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Design, modeling and application of microfluidic single-cell traps for algal and bacterial studies

Summary of the Ph.D. Thesis

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List of publications

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1 Introduction

1.1 Phenotypic heterogeneity in microbes

The fact, that microorganisms occur almost everywhere on Earth points to their remarkable flexibility and adaptability. Niches occupied by microorganisms vary greatly, some of them stay stable for a long time, but in most cases cells experience highly dynamic conditions (frequent fluctuations in nutrients, temperature, osmolarity, pH, etc.). To survive and prevent being outcompeted by other species or genetic variants, microbes have to adapt to the new environmental conditions.

Biological diversity appears not just in complex microbial communities that consist of different species, but also at a lower level of biological organization. Genetically identical cells growing in the same environment often show different phenotypes, and this individuality ensures the survival and increases the fitness of the population under unfavorable or selective conditions. Adaptation to new niches starts with phenotypic adaptation on a short timescale and continues with genetic mutations on a longer timescale.

There are several mechanisms that are responsible for the appearance of phenotypic heterogeneity. In some cases it is caused by stochastic molecular processes in a cell. The molecular composition of a cell and its gene expression pattern changes over time and varies between individuals. Cellular aging can also influence phenotypic diversity through increased noise in gene expression.

Microorganisms live in complex systems where cell-cell interactions are common. These can occur through direct physical contact between cells or through diffusible molecules (example: quorum sensing in bacterial cells). These interactions can cause the appearance of different phenotypes.

1.2 Single-cell analysis of microbial cells

Batch culture studies have been the most common methods in microbiology to study the behavior of microorganisms for a long time. The main disadvantages of these methods are, on the one hand, the oversimplification (by applying homogeneous environment). In nature microorganisms

face variable surface topologies (rocks, intestinal villi, leaves and so on) and constant or periodic fluid flow (rain, urination), which may favor heterogeneous phenotypes in the inhabiting populations. On the other hand, the results of bulk analysis of the cells are population averages, that can mask the structure of cell-to-cell differences.

In the last two decades the number of applications of micro- and nanotechnology started to increase in life sciences. Microfluidics and microfabrication technology enable the manipulation of fluid flow at the micrometer scale. In such systems a small amount of sample is enough for detailed analysis.

Microfluidic platforms consist of chambers that are connected by channels, membranes, slits and other integrable elements and can be used to mimic natural, patchy environments for microbes. Another benefit of this technique is the laminar nature of the fluid flow that ensures the possibility of precise calculation of flow and diffusion conditions. These physically and chemically controllable habitats make it possible to study the properties of microorganisms not just on population, but on a single-cell level, as well.

The microfluidic mother machine (MM) allows the observation of continuously growing linear bacterial colonies for unlimited time. The device ensures the study of cellular aging and tracking cell relatedness in small isogenic populations on a single-cell level by trapping dividing bacteria for hundreds of generations. It consists of an array of dead-end side channels that are connected to a main channel with their open end. The growth channels are for single-cell trapping, therefore their width and height is comparable to the size of a bacterial cell, while the length is longer, ensuring the collection of the close relatives after cell divisions. The main channel ensures constant medium supply for trapped cells and the removal of waste products and cells, entering the main channel. The dimensions of the main channel are much bigger compared to the growth channels.

Furthermore, there is still room for the development of various microfluidic platforms for trapping and long-term observation of the cells.

1.3 Single-cell analysis of algal cells

Greean algae have a growing biotechnological importance and *Chlamydomonas reinhardtii (C. reinhardtii)* is a great model organism. While it is a well-studied organism, the relationship between its morphology, life cycle and photochemistry is still undetermined.

Symbiodinium spp. is an essential symbiont for reef invertebrates. As it is well-known, climate change negatively affects coral reefs and for that reason, understanding the stress tolerance and avoidance of *Symbiodinium* is cardinal. Protoplast technology is a great tool to study oxidative stress, although the preparation of viable protoplast is challenging.

Microfluidic techniques may offer a way to study these problems on the single-cell and population level.

1.4 Quorum sensing

In microbial communities cell-cell interactions are common and can occur in different forms. One of them is quorum sensing (QS), a cell density-based communication system between bacteria, that is mediated by diffusible signal molecules. In case of Gram-negative bacteria, the signal molecules usually belong to the family of acyl-homoserine lactones and they differ only in the length and the composition of their acyl side chain, while Gram-positive bacteria use oligopeptides.

When the population density of the cells is low, they produce the signal molecules on a base level. As the cell population grows, the concentrations of the autoinducers increase and cross a threshold. The signal molecule binds to the corresponding intracellular receptor and the receptor-signal complex acts as a transcription factor triggering cells to synchronize their behavior through changing their gene expression.

QS has significant role in coordinated cell behavior, such as virulence and biofilm formation. This process allows bacteria to function as a multicellular organism gaining benefits they could never obtain acting as loners. According to some theories, the evolution of bacterial QS was the first step that led to the development of multicellularity.

The first discovered QS system was the LuxI-LuxR system of marine bacteria *Vibrio fischeri* and *Vibrio harveyi* in the 1970s. In these species

density-dependent bioluminescence was observed. Later it has been shown, that the simple signal-response mechanism described in *Vibrio fischeri* is employed in most Gram-negative bacteria.

1.4.1 Quorum sensing of *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacterium, that easily adapts to various environmental conditions, primarily lives in soil, water and plant environment. It is an opportunistic human pathogen, the most common source of nosocomial infections (urinary tract infections, post-surgery wound infections and also responsible for the death of patients that suffer in cystic fibrosis). This bacterium strain is resistant to conventional antibiotic treatments. Better understanding the processes, that are responsible for the production of virulence factors, can offer alternative strategies for infection treatments. Therefore, *P. aeruginosa* became one of the model organisms of QS research. It has a complex QS system, consisting of four hierarchically coupled signaling mechanisms, namely *las*, *rhl*, *pqs* and *iqs*.

The first identified QS systems in *P. aeruginosa* were the LuxR-LuxI homologue LasR-LasI and RhlR-RhlI systems with autoinducers *N*-(3-oxododecanoyl)-homoserine-lactone (3O-C12-HSL) and *N*-(butryl)-homoserine-lactone (BHL), respectively. The Rhl system is under the control of the Las system. The third QS signal, the quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone was originally studied as an antibacterial molecule. This was the first time that a 4-quinolone was reported as a signaling molecule in bacteria. The fourth intercellular communication system is the IQS with a 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde signal molecule.

The above mentioned systems are hierarchically layered. At the top of this hierarchy is the *las* system. LasR-3O-C12-HSL complex activates the transcription of *rhlR*, *rhlI*, *lasI* (positive feedback loop) and other virulence genes. The RhlR-BHL complex activates the expression of *rhlI* (second positive feedback loop) and also positively regulates PqsR. The production of PQS autoinducer has an influence on the overall expression of the *rhl* QS system. The *iqs* signaling system is also controlled by the central *las* system under nutrient rich conditions. Surprisingly, under phosphate depletion stress conditions *iqs* was found to be able to partially take over

the function of the central *las* system.

Of these four QS systems, the *las* and the *rhl* systems have been studied in detail. However, our knowledge is still imperfect and detailed studies are required.

2 The aims of the work

Microalgae has a high biological and biotechnological significance. We participated in collaborations for single-cell-level studies of two important algal species. *C. reinhardtii* is considered as a model organism of green algae, while *Symbiodinium* is a vital symbiont of corals. In this effort I participated in the development of suitable microfluidic platforms which enable trapping of individual cells and the progenies when needed. Furthermore, the device would allow microscopy observations, fluorescence measurements of the trapped cells, as well as the precise control of their chemical environment. In particular, my aims were the following:

- Design and simulate a microfluidic platform ("Tulip") for the trapping of individual algal cells for short-term monitoring. I aimed to perform model calculations to determine the characteristics of the fluid flow within the device and the effect of the trapped cells on the flow.

- Design and simulate various microfluidic platforms ("Cup" and traps for *Symbiodinium*) for the trapping of individual algal cells and their progenies for long-term monitoring. In addition to calculating the characteristics of the fluid flow within the devices, I also aimed to determine the shear stress acting on the trapped cells.

In another work the QS of *P. aeruginosa* Δ*lasI* mutant was followed by applying the microfluidic MM device. The main goal of the study was to answer the following questions:

- What is the kinetics of the Las QS response on single-cell and population level? This is an important biological question, since most of the studies focus on the buildup of the quorum, although the quorum-on

→ quorum-off transition is just as important, because otherwise the population would stuck in quorum active state.

- How the external addition and removal of signal molecules influence the emergence of phenotypic heterogeneity?
- How cell lineage history influences cell-to-cell differences?
- How the quorum states of the cells influence different cell parameters?
- How repeatable the QS response of cells is to subsequent pulses of signal molecules?

3 Materials and Methods

3.1 Designs and numerical simulations of the devices

All the two-dimensional designs of the devices were created in KLayout, which is an open source CAD software.

To determine the characteristic properties of the fluid flow in the microfluidic devices, numerical simulations were run using Comsol Multiphysics 4.3a software. The velocity magnitude profiles were calculated by the "Laminar Flow" model using time-dependent study.

In case of the "Tulip" traps and the device designed for *Symbiodinium*, 2D models were used with "shallow channel" approximation to calculate fluid flow. For more precise results, spherical model cells were placed inside the traps. To model the "Pot" device, a 3D model was built considering its two-layered geometry. The simulations were run on a computer with an Intel® Core™ i9-10900 processor and 32 GB of RAM.

3.2 Microfabrication and electronmicrosopy of the devices

The microfluidic devices presented in this work were fabricated using photolithography, direct laser writing and soft lithography methods. For the fabrication of the negative master mold a single or a double layer of SU-8 negative photoresist was spincoated on the top of a silicon wafer. In case

of the "Tulip"-shape algae trapping device a single layer of SU-8 2005 resin with a height of 7 μm was used, while in case of the *Symbiodinium* traps SU-8 2015 photoresist with a height of 13 μm was applied. In case of the "Pot"-type algae traps the molds were fabricated in three steps, by aligning two SU-8 layers with different thicknesses: the first SU-8 2005 layer (thickness was 4 μm) contained the traps with the narrow gaps. The second SU-8 2007 layer (height was 7.5 μm) contained the traps with closed walls. The designs of the devices were then exposed to the photosensitive resin using a laser pattern generator. The master molds were sylanized overnight under vacuum, using (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane. Polydimethylsiloxane (PDMS), a silicon rubber was then poured onto the negative mold mixed in 1:10 iniciator:base ratio to prepare the final pattern. PDMS was cured overnight at 40 °C, then it was peeled off, cut into pieces and the inlet and outlet holes (with diameter of 1.5 mm) were punched. PDMS pieces were covalently bound to a glass slide using oxygen plasma treatment.

The dimensions of the devices were verified using a JSM-7100F field emission scanning electron microscope. For these examinations the PDMS replicas were cut into small pieces, covered with a thin gold layer (15 nm) placed into the scanning electron microscope working with 5 kV voltage.

3.3 Bacterial strain, growth conditions, loading of the mother machine device

For the MM experiments we used a mutant *P. aeruginosa* PUPa3 Δ*lasI* strain, which contains a pKRC12 gfp-based HSL-sensor plasmid (Gm^R; pBBR1MCS-5 carrying P_{lasB}-*gfp*(ASV) P_{lac}-*lasR*) that is responsive to 3O-C12-HSL and coding an unstable variant of GFP protein (GFP(ASV)). This mutant can not produce, but can sense externally added signal molecule and through GFP expression the quorum state of the cell can be determined. In this work the term QS-on/off state describes the quorum state of single cells, while quorum-on/off describes the quorum state of the population, presented in the device. It has been shown, that in *P. aeruginosa* PUPa3 strain the Las and the Rhl systems are not hierarchically coupled.

Single colonies of *P. aeruginosa* cells were grown in 3 ml LB medium containing 50 μg/ml kanamycin and 50 μg/ml gentamycin in plastic culture tubes at 30 °C in an incubator shaker at 200 rpm overnight. Next morning the culture was diluted by 1:1000. When the optical density (OD_{600}) of the culture reached 0.1, 3O-C12-HSL was added to the medium in 10 nM or 1 μM concentrations to induce QS. The experiment started when the culture's OD_{600} reached 0.6. 1.5 ml of the bacteria suspension was centrifuged and the pellet was resuspended in 300 μl medium that did not contain signal molecules. Cells were injected into the MM device by a syringe, then signal-off medium was flowing through the system for 16 h. Fluid flow was ensured by a syringe pump, the applied flow rate was 200 μl/h. The fluorescence time-lapse experiment started when the side channels were fully filled with cells in QS-off state.

The experiments with threshold signal concentration were run for 20 h (6 h signal-on, 14 h signal-off periods), while with the saturating signal concentration the experiments took 44 h (6 h signal-on, 16 h signal-off, 6 h signal-on and 16 h signal-off periods). Three independent repeats for both concentrations (10 nM and 1 μM 3O-C12-HSL) were performed.

3.4 Microscopy

To follow the division and GFP expression of trapped cells during the MM experiment fluorescence time-lapse microscopy was used. The experiment was performed at 30 °C using a Nikon Eclipse Ti-E inverted microscope, a Prior Lumen 200 Pro excitation lamp and a cage incubator. A 40× Nikon Plan Fluor objective, a GFP fluorescence filter set and a Prior Proscan II motorized stage were parts of the microscope setup. NIS Elements Ar. Software was used for image acquisition and microscope control and the imaging was done by an Andor NEO sCMOS camera. Images were taken every 5 min.

3.5 Data analysis

For the analysis of time-series data gained from the MM experiments the BACMMAN (BACteria in Mother Machine ANalyzer) software was used, which is a plugin of Fiji environment. Using this software the pixel-

averaged intensity of the cells, division times and cell lineage distances could be determined. Further analysis of the data produced by BACMMAN was performed using R. For the plots the ggplot2 R package was used.

4 Results and discussion

1. I designed and modeled a microfluidic platform for the trapping of individual algal cells for short-term monitoring.

For the short-term trapping and observation of *C. reinhardtii* I designed and fabricated the microfluidic "Tulip" platform. The construction of the device allows us to immobilize individual cells for a few hours for Chl *a* fluorescence measurements. The device consists of three parallel channels with the array of single-cell traps that are laterally shifted to each other. The total number of the traps is 216. All the traps have a wider, funnel-shaped inlet (the width of the inlet is 28 μm) and a narrow outlet (the width is 3 μm), to ensure the culture medium to flow through the traps, but prevent the escape of trapped cells. The height of the device is 7–8 μm, the diameter of a trap is 8.4 μm.

To simulate the characteristic properties of the medium flow in the chip, the "shallow channel" approximation was used. The fluid flow was modeled in a 500 μm x 600 μm area of a single branch of the device.

Based on my results, in the case of empty traps around 10 % of the flow goes through the traps, enabling a cell to enter the trap. The flow velocity is higher between neighboring traps and lower at the entrance of the trap. Spherical model algae were added to the model to see, how trapped cells change medium flow in the device. These were found to completely block the flow and prevent another cell to enter the trap. During the experiments (run by the Laboratory for Molecular Photobioenergetics) 65 – 71 % of the traps captured a single cell.

Both the model calculations and the experiments proved that the device is suitable for the trapping of *C. reinhardtii* cells for a few hours to monitor their fluorescence without the need of the fixation of the cells, this way not risking their damage.

2. I designed and modeled a microfluidic platform for the trapping of individual algal cells and their progenies for long-term monitoring.

To collect the progenies of a trapped cell, two different microfluidic platforms were designed, modeled and fabricated for two different microalgae.

One of the devices, that was designed for *Symbiodinium* spp. is composed of traps with three different sizes: large trap with 60 μm wide inlet, medium-size trap with 40 μm and small trap with 30 μm wide inlet. All the traps have three, 5 μm wide gaps, ensuring that the medium flows through them.

To determine the characteristic properties of the medium flow in the chip the "shallow channel" approximation was used. Different flow rates (20 μl/h, 60 μl/h and 100 μl/h) were tested. The average flow velocity differs by less than 10 % in the three branches that contains traps with different sizes. Between the traps velocity magnitude can reach 2000 μm/s, while at the inlet of the traps it is around 400 μm/s. In order to simulate the shear stress on cells, model cells with 10 μm diameter were placed in the traps at typical positions, that were observed during the experiments. Maximal shear stress with value of 0.4 Pa was calculated for cells placed near to the gaps, which is not higher, than in the nature (in nature in some cases these cells have to tolerate 600 Pa). Plant Stress, Lipid and Phenomics Group successfully applied this platform for protoplast formation.

The other microfluidic platform is the "Pot" device, that was designed for the collection of *C. reinhardtii* cells. The first version of the device contained seven types of pot-shaped traps, that differed in their inlet geometry and the number of the outlet slits. The constructions were evaluated based on their trapping efficiency (experiments performed by the Laboratory for Molecular Photobioenergetics) and two types were found to be the most efficient. Comsol simulations were run on these two.

The device was constructed in two layers, with 5 μm and 7 μm heights. The shallower layer contained the narrow exits, while the higher layer contained the walls of the traps. This way the escape of the smaller daughter cells ($d \approx 3-4$ μm) could be prevented and the required height (12 μm)

for the bigger mother cell was ensured.

For the numerical simulations I built the 3D model of the device and evaluated the fluid flow in a 500 μm x 400 μm size area, in two different z-layers: in the middle of the shallow layer ($z_1 = 2$ μm) and in the middle of the whole device ($z_2 = 6$ μm). I found, that at 6 μm height the values are always higher, because of the parabolic velocity profile for both designs. The velocity magnitude of the flow was higher between the neighboring traps and lower at the entrance of the traps in both z-layers.

On the first place, the number of the slits can increase the trapping efficiency, but also the inlet geometry has an important role. My simulations proved, that traps with funnel-shape inlets were the most efficient.

3. I successfully applied the mother machine microfluidic device to study quorum sensing in bacteria.

QS is primarily considered to be a population-level phenomenon, nevertheless changes in gene expression patterns in response to environmental changes can be observed on the cellular scale. Based on these, it can be concluded, that population- and cell-level processes strongly intertwine. Therefore, for better understanding the QS dynamics on population level the analysis of single-cell data is needed. For that, I used the microfluidic MM device and filled it with a mutant *P. aeruginosa* Δ*lasI* strain, that can not produce, but can sense externally added signal molecules. The GFP production of the cells is QS controlled, so their fluorescence emission gives us information about their quorum state.

During the experiments cells were exposed to 3O-C12-HSL signal molecule for 6 h ("signal-on" period) and it was followed by a longer "signal-off" period, when the molecules were washed out of the system. Two signal molecule concentrations were applied: one was around or below the threshold concentration (10 nM) and the other was a saturating (1 μM) one. In total 9934 cells were analyzed, 2444 in case of 10 nM signal concentration and 7490 in case of 1 μM signal concentration.

In these experiment series it was possible to follow bacterial growth and the cellular reactions to environmental changes on single-cell and population level, as well. I was able to define QS-on and QS-off states for each cell at all time points. For this purpose, I calculated a threshold intensity

based on the single-cell data from the 1 μM signal concentration. The intensities from 0 to 50 a.u. were scanned through with 0.1 a.u. step size and the number of cells, whose intensities exceeded the limit was counted at every time point: $\frac{n_{on}(t)}{n_{all}(t)}$. The time vs. proportion data was smoothened, the minimum and the maximum proportion values were determined for all the intensities. The threshold intensity was chosen by maximizing the difference between the number of QS-on cells in the signal-on and signal-off periods: $\Delta = \left(\frac{n_{on}(t)}{n_{all}(t)}\right)_{max} - \left(\frac{n_{on}(t)}{n_{all}(t)}\right)_{min}$. The calculated threshold was 23.1 a.u.: cells with intensity values below this were in QS-off state, while cells over this threshold were in QS-on state.

Based on the statistics of the states of individual cells the population-level quorum state was defined. For that, the fraction of QS-on cells was calculated. The criteria I applied here was that at least 50 % of cells had to be in QS-on state to declare the population to be quorum-on state.

4. I characterized the kinetics of the quorum sensing response: the buildup and the decay of the quorum in *P. aeruginosa*.

Most of the previous works in the literature focused on the buildup of the quorum in case of the Las system, although the decay is just as important. Reversibility of quorum transitions can offer new strategies for infection treatments. I followed the QS response on single-cell level for one signal-on and one signal-off periods, that included the buildup and the decay of the quorum, as well. For both signal molecule concentrations heterogeneity in the fluorescence response of the cells could be observed. However, timing of the cellular response (fluorescence and quorum buildup lag) seems to be well-defined in the measured QS kinetics.

In case of the addition of 1 μM 3O-C12-HSL, a short buildup lag (0.7 h) could be observed that was followed by a sharp increase in the QS-on fraction. It took 1.5 h to reach the maximum, where almost 100 % of cells were in QS-on state. After the withdrawal of the signal molecules there was a 5.6 h long quorum decay lag before the fraction of QS-on cells started to decrease. The population spent 14 h in quorum-on state. Based on these results I can say, that the population stabilizes in quorum-on state and is reluctant to leave it, the decay is slow.

In case of the 10 nM signal concentration there was a much longer

(2.5 h) quorum buildup lag, that was followed by a slow increase. The population did not reach the quorum-on state, only approached it, no stabilizing effects appeared. After the withdrawal of the signal molecules the fraction of QS-on cells started to decrease within an hour.

5. I demonstrated the repeatability of the quorum sensing response.

The repeatability of the QS response was tested by exposing the cells to subsequent pulses of signal molecules. For these experiments I applied saturating 3O-C12-HSL concentration. Two 6 h long signal-on periods were followed by two 16 h long signal-off periods.

Overall, we can say that cell response was similar for both signal pulses. The fraction of QS-on cells almost reached 100 % in both cases, but not all the cells switched back to QS-off state during the signal-off periods. In the first case 9 % of the cells remained in QS-on state, while at the end of the second pulse this number was higher, 18 %. Also the decay was slower during the second signal wave.

These results raise a question, whether repeated pulses of signal molecules can cause that the population remains in quorum-on state even between the pulses. We will need further experiments to answer this.

6. I demonstrated a monotonic increase in phenotypic heterogeneity between sibling cells in a cell cycle.

Besides measuring the fluorescence intensities, I could also identify cell division events that made it possible to collect information about cell relatedness.

First, I calculated the normalized fluorescence intensity difference between siblings during the cell cycle to gain more information about the emergence of cell-to-cell variability. Interestingly, it showed monotonic increase for both signal concentrations. These findings suggest, that the process(es) behind this phenomenon did not depend on the cell cycle phase, nor the signal concentration. Heterogeneity builds up fast, it develops between closest relatives already during a single cell cycle.

The normalized fluorescence intensity difference was also calculated for all the possible cell pairs from the same side channel concurrently present

in the device. I found, that the difference was lower in case of close relatives and was higher for distant relatives.

These finding support the assumption that shared lineage history of neighboring cells is the main cause of spatially correlated gene expression levels in clonal populations.

5 Conclusion

In the first part of my work I participated in cooperations regarding single-cell-level algal studies. In the literature, there are several examples for microfluidic platforms available for trapping algae, although these are mostly suitable for microcolony analysis and rarely for real single-cell studies. The devices, we constructed here are relatively easy to fabricate and use, only a simple syringe pump is needed for controlling flow in the devices and this flow carries cells to the traps. The "Tulip" microfluidic platform is perfectly suitable for trapping individual *C. reinhardtii* cells for a short-time analysis, while the other two platforms enable the collection and monitoring of *Symbiodinium* and *C. reinhardtii* cells and their progenies for one or two cell cycles.

In the second part of my thesis I studied the QS of a *P.aeruginosa* mutant strain in a microfluidic MM device. I applied two different 3O-C12-HSL signal concentrations: 10 nM is a near threshold concentration, while 1 μM is a saturating signal concentration, but it can be reached by signal producing batch cultures. In our study I followed the QS response on single-cell level for one signal-on and one signal-off periods (it includes the buildup and the decay of the quorum, as well) and quantified different parameters, that characterize the QS kinetics. In case of saturating signal concentration I exposed the bacterial culture with subsequent pulses of signal molecules (two signal-on and two signal-off periods) and showed the repeatability of the QS response. Furthermore, I explored the phenotypic heterogeneity of cells during the QS response.

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