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Laser printing of microbial systems: effect of absorbing metal film

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Abbreviated running headline: Laser printing of microorganisms.

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Significance and impact of the study: Laser induced forward transfer technique (LIFT) is currently used for printing of microorganisms and in biosensor techniques, for single cell isolation, and for culturing of microorganisms from complex substrates. We have studied the influence of absorbing film materials (Au, Ti, and Cr) on the effectiveness laser printing of microorganisms. It was shown that application of Ti and Cr absorbing layers activates bacterial growth and is more effective in LIFT compared to Au films, which actually have a suppressive effect on bacteria cells. The results can improve LIFT protocols for bacteria isolation and culturing of microbial systems.

Abstract

Recently it was shown that laser-induced forward transfer (LIFT) technology and the laser engineering of microbial systems (LEMS) technique (based on LIFT method) are effective for isolation of microorganisms from different complex substrates. These techniques frequently utilize Au as an absorbing layer material. The purpose of present study was to investigate the influence of absorbing film materials (Au, Ti, and Cr) on the effectiveness of laser printing of microorganisms to improve LEMS and LIFT techniques. It was shown that application of Ti and Cr absorbing layers activates bacterial growth after laser printing and is significantly more effective in comparison to Au films, which actually shown a suppressing effect on bacterial cells. Results of this study can be applied for LEMS and LIFT protocols for improving bacteria isolation and microbial growth.

Keywords: LIFT, LEMS, laser printing, bacteria culturing, bacteria isolation, soil, biodiversity.

Introduction

LIFT technology is currently used for transferring a broad range of materials in solid and liquid phases with very high resolution including cellular and biomolecular structures (Delaporte, Allonce, 2016; Papazoglou, Zergioti, 2017). Moreover, this technology can be applied for microorganisms printing. In particular, LIFT was used to transfer microbial cells for realization of biosensors (Ringeisen et al., 2002; Hopp, Smausz, 2004; Barron et al., 2004; Haider et al., 2017). Recently, effectiveness of this technique for bacteria isolation from different complex substrates was demonstrated (Ringeisen et al., 2010; 2015). This technique can be useful for isolation of rare or new (not described earlier) bacterial species, that is important for studies of Earth's microbial biodiversity, as well as for a discovering the novel producers of antibiotics, ferments, and other bioactive substances. Also this method can be used for single cell isolation (Deng et al., 2017) which has great importance for studies of growth, metabolism, and genomics of individual cells in various fields of life sciences and biomedical research (Francois et al., 2003; Gross et al., 2015).

Based on LIFT method, a technological approach for LEMS has been recently developed (Yusupov et al., 2018; Gorlenko et al., 2018). In LEMS, transfer of a microdroplet of gel substrate containing microorganisms occurs by rapid heating, under the action of a nanosecond infrared laser pulse, of a thin absorbing film on which the substrate layer is located (Fig. 1). Such heating leads to the evaporation of the absorbing film and generation of a rapidly expanding bubble and liquid jet. This jet transfers a gel microdroplet containing microorganisms to the acceptor nutrient medium. It was shown that the LEMS technology allows isolate a wider bacterial biodiversity including rare species from complex substrates (soils) in comparison with traditional cultivation methods (Yusupov et al., 2018; Gorlenko et al., 2018).

The effectiveness of LEMS depends on many parameters including laser pulse energy, absorbing film material, gel substrate composition, etc. A lot of them was investigated in detail and adjusted during our previous research (Yusupov et al., 2017; 2018; Minaev et al., 2018; Gorlenko et al., 2018). At the same time, effects of different absorbing film materials on microorganisms' bioprinting have been insufficiently studied. In our previous work, Au absorbing films were applied due to a widespread application of this material in LIFT (e.g. Gaebel et al., 2011; Ali et al., 2014; Pagès et al., 2015).

The aim of present study was to investigate the influence of absorbing film materials (Au, Ti, and Cr) on the effectiveness of microorganisms laser printing and to found possibilities to improve LEMS and LIFT techniques.

Results and Discussion

In the process of laser printing of bacterial strains, the bacterial colonies counts observed with Cr and Ti absorbing films were different by not more than 1.3 times and these differences were statistically insignificant (Fig. 2, 3). The colonies numbers with Au absorbing films in contrast to Cr and Ti absorbing layers were distinctly lower up to 12-14, 170-230, and 20-50 times for *E. coli* mar14-3565, *Spirillum sp.* KBP-89, and *C. michiganense* KBP V-29 bacteria, respectively. In the case of laser printing with Au films, the colonies numbers were up to 35 times lower than the ones at using traditional plating technique, while bioprinting with Ti and Cr films significantly increased the count of growing bacteria in comparison with plating. No significant differences were found in experiments with 50 and 100 nm absorbing layers for all metals studied.

The observed effects on bacteria growth using laser printing with different metals can be explained by different adhesion of the metals to the glass (Benjamin, Weaver, 1962) and, consequently, by differences in the processes of metal nanoparticles (NPs) formation (or its absence) during laser printing (Yusupov et al., 2017; 2018). It was shown, that Au NPs abundance in LEMS process is in direct ratio with the laser pulse energy, and at the energy which we used in current study (24 μJ) the NPs formation occurs (Yusupov et al., 2017). Metal NPs could be transferred to the acceptor substrate (nutrient medium) together with the gel containing microbial cells. It is known, that metal NPs are toxic for bacteria, and their toxicity strongly depends on material, size, form, and other parameters of NPs (Slavin et al., 2017). At the same time, some NPs could stimulate microorganisms' growth (Schacht et al., 2013; Miazek et al., 2015; Chen et al., 2018). That can explain higher bacterial colony counts in case of Ti and Cr absorbing layers compared to traditional plating. Absence of bacterial growth activation at usage of Au absorbing layers may be caused by lack of microbial growth stimulation with Au NPs (Fig. 2a, 2b) or even toxicity of these NPs for some bacteria (Fig. 2c, 2d). The activation and inhibition of bacterial growth can also be induced by laser radiation. In LEMS, most of laser pulse energy is absorbed within a metal layer, but a part of laser radiation reaches the gel with microbial cells. It was shown, that at usage of gold absorbing layer of thickness 50 nm, about 20% of the laser energy reaches the gel (Yusupov et al., 2017). The measurements using the P100D power meter and the sphere photodiode power sensors S140C (Thorlabs, USA) at performing of the matrix of 1600 individual laser pulses found, that at application of titanium or chromium film of 50 nm thickness about 10% or 25% of the energy respectively passes through the absorbing layer (unpublished data). Earlier it was proved, that laser irradiation in different regimes can enhance or inhibit microbial growth and metabolism (Kohli et al., 2001; Nussbaum et al., 2002; 2004). Studies of these effects are planned in the future.

Since it was found that Cr and Ti absorbing layers are significantly more effective compared to Au for laser printing of pure bacterial cultures, we performed experiments with soil to verify our results. For soil laser printing and traditional plating, the highest bacterial colonies yield was observed with a Cr absorbing layer, which is consistent with the results obtained with pure bacterial cultures (Fig. 2, 4). Au absorbing layer suppressed bacterial growth in terms of total count of bacterial colonies. Also, *Micrococcus* and *Myxococcus* which grew at plating were not found after laser printing with Au absorbing film. Nevertheless, laser printing with Au has stimulated *Bacillus* and *Streptomyces* growth (Fig. 4, 5), which is in agreement with our previous studies (Yusupov et al., 2018; Gorlenko et al., 2018). Activation of *Bacillus* and *Streptomyces* with simultaneous inhibition of *Micrococcus* and *Myxococcus* can be due to different sensitivity of these bacteria to the Au NPs. It is known, that at least some bacterial species differ in sensitivity to NPs (Ruparelia et al., 2008). The largest bacterial diversity was presented when the Cr absorbing layer was used: *Streptomyces, Bacillus, Arthrobacter, Rhodococcus, Micrococcus, Pseudomonas, Sphingomonas, Proteus,* and *Enterobacter* genera were cultured. *Myxococcus* was not found after laser printing, which could be caused by its higher sensitivity to NPs or to laser radiation. It should be noted, that *Proteus* and *Enterobacter* (representatives of *Proteobacteria* phylum) and *Rhodococcus* were not cultured by traditional plating from the studied soil sample. So, the results of the soil laser printing confirm our investigations with pure cultures as well as possibility of increasing in the cultured bacteria diversity using the LEMS method.

These results allow optimization of the LEMS protocols for bacteria transfer. Moreover, since LEMS and LIFT are based on similar physical principles (Delaporte, Allonce, 2016; Papazoglou, Zergioti, 2017; Yusupov et al., 2018, Gorlenko et al., 2018), it is possible to use our results for optimization of LIFT of microorganisms. It is known that Au currently is one of the most frequently used materials as an absorbing layer for LIFT (e.g. Gaebel et al., 2011;

Ali et al., 2014; Pagès et al., 2015). Based on the present research we recommend application of other metals for laser transfer of microorganisms.

Concluding, the study of the effectiveness of Au, Ti, and Cr metal absorbing layers application for bacteria cells and soil particles laser printing by LEMS and LIFT techniques has shown that Ti and Cr layers were significantly more effective compared to Au films. Bacteria growth activation after laser printing procedure with Ti and Cr absorbing layers was observed. Possibility of enrichment in biodiversity of cultured soil bacteria by using the LEMS method was verified. Results of the present study can be applied in LEMS and LIFT protocols for bacteria isolation and culturing of microbial systems.

Materials and Methods

LEMS technique

The LEMS method was described in detail previously (Yusupov et al., 2018; Gorlenko et al., 2018). The scheme of the printing process is presented on the Fig. 1. Briefly, laser pulses of nanosecond ytterbium fiber laser YLPM-1-4x200-20-20 (wavelength λ = 1.06 μm, pulse duration τ = 8 ns, pulse energy E = 24 μJ) with a close-to-Gaussian beam intensity profile (M^2 < 1.5) are focused onto absorbing layer at the donor glass plate. Delivering the laser radiation is implemented by means of an Lscan H-10-1064 two-mirror Galvano scanning head (Ateko, Russia) with an SL-1064-110-160 F-theta objective (Ronar-Smith, Singapore), with a focal length of 160 mm that allows focusing of laser radiation into a spot of 30 μm in diameter. Printing of microdroplets is carried out from the donor slide to the collector Petri dish with a nutrient medium located at a distance of 1 mm. The donor glass plate is coated with a 50 or 100 nm thick metal layer covered with a layer of substrate (gel with bacterial cells or soil) with a thickness of 200 ± 30 μm. Homogeneous coverage with the gel substrate

was produced using a blade coater. The gel based on hyaluronic acid (2% solution in phosphate buffer) was used (Yusupov et al., 2018). In the present work, absorbing layers of Au, Ti, and Cr with 50 nm or 100 nm thickness were used.

Samples description and preparation

The objects of this study were *Escherichia coli* mar14-3565, *Spirillum sp.* KBP-89, and *Curtobacterium michiganense* KBP V-29 bacterial strains, as well as urban soil samples. Bacterial strains have been obtained from bacteria collection of Soil Biology Department of Lomonosov MSU (https://depo.msu.ru). Soil was sampled in a park zone of Moscow city, Russia at 55°42’33.9’’N, 37°31’22.2’’E coordinates from 5-10 cm depth.

Bacterial biomass in stationary phase of growth was suspended in sterile distilled water in concentration of 5×10^8 cells ml^{-1}. After that water suspension was mixed with hyaluronic acid gel at 1:10 ratio. Further, the gel containing bacterial cells was spread onto a donor glass, and laser printing of microdroplets was carried out directly onto a surface of nutrient medium in Petri dishes. Applying the laser printing, 160 microdroplets were transferred into each Petri dish. Simultaneously the gel/cells mixture was plated using traditional plating technique (using glass spatula). Experiments were performed in 3 to 9 replicates.

For soil laser printing, 0.2 g of dry soil was mixed with 0.4 ml of water and 0.8 ml of the gel. The soil/gel mixture was processed similar to the gel/cells mixtures. Using the laser printing, 48 microdroplets were transferred into each Petri dish. Experiment was performed in 4 replicates.

Bacteria cultivation and identification

Bacterial cells and soil particles were printed or plated on a surface of solid glucose-peptone-yeast media (Cheptsov et al., 2017). The dishes with *Spirillum sp.* KBP-89, *Curtobacterium michiganense* KBP V-29, and soil were incubated at a temperature of 28 ºC for 7 days; the dishes with *Escherichia coli* mar14-3565 were incubated at 37 ºC for 3 days. The number of bacteria colonies grew up was counted visually. Identification of bacteria cultured from the soil was carried out on the basis of phenotypical cultural, micromorphological, and physiological-biochemical characteristics (Lysak et al., 2003).

The results are presented with calculations of mean values and standard error of the mean (M±m). The error is expressed as confidence intervals at $p<0.05$.

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Conflict of Interest

No conflict of interest declared.

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