

Transfection and imaging of diamond nanocrystals as scattering optical labels

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Abstract

We report on the first demonstration of nanodiamond (ND) as a scattering optical label in a biological environment. NDs were efficiently transfected into cells using cationic liposomes, and imaged using differential interference and Hoffman modulation ‘space’ contrast microscopy techniques. We have shown that 55 nm NDs are biologically inert and produce a bright signal compared to the cell background. ND as a scattering label presents the possibility for extended biological imaging with relatively little thermal or biochemical perturbations due to the optical transparency and biologically inert nature of diamond.

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1. Introduction

The application of optical labels to biological imaging allows specific sites or even single molecules to be discriminated from the cell background in real time, with sub-micron resolution [1]. Labels are generally classified as luminescent or scattering where luminescent labels include fluorescent dyes or quantum dots, and scattering particles include plasmon-resonant particles, also known as immunolabels. Luminescent labels enable high-contrast imaging due to the spectral separation of excitation and emission bands. Scattering labels on the other hand efficiently scatter incident light, and can be differentiated from the cellular background only due to variations in scattered light intensity or its angular distribution.

None of these labels is ideal. Photobleaching and in some cases toxicity are major limitations of fluorescent dyes, and

spectral sensitivity to environmental conditions can also add disturbing variability to experiments. Quantum dots are relatively new photostable labels, and coatings must continue to improve to ameliorate the problems of toxicity, ‘blinking’ and the large size of quantum dots when coated [2]. Scattering labels such as plasmon-resonant gold and silver particles do not suffer photobleaching but cause thermal perturbation and catalytic effects. Moreover, scattering particles generally must be at least 40 nm in size [3]. The large size of scattering labels and quantum dots limits their usefulness in tracking the relatively small biomolecules without hindering their natural function.

The inapplicability of optical labels to acquiring detailed prolonged observations limits our understanding of many biological processes such as viral infection [4], cell mitosis and multi-step cell signalling [5]. A sufficiently small, biologically inert, photostable particle could, for example, be used to track a single virus for its entire development cycle. This technology would allow understanding of processes essential for antiviral drug design as well as the development of gene-therapy vectors where the viruses are used as vehicles for genes [4].

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We present nanodiamond (ND) as a potentially ideal optical label to overcome many of the problems associated with currently available labels. NDs present a blend of merits as a scattering label, which may also be rendered luminescent via a natural nitrogen-vacancy (NV) defect. Due to its high refractive index, a 55 nm ND scatterer is 300-fold brighter than a cell organelle of the same size [6]. As luminescent labels, NDs have unique properties which will enable prolonged observations, as well as efficient suppression of cell autofluorescence. ND is available as small as 4 nm [7], a suitable size for membrane diffusion and tracking biomolecules without inhibiting their normal function.

With a high refractive index of 2.4, NDs are efficient elastic scatterers and do not cause thermal perturbation in cells, as they are transparent for visible light. Moreover, the scattering efficiency is only marginally affected by the refractive index variation in cells. NDs as small as 37 nm have been imaged and sized using total internal reflection microscopy on a clean substrate and also in a weakly scattering polymer [6]. A key property of NDs is their pure carbon composition, which renders sufficiently pure ND biologically inert and also leads to straightforward bioconjugation chemistry [8].

NDs are made luminescent by a colour centre comprising a nitrogen atom adjacent to a lattice vacancy. These NV colour centres are remarkably photostable and emit bright red luminescence with a quantum yield of close to unity [9]. The fluorescence lifetime of NV defects in nanocrystals is 25 ns [10] and can be exploited for effective suppression of the relatively short-lived cell autofluorescence via temporal filtering.

While the development of these potential biological labels includes the photoactivation [11] and biofunctionalisation [8] of NDs, in this paper we present the first demonstration of imaging of ND as optical scattering labels in the crowded morphological environment of live cells. We also report on a vehicle to deliver NDs inside the cells, and also the imaging techniques used to observe these unique scattering labels.

2. Methods and discussion of results

Synthetic diamond particles with a nominal size of 55 nm (Warren Superabrasive) were transfected into cells for use as scattering optical labels, and have been characterised and sized by optical microscopy in previous work [6]. It has been shown that cellular uptake of ND particles via non-specific attachment and endocytosis is possible, however this pathway may result in inefficient particle uptake and sequestration of particles into endocytic vesicles [12,13]. Several other pathways exist for the introduction of particles in cells, including electroporation, microinjection, and the use of cationic liposomes. These techniques have been used as an efficient delivery scheme for both oligonucleotides and quantum dots [13]. In this work, we apply the use of cationic liposomes to the delivery of NDs

into the cytoplasm. It is expected that ND behaves in a similar way to oligonucleotides during transfection, forming complexes with the liposomes, which then fuse to the cell wall allowing the ND to escape into the cytoplasm as shown in Fig. 1 [14].

NDs were added to a lipid-rich serum-free medium (OptiMEM) and cationic liposomes (Lipofectamine) were used to transfect the NDs into cells. A variety of mammalian cell lines (3T3, 293T and CHO) were cultured in plastic six-well plates and either imaged in this configuration or fixed with paraformaldehyde on glass coverslips for differential interference contrast (DIC) imaging. Cell cultures were incubated in the serum-free solution containing ND to encourage the uptake of the lipid–ND complexes. After 5 h in the serum free environment, fresh medium was added to allow the cells to continue to grow overnight before imaging. Both live and fixed samples were imaged, and samples were transfected with a range of ND concentrations.

In order to obtain high-contrast images of NDs, one needs to discriminate optical scatter of the NDs from that of the cell constituents and also from the incident light [15]. One method to achieve such contrast is to make use of the wide-angle optical scattering of NDs, characteristic for a Rayleigh scatterer ($a \ll \lambda$, where a is the particle diameter and λ is the wavelength of incident light), in contrast with relatively low-angle scatter of cell organelles [16].

We have selected DIC and Hoffman modulation contrast (HMC) microscopy to suppress the significant background light and provide improved contrast of the cell constituents and brightly scattering NDs. Both DIC and HMC techniques convert optical path length gradients to intensity variations, effectively eliminating transmitted and background light. In DIC, this is achieved with the splitting and recombination of polarized light with two Nomarski prisms [17]. While this technique can provide excellent contrast and does not impair resolution, the use of polarized light precludes imaging in commonly used plastic vessels, which disturb the polarization of light. Hoffman contrast avoids this problem by using a condenser slit and modulator plate making it a simple and effective technique for live-cell imaging [18]. Unfortunately, these components obstruct the condenser and objective apertures, thus reducing resolution.

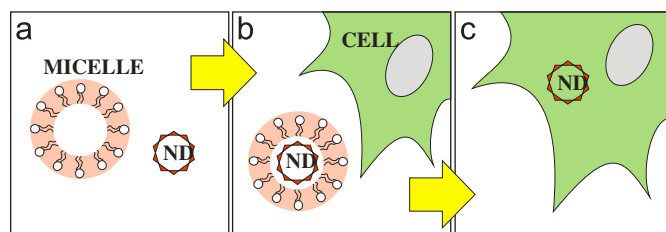


Fig. 1. The transfection process used to introduce NDs into cells. (a) ND is introduced to a lipid-rich medium. (b) Lipofectamine allows lipid–ND complexes to form. (c) Lipid–ND complexes fuse to cell walls due to electrostatic attractions, delivering ND into cells.

The use of Lipofectamine resulted in efficient delivery of NDs into cells, and NDs were clearly visible against the cell background. In both HMC and DIC imaging, NDs were brighter in comparison to a relatively dim cellular background, confirming good contrast between ND particles and cell organelles as expected (Fig. 2). While both contrast enhancement techniques prove sufficient for imaging ND, DIC offers more versatile contrast adjustment and slightly higher resolution than HMC. In DIC microscopy, contrast is adjustable over a wide range by translation of the Nomarski prism and the maximum contrast is fundamentally limited only by the polariser extinction ratios. This near background-free imaging is especially important for imaging small particles. Contrast adjustment in the Hoffman modulation technique is achieved by varying the effective condenser slit width, however, such adjustment is relatively limited.

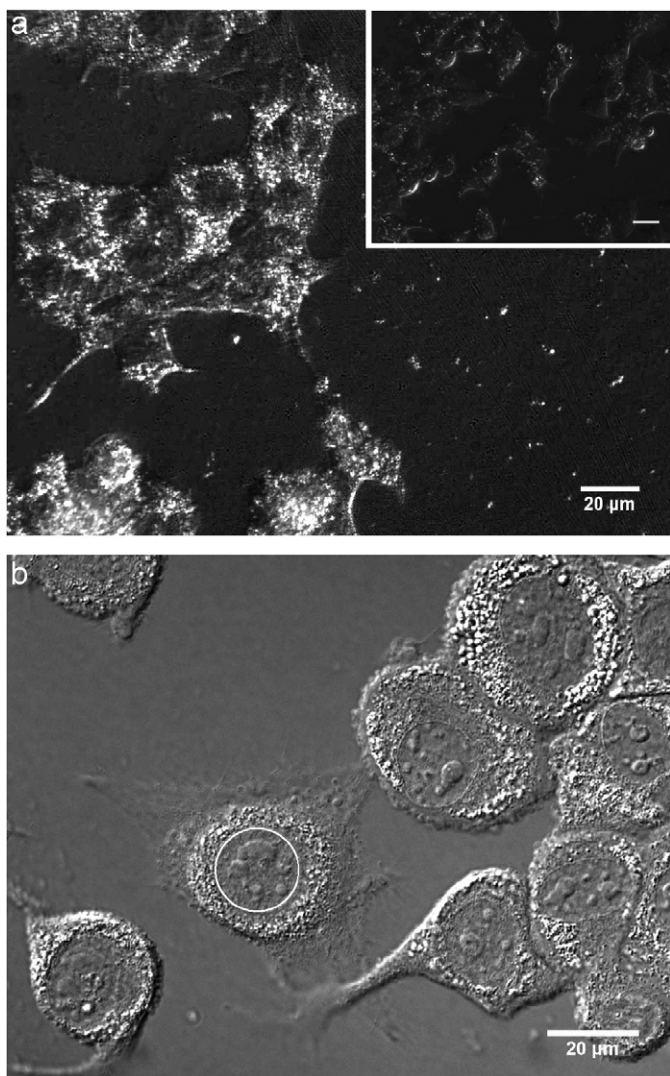


Fig. 2. Contrast enhanced images of nanodiamond particles deposited into cells at a concentration of 10^3 particles per cell. Nanodiamonds contrast strongly with the cell background due to strong elastic scattering. (a) HMC image of live 293T. Inset: negative control. (b) DIC image of fixed 3T3 cells. ND particles are visible as bright rims around the impermeable cell nuclei (a nucleus is labeled with a circle).

The optimal concentration of ND to achieve bright contrast and extensive delivery of ND into cells was on the order of 10^3 particles per cell. Live imaging qualitatively confirmed that NDs do not impede cell viability in low and moderate concentrations, however much higher concentrations of 10^5 particles per cell resulted in a decrease in cell viability.

While the NDs were visible throughout the cytoplasm, it is interesting to note that NDs did not penetrate the nucleus. This is expected as passive transport into the nucleus is limited to particles of 9 nm or less in diameter [19] and while particles of up to 39 nm can be actively transported via the nuclear pore complexes, this requires a nuclear localization peptide sequence [20]. The aggregation of NDs is also apparent in Fig. 2b, however it is unclear whether this aggregation is due to inter-particle interactions or encapsulation into vesicles. Intracellular aggregation has also been observed with quantum dots and as the dispersity of both quantum dots and ND within cells is essential for their use for intracellular labelling this effect warrants further study.

3. Conclusion

We have shown that 55 nm NDs can be used as optical-scattering labels in cells, and produce a bright signal compared to the cell background when imaged with suitable contrast enhancement techniques such as DIC and HMC. NDs can be deposited into cells using a simple transfection technique and are biocompatible with the tested mammalian epithelial cell lines. We also note that 55 nm NDs are distinguishable from cell morphology such as endosomes and vesicles due to their bright elastic scattering property. Using ND as a scattering label presents the possibility for biological imaging with relatively little thermal or biochemical perturbations, due to the optical transparency and biologically inert nature of diamond. For observations on a molecular level, a much smaller label would be ideal, however ND particles smaller than approximately 40 nm are not practical for use as scattering labels due to the dramatic size dependence of the optical scattering cross-section. In this case, luminescent ND particles as small as 4 nm present an ideal label, allowing extended observations of biological processes on a molecular level. The efficient delivery of ND to cells using cationic liposomes and the investigation of ND aggregation in cells are important steps in the development of this potentially ideal optical label. Future directions of our work include biofunctionalisation of the ND for site-specific labelling, and also efficient photoactivation of large quantities of luminescent NDs for imaging in a biological environment.

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