Chapter 3

Simultaneous Measurement of Foveal Spectral Reflectance and Cone Photoreceptor Directionality

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Abstract:
An instrument for simultaneous measurement of foveal spectral reflectance and cone-photoreceptor directionality is described. The key element is an imaging spectrograph (spectral range of 420–790 nm) with its entrance slit conjugate to the pupil plane of a human eye. A 1.9-deg spot on the retina is sampled in 1 s. Video observation of the retina and the pupil facilitates proper alignment. Measurements were performed on 21 healthy subjects. Model analysis of spectra provided densities of photostable ocular absorbers. As an example, macular pigment and melanin are discussed in more detail. Spatial profiles exhibited the optical Stiles–Crawford effect, reflecting cone-photoreceptor directionality.
3.1 Introduction

The transparent media of the human eye allow non invasive probing of the fundus with light. In the past decades, a large number of quantitative reflectometry techniques has been developed. Two important quantifiable aspects of fundus reflectance are its spectral and directional properties. Model analysis of spectral reflectance provides densities of photolabile pigments such as cone visual pigment and of photostable ocular absorbers such as macular pigment, lens, and melanin. Liem et al. provided a review of the clinical importance of visual pigment density. Recently, there has been substantial interest in the photostable macular pigment. It is suggested to reduce the risk for age-related macular degeneration. Intervention may be possible because macular pigment density has been demonstrated to increase after dietary supplementation of lutein and with consumption of spinach, corn, or a combination of both. The directional characteristics of fundus reflectance provide information on the integrity of foveal cones, giving similar insights as obtained with measurement of visual pigment density. Assessment of cone integrity can serve as a differential diagnostic in visual acuity loss of unknown origin. Reduced directionality is associated with macular edema of various origin. In this paper we describe a new apparatus, the foveal reflection analyzer, that is capable of measuring both spectral and directional properties of fundus reflectance simultaneously. The key element is an imaging spectrograph, with its entrance slit placed conjugate to the pupil plane of an eye. Applications concentrate on the fovea, the fundus region corresponding to the area of the visual field with the highest acuity.

Van Norren and Tiemeijer constructed a simple spectral reflectance model using two reflective and four absorbing layers. The model was applied to spectral reflectance at 14 wavelengths, obtained with a densitometer. Delori and Pflibsen equipped a fundus camera with a spectrograph for measuring spectral reflectance of a bleached retina. They elaborated on the spectral reflectance model, an important addition being scattering in the choroid. Following this line of research, Delori build a spectrophotometer, which measures spectral reflectance, and also intrinsic fluorescence of the fundus. Kilbride et al. obtained fundus images with a television-based reflectometer at several wavelengths. Analysis of the images revealed the distribution of visual and macular pigment. The imaging ability is a large advantage over the other techniques; however, spectral resolution is limited. With imaging spectroscopy, Hammer et al. achieved higher spectral resolution and maintained spatial resolution along a bar-shaped field on the retina.

The second important property of light reflected from the fundus is its directionality. Stiles and Crawford demonstrated in a psychophysical experiment that the
luminous efficiency of a narrow ray versus location in the pupil plane shows a bell-shaped curve, with its maximum near the center of the pupil plane. This is now known as the Stiles–Crawford effect of the first kind (SCE I). Reflectance exhibits a similar bell-shaped dependence on location in the pupil plane, also with a maximum near the center. The psychophysical SCE I and optical SCE originate from directional properties of the receptor cells and their alignment towards the center of the pupil plane. Gorrand and Delori designed a photoreceptor alignment reflectometer for measuring the distribution of reflectance in the pupil. Both apparatus are capable of mapping the light distribution in the pupil plane. Using a home-built scanning laser ophthalmoscope (SLO), DeLint et al. obtained retinal images while scanning the small entrance and exit pupil configuration along a horizontal line in the pupil plane. The images showed the optical SCE versus location on the retina. Van de Kraats et al. simultaneously studied spectral and directional properties of fundus reflectance. With a densitometer they were able to measure spectral reflectance versus location in the pupil plane by jointly scanning a small entrance and exit pupil configuration. Van de Kraats et al. added directional properties, and the properties of the visual pigment, to the van Norren and Tiemeijer reflectance model.

In summary, the devices mentioned above measured either spectral reflectance or distribution in the pupil plane. Simultaneous measurement of both was not possible yet. Van de Kraats et al.’s analysis required a series of measurements, and spatial resolution in the pupil plane was poor. Both photoreceptor alignment reflectometers and the SLO used by DeLint et al. contained a laser as the light source and therefore yielded limited spectral information. In this present paper we describe an apparatus for measurement of foveal spectral reflectance versus position on a horizontal section of the pupil plane.

### 3.2 Apparatus

#### 3.2.1 Overview

A schematic representation of the experimental setup is depicted in Fig. 3.1A. Retinal and pupil planes are indicated with R and P and conjugate planes with R’ and P’. In some cases, adjacent elements or planes are drawn as a single line. At the top right, the entrance beam emerges from a lamp and passes lenses L1 through L4 and the ophthalmic front lens Lf. The entrance beam defines a small entrance pupil in pupil plane P and illuminates a small spot in the retinal plane R. Light reflected from the eye is captured by lens Lf. Separation of the reflected light from the entrance beam
Figure 3.1: (A) Schematic of the apparatus; drawing not to scale. P, pupil plane; R, retinal plane; P’ and R’, planes conjugate to P and R; Lamp, 30 W halogen lamp; L1-L11, lenses; F, spectral filters; Mh, mirror with central hole; Lf, ophthalmic front lens; Mi, insertable mirror; Li, insertable lens; V, video camera; Slit, slit conjugate to P; Prism, direct vision prism; CCD, cooled CCD camera. (B) pupil and retinal plane configuration. Left: The disk represents the dilated pupil. The entrance pupil and bar-shaped exit pupil are drawn to scale. Right: The illuminated field, with cross hairs for fixation, and the concentric sampled field.

is achieved with an ophthalmic mirror Mh. The reflected light is either available for observation with a video camera V, or for analysis with an imaging spectrograph. The mode used depends on the position of an insertable mirror Mi. The spectrograph image is captured with a cooled integrating CCD camera.

The retinal and pupil plane configuration is depicted in Fig. 3.1B. In the retinal plane, the illuminated field measures 2.8 deg. The sampled field is concentric, and measures 1.9 deg. This overlap ensures a complete illumination of the sampled field, despite small errors in focus and aberrations in the optics of the eye. The cross wire is centered on the illuminated field. In the pupil plane, the entrance pupil is placed centered with and below the bar shaped exit pupil. Their separation is 0.7 mm. The bar is defined by the slit of the spectrograph. To avoid confusion, the terms entrance and exit are defined with respect to the eye, not the spectrograph.
3.2.2 Entrance Beam

The entrance beam forms a Maxwellian view system, used for controlled illumination of a small spot on the retina. An image of the coil of the 30 W halogen lamp is relayed to the pupil plane with achromatic pairs L1, L2 and L3, L4, and the ophthalmic front lens Lf (20 D, Nikon). The front lens can be moved in the direction of the beam (z direction) to focus on the retina. Eye reflectance is far lower in the blue wavelength region than in the red. In the parallel beam between L1 and L2, glass filters F are placed (FG3 and BG38, 3 mm both, Schott) to increase the ratio of blue over red light and to block most of the infrared light. The intensity of the light entering the eye is $1.10 \times 10^6$ Troland (Td). An aperture in plane p’ after L2 controls the size of the entrance beam in the pupil plane to $0.8 \times 1.2$ mm (with the front lens focussed at infinity). A cross wire for fixation is placed in plane r’ close to the lamp. Between L3 and L4 the beam is again parallel. Another retinal conjugate plane r’ is available here, where an aperture controls the visual angle of the illuminated field on the retina. A 2.8 deg field is used for measurements. For alignment, the field angle is increased to 16 deg. An additional green filter (VG9, Spindler & Hoyer) is then inserted to increase contrast of the view. The intensity of the alignment beam is $3.35 \times 10^5$ Td.

3.2.3 Capturing Light Reflected from the Eye

Light reflected from the eye is captured by lens Lf. The retinal plane is imaged in the focal plane of Lf, the pupil plane in the plane of Mh. Light reflected from the eye is dim compared with the bright entrance beam. To avoid reflections from the cornea, the entrance and exit light are separated with an ophthalmic mirror Mh conjugate to the pupil plane. When the entrance beam, which passes through the central hole of Mh, is correctly focussed in the pupil plane, reflections from the cornea largely disappear in the same hole. Light reflected from the eye is captured by the remaining part of the mirror. This configuration allows observation of the entire pupil, except for the part covered by the hole. Lens Lf is placed slightly out of center and is slightly tilted to redirect reflections at the front and back glass–air interfaces out of the center of the beam. In this way, the reflections are blocked by the retinal aperture at r’ in the spectrograph and by a small mask on a glass plate in front of L10 in the observation beam.

3.2.4 Video Observation of the Retina or Pupil

For alignment of the subject, a video observation channel is available. When the mirror Mi is inserted, lenses L5 and L10 act together as a relay pair. Both the retinal
and the pupil plane are relayed to between lens L10 and L11. Lens L1, moved in or out of the beam with a magnetic solenoid, controls which plane is focused on the video camera chip V (VCB-3512P, Sanyo). Imaging the retina allows us to focus the cross wire and observe whether the subject fixates correctly. Imaging of the pupil allows us to observe the location of the entrance beam within the pupil and is also used to achieve an optimal focus of the entrance beam in the pupil plane.

3.2.5 Imaging Spectrograph

When the mirror M1 is removed from the beam, light is available for the imaging spectrograph. When lenses L5 and L6 are combined, we can image the pupil plane at infinity. In between L6 and L7, a retinal plane R’ is available, where an aperture controls the size of the sampled region on the retina to 1.9 deg. A slit, 0.90 mm wide, is placed in the focal plane of lens L7, conjugate to the pupil plane. The slit defines a horizontal bar-shaped exit pupil, which measures $0.8 \times 12$ mm in the pupil plane of the eye (with the front lens focussed at infinity). The slit is in the focal plane of lens L8, which images it at infinity. The light traverses a direct vision dispersion prism (Prism, Spindler & Hoyer, Part No. 331120). The spectral image is focused on the chip of a cooled CCD camera (CCD, ST-237, Santa Barbara Instrument Group). The camera is read out in $3 \times 3$ binning mode. The spectral image contains 213 points in the spectral direction and 85 points in the spatial direction.

3.3 Methods

3.3.1 Calibration Frames

Reflectance was routinely calibrated with a surface painted with Eastman 6080 white mounted at the end of a black, anodized tube. This painted surface was calibrated against a freshly pressed BaSO4 surface, which we considered the gold standard. The white reference was placed at 445 mm behind the pupil plane and illuminated with the measuring light. The front lens focus was adjusted to place the reference in a retinal conjugate plane. White reference frames calibrate the spectral output of the lamp, transmission of the optics, sensitivity of the CCD camera, as well as sensitivity variations among CCD camera pixels. To account for stray light in the apparatus and dark current in the CCD camera, reference frames of a dark cloth held at approximately 1 m behind the pupil plane were taken. The integration time of the dark frames matched the integration time of the measured spectrum and the white reference frame. White and dark reference frames were obtained prior to each session. In case a refractive
correction was required during the session, additional dark frames for the new position of the front lens were obtained at the end of the session.

3.3.2 Calculation of Reflectance

Each $3 \times 3$ binned pixel on the CCD is a detective element, measuring counts at a certain wavelength $\lambda$ within the range $\Delta \lambda$, and at a certain pixel position $x$ in the spatial direction. In this subsection, we derive an expression for calculating reflectance $R(\lambda, x)$ from the number of counts in the measurement $C_M(\lambda, x)$, the white reference $C_W(\lambda, x)$, and matching dark references $C_{MD}(\lambda, x)$ and $C_{WD}(\lambda, x)$. $R(\lambda, x)$ is an equivalent reflectance: all sources contributing to the reflected light are considered as if they were diffuse reflectors.\(^{1–3}\) Let $P_h(\lambda)$ be the number of photons per second in the entrance beam, at wavelength $\lambda$ within the range $\Delta \lambda$, either entering the eye or falling on the white reference surface. Part of the photons will be reflected and backscattered, forming a source of photons in the retinal plane. The number of counts in a pixel is proportional to the photon flux through its detection area and the integration time. The flux through the area $A_P$ spanned by the detective element in the pupil plane at distance $d$ from the retinal plane, is proportional to $A_P/d^2$. In the measurement situation, the number of detected counts is given by

$$C_M(\lambda, x) = \frac{R(\lambda, x) \gamma P_h(\lambda) t_M A_M S(\lambda, x)}{2\pi d_{\text{eye}}^2} + C_{MD}(\lambda, x), \quad (3.1)$$

with $t_M$ the integration time of the measurement, $A_M$ the detection area in the pupil plane, and $d_{\text{eye}}$ the axial length of the eye. The constant $\gamma$ is the ratio of the sampled and illuminated area in the retinal plane. $S(\lambda, x)$ is a sensitivity factor containing the transmission of the optics, the quantum efficiency, and the gain of the CCD pixels. The factor $2\pi$ accounts for light being reflected into half of a sphere.

In the case of the BaSO$_4$ white reference surface, 99% of light in the range 400–800 nm is reflected perfectly diffuse or isotropic into a half-sphere.\(^{25}\) A relation similar to Eq. (3.1) holds for the white reference images:

$$C_W(\lambda, x) = 0.99 \frac{\gamma P_h(\lambda) t_W A_W S(\lambda, x)}{2\pi d_{\text{ref}}^2} + C_{WD}(\lambda, x), \quad (3.2)$$

with $t_W$ the integration time, $A_W$ the detection area in the pupil plane, and $d_{\text{ref}}$ the distance between the white reference and the pupil plane.

Combining Eqs. (3.1) and (3.2), the percentage equivalent reflectance is given by

$$R(\lambda, x) = 0.99 \frac{t_W A_W}{t_M A_M} \left( \frac{d_{\text{eye}}}{d_{\text{ref}}} \right)^2 \frac{C_M(\lambda, x) - C_{MD}(\lambda, x)}{C_W(\lambda, x) - C_{WD}(\lambda, x)}. \quad (3.3)$$
The factor $A_W/A_M$, which accounts for changes in scale of the pupil plane with the front lens position, is calculated from pixel scale $S_{\text{PIX}}$ as $(S_{\text{PIX,W}}/S_{\text{PIX,M}})^2$ (for calibration of $S_{\text{PIX}}$, see Subsection 3.3.3). The axial length of the eye $d_{\text{eye}}$ is calculated from the front lens focal adjustment. It is assumed that the cornea and eye lens can be treated as a single flat lens with focal distance 22.29 mm$^2$ and that all ametropia can be attributed to a variation in axial length of the eye.

An estimation of the error in the reflectance value starts with calculation of the error in the raw pixel data:

$$\sigma = \left( \frac{\text{RN}^2 + \frac{N - B}{3}}{3} \right)^{\frac{1}{2}}, \quad (3.4)$$

where RN is the read noise of the camera in counts, $N$ is the number of counts read from the pixel, and $B$ is the bias level of the camera in counts. Typical values are $\text{RN} = 12$, $N$ in the range 150–5000, and $B = 100$. The factor 3 accounts for $3 \times 3$ on-chip binning. When the appropriate error propagation mathematics and Eq. (3.3) are used, an error is attributed to the reflectance.

3.3.3 Spectral and Spatial Calibration

For spectral calibration of the spectrograph, images of a mercury lamp illuminating the wall opposing the setup were obtained. Pixel positions of seven lines were determined [wavelengths in air: 435.8, 491.6, 546.1, $(577.0 + 579.1)/2$, 623.4, 690.8, and 772.9 nm]. The wavelength range covered by the spectrograph was 420–790 nm. Dispersion strongly depended on wavelength. At 420 nm the spectral range covered by one $3 \times 3$ binned pixel was approximately 0.4 nm; at 760 nm it was approximately 6 nm. For calibration of pixel scale $S_{\text{PIX}}$, a transparent film containing periodic vertical dark bars was placed in the pupil plane. Images of the wall opposing the setup revealed the periodic pattern, which enabled us to scale the pupil plane to CCD pixels. Scale was calibrated for the complete range of front lens settings. With the front lens focused at infinity, one $3 \times 3$ binned pixel corresponded to 0.14 mm in the pupil plane. Prior to the calculation of reflectance, the images were binned and interpolated to 5 nm spectral and 0.1 mm spatial resolution to correct for the nonlinear dispersion of the prism and variable scaling of the pupil plane.

3.3.4 Protocol

The research followed the tenets of the Declaration of Helsinki and was approved by the local Medical Ethics Committee. The purpose was explained at the beginning
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of the experiment, and written informed consent was obtained. The pupil of one eye was dilated with one or two drops tropicamide 0.5%. A chin rest and temple pads, connected to a headrest, were used to maintain head position. The headrest can be adjusted in three dimensions. This allowed us to focus on the pupil plane and to position the entrance beam within the pupil. Subjects were instructed to fixate the cross wire at all times. For adjustment of the headrest, the large field with an additional green filter was used. With the large field the pupil lit up more brightly, focus in the pupil plane was more critical, and a larger part of the retina could be seen. The entrance beam was focused in the pupil plane. Care was taken to avoid reflections from the corneal surface of the eye. Fixation was checked in the retinal image. If required, the front lens focus was adjusted. During focal adjustment the headrest was moved as well to maintain a good focus in the pupil plane.

We then searched the maximum in the directional reflectance (e.g., the maximum of the SCE) using the measuring field. During the search, we continuously read the spatial profiles near 540 nm from the CCD while discarding the remaining part of the data, thereby achieving a short readout time. Integration time was reduced to 0.25 s. At 540 nm the directional reflectance shows up prominently. In the horizontal direction in the pupil plane (along the spectrograph slit) the maximum position is readily observed in a profile plot on a computer display. The maximum in the vertical direction was found by a manual search. While we scanned vertically, the latest profile was compared by eye with the highest profile till then. The search typically took approximately 2 min. In this period, visual pigments are bleached away at approximately 97%.25 At the optimal entrance position five spectra were obtained. Prior to each measurement, subjects were instructed to blink once, keep their eyes wide open, and fixate on the cross wire. Integration time was 1.0 s. The entire procedure described above, apart from the cross wire focused on the retina, was repeated five times to test repeatability. Settings of the headrest were changed on purpose in between two runs to increase independence.

3.3.5 Subjects

All subjects (n = 21) were Caucasian, unfamiliar with any eye disease, and had no complaints on visual acuity. The majority [n = 15 (12 females)] fell in the age group 18–27; the mean age was 22. Six of the subjects (male) were aged 40–74. The six older subjects can be considered experienced observers, whereas the younger subjects were all naïve subjects. Fourteen subjects had no refractive correction. For the other cases, refraction was in the range −1.5 to −4 D, except two older subjects having −6 and −7 D.
Figure 3.2: Image of foveal spectral reflectance (female subject, age 20). Surface height represents the equivalent reflectance of the fovea, expressed as a percentage on a logarithmic scale, versus wavelength and position in the pupil plane. Temporal (T) and nasal (N) side are indicated.

3.4 Results

3.4.1 Reflectance Spectrum Image

An example of an image of two-dimensional reflectance is presented in Fig. 3.2 (female subject, age 20). Figure 3.2 shows the equivalent reflectance of the fovea, expressed as a percentage on a logarithmic scale, versus wavelength and location in the pupil plane. Temporal (T) and nasal (N) sides are indicated. The image can be looked at in two ways: first, as spectral reflectance versus location in the pupil plane, and second, as an optical Stiles–Crawford profile versus wavelength.

Adopting the first view, the characteristics of spectral fundus reflectance are recognized: a decrease toward short wavelengths, with steeper decrements at 590 nm,
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510 nm, and at the lowest recorded wavelengths. At the longest wavelengths, ocular pigments, except melanin, are fairly transparent. Below 590 nm, light is efficiently absorbed by blood. The contribution to reflectance from deeper, blood rich layers diminishes, causing the first decrement. Reflectance below 590 nm mainly originates from the receptor cell layer. The second decrement at 510 nm is due to macular pigment. The latter also causes a shallow dip near 460 nm. Absorption by the crystalline lens causes a decline at the shortest recorded wavelengths.

The optical SCE is best observed in the region 510–590 nm. The profile near 540 nm, indicated by the thick line in Fig. 3.2, presents a typical example. Reflectance shows a bell-shaped dependence on position in the pupil plane, with a maximum slightly on the nasal side of the pupil center. At longer wavelengths, the directional part of the reflectance dissolves in the much larger nondirectional part. At shorter wavelengths, absorption by the macular pigment and the crystalline lens leaves the bell shape intact, but strongly reduces the amplitude of the directional reflectance.

3.4.2 Spectral Intersections

For each measurement, the spectrum at the pupil position with the highest reflectance at 540 nm was selected (e.g., the spectrum indicated with a thick line in Fig. 3.2). We fitted the spectra with the van de Kraats et al. fundus reflectance model using a least-squares method. Each data point was assigned a weight of 1 over its error squared. The model describes radiation transfer in the eye with a limited number of reflecting, absorbing, and scattering layers. Spectral properties of the absorbers are taken from the literature. Eight parameters were optimized: reflectance from disks in the outer segments of the photoreceptors $R_{\text{disk}}$, at the inner limiting membrane $R_{\text{ilm}}$, at the cornea $R_{\text{cornea}}$, the optical densities of melanin $D_{\text{mela}}$, macular pigment $D_{\text{mac}}$, the aging component of the lens $D_{\text{lens-a}}$, the thickness of the blood layer $T_{\text{blood}}$, and a parameter accounting for scattering in the choroid $D_{\text{scat}}$. The parameter $R_{\text{cornea}}$ was added to the model. Visual pigment density was assumed zero, and the Stiles-Crawford parameter $SC$ was set at unity. Values for other fixed parameters and a detailed description of the model are given in the original paper.

A sample ($n = 5$) of spectral reflectance curves spanning the full age range is shown in Fig. 3.3A. The solid curves represent model fits. The effect of an increase in lens absorption with age is apparent from the downward trend with age of the spectra below 500 nm. Deviations between the data and the model are largest below 425 nm. Here, both reflectance and the power of the entrance beam are low, resulting in a low signal-to-noise ratio. The mean of all spectra ($n = 5 \times 5 \times 21$) is depicted
Figure 3.3: (A) Spectral intersections at the pupil position with maximum reflectance at 540 nm. Five subjects spanning the full age range are presented; gender and age are indicated. Vertical bars indicate the error associated with each point (hardly discernable and absent for most points). The solid curves represent model fits. (B) Mean reflectance spectrum (solid curve), together with data from Delori and Pflibsen\textsuperscript{2} (1989) and van de Kraats \textit{et al.}\textsuperscript{3} (1996). In the mean spectrum, small dips that are due to macular pigment are present at 460 and 490 nm.
Table 3.1: Spectral reflectance data. \( \lambda \), wavelength; \( R \), mean reflectance; SD, relative between-subjects standard deviation, \( i.e., \) the standard deviation of the subject means divided by the mean; RE, relative error

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<th>( R ) (%)</th>
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with a solid curve in Fig. 3.3B, together with data from the literature (squares, Delori and Pflibsen;\(^2\) circles, van de Kraats \( et \) \( al.\)^\(^3\)). The mean data are also presented in Table 3.1, together with relative standard deviations, \( i.e., \) the standard deviation of the subject means divided by the mean of subject means. Also given is the relative error, which was first calculated for each subject from the mean of the estimated measurement errors and mean of 25 spectra and then averaged over subjects.

Table 3.2 gives a summary of the results of the spectral model fit. The parameter mean is the mean of 21 within-subject mean values. The standard deviation \( \sigma_N \) is the standard deviation of the subject means with respect to the parameter means. \( P_{\text{MIN}} \) and \( P_{\text{MAX}} \) are the lowest and highest within-subject means. In some cases \( P_{\text{MIN}} \) reached zero. This was the lower limit set in the fit algorithm. Apart from estimations for the best fit, the Levenberg–Marquardt method returned 68% confidence intervals for the fitted parameters.\(^62\) The mean of 21 for the within-subject mean confidence interval estimations is also shown in Table 3.2. The study design of five measurement
Table 3.2: Spectral model (Top) and Stiles–Crawford parameters (Bottom). PM: parameter mean; $\sigma_N$: between-subject standard deviation; $P_{MIN}$, $P_{MAX}$: lowest and highest within-subject mean; CI: mean of confidence interval estimations; $CR_T$, $CR_S$, $CR_M$: coefficients of repeatability for the total, within the series, or between the series standard deviation. (N) and (T) stand for nasal and temporal.

<table>
<thead>
<tr>
<th></th>
<th>PM</th>
<th>$\sigma_N$</th>
<th>$P_{MIN}$</th>
<th>$P_{MAX}$</th>
<th>CI</th>
<th>$CR_T$</th>
<th>$CR_S$</th>
<th>$CR_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{scat}$</td>
<td>0.20</td>
<td>0.041</td>
<td>0.11</td>
<td>0.27</td>
<td>0.0066</td>
<td>0.014</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>Thblood ($\mu$m)</td>
<td>68</td>
<td>18</td>
<td>29</td>
<td>104</td>
<td>6.5</td>
<td>15</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>$D_{mela}$</td>
<td>1.1</td>
<td>0.11</td>
<td>0.95</td>
<td>1.4</td>
<td>0.022</td>
<td>0.045</td>
<td>0.033</td>
<td>0.032</td>
</tr>
<tr>
<td>$R_{disk}$ (%)</td>
<td>2.8</td>
<td>0.50</td>
<td>1.6</td>
<td>3.5</td>
<td>0.050</td>
<td>0.54</td>
<td>0.30</td>
<td>0.47</td>
</tr>
<tr>
<td>$D_{mac}$</td>
<td>0.45</td>
<td>0.11</td>
<td>0.28</td>
<td>0.64</td>
<td>0.010</td>
<td>0.084</td>
<td>0.071</td>
<td>0.047</td>
</tr>
<tr>
<td>$R_{ilm}$ (%)</td>
<td>0.034</td>
<td>0.052</td>
<td>0.20</td>
<td>8.6</td>
<td>0.049</td>
<td>0.039</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>$D_{lens-a}$</td>
<td>0.11</td>
<td>0.12</td>
<td>0.48</td>
<td>0.032</td>
<td>0.058</td>
<td>0.043</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>$R_{cornea}$ (%)</td>
<td>0.043</td>
<td>0.028</td>
<td>0.10</td>
<td>4.5</td>
<td>0.029</td>
<td>0.024</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>$x_c$ (mm)</td>
<td>0.43 N</td>
<td>0.63</td>
<td>1.3 N</td>
<td>1.3 T</td>
<td>0.0063</td>
<td>0.20</td>
<td>0.13</td>
<td>0.16</td>
</tr>
<tr>
<td>$\rho$ (mm$^{-2}$)</td>
<td>0.17</td>
<td>0.037</td>
<td>0.12</td>
<td>0.23</td>
<td>0.0037</td>
<td>0.036</td>
<td>0.024</td>
<td>0.028</td>
</tr>
<tr>
<td>A (%)</td>
<td>0.77</td>
<td>0.25</td>
<td>0.30</td>
<td>1.14</td>
<td>0.0060</td>
<td>0.22</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>B (%)</td>
<td>0.52</td>
<td>0.080</td>
<td>0.35</td>
<td>0.65</td>
<td>0.0058</td>
<td>0.084</td>
<td>0.062</td>
<td>0.059</td>
</tr>
<tr>
<td>A/B</td>
<td>1.5</td>
<td>0.46</td>
<td>0.70</td>
<td>2.4</td>
<td>0.026</td>
<td>0.52</td>
<td>0.29</td>
<td>0.45</td>
</tr>
</tbody>
</table>

series with five replacements allowed an estimation of (1) the total standard deviation $\sigma_T$, (2) the standard deviation with respect to the mean of series $\sigma_S$, and (3) the standard deviation of the mean of series $\sigma_M$. The coefficient of repeatability is defined as the 95% range for the difference in two repeat measurements. The coefficients followed from the mean values of $\sigma_T$, $\sigma_S$, and $\sigma_M$ after multiplication by $2\sqrt{2}$.

Figure 3.4 gives an impression of the model predictions for single measurements. Figure 3.4A shows 25 macular pigment density ($D_{mac}$) estimates for all 21 subjects. $D_{mac}$ is known to vary substantially between subjects. The within-subject variability is occasionally large. Six subjects had experience in this type of experiment (1, 3, 12, 13, 17, and 21). They generally showed low variability. In some of the other subjects, variability was equally low (e.g., 2, 10, and 11). Others, however, show a large variability (e.g., 9 and 19). Fig. 3.4B is a similar scatterplot for melanin density ($D_{mela}$). For melanin, within-subject variation is much smaller compared with $D_{mac}$. The stability of the melanin data indicates that the high $D_{mac}$ variability is not related to instrumental errors or head instability of the subject. A probable explanation is given in Section 3.5.
3.4. RESULTS

Subject

Macular Pigment Density

Melanin Density

A

B

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

1.1

1.2

1.3

1.4

1.5

1234 5 6 7 8 9 10 11 1213 14 15 16 17 18 19 20 21

Figure 3.4: (A) Macular pigment ($D_{mac}$) and (B) melanin density ($D_{mela}$) estimates for all 25 measurements per subject. For macular pigment, both between- and within-subject variations are large. For melanin they are much smaller. Macular pigment for the within-subject variation is markedly different between subjects; e.g., subject 11 has little variation, whereas for subject 9 the data are highly scattered. The latter subject had difficulties maintaining rigid fixation during alignment.

3.4.3 Spatial Intersections: Stiles–Crawford Effect Profiles

For each measurement, the profile at 540 nm was selected and fitted with

$$R(x) = B + A10^{-\rho(x-x_c)^2}$$

(3.5)

with $R$ the percentage reflectance, $x$ the location in the pupil plane in millimeters, $B$ the nondirectional background reflectance, $A$ the amplitude of the directional reflectance, $\rho$ a measure for the peakedness, and $x_c$ the center position.\(^{18-20}\) The fit used a least-squares method with each data point assigned a weight of 1 over its error squared.\(^{62}\) Data included in the fit met two conditions: distance to either of the pupil edges more of than 1 mm and distance to the entrance beam of less than 3 mm.
Fig. 3.5: Profiles at 540 nm demonstrating individual differences in the optical SCE. Data are for the same subjects as in Fig. 3.3A; gender and age are given. Solid curves represent fits to the data. Nasal (N) and temporal (T) side are indicated. Pupil edges are recognizable as the sharp drops in the region 3–4 mm. Maximum reflectance shows up near the center of the pupil, with a tendency toward the nasal side.

Optical SCE profiles at 540 nm ($n = 5$) are shown in Fig. 3.5 for the same subjects as in Fig. 3.3A. The solid curves represent fits with Eq. (3.5). At the pupil edges, the profiles drop to zero. The edge of the pupil may span numerous pixels when the bar is far below or above the pupil center because the vertical width of the bar is much larger than the horizontal width of the pixels. As usual, the maxima show up near the pupil center, with a tendency to the nasal side. $^{18–20}$ Table 3.2 gives a summary of the SCE parameter fit results (see explanation in Subsection 3.4.2).

3.5 Discussion

3.5.1 General Discussion

We have demonstrated the feasibility of simultaneous measurement of spectral and directional fundus reflectance. Both aspects hitherto have been studied separately. $^{1–3, 15–20, 31, 50, 59, 61}$ The course of the spectra and directional aspects agree with earlier results. In the model by van de Kraats et al. differences between spectra ob-
tained at the maximum of the optical Stiles–Crawford effect and 2 mm nasally were fitted with a single parameter. The model lacks a quantitative relation between the single parameter and the position in the pupil plane. Furthermore, the model assumes simultaneous scanning of the entrance and exit pupil. In contrast, we aligned the entrance pupil with the maximum of the SCE and obtained spectra for a range of exit pupil positions. Thus the van de Kraats et al. model as it is cannot be applied to entire spectrograph images. To our knowledge, other models including both spectral and directional fundus reflectance are not available. For a more detailed comparison with the literature, intersections of the data set were analyzed: spectra at the pupil position with the highest reflectance at 540 nm and profiles at 540 nm.

3.5.2 Mean Spectrum

In Fig. 3.3B, the mean spectrum (solid curve) is compared with literature data. Differences between the spectra depend on (at least) three factors: composition of the population, size of the illuminated and sampled retinal field, and configuration of the entrance and exit pupil. The large differences between subjects are apparent from Fig. 3.3A, with the largest variation below 500 nm. There is a strong influence of age on the spectra, as absorption in the crystalline lens increases with age. Delori and Pflibsen measured subjects aged 22–38 years.2 The lack of older subjects may explain the somewhat higher reflectance below 500 nm.

Above 600 nm, a strong dependence on illuminated field size is expected. At the longest wavelengths, where blood does not absorb, light scatters laterally in the deeper fundus layers. Hence, the larger the illuminated field size, the larger the reflectance. Delori and Pflibsen illuminated 5 deg and sampled 1.2–1.6 deg.2 Van de Kraats et al.3 illuminated 1.9 deg and sampled 1.6 deg. In the present study, field sizes were 2.8 and 1.9 deg. As can be seen in Fig. 3.3B above 600 nm, the reflectance slightly raises with the increment from 1.9 to 2.8 deg, whereas the leap to 5 deg substantially increases the reflectance in the red. Below 500 nm, field sizes also influence the spectra. Macular pigment is highly concentrated toward the center of the fovea. The slightly larger sampled retinal field in the present study reduces macular pigment content and gives rise to higher reflectance in the blue part of the spectrum. The agreement in the range 520–590 nm is rather remarkable. The present mean spectrum was selected at the Stiles–Crawford maximum. The corresponding pupil plane configuration is similar to the one used by van de Kraats et al.3 small, closely separated entrance and exit pupils. Both are sensitive for directional reflection, and spectra are equally high. Delori and Pflibsen2 used a modified Zeiss fundus camera, which uses a rather large annular entrance pupil and a concentric circular
exit pupil, and should be less sensitive to the directional light. Modification of this arrangement was not reported. It is therefore unclear why the latter spectrum is as high as the other two. Perhaps there is a difference in the absolute calibration of the spectra.

Below 630 nm, mean relative errors in Table 3.1 are smaller than obtained by van de Kraats et al. The errors are larger at longer wavelengths. Van de Kraats et al. included both instrumental errors and errors due to instability of the subject. In this study, integration time was short, largely eliminating errors that were due to movement of the subject. The errors are largest below 430 nm, where the output of the lamp drops and reflectance is low, and above 730 nm, where the spectral filters block almost all deep-red and infrared light.

### 3.5.3 Spectral Model Results

The results of the spectral model fit are given in Table 3.2. Parameters are discussed as in the Table 3.2, upward from the sclera. Deep scatter loss $D_{\text{scat}}$ was 0.20, slightly less than 0.23 found by van de Kraats et al. The mean thickness of the blood layer $T_{\text{blood}}$ was 68 µm. Van de Kraats et al. found 22.7 µm, and Delori and Pflibsen found 168 µm. Delori and Pflibsen used a model with a Kubelka–Munk scattering description of the deeper layers. This increased the estimated blood layer thickness. Melanin density $D_{\text{mela}}$ (1.1 at 500 nm, range 0.95–1.4) is comparable to the densities found by van de Kraats et al. (1.32 at 500 nm, 0.98–1.68). Delori and Pflibsen found a higher mean and a much larger range (2.13 at 500 nm, 0.19–7.9). The former two studies contained only Caucasians, the latter included two Blacks with three to four times higher melanin content. This probably explains the differences at the high end of the range.

Receptor disk reflectance $R_{\text{disk}}$ cannot be discerned from a reflecting layer at the level of the retinal pigment epithelium in a single bleached spectrum at the Stiles–Crawford maximum. The arguments for attributing reflectance at this level in the retina to the disks in the receptor outer segments are given by van de Kraats et al. Mean disk reflectance was 2.8%, similar to van de Kraats et al., who found 2.75%. The results can also be compared with the retinal pigment epithelium reflectance of 2.3% in the model by Delori and Pflibsen. The high mean disk reflectance demonstrates the effectiveness of alignment with the Stiles–Crawford maximum. Mean macular pigment density $D_{\text{mac}}$ was 0.45 at 460 nm, with a range of 0.28–0.64. Van de Kraats et al. found higher values (0.54 at 460 nm, 0.42–0.83). As stated above, this was caused when a smaller retinal field was sampled. Delori and Pflibsen found lower values (0.21 at 460 nm, 0.12–0.31). In a more recent paper, Delori et al. re-
3.5. DISCUSSION

ported 0.23 for a reflectometric method (sampled field of 2 deg). With a method based on the autofluorescence of lipofuscin, a fluorophore posterior to the macular pigment, they found 0.48 (sampled field 2 deg). The cause for the low reflectometric values may reside in their model because it lacks reflectors anterior to the macular pigment, e.g., inner limiting membrane and cornea. Accounting for light that is reflected posterior to the macular pigment gives a higher macular pigment level. Berendschot et al. studied the effect of lutein supplementation on \( D_{\text{mac}} \) in eight male subjects with a reflectometer and a SLO. At baseline, mean \( D_{\text{mac}} \) was 0.47 for the reflectometric and 0.26 for the SLO technique.

Reflectance from the inner limiting membrane \( R_{\text{ilm}} \) is small and problematic to fit. In many cases the parameter reached the lowest allowed value of zero. Mean \( R_{\text{ilm}} \) was 0.034%; even the maximum 0.20% is lower than the mean \( R_{\text{ilm}} \) of 0.26% found by van de Kraats et al. They also included data from dark-adapted spectra in the model fit. Undoubtedly, this allowed a better estimate of \( R_{\text{ilm}} \). The mean age-dependent lens density \( D_{\text{lens-a}} \), added with an age-independent density 0.31, gives a mean lens density of 0.42 at 420 nm, with a range of 0.31–0.79. For some of the young subjects, \( D_{\text{lens-a}} \) reached the lowest allowed value of zero. As expected, \( D_{\text{lens-a}} \) showed a trend with age (data not shown). Van de Kraats et al. found a lens density of 0.54 at 420 nm, with a range of 0.42–0.83 for subjects aged 20–51 years, 32 years on average. Delori and Pflibsen found a lens density of 0.66 for a group of 10 subjects aged 22–38 years, with an unknown mean. The latter result is fairly high given the age of the subjects. The aging algorithm by Pokorny et al. predicts a total lens density at 420 nm of 0.66 at age 22, 0.73 at age 32, and 0.89 at age 50. Compared with these values, reflectometric methods produce systematically low values. Reflectance from the cornea \( R_{\text{cornea}} \) was on average 0.043%. This is low in the absolute sense, but is of equal magnitude as light that is reflected from the fundus at wavelengths below 500 nm, especially in older subjects, e.g., see Fig. 3.3A.

The mean confidence interval states how accurately parameters are determined in the model fit. A large confidence interval indicates large measurement errors or bad convergence of the fit. For most parameters, except \( R_{\text{ilm}} \) and \( R_{\text{cornea}} \), the confidence interval was smaller than \( \sigma_N \) and the range \( P_{\text{MIN}} \) to \( P_{\text{MAX}} \). Apparently, in some cases, \( R_{\text{ilm}} \) and \( R_{\text{cornea}} \) did not strongly contribute to reflectance and were attributed to a large error. The weakness of the current model is that sometimes parameters are optimized in the fit while being insignificant or that parameters reach the lower (physical) limit set to zero.

The coefficient of repeatability derived from the total standard deviation \( CR_T \) contains the total experimental error. \( CR_T \) was smaller or hardly larger than \( \sigma_N \) and smaller than the range \( P_{\text{MIN}} \) to \( P_{\text{MAX}} \). This means that the measurements discriminate
well between subjects. CR_T is much larger than the confidence interval for D_{mac} and R_{disk}. This indicates that the uncertainty in D_{mac} and R_{disk} results mainly from experimental errors. A high within series CR_S points to errors in fixation and movements of the subject. Between series, CR_M is mainly connected to errors in the alignment procedure. For R_{disk}, CR_M is largest; most variation is due to variation in alignment on the optical Stiles–Crawford maximum. For D_{mac}, CR_S is largest. Macular pigment is highly concentrated toward the center of the fovea.\textsuperscript{29–32} This makes D_{mac} sensitive to errors in fixation. The influence of fixation was illustrated in the scatterplot in Fig. 3.4A. Experienced subjects showed low variability; and for some inexperienced subjects (e.g., 9 and 19), variability was large. Figure 3.4B demonstrates that variability in melanin is similar for all subjects. The distribution of melanin pigmentation near the fovea is much smoother than for macular pigment.\textsuperscript{31} Therefore melanin will not be influenced as strongly by fixation errors as will macular pigment. With further automation of the setup and optimization of the protocol, an improvement in the assessment of macular pigment might be expected. It is crucial to observe fixation just before each measurement. Coefficients of repeatability for macular pigment, achieved by Berendschot \textit{et al.}, were 0.27 for a reflectometric technique and 0.17 with a SLO based technique.\textsuperscript{13} Despite the problem with fixation, the present coefficient of repeatability 0.084 was better.

### 3.5.4 Stiles–Crawford Profiles

Profiles at 540 nm clearly showed the optical \textit{sce}. The profiles were fitted with the commonly used Gaussian model [Eq. (3.5)]; results are given in Table 3.2. The results are discussed in relation to three earlier studies. Gorrand and Delori scanned the pupil plane with a small exit and entrance pupil configuration.\textsuperscript{18} A 3-deg retinal field was illuminated with 543 nm He–Ne light, and the central 2 deg were sampled. Burns \textit{et al.} used a small entrance pupil and imaged the entire pupil plane on a CCD camera.\textsuperscript{19} A series of images was obtained for several entrance pupil positions. The image with the highest directional reflectance was selected afterwards. A 2-deg retinal field was illuminated with 543 nm He–Ne light, the central 1 deg was sampled. DeLint \textit{et al.} used an SLO with a small entrance and exit pupil configuration.\textsuperscript{20} The horizontal meridian of the pupil plane was scanned with both pupils, and retinal images were obtained in 514 nm argon light. The series of images show the optical \textit{sce} versus location on the retina. The difference in wavelength with the other studies is considered of minor influence.

In the present study mean $x_c$ was $0.43 \pm 0.63$ mm nasal (given are the mean parameter plus or minus $\sigma_N$). Gorrand and Delori reported $0.86 \pm 0.84$ mm nasal,\textsuperscript{18} and
DeLint et al. reported $0.23 \pm 0.41$ mm nasal. In the scatter plot of $x_c$ given by Burns et al., the trend to the nasal side is also present. The slight tendency toward the nasal side is common to all studies. Our mean peakedness $\rho$ was $0.17 \pm 0.037$ mm$^{-2}$. When compared with the results by Burns et al., $0.0813 \pm 0.013$ mm$^{-2}$, this is on the high side. The higher $\rho$ may result from the larger field size (1.9 versus 1.0 deg) because, for small angles, $\rho$ increases with eccentricity. Gorrand and Delori and DeLint et al. use a double scanning method that results in a higher $\rho$: $0.204 \pm 0.035$ mm$^{-2}$ and $0.226 \pm 0.049$ mm$^{-2}$ for the central 2 $\times$ 2 deg of the SLO images. For a more detailed discussion on the differences in $\rho$ between the different techniques, see Marcos and Burns and Berendschot et al. Mean ratio $A/B$ of directional light $A$ over the nondirectional background $B$ was $1.5 \pm 0.46$. This is lower than for Gorrand and Delori, who reported $2.59 \pm 0.82$, and by DeLint et al. who found $4.9 \pm 1.8$. The latter authors used a small aperture confocal to the SLO spot on the retina, which strongly suppresses the diffuse background component that is present with larger fields. With a 1.3 deg confocal aperture their, mean $A/B$ decreased to 3.0. Interpretation of the confidence interval, CRT, CRS, and CRM was discussed above in Subsection 3.5.3. The confidence intervals are small compared with the natural variation. The experimental errors CRT are much larger. Discrimination between subjects was reasonable. For directional reflectance $A$, CRM is much larger than CRS. Variation in $A$ mainly originates from alignment on the optical SCE maximum, similar to $R_{disk}$ (see Subsection 3.5.3).

### 3.6 Conclusion

Simultaneous measurement of spectral and directional reflectance with a simple chinrest and headrest proved possible. A video observation channel and a fast method for optimization of the optical SCE at 540 nm allowed alignment with respect to the apparatus and on the Stiles–Crawford maximum within a few minutes. Application of a short integration time reduced errors that were due to movement of the subject. Spectral analysis provided densities of photostable ocular absorbers such as macular pigment, lens, and melanin and reflectivity of the disks in the outer segment of the cone receptor cells. Analysis of spatial profiles delivered Stiles–Crawford parameters. Use of all data, not just one spectrum, awaits extension of the fundus reflectance model. Errors in fixation were found to be the main source of within-subject variation in macular pigment density. This might be avoided if fixation is checked just prior to the measurement. The new apparatus provides a novel diagnostic tool. It might also greatly facilitate epidemiological studies of ocular pigments.