FULL ARTICLE

Wide-field hyperspectral 3D imaging of functionalized gold nanoparticles targeting cancer cells by reflected light microscopy

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We present a new hyperspectral reflected light microscopy system with a scanned broadband supercontinuum light source. This wide-field and low phototoxic hyperspectral imaging system has been successful for performing spectral three-dimensional (3D) localization and spectroscopic identification of CD44-targeted PEGylated AuNPs in fixed cell preparations. Such spatial and spectral information is essential for the improvement of nano-plasmonic-based imaging, disease detection and treatment in complex biological environment. The presented system can be used for real-time 3D NP tracking as spectral sensors, thus providing new avenues in the spatio-temporal characterization and detection of bioanalytes.

1. Introduction

Recent years have seen tremendous progress in the development and characterization of noble metallic nanostructures for bio-applications. Since gold plasmonic nanoparticles (AuNPs) are photostable, water soluble and biocompatible in vitro and in vivo, they are widely used as optical biomarkers in biological and biomedical applications for detection of chemical and biological analytes, imaging of cells and tissues, detection and treatment of cancer cells [1]. Determination of NP concentration, spatial three-di-

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3D image of the distribution of functionalized AuNPs attached to CD44-expressing MDA-MB-231 human cancer cells.
quantum dots (QDs) are mostly detected by reflected light microscopy [3]. However, every reflected light microscopy configuration should ensure minimization or elimination of the “parasitic” reflected excitation wavelengths from substrate, sample medium, and substrate-sample medium interface. For example, applying dichroic optics and optical spectral filtering, one can spectrally separate excitation and emission spectra when using quantum dots or fluorophores as biomarkers. In plasmonic NPs imaging, spectral filtering is impossible since detected NPs extinction or scattering signal is of the same spectra as illumination. In this case, necessary signal-to-noise ratio for NPs biomarkers visualization is provided by a dark-field microscopy [4]. This optical method is based on the spatial separation of illumination and detection region leading to the intrinsic limitation on the numerical aperture (NA) of the used objective (NA_{objective} < NA_{condenser}).

In this article, we propose a very simple but efficient reflected light microscopy mode for NPs imaging. The approach allows application of high NA objectives improving NPs spatial localization. However it requires using immersion objectives to assure high NPs contrast by minimizing backscattering light. Reflected light mode is especially interesting for NPs-cell complex imaging because in the backscattering mode, cells generally have a much lower backscattering than NPs.

We designed a reflected light hyperspectral imaging system for spectral NPs characterization at the single-particle level using a tunable filter to sweep the supercontinuum illumination source over a spectral range from 400–1000 nm. This system offers wide-field imaging combined with spectroscopic characterization, 3D localization of AuNPs and low phototoxicity due to the narrow irradiation spectrum. Hyperspectral imaging of AuNPs functionalized with monoclonal antibodies against human CD44 was performed on CD44-expressing (CD44⁺)-MDA-MB-231 human breast cancer cells to confirm the efficiency of the developed set-up.

2. Experimental

2.1 Hyperspectral reflected light microscopy

Hyperspectral imaging system for wide-field and spectral examination of plasmonic NPs was built on the basis of an inverted Eclipse Ti microscope (Nikon, Mississauga, ON) equipped with a 100× oil immersion objective (numerical aperture (NA) 0.5–1.3, Nikon) and darkfield condenser (NA = 0.9, Nikon) for conventional darkfield imaging. This system was designed specifically for this project and works on the principle of reflected light microscopy with illumination provided by a supercontinuum white light laser (Fianium SC450-1, Southampton, UK). The quasi-continuous output from 460 to 1800 nm (Figure 1A) is delivered into a laser line tunable filter (Photon etc., Montréal, QC) for spectral filtering and scanning in the range from 400–1000 nm with a spectral width of about 2.5 nm. The fast scanning time from min to max (600 ms) allows spectral tuning and hyperspectral real-time imaging. The light delivery from the tunable filter to the 90:10 beam splitter (Thorlabs, Newton, NJ) is performed using a liquid light guide with 3 mm core diameter. Series of backreflected images at different spectral range were recorded with a low noise 16 bit CCD camera (ORCA-R2, Hamamatsu Photonics K.K., Japan) and transferred to a computer for further analysis by custom-written LabView software (National Instruments, Austin, TX). Speckle reducer (Optotune AG, Switzerland) improved spatial light distribution in imaging displays [5].

2.2 PEGylated AuNPs targeting CD44-expressing cancer cells

We target cancer stem cells (CSCs) since these cells have the capacity to self-renew, give rise to hetero-
geneous progeny, and migrate and invade into surrounding tissues [6]. CD44 have been targeted with many nanomaterials since this cell surface receptor is expressed strongly by CSCs in breast, bone, colorectal, prostate, pancreas, head and neck cancers [7]. Since MDA-MB-231 cells express strongly CD44 (87% of cells) [8], we used this human breast cancer cell line with functionalized AuNPs targeting specifically CD44 [9]. Rat anti-human CD44 monoclonal antibodies (Hermes-1, 150 kDa, abcam, Toronto, ON) were tethered to AuNPs (50 μg/mL, 100 nm diameter, Nanopartz, A11-100, Loveland, CO) using heterobifunctional poly(ethylene glycol) (PEG) linkers by using the strong covalent binding between sulfur and gold [10]. The anti-CD44 antibodies Hermes-1 were added to a solution of orthopyridyl disulfide-PEG-N-hydroxy-succinimide (OPSS-PEG-NHS, 5kDa, Nanocs, New York, NY) in aqueous Na₂CO₃ 10 mM pH 8.5 (100 μg/mL, antibodies/OPSS-PEG-NHS molar ratio: 1:1.88). This PEG-antibodies solution was mixed by vortexing and kept at 4 °C during 3 h. Aliquots of the solution were kept at –20 °C until use. AuNPs were prepared following a previously reported procedure [9] with modifications. An aqueous solution of citrate-coated AuNPs (2 mL) was treated with Na₂CO₃ 10 mM pH 8.5 (212 μL) and the solution of PEG-antibodies (10 μL) at 4 °C during 1 h. Then, an aqueous solution of 50 μM PEG-SH (5kDa, 247 μL, Sigma-Aldrich, Oakville, ON) was added to the suspension to block the remaining free sites on the gold surface at 4 °C during 1 h. The suspension was purified by centrifugation at 5000 rpm during 2 min. The supernatant was removed and replaced with phenol-free Dulbecco’s modified Eagle’s medium (DMEM, 2 mL) containing 4.5 g/L D-glucose, 584 mg/L L-Glutamine, 110 mg/L sodium pyruvate and supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin, 1% PS, Life Technologies).

MDA-MB-231 human breast cancer cells were seeded onto glass bottom dishes (1 × 10⁵ cells, MatTek, Ashland, MA) and grown in DMEM supplemented with 1% PS and 10% fetal bovine serum (FBS, Life Technologies) in a humidified incubator at 37 °C under a 5% CO₂ atmosphere. When cells reached 80% confluence, they were washed once with phosphate-buffered saline (PBS, Sigma-Aldrich). The cells were then incubated with 8 μg/mL CD44-targeted AuNPs in DMEM supplemented with 1% PS and 100% FBS in the 37 °C incubator during 3 h. Cells were then washed three times with PBS to remove unbound NPs, fixed with ice-cold methanol during 10 min at room temperature and washed three times with PBS.

### 3. Results and discussion

#### 3.1 Darkfield and reflected light microscopy of functionalized AuNPs targeting MDA-MB-231 cells

To illustrate particularities and advantages of reflected light microscopy for spatial localization and chromatic characterization of functionalized AuNPs targeting a cancer biomarker in a cellular environment, comparative tests were performed by conven-

![Figure 2](image-url)

**Figure 2** Microscopy images of fixed CD44⁺-MDA-MB-231 cells incubated with CD44-targeted AuNPs taken at different planes along the optical z-axis (1 μm distance step) in darkfield illumination mode (A1–4) and in reflected light imaging mode (B1–4). For better clarity an embossing filter is applied for images A2, A4, B2, B4.
tional darkfield microscopy. Series of images of 100 nm CD44-targeted AuNPs attached to MDA-MB-231 cells at two different planes along the optical $z$-axis with 1 μm step obtained by darkfield microscopy are presented in the Figure 2(A1–A4). For better clarity an embossing filter is applied for images A2, A4, B2, B4. AuNPs were readily detected and easily identified. However, very slight differences in NPs contrast could be found for 1 μm $z$-translations.

The same cells–AuNPs complex was visualized at the same $z$-positions by the reflected light microscopy at 550 nm to insure optimal NP backscattering detection (Figure 2(B1–B4)). The oil immersion objective is important to eliminate reflection by the glass substrate. Obtained results reveal important improvements for AuNPs visualization in the cellular environment in comparison to darkfield microscopy. Imaging with 100× objective with NA = 1.3 provides higher contrast for NP spatial positioning and spectral characterization in the area covered with or without cells comparing to the darkfield imaging using NA = 0.5. Reflected light imaging mode exploits higher backscattering efficiency of plasmonic AuNPs in comparison to the light scattering from cell membranes and extracellular matrix [9]. Moreover, by scanning in $z$-direction, NPs attached to target cells at different distances from the surface are easily detected (Figure 2B). Thus, the proposed approach could be used for the fast 3D wide-field labeled cells scanning as an alternative to more complicated confocal microscopy [11–12]. In conventional darkfield imaging, single NPs and NP clusters are often difficult to distinguish. Using optical power of high NA objectives, the reflected light imaging mode reaches improved spatial resolution for precise NP localization and cluster visualization.

3.2 Hyperspectral measurements of spectral characteristics of functionalized AuNPs targeting MDA-MB-231 cells by reflected light microscopy

We then investigated the spectral characteristics of functionalized AuNPs in the cellular environment by hyperspectral reflected light microscopy. Scan was performed with laser line tunable filter, 300 ms time constant and 4 nm wavelength steps in 450–640 nm range. Reconstructed intensity image, shown on Figure 3A is obtained from hyperspectral scanning data by integrating spectra obtained for every pixel of the spatial image. Color distribution for cells–AuNPs complexes is calculated by color reconstruction method using standard sensitivity functions from the International Commission on Illumination (CIE) (Figure 3B) [13]. Spatial positions of individual AuNPs are easily detected (A) and chromatic signatures are clearly observed (B).

One of the most important applications of the hyperspectral darkfield microscopy for the characterization of cells–AuNPs complex is the possibility to spectrally characterize and identify functionalized NPs directly in a biological sample. Depending on
the NP aggregation state and localization, a spectral distribution was experimentally measured. Some examples of experimental spectral curves for single 100 nm functionalized AuNPs are presented in Figure 4. Theoretical backscattering spectra calculated by Mie theory with plasmonic peak at 569 nm is red-shifted for about 6 nm that could be explained by AuNPs functionalization and attachment to the cell membrane. Considerably large experimental noise could be further reduced by both software improvement and optical filtering methods to smooth and reduce background noise and speckle effects (Figure 1, speckle reducer). In order to eliminate the influence of the microscopy set-up mechanical vibrations and slight movements of the attached AuNPs in the liquid, we included into off-line analysis software procedure for 2D NP localization for each spectral line image. According to the literature, the most accurate method for tracking small spherical NPs is based on fitting each intensity peak in the image with a 2D Gaussian function [14]. This approach combined with subtracting background signal and reference spectral curve obtained from the close environment (about 20 pixels) surrounding the AuNPs allows spectral characterization of the plasmonic NPs in a biological context. Since AuNPs clusters are characterized by a spectral shift of the plasmonic peak, hyperspectral imaging system will provide more knowledge on spectral characterization of samples in a complex biological environment.

3.3 Evaluation of 3D spatial position of functionalized AuNPs targeting MDA-MB-231 cells

Investigation of specific cellular and sub-cellular activity with the use of NPs as spectral markers requires real-time 3D NP tracking, spatial NP distribution and chromatic signatures of single NPs. Reflected light microscopy is independent of the NA imaging objective while the darkfield approach is limited by the NA. Thus, higher precision for NPs 3D spatial localizations is expected in wide-field imaging. Figure 5 shows microscopy images and corresponding 3D intensity pattern of the point-spread function for AuNPs at different z-positions with 1 μm step. Darkfield microscopy (Figure 5A) and reflected light microscopy at 550 nm illumination wavelength (Figure 5B) show drastic difference for NPs visualization. Reflected light microscopy provides much sharper intensity distribution that strongly depends on the z-position. This feature facilitates accurate NPs z-position and can be explained by the influence of high NA objective and optimal illumination wavelength on the 3D intensity pattern of the point-spread function. Precise and fast tuning of spectral line provided by hyperspectral set-up allows the introduction of additional parameters into data analysis software for 3D NP tracking in real-time imaging.

We then recorded the sequence of optical section images taken at different z-positions with 0.1 μm step. The resulting 2D NP localization for each wide-field image was obtained by fitting each corresponding intensity peak with a 2D Gaussian function. Using similar method for the array of images in the z-direction, we obtained a 3D map of the NP position. Figure 6 shows some examples of experimental intensity distribution of the NP point-spread functions for different z-positions. The full width at half maximum (FWHM) of these functions is about 0.5–0.8 μm for reflected light imaging mode (black line) and at least three times larger (2.5–3 μm) in the darkfield mode (blue line) [15].

Figure 6 Normalized intensity patterns of NP point-spread functions for different z-positions at 550 nm illumination wavelength. Black and blue lines represent reflected light and darkfield imaging mode, respectively.
We can conclude that the proposed wide-field reflected light imaging mode with monochromatic illumination provides efficient high contrast for NP localization and can be applied for real-time analysis of 3D cells–AuNPs complex. Two examples of calculated spatial positions of the functionalized AuNPs on the CD44+-MDA-MB-231 cells are shown in the Figure 7. The 3D distribution of AuNPs attached to cell surface clearly shows cell morphology, with a maximum height of about 10 μm for the fixed cancer cells. The high density of AuNPs on the cell surface also indicates the characteristic elongated morphology of MDA-MB-231 cells.

As shown in this article, the wide-field reflected light microscopy with coherent supercontinuum illumination provides high contrast imaging for real-time 2D NP positioning. It also allows full 3D NP tracking with submicron resolution in z-direction based on the data analysis of images obtained by z-scanning. High intensity illumination with high NA imaging objective could expand microscopy to 3D imaging and spectral characterization of smaller NPs. Spectral measurements take advantage of tunable wavelength excitation for better characterization of NPs with different diameters or chromatic signatures. Accurate spectral and spatial characterization of single and aggregated plasmonic NPs is essential for the improvement of nanoplasmonic-based imaging, disease detection and treatment in complex biological environment.

4. Conclusion

We have experimentally presented a new hyperspectral reflected light microscopy system with a scanned supercontinuum light source. This wide-field and low phototoxic imaging system was applied to verify specific attachment of functionalized AuNPs targeting CD44+ cancer cells. We expect that this hyperspectral imaging set-up will be beneficial for wide bio-applications, such as disease detection and treatment where spatial and spectral analysis are required. Presented optical system capability for 3D spectral NP tracking will be important for real-time visualization of specific cellular activity, NP aggregation, distribution, accumulation and elimination.

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Author biographies Please see Supporting Information online.

References


