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A new morphological cell parameter based on optical phase for the evaluation of cell populations.

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ABSTRACT

In this work, we introduce an opto-geometric parameter for the morphologic characterization of cell populations during their early adhesion process. Using measurements for a cell population of the maximal optical phase for each cell and its substrate-contact-surface, we show, experimentally, that a relationship exists between these variables. This connection is primarily associated with morphological cell characteristics. It is also shown that using the relationship obtained above, we can derive a morphological parameter, which, for the cell populations studied, results in a monodisperse Gaussian-size distribution, which would allow for the use of regular statistical variables. This result is in total contrast with the polydisperse distribution obtained if only the contact surface area between cell and substrate is used. In addition, optical phase measurements where accomplished by phase shifted interferometry using a Mirau-type interference microscope. The cellular system studied consisted of Osteoblast-like cells, plated on 316VM medical-grade stainless steel polished surfaces. These cell populations were studied within the same culture conditions of cell type, plating time and substrate roughness conditions. The existence of a relationship between maximal optical phase and substrate contact area agrees entirely with the accepted spreading model for cell adhesion; in particular, considering the close link between the optical phase time change and cell thickness reduction.

Keywords: Optical phase, Cell adhesion, Interference Microscopy, Cell morphology, Quantitative phase microscopy.

INTRODUCTION

The process of early adhesion of an osteoblast-like bone cell is characterized by a morphologic change in the cell, which transforms from a spheroidal form, when the cell is in suspension, to a flat straight-edged morphology when already adhered to the material's surface (spreading). Difficulties affecting the cells through this stage could condition their subsequent function in the remaining cell processes, both *in-vitro* and *in-vivo* (proliferation, differentiation or apoptosis, as appropriate). Since transformation into solid mechanical surface is essential to the effectiveness of any orthopedic or dental implant, and seeking as full merger as possible between the material surface and bone tissue is crucial, it is necessary that a good understanding of the cell adhesion process be obtained; specifically, osteoblast adhesion is very important in optimization of the bone-biomaterial interaction [1-5].

Optical microscopy is generally used in the study of *in-vitro* morphology. Even though the cells are basically transparent to visible light, the elements that constitute the cytosol, as well as the organelle contained inside the cell, gives the cells a different refractive index from their surrounding environment, which turns them into fundamentally phase objects [6-9]. Techniques such as Phase Contrast (CF) or differential interference contrast (DIC) are widely used in biomedical research laboratories because they allow for the optical phase information to be translated to light intensities. These techniques present one basic drawback: it is not possible to obtain quantitative data regarding the optical phase from the

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intensity values because the relationship between both variables is not simple [8, 10-12]. Over the last two decades, there have been great advances in the use of optical techniques for the quantitative study of the optical phase associated with cellular systems, as it provides information about their morphology and the refractive index of the cell throughout the optical axis. Today, there are many devices designed to obtain information about the optical phase of cellular systems, which can be interferometric or non-interferometric based and can be designed as an standalone devices (4D technology or TIE devices) or as an attachment for a routine lab microscope (such as the commercial SLIM module, from Phi optics) [6, 9, 13-16].

Quantitative studies on cell adhesion to their substrate are usually carried out using indirect methods, which extract the culture medium (protein sets, vitamins, salts and any other molecules in solution, necessary to keep cells alive and reproduce the physiological environment) and count the cells present in solution or quantify cellular products. Such procedures provide neither information about the adhesion process itself, aside from its consequences (which are proportional to accession), nor any morphological information about how the cell changes when adhering to the material [17-20]. From an optical standpoint, cell adhesion used to be restricted to a qualitative observation or, in some cases, to the measurement of the cell to substrate contact area or the maximal cross section of the cell, relating this measurement to the cell spreading process.

In this paper we show that traditional cell size measurements made by qualitative phase microscopes (such as CF or DIC), using their maximal cross section, provides information which is invalid for a cell population characterization. We instead propose a morphological parameter, based on quantitative optical phase measurements, as a better descriptor of the cell population.

MATERIALS AND METHODS

Mirau Interferometer

In a Mirau interferometer, the light originating from the source goes through a partially reflecting mirror which acts as the reference surface; the beam transmitted is reflected by the sample to then interfere with the reference beam. This simple design allows for the manufacturing of objectives with the interferometer in their interior (Fig. 1), which makes the system highly stable in the case of mechanical or thermal disturbances [21]. This compact interferometer is commonly used in the study of polished surfaces or those with small variations in their topography [22]. Cells such as osteoblasts, which are thin and have refractive indices similar to that of their surrounding environment, can thus be suitably studied with this device [23].

Optical Microscopy System

The interferential microscope consists of a conventional Nikon Optiphot incident light microscope, frequently used for metallurgical applications, which employs a 20X Mirau-type interferential objective. The illumination comes from a tungsten lamp with an interferential filter centered at $\lambda = 546.1$ nm of wavelength, and a spectral bandwidth of $\Delta\lambda = 10$ nm. The trinocular microscope head allows for an image to simultaneously be obtained for the observer, and another for a digital camera, which allows for control of the system and the acquisition of digital interferograms (Fig. 1). The phase shifting technique is used to obtain the optical phase [21, 24]. This shifting is performed by means of a piezoelectric transducer, which replaces the traditional support of objectives in the microscope. The control of the piezoelectric system is carried out by means of a frequency-to-voltage circuit converter, connected to a conventional PC sound card. The performance of this homemade controller to drive phase shifting is equal or superior to that obtained with a 16 bit analogic-digital converter [25].

The phase reconstructions are performed introducing an arbitrary optical phase in five steps of $\alpha_i = \pi/2$ rad. The optical phase is experimentally determined from the five acquisitions using the relation (1):

$$\Phi(x,y) = \left(\frac{2(I_2 - I_4)}{I_1 - 2I_3 + I_5} \right) \tag{1}$$

where I_n are the intensity values at each point on the interferogram for each increase of α_i. Although the phase shifting technique requires acquisition times greater than those used for other techniques, these times remain very small in

comparison to the evolution times for the biological systems under study. Additionally, it has been demonstrated that redundant information in the methodology of 5 buckets reduces the effect of errors committed in the introduction of the optical phase [24, 26, 27]. Phase unwrapping is carried out following an iterative technique, following the branchcut algorithm [28, 29].

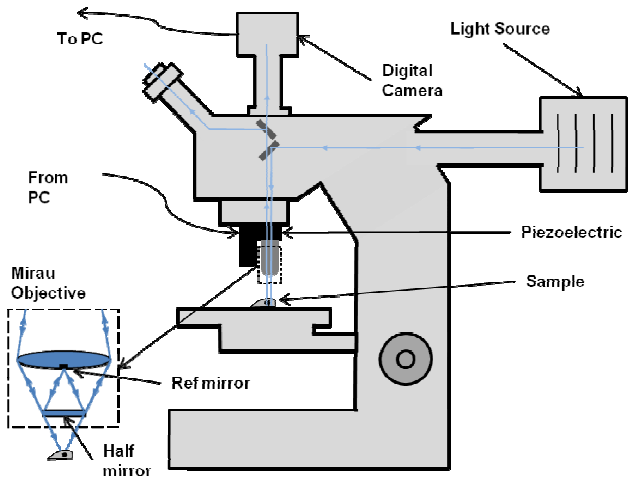

Figure 1: Schematic of the incident light microscope. In detail, the interferential objective.

Cellular System

Osteoblasts are bone cells responsible for the regenerative process of said tissue, where they take part by secreting the organic component of the extracellular matrix after adhesion to its substrate. *In-vitro*, these cells are characterized by the formation of interconnected monolayers of cells, resulting in cells of cuboid morphologies with thicknesses with less than few micrometers. For this study, bone cells were obtained from calvaria (parietal and frontal bones from the skulls of 2-3 day-old Sprague Dowley rats), following the methodology described by Noris-Suárez, et al. Once the soft tissue is eliminated (by scrapping), the calvaria were cut in pieces of around 1mm and submitted to a digestion process using 0.1mg collagenase, 0.25mg trypsin, and 0.5mM EDTA in a D-MEM culture medium. Incubations were performed by means of three incubation series at 37°C for 20 minutes in smooth agitation, and cells collected from the third extraction process were plated in 100 mm culture plates in the D-MEM medium complemented with 10% fetal bovine serum, 100 µg/mL of penicillin (200,000 U/mL), streptomycin (200.000 U/mL), nonessential amino acids and L-glutamine. The culture plates were cultivated in an atmosphere with appropriate CO_2 and humidity levels, and the media were changed every 3 to 4 days [30].

In order to study individual cell sets, approximately $3x10^3$ cells/cm^2 were plated on the substrate discs, in order to have a relatively low cell proportion, compared with $20x10^3$ cells/cm^2 as generally used when cellular confluence is desired[30]. Culture times where measured considering the plating as the origin.

Cell Substrate

The substrate material used to evaluate cell attachment was a surgical stainless steel 316 LVM disk. This kind of material is widely used as an internal fixations metal in the area of orthopedic surgery due to its mechanical properties and acceptable biocompatibility in relation to its cost when compared with other commonly used implantation materials, such as pure titanium or it alloys such as Ti-6Al-7Nb [18, 31-34].

RESULTS

Cell Phase Maps and Morphological Evaluation.

Characteristic phase maps corresponding to different cell culture times can be seen in 3D false color maps in Figure 2.

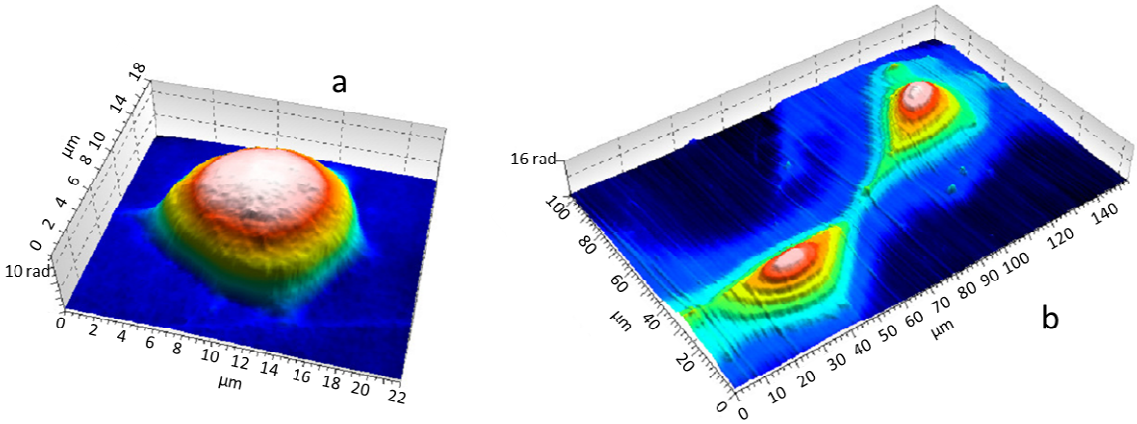

Figure 2: Three-dimensional representation of the optical phase maps (false colors) for osteoblast-like cells. a) (2.5 ± 0.3) hours, b) (23.3 ± 0.3) hours. Morphological change undergone during the first 23 hours of adhesion time can be qualitatively observed.

To measure the maximum cross section (*S*) of any cell on the basis of the phase map obtained, the phase map was converted into a binary system, as shown in Figure 3, establishing as threshold value the point where the phase map is greater than the average value height of the substrate. We then proceeded to count the number of pixels that form the image in the case of Figure 3; all white pixels. With the pixel – micrometer relationship of the image known (made through a simple calibration), the maximum cross section of a cell can be obtained in units of square length.

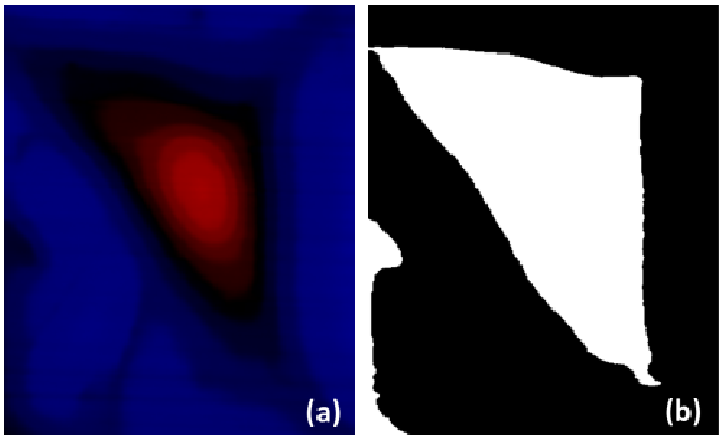

Figure 3: Osteoblast-like cell phase map after (16.6 ± 0.3) hours of culture time. a) False colors phase map, b) binarized image. White image of larger size (cell) has 63,965 pixels corresponding to a surface S = (1317 ± 2) μm^2

Optical phase maps were determined for each individual cell, for culture times between 2 and 24 hours. From the optical phase maps, we can calculate the maximal cross section. Figure 4 shows a couple of examples about the maximal cross section distribution for the cell populations. Even though cell where plated on the same kind of substrate and using the same culture time, it can be seen a non-Gaussian size distribution for the cell population, it can be even be seen multiple preferred sizes, suggesting a polydisperse distribution.

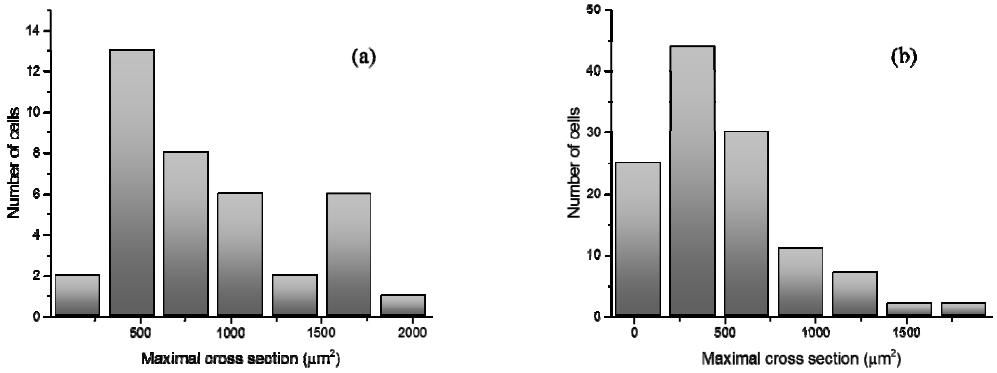

Figure 4: Cross section size histograms for two selected culture times: a) t = (16.5 ± 0.3) h, b) t = (8.1 ± 0.3) h. A not normal distribution can be observed for each one.

In the search for a Gaussian distribution, which would be adequate for a correct statistical analysis, we tried adding a morphological parameter related to cell thickness, choosing the optical phase. We measured the optical phase difference between the average value height of the substrate and the maximal optical phase for each cell; we will define this measure simply as the cell's maximal optical phase.

We attempted a variety of combinations between the maximal cross section and the maximal optical phase in the search for a good morphological parameter for the spreading process characterization for cell distribution, under identical culture conditions. Figure 5 shows the linear relationship between the root of the maximal cross section (S) and the maximal optical phase (Φ) for the same cell populations used in Figure 4.

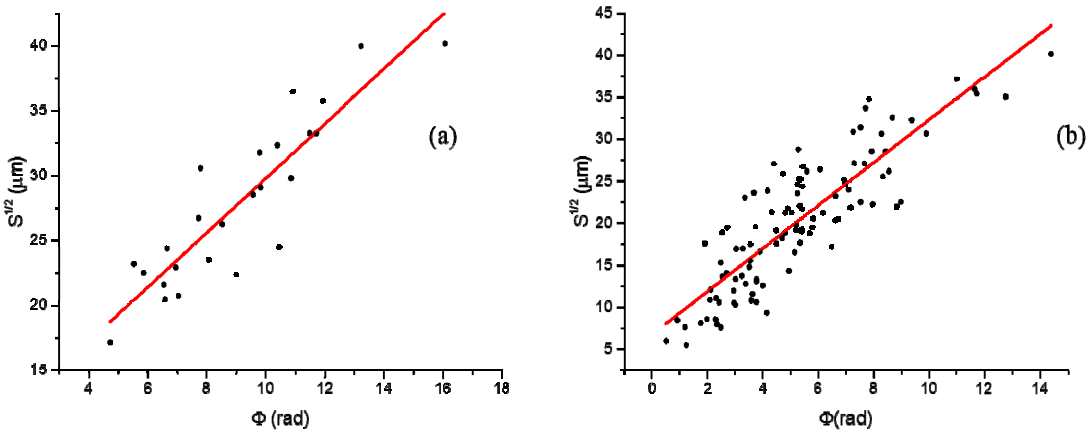

Figure 5: Plot of the root of the maximal cross section (S) vs the maximal optical phase (Φ) for each cell under identical culture conditions for two culture times: a) t = (16.5 ± 0.3) h, b) t = (8.1 ± 0.3) h. The linear relationship between variables suggest a common morphological parameter for the entire population.

The linear relationship observed in Figure 5 showed us that the quotient between these variables is a constant for a representative portion of a cell population plated under the same culture conditions. This proportionality is fulfilled by any population under a specific culture condition, as can be seen in a comparison between plots in figures 5-a and 5-b. This proportionality between cross section and optical face allow us to define the "aspect factor, β" which is given by the relationship (2):

$$\beta = \frac{\Phi}{\sqrt{s}} \tag{2}$$

To verify the utility of the β factor as a parameter for the description of cell populations, we reproduce the plots from Figure 4 but using factor β instead of maximal cross section. Figure 6 shows size distributions for the factor β and the fitted Gaussian curve. It can now be seen how the distribution follows a monodispersed, symmetric and narrowed form, which more suitable for statistical analysis.

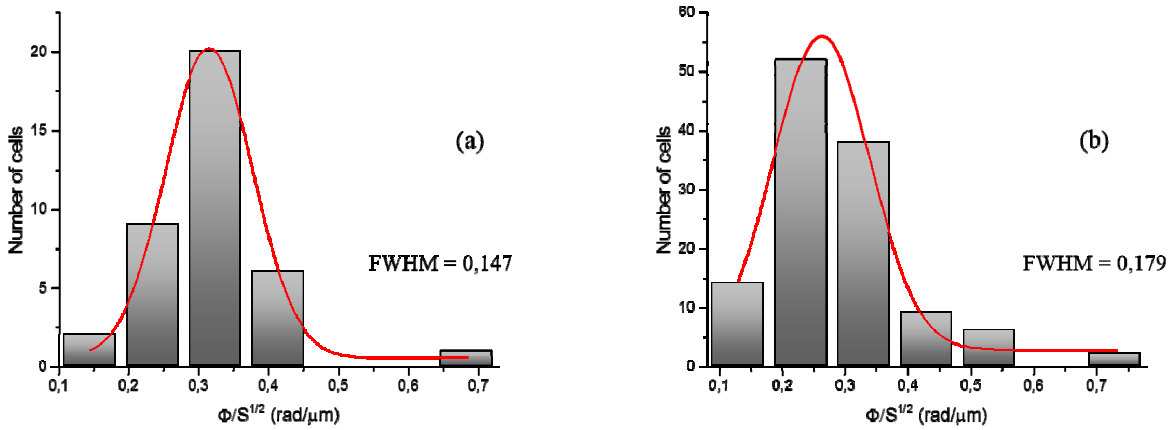

Figure 6: Figure 4: β size histograms for a two culture times (same conditions as Figures 4 and 5): a) t = (16.5 ± 0.3) h, b) t = (8.1 ± 0.3) h. It can be observed that distributions are more symmetrical as compared with the plots in Figure 4. A reduction of the FWHM is observed as the culture time increments.

DISCUSSION

The high variability measured for the maximal cross section of the cell should not come as a surprise. Firstly, we are working with biological systems, and therefore cell size is not necessarily constant. Secondly, cell deposition time over the surface will not be identical for each one: cell plating on the stainless steel disks implies that cells shall be removed from its original substrate (a dish plate, for example), they are then resuspended in culture media, while the cells are in globular form [30]. Cell plating consists of placing one drop of the "cell solution" over the disk, and waiting 30 minutes so as to allow the cells adhere to the surface [35]. During these 30 minutes, cells located at the lower section of the drop will arrive to the disk before those at the upper section, having had more time to develop their spreading process. At the time of measurement, some cells will thus be spread and others will have a form reminiscent of the globular form in suspension. In Figures 6a and 6b, an appreciable increment in the width at half-height from 6a (t = 16.5 h) to 6b (t = 8.1 h) can be observed. A higher width distribution implies a more disperse population of cells. More culture time implies more time for the cells to spread and there will be less cells whose morphology remains globular. This relation between dispersion of the β factor and cell evolution demonstrates the utility of the factor as a cell morphology parameter.

Thickness and optical phase are closely related: even so, the maximal optical phase point for the cell is not necessarily the thickest point of the cell. Optical phase is related not only to thickness, but also to the internal media refraction index. Refraction media is different for each cell organelle, among them the nucleus will barely affect the optical phase due to

its significant size [7, 36, 37]. Structural changes in the cell will affect its thickness, but not its internal media conformation, and therefore, while the β factor does necessarily provide direct information about cell volume or size, it is still a good parameter to detect the occurrence of morphological changes in the cell.

The cell adhesion process on a substrate is a protein-dependent process; actin and myosin are proteins related to the cell's structure. While the spreading takes effect, filament-like structures comprised of actin and located at the cell's border, protrude and "push from inside" the cellular membrane. This action causes the cell to change its form, from globular to its final, spread form on the surface (this is the same model accepted for cell motility). This model thus accepts the fact of a structured cell form; each increment in the cell's cross section will be related to its thickness; this is the result suggested by the existence of the β factor.

CONCLUSIONS

We introduce a morphological parameter, $\beta = \Phi/\sqrt{s}$, where Φ is the maximal optical phase and S is the maximal cross section for a cell. We demonstrate its utility in the description of an osteoblast-like population. The β factor allowed us to obtain a symmetrical and Gaussian-like distribution, an advantage for the application of any statistical analysis. Any cell (any osteoblast-like cell) in a population under same culture conditions complies with the β factor no matter its size.

Calculation of the β factor implies measurement of the optical phase. While such measurements are currently unavailable in any cellular laboratory, there are nonetheless multiple commercial solutions for making this measurement. The results presented in this work thus have the potential to stimulate the use of quantitative phase microscopy as a routine cellular biology laboratory technique.

REFERENCES

[1] Chapekar, M. S., "Regulatory concerns in the development of biologic–biomaterial combinations.," Journal of Biomedical Materials Research., 33, 199-203 (1996).

[2] Anselme, K., and Bigerelle, M., "Statistical demonstration of the relative effect of surface chemistry and roughness on human osteoblast short-term adhesion," J Mater Sci: Mater Med, 17, 471-479 (2006).

[3] Puleo, D. A., and Nanci, A., "Understanding and controlling the bone-implant interface," Biomaterials, 20(23-24), 2311-21 (1999).

[4] Ratner, B. D., Hoffman, A. S., Schoen, F. J. *et al.*, [Biomaterials science: an introduction to materials in medicine] Elsevier Academic Press, San Diego, California(2004).

[5] Medalia, O., and Geiger, B., "Frontiers of microscopy-based research into cell-matrix adhesions," Current Opinion in Cell Biology, 22(5), 659-668 (2010).

[6] Barone-Nugent, E. D., Barty, A., and Nugent, K. A., "Quantitative phase-amplitude microscopy I: optical microscopy," Journal of Microscopy, 206(3), 194-203 (2002).

[7] Curl, C. L., Bellair, C. J., Harris, P. J. *et al.*, "Single Cell Volume Measurement by Quantitative Phase Microscopy (QPM): A Case Study of Erythrocyte Morphology," Cellular Physiology and Biochemistry, 17, 193-200 (2006).

[8] Murphy, D. B., [Fundamentals of Light Microscopy and Electronic Imaging] Wiley-Liss, Inc., EE. UU.(2001).

[9] Popescu, G., Park, Y. K., Choi, W. *et al.*, "Imaging red blood cell dynamics by quantitative phase microscopy," Blood Cells, Molecules, and Diseases 41, 10-16 (2008).

[10] Bennett, A. H., Jupnik, H., Osterberg, H. *et al.*, [Phase Microscopy: Principles and Applications] John Wiley & Sons, Inc., New York(1951).

[11] Danz, R., and Gretscher, P., "C-DIC: a new microscopy method for rational study of phase structures in incident light arrangement," Thin Solid Films, 462-463, 257-262 (2004).

[12] Inukai, N., Tanaka, K., and Takizawa, T., "A convenient technique for live-cell observation on the surface of polytetrafluoroethylene with a phase-contrast microscope," Microscopy, 66(2), 136-142 (2017).

[13] Curl, C. L., Harris, T., Harris, P. J. *et al.*, "Quantitative phase microscopy: a new tool for measurement of cell culture growth and confluency in situ," European Journal of Physiology, 448, 462-468 (2004).

[14] Popescu, G., Park, Y., Lue, N. *et al.*, "Optical imaging of cell mass and growth dynamics," Am J Physiol Cell Physiol, 295, 538-544 (2008).

[15] Zuo, C., Sun, J., Li, J. *et al.*, "High-resolution transport-of-intensity quantitative phase microscopy with annular illumination," Scientific Reports, 7(1), 7654 (2017).
[16] Nguyen, T. H., Kandel, M. E., Rubessa, M. *et al.*, "Gradient light interference microscopy for 3D imaging of unlabeled specimens," Nature Communications, 8(1), 210 (2017).
[17] Keller, J. C., Schneider, G. B., Stanford, C. M. *et al.*, "Effects of implant microtopography on osteoblast cell attachment," Implant Dent, 12(2), 175-81 (2003).
[18] Gupta, A., Majumdar, P., Amit, J. *et al.*, "Cell Viability and Growth on Metallic Surfaces: *in vitro* Studies," Trends Biomater. Artif. Organ, 20(1), 84-89 (2006).
[19] Rosa, A. L., and Beloti, M. M., "TAK-778 enhances osteoblast differentiation of human bone marrow cells cultured on titanium," Biomaterials, 24(17), 2927-32 (2003).
[20] Harrison, M., and Rae, I., [General techniques of cell culture] Cambridge University Press, Cambridge U.K.(2001).
[21] Hariharan, P., [Optical Interferometry] Academic Press, Amsterdam(2003).
[22] Ge, Z., and Kobayashi, F., "High-precision measurement of a fiber connector end face by use of a Mirau interferometer," Applied Optics, 45(23), 5832 - 5839 (2006).
[23] González-Laprea, J., Márquez, A., Noris-Suárez, K. *et al.*, "Study of bone cells by quantitative phase microscopy using a Mirau interferometer," Revista Mexicana de Física 57, 435-440 (2011).
[24] Hariharan, P., Oreb, B. F., and Eiju, T., "Digital phase-shifting interferometry: a simple error-compensating phase calculation algorithm " Applied Optics, 26(13), 2504-2506 (1987).
[25] González-Laprea, J., Cappelletto, J., and Escalona, R., "Frequency to Voltage Converter as a Phase Controller in Phase Shifting Interference Microscopy," International Journal of Optomechatronics, 5(1), 68 - 79 (2011).
[26] Kinnstaetter, K., Lohmann, A. W., Schwider, J. *et al.*, "Accuracy of phase shifting interferometry," Applied Optics, 27(24), 5082-5089 (1988).
[27] Van Wingerden, J., Frankena, H. J., and Smorenburg, C., "Linear approximation for measurement erros in phase shifting interferometry," Applied Optics, 30(19), 2718-2729 (1991).
[28] Venema, T. M., and Schmidt, J. D., "Optical phase unwrapping in the presence of branch points," Optics Express, 16(10), 6985 - 6998 (2008).
[29] Wang, Z., and Li, S., "Phase unwrapping through a branch-cut-based cut-bridging and window-patching method," Applied Optics, 38(5), 805 - 814 (1999).
[30] Noris-Suarez, K., Barrios de Arenas, I., Vasquez, M. *et al.*, "Caracterización biológica empleando células osteoblásticas de vidrios del sistema SiO_2 . $Na_2O.CaO.K_2O.MgO.P_2O_5$, Modificados con Al_2O_3 B_2O_3.," Revista latinoamericana de metalurgia y materiales, 23(1), 82-88 (2003).
[31] Hao, L., Lawrence, J., Phua, Y. F. *et al.*, "Enhanced human osteoblast cell adhesion and proliferation on 316 LS stainless steel by means of CO2 laser surface treatment.," J Biomed Mater Res B Appl Biomater, 73(1), 148-156 (2005).
[32] Hiromoto, S., and Hanawa, T., "Electrochemical properties of 316L stainless steel with culturing L929 fibroblasts," J R Soc Interface, 3(9), 495-505 (2006).
[33] Schmidt, C., Ignatius, A. A., and Claes, L. E., "Proliferation and differentiation parameters of human osteoblasts on titanium and steel surfaces," Biomedical Material Research, 54, 209-215 (2001).
[34] Bordji, K., Jouzeau, J. Y., Mainard, D. *et al.*, "Evaluation of the effect of three surface treatments on the biocompatibility of 316L stainless steel using human differentiated cells," Biomaterials, 17(5), 491-500 (1996).
[35] Dubin-Thaler, B. J., Giannone, G., Dobereiner, H. G. *et al.*, "Nanometer analysis of cell spreading on matrix-coated surfaces reveals two distinct cell states and STEPs," Biophys J, 86(3), 1794-806 (2004).
[36] Rappaz, B., Marquet, P., Cuche, E. *et al.*, "Measurement of the integral refractive index and dynamic cell morphometry of living cells with digital holographic microscopy," OPTICS EXPRESS, 13(23), 9361-9373 (2005).
[37] Bohannon, K. P., Holz, R. W., and Axelrod, D., "Refractive Index Imaging of Cells with Variable-Angle Near-Total Internal Reflection (TIR) Microscopy," Microscopy and Microanalysis, 23(5), 978-988 (2017).