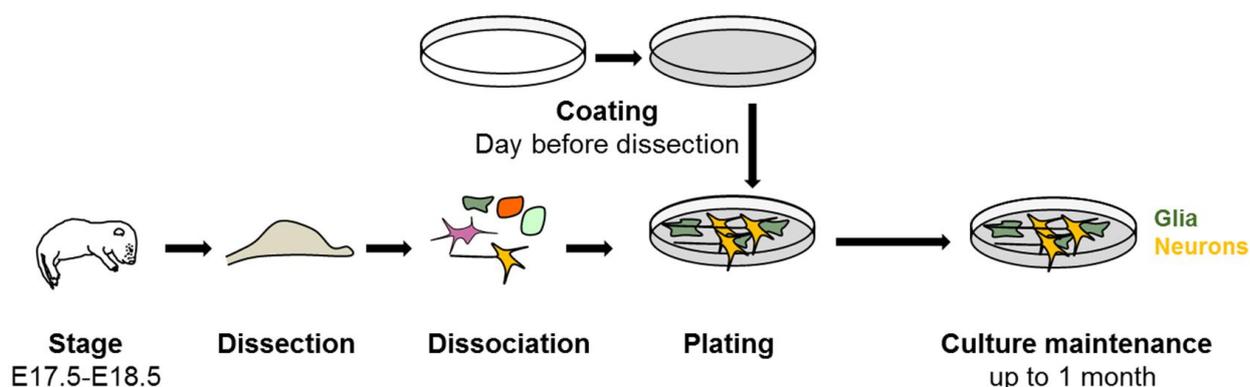




I Introduction

Herein I present a protocol to culture mainly pyramidal neurons in a mixed culture with glia from embryonic (E17 or E18) mouse hippocampus and cortex for up to 4 weeks on Poly-DL-ornithine hydrobromide coated glass coverslips without additional astroglial feeder cell layers. The serum-free neuron maintenance medium does not conduce to the growth and survival of astrocytes [1]. Dissociated neuron cultures have been studied for more than 40 years now and *in vitro* neuron development is well-characterized [2-4]. The cultured neurons express neuronal markers, develop extensive axonal and dendritic arbors, establish spines and form functional synaptic connections [5]. Thus, dissociated neuron cultures became a model to investigate the cellular and molecular mechanisms underlying postnatal neuron development such as neuron polarity formation [6, 7], dendritic outgrowth [8], axon guidance [9] and synaptogenesis [5] in the CNS. This *in vitro* model allows more controlled manipulations than *in vivo* models and is suited for various applications including immunocytochemistry, biochemistry, electrophysiology, Calcium and Sodium imaging and protein and RNA isolation. I use this culture model to study the maturation of the extracellular matrix and its relation to synaptogenesis and receptor recruitment to synapses. The neuron culture is a sequence of the following processes: coverslip coating, dissection, dissociation, cell plating and culture maintenance with feeding.



II Protocol

1. Coating: The day before dissection

- Work in a sterile laminar hood. Transfer glass coverslips to the 12-well tissue culture plate. You can e.g. pick them up with sterile forceps or with a flat Pasteur pipette connected to a vacuum pump and release them into the well by pinching the connection tube to cut off the vacuum.
- Poly-DL-ornithine hydrobromide (Sigma-Aldrich, P8638) stock solution (PO) is prepared in sterile water (1 mg/mL) and stored in aliquots at -20°C. The day before a culture is started, the stock solution is thawed at 37°C and diluted to a final concentration of 80 µg/mL in sterile water.
- For one culture, usually four 12-well plates are prepared, thus 50 mL PO working solution is prepared of which 1 mL is added to each well.
- Press the coverslips down to the bottom of the wells to remove bubbles and prevent them from floating.
- Incubate the culture plate at 37°C in the cell culture incubator overnight.

2. Dissection: Day *in vitro* 0

- Non-guaranteed pregnant female mouse, E17.5 or E18.5* which is delivered 5 days before starting the culture to ensure the female is not stressed at the time of dissection. *E0.5 is noon after the night of the mating.
- Aspirate carefully the PO working solution from the 12-well culture plates without touching the coverslips and let dishes dry in the laminar flow cell culture hood while using UV-light for 15 minutes to sterilize the dissection tools and equipment. Afterwards place the dry 12-well plates back in the 37°C incubator until plating the cells, but preferably not longer than 24 hours.
- Prepare the dissection tools and the dissecting microscope with illumination in the laminar flow cell culture hood, clean everything with 70% ethanol and use the UV light to sterilize the set-up.
- Prepare 100 mm Petri dish with ice-cold HBSS-Glc.
- Sacrifice pregnant mouse at 17 to 18 days gestation with vaporized isoflurane in a sealed jar and subsequent cervical elongation. Add the isoflurane around 20-30 minutes before usage to ensure that it can vaporize completely and add a tissue into the jar to additionally protect the female to accidentally get in direct contact with fluid isoflurane which is irritating.
- Disinfect fur with 70% (vol/vol) ethanol.
- Remove the uterus and put it in a 100 mm Petri dish.
- Remove pups from their embryonic sac, decapitate them and transfer their heads to another 100 mm Petri dish with ice-cold HBSS-Glc. Keep heads and brains always in ice-cold HBSS-Glc.
- Transfer heads, one by one, to a 35 mm Petri dish with around 1 mL ice-cold HBSS-Glc. Pay attention not to make too much medium into the dish to prevent floating of the head.
- It is important to dissect as fast as possible to ensure cell viability and health. Avoid to dissect longer than 60 minutes after decapitating the pups, as culture quality will enormously decrease.
- Hold the head down by curved forceps that you place into the eyes.
- Make a midline incision at the skin surface close to the hindbrain region and gently pull and tease apart the skin on the top of the head with a second pair of curved forceps (Fig. 1).

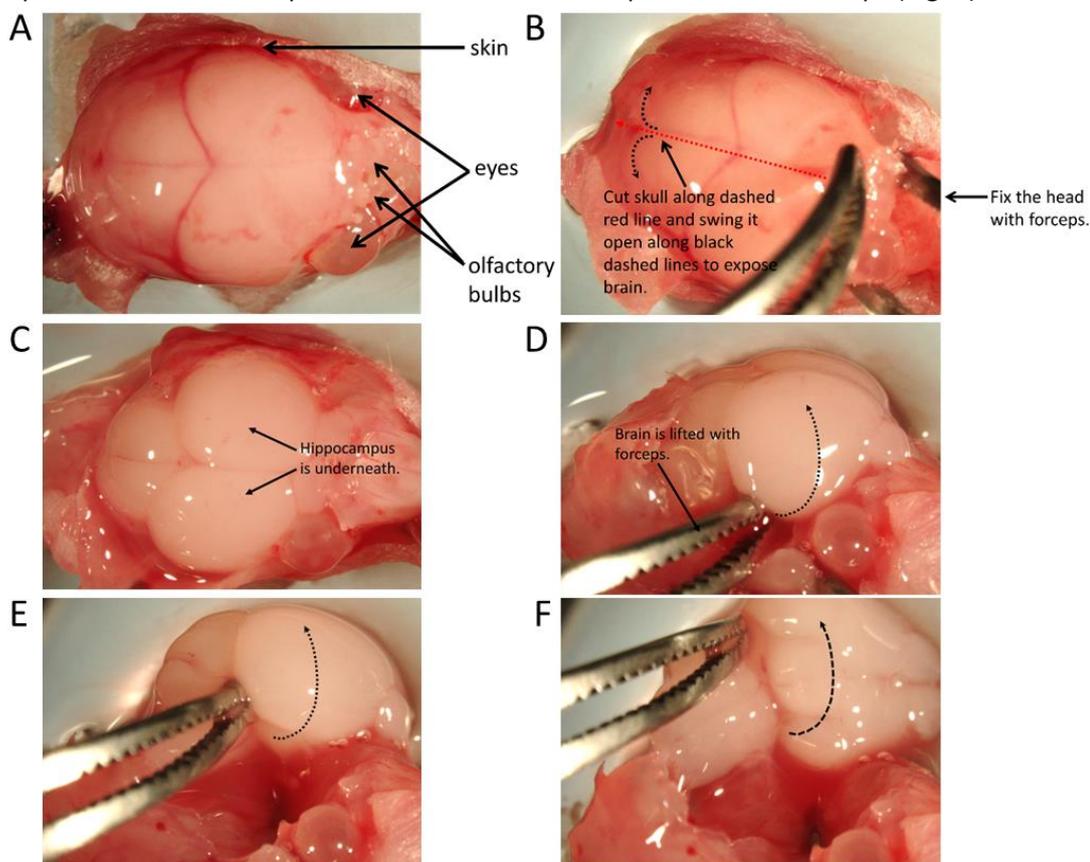


Figure 1: Removal of skin and skull. (A) Top view of opened E18 mouse embryo head. **(B)** Cut skull along red line to expose brain **(C).** **(D-F)** Lift brain with forceps.

Extrabrain E-Protocol: Culture of dissociated mouse hippocampus and cortex cells

- By using fine scissors or forceps, cut open the skull by making an incision beginning at the olfactory bulbs, then swing open the skull to expose brain (Fig. 1A-C). It is important not to damage the brain tissue when removing the skull bone.

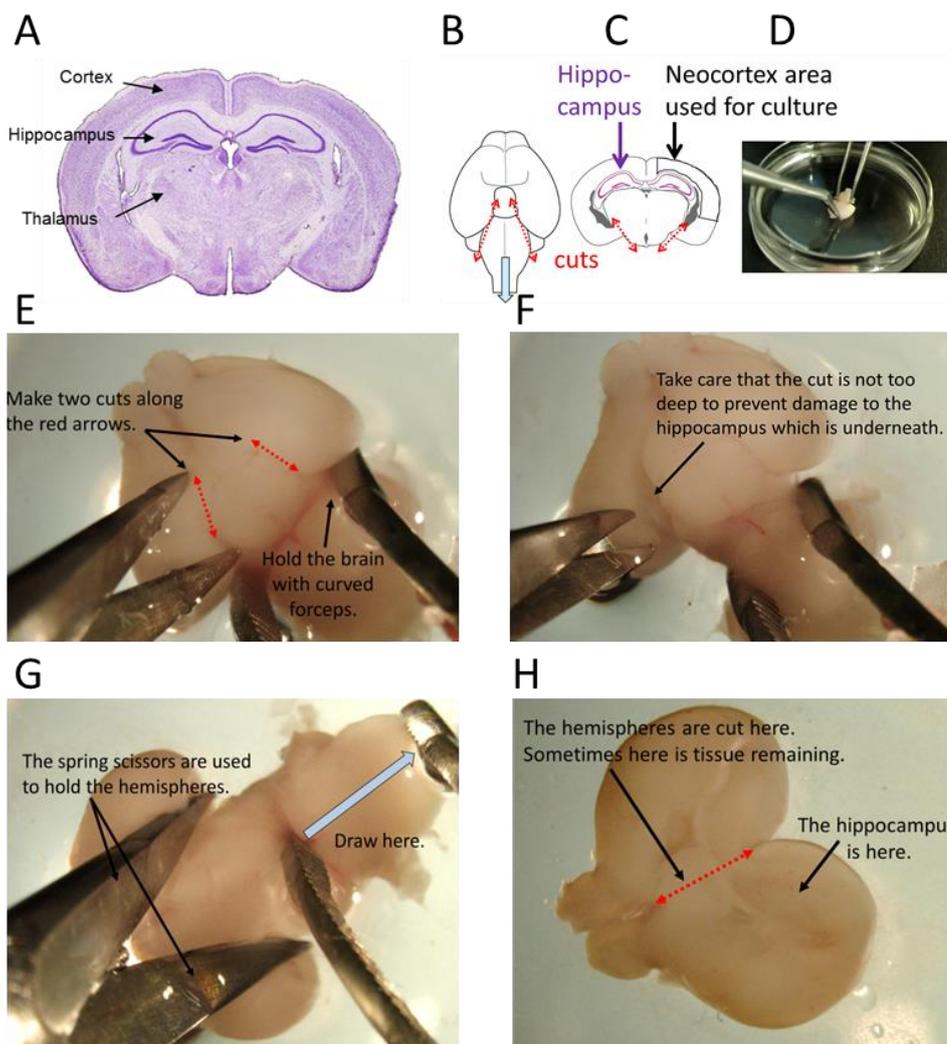


Figure 2: Brain anatomy and steps for separation of the two hemispheres

- Fix the head with forceps and gently place forceps underneath the brain starting close to the olfactory bulbs to scoop the brain out (Fig. 1D-F).
- Gently remove the intact brain and quickly transfer it into a new 35 mm Petri dish with ice-cold HBSS-Glc (Fig. 1F). Ensure that the brains are completely covered by the medium and do not let them dry out at any point. It is important to be extremely fast and careful to avoid contamination.
- Turn the brain to the ventral side. Use the spring scissors to make two cuts (see red arrows) while you hold the brain with curved forceps. Take care not to cut too deeply not to damage the hippocampus.
- Then separate the telencephalon from the brainstem (Fig. 2B-G, bright blue arrow) by drawing the cerebellum away with curved forceps. Discard the cerebellum.
- Then split the hemispheres with a sagittal cut along the midline. For a demonstration see video 1. (Fig. 2D: Video preview)
- Place the brain such that the outer surface of the hemisphere is oriented to the bottom of the dish.
- Remove the meninges/vessel membrane (pia mater) from the cortex. Therefore turn the tissue so the hippocampus faces the bottom of the dish. Pin down the cortex at the side of the olfactory bulb. Use another pair of forceps to peel of the meninges off carefully, ideally as a single piece. Do not forget to remove also the meninges below the hippocampus later (Fig. 3F-G). See video 2 (Fig. 3A). It is important to completely remove the meninges that they cannot contribute any non-neuronal cells to the culture.

Extrabrain E-Protocol: Culture of dissociated mouse hippocampus and cortex cells

- Reorient the tissue so that the hippocampus is on the top. Remove gently, if still present, remaining midbrain and thalamic tissue.
- Now a higher magnification can be used and finer straight or curved forceps whichever is more convenient. Brain hemispheres can be transferred one by one onto a black Sylgard filled 35 mm Petri dish with 1 mL ice-cold HBSS-Glc.
- The hippocampus can be recognized as a banana shaped structure with an opacity different from the neighbouring cortical tissue. The hippocampus is bordered by a sulcus on one and by a ventricle at the other side (Fig. 3B-C).
- Cut hippocampus along the sulcus with fine forceps or with the spring scissors while other forceps are placed into the ventricle to hold the hemisphere (Fig. 3D). See video 2 (Fig. 3A).
- Isolate the neocortex from the ventral telencephalon with the three cuts indicated in Fig. 3E as red dashed lines. If you cut the hippocampus without remaining cortex at the top, you need to add a fourth cut to remove the cortical tissue that was directly above the hippocampus.
- Collect neocortex and hippocampus separately, transfer them gently with a 1000 μ L pipet tip into 15 mL tubes containing HBSS-Glc and leave them on ice.
- The cultures are prepared by pooling tissue from at least two animals.

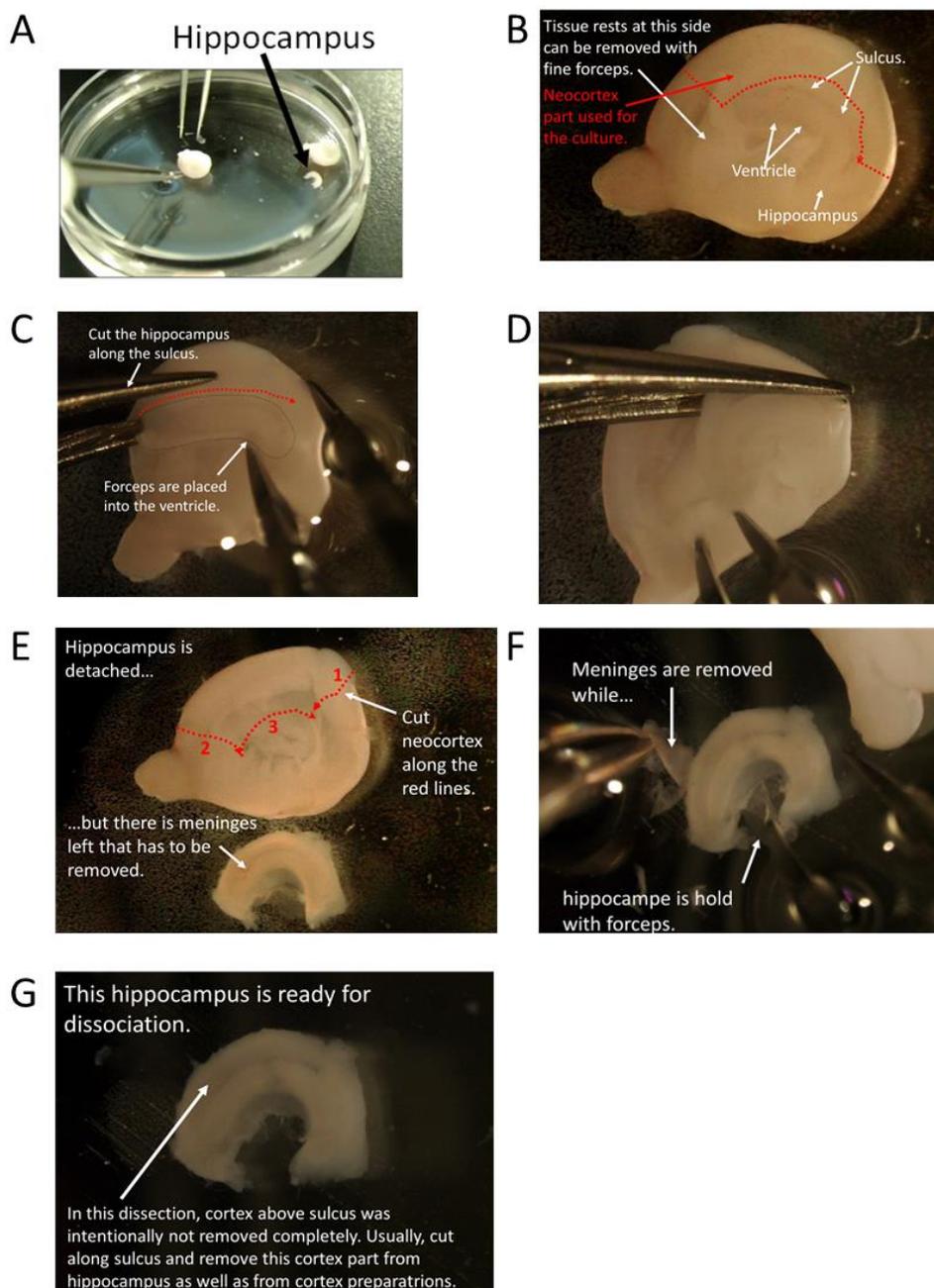


Figure 3: Steps for dissection of the hippocampus and the neocortex from the intact brain hemisphere.

3. Dissociation

- Transfer each brain region (neocortex and hippocampus) into a 15 ml tube containing cold HBSS-Glc medium and work on ice.
- Wait until all tissues settle to the bottom and remove all but 5-10% off the medium. Add 10 mL of fresh ice-cold HBSS-Glc medium to rinse the neocortex/ hippocampus. Repeat this step 3 times. It is important to be very gentle not to break the tissue during this step as smaller tissue pieces are more difficult to handle during this step.
- Optionally: Add 50 μ L DNase I (10 mg/mL) to papain solution directly before usage.
- Replace HBSS-Glc medium with 5 mL of pre-warmed papain solution for each brain region. For hippocampus it is also possible to use trypsin solution (300 μ L of 2.5% Trypsin without EDTA in 2.7 mL pre-warmed HBSS-Glc) which is harsher to the cells.
- Incubate 10-20 minutes at 37°C with pre-warmed papain solution (and 10 min with trypsin solution). It is important that this incubation step does not proceed for longer than 20 or 10 min, respectively.
- Gently aspirate the enzyme solution and rinse three times with 5-10 mL cold HBSS-Glc medium. As the tissue structures have loosened due to enzyme treatment, the treatment has to be extremely gentle not to lose cells.
- Aspirate the medium and add 1 mL pre-warmed attachment medium (AM) plus 10-100 μ L DNase I (1 mg/mL). The serum in the AM medium inactivated the papain/trypsin. Therefore it is important to reach this step fast after enzyme digestion. Two additional washing steps with 10 mL AM medium are also possible.
- Dissociate cells by gently pipetting up and down 10 to 20 times with a 1000 μ L pipette tip to obtain a homogenous cell suspension. Trituration of the cells has to be done as slowly as possible and without any bubbling to minimize cell damage.
- Centrifuge cells 5 seconds at 1000 rpm to spin down non-digested pieces of tissue (or just let the debris sink to the bottom of the tubes).
- Transfer the suspended cells to a new 15 mL tube.

4. Plating

- Dilute cell suspension 1/10 dilution with AM medium and count the cells in a Malassez Hemocytometer to estimate viable cell density. Viable cells appear bright and glossy whereas dead cells appear dark.
- You can expect $5 \cdot 10^5$ to $1 \cdot 10^6$ cells per hippocampus and $1.5 \cdot 10^6$ to $3 \cdot 10^6$ cells per cortex.
- Adjust the cell concentration with pre-warmed AM medium to the desired density.
- Typical densities are 0.8 - $1.5 \cdot 10^5$ cells per 1 mL AM medium per 18 mm coverslip in a 12 well plate.
- If the cell density is too low, the culture cannot develop and will die as well as if the culture is too dense. As a beginner it is recommended to start with higher densities that can be lowered later with increasing experience and quality of the tissue preparation.
- Cells settle down with time, thus remember to gently mix the cell suspension to ensure even plating on the coverslips. It is important to plate the neurons quickly as particularly cortical neurons tend to clump. It is possible to triturate again gently if clumps have formed.
- Incubate cells up to 2 to 4 hours at 37°C to allow them to adhere to the glass.
- Check at a microscope if cells are adherent. Cells that do not settle on the coverslip will not survive.
- Aspirate the AM medium and directly replace it with 1 mL warm maintenance medium (NBC).
- Take care not to expose the cells to a direct stream of medium or vacuum. Instead of medium aspiration or addition directly above cells, allow the medium to trickle down gently at the side walls of the well not to damage and lose neuronal cells.
- Place cells in cell culture incubator, 37°C, 5% CO₂, 95% humidity.

5. Feeding

- Do not handle cells outside the incubator for a long time to avoid creating an alkaline medium and killing cells. When changing medium from multiple plates it is better to take only out and feed one plate at a time and to use a multipipette.
- Hippocampal culture:
 - 1 week after plating: Add 250 μ L pre-warmed NBc with 5% Horse Serum per well.
 - Every week after the first feeding: Add 200 μ L NBc per well to compensate the evaporation of the medium.
- Cortical culture:
 - 1 week after plating: Add 250 μ L pre-warmed NBc medium per well.
 - Every 4-6 days, replace maximum one third of medium with pre-warmed NBc medium.
- It is essential not to replace the entire medium as otherwise suffer an osmotic shock and the neurons miss factors that they secrete over time to promote growth and survival.

The maturation of synapses is achieved around 18 DIV from mouse neurons.

III Material

1. Coating

1.1. Material and Equipment

- \varnothing 18 mm Microscope Cover Glasses (Marienfeld, Ref. 0111586)
- Tissue Culture Multiwell, 12 Well (BD, Ref. 353043)

1.2. Chemicals, Media and Solutions

- Poly-DL-ornithine hydrobromide (Sigma-Aldrich, P8638)
- sterile and pyrogen-free water (Ecotainer, Aqua B. Braun)

2. Dissection

2.1. Animals

- Non-guaranteed pregnant female mouse, e.g. C57Bl6/j, E17.5 or E18.5* (delivered 5 days before starting the culture). *E0.5 is the noon after the night of the mating.

Animals were handled according to European and French regulations.

2.2. Material and Equipment

- Dissecting microscope with Illumination (e.g. Cold Light Source with Double Gooseneck)
- Dissection tools, e.g. standard scissors, standard forceps, 2 curved dissection forceps, e.g. Delicate Forceps - 0.4 mm Tip Angled (F.S.T.), 2 thin straight dissection forceps, e.g. Moria Iris Forceps - 0.5 mm Tip (F.S.T.), Spring Scissors - angled to side or Vannas Spring Scissors
- Two 100 mm Petri dishes
- Two 35 mm Petri dishes and a 35 mm Petri dish, for a beginner glass petri dish coated with 5 mm black Sylgard, used for fixation of the tissue during dissection to preserve the dissection tools.
- Ice in a box

2.3. Chemicals, Media and Solutions

- Vetflurane (1000 mg/mL) – Isoflurane
- 70% (vol/vol) ethanol
- **Dissection and digestion medium (HBSS-Glc), 100 mL**

Components	Initial conc.	Final conc.	Source
Hank's Balanced Salt Solution (HBSS), Ca ⁺ and Mg ⁺ free	10x	1x	Gibco®, Life Technologies, 14060-040
Glucose (in sterile water, filter sterilized)	12% wt/vol	0.6%	
HEPES (pH 7.3)	1 M	20 mM	Gibco®, Life Technologies, 15630-080

Prepare the medium fresh at the day of usage and cool down to 4°C.

3. Dissociation

3.1. Material and Equipment

- 0.22 µm Syringe Filter (Sartorius AG, 16532)
- Hypodermic Syringes without Needle, 15 mL and 50 mL (Terumo®)
- Centrifuge (Eppendorf, 5702)
- Water bath, 37°C
- Ice box

3.2. Chemicals, Media and Solutions

- Dissection and digestion medium (HBSS-Glc), 4°C
- DNase I (Sigma-Aldrich, D5025): Dissolve DNase I in HBSS-Glc to a final concentration of 10 mg/mL, sterilize by 0.22 micron filtration, aliquot and store at -20°C until usage. The solution is stable for 2-3 months.
- “Ready to Use” Papain (Worthington, LK003178): Dissolve content of 1 vial in 5 mL of warm HBSS-Glc and incubate for at least 30 minutes at 37°C. Add DNase I before usage.
- OR “Selfmade” **Papain** (Worthington, LS003126):

Components, volume per 5 mL	Initial conc.	Final conc.	Source
Papain Suspension, 80 µL		20 units/mL	Worthington, LS003126
Cysteine, 100 µL	10 mg/mL	200 µg/mL	Euromedex, Ref. 1622
CaCl ₂ , 30 µL	250 mM	1.5 mM	
EDTA, 12.5 µL	200 mM	0.5 mM	
NaOH, 10 µL	1 M	2 mM	
HBSS-Glc, 4767.5 µL	1x		

Sterilize using 0.22 µm syringe filter with 15 mL hypodermic syringe without needle. Incubate for at least 30 min at 37°C. Add DNase I before usage.

- **Attachment medium (AM), 50 mL:**

Components	Initial conc.	Final conc.	Source
Horse Serum, heat inactivated		10% v/v	A&E Scientific (PAA), B15-124
L-Glutamine	200 mM	2 mM	Gibco®, Life Technologies, 20530-081
Sodium Pyruvate	100 mM	1 mM	Gibco®, Life Technologies, 11360-070
Penicillin-Streptomycin-Glutamine	10,000 U/mL	10 U/mL	Gibco®, Life Technologies, 15140-148
MEM	1x		Gibco®, Life Technologies, 14060-040

Sterilize using 0.22 µm syringe filter with 50 mL hypodermic syringe without needle. Prepare 12.5 mL per culture plate at the day of dissection. Pre-warm the medium at 37°C.

4. Plating

4.1. Material and Equipment

- Dried and UV treated PO Coated Tissue Culture Multiwells
- Malassez Hemocytometer
- Aspiration System
- Sterile Glass Pasteur Pipettes

4.2. Chemicals, Media and Solutions

- Attachment medium (AM)
- **Maintenance medium (NBc), 50 mL:**

Components	Initial conc.	Final conc.	Source
B-27® Supplement, serum free	50x	1x	Gibco®, Life Technologies, 17504-044
L-Glutamine	200 mM	2 mM	Gibco®, Life Technologies, 20530-081
Penicillin-Streptomycin-Glutamine	10,000 U/mL	5 U/mL	Gibco®, Life Technologies, 15140-148
Neurobasal	1x		Gibco®, Life Technologies, 21103-049

5. Feeding

5.1. Material and Equipment

- Multipipette

5.2. Chemicals, Media and Solutions

- Maintenance medium (NBc)

6. General Equipment needed

- Laminar flow cell culture hood
- Cell culture incubator, 37°C, 5% CO₂, 95% humidity
- Water bath, 37°C
- Micropipettes and sterile pipet tips (1000 µL, 200 µL, 20 µL, 10 µL)
- Electronic pipette controller and serological pipettes (5 mL, 10 mL, 25 mL)
- Sterile tubes (1 mL, 5 mL, 15 mL, 50 mL)

IV References

1. Brewer, G.J., Torricelli, J.R., Evege, E.K. & Price, P.J. (2006) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* 35, 567–576
2. Mains RE, Patterson PH (1973) Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J Cell Biol* 59:329–345
3. Chumasov EN et al (1975) Hippocampus morphology in tissue culture. *Tsitologiya* 17:1306–1312
4. Kim SU (1973) Morphological development of neonatal mouse hippocampus cultured in vitro. *Exp Neurol* 41:150–162
5. Chih B et al (2005) Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307:1324–1328
6. Shelly M et al (2010) Local and long-range reciprocal regulation of cAMP and cGMP in axon/dendrite formation. *Science* 327:547–552
7. Wang T et al (2011) Lgl1 activation of rab10 promotes axonal membrane trafficking underlying neuronal polarization. *Dev Cell* 21:431–444
8. Tan ZJ et al (2010) N-cadherin-dependent neuron-neuron interaction is required for the maintenance of activity-induced dendrite growth. *Proc Natl Acad Sci USA* 107:9873–9878
9. Zhu XJ et al (2007) Myosin X regulates netrin receptors and functions in axonal path-finding. *Nat Cell Biol* 9:184–192

Further protocols for dissociated neuron culture:

... with astrocyte feeder layer:

Kaech, S. & Banker, G. (2006) Culturing hippocampal neurons. *Nat. Protoc.* 1, 2406–2415

...from postnatal brains:

Beaudoin, Gerard M J et al (2012) Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nat. Protoc.* 7, 1741–1754