for this would be the constant low freezing rate obtained with the direct transfer. In fact, the only difference that we observed between the freezing methods was a drop of temperature after $-40^\circ$C with the controlled-rate technique. It is obvious that the controlled-rate technique can be modified to avoid this drop, but, because our aim was to find a technique that was easy to use, we used the technique with the noncontrolled rate. On the other side, albumin concentration did not influence the number of living cells at the end of primary culture after cryopreservation. Last, after the first passage, the freezing method no longer influenced cell growth.

In conclusion, human keratocyte cryopreservation can be successfully performed by using 10% Me$_2$SO and human albumin as cryoprotective agents. A freeze–thaw trauma was revealed during primary culture after thawing, which was probably related to cryopreservation-induced cell apoptosis. Direct transfer to a $-80^\circ$C freezer resulted in better postcryopreservation growth in culture than with controlled-rate freezing. A change in albumin concentration from 2% to 10% did not affect the results. Last, cryopreservation did not modify human keratocyte ultrastructure and phenotype.

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The authors thank Carlos Belmonte, University of Alicante, for help in preparing the manuscript, and Visnja Sabolic and Chantal Martinache for technical assistance.

A Comparison of the Optical Stiles–Crawford Effect and Retinal Densitometry in a Clinical Setting

Peter J. DeLint, Tos T. J. M. Berendschot, and Dirk van Norren

PURPOSE. To compare the measurement$^2$ of the optical Stiles–Crawford effect (SCE) to the densitometry of cone visual pigments in a clinical setting. Both tests provide information on outer retinal integrity, but the optical SCE can be performed in far less time.

METHODS. Images acquired with a custom-built scanning laser ophthalmoscope were used to assess visual pigment density and optical SCE. Visual pigment density was regarded as the "gold standard." More than 100 patients with suspected, and some with known, outer retinal pathology were tested. The group included cases of central serous detachment, cone dystrophy, Stargardt’s disease, Best’s disease, and retinitis pigmentosa.

RESULTS. Parameters of the optical SCE of 25 healthy subjects and 106 patients were taken through a stepwise linear regression to predict density. The correlation between predicted density from the optical SCE and the measured density was 0.82. The sensitivity of the optical SCE to detect decreased density was 96%. When only the foveal reflectance was considered, sensitivity was still 84%

CONCLUSIONS. The optical SCE is a sensitive and fast method for detecting cone photoreceptor disturbances. (Invest Ophthalmol Vis Sci. 1998;39:1519–1523)

The Stiles–Crawford effect of the first kind (SCE I) is the phenomenon that light entering the pupil through the center is perceived to be brighter than light entering near the pupil edge.$^1$ The optical characteristics of the photoreceptors generally are thought to be responsible for the SCE I phenomenon. In theory, disturbances of photoreceptor alignment, shape, or composition may influence the SCE.$^2$ Reports on the SCE I in different retinal diseases have demonstrated that it can provide useful insights into photoreceptor disorders.$^3$ The lack of widespread use of the SCE I has been mainly the result of long test times (up to an hour) and of the fact that subjects are required to actively participate with a high level of concentration. Applegate and Lakshminarayan$^4$ suggested that for a test to be of clinical use, it should take no longer than a minute, it should use a simple chin-and-forehead rest, and it should produce analyzed results that are suitable for clinical records.

References


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The reflectance equivalent of the SCE, named the optical Stiles-Crawford effect (SCE), might provide such a test. Light reflected from the fundus is more intense in the center of the pupil than near the pupil edge. The present test involves acquiring up to 30 fundus images at different positions across the (dilated) pupil. The optical SCE measurement can be performed in a few minutes, which makes it suitable for a clinical setting. The optical SCE, performed by imaging the pupil plane, can already be performed in several seconds.

To date there has not been, to our knowledge, an extensive clinical report on other optical objective tests as an indicator of photoreceptor function, except fundus reflection densitometry of the visual pigments. Visual pigment density may in this sense be considered a “gold standard.” The drawbacks of measuring cone visual pigment density are that it requires alignment of the eye at the peak of the SCE and maintenance of the subject’s continuous fixation of gaze and head position for a minimum of 10 minutes. This is strenuous for the subjects, and it is time consuming.

Densitometry is a noninvasive and objective method to measure visual pigment density in living eyes. Density is calculated by taking the log of the reflectance ratio between a dark- and a light-adapted fundus. Fundus reflectance in the central fovea is usually measured first, after a 1- to 2-minute exposure to a bright light. This is followed by a foveal reflectance measurement after 6 to 8 minutes in the dark. Finally, as a check, reflectance often is measured a second time after a 1- to 2-minute period of bright light. Density can be used as an indicator of photoreceptor function. Past reports have shown that visual pigment density can provide valuable clinical information. Retinal conditions may cause a decrease in measured visual pigment density. The density can be reduced because of disturbed alignment or because of changes in the shape or composition of the photoreceptors. The implicit assumption is that these changes all lead to a reduction of the directionally dependent reflectance of the photoreceptors. If this is so, the correlation between density and optical SCE should be high. This is a simplifying assumption that is elaborated in the Discussion section.

The primary aim of the present study was to measure the optical SCE and the visual pigment density in a substantial patient population to determine whether the measurement of optical SCE is a sensitive test to detect possible photoreceptor dysfunction.

**MATERIALS AND METHODS**

Optical SCE and visual pigment density were measured with a custom-built scanning laser ophthalmoscope (SLO) by acquiring fundus images at 514 nm. Fundus images were 22° X 18° (256 X 256 pixels) with the fovea in the center. Only the central 2° X 2° region of the images was analyzed for optical SCE and density measurements. The optical SCE can be determined most reliably in this region, and the major complaint of most patients was a reduced central visual acuity.

Patients were tested after examination by one of the ophthalmologists in our clinic. At the time of the tests most patients were suspected of retinal pathology with no definite diagnosis. Other patients had a definite diagnosis of a retinal disease, such as central serous retinopathy, cone dystrophy, Stargardt’s disease, Best’s disease, or retinitis pigmentosa. All subjects gave their informed written consent after the nature and possible consequences of the study were explained, and all patients were treated in accordance with the tenets of the Declaration of Helsinki. The pupils were dilated with one or two drops of tropicamide 1%. One eye of each patient was examined by SLO. A bite board with a dental compound and two forehead rests ensured proper fixation of the subject’s head. A fixation cross in the scan ensured adequate eye fixation. If necessary, refraction was corrected. Patients with obvious media densities were excluded from the present study. Our custom-built SLO seemed to be more sensitive to vitreous opacities and to other media densities than one of the commercially available SLOs. This is most likely a result of the small diameter of the entrance beam (0.5 mm) and the exit beam (2.0 mm) of our instrument.

The SCE was measured by acquiring a series of 15 to 30 fundus images with the SLO at a light intensity of 5.9 log Td. First, the entrance beam was aligned in the subject’s pupil plane such that the foveal reflectance was at its highest (i.e., the peak of the SCE). A series was made by acquiring an image roughly at every 0.25 mm of the horizontal meridian, from the nasal to the temporal. The SCE was measured by acquiring a series of 15 to 30 fundus images with the SLO at a light intensity of 5.9 log Td. First, the entrance beam was aligned in the subject’s pupil plane such that the foveal reflectance was at its highest (i.e., the peak of the SCE). A series was made by acquiring an image roughly at every 0.25 mm of the horizontal meridian, from the nasal to the temporal.
TABLE 1. Parameters of Optical Stiles-Crawford Effect in a Normal Subject and in a Patient with Central Serous Retinopathy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$</td>
<td>0.179</td>
<td>0.013</td>
</tr>
<tr>
<td>$A'$</td>
<td>0.974</td>
<td>0.145</td>
</tr>
<tr>
<td>$B'$</td>
<td>0.350</td>
<td>0.383</td>
</tr>
<tr>
<td>$x_o$</td>
<td>-0.466</td>
<td>0.765</td>
</tr>
</tbody>
</table>

$B'$ is the average of the lowest two reflectance values in the data set: $A' = A + B - B'$.

TABLE 2. Stepwise Linear Regression of Optical SCE Parameters to Predict Visual Pigment Density of 106 Patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$P^*$ value</th>
<th>Weight Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A'$</td>
<td>&lt;0.001</td>
<td>0.200</td>
</tr>
<tr>
<td>$A'/B'$</td>
<td>&lt;0.001</td>
<td>0.041</td>
</tr>
<tr>
<td>$p$</td>
<td>0.001</td>
<td>0.273</td>
</tr>
</tbody>
</table>

SCE, Stiles-Crawford effect; $P^*$, significance level; $B'$ is the average of the lowest two reflectance values in the data set; $A' = A + B - B'$.

Results of bleached and dark-adapted fovea. The implicit assumption is that the difference in the two images is caused solely by the absorption of visual pigment. At the end of 2 minutes with a light intensity of 5.9 log Td, bleaching more than 95% of the available visual pigments,10 bleached fundus images were acquired. At the end of an 8-minute dark period (2.2 log Td), the dark-adapted images were acquired. The cone visual pigments are more than 95% regenerated after 6 minutes of dark adaptation.10 Next, from the bleached and dark-adapted images a mean background image was subtracted.

Normal values of optical SCE and visual pigment density were obtained in 25 healthy subjects (age, 30.4 ± 11.3 years; mean ± SD). They had no ophthalmologic complaints, had a visual acuity of 1.0 or higher, had no fundus abnormalities, took no drugs, and had no family history of retinal disease. The mean age of the 106 patients was 30.2, with an SD of 13.3.

**RESULTS**

The optical SCE curve for a healthy subject and a patient with a central serous retinopathy is shown in Figures 1A and 1B. The optical SCE fit is described by $p$, $A'$, $B'$, and $x_o$. These correspond to curve shape, height of the curved section, height of the baseline, and the peak position, respectively (Fig. 1A). The continuous line represents the least chi-square fit. The curve in Figure 1B has a lower peakedness and a lower $A'$ in the patient than in the normal subject (Table 1). The amplitude of the baseline is similar in both subjects.

In Figures 2A and 2B the visual pigment densities of the normal temporal pupil edge. The precise position of each image was recorded with a digital slide ruler (accuracy ±0.01 mm) that was attached to the horizontal adjustment. After image acquisition, images with large eye movements were discarded and images with slight eye movements were aligned to a common reference point, usually a retinal blood vessel intersection. From each image a mean background image was subtracted. To quantify such results, the mean reflectance is generally fitted with a model for the optical SCE $f(x) = A(10^{-p(x-x_0)^2}) + B$.

with $A$ representing directionally dependent light, $B$ representing non-directionally dependent (stray) light, $p$ representing curve peakedness, $x$ representing horizontal pupil position, and $x_o$ representing the pupil position at which reflectance is at its maximum. We slightly redefined the amplitude components; $B'$ (an approximation of non-directionally dependent light) equals the average of the lowest two reflectance values in the data set, and $A'$ equals the sum of $A + B - B'$ (see Discussion section). For the remainder of this study we will use the definition of $A'$ and $B'$ to indicate directional and non-directional reflectance. Reflectance calibration of the SLO made it possible to compare the amplitudes of $A'$ and $B'$ from different persons.

Visual pigment density of the fovea (the central 2° × 2° region) also was measured with the SLO at 514 nm. Fundus images were acquired at the peak of the SCE. Visual pigment density was calculated by taking the log of the reflectance ratio of bleached and dark-adapted fovea. The implicit assumption is that the difference in the two images is caused solely by the absorption of visual pigment. At the end of 2 minutes with a light intensity of 5.9 log Td, bleaching more than 95% of the available visual pigments,10 bleached fundus images were acquired. At the end of an 8-minute dark period (2.2 log Td), the dark-adapted images were acquired. The cone visual pigments are more than 95% regenerated after 6 minutes of dark adaptation.10 Next, from the bleached and dark-adapted images a mean background image was subtracted.

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In Figures 2A and 2B the visual pigment densities of the
same subjects as in Figure 1 are shown in a 6° × 5° region with the fovea in the center. The density of the visual pigments at the edges of the images are similar (0.10–0.15). The mean foveal density in the central 2° × 2° region (indicated by the quadrangle) is much lower in the patient (0.05) than in the healthy subject (0.43).

We used a stepwise linear regression to determine which optical SCE parameters could be used best to predict the outcome of the density measurements in a large population sample. The four basic parameters \( \rho, A', B', \) and \( x_c \) were used along with \( A'/B' \). Ocular densities, such as a yellow lens, would result in an underestimation of \( A' \), whereas the ratio of \( A' \) and \( B' \) may be less affected. The combination of \( A', A'/B', \) and \( \rho \) resulted in the best prediction of visual pigment density from the optical SCE (Table 2). Parameters \( B' \) and \( x_c \) provided no additional information. The correlation of the predicted density and the measured density was 0.82 (Fig. 3).

In healthy subjects, a density of 0.26 or lower was defined as abnormal (mean = 0.37 – 2 SDs = 0.11). Using this criterion, the sensitivity (tests with a high specificity seldom miss a particular diagnosis) was 96% (Table 3), and the specificity (tests with a high specificity seldom provide an unjustified diagnosis) was 75%. Positive predictive value (the proportion of true positives among the apparent positives) and negative predictive value (the proportion of true negatives among the apparent negatives) were 71% and 98%, respectively.

An even simpler test to predict visual pigment density might be foveal reflectance measured at the peak of the SCE in a bleached state. We defined the foveal reflectance as the sum of \( A' \) and \( B' \). Applying a linear regression to these values, we again predicted visual pigment density (Table 4). Although the predicted density from the foveal reflectance is less accurate than using the optical SCE parameters, the sensitivity and specificity are reasonably high, 84% and 71%, respectively.

**DISCUSSION**

The optical SCE was found to be a sensitive test and a good predictor of the measured visual pigment density for the central fovea. Only a small percentage (<5%) of low densities are missed by the optical SCE test (i.e., false negatives). This is the same as the percentage missed by the measured density using 2 SDs as criteria. False positives were found to be considerably higher (25%). This might be a result of a higher sensitivity to photoreceptor disturbances of the optical SCE than of the visual pigment density, or of additional changes in reflectance related to diseased retinas not influencing density.

In theory, visual pigment density and optical SCE provide different information about the photoreceptors. Density can provide information on the regeneration rate of visual pigment, maximum density attained, and the spectra of visual pigments. These parameters offer insights into the metabolic ability of the photoreceptors. Causes for a reduced foveal pigment density might be: (1) loss of cone photoreceptors, or shortening of cone outer segments, or both; (2) photoreceptor disorientation; (3) metabolic dysfunction of the cone photoreceptor-retinal pigment epithelium complex; or (4) high stray light levels. To possibly discriminate between causes 1 and 2, both tests would be needed.

The optical SCE test provides data on the structural integrity of the photoreceptors. Numerous conditions likely are needed to allow photoreceptors to exhibit a normal optical SCE. (1) The longitudinal axis of photoreceptors needs to be pointed toward one spot in the pupil, usually near the center. (2) Waveguiding, because of a higher index of refraction inside than outside the outer segment, may enhance the fraction of light reflected from the outer segment (i.e., influencing the amplitude of \( A' \)). (3) Because the diameters of the inner segments in cones are larger than those of their outer segment, the inner segments are thought to be especially efficient in captur-

**Table 3. Contingency Table (2 × 2) of Density from Optical SCE Parameters and Measured Density**

<table>
<thead>
<tr>
<th>Predicted Density</th>
<th>Pathologic Density</th>
<th>Normal Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathologic density</td>
<td>49</td>
<td>2</td>
</tr>
<tr>
<td>Normal density</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>

Sensitivity = 49/(49+2) = 96%; Specificity = 60/(60+20) = 75%; Positive predictive value = 49/(49+20) = 71%; Negative predictive value = 60/(60+2) = 97%.

**Table 4. Contingency Table (2 × 2) of Density from \( A' + B' \) and Measured Density**

<table>
<thead>
<tr>
<th>Predicted Density</th>
<th>Pathologic Density</th>
<th>Normal Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathologic density</td>
<td>43</td>
<td>8</td>
</tr>
<tr>
<td>Normal density</td>
<td>23</td>
<td>57</td>
</tr>
</tbody>
</table>

Sensitivity = 43/(43+8) = 84%; Specificity = 57/(57+23) = 71%; Positive predictive value = 43/(43+23) = 65%; Negative predictive value = 57/(57+8) = 88%.
ing light from one spot in the pupil. In case one or more of these conditions is altered, the directional reflectance of the photoreceptors will be changed.

The measured foveal density of the visual pigments probably will attain only a normal value (>0.26), if the directional properties of the photoreceptors are intact. In general, a low stray light level allows for a high measurement of the visual pigment density inside the receptors. High directional reflectance from the foveal photoreceptors results in a relatively low nondirectional reflectance (i.e., stray light) level. Thus, the directionality of the photoreceptors seems to be, potentially, a suitable test to predict the measured density. Some conditions, however, may, in theory, result in erroneous predictions of density from the optical SCE parameters: a retina may contain "empty" photoreceptors (i.e., photoreceptors that hold no visual pigment); a directional reflector (such as the inner limiting membrane) may mimic photoreceptor reflectance; and the nondirectional component (i.e., stray light) may be increased. However, the existence of empty cones has not, to our knowledge, been demonstrated, and directional reflectors and some sources of increased stray light (namely, vitreous floaters) are easily recognized in moving images and thus can be identified or avoided.

As mentioned in the Methods section, we chose to use \( A' \) and \( B' \) instead of \( A \) and \( B \) to describe directional and nondirectional reflectance. The optical SCE fit in subjects with low \( \rho \) values often had \( B \) values equal to zero. It seemed highly unlikely that the foveas of patients produced no directionally independent light and that all remaining light was directionally dependent. Thus, we redefined the amplitude components using \( A' \) and \( B' \). This description only slightly influences the values of directional and nondirectional reflectance found in healthy subjects (Fig. 1A). In addition, it probably provides a more realistic description of directionally dependent and independent reflectance in the foveas of patients with a disturbed optical SCE.

Reflectance from the central fovea (2° × 2° area) seems to provide an adequate correlation with measured density. This would be an even faster test (image acquisition may take less than 1 second) than the optical SCE. It should be noted that for such a test the wavelength used for measurement is critical. At short wavelengths (\( \lambda < 500 \text{ nm} \)), variations in the density of the eye media and macular pigment add to the variance. At long wavelengths (\( \lambda > 550 \text{ nm} \)), variations in the density of melanin influence the measurement. We used 514 nm; at that wavelength fundus reflection is strongly dominated by receptor reflectance.

In conclusion, the optical SCE provides a fast and sensitive test to examine photoreceptor optics in the foveas of patients. A single measurement, obtained from a calibrated instrument at the peak of the SCE, will likely be more influenced by noise than is an estimate of the maximum amplitude of the optical SCE curve (i.e., \( A' + B' \)). We propose that the optical SCE may serve as a test to screen a selected patient population for possible foveal abnormalities in photoreceptor integrity.

References