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(54) Title: MICROFLUIDIC STRUCTURE FOR CARRYING OUT BIOLOGICAL ASSAYS AND CHIP PROVIDED WITH SUCH STRUCTURE

(57) Abstract: Microfluidic structure (1) for carrying out biological assays, which comprises a first assay channel (C1) and a second assay channel (C2) coplanar, identical and arranged in parallel, a first inlet port (I1) for inserting a first fluid, a second inlet port (I2) for inserting a second fluid, a common outlet (O1), and a distributing channel system (D) comprising a common connection point or pre-chamber (V), a first supply channel (IP1) connecting the first inlet port (I1) with the common connection point (V), a second supply channel (IP2) connecting the second inlet port (I2) with the common connection point (V), a first distribution channel (PC1) connecting the common connection point (V) with the first assay channel (C1), a second distribution channel (PC2) separate from the first distribution channel (PC1) connecting the common connection point (V) with the second assay channel (C2), and a first outlet channel (CO1) connecting the first assay channel (C1) with the common outlet (O1) and a second outlet channel (CO2) separate from the first outlet channel (CO1) connecting the second assay channel (C2) with the common outlet (O1). The invention also relates to a chip (2) having two or more of such microfluidic structures (1) and a method wherein such microfluidic structure is used.

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MICROFLUIDIC STRUCTURE FOR CARRYING OUT BIOLOGICAL ASSAYS AND CHIP PROVIDED WITH SUCH STRUCTURE

TECHNICAL FIELD

The present invention relates to a microfluidic structure for carrying out biological assays. More specifically, the present invention seeks to guarantee reproducibility in biological growth assays and more specifically in the growth of biofilms and their treatment. Another objective is to reduce the assembly time, the test times and the sample volumes involved in the tests. A further objective is to guarantee the stability and uniformity of biofilms during the assays.

STATE OF THE ART

Bacteria form biofilms by adhering to surfaces and developing complex communities. A biofilm is a multicellular aggregate of cells encased in an extracellular polymeric matrix. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings.

In the field of medicine, infections associated with the biofilm growth usually are challenging to eradicate. It is mostly due to the fact that mature biofilms display tolerance towards antibiotics and the immune response. The biofilm matrix provides microorganisms with a protective shield that contributes significantly to several clinical challenges, including symptomatic inflammation, antibiotic resistance, recurrence, and the spread of infectious emboli. In biofilms, cells can survive under harsh environments, for example, at high temperatures or in the presence of antibiotics. The biofilm matrix improves a microbe's opportunities for proliferation inside the body. Further, biofilms often form on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices, causing prevalent infections.

The recent global increase in the prevalence of antibiotic-resistant bacteria and lack of development of new therapeutic agents emphasize the importance of selecting appropriate antimicrobials for the treatment of infections specially those that are chronic. However, to date, the development of completely accelerated drug susceptibility testing methods has not been achieved despite the availability of a rapid identification method, due to the problem of biofilms. Currently, the typical procedure followed in a hospital is the antimicrobial resistance spectrum determination (e.g. antibiogram) in isolated culture bacteria

not when the bacteria is growing in a biofilm.

To date, most antibiotics or antimicrobial molecules have primarily been developed to target bacteria in a planktonic state and all current methodologies to discover anti-biofilm drugs require one-by-one drug testing. However, biofilm formation allows bacteria to develop tolerance to antibiotics and provides mechanisms to evade innate immune systems, being this situation predominant in human and animal infections. Clearly discordant results exist between planktonic and biofilm resistances that implies individual testing of resistance profiles under biofilm growth conditions. Therefore, there is a strong need for an easy methodology to analyse bacterial biofilms and with it, to test antimicrobial molecules effective to eradicate such bacterial biofilms.

Furthermore, biofilms have become problematic in several industries due to the ability to form on plants and during industrial processes. Bacteria can survive long periods of time in water, animal manure, and soil, causing biofilm formation on plants or in the processing equipment. The build-up of biofilms can affect the heat flow across a surface and increase surface corrosion and frictional resistance of fluids. These can lead to a loss of energy in a system and overall loss of products. Along with economic problems, biofilm formation on food poses a health risk to consumers due to the ability to make the food more resistant to disinfectants.

The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium. Hence, an understanding of the mechanical characteristics of biofilms and their dependence on dynamic environmental conditions is critical to the development of strategies for controlled biofilm removal or retention.

Conventional formats for investigating biofilm formation under conditions of shear stress utilize macro-scale flow cells in which the biofilm is formed on a glass slide and fresh medium is continuously perfused through the system. While widely used, this system has the obvious disadvantages of requiring large volumes (limiting when using signals or molecules that are not readily available), is not suited for high-throughput investigation, and does not facilitate spatial and temporal control of biofilm formation. Microfluidic models for biofilm studies have been recently proposed; however, currently available models are either low-throughput, do not produce uniform biofilm structures or require expensive instrumentation and lack flexibility.

Known in the art are microfluidic structures for carrying out biological assays, which comprise assay channels arranged in parallel, inlet ports for inserting samples, a common outlet and a mixing and distributing channel system which connects the inlet ports with the assay channels.

A key characteristic sought in these devices is the reproducibility between channels, i.e. that the same or practically the same conditions must be achieved in all the channels.

Other characteristics sought are ease of manufacture, assembly and handling.

Another objective pursued is to minimize the effect of manipulation by laboratory technicians. Indeed, part of the experimental procedures is done manually, specially injections of samples, and it is desired that these devices allow to minimize the differences in the flows that can be produced by the great variability that may arise in such manipulations.

Another objective sought is the simplification and the cost reduction of the experimental assembly and shorten the volumes of fluids involved, both by the occupation of space and by the storage of the waste generated. Additionally, it is important the reduction of material used in drug screening.

For this specific purpose, the Technical University of Denmark (DTU) has designed a plate comprising three independent inlets, each connected to a single assay channel, each one connected to an outlet port, thus providing three independent parallel circuits on a single plate (Tolker-Nielsen T, Sternberg C, “Growing and analyzing biofilms in flow chambers” Curr Protoc Microbiol. 2011 Chapter 1, 1B.2.1-1B.2.17). The inlet and outlet ports, the assay channel and the connections between the ports and the assay channels are arranged longitudinally.

Although useful, the particular topology of this device entails difficulties to guarantee the reproducibility of the tests between channels, since it is necessary to guarantee equal conditions at the entrances and exits. The DTU mitigate their possible biofilm disturbances by increasing the length of their test chambers.

In addition, their dimensions imply long setting times and large volumes for the fluidic system (each channel about 600 µL) and the different experiments to be done in the system, which the inventors consider to be improvable when new molecules need to be analysed. Furthermore, this system is not suitable for high throughput screening experiments or

multiplexing (i.e. assaying simultaneously several samples without the need to do independent experiments or using independent devices) in an easy way.

US2007202589A1 discloses a microfluidic structure for carrying out biological assays, which comprises a plurality of assay channels arranged in parallel, a first inlet port for inserting a first fluid, a second inlet port for inserting a second fluid and a common area with several culture cells places. The inlet ports are both connected to a common channel, which outlet is connected to a distribution connector, perpendicular to the common channel. The resultant topology provides an addition of the flows in the common channel but implies an uneven distribution of the flows in the assay channels. This uneven distribution is due to both the upstream and downstream conditions of the flows with respect to the assay channels. One severe drawback of this design is that it is very difficult to guarantee homogeneity and thus reproducibility simultaneously in all the assay channels.

US5635358A and US2010170796A1 disclose an analytical device for carrying out biological assays, which comprises a plurality of assay channels arranged in parallel and a port for inserting a fluid, and a distributing channel system which connects the inlet port with the assay channels. The distributing channel system is based in splitting three times the main channel such that eight channels are obtained that simulates a biological system, specifically the circulatory system. In particular, these devices are not adapted to carry out biofilms assays, since the resulting shear stress, even for small flows, is too high.

US6440645B1 discloses a device having a surface microstructure of channels. All the embodiments disclose therein comprise two or more inlet ports connected to a common connection point, which is linked to a channel which ends in an outlet port. The device is aimed at the treatment of one sample obtained by mixing the fluids coming from the inlet ports.

DESCRIPTION OF THE INVENTION

For overcoming the mentioned drawbacks and achieve the afore mentioned aims, the present invention proposes a microfluidic structure for carrying out biological assays, which comprises at least a first assay channel and a second assay channel, the assay channels being coplanar and arranged in parallel, a first inlet port for inserting a first fluid, a second inlet port for inserting a second fluid, a common outlet, a distributing channel system which connects the inlet ports with the first and second assay channels, and wherein the distributing channel system comprises a common connection point, a first supply channel

connecting the first inlet port with the common connection point, a second supply channel connecting the second inlet port with the common connection point, a first distribution channel connecting the common connection point with the first assay channel, a second distribution channel separate from the first distribution channel connecting the common connection point with the second assay channel, and a first outlet channel connecting the first assay channel with the common outlet and a second outlet channel separate from the first outlet channel connecting the second assay channel with the common outlet.

This particular topology allows to overcome the drawbacks of the prior art devices cited in the background section. In particular, this topology allows to obtain balanced flow conditions in the assay channels in assays involving two fluids, namely a biological sample and a growth medium, which, as already said, is a key factor to create similar or almost similar conditions in all the assay channels. As it will be appreciated below, this topology can be implemented in a common base and in a very compact manner.

On one hand the claimed topology allows to obtain identical or near identical fluid conditions simultaneously in all the assay channels and on the other hand it allows to minimize the effect of the application of particular flow sequences such as those disclosed in the background section, that is, sequences where a fluid is injected manually in a short duration of time. This topology makes it possible to reduce the impact on the injection of the way in which each laboratory technician performs the injection or, said in other words, it allows the conditions in the test chambers to be independent of the particular conditions in which the fluid injections are carried out without the disturbance of a formed biofilm. These conditions guarantee the uniformity in all our test channels. In particular, the invention is well suited to carry out a sequence of fluid flows such are those disclosed with reference to Fig. 4, as described in more detail below.

Therefore, contrary to US2007202589A1, where it is impossible to balance the flows between assay channels, thanks to the separate distribution channels it is possible to balance the friction losses that occur between the mixing volume and the assay channels thus not disturbing the channel growing conditions.

In some embodiments, the microfluidic structure has three or more assay channels.

In some embodiments, the assay channels are planar and have a rectangular cross-section, such that a length L, a width W and a height H are defined per channel.

In some embodiments, the connection point is a chamber having a cross-section area comprised between ½·S_{AC} and 3/2·S_{AC}, S_{AC} being the cross-section area of each assay channel. More preferably the chamber has a cross-section area equal to S_{AC}. In the present specification chamber is also called 'pre-chamber'.

In some embodiments, the chamber is cylindrical and planar, such that is coplanar with the assay channels.

In some embodiments, a hydraulic path is defined for each assay channel, each of the hydraulic path going from the common connection point to the common outlet having the same hydraulic losses for a determined flow rate.

In some embodiments, the height H is greater than 150 µm.

In some embodiments, the width W is comprised between 1.9 mm and 2.1 mm.

In some embodiments, the length L is comprised between 8 and 12 mm.

The invention also relates to a chip comprising a microfluidic structure according to the invention, which comprises a base where the inlets, the channels and the outlets are defined, and a cover.

In some embodiments, the base is made of a material selected from PDMS (polydimethylsiloxane), PMMA (poly(methyl methacrylate)), polycarbonate, polystyrene, polypropylene, COC (cyclic olefin copolymer, such as TOPAS®), COP (cyclic olefin polymer, such as ZEONOR) and the cover is made of glass, silicone, titanium, iron, steel, copper, aluminium, or hydroxyapatite, among other suitable materials. More particularly, the base is made of a material selected from PDMS, PMMA, COC or COP (TOPAS®) and the cover is made of glass, COP (TOPAS®) or COC.

In some embodiments, the inlets and outlets have their axis perpendicular to the base.

The chip comprises two or more microfluidic structures, such that each microfluidic structure is independent thus preventing contamination between structures.

Finally, a further aspect of the invention is related to a method of forming a biofilm

starting from a biological sample, using the microfluidic structure of the invention, the method comprising the following steps:

i) Establishing a flow of nutrient medium by using the second inlet port so that the distributing channel system allows a balanced distribution between the assay channels;

ii) Inoculating the biological sample by using the first inlet port, so that the distributing channel system allows a balanced distribution of the sample between the assay channels;

iii) Interrupting both the nutrient medium flow and the biological sample flow for a predetermined time so that the biofilm is formed in the assay channels; and

iv) Establishing a constant flow of nutrient medium using the second inlet port for a predetermined time, so that the distributing channel system allows a balanced distribution of the nutrient medium and therefore a biofilm in similar conditions in all the assay channels is formed.

BRIEF DESCRIPTION OF THE DRAWINGS

To complete the description and in order to provide a better understanding of the invention, a set of drawings is provided. Said drawings form an integral part of the description and illustrate embodiments of the invention, which should not be interpreted as restricting the scope of the invention, but just as an example of how the invention can be carried out. The drawings comprise the following figures:

Fig. 1 shows a plant view of the microfluidic structure according to a first embodiment.

Fig. 2 shows a plant view of the microfluidic structure according to a second embodiment.

Fig. 3 shows a plant view of the microfluidic structure according to an embodiment where the inlet and outlet ports have their axis perpendicular to the general plane of the assay channels.

Fig. 4 is a time diagram of the flows sequence of a biofilm assay for which the microfluidic structure is well suited.

Fig. 5 is a confocal laser scanning microscopy (CLSM) image of the sum and orthogonal views of a *Pseudomonas aeruginosa* PAO1 formed biofilm stained with the LIVE/DEAD BactLight Bacterial Viability kit (Thermo Fisher Scientific) in a channel having a minimum height of 50 microns. A microfluidic structure with a chamber size of 10 mm long x

2 mm wide devoid of a a chamber at the connection point was used, with an estimated flow rate of 3.8 µl/min in each chamber and a velocity of growth medium of 13.6 mm/s. The biomass of this biofilm is 14.58 $\mu m^3/\mu m^2$, and the average thickness is 28.95 µm. Measurements were made by Comstat2 software.

Fig. 6 is a confocal laser scanning microscopy (CLSM) image of the sum and orthogonal views of a *Pseudomonas aeruginosa* PAO1 formed biofilm stained with the LIVE/DEAD BactLight Bacterial Viability kit (Thermo Fisher Scientific) in a channel having a minimum height of 100 microns. A microfluidic structure with a chamber size of 10 mm long x 2 mm wide devoid of a a chamber at the connection point was used, with an estimated flow rate of 3.8 µl/min in each chamber and a velocity of growth medium of 13.6 mm/s. The biomass of this biofilm is 14.67 $\mu m^3/\mu m^2$, and the average thickness is 20.68 µm. Measurements were made by Comstat2 software.

Fig. 7 is a confocal laser scanning microscopy (CLSM) image of the sum and orthogonal views of a *Pseudomonas aeruginosa* PAO1 formed biofilm stained with the LIVE/DEAD BactLight Bacterial Viability kit (Thermo Fisher Scientific) in a channel having a minimum height of 150 microns. A microfluidic structure with a chamber size of 10 mm long x 2 mm wide devoid of a a chamber at the connection point was used, with an estimated flow rate of 3.8 µl/min in each chamber and a velocity of growth medium of 13.6 mm/s. The biomass of this biofilm is 16.40 $\mu m^3/\mu m^2$, and the average thickness is 26.03 µm. Measurements were made by Comstat2 software.

Fig. 8 is a confocal laser scanning microscopy (CLSM) image of the sum and orthogonal views of a *Pseudomonas aeruginosa* PAO1 formed biofilm stained with the LIVE/DEAD BactLight Bacterial Viability kit (Thermo Fisher Scientific) in a channel having a minimum height of 150 microns and in a microfluidic structure having a pre-chamber (V). A microfluidic structure with a chamber size of 10 mm long x 2 mm wide was used, with an estimated flow rate of 3.8 µl/min in each chamber and a velocity of growth medium of 13.6 mm/s. The biomass of this biofilm is 29.55 $\mu m^3/\mu m^2$, and the average thickness is 37.82 µm. The measurements were made by Comstat2 software.

Fig. 9A shows an exploded view of the microfluidic structure.

Fig. 9B is an enlarged photograph of an embodiment of the microfluidic structure of the invention, in which the structure of the invention is present in quadruplicate.

Fig. 10A is a confocal laser scanning microscopy image of the sum and orthogonal views of a *Pseudomonas aeruginosa* PAO1 formed biofilm. A microfluidic structure with a chamber size of 10 mm long x 2 mm wide x 150 microns height with pre-chamber was used, with an estimated flow rate of 3.8 µl/min in each chamber and a velocity of growth medium of 13.6 mm/s.

Figs. 10B and 10C are graphs of the biomass and average thickness of the biofilms shown in Fig. 10A.

Fig. 11A is a confocal laser scanning microscopy image of the sum and orthogonal views of a *Staphylococcus aureus* CECT86 formed biofilm. A microfluidic structure with a chamber size of 10 mm long x 2 mm wide x 150 microns height with pre-chamber was used, with an estimated flow rate of 3.8 µl/min in each chamber and a velocity of growth medium of 13.6 mm/s.

Figs. 11B and 11C are graphs of the biomass and average thickness of the biofilms shown in Fig. 11A.

Fig. 12 shows biofilm biomass of *Pseudomonas aeruginosa* cystic fibrosis isolate (PAET1) (Control) after treatment with 20 µg/ml ciprofloxacin (cpx 20).

DESCRIPTION OF A WAY OF CARRYING OUT THE INVENTION

As shown in Fig. 1, according to a preferred embodiment, the inventive microfluidic structure 1 for carrying out biological assays comprises a first assay channel C1, a second assay channel C2, and a third assay channel C3 that are coplanar, identical and arranged in parallel.

The structure comprises a first inlet port I1 for inserting a first fluid, which is the growth medium, and a second inlet port I2 for inserting a second fluid, for example the biological sample or drug molecules test.

The ends with a common outlet O1, destined to be connected to a waste duct.

For correctly distributing the flows coming from the inlets, the microfluidic structure comprises a distributing channel system D which connects the inlet ports I1, I2 with the three assay channels C1, C2 and C3.

Specifically, firstly the distributing channel system D comprises a common connection point V, a first supply channel IP1 connecting the first inlet port I1 with the common connection point V and a second supply channel IP2 connecting the second inlet port I2 with the common connection point V.

Then a first distribution channel PC1 connects the common connection point V with the first assay channel C1, a second distribution channel PC2 separate from the first distribution channel PC1 connects the common connection point V with the second assay channel C2, and a third distribution channel PC3 separate from the other distribution channels PC1, PC2 connects the common connection point V with the third assay channel C3.

At the exit of the assay channels a first CO1, second CO2 and third CO3 separate outlet channels connect the assay channels to the common outlet O1.

The assay channels C1, C2, C3 are planar and have a rectangular cross-section, such that a length L, a width W and a height H are defined per channel C1, C2, C3, L, W and H being most preferably the same for the three assay channels.

In the embodiments shown, the connection point V is a chamber having a cross-section area S_{AC} which is the cross-section area of each assay channel C1, C2, C3.

The chamber V is cylindrical and planar, such that is coplanar with the assay channels C1, C2, C3.

In another embodiment, depicted in Fig. 2, the chamber V is connected to the inlets with two L-shaped supply channels.

As shown in Fig. 3, the inlets I1, I2 and the outlet O1 have a circular shape having the axis perpendicular to the general plane of the microfluidic structure.

Taking into account the laboratory time, very expensive, that it takes to obtain a biofilm, it is essential for a good comparison to guarantee that during a treatment the biofilm is maintained constant along the different channels. It has been found that a miniaturized microchannel structure without chamber, led to an homogeneous growth of the biofilm in the three assay channels, which is useful to conduct different assays/treatments. However, in

Fig. 7, there is a band of oblique detached biofilm, which shows that a detachment of the biofilm during the inoculation of the treatment occurred.

When the claimed topology is used, with the inclusion of a chamber V, a homogeneous biofilm is obtained in the three assay channels and no detachment of the biofilm occurs (as can be seen in Fig. 8).

In addition, compared with the use of a chip as described in the background, this has allowed to reduce volumes at least in 75%. In the case of assay channels having 2 mm width, 10 mm length and 150 microns height, the volume can be reduced at least to 50 ml for a biofilm assay.

It is pointed out that the invention is not a mere reduction of dimensions, which would not, on its own, achieve the effects of stability, avoiding cross-contamination and flow sought.

What allows to reduce its dimensions is its particular topology that in turn allows to have a pre-chamber V that has been crucial to perform the biofilms tests represented in Fig. 8.

In some embodiments, the hydraulic path defined for each assay channel C1, C2, C3, going from the common connection point V to the common outlet O1 have the same hydraulic losses for a determined flow rate. This is achieved by balancing the linear losses and the losses caused by the direction changes and section changes along the different paths.

According to a preferred embodiment, specially for *Pseudomonas aeruginosa*, the height H of the channels is greater than 150 µm. Remarkably, a structure like the one claimed in which this minimum height is established leads to conditions of shear stress that guarantee the survival of this particular biofilm, or biofilms having similar characteristics. This has been demonstrated by the inventors as can be seen in Figs. 5 to 7.

In Figs. 5 and 6, the formation of biofilms for channel heights of 50 and 100 µm, respectively, can be seen in green. However, the green color is not uniform and dead bacteria can be seen in red, which rules out the trials. However, when a height of 150 µm is established, and as shown in Fig. 7, red spots are no longer seen, that is, the biofilm survives.

Fig. 10A is an image of confocal laser scanning microscopy of the sum and orthogonal views of a *Pseudomonas aeruginosa* PAO1 formed biofilm or different channels stained with the LIVE/DEAD BactLight Bacterial Viability kit (Thermo Fisher Scientific). Figs 10B and 10C are graphs of the biomass and average thickness of the biofilm, calculated with Comstat2 software. The three images and bars are from different channels, showing the uniformity of the biofilm obtained with the microfluidic structure of the present invention.

Fig. 11A is an image of confocal laser scanning microscopy of the sum and orthogonal views of a *Staphylococcus aureus* CECT86 formed biofilm stained with the LIVE/DEAD BactLight Bacterial Viability kit (Thermo Fisher Scientific). Figs 11B and 11C are graphs of the biomass and average thickness of the biofilm, calculated with Comstat2 software. The three images and bars are from different channels, showing again the uniformity of the biofilm obtained with the inventive microfluidic structure.

Preferred dimensions of the microfluidic structure are a width W about 2 mm and a length about 10 mm. These dimensions are sufficient to allow the formation of sufficient biofilm for observation with a confocal or fluorescent microscope.

The resulting structure is very small, but has proven to be very efficient for biofilm growth, stability and analysis.

As shown, the inlets and outlets have their axis perpendicular to the base, such that an easy connection is ensured. This arrangement also prevents contamination upstream.

According to another preferred embodiment, shown in Fig. 9B, the chip supports four microfluidic structures such as disclosed above, such that each microfluidic structure are independent thus preventing contamination between structures.

This particular embodiment allows to carry out multiplexing analysis, while using only one microfluidic structure.

Microfluidic structure manufacturing procedure

Fig. 9A shows an exploded view of the microfluidic structure. Here it can be seen that the structure can be obtained from two pieces only, a first substrate part L1, such as a polymer in which the different channels are practiced, on top of which there is a glass cover L2.

Fig. 9B is an enlarged photograph of an embodiment of the microfluidic structure of the invention, in which the structure 1 of the invention is present in quadruplicate. This structure allows for multiple tests with a single disposable microfluidic structure, in which the same conditions are also available in all devices, which allows valid comparisons between treatments.

According to a preferred embodiment, the manufacture of the microfluidic structure consists of a multistep procedure designed to obtain a deep chamber using photolithographic and soft-lithographic techniques. All procedures are carried out in a clean room facility. Master is designed using CAD software and manufactured over a glass slide. The manufacturing process starts with three consecutive solvent baths of acetone, isopropanol and ethanol. Following the cleaning protocol, next step is pattern Ordyl SY 550. This negative photoresist is laminated on the top of a glass slide using a hot/cold laminator to get a smooth attached film surface (50 µm-high). A second layer of Ordyl SY 550 is laminated on the top of the previous one in order to get 100 µm high. Then, the slide is exposed through an acetate photomask to UV light (5 s, 24 mW cm^2, 345 nm) in the mask aligner and subsequently placed on a hot plate at 65 ºC for 3 minutes.

The 3D master mould manufacturing is finished by developing the Ordyl film using the developer for 3 minutes. To replicate the master, a PDMS prepolymer mixture (a curing agent-to-PDMS ratio of 1:10, Sylgard®184, Dow Corning) is placed in a desiccator with vacuum applied in order to remove bubbles. Then, the mixture is poured on top of the Ordyl master to fabricate a PDMS mould and heated at 65 ºC overnight. The casted PDMS is peeled off carefully and inlet and outlet holes are made using a Harris Uni-Core 1 mm puncher. PDMS structures are permanent bonded to a cover-glass slide using O2 plasma to activate surface and immediately pressed together. PDMS deep chamber chip is ready to use.

In one embodiment, the obtained microfluidic structure has a chamber size of 10 mm long x 2 mm wide x 150 µm.

Method of forming a biofilm using the microfluidic structure

The illustrated and described structures have turned out to be specially adapted to realize a protocol for the growth of biofilms as the one depicted by means of a flow vs time graph shown in Fig. 4.

The procedure consists in first establishing a constant flow of nutrient medium, for example TSB (Tryptic Soy Broth) or LB (Luria Bertani medium), and by means of a pump, whose flow rate can be precisely controlled. This is done using the second inlet I2, for example. This flow rate Q is shown as a constant function that is interrupted to give rise to the inoculation of bacteria or biofilm treatment with antibacterial drugs. This inoculation has been represented as a peak, and represents a manual injection by a laboratory operator.

This stage of inoculation is delicate. The invention allows to control this inoculation stage without a biofilm disturbance. Indeed, by providing the particular topology of the invention, where the connection point is pre-chamber V, it is achieved that the volume of bacteria inoculated reaches in a balanced manner the three test channels, which will allow a valid comparison to be made between them.

After this inoculation, the system is left at rest, about two hours, without flow, to allow the bacteria that start the biofilm to adhere to the glass cover of the assay channels C1, C2, C3.

It is then proceeded to establish a constant flow of nutrient medium for 2 to 3 days, during which the biofilm will be formed, as can be seen in the drawings under the time axis.

This step involves a high volume of nutrient, so that the reduction in dimensions that has been possible thanks to the particular configuration of the microfluidic structure according to the invention.

After the growth stage, a pre-analysis treatment is carried out. At this stage, the biofilm structure is crucial. To this end, the nutrient flow is interrupted and a treatment is injected. This injection is usually done manually and is a particularly delicate stage for which the microfluidic structure of the invention has proved to be specially adapted. If a microfluidic structure as shown in Fig. 11 is used, then one or more independent treatments can be carried out.

Thus, an aspect of the invention is related to a method of forming a biofilm using the microfluidic structure of the invention comprising the steps as explained in section Description of the invention. The resulting formed biofilm in the microfluidic structure can be further analysed in connection with other biological techniques such as microscopy or direct measurement of the biofilm rate by e.g. an impedance system. Examples of aspects of the

biofilm that can be analysed are morphology, grow, biomass, roughness, thickness, or bacterial interactions.

In a particular embodiment, the method of forming a biofilm further comprises applying a treatment to the formed biofilm, so that the method comprises the additional steps following step (iv):

v) Interrupting the nutrient medium flow; and

vi) Inoculating a treatment using the first inlet port so that the distributing channel system allows a balanced distribution of the treatment between the assay channels.

Pseudomonas aeruginosa PAET1 biofilm were cultivated for 3 days at room temperature in flow cell chambers with channel dimension of 10 mm long x 2 mm wide x 150 µm high; then the flow was stopped and treatment with 20 µg/ml of free ciprofloxacin (CPX) was added for 1 additional day without flow. Biofilms were stained with a mixture of 6 µM SYTO 9 and 30 µM propidium iodide at room temperature in the dark for 30 min, according to the specifications of the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes). Confocal scanning laser microscopy was performed with a Zeiss LSM 800 Confocal laser scanning microscope and analysed with the COMSTAT 2 specific biofilm analysis software. The formed biofilm decreased simultaneously in biomass and maximum thickness. Control biofilm without CPX treatment exhibited the highest value for total biomass and average thickness. This can be seen in Fig. 12.

In summary, the present invention arises from the need to be able to carry out tests with biofilms, guaranteeing the reproducibility of the tests, while facilitating the handling, reducing the volumes involved, the experimentation times and therefore the costs.

The microfluidic structure of the invention can be used in basic research for studying bacteria in an *in vivo*-like environment, mimicking natural biological conditions in a three-dimensional culture system. Remarkably, it can also be used in the pharma industry for high-throughput screening of anti-biofilm molecules, and in clinical settings in a microbiological service to evaluate the best antimicrobial treatment for given person (personalised medicine). It can be used as a platform to evaluate biofilm growth of different bacterial species, including clinical isolates from patients infected with bacteria forming biofilms.

The antibiofilm activity of various compounds can be evaluated simultaneously in a high-throughput way with the structure of the invention. Growth complex mixed biofilms can be tested together with eukaryote cells to mimic the realistic conditions during infections.

Different bacterial species can be co-cultured and screened in a single biofilm chip, since infectious diseases are not typically due to a single bacterial growth. The microfluidic structure allows the biofilm growth without cross contamination among the different channels. The microfluidic structure can house hundreds of continuous-flow biofilms allowing independent treatment of them with different drugs, together with their optical characterization and analysis by coupled technologies such as confocal laser scanning microscopy (CLSM).

Thus, the microfluidic structure can be combined with existing quantification tools, such as confocal and fluorescence microscopy, to enable real-time monitoring of biofilm developments.

Remarkably, the microfluidic structure according to the invention can be easily used in an automated analysis unit. In particular, biological fluid analysis devices consisting of two subunits are known, namely a first subunit comprising the pumps and the control electronics of the pumping parameters and a second subunit designed to house the disposable unit in which the biological samples are grown. These two units are connected by means of fluid supply ducts and, possibly, power or signal cables. The subunit housing the disposable unit generally comprises an insertion slot which leads to a housing conveniently provided with the fluid supply ports. For the use of the chip object of the present invention it is only necessary to adapt the dimensions of said slot and the housing, so that the chip of the present invention can be easily used with machines available on the market.

Although it has been described in detail with reference to its use in the analysis of biofilms, the microstructure and chip described can be very advantageous in any type of microfluidic assay in which it is essential to guarantee reproducibility for other cell analysis (yeast, animal cells, etc.).

For example, it can be used in cell live assays, such as cell interaction, cell rolling, cell adhesion, cell migration, cell aggregation, cell invasion, cell exposure and differentiation, analysis of chemoattractants, wound healing assays, bacteria interaction with cells, planktonic growth of bacteria, bacteria co-culture, single cell analysis among others.

In this text, the term “comprises” and its derivations (such as “comprising”, etc.) should not be understood in an excluding sense, that is, these terms should not be interpreted as excluding the possibility that what is described and defined may include further elements.

The invention is obviously not limited to the specific embodiments described herein, but also encompasses any variations that may be considered by any person skilled in the art within the general scope of the invention as defined in the claims.

CLAIMS

1. Microfluidic structure (1) for carrying out biological assays, which comprises at least a first assay channel (C1) and a second assay channel (C2), the assay channels (C1, C2) being coplanar, identical and arranged in parallel, a first inlet port (I1) for inserting a first fluid, a second inlet port (I2) for inserting a second fluid, a common outlet (O1), a distributing channel system (D) which connects the inlet ports (I1, I2) with the first and second assay channels (C1, C2), **characterised in that** the distributing channel system (D) comprises a common connection point (V), a first supply channel (IP1) connecting the first inlet port (I1) with the common connection point (V), a second supply channel (IP2) connecting the second inlet port (I2) with the common connection point (V), a first distribution channel (PC1) connecting the common connection point (V) with the first assay channel (C1), a second distribution channel (PC2) separate from the first distribution channel (PC1) connecting the common connection point (V) with the second assay channel (C2), and a first outlet channel (CO1) connecting the first assay channel (C1) with the common outlet (O1) and a second outlet channel (CO2) separate from the first outlet channel (CO1) connecting the second assay channel (C2) with the common outlet (O1).

2. Microfluidic structure (1) according to any of the previous claims, which has three or more assay channels (C1, C2, C3).

3. Microfluidic structure (1) according to any of the preceding claims, wherein the assay channels (C1, C2, C3) are planar and have a rectangular cross-section, such that a length L, a width W and a height H are defined per channel (C1, C2, C3).

4. Microfluidic structure (1) according to any of the previous claims, wherein the connection point (V) is a chamber (V) having a cross-section area comprised between $\frac{1}{2}\cdot S_{AC}$ and $3/2\cdot S_{AC}$, S_{AC} being the cross-section area of each assay channel (C1, C2, C3).

5. Microfluidic structure (1) according to claim 4, wherein the chamber (V) has a cross-section area equal to S_{AC}.

6. Microfluidic structure (1) according to claim 4 or claim 5, wherein the chamber (V) is cylindrical and planar, such that is coplanar with the assay channels (C1, C2, C3).

7. Microfluidic structure (1) according to any of the previous claims, wherein a hydraulic path is defined for each assay channel (C1, C2, C3), each of the hydraulic path

going from the common connection point (V) to the common outlet (O1) having the same hydraulic losses for a determined flow rate.

8. Microfluidic structure according to claim 3 or any claim dependent thereof, wherein the height H is greater than 150 µm.

9. Microfluidic structure (1) according to claim 8, wherein the width W is comprised between 1.9 mm and 2.1 mm.

10. Microfluidic structure (1) according to claim 9, wherein the length L is comprised between 8 and 12 mm.

11. Chip (2) comprising a microfluidic structure (1) according to any of the previous claims, which comprises a base (L1) where the inlets, the channels and the outlets are defined, and a cover (L2).

12. Chip (2) according to claim 11, where the base (L1) is made of a material selected from polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), cyclic olefin copolymer (COC) or cyclin olefin polymer (COP) and the cover (L2) is made of glass, COP or COC.

13. Chip (2) according to any of claims 11 to 12, wherein the inlets and outlets have their axis perpendicular to the base.

14. Chip (2) according to claim 11 or 12, which comprises two or more microfluidic structures according to any of claims 1 to 10, such that each microfluidic structure is independent thus preventing contamination between structures.

15. Method of forming a biofilm starting from a biological sample, using a microfluidic structure (1) according to any of the preceding claims, the method comprising the following steps:

i) Establishing a flow of nutrient medium by using the second inlet port (I2) so that the distributing channel system (D) allows a balanced distribution between the assay channels (C1, C2);

ii) Inoculating the biological sample by using the first inlet port (I1), so that the distributing channel system (D) allows a balanced distribution of the sample between the assay channels (C1, C2);

iii) Interrupting both the nutrient medium flow and the biological sample flow for a predetermined time so that the biofilm is formed in the assay channels (C1, C2); and

iv) Establishing a constant flow of nutrient medium using the second inlet port (I2) for a predetermined time, so that the distributing channel system (D) allows a balanced distribution of the nutrient medium and therefore a biofilm in similar conditions in all the assay channels (C1, C2) is formed.

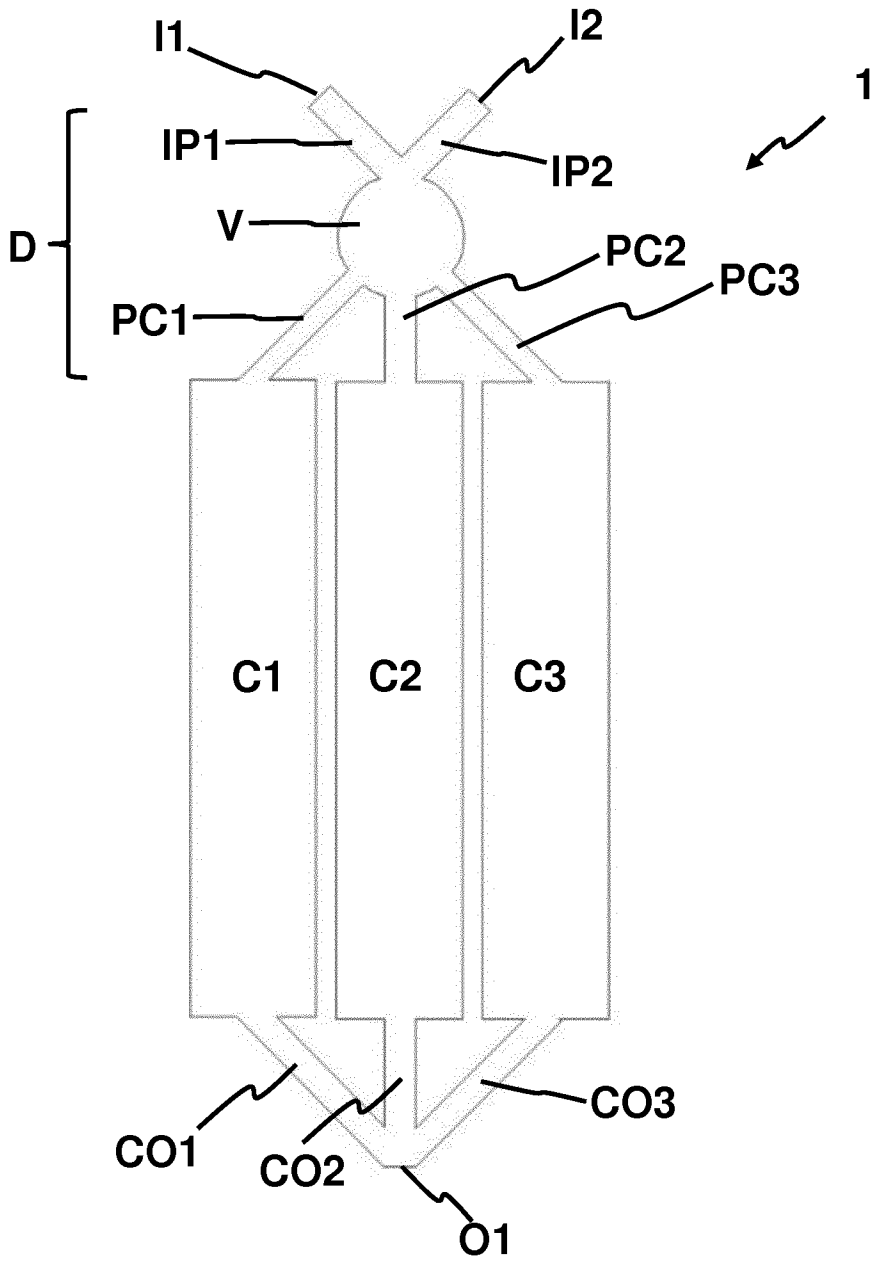


Fig. 1

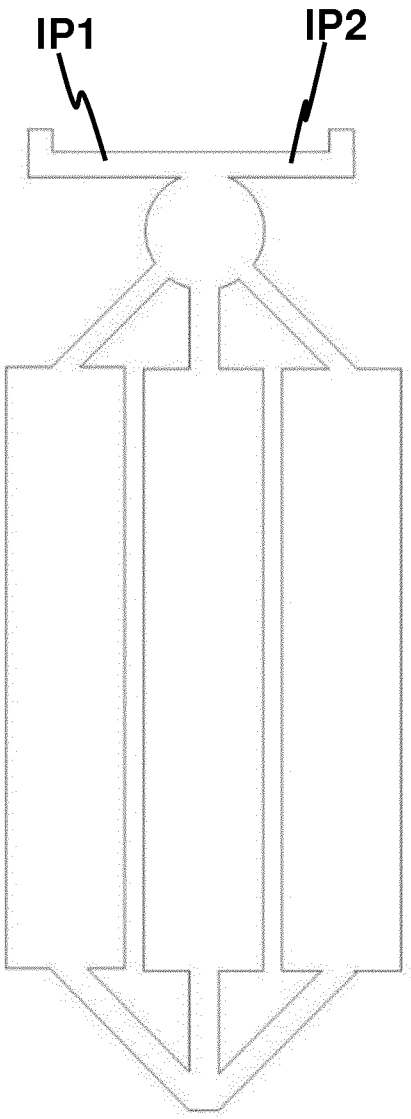


Fig. 2

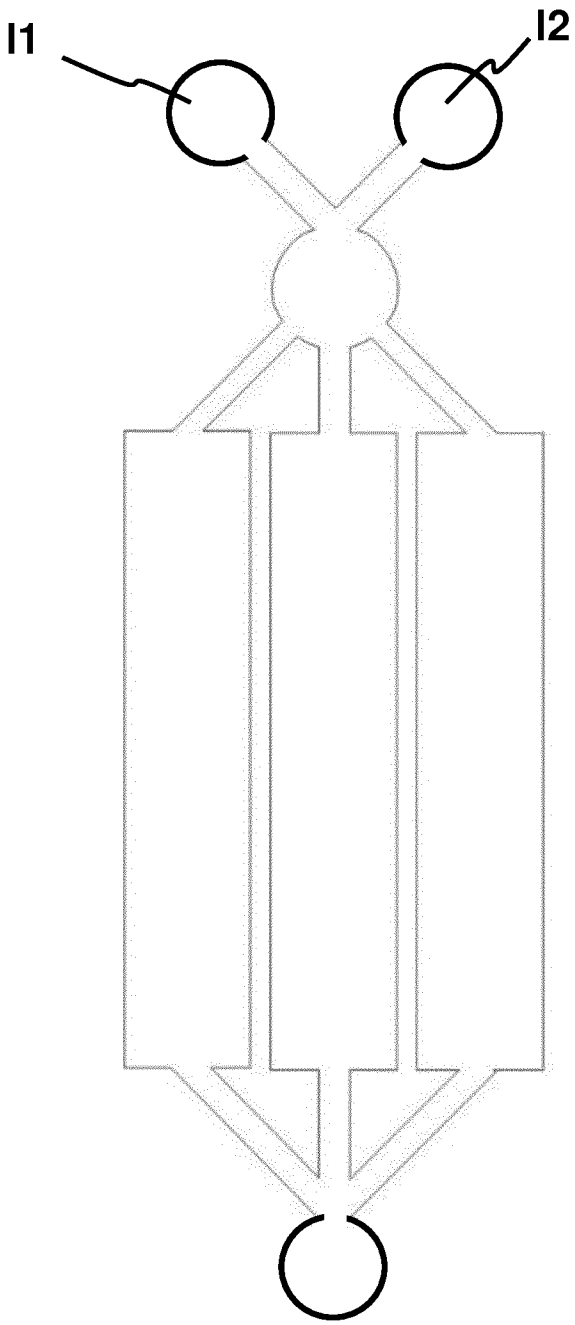


Fig. 3

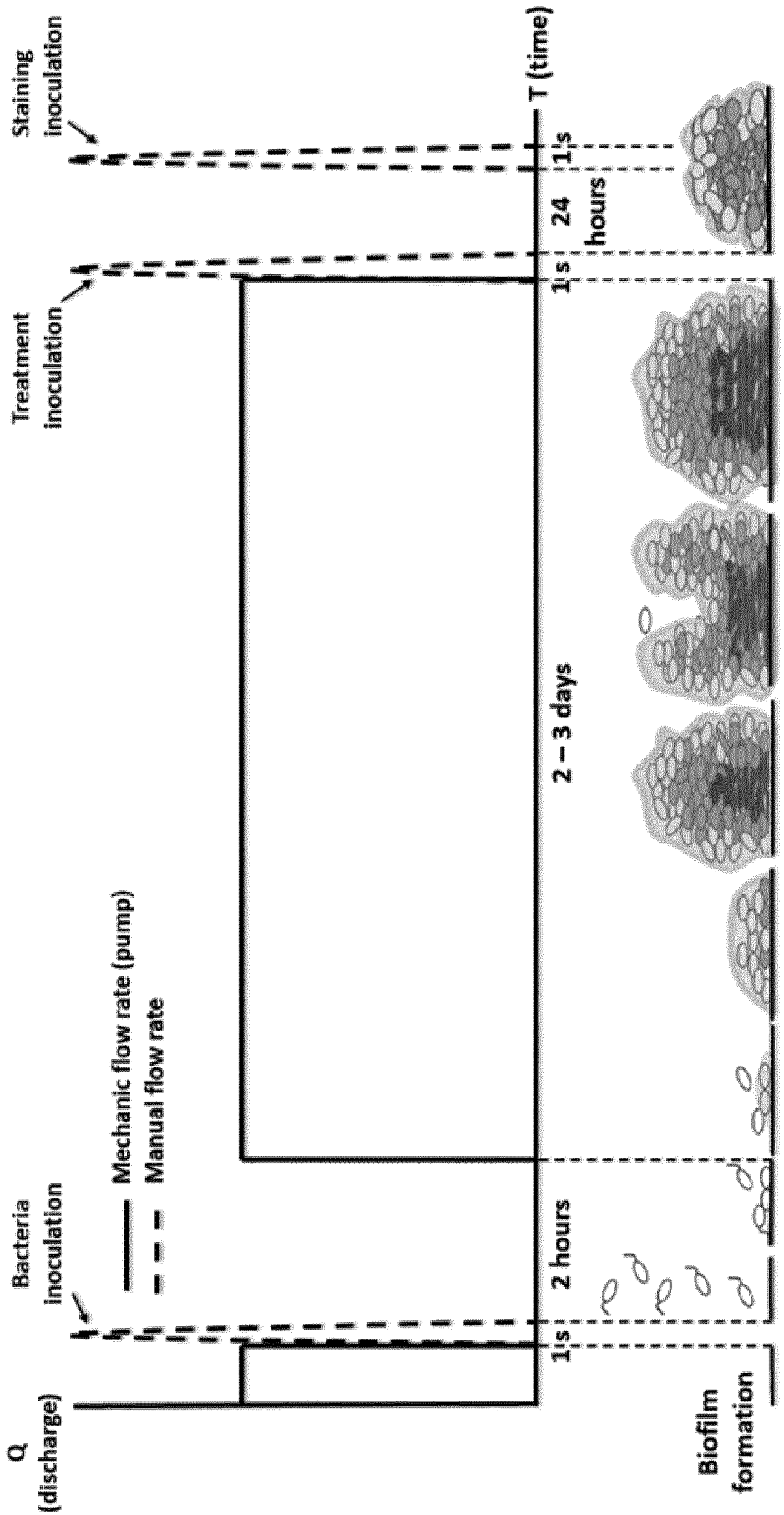

Staining inoculation
T (time)
Treatment inoculation
1's
24 hours
1's
2 – 3 days
Mechanic flow rate (pump)
Manual flow rate
Bacteria inoculation
2 hours
1's
Q (discharge)
Biofilm formation

Fig. 4

Fig. 5

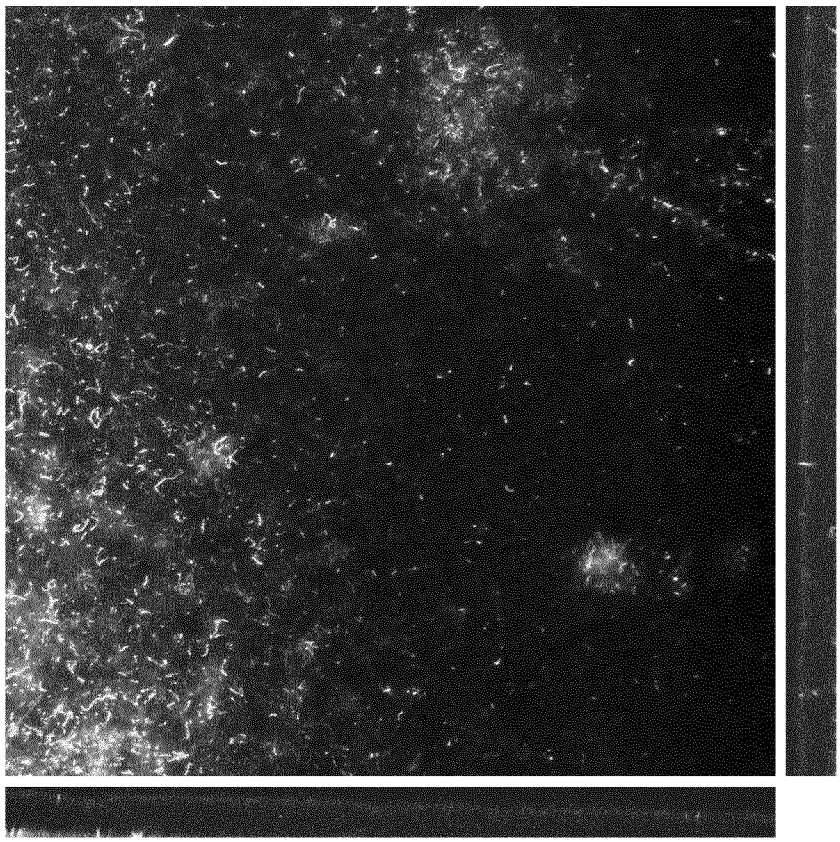


Fig. 6

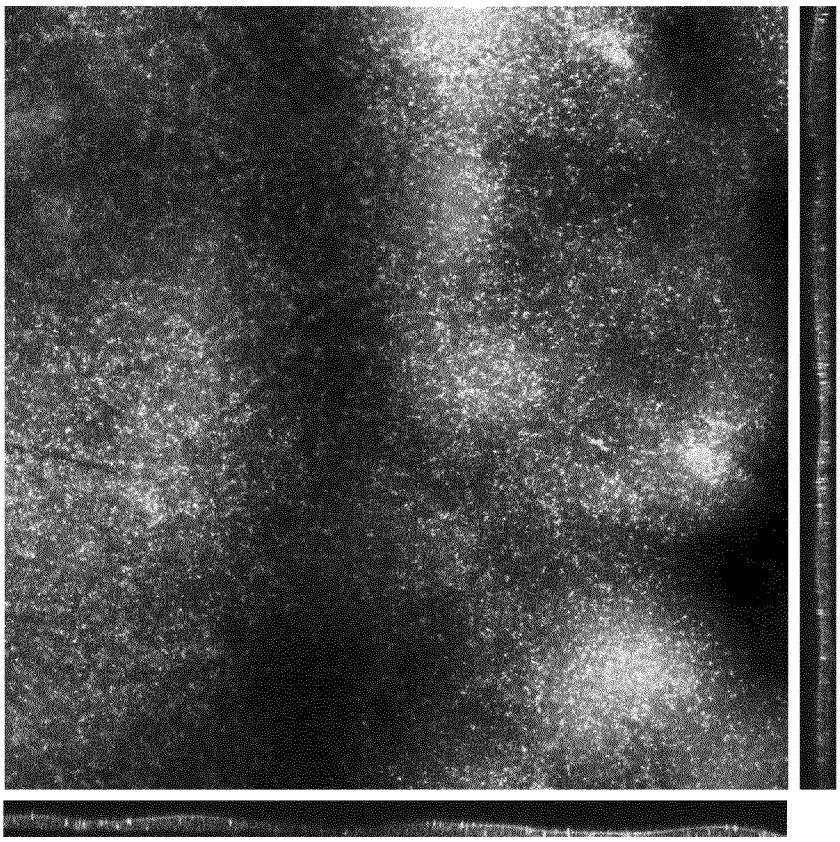


Fig. 7

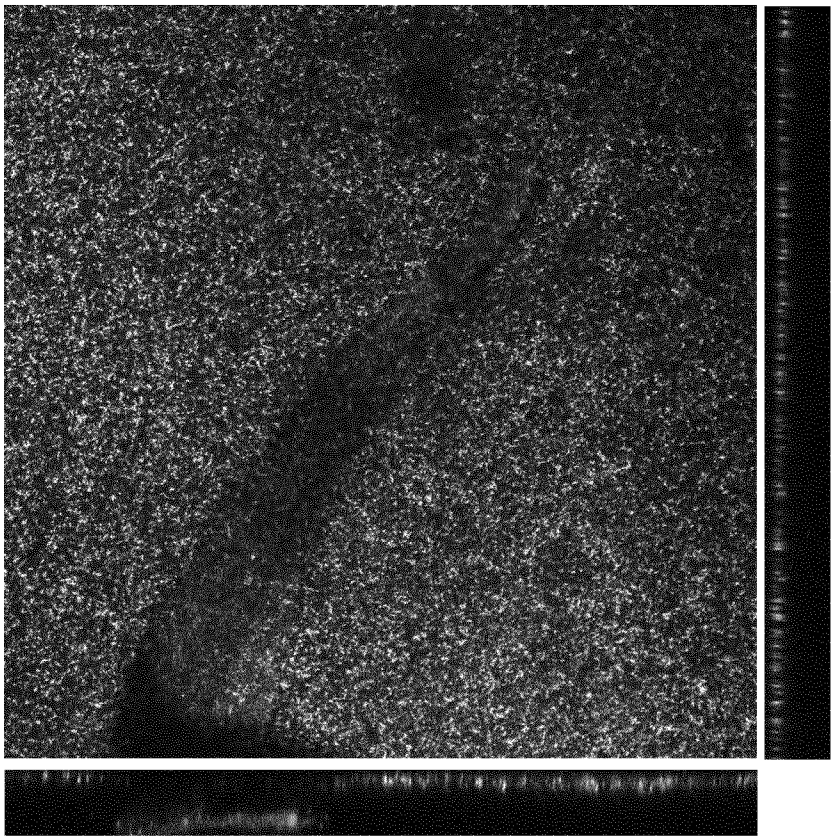
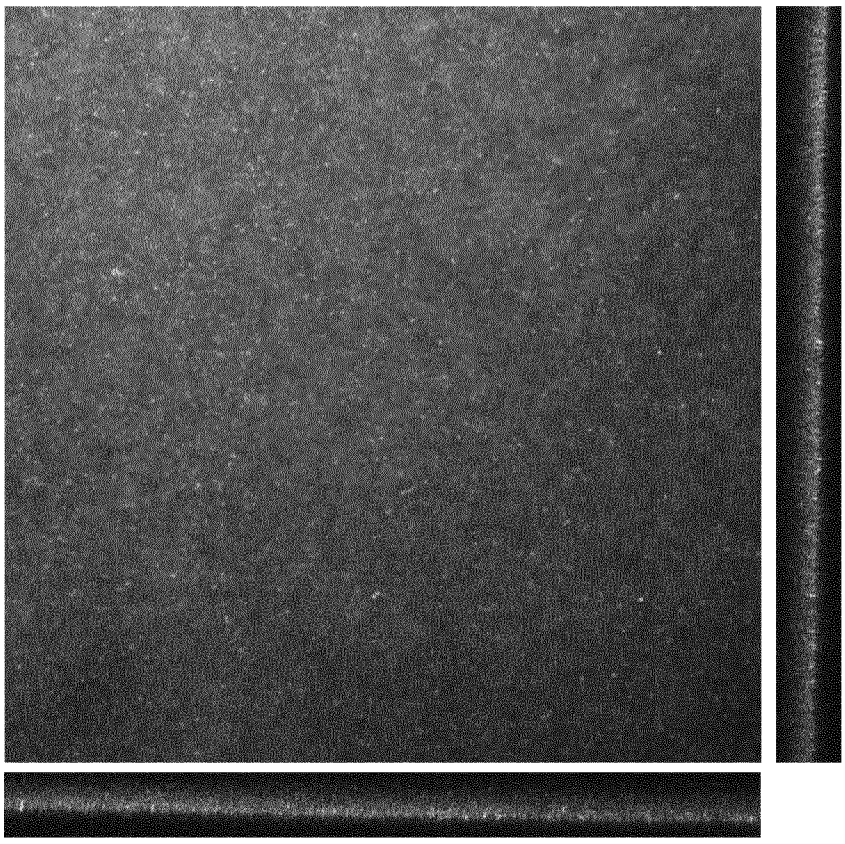


Fig. 8



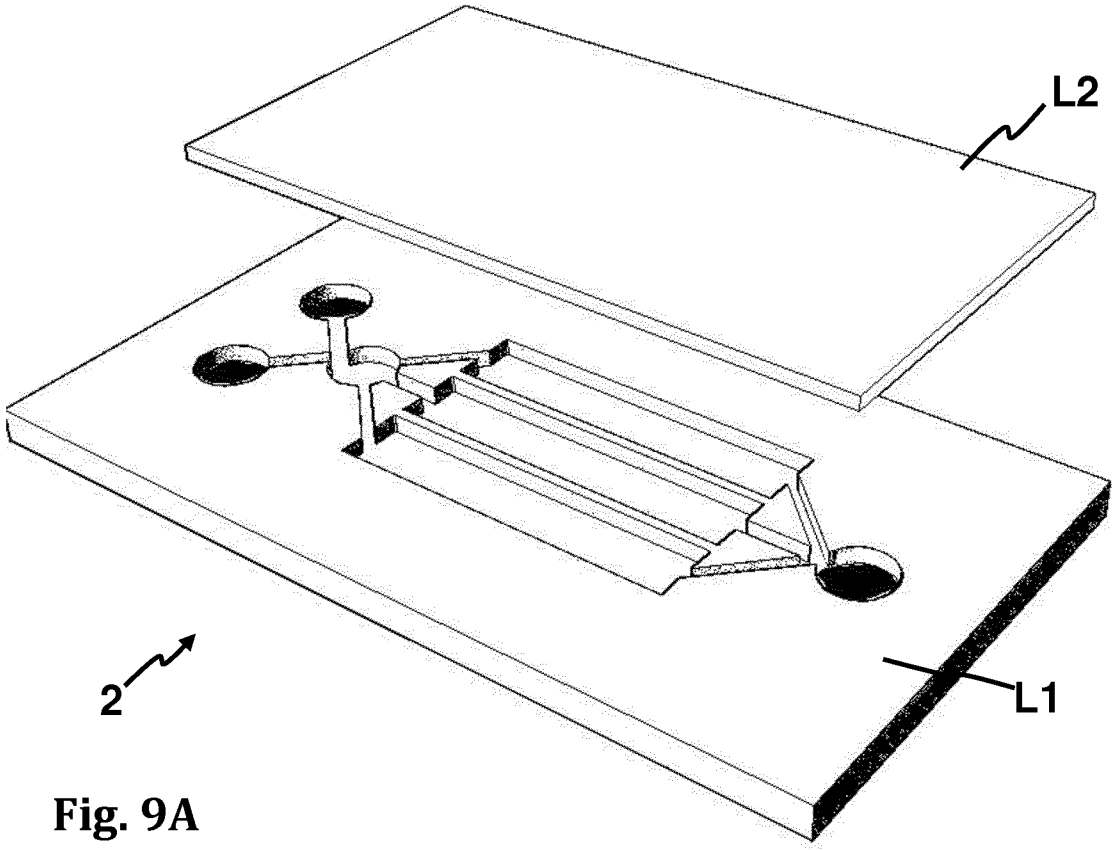


Fig. 9A

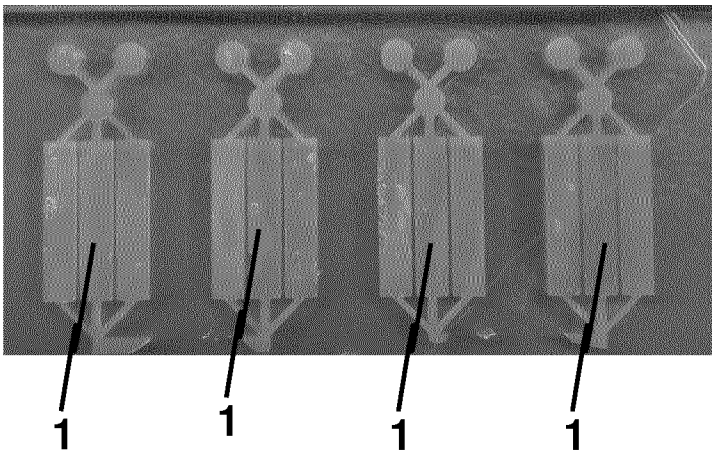


Fig. 9B

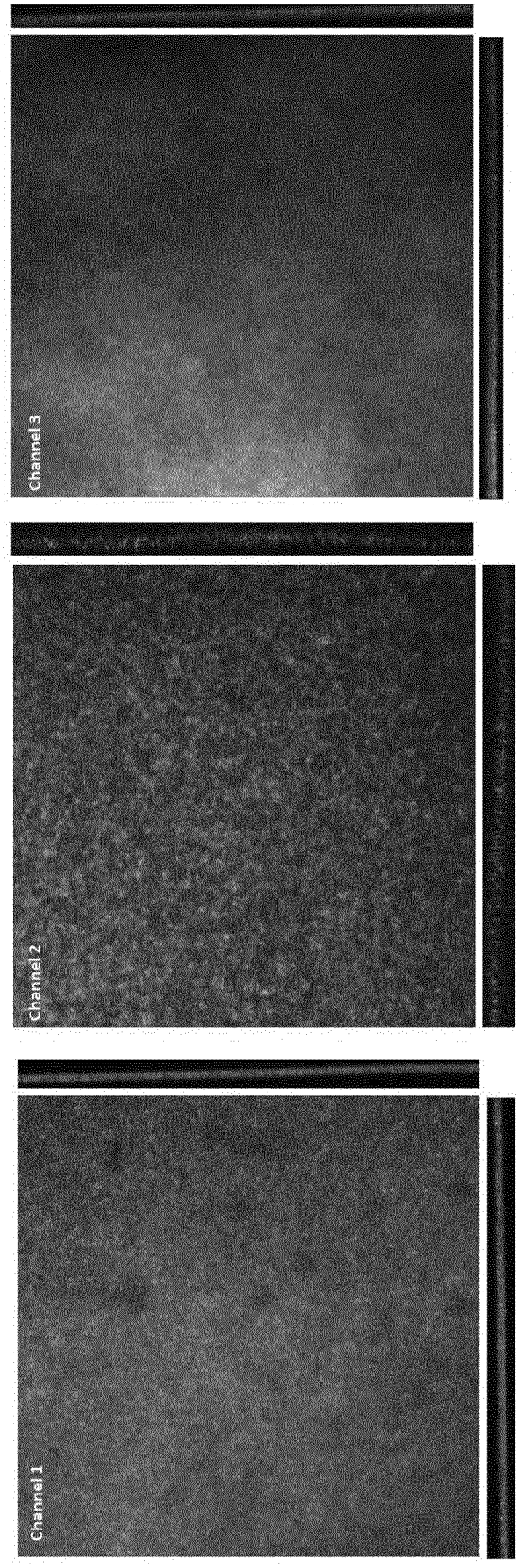


Fig. 10A

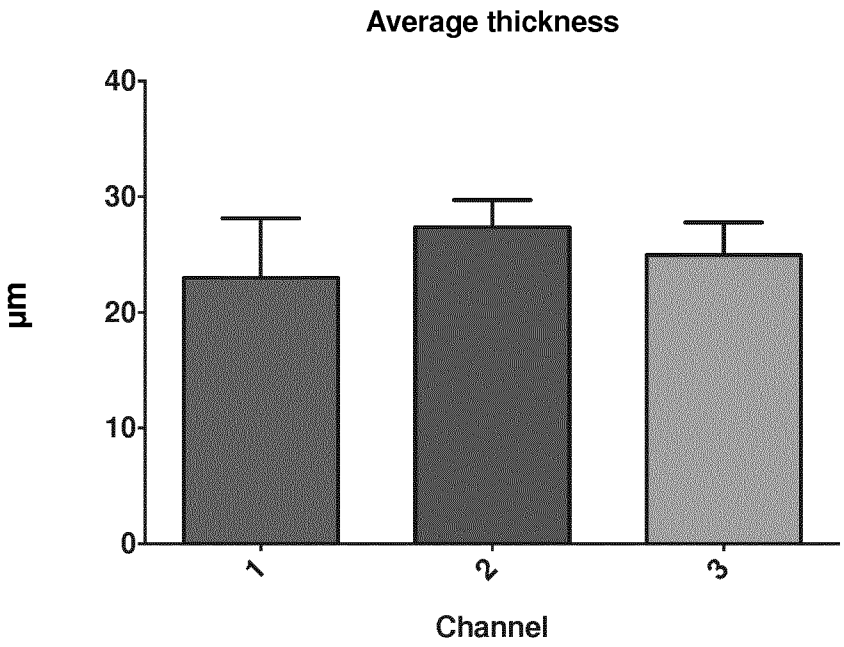


Fig. 10B

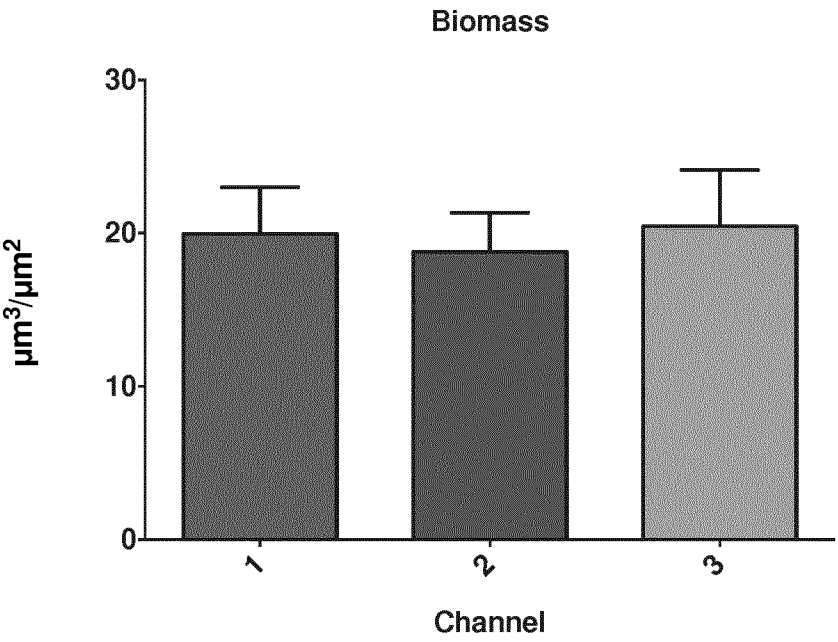


Fig. 10C

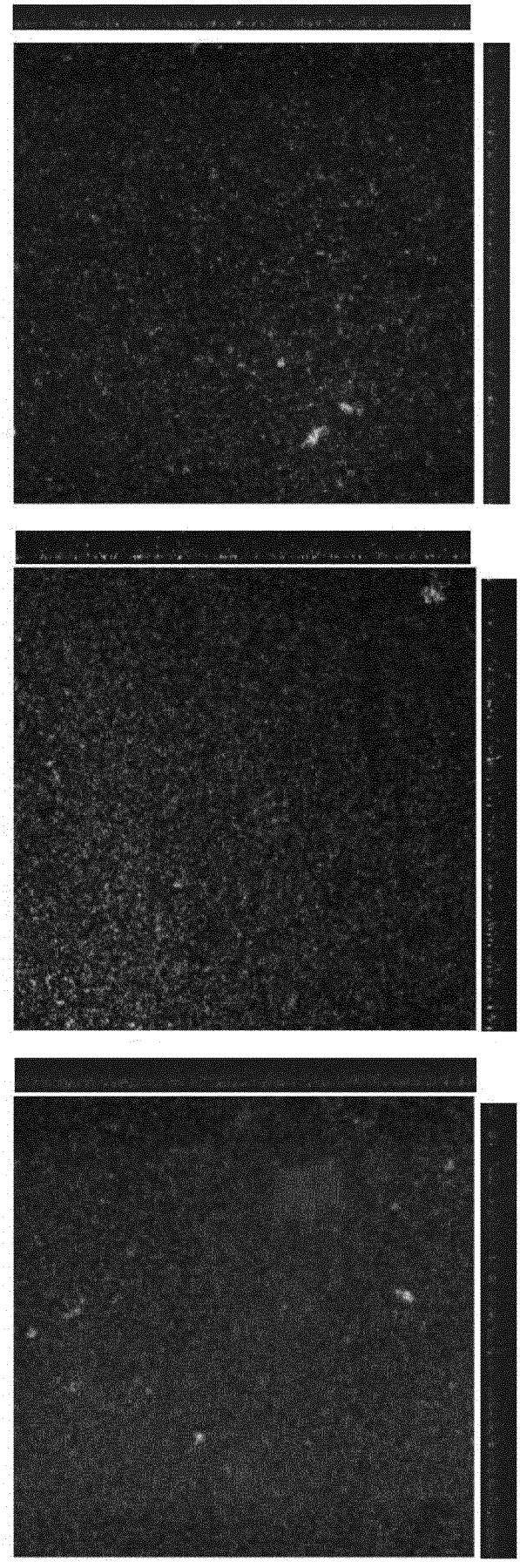


Fig. 11A

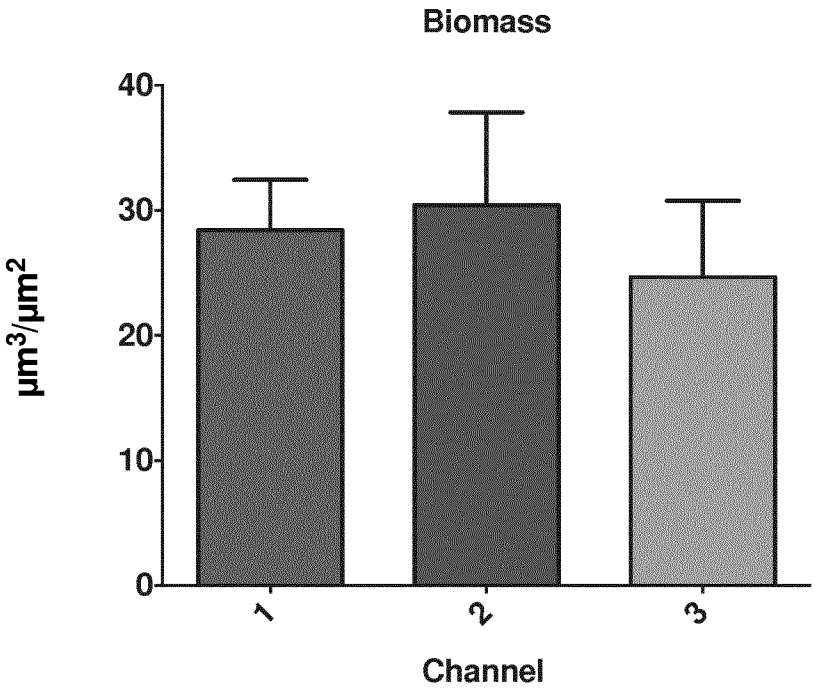


Fig. 11B

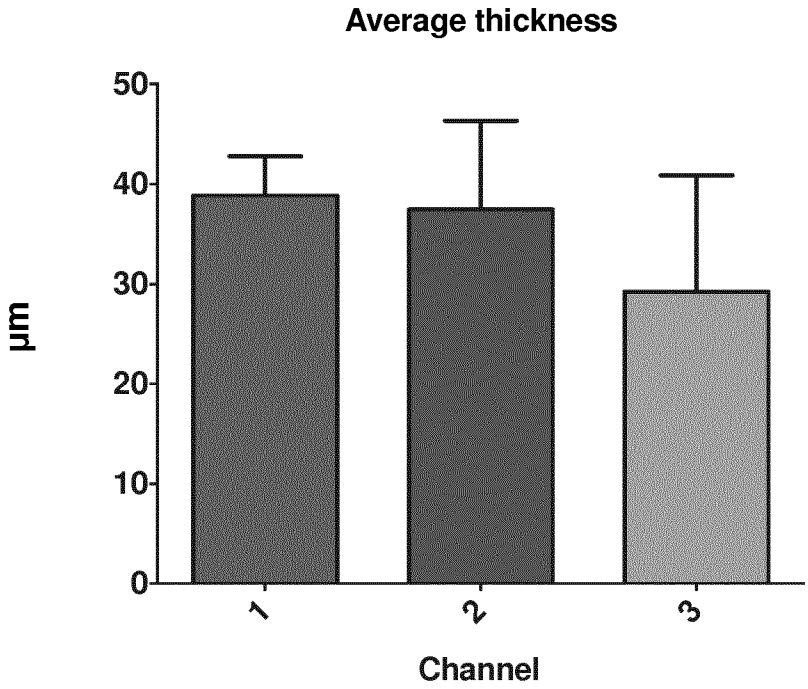


Fig. 11C

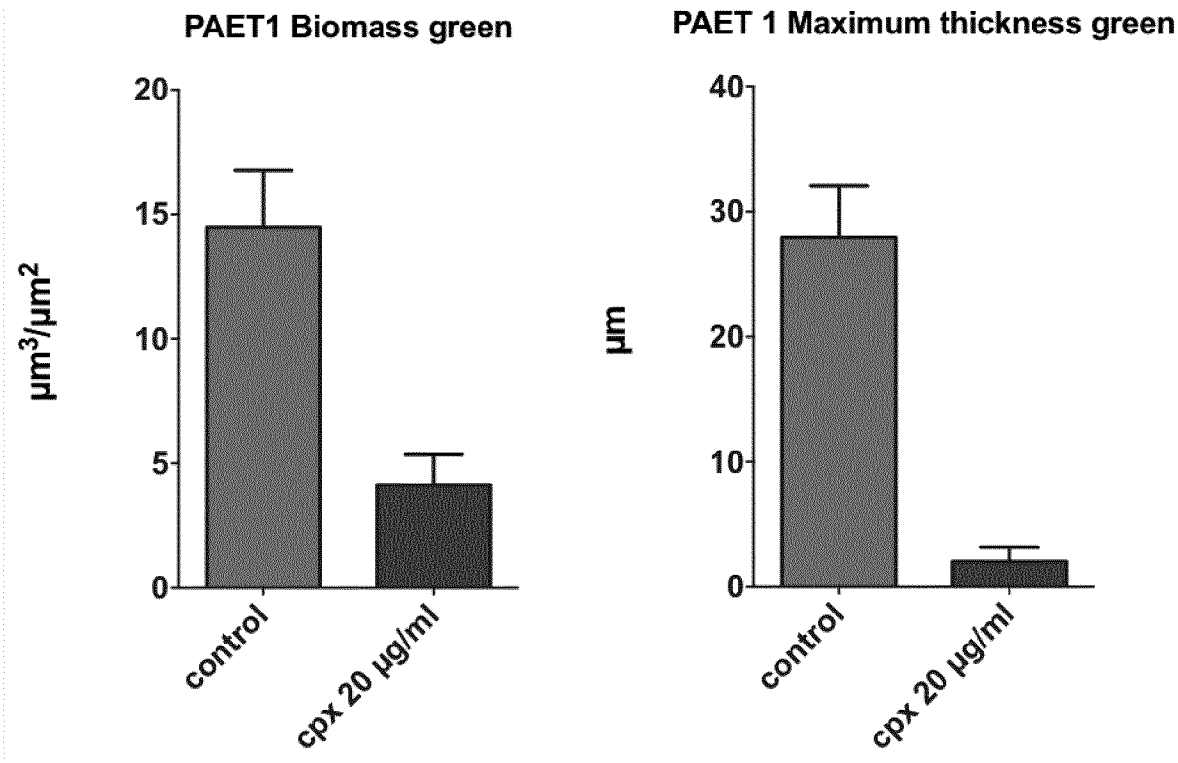

PAET1 Biomass green
µm³/µm²
20
15
10
5
0
control
cpx 20 µg/ml
PAET 1 Maximum thickness green
µm
40
30
20
10
0
control
cpx 20 µg/ml

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/058570

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01L3/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/273470 A1 (HOFFMANN JOCHEN [DE]) 1 October 2015 (2015-10-01) paragraph [0005] paragraph [0037] paragraph [0042] - paragraph [0047]; figure 1	1-15
A	US 2004/180432 A1 (EMERSON STEPHEN G [US] ET AL) 16 September 2004 (2004-09-16) figure 4b	1

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2019	09/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Ueberfeld, Jörn

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/058570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2015273470 A1	01-10-2015	DE 102014206140 A1	01-10-2015
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US 2004180432 A1	16-09-2004	US 5605822 A	25-02-1997
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