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Technical note

Statistical detection of nanoparticles in cells by darkfield microscopy

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ABSTRACT

In the fields of nanomedicine, biophotonics and radiation therapy, nanoparticle (NP) detection in cell models often represents a fundamental step for many *in vivo* studies. One common question is whether NPs have or have not interacted with cells. In this context, we propose an imaging based technique to detect the presence of NPs in eukaryotic cells. Darkfield images of cell cultures at low magnification (10×) are acquired in different spectral ranges and recombined so as to enhance the contrast due to the presence of NPs. Image analysis is applied to extract cell-based parameters (i.e. mean intensity), which are further analyzed by statistical tests (Student's *t*-test, permutation test) in order to obtain a robust detection method. By means of a statistical sample size analysis, the sensitivity of the whole methodology is quantified in terms of the minimum cell number that is needed to identify the presence of NPs. The method is presented in the case of HeLa cells incubated with gold nanorods labeled with anti-CA125 antibodies, which exploits the overexpression of CA125 in ovarian cancers. Control cases are considered as well, including PEG-coated NPs and HeLa cells without NPs.

1. Introduction

Nanoparticle-based therapies in biomedicine are increasingly considered as future alternatives of purely pharmacologic approaches [1,2]. Research in this field is concentrated on the development of new and diverse nanoparticle (NP) types, but also on more effective delivery pathways with higher selectivity and lower side effects [3]. In the field of phototherapies the presence of NPs may drive a significant enhancement of the photo-thermal effect, due to selective photon absorption by NPs in the near infrared range [4]. Furthermore, metal NPs are increasingly considered as both dose-enhancers in radiation therapy and contrast agents in image-based diagnosis, e.g. in MRI techniques, due to their high Z value compared to surrounding tissue or their peculiar magnetic properties [5–7]. In all these cases, development of efficient targeting strategies with NPs is paramount. Most often, *in vitro* trials on eukaryotic cell models are the very first step toward the development of NP-based and/or NP-enhanced strategies, both in diagnosis and therapy. In the case of tumors, besides passive targeting, active targeting by the use of antibodies is one of the most solid solutions to achieve selectivity and specificity [8,9]. The proof of the targeting efficacy is assigned to a great variety of optical and non-optical techniques, such as confocal microscopy and diffusion reflection methods [10,11], TEM/SEM imaging, staining methods, ICP-AES/MS [12,13]. Some of these approaches offer an in-depth analysis of the nano-bio complex, even if drawbacks are present, due to e.g. non-specific labeling (e.g. silver microparticles in silver staining) or the occurrence of false positives in imaging-only-based techniques, as in some cases the bright or black dots may correspond to vesicles or organelles rather than NPs. According to us, two main points arise. The first is that a coupled spectroscopic and imaging detection would provide more reliable indication of the presence of NPs. The second is that a detailed statistical analysis of the sample is needed to accurately account for both the presence and the concentration of NPs. This is especially true when using optical detection methods, as the signal per NP is often hardly greater than the noise level [14]. In addition the nano-bio interfaces are generally affected by a certain variability in the number of internalized or attached NPs [15]. For both reasons, a reliable and accurate detection and quantification of NPs benefits much from a statistical approach, which consists in the repetition of the measurement on a large number of cells and the concomitant application of statistical tests.

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In this context, one promising techniques for a reliable detection of NPs in cell models is darkfield microscopy (DM), a widefield low-cost solution based on the optical scattering properties of the nanoparticulate [16]. Starting from the fact that these properties are size-, shape- and composition-dependent, selective optical filtering coupled with DM may greatly enhance the contrast in the detection of NPs, as demonstrated by previous work [17].

In this communication, we describe a step forward in the investigation of the nano-bio interface by adding statistical methods to the analysis of darkfield micrographs in selected optical windows. Images are recombined to enhance the contrast for the detection of NPs and processed by a cell-by-cell analysis to extract information at the level of the single cell or of a small cluster of cells. Then, data are subject to a *t*-test and a Permutation test analysis, in order to assess the sensibility of the detection method in terms of the minimum number of cells that is needed to acknowledge the presence of NPs in the sample.

The output of this method is intrinsically in terms of the probability that there be NPs in the specific cell type chosen for the experiments. To test out methodology, HeLa cells have been selected for their widespread use in cancer therapy studies and gold nanorods (GNRs) have been chosen as a representative model of plasmonic nanoparticles of much interest for therapeutic and diagnostic applications.

In order to couple the GNRs to the HeLa cells, the surface of the NPs has been modified with polyethylene glycol (PEG) and derivatized with anti-CA125 antibodies, because CA125 is a specific marker of ovarian cancers and is overexpressed on the plasmatic membranes of HeLa cells. Controls include HeLa cells treated with PEGylated GNRs without antibodies or without NPs.

2. Materials and methods

2.1. GNRs synthesis and characterization

2.1.1. Synthesis of GNRs

Cetrimonium bromide (CTAB)-capped GNRs were synthesized by the method described in [18] with the modification by Ratto et al. [4,19]. Typical volumes and aspect ratios are (4000 ± 1800) nm^3 and 4.3 ± 0.8 respectively [19]. As final step, GNRs were transferred at a concentration of 1.6 mM Au into a 10 mM MES buffer at pH 6 containing 120 mM NaCl and 0.005% (v/v) of polysorbate 20. Anti-CA125 conjugated GNRs were prepared as follows: GNRs were first PEGylated and then conjugated with anti-CA125 antibodies as reported in [8]. After purification, GNRs were transferred at a concentration of 4.0 mM Au into sterile PBS.

2.1.2. GNRs spectral characterization

The optical extinction and scattering coefficients of GNRs were estimated and disentangled from an analysis of their colloidal suspensions, by using the method described in [20] by using ad hoc micro-chambers.

2.2. GNRs uptake by HeLa cells

HeLa cells were seeded and allowed to grow for 24 h in 24-well culture plates or on glass coverslips under standard conditions. Cells were then treated overnight with PEGylated GNRs without antibodies or GNRs conjugated with anti-CA125 antibodies at the concentration of 100 μM Au in cell culture medium. Untreated cells served as a negative control. After incubation with the different NPs, cells were washed with abundant PBS in order to remove all unbound GNRs, fixed with a solution of 3.6% paraformaldehyde in PBS buffer for 5 min and rinsed in PBS buffer to remove the excess of the fixative agent. To observe the samples, ad hoc micro-chambers were used as described in [20].

2.3. Darkfield imaging technique

This technique exploits the near infrared plasmonic band of the GNRs as a spectral fingerprint to discriminate light scattered from the NPs with respect to that from the cellular compartments.

The keystone of this technique is to transfer a spectral information into an image, thus preserving the spatial information. For this purpose, we implemented and modified the method introduced in [17] by using an inverted microscope (mod. Ti, Nikon, Italy) equipped with a darkfield condenser (immersion oil, NA = 1.2-1.4, Nikon, Italy) and a CCD camera (Coolsnap HQ^2, Princeton instruments, USA). For each field of view, two darkfield images were acquired in two optical windows that were optimized on the basis of the spectral characterization of the NPs. The first window includes the near infrared plasmon resonance (high-pass filter, RG780, Schott). The second window is centered in an optical region where the contribution from the GNRs is low with respect to that from the cells (510 nm bandpass, 40 nm FWHM, Omega Optical Inc., USA). The ratio of these two images (in the following, we will refer to them as $I_{780}(x,y)$ and $I_{510}(x,y)$) is taken pixel by pixel to obtain a 2D map $R(x,y) = I_{780}(x,y)/I_{510}(x,y)$, where x and y are the pixel coordinates in the image and I is the pixel intensity in gray levels. By definition, R grows with the contribution of near infrared light scattered from the sample, which relates to the presence of GNRs. A characterization of the functional dependence of R from the scattering cross section of the NPs is beyond the scope of this article. Nevertheless, we can state that the R(x,y) images enhance the contrast that arises from local changes in the scattering coefficients, as due to the presence of GNRs. In principle, also $D = I_{780} - I_{510}$ could be used as a contrast-enhancing parameter, the final choice between D and R being mainly due to a preferred methodological approach. In our specific optical setup, R is less prone to dependencies upon erratic causes, such as presence of stray light and illumination inhomogeneities. According to the specific type of plasmonic NPs, this technique can be optimized by choosing a best R parameter as $R = I_{\lambda 1}/I_{\lambda 2}$, where λ_1 and λ_2 are in-resonance and off-resonance, respectively. In the case of non-plasmonic NPs, two equivalent spectral bands might be optimized with the same purpose. The choice of filter characteristics was not driven by possible shift in GNRs resonant peak due to surface functionalization (see also Section 3.1). DM imaging was performed by a 10× objective (Plan Fluor, NA = 0.30, Nikon, Italy). Lateral resolution was ∼900 nm, axial resolution was ∼11 μm (λ = 510 nm). These conditions guaranteed in-focus images on the whole cell thickness (unlike higher magnifications), a sufficient magnification in terms of cell area respect to pixel size and the presence of about 50 cells per field of view. This number proved to be a good compromise between the needs to collect a high cell number ($N \sim 100 \div 500$) and to keep a reasonable imaging and analysis time. Depending upon the specific sample conditions, image acquisition was performed in 10–20 min, while further analysis took up to a few hours. Sample preparation time following GNRs-cell incubation was of about 15 min.

2.4. Image analysis

This analysis was carried out by means of Fiji (http://fiji.sc/Fiji) and Python (https://www.python.org/) dedicated routines. First of all, we give detail on the numerical analysis linked to the ratio operation between the I_{780} and the I_{510} images. As a first step, a manual background region of interest (ROI) identification was performed on both images by means of a threshold operation, in order to take into account the contributions from spurious light and CCD dark current. This operation identifies one or more background ROIs and their complement formed by an ensemble of cell ROIs. After background subtraction, all pixels in the background ROIs