FULL ARTICLE

Hyperspectral darkfield microscopy of PEGylated gold nanoparticles targeting CD44-expressing cancer cells

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Received 15 October 2013, revised 13 November 2013, accepted 17 November 2013
Published online 17 December 2013

Key words: Biomarker, cancer stem cell, darkfield imaging, functionalization, monoclonal antibody, nanosphere, poly(ethylene glycol), supercontinuum

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 in biological and biomedical applications, such as detection of chemical and biological analytes, imaging of cells and tissues, detection and treatment of cancer cells [1]. Nanoplasmonics-based detection and treatment can be achieved with AuNPs functionalized with biomolecules targeting specific biomarkers on cells or tissues. Many nanomaterials have been functionalized with monoclonal antibodies since they display high affinity and selectivity for many established biomarkers [2]. The small size of AuNPs and the complex biological environment

We present a new hyperspectral darkfield imaging system with a scanned broadband supercontinuum light source. We observed the specific attachment of the functionalized gold plasmonic nanoparticles (AuNPs) targeting CD44⁺ human breast cancer cells by conventional and by proposed hyperspectral darkfield microscopy. 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 nanoplasmonic-based imaging, disease detection and treatment in complex biological environment. Presented system capability for 3D NP tracking will also enable investigation of specific subcellular activity with the use of NPs as spectral sensors.

Hyperspectral darkfield microscopy allows spatial mapping of nanoparticles attached to CD44⁺ human cancer cells.

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increase the difficulty to visualize AuNPs and obtain complete information about their physical properties with the target cells or tissues. NP concentration, spatial three-dimensional (3D) position and distribution as well as the spectral characteristics of single and aggregated NPs are very important to visualize and use NPs for selective cell detection and treatment.

For AuNPs imaging and characterization at the single-particle level, we present in this article a hyperspectral darkfield microscopy arrangement using a tunable filter to sweep the illumination source over a spectral range from 400–1000 nm. This system offers wide-field imaging combined with spectroscopic characterization [3], 3D localization of AuNPs [4] and low phototoxicity due to the narrow irradiation spectrum. Hyperspectral imaging of AuNPs functionalized with monoclonal antibodies against human CD44 was performed on human breast cancer cells (CD44⁺ MDA-MB-231 and CD44⁻ BT-474 cells) to confirm the specificity and the efficiency of the developed set-up.

2. Experimental

2.1 Hyperspectral darkfield microscopy

The hyperspectral darkfield microscopy system was built on an Eclipse Ti microscope (Nikon, Mississauga, ON) equipped with a 60× objective (numerical aperture (NA) 0.7, Nikon) or 100× objective (adjustable NA 0.5–1.3, Nikon). The white illumination source used for standard darkfield imaging was replaced in hyperspectral darkfield imaging set-up by a supercontinuum white light laser (Fianium SC450-1, Southampton, UK) producing a quasi-continuous output from 460 to 1800 nm (Figure 1).

The unpolarized light is delivered into a laser line tunable filter (Photon Etc., Montréal, QC) for spectral filtering in the range from 400–1000 nm with a spectral width of about 2.5 nm. This tunable filter achieves fast scanning of the illumination light spectra from min to max in just 600 ms, allowing for hyperspectral real-time imaging. The light delivery from the tunable filter to the darkfield condenser is performed using a microstructured optical fiber and a specially developed darkfield adapter. An optical system of adapter includes optical fiber collimator with a 7 mm focal length lens and an axicon prism to generate the far-field distribution of the annular illumination without any remarkable energy loss [5]. Simultaneously, the focusing center for the hyperspectral darkfield condenser remains fixed because the microstructured fiber supports light delivery in the full spectral range covered by the filter. Series of images at different spectral range were recorded with a low noise 16bit 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).

2.2 PEGylated gold nanoparticles targeting CD44-expressing cancer cells

We target cancer stem cells (CSCs) since these cells have the capacity to self-renew, give rise to heterogeneous 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) while BT-474 cells do not [8], we used these two human breast cancer cell lines to verify the specific attachment of the developed functionalized AuNPs targeting CD44. We confirmed by immunostaining the strong expression of CD44 on MDA-MB-231 cells and its absence on BT-474 cells by using rat anti-human CD44 monoclonal antibodies (Hermes-1 1:100, 150 kDa, abcam, Toronto, ON) and goat anti-rat Alexa488 secondary antibodies (1:100, Life Technologies, Burlington, ON, data not shown). These antibodies against CD44 were tethered to AuNPs (44.4 µg/mL, 100 nm diameter, spi, 4804-AB, West Chester, PA) using heterobifunctional poly(ethylene glycol) (PEG) linkers by using the strong covalent binding between sulfur and gold [9]. A stock solution was generated at 100 µg/mL by
mixing anti-CD44 antibodies and orthopyridyl disulfide-PEG-N-hydroxysuccinimide (OPSS-PEG-NHS, 5 kDa, Nanocs, New York, NY) in a 1:1.88 molar ratio during 3 h at 4°C, then aliquots were kept at −20°C. AuNPs were mixed with 9.55% Na₂CO₃ 10 mM (pH 8.5) and 0.45% OPSS-PEG-Ab during 1 h at 4°C. 10% PEG-SH (2 kDa, 0.1 mg/mL, Sigma-Aldrich, Oakville, ON) was added to block the remaining free sites on the gold surface during 1 h at 4°C. The suspension was centrifuged at 5000 rpm during 10 min, the supernatant was removed and replaced with 2 mL Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies).

MDA-MB-231 and BT-474 human breast cancer cells were seeded onto glass bottom dishes (1 × 10⁵ cells, MatTek, Ashland, MA) and grown in DMEM containing 10% foetal bovine serum (FBS, Life Technologies) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin 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 8.2 μg/mL CD44-targeted AuNPs in serum-free medium during 3 h in the 37°C incubator. Cells were then washed three times with PBS, fixed with cold methanol during 10 min at room temperature and washed three times with PBS.

3. Results and discussion

3.1 Darkfield imaging of AuNPs incubated with MDA-MB-231 or BT-474 cells

MDA-MB-231 and BT-474 cells were incubated with the CD44-targeted PEGylated AuNPs during 3 h, washed with PBS to remove unbound NPs and visualized by conventional darkfield microscopy by using a QIClick digital CCD camera (QImaging, Surrey, BC). This imaging and spectroscopic technique detects light elastically scattered by metallic NPs. CD44⁺ MDA-MB-231 cell membranes scatter white light in darkfield imaging and CD44-targeted AuNPs attached to these cells strongly scatter orange light in a dot pattern (Figure 2A). In the absence of CD44-targeted AuNPs attached to the CD44⁺ BT-474 cells, only the light scattering from their cell membranes is observed (Figure 2B).

Thus, functionalized AuNPs can target efficiently the CD44 biomarker only expressed in MDA-MB-231 cells. In conventional darkfield imaging, the light is scattered by cell membranes and AuNPs. However, single NPs and NP clusters are difficult to distinguish.

Since AuNP clusters are characterized by a spectral shift of the plasmonic peak, we developed an alternative imaging system to conventional darkfield imaging in order to increase the spatial resolution and get more knowledge on spectral characterization of samples in a complex biological environment.

3.2 Hyperspectral darkfield imaging of AuNPs incubated with MDA-MB-231 cells

Darkfield imaging microscopy could benefit from applications of increasingly bright illumination sources like broadband supercontinuum light source system. Operating in the MHz repetition rate range with picosecond pulses, these systems can be utilized effectively for both transient and steady-state lifetime measurements. However, single spatial mode across the output spectrum and high spatial coherence are poorly suited for full-field imaging applications due to coherent artifacts such as speckle effect that corrupts image formation [10]. The influence of this phenomenon is clearly visible in hyperspectral darkfield image of MDA-MB-231 cells with CD44-targeted AuNPs (Figure 3A). Image interpretation is quite complicated since intense light scattering from the cells and interferometric noise completely covers cellular structures and the strong light scattering from 100 nm AuNPs targeting the cells. Consequently, the direct application of supercontinuum light system in a conventional transmitted darkfield illumination is not recommended for cells-AuNPs imaging (Figure 3A, optical set-up). As we would like to develop an imaging system with minimal design change on the inverted Eclipse Ti microscope, we adapted a simplified reflect darkfield illumination approach (Figure 3B, optical set-up). In this case, the darkfield illumination region is formed by the annular light reflected from the backside of the microscopic slides, usually about 0.12–0.17 mm thick. Higher brightness of supercontinuum light source provides sufficient light backscattering intensity to form a good quality image of cells-AuNPs complex.
Cell structure is clearly visible with contrast comparable to the conventional white-light illumination in darkfield microscopy. Drastic changes are obtained for the AuNP visualization. High contrast allows AuNP spatial positioning and spectral characterization in the area covered with cells and the area without cells. Evaluation of the experimental data allows us to estimate the minimal resolved AuNP diameter, with 40 nm for NPs aside of cellular matrix and about 60 nm for those attached to the cells.

### 3.3 Hyperspectral darkfield measurements of spatial and spectral characteristics of AuNPs incubated with MDA-MB-231 cells

We then followed the AuNP spatial position and distribution in z axis direction by using the hyperspectral darkfield microscopy with the supercontinuum source in backscattering configuration. Examples of such images are presented in Figure 4 where S-points correspond to the AuNPs attached to the glass substrate surface, whereas C-points represent AuNPs on the cell surface or inside the cell. We observed that most NPs are located at the level of the glass substrate bottom (Figure 4A) in comparison to the top level of the cell surface (Figure 4B). Since AuNPs were added after the cells were attached to the substrate, then incubated during 3 h and washed three times, most observed NPs are located inside the cells at the bottom level.

Various 3D particle tracking techniques were developed to monitor the AuNPs trajectories with nanometer positioning accuracy [11, 12]. They are usually based on the analysis of the 3D pattern representing the point-spread function of the optical system. The axial position of the object from the imaging focal plane is then precisely found from the ring number and diameter, and the corresponding intensity distribution pattern. As shown in Figures 3 and 4, the wide-field microscopy with coherent supercontinuum illumination provides high contrast imaging for real-time 2D NP positioning. It also allows full 3D NP tracking based on the data analysis of the spherical aberration rings in the defocused images obtained by z-scanning or holographic imaging. Changing focus z-position, we can estimate the NP concentration and distribution at different planes. Figure 5 presents performance of our hyperspectral set-up in 3D NP tracking. Figure 5A and B show holographic images at different defocusing distances for 550 nm illumination wavelength. Two optical configurations were used: the first is for 100× objective in the backscattering set-up described earlier (Figure 3A) and the second is similar to the technique described in literature [11] with 100× objective connected to the substrate with immersion oil. Both configurations show similar experimental trend in the outermost ring diameter dependence on the optical defocusing. However, the first approach provides images with more developed interferometric ring structure with corresponding spatial intensity distribution. This feature facilitates precision of NPs z-positioning and can be explained by the additional contribution of the microscopic slides into aberration properties of the complete optical set-up in the second configuration. Using this method, we estimated an absolute distance ranging from 4.1 to 5.2 μm between AuNPs in points C and S from Figure 4. 3D intensity pattern of the point-spread function depends on the objective NA and illumination wavelength. Precise and fast tuning of spectral line provided by hyperspectral set-up allows introducing additional parameter into data analysis software for 3D NP tracking even in real-time imaging. Sample images at 500, 600 and 800 nm show chromatic de-

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**Figure 3** Scattering (A) and backscattering (B) modes for hyperspectral darkfield illumination with AuNPs and MDA-MB-231 cells.

**Figure 4** Darkfield images taken at different planes along the optical z axis using the 100× objective. Focal plane is positioned to the glass substrate surface (A) or to the top surface of MDA-MB-231 cells (B).
One of the most interesting applications of the hyperspectral darkfield microscopy for the characterization of cells-NPs complex is the possibility to spectrally characterize functionalized NPs directly in a biological sample. Depending on the NP aggregation state and localization, a wide spectral distribution was experimentally measured. Some examples of spectral curves for single 100 nm AuNPs are presented in Figure 5D with optical characteristics close to the theoretical backscattering spectra from Mie theory, with a plasmonic peak at 569 nm. Considerably large noise could be reduced by both software improvement and optical filtering methods to smooth and reduce background noise and speckle effects. Experimentally, we applied vibration to a very flexible optical fiber in the path of illumination that resulted in a much smoother spatial and spectral light distribution in imaging displays. In order to eliminate the influence of the microscopy set-up mechanical vibrations and slight displacement 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 particles is based on the fitting of each intensity peak in the image with a 2D Gaussian function [13]. 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.

Supercontinuum-based microscopic technique for the analysis of gold colloids in cell environment provides high contrast wide-field imaging, accurate spatial localization and spectral characterization of NPs. High intensity illumination with the possibility of localization expands darkfield microscopy to imaging and 3D positioning of smaller NPs. Spectral measurements can benefit from tunable wavelength excitation giving better control of NPs functionalized with fluorescence probe to eliminate the sample autofluorescence. It should be mentioned that additional experimental efforts are needed to obtain spectrally and spatially homogeneous imaging field comparable to the conventional white light illumination used in push-broom hyperspectral imaging systems (CytoViva, PARISS). Accurate spectral and spatial characterization of single and aggregated AuNPs 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 imaging system with a scanned supercontinuum light source. This wide-field and low phototoxic 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 system capability for 3D NP tracking will also enable investigation of specific sub-cellular activity with the use of NPs as spectral sensors.

Acknowledgements The authors thank Le Fonds de recherche du Québec – Nature et technologies and Le Fonds de recherche du Québec – Santé for the financial support. We also acknowledge Jean-Jacques Lebrun and Françoise Winnik for fruitful discussion on the nanoparticle targeting of cancer cells.

Author biographies Please see Supporting Information online.
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