Highly efficient molecular delivery into mammalian cells using carbon nanotube spearing

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Introduction of exogenous DNA into mammalian cells represents a powerful approach for manipulating signal transduction. The available techniques, however, are limited by low transduction efficiency and low cell viability after transduction. Here we report a highly efficient molecular delivery technique, named nanotube spearing, based on the penetration of nickel-embedded nanotubes into cell membranes by magnetic field driving. DNA plasmids containing the enhanced green fluorescent protein (EGFP) sequence were immobilized onto the nanotubes, and subsequently speared into targeted cells. We have achieved an unprecedented high transduction efficiency in Bal17 B-lymphoma, *ex vivo* B cells and primary neurons with high viability after transduction. This technique may provide a powerful tool for highly efficient gene transfer into a variety of cells, especially the hard-to-transfect cells.

Vertically aligned carbon nanotubes grown by plasma-enhanced chemical vapor deposition (PECVD)[1] have ferromagnetic catalyst nickel particles enclosed in their tips[2]. This structure makes nanotubes respond to magnetic agitation. The momentum of the carbon nanotubes can be used to penetrate cell membranes (nanotube spearing) and thereby shuttle macromolecules immobilized on the carbon nanotubes into cells. This should be more controllable than other nanotube-based molecular delivery approaches[3,4] because the magnetic field strength, the nanotube speed and the period of spearing can be used to tune the penetration efficiency. In previous research using direct nanotube treatment of cells[3–6], no cytotoxicity has been observed at low carbon nanotube concentrations (<10 μM), which implies that carbon nanotubes are indeed biocompatible. Considering the nanoscale size, nanotube spearing may be more gentle than the ballistic method[7] for mechanical macromolecule delivery with high cell viability.

Biomineralization[8] and other uptake[9] by endocytosis are alternative approaches used to transport plasmids across the plasma membrane. But before the postuptake DNA plasmids reach the nucleus, their intracellular trafficking has to proceed *via* the endosomal or lysosomal pathway, in which a large number of the plasmids are hydrolyzed. The fates of the plasmids in this trafficking process determine the transduction efficiency and contribute to the difference in transduction efficiency between primary and transformed cells[10]. With nanotube spearing, the nucleus may also be penetrated, and, therefore, receive plasmid DNA directly from the invasive nanotubes. This method of gene delivery will enhance genetic transduction of exogenous DNAs, particularly, in primary cells.

In this study we clearly demonstrated that highly efficient transduction with high viability was achieved in the hard-to-transfect cells including B cells and primary neurons.

RESULTS

Responses of nanotubes to magnetic agitation

We first tested the response of our nanotubes to magnetic agitation. Such a property can be simply demonstrated by placing a container of the carbon nanotube suspension on a magnetic stir plate without a magnetic stirring bar in the container. If the nanotubes are magnetically drivable, a rotating 'cloud' synchronizing with the magnetic field can be seen. We also found that not all nanotubes are responsive to the magnetic agitation. Observations using electron microscopy revealed that the magnetically drivable and nondrivable carbon nanotubes used in our experiments are morphologically different. The drivable ones are always short (<2 μm) and have a magnetic particle with an aspect ratio, defined as the ratio of length to width, of ~ 2.9, whereas the nondrivable ones are much longer (>15 μm) and have an aspect ratio of about 0.7 (**Supplementary Fig. 1** online). It is important to point out that the PECVD nanotubes regardless of their length can be dispersed for hours in ethyl alcohol without using any surfactant.

Nevertheless, in a separate experiment, we tested the effect of a surfactant at saturating concentrations. To suspend the nanotubes, we used 0.8 mg/ml Nanosperse AQ (NanoLab, Inc.), which can be used to keep 1 mg/ml nanotubes stably in water suspension for weeks. Ideal suspensions of both magnetic drivable and nondrivable nanotubes in water were secured so that the behaviors

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PUBLISHED ONLINE 20 MAY 2005; DOI:10.1038/NMETH761

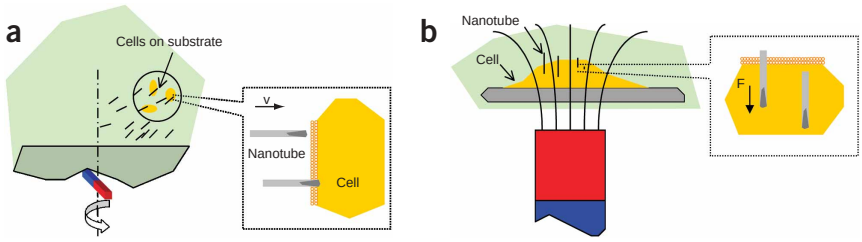

Figure 1 | A two-step procedure of nanotube spearing. (**a**) In the first step, a rotating magnetic field drives nanotubes (short black lines) to spear the cells (yellow) on a substrate. The boxed inset is a close-up of one cell with the nanotube penetrating the membrane. The plasma membrane is illustrated as an assembly of red circles. (**b**) In the second step, a static field persistently pulls nanotubes into the cells. F, magnetic force; v, the velocity of nantobes.

of the nanotubes in magnetic fields were independent of their dispersibility. We obtained this result with or without using the surfactant.

Membrane penetration by nanotube spearing

The spearing of the cell membranes with carbon nanotubes was first demonstrated in MCF-7 cells. The spearing setup and procedure are illustrated in **Figure 1**. The magnetic field drives the nanotubes in medium toward the cells cultured on the substrate. After preliminary spearing by rotating field of nanotubes, the cells were transferred to culture dishes containing nanotube-free medium for enhancing the spearing by a static field of a permanent magnet. The typical membranes of cells without and with nanotube spearing are shown in **Figures 2a** and **2b**, respectively. Parts of the nanotubes that are still outside the membrane are clearly visible (**Fig. 2b**).

We compared the scanning electron microscope (SEM) images of the cell membranes of cells subjected to various combinations of spearing conditions. The process in which cells were speared for 3 min in a rotating magnetic field and for 7 min in a static magnetic field, denoted (3,7), resulted in greater nanotube-embedding in the membrane ($\sim$76% of all cells) than the (10,0) or (0,10) combinations (lower than 10% of all cells, data not shown). We also noted that greater than 90% of MCF-7 cells were viable after the rotating and static field spearing (3,7) based on trypan blue staining. It is important to note that there is no obvious difference in the extent of cell death between the cells with and without spearing (**Supplementary Fig. 2** online).

One concern with carbon nanotube spearing is the intracellular Ni^{2+} contamination owing to the possible incomplete encapsulation of the magnetic particles in the nanotubes. This may alter cellular signal transduction, including the gene expression[11]. A high-resolution transmission electron microscopy study showed that all particles were completely enclosed in the nanotubes by a few layers of graphene sheet and amorphous carbon (**Supplementary Fig. 3** online).

DNA delivery into dividing mammalian cells

As membrane penetration by the nanotubules was strongly implicated by the above data, we sought to spear mammalian cells and thus deliver macromolecules. For these experiments, a mammalian expression vector (pEGFP-c1) containing the EGFP sequence was immobilized on the nanotubes as previously described[6,12,13] (**Supplementary Methods** online). The pcDNA3.1 vector without the gene insert was used as a negative control. The intracellular delivery of plasmid DNA by nanotube spearing was first carried out in Bal17 cells, as described in Methods. After nanotube spearing, Bal17 cells were cultured for 24 h. We used fluorescence microscopy to examine EGFP expression. Approximately 100% of Bal17 cells had a fluorescence signal after nanotube spearing with pEGFP-c1 (**Fig. 3a**), whereas no detectable fluorescence was observed in the negative control Bal17 cells that were cultured in parallel (**Fig. 3b**). Flow cytometry experiments revealed that approximately 85% of the cells were EGFP positive (data not shown). The viability of Bal17 cells before and after spearing was also analyzed by flow cytometry; there was no noticeable difference between the cell viability of control (without spearing) and speared cells at 0, 24 and 48 h after the spearing (**Fig. 3c**). The nanotube spearing did not change the the distribution of cells in G_0/G_1, S, G_2 and M phases of the cell cycle either based on the comparison to control Bal17 cells as determined by propidium iodide staining of nuclei[14].

To understand mechanisms of the nanotube spearing–mediated transduction, we tested different spearing conditions in a series of experiments. The results are summarized in **Table 1**. We used extreme oxidative conditions to remove the Ni particles from the nanotubes (**Supplementary Fig. 4** online). Cells subjected to the spearing protocol (3,7) with these nickel-deprived nanotubes had no fluorescence signal. The incubation, rather than spearing, of the cells with the plasmid-immobilized normal nanotubes (with nickel) did not produce any transduction either. Additionally, no signal was observed upon subjecting cells to the magnetic treatment in the presence of DNA plasmids only. These results indicated that the magnetic actuation of the nanotubes was indispensable to achieve gene expression. Using our experimental conditions, the uptake pathway was not the same as the transducing mechanism. The effectiveness of each of the two spearing steps, that is, using rotating

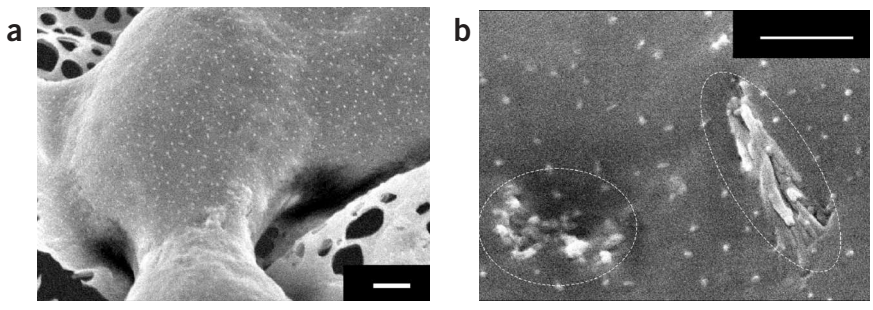

Figure 2 | Nanotube spearing in MCF-7 cells. (**a**,**b**) The cells were cultured on a grid. The membranes shown in the images are of the cells without (**a**) and with (**b**) spearing. Scale bars in **a** and **b** are 1 μm and 500 nm respectively. Dashed ovals in **b** mark the nanotubes in the membrane. The microvilli[25] in the membranes of cells in **a** and **b** have the same site density, which is 15 microvilli/μm^2.

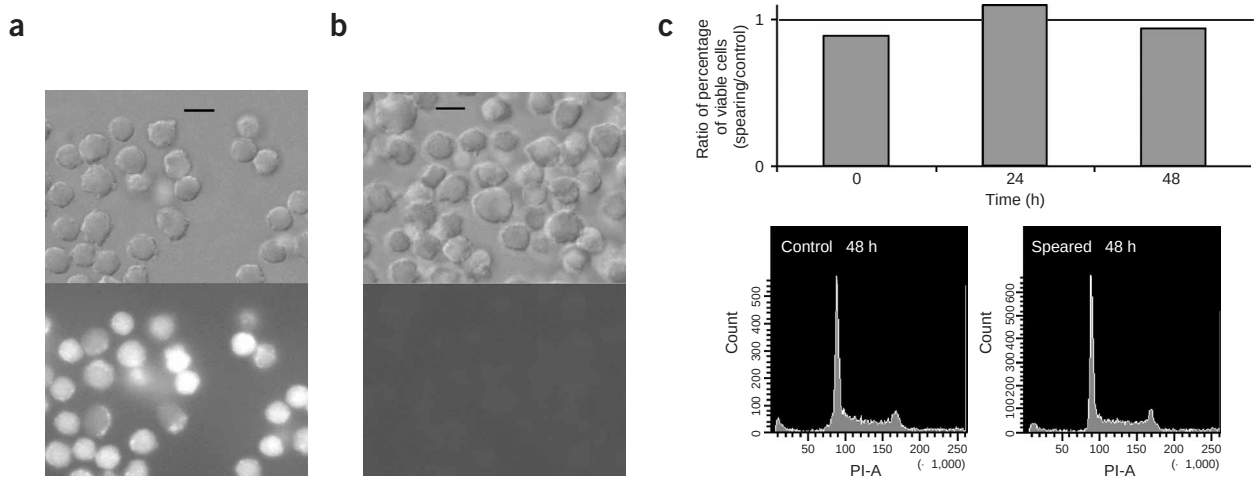

Figure 3 | Transduction of pEGFP-c1 in Bal17 cells by nanotube spearing. (**a**,**b**) Micrographs of cells subjected to the (3,7) spearing protocol by nanotubes with pEGFP-c1 plasmids (**a**), or by nanotubes with the empty pcDNA3.1 vector (**b**). Top panel is the bright field and the bottom is the dark field. Scale bars indicate 10 μm. (**c**) Unspeared (control) and nanotube-speared Bal17 cells were cultured for various times after spearing (0, 24 and 48 h). Cells were stained with propidium iodide and viability was assessed by flow cytometry. In the top panel, the data are presented as a ratio of the percentage of viable cells (speared/control) with a total of 5,000 cells analyzed. In the bottom panel, control and nanotube-speared Bal17 cells were cultured for 48 h, after which cell cycle analysis was done by propidium iodide staining followed by flow cytometric analysis of DNA content. Individual histograms for the control and speared Bal17 cell population are shown versus propidium iodide area (PI-A). The percentages of cells in G_0/G_1, S and G_2/M for control cells were 44.8, 47.5 and 7.7, respectively, whereas the percentages of nanotube-speared cells in G_0/G_1, S and G_2/M were 42.6, 49.7 and 7.7, respectively.

versus static magnetic field, was evaluated in terms of the transduction by two protocols: 15-min exposure to a rotating and 0-min exposure to a static magnetic field (15,0) or vice versa (0,15). Cells exposed to either of these procedures had low levels of fluorescence signals, barely above the background. This suggested the necessity of the two-step protocol for efficient transduction.

DNA delivery in nondividing mammalian cells

According to our original proposal, nanotube spearing may lead to a prominent improvement in transduction of primary cells. The *ex vivo* splenic B cells and primary culture of cortical neurons were used to test this. Mouse splenic B cells were purified from Balb/c mice and cultured as reported[15]. The cortical neurons were separated from embryonic C57/BL6 mice. Almost 100% of the primary B cells expressed EGFP 24 h after the nanotube spearing as determined by fluorescence microscopy, and the amount of fluorescence increased with the spearing time (compare **Fig. 4b** with **Fig. 4a**). Notably, even with the longest spearing time, (20,20), there was no obvious decrease in cell viability as compared to the viability of cells in the samples subjected to the same spearing procedures in nanotube-free medium (**Supplementary Fig. 2**). Cell death observed with primary B cells was due to the absence of fetal calf serum and the B-cell survival factors, such as IL-y, in the medium before and during the spearing, and will be subjected to optimization in the future.

We then carried out experiments with (10,10), (15,15) and (20,20) spearing conditions in primary cortical neurons for EGFP transduction. No fluorescence was observed in 24 h. After 48 h, the neurons subjected to the (20,20) spearing condition had green fluorescence (**Fig. 4c**). As determined by fluorescence microscopy, the percentage of green cells was about 80% (data not shown). We noticed process retraction in the neurons that were subjected to all spearing conditions at 24 h, but they returned to normal after 48 h. Nuclei staining with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) showed a similar cell density in control and speared group even up to 72 h (**Supplementary Fig. 5** online). This suggests that nanotube spearing can perturb cells, but it is minor enough for neurons to recover. The exogenous gene was expressed after the recovery in 48 h.

DISCUSSION

Our results demonstrate highly efficient molecular delivery of plasmid DNA into *ex vivo* neurons and splenic B cells, and transformed mouse B lymphocytes by nanotube spearing technique. In contrast, EGFP expression was not detectable when Lipofectamine 2000 was used as a vehicle for plasmid transduction in both Bal17 cells and *ex vivo* splenic B cells in our experiments (**Supplementary Figs. 6** and **7** online). We suppose the high efficiency of transduction with nanotube spearing results from the unique delivery mechanism: nanopenetration of the cell membrane. We conducted transfection experiments under various conditions (**Table 1**). Our results demonstrate that (i) nanotubes with plasmids, (ii) exposure to a magnetic field and (iii) magnetic response of nanotubes are required to achieve an efficient transduction in the spearing experiments. The incubation experiment clearly excludes the involvement of plasmid uptake by endocytosis and pinocytosis. Therefore, our results strongly suggest the mechanical penetration of cell membranes to be the mechanism of the spearing mediated molecular delivery. Because of the nanoscale of this mechanical impact, the penetration only makes a minor perturbation in cells as vulnerable as primary neurons, and thus, the viability was unchanged after the spearing.

The expression of exogenous genes in B cells and neurons represent several challenges to investigators, with the greatest challenge being the low efficiency of transduction. Our results suggest that the technique of nanotube spearing may be particularly useful for the poorly transducible cells and tissues. In fact, the ability of some proprietary products, such as amaxa's

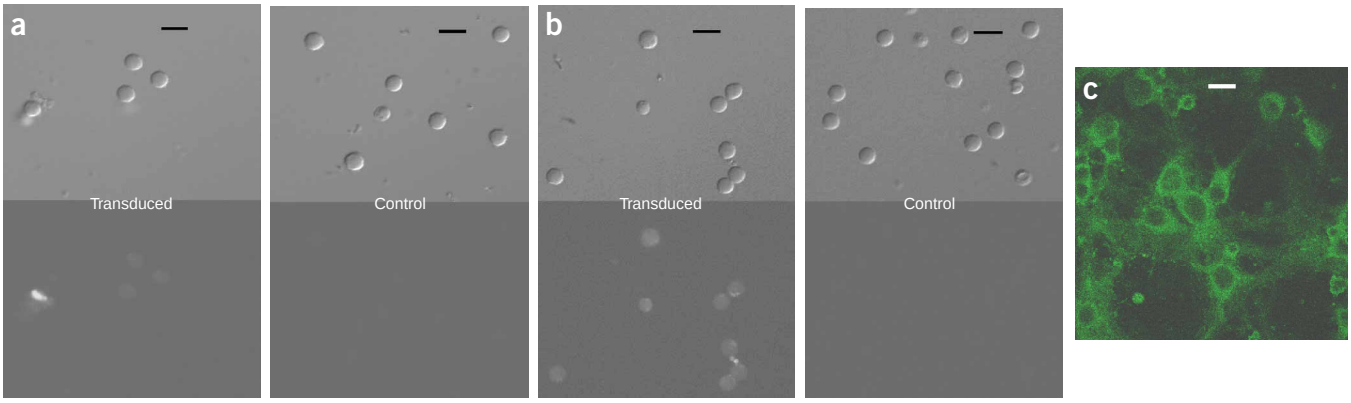

Figure 4 | EGFP transduction in primary cultured cells by nanotube spearing. (**a**,**b**) Primary B cells were speared using protocols (10,10) (**a**) and (20,20) (**b**). In both **a** and **b**, the top panels are bright field micrographs, and bottom panels are dark field micrographs. In the control experiment, cells were speared with nanotubes with empty pcDNA vectors. (**c**) Micrograph of EGFP fluorescence in primary cultured mouse cortical neurons 48 h after spearing using protocol (20,20). Scale bars indicate 10 μm.

Nucleofector[16], to mediate nonviral transfection in primary cells was tested. The efficiency of transfection, however, was never as high as that observed using the nanotube spearing technique. Although this study focused on the delivery of plasmid DNA, the technique can be extended to transport other macromolecules, such as proteins or peptides and RNAi conjugates, into mammalian cells, exploiting the intensively studied surface chemistry for immobilizations. We envision the use of nanotube spearing for *in vivo* applications, such as gene therapy and tissue engineering. Even though researchers have made advances using electroporation and other techniques to enhance plasmid gene delivery *in vivo* into a variety of tissues and tumors[17,18], pressurized vascular and electric field–mediated gene delivery are rather invasive techniques that can cause tissue damage, thereby limiting the efficiency of gene delivery[19–21]. It is therefore conceivable that nanotube spearing can be adapted for *in vivo* molecule delivery with much less invasiveness than other techniques.

It is of interest that another approach, termed magnetofection, can be used to facilitate vector delivery using a magnetic field[22]. The magnetic nanoparticles associated with vectors had been used to enhance local vector concentration on target cells, and make more vectors available for endocytotic uptake. For the magnetofection with non-viral vector, however, the transduction efficiency is not comparable to that of nanotube spearing[22].

According to recent studies, carbon nanotubes can facilitate the delivery of macromolecules in several ways. It has been demonstrated that carbon nanotubes can be internalized by the cell via an unidentified mechanism[3]. Similarly, researchers have observed the cellular uptake of nanotubes by the endocytosis pathway[4]. In both studies, the cells had appreciable signals of the immobilized molecules after incubation with enough carbon nanotubes, for example, 1–5 μM[4]. In contrast, the nanotube spearing technique only requires as little as 100 fM of nanotubes for almost ideal transduction efficiency. So the use of magnetic force resulted in 10^7-fold improvement in the molecular shuttling efficiency. The magnetic force–mediated cell penetration may provide a convenient targetable gene delivery approach in parallel to a previously reported method[6], in which cells had been pinned on an array of nanotube bundles with attached plasmids. Recently, researchers demonstrated cell plasma membrane and nucleus penetration by a silicon nanoneedle attached to an atomic force microscopy tip[23]. The authors envisioned the delivery of macromolecules to a single targeted cell by this technique. But this is a low throughput process, which is not suitable for supporting biochemical assays with a large quantity of cells.

Table 1 | Summary of the nanotube spearing experiments

		Nanotubes				
Treatment	Cell type	With nickel	Without nickel	pEGFP-c1	pcDNA3.1 vector	Result[a]
Spearing (3,7)	Bal17	Yes	No	Yes	No	Fluo
Spearing (3,7)	Bal17	Yes	No	No	Yes	None
Spearing (3,7)	Bal17	No	No	Yes	No	None
Spearing (3,7)	Bal17	No	Yes	Yes	No	None
Spearing (15,0)	Bal17	Yes	No	Yes	No	Weak fluo
Spearing (0,15)	Bal17	Yes	No	Yes	No	Weak fluo
Incubation	Bal17	Yes	No	Yes	No	Nearly background
Spearing (10,10), (20,20)	Primary B cells	Yes	No	Yes	No	Fluo(20,20)
Spearing (10,10), (20,20)	Primary B cells	Yes	No	No	Yes	None
Spearing (10,10), (15,15), (20,20)	Primary neurons	Yes	No	Yes	No	Fluo(20,20)

[a]Fluo, fluorescent signal; none, no signal.

The nanotube spearing technique can be optimized to reduce the amount of DNA required for each transduction. In our experiments, the DNA plasmid was used at saturating concentration, which was about 10^3 times higher than that of the nanotubes. Future quantitative assays of the nanotube functionalization and plasmid immobilization can help to reduce plasmids consumption without affecting the transduction efficiency.

Using the nanotube spearing technique, we have successfully achieved gene delivery into dividing and nondividing cells. The nondividing cells, primary B cells and neurons, are notoriously hard to transfect, and had been effectively transduced only by viral vectors so far. Our nonviral approach, nanotube spearing, yielded a transduction efficiency equivalent to that of the viral approaches. Our technique will immediately benefit the *in vitro* gene delivery for overexpression and knockdown in a variety of cells. In the future, *in vivo* applications that are rarely possible using nonviral techniques, such as gene therapies, genetic vaccination, stem cell–based tissue engineering and drug delivery, may be possible.

METHODS

Nanotube preparation. A silicon wafer was coated with chromium and nickel layers of 350 and 30 nm, respectively. The nanotubes were grown in a hot filament PECVD system[1,24]. A base pressure of 10^{-6} torr was used before the introduction of acetylene and ammonia gases. The growth pressure was 10–20 torr, and the growth time was 1–10 min for proper nanotube length control. The substrate temperature was maintained below 660 °C. The nanotubes were then scraped off from the 2 × 2 cm silicone wafer, and finally suspended in 5 ml ethyl alcohol with an estimated concentration ~1 pM.

The suspension was centrifuged at 10,000*g* for 10 min at room temperature. The supernatant was discarded and the nanotubes were resuspended in 0.5 M HNO_3 that is too dilute to break well-enclosed graphene layer. The container was placed beside a Nd-Fe-B magnet overnight. The nanotubes attached to the wall of the tube next to the magnet were collected and washed three times with deionized water by repeating the centrifuge and resuspension cycle. The nanotubes were stored in 5 ml ethyl alcohol at room temperature.

The nanotubes extracted from 1 ml of the stock were mixed with 5 μg plasmid and 10 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 0.1 M 2-[N-morpholino]ethane sulfonic acid (MES) buffer (pH 4.5) for the aminization between the primary amine groups in the DNA molecules and carboxylic groups on carbon nanotubes. The reaction mixture was left in dark at room temperature for 1 h. The nanotubes were precipitated by the same centrifugation conditions as above and resuspended in 1 ml serum-free culture medium immediately before use (see **Supplementary Methods** online for more details).

Primary cell preparations. Balb/c mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed at Boston College. Mice were cared for and handled at all times in accordance with National Institutes of Health and Boston College Institutional Animal Care Use Committee (IACUC) guidelines. Splenic B cells were purified by depletion of T cells with anti–Thy-1.2 plus rabbit complement; macrophages (and other adherent cells) were removed by plastic adherence. Red blood cells and nonviable cells were removed by sedimentation on Lympholyte M gradients (Accurate Chemical and Scientific Corp.). The resulting B cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 mM HEPES (pH 7.5), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone and 10% heat-inactivated fetal calf serum (Atlanta Biologicals).

Mouse cortical neurons were extracted from an E14 embryonic C57/BL6 mouse. The dissection was conducted under tissue culture hood. The cortices was separated, washed, cut in phosphate-buffered saline, and digested with trypsin and DNase. The cells were disassociated by pipetting up and down, resuspended in Neurobasal Medium (Invitrogen) that contained B27, 1% penicillin G and streptomycin and 2 μM glutamine, and plated on poly(D-lysine)–coated coverslips at the density of 1×10^5 cells/cm^2. The cortical neuron cultures were treated with 10 μg AraC to remove glial cells at day 3 or 4 after the plating. The cells were subjected to nanotube spearing after 24 h of AraC treatment. For a detailed procedure, see **Supplementary Methods**.

Spearing. The cells were dispersed on poly(D-lysine)–coated substrates, such as grids and cover slips. A beaker containing 10 ml serum-free medium supplemented with nanotubes (0.1 pM), was placed on a magnetic stirrer (Fisher Scientific) at room temperature. The cover slips were picked up with tweezers and vertically placed into Dulbecco's Modified Eagle Medium (Invitrogen) with the cells facing the incoming nanotubes. The speed of the stirrer was set at 1,200 r.p.m.

To enhance the nanotube spearing with the static field, the cell dish was laid on a Nd-Fe-B permanent magnet. An adaptor was sandwiched between the dish and magnet with groves machined on the surface to produce a stray field with high gradients in close vicinity to improve the magnetic force.

Electron microscopy. SEM imaging was conducted using JEOL JSA-6340F. Before SEM imaging, MCF-7 cells on grid were fixed in phosphate-buffered saline containing 4% paraformaldehyde, then dehydrated sequentially in ethanol solutions of 50, 70, 95 and 100% (vol/vol in H_2O). After drying in air at room temperature, the sample grid was overlaid with 5 nm–thick gold film.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

The work performed at Boston College is partly supported by the US Department of Energy (DE-FG02-00ER45805), National Science Foundation (NIRT 0304506), and National Institutes of Health (Public Health Service Grant AI-34586). We thank D. Burgess (Boston College, Department of Biology) for the EGFP expressing plasmid. We also thank members of the Burgess' laboratory and Department of Biology for allowing us to use the Confocal Microscopy Facility.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

Received 20 January; accepted 18 April 2005
Published online at http://www.nature.com/naturemethods/

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