Proc. Natl. Acad. Sci. USA
Vol. 73, No. 8, pp. 2928–2931, August 1976
Physiology

Cytoarchitectonic mapping by microdensitometry

(visual cortex/monkey/computer)

A. J. HUDSPETH, J. E. RUARK, AND J. P. KELLY*

Divisions of Biology, Chemistry and Chemical Engineering, and Engineering and Applied Sciences, California Institute of Technology, Pasadena, Calif. 91125

Communicated by James Olds, May 24, 1976

ABSTRACT **Functional differences among various portions of the cerebral cortex are often correlated with differing cortical layering patterns. Convenient, accurate techniques for scoring layering should therefore prove useful in electrophysiological as well as anatomical investigations. We report the application of a computer-controlled scanning microdensitometer as a means of rapid measurement of optical densities in histological sections of monkey visual cortices, areas 17 and 18. The technique readily permits recognition of the previously defined cortical layers and suggests that still finer consistent layering patterns exist; it provides objective "fingerprints" of cortical regions which facilitate comparisons of structure from area to area and from animal to animal. The procedure should serve also to score the positions of autoradiographic grains, degenerating axonal terminals, and other labeled structures, and to allow the comparison of preparations stained by various techniques.**

Regions of the cerebral cortex that are functionally different often display dissimilar patterns of neuronal layering (1, 2). A striking example of this phenomenon is the area 17–area 18 border of the primate visual cortex, where substantial physiological differences between neurons in the two areas (3, 4) can be correlated with an abrupt change in cytoarchitecture. This is to be expected, since the cortical layers are known to be concerned with particular physiological tasks. In the primary visual cortex, input from lower centers terminates in specific subdivisions of layer IV (5), layers V and IV send projections to the midbrain and thalamus, and layers II and III provide input to higher cortical areas (6, 7).

The classical procedure for defining cortical layers is to assess visually variations in cell type, size, and density from the pial surface to the white matter in Nissl-stained sections (8, 9). Unfortunately, this approach to cytoarchitecture is highly subjective, and often fails to identify precisely the zone separating two adjacent cortical areas. Furthermore, layers defined on this basis may contain physiologically significant subdivisions beyond the resolution of such a qualitative technique.

We have examined the layering pattern of the visual cortex in a more objective way by measuring optical density with a scanning microdensitometer under computer control. The brains of three monkeys (*Macaca mulatta*), fixed by perfusion with 10% formol-saline, were available for this study. Serial, 25 μm sections of the celloidin-embedded material were cut in the sagittal plane and stained with 1% thionin. The scanning microdensitometer (Syntex AD-1 Autodensitometer) used in these experiments is generally employed for digitizing crystallographic data; it consisted of a miniature white light source mounted below a movable glass stage with a photomultiplier placed above. The light beam was formed by a 10 μm aperture, which was focused to illuminate a 10 μm $\times$ 10 μm area on the slide. This spot size was chosen because it sufficed for the resolution of layers only one cell in thickness (10). The stage was coupled to paired servo-motors so that it could be moved in calibrated steps along either or both of two orthogonal axes.

After a slide was mounted on the stage, the coordinates of the cortical region chosen for measurement were fed into the Autodensitometer's minicomputer. The light beam surveyed the slide in traverses from the pia to the white matter, and optical density measurements were made at 10 μm intervals. In approximately 5 sec, a total of 300 measurements could be made along a 3 mm traverse through the cortex. The stage was then automatically displaced 10 μm parallel to the cortical surface and another traverse was made. Repetition of this procedure produced a raster of optical density measurements stored on magnetic tape. The entire region scanned was displayed as a hard copy print (Versatec 1200 Matrix Printer/Plotter), from which traverses representing the best oriented regions were selected. Optical density profiles were then produced by averaging the density values at each depth across these traverses with a Nova 800 digital computer (Data General Corporation). The results were scaled before final display to include values ranging from those in the white matter to those in the densest cellular layer.

Fig. 1 illustrates a representative result of this procedure. The region of area 17 from which data were collected in 40 traverses is indicated on the photomicrograph. The averaged optical density plot reflects the pattern of cortical lamination (8) depicted in the photograph. The sharp peak at the extreme left represents the pia mater, and the succeeding trough corresponds to the relatively cell-free layer I. Layers II and III appear as a large increase in optical density upon which smaller peaks are superimposed. Just before the trough associated with the cell-sparse layer IVb, there is a sharp peak indicating the position of layer IVa, which is about one cell in thickness. Of the several peaks associated with layer IVc, the largest undoubtedly indicates the position of layer IVcβ, a sublayer heavily populated with small cells (11). Layer V appears as a zone of low density followed by a large increase in density associated with layer VI.

Although the major features of each 40-traverse scan were similar, there was variation in the small peaks within layers. We analyzed this variability in scans containing from one to 300 traverses. Increasing the number of traverses averaged smoothed the optical density profile by eliminating small peaks (Fig. 2A). Since the structure of the cortex is inherently "grainy," combining more traverses would be expected to average out the inhomogeneities caused by single cells and thus to increase the signal-to-noise ratio. However, if the layers of the cortex are not parallel to the axis of averaging, or if they vary in thickness in the region scanned, comparable points along

* Present address: Department of Anatomy, College of Physicians and Surgeons of Columbia University, New York, N.Y. 10032.

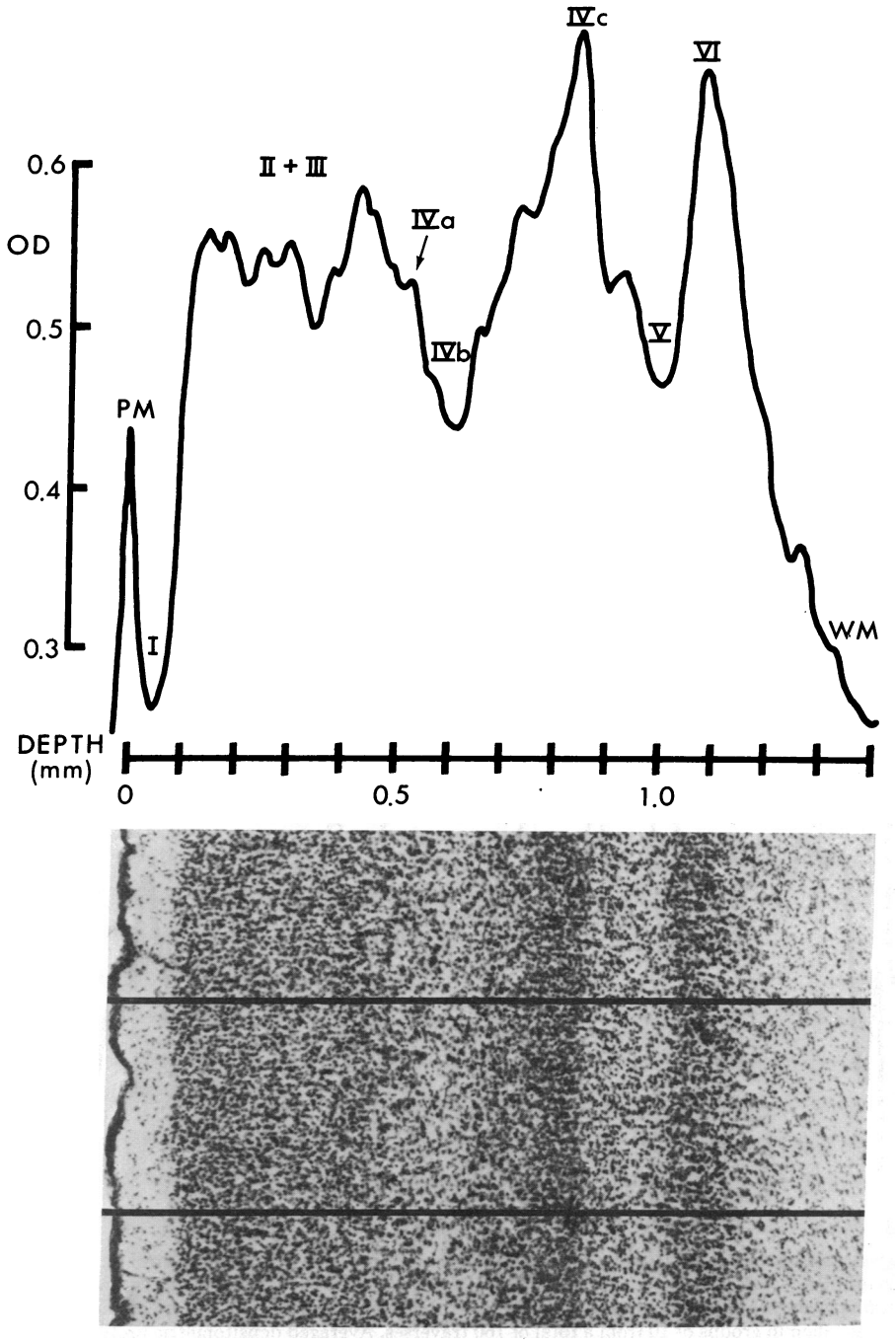

FIG. 1. A traced computer output displaying the averaged optical densities of layers in the specimen of cerebral cortex shown below. The scanning microdensitometer made measurements at 10 μm intervals along each of 40 traverses perpendicular to the cortical surface and 10 μm in width. The area studied, which includes about 5800 density measurements, lies between the parallel lines in the photomicrograph of peripheral macaque primary visual cortex. Readily apparent cortical layers (8), which are bounded by the pia mater (PM) and white matter (WM), are indicated on the densitometric plot with Roman numerals.

each traverse will not be in register. Real layers of small size will accordingly be blurred or superimposed on other layers. To overcome this difficulty, data from all traverses were scaled in the computer to bring layers I and VI into register. Fig. 2B shows the result, an optical density profile of 40 traverses with a high signal-to-noise ratio and good resolution of minor peaks.

Averaged optical density scans of area 17 are similar from region to region in a single slide and from slide to slide in a single animal (Fig. 2C). These profiles suggest that within classical layers, particularly layers II and III, there is a detailed substructure which is not peculiar to individual sections, but is a relatively constant feature. The comparison of area 17 scans from different animals (Fig. 2D) supports this point. Optical density profiles of area 18 (Fig. 3) lack the major peaks associated with layer IV of area 17 and display less substructure in the superficial layers.

The results presented above demonstrate that computerized microdensitometry provides a rapid, reproducible, and objective method for determining the layering pattern of a cortical

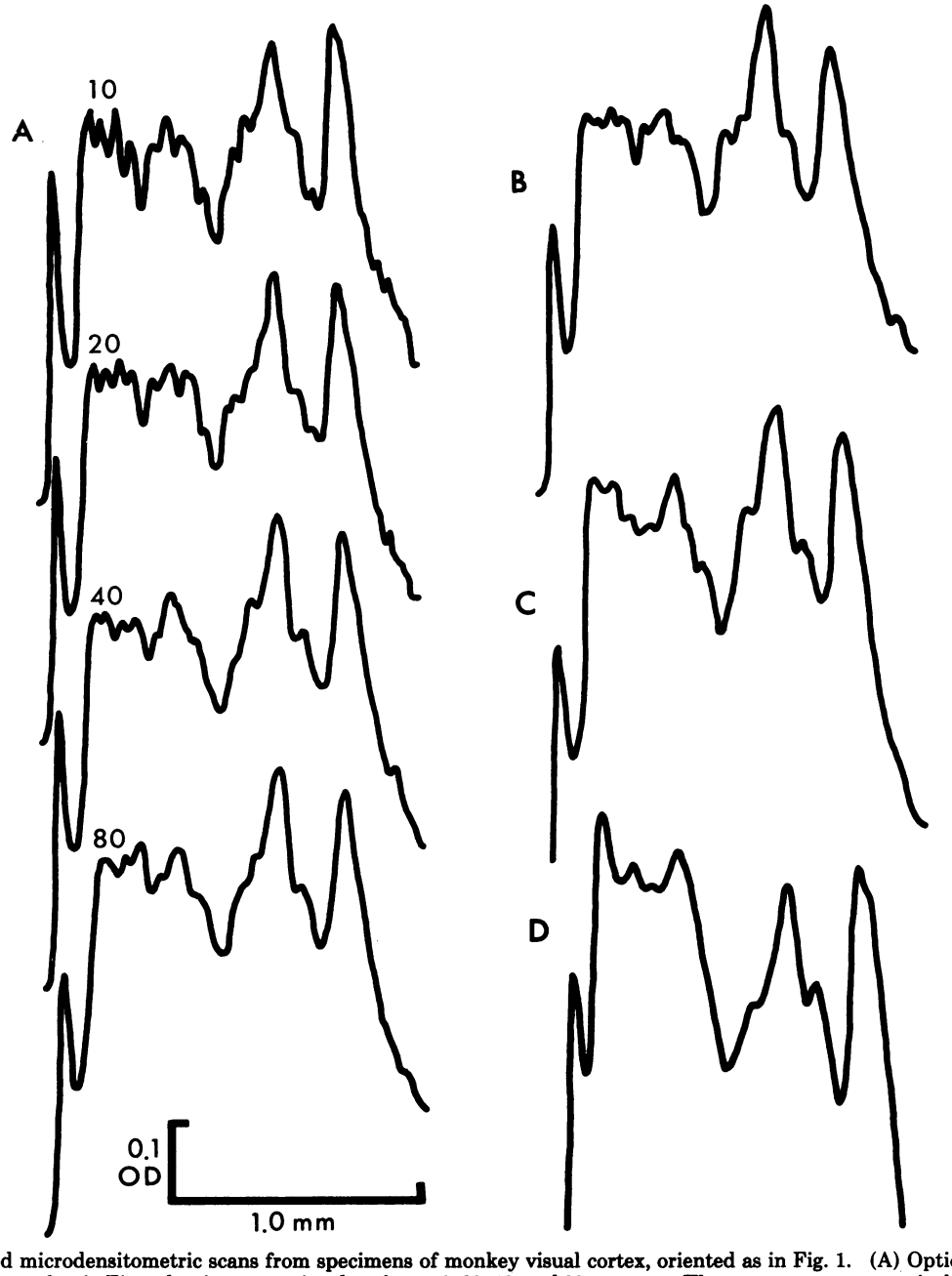

FIG. 2. Averaged microdensitometric scans from specimens of monkey visual cortex, oriented as in Fig. 1. (A) Optical density profiles of the same specimen as that in Fig. 1, but incorporating data from 10, 20, 40, and 80 traverses. The curves grow progressively smoother as more traverses are averaged, but fine layers are obscured as a result of specimen obliquity and variations in cortical thickness. (B) An averaged density scan representing 40 traverses taken in groups of 10 from a total of 160 traverses. Averaged densitometric profiles from each group were linearly scaled in the computer to provide best registration of layer I and layer VI peaks, whereafter the four blocks of data were averaged. Despite the large area over which the data were taken, compensation for variations in cortical thickness and obliquity produces a plot with good resolution of thin layers. (C) An optical density plot from a 40-traverse measurement of another slide of area 17 from the animal employed in the preceding figures, showing the consistency of layering pattern within an animal. (D) A 40-traverse scan from area 17 of another macaque monkey, demonstrating the ability of the technique to resolve comparable layering in various specimens. This plot compares with the foregoing least favorably in the upper cortical layers, where tissue preservation is worst by cytological criteria; it is thus probable that better results will be obtained through better fixation.

area. Moreover, optical density profiles reveal a consistent substructure in cortical layers which visually seem homogeneous in Nissl-stained sections. This substructure might well represent the arborization of axons and dendrites at selective levels within densely cellular layers (12).

Besides providing an objective "fingerprint" of a cortical region, the technique should prove useful in detecting patterns of structure within the plane of cortical layers, i.e. tangential to the surface. Abundant physiological and anatomical evidence suggests that such substructure exists in the visual (13, 14) and somatosensory cortices (1, 15). When applied at the border zones between cortical areas, the procedure should reveal which architectural features persist from one area to the next, and which change abruptly. Finally, microdensitometric scanning should facilitate the comparison of sections stained by various techniques and provide a rapid means of scoring the distributions of autoradiographic grains and degenerating nerve terminals.

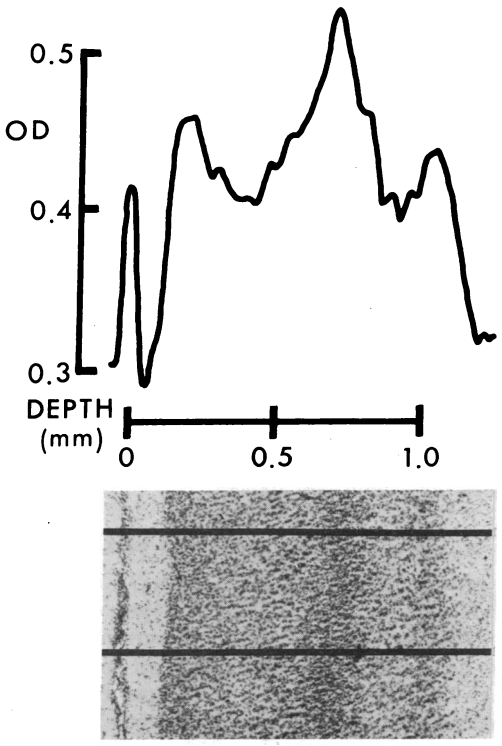

FIG. 3. A plot representing the averaged density of 40 traverses across a portion of macaque area 18 cortex between the lines on the photomicrograph. The specimen and computer output are oriented as in the other figures. The absolute OD scale indicates the lesser prominence of layers in area 18 by comparison to those of area 17, while the overall shape of the plot demonstrates that these two areas are readily separable on the basis of their density "fingerprints."

The authors thank Dr. R. M. Stroud for the use of the microdensitometer and computer facilities and for comments on the manuscript, Drs. T. N. Wiesel and D. H. Hubel for the use of some of the histological material, and Dr. M. Ross for assistance in computer programming. This project was supported by a grant from the California Institute of Technology President's Venture Fund (A.J.H.), by National Science Foundation Grant BMS75-04105 to Dr. R. M. Stroud (J.E.R.), and by grants from The Spencer Foundation and The Hixon Fund (J.P.K.).

1. Mountcastle, V. B. & Powell, T. P. S. (1959) "Central nervous mechanisms subserving position sense and kinesthesis," *Bull. Johns Hopkins Hosp.* **105,** 173–200.
2. Powell, T. P. S. & Mountcastle, V. B. (1959) "Some aspects of the functional organization of the cortex of the post-central gyrus of the monkey: a correlation of findings obtained in a single unit analysis with cytoarchitecture," *Bull. Johns Hopkins Hosp.* **105,** 133–162.
3. Hubel, D. H. & Wiesel, T. N. (1968) "Receptive fields and functional architecture of monkey striate cortex," *J. Physiol. (London)* **195,** 215–243.
4. Hubel, D. H. & Wiesel, T. N. (1970) "Cells sensitive to binocular depth in area 18 of the macaque monkey cortex," *Nature* **225,** 41–42.
5. Hubel, D. H. & Wiesel, T. N. (1972) "Laminar and columnar distribution of geniculo-cortical fibers in the macaque monkey," *J. Comp. Neurol.* **146,** 421–450.
6. Gilbert, C. D. & Kelly, J. P. (1975) "The projections of cells in different layers of the cat's visual cortex," *J. Comp. Neurol.* **163,** 81–106.
7. Lund, J. S., Lund, R. D., Hendrickson, A. E., Bunt, A. H. & Fuchs, A. F. (1975) "The origin of efferent pathways from the primary visual cortex, area 17, of the macaque monkey as shown by retrograde transport of horseradish peroxidase," *J. Comp. Neurol.* **164,** 287–304.
8. Brodmann, K. (1905) "Beiträge zur histologischen Lokalisation der Grosshirnrinde. Dritte Mitteilung: Die Rindenfelder der niederen Affen," *J. Psychol. Neurol.* **4,** 176–226.
9. von Bonin, G. & Bailey, P. (1947) *The Neocortex of Macaca mulatta* (University of Illinois Press, Urbana, Ill.).
10. Bracewell, R. (1965) in *The Fourier Transform and its Applications* (McGraw-Hill, New York), p. 194.
11. Lund, J. S. (1973) "Organization of neurons in the visual cortex, area 17, of the monkey (*Macaca mulatta*)," *J. Comp. Neurol.* **147,** 455–496.
12. Lund, J. S. & Boothe, R. G. (1975) "Interlaminar connections and pyramidal neuron organization in the visual cortex, area 17, of the macaque monkey," *J. Comp. Neurol.* **159,** 305–334.
13. Hubel, D. H. & Wiesel, T. N. (1974) "Sequence regularity and geometry of orientation columns in the monkey striate cortex," *J. Comp. Neurol.* **158,** 267–294.
14. LeVay, S., Hubel, D. H. & Wiesel, T. N. (1975) "The pattern of ocular dominance columns in macaque visual cortex revealed by a reduced silver stain," *J. Comp. Neurol.* **159,** 559–576.
15. Jones, E. G., Burton, H. & Porter, R. (1975) "Commissural and corticocortical 'columns' in the somatic sensory cortex of primates," *Science* **190,** 572–574.