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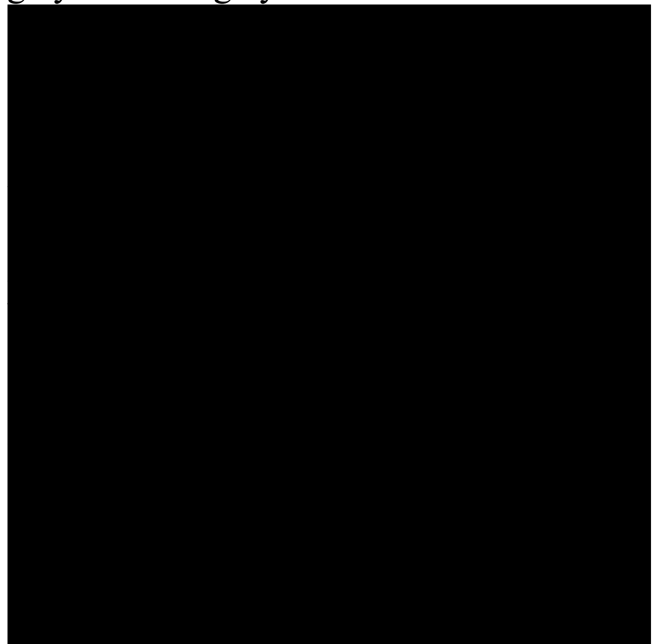
Water-Soluble Quantum Dots for Multiphoton Fluorescence Imaging in	Các Chấm Lượng Tử Tan Trong Nước Phục Vụ Cho Việc Ghi Ảnh Huỳnh Quang

Vivo	Đa Photon Bên Trong Cơ Thể
The use of semiconductor nanocrystals (quantum dots) as fluorescent labels for multiphoton microscopy enables multicolor imaging in demanding biological environments such as living tissue. We characterized water-soluble cadmium selenide-zinc sulfide quantum dots for multiphoton imaging in live animals. These fluorescent probes have two-photon action cross sections as high as 47,000 Goeppert-Mayer units, by far the largest of any label used in multiphoton microscopy. We visualized quantum dots dynamically through the skin of living mice, in capillaries hundreds of micrometers deep. We found no evidence of blinking (fluorescence intermittency) in solution on nanosecond to milli-second time scales.	Việc sử dụng các tinh thể nano bán dẫn (chấm lượng tử) làm các tác nhân gắn nhãn huỳnh quang cho kính hiển vị đa photon giúp chúng ta có thể ghi ảnh màu trong những môi trường sinh học phức tạp chẳng hạn như mô sống. Chúng tôi đã nghiên cứu các tính chất của các chấm lượng tử cadmium selenide-kẽm sunfua tan trong nước để ghi ảnh đa photon trong các động vật sống. Những đầu dò huỳnh quang này (các đầu dò có gắn huỳnh quang, chất thăm dò) có tiết diện hoạt động hai photon cao khoảng 47,000 đơn vị Goeppert-Mayer, lớn nhất trong số các phương pháp gắn nhãn huỳnh quang được sử dụng trong kính hiển vi đa photon. Chúng tôi quan sát các chấm lượng tử chuyển động trong da của chuột sống, trong các mao mạch sâu đến hàng trăm micromet. Chúng tôi không ghi nhận được sự nhấp nháy (các xung huỳnh quang) trong dung dịch ở thang thời gian nano giây đến mili giây.
Quantum dots (QDs) are bright, photostable fluorophores that have a broad excitation spectrum but a narrow Gaussian emission at wavelengths controllable by the size of the material. QDs allow for efficient multicolor imaging of biological samples (1) and should be especially useful for fluorescence imaging in living tissues, where signals can be ob-scured by scattering and competing intrinsic emissions. Multiphoton microscopy enables deep imaging of a variety of biological samples with less overall photobleaching than with wide-field or	

confocal microscopy, and it has now become the primary fluorescence imaging technique in thick specimens (2, 3). For these difficult imaging tasks, we have investigated the two- photon excitation characteristics of QDs and have begun to explore their use for in vivo multiphoton imaging.

For biological applications, robust water-soluble QDs are needed. Several synthesis strategies have been used, such as surface functionalization with water-soluble ligands (4, 5), silanization (6, 7), and encapsulation within block-copolymer micelles (5). Here, we investigated the photophysical properties of water-soluble CdSe-ZnS nanocrystals pre-pared by a synthesis method based on encapsulation of the nanocrystals within an amphiphilic polymer (9).

We measured the two-photon action cross sections of a number of organic and water- soluble QDs of multiple colors. The action cross sections (Fig. 1A) are the product of the nonlinear two-photon absorption cross section ct2P and the fluorescence quantum ef-ficiency (<^f) and provide a direct measure of brightness for imaging (10). Our measured values range from ~2000 to 47,000 Goeppert-Mayer units (GM), depending on the particular preparation and excitation wavelength. An action cross section of 47,000 GM is two to three orders of magnitude larger than those of conventional fluorescent probes

now in use, and is an order of magnitude larger than those of organic molecules designed specifically for enhanced two-photon absorption (11). The measured maximum value of 47,000 GM approaches the theoretical value of 50,000 GM calculated for CdSe QDs (12).

We used fluorescence correlation spectroscopy (FCS) (13) to measure the number of fluorescent particles in the focal volume from which we calculated the sample concentrations (14). The ratio of fluorescent QDs to total QDs is always <1, suggesting in some cases a considerable nonfluorescent fraction (table S1) (15).

Two-photon cross sections for the same core sizes with and without the amphiphilic coat (Fig. 1A, green and red) are nearly identical, indicating that passivation of the core-shell nanocrystals within the amphiphilic micelle appears to have little effect on QD brightness. A comparison between the two-photon action spectrum of the highest cross section preparation and its single-photon absorption spectrum (with wavelength doubled) shows little correspondence between spectral features, other than an overall decrease with increasing wavelength (fig. S1). Moreover, the action cross sections and FCS results of these water-soluble nanocrystals remained roughly constant over a period of >9 months, and we found no evidence for settling or aggregation of QDs in water over this time period.

The excitation intensity dependence of two-photon FCS is shown in Fig. 1B. The

autocorrelation amplitude G(0), which is proportional to the inverse of the number of fluorescent particles in the focal volume, decreases as the excitation power increases. This trend is opposite to triplet transitions or photobleaching effects, which would appear as apparent decreases in concentration [i.e., increases in G(0)] with increasing intensity (16). The power dependence of the fluorescence remains essentially quadratic (Fig. 1B, inset, red circles), but if the flu-orescence counts are normalized by the number of QDs in the observation volume, the fluorescence per QD reaches a limiting value (Fig. 1B, inset, black circles). We show that this observed power dependence is due to saturation of the two-photon excitation probability in the central focal volume along with increased peripheral excitation, resulting in larger effective focal volumes with increasing intensity.

Calculations of the effective two-photon focal volumes for different cross section sizes are shown in Fig. 1C. At cross sections on the order of 200 GM, the volume increase due to saturation effects is less than 10% at powers < 5 to 10 mW (pulse width, 100 fs at 80 MHz) or —0.06 to 0.1 nJ per pulse. However, at 30,000 GM the effective focal volume begins to increase noticeably at only — 1 mW (— 0.01 nJ). This increase in focal volume results in a reduction in optical resolution, leading to "fuzziness" in the images. 1/G(0) values (proportional to the effective focal volume Veff) show a power dependence similar to that given by saturation calculations based on a

30,000-GM probe (black circles, Fig. 1C).

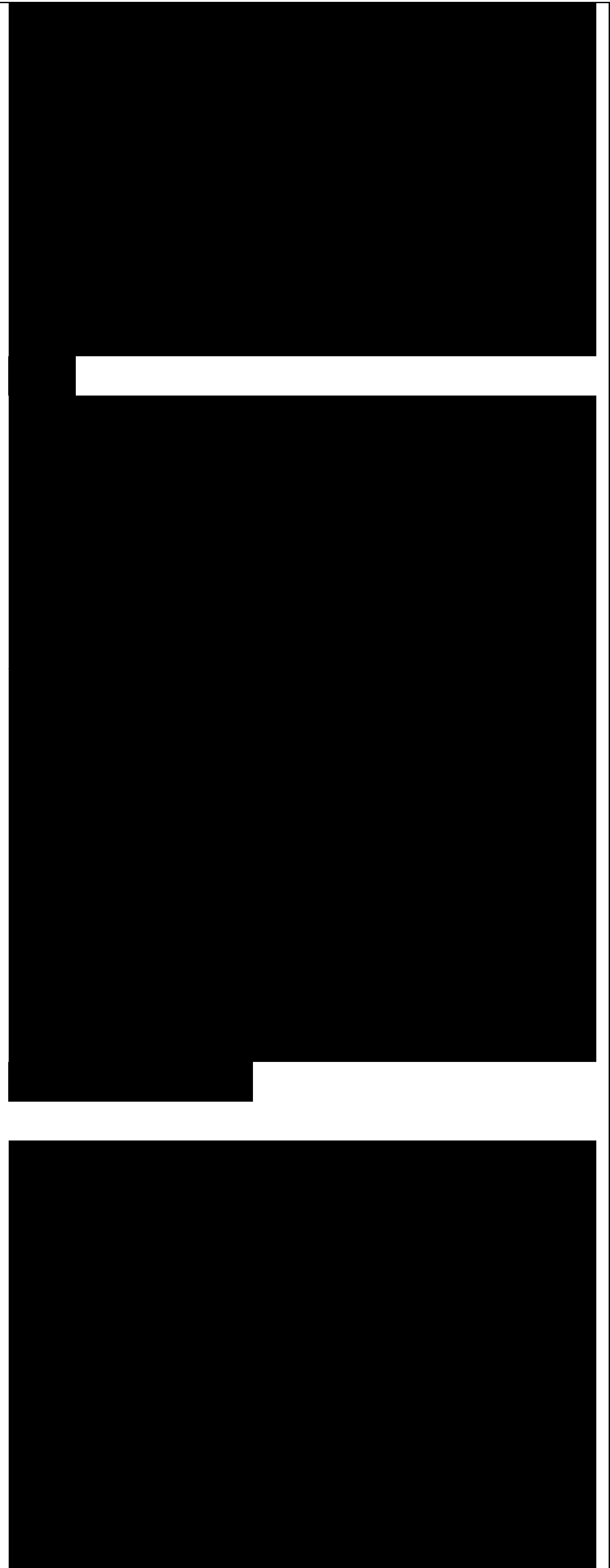
FCS correlation functions of organic QDs in hexane and water-soluble QDs taken at nonsaturating intensities (50 to 200 ^W) indicate slower translational diffusion than would be expected given the physical site of the nanocrystal (Fig. 1D). As derived from the measured diffusion coefficients, the Stokes-Einstein hydrodynamic radii are 14 nm and 9 nm for the water- soluble and organic QDs, respectively. These hydrodynamic sizes are about 2 to 3 times the dry radii [as determined by transmission electron microscopy (TEM)]. The difference between the dry and hydrodynamic radii suggests strong interactions between the polymeric surface coatings of the nanocrystals and the solvent (17).

A previously reported photophysical aspect of QDs has been fluorescence inter- mittency (blinking) on time scales of 200 ^s to hundreds of seconds (18). Characterization of blinking and spectral wandering was previously carried out on immobilized nanocrystals (19-21) where the fluorescence of individual QDs was followed over extended periods of time. However, it is unclear whether the properties of immobilized nanocrystals are the same as those in solution—the more relevant environment for biological studies. Our solution FCS indicates that short-time fluorescence fluctuations are due to molecular diffusion into and out of the focal volume (Fig. 1D), and we find no evidence for temporal fluctuations from other photophysical processes on

time scales up to several milliseconds. Cross-correlation FCS, which eliminates detector after-pulsing artifacts, also shows no intermitten- cy on time scales down to — 100 ns (fig. S2). Thus, on these time scales, blinking is sufficiently rare or heterogeneous that no ensemble property emerges.

The measured cross section values we report here are, to our knowledge, the largest two-photon action cross sections reported to date for a fluorescent probe. For a given excitation intensity, the probability of two- photon excitation is as much as three orders of magnitude higher than with conventional probes, and is six orders of magnitude higher than with intrinsic molecules such as NADH (the reduced form of nicotinamide adenine dinucleotide). Because nonlinear excitation of intrinsic components in cells and tissues in multiphoton microscopy can be the primary source of fluorescence background and phototoxicity, increasing the probe excitation probability relative to that of the intrinsic species increases both contrast and sample viability.

To demonstrate the potential of QDs for deep in vivo multiphoton imaging, we imaged mice intravenously injected with water-soluble QDs. The size of QDs makes them useful for angiography, analogous to the conventional use of fluorescent dextrans for this purpose (22). We examined imaging in the two tissue types we have found to be the most challenging: skin and adipose tissue, both highly scattering tissues with severe



refractive index mismatches. Imaging through the intact skin to the base of the dermal layer in a live mouse at 900 nm, we found the QD-containing vas-culature to be clearly visible (Fig. 2B). On the basis of the volume and concentration of QDs injected and the blood volume of the mice (—1.6 ml), we estimate the concentration in the blood to be — 1 ^M for the experiment shown in Fig. 2, A to C, and —20 nM in Fig. 2, E and F. At the higher concentration of QDs (1 ^M), we were easily able to measure blood flow velocity with the use of line scans (Fig. 2, B and C) [as in (22)] and to clearly detect the heart rate from temporal undulations of the capillary wall (Fig. 3C) directly through the skin.

We compared QDs to conventional methods by injecting 70-kD fluorescein isothiocyanate (FITC)-dextran at its solubility limit, corresponding to ~40 ^M fluorescein in the bloodstream. An image acquired at the same depth as in Fig. 2A with five times as much power (Fig. 2D) shows considerably less detail, and we were only able to acquire blood flow measurements at roughly half the depth accessible with QDs. This in vivo comparison corroborates the action cross section measurements in Fig. 1: Because the cross sections are higher by three orders of mag-nitude, use of QDs enables imaging at greater depths than with standard fluorophores, using less average power. The capillary structure in the adipose tissue surrounding a surgically exposed ovary is shown in Fig. 2, E and F. With 780 nm excitation, the autofluorescence from adipose cells

clearly appears in optical sections near the surface of the fat pad (Fig. 2E); Fig. 2F shows the projected vasculature through a 250-^m- thick region within this tissue.

Presumably, QDs are cleared from the body before breakdown of the highly protective amphiphilic coat. Although cadmium is known to be toxic, the mice used in these imaging experiments showed no noticeable ill effects after imaging and are being main-tained as part of an investigation of long-term QD toxicity—an issue that must be addressed before routine use of cadmium-based QDs for chronic experiments involving valuable transgenic animals can begin.

Quantum dots will become key probes for multicolor fluorescence microscopy and will be especially useful for multiphoton microscopy, where bright probes are needed for the variety of challenging imaging tasks to which multiphoton microscopy is now being applied. We have demonstrated their use as a fluorescence angiography probe, and when conjugated to antibodies or aptamers, they will be bright specific labels useful for tracking cells deep within tissue or for detecting low concentrations of antigens—possibly even directly through the skin, as shown here.