Ex Vivo Culture of Chick Cerebellar Slices and Spatially Targeted Electroporation of Granule Cell Precursors.

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Title:
Ex vivo culture of chick cerebellar slices and spatially targeted electroporation of granule cell precursors

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Short Abstract
The cerebellar external granule layer is the site of the largest transit amplification in the developing brain. Here, we present a protocol to target genetic modification to this layer at the peak of proliferation using *ex vivo* electroporation and culture of cerebellar slices from embryonic day 14 chick embryos.

Long Abstract
The cerebellar external granule layer (EGL) is the site of the largest transit amplification in the developing brain, and an excellent model for studying neuronal proliferation and differentiation. In addition, evolutionary modifications of its proliferative capability have been responsible for the dramatic expansion of cerebellar size in the amniotes, making the cerebellum an excellent model for evo-devo studies of the vertebrate brain. The constituent cells of the EGL, cerebellar granule progenitors, also represent a significant cell of origin for medulloblastoma, the most prevalent paediatric neuronal tumour. Following transit amplification, granule precursors migrate radially into the internal granular layer of the cerebellum where they represent the largest neuronal population in the mature mammalian

brain. In chick, the peak of EGL proliferation occurs towards the end of the second week of gestation. In order to target genetic modification to this layer at the peak of proliferation, we have developed a method for genetic manipulation through *ex vivo* electroporation of cerebellum slices from embryonic day 14 chick embryos. This method recapitulates several important aspects of *in vivo* granule neuron development and will be useful in generating a thorough understanding of cerebellar granule cell proliferation and differentiation, and thus of cerebellum development, evolution and disease.

Introduction

The cerebellum sits at the anterior end of the hindbrain and is responsible for the integration of sensory and motor processing in the mature brain as well as regulating higher cognitive processes[1]. In mammals and birds, it possesses an elaborate morphology and is heavily foliated, a product of extensive transit amplification of progenitors during development that produces over half of the neurons in the adult brain. The cerebellum has been a subject of study for neurobiologists for centuries and in the molecular era has likewise received significant attention. This relates not only to its inherently interesting biology, but also to the fact that it is heavily implicated in human disease including developmental genetic disorders such as autism spectrum disorders[2] and most prominently the cerebellar cancer, medulloblastoma[3], which is the most prevalent paediatric brain tumour. Importantly, it is an excellent model system within which to study fate allocation and neurogenesis during brain development[4]. In recent years, it has also been established as a model system for the comparative study of brain development, owing to the huge diversity of cerebellar forms seen across the vertebrate phylogeny[5-10].

The cerebellum develops from the dorsal half of rhombomere 1 in the hindbrain[11] and developmentally is comprised of two primary progenitor populations, the rhombic lip and the ventricular zone. The rhombic lip extends around the dorsal region of the neuroepithelium of the hindbrain at the border with the roof plate. It is the birthplace of the glutamatergic excitatory neurons of the cerebellum[12-14]. The ventricular zone gives rise to the inhibitory GABAergic cerebellar neurons, most prominently the large Purkinje neurons[14,15]. Later in development (from about embryonic day 13.5 in mouse; e6 in chick[16]), glutamatergic progenitors migrate tangentially from the rhombic lip and form a pial layer of progenitors: a secondary progenitor zone called the external granule layer (EGL). It is this layer that undergoes the extensive transit amplification that leads to the huge numbers of granule neurons found in the mature brain.

Proliferation in the EGL has long been linked to the sub-pial location that results from tangential migration from the rhombic lip[17], with the switch to cell cycle exit and neuronal differentiation of progenitors being associated with their exit from the outer EGL layer into the middle EGL[18]. Extensive tangential migration of post-mitotic granule cells in medial-lateral axis occurs in the middle and inner EGL[19], before final radial migration into the inner granule layer of the mature cerebellar cortex. Migration of cells from the rhombic lip over the cerebellar surface is dependent upon CXCL12 signalling from the pia[20-22] and granule cells express the CXCL12 receptor CXCR4. Their tangential migration is thus reminiscent of that of neocortical tangentially migrating inhibitory interneuron populations[23-25]. Intriguingly, electron microscopic studies[17] have suggested that EGL cells with a proliferative morphology maintain pial contact, linking cell behaviour with proliferative capability in a

manner reminiscent of the basal progenitors of the mammalian cortex[26]. This is reflected in the aforementioned stratification of the EGL into three sublayers that are defined by distinct extracellular environments and where granule precursors have distinct gene expression signatures[18].

Proliferation of progenitors in the oEGL occurs with a normal distribution of clone sizes such that when progenitors are individually genetically labelled at the end of embryonic development in the mouse, they give rise to a median average of 250-500 postmitotic granule neurons[27,28]. Proliferation is dependent upon mitogenic SHH signalling from underlying Purkinje neurons[29-32]. The ability to respond to SHH has been shown to be entirely dependent upon cell autonomous expression of the transcription factor *Atoh1*, both *in vitro*[33] and *in vivo*[34,35]. Likewise, cell cycle exit and differentiation has been shown to be dependent upon the expression of the downstream transcription factor *NeuroD1*[36], which is likely a direct repressor of *Atoh1*[37].

Despite this progress, and considerable advancement in deciphering the cell biological basis of cell cycle exit[38-42], the fundamental molecular mechanism(s) that underlie the decision to exit the cell cycle and to transition from a progenitor to a differentiating neuron, and the associated postmitotic tangential migration in the inner EGL as well as the later switch to radial migration, remain incompletely understood. This is to a large extent because of the experimental intractability of the EGL: it is late developing, and difficult to target genetically since many of the same neurogenic molecules are also crucial earlier in the life of granule precursors at the rhombic lip. To overcome this issue, numerous authors have developed *in vivo* and *ex vivo* electroporation as a method to target the postnatal cerebellum in rodents[43-48]. Here, we pioneer the use of *ex vivo* electroporation in chick to study the EGL, which represents considerable advantages in terms of cost and convenience. Our method of electroporation and *ex vivo* slice culture of chick cerebellar tissue uses tissue dissected from embryonic day 14 chicks at the peak of EGL proliferation. This method allows genetic targeting of the EGL independently of the rhombic lip and will set the stage for genetic dissection of the transition from granule progenitor to postmitotic granule neuron in the cerebellum.

Protocol

Note: All experiments were performed with accordance to King's College London, UK animal care guidelines.

1. Dissection of e14 cerebellum

1.1) Incubate brown fertilised hens' eggs at 38 ^{O}C to embryonic day 14.

1.2) Using egg scissors decapitate chick embryo *in ovo* and remove head to a petri dish containing ice cold PBS (Fig 1A).

1.3) Using standard forceps, make incisions behind each eye all the way through the tissue, removing the eyes and upper jaw. Make a second incision all the way through the pharynx removing the lower jaw (Fig 1B).

1.4) Using standard forceps, remove all skin from surface of skull by peeling it away (Fig 1C) and remove frontal and parietal bones, revealing brain.

1.5) From a ventral aspect, remove pharyngeal cartilage and auxiliary mesenchyme.

1.6) From a dorsal aspect, carefully remove mesenchyme dorsal to the hindbrain taking care not to damage pia, and separate entire brain (Fig 1D).

1.7) Make incision all the way through the tissue between midbrain and hindbrain and separate hindbrain including cerebellum (Fig 1E).

1.8) By making incisions all the way through the tissue at both lateral junctions of cerebellum and alar plate of the hindbrain, remove entire cerebellum, taking care to maintain the integrity of the pia throughout dissection (Fig 1F). Remove the forming choroid plexus (Fig 1G).

1.9) Move the dissected cerebellum into ice cold HBSS.

2. Slice culture of e14 cerebellum

2.1) Transfer the whole cerebellum to the sterile platform of a Tissue Chopper using a spatula or a 3ml Pasteur pipette with the tip cut away to widen the aperture. Remove excess liquid using a pipette.

2.2) Cut entire whole cerebellum in the required orientation at 300 μm thickness using the tissue chopper set with a cutting speed at 50% of the maximum. Tissue integrity is most easily preserved in sagittal section; however, orientation is also an important consideration for cell analysis (Purkinje cell dendrites are sagittally aligned, while granule cell axons run transversely, perpendicular to the plane of Purkinje cell dendrites).

2.3) Using a 3 ml Pasteur pipette, cover the sliced cerebellum in fresh culture medium: Basal Medium Eagle, 0.5% (w/v) D-(+)-glucose, 1% B27 supplement, 2 mM L-Glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin.

2.4) Transfer the culture medium containing the slices to a 60 mm petri dish containing ice cold culture medium using a 3ml Pasteur pipette with cut tip.

2.5) Under a dissecting microscope illuminated with a fiber optic light source, ensure separation of individual slices using watchmaker forceps. Identify slices to be electroporated, based upon their tissue integrity and medio-lateral position.

Note: Each dissection from embryo through to slice incubation in culture medium takes around 10-20 minutes depending upon experience. Perform dissections one at a time to ensure that cerebella spend minimum amount of time between decapitation and *ex vivo* culture.

3. Electroporation of slices

3.1) Place 0.4 µm culture insert into fresh 60 mm petri dish and soak in culture medium.

3.2) Transfer identified slices (up to five per culture insert) onto culture insert using 3 ml Pasteur pipette with the cut tip. Separate and allow to settle onto culture insert in a sagittal orientation.

3.3) Using a pipette, remove excess culture medium so that slices are no longer bathed in solution.

3.4) Transfer culture insert to the electroporation chamber (Fig 2A). Construct an electroporation chamber by fixing the anode of an electroporator to the base of a 60 mm petri dish with insulation tape. Ensure the dish contains approximately 1 ml of culture medium to cover the electrode. Allow the culture insert to rest on the electrode making sure there is always contact between the insert and the electrode.

Note: In this setup, the culture insert with the slices will rest upon the surface of the medium, maintaining the circuit but allowing spatial targeting of the cathode.

3.5) Using a P10 pipette tip, pipette DNA (at a concentration of 1 µg/µL) diluted with 20% fast green over the surface of targeted region of a slice. 20% fast green ensures viscous DNA solution and prevents prohibitively wide dispersal of DNA. Add approximately 5 µL DNA/fast green solution to each slice (Fig 2B).

3.6) Place the cathode over desired targeted tissue and electroporate with 3 x 10 V, 10 ms duration pulses. Avoid direct contact of the cathode with the tissue. Instead, take advantage of surface tension of the liquid to maintain conductance (place the cathode as close to the tissue as possible without actually touching the tissue).

3.7) Repeat DNA delivery and electroporation to multiple regions of EGL on each individual cerebellar slice as desired.

3.8) Upon completion of electroporation, transfer culture insert to 30 mm petri dish.

3.9) To each culture, add 1 ml of pre-warmed culture medium underneath the culture insert such that the culture insert is in contact with medium, but slices are not bathed in it.

3.10) Incubate cultures at 37 ^{O}C/6% CO_2 for up to 3 days. Replace all of the culture medium every 24 hours with fresh pre-warmed medium.

3.11) Following culture, fix slices on culture inserts for 1 hour in 4% paraformaldehyde (or overnight at 4 ^{O}C) in a fresh 30 mm dish with no culture media.

4. Imaging of cerebellar slices

4.1) Following fixation, wash slices 3x five minutes in PBS.

4.2) Using a razor blade, dissect each electroporated and cultured cerebellar slice from the culture insert by cutting around the slice and removing the slice and the region of insert it is adhered to. Do not attempt to remove slice from the culture insert surface.

4.3) Mount slices in approximately 1 ml of mounting medium containing DAPI (if desired) under a coverslip taking care not to introduce bubbles, and image using laser-scanning confocal microscopy.

Note: Imaging parameters can be varied according to the constructs electroporated, and at the discretion of the individual user.

Representative Results
This section illustrates examples of results that can be obtained using slice electroporation and culture of cerebellum from embryonic day 14 chick. The dissection of the cerebellum is illustrated in Figure 1 and the electroporation chamber set up is shown in Figure 2. We show that it is possible to electroporate and successfully culture cerebellar slices, which retain their structure and cellular morphologies in vitro (Fig 3A). Targeted electroporation to individual folia is easily achieved (Fig 3B). We successfully electroporate a number of different plasmids into the EGL cells and show that it is possible i) to label cells with reporter constructs to observe their behaviour (Fig 3C), ii) to test possible genomic regions for functionality in cerebellar cells (Fig 3D), and iii) to manipulate genetically the cells in the EGL by misexpressing proteins of interest (Fig 3E). Additionally, pharmacological manipulations on electroporated slices are possible (results not shown). After culturing it is possible to perform additional tissue analysis such as immunohistochemistry or proliferation assays (Fig 3F). We perform tissue health analysis by calbindin and PH3 immunostaining and show that tissue integrity is maintained for at least 3 div after culture (Fig 4). These results demonstrate that the EGL is now an accessible and easily manipulated structure that can be fully examined and genetically altered in the chick model system.

Figure 1 Dissection of the cerebellum from E14 chick embryos:
A. Decapitate the chick in the egg and remove the head into a petri dish with ice cold PBS. Remove the lower jaw and the eyes by making incision behind the eyes and the pharynx (dashed line). **B.** Remove the skin from the surface of the skull. **C.** Remove the frontal and parietal bones and (**D**) remove the brain from the mesenchyme and cartilage surrounding it. **E.** Under a dissecting microscope identify the location of the cerebellum at the posterior end of the brain. Cut between the midbrain and the hindbrain (dashed lines) to be left with the cerebellum and ventral hindbrain. **F.** Make incisions at the lateral junctions (peduncles) of the cerebellum to separate the cerebellum from the hindbrain (dashed line). **G.** Remove the choroid plexus (asterisk) from the ventral side of the cerebellum until you are left with a whole intact cerebellum with the pia attached. Transfer the cerebellum into ice-cold HBSS before preparing slices with a tissue chopper.

Figure 2 The electroporation chamber set up:
A. A picture of the custom-made electroporation chamber. The chamber consists of an anode of an electroporator placed securely on the base of a 60 mm petri dish. The dish contains approximately 1 ml of culture medium to cover the electrode. The culture insert

should rest on the electrode with constant contact between the insert and the electrode, maintaining the circuit but allowing spatial targeting of the cathode, which is manipulated by hand. **B**. A picture of slices being electroporated. Slices are covered with the DNA/fast green solution. The slices are electroporated as desired: electroporation can be targeted to one folium or multiple locations. After electroporation the insert is placed in a 30mm Petri dish with pre-warmed culture medium and cultured in the incubator. **1.** The anode **2.** The cathode **3.** Culture insert **4.** Petri dish with 1ml media **5.** Dissecting microscope **6.** Individual slices from tissue chopper **7.** DNA solution with fast green dye.

Figure 3 Representative results:
A. A low magnification picture of control electroporation of an RFP encoding plasmid into the EGL at multiple locations. The tissue retains its structure and electroporated cells are clearly visible in a thick subpial layer of the cerebellum**. B**. An example of a targeted electroporation with control GFP plasmid. The targeted folium is indicated by an asterisk. Scale bar A-B = 500µm. **C.** An example where a construct encoding GFP driven by an Atoh1 enhancer has been electroporated into the EGL. The expression of *Atoh1* defines granule cell precursors within the EGL. Various cell morphologies are clearly visible at 3 div and cell behaviour can be monitored. **D.** An example of labelling following the electroporation of a construct containing a putative conserved non-coding element (CNE) of the NeuroD1 gene driving GFP. The CNE reports activity in the cells expected based on endogenous NeuroD1 expression suggesting an active role of this CNE in development. NeuroD1 expression correlates with the initiation of granule cell differentiation. **E.** An example where the tissue can be genetically manipulated by misexpression of NeuroD1 protein and a change in granule cell behaviour can be observed**. F.** An example where the electroporated tissue (control GFP plasmid) can be fixed and stained for markers of proliferating cells, such as phosphohistone H3 (PH3). Scale bar C-F = 50µm

Figure 4 Tissue integrity and proliferation in culture
A-C Calbindin staining of E14 cerebellar tissue electroporated with control GFP plasmid at 1-3 div. Calbindin staining shows that tissue integrity is maintained in culture for at least 3 div. The Purkinje cell layer does not form a monolayer at this stage in chick development but it is clearly seen forming a layer underneath the EGL where granule cell precursors (green) are located. **D**. Phosphohistone H3 (PH3) staining on cerebellar tissue cultured for 2 div. PH3 staining is visible in the EGL (arrows) but also in other cerebellar regions (arrowheads). This staining is representative of all stages in culture examined (1-3 div). Scale bar A-D = 50µm

Discussion
The protocol reported here describes a method for dissecting, electroporating and culturing slices of embryonic day 14 cerebellum from the chick. This protocol enables targeting of electroporation to small focal regions of the EGL, including isolated targeting of individual cerebellar lobes. It enables genetic analysis and imaging at a high resolution and convenience, and at a low cost compared to established techniques in rodents[43-47]. Such analysis is not currently possible *in vivo* due to the extended developmental time period, the paucity of EGL-specific genetic targeting possibilities in mouse, and the commonality of molecular mechanisms between the rhombic lip and the EGL, which means that alterations that may affect EGL biology frequently cannot be analysed since they affect rhombic lip

neurogenesis and abrogate EGL formation[49]. Our system thus represents a major advance in terms of targeting genetic modification to the EGL specifically, and we anticipate it will be applicable to other species beyond the chick that maybe of comparative interest, such as non-model mammals and reptiles.

In performing the slice culture electroporation technique, a number of technical considerations are paramount. Firstly, the robustness of slices to survive in culture without on the one hand undergoing extensive cell death or on the other losing structural integrity limits the thickness of slice to 300 μm in our hands. A second important consideration is the viscosity of the DNA solution, which ensures electroporation of DNA at concentrations that are high enough to induce visible or relevant levels of genetic modification. In *in ovo* electroporation of DNA solutions injected into the early embryonic hindbrain, concentrations of fast green dye of approximately 1% are typical. However, in our protocol, we typically use concentrations of fast green of 20%. This ensures a sufficiently viscous DNA solution to prevent dispersal of the DNA following pipetting but before electroporation, but a sufficiently dilute one to mediate efficient electrical conduction.

In addition to these technical considerations, we observed that proliferative behavior that we observe *ex vivo* does not precisely match that predicted from *in vivo* studies probably due to pial integrity being disrupted by the tissue preparation. Under such conditions, when a GFP expression construct is electroporated, a large proportion of electroporated cells appear to have left the cell cycle after just one day of culture as judged by PH3 staining (Fig 3F). This does not correlate with expected EGL proliferative behavior, where proliferation of clones in both mouse and chick extends over a large time period[27]. The implication that the pia modulates proliferation is supported by the observation that when we electroporate a reporter construct with a *NeuroD1* regulatory element driving expression of GFP all electroporated cells express GFP after one day in culture (Fig 3D). *In vivo*, this construct mirrors endogenous *NeuroD1* expression in marking cells of the inner EGL that are post-mitotic[36,37], while full length NeuroD1 is sufficient to drive cell differentiation (Fig 3E). This suggests that under certain conditions, the proliferative capability of cells may not be maintained as it is in the outer EGL at equivalent stages *in vivo*. PH3 staining does however suggest that there is a lot of proliferation in culture, often localized to the EGL area (Fig 4D). Extensive proliferation outside the EGL indicates possibly enhanced gliogenesis or proliferation of Pax2 GABAergic precursors in white matter. The implication is that interpretation of any experimental procedure will have to take the into account the above proliferative behaviour, the fact that Purkinje cells do not form a monolayer until E18 in chick and that normal development may be compromised after slice preparation (e.g. due to lack of interaction with climbing fibres etc.)

Despite these limitations, our protocol represents a significant step forward in relation to studying many aspects of granule cell biology. The crucial advantage of enabling spatially and temporally specific labeling of the EGL as distinct from the rhombic lip will facilitate multiple examinations of the both the cell biology of granule progenitors and the genetic regulation underpinning it in a manner that is not possible at present *in vivo*. Our technique in chick will complement existing *ex vivo* culture and electroporation protocols in rodents[43-48] and carries the considerable advantages in cost and convenience that are associated with chick. Additionally, it represents a significant advance over existing techniques of culturing

granule progenitors[39]. While it will complement rather than replace the latter, our protocol will open up the control of granule neuron differentiation to a wide variety of pharmaceutical treatments and to the diversity of cell autonomous genetic manipulations that are possible in the chick. It provides a foundation for examining granule cell biology in unprecedented detail.

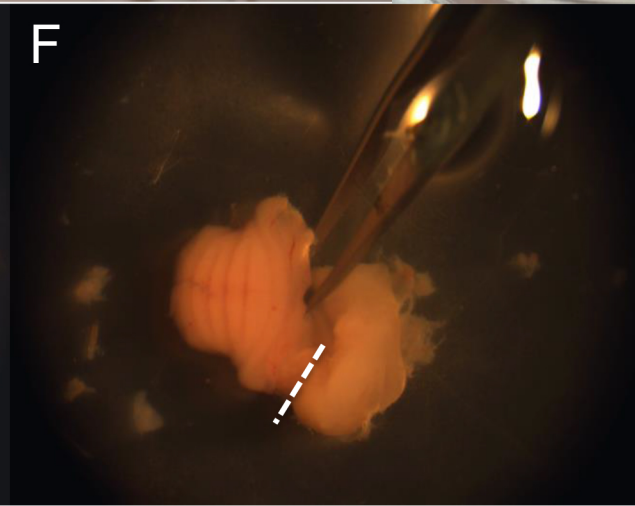**Disclosures**
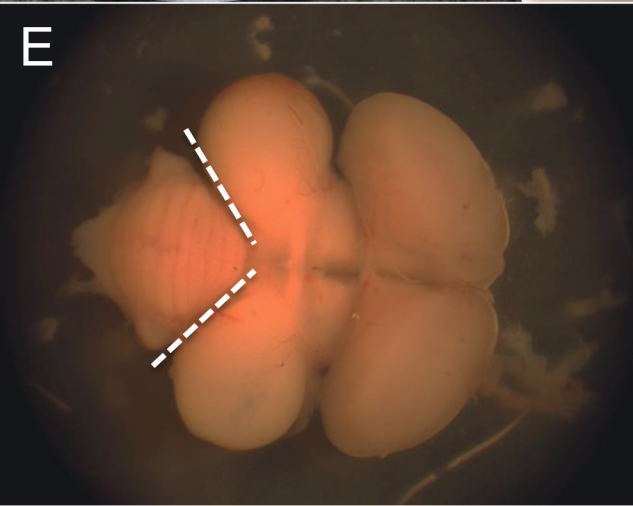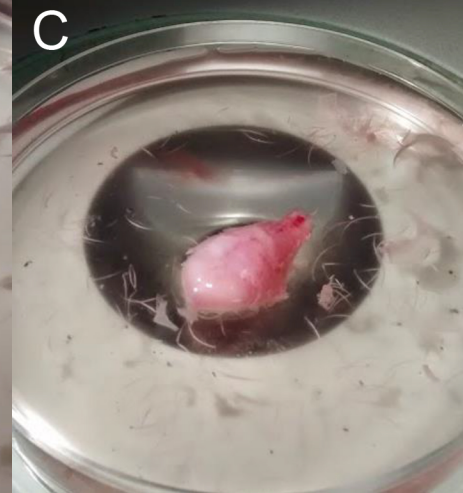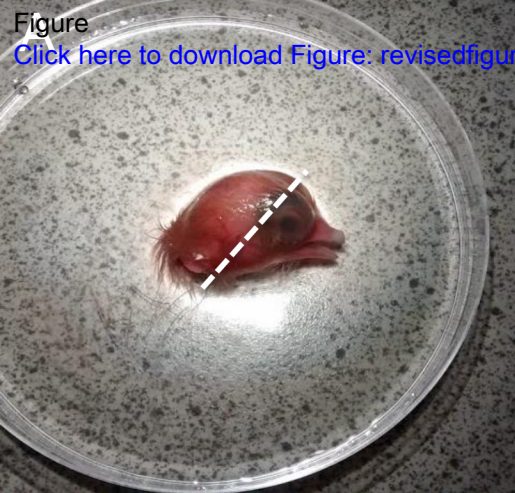The authors have nothing to disclose.

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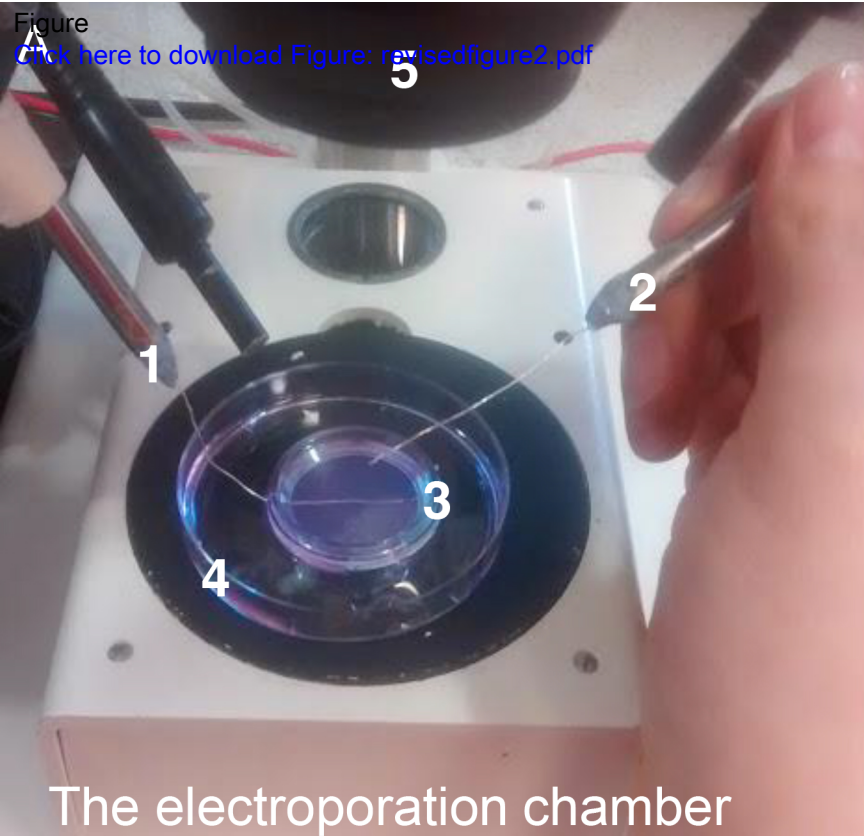
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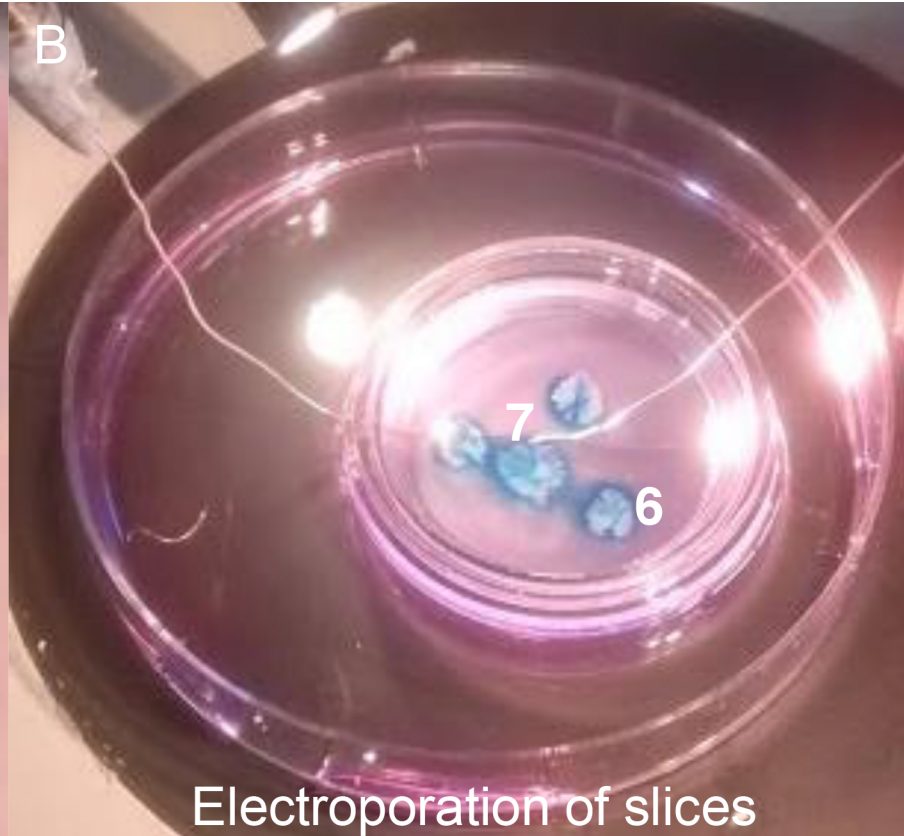
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A

5

2

1

3

4

The electroporation chamber

B

7

6

Electroporation of slices

A Calbindin GFP
PL
EGL
1 div

B Calbindin GFP
PL
EGL
2 div

C Calbindin GFP
EGL
PL
3 div

D PH3
2 div

Excel Spreadsheet- Table of Materials/Equipment
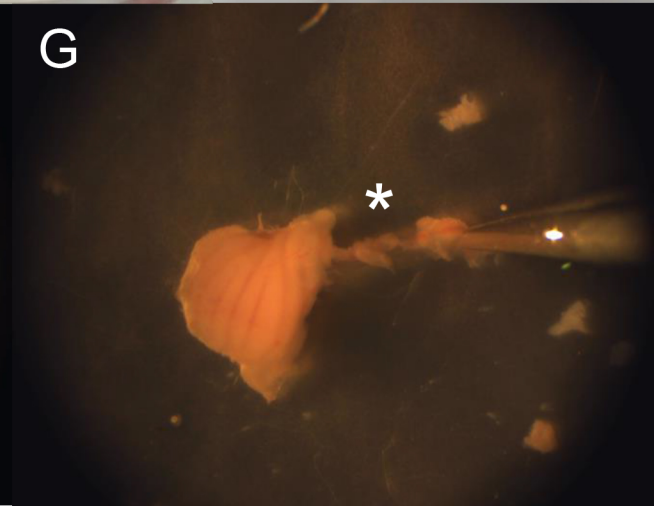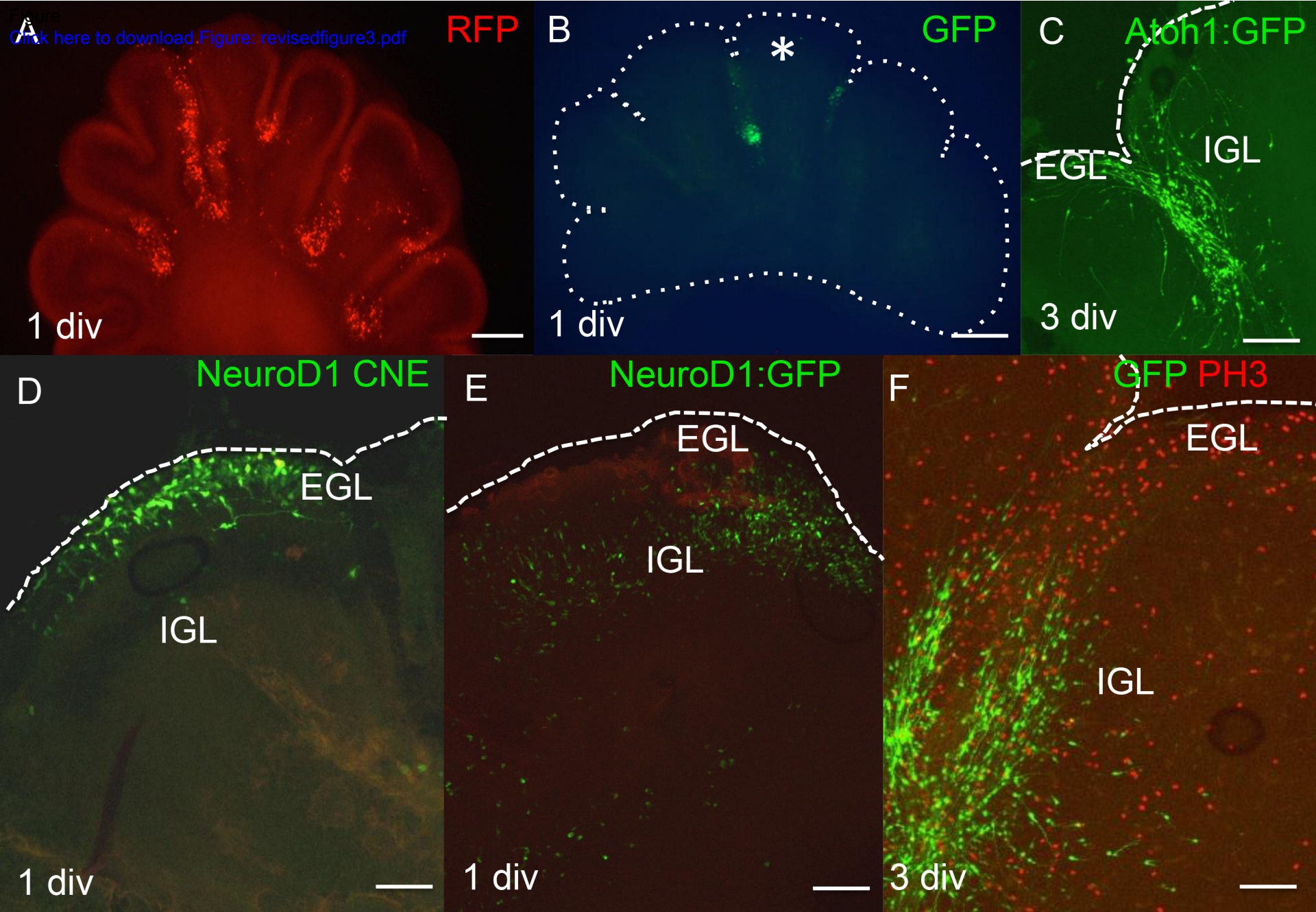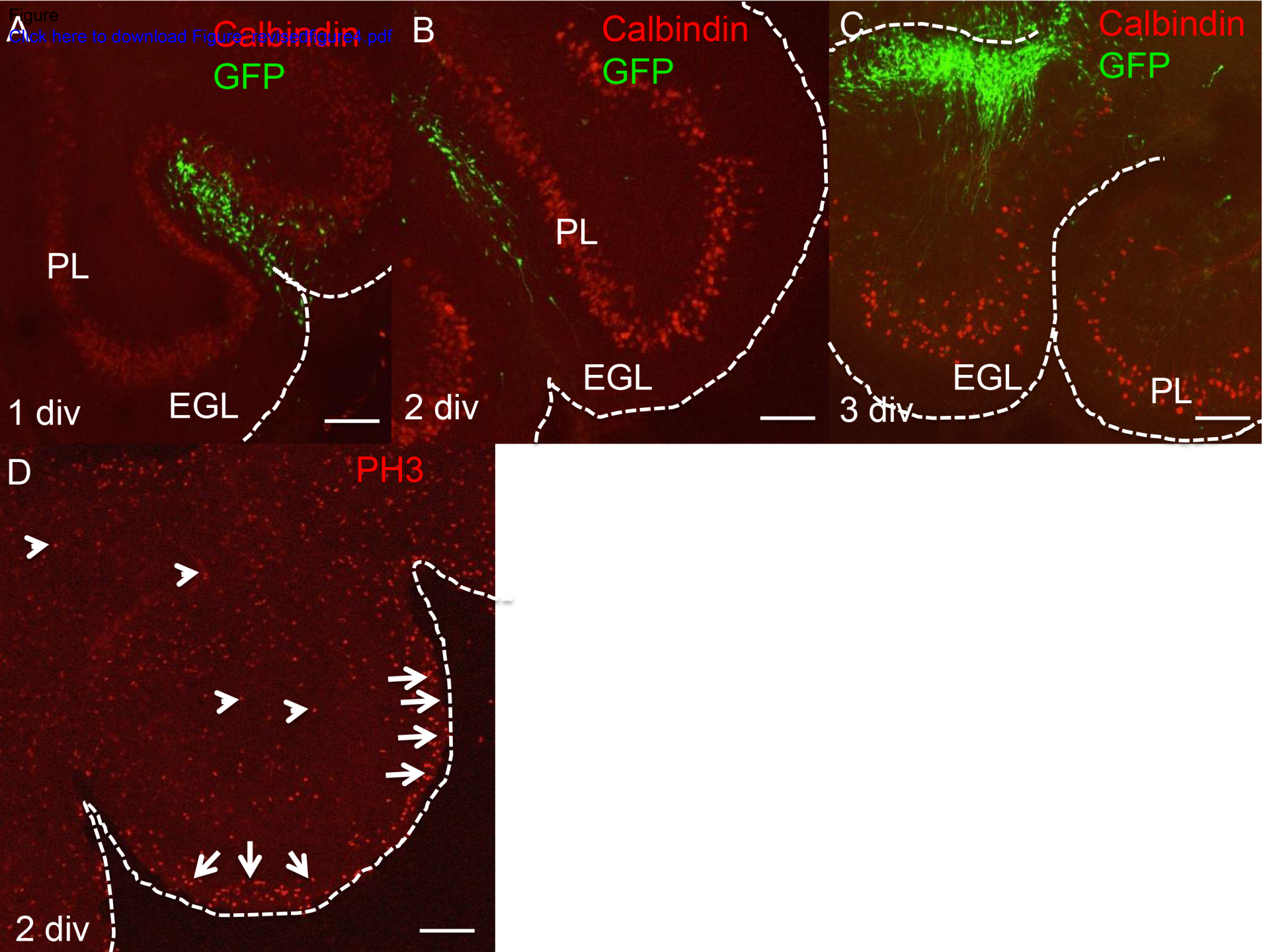
Name of Material/ Equipment	Company	Catalog Number	Comments/Description
McIlwain tissue chopper	Mickle Laboratory Engineering Ltd		Cut at 300µm for best results.
Basal Medium Eagle (Gibco)	Life Technologies	41010-026	
L-glutamine	Sigma	G7513	
penicillin/streptomycin	Sigma	P4333	
0.4µm culture insert	Millipore	PICM0RG50	
TSS20 Ovodyne electroporator	Intracel	01-916-02	Use 3x10v, 10ms pulses for electroporation.