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Laser interference patterning of diamond-like carbon layers for directed migration and growth of smooth muscle cell depositions

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Seeding of cells on functional, biocompatible scaffolds is a crucial step in achieving the desired engineered tissue. The authors show the constructional solutions of the Nd:YAG pulse laser system with the *Q*-switch modulation for direct and interferential shaping of the surface of biocompatible materials. The two-channel interference system is distinguished by high control, simplicity and repetitiveness regarding laser energy level and dimensions of the surface structures. The experiments were conducted on hard, biocompatible substrates of amorphous carbon (diamond-like carbon, DLC) and were preliminarily tested on smooth muscle cell depositions.

Keywords: laser interference lithography, Nd:YAG laser, diamond-like carbon (DLC) structuring, biocompatible scaffolds.

1. Introduction

New materials and modified surfaces work efficiently if it is possible to create them and put them in a suitable place with desired property and technical effectiveness. The surface changes, meaning the changes in topography and microstructure, are intended to fulfil the specified physical, chemical or mechanical requirements such as, for example, the increase in resistance to wear, the decrease in flow resistances, the change in tribological properties, the decrease of the light reflection coefficient, the increase of the adhesion coefficient or protection against corrosion.

The size of the structure ranging between 10 μm and approximately 100 μm is required for some surface properties. This is associated not only with the improvement in oiling of machine parts or filling the printing matrices, but also with the decrease in flow resistances, an example of which is the creation of "shark skin" type ridged surface [1]. In other applications, significantly smaller structures of tens or hundreds of nanometres are required. This is associated, for example, with protection against wear achieved by deposition of hard phases in a plastic wrapping [2], the "lotus leaf" type surface self-cleaning effect [3], local colour changes [4], change in adhesiveness, *e.g.* [5], and sealing effects [6]. As a consequence, multiple surface topography and microstructure modification techniques are required in order to create specified properties in various scales. Moreover, material properties are strongly associated not only with its microstructure, but also its spatial distribution. In many cases, it is actually the key to the surface functionality, often engineered by nature [7].

One of the technologies of creating simultaneous changes in topography and microstructure is the direct interference lithography method [8]. This method is based on irradiating the surface of the materials with a suitable interference pattern by means of a high-energy pulse laser. Such surface illumination by means of specified laser energy may be adjusted in order to execute various processes, from polishing and phase changes to the creation of surface microstructures [9].

In practice, all biomedical implants have a limited lifetime. Therefore, the technological surface processes leading to an increase in the lifetime of existing biocompatible materials and layers or to creation of new ones with enhanced properties tend to be used more often. During the last two decades the amorphous carbon known as diamond-like carbon (DLC) has been such an appealing material. It is characterised by high smoothness, it is chemically neutral, resistant to wear and has a low friction coefficient. Its optical, mechanical, thermal and electrical properties are however a function of the chemical composition, derived from the layer creation methods [10].

2. DLC type layers

The DLC layers are characterised by high tetrahedral (sp^3) carbon atom configuration content, recognised empirically long ago. Their optical properties, especially the refractive and extinction indexes, are particularly appealing due to the ability to be processed by means of laser radiation, especially in the ultraviolet range.

In structuring of DLC layers only the dry etching techniques can be applied. One such technique is etching induced by laser radiation, which is generally conducted in the atmosphere, in the temperature of the surroundings. Structuring of DLC layers in the temperature of the surroundings takes place either as a result of the ablation process at high fluences [11] or due to the reaction between carbon and oxygen below the ablation threshold [12]. In the case of particularly thick DLC layers, between hundreds of nanometres and several micrometres, the removal of material occurs by spallation of thin slices of the layer instead of vaporising occurring during normal ablation process [13]. The thickness of slices being removed is significantly lower than the thickness

of the entire layer, therefore the "spallation" effect differs significantly from the delamination caused by the loss of layer adhesion.

Two types of DLC layers were used in the experiments described in Section 3. Layers with thickness of approximately 500 nm and the $Si_{1-x}C_x$:H = 0.97 composition, deposited by vapour utilising the pulse laser deposition (PLD) method onto the substrate of crystalline silicon with an orientation of [100] [14], were subjected to direct laser processing. Their refractive and extinction indexes strongly depend on the laser radiation wavelength. As implied from the characteristics presented in [14], the absorption within the UV range increases approximately tenfold compared to the absorption in the near-infrared. The BD Falcon Tissue Culture Dish commercial substrates (ref. no. 353003) have been used during processing with the laser interference lithography method.

3. Structuring of DLC layers

Two laser technologies have been used primarily during the last two decades for the direct topographic changes and changes in the microstructure of solids, virtually any materials, meaning the modification of surface layers according to a specified pattern. Those technologies are the direct laser processing and the direct laser interference lithography.

3.1. The direct method

A focused beam of laser radiation pointed directly at the material surface is used in direct laser processing – also referred to as "direct writing". The concurrent movement of the material and the laser beam is solved by using *x*–*y* tables or an optical galvanometric scanner, controlled by means of a computer. This ensures the creation of two- and three-dimensional structures on any surface of the processed material. While using the pulse lasers, "overlapping" of laser spots on the focal surface of the focusing lens shown in Fig. 1 is an important parameter of the process.

Depending on the process (*e.g.*, cutting, welding, perforating, *etc*.) or the required structure, the overlapping of laser beams varies. For precise cutting, the extent of overlapping usually falls within the range between 75% and 90% of beam diameter (Fig. 1).

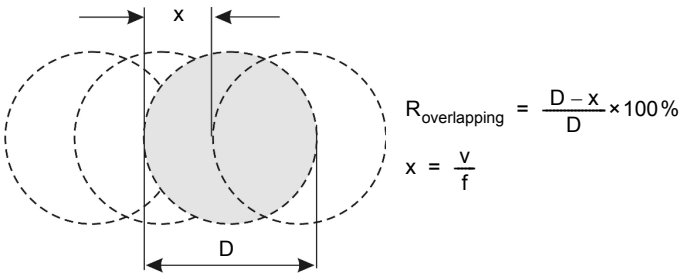

Fig. 1. Scheme of laser beam overlapping and the definition of beam overlapping during laser processing, where v – scanning speed, f – pulse repetition frequency.

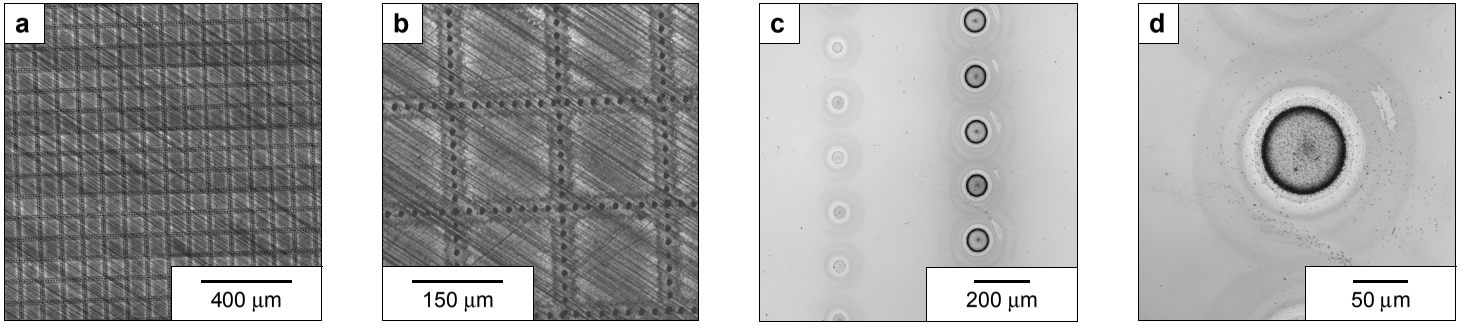

Fig. 2. The structure of lines and "dimples" (diameter of approximately 50 µm, depth of 100 nm) in a 500 nm thick DLC layer, applied by means of the PLD method on the surface of an Si substrate with the diameter of 65 mm: **a**, **b** – controlled by an *x*–*y* table; **c**, **d** – controlled by a galvanometric optical scanner. Digital 3D optical microscope KH 8700, Hirox, Japan.

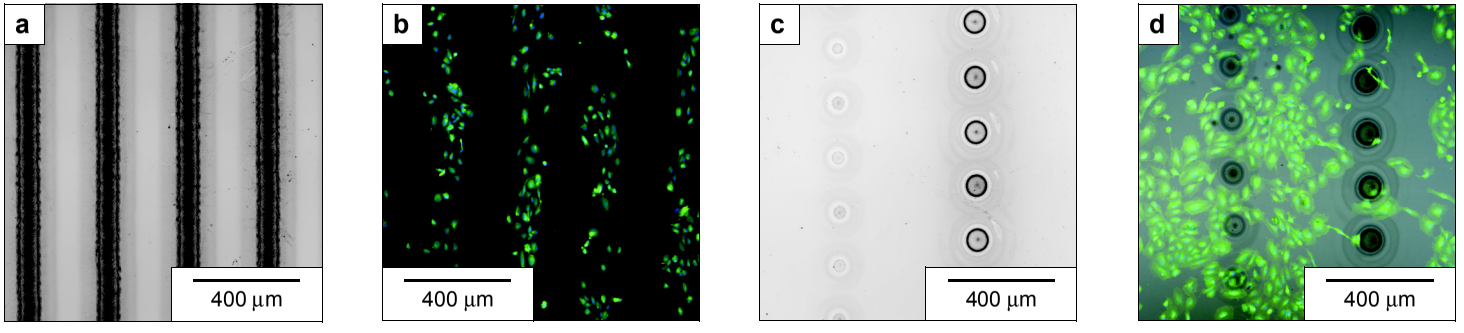

Fig. 3. Examples of two of the many templates applied on a 65 mm Si disc where a 500 nm thick DLC layer has been deposited: **a**, **c** – optical microscopic images of the structures; **b**, **d** – microscopic picture of the directional growth of endothelium cells of human umbilical vein endothelial cells (HUVEC) culture (avoiding or accepting the applied grooves or dimples in the DLC layer), using fluorescent markers. **a**, **c** – digital 3D optical microscope KH 8700, Hirox, Japan; **b**, **d** – confocal laser scanning microscope LSM EXCITER 5 with incubation chamber (Carl Zeiss AG, Germany).

Multiple variants are used for the preparation of the substrate for stem cell seeding, several of them shown in Figs. 2 and 3.

Modification of 500 nm thick DLC layers applied on an Si [100] substrate using the PLD method has been conducted by means of this method. A short pulse laser emitting the wavelength of 266 nm (fourth Nd:YAG laser harmonic) has been used for structuring, its energy being approximately 0.18 mJ in a single pulse and the pulse duration being 60 ps, operating with a pulse generation frequency of 1 kHz. The laser beam was focused by means of a planar-convex lens with a focal length $f = 45$ mm. The structures obtained while moving the material by means of a computer controlled x–y table are presented in Figs. 2**a** and 2**b**. The microscopic images presented in Figs. 2**c**, 2**d** and 3 were obtained using a galvanometric scanner in the laser system. This method has been described in more detail regarding the migration and behaviour of cells in the vicinity of the modified DLC structures in an earlier paper [15]. The main problem during earlier work was associated with the shape of the channel and its influence on the cell–material interaction. The problem which occurred at that time was associated with the deepness of the channel. The cell–material interaction exhibited the impeded cell confluence formation in the area of the channels. Lamellipodia were formed through the channels. The channels did not have a uniform shape following the whole length due to the single laser beam which was applied to create the channels. Cell–material interaction was observed for the channels and for the heat-affected zone, which appeared additionally while the channel was being formed.

3.2. Direct laser interference lithography

3.2.1. General notation of the *n* interference of laser beams

During the last decade, the direct interference lithography method using lasers of high power density between several MW/cm^2 and several GW/cm^2 has been used for periodic modification of the surface of virtually any solid materials [16]. This method enables in a direct manner to create surface structures in the micro- and sub-micron scale with a well-defined long range order.

The full **E** field image of the plurality of interfering beams is obtained as a result of superposition of each individual $\mathbf{E}_j$ field from each laser beam. This resultant field may be written down in the following form:

$$\mathbf{E} = \sum_{j=1}^{n} \mathbf{E}_j = \sum_{j=1}^{n} \mathbf{E}_{j_0} \exp\left[-i(\mathbf{k} \cdot \mathbf{r} - \omega t)\right] \tag{1}$$

where: $\mathbf{E}_{j_0}$ – initial value of component beam electric field vector, **k** – wave number vector, **r** – radial coordinate vector, ω – angular frequency of the electric field.

For two laser beams the intensity of the interferential field may be written down in the following form:

$$I = 4I_0 \cos^2(kx \sin\theta) \tag{2}$$

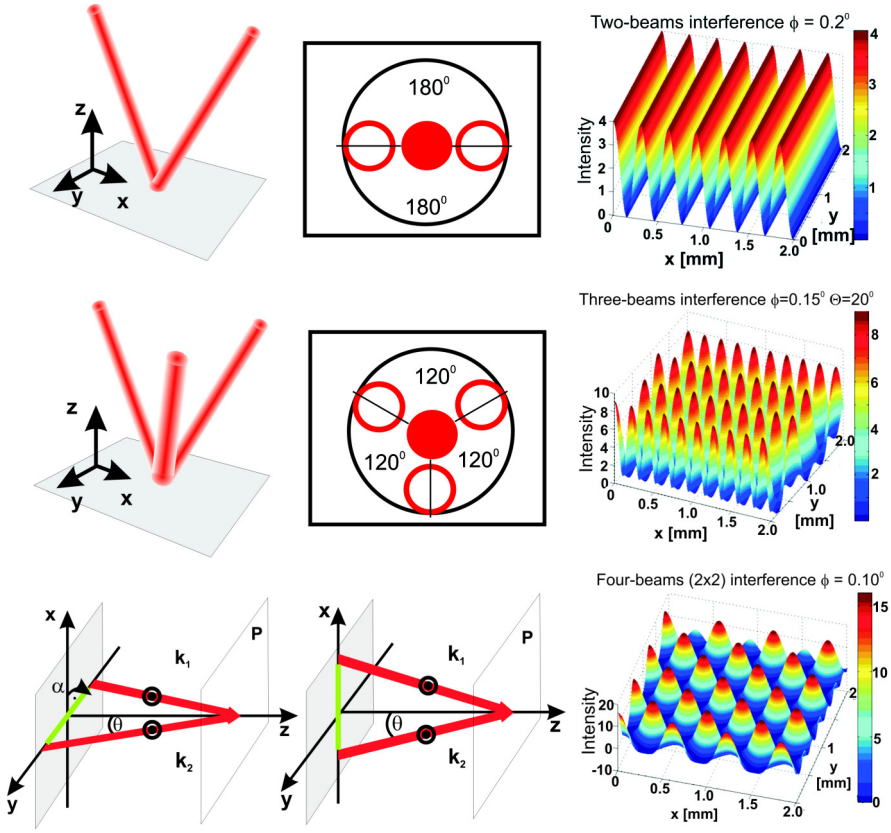

Fig. 4. Interference patterns created by multiple coherent laser beams.

for which the period of the pattern field equals:

$$d = \frac{\lambda}{2\sin\theta} \tag{3}$$

where: I – resultant intensity of the laser beam in Eq. (2), I_0 – initial intensity of the laser beam, θ – half-angle between interfering laser beams.

The geometry and interference images created using two and three laser beams have been shown in Fig. 4. The linear and punctual spatial structures are obtained as a result. The latter are represented in the material in the form of the characteristic dimples.

3.2.2. Laser system and two-beam interferometer

A two-channel Nd:YAG laser system has been built for direct interference lithography with the pulse energy from one channel of approximately 1.5 J and repetition up to 10 Hz. The Nd:YAG laser generator with Q-modulation operated in a "p-branch" type

unstable resonator. The output of the laser beam was realised through a quarter-wave plate and a dielectric polariser, and the diaphragm placed inside the resonator provided a fundamental transverse mode. The output energy equalled approximately 50–60 mJ, and the duration of the pulse depending on the amount of pumping energy ranged between 8 and 10 ns. In the practical laser systems using an yttrium-aluminium crystal doped with neodymium ions, the temporal coherence of the generated beams is sufficient even while generating several or about a dozen longitudinal modes [17]. Splitting into two energetically equal laser beams by means of a dielectric mirror occurred at the generator output. Both laser beams were directed into two channels with two amplifiers in each of them. The diameters of laser rods in the amplifiers equalled 8 and 12 mm, respectively. Two laser beams with a quasi-planar wave front emitted from a two-channel laser were configured in such a way that the whole system formed a "Mach–Zehnder interferometer". As a consequence, such system configuration allowed quick forming of the periodic structure, having properly combined the output beams at the proper angles on a surface area of several cm^2 [9].

The second and third harmonics of the fundamental wavelength of 1064 nm were obtained in the unit of frequency converters, presented in Fig. 5. On the other hand, the optical scheme of superposition of beam overlapping during interference is shown in Fig. 6. Due to the partial depolarisation of the laser beams as a result of the optical birefringence phenomenon in amplifying rods, caused by their thermal heating, two

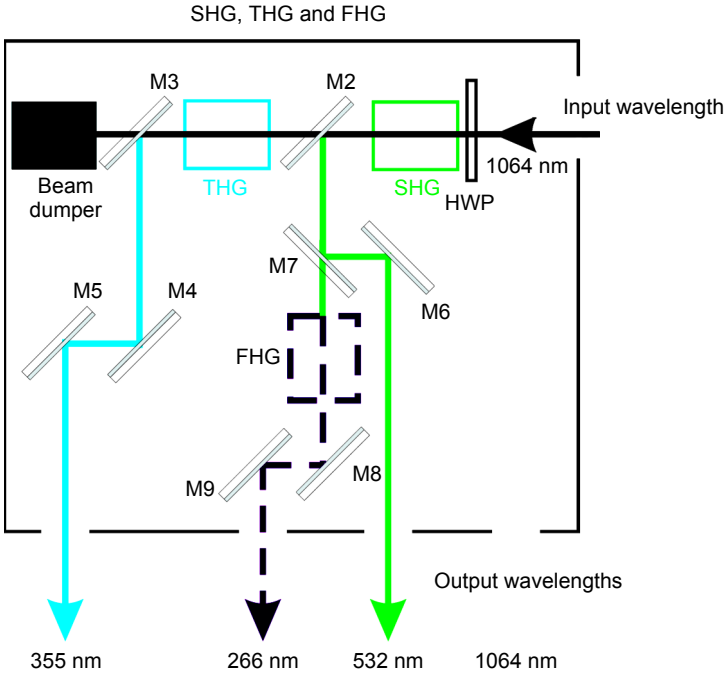

Fig. 5. An optical scheme of a laser frequency conversion system.

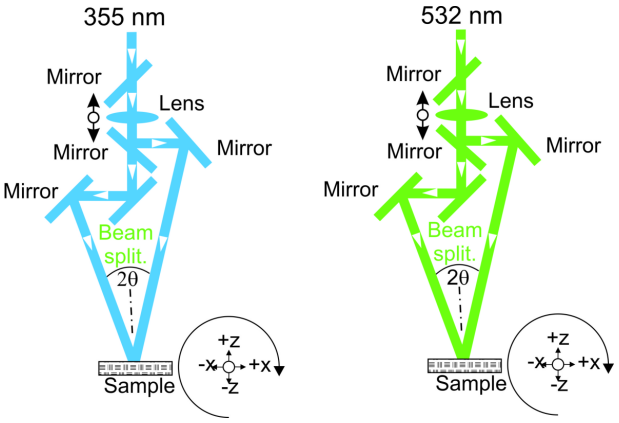

Fig. 6. An optical scheme of the interference of two laser beams (Nd:YAG laser second and third harmonics) after splitting and overlapping in the interference area of the sample.

polarisers were additionally used at the output. The use of frequency converters into the second, third and possibly fourth Nd:YAG laser harmonics, meaning the use of the wavelengths of: 532, 355 and 266 nm respectively, allows the decrease in the period of the structures on the material surfaces. Currently, a wavelength of 1064, 532 and 355 nm is used. The directing mirrors in the system of laser radiation frequency converters were selective mirrors, respectively for each radiation wavelength after processing.

Like in other interferometer systems, cross-like shapes and hierarchical structures may also be created by overlapping linear interferential patterns at various angles. It should be noted that the hierarchical creation of shaped surfaces constitutes a great challenge, especially due to the dependence of the final structure quality on the energy density used in each step (for each grid).

Quick and easy changes in the angle between the interfering laser beams are a primary advantage of the laser system configuration – the "active" Mach–Zehnder interferometer. In practice, this allows obtaining periodic structures on any surface, even of poorly meltable materials, by the proper change of the amplification coefficient in the amplifying channels, and the area of impact is approximate to the output diameter of the final amplifier [9].

3.3. Experimental results of structuring of DLC layers by means of the direct interference lithography method

Figure 7 shows the linear structures created by means of direct interference lithography on the surface of commercial BD Falcon substrate with DLC layers in various magnifications. Dark spots forming the so-called "hot areas" are visible in Fig. 7**a**. They are caused by local nonuniformities of the light intensity in the transverse cross-section of the laser beam or/and local nonuniformities of the material being shaped (surface contaminations, inclusions, *etc*.).

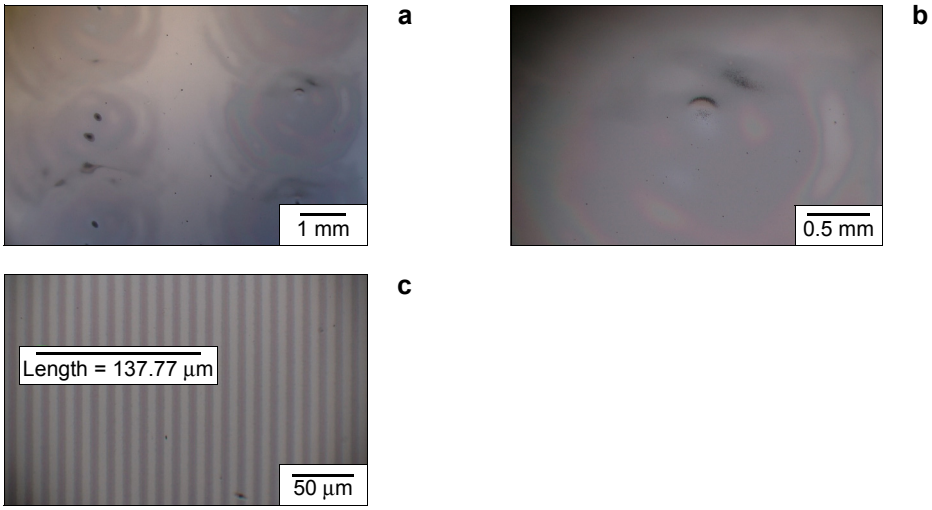

Fig. 7. Photomicrographs of DLC interference structuring: **a** – depiction of substrate scanning in the area of two interfering beams; **b** – an area of one interference pattern; **c** – the area from **b** in a 1000× magnification with the indicated length of ten interference periods. Digital 3D optical microscope KH8700, Hirox, Japan.

The first, preliminary results regarding the seeding of smooth muscle cells (SMCs) on a periodic linear structure are presented in Fig. 8. Figure 8**a** shows cell–material interaction on a non-treated substrate. Linear migration channels in a DLC layer are presented in Fig. 8**b**. Lamellipodia were elongated along the channels (Fig. 8**c**). Modifications of the cell reaction on the substrate are consistent with the theoretical explanation of cell migration. The cell will always migrate to the "better" areas to rise. The first reaction of the cell in response to the substrate changes is the actin polymerisation at the so-called plus end of the cell and the ejection of lamellipodia. In the following step the whole cell is shifted towards the plus end (Fig. 8**d**). The study proved the ejection of cellular lamellipodia along the migration channels. It evidences the possibility of a controlled escalation of the cells following the topography of the substrate.

Smooth muscle cells were purchased from Lonza. Each vial had a concentration of 5×10^5 cells/cm^3. The cells were stored in liquid nitrogen until use. From 1×10^5 to 1.25×10^5 cells were placed in a 25 cm^2 flask. It was possible to prepare 4 or 5 flasks from each vial. The cells were re-suspended in an endothelial cell culture basal medium mixed with cell growth and survival supplements (bullet kit growth mixture purchased from Lonza, including cell growth promoting serum, vitamins, and antibiotics). Before adding the cells, the medium was warmed in a 37°C water bath. The cells were taken from the liquid nitrogen container and placed for 2–3 min inside a 37°C water bath. Under the laminar air flow chamber, a maximum of 1 cm^3 of medium mixes with the bullet kit was added. Everything was then pipetted into a 15 cm^3 Falcon tube and diluted to 4 or 5 cm^3, to receive 1×10^5 or 1.25×10^5 cells/flask, respectively. The re-suspended cells were taken in the amount of 1 mm from the Falcon tube and introduced

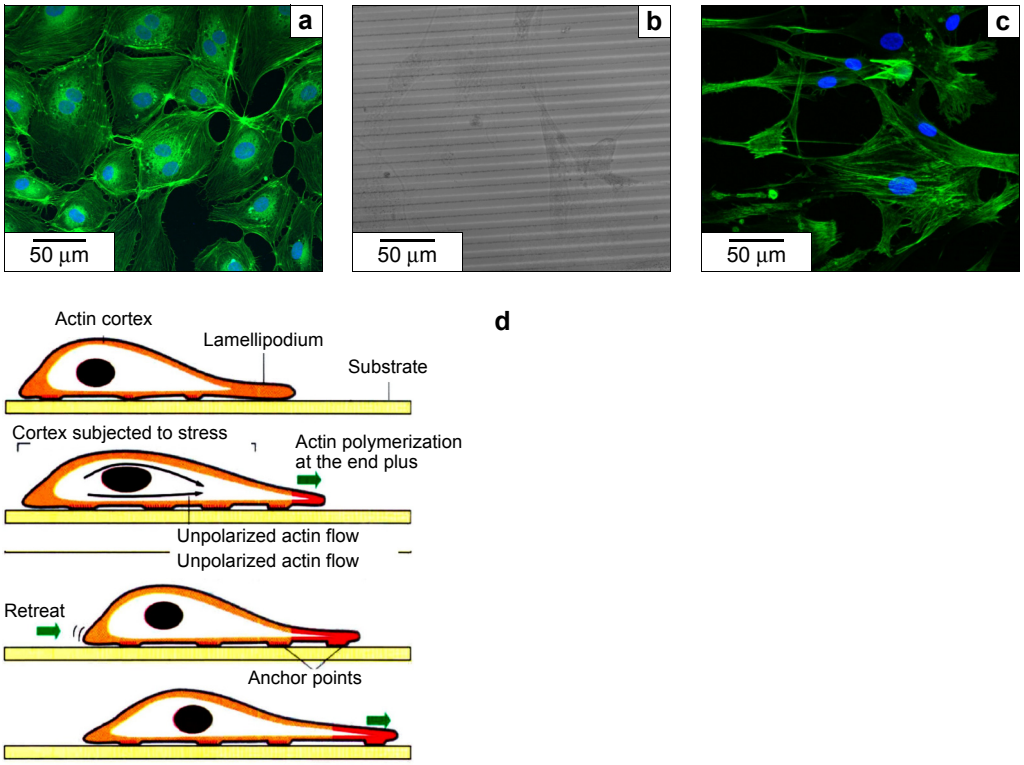

Fig. 8. Microphotographic images of the smooth muscle cells cultivated on DLC layers: **a** – non-treated substrate – image from a confocal microscope using fluorescent markers; **b** – image from a confocal microscope of linear migration channels in DLC obtained using the direct interference lithography method; **c** – confocal microscope image of lamellipodia reaction (elongation); **d** – the scheme of cell movement steps on a DLC substrate. Confocal laser scanning microscope LSM 5 EXCITER with incubation chamber (Carl Zeiss AG, Germany).

into a 25 cm^2 cultivation flask. Each flask was then filled with up to 6 cm^3 of the medium with supplements.

SMCs were deposited directly on the surfaces of the migration channels. The cells were incubated in a 37°C mixture comprising 5% of CO_2 and 95% of air with 100% humidity. After three days the cells were fixed in 4% paraformaldehyde. Then the cells were permabilised in Triton X-100 0.2% detergent for 4 min. The actin cytoskeleton was marked with AlexaFluor Phalloidin, the nucleus with DAPI (Fig. 8**b**). Fluorescent dyes were excited with appropriate laser wavelength, 488 nm (filter 530 nm) for actin cytoskeleton visualization, 405 nm (filter 505–530 nm) to visualize the nucleus of the cell. No cell movement was observed. The experiment was performed under static conditions and no *in situ* analysis was performed. This aspect is the key issue for the further research. Moreover, future work will be associated with the study of the impact of the structure parameters: the distance between the interference stripes and their depth on the behaviour of stem cells.

4. Conclusions

Preparation of the appropriate scaffolds for cell growth and controlled cell behaviour is a challenging task of modern biomaterial engineering. There exist a large number of new methods for direct scaffold fabrication [18], like gas foaming [19], fibre meshes and bounding [20], phase separation [21], and melt moulding [22]. However, they have limitations, particularly associated with the need to better control the precision and resolution for larger area modifications. The method of laser ablation of materials is advantageous in this context, both in the form of direct micropatterning as well as when utilising the interference between two or more laser beams.

The use of a single Nd:YAG laser generator with a *Q*-switch modulator, coupled with a two-channel Nd:YAG amplifier as well as a set of wavelength converters made it possible to construct a system for the interference lithography of the surface of any materials with exceptionally easy experimental scaling of the energy level in the pulse and the interference period (the dimensions of the periodic structures).

In the presented experiments, layers of amorphous carbon (DLC) applied onto silicone and polymers have been used as the substrate. The DLC layers are characterised by the suitable hardness, chemical stability and are neutral relative to the biological cells. As shown in several examples, the precise topography of the substratum surface created by means of laser methods may be a key condition for the control of the quantitative and directed cell growth.

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