Characterization and application of single fluorescent nanodiamonds as cellular biomarkers

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One of the key avenues to understanding how biological systems function at the molecular level is to probe biomolecules individually and observe how they interact with each other directly in vivo. Laser-induced fluorescence is a technique widely adopted for this purpose owing to its ultrahigh sensitivity and capabilities of performing multiple-probe detection (1–3). However, in applying this technique to imaging and tracking a single molecule or particle in a biological cell, progress is often hampered by the presence of ubiquitous endogenous components such as flavins, nicotinamide adenine dinucleotides, collagen, and porphyrins that produce high fluorescence background signals (4–6). These biomolecules typically absorb light at wavelengths in the range of 300–500 nm and fluoresce at 400–550 nm (Fig. 1). To avoid such interference, a good biological fluorescent probe should absorb light at a wavelength longer than 500 nm and emit light at a wavelength longer than 600 nm, at which the emission has a long penetration depth through cells and tissues (5, 7). Organic dyes and fluorescent proteins are two types of molecules often used to meet such a requirement (1, 8, 9); however, the detrimental photophysical properties of these molecules, such as photobleaching and blinking, inevitably restrict their applications for long-term in vitro or in vivo observations. Fluorescent semiconductor nanocrystals (or quantum dots), on the other hand, have gained considerable attention in recent years because they hold a number of advantageous features including high photobleaching thresholds and broad excitation but narrow emission spectra well suited for multicolor labeling and detection (10–14). Unfortunately, most nanomaterials are toxic, and hence reduction of cytotoxicity and human toxicity through surface modification plays a pivotal role in successful application of quantum dots to in vitro labeling, imaging, and diagnosis (10, 15, 16). An additional concern with respect to quantum dots is that their photophysical properties are subject to change, depending on how their surfaces are modified. This limits the scope of their surface modification and functionalization, which often involve complicated surface chemistry. Insulator-based nanoparticles such as nanodiamonds, in contrast, are free of this limitation. We recently demonstrated that diamond crystallites with a nominal size of 100 nm are capable of producing stable fluorescence from color centers after surface treatment with strong oxidative acids (17). The result is not anticipated because the fluorescence of nanodiamonds originates from point defects embedded in the crystal lattice and has little to do with their surface structures. One of the most noteworthy point defects in diamond is the negatively charged nitrogen-vacancy center, (N-V)−, which is the dominant end product of thermal annealing of irradiation-damaged type Ib diamond containing atomically dispersed nitrogen atoms (18). This defect center absorbs strongly at ~560 nm and emits fluorescence efficiently at ~700 nm, which is well separated from the spectral region where endogenous fluorescence occurs (Fig. 1). The material is nontoxic, and, moreover, its fluorescence shows no sign of fading due to photobleaching...

Fig. 1. Comparison of emission spectra of flavin and FND. Note that the entire emission profile of the 35-nm FNDs (red) is well separated from that of flavin (orange) and also coincides with the high quantum efficiency region (gray) of the back-illuminated CCD camera used in this experiment. The absorption (blue) of flavin at 532 nm is negligible (39).


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Abbreviations: FND, fluorescent nanodiamond; ZPL, zero-phonon line; PL, poly-L-lysine.
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particles interrogated. The first ZPL peaking at 576 nm is ascribable to the electronic transition of the neutral defect center (N-V)\(^0\), and the second ZPL peaking at 638 nm corresponds to the \(3\Delta \rightarrow 3E\) transition of the negatively charged defect center, (N-V)\(^-\) (18–21). Both the ZPLs are accompanied by broad phonon sidebands red-shifted by \(\approx 50\) nm. A close comparison of the spectra reveals that these three particles mainly differ in the ratio of (N-V)\(^-\) to (N-V)\(^0\) contents.

To test the photostability of FNDs, we monitored the fluorescence intensities of the individual particles over an extended period (Fig. 3A). In accord with our previous finding for an ensemble of 100-nm diamond powders (17), excellent photostability was observed for the single 35-nm diamonds as well. Under the excitation with 532-nm light at a power density of \(8 \times 10^3\) W/cm\(^2\), the fluorescence intensity of the individual FNDs stays essentially the same over a time period of 300 s. No sign of fluorescence blinking was detected within the time resolution of 1 ms (Fig. 3B). In contrast, single dye molecules such as Alexa Fluor 546 covalently linked to dsDNA photobleached within 12 s (Fig. 3A).

For the 35-nm FNDs, an analysis of the fluorescence intensity for a large number (\(\approx 30\)) of measurements revealed that the brightness of the single nanodiamonds varies \(\sim 5\)-fold among the individuals. Such a variation is likely to result from heterogeneities in size and quantity, as well as the fluorescence quality of the N-V defect centers embedded in different nanocrystallites. Regardless of the difference in absolute intensity, both the 35-nm and 100-nm FNDs show similar photostability (compare time traces in Fig. 3A), which implies that the photostability of nanodiamonds is size-independent. Additionally, the intensity ratio between these two samples is \(\sim 30\)-fold, in accord with their volume ratio of \(\sim 23\):1. Although the fluorescence intensity does not scale quite linearly with the laser energy, this intensity ratio stays almost the same as the laser power density increases from \(1 \times 10^4\) to \(1 \times 10^6\) W/cm\(^2\) (Fig. 3C). All of the observations are consistent with the suggestion that the fluorescence signals detected in this experiment derive primarily from the N-V defect centers distributed evenly in the nanodiamond matrices.

In Fig. 3D we also show the result of the fluorescence lifetime measurements for 100-nm FNDs. The fluorescence decay was obtained by loading the time traces of 30 individual particles. The major component of the decay has a lifetime of 17 ns, which is comparable to that (11.6 ns) measured for bulk diamonds (22) but is substantially longer than that of dye molecules (\(\sim 4\) ns for Alexa Fluor 546 in Fig. 3D) and cell autofluorescence. Such a distinct difference in fluorescence lifetime is expected to be useful for isolation of FND emission from these (and other) background signals using various time-gating methods (23, 24), with which the contrast of single-nanodiamond imaging in biological cells can be further enhanced.

It is instructive to compare the fluorescence brightness of nanodiamonds with quantum dots. From a measurement of \(\sim 100\) particles for each sample, we estimated that the average fluorescence intensity of the 35-nm FNDs is approximately the same as that of quantum dots such as CdSe/ZnS that emit light in the similar wavelength range (see SI Fig. 8). Further information about the fluorescence brightness of FND can be deduced from a close comparison of our result with the single-defect measurements (21, 25), which indicates that the number of defect centers in the individual 35-nm diamonds prepared in this experiment is \(\sim 100\). Note that this number of defect centers is \(\sim 10\)-fold less than expected if all of the nitrogen impurities in the diamond lattice are converted to N-V centers, given a nitrogen concentration of 300 ppm (or \(3 \times 10^{10}\) nitrogen atoms per cubic micrometer) for type Ib diamond (26). This discrepancy suggests that if we can properly optimize our experimental conditions, 10-nm diamonds (or “diamond dots”) with fluorescence brightness comparable to that of quantum dots can be produced. Such fluorescence brightness is
sufficient for single-particle tracking in cells (9), particularly in the extended red region where the autofluorescence background is much reduced.

In a step to demonstrate that nanodiamond is a promising biomarker candidate for in vivo imaging and diagnosis, 35-nm FNDs were incubated together with HeLa cells cultured in DMEM at 37°C on a chamber slide. For the HeLa cell, intense cell autofluorescence was observed at 510–560 nm when exposed to blue light at 476 nm. Switching the laser excitation wavelength to 532 nm and collecting the emission at 650–720 nm where the FND fluorescence resides greatly reduces the fluorescence background signals (see SI Fig. 9). Fig. 4A shows an overlay of the bright-field and epifluorescence images of a single HeLa cell after the FND uptake. Translocation of the FNDs through the cell’s membrane was confirmed by obtaining vertical cross-section images (see SI Fig. 10) of the cell with the wide-field epifluorescence microscope. Although many FNDs are found to form aggregates in the cell, some isolated nanodiamonds can be detected clearly in the cytoplasm (Fig. 4B).

These particles are identified as single nanodiamonds because the spot sizes of their images are diffraction-limited (Fig. 4C) and their fluorescence intensities are comparable to that of the single FNDs spin-coated on the coverglass plate. It is of interest to note that the FNDs uptaken are mainly distributed in the cytoplasm of the HeLa cell and that they are photostable even after continuous excitation of the sample for 20 min at a laser power density of 100 W/cm² (Fig. 4C Inset). Similar to the earlier finding, neither photobleaching nor blinking of the fluorescence sufficient for single-particle tracking in cells (9), particularly in the extended red region where the autofluorescence background is much reduced.

Fig. 4. Observation of single FNDs in a HeLa cell. (A) Bright-field and epifluorescence images of a HeLa cell after uptake of 35-nm FNDs. Most of the uptaken FNDs are seen to distribute in the cytoplasm. (B) Epifluorescence fluorescence image of a single HeLa cell after the FND uptake. An enlarged view of the fluorescence spots (denoted by “1” and “2”) with diffraction-limited sizes (FWHM ~ 500 nm) is shown in Inset. The separation between these two particles is ~1 μm. (C) Intensity profile of the fluorescence image along the line drawn in B Inset. (C Inset) Integrated fluorescence intensity (after subtraction of the signals from cell autofluorescence and background fluorescence from the microscope slides) as a function of time for particle “1.” The signal integration time was 0.1 s. No sign of photobleaching was detected after continuous excitation of the particle for 20 min.
was observed for these FNDs within the limits of our detection sensitivity and time window.

As pointed out in our previous work (27–29) and described in Materials and Methods, the surface of nanodiamonds can be easily functionalized with carboxyl groups and their derivatives for specific or nonspecific binding with nucleic acids and proteins. Such a unique characteristic opens many opportunities for both in vitro and in vivo applications of FNDs. One such example consists of coating carboxylated FNDs with poly-L-lysines (PLs) to facilitate binding of the particles nonspecifically with DNA through electrostatic interactions (27, 29, 30). In this experiment, a single T4 DNA molecule fluorescently labeled with TOTO-1 dye molecules was stretched on an amine-terminated glass substrate with a channel-combing method (31). How the positively charged FND particles interact with the negatively charged DNA molecules was observed directly by a wide-field epifluorescence microscope equipped with a dual-view system.

Two channels were monitored simultaneously to reveal the microscopic details of the interaction between PL-coated FND and DNA: (i) the shorter-wavelength channel detects 545- to 605-nm emission from both FND and the TOTO-1 dyes intercalated within the T4 DNA molecule, and (ii) the longer wavelength channel detects 675- to 685-nm emission from FND only. As shown in Fig. 5 A, the T4 DNA was combed to have a V-shape configuration and stretched to a length of ~60 μm, close to its full contour length of 75 μm (32). An overlay of the images from these two detection channels reveals that the T4 DNA molecule was actually wrapped around the PL-coated FND particle. To quantify the fluorescence decay rates of FND and TOTO-1 separately, the fluorescence intensity (after background subtraction) of each channel was integrated, normalized, and plotted as a function of time. The fluorescence intensity of the TOTO-1 dyes was seen to decrease to one-third of its initial value (owing to photobleaching) after continuous excitation of the sample at 514 nm for 40 s. In contrast, the fluorescence intensity of the FND stayed essentially the same during the same time period of excitation (Fig. 5 B).

Finally, we demonstrate that it is possible to conduct single-particle tracking for the 35-nm FND in the cytoplasm of a live HeLa cell. Fig. 6 A shows the trajectory of the diffusion motion of the particle appearing as a bright red spot indicated in Fig. 6 A. Starting at the coordinate (0, 0), each data point was obtained by fitting the intensity profile of the red spot with a two-dimensional Gaussian function to deduce its center and go beyond the optical diffraction limit (33). The particle’s motion is Brownian and is confined within a small area of 1 × 1 μm² near the nucleus over our observation time window of 13.9 s. Note that in Fig. 6 B the trajectory is plotted as a projection on the x-y plane of the glass slide. Three-dimensional confocal tracking (34) of the single FND is practical if either the bright fluorescence or the strong scattered laser light (35) from the diamond nanoparticle is used as a feedback signal to locate its position in the live cell.

Conclusions
We have characterized in detail the photophysical properties of FND with an average size of 35 and 100 nm at the single-particle level. The material possesses several unique features including facile surface modification, long-term photostability, and no fluorescence blinking. The latter two properties are size-independent and do not show any sign of deterioration even after surface treatment with strong oxidative acids. The easiness of surface functionalization endows nanodiamonds with a significant advantage over other nanomaterials for specific or nonspecific binding with proteins and nucleic acids. Centered in the extended red region (~700 nm), the fluorescence of FND can be detected with minimal interference from cell autofluorescence.
We have demonstrated in this work that a combination of these unique chemical and photophysical properties ensures long-term (short-term as well) single-particle tracking and fluorescent imaging of FNDs as cellular biomarkers.

For future applications where smaller-sized particles are required, it is possible to employ standard separation methods to extract FNDs with a size in the range of 10 nm from the current sample. Each particle of this size is expected to contain up to 30 nitrogen-vacancy defect centers after optimization of our sample preparation conditions and have the photostability similar to that described in this article. A natural extension of the present application to in vivo studies includes the use of FND as a drug or gene carrier, as a device for tumor targeting, and as a fluorescent probe for two-photon confocal microscopy (36). Developed originally for surface finishing industry, the diamond nanoparticle, interestingly, is now finding new and far-reaching applications in modern biomedical science and biotechnologies.

Materials and Methods

Production of FNDs. Synthetic type Ib diamond powders with a nominal size of 35 nm (MSY, Microdiamant) and 100 nm (μm+ MDA, Element Six) were purified in concentrated H2SO4-HNO3 solution (3:1, vol/vol) at 90°C for 30 min. After separation by centrifugation and rinsing extensively with deionized water (Millipore), the diamond powders were resuspended in water to a concentration of 0.1 g/ml as a stock solution. A thin diamond film (~0.5-cm² area × ~50-μm thickness) was prepared by depositing 50 μl of the diamond suspension on a 0.5-mm-thick silicon wafer and air-dried. The dry diamond film was irradiated by a 3-MeV proton beam from a tandem accelerator (9SDH-2; National Electrostatics) at a dose of 1 × 10¹⁰ ions per square centimeter. Formation of nitrogen-vacancy defect centers was facilitated by annealing the proton beam-treated nanodiamonds in vacuum at 700°C for 2 h. To remove graphitic surface structures induced by thermal annealing, the freshly prepared FNDs were additionally cleaned in concentrated H2SO4-HNO3 (9:1, vol/vol) solution at 75°C for 3 days, followed by separation with centrifugation and extensive rinsing with deionized water. Both of the diamond samples were treated following the same protocols.

Surface Modification of FNDs. FNDs (100 nm) were surface-functionalized with carboxyl groups by strong oxidative acid treatment in concentrated H2SO4-HNO3 (9:1, vol/vol) at 75°C for 3 days, subsequently in 0.1 M NaOH aqueous solution at 90°C for 2 h, and finally in 0.1 M HCl aqueous solution at 90°C for 2 h. The resulting carboxylated/oxidized FNDs were separated by centrifugation, rinsed extensively, and resuspended in deionized water. To functionalize the surfaces of FNDs with amino groups, centrifugation and rinsing extensively with deionized water. To functionalize the surfaces of FNDs with amino groups, centrifugation, rinsed extensively, and resuspended in deionized water. To functionalize the surfaces of FNDs with amino groups, centrifugation, rinsed extensively, and resuspended in deionized water.

Observation of Single FNDs, Single Dye Molecules, and Single Quantum Dots on Coverglass Plates. Coverglass plates (Marienfeld Laboratory Glassware) were cleaned by immersing them in concentrated H2SO4-HNO3 (vol/vol, 3:1) solution for 30 min. After separation by centrifugation and rinsing with deionized water, the coverglass plates were spin-coated with the sample was mounted on a modified confocal optical microscope (E600; Nikon) for inspection (37). Excitation of the sample was made through a 100 objective (Plan Fluor, N.A. 1.3 oil; Nikon) by using a solid-state laser (JL-LD532-GTE; Jetlaser) operated at 532 nm. Epifluorescence passing through a 565-nm long-pass filter (E565lp; Chroma Tech) was collected and detected by an avalanche photodiode (SPCM-AQR-15; PerkinElmer). Fluorescence images were first obtained by raster scanning with a piezo-driven nanopositioning and scanning system (E-710.4CL and P-734.2CL; Physik Instrument). After obtaining the images of single FNDs, each particle was moved consecutively to the focal point of the objective to record the time evolution of the fluorescence intensity. The corresponding spectra were acquired by using a monochromator (SP300i; Acton Research) equipped with a liquid-nitrogen-cooled CCD camera (LN/CCD-1100-PB; Princeton Instruments). For fluorescence lifetime measurements, a frequency-doubled picosecond Nd:YAG laser (IC-532-30; High Q Laser) served as the excitation source. The corresponding fluorescence decays were measured with a time-correlated single-photon counting module (SPC-600; Becker & Hickl).

Cell Culture, FND Uptake, and Observation of Single FNDs in HeLa Cells. HeLa cells (10⁵ cells per milliliter) were cultured in DMEM (SH30243.02; HyClone) supplemented with 10% FBS and 1% penicillin/streptomycin. Suspensions of the 35-nm FNDs were first diluted with DMEM without serum to a final concentration of 0.8 μg/ml. After sonication for 30 min to ensure complete dispersion of the FNDs, an aliquot (typically 0.1 ml) of the suspension was added to the wells of a chamber coverglass plate (Lab-Tek II; Nunc) containing the HeLa cells preincubated for 24 h. The chamber slide was then incubated at 37°C in a 5% CO₂ incubator for 5 h for FND uptake. The FNDs not uptaken were removed by washing the sample three times with warm Dulbecco’s phosphate buffer saline, after which the cells were fixed with 4% paraformaldehyde (Sigma). The excess paraformaldehyde was washed away with deionized water. In the experiment of observing the diffusion motion of a single diamond in a live HeLa cell, the sample was treated and inspected following the same procedures described above but with no paraformaldehyde treatment.

The fluorescence images of FNDs in HeLa cells were acquired by using a wide-field epifluorescence microscope (IX70; Olympus) equipped with a Nd:YAG laser (DPSS 532; Coherent) operating at 532 nm (power density ~ 100 W/cm²). The resulting emission was collected by a x100, N.A. 1.35 oil objective (UPLFL ×100; Olympus) and selected by a 565-nm long-pass filter (E565lp; Chroma Tech) for detection. Images were recorded with an electron-multiplying CCD (DV887DSC-BV; Andor) having a pixel size of 16 μm and an exposure time of 0.1 s.
Observation of Single DNA/FND Complexes on a Glass Plate. T4 DNA (165.6 kbp; Wako) was fluorescently labeled with TOTO-1 (T3600; Molecular Probes) at a molar ratio of 4 bp per dye molecule. The length of the T4 DNA molecule extended from 56 μm to ~75 μm after labeling (32). To prepare the DNA/FND complex, 3 μg of PL-coated FNDs were suspended in 200 μl of 0.5× Tris/borate/EDTA buffer (15581-044; Invitrogen) and mixed with T4 DNA at a molar ratio of eight DNA molecules per FND particle. After incubation at room temperature for 10 min, the sample mixture was diluted to a concentration suitable for single-particle detection. 2-Mercaptoethanol (Sigma) was added afterward to the sample solution with a volume ratio of ~3% to avoid rapid photobleaching of TOTO-1.

A microchannel-combing method (31) was used to stretch and fix DNA on a coverglass plate surface-modified with amino groups. The microchannel was constructed with three components: a coverglass plate pretreated with 3-aminopropyltriethoxysilane (APTES; Fluka), a standard microscope slide containing two 1-mm holes separated by 16 mm, and a 3M double-side tape (B-001-020-Y and NSC 94-2120-M-002-009 (National Nanoscience and Nanotechnology Project)).


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