Illumination conditions in microsphere-assisted microscopy

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Abstract

White-light microsphere-assisted microscopy is a full-field and label-free imaging promising technique making it possible to achieve a sub-diffraction lateral resolution. However, the performance of this technique depends not only on the geometrical parameters but also on the illumination conditions of the optical system. In the present work, experimental measurements and computer simulations have been performed in air in order to determine the influence of the two diaphragm apertures of the Köhler arrangement and the spectral width of the light source on both the depth-of-focus of the microsphere and the optimisation of the imaging contrast. Furthermore, the super-resolution phenomenon is demonstrated and the cumulated optical aberrations are shown through the measurement of the optical transfer function for the different arrangements of the illumination part.

Introduction

In classical optical microscopy, the size of the smallest discernible object which can be resolved depends on the nature of light, the central wavelength $\lambda_0$ of the illumination light source, and the numerical aperture $NA$ of the microscope itself. In incoherent imaging, the lateral resolution can be defined as:

$$\delta_{x,y} = \kappa \frac{\lambda_0}{NA}$$  \hspace{1cm} (1)

where the factor $\kappa$ is 0.5 using a broadband light source [1]. This implies that an object with a feature size smaller than 300 nm can therefore not be resolved in air. Several imaging techniques for overcoming the physical barrier of the diffraction of light have recently aroused considerable interest. For example, optical nanoscopy was rewarded with the Nobel Prize for Chemistry in 2014 for high-resolution fluorescence microscopy [2].

As early as the 17th century, i.e. the early days of microscopy, miniature glass spheres were employed by van Leeuwenhoek to visualize the first bacteria [3]. These millimetre-diameter spheres allowed the system to reach a lateral resolution of around 1 µm. In 2011, a super-resolution imaging technique requiring spheres of micrometre size was reported [4]. Microsphere-assisted microscopy now allows a lateral resolution of 50 nm to be reached, depending on optical (illumination wavelength and refractive index of both the microsphere and the surrounding medium) and geometrical (size of the microsphere) parameters. Furthermore, it presents the advantages of being label free and providing a high resolution in the full field [5] in comparison to other far-field imaging techniques such as those based on spatial filtering [6], the Pendry hyperlens [7], the
Figure 1: Generation of the virtual image VI of the object OB through the microsphere MS in microsphere-assisted microscopy. An objective lens, placed above MS, collects the magnified VI. 2-column fitting figure

scanning photonic jet [8], the scattering lens [9] or the submerged microsphere [10]. In addition, microsphere-based ultramicroscopy only requires the use of a classical optical microscope. By placing a glass transparent microsphere on or above an object, a magnified virtual image appears below the object, as shown in Fig. 1. A microscope objective then collects and directs the image onto a camera. Although the phenomenon of microsphere-assisted microscopy is still not fully explained and the claimed resolution values are debatable [12], several experimental results and computer simulations have not only determined the role of evanescent waves [13], the influence of the surrounding medium [14, 15], and the effect of the coherence [16, 17], but also improved the field of view using stitching techniques [11, 18]. Moreover, microsphere-assisted microscopy has been successfully combined with interferometry for the 3D topography reconstruction of nanostructures [19, 20, 21, 22] and for the quantitative phase measurement of cells [23].

Nevertheless, the illumination conditions of white-light microsphere-aided ultramicroscopy have not yet been considered in detail although they play a key role. In this Letter, the influence of both the opening of the diaphragm aperture of the Köhler arrangement and the spectral bandwidth of the light source on the imaging contrast optimization and the control of the imaging depth of the microsphere is studied in microsphere-assisted microscopy for the reflection configuration. For this purpose, the contrast transfer function (CTF) of the imaging system and the depth of focus (DOF) of the microsphere are quantitatively evaluated by placing a glass microsphere in air. The different illumination conditions showed an influence on the CTF due to a potential generation of optical aberrations.

Materials and Methods

A commercial Zeiss optical microscope (Axio Scope.A1) was used for the experimental measurements where the layout is illustrated in Fig. 2(a). The illumination part of the microscope is a continuous-spectrum halogen lamp (central wavelength $\lambda_0 = 650$ nm and bandwidth $\Delta \lambda = 400$ nm) combined with a Köhler arrangement with three focusing lenses, a condenser diaphragm CD and a field diaphragm FD. Furthermore, to experimentally determine the impact of the temporal coherence on the image contrast and the DOF of the microsphere, the spectral width of the light source can be limited using wavelength filters: a cyan filter (SP585/27, MIDOPT, $\lambda_0 = 567$ nm and $\Delta \lambda = 90$ nm) or a blue-line filter (FL441.6-10, Thorlabs, $\lambda_0 = 445$ nm and bandpass of 11 nm). Then, a beam-splitter cube directs the angular incident beam through the microscope objective (Epi plan, Zeiss). The objective has a magnification factor of 50 and a NA of 0.55, giving a theoretical lateral resolution $\delta_{x,y}$ of 593 nm [1]. An object having features smaller than $\delta_{x,y}$, e.g. an array of oval-shaped silver nanodots of 200 nm by 300 nm in size and separated periodically by 200 nm and 300 nm (Fig. 2(b)), can thus not be resolved with the microscope alone as shown in Fig. 2(c) where the objective is focused on the object outside a microsphere. Therefore, a soda-lime-glass microsphere with a diameter of 26 $\mu$m was placed on the elliptic dimers, generating a magnified virtual image (Fig. 2(d)). The size of the glass microsphere provides a good compromise between lateral resolution improvement and lateral field of view. Finally, the highly-resolved virtual image is recorded by the camera (AxioCAM ICC 3, Zeiss) through the tube lens.
In order to retrieve the DOF of the microsphere, the axial intensity distribution generated by the microsphere is sampled by displacing the object and the microsphere along the optical axis using a nano-positioning device (P-611.3S Nanocube, Physik Instrumente). The depth of field of the microscope objective ($\delta_z = 1 \, \mu m$) defines the axial sampling of the measurements. It should be noted that moving both the microsphere and the object does not change the axial intensity distribution due to the collimated beam incident on the microsphere.

The DOF of the microsphere is estimated by calculating the full width at half maximum (FWHM) of the axial contrast curve by analogy with the MTF50 sharpness-perception criterion in photography [24]. 2D rigorous electromagnetic simulations using the finite element method (COMSOL Multiphysics) accompany the experiments in order to help us in understanding the results. The simulation consists of two steps [17]. Firstly, the electromagnetic propagation of the waves emitted by an object placed underneath the microsphere
is calculated. Then, a time-reversed propagation, i.e. in the opposite direction, in free space, allows the retrieval of the virtual image of the object. To calculate the results for temporally incoherent illumination, the numerical operations are repeated over the wavelength spectrum of the light source, i.e. $\lambda$ ranging from 400 nm to 800 nm with an increment of 100 nm, followed by the summing of the back-propagated intensity distributions. In the computing simulations, the DOF is considered as the FWHM of the axial point spread function (PSF) of the microsphere [25].

**Results**

Several virtual images of the nanodots were thus recorded at different axial positions and the imaging contrast was calculated for each intensity slice. The resulting evolution of the imaging contrast is represented in Fig. 3(a) using the white-light illumination for two openings of the field diaphragm. At the best imaging contrast position, i.e. around 50 $\mu$m below the sphere, the measurements show that an increase in value of 70% is achieved by closing the field diaphragm. The virtual images when the two irises of the Köhler

![Figure 3: (a) Experimental measurements of the normalized contrast of the virtual images of dimers at different axial positions when the field diaphragm FD of the Köhler setup is open and closed. The 26-μm-diameter microsphere having a refractive index of 1.52 is illuminated by the white-light source. Its depth of focus is then determined as the length during which the relative contrast is higher than 50%. (b) Virtual images at the best contrast position when (b.i) the condenser diaphragm CD and the FD are widely open, when (b.ii) only CD is closed and when (b.iii) both the CD and the FD are in the closed state. White scale bars represent 5 $\mu$m in the image plane of the microscope. At this axial position, the microsphere performs a magnification of 4.7. (c) Numerical estimation of the depth of focus of the microsphere using the FWHM of the axial intensity point spread functions from two-point sources. The white-dotted line gives their axial position. 2-column fitting figure](https://example.com/fitting-figure.png)
illuminations are open (contrast of 5%), when the condenser diaphragm alone is closed (contrast of 18%), and when the two diaphragms are closed (contrast up to 30%) are shown in Fig. 3(b.i),(b.ii) and (b.iii), respectively.

In addition, closing the field diaphragm of the Köhler arrangement affects the incident illumination cone and, consequently, the DOF of the microsphere. Reducing the incident beam slope allows an increase in the DOF from 32 µm, when the field diaphragm is widely open, to 88 µm, when it is closed. The DOF of the microsphere also depends on the spectral width of the source, decreasing for shorter spectral bandwidths from 88 µm to 50 µm when the width of the spectrum of the halogen lamp is limited by the cyan filter, and to 31 µm using the blue-line filter. This implies that the focus adjustment of the Zeiss optical microscope can be performed along the long DOF of the microsphere. As a matter of fact, the collection of virtual images can be achieved along this spread axial distance as represented in the dotted rectangle in Fig. 2. A numerical model of microsphere-assisted microscopy made it possible to highlight this optical effect, as shown in Fig. 3(c). The object was considered as two emitting point sources placed against the microsphere and separated laterally by 400 nm. In this case, the FWHM of the axial DOF equals 28 µm in the central area of the image. Furthermore, the FWHM of the lateral PSF is 195 nm.

Heretofore, the imaging contrast was estimated along the optical axis using a single-spatial-frequency object. The investigation was further pursued by substituting the array of nanodots for Ronchi rulings placed at the axial position of 50 µm. Fabricated by FIB etching at the MIMENTO Technology Centre (FEMTO-ST institute, Besançon, France), the periodic Ronchi targets consist of square edge profiles allowing the measurement of the contrast transfer function (CTF) of the super-resolution imaging system [26]. The period of the Ronchi rulings varies from 5 µm to 200 nm and its contrast is one. Figure 4 shows the frequency responses of the whole imaging system according to different parameters and conditions.

Without the microsphere, the CTF of the optical microscope is only limited by diffraction of light and the cut-off frequency equals 1.74 lines per µm, corresponding to the cut-off frequency of the objective lens alone (see Eq. 1). Placing the microsphere on the sample allows the lateral resolution to be doubled, performing thus sub-diffraction-limited imaging (Fig. 4(a)). Indeed, despite the contrast of optical microscopy being higher than the contrast of microsphere-assisted microscopy for low frequencies, the cut-off frequency reaches 4.98 lines per µm, i.e. δx,y = 201 nm. However, the CTF curve of the super-resolution imaging system appears now to be deformed. A possible explanation may be the generation of spherical aberrations due to the perfectly-spherical shape of the microsphere. Moreover, the numerical analysis of the frequency response of the microsphere alone, represented by the blue dotted line in Fig. 3(a), allows also the retrieval of this effect. The frequency response modulus of the microsphere alone was calculated with the Fourier transform the lateral PSF, i.e. the image of one point source through the microsphere alone.

Figure 4(b) represents the evolution of the CTF of the super-resolution system by placing the cyan filter in the illumination part and by opening or closing the field diaphragm. By comparing the CTF curves of the microsphere-assisted microscope (red curve in Fig. 4(a) and blue curve in Fig. 4(b)), reducing the central wavelength and the bandwidth of the illumination provides a decrease in the contrast for high frequencies (longer than 1.0 lines per µm) and eases the distortion of the CTF evolution. This could be explained by the reduction in chromatic aberrations. Nonetheless, closing the field aperture gives an enhancement of the imaging contrast, e.g., the contrast of an object having a spatial frequency of 2.5 cycles per µm increases by 68% which is equivalent to the results in white light in Fig. 3(a) where the period of the nanodots equals 400 nm. In this work, the microsphere diameter, i.e. 26 µm, remains larger than the minimal incident beam diameter, as shown in Fig. 3(c), unlike the experiments reported in [11] [19].
Conclusion

In this letter, we have investigated the influence of the illumination conditions of white-light microsphere-assisted microscopy in reflective mode. The imaging contrast and the depth of focus of the microsphere have been studied according to the illumination cone angle by openings the diaphragms of the Köhler illumination, and to the bandwidth of the light source. It has been shown that reducing the spectral bandwidth of the Köhler arrangement improves the contrast and, further, increases the depth of focus of the microsphere. Furthermore, the super-resolution phenomenon has been shown by measuring the transfer function of the super-resolution imaging system. Indeed, using a 26-µm-diameter microsphere in air results in a lateral resolution of 200 nm, corresponding here to an equivalent numerical aperture of 1.61. Finally, the accumulation of the optical aberrations on the imaging contrast has been highlighted.
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References


