Supplementary Information (SI) for Nanoscale.

Significant reduction of cell invasiveness in nanoneedle insertion into a living cell with electron-beam-deposited probe: impacts of probe geometry, speed and vibration

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Confocal microscopy imaging with AFM

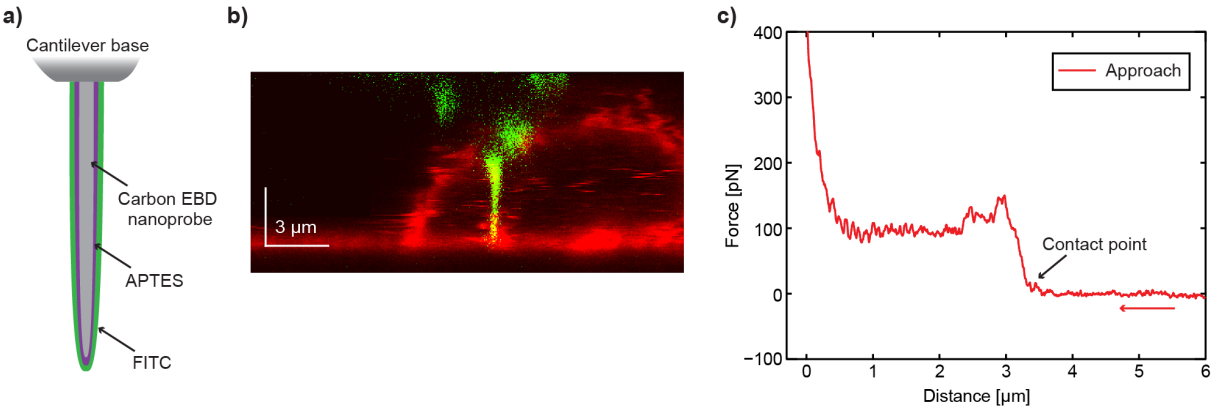

Figure S1. a) Schematic representation of carbon nanoprobe staining, which appears green in the confocal imaging of the cell's cross-section, with the nanoprobe positioned inside b). The confocal image obtained after performing the *F-z* curve measurement, shown in panel c).

The nanoprobe's penetration of the cell membrane was confirmed by a confocal microscopy experiment, as demonstrated in Supplementary Fig. S1. The nanoprobe was inserted into the living cell with an approach speed of 10 µm/s, avoiding the cell nucleus to obtain the *F-z* curve (Fig. S2c). To acquire the confocal image after cell penetration, the nanoprobe was kept inside the cell in contact with the surface during the image acquisition with the feedback on (Fig. S2b), retracting afterward with a speed of 10 µm/s. Fig. S2b shows the confocal image of the cell's cross section with the nanoprobe kept inside. The *F-z* curve, shown in Fig. S2c, also confirmed the penetration event by displaying a force drop after the initial rise. It is important to apply a sufficient set-point force to penetrate the cell membrane and reach the substrate.

Protocol of cell membrane and AFM nanoprobe staining and confocal imaging

On the day of the experiment, the cells (cultured on a 35 mm glass dish (Ibidi)) were rinsed with PBS and kept in the incubator with 2 ml of DMEM (accompanied with 10% FBS and 1% PS), and 1 µl of Cellpaint™ Deep Red (AAT Bioquest) solution. After 30 minutes, the solution was substituted with 2 ml of Leibovitz L-15 medium (Gibco, (-) Phenol red) supplemented with 5% penicillin/streptomycin and imaged with confocal microscopy.

The AFM cantilever with carbon nanoprobe was first placed in the desiccator and treated with 1 ml of APTES (3-Aminopropyltriethoxysilane; >98%) (Tokyo Kasei Kogyo) for 30 minutes. Later the tip was immersed for another 30 minutes in a solution containing 1 mg/ml FITC (Fluorescein Isothiocyanate, Dojindo) and 1 ml of acetone. Finally, rinse the tip with acetone for 10 seconds. As a result of staining, the nanoprobe diameter was increased by 15-20 nm. We quickly transferred the cantilever to the experimental liquid soon after staining to prevent contamination on the nanoprobe.

Confocal imaging was performed with a confocal microscope (Abberior Instruments) combined with JPK Nanowizard ULTRA Speed 2 (Bruker Nano GmbH, Berlin, Germany). The cells and the nanoprobe were observed with a 100x oil-immersed objective lens. Images were taken using Imspector software. The excitation laser wavelength was set at 488 nm and the emission signal was divided into red (630-650 nm) and green (507 nm) signals to produce the confocal images of the stained cell membrane and carbon EBD nanoprobe. We used ImageJ software to analyze the data.

Cell condition after cell penetration experiments

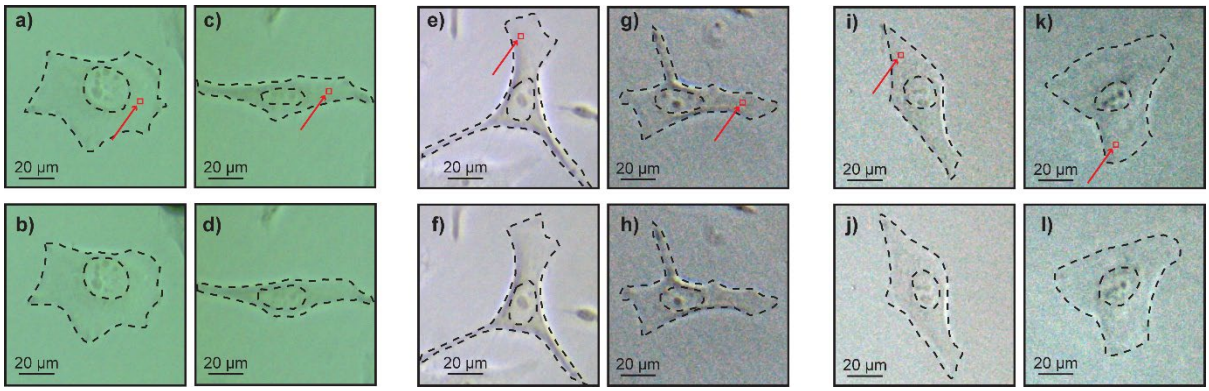

Figure S2. Optical images of cells before (a, c, e, g, i, k) and after (b, d, f, h, j, l) the single *F-z* curve obtained with 120 nm EBD carbon nanoprobes under various conditions: 5 μm/s static (a, b); 5 μm/s dynamic (c, d); 10 μm/s static (e, f); 10 μm/s dynamic (g, h); 30 μm/s static (I, j); 30 μm/s dynamic (k, l). The red arrow with square box indicates the penetration site with EBD nanoprobes.

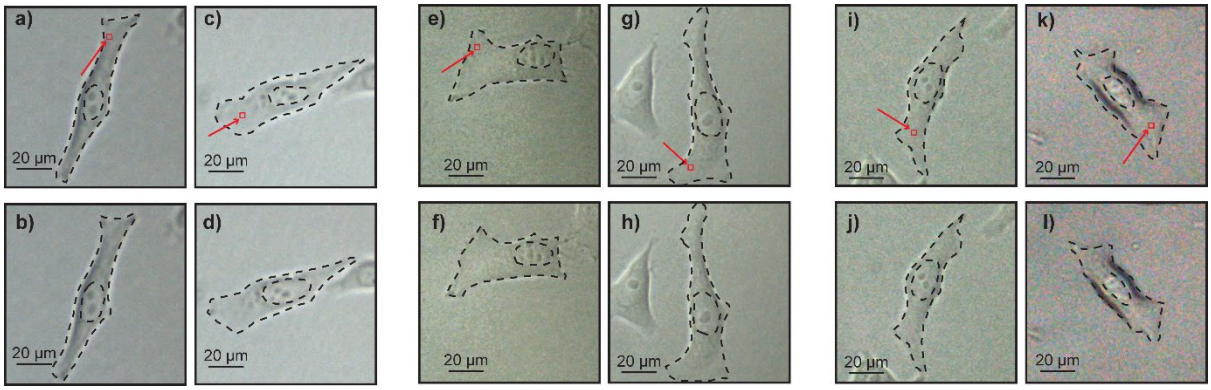

Figure S3. Optical images of cells before (a, c, e, g, i, k) and after (b, d, f, h, j, l) the single *F-z* curve obtained with 80 nm EBD carbon nanoprobes under various conditions: 5 μm/s static (a, b); 5 μm/s dynamic (c, d); 10 μm/s static (e, f); 10 μm/s dynamic (g, h); 30 μm/s static (I, j); 30 μm/s dynamic (k, l). The red arrow with square box indicates the penetration site with EBD nanoprobes.

EBD carbon nanoprobes before and after experiments

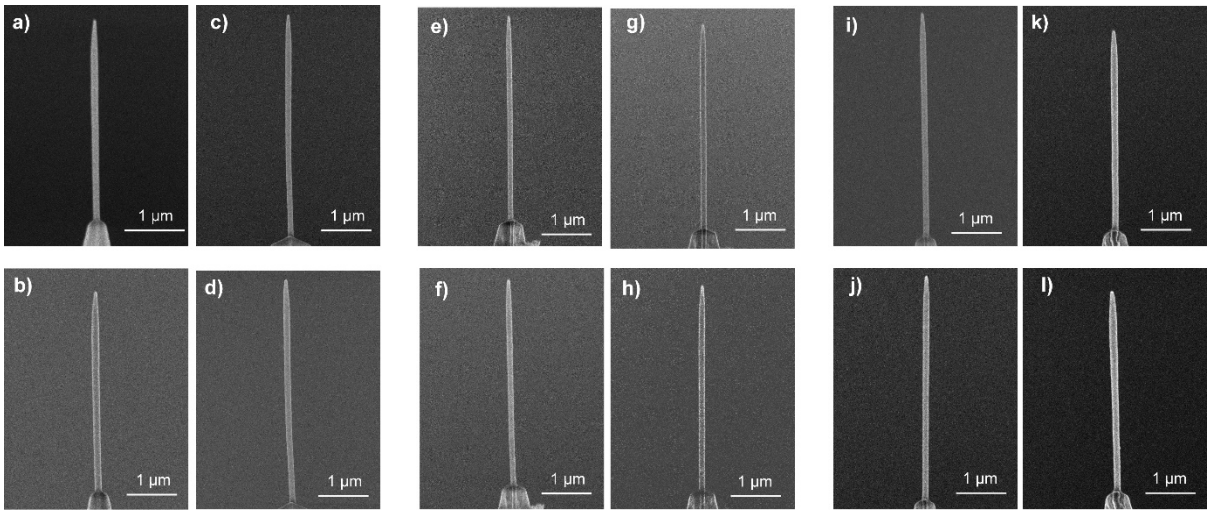

Figure S4. SEM images of 120 nm diameter EBD carbon nanoprobes before (a, c, e, g, i, k) and after (b, d, f, h, j, l) the cell penetration experiment: 5 µm/s static (a, b); 5 µm/s dynamic (c, d); 10 µm/s static (e, f); 10 µm/s dynamic (g, h); 30 µm/s static (I, j); 30 µm/s dynamic (k, l).

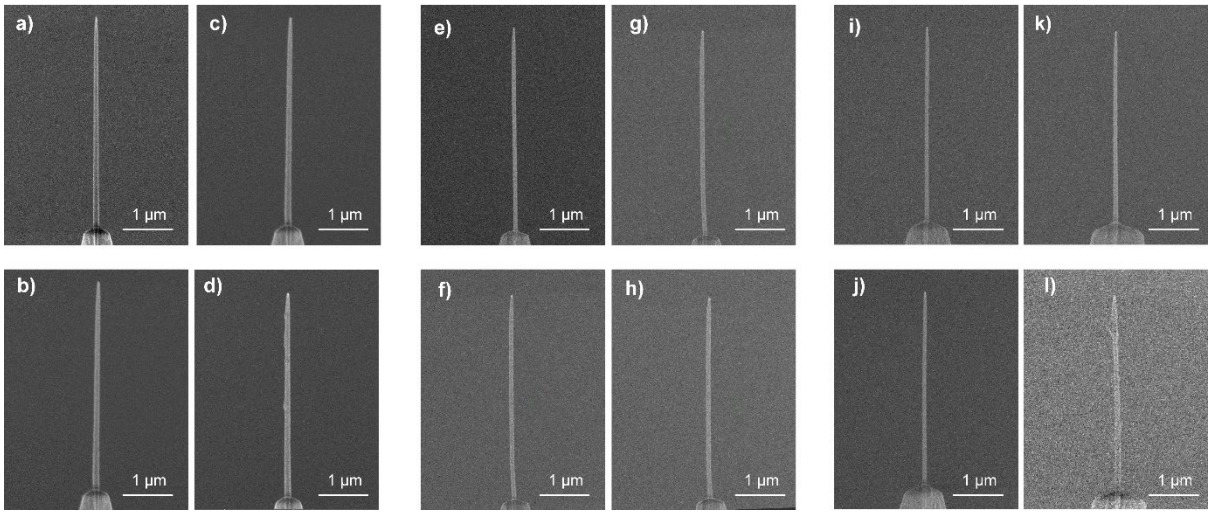

Figure S5. SEM images of 80 nm diameter EBD carbon nanoprobes before (a, c, e, g, i, k) and after (b, d, f, h, j, l) the cell penetration experiment: 5 µm/s static (a, b); 5 µm/s dynamic (c, d); 10 µm/s static (e, f); 10 µm/s dynamic (g, h); 30 µm/s static (I, j); 30 µm/s dynamic (k, l).

Rms measurement during retraction phase of *F-z* curves

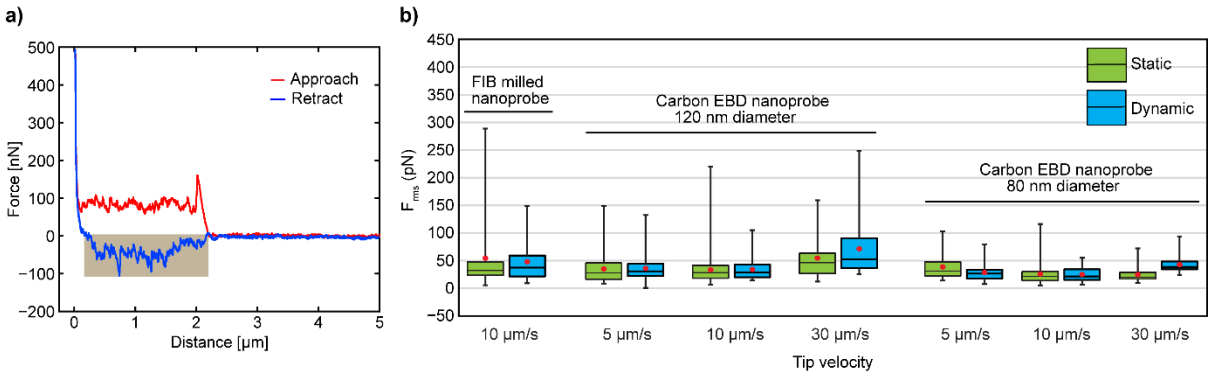

Figure S6. (a) root-mean-square amplitude (F_{rms}) of the retraction F-z profile under the baseline (marked region). (b) Results of the F_{rms} measurement at different speeds by using different methods with FIB-milled and EBD carbon nanoprobes.

Table S1. Statistical data of rms calculation during retraction phase of *F-z* curves with EBD carbon nanoprobes in this study and FIB-milled nanoprobe in the previous study.

Nanoprobe type	Tip speed	Operation modes	Average (pN)	Median (pN)
FIB milled nanoprobe	10 µm/s	static	54 ± 58	32
		dynamic	48 ± 34	37
Carbon EBD nanoprobe 120 nm diameter	5 µm/s	static	35 ± 26	28
		dynamic	36 ± 21	31
	10 µm/s	static	34 ± 27	28
		dynamic	**34 ± 18**	**28**
	30 µm/s	static	55 ± 38	47
		dynamic	71 ± 52	53
Carbon EBD nanoprobe 80 nm diameter	5 µm/s	static	39 ± 22	31
		dynamic	29 ± 26	26
	10 µm/s	static	26 ± 20	23
		dynamic	**25 ± 12**	22
	30 µm/s	static	25 ± 13	**21**
		dynamic	43 ± 15	39

Force offset measurement of *F-z* curves

Table S2. Statistical data of Force offset measurement of *F-z* curves with EBD carbon nanoprobes in this study and FIB-milled nanoprobe in the previous study.

Nanoprobe type	Tip speed	Operation modes	Average (pN)	Median (pN)
FIB milled nanoprobe	10 µm/s	static	95 ± 47	82
		dynamic	78 ± 59	60
Carbon EBD nanoprobe 120 nm diameter	5 µm/s	static	91 ± 75	64
		dynamic	72 ± 51	59
	10 µm/s	static	99 ± 67	79
		dynamic	**59 ± 39**	**52**
	30 µm/s	static	251 ± 127	232
		dynamic	176 ± 129	132
Carbon EBD nanoprobe 80 nm diameter	5 µm/s	static	70 ± 34	77
		dynamic	54 ± 30	48
	10 µm/s	static	44 ± 18	41
		dynamic	**15 ± 11**	**13**
	30 µm/s	static	139 ± 35	130
		dynamic	97 ± 36	89