RESEARCH ARTICLE

Colony development of laser printed eukaryotic (yeast and microalga) microorganisms in co-culture

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Abstract: Laser Induced Forward Transfer (LIFT) bioprinting is one of a group of techniques that have been largely applied for printing mammalian cells so far. Bioprinting allows precise placement of viable cells in a defined matrix with the aim of directed three-dimensional development of tissues. In this study, laser bioprinting is used to precisely place eukaryotic microorganisms in specific patterns that allow growth and microscopic observation of the organisms' micro-colonies. *Saccharomyces cerevisiae* var. *bayanus* and *Chlorella vulgaris* are used as model organisms for this purpose. Growth and development of the micro-colonies are studied via confocal microscopy and the colonies' growth rates are determined by image analysis. The developed protocols for printing of microorganisms and growth-rate determination of the micro-colonies are very promising for future studies of colony growth and development.

Keywords: laser-induced forward transfer, bioprinting, printing of microorganisms, growth rates of micro-colonies, *Saccharomyces cerevisiae, Chlorella vulgaris*

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1. Introduction

Current progress in biotechnology relies heavily on strain characteristics and the development of strains with new properties. Nowadays, molecular biology offers outstanding possibilities with an emerging need for fast screening, selection, and assessment methods. Beyond these requirements, techniques that allow fast industrialisation of selected strains would enable rapid progress up the TRL (Technology Readiness Level) towards fast industrial implantation. Laser printing of microorganisms offers completely new solutions to fill the gap between the creation of GMO *in silico* and industrial production with these organisms.

Laser printing has already been used in microbiology[1], where soil particles were printed unto solid media in order to isolate hitherto unidentified organisms that could only exist in specific configuration in their symbiont. Although this is possible to some extent through classic microbiological methods, the printing process brings in an unparalleled level of precision. Using traditional methods, cell suspensions could be diluted in sterile media and placed manually as droplets on certain positions on the growth matrix; realistically, the volume of a droplet cannot be smaller than 1 μL and the precision of the human hand would require that the droplets placed no closer than 2–3 mm apart. Printing micro-droplets of cell suspensions al-

lows the deposition of, statistically speaking, predetermined number of cells on the growth surface with micrometre positional-precision, and with droplets that can be smaller than 10 pL. This level of precision opens the way for exact growth, selection, and interaction studies with the advantage that replication of the experiment would be only limited by the power of the observation and data analysis techniques. Another advantage of this technique is that by printing microorganisms in grids, the technique can very easily lend itself to developmental models based on grid arrangements such as lattice automata or agent-based models[2–5]. The work presented here is the result of collaborative research between the Department of Bioprocess Engineering and Biomaterials of Centralesupélec Paris in France and the Department of Nanotechnology of the Laser Zentrum Hannover in Germany, two laboratories that are almost 800 km apart.

Two robust microorganisms were used for this work, namely *S. cerevisiae* var. *bayanus* (hereafter referred to as *S. bayanus*)—a wine yeast—and *C. vulgaris*, a well-studied green microalga of the Chlorophyceae group. Both organisms are rather spherical in shape (2–10 μm in diameter), showing good resistance to harsh environmental conditions. *C. vulgaris* has a hard cellulosic cell wall containing some chitin[6] and *S. bayanus* has a hard glucan-based cell wall[7]. Both organisms can grow heterotrophically with glucose as the sole carbon source[8], but *C. vulgaris* is additionally capable of autotrophic (photosynthetic) growth on inorganic carbon in the presence of light[9]. Mixotrophic growth of *C. vulgaris* based on a mixture of autotrophic and heterotrophic growth is also possible[9]. *S. bayanus* can metabolise glucose either respiratively with O_2 or fermentatively in the absence of O_2[10]. The metabolism mode chosen by the organism depends on the conditions but in general, *Saccharomyces* yeasts are Crabtree-positive[10] and "prefer" the fermentative mode of metabolism. Despite this, even when fermenting sugars, yeast requires a small amount of oxygen that is utilised for biosynthesis of cell membrane components[11].

A form of symbiosis could be imagined where an autotrophic organism uses the CO_2 released by a heterotrophic organism and vice versa; the latter uses the O_2 released by the former. This symbiosis of course already exists on a planetary scale with an additional input of CO_2 from geothermic activity. Even under autotrophic conditions, once a culture becomes dense enough to be light-limited, a part of the population starts to metabolise internal storage compounds "heterotrophically", releasing CO_2. The study of such phenomenon with a single organism would be very difficult in liquid culture but with two organisms, the phenomenon of heterotrophic/autotrophic growth could be simulated in low cell density cultures. Before this possibility can be explored, the interaction between the two organisms could be studied with the aid of laser bioprinting.

Different printing techniques, mainly inkjet printing, extrusion printing (also called bio-plotting or syringe-based printing technique), and laser bioprinting have been applied for two and three-dimensional assembly of biological materials including proteins, DNA, microorganisms, and living mammalian cells[12]. Laser printing offers the capability to combine high resolution and ultra-small droplet volumes (below 10 pL) with high sample viscosities and high cell densities due to the absence of a nozzle. In this paper, laser printing is used to print droplets of alginate solution with embedded yeast or microalgae cells in predefined patterns.

In brief, bioprinting of microorganisms paves the way for the development of new and precise methods that could be used to study: (i) the development of microorganisms in solid matrices in the presence of nutrient gradients, (ii) the interaction of the same and different organism-colonies next to each other, (iii) the response to stress and resistance to inhibitors, and (iv) cell communication or quorum sensing. This method provides a relatively simple way to perform experiments with a large number of replicas and could be even applied to strain selection in the future. Printing could also provide the means to perform multifactorial experiments.

To the best of our knowledge, the two eukaryotic microorganisms mentioned above are printed for the first time in order to observe and study these microorganisms in close proximity and potentially different topologies. Here, we report on how *S. bayanus* and *C. vulgaris* can be printed in specific patterns. Their development is observed and measured via confocal microscopy.

2. Materials and Methods

2.1. Strains, Media and Growth Conditions

Chlorella vulgaris (211-11b Göttingen) and *Saccharomyces cerevisiae* var. *bayanus* (DSMZ 3774—referred to as *S. bayanus* hereafter) were used for this work. Bristol medium[13] was used to grow *C. vulgaris* in

shake-flask culture in a photo-incubator (25°C) with an atmosphere enriched in CO_2 (up to 2.0% by volume). The light at the surface of the shaken cultures was 20 µmol photons $m^{-2}s^{-1}$. The rotational speed of the orbital shaking platform was 100 rpm with a rotational diameter of 50 cm. *S. bayanus* was grown in YPD medium under the same conditions. The flasks were only filled to 1/5 of their total volume and stoppered with foam bungs (11901935 - X100; Fisher Scientific).

The Bristol medium was modified with the addition of 5 g/L glucose and its concentration in phosphate was increased five-fold to 0.009 moles/L for better buffering; this medium was solidified, if required, with the addition of agar (1.5% w/v) prior to sterilization by autoclaving.

Petri dishes (60 mm diameter; Nunc™ Cat No. 150326) were filled (20 mL) with solid medium upon which a filter paper (cellulose acetate; 0.2 µm pore; Sartorius Cat. No. 11407--50----ACN) was placed. Printing was performed directly on this filter paper with cells suspended in a saline alginate solution (0.9% w/v NaCl; 2% w/v alginate).

Cell suspensions were prepared via centrifugation of each culture (5 mL) separately. The pellet was then re-suspended in an equal volume of phosphate buffer (100 mM, pH 7.0; containing 9.0 g/L glycerol); centrifuged and re-suspended in a smaller volume of the same buffer. The final volume of buffer added was such as to give a cell concentration of 1.0 M cells/mL (Guava; Viacount flex method). Cell suspensions were cooled overnight to 4°C before being dispatched by post to the printing laboratory in a cool box containing ice blocks.

2.2. Laser Bio-printing

The printing process was as described by Koch *et al.*[14]. The apparatus consists of a pulsed infra-red laser source, a horizontal glass slide “the donor slide”, and a “collector slide”, which in the case presented here, was a filter paper on the agar medium.

The donor slide was coated with a thin layer of laser energy absorbing material (60 nm of gold). A layer of the cell suspension prepared as above was coated onto the absorbing layer. The donor slide was then inverted and held in close proximity (1.0 mm) above the collector slide (Figure 1).

Laser pulses (1064 nm wavelength, 10 ns pulse duration, approximately 20 µJ pulse energy corresponding to laser fluency between 1 and 2 J/cm² at the focal point) are focused through the donor slide on the

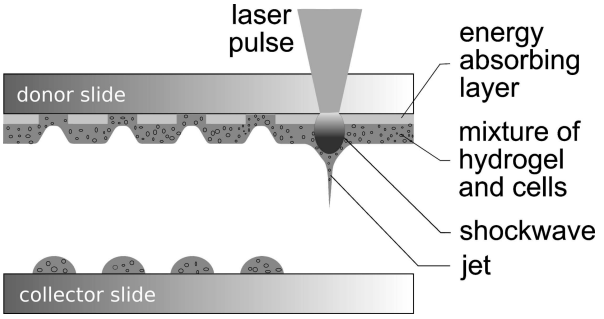

Figure 1. Schematic sketch of the laser-assisted bio-printing. The donor slide is coated underneath with a laser absorbing layer and a layer of biomaterial to be transferred, usually a hydrogel with embedded cells. Laser pulses are focused through the upper glass slide into the absorbing layer. By evaporating this layer a high gas pressure is generated, that propels the biomaterial towards the lower glass slide.

absorbing surface. A high-pressure vapour bubble is thus generated which expands and propels a defined volume of the cell suspension towards the collector slide. The vapour bubble reaches its maximum volume after a few microseconds and collapses when its inner pressure decreases below atmospheric pressure[15]. However, the accelerated biomaterial keeps on moving by inertia to the collector slide and forms a thin jet at the bubble front that lasts for some hundreds of microseconds. At the end, a volume ranging from some picoliters (pL) up to several nanoliters (nL) is transferred to the collector slide surface in the form of a droplet. Biomaterial droplets can be positioned in two-dimensional patterns by moving the donor and receiving slides relative to each other.

The volume of the printed droplets depends on the laser pulse energy, the thickness of the absorption layer, and the biomaterial layer as well as the viscosity of the initial biomaterial layer on the donor slide[16]. The number of cells in each droplet usually depends on the initial cell density in the biomaterial layer on the donor slide and the volume of the printed droplet is subjected to statistical variations. In this case, the conditions used for printing *S. bayanus* were: pulse length (10 ns); pulse energy (18 µJ); droplet volume (180 pL) aiming for a cell concentration of 200 cells per droplet. For *C. vulgaris* the conditions were: pulse length (10 ns); pulse energy (17 µJ); droplet volume (180 pL) aiming for a cell concentration of 200 cells per droplet.

2.3. Microscopy

The development of colonies on the surface of filter papers was observed using a Zeiss confocal micro-

scope (LSM 700 with 4 laser lines) with an inverted stage. A 10X lens and the "tile stacking" function were used to observe the development of the colonies. A laser excitation wavelength of 555 nm was used to observe *C. vulgaris* cells and micro-colonies.

Image analysis was performed using the ImageJ software (http://rsb.info.nih.gov/ij/). The area of the colonies was measured by classical image processing operations:

- conversion to binary using the threshold value obtained by the moments method
- hole filling
- pixel size calibration
- particle analysis

3. Results

S. bayanus and *C. vulgaris* micro-colonies could be easily distinguished by using the natural fluorescence of chlorophyll (emission peak at 668 nm) (Figure 2). After this initial identification, the growth of the micro-colonies could be easily followed by daily observation of the colonies (Figure 3). This required image analysis (Figure 4) could take into account the amount of biomass present in each droplet, especially during the early stages of growth while the droplets were partly "empty".

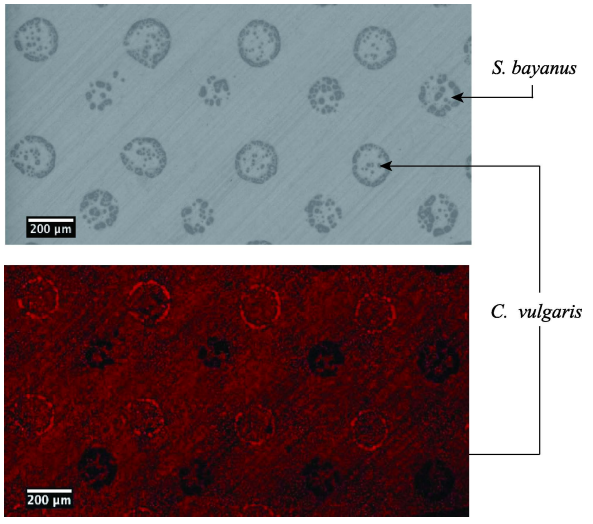

Figure 2. Identification of *C. vulgaris* microcolonies after printing using the natural fluorescence of chlorophyll. Typical cell pattern obtained by the two-step laser printing: *C. vulgaris* can be easily distinguished from *S. bayanus*, thanks to the natural fluorescence of chlorophyll excited at 455 nm. The scale bar indicates 200 µm, the distance between the centres of adjacent printed microcolonies. The red fluorescence indicated the presence of chlorophyll. This picture was taken upon the receipt of the printed samples designated as day 1. The image has been cropped to show a typical part of the bio-printed field.

The results could be analysed by applying the exponential growth model, normally applied to cell populations[17] to the entire micro-colonies. The lag period lasted approximately three days for both microorganisms. Growth took place both within and outside of the initial droplet. Once the droplets were "full", growth continued on the external radius. From day 9 onwards, the colonies started to come into contact with one another and growth could no longer be considered unrestricted. It appeared that *C. vulgaris* dominated *S. bayanus*—maybe thanks to the CO_2 provided by the yeast. In a photo-bioreactor containing defined medium with glucose (10 g/L), photosynthesis over heterotrophic growth was privileged by *C. vulgaris* (data not shown). In this study the plates were lit so it is possible that *C. vulgaris* grew photosynthetically while *S. bayanus* grew heterotrophically.

Growth of the two organisms was followed by measuring the surface areas and radii of their colonies (Figure 4). Following growth by colony radius measurement, *S. bayanus* started to grow first but then reached the stationary phase before *C. vulgaris* (Figure 5). Following colony growth with surface density (area), *S. bayanus* started growing a day later than *C. vulgaris* but reached almost the same extent of growth as *C. vulgaris* (Figure 6). *C. vulgaris* colonies were bigger at the end of the experiment. This was not due to the darker colour of the *C. vulgaris* colonies, as image analysis was performed in such a way as to take this into consideration (Figure 4). Growth of *S. bayanus* peaked between 3 to 7 days (Figure 6); *C. vulgaris* exhibited its fastest growth rate between 2–6 days (Figure 6). The order of growth for the two organisms suggests that *C. vulgaris* benefited from the presence of *S. bayanus* possibly due to the local production of CO_2.

Despite the fact that the growth rate of *S. bayanus* was faster than that of *C. vulgaris* (Figure 7), the internal part of each colony (droplet) was completely occupied within 6 days for *C. vulgaris* and 7 days for *S. bayanus* (Figure 3).

The colonies grew internally within the droplets first, then both internally and externally, and finally only externally to the droplets (Figure 3). Taking this growth pattern into account, it seems that the total surface area of the colonies would be the best parameter for the quantification of colony growth (Figure 6).

The growth of *S. bayanus* stopped at day 9 after which the apparent surface area of the yeast colonies

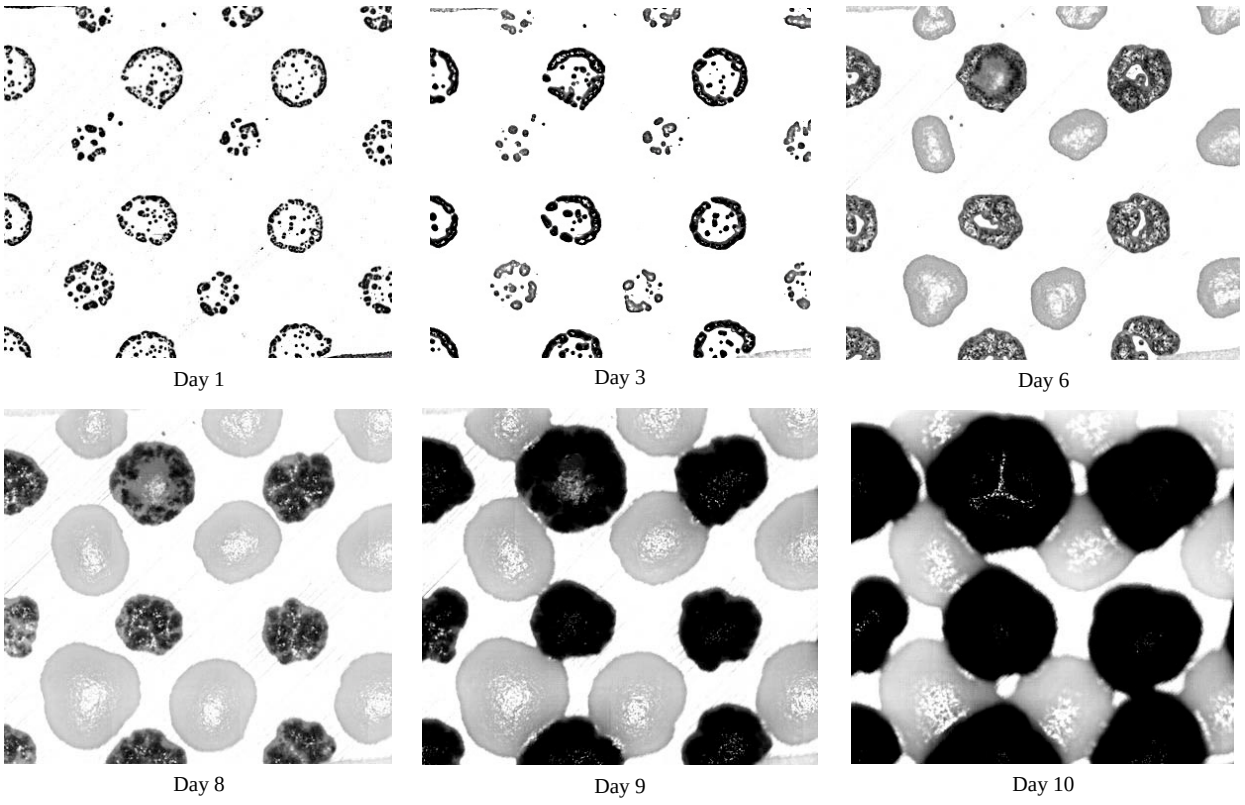

Figure 3. Growth of printed microcolonies as followed by microscopy. Development of the microcolonies in a selected portion of the full printing zone. A typical part of the bio-printed field was chosen for image analysis. The contrast of the images was increased for clarity and better visual definition of the edge of the microcolonies.

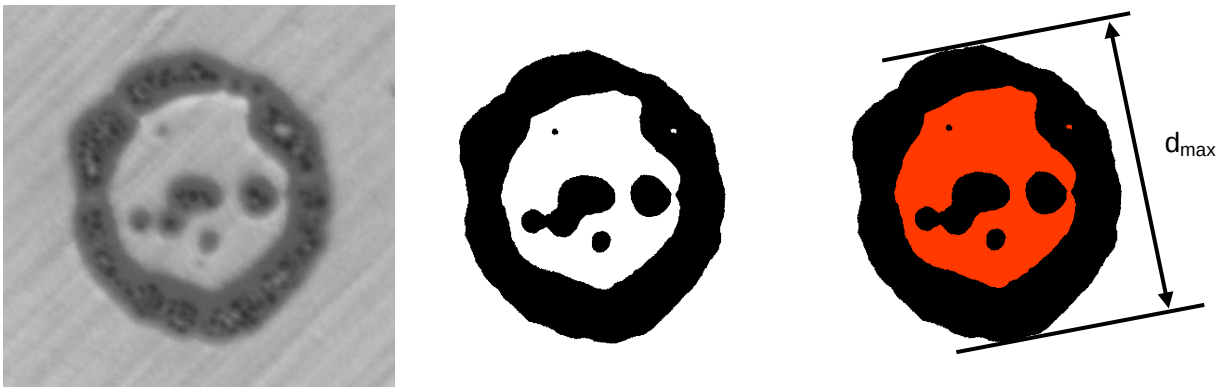

Figure 4. Image processing procedure. Growth parameters: a) initial image; b) binary image obtained with the threshold value given by the moment method; c) colony surface (black), surface fraction (black over black + red) and maximum Feret's diameter (d_{max}). A typical microcolony is shown in order to demonstrate the sequential stages of image analysis. The contrast of the image was increased for visual clarity.

decreased. This decrease was due to the overlapping growth of *C. vulgaris*. There was certainly no evidence that *C. vulgaris* suffered in the presence of *S. bayanus*. On the contrary, the growth rate of *C. vulgaris* seemed to have been boosted by the presence of *S. bayanus*. These observations and speculations have to be confirmed with further experiments, but the production of CO_2 by *S. bayanus* could be a plausible explanation for this increased growth rate of *C. vulgaris*.

The exponential growth rate for the colonies, obtained by identification on the periods of unrestricted exponential growth (Figure 7) was quite similar for

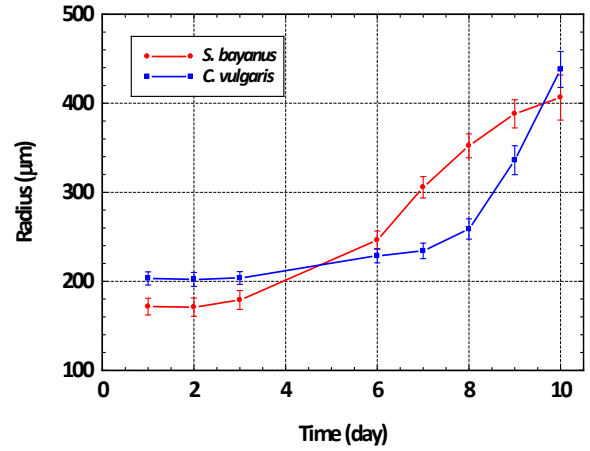

Figure 5. Microcolony growth measured by image analysis. Average radial growth of 10 colonies, error bars represent the standard deviation over these 10 values.

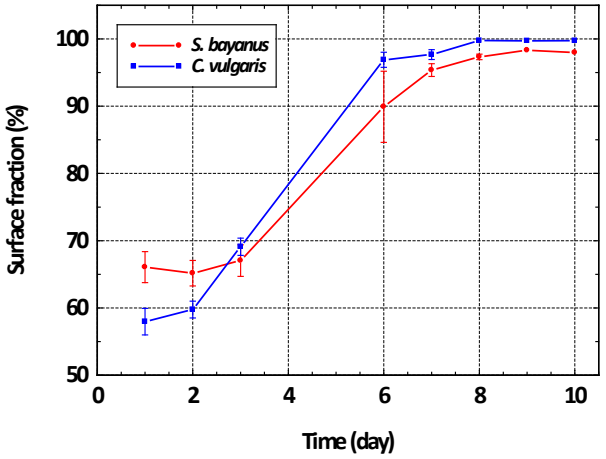

Figure 6. Fraction of the total droplet surface filled with microorganisms. Average values for 10 colonies, the error bars are the standard deviation over the 10 values.

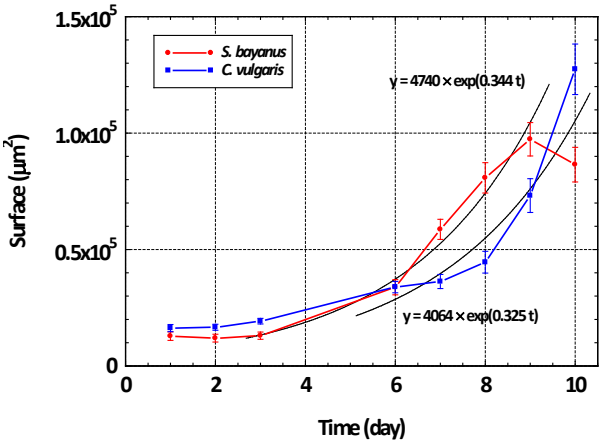

Figure 7. Microcolonies' development as followed by the increase in surface area. Average of 10 colonies, the error bar is the standard deviation over these 10 values.

the two microorganisms (0.344 d^{-1} and 0.325 d^{-1} respectively for *S. bayanus* and *C. vulgaris*) and far below the values usually encountered for these microorganisms (1.19 d^{-1} and 8.32 d^{-1} measured for the two organisms in liquid batch cultures). The authors propose that the apparent "unrestricted growth" observed in this experiment is related to only growth in a part of the colonies such as the cells in contact with the substrate and/or close to the external radius of the colony. Indeed, if one (almost) constant part of the colony grew without restriction, the apparent growth of the entire colony would still present itself as exponential growth, but with a smaller growth rate. This was observed in this study.

4. Discussion and Conclusion

Laser printing and separate observation of the microcolonies in two geographically separate laboratories presented a number of technical uncertainties. Despite these difficulties, both organisms were robust enough to survive the treatment, and the growth rates of their micro-colonies were monitored. The results suggested that for both organisms, the cells across the entire colony grew.

To the best of our knowledge, this is the first time that laser printing has been applied to print microbial micro-colonies of single cell eukaryotes. This technique has recently been used to print earth samples in order to aid in the isolation of new soil organisms[1]. However, the technique has not been applied to the printing of pure culture of eukaryotic microorganisms.

From the results presented here, it is difficult to see whether the two organisms exhibit a symbiotic or a competitive relationship. In any case, in the presence of a heterotrophic carbon substrate and complete exposure to air, there is no need for the two organisms to cooperate.

This protocol is a first step in a series of studies that will aim to study and model the development of these model-organisms in the presence of each other. The interaction of the two organisms in the moments before and after the colonies touch is of particular interest.

This work and laser printing could be applied to strain selection, optimisation of growth and target molecule production through factorial experiments, and even the development of engineered symbioses for better production of target molecules. Additionally, the techniques reported here could be used to study the reaction of organisms to one another and quorum

sensing phenomena. Laser printing has already found several applications in the biomedical area[18,19].

Conflict of Interest and Funding

No conflict of interest was reported by all authors.

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