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Label-free visualization and quantification of single cell signaling activity using metal-clad waveguide (MCWG)-based microscopy

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ABSTRACT

Label-free biosensing methods are very effective for studying cell signaling cascade activation induced by external stimuli. Assays generally involve a large number of cells and rely on the underlying assumption that cell response is homogeneous within a cell population. However, there is an increasing body of evidence showing that cell behavior may vary significantly even among genetically identical cells. In this paper, we demonstrate the use of metal-clad waveguide (MCWG)-based microscopy for label-free real-time monitoring of signaling activity and morphology changes in a small population of cells, with the ability to resolve individual cells. We demonstrate the potential of this approach by quantifying apoptosis-induced intracellular activity in individual cells following exposure to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and by visualizing and quantifying extracellular changes in endothelial cell layer integrity following the activation of the proteinase-activated receptor 1 (PAR1) by thrombin. Results show that averaged signals obtained from a cell population may incorrectly reflect the actual distribution of morphology and kinetics parameters across a cell population by a significant margin.

1. Introduction

Eukaryotic cells express a large diversity of membrane-bound receptors to sense and react to external stimuli such as hormones, cytokines, pathogens and toxins. The activation of these receptors triggers intracellular signaling events regulating cell activities supporting normal tissue and organ functions. The sensitive and non-invasive quantification of cell signaling responses is important in cell biology and pharmacology, and is the basis for many novel developments in biomedical diagnostics, using both commercially available systems and advanced research platforms. Example applications include monitoring of G-protein coupled receptor (GPCR) signaling (Schröder et al., 2010; Scott and Peters, 2010), changes in cell activity induced by toxins and antigens (Chabot et al., 2009; Hide et al., 2002), apoptosis (Arndt et al., 2004; Maltais et al., 2012) and endothelial cell layer integrity (Benson et al., 2013; Tiruppathi et al., 1992). Electrical impedance spectroscopy (Tiruppathi et al., 1992), diffraction gratings (Fang et al., 2006) and surface plasmon resonance (Chabot et al., 2009; Giebel et al., 1999; Jamil et al., 2007; Peterson et al., 2014; Wang et al., 2012; Yanase et al., 2010, 2007) have been used very successfully to monitor and quantify cellular activity in vitro. These label-free platforms do not require the use of exogenous labels that may bias or interfere with the molecular processes under investigation.

Experiments are typically conducted on cell populations ranging from 100 to 4000 cells. As such, measurements represent a statistical average of what may actually be a highly heterogeneous cell response, possibly including multiple cellular phenotypes (Stolwijk et al., 2015). While cell populations are often assumed to respond homogeneously to a specific biochemical stimuli, there is an increasing body of evidence showing that cell behavior varies significantly even among genetically identical cells (Samadani et al., 2006; Slack et al., 2008). Such cellular phenotypic heterogeneity is central to physiological processes relying on lineage selection from distinct clonal population of progenitor cells (Altschuler and Wu, 2010) and during development (Bahar et al., 2006; Colman-Lerner et al., 2005; Raser and O'Shea, 2005; Samadani et al., 2006). However, label-free methods for sensing cell response and signaling events are unable to resolve single cell activity within a population and therefore cannot detect heterogeneity.

Evanescent-field based methods such as surface plasmon resonance (SPR) and metal clad waveguides (MCWG) (Skivesen et al., 2005) are

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label-free and highly sensitive to events occurring within a discrete volume above the sensor surface. These methods, which were originally applied in biosensing to monitor binding kinetics between surface-bound receptors and ligands in solution (Homola, 2003), have since been applied very successfully to study phenomena in a wide range of biological objects. When probing relatively thick microscopic objects such as cells, MCWG-based systems are advantageous compared to SPR owing to their capability for deeper probing into the sample while maintaining relatively high spatial resolution (Söllradl et al., 2017). Indeed, certain cell structures of interest may be located higher up in the cell body (ex: actin cytoskeletal components, > 100 nm; intracellular organelles, > 200 nm) than the effective probing range of conventional SPR systems (~200 nm for visible light systems). In both cases, advanced imaging systems are capable of resolving single cells.

In this paper, we propose a MCWG-based microscopy system designed to make sensitive real-time quantitative measurements of cellular activity in vitro with the ability to resolve individual cells, without the need for fluorescent labeling. The potential of the proposed system is demonstrated in two sets of experiments: 1) quantification of intracellular activity following the activation of apoptotic pathways in an endothelial cell model (EA.hy926) exposed to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and 2) quantitation of the disruption of a confluent endothelial cell monolayer (EA.hy926) resulting from the activation of the protease activated receptor 1 (PAR1) by thrombin. Though the cell populations in the experiments are too small to extract meaningful statistics, the results nevertheless show convincingly how averaged signals obtained from a cell population may not accurately reflect the actual distribution of cell behavior characteristics across a population.

2. Material and methods

2.1. Imaging instrumentation and sensor chip design

The sensor chip design and optical imaging setup based on a high numerical aperture microscope objective (Fig. 1A) are detailed elsewhere (Söllradl et al., 2017). Briefly, light from a fiber coupled LED source (center wavelength: 470 nm) is focused at the back focal plane of the objective and emerges as a collimated beam at the front of the objective, incident onto the backside of the MCWG sensor chip. The lateral offset of the focal spot from the center in the back focal plane of the objective determines the angle of incidence/reflection of the collimated light to/from the chip. A polarizing beamsplitter separates the reflected light into two perpendicular polarization components for recording by CCD cameras (1392×1040, 12 bit, gray-scale), where TM images contain the reflectance-encoded refractive index map and TE images correct for illumination field inhomogeneities. An LED was used as the light source instead of a laser to minimize coherence artifacts in the images and to prevent phototoxic effects (Supplementary Figs. 1 and 2).

Biosensing based on metal-clad waveguide (MCWG) is similar to surface plasmon resonance (SPR) and represents a special case of this broader class of waveguides. MCWG and SPR sensors operate by resonant coupling of incident light to a lossy guided mode in a waveguide consisting of a thin film stack deposited on a glass substrate (the sensor chip), where the biological medium atop the chip acts as the topmost cladding layer of the waveguide. Cellular structures such as the membrane, cytoskeletal components, and organelles have higher refractive indices than the extracellular medium. As a result, when cells cultured atop a MCWG chip change either in morphology or in distribution of intracellular molecular content, the resulting local refractive index changes near the surface perturb the mode characteristics, thereby spatially modulating the reflected light intensity. Fig. 1B shows a typical image of reflected light from a MCWG chip with two human glioblastoma cells (U251) on the top surface (a brightfield micrograph of the cells is shown in Fig. 1C).

The exquisite depth discrimination in SPR and MCWG sensing/imaging results from the strong light confinement by the planar waveguide at the sensor surface. This waveguide-based nature, however, imposes a limit on the achievable imaging spatial resolution in the direction of mode propagation due to the finite decay length of the mode, which is generally greater than the diffraction limit. In the direction perpendicular to mode propagation, imaging spatial resolution is limited by diffraction as with conventional microscopy. There is a direct trade-off between spatial resolution and probing depth into the sample, where decreasing the probing depth will improve resolution and vice-versa (Söllradl et al., 2017). Though slightly more complex to fabricate, MCWG chips (dielectric core waveguides with a metal film

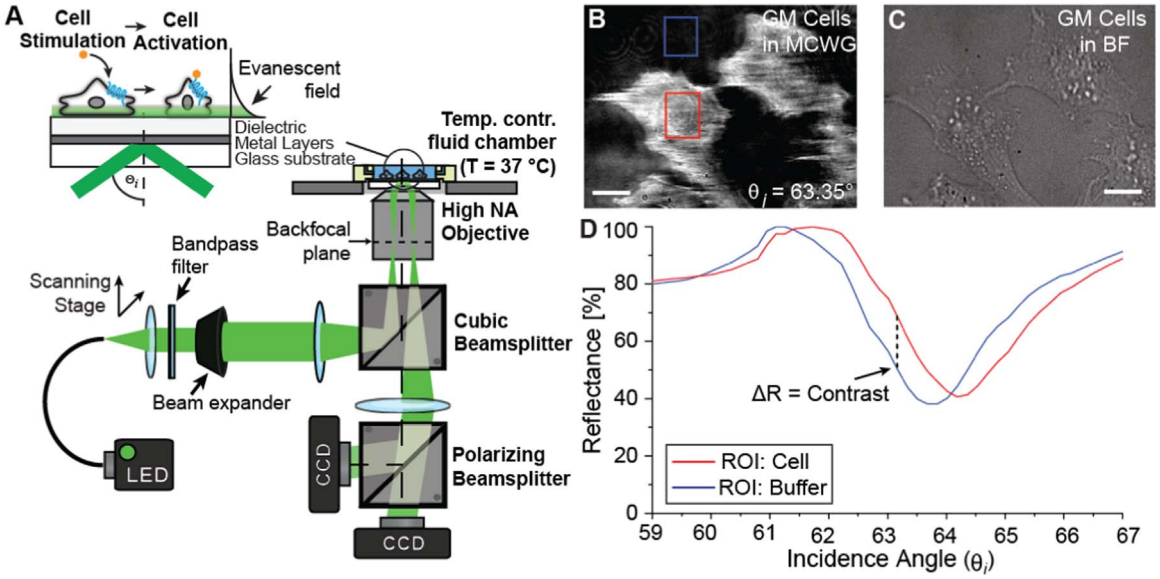

Fig. 1. *Metal clad waveguide imaging of individual cells.* A) Schematic of the metal clad waveguide imaging (MCWG) setup based on a high numerical aperture microscope objective; B) Reflectance image of two glioblastoma cells (U251) cultured on top of the MCWG sensor chip (scale bar = 20 μm, LED center wavelength = 470 nm incidence angle of 63.35°); C) Brightfield image the same two cells (scale bar = 20 μm); D) Mean reflectance as a function of incidence angle for the two rectangular ROI shown in B). The vertical dotted line indicates the operating angle (63.35°) for fixed-angle measurements.