Video Article

Visualization of Cortical Modules in Flattened Mammalian Cortices

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Abstract

The cortex of mammalian brains is parcellated into distinct substructures or modules. Cortical modules typically lie parallel to the cortical sheet, and can be delineated by certain histochemical and immunohistochemical methods. In this study, we highlight a method to isolate the cortex from mammalian brains and flatten them to obtain sections parallel to the cortical sheet. We further highlight selected histochemical and immunohistochemical methods to process these flattened tangential sections to visualize cortical modules. In the somatosensory cortex of various mammals, we perform cytochrome oxidase histochemistry to reveal body maps or cortical modules representing different parts of the body of the animal. In the medial entorhinal cortex, an area where grid cells are generated, we utilize immunohistochemical methods to highlight modules of genetically determined neurons which are arranged in a grid-pattern in the cortical sheet across several species. Overall, we provide a framework to isolate and prepare layer-wise flattened cortical sections, and visualize cortical modules using histochemical and immunohistochemical methods in a wide variety of mammalian brains.

Video Link

The video component of this article can be found at https://www.jove.com/video/56992/

Introduction

Some of the most significant changes in the brain structure across phylogeny can be observed in the cerebral cortex. Despite significant differences, the cortex of animals follows a common pattern and can be broadly divided in two distinct ways, by layers and areas[1]. Cortical layers lie parallel to the surface of the brain and vary in number from 3 layers in reptilian cortices[2] to 6 layers in mammalian cortices[1]. Cortical areas on the other hand are distinct regions of the cortex which largely correspond to distinct functionalities, *e.g.*, the somatosensory cortex is involved in the sensation of touch or the visual cortex in processing visual inputs. These cortical areas can often be subdivided into patches or modules[3], which are regularly repeating anatomical structures, essentially found parallel to the pial surface of the brain. Cortical modules may be confined to a particular layer[4], or extend across several layers[5].

Standard sectioning methods of the brain involve sections normal to the surface of the brain, like coronal or sagittal. While these methods can be used to visualize cortical modules, a multitude of interesting features can be revealed when the cortical modules are visualized tangentially, in a plane parallel to the surface of the brain. For instance, somatosensory modules in the rodent brain representing whiskers, appear as barrels when visualized normal to the brain surface, and thus the regions derive the name barrel cortex. However, on visualizing the barrels in a tangential orientation, they reveal a whisker-map, with the barrels being laid out in a topographic orientation mirroring the exact layout of the whiskers on the external body surface. In certain cases, modular arrangement has even escaped detection for considerable periods, when visualized in a non-tangential manner. The medial entorhinal cortex, is known for the presence of grid cells, neurons which fire in a regular hexagonal pattern when an animal is traversing an environment. Even though it is a heavily investigated area, until recently, the presence of patches or modules of cells in the medial entorhinal cortex, which are physically laid out in a hexagonal pattern[6], had escaped detection. The presence and arrangement of these modules, in the rat brain, was facilitated by making tangential sections of the medial entorhinal cortex and investigating the cytoarchitecture in a layer-wise manner.

Subsequent to sectioning, the particular aspect of visualization of cortical modules can also be realized in multiple ways. Classically, studies have delineated modules based on cell density or fiber layout[1]. Another popular approach is the use of cytochrome oxidase histochemistry, which reveals areas of higher activity[8]. Newer approaches include looking at genetically determined cell types, distinguished on the basis of their protein expression profiles[6,8].

In this study, we highlight methods to isolate the cortex from mammalian brains, obtain flattened tangential sections, and visualize cortical modules based on cytochrome oxidase histochemistry and immunohistochemistry of cell-type specific proteins.

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Protocol

All experimental procedures were performed according to the German guidelines on animal welfare under the supervision of local ethics committees (LaGeSo). Human and bat brain data were derived from Naumann *et al.*[5] The following procedure is performed on a male adult Wistar rat (strain: RJHan:WI).

1. Perfusion and Brain Extraction

NOTE: In order to obtain a homogenously fixed and blood-free brain, transcardial perfusion of the animal is highly encouraged, as residual blood increases unspecific background signal during staining. Nevertheless, it is also possible to obtain flattened sections from un-perfused specimen and stain them. The ease of handling the specimen varies with the concentration of fixative used. Too little fixation increases the risk of handling damage to the brain during flattening and cutting, while too high concentrations lower the flexibility for flattening and the quality of the staining signal.

1. Transcardially perfuse the animal, using a 23G needle. For a more detailed guide, see Gage *et al.*[9]
    1. Use phosphate buffer saline[10] (PBS; 0.02 M; pH 7.4) to flush out blood from the brain and body of the animal. Continue until infusion liquid appears clear, with at least 150 mL PBS being infused through the vascular system.
    NOTE: Best results are achieved at a pressure similar to the range of the physiological blood pressure of the animal being perfused (*e.g.*, rat: 120-130 mmHg). Optionally, add heparin (10 U/mL) to the solution to avoid blood coagulation[11].
    2. To fix the brain, transcardially infuse a minimum of 100 mL paraformaldehyde[12] (PFA, 4%; in 0.1 M phosphate buffer (PB)[13]) until the neck of the animal is stiff.
    CAUTION: PFA is a potential carcinogen.
    NOTE: Best results for histochemical activity staining, such as for cytochrome oxidase, are achieved, when using 2% PFA. For other staining, such as immunohistochemistry, 4% PFA is preferred.

2. Extract the brain from the skull.
NOTE: See Gage *et al.*[9] for details.
    1. Isolate the head using large scissors or bone shears.
    2. Using fine scissors, cut the skin along the midline from neck to nose. Pull apart the skin to expose the skull. Remove the neck and temporal muscles using fine scissors.
    3. Make a midline cut through the interparietal bone starting from the foramen magnum up to the parietal bone.
    4. Use Rongeurs to peel off the interparietal bone. Slide fine scissors along the sagittal suture and cut from the posterior end of the parietal to the anterior end of the frontal bones.
    5. Use Rongeurs to peel off the parietal and frontal bones. Alternatively, lift the bones away using forceps. Use a spoon to cut ventral nerve cords and remove the brain. Transfer the brain to PB (0.1 M; pH 7.4).

3. Fix un-perfused brains by immersion in 4% PFA for 1-3 h at 4 °C (depending on the brain size and probe: Shrew: ~ 1 h, Human: ~ 3 h).
NOTE: For large and gyrencephalic brains like humans, isolate and cut out the area of interest to reduce fixation time.
4. To obtain larger cortical areas in a section, flatten the brain prior to further post-fixation; proceed to step 2. However, to obtain sections of harder to reach areas like the medial entorhinal cortex, post-fix the brain in 4% PFA for 24 h before proceeding to step 2.

2. Brain Dissection and Flattening

1. Separate the brain hemispheres by using a razor blade (lissencephalic brains) or scalpel (gyrencephalic brains).
NOTE: If the brain has not been post-fixed, it is susceptible to damage by handling.
2. Optional step for aiding subsequent section registration and shrinkage estimation:
    1. Puncture the cortex in an area of non-interest, with a 35G needle normal to the cortical surface. Repeat the step two times at defined distances along the cortical sheet. To determine the distance along the cortical sheet, use a pre-cut thread at a fixed length (*e.g.*, 5 mm) and lay it along the cortical surface to determine points of puncture.

3. Lissencephalic brains: Remove subcortical structures by using a dissecting spatula. Critical: Keep the brain moist with PB (0.1 M; pH 7.4) throughout the whole procedure.
    1. Hold the brain by the cerebellum and gently insert the spatula to open a dissection plane in the corpus callosum. The round tip of spatula should point away from the cortex.
    2. Slide the spatula further and pull gently until the spatula is between the thalamus and cortex. Separate subcortical structures with scratching motions.
    3. Inspect the hemisphere for even thickness.
    NOTE: Any uneven regions might result in a trans-layer gradient during sectioning. For tangential sectioning, use a scalpel to make a clean cut through the brain, parallel to the pial surface of the region from which tangential sections are desired.
    4. Optional steps to improve flattening quality:
        1. Make a clean cut through the striatum, nucleus accumbens, and orbitofrontal cortex, since they increase the thickness of the cortex in the lateral region. Also, add a relieving cut at the base of the inner region of prefrontal cortex, which allows unfolding of medial portions of the cortex.

    5. Place the hemisphere (cortex facing down) on a glass slide. Proceed to step 2.6.

4. Gyrencephalic brains: Remove white matter to unfold gyri (for detailed protocols, see: Sincich *et al.*[14]; Tootell and Silverman[15]). Critical: Use PB (0.1 M; pH 7.4) to keep the brain moist at all time.

1. Put the area of interest on a damp filter paper in a large Petri dish, with the cortex facing up.
2. Remove the arachnoid membrane and pia using two forceps.
3. Use a damp cotton swab and insert gently in each sulcus to detach adhesions between gyri.
4. Turn the brain around by using another damp filter paper.
5. Use two curved micro spatulas to unfold single gyri. Use the convex side of the spatulas to tease apart the white matter until reaching close to the concave end of the gyrus. Use a damp cotton swab and proceed with swirling motions to reach the concave end of the gyrus.
6. Tease apart single gyri. If necessary, add small relieving cuts if the tension is too high.
7. Flatten the unfolded brain in the Petri dish or a similar container.

5. Optionally, place larger brains on a filter paper covered sponge, to ensure that regions do not dry out.
6. Place two rolled pieces of clay on both sides. Critical: As the thickness of the clay defines how much the hemisphere can be flattened, ensure that the clay is 10-20% thinner than the un-flattened cortex.
7. Gently press a second glass slide/small Petri dish on the cortex until the hemisphere is fully flat. To obtain the best results for a desired region, place the glass slide first on the respective area. To obtain the best results for lissencephalic brains, place the glass slide tangentially at the lateral portion first and apply concentrated pressure on this region.
8. Put a weight (*e.g.*, a ceramic watch glass) on the glass slides/Petri dish and flatten the hemisphere for 3-5 h at 4 °C in PB.
9. For post-fixation, release the pressure and remove the glass slides. Put the flattened hemisphere in PFA (free floating) for 24 h on a shaker at 4 °C.
   NOTE: Best results for histochemical activity stainings are achieved when using 1% PFA. For other stainings, such as immunohistochemistry, for un-perfused brains, 2% PFA can be used.

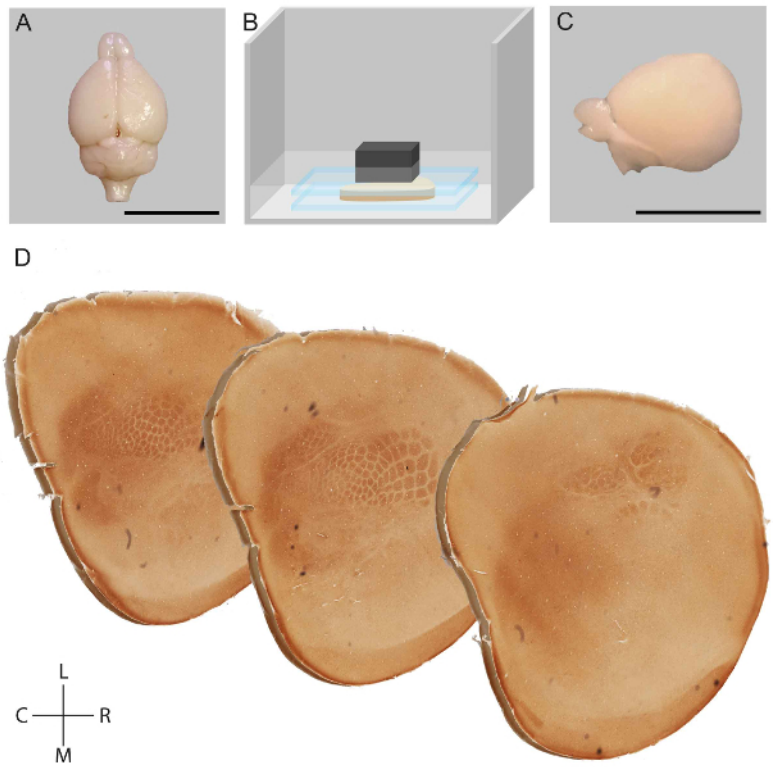


Figure 1: Schematic representation of workflow for flattening of a rat cortical hemisphere and visualization of modules in somatosensory cortex. Subsequent to the transcardial perfusion, the brain of a mouse was dissected (**A**). Subcortical structures were removed and cortex was flattened between two glass slides in phosphate buffer (**B**). Flattened hemisphere (**C**) was post-fixed, tangentially sectioned, and stained for cytochrome oxidase activity (**D**). Scale bars = 1 cm. R: Rostral, C: Caudal, L: Lateral, M: Medial. Figure adapted from Lauer *et al.*[23]
Please click here to view a larger version of this figure.

3. Cutting Tangential Sections

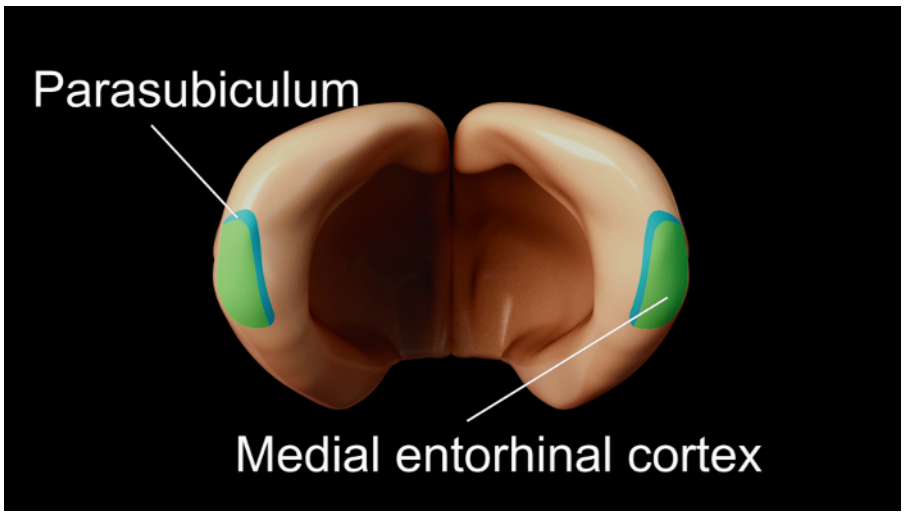
NOTE: Depending on the requirements of the staining protocols, the cutting procedure and thickness can be adapted. A vibratome was used to cut the hemispheres for further histochemical processing (step 3.2) at 80-150 µm. However, for immunohistochemical processing, thinner sections are desired and a freezing microtome was used for sectioning (step 3.3) at 10-60 µm. See **Video 1**.

1. Wash the flattened hemisphere in PB (0.1M; pH 7.4) for 15 min.
2. Cut the hemisphere on a vibratome.

1. Place the hemisphere tangentially on the slicing holder. Optionally, press again gently with a glass slide before fixing it in position (*e.g.*, with superglue).
   NOTE: Minimize the amount of glue used, because cutting artifacts could result due to the small thickness of flattened hemispheres.
2. Cut the hemisphere from the thicker and more stable end towards the thinner end (posterior to anterior, here) at the required thickness. Proceed to step 3.4.
   NOTE: If low fixation concentrations were used, cut it at a slow speed and a high amplitude.

3. Cut the hemisphere on a freezing microtome.
   1. Cryoprotect the brain by immersing it in a 30% sucrose solution (in PB) until it sinks.
      NOTE: Depending on the size of the tissue being cryoprotected, sinking in the solution can range from a few hours to a few days. For larger brains alternative cryoprotection methods might be considered (see Rosene *et al.*[16]).
   2. Form an ice base on the freezing microtome to mount the brain. Construct the ice base by freezing PB on the block face of the freezing microtome. Critical: Ensure that the surface of the ice base is parallel to the microtome blade. To do this, cut the empty ice base using the microtome blade to exactly align the ice base parallel to the cutting surface.
   3. Embed the brain in freezing medium and mount it to the block face of the microtome with the area of interest parallel to the block face. Face the pial surface of the brain towards the block face of the microtome. Critical: Adjust the freezing temperature depending on sample size; higher temperatures ensure better section integrity while sectioning, but larger sections require lower temperatures to freeze sample uniformly.
   4. Cut the brain tangentially at the required thickness (slower and uniform cutting speeds result in best section quality).

4. Wash the sections in PB for 15 min on a shaker.



Video 1: Schematic video of tangential sectioning from a rat medial entorhinal cortex and layout of parasubicular and entorhinal modules. The medial entorhinal cortex of a rodent brain is situated at the posterior end of the cortex and is tilted towards the medial and ventral side. Tangential sections are obtained by orienting a knife along this angle. Consequently, appropriate cell-type specific staining reveals modular structures in the medial entorhinal cortex and adjoining parasubiculum. Video adapted from Ray and Brecht[8]. Please click here to view this video. (Right-click to download.)

4. Visualization of Cortical Modules Using Cytochrome Oxidase Staining

NOTE: Different staining protocols have been developed for histochemical detection of cytochrome oxidase activity, *e.g.*, first by Wong-Riley[17] and later modified by Divac *et al.*[18] This protocol is based on the one by Divac *et al.*[18], since the use of nickel-ammonium sulfate (NiAS) results in a higher contrast and better defined modules in stained cortical areas.

1. Prepare the cytochrome oxidase staining solution (see Divac *et al.*[18]). For 10 mL of solution, add: 10 mL HEPES buffer (0.1 M, pH 7.4), 400 mg sucrose, 12.5 mg NiAS, 2 mg cytochrome C, 6 mg diaminobenzidine (DAB).
   CAUTION: DAB and NiAS are carcinogenic.
   NOTE: Add DAB just prior to incubation of sections.
2. Wash the sections in HEPES buffer (0.1 M, pH 7.4) on a shaker for 15 min.
3. Incubate the sections in the staining solution at room temperature on a shaker.
   NOTE: Observe the speed of the staining. If there is no visible reaction, change to incubation at 37 °C. Depending on the amount of fixation, staining can be observed after 10 min or in several hours.
4. Stop the reaction by adding 4% PFA; this prevents unwanted re-dying and increase of background signal.
5. Wash the sections three times using HEPES buffer for 10 min.
6. Mount and dry the sections on glass slides.
7. Dehydrate the sections with an increasing alcohol row:
   1. Wash the slides in 60% ethanol for 1 min. Wash the slides in 80% ethanol for 1 min. Wash the slides in 96% ethanol for 2 min. Wash the slides in 100% ethanol for 3 min. Wash the slides in isopropanol for 5 min. Wash the slides in xylene for 5 min.

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8. Immediately add a quick hardening-mounting medium, and add a coverslip. Critical: Do not use water-based mounting mediums, as NiAS will be washed out and degrade the staining.
9. Keep the sections at 4 °C for long term storage.

5. Visualization of Cortical Modules Using Immunohistochemical Staining

NOTE: Multiple protocols are available for immunohistochemistry, optimized for the specimen and the type of probe. Adaptations can be made as required, by varying concentrations of antibodies, permeabilizing agents, and incubation times. The following protocol leads to good results for detecting a large range of antibodies and visualization by fluorescent probes.

1. Wash the sections in PBS (0.1 M; pH 7.4) for 15 min.
2. Optional: Perform antigen retrieval to unmask an epitope using a water bath (based on Jiao *et al.*[19]; for alternative methods, see Pileri *et al.*[20]).
    1. Preheat a water bath to 80 °C. Prepare tri-sodium citrate buffer[19] (pH 8.0) and preheat it in the water bath to 80 °C.
    2. Transfer the sections to tri-sodium citrate buffer. Incubate the sections for 30 min at 80 °C
    3. Cool the sections down to room temperature
    4. Wash the sections in PBS for 15 min.
3. Wash the sections two times in PBS-X (0.5% Triton-X, in 0.1 M PBS) for 15 min.
4. Block unspecific epitopes by incubating the sections in a solution of Bovine Serum Albumin (BSA; 2.5%) and Triton-X (0.75%) in PBS for 2 h.
5. Incubate the sections in primary antibody, *e.g.*, Calbindin- D28k (1:5,000, 1% BSA in PBS-X), for 2-3 days on a shaker at 4 °C.
   NOTE: Optimal dilution for the primary antibody depends on the specific antibody used. Refer to manufacturer's information to obtain dilution criteria for a specific antibody. Multiple antibodies can be used together but must be raised against different species.
6. Wash the sections three times in PBS for 15 min each.
7. Incubate the sections overnight in secondary antibody (1:200, 1% BSA in PBS) at 4 °C on a shaker.
   NOTE: Multiple secondary antibodies can be used at different spectra if multiple primary antibodies were used.
8. Wash off the secondary antibody by washing the sections three times in PBS for 10 min each.
9. Mount and dry the sections on a glass slide for microscopy.
   NOTE: The sections should still be moist prior to mounting the coverslip.
10. Apply mounting medium suited for fluorescence dyes on the tissue sections and apply a coverslip.
11. Dry the sections for 1 h.
12. For long term storage, seal the coverslip using nail polish and keep in the dark at 4 °C.

Representative Results

We obtained flattened cortical sections of the somatosensory cortex in a variety of brains, and processed them for cytochrome oxidase histochemistry to visualize the somatotopic modules representing different body parts. This comparative approach allows studying the evolutionary forces that shape cortex, *e.g.*, showing highly conserved representation of mystacial vibrissae in rodents and lagomorpha as barrels[21] (**Figure 2**). In contrast, other body parts such as paws and genitals show variations in their relative size and reflect the specialization to an ecological niche or to sexual selection[22,23].

To understand the architecture of the medial entorhinal cortex, we obtained sections parallel to the pial surface. This was mainly achieved by tangential sectioning of the medial entorhinal cortex in mice, rats, and Egyptian fruit bats. In humans, because of the considerably larger size and more undulations in the entorhinal cortex, we gently flattened the cortex subsequent to making a tangential cut of the entorhinal cortex. Subsequently, all brains were cryopreserved and sectioned on a cryostat at 60 µm. Immunohistochemistry was performed on the obtained sections with an anti-calbindin antibody, to visualize the calbindin-positive pyramidal cell modules in the medial entorhinal cortex[5] (**Figure 3**). The calbindin-modules in the entorhinal cortex show a remarkable periodicity across all these brains, and vary in size by only a factor of 10 across ~ 20,000-fold variation in brain sizes[5].

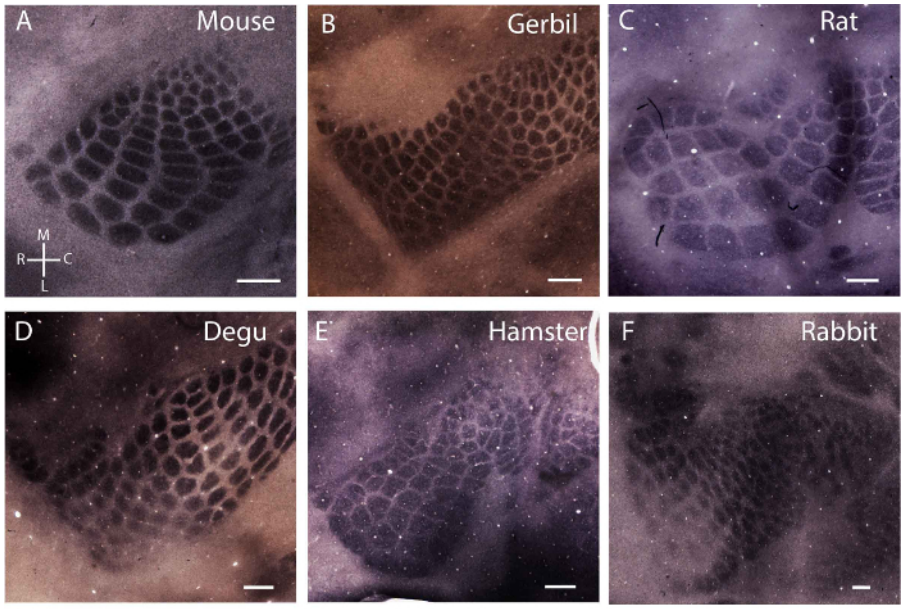

Figure 2: Topographical layout of the barrel cortex modules across mammals identified by cytochrome oxidase histochemistry. Tangential sections of layer IV from the somatosensory cortex of (**A**) mouse, (**B**) Mongolian gerbil, (**C**) rat, (**D**) degu, (**E**) hamster, (**F**) rabbit, showing barrels as a highly conserved somatotopic representation of the mystacial vibrissae. Scale bars = 500 µm. M: Medial, L: Lateral, R: Rostral, C: Caudal; Orientation in A also applies to B-E. Please click here to view a larger version of this figure.

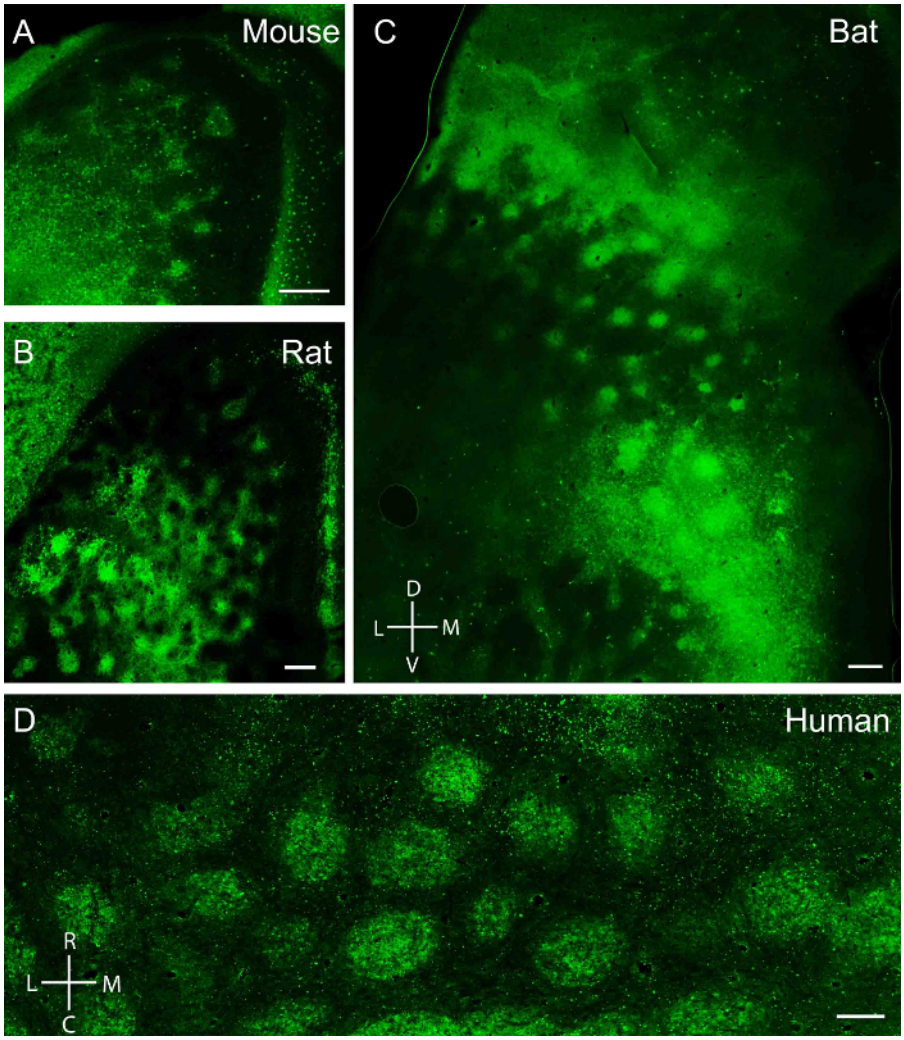

Figure 3: Periodic layout of medial entorhinal cortex modules across mammals identified by calbindin immunoreactivity. Tangential sections from layer II of the medial entorhinal cortex of (**A**) mouse, (**B**) rat, (**C**) Egyptian fruit bat, and (**D**) human, showing a conserved periodic layout of calbindin-positive pyramidal cell modules. Scale bars = 250 µm. M: Medial, L: Lateral, D: Dorsal, V: Ventral, R: Rostral, C: Caudal; Orientation in D also applies to B, C. Please click here to view a larger version of this figure.

Discussion

Modularity in the cerebral cortex has been identified using a variety of techniques. The earliest studies typically identified cortical modules by either visualizing cell dense regions, or an absence of fibers[1]. Subsequent methods have utilized the presence of dendritic bundles[24], afferents from a particular region[25], or enrichment of neurotransmitters[26]. Here we demonstrate two techniques, (i) cytochrome oxidase histochemistry and (ii) immunohistochemical staining.

Cytochrome oxidase staining has been one of the most popular methods to visualize cortical modules, and has been widely used in the primary sensory cortices[27,28]. It visualizes modules by staining darker for areas of higher mitochondrial activity[17], thereby acting as a proxy for anatomical modules which elicit substantial functional responses. This method has been used to visualize somatosensory cortical modules (**Figure 2**) and cortical modules in the visual cortex[27].

One of the drawbacks of traditional histochemical techniques is that their typical visualization by bright-field light microscopy limits the number of modules that can be visualized simultaneously. However, immunohistochemical methods, in conjugation with fluorescent visualization probes can also be utilized to view particular proteins and identify cortical modules. For instance, thalamic afferents to the sensory cortices project in a somatotopic manner. Thus, visualizing these afferents, using VGluT2 immunoreactivity can also be used to visualize the body maps in the primary somatosensory cortex which we have visualized using cytochrome oxidase activity (**Figure 2**). Selective proteins can also be used as cellular markers to identify groups of genetically defined cells. Using immunohistochemistry, we identified clusters of pyramidal cells in the medial entorhinal cortex expressing the calcium binding protein, calbindin[6] (**Figure 3**) and determined that clusters of this particular genetically determined group of cells are conserved across evolution[5], indicating common microcircuit principles underlying their function. Using multiple fluorophores, we also delineated complementary modules in the medial entorhinal cortex[26], demonstrating parallel microcircuits which underlie spatial memory.

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Historically, the brain has been sectioned in a plane normal to its position in the body, in either sagittal, coronal, or horizontal orientations. The identification of cortical modules, like the barrel fields in the somatosensory cortex[4] also prompted sectioning along a tangential and subsequently in flattened cortical preparations[29], though isolated cases of such sectioning were observed much earlier[30,31]. Subsequent to the identification of the barrel cortex, other cortical modules were also identified in the primary visual cortex[27], as well as the presence of a complete body map[32] with a somatotopic representation of the body in primary somatosensory cortex. Flattened sections of the cat visual cortex also revealed the topographic organization of orientation columns without the need for additional reconstructions[33]. Cortical modularity has also been demonstrated in parahippocampal cortices, including the presubiculum and parasubiculum[26,34] and medial entorhinal cortex[6]. Typically flattening a curved object induces distortions in laminar topography - as can be observed by the multitude of projections of the curved earth surface on a flat map[35]. A relatively simplistic method to partially compensate for these distortions is by introducing reference points at defined distances before flattening, and then measuring the distance between them after flattening. These marks then not only subserve in quantitatively estimating distortions introduced by flattening, but also provide an easy reference point to align consecutive sections. Further crucial aspects for preserving laminar fidelity include ensuring homogenous flattening across the section, and finally sectioning exactly tangential to the laminar layout to obtain optimized laminar cortical sections.

The visualization of cortical modules in flattened tangential sections has played an important part in revealing fascinating structure-function relationships in the cerebral cortex. The topographic representation of whisker and body parts in the primary somatosensory cortex and the presence of an anatomical grid in the microcircuits generating functional grid cells in flattened tangential cortical sections reveal intricate microcircuits details which would be hard to comprehend from other orientations. However, our current techniques provide only a two-dimensional slice of these essentially three-dimensional modules. Using immunofluorescence techniques and tissue clearing methods[36,37], it would be possible to visualize these cortical modules as a whole and perhaps reveal further insights into our understanding of cortical maps and modules.

Disclosures

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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