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Source: Zoological Science, 20(9) : 1071-1078

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.1071

ZOOLOGICAL SCIENCE **20**: 1071–1078 (2003)

Visualization of Erythrocytes in the Zebrafish Brain

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ABSTRACT—We found that erythrocytes of zebrafish have cytoplasmic peroxidase activity. Blood in the zebrafish brain was visualized using a standard peroxidase staining method after formaldehyde fixation. The erythrocytes in the brain were heavily stained, but neurons and glias were not stained at all. This easy method enables the distribution of erythrocytes in the whole brain to be determined, and enables the actual number of erythrocytes in each area in the brain to be calculated.

The paths of major, thick blood vessels in zebrafish brain are similar to those in higher vertebrates, however, the distribution of thin blood vessels is different. We also found that the erythrocytes were unevenly distributed in the brain. For example, the density of erythrocytes in the surface layer of the tectum was more than 30-fold higher than in the deeper granular layer. Very few erythrocytes were found in bundles of axons like cranial nerves and the medial longitudinal fascicle. In general, fewer erythrocytes were found in areas near the ventricle, whereas many more were found closer to the surface of the brain.

The distribution of erythrocytes in the brains of sleeping, awake and actively moving fish were compared. In the brains of sleeping fish, most of the erythrocytes were present in large vessels. This was not observed in brains of awake or actively moving fish. We found that the blood supply to motor neurons in the ventral horn of the spinal cord increased during active movement compared to that in awake or sleeping fish.

Key words: blood cells, peroxidase, diaminobenzidine, behavior

INTRODUCTION

Several methods have been developed to visualize the path of blood flow in whole brains (Seylaz *et al.,* 1999). Functional MRI (Magnetic Resonance Imaging) is a useful method (Cheng *et al.,* 2001; Vanduffel *et al.,* 2001) that has been applied to living brains, although resolution is poor. PET (Positron Emission Tomography) is another method that has been used to localize the blood stream (Leonhardt *et al.,* 2000; Tsukada *et al*., 2000), but like MRI, needs expensive equipment. We have developed a new method based on the endogenous peroxidase activity of individual erythrocytes. After formaldehyde fixation, erythrocytes in brain slices can be visualized because neurons and glial cells do not contain peroxidase activity.

The types of cells present in the blood of fish have not been fully determined (Weinreb 1963; Barber *et al.,* 1981; Rowley *et al.,* 1988), however, in higher vertebrates several kinds of blood cells including erythrocytes, thrombocytes (Bielek 1979 and 1981), granulocytes (Cannon *et al.,* 1980; Verburg-van Kemenade *et al.,* 1994), and lymphocytes (DeLuca *et al.,* 1994) have been identified. In contrast to mammalian blood where few, if any immature erythrocytes have been found, immature erythrocytes have clearly been identified in fish blood (Esteban *et al.,* 2000). Compared to the relatively large nuclei and thin cytoplasms of most blood cells, erythrocytes have large cytoplasms, contain hemoglobin and possess relatively small nuclei.

During histological procedures, endogenous peroxidase activity is normally inactivated by a high concentration of hydrogen peroxide before color development with a peroxidase-conjugated secondary antibody. If this step is omitted, peroxidase-containing cells (leukocytes, in the case of mammals) are heavily stained. We found that in fish, this led to the heavy staining of erythrocytes. Using this easy method, we could accurately determine the distribution of erythrocytes in the zebrafish brain.

MATERIALS AND METHODS

Preparation of zebrafish brain

Zebrafish were maintained at 28.5°C under an established cycle of 14 hr of light and 10 hr of darkness (Maeyama and Nakayasu 2000; Miyamura and Nakayasu 2001). Adult fishes (about 5 or 6 mo. after fertilization) were chilled in ice water to induce a state of suspended animation or were anesthetized following established

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methods (Westerfield 1995) using tricaine (3-aminobenzoic acid ethylester) This treatment was approved by our animal care committee (Arata and Nakayasu 2003.).

In order to prepare brains from sleeping (or ordinarily moving) zebrafish, the fish were moved to a small aquarium under normal conditions of aeration. While still in the dark phase of the cycle, a large amount of ice was added to immediately lower the body temperature of the fish. The skulls were removed from the fish heads on ice and the brains were fixed as described in a previous report (Tomizawa *et al.,* 2001a, b). To obtain brains from actively moving fish, a large net was moved around the aquarium for 20 min to stimulate the fish, and the brain was fixed as above. Brains from awake fish were obtained without any pretreatment during the light phase of the cycle.

Morphological studies

Morphological studies were performed as described (Nakayasu *et al.,* 1993, 1995). Briefly, a zebrafish head was fixed with the skull bone in 4% freshly depolymerized paraformaldehyde in BT fix buffer (0.1 M sodium phosphate buffer, pH 7.3, containing 0.15 M $CaCl_2$ and 4% sucrose (Westerfield 1995)) at 4°C overnight, and then washed twice with the same buffer. Prolonged fixation usually decreased peroxidase activity. The brain was carefully removed from the skull and gently washed in 0.1 M sodium phosphate buffer (pH 7.3) containing 4% sucrose, and then placed into a 30% sucrose solution until the brain sank. It was then stored in a 30% sucrose solution containing 30% OCT embedding compound (Tissue Tek) at 4°C for 30 min. The brain was embedded in OCT embedding compound, frozen on a cryostat and cut into sections of about 20 μm in thickness (Tomizawa 2000a, 2000b).

The sections were placed onto glass slides coated with 1.5% gelatin and 0.15% $CrK(SO_4)_2$-$2H_2O$, soaked with PBS (phosphate-buffered saline, pH 7.3), and dried at 55–60°C for exactly 20 min. Incubation for longer periods caused inactivation of peroxidase activity and incubation for less than 20 min resulted in detachment of sections from the glass slide. With the standard histological staining method, sections would now be treated with 0.3% H_2O_2 in order to inactivate endogenous peroxidase activity, however, this step was omitted in the present study. The sections were washed with PBS/Tween/dimethylsulfoxide (DMSO) solution (PBS, pH 7.3, 0.2% Tween 20 and 1% DMSO) containing 0.1% TritonX-100 and then twice with the same buffer without Triton X-100. The slices were pre-soaked with diaminobenzidine (DAB)-heavy metal staining solution (0.1 M Tris-HCl (pH 7.4), containing 0.04% DAB, 0.5% DMSO and 0.45% $Ni(NH_4)_2(SO_4)_2$ for 5 min. H_2O_2 was then added to a final concentration of 0.003%. The peroxidase reaction was stopped by washing sections with 0.1 M sodium phosphate buffer (pH 7.3) and then with distilled water. The slices were dehydrated in a series of increasing alcohol concentrations, cleared in xylene, mounted with Permount and viewed through a Zeiss Axiophot microscope. Blood smears were prepared according to Barber *et al.* (1981).

Photographs were taken with a CCD camera (Photometrics KAF-1400) using V for Windows (Photometrics), arranged using Photoshop (Adobe, Version 6) and printed using a Pictrography 3000 printer (Fujifilm). Calculation of cell numbers was carried out on the photographs using the grid lines in Photoshop Version 6 (Kawai *et al.,* 2001).

RESULTS

DAB staining of blood smears and brain fixation parameters

A smear of zebrafish blood on a glass slide was fixed with freshly depolymerized paraformaldehyde (4%) and stained using standard peroxidase cytochemistry as described in “Materials and Methods”. The most numerous cells (probably a mixture of mature and immature erythrocytes) were spherical in shape and possessed small nuclei and large cytoplasms (Fig. 1 A, insert). The diameter of these cells was about 5 μm. A few unstained cells were smaller in size and possessed large nuclei and thin cytoplasms (Fig. 1), suggesting that they might be lymphocytes. The unstained cells were a minor component of the whole blood cell population (less than 5%). There was also a group of cells that stained very heavily compared to the more abundant cell types. Again, these heavily stained cells (about 10%) were not a major component of the cell popu-

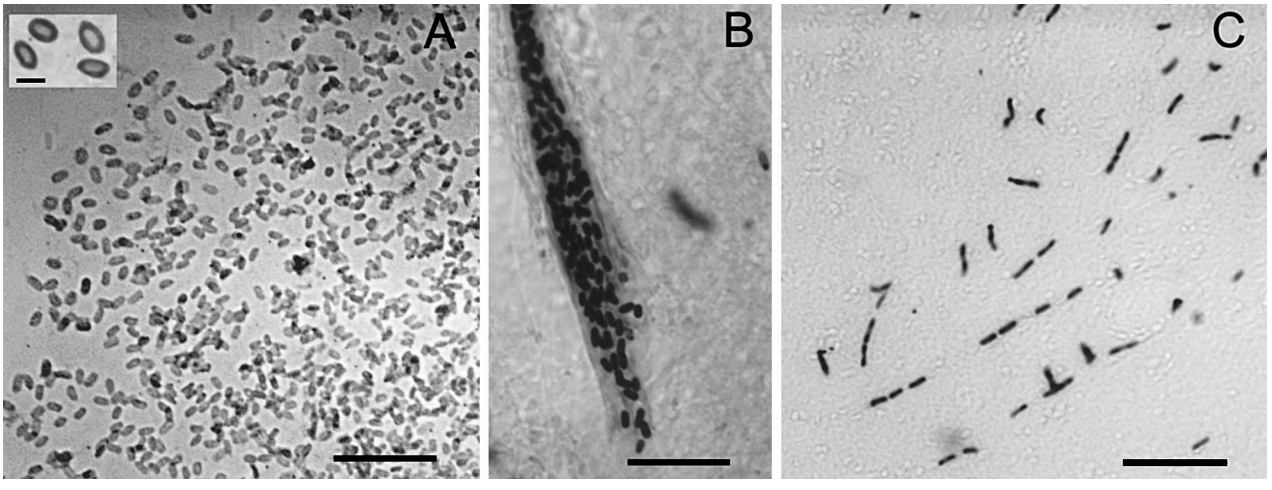

Fig. 1. ***Peroxidase staining of blood cells.***
A. Smeared cells. Insert reveals an enlargement of the stained cells. The peroxidase activity is found in the cytoplasm. B. Blood cells in a large blood vessel in the zebrafish brain (at the level of the tectum). C. Blood cells in a small blood vessel (at the level of the telencepharon). Generally, the staining of the smear sample was weaker than that for *in situ* blood cells. The conditions of fixation were the same between the samples. If fixation time was shortened, the smear sample became more strongly stained. It is possible that the peroxidase activity in the smear sample was partially inactivated by direct contact with the fixative. Scale bar, 40 μm in A, B and C, 5 μm in inset.

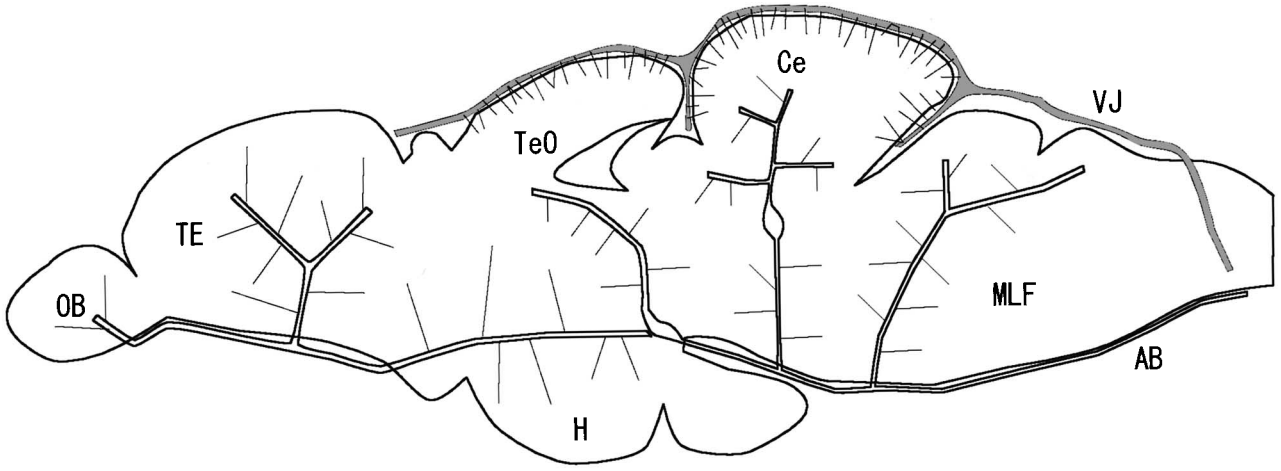

Fig. 2. ***Distribution of large blood vessels in the zebrafish brain (sagittal section).***
VJ, blood vessel jugularis interna; AB, aorta basilaris; OB, olfactory bulb; TE, telencephalon; TeO, tectum opticum; MLF, medial longitudinal fascicle; Ce, cerebellum; H, hypothalamus.

lation. Therefore, the peroxidase-stained cells that made up the major component of zebrafish blood correspond to mature and immature erythrocytes.

DAB staining of brain sections

We then stained sections from zebrafish brain with diaminobenzidine. Erythrocyte peroxidase activity must be maintained during preparation of the samples with the most critical factor being the fixation time. Prolonged paraformaldehyde fixation decreases enzyme activity and subsequent color development. We also found that the optimal time for drying sections on slides was 20 min in an incubator at 55°C.

Cells stained by peroxidase, which were similar in size and different in shape to the erythrocytes seen on the blood smear, were found in the large varicose veins of the zebrafish brain (Fig. 1B). Stained cells were also found in small blood vessels in the brain (Fig. 1 C) and were much more slender than those seen in the large blood vessels. The cells in the smears were weakly stained compared to cells in the brain, probably due to inactivation of peroxidase activity by paraformaldehyde.

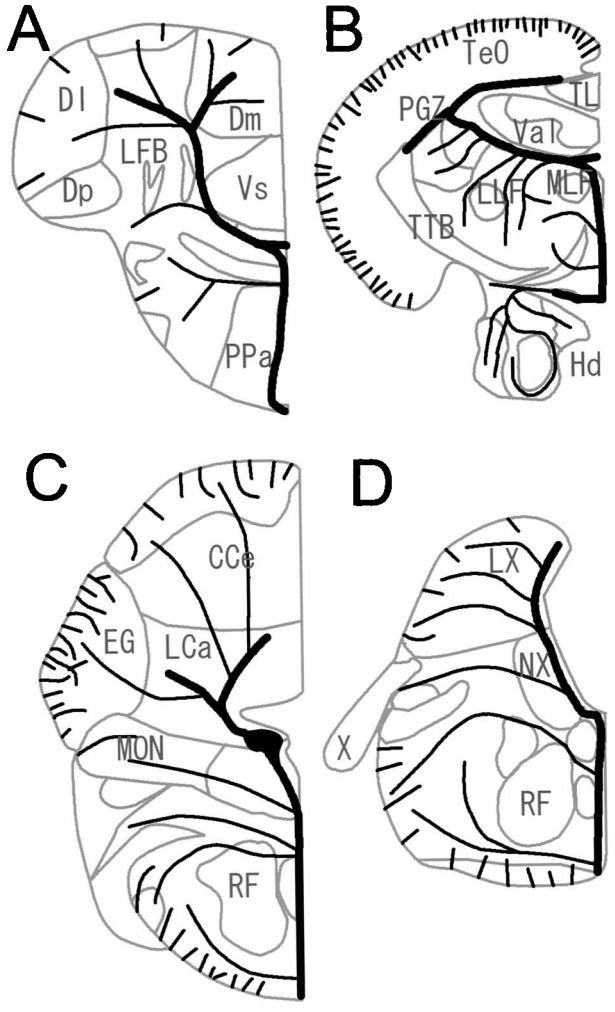

Fig. 3. ***Distribution of large blood vessels in the zebrafish brain (coronal section).***
A: level of telencephalon. Dl, lateral zone of dorsal telencephalon; Dm, medial zone of dorsal telencephalon; Dp, posterior zone of dorsal telencephalon; LFB, lateral forebrain bundle; Vs, supracommissural nucleus of ventral telencephalon. B: Level of tectum. TeO, tectum opticum; PGZ, periventricular gray zone of optic tectum; Val lateral division of valvula cerebelli; LLF, lateral longitudinal fascicle; MLF, medial longitudinal fascicle; Hd, dorsal zone of periventricular hypothalamus C: level of cerebellum. CCe, granular layer; EG, eminentia granularis; MON, medial octavolateralis nucleus; RF, reticular formation; LCa, lobus caudalis cerebelli. D: level of vagal lobe. LX, vagal lobe; NX, vagal motor nucleus; X, vagal nerve; RF, reticular formation.

In the brain, only erythrocytes, not neurons or glial cells, were stained. The erythrocytes within the blood vessels looked like slots. Within the slots, the unstained nucleus was located in the center, and the cytoplasm surrounding the nucleus was strongly stained. Slot thickness was homogenous, suggesting that the thickness of the blood vessels in

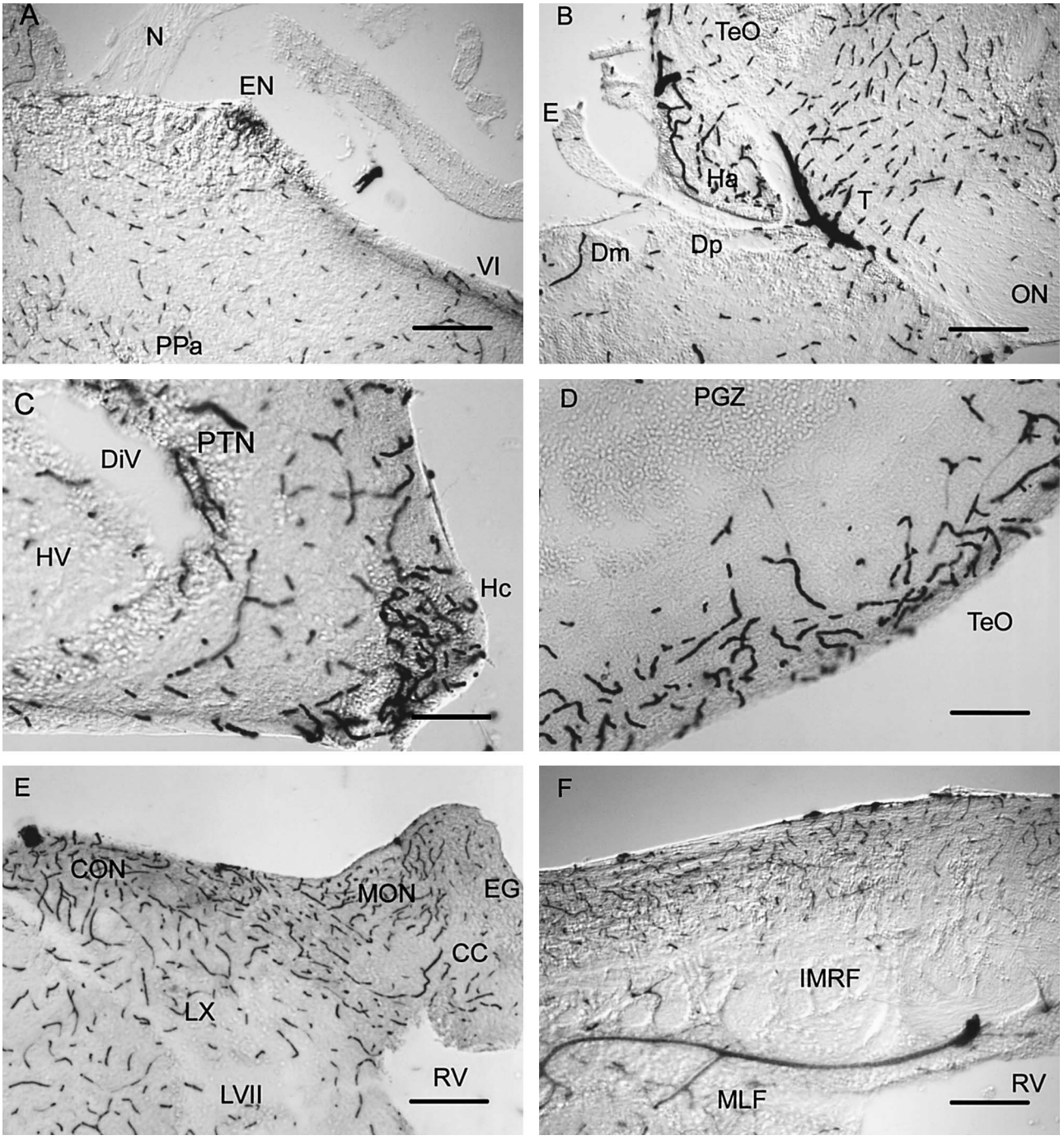

Fig. 4. ***Uneven distribution of blood vessels in the zebrafish brain.***
A, Horizontal section of the caudal telencepharon. EN,entopeduncular nucleus; N, optic nerve; VI, lateral nucleus of ventral telencepharon; PPa, parvocellular preoptic nucleus (anterior part). B, Sagittal section of diencephalon. E, epiphysis; Dm, medial zone of dorsal telencephalon; Dp, posterior zone of dorsal telencephalon; ON, optic nerve; T, thalamus; Ha, habenular nucleus, TeO, tectum. C, Sagittal section of hypothalamus. Hc, caudal zone of periventricular hypothalamus; PTN, posterior tuberal nucleus; DiV, diencephalic ventricle; HV, ventral zone of periventricular hypothalamus. D, horizontal section of tectum. TeO, tectum; PGZ, periventricular gral zone of optic tectum. E, horizontal section of cerebellum and facial lobe. CC, crista cerebellaris; EG, eminentia granularis; MON, medial octavolateralis nucleus; CON, caudal octavolateralis nucleus; RV, rhombencephalic ventricle; LX, lobus vagus; LVII, lobus facialis F, horizontal section of rhombencephalon. IMRF, intermediate reticular formation; MLF, medial longitudinal fascicle. RV, rhombencephalic ventricle. Scale bar, 20 μm in A, B and E, 10 μm in C, D and 40 μm in F.

the brain is practically the same. Several large vessels were also seen in the brain (for example, Fig. 4 F). These large vessels were easily detected because of the abundance of the stained cells. This was especially apparent in brains from sleeping fish, where blood cells appear to have pooled in the large vessels. A tracing of these large vessels is illustrated in Figs. 2 and 3.

Veins in the zebrafish brain

The largest blood vessels were located at the top and bottom of the zebrafish brain (Fig. 2). As in other vertebrates, arteries run at the base of the brain and large veins run on the dorsal side. The large ventral artery branches at four major points; the telencephalon (forebrain branch, Fig. 3 A), the level of the tectum (tectal branch, Fig. 3 B), the level of the cerebellum (cerebellar branch, Fig. 3 C), and the level of the facial lobe (rhombencephalic branch, Fig. 3 D).

These branched arteries then enter the brain (Fig. 3). There are some general rules in the way these branches progress. The large vessels are always crossed by the large nerve bundles. Except for the forebrain branch, they run in the narrow space between the right and left MLF and then branch again just before reaching the ventricle. Small blood vessels in several areas of the brain join each other to form large veins on the dorsal side. The pathways of these small blood vessels were not symmetrical, and seemed to vary among individual fish, however, the paths of the large blood vessels appeared to be almost the same. In large veins, especially in the tectal and cerebella branches, there were large varicosities near the ventricles. These structures could be related to the choroid plexus in higher vertebrates (Weiger *et al.,* 1988). Numerous blood cells were found within these varicosities in sleeping fish, but not in actively moving fish.

Distribution of blood cells in the zebrafish brain.

Because the zebrafish brain is small, it is possible to count the number of erythrocytes in the whole brain. The total number in a typical individual was calculated to be approximately 55,000. Surprisingly, there was a variable distribution of blood cells throughout the brain. As shown in Fig. 4 A, the entopeduncular nucleus (EN) has a very high density of blood cells compared with the other areas in the telencephalon. Practically no blood cells were observed in the optic nerve. Other areas of the brain that contained high densities of blood cells were the habenular nucleus (Fig. 4

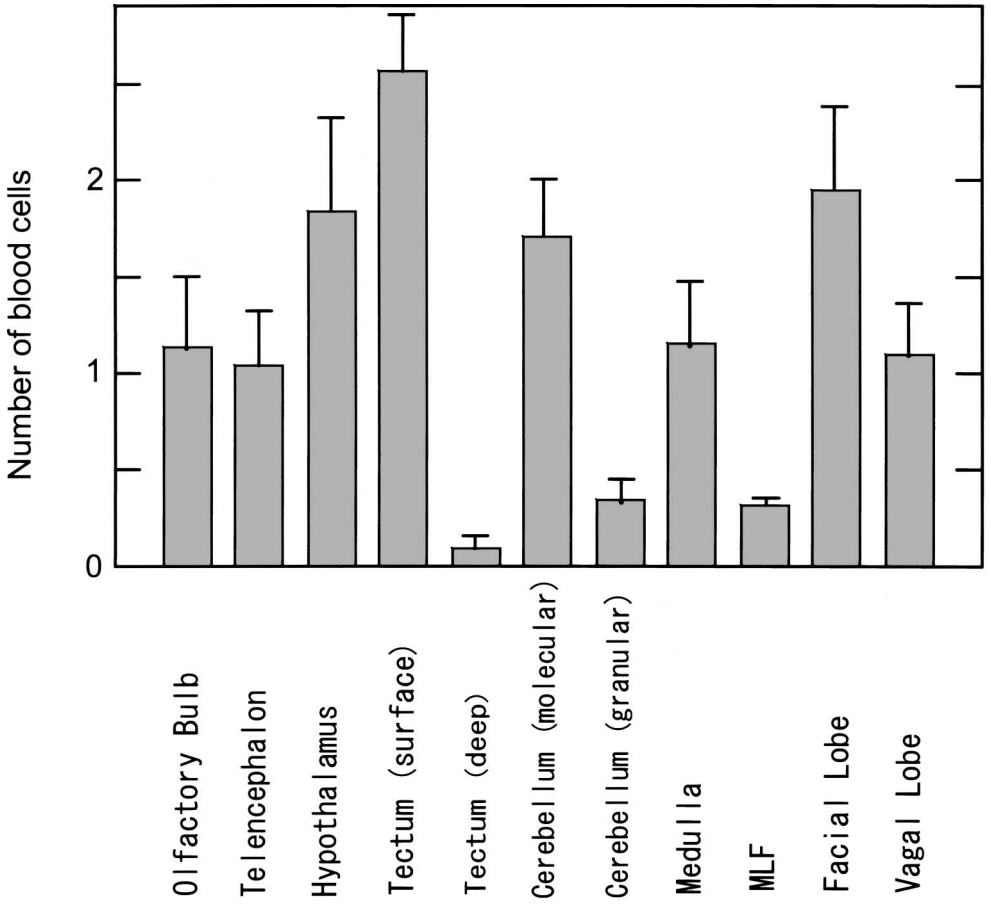

Fig. 5. ***Distribution of blood cells in the zebrafish brain.***
The number of peroxidase-positive blood cells was calculated from 10 photographs of eight different brains. The values represent the total number of peroxidase-positive blood cells in 100 μm^2. Blood cells in large blood vessels were not counted because of cell overlap. Bar, standard errors (n=10).

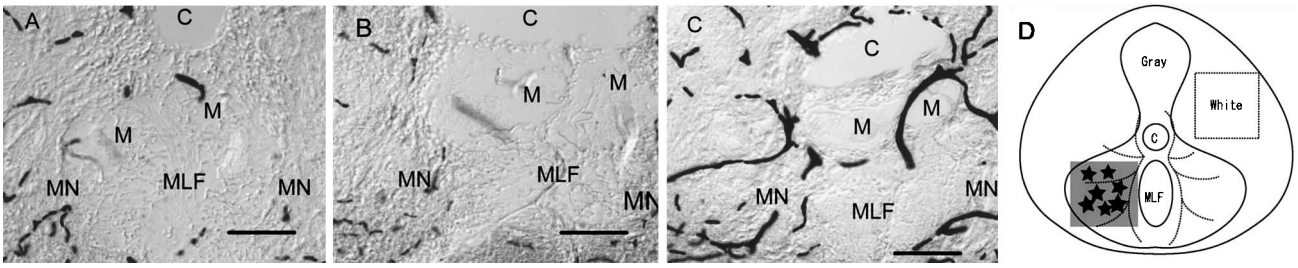

Fig. 6. ***Blood cells in brains from sleeping (A), awake (B) and actively moving fish (C).***
Coronal sections at the level of the most rostral part of the spinal cord. C, central canal; M, Mauthner axon; MLF, medial longitudinal fascicle; MN, area of motor neuron. Scale bar, 20 μm. (D) The most rostral part of the spinal cord. Gray square, area of motor neuron; white, white matter.

B) and the hypothalamus, especially the granular area (Fig. 4 C). The surface layer of the tectum contained a large number of blood cells, however, the deep layer (granular layer) contained very few peroxidase-positive cells (Fig. 4 D). This is very surprising because the deep layer of the tectum contains a large number of neurons. Oxygen and glucose may not be supplied to these granular neurons (as well as the cerebella granular neurons) by the vascular system. In the facial lobe, especially at MON and CON, the cell blood density was also high compared to other areas of the brain (Fig. 4 E). However, a few blood cells were found within the reticular formation and medial longitudinal fascicle (MLF) (Fig. 4 F). Fig. 5 shows a quantitative analysis of the blood cell distribution and that it is very different depending on the area of the brain. Fish tectum is composed of six layers; Stratum marginale (SM), Stratum opticum (SO), Stratum fibrosum et griseum superficiale (SFGS), Stratum griseum centrale (SGC), Stratun album centrale (SAC) and the deepest layer, Stratum periventriculare (SPV). This is particularly true for the five surface layers (SM, SO, SFGS, SGC, and SAC) and the deepest layer (SPV) of the tectum which display very different blood cell densities (about 30-fold, Fig. 5). These values are calculated based on a square measure of each area, however, if the calculation is based on cell number, the value for the five surface layers of the tectum is more than five hundred-fold that of the deepest layer of the tectum where numerous granular neurons are located.

Behavior-related changes in blood cell distribution.

We assessed the blood cell distribution within the brains of zebrafish engaged in activity. Zebrafish display relatively simple patterns of behavior. After illumination in the morning they become active and seek food. To enhance movement, some fish were transferred to another aquarium and chased by a fish net for 20 min before being rapidly fixed in formaldehyde. At night, the fish move very slowly or rest on the bottom of the aquarium.

A comparison of the blood cell distribution in brains from moving and resting fish showed considerable differences. The total number of stained blood cells in the whole brain increases during sleep. The total number of erythrocytes in the brains of active fish is about 55,000 (approxima-

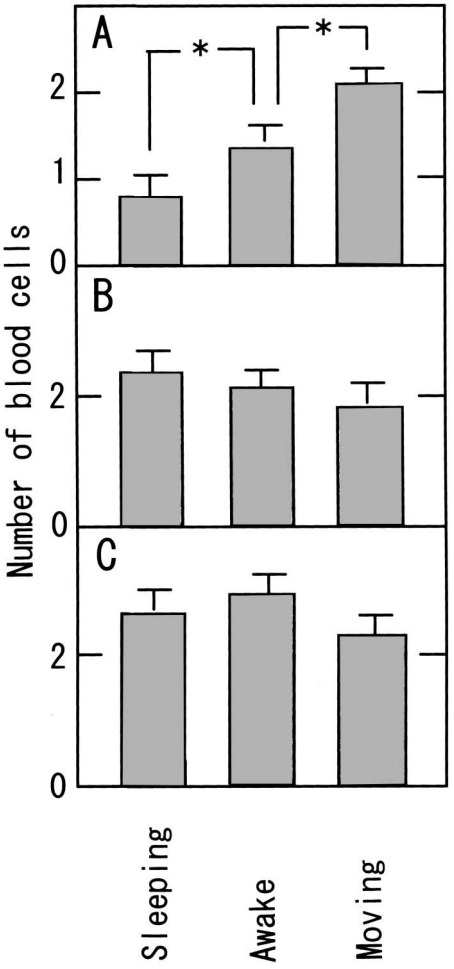

Fig. 7. ***Changes in the distribution of blood cells between sleeping, awake and actively moving fish.***
The number of peroxidase-positive cells was counted on 10 photographs of the most rostral part of the spinal cord. The cells in the motor neuron area (ventral horn, gray square in Fig. 6) (A), white matter (B, dotted square in Fig. 6) and MLF (C) of sleeping, awake and actively moving fish were enumerated. Blood cells in large blood vessels were not counted because of cell overlap. The values represent the total number of peroxidase-positive blood cells in 100 μm^2 of the ventral horn (A) and white matter (B), and the total number of stained blood cells in one section of the MLF (C). Bar, standard errors (n=10). *p<0.05, Student's T test.

tion from three fish) and this increases during sleep to about 68,000 (approximation from three fish). The extra blood cells are found in the large blood vessels. In the small blood vessels, the total number of blood cells and their distribution pattern appeared similar in active and sleeping fish.

In the spinal cord, the number of blood cells near motor neurons in the ventral horn increased during the active phase (Figs. 6 and 7) whereas their distribution in white matter and in the MLF did not change. There was also little difference in the densities of blood cells in the locus coeruleus and raphe nucleus in brains of sleeping and active fish.

DISCUSSION

Peroxidase-stained cells are erythrocytes

We found that the majority of the cells in the blood of zebrafish had peroxidase activity, and that this activity was preserved after standard formaldehyde fixation. These cells must correspond to erythrocytes, as they represented 85–90% of the blood cell population (Barber *et al*., 1981).

The erythrocytes in the brain were heavily stained, but neurons and glias were not stained at all. Based on the small size of the zebrafish brain, this easy and non-expensive method enables one to calculate the actual number of erythrocytes in each area of the brain. Such a result is basically difficult to obtain using ordinary methods, like PET and functional MRI.

The uneven distribution of erythrocytes in the zebrafish brain

The uneven distribution of the erythrocytes in the zebrafish brain was very surprising. For example, the deepest layer of the tectum and granular layer of the cerebellum had very few erythrocytes, which was in contrast to the large number of neurons in these areas. These granular cells are tightly packed, and it is reasonable to assume that they require large amounts of glucose and oxygen. However, there were only a few blood vessels in these areas and the density of erythrocytes was very low.

However, this does not mean that glucose and oxygen are not supplied to these areas. Glucose is not supplied directly to neuronal cells by natural diffusion from the blood vessels but indirectly through glial processes connecting the neuronal cells with the blood vessels. It has been demonstrated that gray matter-specific astrocytes interconnect neuronal cells and blood vessels in the zebrafish spinal cord (Kawai and Nakayasu, 2001). It is possible that a specific glial system interconnects the granular cells and the ventricle containing cerebrospinal fluid. We have found that specific glial cells, probably ependymal cells, found on the internal surface of the tectum, are tightly connected to the granular cells in the deepest layer of the tectum (Yamanari and Nakayasu, unpublished results). Some of the processes of the radial glias also attach to the cerebellar granular cells. Thus, these granular cells may also be supported by ventricle-related glia and not by blood vessel-related glia. In fact, the areas of the zebrafish brain that contained few blood vessels, including the tectum granular layer, cerebella granular layer and reticular formation in the medulla, were located near the ventricle. In contrast, the highly vascular areas, such as the marginal region of the tectum, cerebellum and medulla, were closer to the outer surface of the brain .

Very few blood vessels were found in large bundles of axons like the cranial nerves and MLF. These axons also require glucose in order to maintain the ionic gradients across the plasma membrane. It is possible that other glial cells perform this function. This will require further study as many kinds of astrocytes with different shapes and localizations have been identified that require further characterization.

In the zebrafish brain, some specific areas with high blood cell density were identified (such as the entopeduncular nucleus and periventricular area in the hypothalamus, Fig. 4). These areas might have special functions, such as the secretion of neuro-endocrine substances.

Difference in distribution of blood cells in the brains of awake, active and sleeping zebrafish

We expected that the distribution of erythrocytes would change significantly with changes in fish behavior. An increase in blood cell density in the motor neuron area of the spinal cord was observed. There was an increase to about 150% in actively moving fish compared to those that were merely awake, and an increase to about 210% compared to sleeping fish (Fig. 7). This is not surprising because the consumption of oxygen and glucose increases in the motor neurons with activity. However, this increase is small compared with the increase in impulse generation in the motor neurons. It is probable that ATP is consumed in motor neurons at a much higher rate, and is replenished initially from energy stores in the large motor neurons and glias, followed later by a gradual supply of glucose from the blood stream.

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(Received February 28, 2003 / Accepted June 9, 2003)