Systems Biomimetism: Artificial Cells in a Living World, Living Cells in an Artificial World

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Abstract:
Most biological systems are organized according to complex architectures involving multi-scale hierarchical organizations, non linear interactions, feedback controls, and evolutionary behaviors. To understand the underlying physical, biological, and chemical aspects controlling the organization of living systems, we are developing a multidisciplinary, 'biomimetic' approach where: i) well-defined artificial cell systems such as giant liposomes of controlled size and composition are produced and placed under biologically relevant contions (*artificial cells in a living world*); and ii) living cell systems are investigated under a controlled artificial micro-environment, mainly generated by microfluidic means (*living cells in an artificial world*).

1. ARTIFICIAL CELLS IN A LIVING WORLD

Giant liposomes, phospholipid vesicles with a bilayered membrane and a size in the range 1-100 μm, have been shown to be the best primary model of cells in terms of size, membrane composition, or the ability to encapsulate biologically relevant molecules[1]. They have attracted a strong scientific interest mainly motivated by the perspective of artificial cell synthesis and their relevance for a number of biological, biophysical or biochemical applications. Several techniques for their preparation have been proposed such as spontaneous swelling, freezing and thawing, electroformation, microfluidics, transfer, extraction from membrane, and micro-jetting, each with specific advantages and drawbacks but none of them allowing a simultaneous control of the size and composition of produced liposomes[2]. Recently, our group has developed several methodologies for a precise control of both liposome size[3-5] and composition[6,7], which opens the possibility to generate liposomes of any desired size (in the range 1-100 μm) and composition (inner content, membrane). The generated liposomes were used for different experiments on artificial cell systems, in relationship with biological questions (e.g., gene expression dyanmics).

1.1 Electroswelling from micro-structured phospholipid films.

The most efficient and widely used method to prepare giant unilamellar liposomes is electroformation. It consists of applying AC field between two conductive electrodes (usually in platinum or in tin-doped indium oxide (ITO)) coated by a phospholipid film and separated by an aqueous solution. This technique, which was introduced by Angelova in 1986[8], has several advantages: it is simple, relatively fast (a few hours), and the produced liposomes are 'beautiful' (i.e., spherical) and unilamellar. However, it has two main drawbacks: liposomes are polydisperse in size and the encapsulation potentiality is limited (e.g., high ionic strength, physiological medium or viscous solutions are difficult to be encapsulated). First, we thus focused on improving size control of liposomes in the electroformation process. The lack of control in the classical electroformation method has probably two origins: i) the micro-scale organization of the film on the electrode is not controlled; and ii) the mechanism of electroformation is still poorly understood. Therefore, our strategy was to rationalize the deposition and microscale organization of phospholipid on the electrode in order to i) control the size of the generated liposomes, and ii) to better understand the underlying mechanism of liposome formation under an electric field. Hence, we introduced the use of silicon as a substrate for electroformation and we studied the effect of surface chemistry and surface topology on film organization and liposome generation[5]. We found that density of defects in the film has a strong influence on the electroformation process. Therefore, by controlling the fragmentation of the phospholipid film by using micropillars, a partial control of the liposome diameter was obtained. This was further improved by combining this approach with the micro-localization of the electric field[5].

We saw the importance of the repartition of defects, the localization of the electric field and the fragmentation of the phospholipid film to get a better control in the electroformation of giant liposomes. However, in the approaches described above, the phospholipid film covered the whole surface of the electrode, and a large number of small liposomes were obtained in a non controlled way. An interesting alternative is to use multilayered phospholipid patterns deposited on the electrode. We have developed two methods to generate such phospholipid patterns: i) microcontact printing[4] and ii) capillary assembly[3,9]. In the first case, a multilayer film was prepared on a temporary substrate and transferred to the electrode using a PDMS stamp, which led to very regular multilayer phospholipid patterns deposited on the electrode. After electroformation, each printed spot provided a liposomes and very regular arrays of monodisperse liposomes were obtained on the electrode[4]. Capillary assembly[3,9] is a powerful novel method that we have developed to control the thickness and organization of phospholipid on a solid substrate. It is detailed in the next paragraph.

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1.2 'Capillary assembly': a method for controlled deposition of phospholipid films.

We saw the importance of phospholipid film micro-scale organization on the electrode to get a good control of liposome electroformation. Different strategies were used to affect the phospholipid film organization on a surface, such as surface chemical treatments or micro-structuration[4,5]. However, in all cases the phospholipid was manually spread on the electrode, which led to i) a poor reproducibility of film properties, and ii) no control of the film thickness. In a more general consideration, controlled deposition of phospholipid films on a solid substrate is necessary for biological and biophysical investigations on supported membranes[10]. Various methods are now available to deposit one or a few (up to 5) bilayers on different surfaces in a controlled way, such as small unilamellar vesicles adsorption or Langmuir-Blodgett film deposition[11]. In contrast, much less efforts have been devoted to the deposition of thicker multilayered phospholipid films. To our knowledge, the only available method to pattern phospholipid multilayered film with a controlled thickness is dip-pen nanolithography but it requires specific equipment[12]. To overcome these limitations, we have proposed a novel approach, called 'capillary assembly', to prepare phospholipid films of controlled thickness and organization[3,9]. It consists of dragging the meniscus of a phospholipid solution on a solid substrate at constant speed under controlled temperature and forced convection.

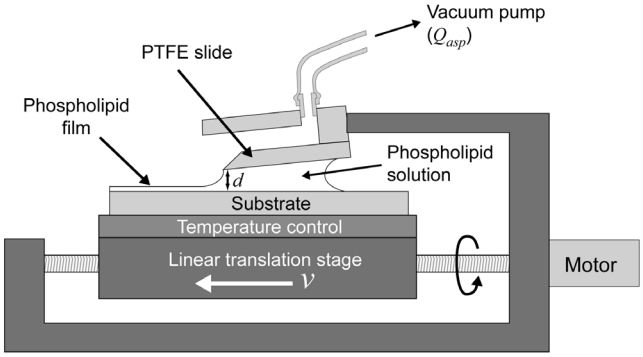

Figure 1. The 'capillary assembly' machine**.** The substrate (e.g., a silicon wafer) is mounted on a computer-controlled linear translation stage equipped with a thermoelectric modulus for temperature contol. A 80 µL droplet of a phospholipid solution is held by a fixed PTFE slide (2 cm width) with a sharp end above which air is pumped at a controlled flow rate (controlled forced convection), while the substrate is moved at a constant speed v and controlled temperature T. Adapted from ref 9.

Figure 1 shows our experimental set-up. First, we used a solvent in total wetting situation for the substrate (n-octane on silicon)[9]. In this case, depending on the deposition speed, capillary assembly led to the deposition of a homogenous dried multilayered phospholipid film with a thickness h in the range of 20-200 nm.

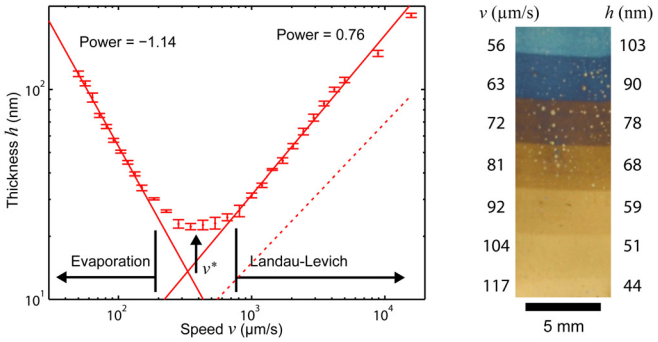

Figure 2. Deposition of phospholipid multilayer films of controlled thickness: Evaporation and Landau-Levich regimes. An organic solution of phospholipid (here, 20 mg/L DOPC in n-octane) was dragged at a controlled speed v and controlled temperature (27°C) on a silicon substrate using the set-up shown in Fig. 1. Left, film thickness h as a function of v. Evaporation and Landau-Levich regimes are observed for $v<v^*$ and $v>v^*$, respectively. The solid lines are power fits providing exponents of -1.14 and 0.76, respectively. Right, real-color photograph of a phospholipid film deposited on silicon with a speed increasing in a stepwise manner (evaporation regime). Speed deposition v and film thickness h (measured by ellipsometry) are indicated on the left and right of the photograph, respectively. Adapted from ref 9.

We found two distinct regimes dominating the film deposition[9] (Figure 2). At low speeds, phospholipid molecules accumulate near the contact line and form a dry film behind the meniscus (evaporation regime). At high speed, viscous forces become predominant and pull out a liquid film that will dry afterward (Landau-Levich regime). Both regimes show robust scaling $h \propto v^{\alpha}$ with α = -1.1 and 0.76, respectively (Figure 2). Although these regimes have been observed separately in the past, they have not been demonstrated in the same material system. Moreover, we established models whose scalings (α = -1 and 2/3) are in close agreement with the observed values. We have also found that the microscale organization of the resulting film is independent of v for a given regime but differs from one regime to another. In the Landau-Levich regime, h is very homogeneous on the microscale with discrete variations of ±5 nm, that is, the thickness of one bilayer.

We showed that capillary assembly can be used to control the thickness of multilayered phospholipid films with good precision. The method can also be used to generate phospholipid patterns of controlled thickness. To this end, we performed capillary assembly using a solvent in partial wetting conditions (trichloroethylene) on a microstructured substrate (hexagonal array of 200 nm deep holes)[3]. Under these conditions, when the phospholipid solution was dragged on the microstructured substrates (Figure 3A), the contact line was moving on the planar parts of the substrate without phospholipid deposition but was pinned on the edges of the microstructures due to the steep increase of the contact angle (Figures 3B and 3C), which resulted in the selective trapping of phospholipid solution in the substrate holes.

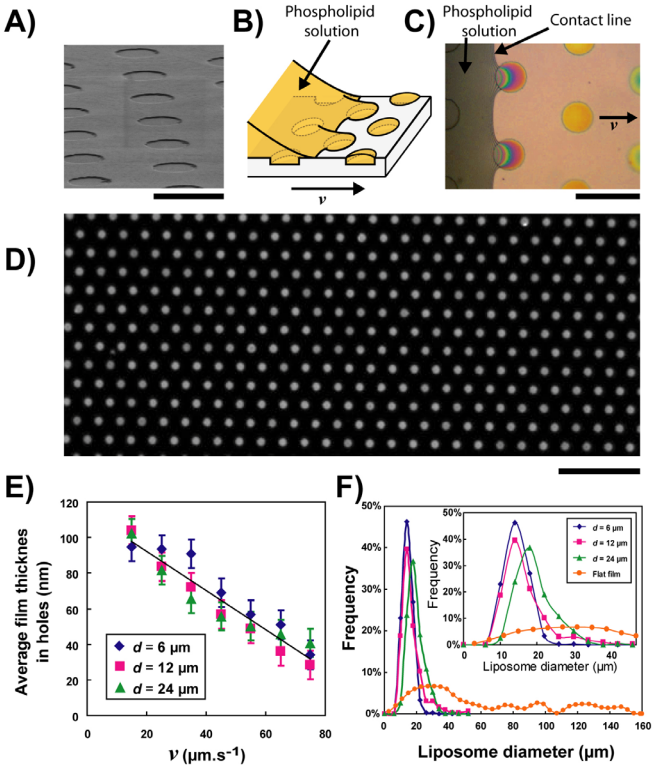

Figure 3. Preparation of phospholipid multilayer patterns of controlled size and thickness by capillary assembly on a microstructured substrate. Regular phospholipid multilayer patterns are obtained by dragging a phospholipid solution on a microstructured silicon substrate (hexagonal array of holes, diameter d, depth 400 nm). A) SEM image of a microstructured silicon substrate (d = 12 µm). The scale bar is 20 µm. B) Principle of the deposition: the phospholipid solution, which is in partial wetting situation on the planar parts of the substrate, is selectively deposited in the microstructures. C) Real color image during the deposition of a phospholipid solution on a substrate (d = 24 µm). The arrow indicates the direction of the substrate translation. The scale bar is 50 µm. D) Fluorescence microscopy image of a phospholipid pattern obtained with d = 12 µm and a deposition speed v = 25 µm.s^{-1}. The scale bar is 100 µm. E) Average film thickness per hole as a function of v for various values of d. F) Distribution of the liposome diameters obtained after electroformation from patterns of various diameters d. For comparison, the distribution obtained by electroswelling a manually deposited phospholipid film on a flat silicon substrate is also represented (orange points). The inset is a zoom for liposome diameters in the range 0 – 50 µm. For all experiments, we used a phospholipid solution (98wt% DOPC, 2wt% NBD-PC) at 0.5 mg.mL^{-1} in trichloroethylene. Adapted from ref 3.

After solvent evaporation, a phospholipid multilayer film was selectively deposited in the microstructures. Following this way, cm-sized patterns were easily obtained with high fidelity (Figure 3D). The thickness of phospholipid film per deposited spot is controlled by deposition speed (Figure 3E) while the pattern size is directly controlled by the microstructure size. To the best of our knowledge, this is the first simple method allowing one to get phospholipid multilayer patterns of controlled size and thickness. Notably, the electroswelling of the generated phospholipid patterns led to the formation of giant liposomes of controlled size with a narrow size distribution (Figure 3F). While capillary assembly has been used in the past to organize colloids or nanoparticles on a solid substrate[13,14], it is the first time that it is applied to pattern molecules. Moreover, regular phospholipid multilayer patterns can be of great interest for the development of novel biosensors or cell screening procedures. Not limited to phospholipid patterning, the method promises to be applicable for high-fidelity patterning of various synthetic or biological molecules, such as proteins, drugs, conductive polymers, or antibodies.

1.3. Phospholipid-coated water-in-oil micro-droplets as novel precursors to liposomes.

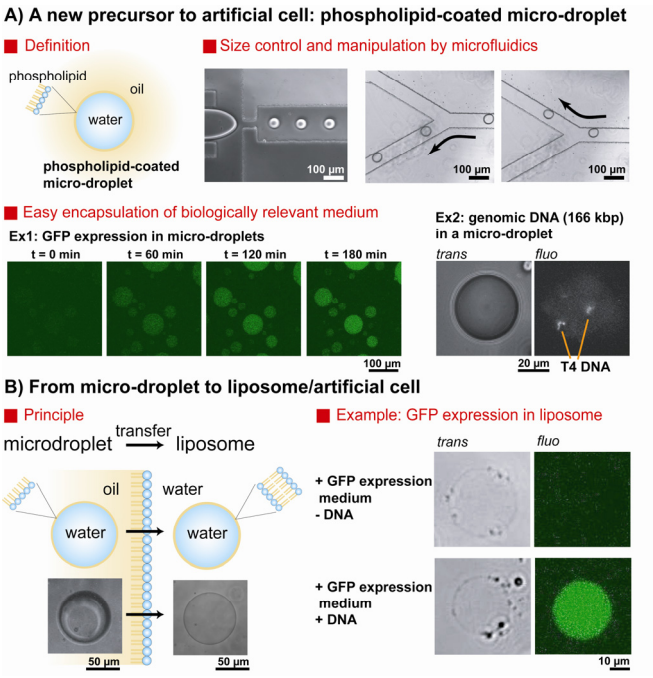

Figure 4. A) Monodisperse phospholipid-coated micro-droplets can be generated and manipulated in a controlled way by microfluidics. Various biologically relevant medium can be encapsulated: Ex1: time-lapse confocal microscopy images of droplets encapsulating GFP expression medium; Ex2: transmission and fluorescence microscopy images of a droplet encapsulating 2 individual molecules of genomic T4 DNA[16]. B) Phospholipid-coated micro-droplets are spontaneously transformed into liposome when they transfer through an oil/water interface[6]. Example of application: GFP expression medium is first encapsulated in a micro-droplet prior to transfer through oil/water interface to obtain a liposome containing the expression medium[7]. Adapted from refs 6,7,15,16.

In the previous parts, all the methods to generate liposomes were based on a phospholipid film initially deposited on a solid substrate (flat, nano- or micro-structured) prior to swelling (spontaneous swelling or electroformation) in an aqueous solution. Regardless of

possible improvements, such approaches have inherent limitations: i) inside and outside compartments of lipomes are identical; ii) the process of swelling is relatively slow (at least a few hours) and kinetic studies inside the liposomes are thus strongly limited; and iii) encapsulation efficiency strongly depends on the medium to be encapsulated. There is thus a need to change the concept of liposome preparation if one wishes to have a real and versatile control of liposome composition.

To this end, we explored the use of phospholipid-coated micro-droplets[15] as novel precursors to liposomes of controlled composition[6,7] (Figures 4A and 4B). These droplets are composed of a water inner compartment separated from the outer oil phase by a phospholipid layer (it can be a monolayer or a multilayer depending on the conditions of preparation). Such droplets have several advantages. The encapsulation is straightforward (a simple emulsification process), biocompatible and any biologically relevant medium (a gene expression medium, cytoskeleton components, etc.) can be encapsulated a priori. Another advantage is that a very small amount of water solution is necessary (a few µL), which is compatible with investigation on expensive, minute biological samples (e.g., a solution of a purified protein). We demonstrated that such droplets can be used as microreactors for biochemical reactions such as protein expression[15] or DNA encapsulation[16] (Figure 4A). We also showed that monodisperse phospholipid-coated micro-droplets can be conveniently generated and manipulated by microfluidics in a controlled way (Figure 4A). Interestingly, we demonstrated that micro-droplets can be transformed into liposomes with the same inner composition by spontaneous transfer through an oil/water interface[6] (Figure 4B). The liposomes generated by this method are mainly unilamellar and their diameter typically ranges from 10 to 100 µm.

1.4. Applications as artificial cell systems.

Various experiments were performed on giant liposomes used as well-defined artificial cell systems. By using the spontaneous transfer method, we could follow for the first time *in situ* protein expression kinetics within individual liposomes[7] (Figure 5). The spontaneous transfer has also been successfully applied by other groups for actin/myosin encapsulation[17] and preparation of asymmetric model membranes[18]. We also prepared a novel artificial system, in which the conformation of individual genomic DNA molecules encapsulated in a cell-mimicking structure was controlled by light, *i. e.*, by an external stimulus[16]. Interestingly, these light-induced DNA conformational genes were applied to control using light transcription and translation reactions[19]. Finally, we also incorporated cyclodextrin moieties in liposome membrane by electroswelling amphiphilic bipolar α-cyclodextrin molecules[20].

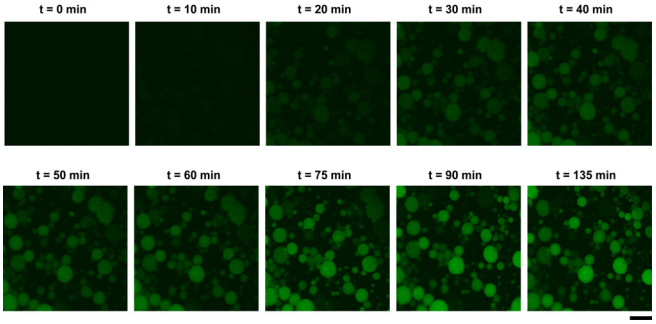

Figure 5. GFP expression in giant liposomes. Time-series confocal fluorescence images of giant liposomes encapsulating a cell-free expression medium and template DNA for GFP expression at a controlled temperature (T = 37°C). The scale bar is 100 µm. Adapted from ref 7.

To conclude, the combination of our developed methodologies in phospholipid deposition, electroformation, microfluidic generation of micro-droplets, and transfer technique thus provides a powerful, readily available platform for the controlled preparation of cell-sized liposomes of any desired size and composition.

2. LIVING CELLS IN AN ARTIFICIAL WORLD

The characterization of single-cell responses and cell-cell interactions within a population under controlled dynamical micro-environmental conditions is a crucial experimental challenge for the understanding of living systems[21]. Various microsystems have been successfully proposed for the investigation of mammalian cells but few can be applied for the most widely studied bacteria, *E. coli* [22]. We have developed novel methodologies to print and culture living bacteria (*E. coli*) with a single-cell resolution[23] and to follow the cell history (e.g., stress response) of all individuals in a growing population under dynamic environmental conditions[24,25].

2.1 Large-scale printing of living bacteria with a cellular resolution.

First, we developed a novel methodology to print arrays of living bacteria with a single-cell resolution[23]. It consists of the microcontact printing of living bacteria from an agarose 'inkpad' to an agarose substrate with PDMS stamps having high aspect ratio. PDMS stamps were fabricated by introducing an improved UV lithography protocol, called reverse *in situ* lithography (RISL). We found that printed bacteria arrays reproduced perfectly the original patterns of the stamp over a very large area (typically cm^2). Figures 6A and 6B show that, for initial concentrations of 10^9 and 10^8 cells/mL, the average number of printed bacteria per spot is 12.1 and 1.4, respectively. For the lower concentration, the distribution is very narrow, with 44.6% of spots having exactly a single *E. coli* cell and other 40.1% having 0 or 2. These results demonstrate that

our methodology can produce very regular arrays of single bacteria over a large scale.

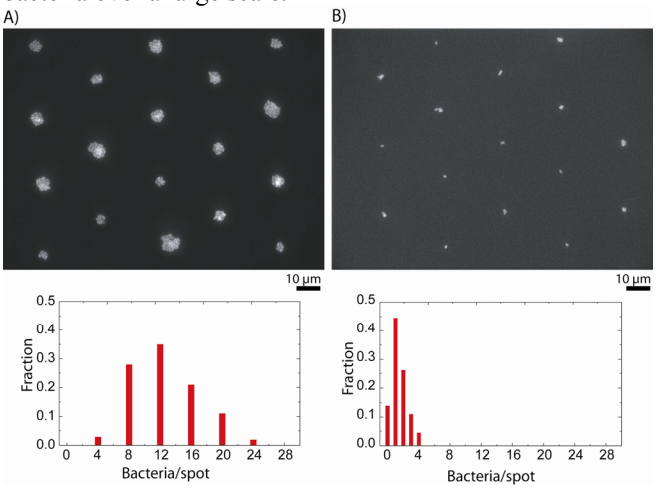

Figure 6. Arrays of living E. coli printed on an agarose gel with a PDMS stamp (diameter 6 µm, period 30 µm, height 12 µm). Top: Fluorescence microscopy. Bottom: Number of bacteria per spot. Bacteria concentrations are 10^9 cells/mL (A) and 10^8 cells/mL (B), respectively. Adapted from ref 23.

Figure 7 shows that patterned bacteria keep living, dividing and growing after printing on the agarose gel. Figure 7B shows the total area of printed bacteria as a function of time. The exponential fit provides a characteristic division time of 20.6 ± 1.3 min per generation, which corresponds well to that in bulk culture conditions (21.4 ± 0.9 min). This demonstrates that bacteria keep their normal physiological behavior after printing. This new method is thus suitable for further systems biology investigations such as cell-cell interaction, competition, evolution, or population dynamics.

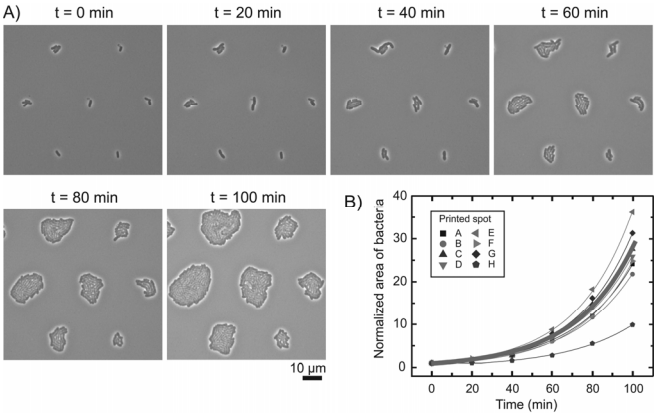

Figure 7. A) Growth of patterned bacteria at T = 37 °C (conditions of Fig. 6B) B) Growth curves of printed bacteria. Each symbol corresponds to one printed spot. Thin lines are single exponential fittings for each printed spot. The thick line is a single exponential averaged fitting curve providing an average division time of 20.6 ± 1.3 min. Adapted from ref 23.

2.2 Single-cell history under a fluctuating micro-environment.

Then, we elaborated a microfluidic device, in which bacteria were grown in a 2D monolayer under dynamical microfluidic conditions[24,25]. It is based on the confinement of bacteria in a sandwich configuration between an agarose gel and a microscopy glass slide. The microfluidic flow (which can bring nutrients, drugs, stress molecules, etc…) is above the gel, *i.e.*, the bacteria are hydrodynamically isolated from the flow (Figure 8A). Phase contrast and fluorescence microscopy (Figure 8B) coupled with an automated image analysis system allowed the single-cell tracking of all individuals. Bacteria growing under a flow of nutriments had an average characteristic division time of 21.5 min (Figure 8C), showing that bacteria kept their normal physiological behaviour inside the microsystem.

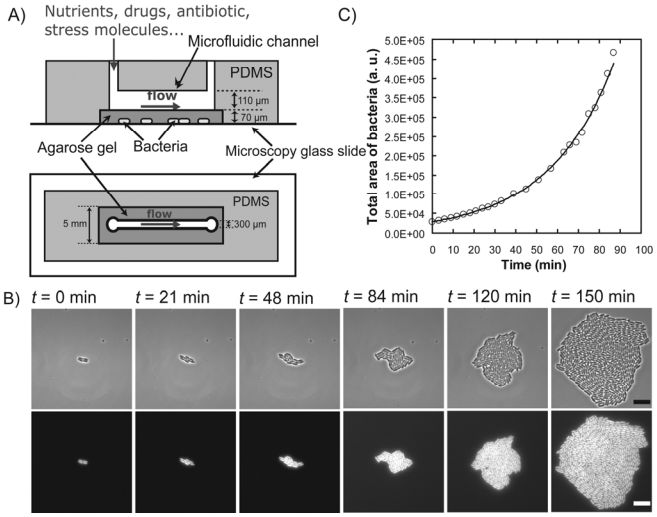

Figure 8. A) Microfluidic device for 2D-culture of bacteria under a flow (nutrients, drugs, stress molecules, etc…). B) Time-lapse microscopy images of E. coli (top: phase contrast, bottom: fluorescence) under a flow of LB culture medium (60 µL/min). A custom-built image analysis software allows the image analysis and single-cell tracking of all individual cells within the growing population. Scale bars are 10 µm. C) Growth curve of bacteria growing under the conditions of B. The solid line is an exponential fitting curve providing a characteristic division time of 21.5 min. Adapted from refs 24,25.

Using this methodology, we established the distribution of single-cell responses to the injection of IPTG of wild type strain of *E. coli* modified to express the gene encoding yellow fluorescent protein (YFP) under the control of $P_L$lacO promoter (induced by IPTG). A typical fluorescence response of growing bacteria is shown in Figure 9A. By combining the individual cell-response data (Figure 9B) to the lineage informations extracted from image analysis, we can build the 'lineage-history tree' shown in Figure 9C. This allowed us to analyze the single-cell response dynamics and signal propagation through successive generations. We established for the first

time that both stochastic effects (fluctuations of repressor concentration) and physiological parameters (growth rate) are determinants of individual cell fate and determine the probability of a given cell to have her progeny switched[24].

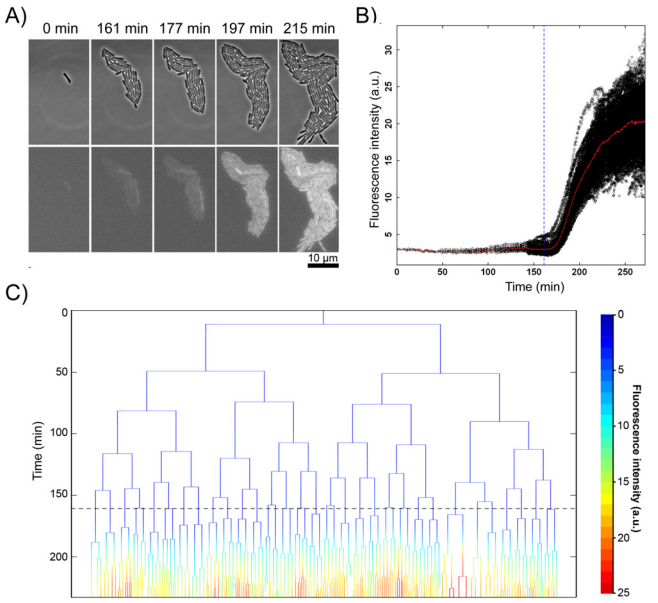

Figure 9. E. coli MG1655 modified to express YFP under the control of $P_L$lacO1, a promoter inducible by IPTG, was grown in the microfluidic device of Fig. 8A under a flow of LB medium (1 mL/h) at 30 °C. IPTG (0.1 mM) was introduced in the LB flow at t = 161 min. A) Time-lapse phase contrast (top) and YFP fluorescence (bottom) of a growing micro-colony. B) Fluorescence intensity of all individual cells (black symbols) and mean single-cell fluorescence intensity (solid red line) as a function of time. The dashed blue line indicates the time of IPTG introduction (t = 161 min). C) Lineage 'history' tree. Individual cells are plotted as vertical lines where the color corresponds to fluorescence intensity as a function of time (vertical axis). At division time, a horizontal line is drawn to connect the mother cell and its two daughters. The dashed line corresponds to IPTG introduction. Adapted from refs 24,25.

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