CLEANING COVERSLIPS FOR NEURON PLATING (from J. Wesseling) PLUS SUGGESTIONS FOR SUBTRATES

Note: Use coverslips from Fisher and do everything using glass material. The cleaning procedure can be substituted by rinsing in 100% ethanol for 2 days, and then go to step 10.

1- Sonicate coverlips in PURE nitric acid (place them in a crystal beaker filled with acid, and inside the sonicator in the hood) for 1 hour
2- Pour nitric acid carefully into another beaker. Discard it by diluting with lots of water and pouring it down the sink
3- Wash 2-3x with MilliQ water (first wash in the hood) to get rid of the acid
4- Sonicate in 100% hydrochloric acid for 1 hour (hood)
5- Rinse about 10x with MilliQ water
6- Sonicate in MilliQ water for 1 hour
7- Rinse 5-10x with MilliQ water
8- Sonicate in 100% ethanol for 1 hour
9- Replace with fresh 100% ethanol. You can keep them for around 2 weeks if you are careful not to let them dry.
10- Place coverslips in plates and let them dry just before coating (about 30 min)
11- Add substrate.

NOTE: Different laboratories use different substrates to support neuron growth.

I cover generously with poly-D-Lys (1 mg/ml Beckton-Dickinson, cat#354210), and leave it o/n. Just before plating rinse 3-4 x with sterile water.

John uses poly-D-Lys (1 mg/ml) + collagen (2 mg/ml). Mix 500 µl water, 20 µl lysine solution and 500µl collagen solution. Add 7 µl per coverslip and distribute evenly by pipetting up and down.

M. Morales and Juan Lerma, and myself on occasions= Coat is Poly-D-lysine and laminin

SUBSTRATE NOTES:

Poly-D- and poly-L-lysine are synthetic molecules used to promote cell adhesion (MW=500-550 kDa. They are positively charged polymers that bind negative charges in glass/plastic surface. PDL is usually preferred because it is not broken down by proteases released by cells in culture.

To aliquot: Allow PDL bottle to equilibrate at RT (Sigma, cat#P0899 or cat#BD, 354210). Dissolve it in water and filter it through 0.22µm. (It should dissolve pretty fast in water, just mix it well. Sometimes I let it sit for 30 min)

We most often do embryonic cultures from E19 fetuses (but works for postnatal cultures, up to P3). An excellent reference book with all kind of details on how to prepare hippocampal neurons: Banker and Gosslin, Culturing nerve cells, 2nd edition.

Note: Our routine is to mate the rat on Thursday (E0), in such a way that we receive it in CIMA at E14 (with pregnancy test done). The culture is then done next Tuesday (E19).

Preparation of coverslips or culture plates

- **If neurons are to be plated on PDL coverslips (Imaging experiments):**

  Day1- Place clean coverslips in a parafilm coated Petri dish, let dry and UV for 5 min

  Coat with a 1 mg/ml PDL solution o/n or for a few hours. (In my hands it is important to defrost the PDL 1-2 h in advance)

  Rinse twice with sterile water

- **For high density cultures in 60 mm dishes (Biochemistry):**

  Day1- Coat with a 100 µg/ml PDL solution o/n (1:10 dilution of Sigma PDL 1mg/ml, cat #0899)

  Day2- Wash twice with water to remove excess PDL

  Add laminin solution (1 µg/ml in Hank's media) for a few hours to o/n

  Rinse once with Hank's and keep in Neurobasal medium till plating

- If neurons are to be plated on PDL + laminin coverslips:

  Day1- Place clean coverslips in 24-well or 12-well plates, let dry

  Coat with a 100 µg/ml PDL solution o/n (around 300 µl)

  Day2- Wash twice with water to remove excess PDL

  Add laminin solution (5 µg/ml in Hank's media) for a few hours to o/n

  Rinse once with Hank's and equilibrate in Neurobasal medium till plating

Note: The procedure to clean the coverlips is crucial, they must be acid-cleaned by sonication and kept under 100% ethanol at all times.

Sterilize all instruments by submerging them in Ethanol 70 %.

Sterile surgical scissors, big and small
Scalpel
Dumont #5 forceps

- 30 minutes before dissection, prepare enzyme solution
    - 10 ml of Hanks Plus or digestion solution (Chi's recipe)
    - 200 μl Papain (1000 u/ml, cat 3126, Worthington Biochemical Corp)
- Heat at 37 °C for 10-30 min and Sterifilter (0.2 μm) once papain is dissolved
- Put 10 ml of dissection medium in 3 small Petri dishes

Dissection procedure

- Decapitate a P0 rat pup using a pair of sterile surgical scissors (1 hippocampus is OK for 1 12-wells-dish at 30-70k density).
- Place head on a paper towel and rinse it with Ethanol 70%.
- Cut head skin (scalp) and skull along the midline using a pair of small scissors.
- Remove skull bones using fine curved forceps to expose the brain.
- Using a scalpel cut between brain and cerebellum.
- Remove brain into a Petri dish containing Hanks Plus (or dissection medium).
- Make a diagonal cut between the cortex and mesencephalon using a scalpel.
- Transfer both brain hemispheres to a small Petri dish containing 10 ml Hanks Plus (or dissection medium).
- Carefully, remove meninges using fine Dumont forceps, and separate hippocampus from cortex
- Using a pair of sharp needles separate hippocampi from Dentate gyrus and transfer tissue to a Petri dish containing 10 ml Hanks Plus (or dissection medium).

Preparation of cell suspension

- Cut hippocampi in small pieces using a pair of small spring scissors.

- Transfer the pieces of tissue to a 15 ml disposable centrifuge tube containing 10 ml of papain solution

- Incubate at 37 °C for 30 minutes with occasional gentle shaking. Add DNAseI (500 ul of sterile 1 mg/ml solution per 10ml) after 15-20 min incubation to break DNA strands released by digestion

- Take tube out of the incubator, let it stand upright and allow the tissue pieces to settle.

- Discard the enzyme solution and wash tissue pieces TWICE with 4 ml N27+5%FBS to neutralize remaining enzyme.

- Aspirate last wash and add 4 ml of N27+FBS

- Dissociate small pieces of tissue into a single cell suspension using a sterile cotton-plugged fire polished pipette or a 5 ml transfer pipette.

- Press the pipette tip against the bottom of the tube and flush tissue and solution back and forth (~7 times) or until no small pieces of tissue are left. Be careful not to overdo it or you'll get a lot of debris.

- Spin to pellet cells at 1,000 rpm for 5 min (to get rid of some debris), and resuspend pellet in 8 mls of N27+FBS. This step is key if you culture cortical neurons form postnatal brain.

Counting and Plating Cells

- Place 10 μl of cell suspension into a hematocytometer and count total number of cells in 16 squares. Multiply that by 10,000 to obtain the number of cells/ ml

- Plate the desired number of cells onto coated- coverslips

  Note: I use 500 μl of media for 24-well plates and 2 ml for 12-well plates. A high-density hippocampal culture would be around 100k/each well in 12-well plate. Lower density cultures of up to 25k grow well w/o need to plate on previously grown astrocyte layers.

- Add 10 μM FUDR (2 μl of FUDR stock in 2ml media) to cultures 3-7 days after plating to suppress the proliferation of non-neuronal cells. For densities between 25-100k, I usually check to make sure astrocytes had proliferated some (w/o taking over the neuronal culture) before adding the FUDR

- Feed once-twice a week by replacing 1/3 of the media with N27 (serum free in this case). I usually keep adding FUDR (1:1000 from stock) to the feeding medium to block glial proliferation. It is a double-edge sword, since glia helps neurons grow healthier.

Hanks Plus

500 ml Hank's (or HBSS) +	10 mM HEPES (5 ml 1M pH 7.2-7.5 stock, Invitrogen)
pH 7.2-7.4	33.3 mM glucose (16.65 ml 1M stock)- *we SKIP this, HBSS has already some glucose*
	5 µg/ml gentamycin (1:10,000 dilution of 50 mg/ml gentamycin= 50 µl, Invitrogen)

Dissection medium (To simplify, dissection can be done in Hank´s Plus!!!)

500 ml Hanks Plus	+ 0.3% BSA (20 ml 7.5%BSA stock, Invitrogen)
	+12 mM $MgSO_4$ (6 ml of 1M stock)

Culture medium (N27)

500 ml Neurobasal medium (Gibco)	+	10 ml B27 supplement (Invitrogen)
		5 ml Glutamax I
		1 µg/ml gentamycin (10µl of 50 mg/ml stock)

Plating medium (N27+5% FBS)

250 ml N27 + 12.5 ml FBS

Digestion solution (Instead, we use papain in Hank´s plus, see recipe above)

$NaHCO_3$ (4.2 mM)	0.176g	in 500 ml
HEPES (25 mM)	2.98g	
NaCl (137 mM)	4 g	
KCl (5 mM)	0.1864g	
Na_2HPO_4 (7 mM)	1.25g	

Adjust pH to 7.4, and filter sterilize.

Reagents

FBS, defined	Hyclone, catalog# SH30070.03 (I´d try to get it directly from Belgium)
Hanks' buffer powder	Sigma, catalog # H-2387. Dissolve powder, add 0.35 g sodium bicarbonate, filter and make sure pH is around 7.2-7.3 before filtering. Check pH after filtering.
DNase I	Sigma, catalog# D-5025. Dissolve the powder in Hanks' buffer at 1mg/ml, and filter it through 0.2um filter before aliquoting.
FUDR stock solution	5-fluoro-2'-deoxyuridine (Sigma, catalog# F-0503) 10mM and uridine (Sigma, catalog# U-3003) 10mM in ddH2O. Filter it through 0.2um filter.
P-D-Lysine (BD #354210, MW 500-550,000kDa)	To reconstitute, equilibrate vial to room temperature. Resuspend in sterile distilled water, let stay for 5-10 min, swirl or vortex (not too strongly) to dissolve. Make 1 ml aliquots and keep at -20/-80ºC.
Laminin (BD #354232)	Thaw laminin slowly at 4ºC (can leave 24h in fridge). Add Hank´s (HBSS) to a final concentration of 500 µg/µl. Make 60 µl aliquots and keep at -80ºC.