Electronic Supplementary Material (ESI) for RSC Advances.

3D Au-SiO_2 Nanohybrid as a Potential Scaffold Coating Material for Neuroengineering

Paromita Kundu*[,a], Andreea Belu[a], Elmar Neumann[a,b], Dirk Mayer[a] and Andreas Offenhäusser[a]

[a]Peter Gruenberg Institute, Bioelectronics (PGI-8) Forchungszentrum GmbH, Leo-brandt Str. 1, Juelich 52425, Germany and JARA- Fundamentals of Future Information Technology, Germany

[b]Helmholtz Nanoelectronic Facility (HNF), Forchungszentrum GmbH, Juelich 52425, Germany

Supplementary information

Experimental Section

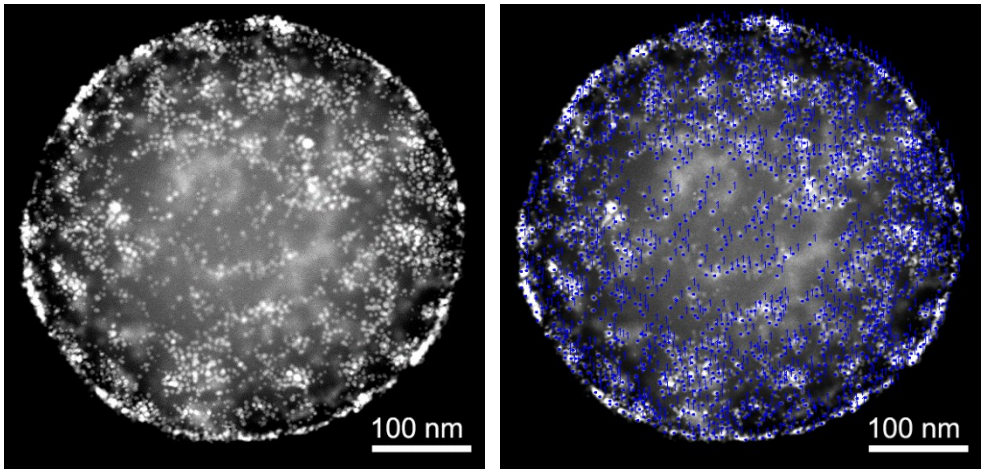
Synthesis of Au-SiO_2 hybrid and surface functionalization: The hybrid was synthesized by the protocol described in earlier report[19] with modification of the time of reaction to obtain larger spheres. The reaction involves formation of amine capped Au nanoparticles of 5-10 nm size in ethylene glycol by microwave route which are transferred to toluene phase after cooling. To 2.5 ml of Au solution, 1 ml ethanolic solution of 10 µL MPTMS {(3-Mercaptopropyl)trimethoxysilane} was added slowly and kept under stirring for 20 mins. To it 40 µL 2M sodium silicate (aq) solution was added and kept under stirring for another 20 mins. Finally left undisturbed for a day approximately. The time of reaction for hydrolysis and formation of SiO_2 core has been increased to more than 20 hours in this case which results in larger spheres than 500 nm. Finally, the product is collected by centrifuge and washed several times with absolute ethanol before drying to powder. In each experiment, the Si/SiO_2 substrates were subjected to O_2 plasma treatment for 30 sec at 200 watt and pressure of 1.4mB before cell culture. In case 2B, after coating the sample on Si/SiO_2 substrate plasma treatment was done for 30 sec and then kept in PEG-silane solution in toluene (5 µL of 2-[methoxy(polyethyleneoxy)propyl]trichlorosilane in 5 ml dry toluene) for 2-3 hours for the exposed SiO_2 surface blocking. After this, the substrate was rinsed well in toluene and ethanol and dried in N_2 purge. 1 mM ethanolic solution of amino-thiol (11-amine-1-undecanethiol hydrochloride) was prepared and the PEG-silane treated substrate was left in amino-thiol solution overnight (approx. 16 hours). The substrate was finally washed with ethanol and milli-Q water before subjecting to cell culture. Each substrate was sterilized in ethanol for 15 mins before cell culture. Si and Si/SiO_2 substrates used for experiments were 1x1 cm and 1.2x1.2 cm, respectively.

Commercially available SiO_2 particles (Sigma-Aldrich, Lot #BCBN1522V, 0.5 – 10 µm size) had been used for comparative cell growth and viability studies.

For preparing the Au-SiO_2 and SiO_2 particles dispersion, about 1 mg of the sample was sonicated for 3 minutes in 1 ml absolute ethanol which resulted in a pink colour and milky white solution, respectively. 20 µl of it was dropcast on substrates in case 1A and for case 2 (A & B) 20 µl X 5 had been used to prepare a dense coverage of Au-SiO_2.

TEM imaging of Au-SiO_2: For TEM studies, an ethanolic dispersion of the sample was dropcast on a formvar coated carbon grid and bright field imaging was carried out in Tecnai G2 microscope operated at 200 kV which is a part of the Ernst Ruska-Centre, Forchungszentrum, Juelich.

Au Nanoparticles count on SiO_2 sphere: The gold nanoparticles count on the SiO_2 sphere in Au-SiO2 hybrid had been performed using cell counter plugin in Image J software. An illustrative example is given below for ~ 500 nm sized Au-SiO_2 nanohybrid where the image before and after counting with appended markers are given. It shows a 2D projection HAADF-STEM image and the total count from it is 1393 considering all the particles on the surface. As it is a 2D projection image one can easily estimate a higher count of nanoparticles to be actually present on the surface.



Cell Culture: Primary cortical neurons were isolated from Wistars rats at the 18th embryonic day, using an enzymatic preparation until a single cell suspension was obtained. Dissociated cells were seeded at a density of 15,000 cells per 1.5 ml volume. Cultures were maintained in an incubator (5% CO_2 and 37ºC). The entire Neurobasal® media, supplemented with 1% (vol/vol) B27 (Invitrogen), 0.25% (vol/vol) L-glutamine (Invitrogen) and 0.1% (vol/vol) gentamycin antibiotic, was changed after 2-3h, followed by a half medium replacement every 3 days after seeding. It is to be noted that no protein coating had been used for the cultures. Activation of control coverslips had been done with oxidizing flame and that for the other substrates were done with 30 sec O_2 plasma, except for the sample with PEG-silane and aminothiol coating. For analysis, the live-dead staining protocol was used to determine the viability of the cells. Calcein dye was used to stain live cells (green) together with ethidium homodimer for the dead cells

(red), using 3 µL of each in 3 ml PBS solution for each substrate, and observed under fluorescence microscope (Carl Zeiss). The cell counting was performed using cell counter feature in Image J.

Immunostaining and Image Analysis: The blocking buffer (BB) used for immunostaining was prepared by mixing 1% BSA and 2% goat serum in PBS. For immunostaining, the substrate with cells from culture were rinsed twice with warm PBS (37^{o}C) and fixed with 4% PFA solution for 12 mins. Samples were rinsed thereafter thrice with PBS. Following this, the permeabilization was carried out with 0.3% Triton-X, prepared in blocking buffer solution, under room temperature condition for 15 mins and washed thrice with PBS. Then to prevent the unspecific binding of the antibodies, the substrates were stored in BB overnight at 4^{o}C. This was followed by rinsing the samples thrice with PBS and treatment with the 1st antibodies for specific binding where Tau-1$_{(mouse)}$ (1:200 in BB) and β-III tubulin$_{(rabbit)}$ (1:500 in BB) were used for the axon specifically and cytoskeleton microtubules, respectively, in consequetive steps. After rinsing with PBS sufficiently, they were stained with 2nd antibodies where anti-Tau$_{(mouse)}$ Alexa Fluor 488 (green) (1:500 in BB), anti-tubulin(rabbit) Alexa Fluor 546 (orange/yellow) (1:500 in BB), sequencially and finally, Phalloidin (red) (1:40 in BB) and DAPI (4',6-diamidino-2-phenylindole) (blue) (1:1000 in BB) were used to characterise the axon, cytoskeleton, F-actin for focal adhesion and the nucleus, respectively. Samples were finally rinsed with PBS and mounted on slide to observe with 40x and 63x oil objective apertures. The image analysis were performed using Neurite J plugin in Image J software for neurite lengths and numbers and focal adhesion area were measured using freehand boundary tool to designate the soma area in Image J.

Cell Fixation: Cells were fixed for electron microscopy and focus ion beam (FIB) cutting using the method as follows: cells were rinsed twice with pre-warmed PBS (37^{o}C) and then fixed with 3.2% glutaraldehyde in PBS (pH 7.4) for 15 minutes. Samples were rinsed twice with PBS and subsequently dehydrated with increasing concentration of ethanol (10%, 30%, 50%, 70% for 10 min respectively, 90% and 95% each 5 min for three times, 100% for storage). Subsequently, the sample was dried using critical point drying procedure to preserve the cell morphology.

FIB-SEM Imaging: For this study, samples from case 1B are used for FIB sectioning and subsequent SEM imaging, carried out in a FEI HELIOS NanoLab™ 600i Dualbeam instrument using 30kV and 0.79nA or 80pA for ion-beam milling and 3kV and 21pA (TLD, SE) for electron beam imaging. Prior to these, the neat sample surface had been coated with a thin layer of iridium (Ir) by sputtering and a thick protective EBID (e-beam induced deposition) and IBID (ion-beam induced deposition) Pt layer had been deposited on the region of interest before FIB sectioning to avoid artefacts like curtaining effects.

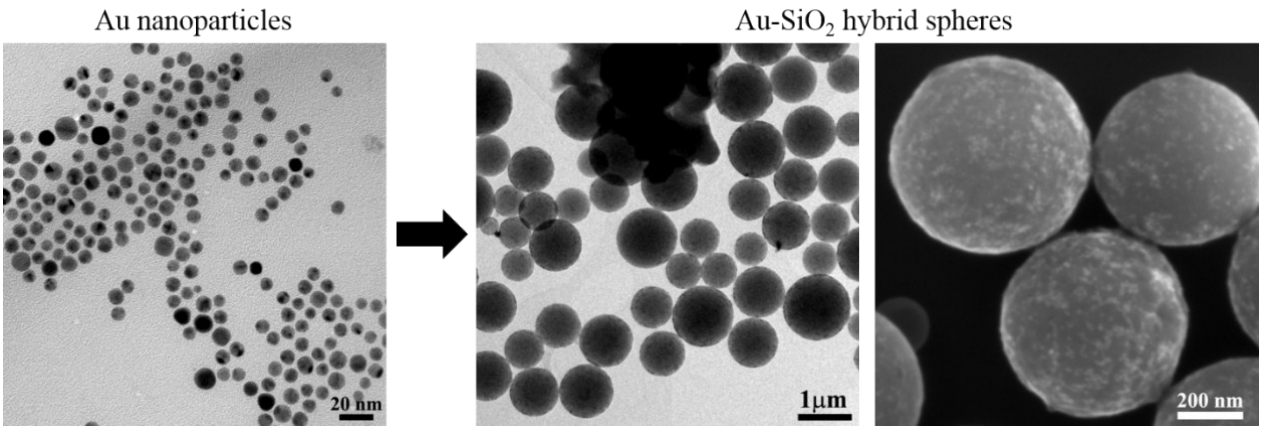


Figure S1. Au nanoparticles of 5-10 nm formed the 500 nm – 1 μm size Au-SiO_2 hybrid as shown in the low magnification TEM and high magnification SEM images.

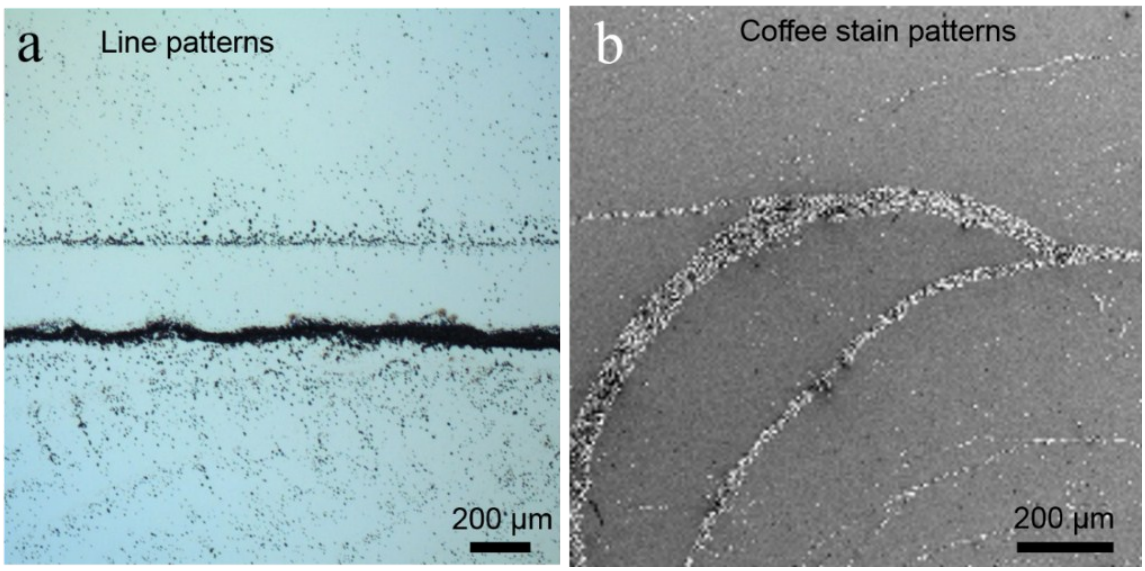


Figure S2. (a) Visible light microscope image showing the line patterns with dispersion on either side of the lines and (b) shows the SEM micrograph of coffee stain patterned region on the substrate.

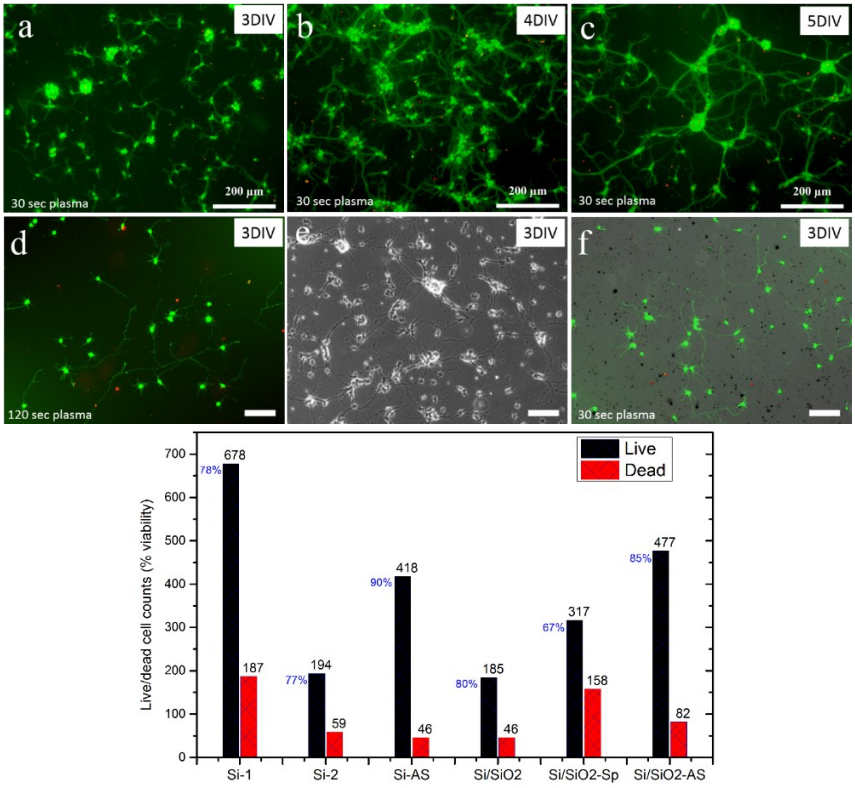


Figure S3. Cell culture on Si substrates under different conditions of O_2 plasma shows the poor adhesion of neurons with 30 sec plasma and clustering increases with more days in vitro as in a, b, c. Instead, 2 mins plasma treated substrate offers better platform for cell attachment and growth (d). (e) shows the control experiments on flamed coverslips and clustering has been seen majorly. An improvement in cell attachment, density and singularity was observed for Au-SiO_2 coated Si substrate even with 30 secs plasma treatment as in (f). The scale marker denotes 100µm. Cell viability analysis (4DIV) have been done with two separate cultures using live-dead staining and fluorescence imaging where the sample designations are as follows: Si-1and Si-2 are blank Si substrates treated with 30 secs and 120 secs O_2 plasma, respectively; rest are Si-AS (AS is Au-SiO_2), Si/SiO_2, Si/SiO_2-Sp (Sp is commercial SiO_2 particles) and Si/SiO_2-AS where 30 secs plasma treatment was done in each case. Details of O_2 plasma treatment are mentioned in the experimental section.

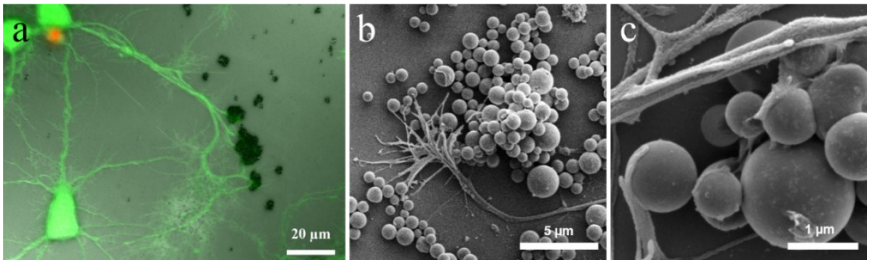


Figure S4. (a), (b) and (c) shows the different cases of the neurites extending and attaching to the Au-SiO_2 spheres specifically.

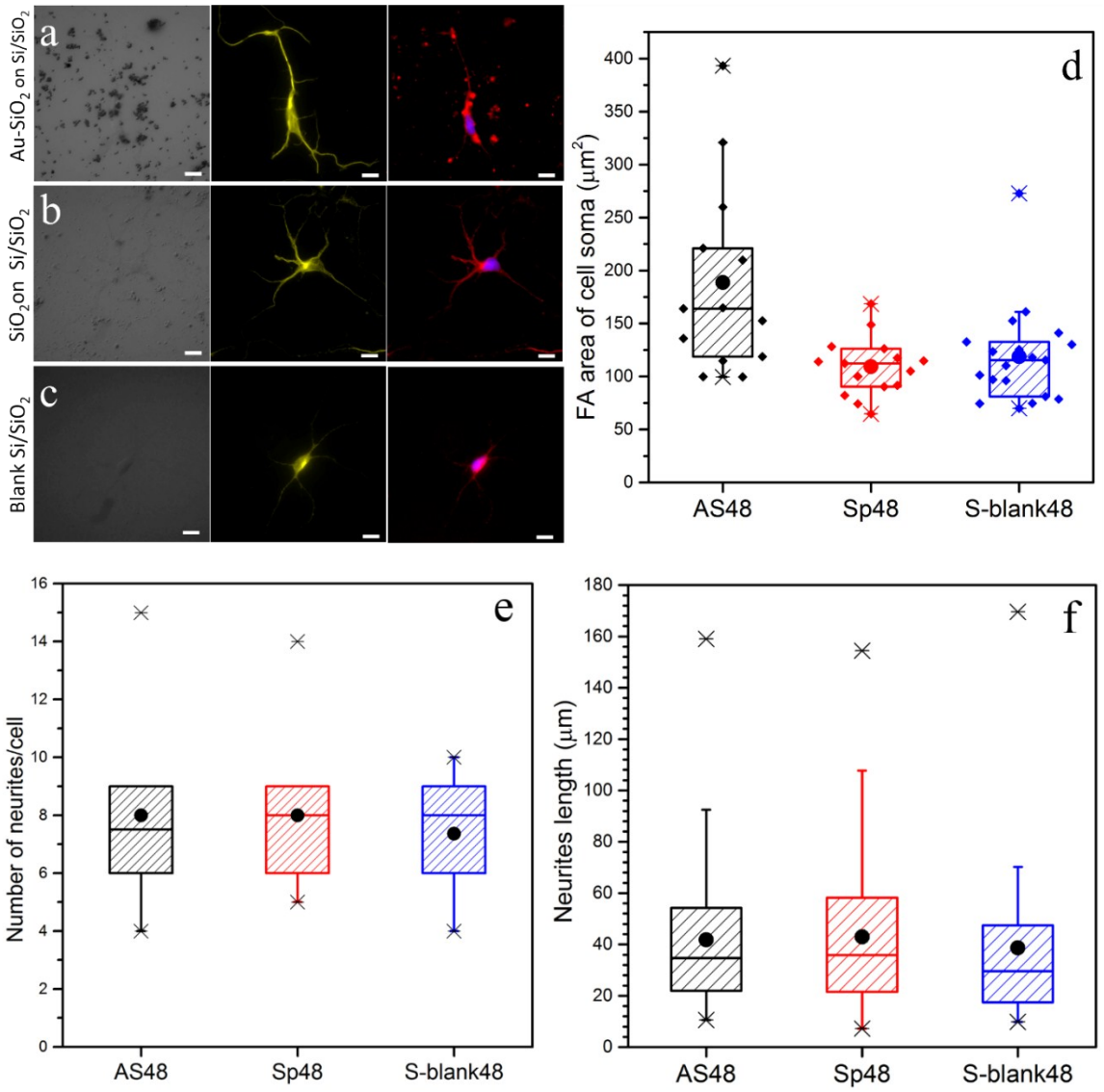


Figure S5. Bright field (showing nanoparticles) and the corresponding fluorescent images as a result of cells Immunostaining (yellow – neurites; red – focal adhesion; blue – nucleus) shows the focal adhesion (FA) of soma and guidance of the neurites by (a) the Au-SiO_2 hybrid (AS48), (b) commercial SiO_2 powder particles (Sp48) and (c) the cell adhesion on blank Si/SiO_2 (S-blank48) substrate. (d) FA area compared for the three cases showing higher mean value in case of AS48. The lowest value of FA for SiO_2 particles also supports the lowest viability of the cells on those substrates. It is probably due to the poor adhesion that the cells survival is less on SiO_2 particles coated substrates. (e) and (f) shows the average number of neurites per cell and average neurites length in each case, respectively, where the mean length of the longest neurite (axon) were ~90 μm (AS48), ~84 μm (Sp48) and ~87 μm (S-blank48). The scale bar in the images denotes 10 μm.

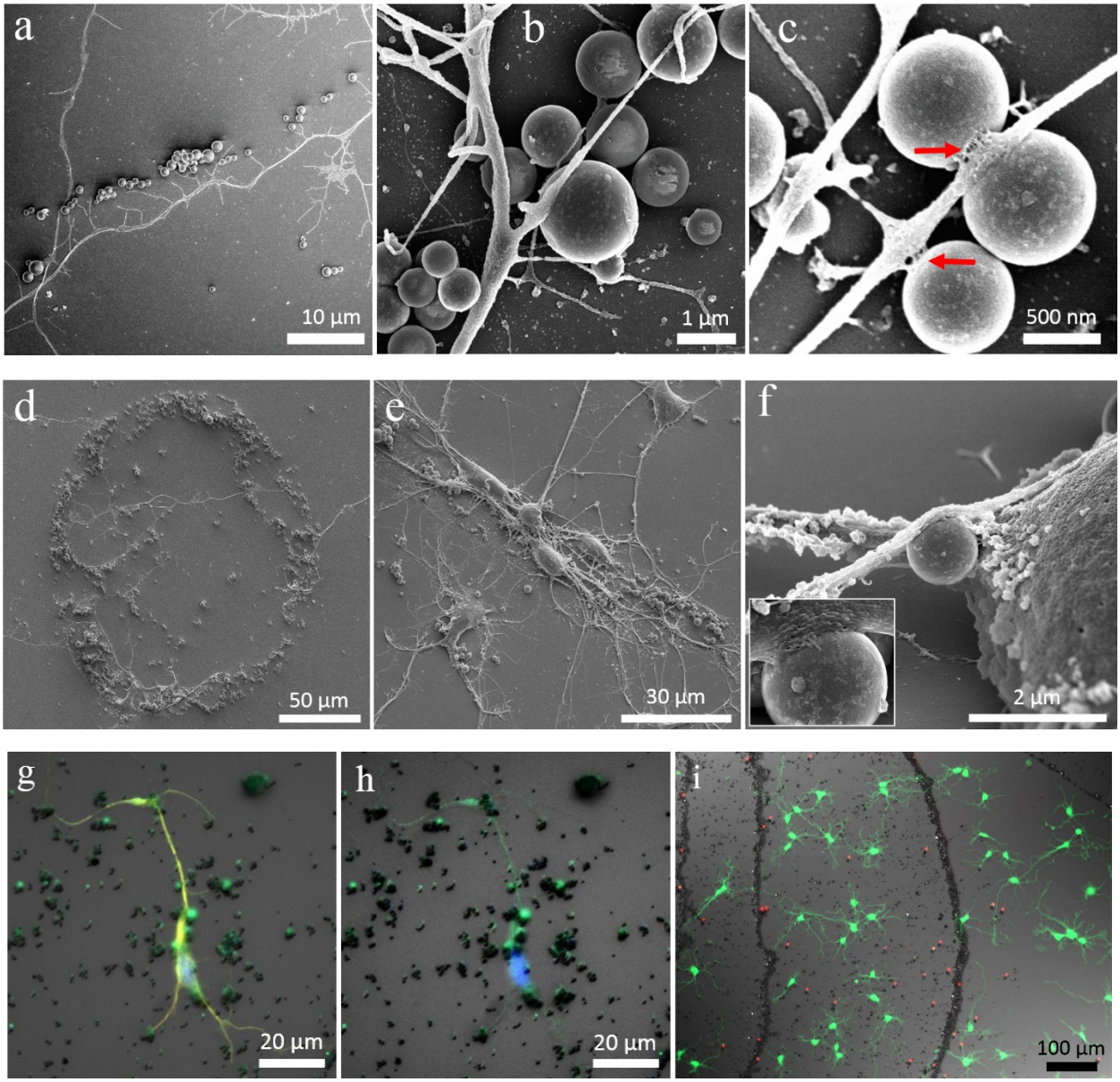


Figure S6. SEM micrographs showing that the neurites extends the filopodia towards the hybrid spheres as in (a), they strongly attach to the Au-SiO_2 surface as in (b) and a magnified view of the attachment (pointed by arrows) is shown in (c).In (d-e), the low magnification images shows the selective cell adhesion to the nanohybrids particles and neurite guiding. (f) shows the attachment of the particles to the neurites which pulls it up from the substrate and inset shows a tight binding of the neurite structure to the nanohybrid surface. (g) & (h) are the composite of bright field and fluorescence images of immunostained cell showing the neurite (yellow) and axon (green) guidance by the hybrid particles. The nucleus is stained blue. (i) Live-dead stained cells on SiO_2 particles patterns and their guiding behavior towards the particles.

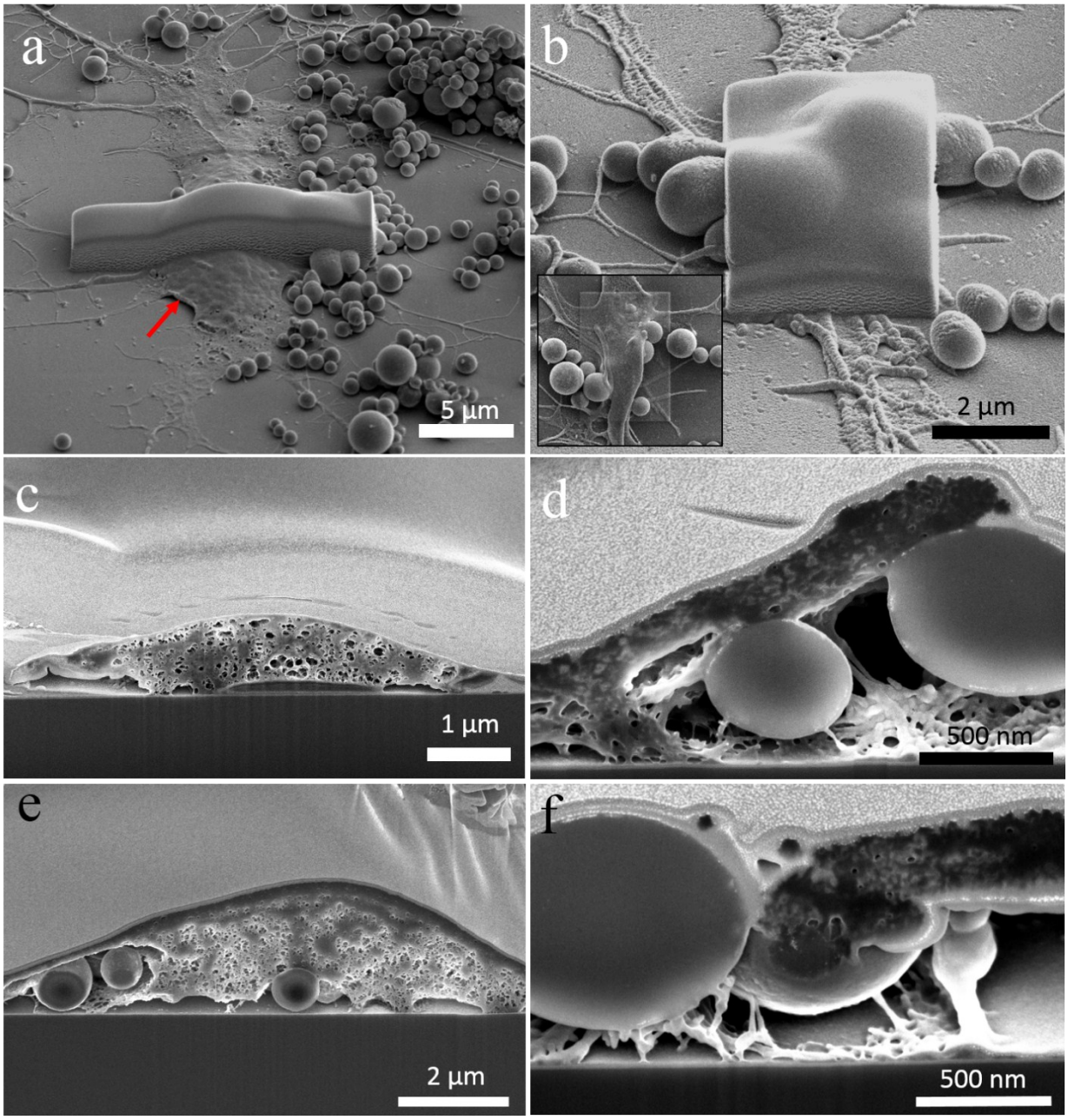


Figure S7. SEM images of the selected portion of cell-nanohybrid assembly which has been coated with a thick conductive Pt containing layer deposited by EBID and IBID sequencially for FIB sectioning. (a) a selected region of the cell body and (b) a selected portion of the neurite, interacting with the nanohybrid. The red arrows mark the places of detachment or delamination of the cell from the substrate, possibly during the process of fixation and the inset in (b) shows the region before IBID. (c) shows the adhesion of the cell to the Si/SiO_2 surface, including the areas of detachment from the surface. (d-f) shows the cell material interaction from different sides of the sphere and their uptake at different places.