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Perspective

Label-free analysis of GPCR-stimulation: The critical impact of cell adhesion

S. Lieb[a], S. Michaelis[b], N. Plank[a], G. Bernhardt[a], A. Buschauer[a], J. Wegener[b,*]

[a] Institute of Pharmacy, University of Regensburg, D-93053 Regensburg, Germany
[b] Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, D-93053 Regensburg, Germany

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ABSTRACT

Label-free cell-based assays have been attracting growing attention in drug research. Optical approaches based on evanescent electric fields (e.g. EPIC, RWG/DMR, SPR) and electrochemical impedance analysis (ECIS, xCELLigence) are by far the most widespread techniques for such purposes. We compared three label-free approaches (ECIS, RWG/DMR and SPR) with respect to the activation of the human histamine H_1 receptor (H_1R) expressed by U-373 MG glioblastoma and genetically engineered HEK 293T cells. HEK 293T cells were either expressing the hH_1R alone or in combination with the adhesion protein hMSR1. The β_2-adrenergic receptor (β_2-AR) expressed by bovine aortic endothelial cells (BAEC) served as a second cell model. Reduced cell adhesion to the surface of the sensing devices affected both, the optical and the impedance-based readout, but became much more obvious in case of RWG- or SPR-based assays. By contrast, the co-expression of hH_1R and hMSR1 in HEK 293T cells strongly enhanced the signal compared to hH_1R expression alone. As the sensitivity of the optical readouts is confined to a distance of 100–200 nm from the surface, depending on the wavelength of the incident light, this observation is in accordance with tighter adhesion of the co-transfectants, inducing a shorter distance between the cell membrane and the substrate. Combining ECIS and SPR, allowing for simultaneous registration of both signals for a single cell population, provided a direct correlation of both readouts, when H_1R or β_2-AR stimulation was investigated for the same cell populations. Cell adhesion was found to have a critical impact on the results of label-free cell monitoring, in particular when techniques based on evanescent electric fields are applied.

1. Introduction

G-protein-coupled receptors (GPCRs) constitute the largest family of membrane-bound signaling proteins, which transmit extracellular stimuli across the cell membrane into the cytoplasm by activating heterotrimeric G-proteins [1,2]. GPCRs are involved in a plethora of diseases, including inflammation, pain, cardiovascular diseases and neurological disorders [3–6]. They represent the biological targets of more than 30% of all marketed drugs [7]. In addition to those GPCRs addressed by approved drugs, the discovery and characterization of orphan receptors still holds a great therapeutic potential [8].

Various functional assays have been designed and successfully applied to determine the ligand-dependent, GPCR-mediated activation or inhibition of cellular signaling cascades, e. g. by quantification of second messengers such as cyclic AMP, inositol trisphosphate or calcium ions [9]. These assays require specific molecular probes (e.g. Fura-2/AM), and the analytical readout is most often based on fluorescence or luminescence. By contrast, label-free technologies provide a holistic, unbiased and time-resolved approach to monitor the cellular response to a compound of interest (agonist/antagonist). These techniques are becoming more and more accepted and appreciated in drug discovery [10], in particular, due to their applicability to orphan GPCRs, for which the endogenous ligands and the physiological signaling pathways are still to be identified.

The most widely applied devices to perform label-free cell-based assays rely on measuring (i) the electrochemical impedance Z of cell-covered planar electrodes (Fig. 1A) [11–13] or (ii) the resonance wavelength λ of cell-covered optical waveguides (resonance waveguide = RWG) during experimental stimulation of the cells (Fig. 1B) [14–17]. Although the surface plasmon resonance (SPR) technique (Fig. 1C) was developed earlier, it has not yet received

* Corresponding author.
E-mail address: Joachim.Wegener@ur.de (J. Wegener).

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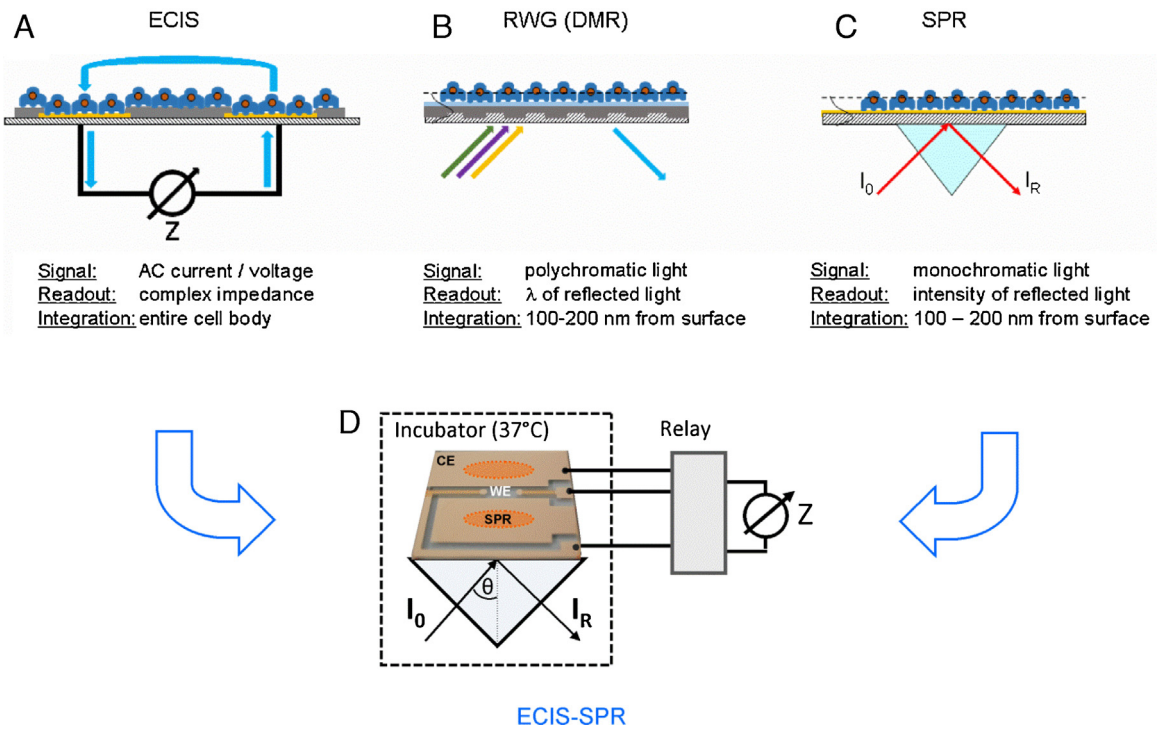

Fig. 1. Schematic overview of the most widely applied label-free approaches to monitor cell-based assays. (A) *Electric Cell-Substrate Impedance Sensing* (ECIS) reads the electrical impedance of cell-covered electrodes with non-invasive AC currents and voltages. The impedance signal Z is sensitive to coverage of the electrode with cells and the geometric shape of the cells on the electrode. (B) Surfaces that hold a *resonant waveguide* grating (RWG) reflect monochromatic light of wavelength λ when exposed to polychromatic incident light. The resonance wavelength of the reflected light is determined by the refractive index close to the surface, making the device sensitive to any change in mass distribution in the lower part of the cell body. (C) Monochromatic incident light excites *surface plasmons* under *resonance* (SPR) conditions along a gold-covered surface. The intensity of the reflected light – not dissipated into plasmonic energy – is determined by the refractive index close to the surface. Thus, SPR provides very similar information as RWG. (D) Setup for simultaneous monitoring of the cellular response by ECIS and SPR. The sensor surface holds two elliptical fields for SPR sensing and two much smaller working electrodes for ECIS recordings. The relay allows switching between the individual electrodes. RWG = resonance waveguide; ECIS = electric cell-substrate impedance sensing; SPR = surface plasmon resonance; I_0 = intensity of the incident light; I_R = intensity of the reflected light; Z = impedance; CE = counter electrode; WE = working electrode.

the same attention for the analysis of cell-based assays as RWG- or impedance-based approaches.

In SPR the intensity of the reflected light is measured. Intensity readings report on the generation of surface plasmons along a gold-coated growth surface. Whereas ECIS is sensitive to the dielectric properties of the cell bodies on the electrode, RWG and SPR report on the integral refractive index within a distance of 100–200 nm from the surface. The refractive index is affected by the cell bodies and the distribution of mass along the surface.

The basic concepts of all these approaches have been described in detail elsewhere [12,13,16]. Thus, we only give a short summary to emphasize those similarities and differences that are important for the main conclusion of this study.

The use of electrochemical impedance measurements to monitor cell-based assays was first described by Giaever and Keese [15] and is referred to as electric cell-substrate impedance sensing or short ECIS. In ECIS the cells are grown on the surface of thin gold-film electrodes deposited on the growth surface. When cells attach and spread on the electrode, the impedance increases since the dielectric cell bodies behave like insulating particles forcing the current to flow below, between or through the cells, depending on the AC frequency used for the measurement. Accordingly, the technique is sensitive to electrode coverage and cell morphology. When confluent cell layers are studied, cell morphology determines the impedance readout entirely. This phenomenon is based on the fact that cell size as well as the tightness of cell–cell and cell-substrate contacts determine the geometric and resistive properties of the ionic current pathways around the cell bodies. Any change in cell shape causes a corresponding change in the geometry of the current pathways and, thus, the measured impedance. Since it is well-known that GPCR activation may lead to actin cytoskeleton remodeling [14], it is straightforward to apply ECIS as monitoring device in cell-based assays to investigate GPCR pharmacology.

In RWG devices [17] the surface for cell growth is manufactured with an optical grating. As a consequence polychromatic incident light is reflected as monochromatic light while an evanescent electric field is generated at the substrate surface. The evanescent electric field penetrates approximately 100–200 nm into the sample. The integral refractive index within this "sensitive sheet" affects the resonance condition and, thus, determines the wavelength of the reflected light. Since the plasma membrane of adherent mammalian cells can be as close as 25 nm to the surface (strongly dependent on cell type), the resonant wavelength is affected by any mass redistribution, provided that it is localized in the lower part of the cell body close to the membrane. With respect to this interpretation of refractive index changes the approach is often referred to as "DMR" for dynamic mass redistribution.

In SPR [16] a high quality glass slide is covered with a thin layer of gold. When the interface between glass and gold is hit by a monochromatic laser beam under resonance conditions (wavelength, angle of incidence) the evanescent electric field couples into the conduction band electrons of the metal and excites surface plasmons (i.e. electron density fluctuations). These plasmons generate an evanescent electric field with a similar penetration depth as the one provided by the resonance waveguide grating in the range of 100–200 nm (dependent on wavelength). The refractive index within this "sensitive sheet" affects the resonance condition and thereby modulates the intensity of the refracted light. Accord-