Journal of Visualized Experiments

Characterization of the interaction of primary cells from the rat inner ear with polymer films as coatings for cochlear implant electrode surface

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE56792R3
Full Title:	Characterization of the interaction of primary cells from the rat inner ear with polymer films as coatings for cochlear implant electrode surface
Keywords:	Cell adhesion, poly(N,N-dimethylacrylamide) (PDMA), poly(2-ethyloxazoline) (PEtOx), poly([2-methacryloyloxy)ethyl]trimethylammoniumchloride) (PMTA), scanning electron microscopy (SEM), spiral ganglion neurons (SGN), immunocytochemistry
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Hannover, 15.06.2017

Dear Dr. Myers

Hereby, we submit our manuscript entitled **Blocking fibroblast overgrowth by polymer coatings of cochlear implant electrode surface – an option for improving electrode-nerve-interface.**

The main focus of the manuscript is the methodical description of qualitative and quantitative characterisation of the interaction of fibroblasts, glial cells and spiral ganglion neurons (SGN) from postnatal rats with ultrathin polymer films immobilised onto glass surfaces.

In general, insertion of electrode arrays into the inner ear of hearing impaired people induce overgrowth of connective tissue and scar formation, which increase the impedance and, thus, diminish the interactions between neural probes as like cochlear implants and the target tissue. The modification of the carrier material of the electrodes with cell selective polymer coatings may be an option to improve the electrode nerve-interface.

For the first time, we could show by scanning electron microscopy and immunocytochemical staining of neuronal and intermediary filaments that glial cells of the spiral ganglions predominantly attached on PMTA films, but not on PDMAA and PEtOx monolayers. Hereby, strong survival and neurite outgrowth were only found on PMTA, whereas PDMAA and PEtOx coatings inhibited both cell attachment and neuritogenesis. In conclusion, survival and neuritogenesis of SGN correlated with the extent of the glial cell growth on different coatings.

Finally, we think that our manuscript is suitable for publication in “JoVE”.

Yours sincerely, Kirsten Wissel

TITLE:

Characterization of the Interaction of Primary Cells from the Rat Inner Ear with Polymer Films as Coatings for Cochlear Implant Electrode Surface

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KEYWORDS:

Cell adhesion, poly(N,N-dimethylacrylamide) (PDMA), poly(2-ethyloxazoline) (PEtOx), poly([2-methacryloyloxy)ethyl]trimethylammoniumchloride) (PMTA), scanning electron microscopy (SEM), spiral ganglion neurons (SGN), immunocytochemistry

SUMMARY:

Here, we present an immunocytochemical and electron microscopical protocol that enables qualitative and quantitative characterization of the interaction of the primary spiral ganglion neurons and other cell types within ultrathin polymer films.

ABSTRACT:

The spiral ganglion (SG) cells prepared from the inner ear of postnatal rats represent one of the key cell culture models in hearing research. Numerous projects in hearing research aim at improving nerve-electrode-interactions by inhibiting the formation of connective tissue following cochlear implant insertion, *i.e.*, by coating the carrier material with ultrathin polymer films for selective cell attachment. Here, we established scanning electron microscopy (SEM) and immunocytochemical (ICC) staining to enable the characterization of the interactions of fibroblasts, glial cells, and spiral ganglion neurons (SGN) growing on polymers, *i.e.*, poly(N,N-

dimethylacrylamide) (PDMAA), poly(2-ethyloxazoline) (PEtOx), and poly([2-methacryloyloxy)ethyl]trimethylammoniumchloride) (PMTA). For this purpose the primary cells dissociated from the SG of postnatal rats were cultivated for 48 h on the polymer films. ICC was used to demonstrate the preferences of cell adhesion on the polymer coatings. It could be shown that glial cells and SGN mainly adhered on PMTA monolayers forming long processes, but not on PDMAA and PEtOx films. Also, SEM imaging showed that only PMTA enabled SG neuron survival and neurite outgrowth. In conclusion, the ability of the SGN to survive and to form neurites was associated with glial cell adhesion on different coatings.

INTRODUCTION:

Numerous projects in hearing research aim at improving nerve-electrode-interactions by inhibiting the formation of connective tissue following cochlear implant insertion by either application of drugs like dexamethasone[1-4], coating the carrier material with ultrathin polymer films[5-8], or nano structuring of the carrier materials for selective cell attachment[9-12]. To characterize biocompatibility, protein binding affinity, and effects on cell morphology, adhesion, and motility, *in vitro* cell culture assays represent the method of choice prior to *in vivo* experiments in laboratory animals.

Even though homogenous cell lines provide reliable experimental data, they do not represent the complex tissue reactions. In contrast, primary cells such as dissociated cochlear SG cells in culture may mimic the natural tissue environment. So far, SG cells from both mice and rats are well-established in *in vitro* cell culture systems to examine the signal pathways that protect the auditory neurons against apoptosis-inducing agents and that influence neuronal interactions with growth factors[13-21]. However, the investigations described herein have been confined to the SGN and did not include the biological activities of other cell types such as glial cells, satellite cells, and fibroblasts. Due to their differentiated character, primary cells may require refined techniques to study their behavior on different surfaces as well their intercellular interactions.

This study presents fast, simple, and reliable ICC techniques that enable the examination of SGN interactions with non-neuronal cells (such as Schwann cells and fibroblasts) not only on standard tissue culture plates, but also on modified surfaces like ultrathin polymer layers. The polymers PDMAA, PEtOx, and PMTA were manufactured according to Prücker *et al.*[22]: a photoactive benzophenone derivative was coupled with a chlorosilane anchor for immobilization onto the glass surface. The subsequent spin-coating of the polymers PDMAA, PEtOx, and PMTA on the photoactive group and exposure to UV light resulted in a photochemical attachment of the polymers onto the substrate. As follows, the primary cells were dissociated from the SG of postnatal rats and cultivated for 48 h on the polymer films. ICC methods and SEM were used to demonstrate the preferences of cell adhesion on the polymer coatings.

PROTOCOL:

Neonatal Spraque-Dawley rats (P3-5, n = 18 per experiment, n = 5 independent experiments) were used for SG dissection in accordance with the institutional guidelines for animal welfare of Hannover Medical School following the standards described by the German “Law on protecting animals” (Tierschutzgesetz).

1. Dissection of the Cochlear SG from Neonatal Rats[13-14]

1.1. Sacrifice the postnatal rats, which should not be older than a maximum of 5 days, decapitating the heads quickly using a scissor with pointed tips.

1.2. Fix a head with a curved standard pattern forceps by inserting it into the nose of a rat.

1.3. Remove the mandible and pull the skin, starting at the side of the neck, by using Adson-Brown forceps.

1.4. Open the skull along the midline and separate it into two halves using a scissor with pointed tips.

1.5. Scrape away the brain by using the handle of one of the forceps and transfer the two head halves into a Petri dish with ice-cold phosphate buffered saline (PBS) tablets.

1.6. Extract the cochlea out of the temporal bone by using the forceps Dumont #5 and #3c under microscopic view with 4x magnification.

1.7. Open the bony capsule, carefully exposing the cochlear parts of the membranous labyrinth by using the forceps Dumont #5 and #3c.

1.8. Remove the organ of Corti and the stria vascularis from the modiolus by using the forceps Dumont #5 and #3c.

1.9. Separate the entire SG from the modiolus of each head half and place them in ice-cold Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS).

2. Enzymatic Dissociation of the SG and Cell Cultivation Set-up[23]

2.1. Preparation of the enzyme solution and the cell culture medium

2.1.1. Prepare the enzyme solution with 0.1% trypsin and 0.01% DNase I in Ca^{+2}/Mg^{+2}-free HBSS and keep it at 37 °C in the incubator.

2.1.2. Prepare 15 mL of cell culture medium with neuro medium supplemented with 25 mM HEPES, 30 U/mL penicillin, 0.15% glucose, 8.75 µg/mL insulin, and 1x N2-supplement.

2.2. Dissociation procedure

2.2.1. Incubate the SGs in 2 mL of the enzyme solution at 37 °C for 8 min, shake the cells gently, and incubate for an additional 8 min at 37 °C.

2.2.2. Stop the enzymatic activity by adding 200 µL fetal bovine serum (FBS).

2.2.3. Discard the supernatant by using a pipette. Wash the cell clusters three times with cell culture medium by short pulse centrifuging (4,000 rpm for 3 s) and removal of the supernatant by pipetting at room temperature.

2.2.4. Disrupt the cell clusters carefully by pipetting up and down the suspension using 1,000 µL and 200 µL filter tips, consecutively, until any clusters are seen.

2.2.5. Determine the cell yield by using the Neubauer cell count-chamber following staining of a 1:5 or 1:10 dilution of the cell suspension with 10% trypan blue to exclude apoptotic cells from the cell count.

3. Cell Seeding

3.1. Coating of the glass plates

3.1.1. Place the glass plates (8 mm x 8 mm) with and without polymer coating (*e.g.* PDMAA, PEtOx, PMTA) with a curved standard pattern forceps into the wells of a 48 well-microtiter plate.

3.1.2. Pipette 100 µL of 70% ethanol into the wells and incubate all glass plates for 10 min each and rinse them with PBS, pH 7.5, at room temperature using a pipette. Repeat this wash two more times.

3.1.3. Coat the glass plates without polymer for the positive control (PosCtrl) with 100 µL of 0.1 mg/mL poly-DL-ornithine for 1 h at room temperature. Then rinse them once with PBS, pH 7, at room temperature.

3.1.4. As follows, coat PosCtrl with 100 µL of 0.01 mg/mL laminin at 37 °C for 1 h and rinse them once with PBS, pH 7.5, as described in step 3.1.3.

3.2. Experimental set-up

3.2.1. Add 250 µL/well of the cell suspension containing 2 x 10^4 cells in serum-free culture medium, for each polymer coating, positive control, and negative control (glass plate without polymer and ornithine/laminin coating) by using a pipette. Use for each assay n = 5 wells.

3.2.2. Add 250 µL of serum-free culture medium with 20% FBS to each well by using a pipette (total volume now is 500 µL).

3.2.3. Cultivate the cells in the 500 µL culture medium in a humidified incubator at 37 °C and 5% CO_2 for 48 h.

4. ICC Staining

4.1. ICC detection of cell specific antigens to examine the population composition of the SG following cultivation on the polymer films

4.1.1 Fixation of the cells

4.1.1.1. Remove the PBS (pH 7.5), by pipetting and fix the cells by adding 200 µL/well of methanol.

4.1.1.2. Incubate for 10 min at room temperature and rinse 3x with 250 µL PBS, pH 7.5, by using a pipette.

4.1.2. Preparation of the permeabilization and antibody dilution buffers

4.1.2.1. Prepare the permeabilization buffer with Triton X-100 0.1% (w/v) to PBS, pH 7.5 (PBSTx).

4.1.2.2. Prepare the antibody dilution buffer with a final concentration of 1% bovine serum albumin (BSA) in PBS.

4.1.3. Antibody incubation

4.1.3.1. Permeabilize the cells with 0.1% PBSTx for 5 min, remove the permeabilization solution, and wash three times with PBS, pH 7.5, by pipetting.

4.1.3.2. Dilute the primary antibodies in the antibody dilution buffer as described in **Table 1** and prepare a master mix containing enough of the respective diluted primary antibody.

4.1.3.3. Pipette 100 µL of the desired antibody solutions onto the glass plates and incubate them for 1 h at room temperature.

4.1.3.4. Remove the antibody solution by pipetting and rinse the glass plates 3x with PBS, pH 7.5, for 5 min each.

4.1.3.5. Prepare a 1:400 dilution of the fluorescently labeled secondary antibodies in antibody dilution buffer (**Table 2**) and prepare a master mix containing enough of the respective diluted secondary antibody.

4.1.3.6. Pipette 100 µL of the desired secondary antibody solutions onto the glass plates, incubate for 1 h at room temperature protected from light, and wash the glass plates 3x with PBS, pH 7.5, as described in step 4.1.3.4.

4.1.3.7. Pipette 20 µL of the mounting gel containing 4,6-Diamidino-2-phenylindole (DAPI) onto the glass plates, remove them from the microtiter plate, and place them upside down on coverslips (24 x 60 mm^2).

Note: The control experiments to verify the specificity of the immune staining have been performed in parallel by incubating the fixed cells with secondary antibodies only. Hereby, the primary antibodies were exchanged with 1% BSA in PBS.

5. Distribution and Morphology of the Adherent SG Cells on the Uncoated and Coated Glass Plates[24]

5.1. Fixation of the cultivated cells

5.1.1. Remove the culture medium with a pipette.

5.1.2. Using a pipette gently load the glass plates with the adhering cells with 2.5% glutardialdehyde in 0.1 M sodium cacodylate, pH 7.3. Incubate for 1 h at room temperature.

5.2. Dehydration of the cells

5.2.1. Remove the solutions with the pipette the wells with the containing glass plates and fill them with 30% acetone in 10 min. Repeat twice.

5.2.2. Dehydrate the cells twice with 50%, 70%, 90% acetone in 10 min, respectively, as described in 5.2.1.

5.2.3. Rinse the cells with 100% acetone 6x within 30 min.

5.2.4. Put the specimens in the critical point dryer (CPD) and exchange 8x acetone against fluid CO_2.

5.2.5. Deflate the CO_2 at the pressure of 7.3 MPa and the temperature of 31 °C in the CPD.

5.3. Mounting and preparation of the samples for SEM

5.3.1. Transfer the glass plates containing the dried cells with standard forceps and fix them with fluid carbon in xylol (*e.g.* Leit-C) on top of the aluminum holder.

5.3.2. Put the aluminum holder on the desk of the high-resolution sputter coater.

5.3.3. Evacuate the chamber to 9×10^{-2} mbar.

5.3.4. Flush with argon 3x by opening the exit valve and closing the valve, followed by floating with argon from the gas cylinder.

5.3.5. Keep the voltage at 1 kV; a current of 20 mA will be induced by the floating argon.

5.3.6. Ionize the gold target for 120 s to produce a 20 nm thick gold layer on the specimens.

5.4. Analysis of the specimens with SEM

5.4.1. Analyze the specimens at 10 kV with low magnification (x156) and higher magnification (until x1,250).

5.4.2. Save micrographs of the cells in all regions of the uncoated and coated glass plates with the SEM software (Gebert and Preiss)[24].

REPRESENTATIVE RESULTS:

The aim of establishing the ICC protocol was the differentiation of the cell types accompanying the adhesion and neurite outgrowth of the SG neurons. As shown in **Figure 1A–H**, the method detected not only the expression of the neurofilaments in SGN, but also vimentin in both fibroblasts and glial cells on the polymer films (**Figure 1A, C, E, G**). Double staining with anti-vimentin and glial cell specific anti-p75 NGFR antibody enabled the identification of the glial cells adhering on PMTA, PDMAA, and PEtOx (**Figure 1B, D, F, H**). Whereas both the positive control and PMTA allowed attachment and growth of all cell types of the SG, the PDMAA and PEtOx presented mainly fibroblasts rather than glial cells (**Figure 1F, H**). Moreover, specific staining of the glial cells and the fibroblasts allowed the verification of the inhibition of the glial cell attachment on both PDMAA and PEtOx films by relating the number of fibroblasts and glial cells to the total number of DAPI stained cell nuclei[7]. Additionally, SEM revealed not only the distribution of the different cell types but also their interactions. On the PMTA coatings the SG cell types demonstrate their typical morphology compared to the control (**Figure 2A, B**). In contrast, flat cells with broad lamellopodia are connected to the PEtOx film (**Figure 2C**). The PDMAA surface is cell-free (**Figure 2D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative fluorescence microscopic view of the SGN and non-neuronal cells following cell specific labeling. The SG cells were double-labeled either with anti-neurofilament (NF, yellow) and anti-Vimentin (Vim, red) antibody, or anti-p75 neurotrophic growth factor receptor (p75-NGFR, denoted as p75, yellow) and anti-Vim antibody, which demonstrated the adherence of all cell types on the glass plates (**A**), PMTA (**C**), PDMAA (**E**), and PEtOx (**G**). Positive staining of the p75-NGFR antigen clearly demonstrated glial cells attaching to glass plates (**B**), PMTA (**D**), PDMAA (**F**), and PEtOx (**H**). This figure has been modified from Hadler *et al.*[7]

Figure 2: SEM of the SG cells adhering on the polymer films and glass plates coated with ornithine/laminin. The SG cells retained their different cell morphology not only on ornithine/laminin coated glass plates (positive control, **A**) but also on PMTA layers (**B**). In contrast, cells with broad flat lamellopodia were predominately represented on PEtOx films (**C**). The adhesion of SG cells was clearly reduced on the PDMAA coated surfaces (**D**). This figure has been modified from Hadler *et al.*[7]

Table 1: Primary antibodies used to determine specific antigens in the SGN, fibroblasts, and

glial cells.

Table 2: Secondary antibodies used in this study.

DISCUSSION:

This study represents for the first time the differential interactions of SGN, glial cells, and fibroblasts on varying polymer films. Staining of the specific intermediary filaments and the neurotrophic growth factor receptor enabled not only a strong distinction between the cell types and their morphology following adhesion, but also the quantitative determination of the interesting cell types. Hereby, as described in Hadler *et al.*[7], the growth of fibroblasts was clearly scaled down on the PMTA layers in comparison to the glial cells. This finding indicates these surfaces as preferential substrate for glial cell attachment and glial cell driven formation of the ECM rather than for fibroblasts. As well, adhesion and survival of the SGN was found to be strongly associated with glial cell growth. It is supposed that the glial cells are not only involved in trophic support and neuronal protection, but also in providing myelin proteins to induce regeneration of the nerve fibers[25-26]. Moreover, the SEM examination revealed that both PDMAA and PEtOx films inhibited the attachment of the glial cells and, in consequence, the adhesion and survival of the SGN.

Possible critical steps in the protocol refer to the dissection of the cochlear SG from postnatal rats, which require strong surgical skills and knowledge of the inner ear anatomy; especially, for the dissection of cochlear parts of the membranous labyrinth under microscopic view due to the rat cochlea fine-structure. Additionally, the postnatal rat should no older than five days, since the bony capsules become hard and, in consequence, the quality of the dissection of the SGs may be degraded. It is critical to ensure that during the enzymatic dissociation procedure the homogenous cell suspension is free of any cell clusters.

Prior to ICC detection of the specific antibody-antigen interactions it has to be considered that the choice of the fixation solution depends not only on the protein structure of the desired antibodies, but also on the chemical structure of the surface for cell adhesion. In general, depending on the cell culture surface the SGN may be alternatively fixed with methanol/acetone and 4% paraformaldehyde (PFA).

There are several reasons to limit the number of SGN for each experiment: (i) in general, it is best practice to limit the number of animals used for any experiment, (ii) the number of postnatal rats is varying and depends on the number of pregnant female rats, (iii) and the extraction of the cochlear tissues is time-consuming and labor-intensive. Thus, flow cytometry techniques[27] may not be useful for cell sorting of the SGN cultures because it requires higher cell numbers.

For the SEM analysis, it is critical that the cells dry in contact with the material. Therefore, the chemical stability of the test material not only during the cell culture but also the chemical resistance of the surfaces in acetone must be tested beforehand. Size and/or light transmissibility is not important, and therefore, complete biomaterials after explantation can be analyzed with a high resolution. Nevertheless, only the cells as well formed extracellular matrices on free or

dissected surfaces of the specimens can be evaluated. Cell-to-cell contacts as well as interactions between the cells and the materials can be precisely documented. If the cell types are difficult to separate, immunochemical surface labeling with gold allows a detailed analysis.

By this consideration, there is no alternative to immunostaining and cell counting under microscopic view. Also, labeling of the antibody-antigen-interaction visualizes morphology, interactions, and function of the cells adhering on the polymer films or other surface types.

ICC staining of specific antigens together with SEM techniques allows not only identification and quantification of primary cell culture assays, but also cellular behavior and interactions with various substrates to characterize their biological effects and toxicity. Furthermore, high-resolution microscopy may reveal new insights of signal pathways and metabolic activity of the cells of interest.

ACKNOWLEDGMENTS:
The support of S. Zwittian and G. Preiss, Institute of Cell Biology, Hannover Medical School for was greatly appreciated. This study was funded by the Deutsche Forschungsgemeinschaft (SFB 599, subproject D2).

DISCLOSURES:
The authors have nothing to disclose.

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Figure1

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Fig1_JoVE_Immunostaining_rev.tif

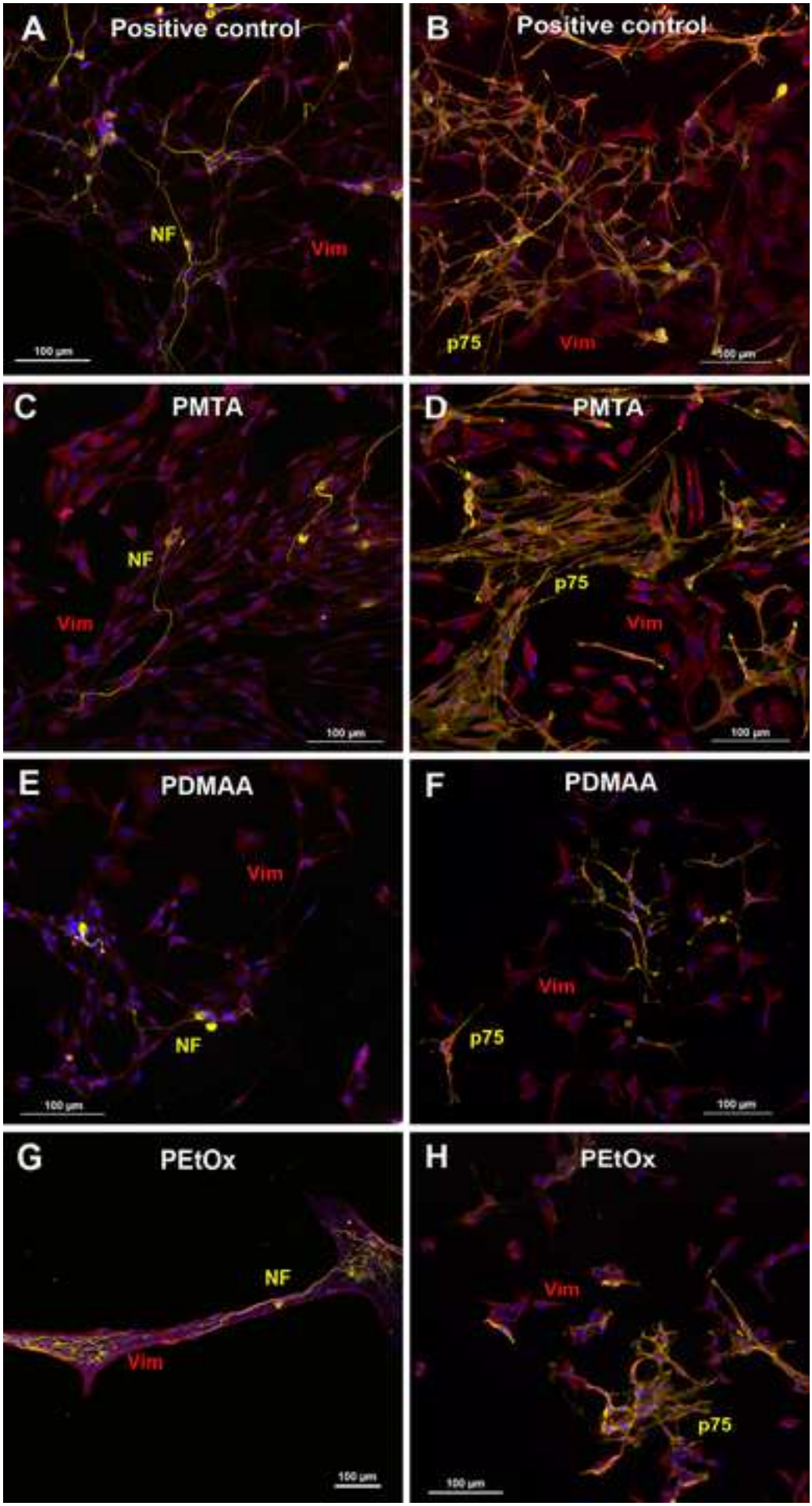

Figure2

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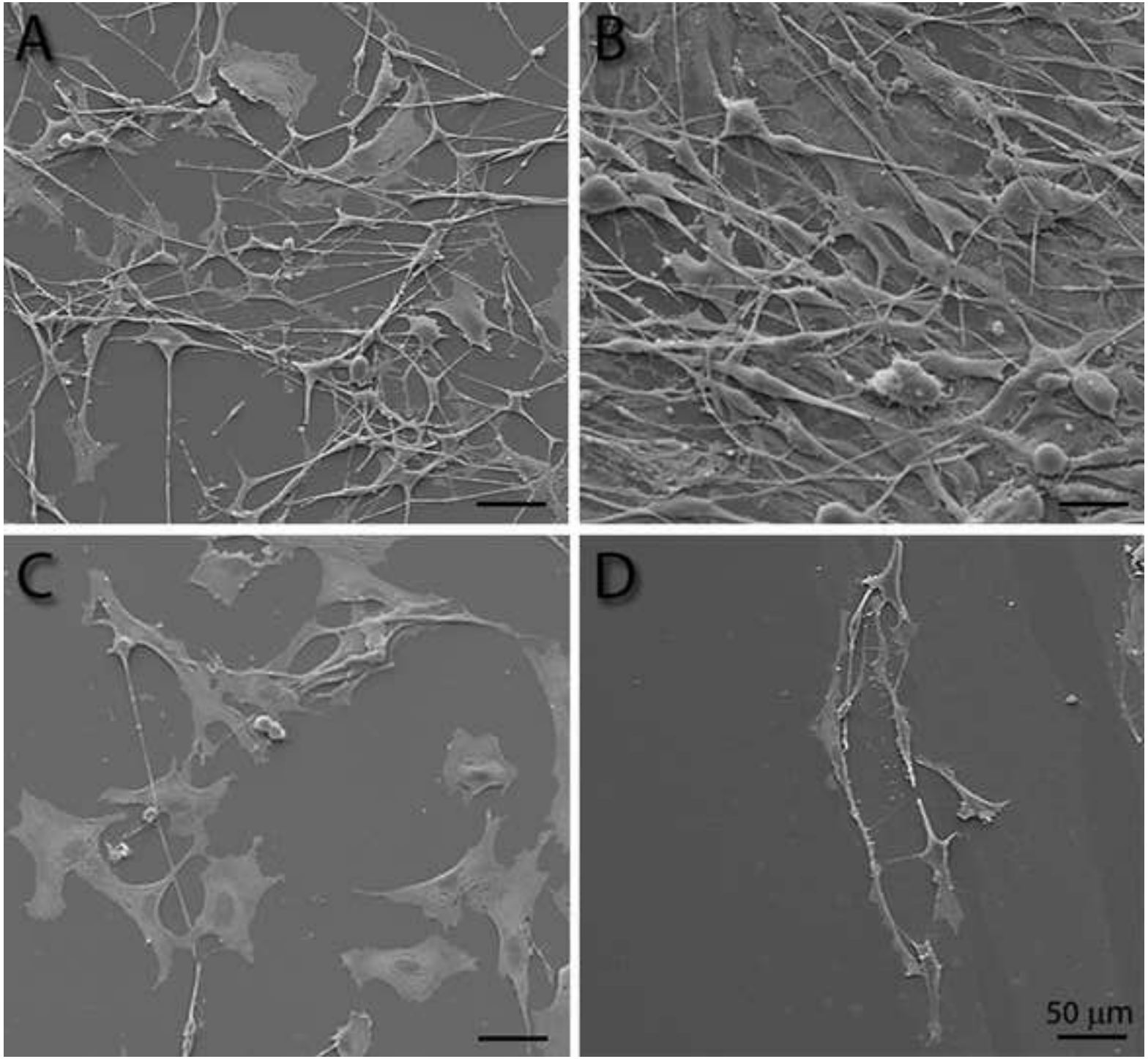

Table

Primary antibody	Host	Description	Cell specificity	Dilution
Neurofilament 200kD, monoclonal	Mouse	Intermediar filament	Neurons	1:400
p75, polyclonal	Rabbit	Neurotrophic growth factor receptor	Glial cells	1:500
Vimentin clone V9, monoclonal	Mouse	Intermediar filament	Fibroblast, glial cells	1:200
Vimentin, polyclonal	chicken	Intermediar filament	Fibroblast, glial cells	1:750

Table

Secondary Ab, IgG (H+L)	**Host**	**Description**	**Abs./em. [nm]**
Anti-chicken	Goat	Texas Red (**GaCh TR**)	586/605
Anti-mouse		New Dylight 488 (**GaM 488**)	493/518
Anti-rabbit		Alexa Fluor 594 (**GaRb 594**)	591/616

Materials Table

Click here to download Materials Table JoVE_Materials_Wissel rev.xlsx

Name of Material/ Equipment	Company	Catalog No.	Comments/Description
Animals			
Sprague-Dawley rats	Charles River, Sulzfeld, Germany		Inbreeding strain, ordered and provided by the Laboratory Animal Sciences, Medical School Hannover, Germany
Laboratory equipment			
Transmission light microscope Leica MZ-6	Leica, Germany		Preparation of the cochlear structures
Inverse microscope: Keyence BZ 9000 Biorevo	Keyence International, Mechelen, Belgium		Fluorescence microscopy
SEM 505	Philips, NL		Scanning electron microscopy
CPD 030	Balzers Union, Balzers FL		Critical point drying
Polaron E5400	Watford Hertfordshire, England		High resolution sputter coater
Laboratory tools			
Standard Pattern Forceps, curved/12 cm	Fine Science Tools, Heidelberg, Germany	11001-12	Dissection of the spiral ganglia from postnatal rats
Adson-Brown Forceps, Shark Teeth	Fine Science Tools, Heidelberg, Germany	11627-12	
Forceps Dumont #3c, straight/11 cm	Fine Science Tools, Heidelberg, Germany	11231-20	
Forceps Dumont Medical #5, straight	Fine Science Tools, Heidelberg, Germany	11255-20	
Scissor, pointed-pointed, straight/11 cm	Wirtschaftsgenossenschaft deutscher Tierärzte (WDT) eG, Garbsen, Germany	27040	
Plastic and glass material			
48well-microtiter plate	Nunc/thermo Scientific	150787	
Coverslips Menzel-Gläser 24 x 60 mm	VWR International GmbH, Darmstadt, Germany	631-0973	

Buffers, enzymes, proteins, chemicals, cell culture supplements			
Acetone	Mallinckrodt Baker R.V., Griesheim, Germany	9002-02	
Argon	Linde Gas, Pullach, Germany	Argon 5.0	
Bovine serum albumine	Sigma-Aldrich, St.Louis, USA	A3294	Lyophilized powder, initial fraction by heat shock, fraction V.
DNase I	Roche, Basel, Switzerland	11284932001	
Fetal bovine serum (FBS)	Biochrom, Berlin, Germany	S0415	
Glucose 40 % concentrate			Our lab is receiving glucose by the hospital pharmacy.
Glutardialdehyde	Polysciences, Warrington, PA, USA	01909-10	
Hank's balanced salt solution (HBSS)	Invitrogen/Fisher Scientific, Waltham, MA, USA	14170-070	Ca2+/ Mg2+-free
HEPES	Invitrogen/Fisher Scientific, Waltham, MA, USA	15630-056	1 M
Insulin from bovine pancreas	Sigma-Aldrich, St.Louis, USA	I0516	
Leit-C	Plano, Wetzlar, Germany	G3300	fluid carbon in xylol
N2-Supplement	Invitrogen/Fisher Scientific, Waltham, MA, USA	17502-048	100x
Natural Mouse Laminin	Invitrogen/Fisher Scientific, Waltham, MA, USA	23017-015	
Panserin 401 neuro medium	PAN Biotech, Aidenbach, Germany	P04-710401	Neuro medium for cultivation neuronal cells
Phosphate buffered saline (PBS) tablets	Invitrogen/Fisher Scientific, Waltham, MA, USA	18912-014	Ca2+/ Mg2+-free
Penicillin G Sodium salt	Sigma-Aldrich, St.Louis, USA	PENNA-10MU	

Poly-DL-Ornithin hydrobromid	Sigma-Aldrich, St.Louis, USA	P8638	
Prolong anti-fade Gold with DAPI	Invitrogen/Fisher Scientific, Waltham, MA, USA	P36941	DAPI containing mounting gel
Sodium cacodylate	Sigma-Aldrich, St.Louis, USA	C4945-10G	
Triton-X 100	Sigma-Aldrich, St.Louis, USA	T8787	
Trypan Blue	Sigma-Aldrich, St.Louis, USA	T 8154	
Trypsin	Biochrom, Berlin, Germany	L2123	1:250 in PBS, Ca2+/ Mg2+-free
Primary antibodies			
Neurofilament 200kD, mouse, monoclonal	Novocastra, Newcastle upon Tyne, UK	NCL-NF200	
p75, rabbit, polyclonal	Abcam, Cambridge, UK	38335	
Vimentin clone V9, mouse, monoclonal	Dako GmbH, Jena, Germany	M0725	
Vimentin, chicken, polyclonal	Abcam, Cambridge, UK	ab24525	
Secondary antibodies			
Goat anti-chicken, conjugated with Texas Red	Santa Cruz Biotech., Dallas, TX, USA	sc2994	
Goat anti-mouse, conjugated with New dylight 488	Jackson-Immunoresearch Laboratories Inc., West Grove, PA, USA	115-485-008	
Goat anti-rabbit, conjugated with Alexa Fluor 594	Jackson-Immunoresearch Laboratories Inc., West Grove, PA, USA	111-515-144	

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Author(s): Brandes G, Hadler C, Warnecke A, Bohlmann J, *

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Department: Dept. of Otorhinolaryngology

Institution: Medical School Hannover

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Hannover, 20.12.2017

Second revision of the manuscript *"Characterization of the interaction of primary cells from the rat inner ear with polymer films as coatings for cochlear implant electrode surface"*

Dear Editor,

Thank you for further critical comments! Please find attached the revised version of our manuscript for publication in JoVE. Additional to the revised manuscript a separate document addressing all the editorial comments and changings presented as mark ups ed uploaded. Also, the material list was revised and submitted as excel-table.

We hope to submit an improved manuscript that is now suitable for publication in JoVE.

Sincerely, Kirsten Wissel

Comments of the editor:

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. For example, the caption of figure 1 should be revised to avoid any overlap with your previous paper https://doi.org/10.1371/journal.pone.0157710

Response:
We thank the editor for the critical remark and revised the manuscript for overlapping sections. Please, find the corrections in following sections: Table 1, figures 1 and 2.

3. Please provide an email address for each author on the first page.

Response:
The e-mail addresses have been added on the first page.

4. Please define all abbreviations before use.

Response:
We considered the editor's comment and defined the abbreviations. Hereby, the abbreviations have been added to the list of keywords and in the abstract sections and "Introduction". Protocol related abbreviations are described within the respective section and chapters.

5. Please use SI units, e.g. please use "µL" instead of "µl".

Response:
We considered the editor's comment and included the SI units into the protocols.

6. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response:
As recommended by the editor the short abstract has been rephrased. Please, refer to this section on page 1.

7. Please rephrase the Long Abstract to more clearly state the goal of the protocol. Please attention that in the final form, Long Abstract will be used as the Abstract. The Short Abstract will be used as Highlight for the databases.

Response:
As recommended by the editor the long abstract has been rephrased. Please, refer to this section on page 1.

8. 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.

Response:
We are following the editor's instructions and have removed the company names and trademark symbols. Please, refer to the protocol section.

9. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Response:
The critical view of the editor is very appreciated and the section Introduction has been rephrased.

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response:
We are following the editor's comments and have assigned [Neonatal Spraque-Dawley rats ….described by the German "Law on protecting animals" (Tierschutzgesetz)] as "Note". Following revision of the section "Protocol", we can confirm, that all text in this section is written in the imperative tense.

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response to 11.-21.:
We considered the critical note of the editor and completed the protocol section as follows:

12. Protocol: Please move the ethics statement before your numbered protocol steps.
As described in comment no. 10 the ethics statement is assigned as "Note" before numbering the protocol steps.

13. Protocol: Please specify what tools are used, what incisions are made, where and how large, etc.

We agree with the editor to give a more detailed description of the dissection of the spiral ganglions. Please refer to chapter "1. Dissection of the cochlear spiral ganglion (SG) from neonatal rats".

14. Protocol: How were the rats euthanized?
As described in the protocol step 1.1. the 3-5 days old rats were sacrificed by cutting quickly the head with a scissor.

15. Protocol: 1.2, 1.3, etc.: How? Using what? Please describe the steps clearly, or refer to an appropriate reference or protocol.

Please refer to the response to comment **13**.

16. Protocol: 3.1.2: Incubate at which temperature?
As described in the protocol step 3.1.2. the coated and uncoated glass plates were incubated at room temperature.

17. Protocol: 4.1.3.6: How is this done?
The control experiments have been done in parallel to specify the quality of the antibodies: The immune staining procedure was identic, only the primary antibodies has been omitted. Please refer to "Note" following step **4.1.3.6.**

18. Protocol: 4.2.4: How is this done?
Here we disagree with the editor, since this protocol step is not present.

19. Protocol: 5.1.1: How?
We added this protocol step as follows: "Remove the culture medium with a pipette".

20. Protocol: 5.1.3: Fix with what? How?
This protocol step was included into the protocol step 5.1.2.: "Gently overwhelm the cells with 2.5% glutardialdehyde in 0.1 M sodium cacodylate, pH 7.3., for 1h at room temperature".

21. Protocol: 5.3.5: Please use another word besides wash.
"wash" was exchanged by "flush". Additionally, this protocol step was explained more detailed: "Flush with argon three times by opening the exit valve and closing, followed by floating with argon from the gas cylinder".

22. After revising the protocol, please include a blank line between each step. Please attention that there is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response:
Here we do not understand this recommendation of the editor. Our manuscript has been prepared accordingly to those provided by the journal (Hemant Suryawanshi et al. (2015) A Simple Alternative to Stereotactic Injection for Brain Specific Knockdown of miRNA). Herein, any additional blank lines have been found. Also, we used the submission template provided by JoVE.

23. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response:
As recommended by the editor we have proven this.

24. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response:
As recommended by the editor we have proven this.

25. Please use the micron symbol (μ) for microns instead of the letter U: Figure 1 scale bar, etc.

Response:
The comment of the editor has been considered and the micron symbol has been added in figure 1.

26. Please move the manufacturer's column in Table 1 and 2 to the Table of Materials.

Response:
The comment of the editor has been considered and the manufacturer's column has been deleted in tables 1 and 2.

27. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Response:
As recommended by the editor the material table has been revised and completed.

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Response:
As appreciated by the editor the copyright permission has been submitted as doc-file to the Editorial Manager account. As well the citation was added to the figure legends.

Additional note: The title has also been rephrased to give a more clear article description.

Comments of the reviewers

Reviewer #1:

Manuscript Summary:
This article studies the adhesion properties of cultured spiral ganglion cells on different materials that could be used in cochlear implant design in order to drive neurite outgrowth toward the array electrodes. The protocol is well described.

Major Concerns:
The protocol and the figures were already published by the same team in
PLoS One. 2016 Jul 8;11(7):e0157710. doi: 10.1371/journal.pone.0157710. eCollection 2016.
Polymer Coatings of Cochlear Implant Electrode Surface - An Option for Improving Electrode-Nerve-Interface by Blocking Fibroblast Overgrowth.
Hadler C1, Aliuos P2,3, Brandes G4, Warnecke A2,3, Bohlmann J2, Dempwolf W1, Menzel H1, Lenarz T2,3, Reuter G2, Wissel K2.

Response:
Herein, the focus of this manuscript is the description of the immunocytochemical and electron microscopical technique to characterize the interactions between primary cells and different kind of surfaces. The coating of the glass plates with ultrathin polymer films presents a good example how different types of cells may specifically interact with distinctly chemically modified surfaces.
As well, the copyright permission for figures has been submitted as doc-file to the Editorial Manager account.

Minor Concerns:
If the fact that the protocol and data are already published elsewhere does not bother the editor, i would add a short paragraph on how the coating with different polymer is made in the mat med. I would also add a paragraph in the discussion on how the polymer would be placed on the cochlear implant design and why such a coating would be clinically relevant for the patients.

Response:
The comment of the reviewer is very appreciated, however, as described above characterization of the interactions of the cells with modified surfaces is focused in this manuscript and not the preparation of the polymer films. The manufacturing of the ultrathin polymer layers has been successfully modified and already published in Hadler et al., 2017 [Hadler C, Wissel K, Brandes G, Dempwolf W, Reuter G, Lenarz T, Menzel H. Photochemical coating of Kapton® with hydrophilic polymers for the improvement of neural implants. Mater Sci Eng C Mater Biol Appl. Jun 1; 75:286-296 (2017)].
Nevertheless, a short description of the polymer film fabrication and immobilization has been added to this manuscript in the section “Introduction”.

Reviewer #2:

The Manuscript is well writing. However, it is important to add some details and graphical help to have an impact on the transfer of knowledge. Minor revision will be needed in the following points.

***Dissection of the cochlear spiral ganglion (SG) from neonatal rats Section**

1. Can the authors add a graphical representation of the dissection process please? This will be particularly helpful for the readers to identify the last main steps detailed in 1.7, 1.8 and 1.9, (In addition to the future video).

Response:

On one side we agree with the reviewer to present the dissection of the cochlear spiral ganglions as graphics helping to get an idea of the anatomical structures of the inner ear. On the other side, creation of this kind of artworks may not entirely match the effective extraction of the cochlear tissues. Thus, we think it is much more helpful to produce the video and to follow the instructions step by step.

***Coating of the glass plates without polymer layers used as positive control Section.**

1 Can the authors explain why the laminin layer on top of the poly-DL-ornithine coating is needed please? Some protocols of primary cell dissociation have shown cell adhesion with the positive controls only coated with poly-DL-ornithine.

Response:

The critical comment of the reviewer is appreciated. To establish the spiral ganglion neuron (SGN) cultivation in our laboratory, varying concentrations of both ornithine and laminin as well combinations of both adhesion proteins were tried to increase the survival rate of the SGN. By these experiments it was demonstrated that coating of the tissue culture plate with D,L-ornithine as well as with laminin as described in this manuscript resulted in more stable SGN survival and neurite outgrowth.

***In the results Section**

1. Can the authors add the quantification results presented in bar or pie charts, for instance please?. This is important to complement the fluorescent micrographs. It is advisable to perform the statistical analysis of the results to be able to claim the reduction in fibroblast adhesion by the polymer coatings.

Response:

Here, we are not agreeing with the reviewer, since the focus of the manuscript is on developing a simple and fast method to highlight and to characterize interactions between cells and modified surfaces. For further informations, please refer to the article by Hadler C, Aliuos P, Brandes G, Warnecke A, Bohlmann J, Dempwolf W, Menzel H, Lenarz T, Reuter G, Wissel K. Polymer Coatings of Cochlear Implant Electrode Surface - An Option for Improving Electrode-Nerve-Interface by Blocking Fibroblast Overgrowth. PLoS One. 11(7):doi: 10.1371/journal.pone.0157710. (2016).

Reviewer #3:

Manuscript Summary:

The manuscript appears to focus on one aspect of a previously published paper on interactions of cochlea related cells with materials. The paper describes a method for cell harvest, dissociation and assessment via immunohistological techniques and SEM.

Major Concerns:

The major disconnect in the paper is between the actual protocol and the Introduction or context. From the Abstract and Introduction the reader is expecting a protocol related to polymer coating of substrates and assessments of mixed cell populations, however the actual paper is just the culture of the cells and assessment. In addition these techniques are all known within the field. While this paper will be of use to newcomers, the more interesting aspects would be associated with techniques used to ensure polymer coating across sample types are consistent and possible issues associated with cell assessment that are related to the materials.

Response:

We consider the critical comments of the reviewer. As first, the sections “Abstract” and “Introduction” has been rephrased to a more clear statement of the aim of the study. Indeed, the immunocytochemical as well electron microscopical technique are very well characterized, however, we modified and used these techniques , i.e. to enable specific characterization in primary cultures, especially of spiral ganglion cells, for studying cellular interactions between cells and coated surfaces, i.e. coated with polymer films.

Minor Concerns:

Given that these methods are largely related to known and published techniques, it is necessary to reference the papers from which these protocols were adapted. Only Step 2 seems to be referenced, however all steps are related to known techniques.

Response:

We consider the recommendation of the reviewer and added references to the protocol steps. The present protocols were adapted to the special project.

Wissel, Kirsten Dr.

Von: noreply@salesforce.com im Auftrag von one_production <one_production@plos.org>
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Polymer Coatings of Cochlear Implant Electrode Surface – An Option for Improving Electrode-Nerve-Interface by Blocking Fibroblast Overgrowth Plos One ========== Dear Madam/Sir, we are going to submit a methodical manuscript concerning immunocytochemical detection and scanning electron microscopic of spiral ganglion neurons, glial cells and fibroblasts growing on polymer surfaces. For that few figures, in particular figures 3a-h and 5a-d, are considered for submission in the "Journal of Visualized Experiments" (JoVE). Thus, we kindly ask you for permission to leave the figures 3 and 5 to JoVE.

Thank you very much in advance!

Best regard, Kirsten Wissel

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