THE MICRO PETRI DISH: A HIGHLY SUBDIVIDED MICROBIOLOGICAL GROWTH FORMAT FABRICATED BY MICRO-ENGINEERING A HIGHLY POROUS CERAMIC FOR DIAGNOSTICS, COUNTING AND SCREENING APPLICATIONS

Colin Ingham,[1,2 *], Ad Sprenkels[3], Johan Bomer[3], Albert van den Berg[3], Willem M. de Vos[1,2] and Johan E.T. van Hylckama Vlieg[1,4]

[1] Wageningen Centre for Food Sciences, Wageningen, NL

[2] Laboratory of Microbiology, Wageningen University, Wageningen, NL.

[3] MESA+ Research Institute, University of Twente, Enschede, NL.

[4] NIZO Food Research, Ede, NL.

Email: colin.ingham@wur.nl

Abstract

A microbial culture chip with an unprecedented number of culture areas (millions) has been manufactured by microengineering a porous aluminium oxide support. These chips have great potential in high throughput and diagnostic microbial culture.

Keywords: Microbial culture chip, porous aluminium oxide

1. Introduction

Microbial culture faces new challenges. Traditional growth formats such as the Petri Dish, whilst used for centuries, do not suit modern needs such as automation and high throughput. Furthermore, microbial culture from a few cells to the level of a visible colony or turbidity measurements is slow; taking from overnight for a rapidly growing organism such as *Escherichia coli* to >3 weeks for *Mycobacterium tuberculosis*. To meet the needs of microbial diagnostics, and industries such as manufacturing and food production, new growth formats are required. It is desirable that such formats are cheap and do not require complex technologies, such as robotics. MEMS techniques may help provide solutions here.

Microbial culture using porous materials as an inert support is well known. For example, dialysis membranes or nitrocellulose filters have been used for the growth of bacteria and fungi with the nutrients supplied through the pores from beneath (1, 2). However, such membranes have never been developed into high throughput or highly miniaturized formats. This may be due to the fact that the microengineering of these flexible and uneven filters and membranes is challenging. One alternative material that could be used is porous aluminium oxide, also know as Anopore. Microbial culture is possible on strips of this material with the nutrients supplied from beneath, allowing organisms on the upper surface to divide and create microcolonies (2-4). Growth of bacteria or fungi can be monitored at the single cell and microcolony level using a variety of microscopy techniques including fluorescence. The imaging properties are excellent due to a flat surface with low background fluorescence and the material is translucent when wet (2, 3). Furthermore, the high degree of porosity and inert nature of this material are useful. However, whilst aluminium oxides are used extensively within the MEMS and nanotechnology disciplines, highly subdivided microbial growth formats (100,000 to millions of culture areas) have never been made.

2. Theory

The challenge was to create a method of modifying a strip of porous aluminium oxide to create an array of highly precise culture areas that support microbial growth. The format needed to support culture of all culturable organisms and facilitate microbial cell analysis. Such a "culture chip" can be thought of as a large number of miniaturized Petri dishes. It is important that such chips should be relatively simple and behave in a reproducible and unbiased way; for example in viable counts or screening for mutants with altered properties.

3. Experimental

The solution to the problem was to bond an acrylic polymer over a sheet of porous aluminium oxide. Then reactive ion etching, directed by means of a shadow mask, was used to remove the acrylic polymer in selected locations leaving the material in place to provide cross-walls to segregate the different test areas. Finally, a sputtered layer of platinum was used to mask autofluorescence common to polymers. The solution was validated by culture of a series of very different microorganisms on the culture chip to utility, as described below.

Microengineering - Briefly: 36 x 8 mm strips of porous aluminium oxide (Anopore from Whatman, UK) were laminated over the entire surface using an acrylic film (from Ordyl). A silicon shadow mask was constructed and sputtered with a protective layer of 150 nm aluminium oxide. The mask was used to direct reactive ion etching (using a PlasmaTherm 790 parallel-plate reactive ion etcher) to remove the laminate in an array of square culture areas (7 x 7 to 150 x 150 µm). The culture areas of exposed aluminium oxide were separated by 20 µm wide and 10 µm high walls of intact laminate that acted as barriers against microbial cross-contamination then sputtered with platinum.

Culture and validation - Bacteria and fungi were cultured upon ethanol-sterilized chips placed upon the appropriate culture medium. Culture conditions were 6 h at 37 ºC on L-agar for *Escherichia coli*, 8 h at 37 ºC (anaerobic conditions) for *Lactobacillus plantarum* WCFS1 and 7 h at 37 ºC on Sabouraud agar for the yeast *Candida albicans*. Imaging was by transmission or fluorescence microscopy scoring compartments as supporting growth imaged by a Kappa CCD camera mounted on an Olympus BX41 microscope with the R, ImageJ and Excel software packages used for analysis.

4. Results and discussion

By etching techniques discrete growth compartments were constructed on the surface of the aluminium oxide. The result is a highly regular array (C.V. of dimensions < 5%) of miniaturized culture areas capable of supporting growth combined with *in situ* imaging (Fig 1) at densities ranging from 8200 to 360,000 growth areas cm^{-2}. This allows the creation of chips with 7 to 40 million of culture areas on a 96 well footprint: well within the range required for high throughput screening applications. Culture, containment of microbial growth within discrete areas has been demonstrated for *Lactobacillus plantarum* strain WCFS1 (Fig 1) and other micro-organisms suggesting a general applicability. For all organisms in the test panel > 99.5% of the culture areas could support growth and there were no obvious spatial biases across chips in terms of culturability.

5. Conclusions

Highly subdivided culture chips were created using MEMS techniques and shown to support microbial culture. The grid forms a highly regular framework for software to interpret

growth patterns and allows an unprecedented level of massively parallel culture. Additionally, unlike other miniaturized growth there are no problems with desiccation or aeration. We suggest that such culture chips are amenable to automation and may be valuable tools for high-throughput single cell biology, microbial diagnostics and counting, and screening.

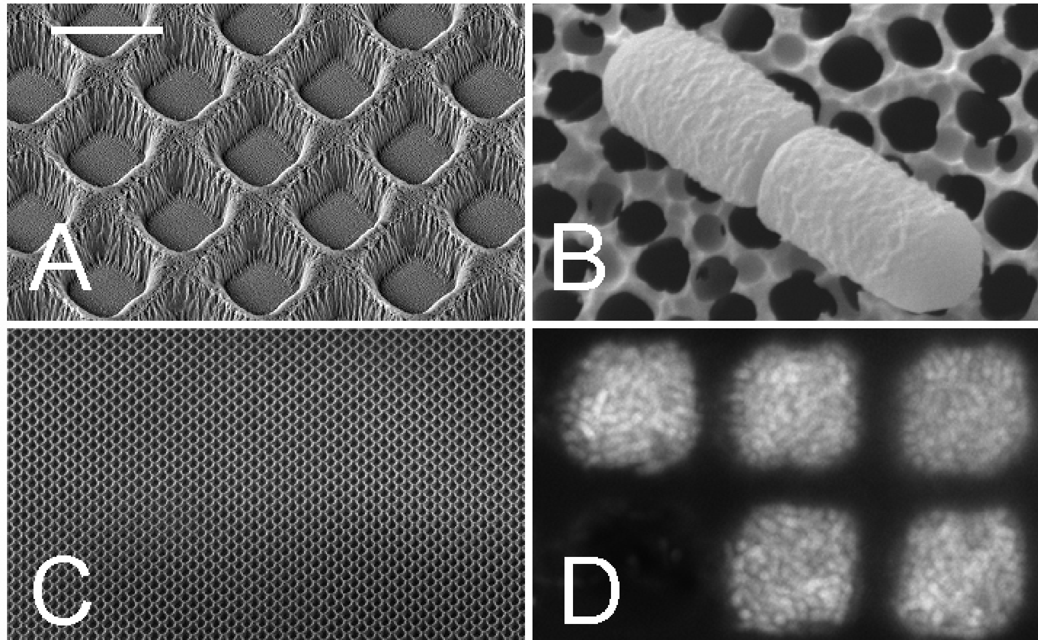

Figure 1. Example of culture format. (A) Scanning electron micrograph with porous base material marked. (B) Culture of *Lactobacillus plantarum* WCFS1 on chip surface imaged by SEM showing detail of aluminium oxide. (C) Overview of A. (D) Culture of *L. plantarum* WCFS1 within this format stained after growth with the fluorogenic dye Syto 9 and imaged by fluorescence microscopy. The inoculum was only a few bacteria per compartment, the organism has multiplied over a period of hours to fill the compartment but the walls are effective at protecting empty growth areas (such as the unstained area in the bottom left corner).

Acknowledgements

Thanks to Lion*iX* B.V. for help with chip manufacture.

References

1. Nordbring-Hertz, B., Veenhuis, M. & Harder, W. (1984). Dialysis membrane technique for ultrastructural studies of microbial interactions. *Appl Environ Microbiol* 47, 195-197.
2. Jones, S.E., Ditner, S.A. Freeman, C. Whitaker, C.J. & Lock, M.A. (1989). Comparison of a new inorganic membrane filter (Anopore) with a track-etched polycarbonate membrane (Nuclepore) for direct counting of bacteria. *Appl Environ Microbiol* 55, 529-530.
3. McKenzie, C.H., Helleur, R. & Deibel, D. (1992). Use of inorganic membrane filters (Anopore) for epifluorescence and SEM of nanoplankton and picoplankton. *Appl Environ Microbiol* 58, 773-776.
4. Ingham, C.J., M. van den Ende, D. Pijnenburg, P.C. Wever and P.M. Schneeberger. 2005. *Appl. Env. Microbiol.* 71, 8978-8981.