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A system to measure the pupil response to steady lights in freely behaving mice

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Abstract

Background—Transgenic mice are widely used for the study of basic visual function and retinal disease, including in psychophysical tests. Mice have a robust pupillary light reflex that controls the amount of light that enters the eye, and the attenuating effects of the pupil must be considered during such tests. Measurement of the size of pupils at various luminance levels requires that mice remain stable over prolonged periods of time; however, sedation of mice with anesthesia and/or manual restraint can influence the size of their pupils.

New Method—We present a system to measure the pupillary light response to steady lights of freely behaving mice using a custom-built, portable device that automatically acquires close-up images of their eyes. The device takes advantage of the intrinsic nature of mice to inspect objects of interest and can be used to measure pupillary responses in optomotor or operant behavior testing chambers.

Results—The size of the pupils in freely behaving mice decreased gradually with luminance from a maximal area in the dark of 3.8 mm² down to a minimum 0.14 mm² at 80 scotopic cd/m². The data was well fit with a Hill equation with Lo equal to 0.21 cd/m² and coefficient $h=0.48$.

Comparison with existing methods—These values agree with prior measurements of the pupillary response of unrestrained mice that use more laborious and time consuming approaches.

Conclusions—Our new method facilitates practical, straightforward and accurate measurements of pupillary responses made under the same experimental conditions as those used during psychophysical testing.

Keywords

Mouse; pupillary reflex; freely behaving mice; visual behavior; optomotor response; operant behavior; habituation

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1. Introduction

Transgenic mice are widely used for the study of basic visual function and retinal disease (Nishina and Naggert, 2003; Pinto and Enroth-Cugell, 2000). These studies compare structure-function changes at the cellular and retinal levels by combining traditional approaches such as immunohistochemistry, electrophysiology and biochemistry (Huberman and Niell, 2011; Pinto and Enroth-Cugell, 2000). More recently, these traditional approaches have been complemented with psychophysical tests that assess the limits of vision in mouse (Busse et al., 2011; Histed et al., 2012; Naarendorp et al., 2010; Prusky et al., 2004) and can track visual changes in models of retinal degeneration (McGill et al., 2012; Umino et al., 2006), retinal transplantation (Pearson et al., 2012) and gene therapy treatment (Boye et al., 2013; Pang et al., 2011). A primary objective of these visual psychophysical assays is to determine the minimal amount of light or contrast that is necessary to elicit a visual response in animals (also known as visual threshold) (Stebbins, 1970). However, mice have a robust pupillary light reflex that controls the amount of light that enters the eye (Pennesi et al., 1998): as luminance increases the iris muscles constrict and close the pupil. Hence, the attenuating effects of the pupil must be considered, particularly when it is important to know the precise level of retinal illumination (Lyubarsky et al., 2004).

Characterizing the response of the pupil to light is a straightforward procedure with human subjects, but can be a challenging undertaking in mice. Measurement of the size of mice pupils at various luminance levels requires that mice remain stable over prolonged periods of time. This is generally accomplished by gently restraining the mice by hand while the measurements are performed (Guler et al., 2008; Hattar et al., 2003; Lucas et al., 2003).

Intrinsically photosensitive melanopsin-containing retinal ganglion cells (ipRGCs) mediate the pupillary light reflex (Berson et al., 2002; Hattar et al., 2002; Provencio et al., 2000); ipRGCs also receive input regarding luminance from rod and cone photoreceptors (Altimus et al., 2010; Dacey et al., 2005; Guler et al., 2008; Lall et al., 2010; Viney et al., 2007; Weng et al., 2013). However, the size of pupils in restrained mice can be influenced by an autonomic response triggered when restrained (Bitsios et al., 1996) irrespective of background illumination. An option that reduces animal anxiety for assaying the pupillary light reflex when restrained is to immobilize (Pennesi et al., 1998) or sedate the mice during the procedure (Kuburas et al., 2014; Thompson et al., 2011). Small amounts of ketamine/xylazine anesthesia do not interfere with the pupillary light response to *brief* light flashes (Thompson et al., 2011) but the effect of ketamine/xylazine anesthesia on the size of the pupil in *steady* lights has not been determined. More importantly, it is unknown whether the pupil size determined with either of these procedures is the same as the size of the pupil of freely behaving mice exposed to similar luminance environments.

In view of these uncertainties, in an earlier study of the optomotor response, we measured the pupillary light response of unrestrained mice standing on a pedestal inside the enclosure formed by the four video LCD monitors that provided the visual stimulation (Umino et al., 2012). However, a practical difficulty we faced with this setup is that mice do not always orient themselves in the direction of the camera, making the acquisition of high quality images of the pupil a problematic, time demanding task. To address this problem we built a

portable device that automatically acquires eye images of unrestrained mice as they explore an object of interest. Here we describe this automated system and demonstrate its application for the measurement of steady-state pupil responses in mice. To determine the accuracy of this method we compare pupillary responses measured with the new system to the responses obtained previously in unrestrained mice standing on a pedestal (Umino et al., 2012) and those obtained in gently restrained mice under a Ganzfeld illuminator. Finally, we present an empirical formula that describes the relationship between size of the pupil and luminance in freely behaving C57BL/6J mice within the OptoMotry® enclosure.

2. Methods

Adult (3-4 months of age), female C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were maintained at SUNY Upstate Medical University (Syracuse, NY). Mice were fed ad libitum a standard diet and maintained on a 14-hour light/10 hour-dark cycle (lights on at 6 am). All measurements were performed in the early evening hours (6pm to 10 pm), when mice increase their levels of activity. Mice were dark-adapted for 2 hours prior to the measurements of their pupil areas and handled in dim red lights to minimize the effects of light adaptation. The procedures in this study were approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (2011, National Academies Press, Washington, DC) and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.1. Measurement of the pupillary light response to steady lights in freely behaving mice

We measured the pupillary light response to steady lights of freely behaving mice using a custom-built, low-cost portable device that automatically acquires eye images of unrestrained mice (Fig. 1A). The device was designed to fit inside illuminated enclosures such as those used for optomotor (Fig. 1B) or operant behavior testing and consisted of a transparent plexiglas box (11 cm × 11 cm × 10.5 cm) with a small circular aperture (0.6 cm in diameter) drilled on a side wall at 1.25 cm from the elevated floor and 0.3 cm from the side panel (Fig. 1C). During experiments a plexiglas divider was introduced to reduce the 'behaving surface' by approximately half of the chamber floor dimension in order to minimize the area that the animal was allowed to explore. The floor was elevated 1.25 cm from the bottom of the chamber and was perforated by uniformly spaced holes 0.6 cm in diameter. Four aeration holes were drilled into the lid of the chamber.

Mice placed inside the enclosure spend significant amounts of time exploring the aperture on the side panel (Fig. 1A) which provides the opportunity to acquire close-up images of their pupils. High resolution, infrared images of the pupil were captured using a CCD Webcam (Logitech HD Pro Webcam C920, Newark, CA,) after replacing the IR blocking filter in the camera's light path with an IR high pass filter (Edmund Optics, Barrington, NJ). A cosmetic blue LED located inside the camera and the autofocus feature were inactivated. Infrared illumination was provided with an array of IR LEDs (940 nm emission wavelength) aligned with the camera's view (Fig. 1A, C). The webcam was attached to the lid of the chamber and focused at the level of the aperture and oriented to provide a frontal view of the

pupil as the mice explored the aperture. Size calibrations were performed with a graticule positioned at the level of the aperture. Calibrations were repeated with the graticule positioned 5 mm proximal and 5 mm distal relative to the aperture in order to cover all possible locations where the eye may be positioned as the mouse inspects the aperture. Images remained on focus and the same calibration factor (39 pixels/mm) applied to all three locations indicating that the quality and size of the image does not vary significantly within the range of possible eye locations. Video acquisition was triggered with motion detection software (Webcam Zone Trigger, Montreal, QC) every time the nose of the mouse crossed the plane of the aperture. Each video clip was a minimum 5 seconds in duration but automatically extended to longer recording times as long as activity continued to be detected at the aperture. Video resolution was 1920×1080 pixels per frame acquired at 15 Hz. Off-line analysis of pupil areas is described below.

2.2 Illumination

For the purpose of this study, the portable device was placed inside an enclosure formed by four video LCD monitors that provided white (broad spectral) light stimulation, an environment routinely used to measure the optomotor response of mice (OptoMotry®, Cerebral Mechanics, Lethbridge, AB, Canada). Monitor luminance was 29 photometric cd/m² producing 230 photometric lux at the expected position of the pupil. To determine the specific activation of each photoreceptor type in the retina (Lucas et al., 2014) we measured the spectral radiance of the incident light using a PR-650 Spectra Scan portable spectroradiometer (Photo Research, Chatsworth, CA). The spectrum exhibited the characteristic peaks of the fluorescent lamps powering the monitors (Fig. 1D). With this information we applied the MouseToolbox software (<http://www.eye.ox.ac.uk/team/principal-investigators/stuart-peirson>) to determine the illumination in terms of specific photopigment complements. Hence, in the absence of optical filters, the illumination at the cornea produced 6.1 α-opic_{sc}lux (S-cone), 126 α-opic_zlux (melanopsin), 144 α-opic_rlux (rods), and 156 α-opic_{mc}lux (M cone).

Light levels were controlled with optical density filters (Lee Filters, Burbank, CA) laid over the computer monitors as described previously (Umino et al., 2008). The filters attenuate approximately 90% of incident light in the visible range but their attenuation decreases sharply at wavelengths > 700 nm (Fig. 1D). As a result, the relative contribution of the emission peak at 725 nm increases as the number of filters increases (Fig. 1E). Luminance values expressed in terms of scotopic cd/m² were estimated by evaluating the product of the filter(s) transmittance by the spectral radiance and the normalized absorption spectrum for rhodopsin at each wavelength (Figs. 1C and D). The product was then integrated across the spectrum and the conversion factor $K_n = 6.72 \times 10^{-16}$ lm s/photon (Rodieck, 1998) applied to convert photons into lumens.

2.3 Image analysis

Individual frames in the video clips (Fig. 2A-C) were imported and analyzed off-line using Image J analysis software. Our preliminary observations indicated that on occasion the pupil size increases shortly after the mouse reaches the aperture on the side panel. Because our goal was to determine the size of the pupil as mice move freely in the chamber and not with

pupil sizes that can be associated with increased interest or arousal as mice explore the aperture on the side panel (Binda et al., 2014; Zenon et al., 2014) we limited the analysis of the pupil to the first five frames in each video (333 msec). Frames were discarded when images were out-of-focus, presented motion artifacts, illumination artifacts (e.g., reflection of the IR LED lights obscuring the pupil), or angled views of the eye (Fig. 2C). Following this ‘triage’ process, the remaining frames were analyzed as described below.

We implemented a simple macro that applied ImageJ selection tools and user assistance to measure the pupil areas in the images. The analysis consisted of the following steps : a) switch from full (Fig. 2A or 2B) to close-up view (Fig. 2D) of the eye; b) determine the minimal pixel value within the pupil; c) apply this value to a parametric formula (see below) that determines the threshold value and apply this threshold to the image (Fig. 2E) ; d) connect pupil regions separated by overlying whiskers using the built in ‘line’ selection function; e) trace contour of pupil regions with the ‘wand’ selection tool (Fig. 2F); f) apply the ‘convex hull’ selection tool to enclose the pupil (Fig. 2G); g) fit the enclosed region with an ellipse and determine aspect ratio (major axis/minor axis) (Fig. 2H); h) apply the built in Measure command to determine the area of the ellipse. The macro used for the analysis is available upon request.

An important matter is the definition of threshold level applied to the pupils. A unique threshold level cannot be applied across-the-board to all pupil images because the IR LEDs provide a non-uniform illumination resulting in pixel values that depend on both spatial location and orientation of the eye. To circumvent this problem we defined an adjustable threshold that accommodated to differing light levels in each image. We first measured pixel values in the pupil and iris of mice in multiple visits to inspect the aperture (Fig. 2I). Histogram plots showed a significant difference between the minimal pixel values measured in the pupil and iris (Fig. 2J). Indeed, minimal pixel values in the iris (MPI) follow the empirical relation:

$$\text{MPI} = \text{MPP} + \Delta * \log (\text{MPP}) \quad (\text{Eq. 1})$$

when plotted as function of minimal pixel values in the pupil (MPP) (Fig. 2K, black line), where $\Delta = 20$ ($R^2 > 0.97$). We arbitrarily defined threshold level (THL) as

$$\text{THL} = \text{MPP} + \Delta_t * \log (\text{MPP}) \quad (\text{Eq. 2})$$

where $\Delta_t = 16$ (80% of its original value) (Fig. 2K, grey line). We applied this definition of threshold systematically to the analysis of the images in this study.

2.3. Curve fits and statistical analysis

Pupil area was plotted as a function of luminance and fit with the Hill equation:

$$A = \frac{A_{Dark} L_o^h + A_{Min} L^h}{L_o^h + L^h} \quad (\text{Eq. 3})$$

where A is pupil area as a function of intensity or luminance, A_{Dark} is the area of the pupil in dark-adapted conditions, A_{Min} is the area of the pupil attained in the brightest light stimulus, h is Hill's coefficient, L is the luminance of the light stimulus, L_o is the stimulus required to reduce the pupil area (A) to 50% of its dynamic response range such that $A = (A_{Dark} - A_{Min})/2 + A_{Min}$. We use L_o as an inverse measure of the sensitivity of the pupillary response to steady light stimulation. A relatively low value of L_o indicates higher response sensitivity and vice versa.

A second order polynomial equation used to fit the relation between visit times (VT) and the respective visit number (VN):

$$VT = a_1 * VN + a_2 * VN^2 \quad (\text{Eq. 4})$$

Where a_1 and a_2 are the linear and second order coefficients, respectively. Fit parameters were estimated using the Levenberg-Marquadt method for nonlinear least squares minimization (SigmaPlot, Systat Software, San Jose, CA).

Student's t-test (two sided) was used to compare mean sensitivity (L_o) between experimental procedures. Repeated measures ANOVA was applied to infer statistical significance in tests of pupil areas in light and dark conditions. Data points in plots represent mean $\pm$ SEM. The number of mice used for each experiment (n) is indicated in the text. Fits, regression analysis and statistical inferences were performed with SigmaPlot.

3. Results

3.1. The pupillary light response of freely behaving mice

We built a portable device that automatically acquires eye images of unrestrained mice as they explore an object of interest (see Methods). Here, the object of interest was a small opening on a side panel of the plexiglas chamber containing the mice. After discovering its presence, freely behaving mice will regularly visit and inspect the opening in the side panel. More importantly, the exploratory behavior proceeds with highly reproducible head motions which allowed us to set up and focus a camera to automatically acquire images of their pupils as mice explored the opening on the panel. To measure the pupillary responses to steady lights we placed the plexiglas chamber containing the mice within the OptoMotry[®] enclosure and illumination was controlled with optical density filters. Animals were placed inside the chamber and images of their pupils were captured automatically under a steady luminance of 80 scotopic cd m^{-2} producing 144 α -opic μlux (rods) (see Methods for excitation levels of other photoreceptor types in the retina). Trials were 20 minutes in duration. We used long trial periods to match the duration of standard optomotor or operant

behavioral assays. Images of the pupil were measured off-line, averaged, and plotted as a function of time.

Pupil areas of mice (3-4 months of age, $n=6$) exhibit two forms of variation – slow and fast – when mice arrive to explore the opening in the side panel in 80 scotopic cd m^{-2} . Pupil area values change randomly from sample-to-sample about a steady mean (M35, M40) or follow slowly increasing (M38, $R^2 = 0.33$, slope = $1 \times 10^{-4} \text{ mm}^2/\text{sec}$, $p = 0.02$) or decreasing (M39, $R^2 = 0.14$, slope = $-8 \times 10^{-5} \text{ mm}^2/\text{sec}$, $p = 0.04$) linear trends (Fig. 3A). In repeated trials a slow trend is present ($p < 0.05$) in approximately 40% of the sessions, often in the form of a decrease in pupil area. Individual histograms of the sample-to-sample variation in pupil areas were well fit by Gaussian distributions ($p > 0.05$) (Fig. 3B) consistent with the random nature of these variations.

We examined if the ‘fast’ variations in pupil area arose from distance effects. It follows from first principles that the angular size of an image changes in inverse relation to the distance of the object to the camera. In such case, pupil area should correlate inversely with the size of other eye dimensions, as for example, the distance between the inner and outer cantus of the eye. Plots of pupil areas vs inter-cantus distance do not show a significant trend (Fig. 3C), dismissing distance effects as a source of variation in pupil areas. We also explored the possibility that the changes in measured pupil areas arise from views that are at an angle with the line of sight. When viewed at an angle, the pupil appears elliptical and the area decreases (Mathur et al., 2013). Plots of pupil area vs aspect ratio (ratio of the major to the minor axis in the ellipse fitting the pupil) showed a significant correlation in only one (M39) out of six mice tested (Fig. 3D), ruling out angled views as a major source of variability. We conclude that the variability in the size of the pupils likely results from a dynamic modulation of the pupil in freely behaving mice (Fig. 3E).

3.2 Time between visits

One important question is whether mice maintain the level of exploratory behavior in repeated trials or they show signs of habituation (Rankin et al., 2009) commonly observed in rodent behavioral assays (for example, Bolivar, 2009). To determine if mice exploring the aperture exhibit intra-session habituation we determined the time at which each mouse visited the aperture, and plotted this time as a function of corresponding visit number (Fig. 4A). At first, time to visit increases linearly, with small, rather regular increments. Signs of habituation were evident with increasing number of visits: the time between visits increased and the size of the steps increased, giving the function a slight upward curvature. Occasional steep steps (arrows in Fig. 4A) indicate prolonged periods when the mouse did not visit the aperture. During these times when mice did not visit the aperture, mice often directed their attention to other aspects of the chamber or engaged in grooming behavior. We also observed a case in which the overall frequency of the visits slowed altogether, giving rise to a major change in the slope of the function (M36). The functions were well fit ($R^2 > 0.97$) with second order polynomials (Methods) where $a_1 \gg a_2$ (Fig. 4B), which is consistent with the notion that initially the average behavior is linear, with visits to the aperture at constant time intervals (Fig. 4C). Similar behavior was observed in two separate sessions (Fig. 4A-C, black vs grey symbols) although the frequency and time of the ‘steep steps’ do not appear to

be predictable. On average the fitting coefficients a_1 and a_2 did not differ in the two sessions (paired t-test, $p > 0.05$), consistent with little intersession habituation.

To further characterize the temporal aspects of the response we analyzed the times between visits. Histograms of the times between visits show that short intervals were more frequent than long intervals (Fig. 4D). In general, all histograms were well fit by a decaying exponential function ($R^2 > 0.7$), consistent with randomly recurring visits every 29.3 ± 4.4 sec, the average value of the exponential time constant. Furthermore, mice averaged 18 ± 3 ($n = 6$) visits during the first 10 minutes of each session (Fig. 4E), or approximately 33 sec between visits.

3.3 Pupil areas in the dark

Next we investigated the behavior of mice in the dark (Fig. 5). Mice were dark-adapted for two hours prior to the tests and images of their pupils acquired in 20 min sessions. Darkness did not deter the mice from visiting the aperture in the side panel. Pupils were relatively dilated (range 3.5 to 4.5 mm²), and did not exhibit a significant long-term trend toward change in area (Fig. 5A). Only M38 exhibited a decrease in area over time ($R^2 = 0.23$, slope = -5.4×10^{-4} mm² sec⁻¹, $p = 0.011$), whereas the pupils of the other mice remained relatively constant ($p > 0.05$). As in the light, mice in the dark exhibit a significant visit-to-visit variation in their pupil areas (compare Figs. 4A and 5A). However, the pupil areas seem to be largely controlled by the opening and closing of the eyelids rather than by obvious contractions of the iris (Fig. 5B).

Surprisingly, mice in the dark continued to visit the opening in the side panel as frequently as in the light (Fig 5C). Plots of time-to-visit vs visit number exhibited linearity at the origin followed by a slight curvature, the latter indicative of a progressive slowing down in the time between visits. The relation between time-to-visit and visit number were well fit by second order polynomials (Fig. 5C; $R^2 > 0.98$) and did not differ significantly from those measured in the light (Fig. 4B). Indeed, although the number of visits was slightly higher in the dark than in light (compare Fig. 4E), this difference is not statistically significant within the limited power of our tests (paired two-tailed t-test, $p = 0.21$, power = 0.34).

3.4 Reproducibility of the results

In view of the variability in the pupil areas noted above, the next question is how many samples are necessary to obtain a representative measure of the area for each mouse. We reasoned that the number of visits will be sufficient when the cumulative mean areas and cumulative standard deviation reach their steady values. We defined the cumulative mean area at sample number n as the average of all pupil areas in samples 1 through n . Similarly, the cumulative standard deviation at sample n is the standard deviation estimated using samples 1 through n . To illustrate this point we show in Figure 6A (top panel) the area plotted as a function of sample number in two mice from Fig. 4. The pupil areas of the two light-adapted mice varied significantly over the course of the session. The middle and bottom panels of Fig. 6A show the corresponding cumulative mean area and standard deviations. After initial variation, both the cumulative mean area and the cumulative standard deviation values asymptote at a steady level for samples numbers > 10 . Similar

results were observed with the population averages ($n = 6$) measured in the light (Fig. 6B). However we observed that the cumulative standard deviation settled more slowly in the dark than in the light, requiring approximately 15 samples before reaching steady state (Fig. 6C).

Given that this group of mice visited the aperture approximately 18 times in ten minutes (resulting in 15 samples or high quality images of the pupil (Fig. 4E)), we proceeded to test the reproducibility of pupil areas in light conditions, as in Fig. 3 (80 scotopic cd m^{-2}), in three different sessions, each ten minutes in duration. The averages for each session were 0.649 ± 0.028 , 0.645 ± 0.018 and 0.659 ± 0.017 mm^2 , respectively.

Test with a One Way Repeated Measures ANOVA did not detect a statistically significant difference ($p = 0.86$) in the values of the pupil areas among sessions. These results suggest that ten minute sessions with a group of 6 mice are sufficient to generate reproducible results.

3.5 Pupil areas as a function of light intensity

We measured the size of the pupil in freely behaving mice exposed to different light levels. Figure 7A shows pupil areas plotted as a function of sample number for a representative mouse (M40). Recordings at each light level were carried out over the course of 10 minute sessions performed on different days. Pupil areas and associated variations in size decreased gradually with light level from a maximum in the dark to a minimum in bright lights (80 scot cd/m^2).

Next, we estimated the average pupil area for each session and plotted it as a function of luminance intensity (circles, Fig. 7B). The maximal and minimal areas (straight and inverted triangles, Fig. 7B) measured at each light intensity decreased in proportion to the average area of the pupil with increasing luminance. On average, pupil areas decreased gradually with light intensities from a maximal area of A_{Dark} of 3.92 ± 0.37 mm^2 in the dark down to A_{Min} of 0.19 ± 0.012 mm^2 at 210 scotopic cd/m^2 (Fig. 7C, D). The data are well fit with a Hill equation (see Methods), with L_o equal to 0.2 scotopic cd/m^2 and Hill coefficient $h=0.47$ ($R^2=0.99$). The average maximal and minimal pupil areas measured at each intensity were also well described by Hill equations with L_o values of 0.36 and 0.1 respectively and a common Hill coefficient of 0.47 ($R^2>0.98$). Furthermore, close analysis of the variations in pupil areas reveal two important properties: 1) the ratios between maximal and minimal areas remain approximately constant over the entire intensity range (25% decline over 8 log unit luminance range, see Fig. 7E); and 2) the amplitude of the variations in pupil area at each intensity level (expressed as the difference between maximal and minimal areas), increases linearly with the area of the pupil (Fig. 7F). These findings also suggest a highly coordinated ‘duplex’ control of the variations in pupil area by the iris (in bright lights, see Fig. 3) and the eyelids (in dark/dim conditions, see Fig. 5).

3.5 Comparison with other methods

We compared pupil responses of mice to steady lights measured with three different methods including, a) mice exploring the opening in the side panel ($n = 6$), b) freely behaving mice standing on a pedestal as reported previously ($n = 4$), (data from Umino et al., 2012), and c) gently restrained mice receiving 30 seconds of green monochromatic (narrow band filter

centered at 500 nm) Ganzfeld illumination ($n = 5$, Fig. 8). A-D), as performed routinely to measure pupil areas of mice (Lucas et al., 2001; Xue et al., 2011). Pupil areas were plotted as a function of luminance for each mouse and the individual data fit with Hill equations ($R^2 > 0.95$). Next we applied a one-way between subjects ANOVA to compare the effects of methodology on two different parameters: L_o , an inverse measure of the sensitivity of the pupillary response to steady light and h , the Hill coefficient (see Methods section for definitions). A logarithmic transformation on the data was applied to pass requirements of normality ($p = 0.495$) and equal variance ($p = 0.37$). There was a significant effect of 'method' on L_o at the $p < 0.05$ level for the three conditions [$F(2, 5) = 79.9$, $p < 0.001$]. Post-hoc comparisons using the Bonferroni test indicated that the mean value for L_o in exploring mice (mean = -0.68 , SEM = $0.18 \log \text{scot cd/m}^2$, $n = 6$) did not differ significantly from that measured in freely behaving mice on the pedestal (mean = -1.40 , SEM = $0.11 \log \text{scot cd/m}^2$, $n = 4$), confirming that the two approaches are equally useful in measuring the natural pupillary response of freely behaving mice to steady lights. However, the value of L_o measured for restrained mice (mean = -3.18 , SEM = $0.17 \log \text{scot cd/m}^2$, $n = 5$) was significantly different from that for mice exploring an aperture or mice standing on a pedestal (Fig. 8E). A one-way ANOVA indicates that the Hill coefficients (0.5 ± 0.021 , 0.38 ± 0.1 , and 0.37 ± 0.02 for mice exploring the aperture, standing on a pedestal, and receiving Ganzfeld illumination, respectively) did not differ significantly with methodology ($p = 0.156$). Taken together these results suggest that the methodology used can shift the response functions along the intensity axis without influencing their slopes (Fig. 8F). The probable causes underlying these differences in sensitivity are listed in the Discussion.

To test the reproducibility of the responses measured for mice exploring the aperture, we compared the plots of the pupillary areas vs luminance measured by two different observers and two sets of C57BL6/J mice (Fig. 8E, Gp#1 and Gp#2, respectively). The two data sets match closely and are not significantly different ($L_o = 0.31$, SEM = $0.14 \log \text{scot cd/m}^2$, $n = 6$ for Gp#1 and $L_o = 0.19$, SEM = $0.03 \log \text{scot cd/m}^2$, $n = 5$ for Gp #2, $p = 0.75$) confirming that the approach produces reproducible results.

4. Discussion

The attenuating effects of the pupil must be considered in behavioral studies where it is important to know the precise level of retinal illumination (Lyubarsky et al., 2004). However, measuring pupillary responses to light in unrestrained, freely behaving mice can be challenging and problematic. To address some of these challenges, we implemented a new, practical system that automatically acquires images of the eye in freely behaving mice, and show that this system provides accurate and reproducible measures of the pupillary responses to steady lights. Our results indicate that the experimental conditions used to measure the pupil size must be considered for accurate estimations of retinal illuminance in behavioral studies as we detail below. We present an empirical formula that describes the relationship between size of the pupil and luminance in freely behaving mice within an OptoMotry® enclosure.

4.1. Exploratory behavior

We have measured the pupil of mice standing on a pedestal within the optomotor enclosure (Umino et al., 2012). This is a laborious and time-consuming approach that requires that mice orient their heads in a precise direction to acquire an image of their pupils. Here, we took advantage of the exploratory behavior of mice to acquire images of the mice as they instinctively investigated a small opening in the side panel of the chamber. Mice repeat this behavior spontaneously and frequently over the course of 20-minute trials. At the start of a trial, mice visit the aperture regularly, with approximately constant time intervals between visits. However, some signs of habituation are evident during the late phases of each trial as mice turn their attention to other elements in the chamber, and the time intervals between their visits increase in duration. This behavior is well described by a second order polynomial function. Interestingly, the overall frequency of visits measured in a trial did not change from day to day, suggesting little day-to-day habituation and underscoring the reliability of this test for long-term studies of mice exploratory behavior.

Mice performed this exploratory behavior efficiently both in dark and light conditions. These results suggest that mice do not rely entirely on their sense of vision to perform this task but may also involve vibrissal sensing (Mitchinson et al., 2011) and /or olfactory cues (Yang and Crawley, 2009) to reach the aperture. Indeed, based on our limited number of observations ($n = 6$) we found that the frequency of visits to the aperture does not change in light vs dark conditions, suggesting that mice use cues other than vision during the exploratory behavior. This lack of difference between dark and light conditions is a surprising result, given that rodent activity levels in light are generally inhibited relative to that in dark conditions (negative masking, Thompson et al., 2011) but may be enhanced in young mice exposed to dim lights (positive masking, Mrosovsky et al., 2000). Further studies with improved statistical power are necessary to confirm our results.

The frequency of visits was significantly lower in the morning and noon hours than in the evening hours (not shown), consistent with the circadian modulation of activity levels in rodents (Jud et al., 2005). Hence, all the tests performed for this study were carried out in the early evening hours, when activity in mice rises to its peak levels. Together, these results suggest that our behavioral assay has the potential for use as a practical assay of mouse activity levels.

4.2. Pupil areas as a function of steady illumination

The relation between pupil size and steady illumination is well described with a simple Hill equation in freely behaving mice (Methods). The Hill equation can be applied in conjunction with the appropriate conversion factors (Lobanova et al., 2007; Lyubarsky et al., 2004; Saszik et al., 2002) to estimate retinal illumination in freely behaving mice when the luminance values are known. However, each mouse we tested exhibits significant variations in the size of their pupil during repeated measurements at a given intensity. The amplitude of the variations in each mouse was directly related to the mean size of its pupil and the pupil size appeared to be controlled by two mechanisms. At dim light levels, the iris was maximally dilated, and mice controlled the illumination through their pupil by opening or closing their eyelids; while at bright light levels the pupil size was controlled directly by

contracting or dilating the iris. When controlled by the iris, pupil size responds both to illumination levels (Do and Yau, 2010; Lucas et al., 2014) and to the cognitive or arousal state (Binda et al., 2014; Zenon et al., 2014). The mechanisms that control the pupil size via the eyelids in dim lights (<0.001 cd/m²) remain unclear. Surprisingly, the relative amplitude of the variations in pupil size was approximately constant across the entire illumination range tested (8 log units) suggesting that a connection may exist between the neural mechanisms driving both the eyelids and the iris. Further studies are necessary to understand these findings.

4.3 Comparison to results using other methods

The pupil responses we documented were similar when measured either with mice standing on a pedestal or exploring the opening in the side panel (Fig. 8), suggesting that any curiosity associated with these particular behavioral tasks does not seem to contribute significantly to steady state pupillary responses. The sensitivity to steady lights we report here in unrestrained mice was approximately 1 to 2 orders of magnitude less sensitive than that of restrained, alert mice receiving 30 seconds of Ganzfeld illumination (our measurements and others' (Lucas et al., 2001; Xue et al., 2011)). To convert between irradiance and luminance values reported in these various studies we applied the relation that 1 scotopic cd m⁻² produces a luminous flux at the plane of 4700 photons μm^{-2} sec⁻¹ at $\lambda = 507$ nm (Lyubarsky et al., 2004).

The differences in sensitivity between unrestrained and restrained mice may arise as a result of the slow decay (lasting minutes) in the amplitude of the pupillary response to steady illumination. Gooley et al. (2012) have shown in humans that the slow decay in the pupillary response produces a progressive increase in *Lo* by up to approximately 2 log units. Because restrained mice received 30 seconds of Ganzfeld illumination while freely behaving mice received 10-20 minutes of steady illumination, it is possible that the observed low sensitivity is the result of the prolonged trial times. Interestingly, we did not observe slow, long-term changes in pupillary responses to steady lights that may reflect slow decay effects. A possible explanation and a drawback of our method is that it lacks the temporal resolution needed for the detection of the initial decay in pupillary responses. Temporal resolution is limited by the time between visits to the aperture which is on the order of 30 seconds, preventing the measurement of responses to transient light steps (or fast sinusoidal modulation). Other factors that may also contribute to the differences in sensitivity between the two methods, include differences in the distribution of light on the retina (Atchison et al., 2011; Park and McAnany, 2015; Stanley and Davies, 1995; Watson and Yellott, 2012) and differences between the narrowband vs broadband spectral radiance of the Ganzfeld illuminator and the OptoMotry[®] enclosure (differentially activating the various opsins in the retina (Lucas et al., 2014)). Finally, we cannot rule out anxiety or distress-associated factors that modulate the pupil (Bitsios et al., 1996) in restrained mice that would not be present in freely behaving mice. In summary, we conclude that the pupillary response and behavioral sensitivity must be established under the same experimental conditions for an accurate estimation of retinal illuminance that is appropriate to determine visual threshold.

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Highlights

- We present a system to measure the pupillary response to steady lights of freely behaving mice.
- Our method uses a low-cost, portable device that automatically acquires close-up images of mouse eyes.
- Our method takes advantage of the intrinsic nature of mice to inspect objects of interest
- Our method does not require manual restraint, anesthesia or training of mice.
- Our method can be used in optomotor or operant behavior testing chambers.

Figure 1. Recording chamber and illumination used for measurement of pupil areas in freely behaving mice

(A), A freely behaving mouse inside a custom-built chamber explores an opening in the side wall of the chamber (1). Outside the chamber and suspended from the lid of the chamber is a video camera (2, 3) that captures infrared images of the lateral view of the mouse every time its nose crosses the aperture. LEDs provide IR illumination (4). Parallel guides (5) hold a plexiglas divider to reduce the area of the chamber during experiments. Aeration holes are located on the lid of the chamber (6).

(B), Recording chamber within the OptoMotry[®] enclosure; (C), Drawing plans of experimental chamber. Upper row: top views of lid and elevated floor; bottom row: side views of chamber and camera arrangement. Dimensions are indicated in centimeters. (D), Ambient light levels were controlled by placing the chamber within the Optomotory[®] enclosure formed by four LCD monitors powered by fluorescent light sources. The graph shows the radiance spectrum of the monitors and the transmittance of the optical density filters, both plotted as a function of wavelength. Radiance units were photons/sec/sr/ $\mu\text{m}^2/\text{nm}$. Filter transmittance (filter transm in the figure) is approximately flat and less than 10% over most of the visible spectrum, but increases rapidly for wavelengths above 700 nm. Indicated in green is the absorption spectrum for rhodopsin used for our luminometric calculations. The absorption spectrum was estimated according to Govardovskii et al. (2000), with $\lambda_{\text{max}} = 498 \text{ nm}$ (Nikonov et al., 2006).

(E), Estimated radiance spectra in the presence of 0, 2, 4, and 6 optical density filters respectively. Logarithmic ordinates were used in order to better resolve the large range of radiance values. Note that the relative contribution of the emission peak at wavelengths >700 nm increases with the number of filters.

Figure 2. Measurement of pupil areas

Representative IR images of the side view of the mouse captured with the video camera as they visit the aperture on the side panel, (A) in the dark and (B), in 80 scotopic cd/m². (C), Not all visits to the aperture produced images of the pupil that could be processed. We discarded images when mice rotate their heads as they inspect the aperture and resulting in angled views of the pupil. (D-H) Steps followed to determine pupil areas. (D), Close-up view of an eye divided into two separate regions by an overlying whisker. The reflection of the IR LED light also falls within the image of the pupil; (E), Red pixels are pupil pixel levels below the threshold level estimated with function in (K); (F), Separate regions above and below the whisker are joined with the ‘line’ tool and traced using the ‘wand’ tool in Image J; (G), The contour of the pupil is determined by applying the ‘convex hull’ tool; (H), The contour of the pupil was fit with an ellipse. The area enclosed by the ellipse and its aspect ratio were determined. (I), Image frames were processed to determine the threshold levels as detailed in the Methods section. Close up view of the eye reveals the dark pupil surrounded by the ‘grey’ iris. Circles indicate regions of interest in the pupil (blue) and iris (red) used to determine the threshold. (J), Histogram illustrating differences in pixel levels recorded in the pupil (blue) and iris (red). (K), Minimum iris pixel levels increase monotonically with minimum pupil pixel levels. The grey function indicates threshold levels applied to define the edges of the pupil.

Figure 3. Dynamic modulation of pupil areas in freely behaving mice

(A), Pupil areas of six mice plotted as a function of time of visit to the aperture. Areas measured during the first (black circles) and second (grey triangles) sessions overlap closely. Linear regression to areas measured during the first session is shown. No consistent trend was observed across mice. Luminance level was 80 cd/m². (B), Histograms of pupil areas for each mouse were well fit by normal distributions. Data for the two sessions were included in the analysis. (C), Pupil area plotted as a function of inter-canti (IC) distance. (D), Pupil area plotted as a function of the aspect ratio of the ellipse fitting the pupil contour. (E), Close-up images showing maximal and minimal pupil areas for each mouse.

Figure 4. Analysis of time-between-visits

(A), Plots of time-to-visit vs visit number measured in two separate sessions (black circles and grey triangles, respectively). Continuous lines are fits with second order polynomial functions. Luminance was 80 cd/m². Arrows point to prolonged periods when the mouse did not visit the aperture. (B), Bar graph comparing average values of a_1 (linear) and a_2 (second order) coefficients in the polynomial function fitting the time-to-visit data in two sessions performed in light (Light 1 and Light 2 respectively) and one session performed in dark conditions. No significant differences were observed (t-tests, $p > 0.05$, $n = 6$). (C), Average time-to-visit vs visit number plots measured in the two separate sessions. (D), Representative histogram of time between visits fit with decay exponential function. The histogram includes data from the two separate sessions. (E), Average number of visits did not differ between light and dark sessions (t-tests with Light 1 as control, $p > 0.05$).

Figure 5. Dynamic modulation of pupil areas in darkness

(A), Individual plots of pupil area vs time show variability in the pupil areas. Linear regression does not show a consistent trend across mice. (B), Close-up images of maximal and minimal pupils for indicated mice. In darkness, pupil area is largely controlled by partial opening and closing of the eyelids. (C), Individual plots of time-to-visit vs visit number. Data was fit with a second order polynomial function. (D), Average time-to-visit vs visit number plots measured in dark and compared to that measured in light conditions.

this axis should read area measured number....not visit number

Figure 6. Population statistics

(A), Pupil area statistics for two representative mice, M37 and M40, measured at 80 scotopic cd/m². *Top*: Plot of pupil area vs sample number. *Center* and *bottom*: Cumulative mean area and standard deviation for the same mice. (B), Population averages plotted as function of sample number; (C), Population averages of same mice in darkness. *Top*: Mean pupil area; *Center*: Population average of the cumulative mean area; *Bottom*: Population average of the cumulative standard deviation (error bars represent SEM, n = 6 mice).

Figure 7. Light modulates the amplitude range of the dynamic modulation of the pupil
 (A), Plot illustrates the variation in pupil areas vs visit number for selected light illumination levels (mouse M40). (B), Corresponding pupil area for the same mouse averaged over 10-12 samples at each luminance level. Error bars indicate the standard deviations at each luminance level. Grey triangles delimit the range of pupil areas observed at each light level. Normal and inverted triangles indicate maximal and minimal pupil areas respectively. (C), Population averages (mean $\pm$ SEM, $n = 6$) for mean pupil area (black circles), maximal (grey triangles) and minimal pupil (inverted triangles) respectively. Data fit with Hill equation (Methods Eq. 3). (D), Same as (C) in logarithmic units to reveal the smaller responses. The dynamic range of the pupil is modulated by the eyelids over the 'flat' region of the intensity-response curve (dark to ~ -3 log cd/m²) while the iris modulates the variations at higher intensities in the asymptotic region of the curve (> -3 log). (E), The ratio of maximal to minimal pupil areas varies by less than 25% over an 8 log unit illumination range. (F), The amplitude of the dynamic modulation (maximal – minimal pupil area) is directly proportional to the mean area of the pupil.

Figure 8.

Comparison of pupil responses to steady lights measured in mice exploring the aperture on the side panel vs freely behaving mice standing on a pedestal within the OptoMotry[®] enclosure and gently restrained mice held under a Ganzfeld illuminator. (A), Unrestrained mouse standing on the pedestal; (B), Close-up view of the eye acquired with IR illumination; (C, D) Pupil images of restrained mouse exposed to Ganzfeld illumination of 0 and 0.15 scot cd/m² respectively; (E), Average value of the Hill parameter, L_0 , for exploring mice (Group#1), mice standing on the pedestal, and restrained mice under the Ganzfeld illuminator. One-way ANOVA analysis, multiple pairwise comparisons (***, $p < 0.001$); (F), Pupil areas plotted as a function of steady luminance provided by the OptoMotry[®] environment (Explore and Pedestal) or the Ganzfeld illuminator and fit with Hill equations ($R^2 > 0.98$ in all cases). Fit parameters (h , L_0 , A_{max}) in each condition were as follows: Explore Group#1: 0.48, 2.11×10^{-1} sc cd/m², 3.80 mm²; Explore Group#2: 0.58, 1.78×10^{-1} sc cd/m², 4.02 mm²; Standing on pedestal: 0.51, 3.61×10^{-2} sc cd/m², 3.74 mm²; exposed to Ganzfeld: 0.37, 0.71×10^{-4} sc cd/m², 4.71 mm², respectively. For the fits, the value of A_{min} was fixed at 0.1 mm². Pedestal data is from (Umino et al., 2012).