Human Lens Transmission of Blue Light: A Comparison of Autofluorescence-Based and Direct Spectral Transmission Determination

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Key Words

Autofluorescence • Human lens • Blue light transmission •
In vivo determination

Abstract

Purpose: Direct measurement of the transmission of light through the human lens is not possible in vivo unless invasive techniques are used. In the current study, a reliable in vivo estimate of the transmission of blue light through the lens was assessed by comparing an indirect and noninvasive method based on autofluorescence measurements with a direct method. **Methods:** Total transmission of blue light was measured in human donor lenses using a direct method applicable only in vitro and compared with transmittance estimates made by an in vivo applicable autofluorescence technique. **Results:** Human lens transmission of blue light decreases with age by 0.7–0.8% per year at 480 nm. The comparison of methods showed that the autofluorescence-based method correlated significantly with the direct measurements ($R = 0.83$, $p < 0.001$) and acceptable agreement between the two methods was found. **Discussion:** In conclusion, the human lens transmittance of blue light can be measured reliably in vivo. This enables the possibility to correct for retinal light intensities when studying the mechanisms of

the circadian rhythm in clinical studies and related disorders and in addition when working with clinical and experimental methods affected by retinal blue light intensities.

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Introduction

As the human body ages, the transmission of blue light to the retina decreases due to an increased absorption in the lens of the eye preferentially in the blue spectral region [1]. The decrease in transmission is caused by the accumulation of yellow chromophores acting as an effective filter for blue light causing impaired color vision [2]. The lens proteins are extremely long lived and robust [3] but signs of denaturation are detectable from an early age in the form of insoluble yellow fluorescent protein aggregates [4]. Ultimately, this process may contribute to cataract but it is first and foremost a sign of normal ageing. There are several reasons to measure this decrease in transmission that occurs naturally with age and is accelerated with diseases, e.g. diabetes [2, 5].

For instance, in order to compare different age groups, corrections for lens transmission should be made when using ophthalmologic examinations operating in the

short wavelength spectral area [6] or when studying scotopic vision [7, 8]. Measurements of the transmission should also be applied when studying the ageing lens itself and how these changes may affect general health and well-being through alterations in regulation of the circadian rhythm. A newly discovered subset of intrinsically photosensitive retinal ganglion cells, sensitive only to blue light [9], pass information about the external luminance to the suprachiasmatic nucleus acting as the internal clock [10–12]. The age-induced decrease in blue light reduces retinal blue light intensities. Hypothetically, less stimulation of the intrinsically photosensitive retinal ganglion cells may result in a demodulation of the circadian rhythm. On the other hand, the retina is susceptible to blue light phototoxic damage [13–15], which is why there is reason to investigate the protective effects of blue light absorption by the lens.

Various methods to estimate the lens transmission have been described. Most of these are based on the autofluorescence capabilities of the lens [16, 17] but in addition, lens transmission can be estimated based on reflected light from the retina, by a psychophysical approach [18] or by measurements of visually evoked potentials [19]. In this methodological study, we have assessed the reliability of an indirect noninvasive method of estimating the transmission of blue light in the human lens using a commercially available instrument based on autofluorescence measurements and compared this method with direct measurements on human donor lenses *in vitro*.

Materials and Methods

Human Donor Lenses

Human donor lenses were provided by the Lions Eye Institute for Transplant and Research, Tampa, Fla., USA. Lenses were received and stored at 5°C in Optisol GS transport medium and were used for the experiments within 1 day of arrival and no later than 1 week after death. All lenses showed normal age-related optical changes but did not have any localized opacities in the central part of the lens as such opacities may affect lens autofluorescence measurements [20]. For all measurements, the lenses were placed in 5-mm path length quartz cuvettes containing a neutral, saline solution (in g/l: NaCl 8.00, KCl 0.40, Na₂HPO₄ 0.10, glucose 1.00, Hepes 2.38, buffered to a pH of 7.4 using 0.5 M NaOH) [21].

Direct Determination of Spectral Transmission *in vitro*

The transmission of the human lenses was measured in the most central part of the lens along the visual axis with the lenses placed with the anterior surface of the lens facing towards the white light source. A broad-band supercontinuum white light la-

ser with a beam diameter of 1 mm was used as the light source (SuperK Blue, Koheras A/S, Denmark). After passing through the cuvette containing the human lens, all the transmitted light was collected by an integrating sphere (FOIS-1, Ocean Optics, The Netherlands) that was coupled to a spectrometer (USB4000, Ocean Optics) by an optical fiber (P600-2-UV-VIS from Ocean Optics). The spectrometer was controlled by a computer program (Spectra Suite, Ocean Optics). This method, employing an integrating sphere, measures the total transmitted light including scattered light (transmission).

Transmission was calculated according to Beer-Lambert's law as the ratio between the intensity of the incident light and the light leaving the material. The formula was rewritten to correct for background light levels that were measured prior to lens transmission measurements. The light measurements provided values for transmission within the spectral region from 400 to 800 nm at approximately every 0.21 nm. Data were limited to 3 intervals: red (655–665 nm – T_{660}), green (525–535 nm – T_{530}) and blue (475–485 nm – T_{480}). The latter was chosen since the absorption peak for melanopsin is located at 480 nm [22]. These measurements were used as the golden standard when comparing the transmittances found with the indirect method.

Indirect, Autofluorescence-Based Transmittance Measurements

An autofluorescence profile of the lens was obtained along the visual axis using a commercially available fluorometer (Fluorotron Master, Ocumetrics, Mountain View, Calif., USA). This device projects a beam of blue light in the form of a vertical slit into the eye. At the same time, a detector filtered to allow only fluoresced light is focused on the same point in the eye (fig. 1). The Fluorotron Master was fitted with an anterior segment adapter and records fluorescence in 149 steps along the visual axis of the eye. The excitation wavelength was from 430 to 490 nm and the fluorescence detection was from 530 to 630 nm. The excitation spectrum is shown in combination with the human spectral transmission (age 18 and 76 years) and the absorption curve for melatonin suppression (fig. 2). Autofluorescence measurements were performed in a dark room with the cuvettes containing the lenses placed in a special build holder that allowed for visual control of the correct positioning of the lens relative to the excitation source. All autofluorescence profiles were recorded along the visual axis in the most central part of the lens. For each lens, the autofluorescence profile was recorded 3 times with the anterior lens surface facing the instrument and 3 scans were recorded with the posterior surface facing the instrument. The raw data were exported and the anterior and posterior lens peaks (P_{Ant} and P_{Post}) were identified for each scan and the mean of the peak value $\pm$ the 3 neighboring measurements were used to calculate the fluorescence intensity of the lens peaks (fig. 3).

The autofluorescence-based transmittance (T_{FL}) can be calculated from P_{Ant} and P_{Post} based on Beer-Lambert's law. Due to absorption in the lens P_{Post} seems lower than P_{Ant} and the ratio between these is thus the transmittance. However, this requires that the emitted fluorescence intensities in the anterior and posterior part of the lens are equal or that the intrinsic peak ratio equals 1. Congruently to previous work [23, 24], recordings of fluorescence profiles of the lens with the anterior surface facing the fluorometer (similar to *in vivo* measurements) and with the posterior surface facing the fluorometer (opposite to *in vivo* measurements)

Fig. 1. A schematic of the mechanism behind the Fluorotron Master. The light source filtered to allow only blue light to pass excites the lens by a vertical slit; note how the intensity of the transmitted blue light is reduced by absorption through the lens. The detector filtered to allow only fluoresced light records the emitted fluorescence from the exact point excited by the light source, which is repeated 149 times along the visual axis. The diagram beneath the eye shows a typical lens autofluorescence profile. Inside the enlargement of the lens, the absorption of photons (A) by the lens proteins resulting in fluorescence (F) is illustrated by a Jablonski diagram.

Fig. 2. The Fluorotron Master (FM) excitation spectrum in combination with the spectral absorption curve for melanopsin [18] illustrating that these peak in the same spectral area. This also shows that the transmittance based on autofluorescence measured with the Fluorotron Master is specific for blue light. Shown is also the spectral transmittance of a young and elderly human lens as an example of the reduced blue light transmission with age.

Fig. 3. An autofluorescence scan through the anterior chamber of the eye. The marked peaks represent the anterior and posterior lens peaks (P_{Ant} and P_{Post}). The dashed line shows the same lens when recorded with the posterior surface facing the Fluorotron Master illustrating the difference between the measured posterior peak and the actual fluorescence in the posterior lens segment ($P_{PostIntrinsic}$). The transmittance can be calculated as the ratio between P_{Post} and $P_{PostIntrinsic}$. As the latter cannot be measured in vivo, it was empirically estimated from P_{Ant} .

Table 1. Data (mean $\pm$ SD) used for calculating the transmittance as well as the direct transmission measurements

Age group	n	Age	P_{Ant}	P_{Post}	$P_{PostIntrinsic}$	$Corr$	T_{FL}	T_{480}	T_{530}	T_{660}
18–39	7	23.1 $\pm$ 5.1	131 $\pm$ 26	97 $\pm$ 26	144 $\pm$ 27	1.10 $\pm$ 0.03	0.71 $\pm$ 0.09	0.75 $\pm$ 0.04	0.88 $\pm$ 0.03	0.97 $\pm$ 0.02
40–59	4	50.3 $\pm$ 5.6	607 $\pm$ 218	256 $\pm$ 46	729 $\pm$ 226	1.20 $\pm$ 0.13	0.38 $\pm$ 0.11	0.42 $\pm$ 0.09	0.69 $\pm$ 0.06	0.97 $\pm$ 0.02
60–80	21	68.9 $\pm$ 5.6	533 $\pm$ 178	241 $\pm$ 74	697 $\pm$ 243	1.31 $\pm$ 0.39	0.35 $\pm$ 0.07	0.35 $\pm$ 0.12	0.62 $\pm$ 0.12	0.93 $\pm$ 0.10

To show the age variation the lenses have been split into three age groups: 18–39 years of age, 40–59 years of age and 60–80 years of age. P_{Ant} , P_{Post} and $P_{PostIntrinsic}$ show the average measured values for these parameters for the three age groups. $Corr$ (C) or the intrinsic peak ratio show the statistically established $P_{PostIntrinsic}/P_{Post}$ ratio used to calculate the transmittance. T_{FL} and T_{480} show the average values for the autofluorescence-based method and the direct spectral measurement. Lastly, since the direct spectral determination of the transmission provided transmission values at a wide spectral area, values are shown for green (T_{530}) and red (T_{660}) light also.

Table 2. The mean autofluorescence-based transmittance measurement (T_{FL}) and direct determined transmission of blue light

Transmission	Mean	Slope	Intercept	R ² correlated to T_{FL}
T_{FL} (95% CI)	0.43 (0.10 to 0.76)	–0.007 per year (–0.009 to –0.006)	0.84 (0.74 to 0.94)	–
T_{480} (95% CI)	0.45 (0.07 to 0.83)	–0.008 per year (–0.01 to –0.006)	0.90 (0.78 to 1.02)	(0.71)
T_{530} (95% CI)	0.69 (0.43 to 0.95)	–0.005 per year (–0.006 to –0.003)	0.96 (0.87 to 1.06)	(0.65)
T_{660} (95% CI)	0.95 (0.87 to 1.02)	–0.001 per year (–0.001 to –0.000)	0.99 (0.95 to 1.03)	(0.17)

The direct determined transmission of blue light (T_{480}) and the indirect method (T_{FL}) are neither statistically different with regard to mean, intercept nor slope ($p > 0.1$). R^2 shows the coefficient of determination when the method is compared to T_{FL} . The table also shows the mean transmission and the age correlation of

green and red light. The R^2 values are put in parentheses because the comparison of T_{FL} with these values is somewhat futile since we can assume that T_{FL} will provide transmittance values in the blue spectral region given the excitation spectrum.

revealed that the intrinsic peak ratio differed from 1 and that a correction was needed. Since the transmittance could not simply be calculated as the ratio between P_{Post} and P_{Ant} , the transmittance was calculated as the ratio between P_{Post} and the intensity of the emitted fluorescence from the posterior lens segment ($P_{PostIntrinsic}$).

$$T_{FL} = \frac{P_{Post}}{P_{PostIntrinsic}} \quad (1)$$

$P_{PostIntrinsic}$ is not measurable in vivo and for the method to be useful in clinical settings it had to be estimated from P_{Ant} using the intrinsic peak ratio, i.e. the ratio between the $P_{PostIntrinsic}$ and P_{Ant} (C) established statistically from the autofluorescence scans recorded for all the human donor lenses included in the study.

$$C = \frac{P_{PostIntrinsic}}{P_{Ant}} \Leftrightarrow P_{PostIntrinsic} = P_{Ant} C \quad (2)$$

Statistical Methods

Statistical analyses were performed using Sigmapstat v. 3.5, Systat Software, Inc., USA and for all tests a significance level of 0.05 was chosen and variance was described as 95% confidence

intervals (95% CI). On average, the methods were compared by a paired t test. The age relation was tested with linear regression analysis and the produced slopes were tested for parallelism [25]. The two methods were also correlated and the coefficient of determination was assessed. Finally a comparative plot was constructed to consider the agreement between the two methods [26].

Results

A total of 32 lenses from 21 donors were available for direct determination of spectral transmission as well as regular transmittance indirectly measured with the fluorometer. The average age of the lenses was 56.6 years (from 18 to 80 years; table 1).

Direct Determination of Spectral Transmission in vivo

The average transmission of blue light (475–485 nm) was 0.45 with a considerable range (95% CI 0.066–0.826)

Fig. 4. The $P_{Ant}/P_{PostIntrinsic}$ ratio related to age. This correlation was used as an age-dependent correction factor (C) when the blue light transmittance based on autofluorescence measurements of the lens was calculated thus creating an age-corrected blue light transmittance value.

Fig. 5. A Bland-Altman plot where differences of measurements are plotted against the means of the measurements. The dashed lines represent 95% CI. The plot shows the calculated and corrected autofluorescence-based transmittance (T_{FL}) compared to the white light transmittance at 480 nm (T_{WL}). It shows that the mean difference between the two methods is close to 0 (0.0208, SD 0.106) proving a good mean agreement. The limits of agreement were -0.19 to 0.23 . Consequently the autofluorescence-based method is good when dealing with a large data set.

due to a significant decrease in transmission with age ($R = 0.81$, $p < 0.001$).

$$T = 0.89 - 0.0080 \cdot \text{Age (years)} \quad (3)$$

Consequently, the average transmission of blue light for an 18-year-old lens was 0.75, whereas the blue light transmission for an 80-year-old lens was 0.26 or approximately one third of the 18-year-old (table 2).

Intrinsic Peak Ratio (C)

The average intrinsic peak ratio ($P_{PostIntrinsic}/P_{Ant} = C$) was 1.27 (95% CI 0.62–1.91) but strongly dependent on age ($R = 0.39$, $p = 0.027$) meaning that more fluorescence was emitted from an 80-year-old posterior lens segment than from an 18-year-old posterior lens segment relative to the fluorescence emitted from the anterior segment (fig. 4):

$$C = 0.90 + 0.0065 \cdot \text{Age (years)} \quad (4)$$

Equation 4 was incorporated into equation 1 according to equation 2 producing equation 5:

$$T_{FL} = \frac{P_{Post}}{P_{Ant} (0.90 + 0.0065 \cdot \text{Age (years)})} \quad (5)$$

Indirect Measurement of Regular Lens Transmittance in vitro Using Autofluorescence-Based Method

The average transmittance was 0.43 (95% CI 0.10–0.76) but a significant decrease with age was found ($p < 0.001$, $R = 0.85$):

$$T_{FL} = 0.841 - 0.007 \cdot \text{Age (years)} \quad (6)$$

The 95% CI for the intercept was 0.74–0.94, and -0.0089 to -0.0057 for the slope. The analysis showed that an 18-year-old's transmittance is reduced by 2/3 at the age of 80.

Comparison of Direct and Indirect Measurements of Lens Transmission

On average, the difference between T_{480} and T_{FL} was less than 3%. Lens transmission measurements obtained using the golden standard and the fluorescence-based method were not statistically different when analyzed with the paired t test ($p = 0.275$) and neither was a significant difference found between the slopes produced by the linear regression analysis ($p > 0.1$). A significant correlation between the two methods was found ($R = 0.84$, $p < 0.0001$) as well as a high coefficient of determination ($R^2 = 0.71$; table 2). The Bland-Altman comparative anal-

ysis of T_{FL} and T_{480} showed that the average difference between the two methods was close to 0 (0.021). The limits of agreement were from -0.19 to 0.23, which means that at least 95% of the found differences between measurements were located within the interval. There was no tendency in the distribution of data in the Bland-Altman plot as the data were randomly distributed within the limits of agreement (fig. 5).

Discussion

The aim of this study was to investigate the possibility of estimating the lens transmission in vivo using a noninvasive method based on lens autofluorescence measurements with a commercially available instrument. This indirect determination of the regular transmittance was compared to a direct determination of the total spectral transmission at 480 nm. Our results showed a good agreement between the two methods demonstrating that the blue light transmission can be assessed in vivo. A strong correlation between age and loss of blue light transmission was found in this study similar to previous work [16]. Furthermore, an age-dependent correction factor was found. However, this correction is specific for these data (healthy subjects) and precautions should be taken in the application of this method on e.g. a diabetic or cataractous patient group. The study showed that the transmission at 480 nm was reduced by 2/3 at the age of 80.

An objective, quantitative method for measuring the blue light transmission is very relevant. Hypothetically, decreasing lens transmission may, through a reduction of retinal blue light intensities, impair the entrainment of the circadian rhythm and elderly who have a proven low transmission [4, 27] have been shown to suffer more frequently from sleep disorders, which may be caused by demodulation of the circadian rhythm or even phase shift [28]. Studies involving cataract patients have shown an improvement in sleeping patterns after cataract extraction indicating an improvement in circadian functionality [29]. Studies on this topic and the potential harm of blue light could benefit from measurement of the human lens transmission so that retinal light intensities may be assessed. A fast and reliable method for measuring the blue light transmission through the human lens is thus relevant when working with clinical as well as experimental methods affected by retinal blue light intensities and/or working with subjects of different age groups. However, a degree of cooperation is required from subjects, which is why other methods [19] should be applied on very young subjects.

In conclusion, we have shown that the transmission of blue light through the human lens can be assessed in vivo using an autofluorescence-based technique and that this method has a wide range of relevant application possibilities.

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