Preparation of organotypic hippocampal slice cultures for long-term live imaging

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This protocol details a method to establish organotypic slice cultures from mouse hippocampus, which can be maintained for several months. The cultures are based on the interface method, which does not require special equipment, is easy to execute and yields slice cultures that can be imaged repeatedly – from when they are isolated at postnatal day 6–9, and up to 6 months *in vitro*. The preserved tissue architecture facilitates the analysis of defined hippocampal synapses, cells and entire projections. Monitoring of defined cellular and molecular components in the slices can be achieved by preparing slices from transgenic mice or by introducing transgenes through transfection or viral vectors. This protocol can be completed in 3 h.

INTRODUCTION

Recent advances in gene delivery and live imaging technology have had a marked impact on the range of experimental tools that are available to life scientists[1,2]. For research in neuroscience, these developments have meant that studying the structure and function of biologically relevant neuronal circuits can now be approached in a non-invasive way and with unprecedented analytical power. To fully exploit these technological developments, adequate biological preparations to investigate neuronal circuits have to be established in parallel. Fortunately, preparations that were developed by physiologists more than a decade ago[3–5] could be readily adapted for live imaging studies of defined neuronal circuits[6,7].

Advantages of the method

Key features of the hippocampal organotypic slice cultures[4] include: well-defined cellular architecture of the hippocampal circuit, which preserves the organization *in vivo*, and allows the identification and manipulation of defined neurons and synapses[3–7]; presence of axonal projections (mossy fiber axons extending from dentate gyrus granule cells to the distal end of CA3), which can largely be recovered in the slices in their original state (that is, without lesioning) and which establish stereotype numbers of readily identifiable presynaptic terminals onto excitatory and inhibitory neurons in the hilus and in CA3 (refs 6–9); a long-term thickness of 100–150 μm, preserving three-dimensional organizations of connectivity[4,5]; maturation of the slice cultures, closely reflecting the corresponding schedule *in vivo*[8,9]; option to prepare the slices from mice of any genetic background, including those of poor postnatal viability.

Critical aspects

The main critical issues relate to the extent to which organotypic slice cultures reproduce the properties of hippocampal circuits *in vivo*[5]. This information is important for deciding whether the approach is appropriate for addressing the particular experimental issues that might be in mind. These issues have been investigated in much detail by physiologists, who have demonstrated extensive similarities, but also a few discrepancies, to properties of the corresponding circuits in the adult brain[5]. With respect to development, the slice cultures exhibit a temporal profile of excitatory and inhibitory miniature synaptic events, which closely match, qualitatively and quantitatively, the corresponding times *in vivo*[8]. This indicates that features that are critical to hippocampal circuit development and maturation are well established at 1 week postnatally, and are stable under organotypic culture conditions. A further critical issue involves the unavoidable separation of the hippocampal slices from their natural inputs, outputs and neuromodulatory systems. It turns out that most neuronal excitability and network properties are well preserved, in spite of the fact that the actual activity in the slices must be significantly different from the *in vivo* situation[5]. Predictably, synaptic connectivity in the slice cultures is initially greatly reduced due to the isolation procedure but, during the first 2–3 weeks *in vitro*, synapse numbers recover to a level comparable to that *in vivo*[5], and the cultures are stable with respect to total synapse numbers from about 3 weeks *in vitro*[6]. As a result, the degree of connectivity between some of the individual neurons that are present in the slices (e.g., pyramidal neurons in CA3) is higher than *in vivo*, a fact that facilitates the analysis of synaptically connected neurons[5]. With the exception of the molecular layer of the dentate gyrus, in which the occasional recurrent mossy fiber collaterals can produce an excitation level that is higher than that of normal granule cells, this higher connectivity does not seem to produce aberrant patterns of activity[5]. The slices can, however, be electrically labile, and gentle handling is important to avoid epileptic-like discharges. One way to avoid higher excitability in granule cells is to prepare slices from P20-30 mice when the circuits are more stable[10]. Finally, attempts to investigate adult neurogenesis in hippocampal slice cultures have suggested that the phenomenon is much less frequent than *in vivo*. This might be influenced by the culture medium, but the issue requires further investigation.

Possible results and outlook

Organotypic slice cultures from ~1-week-old mouse hippocampus appear to reproduce most anatomical and functional properties of the corresponding hippocampal circuits *in vivo* for at least 6 months *in vitro* due to the intrinsic properties of their neurons. Accordingly, limitations to their applications might be confined to studies of hippocampal input–output relationships. This leaves an

exciting range of possibilities for the exploration of mechanisms that control the assembly and function of neuronal circuits. Some of these include: time-lapse imaging from the sub-second to the months range, and from individual molecules to entire neuronal projections and circuits; imaging of neuronal[6,7,11,12] and glial[13] subtypes; molecular manipulation using transfection[14,15] or viral approaches[16,17] to knock down or overexpress genes, silence or activate neurons, render neurons responsive to light or selective drugs and to highlight sub-circuits; combined physiology-imaging methods; manipulations to investigate lesion-induced plasticity and pathways of neurodegeneration and repair (e.g., amyloid- or epilepsy-related); following the insertion of new neurons, the development of axons and their connections, or the insertion of exogenously added stem cells; *post-hoc* analysis using, for example, tracers, electron microscopy and single-cell genomic methods.

MATERIALS

REAGENTS

- Animals: 6–9-day-old mouse pups. You can prepare slices from six pups within one session, but for the beginner it may be preferable to start with two or three pups ! CAUTION All animal experiments must comply with national regulations.
- Hand sterilizing solution, e.g., Sterilium (Bode) or equivalent
- Penicillin/streptomycin (Invitrogen, cat. no. 16050-122)
- HEPES
- Hank's balanced salt solution (HBSS; Invitrogen, cat. no. 24020-083)
- Horse serum
- 2× MEM (liquid Eagle's with Hank's Salts and 25 mM HEPES; Gibco, cat. no. 04195120M)
- Tris-(hydroxymethyl)aminomethane

EQUIPMENT

- Dissection microscope (e.g., ZEISS Stemi 2000-C binocular with 10×23 objectives, but any 5–10× magnifying dissection microscope is suitable)
- McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd.)
- Sterile dissection hood
- Razor blades that can be fixed in the McIlwain tissue chopper
- Filter paper (e.g., Schleicher & Schuell, cat. no. 300009, or Whatman paper)
- Small (35 mm × 10 mm) and large (100 mm × 20 mm) cell culture dishes (Corning, cat. no. 430165 and 430293, respectively)
- 6-well culture plates (Corning, cat. no. 3516)
- Culture plate inserts: 0.4 µm Millicell membrane, 30 mm diameter (Millipore, cat. no. PICM03050)
- Vacuum filter sterilizer for medium (e.g., Vacuum-driven disposable filtration system, 0.22 µm pore width; Millipore, cat. no. SCGPU02RE)
- Cell culture incubator at 35 °C, 95% air, 5% CO_2

EQUIPMENT SETUP

Dissection tools Scalpel, two round-ended spatulas, small scissors, large scissors, one pair of fine straight forceps, two pairs of curved fine forceps and two glass Pasteur pipettes that have to be custom designed as follows: one pipette is fire-polished at the tip so that it adapts a round shape, has no sharp edges, but still has a small opening; the second pipette is cut at the intersection of the fine and thick tube using a canula opener (glass cutter), and the resulting large opening of this pipette is fire-polished to smooth the edges.

REAGENT SETUP

Penicillin/streptomycin solution Dissolve 1.6 g penicillin G (100 U ml^{-1}) and 2.5 g streptomycin (0.1 mg ml^{-1}) in 200 ml H_2O, filter-sterilize and store at –20 °C in 2-ml aliquots. Note that penicillin can reduce GABAergic neurotransmission in slices[18]. Signs of epileptic activity have, however, not been detected under these culture conditions.

Horse serum Heat-inactivate the complement system of the horse serum at 56 °C for 30 min; aliquots can be stored at –20 °C for at least 1 year.

? TROUBLESHOOTING

Dissecting medium 50 ml MEM 2×, 1 ml penicillin/streptomycin solution, 120 mg Tris (hydroxymethyl)aminomethane (final concentration: 10 mM); add up to 100 ml with ddH_2O. ▲ CRITICAL Prepare within 24 h of the experiment, filter-sterilize through a 0.22 µm membrane and keep it at 4 °C until dissection.

Culture medium 50 ml MEM 2×, 1 ml penicillin/streptomycin solution, 120 mg Tris (hydroxymethyl)aminomethane (final concentration: 10 mM), 910 µl of a 7.5% $NaHCO_3$ aqueous solution, 50 ml heat-inactivated horse serum, 50 ml 1× HBSS; add up to 200 ml with ddH_2O. ▲ CRITICAL Filter-sterilize through a 0.22 µm membrane and keep at 4 °C. Pre-heat only the medium that is needed for a medium change on the same day. Culture medium can be stored at 4 °C for at least 1 month.

PROCEDURE

Preparation of membrane inserts and culture medium ● TIMING 10–30 min

1| Prepare the culture medium and filter-sterilize it.

2| Add 1 ml culture medium per well of a 6-well plate; prepare 3–4 wells for each pup to be dissected (one pup should yield 6–8 usable hippocampal slices and about two slices are cultured on one membrane).

3| Add one culture plate insert into each prepared well, so that the insert membranes touch the medium, but are not covered by it.

4| To warm up the medium, put the prepared 6-well plates into the cell culture incubator.

Preparation of dissection medium and chambers ● TIMING 10–20 min preparation + 15 min sterilization

5| Prepare the dissection medium, filter-sterilize and keep it at 4 °C.

6| Cut a small triangle (about 4 × 4 × 4 cm) out of the filter paper, take the cover of a 100-mm cell culture dish and place the filter paper triangle into it. Prepare 1 cover + filter paper for each pup to be dissected.

7| Sterilize the covers containing the filter papers under ultraviolet light in the culture hood for 15 min.

8| Add 1 ml of cold dissection medium on top of each filter paper and cover with the bottom of the cell culture dish under sterile conditions.

9| Keep these 'dissection chambers' at 4 °C until dissection.

Preparation of dissection material ● TIMING **5–15 min**

10| Clean all dissection tools with 70% ethanol, fire-sterilize them inside the dissection hood and keep them there. Clean a fresh razor blade with a chloroform:isoamylalcohol (49:1) solution, followed by 100% ethanol and 70% ethanol and fix it in the McIlwain tissue chopper placed inside the dissection hood.

! CAUTION Chloroform is toxic; avoid inhalation, ingestion or contact with skin, eyes or mucous membranes.

11| Fix the plastic platform on the McIlwain tissue chopper and clean it with 70% ethanol; switch the chopper on and adjust the cutting thickness to 400 µm.

12| Keep the dissection medium on ice inside the dissection hood.

13| Put one 35-mm cell culture dish per pup to be dissected under the dissection hood.

Hippocampus dissection and cutting of coronal sections ● TIMING **15–30 min/pup**

14| Place one of the prepared, cold 'dissection chambers' under the dissection microscope and remove the top plate so that the filter paper covered with cold dissection medium is exposed.

▲ CRITICAL STEP Steps 14–38 are carried out under sterile conditions inside a dissection hood unless otherwise mentioned.

15| Decapitate one pup outside the dissection hood using large scissors. Note that anesthesia of pups is notoriously difficult (dry ice is one possibility), and that decapitation as described above is usually advised. Nevertheless, make sure that the procedure complies with local regulations.

16| Flush the head with 70% ethanol and transfer it into the hood.

▲ CRITICAL STEP The fur of the pup is a potential contamination source.

? TROUBLESHOOTING

17| Sterilize your gloves with Sterilium or 70% ethanol before you proceed.

▲ CRITICAL STEP Proceed carefully for Steps 18–24. The delicate nervous tissue of the brain is easily damaged by the sharp dissection tools or the edges of the cut skull.

? TROUBLESHOOTING

Dissection

18| Make an incision into the skin along the midline of the head, starting at the neck up to between the eyes using the small scissors (see **Figs. 1–3**).

19| To hold the head more easily and to expose the skull, flip the skin around the head and pull it to the lower side, where you pinch it between your fingers.

20| Use the small scissors and the fine straight forceps to remove neck muscles and the first vertebrae.

21| Insert the lower part of the small scissors carefully into the foramen magnum and cut the skull along the midline from the foramen magnum to the front until you reach between the eyes.

22| Make two lateral cuts starting from the midline towards the sides.

23| Peel away the skull using the fine straight forceps.

24| Hold the head upside down above the prepared filter paper, which should be covered with cold dissection medium.

25| Introduce the spatula carefully between the brain and the skull and remove the brain from the skull, cut the cranial nerves and, if necessary, the olfactory bulbs with the spatulas.

26| Let the brain drop gently onto the filter paper covered with dissection medium.

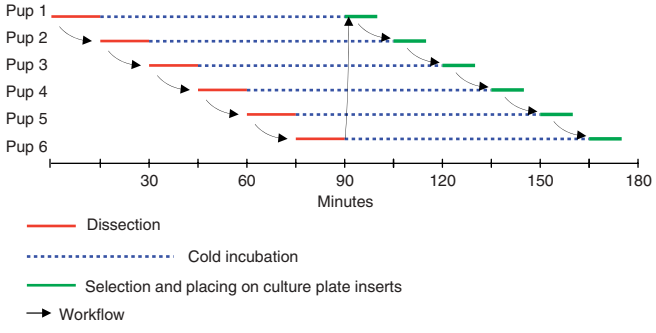

Figure 1 | Workflow diagram. Slices from pups 1 to 6 are prepared sequentially: dissection, cold incubation and slice selection are performed in a staggered way.

27| Immediately put a few drops of cold dissection medium onto the exposed brain.

28| Put the brain upside up with the ventral side lying on the filter paper.

29| Use the scalpel to cut off the forebrain and the cerebellum by coronal cuts.

30| Using the scalpel, separate the two hemispheres cutting along the inter-hemispheric fissure.

31| Place one brain hemisphere on the frontal or caudal cutting surfaces; the intersection between cortex, midbrain and brainstem becomes visible.

32| Separate the cortex with the underlying hippocampus from the brainstem, midbrain and striatum using the two spatulas.
▲ CRITICAL STEP Do not touch the hippocampus with the spatulas.
? TROUBLESHOOTING

33| Place the cortex upside down, so that the hippocampus is exposed.

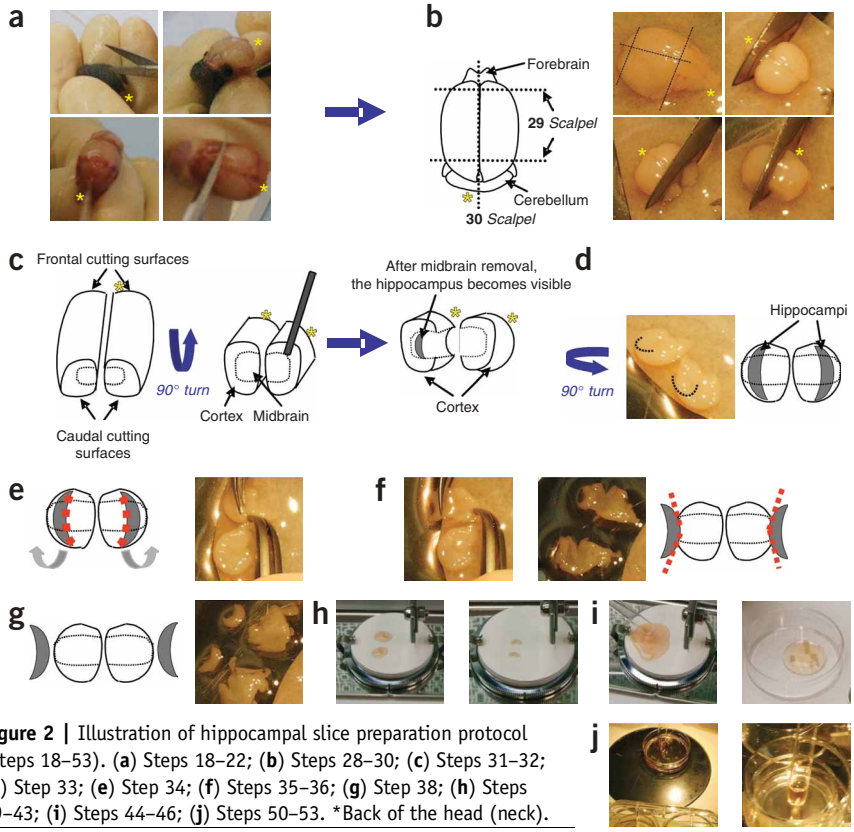

Figure 2 | Illustration of hippocampal slice preparation protocol (Steps 18–53). (**a**) Steps 18–22; (**b**) Steps 28–30; (**c**) Steps 31–32; (**d**) Step 33; (**e**) Step 34; (**f**) Steps 35–36; (**g**) Step 38; (**h**) Steps 39–43; (**i**) Steps 44–46; (**j**) Steps 50–53. *Back of the head (neck).

34| Use the curved forceps to cut the connections of the hippocampus to the ventral side (fimbria); leave it connected to the cortex by the subiculum.

35| Flip the hippocampus over and out.

36| Using the scalpel, cut the connection of the hippocampus to the enthorinal cortex (subiculum).

37| Flush the dissected hippocampus with ice-cold dissection medium.

38| Prepare the second hippocampus of the opposite hemisphere in the same way (see **Fig. 2**).

39| Use the wide-bore, custom-made Pasteur pipette to suck one hippocampus into the pipette along with some dissection medium and transfer it to the plastic platform on the McIlwain tissue chopper.

40| Repeat the same for the second hippocampus.

41| Using the narrow-bore pipette, align the two hippocampi perpendicularly to the chopper blade.
▲ CRITICAL STEP Avoid touching the slices; instead, use medium to push and pull them into the right position.
? TROUBLESHOOTING

42| Remove all dissection medium around the hippocampi.

43| Chop rapidly into 400 μm thick transverse sections.

44| Float the freshly cut sections immediately with cold dissection medium.

45| Use the wide-bore pipette to transfer the sections into a 35-mm cell culture dish.

46| Separate the sections by shaking the dish gently. If the sections stick together, remove all dissection medium and shake harshly but not for too long; alternatively, use the narrow-bore pipette and try to separate the sections by the flow of some dissection medium.
▲ CRITICAL STEP Use great care as the sections are very easily damaged!

47| After separation, fill the 35-mm dish with cold dissection medium so that all sections are covered; push floating sections down to the bottom of the dish by dropping medium onto their tops.

Cold incubation ● TIMING 30 min–1.5 h/pup

48| Cover the 35-mm cell culture dish with its cover and label it with the number of the pup and the exact time. Incubate the separated slices for a minimum of 30 min at 4 °C (up to 1.5 h). Repeat Steps 14–48 for all pups (see **Fig. 1**).

▲ **CRITICAL STEP** After incubation of each pup, clean and fire-sterilize all dissection tools and the chopper platform; take a fresh and cold 'dissection chamber'; change and sterilize your gloves!

? TROUBLESHOOTING

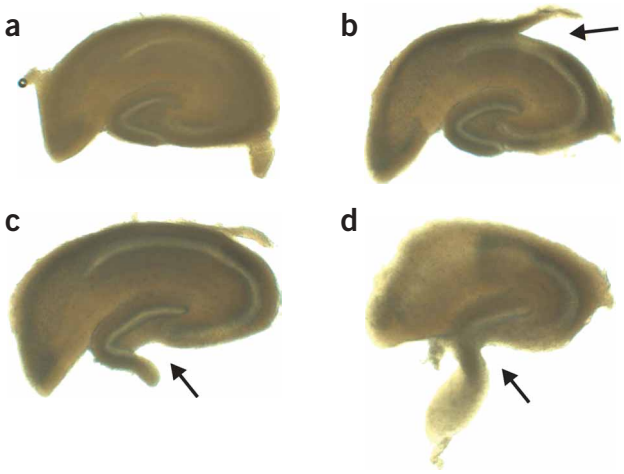

Figure 3 | Selection of slices for culturing. (**a**) Optimal slice with nice cell layers in the dentate gyrus and CA1-3 and smooth margins; (**b**) slice in which the CA1 region was lesioned during preparation (arrow); (**c**) slice in which the dentate gyrus was lesioned (arrow); (**d**) slice in which the dentate gyrus detached from the rest of the section (arrow). Only the slice in **a** should be selected for culturing.

Selection and incubation of hippocampal slices ● TIMING 10–20 min/pup

49| After completing the dissection of the last pup, start the selection of slices from the first dissected pup (see label) and proceed with the selection in the same sequence as in the dissection (see **Fig. 1**).

▲ **CRITICAL STEP** Make sure that slices from each pup were cold incubated for at least 30 min (check the time on the label). Place the first 35-mm dish (slices of the first pup) under the dissection microscope in the dissection hood.

50| Remove the lid of the 35-mm dish and select the best slices for culturing according to the following criteria (see **Fig. 3**) Slices should have smooth margins and be clearly visible, have uniform and well-defined cell layers in the dentate gyrus and in CA1-3; the dentate gyrus should be tightly connected to the rest of the slice, and the fimbria should be intact.

? TROUBLESHOOTING

51| Collect one pre-heated 6-well plate containing culture medium and a cell culture insert from the 35 °C incubator.

52| Using the wide-bore pipette, transfer the selected slices individually onto the membranes along with some dissection medium. Alternatively, some labs use thin spatulas for the transfer, but we have no direct experience with this alternative method.

53| Using the narrow-bore pipette, orientate the slices to the middle of the membrane by pushing and pulling them with the stream of dissection medium.

54| You can place up to four slices on one membrane but the number of slices on one membrane has to be adapted to the planned experiments.

▲ **CRITICAL STEP** For live imaging, you should only place one or two slices on each membrane. Place the slices as close to the center as possible, so that the plastic edge of the membrane insert will not hinder the microscope's access to the top of the slices. Adapt the slice number that you put on one membrane to the estimated time required for later imaging of all slices on one culture plate insert. The time you can keep one culture plate insert outside the incubator during imaging is restricted to a maximum of 30 min (see also Imaging Protocol[19]). Keep a minimal distance of 2 mm between the slices to avoid fusion when flattening out during the culture period.

55| Using the narrow-bore pipette, remove all dissection medium around the slices.

▲ **CRITICAL STEP** This is critical because any remaining dissection medium covering the slices hinders oxygen exchange.

56| To avoid cooling, put the 6-well culture plate back into the incubator immediately after having placed the slices.

57| After 3–4 d, remove all culture medium below the insert and replace it with 1 ml of fresh, 35 °C-warmed culture medium.

58| Replace the culture medium every 3–4 d.

59| The slice cultures can be maintained for several months. Criteria to verify viability: the slices must be transparent, firmly attached to the membrane and the dentate gyrus must be visible to the naked eye. In addition, if neurons are labeled with fluorescent markers, microscopic examination should reveal an absence of axonal and dendritic beading. If necessary, cell death in the slices can be assayed with propidium iodide[6].

● TIMING

Steps 1–4: 10–15 min **(For trained experts)**; 20–30 min **(For beginners)**.
Steps 5–9: 10 + 15 min **(For trained experts)**; 20 min **(For beginners)**.
Steps 10–13: 5–10 min **(For trained experts)**; 15 min **(For beginners)**.
Steps 14–47: 15 min per pup **(For trained experts)**; 30 min per pup **(For beginners)**.
Step 48: 30–90 min **(For trained experts)**; 30–90 min **(For beginners)**.
Steps 49–56: 10 min per pup **(For trained experts)**; 20 min per pup **(For beginners)**.

? TROUBLESHOOTING

See **Table 1** for troubleshooting advice.

TABLE 1 | Troubleshooting for slice culture preparation.

PROBLEM	POSSIBLE REASON	SOLUTION
Slices become contaminated soon after preparation	Contamination by the fur or blood of the pups	Use 70% ethanol to repeatedly flush the head after decapitation
		Change and sterilize the gloves after having touched the pups' fur and blood
Slices detach from the membrane of the cell culture plate insert soon after preparation	Check for the correct composition and pH of the culture medium	Always adjust the pH of the culture medium to 7.2
	Change the horse serum	Inactivate the horse serum carefully and note the batch number, if possible; keep using a batch that has worked as long as possible (make many aliquots)
Slices die prematurely during the culturing period	Slices can become epileptic if treated too harshly	Always move slices slowly and smoothly
		Avoid strong vibrations
Axons and/or dendrites assume a beaded appearance	Wrong medium composition	Strictly follow the indications in the protocol concerning media and times during preparations and handling
	Wrong pH of culture medium	
	Treatment during preparation too harsh	
	Slice preparation took too long	
	Media were not at the right temperature	
	Incubator at wrong temperature or atmosphere	
Aberrant axonal projections	The slices that were selected for culturing did not show the right morphology or were injured	Select only slices in which the cell layers can be clearly seen and have the expected shape
		Make sure that you do not touch the slices with any sharp tools
	The cutting angle was not perpendicular to the long axis of the hippocampus	Make sure that you cut and then select slices that were cut perpendicularly to the long axis of the hippocampus

ANTICIPATED RESULTS

Critical factors to reproducibly achieve good yields and quality of slice cultures are speed, avoiding physical damage of the hippocampus and avoiding contaminations during the preparation (see **Figs. 1–3**). These requirements mainly depend on training and concentration. We therefore recommend that beginners practice repeatedly during the first 2–4 weeks, in order to become confident and to acquire good experimental skills. A trained user should produce 6–8 good quality slices per pup, which can be maintained and imaged for at least 6–10 weeks.

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