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Postnatal development of the rat corticospinal tract,
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Summary

The corticospinal tract within the spinal cord of the neonatal rats, ranging from postnatal day 1 (P1) to P11, was investigated by using anterograde labeling with DiI optimized by confocal laser scanning microscopy. This method enabled us to observe a thick section containing a large population of the labeled axons and to acquire fine structure images with sufficient resolution. As advantages, novel views of diversity emerged in the developing axons and their collaterals. First, the individual parent axons showed unrestrained trajectories within the main bundle which constantly occupied the ventral part of the dorsal column. Second, there was an aberrant bundle which ran ventrally apart from the main bundle and converged into it. Third, several projection collaterals branched from the parent axons and left the main bundle in various directions, whereas the majority of them branched off at approximately a right angle. In addition, the main bundle consisted of a small number of pioneer axons and numerous follower ones. The pioneer axon tips were first found in the cervical level on P1, in the thoracic level on P2, in the lumbar level on P5 and in the sacral level on P7. The follower axon tips were observed in a wide range from away from the pioneer tips to just behind them. Projection collaterals were arborized within the gray matter and their arbors entered the dorsal horn, the intermediate substance or the ventral horn in the transverse plane.

Introduction

During neurogenesis of the long tract, constituent axons should approach their target area, recognize and accurately attach onto the target neurons and finally form a functional connection. In order to reach the target neuron numerous decision points may exist in the growing axons. Therefore, their morphological expression including the growth cone and branching region should be informative in terms of analyzing the underlying mechanism forming a certain nerve pathway.

The neonatal rat corticospinal tract used in this study has the following advantages in investigation of axonal development: The axons can be easily and specifically traced in the spinal cord, since this tract originates from the cerebral sensorimotor cortex, descends in the dorsal column and terminates within the spinal gray matter. In rodents, the axons complete their projection throughout the spinal cord during the first two weeks after birth, thus the materials obtained during the neonatal period are sufficient for observing the developing axons (Schreyer and Jones, 1982; Gribnau et al., 1986; Kalil and Norris, 1992). Furthermore, the axons at different spinal levels in a given specimen represent the corresponding chronological phases in the developmental process.

A labeling technique using a lipophilic fluorescent tracer, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI), has been a powerful strategy for investigating developing neurons, their

neurites, axonal growth cones or collaterals (O'Leary and Terashima, 1988; Honig and Hume, 1989; Kalil and Norris, 1992). DiI-labeled axons have previously been optimized under a fluorescence microscope, but limitations in terms of spatial and contrast resolution have meant that only a small population of labeled axons could be investigated. Recently the high resolution of the confocal laser scanning microscope has brought a great improvement in visualization of fluorescent materials, even in thick sections (White et al., 1987; Fine et al., 1988; Schotton, 1989; Wilson, 1989). The author could successfully observe each axon within a large population of the corticospinal axons labeled with large amounts of DiI solution. Thus in the present study, both general findings of the entire corticospinal tract and detailed structure of the individual axons were observed without out-of-focus blur even at high magnification.

Materials and methods

A total of 42 litters of pups born from 11 Wistar rat mothers were obtained in our breeding colony. Gestation was confirmed by spermatozoa from a vaginal smear the morning after mating and this day was recorded as embryonic day 0 (E0). Four mothers were delivered at E21, seven at E22 and one at E24. The ages and numbers of animals injected with DiI and perfused are listed in Table 1. In this study, postnatal day 0 (P0) refers to the first 24 hours after birth. Animal age is given as the day of sacrifice, since

axonal extension possibly progresses after DiI injection.

Pups between the ages of P0 and P7 were anesthetized by means of hypothermia in crushed ice, and chloral hydrate was used for older pups (3.5 mg/10 g body weight). For DiI injection, a small burrhole was made over the hindlimb area of the right sensorimotor cortex according to the maps of Zilles (1985) and Paxinos and Watson (1986). DiI (Molecular Probes: Eugene, OR, USA) was dissolved in N,N-dimethylformamide at a concentration of 25% and 0.6 to 1.0 μ l of that solution was injected into the cortex with a Hamilton's microsyringe under an operating microscope. After DiI injection each animal was returned to its mother's cage. The survival period was 24 hours with normal feeding for those animals perfused at P1, or 48 hours in other cases. After these survival times, the animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). The calvarium and vertebral lamina were removed immediately after perfusion and immersed in the same fixative for more than 24 hours. The length of the spinal cord was defined as the distance between the pyramidal decussation and the fourth sacral segment (S4) which corresponded to the caudal end of the conus medullaris. The coccygeal cord was omitted.

The spinal cord and hindbrain were sectioned sagittally or transversely at a thickness of 100 μ m with a Microslicer (Dosaka E.M., Kyoto, Japan). Serial sagittal sections were prepared to observe DiI-labeled axons mainly within the white matter. The observation sites were noted according to

the spinal cord level which was estimated from the ratio of the distance from the pyramidal decussation to the total length of the spinal cord, as the levels presumed from the ratio values were considered to represent approximately the true levels. Transverse sections at certain spinal segments, namely, the cervical enlargement (C6), the mid-thoracic level (T8), the lumbar enlargement (L3) and the sacral segment (S1), were selected to confirm the existence of descending fibers in the dorsal column and to trace the collateral branches extending into the gray matter from the bundle of the parent axons.

Images of DiI-labeled axons were obtained with a confocal laser scanning microscope (MRC-500, Bio-Rad, Hemel Hempstead, UK). Tomographic images of appropriate optical thickness were obtained and an image sequence was accumulated if necessary. The transverse section adjacent to the one observed in the confocal system was osmicated and embedded in Epon. Semithin sections (1- μ m) were stained with heated 1.0% toluidine blue in 1.0% borate solution for light microscopic observation.

Results

Trajectory of the corticospinal axons

The labeled corticospinal axons formed the compact fascicle and passed through the internal capsule and brain stem to the pyramidal decussation where they curved

dorsally. In sagittal sections they descended through the dorsal part of the spinal cord alongside the central canal to the lumbar enlargement (Fig. 1 A,C,D). Below the sacral segments they gradually drew nearer to the dorsal surface of the spinal cord (Fig. 1 D,E). In the transverse sections the labeled fascicle occupied the ventral part of the dorsal column only on the side contralateral to the DiI-injected hemisphere (Fig. 2). However, the trajectories of individual axons were not parallel to each other and there was no constant relationship between neighboring axons. Thus, their arrangement was not strictly somatotopic, but quite unrestrained within a definite width of the labeled bundle (Fig. 1B).

The growth cones of the pioneer and follower axons

There were numerous varicosities along the entire length of the labeled axon. The leading front of the labeled bundle was always composed of a small number of axons at the furthestmost site and numerous ones advancing behind them. These were regarded as the "pioneer axons" and "follower axons," respectively. Both the pioneer and follower axons possessed enlargements at their tips, which were considered the growth cones. There was no definite difference in the configuration of the growth cones between the pioneer and follower axons.

The growth cones ranged in size from about the same as the varicosity to several times as large. Their

configurations varied as follows: fusiform, oval, like a string of beads, flat, and lamellipodial with or without lateral protrusions (Fig.3 A-G). Within the gray matter, sometimes growth cone-like enlargements were observed at the tips of the collateral branches (Fig. 3H). Their shapes, however, seemed more uniform and simpler than the growth cones of the parent axons in the white matter.

The growth cones of the follower axons were distributed variously along the growing direction. The most proximal location of the follower growth cone varied between different specimen observed even at the same stage. The distance between the pioneer and follower tips, for example, ranged from 600 μ m within a single spinal segment of P6 (Fig. 4A) to 12 mm over ten segments on P5. In some pups the follower tips could be confirmed as being just behind the pioneer tips or even a long distance away from them (Fig.4 B,C,D). Therefore, the initial time or the speed of extension should be vary among different follower axons.

Advancement of the leading front of the corticospinal axons

Figure 5A shows the length of the spinal cord and the real distance between the furthestmost tip of the DiI-labeled corticospinal axon and the pyramidal decussation in each animal whose spinal cord was sagittally sectioned. Figure 5B shows the correlation between the postnatal day of sacrifice and the level of the spinal cord where the leading front of the labeled axons was positioned. In contrast to the

follower growth cones, the pioneer axon tips could be arranged in a stereotyped pattern according to the postnatal day. The pioneer axons reached the cervical enlargement on P1. Subsequently they passed through the thoracic cord between P2 and P4, progressed in the lumbar enlargement on P5 to P7 and reached the sacral segment after P8. The speed of extension averaged about 4 mm/day. The results of such a time schedule of the axonal extension were also determined by observation of certain spinal segments in a transverse plane at every developmental stage (Fig.2).

Collateral branching within the white matter

All of the projection domains of labeled fibers extending into the gray matter were collateral branches which were given off from the main "parent axons" within the bundle. The growth cone of the parent axon itself never turned toward the target area. There were collateral sproutings or short branches arising from varicosities proximal to their tips (Fig.6 A,B). The collaterals commonly extended ventrally into the gray matter in a sagittal section. Collaterals branching dorsally were rare (Fig. 6F). Branching patterns within the bundle varied as follows; 1) single and rectangular branching (the most common), 2) branching with immediately arborized secondary branches, extending in different directions, 3) two adjacent branches close to each other from the common parent axon (Fig.6 C,D,E). The direction of the projection collaterals which

were leaving the bundle varied as follows: 1) perpendicular to the ventral (the most common), 2) oblique to the ventro-caudal, 3) oblique to the ventro-rostral, 4) descending along the ventral margin of the bundle and turning toward the ventro-caudal (Fig.7 A-E). The projection collaterals which originated from the different parent axons passed through a common field then extended in different directions, which might suggest their termination in different targets (Fig.7 C,D).

The projection collaterals were most frequently observed at the cervical enlargement in sagittal sections of animals after P2. The collateral branching always occurred within the bundle consisting of the majority of follower axons. The distance between the branching site and the pioneer growth cone ranged from 1 mm to 20 mm. In one animal on P5 a short branch was observed within the lower thoracic segment at the shortest distance, 1 mm, from the pioneer tip (Fig. 7F). Therefore entering the gray matter should be initiated just after the pioneer axon arrives. In another animal on P5, in contrast, a similar short branch was found at the upper cervical level at the longest distance, 20 mm, from the pioneer tip which reached the lower thoracic level (Fig. 7G). The majority of the short branches were found at a distance of more than 6 mm from the pioneer tip. If such short branches, no more than several hundred micrometers in length, represented the early stage of the collateral extension, collaterals should start sprouting toward the gray matter after a time lag of more than 1.5 days after

arrival of the pioneer axons, based on the calculation that 4 mm/day is the average speed of the pioneer growth. Because the collaterals occur at various distances from the pioneer tip, the time lag might range from several hours to 5 days, and was commonly more than 1.5 days.

The projection collaterals within the gray matter

Arborization of the projection collaterals, of which the secondary or multiple branches were widely distributed within the gray matter, could be optimized on the superimposed image of serial optical sections out of a single sagittal specimen. Figure 8 indicates the projected image which was produced from 12 sequential tomograms in 4- μ m steps in the cervical enlargement of P7. It could be traced beyond the fourth branching spreading into the gray matter at a volume of $150 \times 90 \times 48 \mu\text{m}^3$. In transverse sections massive projection collaterals were observed in the gray matter of the cervical enlargement in the specimens taken after P5, whereas only a few of them could be found at that level on P3 and P4. There were also sparse collaterals within the gray matter of the thoracic and lumbar segments only in the P11 specimens. Many of them extended laterally in the gray matter and some of them showed ventral or dorsolateral extension. They never entered the contralateral gray matter across the midline. The extending collaterals added arborizing within the gray matter and the tips of these arbors were observed in a limited territory of the

target area, namely, in the dorsal horn, the intermediate substance or the ventral horn (Fig.9 A-C). This suggests that a set of collateral arbors emanating from the common parent axon might tend to terminate the preferential phenotype of the target neurons.

The aberrant bundle occurring ventral to the main fascicle

In the caudal part of the lumbar enlargement to the sacral segments of all animals after P8, there was an accessory or aberrant bundle which ran ventrally away from the main bundle, before converging again (Fig. 1E). Such a pattern was regularly repeated over several spinal segments, forming a wavy curve. Small separated bundles which converged into the ventral part of the main bundle were found in the upper cervical and thoracic segments of P4 (Fig.1 A,C). At least in these spinal segments individual axons can take their trajectories apart from the proper descending bundle unrelated to the target projection.

Discussion

Spatial features and time course of the corticospinal outgrowth

In this study developing corticospinal axons could be observed from both spatial and temporal viewpoints by using the DiI-labeling method. Compared with findings of axons in

previous studies using horseradish peroxidase (HRP) or wheat germ agglutinin-conjugated HRP (WGA-HRP) labeling, detailed morphology of DiI-labeled axons, especially collateral branching patterns, was more clearly demonstrated and easily traced with a high contrast (Schreyer and Jones, 1982; Gribnau et al., 1986). A confocal laser scanning microscope enabled the observation of a thick section containing a large population of the labeled axons and the acquisition of fine structures even at a high magnification. Because of these advantages over conventional fluorescence microscopy, an unrestrained course of constituent parent axons which could be distinguished within the labeled bundle and various features in the collateral branching and projecting patterns were consequently disclosed. In addition, the accessory or aberrant bundle, which appeared in the ventral side of the main bundle, was first described in this study. A general image of the aberrant bundle could be obtained by labeling a large number of the corticospinal fibers.

The bundle of the growing parent axons passed definite gateways within the white matter, e.g. the internal capsule, cerebral peduncle, pyramid, pyramidal decussation and the ventral part of the dorsal column, especially the part enclosed by the gracile and cuneate tract. In contrast to the constant location of the main bundle, there was no somatotopic or restrictedly localized arrangement in the trajectories of the individual axons.

This pathway during development was composed of two types of axons, the pioneer and follower axons. There was no

obvious difference in shape between the pioneer and follower growth cones, meaning that both of them probably had pathfinding functions, even though the manner might differ. The pioneer axon advanced regularly and constantly at a rate of 4 mm/day. This finding is generally consistent with those of previous studies using HRP or WGA-HRP (Schreyer and Jones, 1982; Gribnau et al., 1986). The follower axons, in contrast, showed various patterns in their extension in a staggered manner as suggested in previous reports (Schreyer and Jones, 1982; Gribnau et al., 1986; Stanfield, 1992).

The aberrant bundle showing an undulated course in the sacral segments of the specimens after P8 did not send projection collaterals toward the gray matter while it ran apart from the main bundle. Thus the parent axon in the aberrant bundle held the properties of fasciculating extension in the caudal direction even when apart from the main bundle.

All of the domains of the labeled fibers projecting toward the target gray matter consisted of collaterals which branched off from the parent axon within the main bundle. The most common pattern of the collaterals was rectangular branching out of the parent axon and extending straight to the target. It was similar to the developing corticopontine projection formed by interstitial budding (O'Leary and Terashima, 1988). The collaterals branched at a right angle at the shortest distance from the target area, so they could easily recognize some diffusible signals inducing to the target area, if present. However, other collaterals often

branched caudally or rostrally from the parent axons. These projection collaterals could explore the way to the target as well, even if the branching point was not the nearest to the target.

In the cervical enlargement the projection collaterals were much more numerous than those below the thoracic segments. This might be due to the fact that, first, there may be a much larger population of the target neurons in the cervical segments related to early innervation to the forelimbs and, second, the projection collaterals in the lumbar enlargement should not yet reach the maximum number during the period of this study (Gribnau et al., 1986).

Frequently a single parent axon sent multiple collaterals into different levels and areas, and each projection collateral showed repeated arborization within the gray matter. There are two possible reasons for these phenomena. One is that a single neuron projects to multiple target neurons even at different levels, as found in the studies on rodent and primate corticospinal tract (Kuang and Kalil, 1990; Shinoda et al., 1981). The other is that axons or their collaterals might be overproduced for the appropriate connection to the target neurons as suggested previously (Rakic and Riley, 1983; O'Leary and Terashima, 1988; O'Leary and Koester, 1993).

To analyze the dynamics of developing axons, it is important to simultaneously observe the furthestmost axon tips and events behind them in a single specimen. Such a spatial distance could reveal a time delay after the pioneer

axons had passed. The short branches, which were expected to be sprouting sites of the projection collaterals, were found at various distances from the pioneer tips. This distance represents the time lag necessary for forming a collateral projection, and is referred to as the "waiting period" (O'Leary and Terashima, 1988; Kalil and Norris, 1992). In the rat corticospinal tract the waiting period was found to be approximately two days (Schreyer and Jones, 1982; Gribnau et al., 1986). In these studies using HRP or WGA-HRP as a tracer, the waiting period was estimated by comparative observation of pups on different postnatal days. In contrast, by the DiI-labeling method the waiting period was estimated on the basis of the distance between the tip of the growing axon and the interstitial collateral in the rat corticopontine projection (O'Leary and Terashima, 1988). In the hamster corticospinal tract, the waiting period was considered to be no more than a few hours, when anterograde labeling of DiI was used (Kalil and Norris, 1992). In this study the waiting period varied from several hours to 5 days, and the variation might depend mainly on the staggered outgrowth of the follower axons.

Mechanisms to guide to the target and establish the specific connection

The chemoaffinity hypothesis is that the matching of molecules between the projection and target neurons plays a roll in forming specific connections between them with

precise topography (Sperry, 1963). On the basis of this concept the presynaptic neuron could be considered to interpret the positional information by the gradient of some signals from the postsynaptic neuron (Gaze and Keating, 1972). In co-culture studies of the cortical and pontine or spinal explants, diffusible target-derived chemoattractants which preferentially guide axons of the projection neurons were proposed (Joosten et al., 1991; O'Leary and Koester, 1993). However, it is unlikely that the collateral projections are guided only by a chemotropic factor, because multiple collaterals extend from a single parent axon toward different target areas, as also shown in the present study, and many collaterals from different origins passed commonly through a limited field and then terminated at dispersed areas. Thus chemoaffinity could prepare the general organization between the projection collaterals and target areas, whereas fine tuning would be needed to reach the appropriate target neuron by other mechanisms.

Another hypothesis is that the molecular cues, which are presumed to be present on the cell surface or the extracellular matrix, might navigate the pathway of the growth cones by direct contact (Dodd and Jessell, 1988; Jessell, 1991). In the rat corticospinal tract, the immature glial cells differentiate almost simultaneously within the fascicle area, when the pioneer growth cones are passing (Joosten, 1989; Schwab, 1991). The pioneer axons are possibly guided by maturing changes in the cell environment around the growth cones. It is controversial whether the

follower axons were guided by the same mechanism as the pioneer axons or by a different mechanism (Gorgels, 1991; Kalil and Norris, 1992; Stanfield, 1992). At least there was a definite difference in the time course of outgrowth between the pioneer and follower axons.

According to the selective stabilization theory, a functional connection in the adult central nervous system is not perfectly formed according to the genetic plan, but is established by competition or selection among exuberant synapses during early development (Changeux and Danchin, 1976). This mechanism is supported by the phenomenon of collateral elimination (O'Leary and Terashima, 1988; Schreyer and Jones, 1988). The total number of corticospinal axons at the medullary pyramid reached a peak on P4 and rapidly diminished until P14 (Gorgels, 1990). Consequently, the "inappropriate" synapses should be pruned and the "functionally normal" synapses should be maintained. The neuronal connection might be further remodeled to establish the final network of the corticospinal tract. Although the projection collaterals were constantly given off from the parent axons, it is still unclear whether the parent axon extending caudally is eliminated or continues to send other collaterals after P11.

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Figure Legends

Fig.1 The bundle of the DiI-labeled corticospinal tract in sagittal sections. The compact bundle descended straight through the cervical (A) and thoracic (C) cord on P4. It passed the lumbar enlargement (D) and gradually drew nearer to the dorsal surface of the sacral cord (E) on P8. The constituent axons (B) took unrestrained trajectories, which were not parallel to each other, within the bundle at the upper cervical segment on P10. Arrows indicate the aberrant courses which ventrally separate from the main bundle not related to the target projection. Left, rostral; up, dorsal. Scale bars: 250 μm for A,C; 100 μm for B; 1 mm for D,E.

Fig.2 DiI-labeled corticospinal tract in transverse sections. In the left column (A,D,G) sections were at the cervical enlargement, the middle column (B,E,H) at the mid-thoracic level, and the right column (C,F,I) at the lumbar enlargement. The labeled axons were enclosed in the ventral part of the dorsal column only on the side contralateral to the DiI-injected hemisphere. On P4 (A,B,C) they were observed only at the cervical enlargement (A). On P5 (D,E,F) they passed the mid-thoracic level (E) and on P8 (G,H,I) they progressed below the lumbar enlargement (I). Arrows indicate the dorsal median septum. Scale bar, 100 μm .

Fig.3 Various configurations of the growth cones (GC) of the pioneer (A-C) and follower (D-G) axons in the sagittal section (left, rostral; up, dorsal). Simpler GC-like enlargement (H) at the projection collateral tip in the transverse section (left, medial; top, dorsal) of the cervical enlargement on P4. A: The flat (arrow) and oval (arrowhead) GC at the mid-thoracic level on P4. B: The lamellipodial GC at the upper cervical level on P1. C: The lamellipodial GC with the lateral protrusion (arrows) at the mid-thoracic level on P6. D: The oval GC at the lower thoracic level on P5. E: The fusiform GC at the cervical enlargement on P3. F: The string of bead-like GC at the upper cervical level on P3. G: The lamellipodial GC with the lateral protrusion at the cervical enlargement on P4. Scale bars: 50 μm for A,B,D,E,F; 25 μm for C,G,H.

Fig.4 The pioneer and follower growth cones (GC) in the sagittal sections (left, rostral; top, dorsal). Numerous follower GC (A, arrowheads) within 600 μm from the pioneer GC (A, arrows) at the lower thoracic level in a 6-day-old pup. The follower GC at 3 mm (B) and 1 mm (C) proximal to the pioneer GC (D) at the upper lumbar level in another 6-day-old pup. Scale bars: 100 μm for A; 50 μm for B,C,D.

Fig.5 A: The length of the spinal cord (open circles) and the real distance between the furthestmost tip of the labeled corticospinal axon and the pyramidal decussation (solid circles) in animals whose spinal cords were sagittally sectioned from P1 to P10. B: The correlation between postnatal day and the spinal level of the leading front of the corticospinal axons. The pioneer axons which were at the cervical enlargement on P1 regularly progressed in the thoracic cord between P2 and P4, passed through the lumbar enlargement between P5 and P7, and reached the sacral segments after P8.

Fig.6 Various manners of branching of the collaterals in sagittal sections (left, rostral; top, dorsal). Arrowheads indicate the branching points on the parent axons. A (arrows): Side protrusions arising from some varicosities in the upper cervical level on P4. B (arrow): A short branch with secondary arborization at the upper cervical level on P4. C: Single, rectangular branching to the parent axon in the cervical enlargement on P7. D: Branching with secondary arborization in the cervical enlargement on P5. E: Two adjacent branches from the common parent axon at the upper cervical level on P4. F: Dorsal branching at the upper lumbar level on P7. Scale bars: 25 μ m for A,B; 100 μ m for C,D; 50 μ m for E,F.

Fig.7 Various courses (indicated with arrows) and locations of the projection collaterals in sagittal sections (left, rostral; top, dorsal). Perpendicular extension to the ventral (A,B, different planes of focus) at the upper cervical level on P2. Ventro-caudal (C) and ventro-rostral (D) extension in the cervical level on P6 (Note many other collaterals which were given off from the different parent axons). E: Turning course at the ventral margin of the original bundle in the cervical enlargement on P5. F: A short branch, about $100\ \mu\text{m}$, at the lower thoracic level only 1 mm behind the pioneer tip on P5. G: A short branch, about $200\ \mu\text{m}$, at the upper cervical level 20 mm behind the pioneer tip on P5. Scale bar, $100\ \mu\text{m}$.

Fig.8 Multiple arborizations of the projection collateral from the single parent axon in a sagittal section (left, rostral; top, dorsal). The arrow indicates the branching point from the parent axon. The collateral arbors emanating from more than the fourth branching spread in the gray matter over a volume of $150 \times 90 \times 48\ \mu\text{m}^3$ in the cervical enlargement 29 mm behind the pioneer tip on P7. Scale bar, $50\ \mu\text{m}$.

Fig.9 The projection collaterals within the gray matter of the cervical enlargement in transverse sections. The collateral arbors extending toward the dorsal horn on P7 (A), toward the intermediate substance on P5 (B), and toward the ventral horn on P7 (C). The arrows indicate the dorsal median septum. Scale bars: 50 μm for A; 100 μm for B,C.



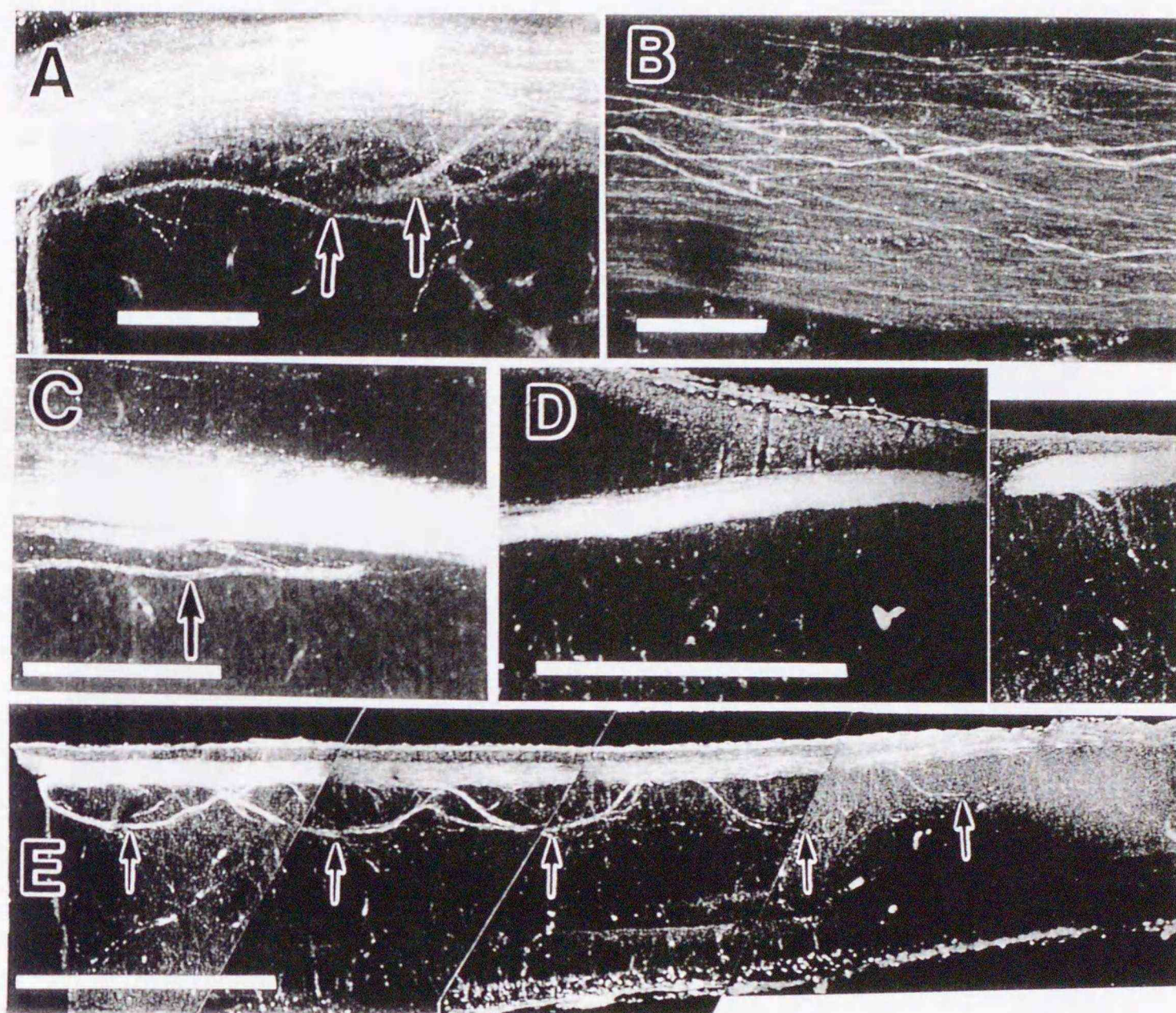


Figure-1
Nagashima, M
bottom

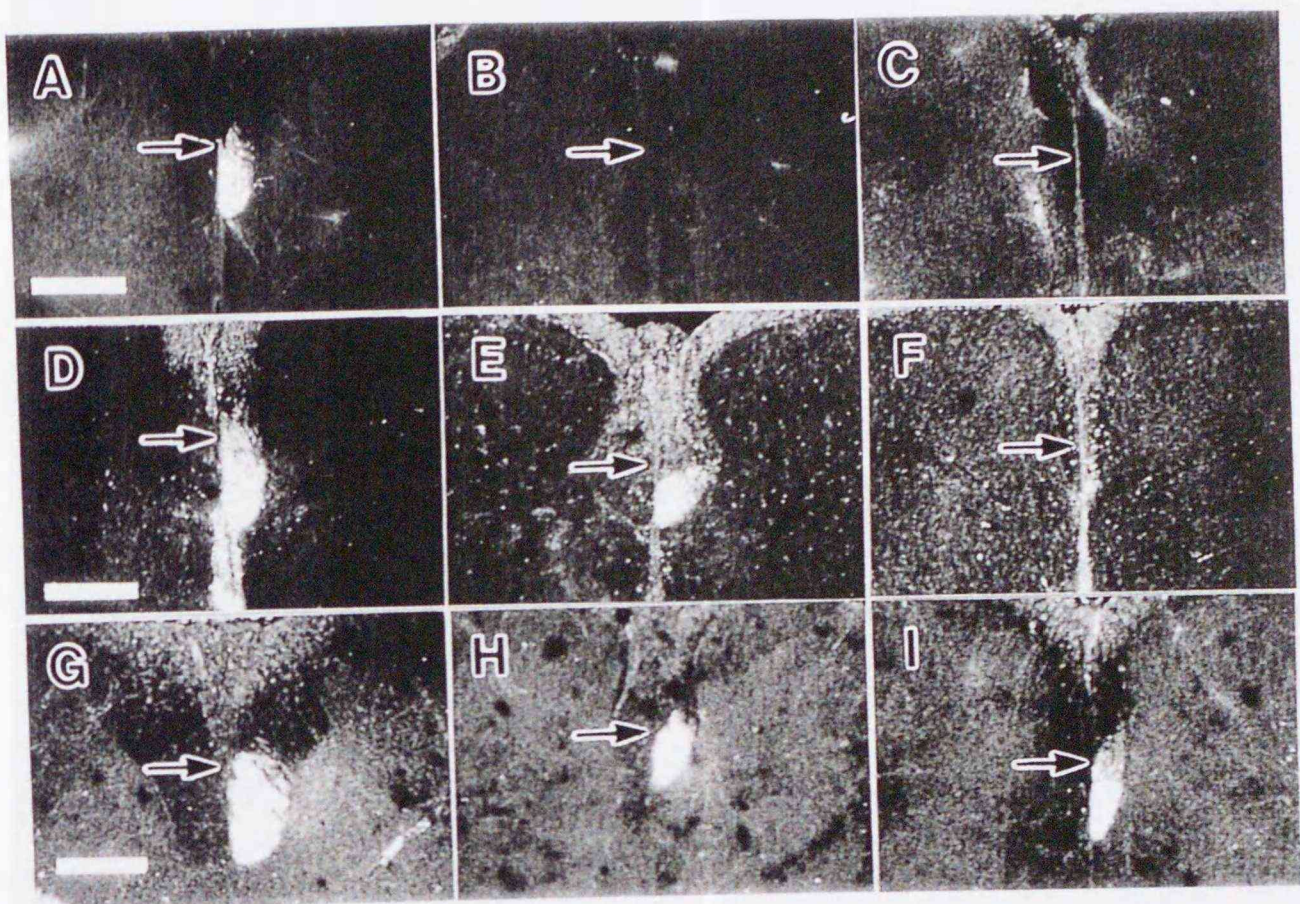


Figure-2
M. Nagashima
bottom

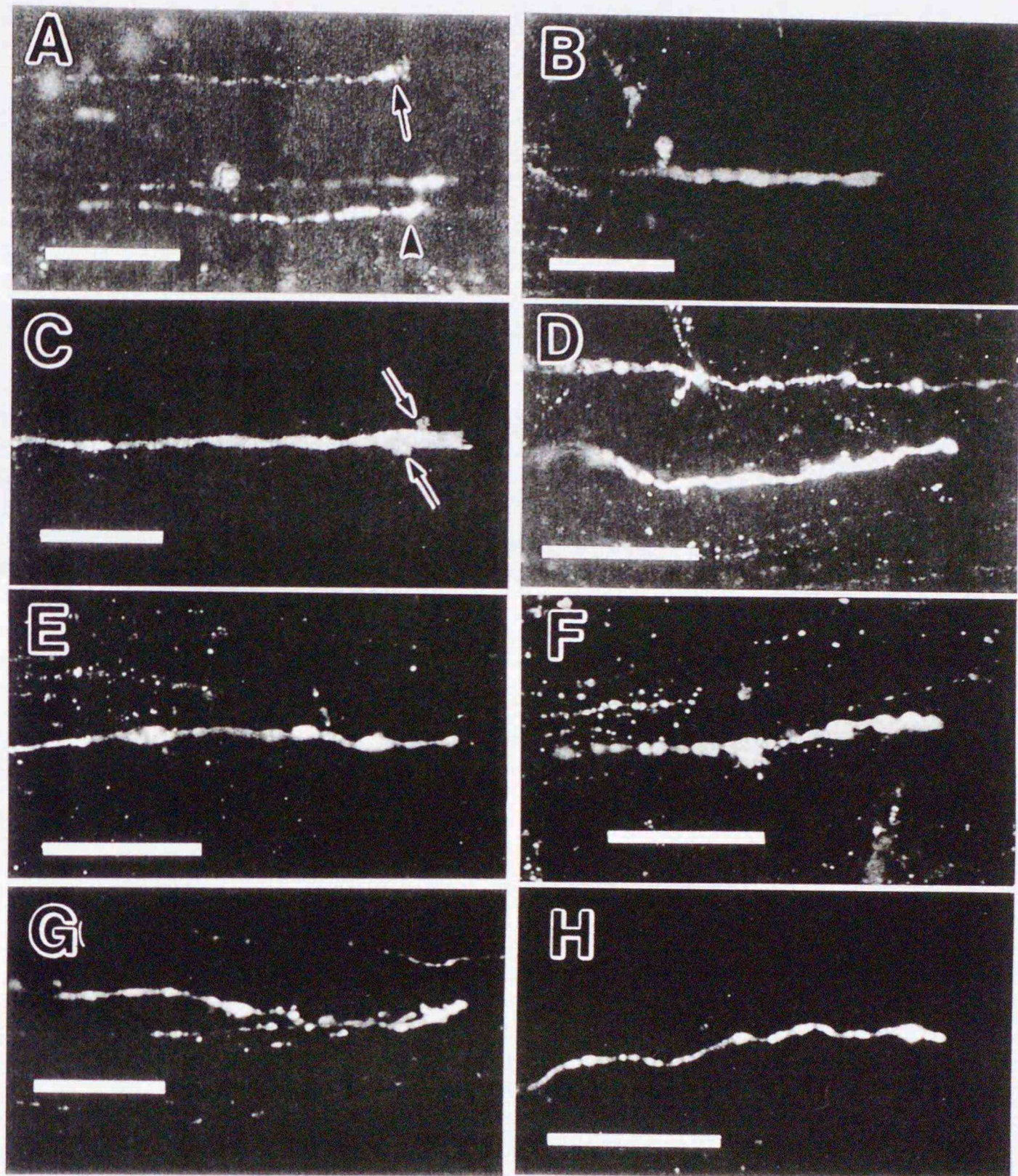


Figure-3
M. Nagashima
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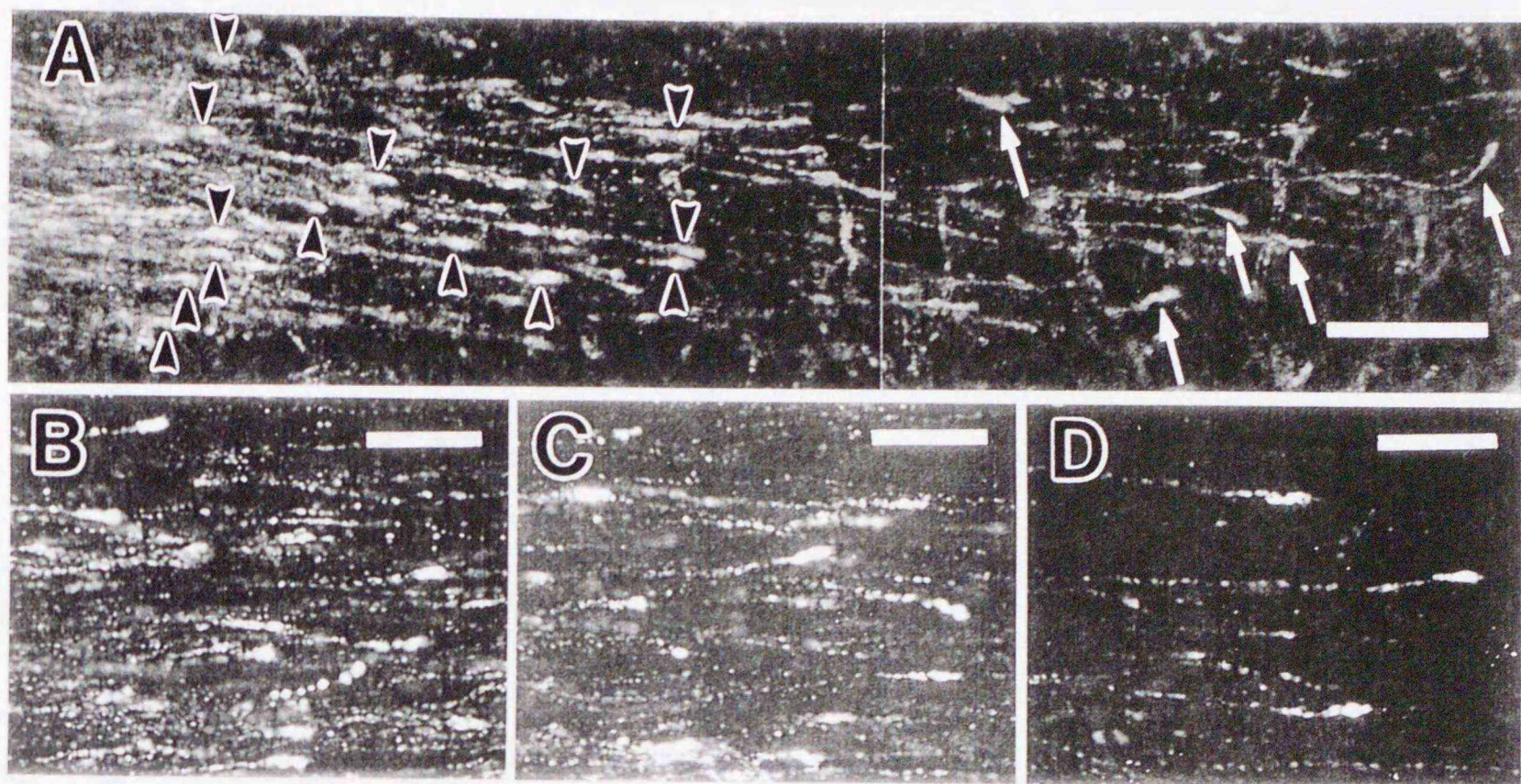


Figure-4
M. Nagashima
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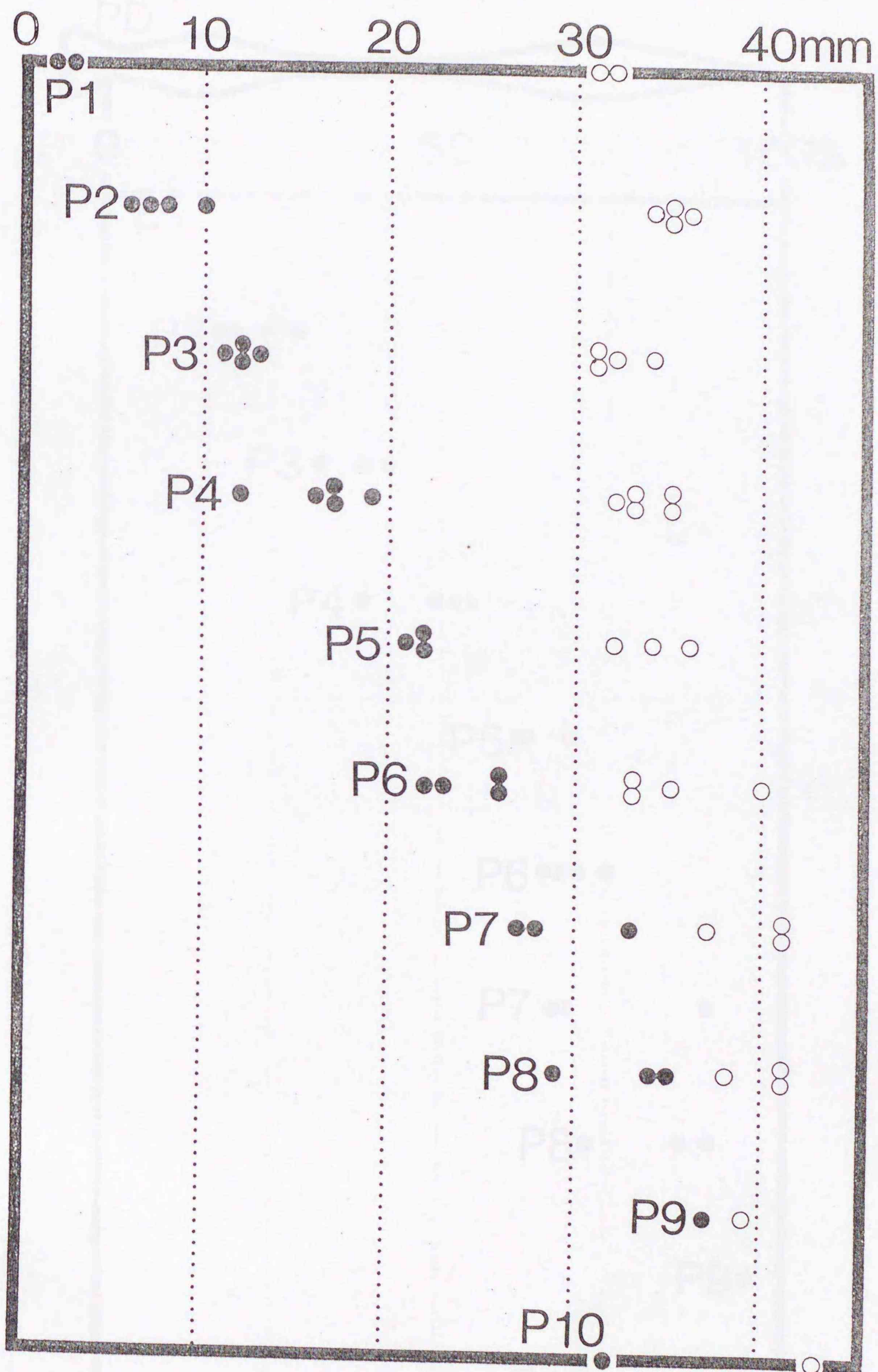


Figure-5A
M. Nagashima

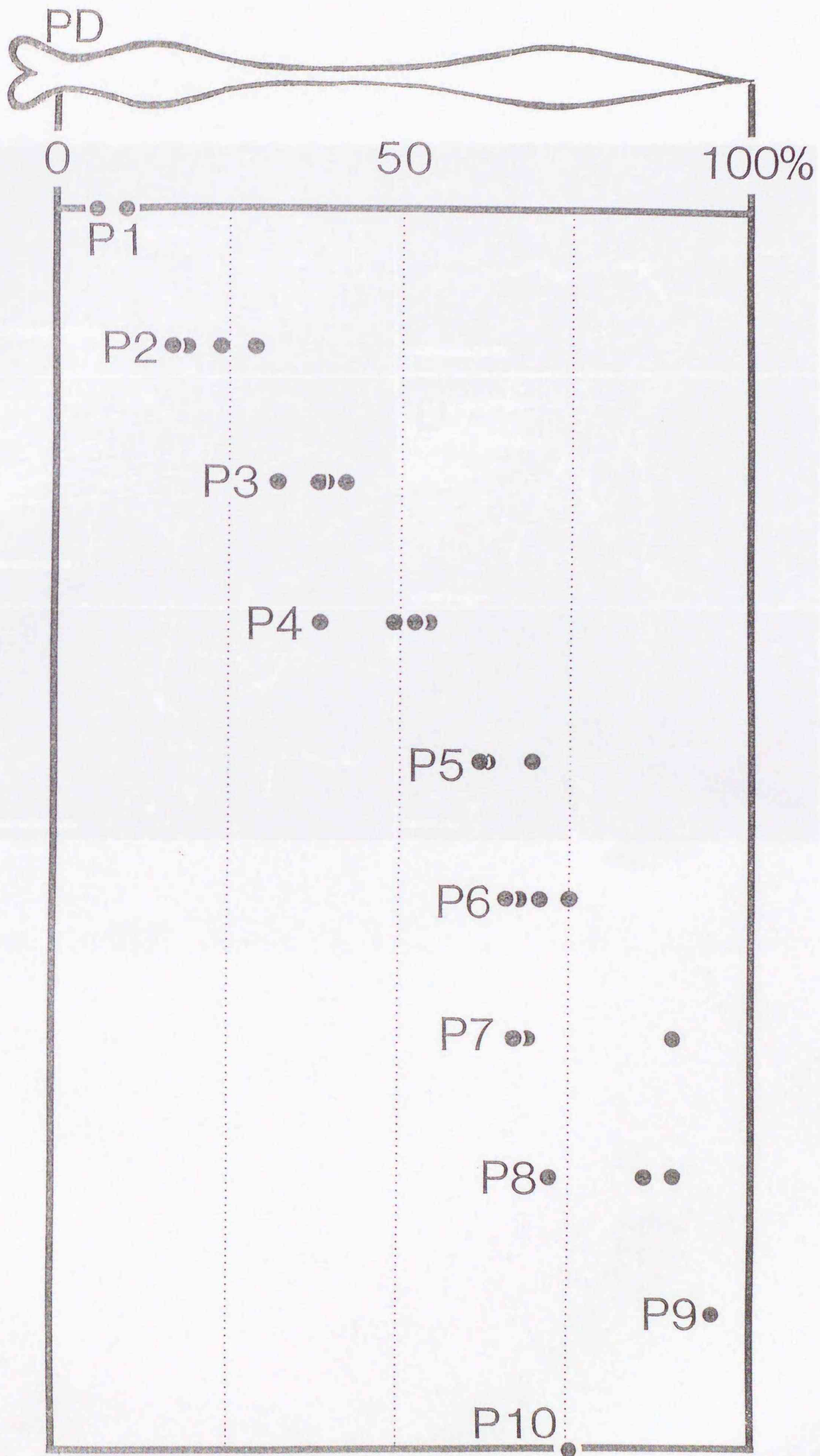


Figure-5B
M. Nagashima

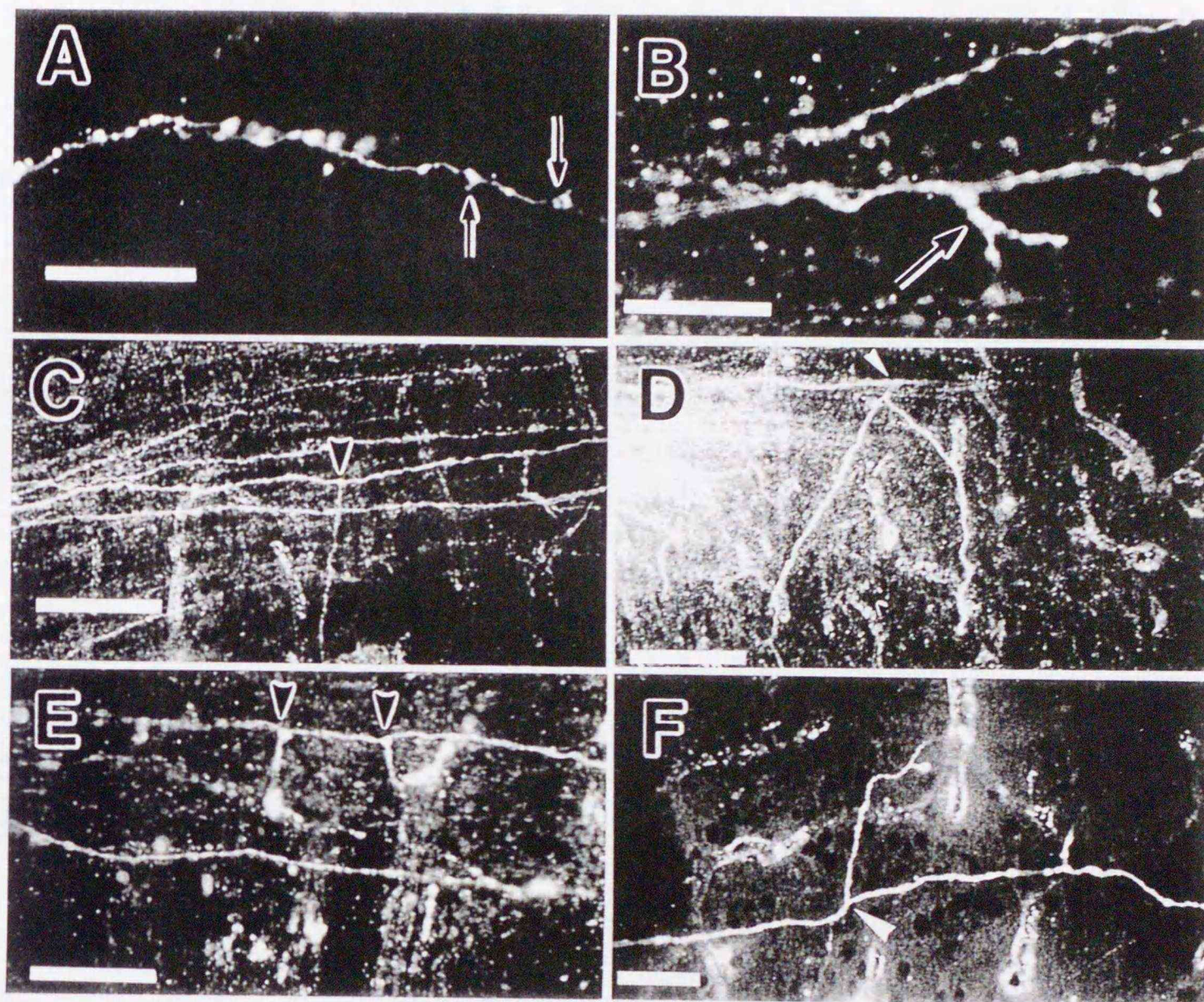


Figure-6
M. Nagashima
bottom

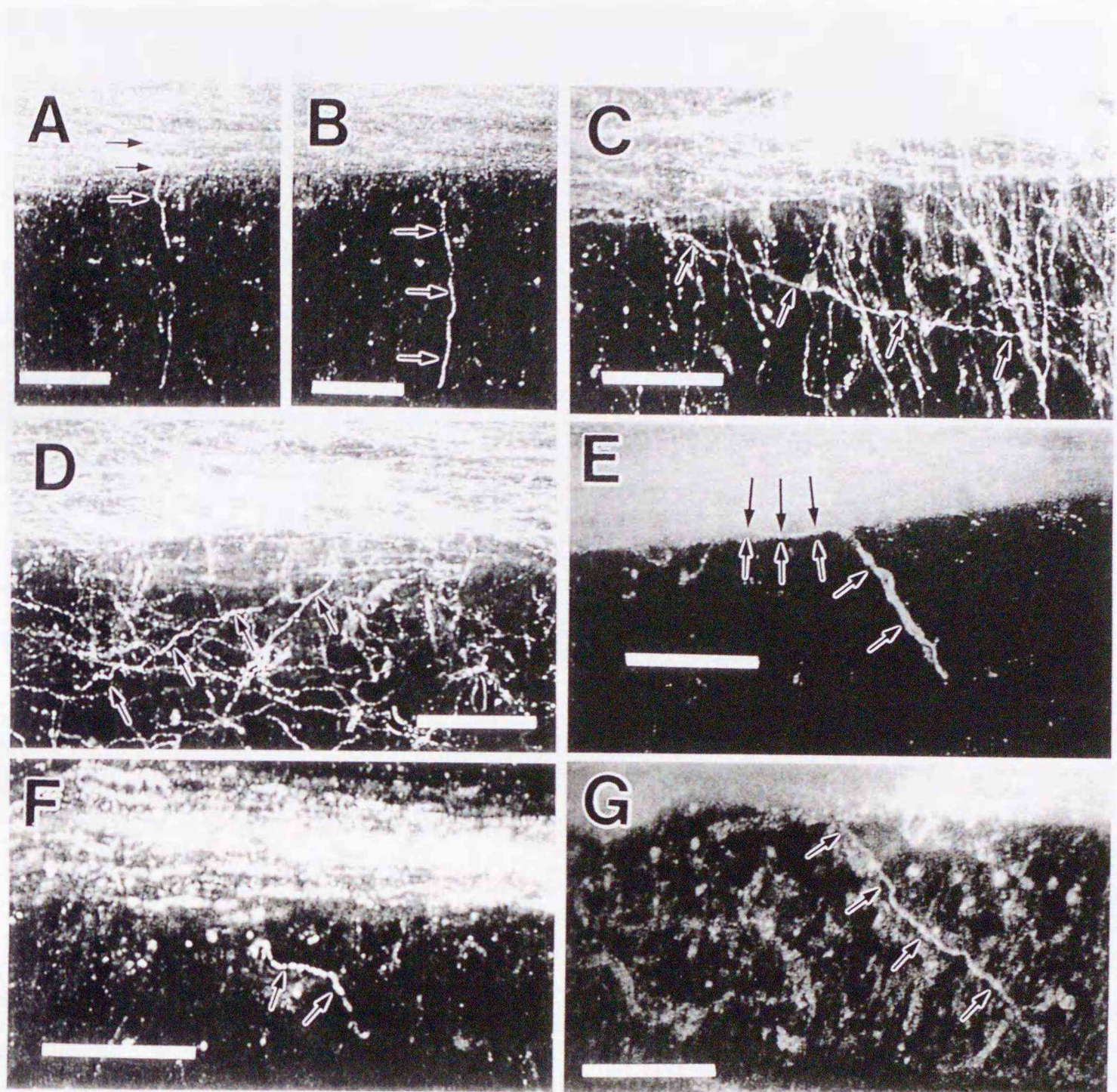


Figure-7
M. Nagashima
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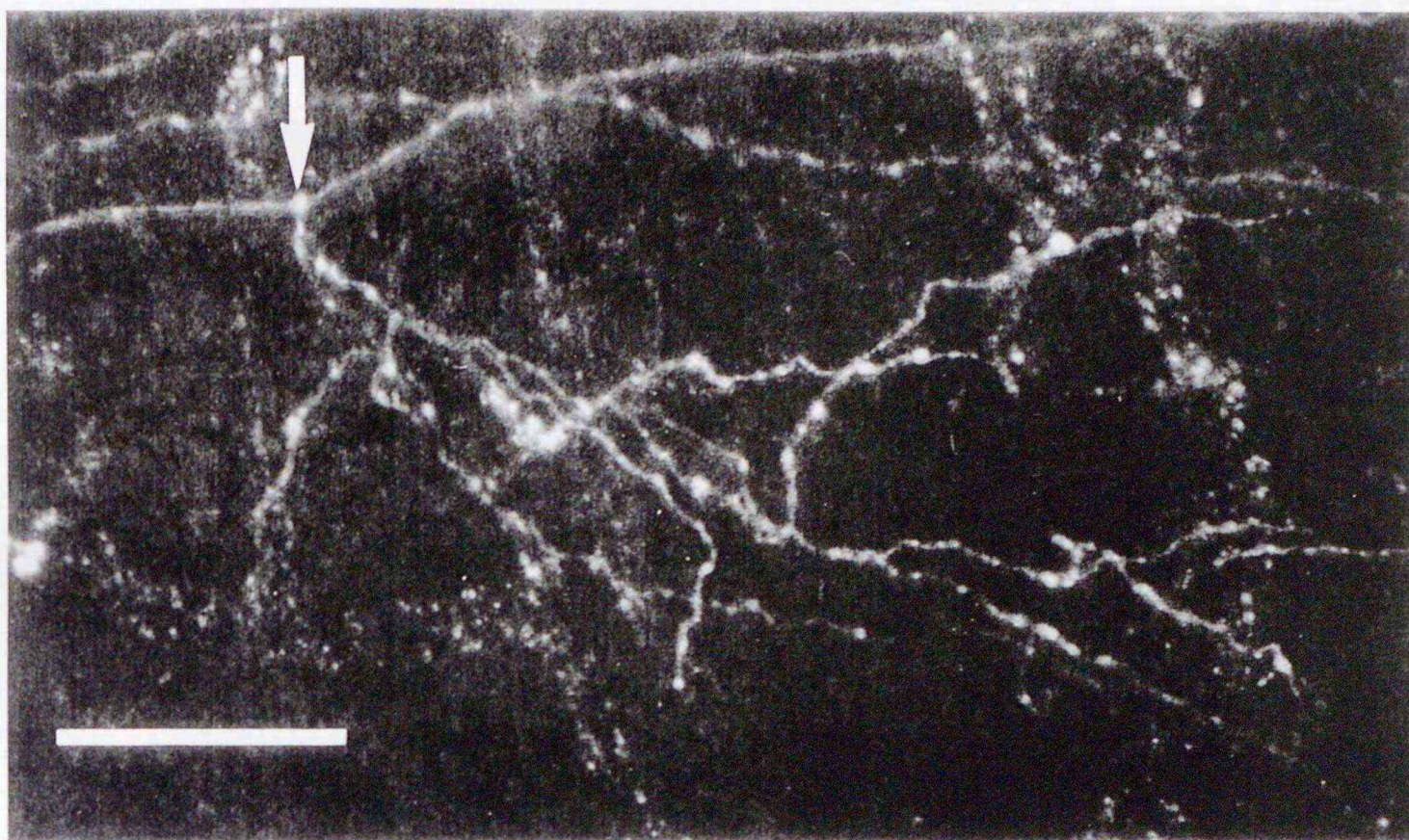


Figure-8
M. Nagashima
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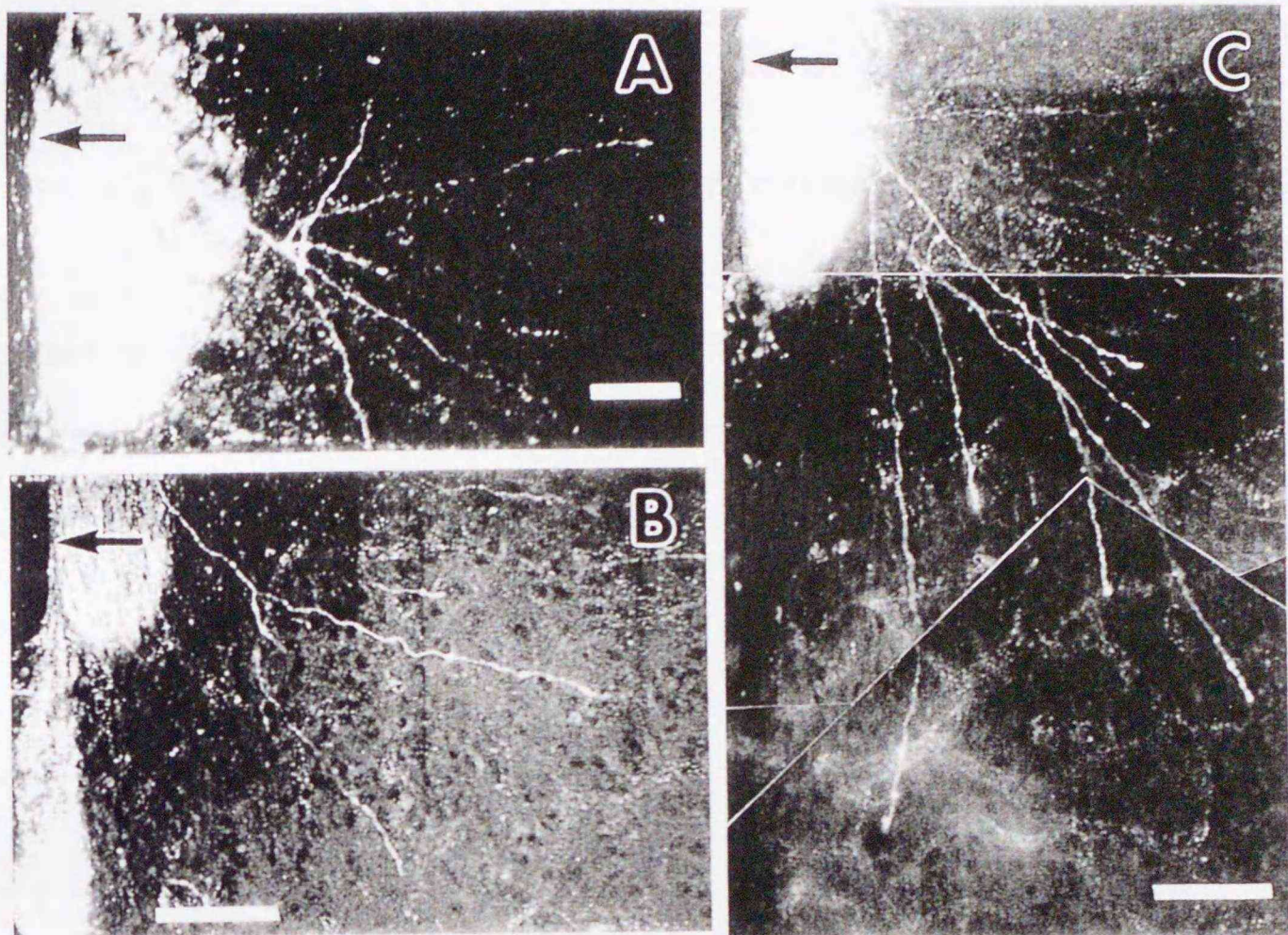


Figure-9
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Table-1

Age at perfusion and number of cases.

Postnatal days Number of cases

P1	2
P2	4
P3	5
P4	7
P5	5
P6	4
P7	4
P8	5
P9	1
P10	2
P11	3

Total 42

Sum of positive and negative values

Positive and negative values

1	10
2	15
3	20
4	25
5	30
6	35
7	40
8	45
9	50
10	55
11	60
12	65
13	70
14	75
15	80
16	85
17	90
18	95
19	100
20	105

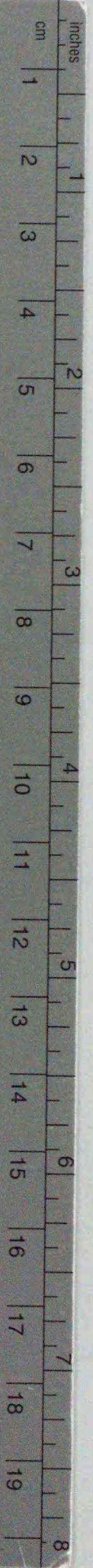
Table 2

THE UNIVERSITY OF CHICAGO

LIBRARY OF THE UNIVERSITY OF CHICAGO

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85	99
86	100

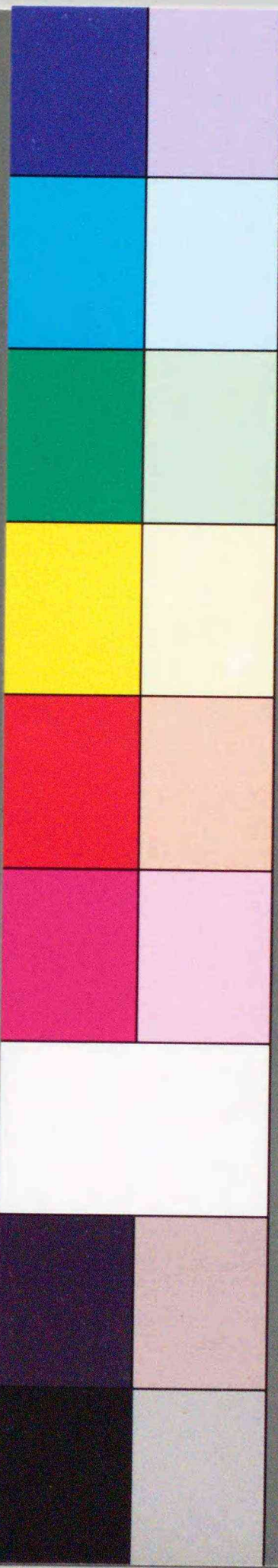
THE UNIVERSITY OF CHICAGO



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Kodak Gray Scale



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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19

