Materials and protocols - iGEM Pasteur Paris 2018 team

Antoine EHRET - Andreas FYRILLAS - Ellyn REDHEUIL

Here are some of the materials and protocols the iGEM Pasteur Paris 2018 team used for their experiments during the iGEM 2018 competition. This document has been made for the PSE (Scientific Team Project) course at ESPCI Paris and does not contain all the protocols used by the team. You can find all of them on the team website: `http://2018.igem.org/Team:Pasteur_Paris/Experiments`.

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DNA Assembly and Microbiology

iGEM Pasteur Paris team - Institut Pasteur

Axelos Etienne Dejoux Alice Dumont Claire Ehret Antoine
Fyrillas Andreas Ghaneme Aymen Guerassimoff Léa
Jaoui Samuel Picaud-Lucet Emma Porte Sarah
Redheuil Ellyn Richard Charlotte Starck Thomas
Thomas Florian Trang Kelly Madelénat Manon
Paillarès Éléa Naccache Jonathan Sachet Gabriela

2018

I. Agarose gel preparation

Aim Prepare an 8%agarose gel for the electrophoresis of DNA samples.

Materials

- *UltraPure*^{TM} Agarose (Invitrogen, 16500-100)
- *UltraPure*^{TM} 10X TAE Buffer (Invitrogen, 15558)
- Gel Green Nucleic Acid Stain (Biotium, 41005)
- Scale
- Microwave
- Spatula
- Erlenmeyer (250 mL)
- Measuring cylinder
- *PowerPac*^{TM} Basic (Bio-Rad, 1645050)
- Owl Scientific (Thermofisher) B2 Series gel block (plus Biorad powerpack adaptor)

Procedure

1. Prepare 600 mL of TAE 1X by diluting 60 mL of 10X buffer in 540 mL of deionized water.
2. Weigh 0.6 g of agarose on a scale.
3. Place the agarose in an Erlenmeyer.
4. Fill the Erlenmeyer with 75 mL of TAE 1X.
5. Heat the Erlenmeyer for 2 min 30 s at 350W in the microwave oven.
6. Stir and place it again in the microwave for an additional minute.
7. Let the mixture cool down to around when it just warm to touc and add 5 μL of Gel Green.
8. Pour the agarose in the horizontal electrophoresis system. Don't forget to place the comb before!
9. Let the gel cool down for 20-30 minutes before deposing the samples.

II. Bacterial stock

Aim Store a bacterial culture at -80^oC.

Materials

- Desired bacterial cultures on petri dish
- Sterile LB media
- Appropriate antibiotics : Carbenicillin (50 mg/mL) or Chloramphenicol (25 mg/mL)
- Glycerol 50% v/v sterile
- Dry Ice
- Falcon 15 mL and 50 mL
- Erlenmeyer (125 mL)
- Sterile cryotube
- Inoculation loop
- Pipette p200 + p20 and associated cones
- Plastic graduated pipette (25 mL)
- Electric Pipetman (propipet)

Procedure In advance :

- Prepare a stock solution of LB + desired antibiotic in 50 mL falcon tube depending on how many culture you want to stock in glycerol.
- Prepare a sterile stock solution of glycerol 50 %.

1. In 15 ml sterile falcon, add 5 mL of LB medium.
2. Vortex the stock solution of antibiotic and add 5 μL to the LB.
3. Using an inoculation loop, gently touch a colony of transformed bacteria from the petri dish, plastic side facing you.
4. Immerse and dip the inoculation loop in the liquid media and stir.
5. Place the liquid culture in the incubator at 37^oC and 180 rpm for 16 h.
6. After 16 h, centrifuge the tubes 5 minutes at 3000 rpm.
7. Discard supernatant.
8. Resuspend the pellet in 5 mL of fresh sterile LB medium.
9. Centrifuge the tubes for 5 minutes at 3000 rpm.
10. Discard supernatant.
11. Resuspend the pellet in 1 mL of fresh sterile LB medium.
12. In a 125 mL Erlenmeyer, add 1 mL of bacterial culture in 24 mL of LB + desired antibiotic.
13. Incubate the culture at 37^oC and 180 rpm.
14. Measure the optical density at 600 nm (OD600) every hour for the first 3 h and then every 20 minutes.
15. When the OD600 reaches 0.6 to 0.7, withdraw 5 mL of the bacterial liquid culture and add 5 mL of glycerol 50%.
16. Vortex the tube.
17. Aliquot the 10 mL into 1 mL volumes in the sterile cryotubes.
18. Place into dry ice and freeze at -80^oC.

III. Gel extraction

Aim Extract a specific DNA band from an agarose electrophoresis gel.

Materials

— QIAquick Gel Extraction Kit (Qiagen, 28706)
— Scale
— Scalpel
— Heating block
— Water bath
— UV light box, equipped with blue diodes (long wavelength)

Procedure According to the QIAquick Gel Extraction Kit's manual

1. Using a UV light, excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Weigh the gel slice in an Eppendorf tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg 100 μL).
2. Incubate at 50^oC for 10 minutes (or until the gel slice has completely dissolved). To help dissolve the gel, mix by vortexing the tube every 2 to 3 minutes during the incubation.
3. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
4. Add 1 gel volume of isopropanol to the sample and mix.
5. To bind DNA, pipet the sample onto the QIAquick column and spin in a microfuge for 1 min. (Optional) : Add 0.5 mL of Buffer QG to QIAquick column and spin again.
6. To wash the column, add 0.75 mL of Buffer PE to QIAquick column and spin. Transfer QIAquick column to a clean 1.5 ml microcentrifuge tube or to a provided 2ml collection tube. Centrifuge for 1 min at 13,000 rpm (17,900 x g).
7. Place QIAquick column in a clean 1.5 mL microcentrifuge tube.
8. To elute DNA, add 50 μL) of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the centre of the QIAquick membrane and centrifuge the column for 1 minute at 13,000 rpm (17,900 x g). Alternatively, for increased DNA concentration, add 30 μL) elution buffer, let stand for 1 min, and then centrifuge for 1 minute.

IV. Ligation

Aim Perform the ligation of one or more inserts in a plasmid using the In-Fusion cloning kit.

Materials

- Stellar competent cells (Takara Clontech - Ozyme)
- Linearized plasmid
- Purified insert(s)
- 5X In-Fusion HD Enzyme Premix (Takara Clontech - Ozyme)
- Control plasmid pUC19
- Control insert
- Deionized water
- Water bath at 50^oC
- 1.5 mL Eppendorf tubes
- Heating block at 80^oC
- Dry ice

Procedure

1. Set the mix between insert and linearized vector in molar ratio 2 :1 and complete with distilled water to reach a reaction volume of 16 μL. The optimal quantity of vector is 100-150 ng.
2. Pre-heat vector and insert for 5 minutes at 80^oC.
3. Put on ice for 3 minutes.
4. Add 4 μL 5X In-Fusion HD Enzyme Premix and let the cloning occur in a water bath at 50^oC.
5. Set on ice and proceed to transformation in Stellar competent cells.

V. Liquid culture

Aim Start a liquid culture of transformed bacteria to amplify a plasmid.

Materials

- Petri Dish with LB agar media + antibiotics carbenicillin 50 μg/mL or chloramphenicol 25 μg/mL
- Sterile LB culture medium
- Antibiotics : Carbenicillin 50 mg/mL or Chloramphenicol 25 mg/mL
- Shaking incubator at 37^oC
- Sterile 250 mL Erlenmeyer or 50 mL Falcon tube
- Inoculation loop
- Plastic graduated pipette (10 mL or 20 mL)
- Electric Pipetman (propipet)

Procedure

1. Add 5 mL of sterile LB culture medium + appropriate antibiotics to a 15 mL falcon tube (or Erlenmeyer).
2. Using an inoculation loop, touch a colony of transformed bacteria from the petri dish. Immerse the inoculation loop in the liquid medium and stir energetically.
3. On a new petri dish + antibiotic, spread the rest of the bacterial colony in a zig-zag movement to have a copy plate.
4. Place the liquid culture in the incubator at 37^oC for 14 hours at 180 rpm. Maintain the lids on top using tape but do not close the tubes. The liquid cultures are then good to use for further experiments.
5. Place the petri dish in the incubator at 37^oC for 14 hours and then store it at 4^oC.

VI. Midiprep for plasmid extraction

Aim Retrieve and purify the amplified plasmids from a culture of transformed bacteria.

Materials

— 25 mL of overnight liquid bacterial culture of a transformed colony
— $QIAfilter^{TM}$ Plasmid Midi Kit (Qiagen, 12243)
— Centrifuges (Eppendorf 5804R, Sigma 1-14 microfuge)
— Falcon tube 50 mL
— Electric pipetman (propipet)

Procedure According to the $QIAfilter^{TM} PlasmidMidiKit's$ manual

1. Harvest 25 mL of overnight bacterial culture by centrifugation in a 5804R at 6000 x g for 15 minutes at 4^oC.
2. Re-suspend the pelleted bacterial cells in 4 mL Buffer P1.
3. Add 4 mL Buffer P2 and mix thoroughly by inverting the tube 4-6 times and incubate at room temperature for 5 minutes.
4. Add 4 mL Buffer P3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Pour the lysate into the barrel of the QIAfilter cartridge. Incubate at room temperature for 10 minutes, without inserting the plunger.
6. Equilibrate a QIAgen-tip 100 by applying 4 mL Buffer QBT and allow the column to empty by gravity flow into a collector tube.
7. Insert the plunger into the Cartridge and filter the cell lysate into the previously equilibrated QIAgen-tip. Let the cleared lysate enter the resin by gravity flow.
8. Wash the QIAgen tip with 2 x 10 mL Buffer QC.
9. Elute DNA with 5mL Buffer QF.
10. Precipitate DNA by adding 3.5 mL of isopropanol at room temperature (RT) to the eluted DNA.
11. Mix and aliquot in 1.5 ml Eppendorf tubes, and centrifuge immediately at 15,000 x g in Sigma 1-14 microfuge for 30 minutes at 4^oC. Carefully decant the supernatant.
12. Wash DNA with 2mL of 70 % ethanol at RT, and centrifuge at 15,000 x g for 10 minutes. Carefully decant the supernatant.
13. Air-dry the pellet for 5-10 minutes, and re-dissolve DNA in buffer TE.

VII. Miniprep for plasmid extraction

Aim Retrieve and purify the amplified plasmids from a 5 mL culture of transformed bacteria.

Materials

— 25 mL of overnight liquid bacterial culture of a transformed colony
— $QIAprep^{TM}$ Spin Miniprep Kit (Qiagen, 27106)
— Centrifuge
— 1.5 mL Eppendorf tubes
— Electric pipetman (propipet)

Procedure According to the QIAprep^{TM} Spin Miniprep Kit's manual

1. Pellet 5 mL of overnight bacterial culture by centrifugation in a 5804R at 6000 x g for 3 minutes at room temperature.
2. Re-suspend the pelleted bacterial cells in 250 μL Buffer P1 and transfer to a 1.5 mL Eppendorf tubes.
3. Add 250 μL Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 minutes.
4. Add 350 μL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 minutes at 13,000 rpm in a Sigma 1-14 micro-centrifuge.
6. Apply 800 μL supernatant from step 5 to the QIAprep2.0 spin column by pipetting. Centrifuge in the microfuge for 30-60 s and discard the flow-through.
7. Wash the QIAprep2.0 spin column by adding 0.75 mL Buffer PE. Centrifuge for 30-60 s and discard the flow-through. Transfer the QIAprep2.0 spin column to the collection tube.
8. Centrifuge for 1 minute to remove residual wash buffer.
9. Place the QIAprep2.0 column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 μL Buffer EB. Let stand for 1 minute, and centrifuge in the microfuge for 1 minute at 13,000 rpm.

VIII. Transformation of *E. coli* BL21 (DE3) pLysS

Aim Transform a plasmid in E. coli BL21 competent cells for protein expression.

Materials

- LB agar plates covered with appropriate antibiotics
- SOC Outgrowth Medium (NEB, B9020S)
- $BL21(DE3)pLysSSingles^{TM}CompetentCells$ – Novagen (Merck, 70236)
- Plasmids of interest
- Shaking incubator at 37^oC (inFORS minitron HT)
- Stationary incubator at 37^oC
- Water bath at 42^oC
- Ice bucket filled with ice
- Micro centrifuge tubes
- Sterile spreading rake

Procedure

1. Remove agar plates from storage at 4^oC and let them warm up to room temperature.
2. Take competent cells out of -80^oC and thaw on ice for approximately 5 minutes.
3. Add 1 μL of purified plasmid DNA to the tube of competent cells. Tap gently to mix and return to ice.
4. Incubate the competent cell/DNA mixture on ice for 5 minutes.
5. Heat shock each transformation tube into a 42^oC water bath for 40 seconds.
6. Put the tubes back on ice for 2 minutes.
7. Add 650 μL of SOC medium (without antibiotic) to the bacteria and grow at 37^oC and 180 rpm for 60 minutes.
8. Plate 250 μL of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
9. Incubate plates at 37^oC overnight.

IX. Transformation of *E. coli* DH5-alpha

Aim Transform a plasmid in E. coli DH5-α competent cells for amplification and storage.

Materials

- LB agar plates covered with appropriate antibiotics
- SOC Outgrowth Medium (NEB, B9020S)
- DH5-α Competent E. coli (High efficiency) (NEB, C2987 I)
- Plasmids of interest
- Shaking incubator at 37^oC
- Stationary incubator at 37^oC
- Water bath at 42^oC
- Ice bucket filled with ice
- Micro centrifuge tubes
- Sterile spreading rake

Procedure

1. Remove agar plates from storage at 4^oC and let them warm up to room temperature.
2. Take competent cells out of -80^oC and thaw on ice for approximately 5 minutes.
3. Add 1 μL (diluted to approx.. 10 ng/μL) of purified plasmid DNA to 100 μL of competent cells.
4. Mix by flicking the bottom of the tube and return to ice.
5. Incubate the competent cell/DNA mixture on ice for 30 minutes.
6. Heat shock each transformation tube into a 42^oC water bath for 40 seconds.
7. Put the tubes back on ice for 3 minutes.
8. Add 650 μL of SOC medium (without antibiotic) to the bacteria and grow at 37^oC and 180 rpm for 40 minutes.
9. Plate 250 μL of the transformation onto a 10cm LB agar plate containing the appropriate antibiotic.
10. Incubate plates at 37^oC overnight.

Protein characterization

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I. Fast Protein Liquid Chromatography

Aim Purify the proteins from a bacterial sample containing our protein of interest with a His-tag (in our case : NGF)

Materials

- Buffer A (5% glycerol, 50 mM Tris, 200 mM NaCl)
- Buffer B (5% glycerol, 50 mM Tris, 200 mM NaCl, 250 mM imidazole)
- Phenylmethylsulfonyl fluoride (PMSF)
- Sonicator Branson sonifier 450
- High load centrifuge (Avanti J-265 XPI, rotor JLA-14.50, Beckman Coulter)
- Fast Protein Liquid Chromatography (FPLC) ÄKTA pure (General Electric) with software Unicorn 6.3
- Ni-NTA Superflow Cartridge 5mL, (QIAGEN, 1034558)

Procedure

1. Resuspend the bacterial pellet of the 800 mL culture in 10 mL buffer A and add 10 μM PMSF to prevent protein degradation.
2. Sonicate the bacteria during 5 cycles of 45 seconds with 1 min intervals at power 6 and 50% duty control.
3. Centrifuge at 15,000xg for 25 min at 4^oC.
4. Realize the FPLC with the following sequence :
   - Inject the lysis supernatant at 0.5 mL/min,
   - Wash with buffer A for 90 min,
   - Wash with 5% buffer B in buffer A for 30 min,
   - Increase the proportion of buffer B gradually until reaching 100% during 70 min.

II. Gel Transfer for Western Blotting

Aim Transfer the proteins from an SDS-PAGE gel to a nitrocellulose membrane for western blotting.

Materials

— Gel from an SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis)

— iBlot® 2 NC regular stacks (Thermofisher-Invitrogen, IB23001)

— iBlot® 2 gel transfer device (Thermofisher-Invitrogen)

Procedure According to the $iBlot^{TM}$ 2 Dry Blotting System USER GUIDE .

1. Unseal the Transfer Stack. Set the Top Stack to one side and discard the white separator.
2. Keep the Bottom Stack in the plastic tray.
3. Place the Bottom Stack (in the tray) on the blotting surface. Align electrical contacts on the tray with the corresponding electrical contacts on the blotting surface of the iBlot® 2 Gel Transfer Device.
4. Wet the pre-run gel(s) and place it on the transfer membrane of the Bottom Stack.
5. Place a pre-soaked (in deionized water) iBlot® Filter Paper on the gel and remove air bubbles using the Blotting Roller (segment of a 10 ml plastic pipette in our case).
6. Place the Top Stack, over the pre-soaked filter paper. Remove air bubbles using the roller.
7. Place the Absorbent Pad on top of the Top Stack such that the electrical contacts on the blotting surface of the iBlot® 2 Gel Transfer Device.
8. Close the lid of the device.
9. Select the method P0 and touch Start Run. The transfer lasts for 7 minutes.

III. Immunodetection on membrane

Aim Mark the nitrocellulose membrane from the transfer with an anti-his-tag antibody to detect 6his-tagged NGF.

Materials

- Phosphate-buffered saline 1X (PBS) (Sigma, P4417)
- Tween20 (VWR, 437082Q)
- Bovine serum albumine (BSA) (Sigma, A3608)
- 6x-His tag antibody, Alexa Fluor® 647 conjugate (HIS.H8) (Invitrogen, MA1-21315-A647)
- Odyssey Gel Imaging Scanner (Li-COR, Nevada)
- Western blotted nitrocellulose membrane
- Appropriate recipient for the membrane and low agitation horizontal orbital shaker

Procedure

1. Wash the transfer membrane in PBST (1L phosphate buffer saline 1X + 1mL Tween20) for 10 min.
2. Incubate the membrane with 10 mL saturation buffer (95 mL PBS 1X, 3% BSA, 100 μL Tween20) for 1 hour at room temperature.
3. Wash the membrane with PBST for 1h.
4. Incubate the membrane with the antibody diluted at 1 :500 in 10 mL antibody incubation buffer (10 mL PBS, 3% BSA) for 1 hour at room temperature or overnight at 4^oC.
5. Wash 3 to 5 times for 5 minutes in 10 mL PBST at a higher agitation to decrease background noise.
6. Reveal the Western Blot using the Odyssey X-ray scanner.

IV. IPTG induction and protein expression

Aim Derepress the T7 promoter using IPTG to activate NGF or RIP expression.

Materials

- Desired transformed bacterial cultures on petri dish
- Sterile LB medium
- Appropriate antibiotics : Carbenicillin (50 mg/mL) or Chloramphenicol (25 mg/mL)
- 100 mM Isopropyl β-D-1-thiogalactopyranosid (IPTG) (Sigma-Aldrich, I6758)
- Erlenmeyer (V = 125 ml)
- InFORS minitron HT shaking incubator
- Inoculation loop
- Pipette p200 + p20 and associated cones
- Plastic graduated pipette (25 ml)
- Electric Pipetman (propipet)

Procedure

1. Resuspend a single transformed colony from a petri dish in 10 mL of LB medium containing the desired antibiotic.
2. Incubate at 37^{o}C and 180 rpm until the OD600 reaches 0.4 to 0.8 in an inFORS minitron.
3. Induce the protein expression with IPTG to a final concentration of 0.1 to 0.5 mM depending on the characteristics of the expressed protein.
4. Incubate at 37^{o}C and 180 rpm for 3 to 5 hours.
5. Check for protein expression either by staining an SDS-PAGE gel, performing a Western Blot or checking for protein activity, or by mass spectrometry. Compare protein expression in both the total cell extract (soluble + insoluble) and the soluble fraction only.

For large scale protein expression, inoculate 800 mL of liquid LB medium containing the desired antibiotic with a freshly grown colony or 10 mL of fresh liquid preculture. The overnight preculture is first pelleted, washed with LB, twice, then resuspended in 10 mL LB. Then perform steps 2 to 5 using the optimal time/temperature determined in a small-scale trial.

V. Purification of His-tagged protein

Aim Purify the proteins from a bacterial sample containing our protein of interest with a His-tag (in our case : NGF)

Materials

- Buffer A (5% glycerol, 50 mM Tris, 200 mM NaCl)
- Buffer B (5% glycerol, 50 mM Tris, 200 mM NaCl, 250 mM imidazole)
- High speed centrifuge (Avanti J-26 XP, rotor JLA-8.100, Beckman Coulter)
- Peristaltic pump (P1 Pharmacia – GE Healthcare)
- Ni-NTA Superflow Cartridge 5 mL, (QIAGEN, 1034558

Procedure

1. Centrifuge 800 mL of induced bacterial culture at 6,238xG during 25min in a JLA-8.100 rotor and Avanti-J26 XP centrifuge.
2. Carefully take aside 500 mL of supernatant without taking any bacteria from the pellet.
3. Using the peristaltic pump at 2.5 mL/min, flow 500 mL of supernatant through a 5 mL Ni-NTA superlfow cartridge in a closed loop overnight.
4. Wash the column with 30 mL Buffer A.
5. Elute the fixed proteins with 10mL Buffer B in 10 different fractions of 1 mL.

VI. SDS-Page

Aim Separate proteins from a sample according to their molecular mass by electrophoresis (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis)

Materials

— Spatula
— Measuring cylinder
— NuPAGE^{TM} 4-12% Bis-Tris Gel (Invitrogen, NP0322BOX)
— Power Pac 300 (BIO-RAD)
— Mini Gel Tank (Invitrogen, A25977)
— NuPAGE® MOPS SDS Running Buffer 20X (Novex, NP0001)
— Protein sample
— Dry heater block at 90^{o}C
— 2X SDS-PAGE Sample Buffer (4% SDS, 20% Glycerol, 0.01% bromophenol blue)

Procedure

1. Prepare protein samples by mixing them with 2X SDS-PAGE Buffer (50% v/v).
2. Prepare 500 mL of 1X Running Buffer by diluting 25 mL of 20X Buffer in 475 mL of distilled water.
3. Install the precast gel on the mini gel tank.
4. Heat the sample at 90^{o}C for 5 minutes, place on ice.
5. Deposit up to 20 μL of protein in each well.
6. Run the gel using the Power Pac 300 for 10 min at 85V and then for 40 min at 125 V.

Microfluidics: membrane filters

iGEM Pasteur Paris team - Institut Pasteur

Axelos Etienne Dejoux Alice Dumont Claire Ehret Antoine
Fyrillas Andreas Ghaneme Aymen Guerassimoff Léa
Jaoui Samuel Picaud-Lucet Emma Porte Sarah
Redheuil Ellyn Richard Charlotte Starck Thomas
Thomas Florian Trang Kelly Madelénat Manon
Paillarès Éléa Naccache Jonathan Sachet Gabriela

2018

Introduction

Soon enough we realized that we would need something to confine the bacteria, so that it doesn't attack the neurons during our experiments, or escape the device in a real prosthesis system. The solution came as a nanoporous membrane, that would also be used as the conductive element in our system to transmit the neuron's impulses to an electrode. The goal here is to coat alumina oxide membranes with different types of conductive polymers.

Figure 1 – *White alumina oxide membranes before coating*

I. PEDOT :PSS coating

An aqueous solution of PEDOT :PSS can be prepared [1]. We decided to dip the membranes in this solution during the polymerization.

I.1. Materials

- EDOT (3,4-Ethylenedioxythiophene, Sigma-Aldrich, 483028-10G)
- PSS (Sodium 4-vinylbenzenesulfonate, Sigma-Aldrich, 94904-100G)
- Deionised water
- Sodium persulfate (Sigma-Aldrich, 216232-500G)
- Iron(III) sulfate hydrate (Sigma-Aldrich, F0638-250G)
- Alumina Oxide Membrane Filters, 0.2 micron pores, 13 mm (Sterlitech)
- Stripette (Corning Costar, 5 mL) + pipette filler

- Analytical balance (Mettler Toledo NewClassic MF ML204 /01)
- Magnetic stirrer with heating plate (yellowline MSH basic)
- Fume hood (Delagrave SA OPTIMUM 1500)
- Gloves (Kimtech purple nitrile)
- Forceps (Bochem art. 1013)
- Glass beaker (600 mL)
- Petri dish

I.2. Procedure

Step 1 : Pour 0.8 g EDOT, 2 g PSS and 208 mL water in the glass beaker.
Step 2 : Put the membranes in the solution.
Step 3 : Stir for 10 minutes.

Figure 2 – *Solution with membranes after 10 minutes of stirring*

Step 4 : Add 2 g of sodium persulfate and 0.015 g of iron(III) sulfate hydrate.
Step 5 : Stir for 24 hours.

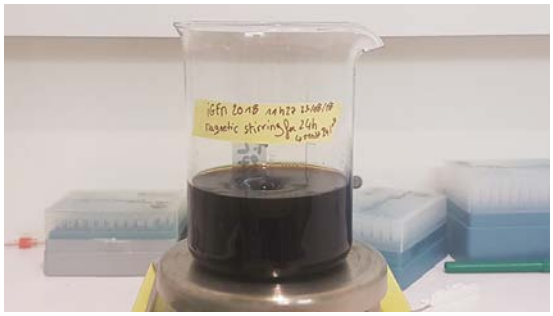

Figure 3 – *Solution after 24 hours of stirring*

Step 6 : Wash membranes with water and let them dry at room temperature in a Petri dish.

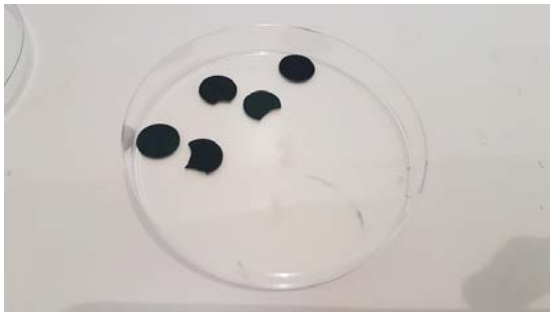

Figure 4 – *PEDOT :PSS coated membranes*

II. PEDOT :TS AND PEDOT :CL COATING

PEDOT :Ts and PEDOT :Cl polymers can be obtained by vapor phase polymerization on alumina oxide membranes [2].

II.1. Materials

- EDOT (3,4-Ethylenedioxythiophene, Sigma-Aldrich, 483028-10G)
- Iron(III) p-toluenesulfonate hexahydrate for PEDOT :Ts (Sigma-Aldrich, 462861-25G) or Iron(III) chloride for PEDOT :Cl (Fischer Scientific, 217091000)
- 1-butanol (Sigma-Aldrich, B7906-500ML)
- Deionised water
- Alumina Oxide Membrane Filters, 0.2 micron pores, 13 mm (Sterlitech)
- Paper masks (see figure 5)

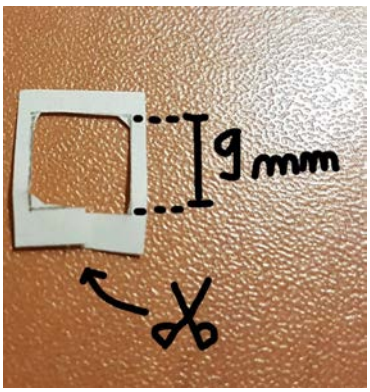

Figure 5 – *Paper mask*

- Stripette (Corning Costar, 5 mL) + pipette filler
- Analytical balance (Mettler Toledo NewClassic MF ML204 /01)
- Magnetic stirrer with heating plate (yellowline MSH basic)

- Fume hood (Delagrave SA OPTIMUM 1500)
- Gloves (Kimtech purple nitrile)
- Forceps (Bochem art. 1013)
- Glass beaker (600 mL and 50 mL)
- Petri dish

II.2. Procedure

Step 1 : Prepare homogenous oxidant solution.
- for PEDOT :Ts : 1.58 g Iron(III) p-toluenesulfonate hexahydrate and 10 mL butanol

Figure 6 – *Oxidant solution for PEDOT :Ts*

- for PEDOT :Cl : 1.35 g Iron(III) chloride and 10 mL butanol

Figure 7 – *Oxidant solution for PEDOT :Cl*

Step 2 : Dip membranes in oxydant solution.
Step 3 : Let membranes dry at 40°C.

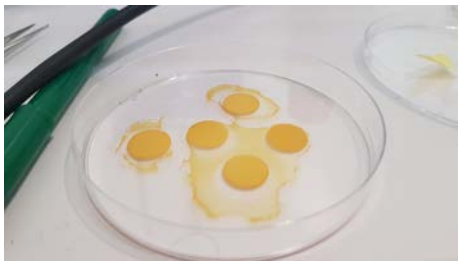

Figure 8 – *Membranes after being dipped in oxidant solution*

Step 4 : Place membranes in paper masks on Petri dish lids.

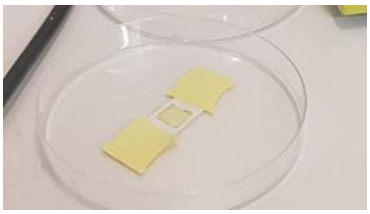

Figure 9 – *Membrane in paper mask*

Step 5 : Pour 200 μL EDOT in 50 mL beakers.
Step 6 : Place Petri dish lids on top of the 50 mL beakers, membranes facing the inside of the beakers.
Step 7 : Heat the beakers at 40°C and stop when membranes darken (takes about 6 minutes).

Figure 10 – *Vapor phase polymerization of PEDOT :Ts*

Step 8 : Wash membranes with butanol and water.
Step 9 : Let membranes dry at room temperature.

Figure 11 – *PEDOT :Ts coated membranes*

Figure 12 – *PEDOT :Cl coated membranes*

References

[1] Jikui Wang, Guofeng Cai, Xudong Zhu, Xiaping Zhou, *Oxidative Chemical Polymerization of 3,4-Ethylenedioxythiophene and its Applications in Antistatic coatings*, Journal of Applied Polymer Science, 2012, Vol. 124, 109-115 .

[2] Alexis E. Abelow, Kristin M. Persson, Edwin W.H. Jager, Magnus Berggren, Ilya Zharov, *Electroresponsive Nanoporous Membranes by Coating Anodized Alumina with Poly(3,4-ethylenedioxythiophene) and Polypyrrole*. 2014, 299, 190–197.

PDMS Well Chip

iGEM Pasteur Paris team - Institut Pasteur

Axelos Etienne Dejoux Alice Dumont Claire Ehret Antoine
Fyrillas Andreas Ghaneme Aymen Guerassimoff Léa
Jaoui Samuel Picaud-Lucet Emma Porte Sarah
Redheuil Ellyn Richard Charlotte Starck Thomas
Thomas Florian Trang Kelly Madelénat Manon
Paillarès Éléa Naccache Jonathan Sachet Gabriela

2018

Introduction

The well chip was designed and assembled by our team. It was used to test the biocompatibility of our membranes, as well as the culture of bacteria in the presence of current. Here we show how the molds were made, how the chip itself was assembled, how well's conductivity was measured and how biofilm culture was performed on it.

I. Molds

Molds were made of aluminium according to the following plans (Figure 1). Part 1 Mold's center cylinder part is detachable from the bottom to make the demolding ot PDMS easier.

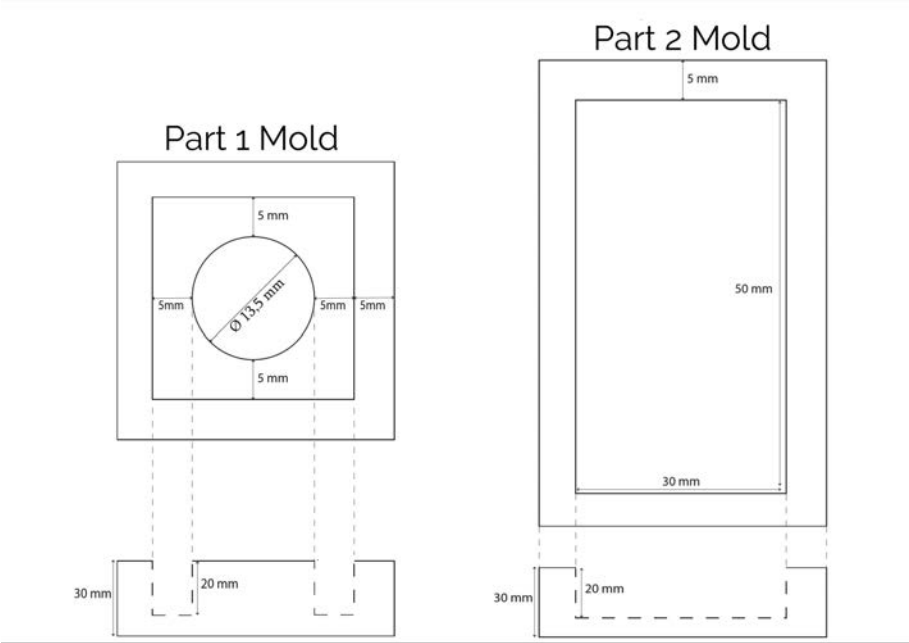

Figure 1 – *Mold plans*

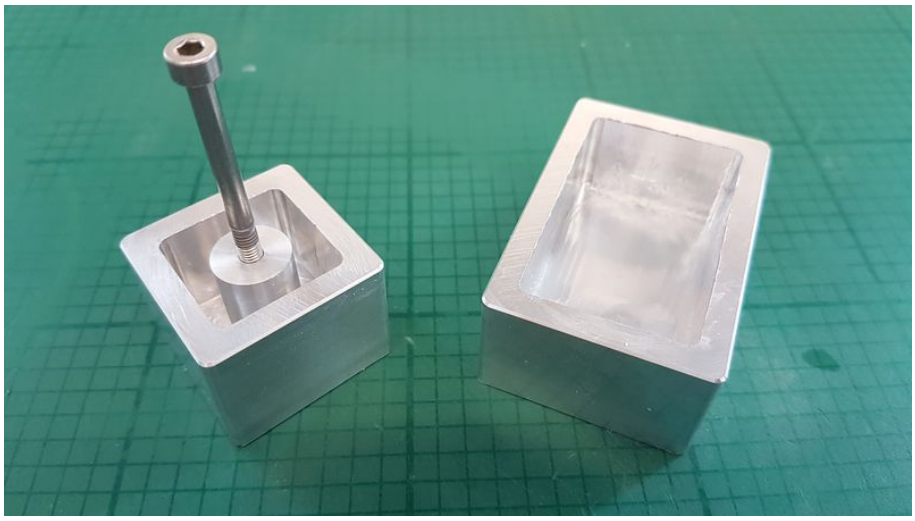

Figure 2 – *Molds*

II. Chip fabrication protocol

II.1. Materials

- Molds
- Syringe (Terumo syringe without needle, 10 mL)
- Platinum 24 mm x 2 mm strip (mechanically flattened 24mm long 0.7mm diameter platinum wire)
- Polycarbonate gold-coated membrane filters, 0.4 micron, 13mm diameter (Sterlitech) or polymerized membrane (see Microfluidics : Membranes, **link to protocol**)

Refer to sections 1 and 2 of Microfluidics : General Protocols (**Link**) for further needed materials.

II.2. Procedure

Step 1 : Prepare 20 g of PDMS monomer using section 1 of Microfluidics : General Protocols (**Link**). Replace step 5 by : Fill the syringe with PDMS. Fill part 1 mold until it's full and part 2 mold until the PDMS layer is more or less 1 cm thick. Keep the PDMS that is left.
Step 2 : Demold the chip following section 2 of Microfluidics : General Protocols (**Link**). Ignore step 2.

Step 3 : Put membrane and platinum strip on PDMS part 1. Refer to figure 2 for their position.

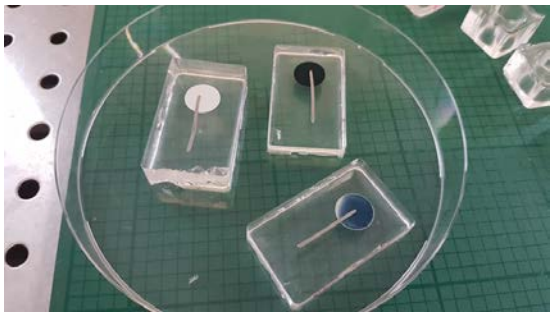

Figure 3 – *Multiple PDMS parts 1 with membrane and platinum strip*

Step 4 : Refer to section 3 of Microfluidics : General Protocols (**Link**) to bond PDMS part 2 to the PDMS part prepared in the previous step. It should look like figure 4.

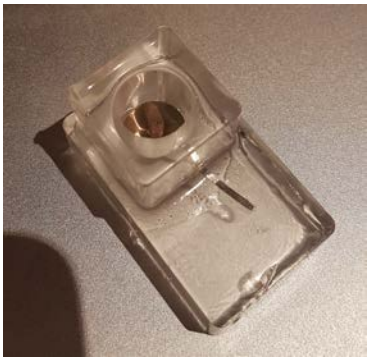

Figure 4 – *PDMS well chip*

Step 5 : Apply a small layer of PDMS with the syringe. Refer to figure 5 . This way, the well is watertight.

Figure 5 – *Apply PDMS on the red zone*

Step 6 : Put the chip in the stove for 3 hours.

III. Well conductivity measurement

III.1. Materials

- Oscilloscope (Tektronix TDS 2002C)
- Function generator (GW Instek SFG-2010)
- Solderless breadboard assembly (Twin Industries TW-E41-102B)
- 4 Electric wires with banana connectors
- 1 coaxial cable (RG58C/U BNC Plug Coaxial Cable Assemblies - 50 Ω Transmission Line with BNC Male Connectors)
- Male BNC to 2 female banana connectors converter (TNP BNC Male Plug to 2x 4mm Dual Banana Female Jack Socket Binding Post RF Coax Coaxial Splitter Connector Adapter Adaptor)
- BNC Splitter (BNC Male Connector to BNC Double Female (T-Shape) Adaptor)
- 1 kΩ resistor

III.2. Procedure

Reproduce the following electric circuit (figure 6 and 7).

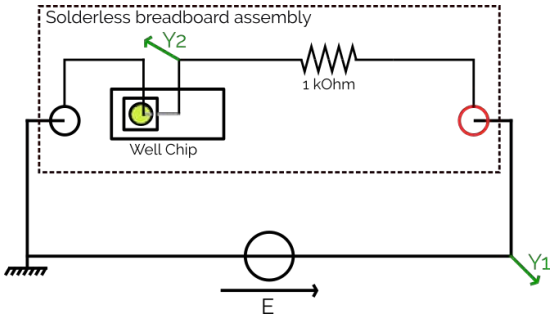

Figure 6 – *Electric circuit for well conductivity measurement, Y1 and Y2 being the oscilloscope inputs*

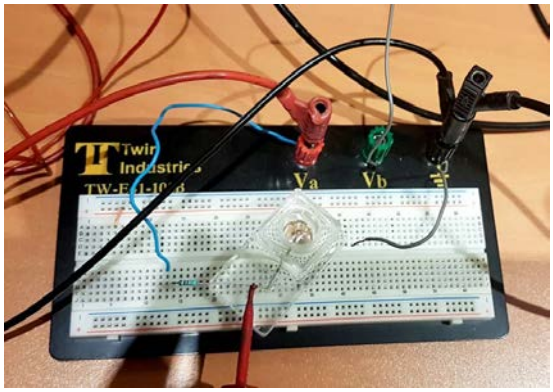

Figure 7 – *PDMS well chip on breadboard assembly*

IV. Biofilm assay

IV.1. Materials

- BL21 liquid culture, see Molecular Biology : DNA Assembly and Microbiology (**Link**)
- PDMS well chip
- Crystal violet (Thermofisher, 0.1 % in water)
- Distilled water
- Acetone
- Ethanol 96%
- P1000, P200, P20 (Gilson) + tips
- Gloves (Kimtech PFE)
- Biochrom WPA CO8000 Cell density meter
- glass jar of bleach
- plastic jar
- Falcon tube 15 mL

IV.2. Procedure

According to Dr Jean-Marc Ghigo.

IV.2.1 Biofilm formation

Step 1 : Pour 600 μL of liquid culture in the well.
Step 2 : Incubate well at 37 degrees Celsius for 24 hours.

IV.2.2 Well wash

Step 1 : Discard the supernatant in microbiological waste bin. Do not pipet.
Step 2 : Immerse well in the plastic jar with distilled water (let the water softly enter the wells).

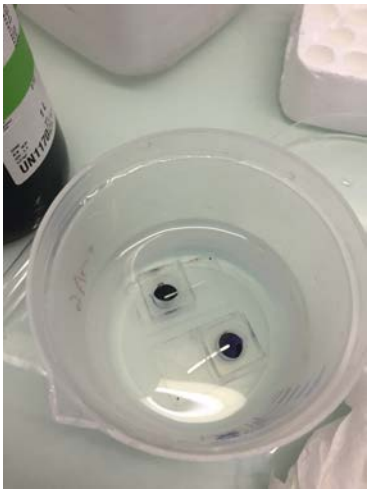

Figure 8 – *Immersed well*

Step 3 : Take the well out of the water and discard water sharply over the waste container.
Step 4 : Repeat this operation twice.
Step 5 : Bang on blotter paper to eliminate residual water.

IV.2.3 Crystal violet staining

Step 1 : Add 125 μL Crystal violet in the emptied well.
Step 2 : Wait 15 minutes for staining.

Figure 9 – *Wells with crystal violet*

Step 3 : Wash 3 times with distilled water as described before.
Step 4 : Bang on blotter paper to eliminate residual water.
Step 5 : Suspend colored biofilm by adding 150 μL ethanol/acetone solution (80 :20).
Step 6 : Transfer 50 μL of the solution in a falcon tube and add 1.5 mL of ethanol/acetone solution (80 :20).

Figure 10 – *Solution ready for optical density measure*

Step 7 : Read optical density of 1 mL of the falcon tube's solution at 600 nm.

Microfluidics: microchannel chip

iGEM Pasteur Paris team - Institut Pasteur

Axelos Etienne Dejoux Alice Dumont Claire Ehret Antoine
Fyrillas Andreas Ghaneme Aymen Guerassimoff Léa
Jaoui Samuel Picaud-Lucet Emma Porte Sarah
Redheuil Ellyn Richard Charlotte Starck Thomas
Thomas Florian Trang Kelly Madelénat Manon
Paillarès Éléa Naccache Jonathan Sachet Gabriela

2018

Introduction

We used the microchannel chip to test the effect of NGF on neuron's growth. Institut Curie allowed us to use their chip design and their molds for our experiments. We then proceeded to enhance the chip with a few customizations. We integrated nanoporous membrane in the chip to prevent our bacteria to come in contact with the neurons.

I. Molds

We were not involved in the process of mold fabrication. We made a short animation to explain the different steps (**Link**).

II. Basic Microchannel Chip Fabrication

II.1. Materials

- Molds

Refer to sections 1 and 2 of Microfluidics : General Protocols (**Link**) for further materials.

II.2. Procedure

Step 1 : Prepare 80 g of PDMS monomer using section 1 of Microfluidics : General Protocols (**Link**).
Step 2 : Demold the chip following section 2 of Microfluidics : General Protocols (**Link**).

III. Membrane Microchannel Chip Fabrication

The goal here is to insert a membrane in one of the chambers of the microfluidic chip in order to isolate the neuron's chamber from the bacteria's one.

III.1. Materials

- Basic microchannel chip
- Polycarbonate gold-coated membrane filters, 0.4 micron, 13 mm diameter (Sterlitech)
- Razor blade (OEMTOOLS 25181 Razor Blades, 100 Pack)

- Scissors
- Forceps

III.2. Procedure

Step 1 : Make a cut in the basic microchannel chip with a razor blade (see figure below). Do not cut the chip in half!

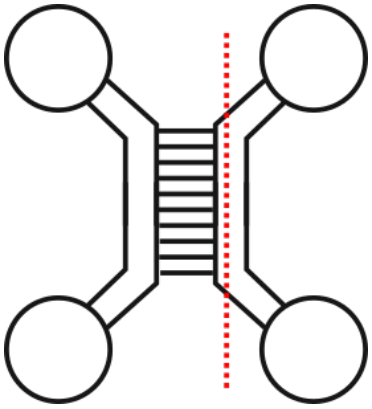

Figure 1 – *Cut along the red line*

Step 2 : Stretch the cut and insert the membrane using the forceps. Cut with a razor blade the exceeding part of the membrane.

Figure 2 – *Cut microfluidic chip with membrane inserted*

IV. Microchannel Chip Bonding

IV.1. Materials

- Basic or membrane microchannel chip
- Distilled water
- Imaging Dish (Ibidi μ-dish 35 mm, high glass bottom)
- Fridge

Refer to section 3 of Microfluidics : General Protocols (**Link**) for further materials.

IV.2. Procedure

Step 1 : Bond microfluidic chip to the bottom of an imaging dish using section 3 of Microfluidics : General Protocols (**Link**).
Step 2 : Fill the chip with distilled water. If water leaks out of the chip, unstick it from the imaging dish and retry step 1.
Step 3 : Store in fridge.

V. Double Membrane Microchannel Chip

Here we have to insert a membrane underneath the neuron's chamber of a membrane microchannel chip, in order to be able to expose the neurons to current.

V.1. Materials

- Membrane microchannel chip
- Distilled water
- Imaging Dish (Ibidi μ-dish 35 mm, high glass bottom)
- Syringe (Terumo syringe without needle, 10 mL)
- Conductive silver paste (MG Chemicals 8330S-21G)
- Wooden toothpick
- Petri dish
- Stove
- Fridge

Refer to section 1 and 3 of Microfluidics : General Protocols (**Link**) for further materials.

V.2. Procedure

Step 1 : Prepare 5g of PDMS following section 1 of Microfluidics : General Protocols (**Link**).
Step 2 : Prepare 1 mL of conductive silver paste following the manufacturer's instructions (Preheat parts A and B in stove at 70 degrees Celsius, put 0.5 mL of part A and 0.5 mL part B in a Petri dish, mix with the toothpick).
Step 3 : See figure below for more information about the position of each element. Deposit a small layer of conductive silver paste on the border of the bottom of the imaging dish. Stick a piece of gold membrane cut with the scissors in the silver paste. Put 10 minutes in stove at 70 degrees Celsius. Put another small layer of silver paste on top of the previous one.
Step 4 : Bond membrane microchannel chip to the bottom of the imaging dish following section 3 of Microfluidics : General Protocols (**Link**). The extremity of the gold membrane piece has to be in one of the holes of the neuron's chamber (see figure 3).
Step 5 : Deposit a small layer of PDMS on the side where the membrane sticks out of the chip. Wait 2 minutes. Fill chip with distilled water. If water leaks out, unstick the chip, discard imaging dish and restart from step 3.
Step 6 : Store in fridge.

VI. Chip Sterilization

Unwanted living organisms in microfluidic chips can be a big deal, especially when these chips have to stay for 3 days filled with cultur medium in an incubator. The chips need to be exposed to UV rays in order to eliminate these unwanted organisms. We took extra securitiy measures, because we also needed to transport our chips from Institut Curie's lab at IPGG to Institut Pasteur.

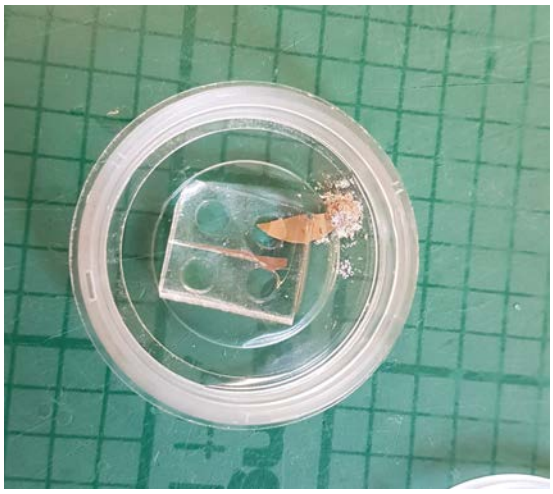

Figure 3 – *Bonded double membrane microchannel chip*

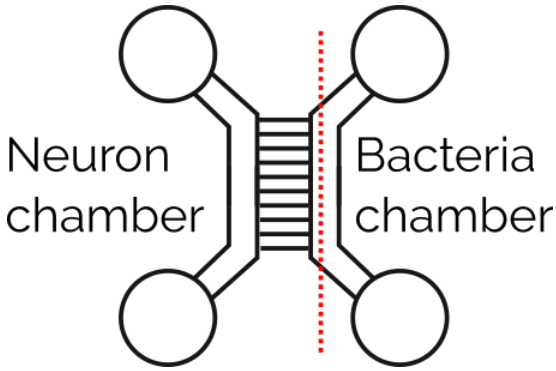

Figure 4 – *Neuron and bacteria chambers*

VI.1. Materials

- Bonded (to imaging dish) and water-filled microchannel chips
- Big Petri sish (150 mm diameter)
- Gloves (Kimtech PFE)
- UV curing unit (DWS)
- Wrapfilm for food use (Ecopla France film pro)
- Parafilm (Bemis parafilm "M")
- Fridge

VI.2. Procedure

Step 1 : Open imaging dishes containing bonded microchannel chips and put them in the UV curing unit with their corresponding lid.

Figure 5 – *Imaging dishes and their lid in UV curing unit*

Step 2 : Expose to UV rays for 20 minutes.
Step 3 : With gloves, put exposed dishes in a big Petri dish.
Step 4 : Seal Petri dish with parafilm.
Step 5 : Cover Petri dish with 3 layers of wrapfilm.
Step 6 : Expose 15 minutes to UV rays.
Step 7 : Cover Petri dish with 2 additional layers of wrapfilm.
Step 8 : Store in fridge.

Cortical Cell Culture on Microfluidic Chip

iGEM Pasteur Paris team - Institut Pasteur

Axelos Etienne Dejoux Alice Dumont Claire Ehret Antoine
Fyrillas Andreas Ghaneme Aymen Guerassimoff Léa
Jaoui Samuel Picaud-Lucet Emma Porte Sarah
Redheuil Ellyn Richard Charlotte Starck Thomas
Thomas Florian Trang Kelly Madelénat Manon
Paillarès Éléa Naccache Jonathan Sachet Gabriela

2018

I. PDMS Chip Coating

I.1. Materials

- PDMS microchannel chip, see Microfluidics : microchannel chip (**Link**)
- Pipettes + tips
- Gloves
- Falcon tubes
- Eppendorf tubes
- Automated aspiration system
- Incubator
- UV box
- Poly-L-lysine solution (PLL) (Sigma-Aldrich, ref : P8920-100ML)
- Laminin form engelbreth-holm-swarm murin (Sigma-Aldrich, ref : L2020-1MG)
- Phosphate buffered saline tablet (PBS) (Sigma-Aldrich, ref : P4417-50TAB)

I.2. Procedure

After sterilization under UV, see section 6 of Microfluidics : microchannel chip (**Link**), manipulations until the fixations are realized in sterile conditions.

Drain water with aspiration system (Figure 1).

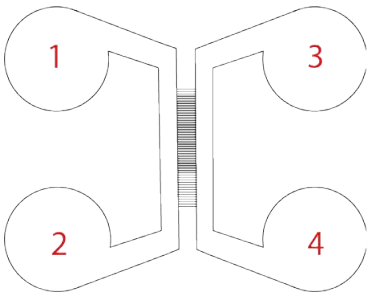

Figure 1 – *Order without creation of flow*

Add 50 μL of 10 μg/mL Poly-L-Lysine (PLL) into the top wells and allow the flow through (Figure 2a). After 5 minutes, fill the bottom wells (Figure 2b). Place the device in an incubator overnight at 37°C.

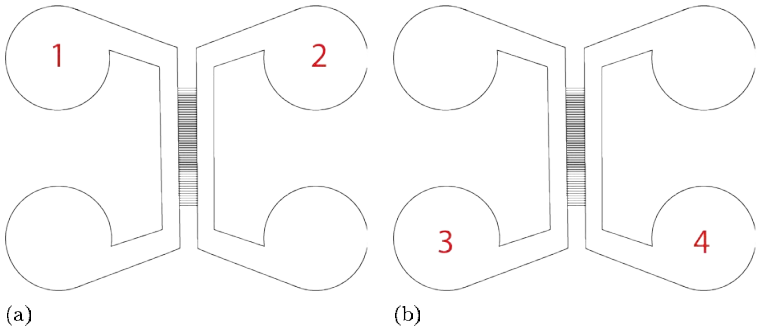

Figure 2 – *Order to create a flow*

Drain the device with aspiration system (Figure 1), and rinse with 30 μL of PBS 1X to wash out the excess fluid of PLL. Add 20 μL of 1mg/mL laminin into the top wells only (Figure 2a). Place the device containing laminin in an incubator at 37°C for a minimum of 2 hours.

II. Enzymatic digestion

II.1. Materials

- Bunsen burner
- Sterile Eppendorf tubes
- Sterile 15 mL Falcon tube
- Pipettes + tips
- Centrifuge
- E18 rat cortex (2 pieces of tissue) stored in Hibernate EB (BrainBits)
- Sterile papain (BrainBits PAP 6mg)
- Hibernate E-Ca (BrainBits HE-Ca 5mL)
- Sterilized Pasteur pipette (BrainBits FPP)
- Appropriate cell culture medium

Reagents used for the different cell culture mediums :
FBS,HI,Qualified, USA origin (Fisher Scientific, ref : 11570516)
HBSS,W/OCA,MG,PH red 1X (Fisher Scientific, ref : 11530476)
Glutamax 1 100X (Fisher Scientific, ref : 11574466)
B27 supplement 50X (Fisher Scientific, ref : 11530536)
Neurobasal MED SFM (Fisher Scientific, ref : 11570556)
Dulbeccos modified eagles medium 1X (DMEM) (Fisher Scientific, ref : 21969-035-500ML)

II.2. Procedure

According to BrainBits protocol (**Link**).
Prepare cell dissociation solution by dissolving 6 mg sterile papain in 3mL of Hibernate E-Ca for a final working concentration of 2 mg/mL papain. Incubate for 10 minutes at 30°C. Then, carefully transfer and save Hibernate EB solution to a sterile tube leaving the tissue with minimal Hibernate EB volume. Add 2 mL of cell dissociation solution to the tissue and incubate for 10 minutes at 30°C, gently swirl every 5 minutes. Then, carefully remove cell dissociation solution and return the saved Hibernate EB medium. Fire polish the tip of a sterilized Pasteur pipette to an opening of about 5 mm. With this pipette, triturate tissue for about 1 minute and let undispersed pieces settle for 1 minute. Then, transfer supernatant containing dispersed cells to a sterile 15mL, leave about 50 μL of Hibernate EB solution containing debris. Spin 1100 rpm for 1 minute, and discard supernatant leaving 50 μL of Hibernate EB solution containing the pellet. Resuspend pellet in 1mL of appropriate cell culture medium.

III. Cell counting

III.1. Material

- Sterile Eppendorf tubes
- Neubauer chamber
- Light microscope
- Pipettes + tips
- Trypan blue solution cell culture (Sigma-Aldrich, ref : T8154-20ML)

III.2. Procedure

Aliquot 10 μL of cell solution into the tube containing 10 μL of Trypan Blue. Place 10 μL of the suspension in the Neubauer chamber. Count all the cells that are not colored in blue in the center square of the chamber, about 0.1 μL. The cellular concentration is determined by the total number of cells in 0.1 μL multiply by 10,000. Then, multiply the result by the dilution realized with the Trypan Blue.

IV. Seeding neurons into the microfluidic chip

IV.1. Material

- Prepared tissues
- Appropriate cell culture medium
- Microfluidic chips coated with PLL, products of section 1
- Pipettes + tips
- Automated aspiration system
- Incubator 37°C, 5% CO_2

- Ethylenediaminetetraacetic acid solution (EDTA) (Sigma-Aldrich, ref : 03690-100ML)

IV.2. Procedure

Drain the laminin with aspiration system (Figure 1). Dilute cells with appropriate cell culture medium and plate 1.7 μL at 40,000 cells/cm^2 (top wells only) (Figure 2a). For the microfluidic chip seeding, tilt the tip in the direction of the chamber in order to help the migration of the neurons. Incubate for 5 minutes at room temperature, and add 50 μL of appropriate cell culture medium at 37°C (Figure 1). The culture medium needs to be prepare at least 1 hour before. Add about 1 mL of EDTA around the chip to avoid contamination and prevent evaporation. Then, incubate the device at 37°C with 5% CO_2. Change half the cell culture medium every 3-4 days, use a P1000 pipette and not the aspiration system.

V. Fixation and staining of neurons

V.1. Material

- Microfluidic chips seeded with neurons, products of section 4
- Automated aspiration system
- Pipettes + tips
- Medium B (32% paraformaldehyde solution (PFA) (Bio-Rad, ref : 15714S) + 4% sucrose)
- Medium C (PBS + 0.5% triton X100 (Sigma-Aldrich, ref : T9284-100ML))
- Medium D (PBS + 1% bovine serum albumin solution (BSA) (Sigma-Aldrich, ref : A7979-50ML))
- Anti-MAP2, clone AP20, Alexa Fluor (Merck, ref : MAB3418A5)
- Anti-β-tubulin antibody coupled with Alexa Fluor 448nm
- Phosphate buffered saline tablet (PBS) (Sigma-Aldrich, ref : P4417-50TAB)

V.2. Procedure

After 6-7 days of incubation, remove the medium with a P1000 pipette (Figure 1) and fixate cells (Figure 3) in 50 μL of fresh medium B (4% PFA) for 30 minutes at room temperature.

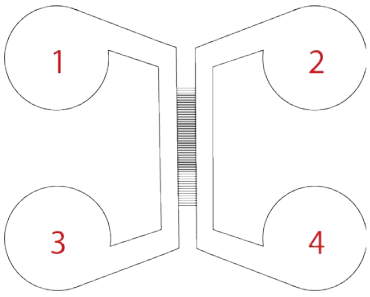

Figure 3 – *Order to fixate the cells*

Then, drain with the aspiration system (Figure 1), and wash with 50 μL of 1X PBS for 5 minutes (Figure 2a) x2 and replace the EDTA around the chip by 1mL of 1X PBS. Drain with the aspiration system (Figure 1), and permeabilize in 50 μL of medium C for 10 minutes at room temperature. Then, drain with the aspiration system (Figure 1), and block with 50 μL of solution D for 30 minutes at room temperature. Prepare in 1X PBS, 500X anti MAP2 antibody coupled with Alexa Fluor 555nm and 500X anti β-tubulin antibody coupled with Alexa Fluor 448 nm. Drain

with the aspiration system (Figure 1), and drop 20 μL of the suspension on the top wells (Figure 2a) and incubate 1 hour at room temperature in the dark. Then, drain with the aspiration system (Figure 1), and wash with 1X PBS and store at 4°C fluorescent microscopic analysis.