils. (B) Principle of the topographical gelation using a PDMS ceiling: gray levels of the images are interpreted as different UV powers, leading to smaller or bigger inhibition zones and thus different hydrogel heights. (C) Image loaded in the Leonardo software (top) and scheme of the resulting 3D hydrogel structure[4]. (D) Wide-field image of HEK 293T cells that have aggregated in the cuvettes[4]. (E) Confocal images of the spheroids[4].

polymerized monomers and the photo-initiator, then the ceiling PDMS (stencil n°2) was removed.

3) Cell seeding and spheroid formation

A human embryonic kidney cell line (HEK 293T) was cultured in complete cell culture media (DMEM, FBS 10%).

Cells were detached and dissociated using trypsin-EDTA (Thermo Fischer Scientific), centrifuged and resuspended in medium. They were finally seeded on the hydrogel structures and let overnight to sediment into the cuvettes and aggregate as spheroids.

4) Imaging and characterization

The spheroids were fixed with PFA 4% for 15 min and permeabilized by a 1% solution of Triton-X100 in PBS for 15 minutes before saturation with a 2% BSA solution for one hour. Finally, a solution of phalloidin-A647 (1/200), rabbit anti-lamin antibodies (1/500) in PBS 1% BSA was incubated for 2 hours. Finally, anti-rabbit-A568 secondary antibody was incubated (1/200) in PBS 1% BSA for 2 hours.

The spheroids were imaged in 3D with a home-made DMD-based confocal microscope[5].

Beyond their high homogeneity in shape and size, we can see on the **Figure 3C to 3E** that it is also possible to impose different shapes to the 3D cell aggregates.

Spheroids made from micropatterns

The second method to make spheroids is to use PRIMO to micropattern small groups of cells that will eventually grow in 3D thanks to the spatial confinement. The process of this micropatterning technique, based on the LIMAP technology[6] is shown in the **Figure 4A**.

1) Micropatterning on glass coverslip

Briefly, a H-coated Nexterion glass coverslip (Schott) was covered with a PDMS stencil (Alvéole) in which were deposited

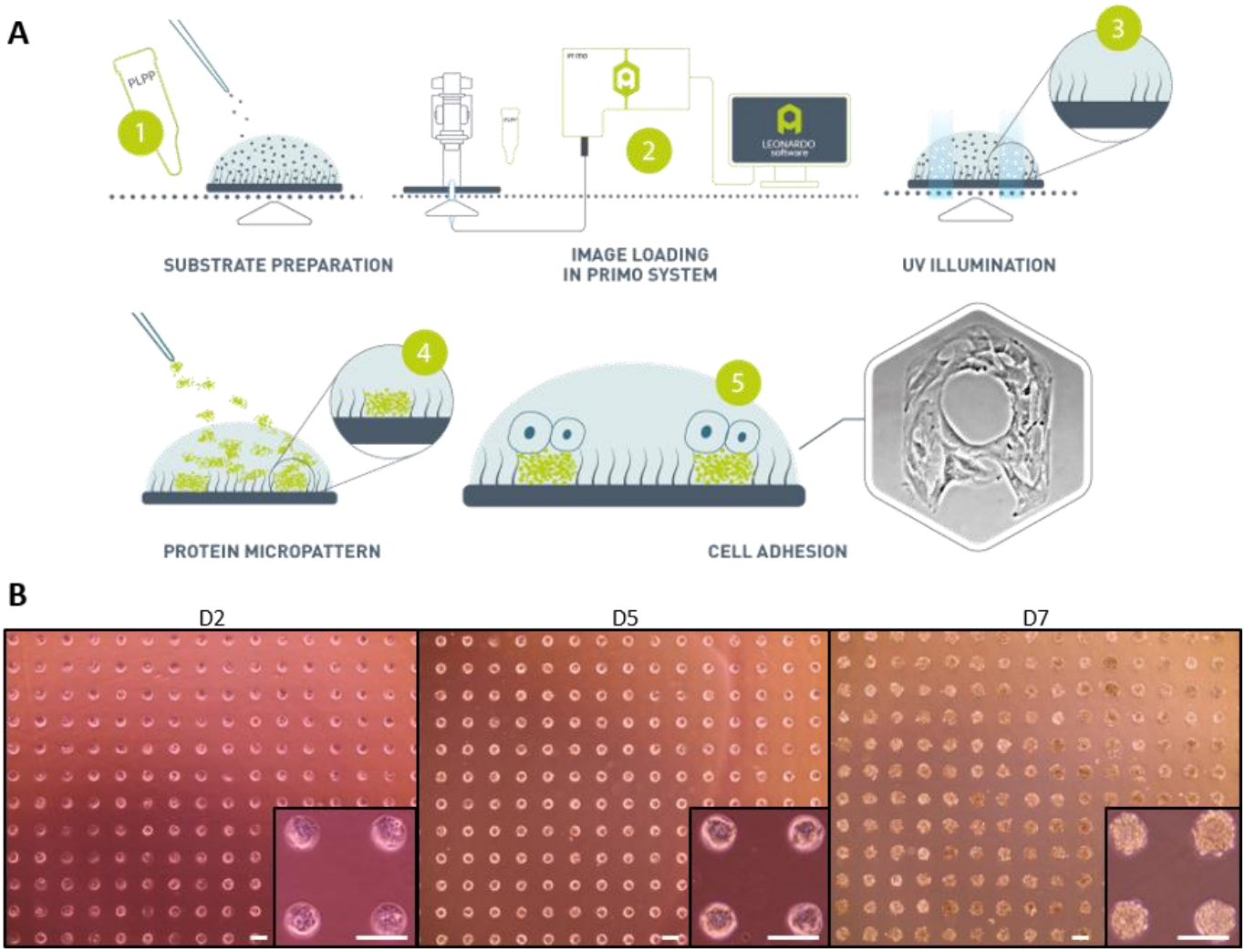

***Figure 4**: Production of spheroids using micropatterns. (A) Keys steps of the micropatterning process using PRIMO, Leonardo software and the PLPP gel photo-initiator, followed by cell seeding. (B) Growth of Huh-7 from 2D micropatterns (Day 2) into 3D spheroids (Days 5 and 7). Scale bar = 200 µm*

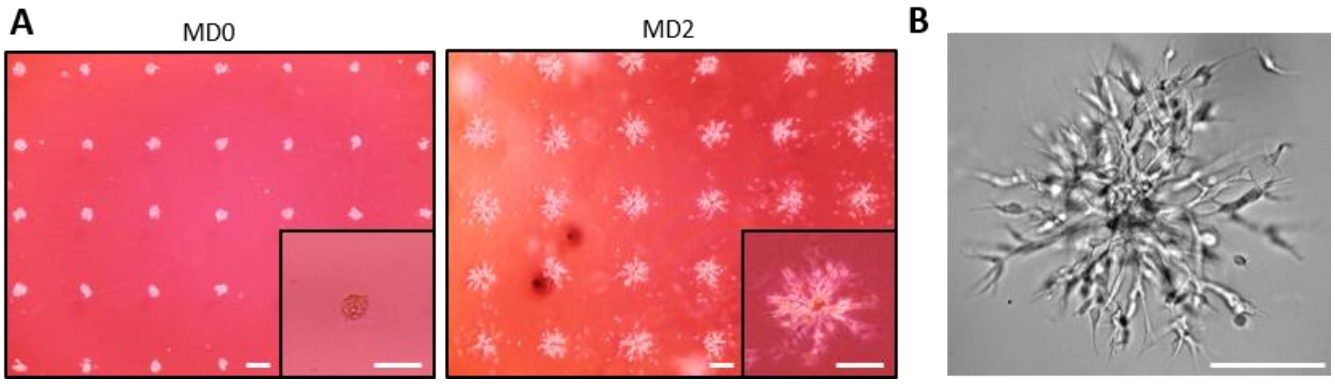

Figure 5: 3D spheroid invasion assay with spatially organized glioblastoma spheroids embedded in Matrigel. (A) Evolution of the U-87 MG spheroids from the day they are embedded in Matrigel (MD0) to the moment they invade the gel in 3D (MD2). (B) Wide-field image of a U-87 MG spheroid invading the Matrigel. Scale bar = 200 µm.

3 µL of PLPP Gel (Alvéole) **(Fig. 4A step 1)**, 0.5 µL of Surfactant Mix (Alvéole) and 10 µL of milliQ water. After homogenization of the mixture, it was let to dry at room temperature during 15 minutes.

After complete drying of the gel, the sample was placed onto the microscope holder and the patterns were projected onto the surface at a UV dose of 45 mJ/mm2 **(Fig. 4A steps 2 and 3)**. Leonardo software allowed to replicate the patterns to cover the entire surface of the surface of the substrate.

After rinsing with PBS, some 10 µg/ml Alexa-647 conjugated fibrinogen (Invitrogen) was incubated for 10 minutes at room temperature and adsorbed only on the previously illuminated areas **(Fig. 4A step 4)**. The protein solutions were finally rinsed profusely with PBS.

2) Cell seeding and spheroid formation

Human hepatocyte cell line (Huh-7) was cultured and dissociated as explained previously for HEK 293T cell. Cells were seeded in excess onto the sample and were allowed to adhere for 30 min before washing with medium **(Fig. 4A step 5)**. They were then let in the incubator for a few days and started to form 3D structures after 2 to 3 days.

As shown on the **Figure 4B**, this technique allowed to produce hundreds of similar spheroids on a single coverslip (in this case we made an array of 2500 spheroids of 150 µm in diameter on a 2x2 cm coverslip). To collect them, a gentle manual agitation of the sample was sufficient to detach them.

Example of application: 3D spheroid invasion assay

Invasion of surrounding normal tissues is generally considered to be a key hallmark of malignant tumors. A common experiment to study the invasive capacity of a tumor in vitro is to perform a 3D spheroid invasion assay. The principle is to make spheroids, culture them inside a hydrogel and observe and measure the cells invading the surrounding environment.

We performed this experiment by first producing an array of human glioblastoma (U-87 MG) spheroids as explained in the "Spheroids made from micropatterns" part. After two days of growing, we covered them with a hydrogel (Corning Matrigel matrix) to allow their migration in 3D.

In the **Figure 5** we see that the spheroids are round at the beginning of the experiment, and that after just twos day in the Matrigel they invade it in 3D. On the contrary to classical 3D invasion assay, we have here a well-organized array of identical spheroids that allows the precise monitoring and measurements of the glioblastoma's invasion.

Conclusion

Here we have presented two methods for the production of spheroids. The hydrogel cuvettes technique and the micropattern techniques have different pros and cons and thus one or the other can be more relevant depending on the experiment:

- On the one hand, the **cuvette technique** allows longer culture time and is compatible with all cell types.
- On the other hand, the **micropatterning technique** gives an easier way to collect the spheroids or to image them since there are directly in contact with the glass coverslip. However, it does not work for all cell types since they do not all grow in 3D from a 2D pattern.

However, they still share some common features. Firstly, they both allow the generation of **uniform and reproducible spheroids with a good throughput**. It is interested to note that

unlike other spheroid production methods that produce one spheroid per well/condition, our two techniques have the ability to **generate multiple spheroids** in each well/condition thus improving the robustness of assays and data analysis. Secondly, they both improve the data analysis by making it possible to **organize in space the spheroids** to be able to easily monitor their behavior or their response to a drug.

Finally, beyond all the applications in cancer research and drug screening linked to spheroids, an application that is still very poorly explored is **the control of the initial shape of organoids** to study or to improve their self-organization. With PRIMO, it is possible to test different hydrogel cuvette shapes just by changing the grayscale image. Thus, it could also represent a powerful tool to develop more relevant in vitro disease model.

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