HIGH-THROUGHPUT LINEAGE TREE INVESTIGATIONS OF BACTERIA MICROCOLONIES USING ARRAYS OF MONOLAYER GROWTH CHAMBERS

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ABSTRACT

A disposable, microfluidic chip-system for high-throughput lineage tree investigations of bacterial microcolonies is presented. The device enables hydrodynamic trapping of multiple single bacteria cells of various industrially relevant bacteria strains in monolayer growth chambers. The chambers are arranged in array-like structures allowing systematic high-throughput cultivation, analysis and lineage tree generation. This work paves the way for future system biological investigations such as cell-to-cell heterogeneity studies with the main goal of collecting statistical data. As an example, the complete "lineage tree generation pipeline" for bacteria microcolonies, starting from microfluidic cultivation, over microscopy data acquisition, semi-automated image analysis to the lineage tree is demonstrated for *Gluconobacter oxydans*.

KEYWORDS

Single Cell Analysis, Lineage Tree Generation, Systems Biology, Bacteria, Image Analysis

INTRODUCTION

Investigating growth and division behavior of bacterial cells has been the research interest for many decades [1]. Since the last decade there is an increasing interest on single cell analysis to gain time resolved data, rather than relying on average information. Fields of interest range from stochastic gene expression [2] to metabolite production on single cell level [3] including growth studies at defined environments [4]. Typically, simple agar-pads are used to investigate bacteria on single cell level and observe growth by time-lapse microscopy [2]. Despite continuous improvements, studies under constant environmental conditions are limited in these devices. In particular, defined medium changes and high-throughput investigations are not possible. Microfluidic systems have tackled these limitations. Systems have been developed to observe single cells in dead-end growth channels [5] and larger colonies in monolayer growth areas [6]. For investigation of cell lineage relationships over time, such solutions are problematic due to a continuous loss of daughter cells. No system has been reported that allows the investigation of microcolonies on single cell level, starting from one mother cell to larger colonies without losing single bacteria cells. Furthermore, most systems are specialized on trapping and cultivation of specific bacteria species, making the investigation of various bacteria strains difficult.

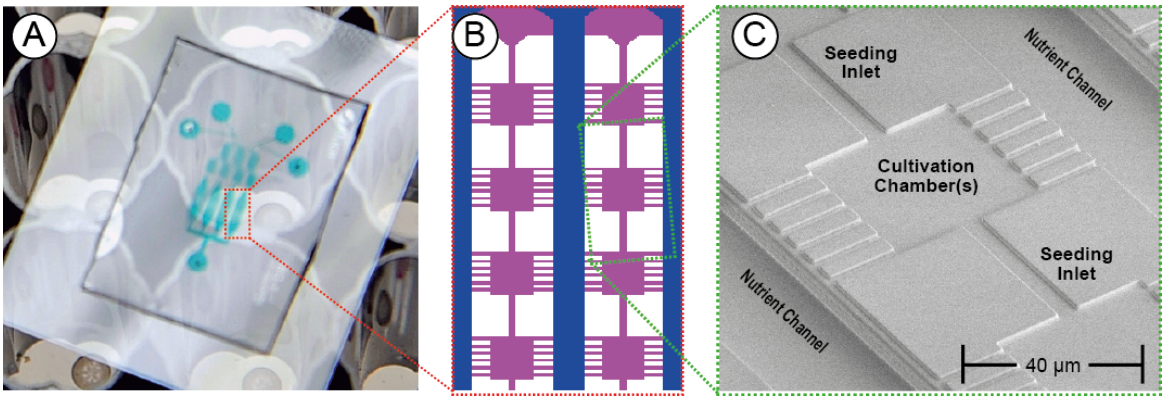

Figure 1: Microfluidic chip system. (A) Chip containing inlets, one gradient generator, 4 parallel trapping arrays and one outlet. The chip is sealed with a 170 µm thick glass plate suitable for high-throughput microscopy (B) Design of an array of monolayer cultivation chambers. (C) SEM of one cultivation chamber with a central seeding inlet channel. The parallel arranged nutrient channels supply the growth chambers through narrow channels with fresh medium.

PRINCIPLE

We present a microfluidic system (Fig. 1A), containing several arrays (Fig. 1B) of monolayer growth chambers (Fig.1C). Using common soft lithographic methods, disposable poly(dimethylsiloxane) (PDMS) microfluidic chips were fabricated. To manufacture the mould for PDMS casting, a two layer SU8 process was carried out on a silicon wafer. For typical processing parameters the reader is referred to the literature [7].

The chip consists of seeding inlets, for bacteria suspension and growth medium. Monolayer growth chambers are arranged in 4 lanes with 4 arrays of 10 chambers each (total of 160 chambers per chip) (Fig. 1B). Due to the reactor height of 1 µm, cell growth is restricted to a monolayer, ideal for time lapse microscopy (Fig. 1C). In addition, the reactor height minimizes bacteria migration which is an unwanted side effect when tracking of cells is desired. After seeding, cells are provided with nutrients through the side channels which are connected to the main channels. This allows for diffusive nutrient transport without convective flow across the growing microcolony.

RESULTS AND DISCUSSION

As a proof of principle, industrially relevant bacterial strains, *e.g.*, *G. oxydans*, *E. coli*, and *C. glutamicum*, were cultured on single cell level and high resolution time-lapse microscopy was performed. Figure 2A shows a growing *G. oxydans* colony right after the start of the experiment (Fig. 2A left), after 7 hours of the experiment (Fig. 2A middle) and after 11 hours of cultivation (Fig. 2A right). It can be seen that the colony is not affected by media flow during the cultivation time allowing image based single cell tracking and counting.

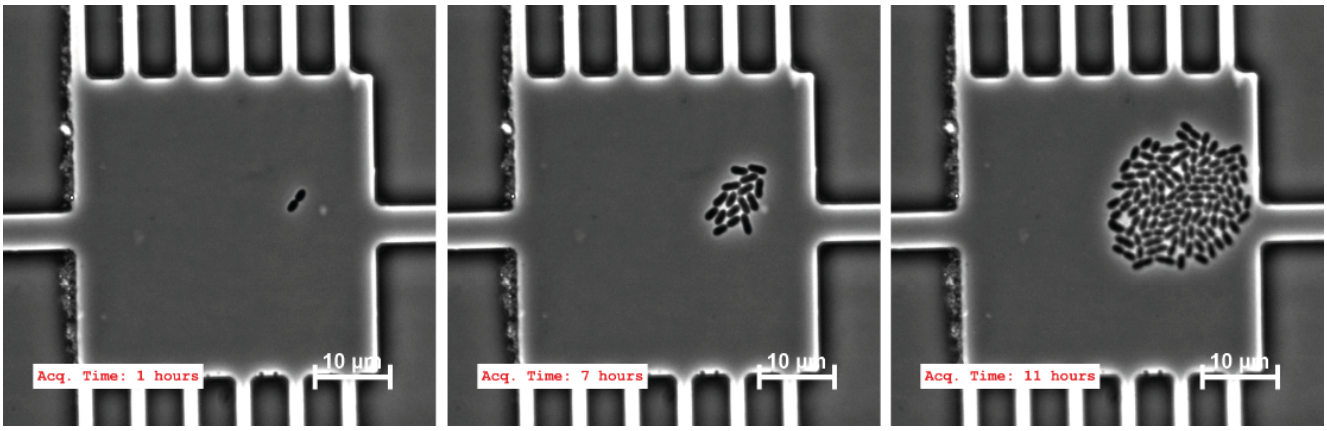

Figure 2: Time-lapse images of a growing *G. oxydans* microcolony in a monolayer growth chamber. (left) Single "mother cell" during first division event (middle) Growing colony after several divisions (right) Colony after 11 hours, before the experiment was finished.

Lineage tree investigation was done by semi-automated image analysis using an in-house developed image analysis software (cf. Fig. 3B for an overview). In detail, the image sequence obtained by time lapse microscopy was pre-processed to align images properly. This was done with a Lukas-Kanade-based alignment algorithm. Cells were identified in each image by a local adaptive thresholding procedure in combination with the Watershed segmentation algorithm [8]. After manual checking and, if necessary, correcting the segmentation results, cells were identified for tracking and lineage tree reconstruction. To track cells over the image sequence, relation score matrices for all cells in consecutive images are constructed. A linear optimization problem is solved to obtain the actual cells' frame-to-frame correspondence[9]. From this information lineage trees were generated. In Fig. 3A the lineage tree derived from the *G. oxydans* microcolony shown in Fig. 2 is depicted. It can be seen that this specific strain exhibited a homogeneous division pattern in terms of morphology and individual division times.

CONCLUSIONS

We have described a microfluidic system for lineage tree data generation of bacteria microcolonies. The device incorporates several monolayer growth chambers, allowing for culturing several colonies in parallel. The results clearly prove that the device can be used to investigate various bacteria strains with respect to growth and division behavior as well as the response onto various environmental conditions with high-throughput. Using automated time-lapse microscopy and semi-automated image analysis, lineage tree analysis is performed .The developed system opens up new possibilities for future studies on single cell heterogeneity of various bacteria strains.

This tool will be used in different disciplines, ranging from microbiology to biotechnology and lays the foundation for future system biological investigations such as heterogeneity studies of bacterial populations.

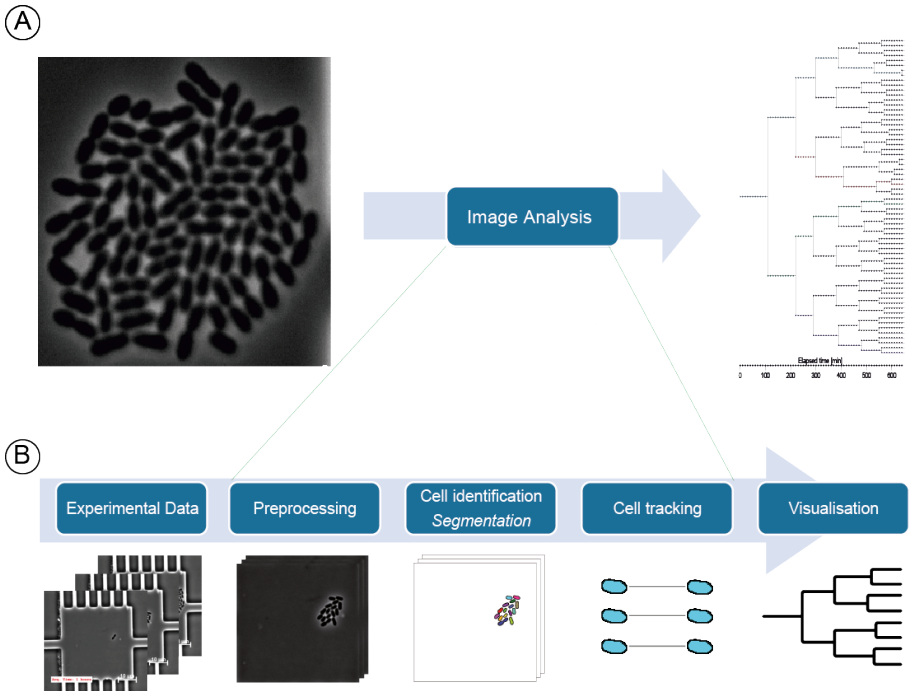

Figure 3: Lineage tree generation from microfluidic micro colony growth experiments. (A) Semi-automated image analysis was performed to derive the lineage tree for *G. oxydans*. (B) Image analysis pipeline: core steps are time-lapse microscopy, image pre-processing, cell identification, tracking and lineage tree visualization.

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