Culturing hippocampal neurons

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We provide protocols for preparing low-density dissociated-cell cultures of hippocampal neurons from embryonic rats or mice. The neurons are cultured on polylysine-treated coverslips, which are suspended above an astrocyte feeder layer and maintained in serum-free medium. When cultured according to this protocol, hippocampal neurons become appropriately polarized, develop extensive axonal and dendritic arbors and form numerous, functional synaptic connections with one another. Hippocampal cultures have been used widely for visualizing the subcellular localization of endogenous or expressed proteins, for imaging protein trafficking and for defining the molecular mechanisms underlying the development of neuronal polarity, dendritic growth and synapse formation. Preparation of glial feeder cultures must begin 2 weeks in advance, and it takes 5 d to prepare coverslips as a substrate for neuronal growth. Dissecting the hippocampus and plating hippocampal neurons takes 2–3 h.

INTRODUCTION

Perhaps the greatest appeal of nerve cell culture is that it makes living neurons immediately accessible to observation and manipulation. In addition, low-density cultures are far less complex than neural tissue, making them an ideal preparation for investigating the subcellular localization and trafficking of neuronal proteins. Advances in fluorescence imaging over the last decade and the development of new methods for manipulating DNA expression provide new opportunities to exploit nerve cell cultures.

Continuous (clonal) cell lines from the central nervous system are not widely used, because such cells do not form well-defined axons or dendrites and do not make synapses. Instead, most neuroscientists work with primary cultures. In principle, primary nerve cell cultures could be prepared from any region of the brain or spinal cord. What is it that accounts for the widespread popularity of hippocampal cultures? One reason is that the nerve cell population in the hippocampus is relatively simple compared with most other regions of the central nervous system. Pyramidal neurons, the principal cell type in the hippocampus, account for the vast majority of the total neuronal population. The hippocampus also contains a variety of interneurons, but they are comparatively few in number and most are morphologically distinguishable in culture[1]. Another factor is that cultured pyramidal neurons express many of their key phenotypic features in cell culture. They form well-developed dendrites studded with spines and make extensive, synaptically connected networks. The stages of hippocampal neuron development in culture have been well-characterized and are reasonably consistent from laboratory to laboratory. Finally, as hippocampal cultures have been investigated for over 20 years, there is already an extensive database that provides the starting point for new experiments.

Hippocampal cultures are most often prepared from rats, but the same protocol also works well for mouse cultures. This provides the opportunity to apply live-cell assays to cultures prepared from knockout animals. In the case of knockout animals that die at birth from systemic defects, cell culture affords an opportunity to study those aspects of neural development that occur postnatally. Cultures are prepared from late-stage embryos because at that time the generation of pyramidal neurons is essentially complete, but the generation of dentate granule neurons has not yet begun[2]. Embryonic tissue is also easy to dissociate and contains comparatively few glial cells, the major source of contamination in neuronal cultures.

Ideally, one would like to grow neuronal cultures in a chemically defined medium, adding whatever protein factors are necessary for neuronal growth and survival. Although this goal has been attained for cultures prepared from the peripheral nervous system, present methods for maintaining hippocampal neurons still depend on paracrine trophic support from nearby neurons and glia. The lower the cell density, the less the paracrine support and the more difficult it is to maintain the culture. Current protocols for culturing hippocampal neurons differ in the strategies they use to provide trophic support. Our approach is admittedly the most complicated. We use astroglial monolayer cultures, grown in culture dishes, to support dissociated hippocampal neurons, which are grown on the underside of coverslips suspended above the glia. The configuration of such 'sandwich' cultures (where neurons grow in the narrow space between the coverslip and the surface of the culture dish) creates a microenvironment where factors secreted by neurons and glia become concentrated and the oxygen tension is reduced[3]. When the cells have reached the desired stage of development, the coverslips can be removed, yielding a population of neurons with very few contaminating glia. The advantage of this approach is that neurons survive at very low densities, resulting in cultures with widely spaced cells whose processes are essentially confined to a single plane, an ideal situation for imaging experiments. Plating cells at substantially higher densities yields cultures that are suitable for biochemical analysis, such as 1- and 2-dimensional PAGE, immunoblotting, immunoprecipitation, microarray analysis and real-time PCR.

An alternative strategy, developed by Greg Brewer and his colleagues, uses a medium specifically formulated to promote neuronal survival while suppressing glial proliferation[4] (additional information at http://www.brainbitsllc.com). When this method is used, neurons survive for long periods without the need for astroglial feeder cultures, but the phenotypic differentiation of pyramidal neurons using this system has not been fully characterized. A third method involves plating hippocampal neurons directly onto an astroglial monolayer[5] or growing cultures under conditions that foster the proliferation of hippocampal glia, which rapidly

form a monolayer beneath the neurons[6]. Such cultures, which are often prepared from early postnatal rats, are well suited to physiological studies, but do not lend themselves to live-cell imaging or immunostaining because the neurons and glia are intermixed, much as they are in neural tissue.

Our protocol for culturing hippocampal neurons (shown schematically in **Fig. 1**) involves preparing a suspension of dissociated hippocampal neurons, plating them onto polylysine-treated glass coverslips, then transferring the coverslips with adhering neurons into dishes containing a monolayer culture of type 1 astrocytes. The astroglia provide trophic support, allowing the neurons to be cultured at low density. In practice, these steps are performed in the opposite sequence, as the astrocyte cultures and polylysine-treated coverslips must be prepared in advance. In the following sections, we provide a protocol for making astroglial feeder cultures (Steps 1–13), treating coverslips with polylysine (Steps 14–21) and preparing cultures of hippocampal neurons (Steps 22–32). Of course, the timing of all of these steps must be coordinated with the availability of a pregnant rat or mouse at the correct stage of gestation.

For glial support, primary cultures of type 1 astroglia are prepared from the cortices of newborn rat pups. One preparation will generate enough glial cells to make feeder cultures for several preparations of hippocampal neurons. The extra cells are frozen down until needed. Even when culturing mouse neurons, we use rat astroglia. Rat glia are easy to culture, and the resulting 'hybrid' cultures develop very well. Glial cultures are grown in medium supplemented with horse serum. We screen different lots of horse serum, evaluating their ability to support good glial growth and the effectiveness of the resulting glial preparations in supporting neuronal cocultures.

The state of the substrate is remarkably important for proper development and maturation of the neurons. Unfortunately, the variables that make for the optimal substrate are not well understood. As a result, we are compulsive about preparing coverslips the same way every time (Steps 14–21). Not all glass coverslips work well for culturing neurons by this method. We use coverslips cut from glass manufactured in Germany by Schott Desag (designated 'D' by the supplier). Cells cultured on inappropriate glass grow well initially but detach after several days.

Hippocampal neurons are isolated from embryonic day 18 (E18) rat embryos, which typically are about 25 mm in length. Neurons can also be prepared from E17 mouse embryos using the same protocol. Note that, as we use this terminology, embryos reach E1 the day after the dam is found to be sperm-positive. Some suppliers consider embryos to reach E1 at the time the dam is found to be sperm-positive. We try to complete the dissection and plating within 2.5 h. It speeds things up to have one person dissect out the brains while a second, working under a dissecting microscope, removes the hippocampus. Ordinarily, the dissected hippocampi are dissociated and the neurons put into culture immediately, but media have been formulated for storage of embryonic tissue[7] (additional information at http://www.brainbitsllc.com). This can be useful for those working with mouse models, as individual embryos can be genotyped before culturing and tissue can be shipped from one laboratory to another.

Hippocampal cultures can be grown in either N2 (ref. 8) or Neurobasal/B27 (Invitrogen) Medium (**Table 1**). We routinely prepare cultures in N2 because the development of hippocampal neurons has been best characterized in this medium. Our impression is that early development occurs more rapidly and more synchronously in N2, but long-term survival is more consistent in Neurobasal/B27. Even for long-term studies, we continue to use N2 because it is our sense (admittedly subjective) that maturation in 'good' N2 cultures is better than it is in Neurobasal/B27. But there are also more 'bad' cultures with N2. Choosing to work with primary neuronal cultures means accepting the fact that there will be some bad culture days.

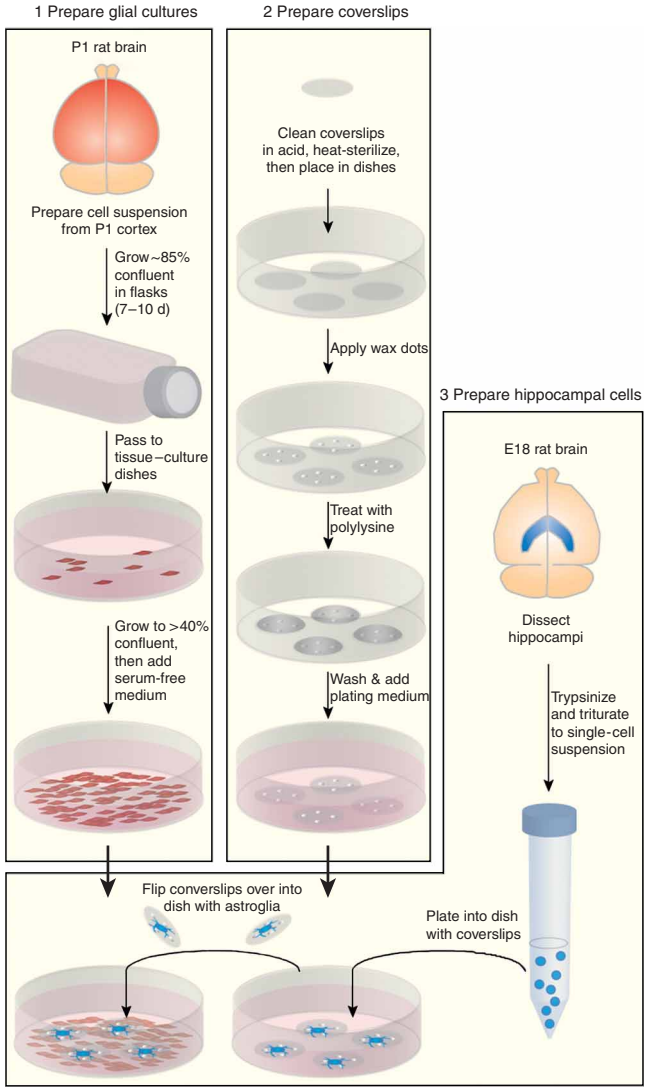

Figure 1 | Flowchart showing the three-part protocol for preparing hippocampal cultures.

Overview of procedure

Steps 1–12, prepare cortical astroglia cells and expand in flasks
Step 13, expand glial feeder layers in dishes for coculture
Steps 14–18, clean and sterilize glass coverslips
Steps 19–21, treat glass coverslips with polylysine
Step 22, pre-condition Neuronal Maintenance Medium
Steps 23–31, dissect hippocampal neurons and plate for coculture
Step 32, culture maintenance

TABLE 1 | Media.

CMF-HBSS	Calcium-, magnesium-, and bicarbonate-free Hank's balanced salt solution (BSS) buffered with 10 mM HEPES, pH 7.3	
	Component	Source
	10× Hanks' BSS	Invitrogen 14185-052
	1 M HEPES buffer, pH 7.3	Invitrogen 15630-080
Glial Medium	Minimal essential medium (MEM) supplemented with glucose (0.6% wt/vol), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and containing 10% (vol/vol) horse serum	
	Component	Source
	MEM with Earle's salts and L-glutamine	Invitrogen 11095-080
	D-Glucose	Sigma G8769
	Penicillin-streptomycin	Invitrogen 15140-122
	Horse serum	Invitrogen 16050, SAFC Biosciences 12449, or Atlanta Biologicals S12150
Neuronal Plating Medium	MEM supplemented with glucose (0.6% wt/vol) and containing 10% (vol/vol) horse serum or 5% (vol/vol) fetal bovine serum	
	Component	Source
	MEM with Earle's salts and L-glutamine	Invitrogen 11095-080
	D-Glucose	Sigma G8769
	Horse serum or fetal bovine serum	Horse serum, see above; fetal bovine serum, Invitrogen 16000
Neuronal Maintenance Media		
N2 medium	MEM containing the N2 supplement described in ref. 22. It is prepared by combining nine parts MEM supplemented with glucose (0.6% wt/vol) and one part 10× N2 supplement.	
	10× N2 supplement contains the following ingredients prepared in MEM	
	Component	Source
	10 mM sodium pyruvate	Sigma P2256
	1 mM putrescine	Sigma P5780
	0.2 μM progesterone[a]	Sigma P8783
	0.3 μM selenium dioxide[b]	Sigma 200107
	1 mg ml^{-1} bovine transferrin	Sigma T1428
	50 μg ml^{-1} insulin[c]	Sigma I5500
Neurobasal/B27 Medium	Prepare according to the manufacturer's instructions by supplementing Neurobasal Medium with GlutaMAX-I and B27 supplement.	
	Component	Source
	Neurobasal Medium	Invitrogen 21103-049
	GlutaMAX-I supplement	Invitrogen 35050-061
	B27 serum-free supplement	Invitrogen 17504-044

[a]Dissolve 63 mg progesterone in 100 ml EtOH, then dilute 1:100 in H_2O to make a 20 mM stock solution.
[b]Dissolve 33 mg SeO_2 in 100 ml H_2O, then dilute 1:100 in H_2O to make a 30 μM stock solution.
[c]Dissolve 50 mg insulin in 10 ml 0.01N HCl, made by adding 86 μl concentrated HCl to 100 ml H_2O.

MATERIALS

REAGENTS

- Cell-culture media (see **Table 1**)
- 2.5% (wt/vol) trypsin (Invitrogen, cat. no. 15090-046); aliquot for single use and store at –20 °C
- DNase, 10 mg ml^{-1} in CMF-HBSS (Roche Applied Science, cat. no. 10104159)
- Trypsin/EDTA (Invitrogen, cat. no. 25300-054)
- Cell-freezing medium (Recovery; Invitrogen, cat. no. 12648-010)
- Paraffin, sterilized by autoclaving
- Polylysine solution, 1 mg ml^{-1} in borate buffer (prepared from poly-L-lysine, molecular weight 30,000–70,000 kDa; Sigma-Aldrich, cat. no. P2636)
- Borate buffer, 0.1 M, pH 8.5 (prepared from boric acid and sodium tetraborate)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668)
- Rat Neuron Nucleofector Kit (Amaxa, cat. no. VPG-1003).

EQUIPMENT

- Laminar flow hood, able to accommodate a dissecting microscope
- Tissue culture incubator at 35.5 °C with humidified, 5% CO_2 atmosphere
- Water bath at 37 °C
- Dissecting microscope
- Dissecting tools (sterilized): fine-tipped forceps (for example, Dumont no. 5), microdissecting scissors (Vannas-style spring scissors)
- Hemacytometer for counting cells
- Cell strainer with 70-μm mesh (BD Biosciences, cat. no. 352350)
- Freezing vials; for example, Nunc CryoTubes 366656
- Cell-freezing device; for example, Nalgene 5100 Cryo 1 °C Freezing Container
- Liquid nitrogen storage
- Glass coverslips, 18 mm diameter, no. 1D (Fisher Scientific, cat. no. 12-545-84-1D)
- Ceramic coverslip-staining rack, such as Thomas Scientific, cat. no. 8542E40
- Sterile plasticware: 5-, 10- and 25-ml serological pipettes, 60-mm tissue culture dishes and bacteriological dishes, 75-cm^2 tissue-culture flasks (for example, Primaria, BD Biosciences, cat. no. 353824), 15-ml and 50-ml conical centrifuge tubes

- Sterilization filter units: Steriflip-GP filter unit (Millipore, cat. no. SCGP00525) and Stericup-GP filter unit (Millipore, cat. no. SCGPU02RE)
- Sterile glass Pasteur pipettes
- Nucleofector Device (Amaxa, cat. no. AAD-1001)

PROCEDURE

Preparation of cortical astroglia cells and expansion in flasks ● TIMING ~1 week

1| Kill three or four postnatal rat pups using an approved method of euthanasia. We normally use 1-day-old pups.

2| Working in a laminar flow hood, remove the brains and place them in a dish containing CMF-HBSS (**Table 1**). The tissue needs to remain submerged at all times.

3| Under a dissecting microscope, remove the cerebral hemispheres and carefully strip away the meninges with fine forceps. ▲ CRITICAL STEP Be thorough when removing the meninges—meningeal tissue tends to hide in fissures. The meninges contain fibroblasts that can overgrow astroglial cultures.

4| Transfer the hemispheres to a drop of CMF-HBSS and chop the tissue as finely as possible with scissors.

5| Transfer the tissue pieces to a 50-ml conical centrifuge tube in a final volume of 12 ml CMF-HBSS and add 1.5 ml each of 2.5% trypsin and 1% (wt/vol) DNase. Incubate in a 37 °C water bath for 5 min, swirling the tube occasionally.

6| Triturate by passing the solution up and down 10–15 times in a 10-ml pipette. Return to the water bath for another 10 min, swirling occasionally. Triturate another 10–15 times with a 5-ml pipette until most chunks disappear.

7| Pass the cell suspension through a cell strainer to remove chunks of undissociated tissue and collect in a 50-ml conical tube containing 15 ml Glial Medium (**Table 1**).

8| Centrifuge the cells for 5–10 min at 120*g* to remove enzymes and lysed cells. Discard the supernatant and resuspend the cell pellet in 15–20 ml Glial Medium (**Table 1**). Determine the cell density using a hemacytometer—the yield should be on the order of 10^7 cells per brain.

9| Plate 7.5×10^6 cells per 75-cm^2 flask and add Glial Medium to a final volume of 15–20 ml.

10| After 1 d, feed cultures with Glial Medium. First, swirl the flask to remove loosely attached cells, then aspirate off the medium and replace with fresh medium. When inspecting cultures at this stage, do not be alarmed by the sparseness of the culture and the amount of cell debris. The astrocytes you want to expand remain firmly attached to the flask surface and will proliferate quickly.

11| Feed the culture every 2–3 d. Before removing the medium, slap the flask 5–10 times against your hand to dislodge loosely attached cells.
▲ CRITICAL STEP Contaminating cells such as O2A progenitors and microglia (macrophages) sometimes grow on the surface of the astrocyte monolayer. Removing loosely attached cells at this step helps to minimize their number. Microglia (recognizable as round, phase-bright, loosely attached cells) release cytokines that are highly neurotoxic. Preparations with high numbers of microglia should be discarded.

12| Harvest the astroglia when the cells are near confluence (usually within 7–10 d). Rinse each flask briefly with 3–5 ml of trypsin/EDTA, then add 2–3 ml of trypsin/EDTA and incubate at 37 °C until the first cells just begin to lift off (usually in less than 2 min). Add 5 ml Glial Medium to stop the trypsinization, then release cells by repetitive pipetting, transfer the cell suspension to a conical centrifuge tube and pellet the cells at 120*g* for 7 min. Resuspend in Glial Medium and passage into 60-mm dishes (10–30 dishes per flask), as per Step 13.
■ PAUSE POINT Extra cells can be resuspended in cell-freezing medium at $\sim 2 \times 10^6$ ml^{-1}, frozen in cryotubes (1 ml) and stored in a liquid nitrogen freezer, then thawed and plated into 60-mm dishes as needed.

Prepare glial feeder layers in dishes for coculture ● TIMING ~1 week

13| Approximately 1 week before the animal reaches the correct gestational day (henceforth called dissection or D-day), start the glial feeder cultures by seeding $\sim 10^5$ cells per 60-mm dish. Replace the medium in 60-mm dishes with fresh Glial Medium every 3–4 d. Cultures are suitable for coculturing with neurons when they reach 40%–70% confluence.

Clean and sterilize glass coverslips ● TIMING 2 d

14| At least 5 d before D-day, place 18-mm coverslips in ceramic racks, rinse in water to remove dust then place in concentrated nitric acid (70% wt/wt) for at least 18 h (or over the weekend). Acid can be reused many times; it should be replaced at the first sign that the polylysine solution does not spread easily to cover the glass coverslip (see Step 19). It is easiest to handle coverslips using fine forceps (for example, Dumont no. 5).

15| At least 4 d before D-day, rinse the coverslips in the racks in four changes of distilled water over a period of 2 h. Remove any remaining water droplets by aspiration or by rinsing in absolute ethanol, then air-dry.

16| Place racks in a glass beaker covered with aluminum foil and sterilize in an oven at 225 °C for 6–16 h. Freshly cleaned glass surfaces readily adsorb contaminants from the air.
▲ CRITICAL STEP Make sure that the oven is clean and do not store cleaned coverslips for more than a few days.

17| Working in a laminar flow hood, place coverslips in 60-mm bacteriological Petri dishes, four or five per dish.

18| Apply wax dots to the coverslips. The dots serve as 'feet' to suspend the coverslips above the glial feeder layer. Melt paraffin in a suitable bottle and maintain at about 100 °C on a hot plate. Dip a Pasteur pipette into the paraffin, then rapidly touch it to three or four spots near the edge of a coverslip. The wax will harden into a small dot about 0.5 mm high and 2 mm wide.
▲ CRITICAL STEP This step takes some practice—expect some difficulties the first time you try. The temperature of the wax is critical. If it is too hot, the wax spreads too much and the dots are too thin; if it is too cool, the dots do not adhere well and are likely to float off when the coverslips become wet.

Treat glass coverslips with polylysine ● TIMING 1 d

19| At least 2 d before D-day, coat coverslips with polylysine. Immediately before use, dissolve poly-L-lysine at 1 mg ml^{-1} in 0.1 M borate buffer (pH 8.5) and filter-sterilize. Add about four drops of the polylysine solution to each coverslip. When properly cleaned, the coverslips are very hydrophilic and this small volume will spread evenly over the entire surface. The hydrophobicity of the bacteriological plate will prevent the solution from spreading onto the dish. Let stand for 12–24 h at room temperature (~20–25 °C), covered to protect the dishes from evaporation.

20| Remove the polylysine solution and rinse twice with sterile water, 2 h each.
▲ CRITICAL STEP Never let polylysine-treated coverslips dry during Steps 19 and 20. If stored in a laminar flow hood, cover them or turn off the blower.

21| Remove the rinse solution and add 6 ml Neuronal Plating Medium (**Table 1**). Place the dishes in a CO_2 incubator (where they can be stored for several days before use).

Preconditioning of Neuronal Maintenance Medium ● TIMING 1–2 d

22| One to two days before D-day, remove the medium from the glial feeder cultures and add 6 ml Neuronal Maintenance Medium, either N2 or Neurobasal/B27 (**Table 1**), for preconditioning.
▲ CRITICAL STEP This preconditioning step is important for optimal neuronal growth.

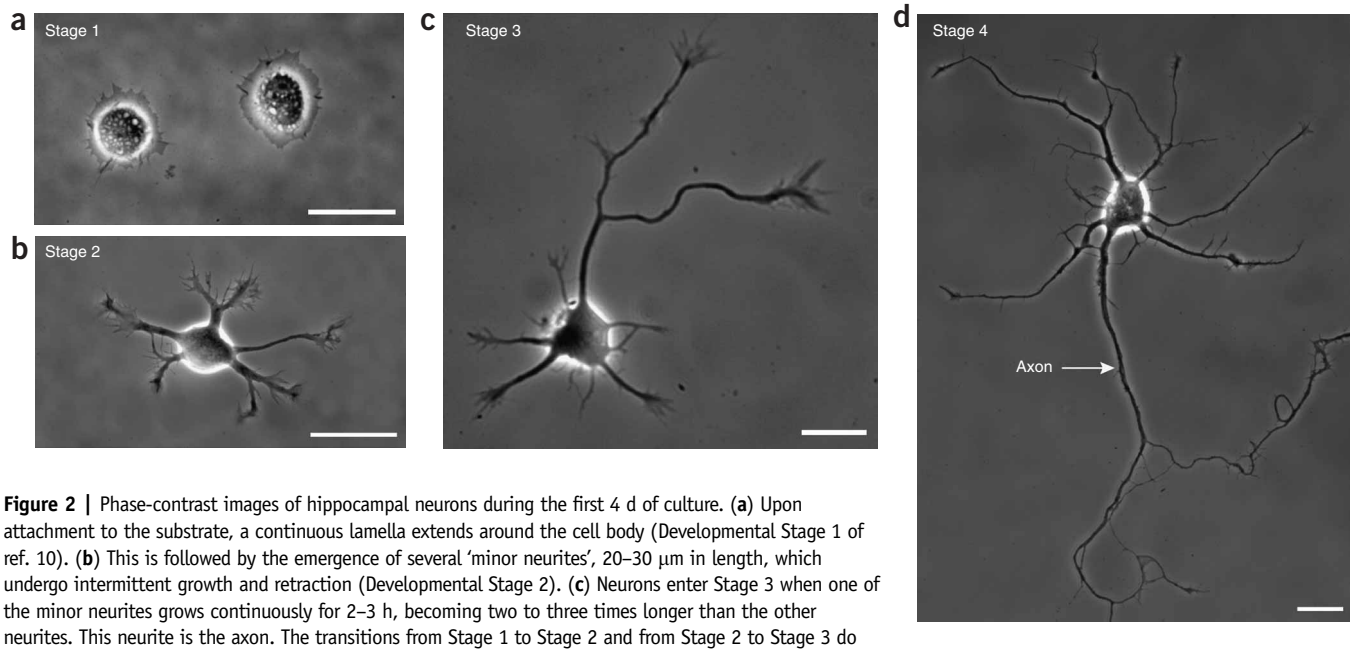

Figure 2 | Phase-contrast images of hippocampal neurons during the first 4 d of culture. (**a**) Upon attachment to the substrate, a continuous lamella extends around the cell body (Developmental Stage 1 of ref. 10). (**b**) This is followed by the emergence of several 'minor neurites', 20–30 μm in length, which undergo intermittent growth and retraction (Developmental Stage 2). (**c**) Neurons enter Stage 3 when one of the minor neurites grows continuously for 2–3 h, becoming two to three times longer than the other neurites. This neurite is the axon. The transitions from Stage 1 to Stage 2 and from Stage 2 to Stage 3 do not occur synchronously across the cell population. Under optimal conditions, half of the neurons reach Stage 3 24 h after plating; more than 80% reach Stage 3 by 36–48 h. Scale bars, 25 μm. (**d**) Phase-contrast image of a Stage 4 neuron. By this stage, the dendrites have begun to grow and branch and to show their characteristic taper. The axon extends for many hundreds of micrometers, far beyond the field of view illustrated. This comparatively rare image was chosen because the axon and dendrites are easily distinguishable. More typically, recurrent branches of the axon intersect the dendrites and run along them. The change in dendritic morphology between Stage 3 and Stage 4 is more easily appreciated by immunostaining for the dendritic marker protein MAP2. The cell illustrated is 4 d old. Scale bar, 25 μm.

Dissection of hippocampal neurons and plating for coculture ● TIMING **2–3 h**

23| On D-day, kill the pregnant dam using an approved method of euthanasia, dissect out the uterus and place in a sterile Petri dish.

24| Working in a laminar flow hood, remove the fetuses from the uterus, dissect out their brains and place them in a dish containing CMF-HBSS. The tissue needs to remain submerged at all times.

25| Using a dissecting microscope, remove the meninges from the medial aspect of the cerebral hemispheres, then dissect out the hippocampus. Collect the hippocampi in a dish containing CMF-HBSS (**Table 1**). Details and photographs of the dissection may be found in ref. 9.

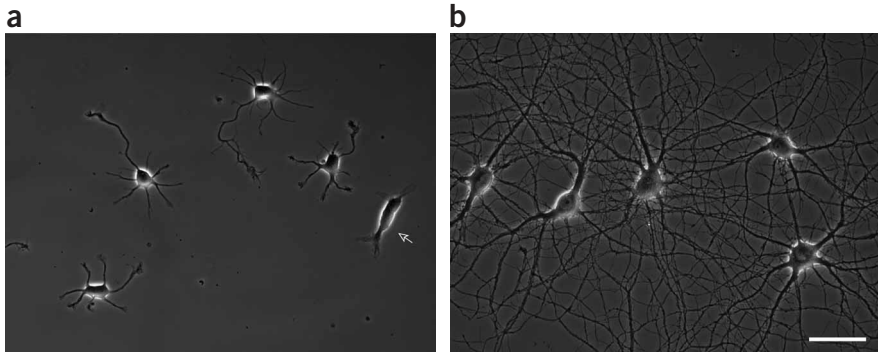

Figure 3 | Low-magnification phase-contrast images of hippocampal cultures after 1 d (**a**) and 13 d in culture (**b**). Both cultures were plated at our standard density of 150,000 cells per 60-mm dish (Step 29). After 1 d in culture, some neurons have reached Developmental Stage 3 and others remain in Stage 2. There are also occasional non-neuronal cells (arrow); addition of antimitotic drugs a few days after plating (Step 31) nearly eliminates non-neuronal contamination over the course of a week. After 13 d in culture, nerve cell bodies have increased markedly in size and an extensive, intertwined network of axons and dendrites has developed. Both images were taken at the same magnification. Scale bar, 50 μm.

26| When all of the hippocampi have been removed, place them in a 15-ml conical centrifuge tube and bring the volume to 4.5 ml with CMF-HBSS. Add 0.5 ml of 2.5% trypsin and incubate for 15 min in a water bath at 37 °C.

27| Gently remove trypsin solution, leaving the hippocampi at the bottom of the tube; add 5 ml of CMF-HBSS and let stand for 5 min at room temperature. Repeat this twice to allow residual trypsin to diffuse from the tissue. Bring the final volume to 2–3 ml.

28| Dissociate the hippocampi by repeatedly pipetting them up and down in a Pasteur pipette. Begin with five to ten passes through a regular pipette, then continue with five to ten passes through a Pasteur pipette that has been flame-polished so that its tip diameter is narrowed by half. Expel the suspension forcefully against the wall of the tube to minimize foaming. By the end, there should be no chunks of tissue left.

▲ CRITICAL STEP The diameter of the flame-polished pipette is important. Too narrow a tip will damage the cells; too wide a tip will not fully dissociate the tissue. Pipette only as many times as is necessary to obtain a homogeneous solution with no tissue pieces. Low cell viability (see Step 29) usually means one of two things: if you are a beginner, low viability probably means the flame-polishing resulted in a tip that was too narrow. If you are an old hand and cell viability suddenly drops after you have had many consistent cell preparations, it probably means the trypsin has lost activity and should be replaced.

29| Determine the cell density by adding a drop of the cell suspension to a hemacytometer. Also determine the total yield, which should be 400,000–500,000 cells per hippocampus. The viability, according to exclusion of trypan blue, should be 85%–90%. Adjust the cell density to about 1–1.5 million cells ml^{-1}. At this point, cells may be transfected using the nucleofection protocol (see **Box 1**). Using a micropipette, add the desired number of cells to each of the dishes containing the polylysine-treated coverslips in Neuronal Plating Medium. Our standard plating density is 150,000 cells per 60-mm dish, but a range from 50,000 to 500,000 is practicable. This plating method ensures an even density of cells across the entire coverslip, although cells that do not settle onto the coverslips are lost.

30| After 3–4 h, examine the dishes to ensure that most of the cells have attached, then transfer the coverslips to the dishes with the glial feeder layer in N2 or Neurobasal/B27 (**Table 1**). Invert the coverslips so that the paraffin feet are resting on the bottom of the dish.

31| Three days after plating, add cytosine arabinoside (1-β-D-arabinofuranosylcytosine) to a final concentration of 5 μM to curb glial proliferation.

Culture maintenance ● TIMING **up to 4 weeks**

32| One week after plating, and every week thereafter, feed the cultures by replacing one-third of the volume with fresh Neuronal Maintenance Medium.

▲ CRITICAL STEP Never change the culture medium completely; the neurons depend on a 'conditioning' of the medium by glial cells for their long-term survival.

? TROUBLESHOOTING

● TIMING

Steps 1–12, prepare cortical astroglia cells and expand in flasks, ~1 week
Step 13, expand glial feeder layers in dishes for coculture, ~1 week
Steps 14–18, clean and sterilize glass coverslips, 2 d
Steps 19–21, treat glass coverslips with polylysine, 1 d
Step 22, precondition Neuronal Maintenance Medium, 1–2 d
Steps 23–31, dissect hippocampal neurons and plate for coculture, 2–3 h
Step 32, maintenance of culture, up to 4 weeks

BOX 1 | EXPRESSING COMPLEMENTARY DNA CONSTRUCTS IN CULTURED NEURONS

Over the past decade, a number of different methods have been developed for reliably expressing cDNA constructs in cultured hippocampal neurons[24]. This technology has greatly extended the usefulness of hippocampal cultures for a variety of applications. Each method has its own advantages and disadvantages. Viral expression systems offer consistently high transfection efficiencies but require generation of a new virus for every construct to be expressed. Calcium phosphate–mediated transfection was one of the first methods to be used successfully to transfect neuronal cultures. The crucial parameters that affect success with this method have been carefully worked out, so that many laboratories continue to rely on this approach[25]. More recently, a variety of proprietary products, mostly lipid-based, have been developed for transfecting cDNAs into cultured cells, and several of these work well on cultured neurons. Most give high levels of expression, but the transfection efficiency in neuronal cultures is comparatively low (1%–10%). We have found Lipofectamine 2000 (Invitrogen, cat. no. 11668) to be satisfactory for our applications. In our hands, the transfection efficiency varies over the lifetime of the culture—we get the best results between 3 and 10 d in culture. At these stages, 1%–3% of the neurons express the transgene, which is ideal for our purposes. Expression typically begins within 4 h of adding the lipid-DNA complexes to the cells, and the number of cells expressing the construct increases for about 8 h; expression can continue for the life of the culture. 'Nucleofection', a recent approach that combines electroporation with the use of a proprietary solution to enhance nuclear import of DNA constructs, gives transfection efficiencies comparable to those obtained with viral expression systems[26], with typically more than half the cells expressing the transgene. This method is applied to cells in suspension, immediately before plating. In our laboratory, lipid-based transfection (A) and nucleofection (B) have become the methods we routinely use for expressing DNA constructs in hippocampal cultures; protocols for both are given below.

Over the years, we have tried many different expression vectors, with mixed success. We have finally settled on two vectors that work consistently in our hands. The first, CAG, includes the CMV immediate early enhancer and the chicken β-actin promoter[27]; it can be used to express transgenes in hippocampal cultures at all stages of development. We routinely use this composite promoter when we express genes by electroporation before plating. The second vector (generously provided by J. Adelman, Vollum Institute) uses the CMV immediate early promoter and contains an intron upstream of the open reading frame, which is thought to enhance nuclear export of the spliced mRNA. This vector gives higher levels of expression than CAG between 3 and 10 d in culture but does not give reliable expression outside of this time window. We are also careful to insure that the bases immediately upstream of the start codon conform to a consensus sequence for translation initiation ($GCCA^{A}/_{G}CC$**ATG**G, where the start codon is in bold)[28] and that the base after the stop codon is unchanged to favor proper chain termination[29]. Run-of-the-mill 3′ untranslated region and SV40 polyadenylation sequences from commercially available vectors are included downstream[29]. Routine purification of plasmid DNA includes steps to eliminate endotoxin contamination.

(A) Lipid-based transfection.

(i) In a sterile microfuge tube, dilute 3–5 μl Lipofectamine 2000 into 100 μl minimal essential medium (MEM); let stand at room temperature for 5 min.

(ii) In a second microfuge tube, dilute 0.5–3 μg plasmid DNA into 100 μl MEM.

(iii) Mix the lipid solution with the DNA solution and let stand at room temperature for an additional 20–30 min.

(iv) Select the dish to be transfected and flip the coverslips so the neurons are face up. Distribute the lipid-DNA complexes over the dish and return it to the incubator.

(v) After 60–90 min, flip the coverslips back over and return the dish to the incubator. If you plan to maintain the culture more than a day, the Lipofectamine reagent should be removed. This is best done by transferring the coverslips to a new glial support dish. When transfecting cultures more than 1 week old, some investigators add NMDA-receptor blockers such as APV (0.1 mM) or kynurenic acid (0.5 mM) to reduce excitotoxic damage.

(B) Electroporation.

(i) Gently pellet at least 500,000 freshly isolated neurons (from Step 29) in 2.5 ml Neuronal Plating Medium in a 15-ml conical centrifuge tube (120*g* for 5–7 min). Although the manufacturer's instructions call for using at least 5×10^6 cells per cuvette, we routinely use 500,000 cells with good success.

(ii) Add 1–5 μg of plasmid DNA to a microfuge tube.

(iii) Carefully aspirate the supernatant from the cell pellet and resuspend the cells in 100 μl of Nucleofector solution from the Rat Neuron Nucleofector Kit (Amaxa, cat. no. VPG-1003). Transfer the cell suspension to the tube containing the DNA, mix gently but thoroughly by pipetting slowly up and down, then transfer this mixture into a cuvette and pulse in the Nucleofector Device (Amaxa, cat. no. AAD-10001) according to the manufacturer's instructions (Nucleofector Program O-03).

(iv) Promptly add about 0.5 ml Neuronal Plating Medium to the cells in the cuvette and transfer the desired number of cells to a dish containing coverslips in plating medium.

? TROUBLESHOOTING

See **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible explanation	Solution
Slow initial development. Ideally, >80% of cells extend axons within 2 d.	Insufficient glial conditioning of medium or suboptimal substrate.	Add Neuronal Maintenance Medium to glial feeder cultures earlier. Prepare plating medium using different lots of serum, which contain axon-promoting substances that bind the substrate.
Neurons initially grow well, but begin to detach after a few days; no cell survive past 1 week. Neurites fasciculate; in extreme cases, cell bodies clump together.	Coverslips manufactured from unsuitable glass. Happens rarely, but with devastating consequences. Poor adhesion to the substrate. Coverslips not cleaned properly or stored too long before use.	Confirm with supplier that coverslips were prepared from glass manufactured by the German company Schott Desag. Test growth on coverslips from another source. Prepare new coverslips using fresh acid. If cleaned properly, polylysine solution will immediately spread over the entire coverslip.
Suboptimal neuronal survival or maturation.	Glial feeder layers contain high percentage of microglia (which release toxins) or fibroblasts (which do not support neuronal development).	Discard any glial cultures with significant numbers of microglia (small, round, phase-bright, loosely attached cells). Confirm that nearly all cells stain brightly with astroglial markers like glial fibrillary acidic protein. Prepare new glial cultures. Screen other lots of horse serum.

ANTICIPATED RESULTS

The development of hippocampal neurons in culture has been the subject of a remarkable number of studies in this and the previous century (for review of earlier literature, see ref. 10). One focus of many studies concerns the development of neuronal polarity. This seminal event in neuronal differentiation occurs during the first 2 d in culture (**Fig. 2**). Shortly after plating, hippocampal neurons extend a lamella all around the cell body (Developmental Stage 1, in the terminology of ref. 11). Then, this lamella coalesces at distinct spots around the cell periphery, where minor neurites begin to extend. This corresponds to Developmental Stage 2. At this stage, the growth cones at the tips of the neurites show their characteristic motility and neurites extend and retract over short distances, but there is little net growth and the cell retains its symmetric appearance. Most axonal and dendritic markers stain all neurites equally, and all neurites have the capacity to become axons or dendrites. Neurites may develop larger growth cones, and some axonal markers may become concentrated in a single neurite, but that does

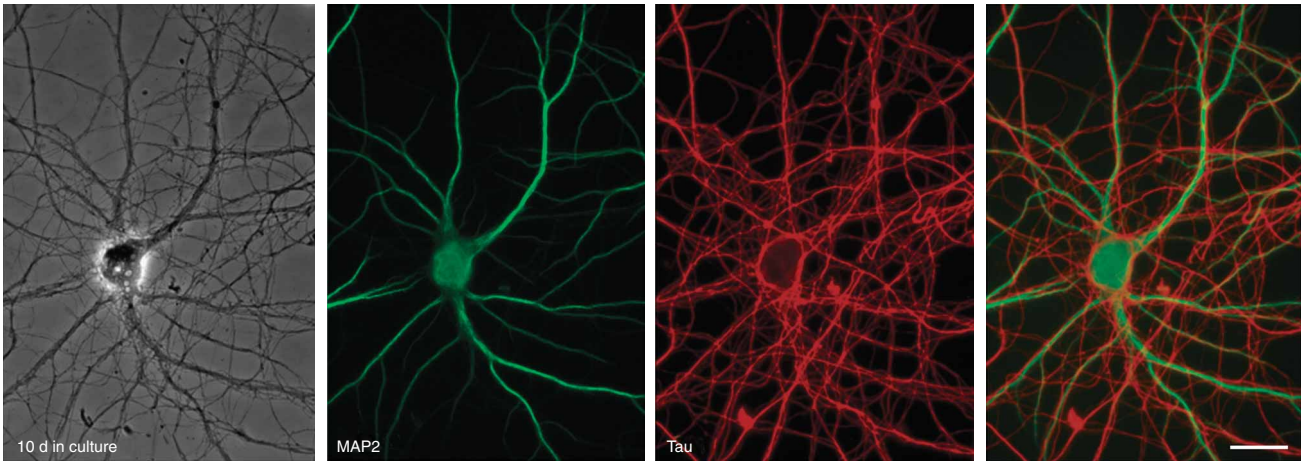

Figure 4 | Using selective markers to visualize axons and dendrites. This 10-day-old culture was fixed and immunostained with antibodies that differentially label the axonal and dendritic microtubule cytoskeleton. The microtubule-associated protein MAP2 decorates only dendritic microtubules. Good monoclonal and polyclonal antibodies against MAP2 are commercially available, such as clone HM-2 (Sigma-Aldrich, cat. no. M9942) or the chicken polyclonal antibody used here at 0.6 μg ml^{-1} (Abcam, cat. no. 5392); they can be used to selectively stain dendrites throughout the development of the culture. Antibodies directed toward differentially phosphorylated isoforms of tau specifically label axons. The de-phosphoepitope recognized by clone tau-1 (Chemicon, cat. no. MAB3420) is abundant during the first week in culture, whereas the phosphoepitope recognized by clone PHF-1 (generously provided by P. Davies, Albert Einstein College of Medicine) labels axonal tau in older cultures, as shown here. Scale bar, 25 μm.

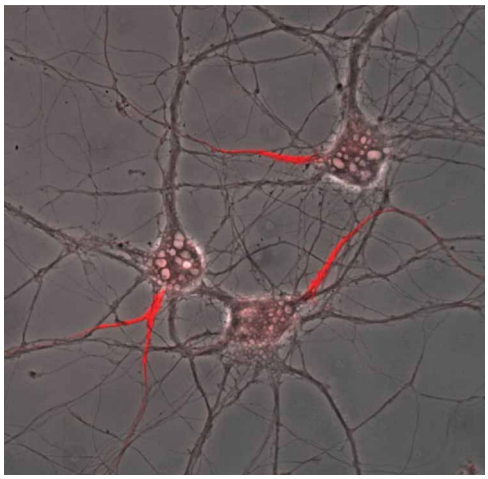

Figure 5 | Initial segment markers can be used to visualize the origin of the axon. This 8-day-old culture was stained with antibodies against ankyrin G (Santa Cruz, cat. no. sc-12719). Staining for sodium channels (Sigma-Aldrich, cat. no. S8809) similarly marks the initial segments. As these proteins are relatively low in abundance, it is necessary to use a biotin-streptavidin detection method.

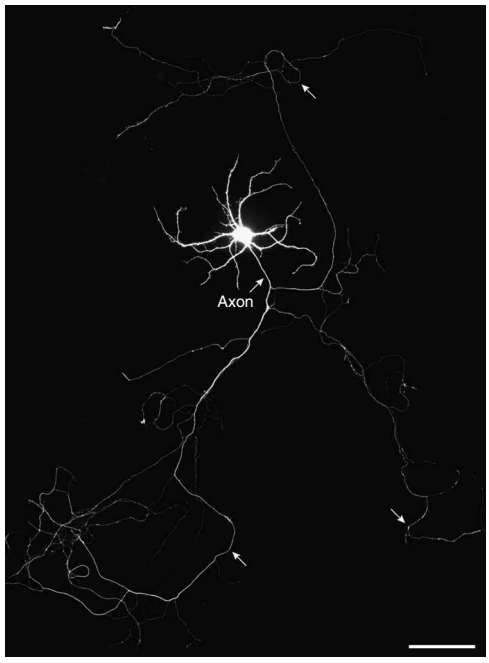

Figure 6 | Expressing GFP enables visualization of the complete arborization of an individual cell. The dendrites, which extend radially from the cell body, are tapered and can be followed all the way to their ends (reaching about 100 μm from the soma in this 8-day-old cell). The axon (arrows), which gives rise to several branches, is far longer, sometimes winds in circuitous patterns and maintains a uniform diameter over long distances. In this cell, the axon and dendrites are well separated, but in most cases, branches of the axon intertwine with some of the dendrites. Scale bar, 100 μm.

not necessarily indicate that this neurite will become the axon—an hour later, a different neurite may show these features[12]. Cells remain in Stage 2 for 12–36 h. Then an abrupt transition occurs. One neurite grows for an extended period without retracting, until it becomes two or three times longer than the other neurites. This event marks the beginning of Developmental Stage 3; the long neurite will continue to elongate rapidly and acquire axonal characteristics, whereas the remaining neurites grow more slowly and become dendrites. A number of exciting studies over the past few years have begun to define the signaling events that channel growth to a single neurite and specify neuronal polarity[13,14].

After 3–4 d in culture, the remaining minor neurites begin to grow and acquire the taper that is characteristic of dendrites. At roughly the same time, the first synapses occur[15]. These events mark the beginning of Developmental Stage 4 (**Fig. 2d**). With time, the dendrites become more highly branched, an extensive network of synaptic connections forms and the dendrites of pyramidal neurons become studded with spines (see below). Such cells are said to be in Stage 5 of development, although the transition between Stages 4 and 5 is very gradual and there is no clear consensus on the characteristics that define a Stage 5 cell. Images of 1-day-old and 13-day-old cultures taken at the same magnification illustrate the extent of growth that occurs (**Fig. 3**).

A second major focus of experimentation using hippocampal cultures concerns later events: dendritic development and branching, synaptogenesis and synaptic plasticity. For such studies, most investigators use cultures between 1 and 4 weeks old. The peak of dendritic growth and synapse formation occurs during the second and third weeks in culture, with dendritic spines first appearing at about 2 weeks in culture. At this stage, hippocampal cultures have formed such a dense network (**Fig. 3**) that visualizing dendrites and spines requires immunostaining or expression of an appropriate fluorescent

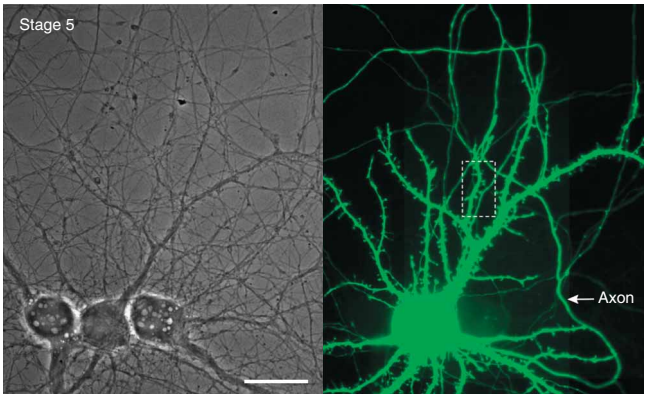

Figure 7 | A Stage 5 neuron in a 3-week-old culture. Using phase contrast (left), one can identify cell bodies and follow the proximal course of some dendrites. It is impossible to identify the axon that arises from a particular cell. Expressing GFP (right) allows neurites to be traced in their entirety. The axon of this cell emerges near the base of a dendrite. Numerous dendritic spines are characteristic of pyramidal cells at this stage of development. Some branches of the axon of this cell can be seen to contact dendritic spines (boxed region), probably forming autapses. Image courtesy of Michael A. Silverman. Scale bar, 25 μm.

marker (**Fig. 4**). Staining with dendritic markers is useful at all stages of development, but after a week in culture, staining with axonal markers is no longer very revealing. Dendrites become so ensheathed with axons that they also appear positive. The initial segment at the origin of the axon can be visualized by immunostaining for ankyrin G (**Fig. 5**) or sodium channels. Expressing soluble green fluorescent protein (GFP) in a small fraction of the cells offers a nice way to visualize the entire axonal and dendritic arbors of the labeled cells at all stages of development (**Fig. 6**). Axons and dendrites are easily distinguished by their morphology. Dendrites emerge gradually from the cell body, taper with distance, generally have a radial orientation and terminate 200–300 μm from the cell body. Axons are thinner at their origin, show less taper, follow a meandering course over several millimeters and often loop back on themselves. By 3 weeks in culture, pyramidal-cell dendrites are studded with spines (**Fig. 7**). Expressing appropriate GFP-tagged chimeric constructs can allow visualization of protein trafficking and the assembly of supramolecular structures[16,17]. For example, expressed MAP2-GFP selectively labels dendrites[18] and GFP-tagged synaptic vesicle proteins or components of the postsynaptic specialization can be used to visualize the dynamics of synapse formation in real time[19–22].

Eventually, of course, all the neurons die. This period of cell death typically begins after 3 or 4 weeks, and only the most resolute attempt to work with cultures beyond 4 weeks. This protocol was provided directly by the authors. A more detailed discussion and a historical perspective are provided in ref. 23.

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