Amplitude and phase images of cellular structures with a scanning surface plasmon microscope

L. Berguiga,1,2 T. Roland,1,2 K. Monier,1,2 J. Elezgaray,3 and F. Argoul 1,2,*

1 USR3010, UMR 5672, CNRS, Ecole Normale Supérieure de Lyon, 46 Allée d’Italie, 69364 Lyon, France
2 Université de Lyon, F-69000 Lyon, France
3 CBMN, UMR 5248, CNRS, 2 rue Robert Escarpit, 33600 Pessac, France
*francoise.argoul@ens-lyon.fr

Abstract: Imaging cellular internal structure at nanometer scale axial resolution with non invasive microscopy techniques has been a major technical challenge since the nineties. We propose here a complement to fluorescence based microscopies with no need of staining the biological samples, based on a Scanning Surface Plasmon Microscope (SSPM). We describe the advantages of this microscope, namely the possibility of both amplitude and phase imaging and, due to evanescent field enhancement by the surface plasmon resonance, a very high resolution in Z scanning (Z being the axis normal to the sample). We show for fibroblast cells (IMR90) that SSPM offers an enhanced detection of index gradient regions, and we conclude it is very well suited to discriminate regions of variable density in biological media such as cell compartments, nucleus, nucleoli and membranes.

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References and links
1. Introduction

Since the earliest discovery of cellular structures, biologists have been fascinated by the observation of cells with optical microscopy. Thanks to the development of fluorescent labeling technologies and as well as sophisticated optical microscopes, the investigation of dynamical processes in living cells could be achieved since the nineties. However, these fluorescence techniques rely on exogenous contrast agents or fixation processes. Since we do not discuss much fluorescence imaging techniques in this paper, as we rather focus methods based on optical phase shift based methods, we refer the reader to reference [1] where a very complete survey of fluorescence nanoscopic microscopy techniques has been recently published.

In place of these contrast agents, optical phase shift has been used as an endogenous contrast agent in different techniques such as Phase Contrast, Differential Interference Contrast (DIC) microscopies [2]. Even though these methods allowed the discrimination of transparent objects such as living cells, it was not until the development of Quantitative Phase Imaging, Spatial Light Interference Microscopy and Diffraction Phase microscopy techniques that sub-nanometer changes in optical path lengths could be measured [3, 4]. It is now well established experimentally that the combination of both phase and amplitude signals offers the possibility to recover a maximum of information from experimental images, and to push the sensitivity of optical microscopy even further.

We show in this paper that the combination of phase and amplitude imaging can enhance even more both sensitivity and resolution if coupled with Surface Plasmon Resonance (SPR) microscopy. The physical phenomenon occurring at SPR is characterized by a sharp drop of the reflected light near a resonance angle $\Theta_p$ [5, 6]. SPR detection methods capture the shift of the plasmon resonance produced by a local modification of the refractive index of a dielectric medium in contact with gold, for instance after the adsorption of molecules, cells, nanobjects. . . The plasmon wave changes both in amplitude and in phase when it meets a medium of different refractive index. Besides its performance in biosensors developed since the early nineties [7, 8], the sensitivity of SPR is also well suited for capturing very small index variations in structured objects such as biological samples.

Imaging the modification of the SPR due the spatial modulation of an adsorbed layer has been suggested by many authors [9–13]. These systems were launching the plasmon by prism-coupled devices, and collected either the modulus $|r|$ or the phase $\phi$ of the reflectance $r$. In the early 2000’s, a new generation of Interferometric SPR Imaging (ISPRi) systems was proposed to reconstruct SPR phase images [14–16]. The system of Grigorenko and coworkers [15] could work either in a Zernike mode for phase contrast, or in the fringe mode, or finally in a conventional amplitude SPR microscope, by occulting the reference beam of the interferometer. Although enhanced sensitivity and higher resolution over conventional SPR microscopy were achieved with ISPRi, the spatial resolution remained much larger than the wavelength due to the lateral propagation of the plasmon [17, 18]. The surface plasmon wave has a propagation length along the gold-dielectric medium interface which prevented (sub-wavelength) high resolution imaging in prism-coupled devices [19, 20]. Other groups have also invested much instrumental efforts in developing ISPRi systems [22, 23] and pushed down its sensitivity to more than 100 deg.RIU$^{-1}$.

The vertical resolution of SPR is theoretically in the sub-nanometer range, which is at least
one order of magnitude better than that of Interference Reflection Microscopy (IRM). The vertical confinement of the electromagnetic field due to evanescent waves is used in Near-field Scanning Optical Microscopy (NSOM) and Total Internal Reflection Microscopy (TIRM). NSOM relies on a local optical probe which scans the sample whereas TIRM uses a prism or a diaphragmed objective lens to narrow the range of incident angles to those corresponding to evanescent waves. The emergence of optical nanoantennas is expected to push down the resolution of near-field scanning microscopy (NSOM) to about 50 nm in a near future [24] and therefore to make this scanning probe technique very attractive for biological applications. As compared to SPPM, this method remains an invading method since a nanoscale probe (optical tip) used to scan the sample. Moreover, it is better suited for interrogating events confined to the cellular surface (cortex, membrane, wall). However, coupling NSOM with SSPM could take advantage of both techniques, since one may use NSOM as a very local perturbation and record with SSPM the real time response of the internal structure of the cell. TIRM has been very often applied to surface imaging fluorescent markers or particles in the well known method of Total Internal Reflection Fluorescence Microscopy (TIRFM), more recently a very elegant and original implementation of a TIRM has been proposed [25], based on speckle averaging that allows high resolution imaging of cellular adhesion, without staining. This wide field microscopic method allows high speed image sampling. It is therefore very well suited for real time imaging of cells in liquid medium. Compared to SSPM, this method is faster, however its resolution and sensitivity are lower since it does not use neither SPR nor heterodyne interferometry coupled to \((X, Y, Z)\) scanning. SSPM offers a better discrimination of internal cellular structures like the cell nucleus, or the cytoskeleton fibers, at the cost of sampling speed. The Surface Plasmon Resonance Microscope (SPRM) captures the interference of the light scattered by cell bodies with the evanescent field, thus making possible to estimate the cell/substrate distance from the intensity of the reflected light. Contacts between the cell membrane and the substrate can thus be visualized at high contrast with a vertical resolution \((Z)\) in the nanometer range [26]. However in these systems, the lateral resolution along the direction of surface plasmon propagation is still in the order of several micrometers.

The introduction of a high numerical aperture objective lens on SPR microscope benches opened the way to high resolution surface plasmon microscopy in the early 2000’s. Two groups of methods can be distinguished, based respectively on a Wide Field SPR Microscopy (WSPRM) [27–30] and on Scanning Surface Plasmon Microscopy (SSPM) [27, 28, 31–37].

2. Objective lens-coupled surface plasmon microscopes

2.1. Wide-field systems

Objective lens-coupled SPR microscopes allow a direct imaging of the gold interface and improve the spatial resolution, without the need of readjusting the incidence angle, as required by prism-coupled SPR imaging. These systems can achieve an in-plane resolution of 1 \(\mu\)m or better, and they can be used to discriminate different parts of the cell, in direct contact with the gold surface, depending on their density. By the reconstruction of refractive index maps, Moh and colls. [38] have shown that the portions with the highest refractive index correspond to the location of the cell nucleus. Jamil and colls [39] reported the first WSPRM for the examination of a cell surface with a sub-micron lateral resolution. Their study showed that this technique is well suited to investigate interfacial cellular signaling mechanisms without the use of label.
2.2. Scanning Surface Plasmon Microscope: SSPM

2.2.1. Description of the SSPM system

This high resolution microscopy method is different from standard SPR imaging methods, as it combines the following points:

- Focusing of light by a high numerical aperture (NA) objective lens (Olympus 60x, NA 1.45 for SSPM imaging in air), the resolution of this microscope has been estimated from calibrated nanoparticles in air: 220 ± 20 nm and in water: 135 ± 20 nm [37] (λ = 633 nm).

- Polarization of light (radial, azimuthal, linear) is obtained with a polarization converter made of 3 liquid crystal stages (Arcoptix), allowing fast switching from radial to azimuthal polarization. Linear polarization is obtained by removing the converter.

- Interferometric heterodyne detection to capture the reflected field, integrated over the objective pupil, and to remove the background noise for higher signal to noise ratio.

- Scanning in (X, Y) and Z directions: for fixed Z, 2D images in (X,Y) plane are reconstructed (a 80×80 μm² image is captured in 50 s); for fixed (X,Y), V(Z) curves are captured.
recorded by varying the focus position \( Z \). \( V(Z) \) curves model the variation of the integral of the back reflected beam over the objective pupil versus the distance \( Z \) of the objective focus to the gold interface. The three axis piezo scanning of this microscope has been purchased from Physik Instrument (PI).

- Conditioning of the incoming light to enhance SSPM sensitivity, for instance by a diaphragm that increases the fraction of light that contributes to SPR excitation. This optical element was switched off in the experiments described in this paper.

2.2.2. SSPM set-up

The experimental device performing linear, radial or azimuthal polarization of high resolution surface plasmon imaging is illustrated in Fig. 1A. A simplified drawing of the objective lens system, the glass coverslip and its metal coating and the incoming and back-reflected beams is sketched in Fig. 1B. At focus (\( Z = 0 \)) and out of focus (\( Z > 0 \)) the incoming rays (A) are reradiated in rays (B') that contain amplitude and phase information about SP propagation inside the confined area. When the focus is in the glass coupling medium (\( Z < 0 \)) surface plasmon waves propagate outward the focal spot and do not contribute to the image contrast. The radial symmetry of the high NA objective lens produces a confinement of the SP waves in a very small zone. The rays (C) which are not coupled to SP are reflected back (D) without SP phase retardation. Three different polarization modes are accessible in this SSPM: a radial polarization (Figs. 1C(a-d), 1D(a)), an azimuthal polarization (Figs. 1C(b-e), 1D(b)) and planar polarization (Figs. 1C(c-f), 1D(c)). The first one excites the plasmon resonance in all directions, the second one does not excite the surface plasmon resonance, and the third one excites the surface plasmon resonance only for wavevectors in two angular cones, visible as two black crescents in Fig. 1D(c).

When the objective lens is scanned along its axis (\( Z \)), a \( V(Z) \) signal is detected by the photomultiplier. The measurement of \( V(Z) \) versus \( Z \) is at the basis of SSPM image contrast [31, 33, 35, 37]. An example of theoretical \( V(Z) \) curves is shown in Fig. 2, computed from a gold film in contact with air, on which a 50 nm film with a variable refractive index (\( n_C \) varying from 1 to 1.6) has been added. SPR is excited when the electric field vector is parallel to the plane of incidence, this corresponds to \( p \) polarization. When the electric field vector is perpendicular to the plane of incidence, there is no SPR, this corresponds to \( s \) polarization. Experimentally, a radial (resp. azimuthal) polarization (shown in Fig. 1C.a (resp. 1C.b)) allows a \( p \) (resp. \( s \)) polarization for all radial angles \( \varphi \). In the sequel, we will use indifferently \( p \) for radial polarization, and \( s \) for azimuthal polarization. Note that slow mode oscillations superimpose to \( V(Z) \) for positive values of \( Z \) (Fig. 2(a)) when the light is \( p \) polarized. On Fig. 2(b) and (d), the phase \( \Phi(Z) \) of \( V(Z) \) is represented for each polarization. Besides a global linear increase of this phase with the focus \( Z \), modulations of the phase superimpose to this linear trend for positive values of \( Z \) in \( p \) polarization. These oscillations (both in modulus and phase of \( V(Z) \)) are due to the amplification and damping of the back reflected signal after plasmon excitation. When the index of the dielectric layer on gold changes, both the amplitude and the phase of \( V(Z) \) change drastically in \( p \) polarization for \( Z \) larger than 500 nm. \( V(Z) \) curves (amplitude and phase) do not change monotonously with the index of the dielectric layer on gold, a likely explanation of this behavior is related to the fact that the whole angular range of angles from 0 to the numerical aperture of the objective lens is considered, without diaphragming. We have chosen this configuration to keep a maximum light intensity. The characterization of the influence of an angular diaphragming on the SSPM imaging will be performed in a forthcoming publication.

The \( V(Z) \) response depends on the variation of the local dielectric properties of the medium in contact with gold inside the evanescent field, with consequently a modification of the slow
Fig. 2. Theoretical $V(Z)$ curves illustrating the SSPM response of a 45 nm gold film in air, on which a 50 nm film with variable refractive index has been added. (a) $|V(Z)|$ in $p$ polarization mode. (b) $\Phi(Z)$ in $p$ polarization mode. (c) $|V(Z)|$ in $s$ polarization mode. (d) $\Phi(Z)$ in $s$ polarization mode. $\lambda = 633$ nm, $\varepsilon_{\text{Au}} = -11.81 + 1.214 \, i$, $\delta_{\text{Au}} = 45$ nm, $\varepsilon_{\text{Cr}} = -1.1125 + 20.793 i$, $\delta_{\text{Cr}} = 3$ nm. The color coding of the curves depends on the index of the dielectric layer: black: $n_C = 1$; magenta: $n_C = 1.2$; light blue: $n_C = 1.3$; dark blue: $n_C = 1.4$; green: $n_C = 1.5$; red: $n_C = 1.6$.

mode oscillations. For a given value of $Z$ the variations of $|V(Z)|$ will be either positive or negative, depending on the local index of the medium. The amplitude and phase of these modulations change with $Z$, according to the formula:

$$V(Z) = \int_{\theta_{\text{min}}}^{\theta_{\text{max}}} P^2(\sin \theta) \mathcal{Q}(\theta) \exp(2j\kappa_0 Z \cos \theta) \cos \theta \sin \theta \, d\theta$$

where $P$ is the pupil function of the objective lens, $n_0$ is the index of the coupling medium (lens, glass coverslip and matching index oil), $\kappa_0$ the wave number inside glass, $\mathcal{Q}(\theta)$ is proportional to the back reflected field, integrated over the radial angle $\varphi$. This quantity depends on the light polarization. For a planar dielectric layer:

$$\mathcal{Q}(\theta) = \int_0^{2\pi} r^p,s(\theta) \cos^2 \varphi \, d\varphi = \pi r^p,s(\theta)$$

By a first order approximation of the variation of the reflectivity $r^p$ with a modification of the local dielectric index, we have shown that the contrast of the SSPM images is proportional to an integral function of $\partial r^p / \partial \varepsilon$ [40].

$$\Delta V(Z) \sim \frac{\partial Y}{\partial (\varepsilon_C, \delta_C)} \int_{\theta_{\text{min}}}^{\theta_{\text{max}}} P^2(\sin \theta) \left[ \frac{\partial r^p(\theta)}{\partial \varepsilon} \right] \exp(2j\kappa_0 Z \cos \theta) \sin \theta \, d\theta .$$
Fig. 3. Three dimensional view of the plane sections in Z direction from the 3D map of |V(X,Y,Z)| that give the images I(X,Y) for fixed Z value. This (X,Y,Z) scan has been done on a 50 nm gold nanoparticle. Images were acquired using a radially polarized light.

$\rho_P$ can be approximated by a Lorentzian function of the surface plasmon wave vector $k_{SP}^X$ along direction X (plane of observation). In the limit of small thickness $d_C$, such that $k_Cd_C \ll 1$ \[5,41\], it reads:

$$\Delta k_{SP}^X = \left(\frac{\omega}{c}\right)^2 d_C \left(\frac{\varepsilon_C - 1}{\varepsilon_C}\right) \left[\frac{\varepsilon_m'}{1 + \varepsilon_m'}\right]^2 \frac{1}{(1 - \varepsilon_m')^{1/2} \varepsilon_m'}.$$

(4)

This term is a linear function of $d_C$, with a real positive slope when $\varepsilon_C$ is real. When the index of the additional layer changes with the position (X,Y), the whole term $G_C = \frac{d_C}{\varepsilon_C}(\varepsilon_C - 1)(\varepsilon_C - \varepsilon_m')$ must be considered, depending on both the thickness of the layer and its index. This simplified formulation of $\Delta k_{SP}^X$ will be suited also for the study of inhomogeneous layers such as cellular objects, because their internal index and thickness may change in space. Thickness and refractive index are interconnected when imaging an inhomogeneous media, and they will both modify locally the plasmon wave propagation. When the adsorbed object has a complex dielectric constant (absorption of light), it involves also a phase shift of this wave, leading to a lengthening or a shortening of the plasmon resonance pathway.

2.3. Principle of image reconstruction

SSPM images are captured by a two dimensional scan (X,Y) of the microscope lens in the area of interest, for a fixed position Z of the objective. From a stack of (X,Y) images at different foci Z, one can reconstruct a 3D map of V(X,Y,Z) values, as illustrated in Fig. 3. This 3D map is used afterwards to reconstruct V(Z) curves for each (X,Y) position. These V(Z) curves are low-pass filtered by a gaussian function to keep the slow mode oscillations only and get rid of high frequency noise. They are also used to compensate a tilt of sample coverslip with respect to the objective lens, with a planar interpolation of the 2D map of the maxima of all the |V(Z)|

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curves. Although this compensation is critical to have a correct reconstruction of SSPM images, we do not discuss this aspect in this paper and we focus instead on the possibility to combine amplitude and phase images. The set of images reported in Fig. 3 has been reconstructed from a 50 nm gold nanoparticle. We note on this figure that the contrast of the nanoparticle image through the SSPM depends on the focus $Z$ [35, 37].

3. Cellular imaging with the SSPM

3.1. Materials and methods

3.1.1. Preparation of the gold coated coverslips

Glass coverslips (Cs) were cleaned in a caustic detergent for one hour at 50°C, and rinsed two times with distilled water (at least 2 hours each) at room temperature. After this, the Cs were quickly rinsed with ultrapure water ($18 \text{ M}\Omega\cdot\text{cm}^{-1}$), absolute ethanol and dried under a nitrogen flow. After cleaning the glass coverslips, a thermal evaporation system was used to deposit thin layers of chromium ($\delta_{Cr} = 3 \text{ nm}$) and gold ($\delta_{gold} = 45 \text{ nm}$ with 0.1% accuracy) on them. If not used immediately, gold-Cs were stored into individual closed vessels filled with absolute ethanol to prevent pollution due to impurities.

3.1.2. Cell culture

IMR–90 cells were obtained from a primary culture of fibroblasts of human fetal lung. The cells were cultivated at 37°C in the growth medium (EMEM, 10% SVF, NEaa) and an atmosphere saturated with 5% of CO$_2$. They were detached by addition of trypsin, counted, and resuspended in the growth medium at a concentration of 100 000 cells/ml. The gold coated glass coverslips were deposited inside tissue culture dishes and a volume of 3 ml of cell suspension was poured into each well (i.e. $\sim$300 000 cells). Cells were incubated during 48 h so that the cells are dense enough and still, not at confluence. Each coverslip was subsequently rinsed with 1X PBS, treated with absolute methanol (at -20°C) and incubated 30 min on ice. Methanol was eliminated and the samples were then incubated 2×5 min in 1X PBS, 0.1% Triton, before a series of 5 min dehydration steps (70%, 90% and 100%) in absolute ethanol. Finally, the coverslips were dried under nitrogen flow and stored under vacuum at ambient temperature. SSPM images where recorded on fixed cells in air, with a 1.45 NA objective lens.

3.2. Amplitude images

Figures 4 and 5 show two sets of SSPM images recorded for two resolutions. The first one with a $X,Y$ scanning on 80 $\mu$m and the second one on a smaller domain (30 $\mu$m) centered on the nucleus of the cell. The $|V(Z)|$ curves selected on different points of the surface corresponding either to gold or to cellular elements are very well distinguishable and lead to highly contrasted SSPM images that allow the discrimination of internal structures of the cell, that is some of the internal structures of the nucleus, and more precisely the nucleoli (zoom of Fig. 5).

The pattern of the cell changes progressively when $Z$ goes from 0 to 1.4 $\mu$m. Figure 4(b) shows one fibroblast with two neighbouring cells that seem to pass over its crawling protrusions. At $Z = 0 \mu$m the fine structure of the actin cytoskeleton is revealed as a network of entangled fibers, and very sharp excrescences are observed. In Fig. 5 we focus on the nucleus of this cell. At $Z = 0 \mu$m the contours of the nucleoli are fuzzy and poorly contrasted, and the cytoplasm gives a maximum of reflected intensity. At this focus, the depth of penetration of the evanescent field inside the cell is minimum and therefore the parts of the cell which are closer to the gold surface mostly contribute to the plasmon reflected field. For increasing $Z$ values, the zones concerned by the plasmon excitation progressively shift further away from the gold surface, these zones correspond to the nucleus, which is thicker that the cytoplasmic
Fig. 4. SSPM (X, Y) amplitude images reconstructed from IMR90 fibroblasts for different foci Z in radial polarization. (a) Selection of three $|V(Z)|$ curves, corresponding to the colored symbols of Fig. 4(b). (b) Z = 0 μm. (c) Z = 0.4 μm. (d) Z = 0.8 μm. (e) Z = 1.2 μm.
Fig. 5. SSPM (X,Y) amplitude images reconstructed from IMR90 fibroblasts for different foci Z in radial polarization, on the same cell as in Fig. 4, zooming on the nucleus. (a) Selection of three |V(Z)| curves, corresponding to the colored symbols of Fig. 4(b). (b) Z = 0 μm. (c) Z = 0.4 μm. (d) Z = 0.8 μm. (e) Z = 1.2 μm.
zone (Fig. 5(c)). This can be explained by the fact that the excitation of plasmon is performed by a high aperture objective lens and a wide range of angles in that case (we do not diaphragm the incoming beam). There is therefore a combination of incidence angles, and although the narrow range of angles corresponding to surface plasmon resonance gives an amplification of the electromagnetic field, the other incidences contribute also this field farther from the surface. When the defocus $Z$ increases these components superimpose to the evanescent field to increase its penetration depth.

In Fig. 5(b), we note that the zone surrounding the nucleus is fragmented with invaginations coming from the nuclear membrane into the cytoplasm. This cloudy pattern above the nucleus could correspond to the transgolgi network. A fluorescent staining of this zone will be done in the future to check the relevance of our observation. In the lower part of this image, the membrane of the nucleus is not surrounded by cytoplasm and it seems in direct contact with the outer cell membrane, actually this zone appears as a straight barrier delineating the nucleus. The tension of this barrier is likely to be much stronger than the nucleus membrane tension on the other side. The adhesion energy of the cell on the coverslip is high enough in the case of our gold coated samples to allow a strong linear tension on the outer cell membrane. Different treatments of the gold surface will be tested in the future to play on the tensional stress of these cells.

Progressively, details in the nucleus are revealed as $Z$ increases beyond 0.8 μm and diffraction effects superimpose to spread the response function of each detected object and additional circular fringes appear around each nucleolus. We have recently shown by solving Maxwell equations with multipolar expansions of the electric field that the optical signature of SSPM is not limited by the attenuation length of the plasmons induced in the metallic layer, but is rather diffraction limited [35]. The variation of the $Z$ parameter allows an optimization of the contrast of the image without altering the localization properties of the plasmon.

### 3.3. Phase images

As mentioned before, the $V(Z)$ signal captured by the SSPM contains also a phase information from which images can be reconstructed. Figure 6 shows the phase images corresponding to the amplitude images presented in Fig. 5. The phase response of the plasmon microscope is very different from what can be obtained with other phase microscopes, since the SPR produces on the reflectivity signal a very sharp jump in the phase at resonance [5]. This property amplifies the phase response of the dielectric medium index changes in the vicinity of the gold surface. The phase of the SSPM response is also more sensitive to external phase noise, and its measurement requires therefore a very good stability of the optical and electro-optical devices, and a correct compensation of the coverslip tilts. The four phase images shown in Fig. 6 illustrate the evolution of the phase response with $Z$, in particular the increase of their contrast with $Z$. More precisely the contrast increases drastically for $Z$ values beyond 0.5 μm. Another interesting observation is the fact that the phase can delineate more precisely zones of the nucleus with different indices, as compared to amplitude alone. The phase images bring much finer contour details on the cell membrane adhering on the gold surface (Fig. 6(b)-(d)), this contour remaining very contrasted for different $Z$ foci.

### 3.4. Influence of the beam polarization on the contrast of SSPM images

The comparison of amplitude and phase images obtained with this microscope with radial and azimuthal polarizations gives the demonstration of the interest of surface plasmon for better sensitivity and better discrimination of the cellular objects with $Z$. Indeed, in azimuthal polarization the excitation of surface plasmon is null, no field amplification can be expected, and the image contrast is diminished. In Fig. 7, we compare these two polarizations on the same zone,
Fig. 6. SSPM (X,Y) phase images corresponding to the amplitude images of Fig. 5; radial polarization. (b) \( Z = 0 \, \mu \text{m} \). (c) \( Z = 0.4 \, \mu \text{m} \). (d) \( Z = 0.8 \, \mu \text{m} \). (e) \( Z = 1.2 \, \mu \text{m} \).

3.4.1. Amplitude images

The two amplitude images shown in Fig. 7(a) and (d) have been reconstructed from two stacks of SSPM images recorded at different times for \( p \) and \( s \) polarization respectively. They cannot be exactly superimposed, however we have selected six points (colored markers) on each stack, in similar zones, corresponding to the nucleus. The evanescent field is not identical with and without plasmon excitation, therefore an exact comparison of images for a given \( Z \) focus may be an impossible task.

Below these amplitude images we report the corresponding \( |V(Z)| \) and \( \Phi(Z) \) for the same positions, and with the same color coding. The \( |V(Z)| \) of Fig. 7 curves differ markedly from radial to azimuthal polarization, since the slow mode oscillations, typical of SPR, vanish in azimuthal polarization. However, amplitude images can be obtained in azimuthal polarization and the nucleoli can also be identified in that case, though less contrasted, their contours being more difficult to distinguish from the background. When \( Z \) increases from \( Z = 0 \, \mu \text{m} \) to positive values, discriminating the nucleus from the cytoplasm is much more difficult, due to smeared nuclear contours. The ability of the microscope to span different depths and to separate objects with the vertical focus position \( Z \), depending on the amplitude of the \( V(Z) \) function has mostly vanished. The comparison of the magenta and orange \( |V(Z)| \) curves in Fig. 7 shows that despite
Fig. 7. SSPM characterization of a nuclear zone of an IMR90 fibroblast: comparing radial (left column: (a)-(c)) and azimuthal (right column: (d)-(f)) polarization modes. (a) Amplitude image for $Z = 0.6 \, \mu m$ (radial polarization). (b) $|V(Z)|$ curves corresponding to the colored markers in (a). (c) $\Phi(Z)$ curves corresponding to the colored markers in (a). (d) Amplitude image for $Z = 0.6 \, \mu m$ (azimuthal polarization). (e) $|V(Z)|$ curves corresponding to the colored markers in (d). (f) $\Phi(Z)$ curves corresponding to the colored markers in (d).
these two zones correspond to similar zones on the border of a nucleolus, their response is quite different on the $|V(Z)|$, and this difference is greater for $Z < 1.2 \, \mu m$. To interpret quantitatively the reason of these differences, we plan to combine the SSPM imaging with Atomic Force Microscopy (AFM) to have more information on the topography of the different compartments of the nucleus and to estimate the thickness and density of the nucleoli.

3.5. Phase images

In Figs. 7(c) and (f) we report also $\Phi(Z)$ curves corresponding to the same color as for $|V(Z)|$ in Figs. 7(b) and (e) respectively. The difference of the $\Phi(Z)$ in radial and azimuthal polarizations is even more striking than the amplitude. The comparison of these experimental $\Phi(Z)$ curves with the theoretical curves of Fig. 2 is very impressive, despite the fact that the model used for computing the $V(Z)$ curve is a simplification of the real optical system: we cannot know precisely neither the pupil function $P$ of the objective lens and its optical coupling with the coverslip, nor the alignment of the incoming light beam into the objective. The phase response behaves like an on and off switch, depending on the polarization, and the differences of the phase increase drastically for $Z$ larger than 0.8 $\mu m$. The modulations of the phase can be very large and give very strong contrast as illustrated in Fig. 8 for two $Z$ values (0.8 $\mu m$ and 1.2 $\mu m$) ((a) and (b) correspond to radial polarization, (c) and (d) correspond to azimuthal polarization). Note that the grey coding for the different images is not the same. The phase images in azimuthal polarization are one order lower in dynamics than in radial polarization. Although the phase images in azimuthal polarization are very noisy and the nuclear contour is very fuzzy, one can still distinguish internal bodies, but it is hard to say if they actually belong to the nucleus, and it will be very unlikely that we can extract some information on the depth of these bodies by the comparison of the two images at $Z = 0.8 \, \mu m$ and 1.2 $\mu m$.

4. Conclusion

We have shown here that surface plasmon microscopy offers the possibility to image at high resolution internal compartments of fibroblasts, since it can resolve nucleoli, nucleus, cytoplasm, cytoskeleton and internal and outer membranes without the need of exogenous contrast agents. At the best of our knowledge, it is the first time that highly resolved surface plasmon microscopy of the phase has been achieved. Two polarization modes (respectively radial and azimuthal) have been chosen since they can clearly demonstrate the importance of surface plasmon for amplitude and phase contrast enhancing. The dependence of the SSPM response versus the focus $Z$ suggests that the contrast may be adjusted by tuning $Z$, depending on the index and the size of the zone of interest. Moreover the comparison between images obtained with azimuthal (no plasmon excitation) and radial (excitation of the plasmon) polarizations clearly shows the critical role played by SPR in the improvement of the contrast. A more thorough interpretation of the behavior of the amplitude and phase response of this microscope with the phase requires a computation of the SSPM response from model index gradient objects, in three dimensions. Thanks to a three dimensional Maxwell equation model of SSPM [35], we hope to elaborate on this issue in a near future.

Finally let us emphasize that phase imaging in radial polarization improves dramatically the ability of SSPM amplitude images to detect the edges of internal cellular bodies. Since the thickness of the sample probed by the evanescent field excited by SPR depends on the focus $Z$, we expect that further developments in the processing and comparison of amplitude and phase data will allow us to get additional information on the thickness of these sub-cellular structures in the future. In particular we also plan to compare the thickness estimated from the SSPM, with that measured with AFM. All these experiments have been performed on dried cell samples, fixed with ethanol. The operation of this microscope in liquid environment is possible since we
have already performed the imaging of dielectric nanoparticles embedded in water [33,37]. We are presently working on the setting up of cell culture dishes on the SSPM stand, to work on living cells.

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