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TITLE:

Fabrication of Magnetic Platforms for Micron-Scale Organization of Interconnected Neurons

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magnetic nanoparticles, perpendicular magnetization anisotropy (PMA), magnetic targeted drug delivery, magnetic cell manipulation, neural culture, neural engineering, bio-interfacing, photolithography, magnetic multilayers

SUMMARY:

This work presents a bottom-up approach to the engineering of local magnetic forces for control of neuronal organization. Neuron-like cells loaded with magnetic nanoparticles (MNPs) are plated atop and controlled by a micro-patterned platform with perpendicular magnetization. Also described are magnetic characterization, MNP cellular uptake, cell viability, and statistical analysis.

ABSTRACT:

The ability to direct neurons into organized neural networks has great implications for regenerative medicine, tissue engineering, and bio-interfacing. Many studies have aimed at directing neurons using chemical and topographical cues. However, reports of organizational

control on a micron-scale over large areas are scarce. Here, an effective method has been described for placing neurons in preset sites and guiding neuronal outgrowth with micron-scale resolution, using magnetic platforms embedded with micro-patterned, magnetic elements. It has been demonstrated that loading neurons with magnetic nanoparticles (MNPs) converts them into sensitive magnetic units that can be influenced by magnetic gradients. Following this approach, a unique magnetic platform has been fabricated on which PC12 cells, a common neuron-like model, were plated and loaded with superparamagnetic nanoparticles. Thin films of ferromagnetic (FM) multilayers with stable perpendicular magnetization were deposited to provide effective attraction forces toward the magnetic patterns. These MNP-loaded PC12 cells, plated and differentiated atop the magnetic platforms, were preferentially attached to the magnetic patterns, and the neurite outgrowth was well aligned with the pattern shape, forming oriented networks. Quantitative characterization methods of the magnetic properties, cellular MNP uptake, cell viability, and statistical analysis of the results are presented. This approach enables the control of neural network formation and improves neuron-to-electrode interface through the manipulation of magnetic forces, which can be an effective tool for in vitro studies of networks and may offer novel therapeutic biointerfacing directions.

INTRODUCTION:

Micropatterning of neurons holds great potential for tissue regeneration[1–5] and the development of neuro-electronic devices[6–8]. However, the micron-scaled positioning of neurons at high spatial resolution, as in biological tissues, poses a significant challenge. Forming predesigned structures at this scale requires the guidance of nerve cell processes by locally controlling soma motility and axonal outgrowth. Previous studies have suggested the use of chemical and physical cues[9–12] for guiding neuronal growth. Here, a novel approach focuses on controlling cell positioning by magnetic field gradients[13–17], turning cells loaded with MNPs into magnetic-sensitive units, which can be remotely manipulated.

Kunze et al., who characterized the force needed to induce cellular responses using magnetic chip- and MNP-loaded cells, proved that early axonal elongation can be triggered by mechanical tension inside cells[18]. Tay et al. confirmed that micro-fabricated substrates with enhanced magnetic field gradients allow for wireless stimulation of neural circuits dosed with MNPs using calcium indicator dyes[19]. Moreover, Tseng et al. coalesced nanoparticles inside cells, resulting in localized nanoparticle-mediated forces that approached cellular tension[20]. This led to the fabrication of defined patterns of micromagnetic substrates that helped to study cellular response to mechanical forces. Cellular tension arising from the application of localized nanoparticle-mediated forces was achieved by coalescing nanoparticles within cells[20]. A complementary metal oxide semiconductor (CMOS)–microfluidic hybrid system was developed by Lee et al. who embedded an array of micro-electromagnets in the CMOS chip to control the motion of individual cells tagged with magnetic beads[21].

Alon et al. used micro-scale, pre-programmed, magnetic pads as magnetic “hot spots” to locate cells[22]. Specific activity could also be stimulated within cells using micro-patterned magnetic arrays to localize nanoparticles at specific subcellular locations[23]. Cellular MNP uptake has been successfully demonstrated in leech, rat, and mouse primary neurons[24–26]. Here, this has been

demonstrated on a rat PC12 pheochromocytoma cell line, which has been previously reported to show high uptake of MNPs[27]. In recent years, there have been various medical applications of MNPs, including drug delivery and thermotherapy in cancer treatments[28–31]. Specifically, studies deal with the application of MNPs and neuron networks[32–35]. However, the magnetic organization of neurons using MNPs at a single-cell level deserves further investigation.

In this work, a bottom-up approach has been described to engineer local magnetic forces via predesigned platforms for controlling neuronal arrangement. The fabrication of micron-scale patterns of FM multilayers has been presented. This unique, FM multilayered structure creates stable perpendicular magnetization that results in effective attraction forces toward all the magnetic patterns. Via incubation, MNPs were loaded into PC12 cells, transforming them into magnetic sensitive units. MNP-loaded cells, plated and differentiated atop the magnetic platforms, were preferentially attached to the magnetic patterns, and the neurite outgrowth was well-aligned with the pattern shape, forming oriented networks. Several methods have been described to characterize the magnetic properties of the FM multilayers and the MNPs, and techniques for cellular MNP uptake and cell viability assays have also been presented. Additionally, morphometric parameters of neuronal growth and statistical analysis of the results are detailed.

PROTOCOL:

NOTE: Perform all biological reactions in a biosafety cabinet.

1. Magnetic platform fabrication

1.1. Lithography

1.1.1. Cut glass slides into 2 x 2 cm^2 using a scriber pen. Clean the glass slides in acetone and then isopropanol for 5 min each in an ultra-sonication bath. Dry with ultra-high purity (UHP) nitrogen.

1.1.2. Coat the glass with photoresist using spin-coating at 6,000 rpm for 60 s, to attain 1.5 µm thickness, and bake at 100 °C for 60 s. Expose the sample to a light source, using an appropriate wavelength for photoresist, with a desired pattern, using a photomask or mask-less lithography.

1.1.3. Develop for 40 s in a developer, diluted in distilled water (DW) according to the manufacturer's instructions; wash in DW for 45 s, and dry with UHP nitrogen gas. Inspect the pattern under an optical microscope.

1.2. Sputter deposition

1.2.1. Insert the sample into the main chamber of the deposition system and wait for base pressure (~5×10^{-8} Torr). Open the gas flow; set the argon flow for standard sputtering (28 sccm [standard cubic cm per min) herein. Ignite the sputter targets, then set the sputter pressure to 3 mTorr.

1.2.2. Increase the power on each target until the desired rate is achieved.

NOTE: Pd Rate: 0.62 A/s = 1.0 nm in 16 s; $Co_{80}Fe_{20}$ Rate: 0.32 A/s, 0.2 nm in 6.25 s.

1.2.3. Turn on rotation. Deposit the FM multilayer, alternating between the $Co_{80}Fe_{20}$ and Pd targets, by opening and closing the target shutters, respectively. Deposit 14 bilayers of $Co_{80}Fe_{20}$ (0.2 nm)/Pd (1.0 nm), and finish with an additional 2 nm Pd capping layer.

1.2.4. Lift-off: Soak the sample in acetone for 30 min, and rinse with isopropanol. Then, dry with UHP nitrogen, and keep the sample in a clean and dry environment until use.

2. Characterization of magnetic device via transport measurements

2.1. Use a Si substrate or glass slide with a cross-shaped magnetic bar of 100 µm width, deposited with FM multilayers (see **Figure 1C** inset). Attach the sample to the holder using double-sided tape.

2.2. Using a wire bonder, bond 4 wires to the sample, one on each leg of the cross electrode. Set the sample-holder and sample inside the transport measurement system with a magnetic field so that the magnetic field is perpendicular to the sample. Perform measurements at room temperature.

2.3. Perform transverse voltage (VT) measurement of the device; follow the markings in **Figure 1C** (inset): apply a current of 1 mA between contacts 1 and 3; measure the VT between contacts 2 and 4; then, apply a current between 2 and 4, and measure the voltage between 1 and 3. Finally, calculate the difference between the voltages of both the measurements and divide by 2 to obtain VT. Use a switching system to automatically change between the two measurement configurations.

2.4. Sweep the magnetic field between 0.4 T to -0.4 T in steps of 5 mT and measure the VT as a function of the field. Plot the transverse resistance (VT/I) vs. the magnetic field to determine the anomalous Hall signal, which is proportional to the perpendicular magnetization in the film.

3. Characterization of MNPs and magnetic multilayers by magnetometry measurements

3.1. Magnetometric measurement for FM multilayers

3.1.1. Deposit the FM multilayer on the Si substrate (see section 1.2). Cut the sample into 6 squares of 4 x 4 mm^2 size. Stack the samples one on top of the other and arrange them in the capsule perpendicular to the direction of the magnetic field (see **Figure 1D** inset).

3.1.2. Insert the capsule into the magnetometer and measure the magnetization at room temperature. Sweep the magnetic field between -0.4 T and 0.4 T.

3.1.3. Calculate the total volume of the magnetic material, considering the thickness of the magnetic layer, the size of the samples, and the number of substrates. Divide the magnetization by the total volume of the magnetic material.

3.1.4. Plot the magnetization (per unit volume) vs. the magnetic field. Subtract the diamagnetic background of the substrate from the high magnetic field response and extrapolate the saturation magnetization of the FM from the graph.

3.2. Magnetometric measurement for MNPs

3.2.1. Insert a designated mass of MNPs into a synthetic polymer capsule. Consider a larger volume if measuring small magnetization saturation values.

3.2.2. If the MNPs are suspended in a solvent, dry the MNPs by leaving the capsule open overnight. Insert the capsule into the magnetometer and measure the magnetization at room temperature. Sweep the magnetic field between -0.2 T and 0.2 T.

3.2.3. Calculate the total mass of the MNPs by multiplying the designated volume by the particle concentration. Normalize the results to 1 g.

3.2.4. Plot the normalized magnetization (per gram) vs. the magnetic field. Extrapolate the magnetization saturation of the MNPs from the graph.

4. Collagen-coating protocol

4.1. Coating plastic dishes

4.1.1. Prepare 0.01 M HCl by adding 490 µL of HCl to 500 mL of autoclaved double-distilled water (DDW).

NOTE: Perform this step only in the chemical hood.

4.1.2. Dilute collagen type 1 (solution from rat tail) 1:60–1:80 in 0.01 M HCl to obtain the final working concentration of 50 µg/mL. Place 1.5 mL of the diluted solution in a 35 mm culture dish. Leave the dish in the hood for 1 h, covered.

4.1.3. Remove the solution, and wash 3x in sterile 1x phosphate-buffered saline (PBS). The dish is ready for cell seeding.

4.2. Coating glass slides

4.2.1. Dilute collagen type 1 (solution from rat tail) 1:50 in 30% v/v ethanol. For coating a 35 mm dish, add 20 µL of collagen to 1 mL of 30% ethanol.

4.2.2. Cover the dish with the solution, and wait until all the solution evaporates, leaving the dish uncovered for a few hours. Wash 3x in sterile 1x PBS; the glass slide is ready for cell seeding.

5. Cellular MNP uptake and viability

5.1. Cellular MNP uptake

5.1.1. Prepare basic growth medium for PC12 cell culture by adding 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 0.2% amphotericin to Roswell Park Medical Institute (RPMI) medium, and filter using a 0.22 µm nylon filter.

5.1.2. Add 1% horse serum (HS), 1% L-glutamine, 1% penicillin/streptomycin, and 0.2% amphotericin to RPMI medium to prepare PC12 differentiation medium, and filter using a 0.22 µm nylon filter.

5.1.3. Grow cells in a non-treated culture flask with 10 mL of basic growth medium; add 10 mL of basic growth medium to the flask every 2–3 days, and sub-culture the cells after 8 days.

5.1.4. For cellular uptake, centrifuge the cell suspension in a centrifuge tube for 8 min at 200 × *g* and room temperature, and discard the supernatant.

5.1.5. Resuspend the cells in 3 mL of fresh basic growth medium. Again, centrifuge the cell suspension for 5 min at 200 × *g* and room temperature, and discard the supernatant. Resuspend the cells in 3 mL of fresh differentiation medium.

5.1.6. Aspirate the cells 10x using a syringe and a needle to break up cell clusters. Count the cells using a hemocytometer, and seed 10^6 cells in a regular uncoated 35 mm dish.

5.1.7. Add to the dish the calculated volume of MNP suspension and volume of differentiation medium to achieve the desired MNP concentration and total volume. Mix the cells, MNPs, and differentiation medium; incubate the dish in a 5% CO2 humidified incubator at 37 °C for 24 h.

5.1.8. Centrifuge the cell suspension for 5 min at 200 × *g* at room temperature, and discard the supernatant. Resuspend the cells in 1 mL of fresh differentiation medium, and count the cells using a hemocytometer.

5.2. MNP-loaded cell differentiation

5.2.1. Perform uptake protocol (section 5.1). Seed 8 × 10^4 MNP-loaded cells on a 35 mm, collagen type I-coated dish in the presence of differentiation medium (see collagen coating protocol in section 4.1). After 24 h, add 1:100 fresh murine beta-nerve growth factor (β-NGF) (final concentration 50 ng/mL).

5.2.2. Renew the differentiation medium and add fresh murine β-NGF every 2 days. Image the cells every 2 days using optical microscopy. After network formation (6–8 days for PC12 cells), image the cells using confocal microscopy, and observe the fluorescence of the particles.

5.3. Viability assay for MNP-loaded cells: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cell viability test.

5.3.1. Prepare the basic growth medium according to step 5.1.1. Cultivate the PC12 cells with MNPs at different concentrations (0.1 mg/mL, 0.25 mg/mL, and 0.5 mg/mL in basic growth medium) and also without MNPs for the control in triplicate in a flat 96-well plate (total volume of 100 µL/well). Incubate the cells for 24 h in a 5% CO_2, humidified incubator at 37 °C.

5.3.2. Prepare blank wells containing medium without cells for background correction. Thaw the XTT reagent solution, and the reaction solution containing *N*-methyl dibenzopyrazine methyl sulfate) in a 37 °C bath immediately prior to use. Swirl gently until clear solutions are obtained.

5.3.3. For one 96-well plate, mix 0.1 mL of activation solution with 5 mL of XTT reagent. Add 50 µL of the reaction solution to each well, slightly shake the plate for an even distribution of the dye in the wells, and then incubate the plate in an incubator for 5 h.

5.3.4. Measure the absorbance of the sample against the blank wells using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 450 nm. Measure the reference absorbance using a wavelength of 630 nm and subtract it from the 450 nm measurement.

5.3.5. As slight spontaneous absorbance occurs in the culture medium incubated with the XTT reagent at 450 nm, subtract the average absorbance of the blank wells from that of the other wells. Subtract signal values from parallel samples of MNPs at the same tested concentrations as the cell samples.

5.4. Viability assay for MNP-loaded cells: resazurin-based cell viability test

5.4.1. Prepare basic growth medium according to step 5.1.1. Cultivate the PC12 cells with MNPs at different concentrations (0.1 mg/mL, 0.25 mg/mL, and 0.5 mg/mL in basic growth medium) and without MNPs as control in triplicate in a flat 96-well plate for 24 h. Incubate the cells for 24 h in a 5% CO_2 incubator at 37 °C. Prepare blank wells containing medium without cells.

5.4.2. Wash the cells with 1x PBS. Add the resazurin-based reagent (10% w/v) to the medium and incubate for 2 h in a 37 °C incubator.

5.4.3. Place 150 µL aliquots of the samples in the ELISA reader, and measure the absorbance at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Subtract the signal values from the parallel samples of MNPs at the same tested concentrations as the cell samples.

6. Characterization of MNP concentration inside the cells using inductively coupled plasma (ICP)

6.1. Prepare basic growth medium according to step 5.1.1. Cultivate the PC12 cells with MNPs at different concentrations (0.1 mg/mL, 0.25 mg/mL, and 0.5 mg/mL in basic growth medium) and without MNPs as control in triplicate in a flat 96-well plate (total volume of 100 µL/well). Incubate in a 5% CO_2, humidified incubator at 37 °C for 24 h.

6.2. Transfer the suspension to a centrifuge tube (from each well separately), centrifuge cells at 200 × *g* for 5 min at room temperature and discard the supernatant. Resuspend the cells in 1 mL of fresh differentiation medium, and count the cells using a hemocytometer.

6.3. Lyse the cells by treatment with 100 µL of 70% nitric acid to each well separately for at least 15 min. Add 5 mL of DDW to the lysed cells and filter the solutions.

6.4. Measure the iron concentration using ICP and use the cell count to record Fe concentration per cell.

7. Cell differentiation and growth on magnetic platform

7.1. Clean the patterned substrate with 70% v/v/ ethanol and place the substrate in a 35 mm culture dish in the hood. Place a large magnet (~1500 Oe) below the patterned substrate for 1 min and remove the magnet by first moving the dish up and away from the magnet, and then take the magnet out of the hood. Turn on the ultraviolet light for 15 min.

7.2. Coat the substrate with collagen type 1 according to section 4.2. Suspend the cells after cellular MNP uptake (section 5.1), seed 10^5 cells in a 35 mm culture dish, and add 2 mL of differentiation medium. Incubate the culture in a 5% CO_2, humidified incubator at 37 °C.

7.3. After 24 h, add 1:100 fresh murine β-NGF (final concentration of 50 ng/mL). Renew the differentiation medium and add fresh murine β-NGF every 2 days. Image the cells every 2 days using light microscopy, and after network formation, perform immunostaining on the cells (section 8.1).

8. MNP-loaded cell staining

8.1. Tubulin immunostaining

8.1.1. Prepare 4% paraformaldehyde (PFA) solution by mixing 10 mL of 16% w/v PFA solution, 4 mL of 10x PBS, and 26 mL of DDW. Prepare 50 mL of 1% PBT by adding 500 µL of a non-ionic surfactant to 50 mL of 1x PBS. Prepare 50 mL of 0.5% PBT by mixing 25 mL of 1% PBT with 25 mL of 1x PBS. Prepare blocking solution by mixing 1% bovine serum albumin and 1% normal donkey serum in 0.25% PBT.

NOTE: Use PFA only inside the chemical hood.

8.1.2. Remove the supernatant medium from the cells. Fix the MNP-loaded cells in 4% PFA for 15 min at room temperature inside a chemical hood. Wash the MNP-loaded cells 3x in 1x PBS, 5 min each wash, inside a chemical hood.

8.1.3. Permeabilize the MNP-loaded cells with 0.5% PBT for 10 min. Incubate the MNP-loaded cells first in blocking solution for 45 min at room temperature and then with rabbit anti- α-tubulin antibody in blocking solution overnight at 4 °C. Wash MNP-loaded cells 3x in 1x PBS, 5 min each wash.

8.1.4. Incubate the MNP-loaded cells with Cy2-conjugated donkey anti-rabbit secondary antibody for 45 min in darkness and at room temperature. Wash the MNP-loaded cells 3x in 1x PBS, 5 min each wash.

8.1.5. Perform confocal imaging. For tubulin, use an excitation wavelength of 492 nm and an emission wavelength of 510 nm. For the MNPs (rhodamine), use an excitation wavelength of 578 nm and an emission wavelength of 613 nm.

8.2. Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI)

8.2.1. Wash the MNP-loaded cells 3x in 1x PBS, 5 min each wash. Remove excess liquid around the sample, add 1 drop (~50 µL) of mounting medium containing DAPI to cover an area of 22 mm x 22 mm, and incubate for 5 min in darkness and at room temperature.

8.2.2. Wash the MNP-loaded cells 3x in 1x PBS, 5 min each wash. Perform confocal imaging. For DAPI, use an excitation wavelength of 358 nm and an emission wavelength of 461 nm. For the MNPs (rhodamine), use an excitation wavelength of 578 nm and an emission wavelength of 613 nm.

9. Measurements and statistical analysis

9.1. Morphometric analysis of MNP-loaded cell differentiation

9.1.1. To measure the number of intersections at various distances from the cell body, acquire phase images of cultured cells up to 3 days after treatment with NGF.

NOTE: If done later, the cells may develop networks, preventing single-cell resolution measurements.

9.1.1.1. Open the images in the image processing program, ImageJ, and use the NeuronJ plug-in, which enables a semi-automatic neurite tracing and length measurement[36]. Using the neurite tracer plug-in, trace the neurites and convert the data to binary images. Define the center of the soma.

9.1.1.2. Perform Sholl analysis, available in the NeuronJ plug-in. Define the maximal radius. Repeat the experiment three times. Analyze more than 100 cells in each experiment.

9.2. Cell localization analysis

9.2.1. To determine the percentage of cells localized on the magnetic area after 3 days of incubation, acquire confocal microscopic images of cells with and without MNP uptake. Use DAPI staining (section 8.2).

9.2.2. Manually count the cells atop or partially atop the pattern (touching cells) and the cells that are not. Repeat for three experiments. Analyze more than 400 cells with MNP and without uptake.

9.2.3. Calculate the relative proportion of the cells that are atop the magnetic patterns out of the total number of cells, with and without MNPs. Additionally, calculate the percentage of the magnetic pattern's effective area by adding the cell body diameter to the pattern width to determine the random probability of cells landing on a magnetic pattern.

9.2.4. Perform a single sample *Z*-test to analyze whether the cell distribution is a result of isotropic cell landing, or if there is a preferred bias to the magnetic pattern.

9.3. Growth directionality analysis

9.3.1. To quantify the effect on neurite outgrowth directionality, acquire confocal microscopic images of the cells with and without MNP treatment after 8 days of incubation. Perform immuno-staining (section 8.1).

9.3.2. Using ImageJ software, measure the angle between the cell neurite and the magnetic stripes in both conditions.

NOTE: Analyze only neurites that originate from somas located on the magnetic stripes.

9.3.3. Plot the distribution of the neurites' angles relative to the direction of the stripes ($\Delta\theta$). Perform a Chi-squared test of the distribution of $\Delta\theta$ to demonstrate that the distribution is not normal or uniform.

REPRESENTATIVE RESULTS:

Magnetic platforms with different geometric shapes were fabricated (**Figure 1A**). Magnetic patterns were deposited by sputtering: 14 multilayers of $Co_{80}Fe_{20}$ and Pd, 0.2 nm and 1 nm, respectively. Electron microscopy revealed the total height of the magnetic patterns to be ~18 nm (**Figure 1B**). This unique FM multilayer deposition creates a stable platform with perpendicular magnetization anisotropy (PMA) relative to the substrate plane that enables the attraction of the MNP-loaded cells toward the entire magnetic pattern and not only to the

edges[22,37]. The parameters of the FM multilayer structure were characterized by magneto-transport measurements for which a cross-shaped device of FM multilayers was fabricated (**Figure 1C** inset), and the magnetization perpendicular to the substrate was measured via the anomalous hall effect (AHE)[38], where the AHE resistance is proportional to the perpendicular magnetization. The AHE vs magnetic field measurement showed a hysteresis loop indicative of PMA ferromagnets (**Figure 1C**). The remnant magnetization of the FM multilayers (magnetic moment at zero field) was identical to the magnetization saturation (M_S) at high fields. In addition, the coercive field of the FM multilayers was ~500 Oe, reaching saturation at 1,200 Oe, which enabled easy magnetization of the device and ensured stability against the influence of unintentional magnetic fields. The M_S value of the multilayers was measured using a magnetometer (**Figure 1D**), as described in section 3.1. The M_S was 270 emu/cm^3, which is at par with previous measurements of similar structures.

Fluorescent iron oxide (γ-Fe_2O_3) MNPs were prepared according to a previous publication[39]. The MNPs were synthesized by nucleation, including the covalent conjugation of six-layered thin films of iron oxide to rhodamine isothiocyanate and coating with human serum albumin. The dry diameter size of the MNPs was ~15 nm with zeta potential of -45, according to the transmission electron microscopic measurement. The magnetometric measurements of the MNPs (**Figure 2A**) show that the magnetization curve had no hysteresis, indicating superparamagnetic behavior of the MNPs, a low saturation field of 500 Oe, and relatively high M_S of 22 emu/g. To control cell localization using magnetic patterns, PC12 cells were incubated in medium mixed with iron oxide fluorescent MNPs for 24 h, transforming them into magnetic units. The MNP concentration in the medium can be varied; it was 0.25 mg/mL in the plating experiments. Confocal microscopic images were taken after DAPI staining (**Figure 2B**). The MNPs were internalized into the PC12 cells' soma, but not into the nuclei, which was reflected by the dark shadow in the center. The results show that there was no red florescence in the nuclei, which indicates that the MNPs were not internalized into the nuclei or had attached to the outer surface of the cells. Using ICP measurements, it was possible to quantify the amounts of MNPs internalized into the PC12 cells. The iron concentration inside the cells increased with the increase in MNP concentration in the medium (**Figure 2C**).

The viability of MNP-loaded PC12 cells for different concentrations of MNPs was evaluated using XTT- and resazurin-based assays. **Figure 3A** shows PC12 cells after MNP treatment, growing and differentiating atop a collagen-coated plastic dish. To examine the impact of internalized MNPs on differentiation, Sholl morphological measurement was performed. No significant difference was observed in cell morphology between the MNP-loaded cells and control cells (*t*-test, $p > 0.05$, $n = 3$) (**Figure 3B**). The metabolic activity of the PC12 cells was measured using XTT- and resazurin-based assays after cell incubation with different MNP concentrations. The results were normalized to the control measurement of PC12 cells without MNPs. These concentrations of MNPs showed no significant cytotoxicity toward the cells, evident in the lack of significant differences in cell viability for any of the preparations (*t*-test, $p > 0.05$, $n = 3$) (**Figure 3C,D**). The effects of MNPs on cell plating and development were determined by comparing MNP-loaded cells to non-loaded cells, plated and grown on identical magnetic substrates. Cells were seeded and left to adhere to the substrate. Every 2 days, cells were treated with fresh medium and NGF

as described in section 7. **Figure 4** shows PC12 cells with and without MNP treatment, growing and differentiating on a magnetic substrate with 20 μm wide stripes and 100 μm spacing. After 3 days of growth, the cells were immunostained, DAPI-stained, and images were taken.

The magnetized cells were found to attach to the magnetic patterns and grow branches according to the patterns, while cells without MNP treatment grew with no affinity to the magnetic devices (**Figure 4A,B**). **Figure 4C** presents the positioning of cells and network formation on a substrate with hexagonal geometry of a side length of 200 μm and line width of 50 μm. Cells were imaged after 6 days. The magnetized cells were located on the magnetic pattern, with similar affinity to the striped substrates. Cell positioning was quantified by counting the cell bodies located on the magnetic patterns or attached to them. The relative proportion of cells was calculated from the total cell population. This was done for cells with and without MNP treatment. The effective area of the magnetic response was calculated by adding the cells' diameter (~10 μm for PC12 cells) to both edges of the magnetic pattern. For the magnetic stripe patterns, the effective magnetic area ratio was 33%, which corresponded to the probability of the cells landing randomly on the magnetic stripes. The results showed that 75% of MNP-loaded cell bodies were in contact with the magnetic stripes, whereas only 35% of the un-magnetized cells were located on the stripes (in statistical agreement with an unbiased distribution) (**Figure 4D**). Statistical analysis indicated that the measurement was not derived from arbitrary cell landing (one-sample *z*-test, $p < 10^{-6}$, $n = 430$).

In contrast, the statistical analysis of the magnetic hexagons revealed that 92% of the MNP-loaded cells' soma were attached to the magnetic patterns, compared to 38% for cells without MNP treatment (**Figure 4E**). The effective area ratio of the hexagons was 32% of the substrate. Statistical analysis for the hexagons also indicated that the measurement was not derived from arbitrary cell landing (one-sample *z*-test, $p < 10^{-6}$, $n = 370$). The results revealed a clear preference of the MNP-loaded cells for the magnetic pattern, while cells without MNPs adhered randomly to the entire substrate. In addition to the cell-positioning effect, these magnetic platforms were found to also control the directionality of the growing neurites. **Figure 5A** shows MNP-loaded cells with neurites, aligning according to the stripes' orientation. In contrast, the control measurement of cells without MNPs showed neurite growth across the platform regardless of the magnetic patterns.

To evaluate the magnetic effect on neuronal growth directionality, the angle between the neurites and the magnetic stripes was measured. The data revealed that 80% of the neurites of MNP-loaded cells exhibited correlation with the magnetic stripes' orientation, within $\Delta\theta < 15°$ relative to the stripes' direction. However, only 32% of the neurites of cells without MNPs developed in that range. Cells without MNP treatment showed no correlation with the magnetic stripes and grew according to a uniform angle distribution (**Figure 5B**). Statistical analysis of the distribution of $\Delta\theta$ revealed that it was not normal or uniform (Chi-square test, $p < 0.001$). The effect of the hexagonal geometry on neurite growth was demonstrated as well. **Figure 5C** shows fluorescence images of neural network development of magnetized and un-magnetized PC12 cells atop a magnetic pattern of hexagons and large circles between the edges. The side length of the hexagon was 200 μm and the lines width was 10 μm; the circle diameter was 30 μm. The

cell somas showed high affinity for the circles and developed a well-oriented neural network along the hexagonal outlines. A zoom-in image demonstrates cells attached to a magnetic circle and neurites growing along those outlines (**Figure 5D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Characterization of the magnetic devices. (**A**) Optical microscopic images of magnetic devices with various geometric shapes. Scale bar = 200 µm. (**B**) Scanning electron microscopic image of $Co_{80}Fe_{20}$/Pd multilayers and a schematic of the multilayers. The total height of the magnetic patterns is 18 nm. Scale bar = 100 nm. (**C**) Anomalous Hall effect measurement of the magnetic device showing the coercive and remnant magnetic fields of the FM. Inset: image of device with marked electrodes. (**D**) Magnetometry of multilayer device shows the magnetization saturation value calculated per volume. This figure has been modified from Marcus et al.[37]. Abbreviations: AHE = Anomalous Hall effect; FM = ferromagnetic; B = magnetic field.

Figure 2: PC12 cell uptake of MNPs. (**A**) Magnetometric measurement of MNPs at room temperature. (**B**) Confocal microscopy image of MNP uptake by PC12 cells. Nuclei stained with DAPI; MNPs labeled with rhodamine enter the cells. Scale bar = 10 µm (**C**) ICP measurement of the internalized iron oxide MNPs (pg) by PC12 cells after 24 h of incubation with several MNP concentrations. This figure has been modified from Marcus et al.[37]. Abbreviations: MNPs = magnetic nanoparticles; DAPI = 4',6-diamidino-2-phenylindole; ICP = inductively coupled plasma.

Figure 3: MNP-loaded PC12 cell viability. (**A**) Confocal microscopic images of differentiated PC12 cells incubated with MNPs. Arrows show differentiated neurites with internalized MNPs. Scale bars = 50 nm. (**B**) Sholl analysis of neurite outgrowth of PC12 cells after 3 days of differentiation. (**C**) XTT viability assay of PC12 cells treated with increasing concentrations of MNPs after 24 h of incubation. Measurements are normalized to control. (**D**) Resazurin-based viability assay of PC12 cells treated with increasing concentrations of MNPs after 24 h of incubation. Measurements are normalized to control. There is no statistical significance in both analyses. This figure has been modified from Marcus et al.[37]. Abbreviations: MNPs = magnetic nanoparticles; XTT = 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

Figure 4: Cell localization atop magnetic devices. (**A**) Fluorescent images of PC12 cells loaded with MNPs growing on the magnetic stripes: (i) α-tubulin labeling, (ii) DAPI staining, and (iii) merged image. Scale bar = 100 µm. (**B**) Fluorescent images of PC12 cells without MNP treatment, growing on top of the magnetic stripes as in (**A**). (**C**) Fluorescence images of PC12 cells, with and without MNPs, on the hexagonal magnetic pattern. Scale bar = 200 µm. (**D**) Percentage of cell bodies, with and without MNPs, located on the magnetic stripes. Error bars represent standard deviation. The dotted line represents the probability of cells landing on the magnetic stripes. (**E**) Percentage of cell bodies, with and without MNPs, located on the magnetic hexagons. Error bars represent standard deviation. The dotted line represents the probability of cells landing on the magnetic patterns for a random distribution. There is no statistical significance in both analyses. This figure has been modified from Marcus et al.[37]. Abbreviations: MNPs = magnetic nanoparticles; DAPI = 4',6-diamidino-2-phenylindole.

Figure 5: Neuronal network directionality. (**A**) Confocal microscopic images of α-tubulin-labeled PC12 cells with MNP treatment (left) and without MNP treatment (right), growing atop magnetic stripes. The stripes are marked. (**B**) Polar histograms present the neurite directionality effect on PC12 cells (left) with MNP treatment and (right) without MNP treatment. The deviation of the orientation angle is defined as the difference in the angle between the neurites and the magnetic stripe orientation (15° bins). (**C**) Fluorescence images of PC12 cells grown atop a magnetic hexagonal pattern, (left) with MNP treatment and (right) without MNP treatment. Scale bar = 200 µm. (**D**) Zoom-in image of a magnetized cell developing neurites according to the magnetic pattern. Scale bar = 30 µm. This figure has been modified from Marcus et al.[37]. Abbreviation: MNPs = magnetic nanoparticles.

DISCUSSION:

The representative results demonstrate the effectiveness of the presented methodology for controlling and organizing neuronal network formation at the micron-scale. The MNP-loaded PC12 cells remained viable and were transformed into magnetic sensitive units that were attracted by the magnetic forces from the FM electrodes to specific sites. This is best demonstrated in **Figure 5C**, where the cells preferentially adhered to the larger vertices of the hexagons and not the thin lines. Moreover, branching of the cells also developed favorably following the magnetic patterns. All control experiments demonstrated unambiguously that the magnetic forces directed the localization of the cell bodies and outgrowths. Although it was demonstrated that topographic cues can be used for directing neurite outgrowth[40,41], this is not the case here, as cells without MNPs showed no response to the shape of the pattern.

A bottom-up approach was employed to engineer the local magnetic forces, using standard photolithography and sputtering depositions that are available in many research facilities, facilitating the adoption of these techniques by many researchers. The bottom-up approach allows freedom in the design of complex patterns and shapes according to the researchers' needs, with micron-scale resolution for centimeter length areas. Although the results were demonstrated on glass slides, in principle, it is possible to prepare the devices atop other biocompatible materials that are suitable for in vivo therapeutic applications such as flexible electrode arrays for neuronal recording and stimulation[42,43].

These unique PMA platforms, attained by multilayer deposition, produce a strong magnetic field along the entire magnetic pattern and not only on the edges, as observed earlier[22]. Additionally, FMs were designed with a large remnant magnetization saturation, i.e., even when the external magnetic field is removed, the electrodes remain fully magnetized and keep attracting the cells without the need of an external magnet. However, an external magnetic force can assist in fully magnetizing the MNPs in the cells, thus increasing the force of attraction and efficiency during plating. An important consideration in FM design was the number of repetitions. While more repetition will increase the total magnetic moment, which is favorable, adding many layers will also increase layer intermixing, causing less stable PMA and finally result in an in-plane magnetization[44,45] easy axis and attraction to different edges of the electrodes[22,37]. Therefore, it is necessary to optimize the FM number and composition of the multilayers to ensure stable PMA

with maximal magnetic fields.

The MNPs presented in the representative results showed almost no toxicity toward the PC12 cells at the tested concentrations, nor did they affect cellular behavior, despite being able to enter the cells and having a relatively high magnetic moment. The attraction force on a cell depends on the number of MNPs in the cell and the magnetization of each MNP. Ideally, both should be high; however, there may be a tradeoff between the two. With some commercial MNPs, cell viability was good, but the magnetic moment was too weak. For other particles fabricated in the laboratory, the magnetic moment was high, but the MNPs tended to aggregate and cell viability was low. Thus, it is important to test cell viability and characterize their magnetization when choosing MNPs. The MNPs used here are also fluorescent, which makes it easy to track their location in the cells. The results show neurites developing according to the shape of the magnetic pattern, and the fluorescence indicates the presence of MNPs along the neurites.

The internalization mechanism of MNPs into cells has been previously investigated[46]. Cellular uptake of MNPs occurs via endocytosis according to their size, shape, and surface chemistry. Previous studies examined the uptake of different types of MNPs into neurons[24]; cellular uptake of coated MNPs was better than the cellular uptake of uncoated MNPs. As shown in **Figure 3A,B**, MNPs were internalized into the cytoplasm, but remained outside the nuclei and were transferred to the neurites during their development. Additionally, MNPs conjugated to NGF that activated the NGF signaling pathway were also internalized into cells via endocytosis[47,48].

To conclude, this paper presents an effective toolbox of magnetic manipulation for research requiring biological element organization. The use of magnetic forces enables the positioning of cells, directing neurite growth. This method enables the design of platforms with complex geometric shapes. The forces of magnetic attraction can be engineered to manipulate the neural network formation remotely by changing the magnetic force landscape over time. This entire methodology can be easily extended to control other factors or chemicals that can be coupled to the MNPs and bring them to predesigned points of interest, all with micron-scale resolution.

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DISCLOSURES:
The authors declare no competing financial interests.

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Figure 1

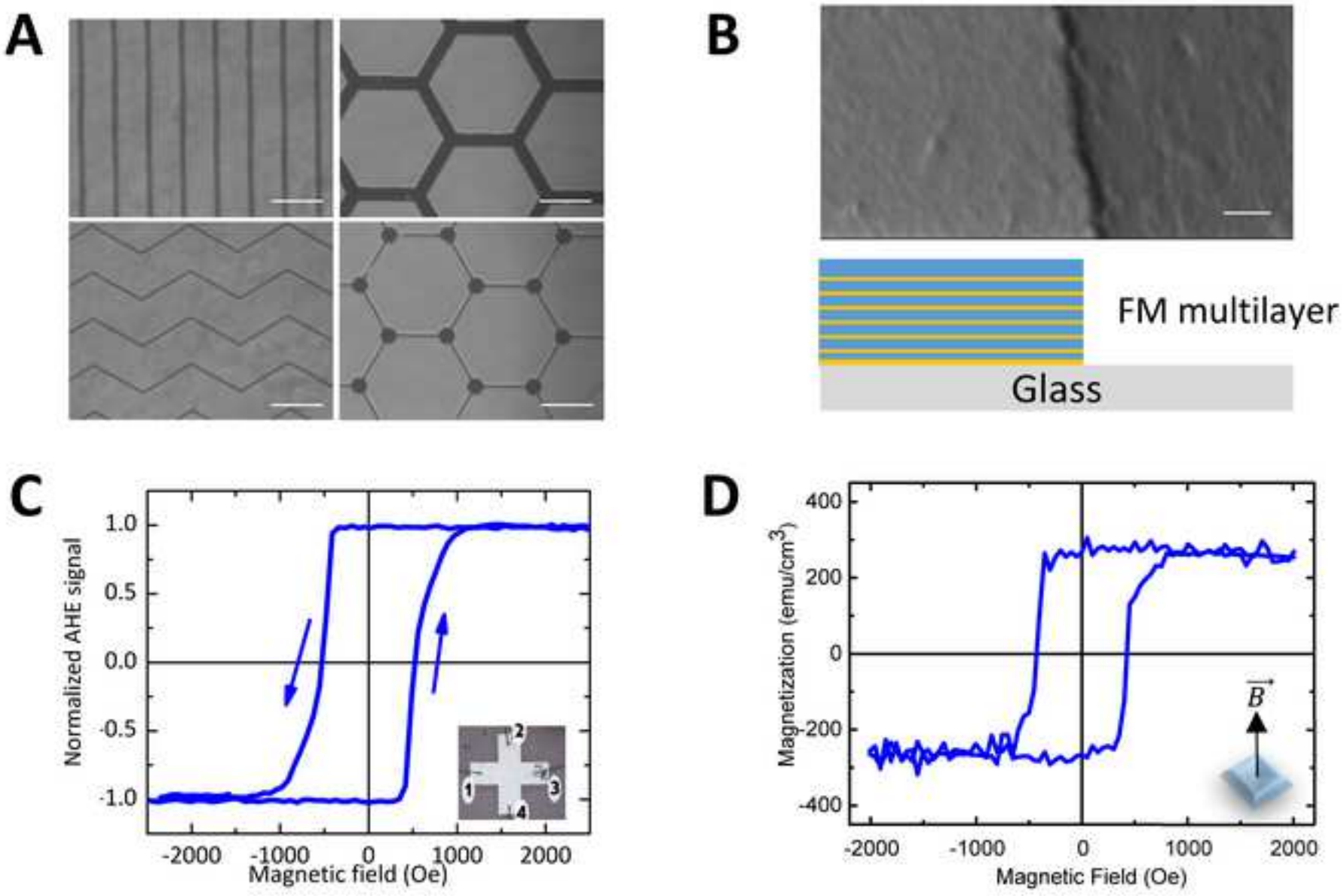

Figure 2

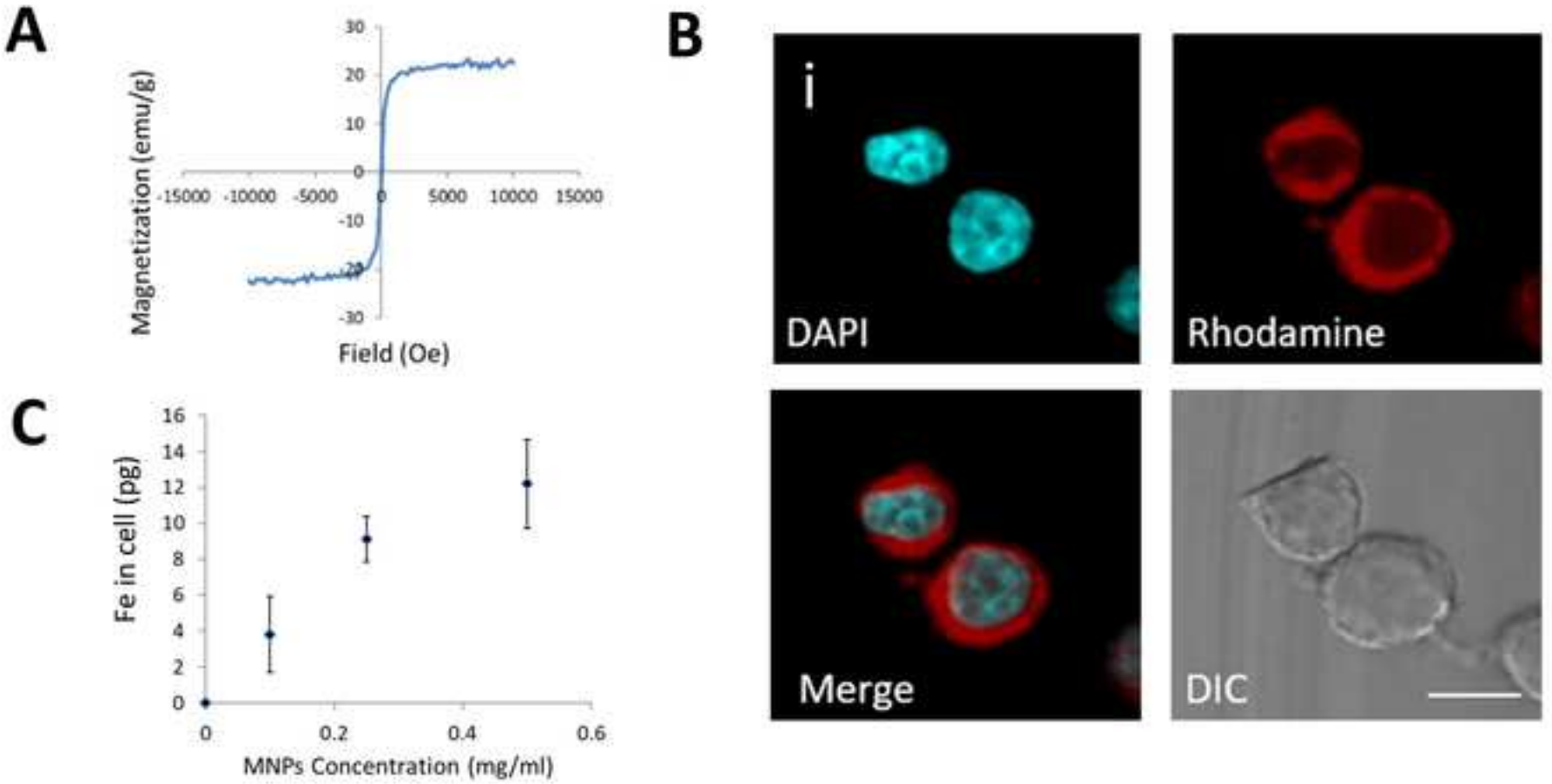

Figure 3

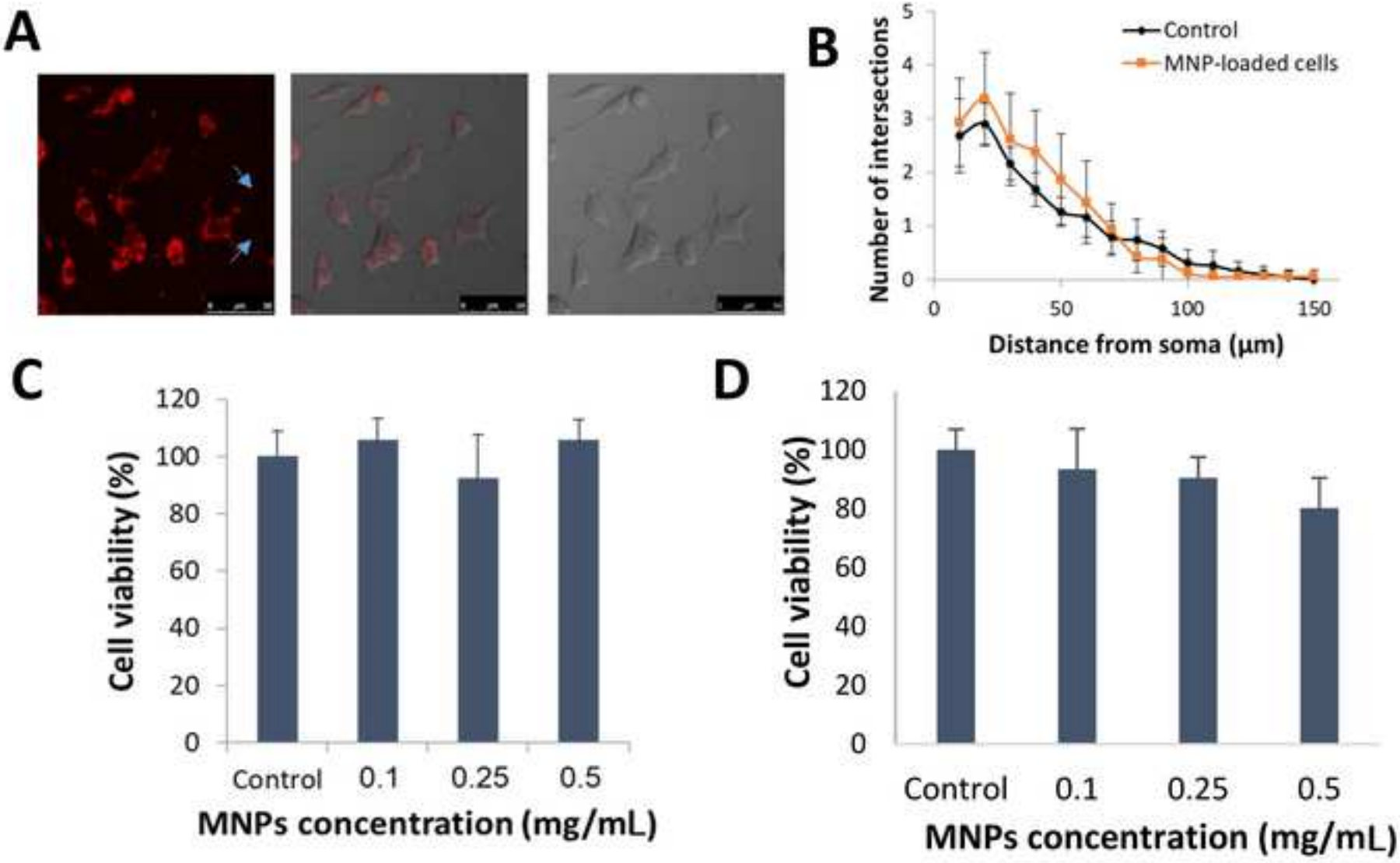

Figure 4

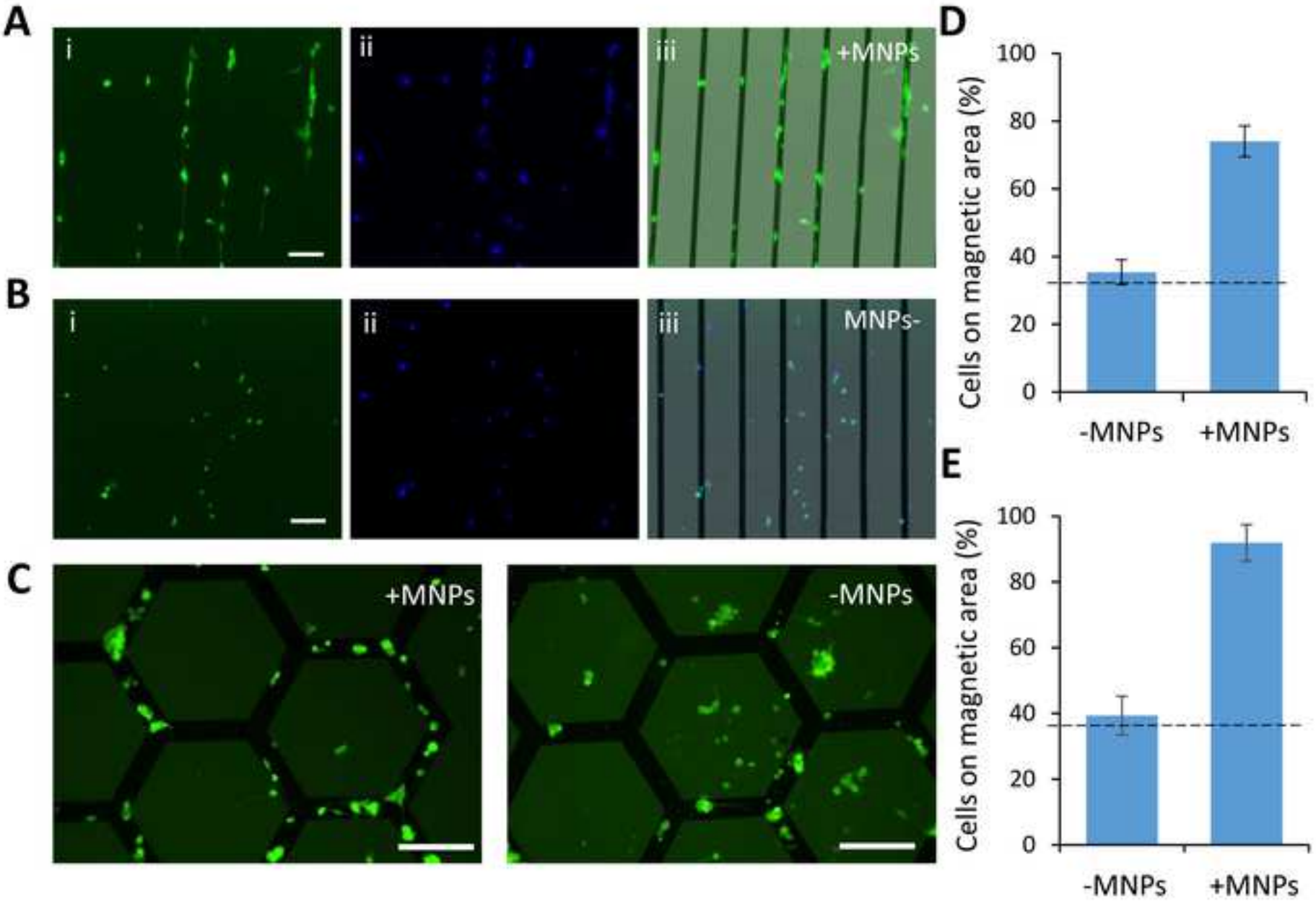

Figure 5

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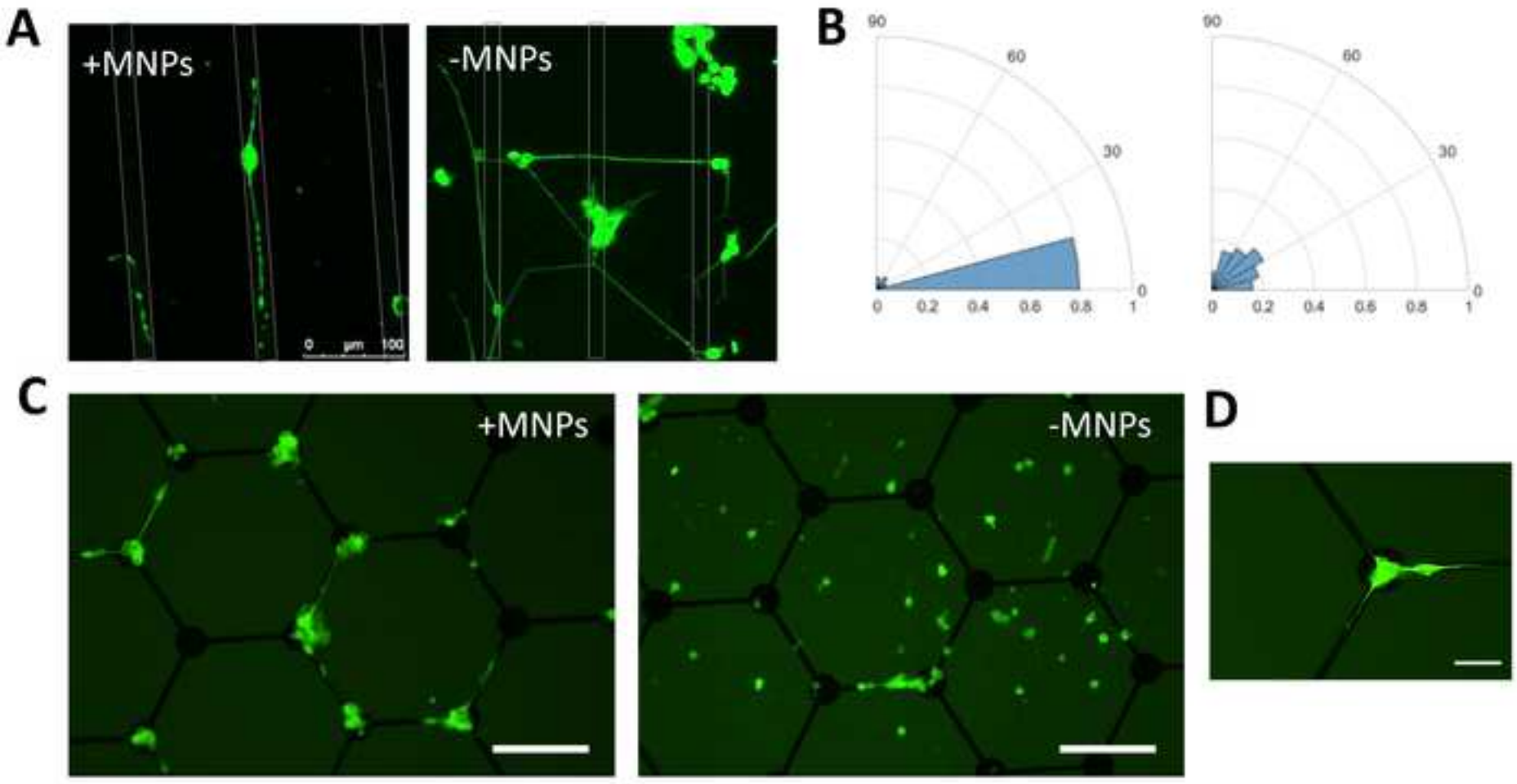

Name of Material/ Equipment	Company
16% Paraformaldehyde (formaldehyde) aqueous solution	ELECTRON MICROSCOPY SCIENCES
6-well cell culture plate	FALCON
96-well cell culture plate	SPL life sciences
Amphotericin B solution	Biological Industries
AZ 1514H photoresist	MicroChemicals GmbH
AZ 351 B developer	MicroChemicals GmbH
Bovine serum albumin (BSA)	Biological Industries
Cell and Tissue cultur flask	Biofil
Cell culture dish	Greiner Bio-One
Cell Proliferation Kit (XTT-based)	Biological Industries
Centrifuge tube	Biofil
Co80Fe20 at% sputter target	ACI Alloys
Collagen type I	Corning Inc.
Confocal microscope	Leica
Cy2-conjugated AffiniPure Donkey Anti-rabbit secondary antibody	Jackson ImmunoResearch Laboratories, Inc.
DAPI fluoromount-G	SouthernBiotech
Disposable needle	KDL
Disposable syringe	Medispo
Donor horse serum	Biological Industries
ELISA reader	Merk Millipore
Ethanol 70%	ROMICAL LTD
Ethanol absolute (Dehydrated)	Biolab-chemicals
Fetal bovine serum (FBS)	Biological Industries
Fresh murine β-NGF	Peprotech
GMW C-frame electromagnet .	Buckley systems LTD
Hydrochloric acid 32%	DAEJUNG CHEMICAL & METALS
ImageJ	US National Institutes of Health, Bethesda
Inductively coupled plasma (ICP)	Ametek Spectro

Keithley source-measure	Keithley
Keithley switching system	Keithley
L-glutamine	Biological Industries
Light microscope	Leica
Maskless photolithography	Heidelberg Inst.
Microscope Slides	BAR-NAOR
Nitric acid 70%	Sigma-Aldrich
Normal donkey serum (NDS)	Sigma
PBS 10x	hylabs
PC12 cell line	ATCC
Pd sputter target	ACI Alloys
Penicillin-streptomycin nystatin solution	Biological Industries
PrestoBlue cell viability reagent	Molecular probes
Rabbit antibody to α-tubulin	Santa Cruz Biotechnology, Inc.
RF magnetron sputtering system	Orion AJA Int.
RPMI 1640 with l-glutamine	Biological Industries
Sonication bath	KUDOS
SQUID magnetometer	Quantum Design, CA
Triton X-100	CHEM-IMPEX INTERNATIONAL
XTT cell viability reagent	

Catalog Number	Comments/Description
15710	
353846	
30096	
03-028-1B	
03-010-1B	
TCF002250	75.0 cm^2 250 mL Vent cap, Non-treated
627-160	35 mm
20-300-1000	
CFT021500	50 mL
	99.95%
354236	Rat Tail, concentration range 3-4 mg/mL
	TCS SP5
711-165-152	
0100-20	
	23 G
1160227640	10 mL
04-124-1A	
	BioTek synergy 4 hybrid microplate reader
19-009102-80	
52505	
04-127-1A	
450-34	
	3470, 45 mm
4170-4100	
	NeuronJ plugin
	SPECTRO ARCOS ICP-OES, FHX22 MultiView plasma

	2400
	3700
03-020-1B	
	DMIL LED
	MLA150
BN1042000C	
438073	
D9663	
BP507/1LD	
CRL-1721	
	99.95%
03-032-1B	
A-13261	resazurin-based
	Orion 8
01-100-1A	
SK3210HP	Frequency: 53 kHz. Ultrasonic power: 135 W

1279 non-ionic surfactant

A

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the version and corrected accordingly.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side.

Editorial changes performed.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Protocols numbering was adjusted according to instructions.

4. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 240-244, 261-264, 291-296, Figure 5's legend ("magnetic stripes…200 µm"

The lines were revised as you can see in the new manuscript. The new lines are 215-220, 232-233, 251-255 and 509-517 accordingly.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: B-351 developer, Orion AJA Int., MPMS SQUID (Quantum Design, Inc.), BioTek Synergy 4 Hybrid Microplate Reader etc

All materials and equipment names have been moved to the table of materials. All commercial language was removed.

7. Line 209: what do you mean by collagen type I rat tail? If you are using animals in this study, please add an ethics statement indicating approval of your study design and experiments by appropriate institutional ethics committees at the beginning of the protocol.

We are not using animals in the study. This refers to a solution that we purchase. We changed text to 'collagen type 1 (solution from rat tail)'.

8. Line 264: Do you use trypan blue to count viable cells or are you counting cells based on uptake of MNPs? Please clarify.

Please reference the relevant viability assay for MNP-loaded cells (lines 277-306) if this is what is used at this stage.

Regarding line 264 (now 233): In this step we perform regular cell counting with a camera in order to seed cells in plate and examine their differentiation and viability.

Regarding line 277 (now 243): We used two viability assays for the MNPs loaded cells. protocol 3.1 refers to XTT and 3.2 to Presto Blue.

9. Please clarify when the XTT and Prestoblue assays are used.

We preform viability assays for cells with MNPs (after uptake) to check for what concentrations the MNPs are not toxic to the cells. We do not perform this procedure routinely. We modified line 418 to clarify this issue:" The viability of MNPs-loaded PC12 cells for different concentrations of MNPs are evaluated and verified using XTT and Presto Blue assays".

10. Lines 341-346: If you don't plan to show how to use a Gauss meter or calculate the medium's viscosity, cell diameter, and cell velocity for filming the video, please cite references to allow readers to replicate these techniques.

We found that this methodology, while interesting, is more complicated to most researchers, and does not provide much data. For sake of clarity and focus on the important methods we removed it from the revised manuscript.

11. After including a one-line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will help determine what needs to be filmed.

The protocols we would like to film are:
1. Magnetic platform fabrication (1.1 and 1.2),
2. Characterization of magnetic device via transport measurements,
4. Collagen coating protocol
5.1. Cellular MNPs uptake
7. Cell differentiation and growth on magnetic platform

12. Please remove the embedded figures from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Please include only the figure legends (figure titles and a description) after the Representative Results of the manuscript text.

Figures were removed and figures legends were removed to the correct section according to the instructions.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please make volume numbers bold and remove the

abbreviated journal names; journal names should be written fully with the first letter of each word in the name capitalized.

All references were corrected.

Reviewers' comments:

We thank the reviewer for the relevant comments that helped us improve our paper. The response to all comments, and the related paper modifications appear below.

Reviewer #1:
Manuscript Summary:
Q: This work entitled: "Fabrication of Magnetic Devices for Micron-Scale Organization of Neuronal Networks" describes an innovative protocol to fabricate magnetic micro features on a glass carrier utilizable for patterning cells, specifically PC12 cell lines. The design and fabrication of the magnetic micro features are innovative, highly relevant to neuronal cell growth studies and other cell patterning applications. They are easy to handle and the protocol introduce here is timely. Before publication of this protocol, however, a few major and few minor concerns must be addressed.

We thank the reviewer for recognizing the relevance and innovative quality of our research.

Major Concerns:
Q: Title: The paper describes the fabrication of magnetic micropatterns / features /attracting elements for the organization of neuronal networks in vitro. It is not really a device. The title should be adjusted.

According to the reviewer's comment, we agree with the need to correct the title and changed 'device' to 'Platforms'.

Q: Similarity: The paper is very similar to the work published by the same author: https://doi.org/10.1002/admi.202000055 A clear statement is needed in the introduction and conclusion how this work here is different from the already published work.

The focus of this paper is on providing all the protocols and the know-how to enable researchers to use the methodologies. These were used by us in previous studies and are now summarized according to the guidelines of JoVE.

Q: Summary: Micro-electrodes are mentioned here, however, this is highly confusing as no electrical field is used to induce / operate the magnetic patterns.

Changed to ‘micro-patterned platform’

Q: Abstract: Line 48 - 50 this is an overstatement as literature does show single cell positioning through PLL patterns and other methods.

The abstract has been modified, stating that single cell positioning is scarce. A reference to the PLL method was added in the intro. We do not claim that ours is the only reported method, rather that these methods are rare. We believe that the flexibility offered by using magnetic forces (e.g., turning them on and off, and not chemically modifying the surface) is an important addition to researchers' toolkit.

Q: Intro: Line 71 - 74 a variety of references are missing who have used magnetic attracting elements for neuronal studies.

We added references 13-17 and 32-35 that represent prior knowledge. According to the reviewer recommendation, we cite Gahi et al., Tay et al., Kunze et al. and Roet et al. and more.

Q: Line 82 what is a strong magnetic force, here, in this context?

The meaning in strong magnetic force is that the remanent magnetization of the magnetic patterns is significant (equals the saturation of 270 emu/cm^3) so the magnetic force is stable and do not require an external magnetic field. For clarify, we changed in the text to “effective attraction forces” (line 101).

Q: Main text: Line 503: The usage of a device is confusing.

Changed to 'platforms' to avoid confusion throughout the text.

Q: Line 512: The usage of electrode is confusing

Changed to ‘cross-shape device’ and directed readers to the inset figure.

Q: Additional information about the nano-particles are needed (size, coating, biologically active, surface charge).

We add additional information about the particles (lines 400-407)

‘Fluorescent iron oxide (γ-Fe2O3) magnetic nanoparticles (MNPs) were prepared according to a previous publication[57]. The MNPs were synthesized by nucleation. The particles preparation includes six-layered thin films of iron oxide covalently conjugated to rhodamine isothiocyanate (RITC) and coated with Human Serum Albumin (HSA). The MNPs dry diameter size is 15+-3 nm with zeta potential of -45, according to TEM measurement.’

Q: How long were cells incubated with the nanoparticles?

The cells were incubated for 24 hours. This is mentioned in line 409 after the description of the particles. No changes were made.

Q: Was their incubation time with and without the magnetic field? What was the exact experimental procedure for aligning nanoparticle loaded PC12 cells on the magnetic features? How long was the magnetic field applied? How long were cells grown in vitro. Was the magnetic field applied during the entire duration?

To clarify, cell uptake is done without any magnetic fields. Otherwise, the MNPs would be attracted to the magnets instead of entering the cells. We are concerned that talking about fields during uptake will confuse the readers.

We emphasize, since the magnetic multi-layers are stable, there is no need for an external field during plating or growth.

To clarify, we added to the text (line 430): 'Cells were seeded and left to settle down and adhere to the substrate. Every 2 days, cells were treated with fresh medium and NGF as describe in protocol 7.'

The cells grow on the substrate between 3-8 days (depending on the goal of the experiment), in each image we note the time. The magnetic field is permanent from the magnetic pattern so is applied during the entire growth time.

Q: Fig.1 A: Scale bars for all images are needed. 1B: What is the height of the final magnetic structure?

We added scale bars in Fig. 1A. The height is mentioned in line 382. We added it to the legend for clarity.

Q: Line 541 - 543: This has been shown in numerous previous publications in the same field, hence what Reference is it based on?

We found that this methodology, while interesting, is more complicated to most researchers, and does not provide much data. For sake of clarity and focus on the important methods, we removed it from the revised manuscript.

Q: Fig.5 D: Scale bar needed.

Done.

Q: References: Reference 63 and 55 are identical, same for 57 and 62; the citation of patents needs to be corrected and the pre-print reference needs to be updated or replaced with the fully published version

The identical references were removed, and the references list was corrected according to JoVE instructions.

Q: The two major issues are the high similarity to an already published article from the same group and missing references covering the modulation of neuronal growth and function, interaction with nanoparticles and magnetic element fabrication and applications with neurons in vivo and in vitro (e.g., Gahl et al.; Tay et al., Kunze et al., Christianzen et al., Roet et al., and additional)

We commented about the similarity issue above. We thank the reviewer for pointing out relevant papers. We added some of the suggested and others we thought may be relevant.

They now appear as refs. Number 16-19 and 32 in the modified manuscript.

Minor Concerns:
Q: Line 357: What is ICP?

Inductively coupled plasma. It is now also defined in the protocol.

Q: Line 658: Space before comma

Changed.

Reviewer #2:
The manuscript titled "Fabrication of Magnetic Devices for Micron-Scale Organization of Neuronal Networks" is well written and provides insights into a novel way of guiding neuronal growth in vitro and possibly in vivo in the future. Methods offer a detailed explanation of the methodology developed during this research, and results showed the rigor and validity of the experiments. Few minor points need to be addressed, which will improve the readability of the manuscript and make it ready for publishing.

We thank the reviewer for the relevant comments that helped us improve our paper. The response to all comments, and the related paper modification appears below.

1. Title and Abstract
Q: * A short and informative title was provided. It briefly explained the main accomplishment of the manuscript.

The title was corrected following the reviewer's comment.

Q: * The abstract organization can be improved by sequentially providing the introduction, main methods used, and the main results. Authors are recommended to improve the organization of the abstract to enhance readability.

We edit the abstract according to the recommendation. The new abstract includes an introduction, main methods, and results as you can see in lines 45-64.

2. Introduction
Q: * The introduction is relatively short, and authors should summarize how other researchers have provided patterning to the growing neurons in a little bit more detail.

The introduction has been expanded with more details on other researchers on patterning of neurons, the relevant references appear in numbers 1-5.

Additionally, we added a new paragraph which summarizes previous works on neurons and magnetic manipulation as you can see in lines 76-88.

Q: * Can this technique work for other cell types? Please explain that if it is possible.

We examine this method on PC12 only, but as we noted and cite in the introduction, there are many publications of cells with MNPs, so our method is suitable for other cells too.

Q: * A brief literature search provides a reference on how other researchers have used magnetic particles with neurons. Please include those as well.

We cite the relevant references. Numbers 32-35.

Q: * Please also provide a brief overview of the results produced during research at the end of the introduction section.

we added to the introduction a brief overview of the results, lines 98-108.

3. Methods

Q: Methods are well explained step-by-step. The study can be repeated independently based on the instructions provided in the methods section.

Thank you.

4. Results

Q: * Please explain the reasoning behind creating multilayers of Co80Fe20 and Pd?

In order to attract magnetic elements to the patterns the magnetization should be perpendicular to the substrate. Multilayers of magnetic materials and Pd (or Pt) is a common way to induce a perpendicular magnetic easy axis, with remnant magnetic field similar to the saturation field, which means we do not need to use external fields in the experiments, and the MNPs loaded cells attract to the entire pattern (not only to the edges). We cite our previous work when we do not use magnetic multilayers, for comparison (Ref number 22). The use of a CoFe alloy results in a ferromagnetic material that can reach higher saturation magnetization (due to Fe), while maintaining perpendicular magnetism (due to Co).

To clarify, we add some changes in the text. (lines 380-386)

Q: * It looks like that the rhodamine nanoparticles are present at the outside periphery of the cells (Fig 2B). Are the particles inside the cells or just sticking to the outer part of the soma?

The particles are inside the cell but outside the nuclei. This is demonstrated by the staining. As is evident the Rhodamine has low fluorescence from where the nucleus is located. If the particles were outside, we would have seen a constant Rhodamine signal. If NPs exists in the

entire cell except the nucleus, then there would be more fluorescence inside the cell but out of the nucleus – which is what we see (nucleus position is marked with DAPI).

We modify the text in lines 408-417.

Q: * Neurites from the soma of PC12 is not visible in Fig 3A. Will it be possible to provide other images where it is easier to visualize?

The florescence in figure 3a (left) is from the Rhodamine of the nanoparticles. There are a few neurites that clearly have MNPs. However, if the concentration of the particles inside the neurites is low it will be difficult to observe them. So, we do not expect to be able to see them in long elongated neurites. We added arrows to draw attention to these places in the image and added in the caption " Arrows show differentiated neurites with internalized MNPs.".

Q: * Does the MNPs transport into neurites when the cells are differentiating? Please provide some example images. How are the neurites guided into the magnetic patterns if the MNPs are not getting transported into the neurites?

The results show that the neurites are influenced by the magnetic pattern and grow accordingly so it indicates that the MNPs transport to them. Moreover, it can be seen from image 3a that the particles transport to the neurites after differentiation. As written above, we added arrows in the image and added in the caption " Arrows show differentiated neurites with internalized MNPs.".

Q: * What happens to the neurites if the magnetic field is removed? Do they still keep on growing on the patterns, or they come out of the patterns and start growing throughout the substrate?

Since the magnetic field that guide the neurites is from the magnetic pattern, and not from external source, it is not simple to “turn it off”. In principle, it is possible to de-magnetize and re-magnetize the pads by applying an external field modulation sequence, thus turning on and off the magnetic forces for additional control. Actually, one of our current studies is on this issue.

Q: * Does it look like that the cells were plated a low density? Please explain.

This is correct. We plate the cells at low density since we wanted to study effects of the substrate on single cells. A high density would result in interconnections (and chemical cueing). This will hamper our ability to perform statistical analysis, e.g. Sholl analysis, for single cells.

Q: * Have you tested the growth of PC12 cells on the un-magnetized substrate but with patterns? How do the neurites grow on such a substrate?

We showed in the article that cells without MNPs treatment that grow atop our magnetic platforms do not attract to the magnetic patterns and the neurites develop randomly (figures 4 and 5). This control sample indicate that the effect is related to the magnetic attraction and not

comes from topography or chemical effects. The comparison to cells growing atop platforms with patterns from non-magnetic material will not provide the control for testing the ability of MNPs to direct neurites, which is the aim of this method. In general, we and others have studied growth of neuron-like cells on non-magnetic patterns, and for thicknesses <20nm there is nearly no affect.

Q: * Did the MNPs show no cytotoxicity for all the concentration of MNPs used?

The concentrations we tested don't show toxicity for the cells but high enough so the cells after uptake attract to magnetic field. The final concentration we work with during all experiments is 0.25 mg/ml (which is considered reasonable), noted in line 410.

Q: * How long the magnetization last after removing the external magnets?

The magnetization of the magnetic pattern is stable duo to the coercivity of the magnetic multilayers, so the magnetization does not change unless external magnetic fields are applied (or by heating the substrate to a few hundred C). We measured the magnetic platforms after a few weeks and the magnetization did not change.

5. Discussion

Q: * Please only discuss the results which are presented in this manuscript. Please remove the details from line 632-635. Please provide the results if that discussion is considered essential.

The purpose in this discussion is to emphasis the importance of performing viability test and magnetic characterization when choosing MNPs. There are several commercial MNPs but not all of them are suitable for this experiment. Researchers planning to use our protocol must have this information, or we are setting them up for failure.

Reviewer #3:

Manuscript Summary:

Q: Thank you for assigning me to review the manuscript "Fabrication of Magnetic Devices for Micron-Scale Organization of Neuronal Networks". The authors Sharoni et al. report about their method to fabricate magnetic patterns on substrates such as glass, that can be used to control the position of cell attachment. Further, they show that the neurite growth takes preferentially place along with the magnetic patterns. As the cell viability and the response to NGF seems to remain unchanged, the magnetic patterning can be used to position cells in a defined location which could enhance the reproducibility in some applications.

We thank the reviewer for reviewing our manuscript and useful comments.

Major Concerns:

Q: The presented method is interesting as the positioning of the cells seems to work very efficiently. Therefore, the manuscript could be helpful for many labs with different aims.

Although the main information of the manuscript is convincing, several aspects raise major and minor concerns and need to be revised before the manuscript can be published.
* The language of the manuscript needs to be improved. It is partially difficult to understand the content as the language is not precise enough. In addition, spelling errors and inconsistent use of abbreviations hamper the reading experience. Examples:
I) Inconsistent and incomprehensible usage of the abbreviations MNP and MNPs: the plural MNPs is often falsely used e.g.: line 231: cellular MNPs uptake and viability. The authors should check carefully where the plural or singular should be used.
II) Lines 85-88: The magnetized cells show a strong attraction of soma to the patterns during plating, and the neurites developing process, which develops nicely, also grow preferentially according to the predesigned structures.
III) Line 266: MNPs loaded cells plating and differentiation.
IV) Line 534: After MNPs cellular uptake, the cells are suspended in a plastic plate with regular medium.
V) And more…

We edited the paper for grammatical and spelling errors, corrected the aforementioned errors and others. We thank the reviewer for pointing these out.

Q: * The term „neural network" is used throughout the manuscript. A key property of a network is the interconnection of the separate units and their functional transmission of signals or information. In the manuscript, basically, the neurite outgrowth of single cells is shown but not if they form a type of network. Either the term "neural network" should be avoided, or necessary experiments to prove the network formation has to be performed.

We agree with the reviewer and changed the title of the manuscript to “interconnected neurons” instead of neural network. In addition, we modified the text and avoid the use of neural network.

Q: * Line 284 ff.: It is written, that for the viability assay MNP concentrations in the range of 0.1-0.5 mg/ml were measured. If this is true, the positive control of cells without MNPs is missing and needs to be performed. For a convincing viability assay, it is important to know how the viability is for cells without the substance that should be tested as it represents the viability in normal conditions.

Of course viability tests for control sample with no MNPs are important. Control samples were measured, and all the results were normalized to the control, as presented in figures 3c and 3d in the viability assay results. This has been clarified in the protocol, lines 245 and 268 (revised version): "Cultivate the PC12 cells with MNPs at different concentrations (0.1 mg/mL, 0.25 mg/mL and 0.5 mg/mL in basic growth medium) and cultivate cells without MNPs for control sample in triplicate in a flat 96-well plate, each well should have a total volume of 100 µL."

Q: * Line 327 ff.: The method of the "characterization by constant velocity measurements" is

not convincing. How long after seeding the cells into the dish has the measurement been performed? What is the impact of external factors such as motions caused by touching the dish or moving the stage of the microscope? How can the exact position of the dish be located to apply the proper value for the magnetic field gradient?

We found that this methodology, while interesting, is more complicated to most researchers, and does not provide much data. For sake of clarity and focus on the important methods we removed it from the revised manuscript completely.

Q: * Line 83: "MNPs are loaded into PC12 cells via incubation, …". What is the mechanism for MNP uptake? Are the MNPs afterward present in the cytoplasm or inside endosomes?

According to this study and others performed in our lab, we believe that the MNPs transfer the cell via endocytosis, and are present inside of the membrane but outside the nuclei.

We added to the discussion: The internalization mechanism of MNPs into cells have been previously investigated [46]. Cells uptake MNPs via endocytosis according to their size, shape, and surface chemistry. In previous studies we examined different types of MNPs uptake with neurons[24]. We found that coated MNPs uptake into the cells better then uncoated MNPs. As we display in Figure 3B and 3A, MNPs internalized into the cells' cytoplasm but outside the nuclei, and transfer to the neurites during the developing process. Additionally, we have shown that MNPs conjugated to NGF that activated NGF pathway also have been internalized into cells via endocytosis[47, 48].

Minor Concerns:
Q: * Line 114 ff.: Sonication bath (70% power): unprecise information as the model of the sonification bath is not mentioned. What are the frequency and amplitude?

Our sonication bath is from KUDOS company. Model: SK3210HP. Frequency of 53kHz and ultrasonic power of 135W. All the details now appear in the Table of Materials section.

Q: * Line 114 ff.: What are the specifications of the glass slides

We use microscope slides of BAR-NAOR company. Pre-cleaned 26x76 mm and thickness of 1.0-1.2mm. All the details now appear in the Table of Materials.

Q: * Example: line 135, 136, ff.: Inconsistent use of chemical symbols (Co80Fe20) vs. full names (Palladium)

Corrected to chemical symbols only. Lines: 130-133.

Q: * Misleading mentioning of product names: line 209: …collagen type 1 rat tail… as only the collagen is used which was isolated by the manufacturer from rat tails, the addition "rat tail" should be written before "collagen " or in brackets

Changed to: “collagen type 1 (solution from rat tail)”

Q: * Line 206 ff.: Concentrations and molarities, etc. should are usually written in front of the substance name (HCl 0.01 M vs. 0.01M HCl; PBS 1x vs. 1x PBS, etc.)

Changes have been made throughout the paper.

Q: * Line 235: …penicillin streptomycin… The authors should indicate that it is not a single substance but a mixture of penicillin and streptomycin (e.g. penicillin/streptomycin)

Done. Line 210.

Q: * Line 240 ff.: Grow cell suspension in a culture flask… The authors should specify if surface-treated or non-treaded flasks were used. Even with the article number in the table, it is not possible to identify. Which size was used? What happened to the cells after 8 days? How was the passaging for the maintenance culture performed?

We use non-treated culture flask with vent cap, size of 75 cm^2, 250ml. All the details are now clearly mentioned in the attached table. After 8 days, the cells reproduce to a point of high density in the flask so they need to be treated, the procedure is to split them. Since PC12 cells grow in suspension and do not attach to the flask, transferring to the maintenance culture is performed with a regular pipette.

Text was modified to clarify these issues. Lines 215-219: “Grow cells in a non-treated culture flask with 10 mL of basic growth medium; every 2-3 days add 10 mL of basic growth medium to the flask, after 8 days split cells. For cellular uptake, transfer the cell suspension to a centrifuge tube using regular pipette; centrifuge cells for 8 min at 200 x g and RT. Discard the supernatant.”

Q: * Line 247 ff.: Suspend the cells in 3 mL of fresh differentiation medium. In this section, it seems that no NGF was added to the differentiation medium. The authors should explain why they used the differentiation medium without NGF. Doe the incubation for 24 h in low serum conditions (differentiation medium) without NGF addition on the cell viability compared

The uptake process is performed with differentiation medium but without NGF since we want the cells to differentiate, but not to attach to the plate and start to grow neurites. After incubation of 24 h with the MNPs, we seed the MNPs loaded cells with differentiation medium and NGF to initiate neurite growth, as detailed in the protocol. The viability test examines the toxicity of the MNPs for the cells, so we test the cells after 24 h of incubation with the particles without the presence of NGF.

Q: * Line 375: what is a "large magnet". What are the magnetic properties of the magnet?

As mentioned above we removed this section from the revised manuscript.

Q: * Line 384: It is not clear if 50 ng/ml is the final concentration or the concentration of the NGF stock solution.

50 ng/ml is the final concentration of the NGF in the plate. The concentration of the NGF stock is mentioned in the Table of materials. For clarify, we changed in the text: “After 24 h, add 1:100 fresh murine β-NGF (final concentration of 50 ng/mL).” (line 238)

Q: * Line 387: The network is formed usually after 8 days. When is the network formed the other cases? What factors influence network formation and are considered here as "usually"?

In PC12 cells network formation is observed within 6-8 days. We changed the text accordingly (line 241).

Q: * Line 433: How are the measured "intersections" are defined?

We map the dendritic branching of cells using a dedicated software, ImageJ. The "intersections" are related to the branching points, referring to the number of branches crossing a circle with a specific radius from the cell center. The term "intersections" is the convention used in Sholl analysis and is an important morphological characteristic.

Q: * Line 534: What is meant by "regular medium"?

Changed to basic growth medium. The preparation protocol detailed in line 208.

Q: * Line 539: What determines the "drag force"?

This section was removed (as explained above).

Q: * Figure 1 B: The orientation of the microscope picture is not clear. Is the brighter gray area the first CoFe/Pd layer and the dark gray area the lower CoFe/Pd or the glass? Or does the microscopic picture show the xy-orientation, while the sketch shows the xz-orientation? Is the CoFe/Pd pattern an elevated structure on the glass or are the spaces between the pattern filled with some substrate?

In this study the pattern is elevated (a total of 18nm) above the substrate. We modified Fig. 2B so the SEM image and cartoon are aligned and believe the image is clear now. The brighter gray area is the CoFe/Pd multilayers and the dark gray is the glass substrate.

Q: * Figure 2 D: descriptions of the axis of the graph are missing. Which magnification is used to capture the images?

This section was removed (as explained above).

Q: * Figure 3: A) Scale bar is difficult to identify.

Scale bar size added to the figure legend.

Q: C+D) what is the normalized control in these graphs?

The control is PC12 cells without MNPs and the results are normalized to this. (100% is for cells without MNPs)

To clarify, we modify the text in line 425: “The results are normalized to the control measurement of PC12 cells without MNPs.”

Q: * Figure 4: B) DAPI-staining is hardly visible.

We improved the image. See the new attached figures.

Q: C) The label "MNPS" should be changed to "MNPs".

Done.

Q: D+E) The layout of both graphs should be harmonized. Now, the order of "+MNPs" and "-MNPs" are presented in the opposite order.

Done.

Q: * Lines 613-615: It is written that the technique could be in principle used for "implanted and in-vivo therapeutic applications". Would it be possible to give an outlook for an in vivo application that facilitates this technique? Is the CoFe/Pd biodegradable within the organism? Does the method only work with monolayers of cells? How would these cells integrate into the neighboring tissue?

The in vivo application we envision is related to bio-interfacing therapeutic solutions such as flexible electrodes array for measuring and inducing electrical activity. Our design improves neuron to electrode interface by attracting cells preferentially to electrodes and may increase signal to noise ratio. It is possible to design our multilayers atop biocompatible substrate that will be suitable for therapeutic applications (see ref 42 43). It may be possible to develop similar magnetic platforms which are biodegradable, but this is not part of our research scope.

We modified in the text (line 535):” in principle it is possible to prepare the devices atop other biocompatible materials that are suitable for in-vivo therapeutic applications such as flexible electrodes array for neuronal recording and stimulation”

Q: * Lines 640-646: Although an unbiased statistical analysis has high importance, it should be taken for granted and is not needed to be mentioned in the discussion.

We wanted to emphasis the importance of unbiased statistical analysis in this experiment, but we agree that it is essential for any research, so we removed this part from the discussion.

Q: * Lines 824 ff.: literature 54 and 55 are mentioned again as 62 and 63.

The references list has been revised and the duplicate was removed.

Q: * Recent publications demonstrate magnetic patterning and should be therefore mentioned and included in the discussion. Examples: Goranov et al., 2020, Scientific Reports; Bongaerts et al., 2020, IJMS; …

According to the reviewer recommendation, we cite Goranov et al., 2020, Scientific Reports; Bongaerts et al., 2020, IJMS; in references number 17 and 13 accordingly.

Q: * Material/Equipment list: As ImageJ was used, the corresponding publication should be cited.

We added the following citation: Abramoff, M.D., Magalhaes, P.J., Ram, S.J. "Image Processing with ImageJ". Biophotonics International, volume 11, issue 7, pp. 36-42, 2004. (ref num 36)

Q: * Which permanent magnets were used?

This section was removed (as explained above).

Reviewer #4:

Manuscript Summary:

This manuscript describes the protocol for the fabrication of a cell culture substrate with magnetic patterns and protocols for the characterization of cell positioning and neurite outgrowth in magnetic nanoparticle-loaded PC12 cells on this substrate. The protocols and the representative results are sufficiently detailed and clear. However, there is room for improvement in terms of language and style as well as the results presented.

We thank the reviewer for the relevant comments that helped us improve our paper. The language and style of the manuscript have been revised and modified. The response to all comments, and the related paper modification appear below.

Major Concerns:

Q: 1. Panels of Fig. 1 need to be better referenced in the manuscript text: Fig. 1A should be referenced in the protocol section, as claimed in line 504. In fact the reader is informed on the details of the linear geometry later in the text (lines 555-556), but not informed on the hexagonal geometry. This information could be provided in the caption of Fig. 1.

In figure 1A, we display different geometric shapes that we design and fabricate. The parameters of the patterns are mentioned when the results are displayed. The missing

parameters for the hexagons now appear in line 438: " hexagonal geometry of 200 µm long sides and the lines' width is 50 µm." and in line 469: "The long side of the hexagon is 200 µm and the lines' width is 10 µm, the circles diameter is 30 µm."

Also, protocol 2.1 mentions a cross-shaped electrode and refers to Fig. 1B for the deposition of the multilayered substrate; however, the reader only sees the cross-shaped electrode in the inset of Fig. 1C. It would be better to provide a photograph of this electrode with bonded wires (as described in protocol 2.3).

The mention for the cross-shaped electrode changed to Figure 1C inset in the protocol and the image replaced to a photograph of the electrode with bonded wires, according to the reviewer recommendation.

Q: 2. The introduction has over-citation. Providing 17 citations for previous studies (line 70) is not very helpful, considering that the authors do not discuss any of the studies cited (which is normal in a protocol article). Same comment on the 19 citations given for the medical uses of magnetic nanoparticles (line 77). There are many good review papers for both subjects the authors could cite.

The references have been revised, see update list.

Q: 3. In protocol 6.2, the authors estimate the Fe concentration per cell by ICP measurements. Can the authors estimate the number of nanoparticles per cell from this concentration value? It would be easier to compare with the number of particles per cell they estimated in protocol 6.1 by magnetically-induced particle movement.

We found that this methodology, while interesting, is more complicated to most researchers, and does not provide much data. For sake of clarity and focus on the important methods we removed it from the revised manuscript.

Q: 4. The authors confirm by z-test that cell landing to circular magnetic areas are statistically not random (line 571). They should conduct a similar statistical test to confirm that the same is true on hexagonal patterns.

We performed statistical analysis for the hexagon pattern and confirm that the results are similar to the results of the lines.

We added this information to the text in line 452: “Statistical analysis for the hexagons also indicates that the measurement is not derived from arbitrary cell landing (One-Sample z-test, $p<1*10^{-6}$, n=370).”

Q: 5. The authors demonstrate the orientation of the PC12 neurites with the magnetic patterns (Fig. 5). The authors also demonstrate that loading of PC12 cells with magnetic nanoparticles does not affect the neurite elongation behavior on glass coverslips (Fig. 3B). However, a key piece of information is missing: If and how does magnetic patterning affect neurite elongation?

This can be easily answered by analyzing the dataset obtained to generate Fig. 5B. The authors should show if constraining the neurites on magnetic patterns increases their elongation rate (or total length) as observed for linear geometric constraints, or decreases their elongation rate (or total length) as one could imagine that the neurite needs to act against magnetic forces in the -z direction, or these two effects cancel each other.

The suggestion of the referee is very good, and we plan to apply it in our future studies. In the current study this is difficult to perform since, while the average cell concentration is not very high, once they are attracted to the magnetic lines, their effective concentration is much higher. This results in neurites extending toward cells, and much fewer instances where the total (undisturbed) length could be extracted and analyzed with statistical significance.

Q: 6. There are many redundancies in the protocol making it bulky. These include basic dilutions, e.g., collagen (lines 212-214), PBS 1x (lines 216, 226, 393), 30% ethanol (lines 221-222), 4% PFA (line 394), concentration calculations (lines 253-256), and environment- or software-specific task (lines 118 and 441, respectively).

The protocols revised and modified according to the reviewer suggestions and the JoVE editorial guidelines. However, some of the lines are easier to understand by giving specific quantities, such as 4% PFA preparation.

Minor Concerns:
Q: 1. The numbering style of the protocol is poor. In section 1, the authors use letters for the second level, for the remaining sections they use numbers. Apart from this discrepancy, tabs are also not uniform, making the protocol hard to follow. The authors could use different typeface for different levels, or use extended numbering, e.g., 5.3.1.6.

The numbering style of the protocol has been corrected according to JoVE guidelines, see revised manuscript.

Q: 2. Section 5 could be better organized. Protocols 5.3.1.1-4 and 5.3.2.1-4, and 5.3.1.10 and 5.3.2.7 are identical. A better organization could save space and probably one level of sub-sectioning.

Duplicates have been removed.

Q: 3. Consider revising sentences starting in lines 80, 532, 580, and 640 for clarity.

Line 80: abstract was rewritten.

Line 532 and 640 removed from text.

Line 580 modified, now appears in line 458: “In contrast, the control measurement of cells without MNPs, show neurites growth across the platform regardless of the magnetic patterns.”

Q: 4. What does PMS stand for in line 290?

The reaction solution contains PMS. We added to the protocol: N-methyl dibenzopyrazine methyl sulfate (line 251)

Q: 5. Terminology: Biological hood (line 110) - biosafety cabinet; rounds (line 119) - rotations; dish (line 228) - glass slide; suspend (lines 244, 247, 263) - resuspend; MNPs moment (line 542) - MNPs; cell suspension (line 240) - cells; hemocytometer cell counter (line 250) - hemocytometer.

We thank the reviewer for the terminology corrections. We incorporated the requested changes, and additional grammatical errors, during editing of the manuscript.

Q: 6. Typos: Technic (line 92) - technique; manufacture (line 212) - manufacturer; preform (line 268) - perform; triplicates (lines 285, 315, 361) triplicate; incubated (line 320) incubate; decrease (line 537) - decreases; rigors (line 640) - rigorous or rigor depending on what the authors are trying to say.

All the corrections have been performed.

Q: 7. Protocols 1.a.2 and 1.a.3 should end with period.

Done.

Q: 8. Protocol 1.b.4 needs space between numbers and units.

Done.

Q: 9. Abbreviation of ICP comes before the term itself (lines 357 and 367). Abbreviation of AHE in Fig. 1 caption should be spelled out.

The ICP full name now appears in lines 280 and 415 before the abbreviation. The abbreviation of AHE was removed from the figure caption.

Q: 10. In sentence starting in line 536, the authors could provide the nature of the function between the field gradient and the distance between nanoparticle and the magnet. Is the variation of field gradient significant over the area where the imaging was done considering the distance between the sample and the permanent magnet is likely much larger than the distances taken by the cell under force?

This section was removed (as explained above).

Q: 11. Consider replacing the order of "-MNPs" and "+MNPs" in Fig. 4E to be consistent with Fig. 4C and Fig. 4D.

Done.

Q: 12. Consider stating that there is statistical significance (or not) in panels Fig. 3C, 3D, 4D, and 4E.

Added to figures caption: " There is no statistical significance in both analyses."

Q: 13. In parts of the text there is inconsistent use of verb tenses.

The text has been revised and modified.

Q: 14. The authors should describe how they recovered plated cells into suspension, e.g., trypsinization (in line 261 and similar).

Since PC12 cells do not attach to the flask in medium growth and develop in suspension, we transfer the cells suspension using a regular pipette.

For clarity, we changed in the text line 217: " For cellular uptake, transfer the cell suspension to a centrifuge tube using a regular pipette."