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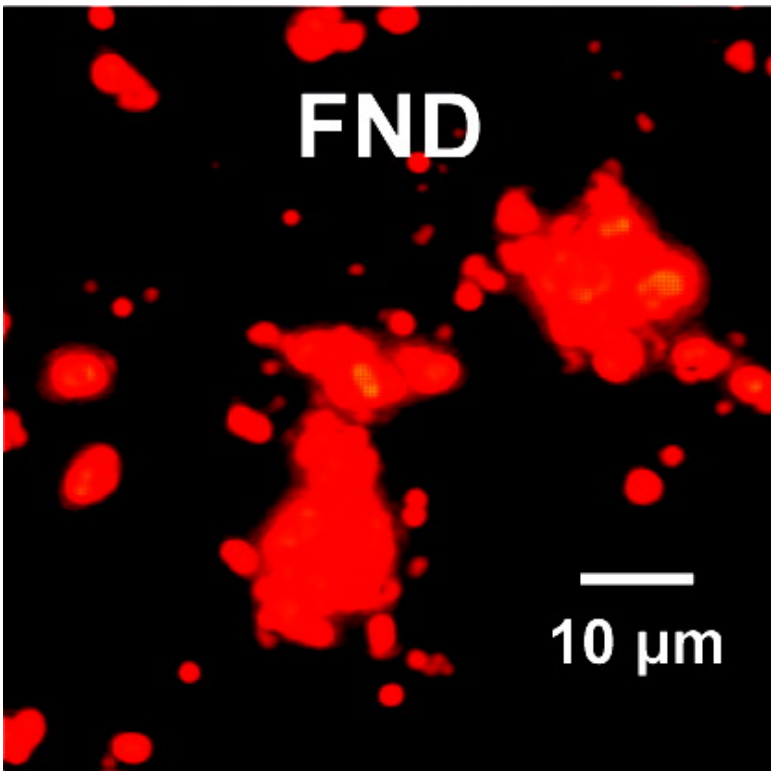

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Bright Fluorescent Nanodiamonds: No Photobleaching and Low Cytotoxicity

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Semiconductor quantum dots are finding increasing applications in modern biotechnology.[1,2] The materials hold great promise as fluorescent probes for intracellular processes at the single particle level. However, photobleaching and cytotoxicity have been two major concerns of these nanoparticles. Although silica-coated CdSe quantum dots have been demonstrated to be photostable for hours,[3] safety is always an issue whether toxic ions would be released during the course of diagnosis and treatment.[4,5]

Nanosized diamond powders are interesting alternatives. The materials are chemically inert, but can be surface-functionalized easily with carboxyl groups and their derivatives for specific or nonspecific binding with nucleic acids and proteins.[6] Moreover, they are optically transparent and capable of fluorescing from point defects.[7] Of all defects in diamond, the negatively charged nitrogen-vacancy center $(N-V)^-$ is most noteworthy.[8] It is the dominant end product of thermal annealing of irradiation-damaged diamond containing atomically dispersed nitrogen atoms.[9] The defect center absorbs strongly at ~560 nm and emits fluorescence efficiently at ~700 nm. The absorption cross-section at the band center is in the range of $\sim 5 \times 10^{-17}$ cm^2, comparable to that of a dye molecule.[9] The fluorescence quantum efficiency is $\phi \sim 1$, with a lifetime of 11.6 ns at room temperature.[10] To create these color centers, a type Ib diamond crystal (containing typically 100 ppm nitrogen) is first irradiated by a high-energy (~2 MeV) electron beam and subsequently annealed at high temperatures (~800 °C). The annealing brings the irradiation-created vacancy to a site close to the nitrogen atom, forming a $(N-V)^-$ center. The center acting as an ion embedded in an inert solid matrix hardly photobleaches,[8,11] allowing long-term observation of a single diamond nanocrystal. Here, we report a method to create high concentrations of $(N-V)^-$ centers in nanodiamonds with a proton beam and demonstrate the utility of this novel material for biological applications.[1]

Synthetic type Ib diamond powders with a nominal size of 100 nm (Micron+ MDA, Element Six) were purified in strong oxidative acids and suspended in deionized water.[12] A thin diamond film was prepared by depositing an aliquot (50 μL) of the suspension (0.1 g/mL) on a silicon wafer and dried in air. The air-dried diamond film was then irradiated by a 3 MeV proton beam from a NEC tandem accelerator (9SDH-2, National Electrostatics Corporation) at a dose of 5×10^{15} ions/cm^2.[13] Annealing of the ion-irradiated film at 800 °C in vacuum for 2 h produced fluorescent nanodiamonds (FND). Optical images of the FND were obtained with a laser scanning confocal fluorescence microscope (C-1, Nikon), and the corresponding spectra were acquired with a multichannel spectral analyzer (C7473, Hamamatsu). Cytotoxicity tests were performed with human kidney cells of the 293T line using an in vitro toxicology assay kit (TOX-1, Sigma) based on the reduction activity of methyl thiazolyl tetrazolium (MTT). Prior to incubation with FND, the 293T cells were cultured in Dulbecco's modified Eagle's medium (SH30243.02, HyClone) for 24 h. The ensuing incubation was conducted in a CO_2 incubator at 37 °C for 3 h, after which the cells were either fixed on a chamber slide with 4% *para*-formaldehyde for microscopic inspection or tested with the MTT assay in a 3.5 cm dish (see Supporting Information for further details).

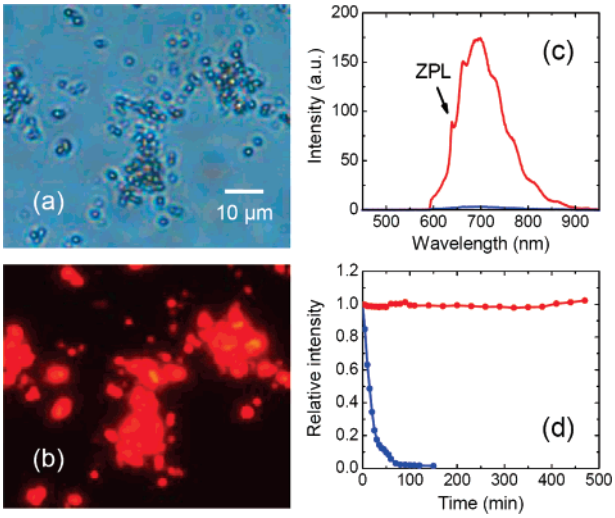

Figure 1. (a) Bright field and (b) epifluorescence images of FND. Both images were obtained with a 40X objective. (c) Fluorescence spectra of annealed nanodiamonds with (red) or without (blue) proton beam irradiation. The excitation was made at 510−560 nm, and the emission was collected at a wavelength of >590 nm. (d) Photostability tests of FND (red) and fluorescent polystyrene nanospheres (blue) excited under the same conditions. The fluorescence intensity was obtained by integrating over the wavelength range of 590−900 nm for each sample.

Figure 1a shows a bright field image of neat FND, which formed aggregates on a glass slide. Excitation of the particles with the yellow lines from a 100 W mercury vapor lamp produced intense red emission (Figure 1b). Spectral analysis of the emission revealed a weak zero-phonon line (ZPL) at 638 nm, accompanied with broad phonon sidebands spanning from 600 to 800 nm (Figure 1c). A comparison of this spectrum with that observed in the control experiment using samples not irradiated but annealed under the same conditions suggests a 10^2-fold enhancement in fluorescence intensity due to the proton beam irradiation (Figure 1c). In accord with previous findings,[8,11] no sign of photobleaching was found for FND even after 8 h of continuous excitation with the Hg lamp (Figure 1d). By contrast, the 0.1 μm red fluorescent polystyrene nanospheres (F8801, Molecular Probes), with excitation/emission maxima of 580/605 nm and containing $\sim 10^4$ dye equivalents, photobleached within 0.5 h under the same excitation conditions.

High concentrations of vacancies were created by the ion irradiation damage. According to the SRIM Monte Carlo simulation,[14] a 3 MeV proton would produce 12 vacancies at an estimated

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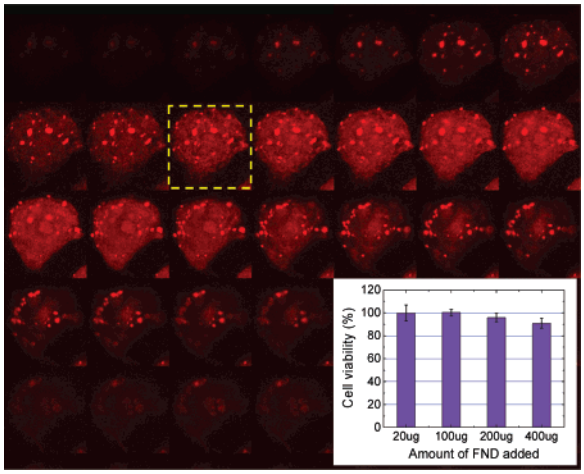

Figure 2. Confocal fluorescence images of a single 293T human kidney cell after FND uptake. The cross-sectional image in each three-dimensional scan (as indicated by the yellow dashed square) has a vertical thickness of 0.25 μm and an area of 42 $\times$ 42 μm^2. The bright red spots correspond to FND. Inset: Cytotoxicity tests of FND with the 293T cells and the MTT reduction assay.

displacement energy of 35 eV for the carbon atom in diamond.[15] The penetration depth of the proton beam is 48 μm. With an irradiation dose of 5 $\times$ 10^{15} ions/cm^2 as used in this experiment, the concentration of the nitrogen-vacancy defect centers created in these nanodiamonds is $\sim$1 $\times$ 10^7 centers/μm^3, corresponding to the production of $\sim$1 $\times$ 10^4 vacancies per 100 nm particle. This amount of vacancies is of the same magnitude as that ($\sim$2 $\times$ 10^4) of nitrogen atoms in each type Ib diamond nanocrystal. It suggests that the number of N$-$V defects created per particle is on the order of 10^4, assuming no vacancy annihilation.[16] Such a high concentration of defect centers,[17] each of which has fluorescence brightness comparable to that of a dye molecule, enables visualization of the individual nanocrystals by epifluorescence (Figure 1b).

Apart from bright fluorescence and high photostability, low cytotoxicity is an additional merit of FND. As revealed by the MTT assay (inset in Figure 2), adding up to 400 μg (or $\sim$1 $\times$ 10^{11} particles) of FND to 1 mL of the culture medium does not diminish the cell reduction activity significantly. This is in line with our expectation for diamond that the material is chemically inert and does not release any toxic chemicals even in harsh environments. Figure 2 shows the result of the FND uptake experiment (see also Supporting Information). Uptake of the fluorescent nanodiamonds is confirmed by generating the vertical cross-sectional images of one of the 293T human kidney cells with the confocal fluorescence microscope. Observation of the bright red spots inside the reddish envelope is indicative of FND translocation through the cell's membrane, possibly by endocytosis. The FND particles appear to form aggregates within the cell, but no bright spots are seen in the central region where the nucleus resides. It suggests that the 100 nm sized diamonds are not able to enter the nucleus. To facilitate further the FND uptake, use of smaller (<50 nm) and/or surface-derivatized nanodiamonds is desired and deemed feasible.

A 5 mW green He$-$Ne laser (543 nm) was used as the light source for confocal fluorescence imaging of FND in this work. More intense fluorescence can be obtained with stronger laser excitation. Gruber et al.[8] have reported that the (N$-$V)$^-$ centers are so stable that no detectable change in their fluorescence spectrum was observed as a function of time. Even under the excitation intensity of 5 MW/cm^2, none of the single defect centers showed any sign of photobleaching. Compared to the spectra of these single centers,[8,11] our spectrum (Figure 1c) is broader and extends farther into the near-infrared (wavelength > 800 nm). The emission in the deep red region matches well with one of the spectral windows for in vivo imaging (700$-$900 nm)[18] and can have broad biological applications.

Finally, we note that it is possible to produce FND with different colors of emission,[19,20] such as that derived from the H3 defect center (N$-$V$-$N) of a type Ia diamond. Similar to that of (N$-$V)$^-$, the green emission (e.g., 531 nm) from this defect center is bright and also has a quantum efficiency close to 1.[19] Together with the nontoxic nature of the material and the brightness of the fluorescence, this multicolor emission capability endows FND with attraction for applications in life science research.

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Supporting Information Available: Materials and methods and confocal fluorescence images of FND uptake (PDF). The material is available free of charge via the Internet at http://pubs.acs.org.

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