Retinal photographs taken by this technique do show differences in speckle contrast between blood vessels and surrounding areas. However, the differences are difficult to observe visually and would certainly be difficult to quantify, as can be seen from the examples presented in Fig. 2. Some method is needed to enhance these contrast variations, and for this reason we have added a second step to the technique.

Enhancement of the photographs. We use a standard high-pass optical filtering technique to convert the speckle contrast variations to intensity variations. This has a similar effect to that observed in dark-ground microscopy—areas with fine detail, in our case with speckle, are reproduced with a higher intensity than areas lacking such fine detail. Thus blood vessels in which flow is occurring will be reproduced darker than other areas, including blood vessels in which the flow is impeded.

Early results with this optical filtering technique are presented in Fig. 3. This shows the filtered versions of the photographs of Fig. 2, and the enhancement of the blood vessels is clearly evident. Other methods of optical filtering are being attempted to improve the final photographs.

Conclusions. We stress that this project is only at the feasibility testing stage. As stated above, exposures of the order of 1/1000 sec would be needed to map velocities greater than about 1 mm/sec. At present we are using a 50-mW helium-neon laser, and with the high-resolution film necessary for the recording of the speckle photographs we find that we need an exposure time of at least 1/60 sec. At these speeds the blood flow has completely blurred the speckle, so that all we see in our photographs is the difference between areas of flow and areas of no flow. In order to use exposure times short enough to resolve the velocities encountered in retinal blood vessels, we need more light. For this reason we are looking at the possibility of using a pulsed laser. We are also considering the advantages that may be gained by using a different laser wavelength.

The results so far are encouraging, in that we can differentiate between areas of flow and areas without flow (Fig. 3). However, more work needs to be carried out before we are in a position to quantify the information about blood-flow velocities. It will also be necessary to improve the quality of the photographs, and ways of achieving this are also being investigated.

In conclusion, it is unlikely that this technique will ever be able to compete with laser Doppler anemometry so far as precision is concerned, but we believe that the advantage of offering a "map" of blood velocities, even if it is only semiquantitative, makes the method attractive as an additional aid in ophthalmology.

The authors acknowledge the contributions of A. Penkney and M. Peukert to this project.

From the Universitaet Essen, Essen, Federal Republic of Germany. Supported by the Deutsche Forschungsgemeinschaft. Submitted for publication Aug. 26, 1981. Reprint requests: Prof. Dr. A. F. Fercher, Fachbereich 7/Physik, Universitaet Essen, Postfach 103764, D-4300 Essen 1, Federal Republic of Germany.

Key words: laser speckle, laser photography, retina, blood flow, flow velocity, velocity measurement

REFERENCES

Localization of substance P–like immunoreactivity in the anterior eye segment of squirrels: an immunohistochemical analysis. YOSHIKI SHIMIZU, YASUAKI KUWAYAMA, MASAKATSU FUKUDA, ICHIBO ISHIMOTO, SADAO SHIOSAKA, SHINOBU INAGAKI, HIROSHI TAKACI, MASASHIRO SAKANAKA, EMIKO SENBA, YUKIKO KAWAI, KENICHI TAKATSUKI, AND MASAYA TOHYAMA.

Localization of substance P (SP)–like immunoreactivity in the anterior eye segment of the squirrel was examined immunohistochemically. The present study demonstrates

0145-0404/82/02/259+05$0.50/© 1982 Assoc. for Res. in Vis. and Ophthal., Inc.
Fig. 1. Fluorescent photomicrographs showing SP-positive fibers in the ciliary body. Numerous SP-positive fibers were located in the ciliary muscle fibers (A). In some instances, they formed a dense fiber band in the muscle layer (B), from which several SP-positive fibers branched to enter the ciliary process (CP) (B, arrowheads). C, Several SP-positive fibers around the ciliary cleft (CC) (arrows). (All ×530.)

The existence of substance P (SP) in the eye has recently been suggested by several authors. \(^6\) The existence of substance P (SP) in the eye has recently been suggested by several authors. \(^6\)
Fig. 2. Fluorescent photomicrographs showing SP-positive fibers in the iris. A, Midportion; B, peripheral portion. The majority of these were located in the stroma near the anterior chamber (upward). (×530.)

In the retina, it has been demonstrated that SP is localized in the amacrine cells. However, little is known of the distribution of SP in the anterior eye segment (cornea, ciliary body, and iris), although the existence of SP-positive fibers in the cornea was recently reported. The present study was aimed at elucidating the localization of SP in the anterior eye segment, particularly in the ciliary body and iris, by means of an indirect immunohistochemical method.

Materials and methods. Experiments were carried out on 10 squirrels. The animals were perfused through the heart with 30 ml of ice-cold saline followed by 200 ml of Zamboni's fluid while under pentobarbital anesthesia (30 mg/kg, i.p.). After perfusion, the eyes were enucleated, post-fixed in the same fixative for 15 to 20 hr at 4°C, and washed overnight in phosphate-buffered saline (PBS) containing 30% sucrose. Serial frozen sections were cut on a cryostat with a section thickness of 10 μm. The slides were pretreated with chrome-alum gelatin to prevent detachment of the sections during incubation.

The indirect immunofluorescence technique of Cooms was used. Half the sections were incubated for 60 min in a humid atmosphere at 37°C with SP
antiserum pretreated with bovine serum albumin at a dilution of 1:80, after which they were rinsed three times in succession in ice-cold PBS for 10 min each time. After the buffer rinse, the sections were incubated with fluorescein isothiocyanate-conjugated antibodies (Japan Immunoresearch Laboratory Co. Ltd.) at a 1:60 dilution under the same conditions as above, rinsed three times at 4°C with PBS for 10 min each time, and mounted in PBS-glycerine mixture. As a control serum, antiserum to SP, which was absorbed by excess SP (60 μg/ml), was used. The remaining half of the slides were first incubated in the control serum and then processed as described above. (As to the specificity and nature of the antiserum to SP, see also Inagaki et al.). After observation, the sections were stained with cresyl violet for exact identification of the labeled region.

**Results.** The specificity of the immunoreaction was checked by comparing sections stained with SP-antiserum and control serum. Since the structures stained with SP-antiserum were not seen in the control sections, the structures stained by SP-antiserum were considered specific. These structures should correctly be described as showing SP-like immunoreactivity, but in this study we use the simpler term “SP-positive.”

**Ciliary body.** Numerous SP-positive fibers were seen in the ciliary body (Fig. 1, A and B). The greatest number of SP-positive fibers were located among the ciliary muscle-fibers (Fig. 1, A and B), some of which often formed dense fiber bands (Fig. 1, B). In addition, a few SP-positive fibers were observed around the ciliary cleft (Fig. 1, C, arrows). Dense fiber bands situated in the ciliary muscles often gave rise to several SP-positive fibers that entered the ciliary process (Fig. 1, B, arrowheads). These fibers transversed the stroma to reach the area just beneath the pigment epithelial cell layer, giving off several branches along their course.

**Iris.** A substantial number of SP-positive fibers were demonstrated in the iris (Fig. 2). In some instances, these fibers were directly continuous with those seen in the ciliary body. The majority of these fibers were located in the stroma near the endothelial cell layer (Fig. 2), although a few SP-positive fibers were scattered in other parts of the stroma such as around the vessels (Fig. 2, B). Moreover, a few SP-positive fibers were directed medially to distribute in the sphincter muscles of the iris.

**Cornea.** SP-positive fibers seen in the cornea of the squirrel ran mainly in the stroma just beneath the epithelial cell layer. In some instances, SP-positive fibers leaving the stroma to enter the epithelial cell layer were also identified (Fig. 3, arrows).

**Ciliary and trigeminal ganglia.** To provide the immunohistochemical basis for postulating the origins of SP-positive fibers in the anterior eye segment, additional observations were made to examine whether the ciliary and trigeminal ganglia contain SP-positive cells.
A significant number of SP-positive cells were seen in the trigeminal ganglion, and no SP-positive cells were seen in the ciliary ganglion.

Discussion. Previous pharmacologic studies with the rabbit have shown that SP affects the constriction of the pupillary sphincter muscle. However, the present study with squirrels failed to demonstrate a conspicuous number of SP-positive fibers to the pupillary sphincter, although a few SP-positive fibers do exist in this area. This discrepancy might be explained by species differences since another of our studies with rats has demonstrated numerous SP-positive fibers to the pupillary sphincter.

The present study has shown the existence of a small number of SP-positive fibers around the ciliary cleft. This fact suggests that SP might play some role in the aqueous outflow system.

With regard to the origins of these fibers, the trigeminal ganglion cells are mentioned as a possible site because a substantial number of SP-positive cells are located in this ganglion whereas none is demonstrable in the ciliary ganglion of the squirrel. However, since trigeminal ganglion cells are primary sensory afferent neurons, other origins cannot be excluded.

The present study demonstrates the presence of SP-positive fibers in the cornea of the squirrel, which is in good agreement with the results of a previous study. Because the branches of these fibers are located in the epithelial cell layer, these fibers are presumed to have some effect on sensory transmission. There is little evidence regarding the origins of these fibers, although a recent study denied the possibility that trigeminal SP-positive cells are the origin of corneal SP.

In any case, although it is certain that SP plays an important role in the physiologic control of anterior eye segment function, the precise role of SP in this area remains obscure. To explore the function of SP in this area, further anatomic and physiologic studies are needed.

We thank Profs. R. Manabe and Y. Shiotani for their helpful criticism of the manuscript.

From the Department of Ophthalmology (Y. S., Y. K., M. F., I. I.) and Department of Neuroanatomy, Institute of Higher Nervous Activity, Osaka University Medical School, Osaka, Japan. Supported by grant 577660 from the Japan Ministry of Education. Submitted for publication May 1, 1981. Reprint requests: Dr. Y. Shimizu, M. D., Department of Ophthalmology, Osaka University Medical School, 1-1-50, Fukushima, Fukushima, Osaka 553, Japan.

Key words: substance P, anterior eye segment, squirrel, immunohistochemistry

REFERENCES


Ca++-induced cataract. K. R. HIGHTOWER AND V. N. REDDY.

Cataracts in cultured rabbit lenses were produced by elevation of internal calcium. Experimental procedures were successful in increasing levels of total and bound Ca++. open without significant change in sodium, potassium, or water content. Although the excess in calcium was predominantly associated with water-soluble proteins and was freely diffusible, a significant amount was...