Super-resolution endoscopy for real-time wide-field imaging

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Abstract: Resolving subcellular structures in vitro beyond optical diffraction barrier by a light microscope has achieved significant development since the advancement of super-resolution fluorescence microscopes, such as stimulated emission depletion (STED) microscopy, stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM). However, the resolution of observation in deep and dense in vivo tissues is still confined to cellular level presently, and hence, exploring image details at subcellular level or even beyond organelle level in vivo has continued to attract much research attention. Currently, endoscopy provides an effective way to achieve in vivo observations and is compatible with mature optical microscopy technologies, but its resolution is usually confined to ~1 µm. Here we report a new endoscopy method by functionalizing graded-index (GRIN) lens with microspheres for real-time white-light or fluorescent super-resolution imaging. The capability of resolving objects with feature size of ~\(\lambda/5\), which breaks the diffraction barrier of traditional GRIN lens based endoscopes by a factor of two, has been demonstrated by using this super-resolution endoscopy method. Further development of such a super-resolution endoscopy technique may provide new opportunities for in vivo life sciences studies.

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References and links
1. Introduction

Investigation of deep live tissues based on high- or super-resolution optical imaging are limited to in vitro cell culture or tissue slices [1] due to scattering and absorption induced attenuation of light propagation [2]. By taking the advantages of penetration ability of near-infrared light and objectives with high numerical aperture (NA), two-photon fluorescence microscopy enables three-dimensional tissue imaging as deep as 500 – 1000 µm [3,4] with resolution of cellular level [3–6]. In order to extend the penetration depth of two-photon fluorescence microscopy, several strategies have been proposed, such as enhancement of the fluorescence signals generation, improvement of optical signal collection and use of endoscope [2]. Among them, endoscope based approaches provide an effective way to achieve in vivo observation deeper than 1000 µm and are compatible with matured optical microscope technologies, such as wide-field epifluorescence, laser-scanning confocal and
two-photon fluorescence [1,7–10]. By using these microscopy methods and combining them with gradient refractive index (GRIN) lenses, which are commonly used in endoscopy, in vivo fluorescence imaging in muscles [11], esophagus [9], vasculature [9], trachea [9], thalamic [1] and hippocampus [10] has been achieved. Generally, the NA of GRIN lenses is smaller than 0.6 [10], which means a resolution barrier of $0.6\lambda/\text{NA}=\lambda$, according to Rayleigh criterion, where $\lambda$ is the illuminating wavelength. Improved resolution, $\sim 0.72\lambda$ (NA = 0.6), can be obtained when two-photon microscopy is applied. However, the actual image resolution is confined to $\sim 1.6 \, \mu m$ [1,2,4,7–12] since spatial aberration degrades the effective NA of GRIN lenses [10,12]. By introducing plano-convex lenses with high NA of $\sim 0.8$ into GRIN based endoscopy and correcting aberrations to enhance the excitation and collection of signals, Barretto et al. improved in vivo two-photon fluorescence imaging resolution to $\sim 1 \, \mu m$ [10]. Though the resolution has been improved by almost two times, resolving subcellular structures beyond optical diffraction limit [13] is still a problem. Since optical super-resolved fluorescence microscopy, such as stimulated emission depletion (STED) microscopy [14], stochastic optical reconstruction microscopy (STORM) [15] and photoactivated localization microscopy (PALM) [16], have achieved success in breaking the diffraction limit in the far field for in vitro observations, resolving structures at subcellular level or even beyond organelle level in deep live tissue has attracted research attention recently. Gu et al. demonstrated that STED can be combined with fiber-optical endoscope (NA = 0.35) with an azimuthally-polarized beam and achieved fluorescent imaging resolution of 310 nm in vitro and its resolution relied heavily on the NA of endoscopic probes [6].

In the past few years, studies have shown that dielectrics with specific morphology have the capability to provide image resolution beyond diffraction limit [17–24]. Lee et al. demonstrated that high resolution imaging, i.e., $\lambda/2.2$ ($\lambda = 472\text{nm}$), can be obtained by nanoscale dielectric plano-spherical convex lenses due to the curvilinear trajectories of light that induced significantly short near-field focal lengths [17]. Wang et al. reported the observation of a gold-coated anodic aluminum oxide membrane by SiO$_2$ microspheres with refractive index (n) of 1.46 (2µm<diameter<9µm) and pushed the lateral resolution to 50 nm ($\lambda/14$ at $\lambda = 750 \, \text{nm}$) [18]. Observations of subcellular structures like centrioles, mitochondria, chromosomes [22] and 75-nm adenoviruses [24] have also been demonstrated by this microsphere based super-lens. Researchers have also proved that the super-resolution capability of microsphere can be enhanced by semi-immersing microspheres in liquid [25].

In this study, we report for the first time that the microsphere based super-resolution imaging capability can be introduced into GRIN lenses based endoscopy systems to overcome the optical diffraction limit of traditional GRIN lens based endoscopes. Our in vitro experimental results demonstrated that resolving objects with feature size of $\sim \lambda/5$ (which shatters the diffraction barrier by a factor of two) is possible by simply attaching microspheres to the proximal end of an endoscope to observe nanoparticles. Experimental data from super-resolved white-light imaging also shows that this endoscopy system is not limited to fluorescent applications.

2. Methods

2.1 Endoscope, microsphere, sample and microscopy

Two different kinds GRIN lens based endoscopes were used, i.e., singlet and doublet probes (Fig. 1). The singlet probes have different parameters: 1.8 mm-diameter GRIN lenses (0.46 numerical apertures, 1 pitch, Feteco) and 1.0 mm-diameter GRIN lenses (0.11 numerical apertures, 1 pitch, Gofoton). The doublet probes are composed by fusing a 0.42 numerical aperture, 0.28 pitch GRIN objective lens to a 0.11 numerical aperture, 1 pitch GRIN relay lens (Gofoton). The barium titanate glass microspheres with diameter of 30 – 100 µm were obtained from Cospheric. The GRIN lens, microsphere and sample are contact by applying pre-stress on them. The fluorescent nanoparticles with diameter of 100 nm, which have
emission maximum wavelength of 515 nm and excitation wavelength of 505 nm, were obtained from Life Technologies (F-8803). AFM-scanned results were obtained by Dimension 3100 (Veeco, Inc). The control results shown in Fig. 2(b) was imaged by a high-resolution optical digital microscope with magnification of 3500 × using a 0.5 numerical aperture objective lens (OL-350II, KH-7700, Hirox, Japan). For white-light imaging by super-resolution endoscope, an OPTEM optical microscope with magnification of 5 × (NA = 0.09) or 8 × (NA = 0.15) was used (Zoom 160, OPTEM, USA), which was mounted with a charge-coupled device (CCD, DH-SV1411FC, DaHeng, China). The blue color of the images shown in Figs. 2(c), 2(d) and Fig. 3 is due to an improper color adjustment of the CCD. Figures 2(e) and 2(f) were transformed to gray image by an open source software (ImageJ). For fluorescent imaging, a Nikon microscope (Ti-U Nikon, Japan) fitted with a 10 × objective lens (NA = 0.30, Plan Fluor) and a CCD of DS-Fi1c (Nikon, Japan) was utilized. The additional transmission illumination was generated by a LED light source (LFP-10WP-R, SHIBUYA, Japan).

2.2 Simulation method

Simulation results were calculated by Lumerical FDTD Solutions. To simplify the simulation model and concentrate on studying the influence arising from the coupling between microsphere and GRIN lens, the GRIN lens was treated as a uniform material with refractive indexes of 1.60 in the simulation. Plane wave illumination is used to simplify the illumination condition generated by the endoscopy due to 1) the small diameter of the microsphere compared with endoscope, and 2) the microsphere is located at the middle area of the end of GRIN lens. The refractive indexes of BTG microsphere, medium (water) used in these simulations are 1.90 and 1.33, respectively. The wavelength of incident light is 600 nm.

3. Results

Figure 1(a) provides a schematic of microsphere based super-resolution endoscope which is constructed by attaching barium titanate glass (BTG) microspheres with high-refractive index of ~1.90 to the proximal end of the endoscope. To initiate imaging, the endoscope was inserted into water and making the microsphere to be in contact with the sample and GRIN lens. For imaging in reflection mode, the GRIN lens bridges a light path for transmission illumination or super-resolved magnified virtual images generated by microspheres that
project the samples’ near-field sub-diffraction limit details into far-field. Single GRIN lens (NA = 0.46 or 0.11) and doublet endoscope probes composing of an objective (NA = 0.42) and a relay lens (NA = 0.11), as shown in Fig. 1(b), were both used to construct the super-resolution endoscopes. To demonstrate our concept, samples were imaged by white-light and fluorescence modes, respectively.

3.1 White-light imaging by super-resolution endoscopy

In these experiments, digital versatile disc (DVD) consisting of ~450 nm width and ~110 nm height stripes spaced by ~270 nm gaps as shown in Fig. 2(a) was first imaged by a digital microscope with high resolution and magnification of 3500 × without use an endoscope; (c) and (e) DVD surface image by a conventional white-light microscope through a GRIN lens based endoscope; (d) and (f) DVD surface image by a conventional white-light microscope through a microsphere based super-resolution endoscope. The singlet GRIN lens used in (c) and (d) corresponds to the second endoscope probe in Fig. 1(b), which has diameter of 1 mm, length of 1 pitch and NA of 0.11. The singlet GRIN lens used in (e) and (f) is the first endoscope probe in Fig. 1(b), which has diameter of 1.8 mm, length of 1 pitch and NA of 0.46. The diameter of imaging microsphere used in (d) is ~92 µm.

In these experiments, digital versatile disc (DVD) consisting of ~450 nm width and ~110 nm height stripes spaced by ~270 nm gaps as shown in Fig. 2(a) was first imaged by a digital microscope with high resolution and magnification of 3500 × [Fig. 2(b)] as control. For the case of reflection mode white-light imaging by super-resolution endoscopy, the objective lens of a conventional optical microscope focused illumination near the distal end (top surface) of endoscope probes as shown in Fig. 1(a), which refocused illumination on the sample surface or the virtual image plane of microspheres. Without attachment of microspheres, each endoscope used in our experiments cannot resolve DVD morphology details [Figs. 2(c) and 2(e)] because of the diffraction limit. For the white-light illumination with peak wavelength of 600 nm, the Rayleigh criterion confined best resolutions are 3327 nm, 871 nm and 796 nm for GRIN lenses with NA of 0.11 [Fig. 2(c)], 0.42 [Fig. 3] and 0.46 [Fig. 2(e)] in air, respectively. In water, these diffraction-limited resolutions can be improved to about 2502 nm, 655 nm and 598 nm. However, when the microsphere-functionalized endoscopes were used, ~270 nm gaps on DVD surface could be resolved by collecting magnified virtual images generated by the microspheres [Figs. 2(d), 2(f) and Fig. 3]. In these observations,
diffraction-limited resolution barrier is shattered by a factor of ~2 (for GRIN lens with NA = 0.46) to ~9 (for GRIN lens with NA = 0.11) with magnification of 1.6 × to 6.8 ×. Comparing Fig. 2 and Fig. 3, the results obtained by microsphere-functionalized doublet endoscopes are better than those of singlet ones, which can be explained by the fact that imaging property of GRIN lens could be influenced by its pitch and the coupling condition between an endoscope and a microscope [12]. There is no apparent distortion observed by using this super-resolution endoscopy, which could be attributed to the positive and negative spherical aberration compensation of microsphere [26].

![Fig. 3. Microsphere-functionalized doublet endoscopy imaging in white-light mode. DVD surface was imaged by a conventional white-light microscope through a microsphere-functionalized doublet endoscope probe composed by fusing a 0.42 numerical aperture, 0.28 pitch GRIN objective lens to a 0.11 numerical aperture, 1 pitch GRIN relay lens, which corresponds to the third probe shown in Fig. 1(b). The diameter of imaging microsphere is ~55 µm.](image)

3.2 Fluorescent imaging by super-resolution endoscopy

Fluorescence microscopes are widely used in life science studies due to its many applications, including detecting specific molecules and live-cell imaging [13]. To explore the fluorescent imaging capability of the microsphere-functionalized super-resolution endoscope, we selected 100 nm fluorescent nanoparticles as observation samples as shown in Fig. 4(a). Results of imaging through a single compound doublet endoscope and a microsphere-functionalized super-endoscope are presented in Figs. 4(b) and 4(c), respectively. Considering the diffractive limit, the Rayleigh criterion confines the best resolution of the compound doublet endoscope probe, whose NA is determined by the objective GRIN lenses [12], to ~562 nm in water at wavelength of ~515 nm; this restricts the system from resolving single 100 nm fluorescent nanoparticle as shown in Fig. 4(b). When the super-resolved capability of the microsphere is introduced to the system, a single nanoparticle (with diameter of ~100 nm) could be distinguished from others as shown in the magnified virtual image generated by the microsphere in Fig. 4(c). This magnified virtual image cannot be directly used to estimate the total system resolution. Thus, we first calibrated the microsphere based super-resolution endoscope by observing 100 nm fluorescent particles dispersed on a DVD surface whose feature size can be measured by an AFM scanning image as shown in Fig. 2(a). An additional white light transmission illumination was introduced to the optical system as shown in Fig. 1(a) to make observation of fluorescent and non-fluorescent objects simultaneous [Fig. 4(d.1)] or separately [Figs. 4(d.2) and 4(d.3)]. Figure 4(d.2) shows the imaging of DVD surface under transmission illumination, which was used to calibrate the microsphere based super-resolution endoscope. Figure 4(d.3) shows the fluorescent imaging of 100 nm
fluorescent particles without introducing additional transmission illumination. The calibrated results shown in Fig. 4(d.3) demonstrated the full width at half maximum (FWHM) of the 100 nm particle images has a value of 290 ± 13 nm, which overcomes the diffraction-limited resolution barrier of traditional GRIN lens based endoscope by a factor of ~2.

Fig. 4. Microsphere based super-resolution endoscopy imaging in fluorescence mode. (a) Upper: AFM-scanned image of 100 nm fluorescent nanoparticles. Lower: the cross section image of upper image marked by dotted dark line. (b) 100 nm fluorescent nanoparticles imaged by a conventional fluorescent microscope through a doublet endoscope probe. (c) 100 nm fluorescent nanoparticles imaged by a microsphere-functionalized super-endoscope as described in this paper. The diameter of this imaging microsphere is ~80 µm. The objective lens of fluorescent microscope used in (b) and (c) has a magnification of 10 × (NA = 0.30). The endoscope used in (b) and (c) corresponds to the third probe illustrated in Fig. 1(b), which is composed by fusing a 0.42 numerical aperture, 0.28 pitch GRIN objective lens to a 0.11 numerical aperture, 1 pitch GRIN relay lens. (d) Images used to calibrate the system, which were obtained by combining an optical microscope and a super-resolution endoscope. (d.1), (d.2) and (d.3) share the same scale bar as shown in (d.3).

4. Discussion

For the super-resolution endoscope presented in this paper, a microsphere is made to be in contact with a GRIN lens. To study the influence of the contact area on the imaging performance of a microsphere, we conducted numerical calculations to simulate the $|E|^2$ intensity distributions around a microsphere as shown in Fig. 5 and a microsphere foci properties as shown in Fig. 6. From Figs. 5(a) and 5(b), attaching a microsphere to GRIN lens has negligible influence on the spatial distribution of $|E|^2$ intensity and therefore the near-field focusing effect of the microsphere; hence, having a GRIN lens in contact with a microsphere will not induce deterioration of super-resolution capability of the microsphere. Quantitative calculations of the FWHM and the distance between the center of microsphere and focus spot further demonstrate that the effect of contact area is negligible [Fig. 6(b)]. The focus spot size, which is related to FWHM, increases with the diameter of microsphere, but is below the
Rayleigh diffraction limit when the microsphere diameter falls into the range of 10 – 90 µm; some researchers believe that this sub-diffraction-limit focus spot size enables microspheres to have super-resolution capability [18,27]. Compared with the single microsphere case (i.e., the GRIN lens is not used), microsphere based super-resolution endoscope enhances the maximum intensity of focus by almost 20% [Fig. 5(a)], which could observe more near-field sub-diffraction limit details from the transformation of evanescent waves to propagating waves based on Lee et al.’s analysis [28].

![Image](https://via.placeholder.com/150)

Fig. 5. Contact of microsphere and GRIN lens induces difference in $|\langle E \rangle|^2$ intensity distribution. The $|\langle E \rangle|^2$ intensity (V$^2$/m$^2$) distributions simulated for a single microsphere (a.1), (b.1) and a microsphere attached to a GRIN lens (a.2), (b.2). D is the distance between the center of microsphere and focus spot.

Different from other optical super-resolved microscopes, the capability of breaking the diffraction barrier by microspheres shows little dependence on the numerical aperture of objective lenses due to its increased acceptance cone [19]. This characteristic allows the combination of microspheres and GRIN lenses with low numerical aperture to endow real-time super-resolution capability to endoscopy. Microspheres with diameter of 2 – 200 µm have already been demonstrated to project sub-diffraction limit details into far-field and the field of view (FOV) increases linearly with diameter (FOV > 30 µm when diameter > 200 µm) [20]. As a note, only the light path through the endoscope and the microsphere can realize super-resolution imaging, and therefore, the final FOV is confined by the FOV of the microspheres in our experiments. Hence, the method discussed here could serve as a flexible technique to modify endoscopes with commonly used diameter of 0.35 – 2 mm [12] in order to obtain proper FOV for real-time super-resolution imaging.
5. Conclusion

In conclusion, our findings have demonstrated a new super-resolution endoscopy method that can be realized without using a complex or bulky microscope system in both white-light or fluorescent modes, i.e., this method requires only the attachment of a microsphere on the end of a GRIN lens based endoscope. Shattering the diffraction barrier by a factor of two by resolving objects with feature size of $\sim \lambda/5$ has been demonstrated by using this super-resolution endoscopy method to resolve 100 nm nanoparticles in fluorescent mode. This new endoscopy method has potential applications of in vivo life sciences studies in deep live tissue to resolve details at subcellular level or even beyond organelle level.

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