Calibration of infrared video-oculography by using bioadhesive phosphorescent particles for accurate measurement of vestibulo-ocular reflex in mice

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Eye movements are known to be well-quantitative indices to analyze a variety of neurological deficits as well as mechanisms for controlling motor systems in both scientific and clinical researches. In this study, a novel method we developed to measure the eye movement in mice, using bioadhesive polymer-coated phosphorescent particles, was applied for the calibration of the conventional video-oculography that tracks the pupil. We tested the vestibulo-ocular reflex, a reflexive eye movement observed across various species, and compared the values measured by the new method with ones measured by the conventional video-oculography. We found the difference between them was significant when the size of the pupil became small whereas those values were similar when the size of the pupil was relatively large. Our results suggest that there is a calibration factor related to the size of the pupil to improve the accuracy of the video-oculography and a potential benefit of our novel method to measure eye movements in rodents.

Keywords: Vestibulo-ocular reflex, Bioadhesive phosphorescent particles, Video-oculography

I. INTRODUCTION
Measurement of eye movements has been widely used as a highly quantitative tool to address motor control processes and to detect deficits in the neural circuit in the brain. Among a variety of different types of eye movements, the vestibulo-ocular reflex (VOR) is a reflexive eye movement that is occurred in most of vertebrates from fish to human beings.¹

In order to elucidate mechanisms for motor control and motor learning, there have been a number of studies utilizing the VOR measurement in experimental animals with several reasons. For example, the neural circuit to regulate the VOR is a relatively simple compared to other motor systems. In addition, it is easy to quantify inputs to induce the VOR and its outputs, and the VOR can be altered by motor learning during training for tens to hundreds of minutes.²

Recently mice have become an increasingly common choice for neuroscience experiments including approaches to neural mechanisms for the oculomotor system, due to their suitability for molecular-genetic manipulations. Since the late 1990’s, several different methods to measure eye movements in mice have been developed.³⁴ The search coil technique and the infrared video-oculography are two major methods in this research fields. The search coil technique is to estimate the angular velocity of the eye by measuring currents induced in the small coil which is implanted between
the conjunctiva and sclera. When the mouse with the eye coil is located in the magnetic field, the amount of induced currents in the coil will be changed when the eye is rotated. This technique has been widely used for measuring eye movements in experimental animals due to its high sensitivity and enabling us to do the calibration quite accurately. However, a potential problem was reported that the implanted eye coil itself could disturb the eye motion, in particular, for relatively small size of animals such as mice. The infrared video-ocularography is to track the pupil position by when the infrared light LED illuminates the surface of the eye and to calculate the angular velocity of the eye. This technique gives us the opportunity to measure eye movements in mice less invasively. However, since it needs to detect the pupil area distinct from corneal area around it, it is required to shrink the pupil in the darkness, the situation required to measure the VOR, by instilling miotic agents such as pilocarpine hydrochloride. A couple of potential problems, e.g. side effects of miotic agents that could alter the eye movements or a variety of effects of miotic agents across lines of transgenic mice, have been pointed out. In the present study, using a novel method to measure eye movements we developed, we measured the VOR in mice and compared with the VOR measured by using a conventional video-ocularography quantitatively. This new method, using bioadhesive polymer-coated phosphorescent particles, is less invasive than the search coil technique and no drug treatment is required. We found a physiological calibration factor, which is potentially available to improve the accuracy of the conventional video-ocularography. Details of developing a bioadhesive marker to measure eye movements were described in the proceedings previously.

II. METHODS AND MATERIALS

II-1. Preparation of bioadhesive phosphorescent markers

Preparing bioadhesive phosphorescent particles was described previously. Briefly, LumiNova® BG series, BG-300M (Nemoto Lumi-Materials Co., Ltd), which is a long afterglow Sr₂Al₁₄O₂₅:Eu,Dy phosphor, was used as a phosphorescent material. Ten grams of its particles, 32-45 μm in diameters, were agitated in the truncated conical coating pan. Ten milliliters of 2% carboxyvinyl polymers, Carbopol® 934 (Serva Electrophoresis GmbH) in ethanol solution, were sprayed uniformly on the particles to coat them in the pan. The coated particles were dried by warm air at 60°C. The coating procedure was repeated five times or ten times.

II-2. Animal surgery

Experiments were performed on adult female C57BL/6J mice (12-18-month-old, 25-35 g). On surgery, while the mouse was under gas anesthesia using isoflurane, a head post (M4 x 15, YAHATA) was attached to the top of the skull using stainless anchor screws (Size 000 x 3/32 (0.86 mm in diameter, 2.4 mm in length), J.I. Morris) and dental acrylic (UNIFAST II, GC corporation). Mice were allowed to recover from surgery for 5–7 days before oculomotor testing.

II-3. VOR experiments

For oculomotor experiments, the head of the mouse was immobilized by attaching the implanted head post to a restrainer and its body was loosely restrained in a vinyl chloride pipe (50 mm in diameter, 120 mm long) (Fig. 1A). The restrainer was attached to a turntable (60 cm in diameter, Santo Co., Ltd) with a direct drive servomotor (NMR-CTFIA2A-841A, NIKKI DENSO), which delivered a vestibular stimulus by rotating the mouse about an earth vertical axis. One drop (about 1 μL) of 3% pilocarpine hydrochloride (Sanpilo®, Santen Pharm. Co., Ltd) was applied on the right eye of mouse. After the tear fluid including the excess of the miotic agent was removed with a piece of filter paper to dry up a small area of the cornea, bioadhesive phosphorescent particles were placed on its surface as markers (Fig. 1B).

The eye of the mouse was illuminated by the infrared LED (peak wavelength: 850 nm, M850L3, Thorlabs), which was attached to the turntable. The
infrared CCD camera (GV200, Library Co., Ltd) equipped with a zoom lens was attached to the turntable and was focused on the surface of the right eye to sample images of the pupil and markers. Sinusoidal horizontal vestibular stimulus was used to induce the VOR in darkness or in light, at frequencies of 0.5 Hz, with a peak velocity of ±10°/s. The angular velocity signals of turntable were sampled at 1 kHz, digitized by the I/O interface, filtered at 10 Hz of cut-off frequency (Spike2, CED) and stored on the PC. Sampling of the pupil and marker images was processed by the CaptureEx (Library) at a sample rate of 200Hz, with 640 x 480 pixels. Move-tr2D, an eye tracking system (Library), was used to track the center of the pupil and that of the marker and to digitize their horizontal position as well as the size of the pupil. The angular velocity of the pupil was calculated based on the method described previously. The angular velocity of the marker was calculated by differentiating all the values of central angles formed by positions of the center of the marker in the two consecutive time windows, assuming the eye ball a true sphere. Then all the angular velocity data were fitted with the sinusoidal function and the peak amplitude and phase of each parameter were calculated using Matlab (Mathworks) (Fig. 2). The VOR gain was calculated as the ratio of the pupil/marker to the turntable velocity amplitudes. The VOR phase was calculated as the difference between the peak pupil/marker velocity phase and the peak head velocity phase in the opposite direction.

Fig. 2. Representative eye velocity traces induced by vestibular stimulation. The earth-horizontal sinusoidal rotation at frequencies of 0.5 Hz with a peak velocity of ±10°/s was given (top panel). Both the bioadhesive marker (middle) and the pupil (bottom) were well-tracked by the CCD camera. The gray lines represent nonlinear least-squares fit of data to the sine curve. The arrow heads indicate when the mouse made a blink appeared in both the marker and pupil trackings.

Fig. 1. Experimental setup for VOR measurement. A. Eye movement measurement system for video-oculography. B. A screenshot of a bioadhesive phosphorescent particle (located in the white box) on the mouse cornea.
All procedures for animal care and experimental protocols were carried out in accordance with “Fundamental guidelines for proper conduct of animal experiment and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (2006)” and “Basic policies for the conduct of animal experimentation in the Ministry of Health, Labor and Welfare (2006),” and were reviewed and approved by The Institutional Animal Care and Use Committee at Tokai University.

III- RESULTS

III-1. Comparison of the VOR gains based on detecting the pupil and phosphorescent marker position

The VOR gain based on the peak angular velocity calculated with the position of the pupil (VGp) and that calculated with the position of the phosphorescent marker (VGM) were plotted on the abscissa and ordinate, respectively (Fig. 3). The angular velocity of the pupil was larger than that of the marker in 6 out of 8 measurements and the average of VGp (0.67 ± 0.02) was significantly larger than that of VGM (0.57 ± 0.03) (p < 0.05 by paired t-test).

Fig. 3. Comparison of the VOR gain in two different measurements. Values of the VOR gain based on the pupil tracking (VGp) were larger than the one based on the marker tracking (VGM). The diagonal line indicates a slope of 1.

III-2. The effect of the pupil area to the calculation of the VOR gain

Calculation of the VOR gain described by Sakatani and Isa9 can be affected by the area of the pupil (See Discussion). As shown in Fig. 4, the ratio of VGM to VGp was positively correlated with the size of the pupil relative to the size of the eye ball (p < 0.05, R = 0.75, slope = 0.86, intercept = 0.56), and the former values were around 1 as the latter values became larger. This result suggests that there is a calibration factor related to the area of the pupil to calibrate the VOR gain using video-oculography.

Fig. 4. Correlation between the size of the pupil and VOR gain. The ratio of VGM to VGp was positively correlated with the diameter of the pupil (φp) relative to the diameter of the eye ball (φE).

III-3. Stability of the phosphorescent marker on the cornea

To confirm the stability of the phosphorescent marker on the cornea during measuring the VOR, the differences of the VOR phase calculated with the position of the pupil (VPp) and that calculated with the position of the phosphorescent marker (VPm) were compared. The mean difference between VPp and VPm was 2.3 ± 6.9 ms, not significant from zero (p > 0.7), indicating that the phosphorescent markers were stable enough on the cornea without slipping during VOR measurements.
IV. DISCUSSION

Eye movements are well-quantitative motor systems and are driven by relatively simple neuromuscular circuits compared to other motor systems. Therefore, development of techniques to measure eye movements accurately not only for clinical scenes but also for research using experimental animals is demanded. In our present study, we showed the ability of the carboxyvinyl polymer-coated phosphor particles as a bioadhesive marker to measure the eye movement in mice as we described previously. The main conclusion was that we found an important calibration factor for the conventional video-oculography by using this novel technique.

The video-oculography, one of the major eye tracking systems in experimental animals, captures the position of the center of the pupil by illuminating the eye ball with the infrared light and then calculates the angular velocity of the eye. Recently people widely use this system to measure eye movements in mice because of its less invasiveness than the search coil method. However, there have been a couple of issues reported for the video-oculography that need to be addressed to improve its acuity. First, the calibration of video-oculography is not simple. Since the fovea in mice is not well-developed, the visual fixation cannot be used for its calibration unlike human or monkey research. In addition, since the white region of the eye ball covered with the sclera is not visible from outside in rodents, it is required to capture the image of the pupil illuminated with the infrared light for eye tracking. The pupil is located under the cornea, not on the surface of the eye ball, resulting in its effective radius from the center of the eye ball to the center of the pupil fluctuated depending on the input from the pupillary sphincters. Second, in order to measure the VOR, a miotic agent should be instilled into the eye to capture the pupil image because the pupil becomes mydriatic in the darkness. The size of the pupil is regulated by the autonomic nervous system and it is possible that the effects of miotic agents can be various between lines of mice. Our novel method to measure eye movements in mice using the carboxyvinyl polymer-coated phosphor particles as a bioadhesive marker has a couple of advantages; first, the marker particles are adhered on the corneal surface so that the effective radius of the marker is equivalent to the radius of the eye ball to calculate the angular velocity of the marker. In addition, no drugs are required to be administrated to measure the eye movement with this method. It is expected that these advantages could decrease the complexity of the calibration for the video-oculography. Alternative method with a similar idea using titanium dioxide pigments as markers was reported but no VOR measurement was examined.

In order to estimate the peak angular velocity of the pupil to calculate the VOR gain, we used the calibration method proposed by Sakatani and Isa. This calibration seems reasonable and accurate considering the fluctuation of the effective radius of the pupil at the each time points of measurement. In fact, when the ratio of the size of the pupil to the size of the eye ball (3.3 mm on average) was around 0.4 to 0.5, the estimation of VOR gains was not significantly different between VGp and VGm (Fig. 4). On the other hand, the VGp that is calculated based on the position of the pupil seems overestimated when the size of the pupil was relatively small (< 0.3 of the ratio of the pupil to the eye ball). Our results suggest a new calibration factor according to the size of the pupil to estimate the VOR gain, probably caused by the refraction of the cornea that was not considered in the previous studies.

It is demanded to measure eye movements continuously for longer time in the case to use a mouse model of human diseases. Long time illumination of the infrared light to the cornea for video-oculography could occur dryness of the cornea. In this study, we needed to illuminate the surface of the eye ball to detect the carboxyvinyl polymer-coated phosphor particles on the cornea because the intensity of afterglow of the phosphor particles we used was not strong enough. Improving the intensity of afterglow of the phosphor particles as well as the ability of its bioadhesiveness...
can achieve long-time measurements of eye movements in rodents without using the infrared LED nor miotic agents in future.

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REFERENCES

1 M. Ito, Annual review of neuroscience 5, pp. 275-296 (1982).
2 E. S. Boyden, A. Katoh and J. L. Raymond, Annual review of neuroscience 27, pp. 581-609 (2004).
3 A. Katoh, H. Kitazawa, S. Itohara and S. Nagao, Proceedings of the National Academy of Sciences of the United States of America 95, pp. 7705-7710 (1998).
4 S. K. Koekkoek, A. M. v Alphen, J. vd Burg, F. Grosveld, N. Galjart and C. I. De Zeeuw, Genes and function 1, pp. 175-190 (1997).
5 J. S. Stahl, A. M. van Alphen and C. I. De Zeeuw, Journal of neuroscience methods 99, pp. 101-110 (2000).
6 A. Katoh, E. Takeuchi, T. Hatanaka, E. Sasagawa, T. Iwasato, S. Itohara, Neuroscience 2014 Abstracts, Washington, USA: Society for Neuroscience, 62. 23 (2014).
7 T. Kimura, Uchida, M., Yamaki, T., Hatanaka, T., Katoh, A., Natsume H., 134th Annual Meeting Abstracts. Kumamoto, Japan: the Pharmaceutical Society of Japan, 29pmL-053 (2014).
8 L. A. Felton, International journal of pharmaceutics 457, pp. 423-427 (2013).
9 T. Sakatani and T. Isa, Neuroscience research 49, pp. 123-131 (2004).
10 M. de Jeu and C. I. De Zeeuw, Journal of visualized experiments : JoVE (65), e3971 (2012).
11 B. van Alphen, B. H. Winkelman and M. A. Frens, Investigative ophthalmology & visual science 51, pp. 623-630 (2010).