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# In-situ and label-free optical monitoring of the adhesion and spreading of primary monocytes isolated from human blood: Dependence on serum concentration levels

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ABSTRACT

Adhesion and spreading of primary monocytes isolated from human blood were monitored utilizing optical waveguide lightmode spectroscopy (OWLS); a highly sensitive label-free biosensor technique using evanescent optical waves generated at a biocompatible surface. Appropriate development on a custom built setup enabled the OWLS cuvette to be operated as a 1.5 ml mini-incubator, controlling both temperature and $CO_2$ levels. The incubator-equipped OWLS is readily applicable for delicate and long-term studies on sensitive primary cells, demonstrated here through monitoring the serum dependence of the adhesion and spreading of human monocytes. Moreover, the custom-built setup enables the simultaneous monitoring of the position and overall width of the OWLS resonant peaks. This unique feature makes it possible to distinguish the refractive index variations induced by the adsorption of secreted material from refractive index changes provoked by cellular spreading. A definite attachment and spreading activity was observed on the substratum (glassy silica–titania), when the serum level of the culturing medium was 0.0–0.01%. Increasing serum concentration resulted in a steep fall in monocyte surface adhesion and spreading. 1.0% serum level practically abolished all spreading activity measured by OWLS, and the number of attached cells was significantly decreased, too. Serum addition to fully spread cells provoked a reduction in the cell–substratum contact area, clearly detectable by the biosensor. Cell spreading was inhibited by pre-coating the sensor surface with considerable amounts of serum proteins. These findings suggest that monocyte spreading is inhibited by the adsorption of serum biomolecules to the substratum, rather than by soluble factors present in the serum. All of these results were obtained completely noninvasively with real time monitoring; demonstrating the capabilities of OWLS to sensitively monitor the adhesion properties of immune cells isolated from human blood. The current study is, therefore, a significant step towards the application of label-free optical biosensors in medical diagnostics.



## 1. Introduction

Monocytes are a type of white blood cells (leukocytes) playing regulatory and effector roles, both of pivotal importance in innate immune function (Dale et al., 2008). Initially they reside in the blood and monitor foreign substances, but further roles require that monocytes leave the circulatory system by penetrating through the epithelium of the blood vessels and migrate to the neighboring tissues. These monocytes differentiate to dendritic cells (DCs) and macrophages (MFs) to either replenish the declining resident population of those cells under normal conditions or to give rise to a locally increased population of DCs and MFs, which determine the immune response at inflammation sites. Adhesion of monocytes to the endothelial cells (Beekhuzien and van Furth, 1993) and to components of the extracellular matrix (ECM) is essential for the complex, multi-step process of transmigration (Ley et al., 2007). Compared to non-leukocytes, monocytes exhibit some unique features in their adhesion, which is associated with their function (Gahmberg, 1997; Harris et al., 2000; Ley et al., 2007; van Kooyk and Fidgor, 2000); they express leukocyte-specific $\beta_2$ integrins, which can be rapidly activated upon stimulation

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(*e.g.* by inflammatory cytokines) (Gahmberg, 1997; Harris et al., 2000; Ley et al., 2007), and provoke the cell to undergo from weakly to highly adherent state.

Several diseases are associated with malfunctions in the tightly regulated adhesion of monocytes to the endothelium or to the ECM. Leukocyte adhesion deficiency (LAD) syndrome is characterized by insufficient leukocyte expression of $\beta_2$-integrins leading to life-threatening infections in patients (Anderson and Springer, 1987; Beekhuzien and van Furth, 1993). Monocyte adherence has been reported to be also impaired in patients with diabetes mellitus or alcohol-induced cirrhosis (Kelly et al., 1985), which is the reason why these patients are more prone to infections. Monocytes specifically adhering to damaged arterial endothelial cells and forming foam cells have major importance in the pathogenesis of atherosclerosis (Beekhuzien and van Furth, 1993; Huo and Ley, 2001).

Due to the extensive research of the last decades on leukocyte extravasation, much is known about the activators, mediators and regulators of the distinct steps of the leukocyte adhesion cascade (Ley et al., 2007). However, *e.g.*, the complex regulatory function of the ECM on lymphocyte migration and its modulating role during the inflammatory immune response have gained attention only very recently (Korpos et al., 2010; Schor et al., 2000; Sorokin, 2010; Vaday and Lider, 2000).

In order to achieve a more detailed understanding of the mechanism of monocyte adhesion, we wish to move in the direction of increased precision in the quantification of the cells' behavior when they encounter the ECM. This precision should, ideally, be obtained in real time, and with negligible perturbation of the system. These criteria can be met by using a high-resolution label-free biosensing technique, optical waveguide lightmode spectroscopy (OWLS) (Ramsden, 1998).

OWLS is a high performance surface-sensitive technique allowing label-free real-time monitoring of processes accompanied by refractive index changes in the close vicinity of a waveguiding sensor chip (a thin waveguiding layer supported on a thicker optical glass slide and having a shallow diffraction grating embedded into the structure). The successful incoupling of a linearly polarized monochromatic laser beam via the grating is characterized by a sharp resonance peak when plotting the intensity of the incoupled light against the incident angle of the illuminating beam. The sensing principle is based on the perturbation of the evanescent waves of the guided lightmodes; refractive index variations alter the discrete incoupling angles so the position of the resonance peak will be shifted (Tiefenthaler and Lukosz, 1989; Vörös et al., 2002).

In the present OWLS configuration (the so-called conventional configuration in which the refractive index of the substratum is greater than that of the covering medium) the evanescent field penetrates to a 100–200 nm thick layer above the planar waveguide, making OWLS suitable for monitoring the contact area of living cells (Aref et al., 2010a, 2010b; Hug et al., 2002; Ramsden et al., 1994). Using reverse symmetry waveguides (Horvath et al., 2002), supported on substrata with *lower* refractive index than that of the covering medium, the penetration depth of the evanescent field can be increased and fine-tuned (Horvath et al., 2008, 2005b, 2003b), making it possible to monitor changes deeper inside cells (Horvath et al., 2008). Another advantage of OWLS over other sensing platforms (Ramsden, 1997) is that it enables kinetic readout of multiple parameters characterizing the OWLS's resonance peaks (Cottier and Horvath, 2008). Comparative analysis of the temporal evolution of these parameters enables to distinguish the refractive index variations caused by secreted molecules from refractive index changes provoked by cellular spreading (Ramsden and Horvath, 2009). Noted that the substratum is the high refractive index waveguiding film, typically about 200 nm thick, for which a wide range of biocompatible materials (e.g., titania, niobia (Starikov et al., 2007)) are available. Moreover, these substrata can be easily surface-modified or coated with a thin layer of virtually any material, provided it is transparent at the wavelength of the guided light. This gives a tremendous flexibility to the technique.

Given that high sensitivity, excellent temporal resolution, and label-free detection principle are further characteristics of the technique, we believe that OWLS has an outstanding potential to contribute to a deeper understanding of monocyte adhesion. Although OWLS has previously been used to investigate the adhesion and spreading of various cell lines (Aref et al., 2010a, 2010b; Horvath et al., 2008, 2005a; Hug et al., 2002, 2001; Li et al., 1994; Ramsden and Horvath, 2009; Ramsden et al., 1994), its potentials have never hitherto been exploited in primary cell studies.

The purpose of this paper is twofold. Firstly, we describe a significant instrumental development of a previously constructed (Horvath et al., 2008, 2003a, 2002) OWLS setup. We developed the OWLS sample cuvette into a mini-incubator that enables control over the temperature and atmosphere of the living cell environment while retaining all the other convenient functions. This upgraded OWLS can be readily used for the long term monitoring of sensitive cells. Secondly, we utilized this incubator-equipped setup to characterize monocyte adhesion and spreading as a function of the serum content of the culture medium. Analysis of the OWLS data enables the underlying cause of the observed dependence now be understood in its details.

Beyond these advances, we consider these experiments as the next step towards medical diagnostic applications, especially those involving the monitoring of primary cells noninvasively.

## 2. Materials and methods

### 2.1. Sensor chip cleaning and pretreatment

Prior to experiments, the applied OW2400 waveguide sensor chips (Microvacuum Ltd., Hungary) underwent a cleaning routine. First, they were immersed into chromic acid (Merck) for 3 min, followed by subsequent rinsing with Milli-Q water (MQ), potassium hydroxide (KOH), and MQ again. Afterwards the chips were placed into an ultrasonicator for at least 30 min and the bathing MQ water was changed every 3 min over them. Prior to experiments the cleaned waveguides were incubated in serum-free Roswell Park Memorial Institute (RPMI, Sigma) medium overnight.

### 2.2. Monocyte isolation from human blood

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat obtained from healthy donors and provided by the Hungarian National Blood Transfusion Service by density gradient centrifugation on Ficoll-Paque (GE Healthcare). Informed consent was provided for the use of blood samples according to the Declaration of Helsinki. Unlabeled monocytes were isolated by negative magnetic separation using the Miltenyi Monocyte Isolation kit II (Miltenyi) according to the manufacturer's instructions. In short, non-monocytes are indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies and anti-biotin MicroBeads. Highly enriched unlabeled monocytes are obtained by depletion of the magnetically labeled cells. Cells were cultivated in RPMI-10% FBS (37 °C, 5% $CO_2$ atmosphere) media in Teflon coated flasks to avoid spontaneous monocyte attachment to the culture dish. OWLS experiments were carried out within one day after their isolation to exclude the spontaneous differentiation into MFs or DCs.