The Effects of Diabetic Retinopathy Stage and Light Flicker on Inner Retinal Oxygen Extraction Fraction

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PURPOSE. We determined the effects of light flicker and diabetic retinopathy (DR) stage on retinal vascular diameter (D), oxygen saturation (SO2), and inner retinal oxygen extraction fraction (OEF).

METHODS. Subjects were categorized as non-diabetic control (NC, n = 42), diabetic with no clinical DR (NDR; n = 32), nonproliferative DR (NPDR; n = 42), or proliferative DR (PDR; n = 14). Our customized optical imaging system simultaneously measured arterial and venous D (DA, DV) and SO2 (SO2A, SO2V) before and during light flicker. Inner retinal OEF was derived from SO2 values. Light flicker-induced ratios of metrics (DA-R, DV-R, SO2A-R, SO2V-R, OEFR) were calculated.

RESULTS. Arterial D was larger in NPDR compared to NC (P = 0.01) and PDR (P = 0.002), whereas DV was similar among groups (P ≥ 0.16). Light flicker increased DA and DV (P ≤ 0.004), but DA-R and DV-R were similar among groups (P > 0.09). Arterial SO2 was higher in all groups compared to NC (P ≤ 0.02) and higher in PDR compared to NDR and NPDR (P < 0.001). Arterial SO2 did not change with light flicker (P ≥ 0.1). Venous SO2 was higher in NPDR and PDR compared to NC and NDR (P ≤ 0.02). Light flicker increased SO2V in NC, and PDR (P ≤ 0.003), and SO2V-R was lower in NPDR compared to NC and NDR (P ≤ 0.05). Inner retinal OEF was lower in NPDR compared to NDR and PDR (P ≤ 0.02). Light flicker decreased OEF (P ≤ 0.05), but OEFR was greater in NPDR compared to NC and NDR (P ≤ 0.05).

CONCLUSIONS. The findings of alterations in retinal D, SO2, OEF, and their light flicker-induced responses at stages of DR may be useful to elucidate the pathophysiology of DR.

Keywords: diabetic retinopathy, light flicker stimulation, inner retinal oxygen extraction fraction, retina

Diabetic retinopathy (DR) is an established technique to assess the functional capacity of the retina to respond to a physiological challenge. In healthy human subjects, light flicker has been shown to stimulate retinal neural activity,1 augment DA and DV,1 alter SO2,12 and decrease inner retinal oxygen extraction fraction (OEF).13 Inner retinal OEF is defined as the ratio of inner retinal oxygen metabolism (MO2) to oxygen delivery (DO2). Thus, the light flicker-induced OEF response provides information about the ability of the retinal vasculature to address changes in inner retinal oxygen metabolism.15 In DR, reductions in light flicker-induced responses of DA and DV have been demonstrated compared to healthy subjects. However, to our knowledge, neither OEF nor its flicker-induced response has been reported previously in DR. Furthermore, light flicker-induced responses of DA and DV are not assessed simultaneously, potentially affecting results due to intertest variability. Simultaneous assessment of retinal D, SO2, and OEF and their flicker-induced responses at stages of DR may be useful to elucidate the pathophysiology of DR. In the current study, we tested the hypothesis that D, SO2, OEF, and their light flicker-induced responses are altered at stages of DR.

METHODS

Subjects

The study was approved by an Institutional Review Board at the University of Illinois at Chicago. Before enrollment, the research study was explained to the subjects and informed consents were obtained according to the tenets of the Declaration of Helsinki. A total of 130 subjects participated in the study. Subjects’ eyes were classified by clinical examination as nondiabetic control (NC; n = 42), diabetic without clinical retinopathy (NDR; n = 32), proliferative diabetic retinopathy (PDR; n = 42), or proliferative diabetic retinopathy (PDR; n = 14). All PDR subjects had received panretinal photocoagulation (PRP) treatment. Exclusion criteria included history of
stroke or myocardial infarction (within 3 months before imaging), active angina, sickle cell disease, glaucoma, age-related macular degeneration, retinal vascular occlusion, refractive error >6 diopters, or intraocular surgery or cataract surgery performed within 9 months of imaging.

Before imaging, subjects' pupils were dilated using 1% tropicamide and 2.5% phenylephrine. Subjects were seated in front of a modified slit-lamp biomicroscope with their heads resting on a chin and forehead support. During imaging, a light emitting diode was presented to the fellow eye as a fixation target. Subjects were continuously light adapted during imaging due to the instrument's retinal illumination light. Retinal imaging was performed before and during light flicker stimulation. One eye per subject was selected based on the exclusion criteria. If both eyes qualified, the eye with better image data was selected.

Instrumentation

Our previously described optical imaging system was used to simultaneously quantify retinal vascular D and SO2 before and during light flicker stimulation. Briefly, a rapid switching filter wheel was fitted with three bandpass filters and inserted into the illumination path of the slit-lamp biomicroscope. Retinal reflectance images were acquired at 606 and 570 nm wavelengths within 3 seconds before and during light flicker stimulation. Light flicker stimulation was provided at 10 Hz for 60 seconds using light at 530 nm. Images from 606 and 570 nm wavelengths were registered using an automated algorithm and averaged to generate a single mean image at each wavelength. Retinal vessels within a circumpapillary region of interest were segmented (Fig. 1) and vessel centerlines were generated. Vessel D was measured along the vessel centerlines and vessel SO2 (Fig. 1) was calculated from optical density ratio measurements. Measurements of D and SO2 from each vessel within the circumpapillary region of interest were averaged to yield a mean D and D, and arterial and venous SO2 (SO2A, SO2V). These mean values were determined for each subject before and during light flicker stimulation.

Effects of DR and Light Flicker on OEF

FIGURE 1. Example of a retinal reflectance image acquired using light at 570 nm wavelength in a representative nondiabetic control subject. Retinal vessels were segmented and a mean diameter was measured for each vessel (red lines) within the circumpapillary region of interest enclosed by two concentric green circles (Left). A mean retinal vascular oxygen saturation was calculated for each vessel using reflectance images acquired with light at 570 and 606 nm wavelengths. Oxygen saturation is overlaid on the retinal image in pseudocolor for illustrative purposes (Right). Color bar: oxygen saturation in percent.

Inner retinal OEF quantifies the ratio of MO2 to DO2. With Fick’s equation, MO2 and DO2 can be expanded using retinal blood flow (BF) and oxygen content. Since the solubility of oxygen in blood is minimal, SO2 is used to estimate oxygen content. Furthermore, since BF is a determinant of MO2 and DO2, the ratio defined by OEF is independent of BF. Thus, OEF was calculated as \( \frac{[SO2_A - SO2_V]}{SO2_A} \) and used to provide information on the ratio of MO2 to DO2 without providing absolute measurement of either terms. Inner retinal OEF was calculated before and during light flicker stimulation. Light flicker-induced metric ratios (DAR, D VR, SO 2AR, SO 2VR, and OEFR) were calculated by dividing the value of the metric during light flicker by the value before light flicker.

Data Analysis

The distributions of metrics D, D, SO2A, SO2V, and OEF were evaluated to assess data normalcy and identify outliers. Regression diagnostics including Cook’s distance were performed to assess the linear relationship between DR stage and each metric to identify data points that were outliers, had leverage, or were influential. Three outliers were identified, which were removed from further analyses. Subsequent testing for each metric indicated normalcy. The effect of light flicker on measurements of metrics (D, D, SO2A, SO2V, and OEF) within each DR stage group (an intragroup comparison) was performed by paired t-test using metric values before and during light flicker. Descriptive statistics were compared for demographic variables using the \( \chi^2 \) test and t-tests. The independent effects of DR stage group (an intergroup comparison) on baseline measurements of metrics (D, D, SO2A, SO2V, OEF) and metric ratios (D,R, D,R, SO2A,R, SO2V,R, and OEFR) were assessed using linear regression analysis. Multivariable linear regression models were constructed using a priori-selected covariates (age, race, sex, eye examined) from univariate models to compute the parameter estimates and 95% confidence intervals. All statistical tests were 2-sided and significance was set to \( P \leq 0.05 \). All statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).
RESULTS

Demographic data for subjects are presented in Table 1. The distribution of races differed significantly among DR stage groups ($P < 0.001$). Mean ages of NC subjects (59 ± 13 years; mean ± SD), NDR subjects (56 ± 12 years), NPDR subjects (56 ± 10 years), and PDR subjects (51 ± 11 years) were not significantly different ($P = 0.2$). Unadjusted means of metrics ($D_A, D_V, SO_2A, SO_2V,$ and OEF) before and during light flicker stimulation as well as metric ratios ($D_{AR}, D_{VR}, SO_{2AR}, SO_{2VR},$ and OEFR) stratified by DR stage group are presented in Table 2. For each DR stage group, means were statistically adjusted for covariates (age, race, sex, eye examined). Differences in estimated means of metrics ($D_A, D_V, SO_2A, SO_2V,$ and OEF) before light flicker between DR stage groups are provided in Table 3. Similarly, differences in estimated means of metric ratios ($D_{AR}, D_{VR}, SO_{2AR}, SO_{2VR},$ and OEFR) between DR stage groups are presented in Table 4. Unadjusted mean metrics ($D, SO_2,$ and OEF) before and during light flicker stimulation in each DR stage group are shown in Figures 2 to 4, respectively.

Effect of DR Stage: Intergroup Comparison

Differences in estimated means of metrics ($D_A, D_V, SO_2A, SO_2V,$ and OEF) before light flicker between DR stage groups are provided in Table 3. Similarly, differences in estimated means of metric ratios ($D_{AR}, D_{VR}, SO_{2AR}, SO_{2VR},$ and OEFR) between DR stage groups are presented in Table 4. Unadjusted mean metrics ($D, SO_2,$ and OEF) before and during light flicker stimulation in each DR stage group are shown in Figures 2 to 4, respectively.

### Table 1. Characteristics of Subjects Stratified by DR Stage Group

| Variable | Total, $n = 130$ | NC, $n = 42$ | NDR, $n = 32$ | NPDR, $n = 42$ | PDR, $n = 14$ | $P$ Value* |
|----------|-----------------|-------------|-------------|-------------|-------------|------------|
| Sex      |                 |             |             |             |             |            |
| Male     | 48              | 14          | 10          | 19          | 5           | 0.59       |
| Female   | 82              | 28          | 22          | 23          | 9           |            |
| Race     |                 |             |             |             |             |            |
| African American | 51     | 5           | 21          | 19          | 6           | $< 0.001$ |
| White    | 50              | 33          | 5           | 9           | 3           |            |
| Hispanic | 29              | 6           | 6           | 14          | 5           |            |
| Age, y   |                 |             |             |             |             |            |
| < 30     | 2               | 1           | 0           | 0           | 1           | 0.33       |
| 30–39    | 9               | 2           | 5           | 3           | 1           |            |
| 40–49    | 22              | 7           | 6           | 5           | 4           |            |
| 50–59    | 38              | 10          | 9           | 15          | 4           |            |
| 60–69    | 43              | 12          | 10          | 17          | 4           |            |
| ≥ 70     | 16              | 10          | 4           | 2           | 0           |            |
| Eye      |                 |             |             |             |             |            |
| Right    | 86              | 29          | 22          | 25          | 10          | 0.74       |
| Left     | 44              | 13          | 10          | 17          | 4           |            |

Number of subjects in each category is listed. *$P$ values derived using $\chi^2$ test of proportions.

### Table 2. Unadjusted $D_A, D_V, SO_2A, SO_2V,$ and OEF Before and During Light Flicker Stimulation and Their Light Flicker–Induced Ratios ($D_{AR}, D_{VR}, SO_{2AR}, SO_{2VR},$ and OEFR) Stratified by DR Stage Group

| Metric | Flicker Condition | NC | NDR | NPDR | PDR |
|--------|-------------------|----|-----|------|-----|
| $D_A, \mu m$ | Before flicker | 86 ± 8 | 90 ± 12 | 92 ± 11 | 82 ± 11 |
|         | During flicker   | 91 ± 7* | 94 ± 12* | 96 ± 12* | 86 ± 12* |
| $D_V, \mu m$ | Before flicker | 1.07 ± 0.05 | 1.04 ± 0.04 | 1.05 ± 0.06 | 1.05 ± 0.05 |
|         | During flicker   | 112 ± 15* | 112 ± 15* | 115 ± 15* | 110 ± 10* |
| $SO_2A, %$ | Before flicker | 0.92 ± 6 | 0.96 ± 5 | 0.98 ± 7 | 108 ± 8 |
|         | During flicker   | 92 ± 5 | 95 ± 6 | 97 ± 7 | 107 ± 7 |
| $SO_2V, %$ | Before flicker | 1.00 ± 0.04 | 0.99 ± 0.04 | 0.99 ± 0.04 | 0.99 ± 0.03 |
|         | During flicker   | 60 ± 9 | 61 ± 7 | 66 ± 7 | 66 ± 7 |
| $OEFR$  | Before flicker   | 0.87 ± 0.15 | 0.89 ± 0.12 | 0.96 ± 0.15 | 0.89 ± 0.08 |
|         | During flicker   | 0.87 ± 0.08 | 0.89 ± 0.07 | 0.91 ± 0.07 | 0.94 ± 0.08 |

Mean ± SD are reported. Significant differences in metrics compared before and after light flicker in each DR stage group are indicated by superscript.

* $P < 0.05$; derived using paired $t$-tests.
† $P < 0.1$; derived using paired $t$-tests.
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Table 3. Differences in Estimated Means of DA, DV, SO2A, SO2V, and OEF Before Light Flicker Between Each DR Stage Group and a Reference Group

| Metric   | Reference | NDR | NPDR | PDR |
|----------|-----------|-----|------|-----|
| DA, μm   | NC        | NS  | 5.4* | NS  |
|          | NDR       | NS  | NS   | NS  |
|          | NPDR      | NS  | −7.0*| NS  |
| DV, μm   | NC        | NS  | NS   | NS  |
|          | NDR       | NS  | NS   | NS  |
|          | NPDR      | NS  | −9.2*| NS  |
| SO2A, %  | NC        | 3.5*| 5.6* | 16.3*|
|          | NDR       | NS  | 12.8*| NS  |
|          | NPDR      | 10.7*|   |   |
| SO2V, %  | NC        | 6.1*| 6.9* | NS  |
|          | NDR       | 5.3*| 6.1* | NS  |
|          | NPDR      | NS  | NS   | NS  |
| OEF      | NC        | −0.03†| NS  | NS  |
|          | NDR       | −0.04*| NS  | NS  |
|          | NPDR      | 0.06*|   |   |

Elements of the table were omitted to prevent duplicate comparisons. Differences with P ≥ 0.1 are denoted as not significant (NS).

* P ≤ 0.05; multivariable linear regression, adjusted for age, race, sex, and eye examined.
† P < 0.1; multivariable linear regression, adjusted for age, race, sex, and eye examined.

Effect of Light Flicker Stimulation: Intragroup Comparison

Unadjusted means of DA, DV, SO2A, SO2V, and OEF before and during light flicker as well as their metric ratios are shown in Table 2. Arterial D also was larger in NDR compared to PDR subjects (P = 0.02). There was no significant difference in DA between DR stage groups (P ≥ 0.16). Arterial SO2 was higher in NDR, NPDR, and PDR compared to NC subjects (P ≤ 0.02). Additionally, SO2A was higher in PDR compared to NC and NPDR subjects (P < 0.001). Venous SO2 was higher in NPDR and PDR compared to NC (P ≤ 0.01) and NDR subjects (P ≤ 0.02). In NPDR subjects, OEF was significantly lower compared to NDR and PDR subjects (P ≤ 0.02) and tended to be lower than NC subjects (P = 0.07).

Effect of Light Flicker Stimulation: Intergroup Comparison

Differences in estimated means of DA, DV, SO2A, SO2V, and OEF between DR stage groups are provided in Table 4. There was a trend of diminished DA in NDR compared to NC subjects (P = 0.09) and diminished DV in NPDR compared to NC subjects (P = 0.09). The light flicker–induced ratio of SO2A was similar between DR stage groups (P ≥ 0.18). The light flicker–induced ratio of SO2V was lower in NPDR subjects compared to NC and NDR subjects (P ≤ 0.05). The light flicker–induced ratio of OEF was higher in NPDR compared to NC and NDR subjects (P ≤ 0.03), corresponding to a diminished light flicker–induced OEF response in NPDR.

Discussion

Using our previously developed optical imaging system, simultaneous measurements of retinal D, SO2, and OEF were obtained before and during light flicker in nondiabetic control and diabetic subjects at stages of DR. Metrics before light flicker stimulation and their light flicker–induced responses were compared after adjusting for age, race, sex, and eye examined. The results confirmed our hypothesis that D, SO2, and OEF and their light flicker–induced responses are altered at stages of DR. Arterial D was increased in NPDR and decreased in PDR and the light flicker–induced vasodilatory responses tended to be decreased in NDR and NPDR. Arterial SO2 and SO2V were increased in NDR, NPDR, and PDR and the light flicker–induced increase in SO2V was diminished in NPDR. Correspondingly, in NPDR subjects, OEF was decreased and the light flicker–induced decrease in OEF was diminished.

Effect of DR Stage: Intergroup Comparison

In the current study, DA was significantly larger in NPDR compared to NC and PDR subjects. This finding is consistent with previous studies that found that retinal arterial dilation is related to the incidence of DR.2,7,8,22 However, DV was not significantly different among DR stage groups in the current study, in contrast to the findings of Klein et al.3 and Kifley et al.,22 who reported that dilation of retinal veins was related to DR progression. The discrepancy between these results is likely due to the smaller sample size in the current study, study design, and differences in covariate corrections. In the current study, SO2A was higher in all DR stage groups compared to NC subjects. Additionally, SO2A was higher in PDR compared to
NDR and NPDR subjects. Venous SO2 was higher in NPDR and PDR compared to NC and NDR subjects. These findings are in agreement with previous studies that found increased SO2A and SO2V in DR6,23 and increasing SO2V with DR progression.5,23,24

In NPDR subjects, OEF was significantly lower compared to NDR and PDR, and tended to be lower than NC subjects. Since OEF is defined as the ratio of MO 2 to DO 2,13 these results indicated differences in MO 2 and DO 2. Inner retinal oxygen DO2 is determined by arterial blood oxygen content and BF, which is, in turn, related to D. Although DO2 was not directly measured in the current study, DO2 was likely increased in NPDR since D A was larger, consistent with previous reports of increased BF in NPDR subjects.25,26 On the other hand, MO2 may have been reduced during NPDR. Retinal hypoxia is implicated in DR,27,28 which depending on severity, may cause a reduction in MO2 as shown under severe hypoxia in rats.29 However, a decrease in MO2 is only speculative, since direct measurement of MO2 has not been reported previously in DR subjects, to our knowledge. Therefore, it seems likely that the observed reduction of OEF in NPDR was primarily due to an increase in DO2. In PDR subjects, OEF was not significantly different from that of NC subjects. Since all PDR subjects had received PRP treatment, inner retinal oxygenation was presumably improved30 due to a loss of oxygen-consuming outer retinal tissue and the resultant increased oxygen flux from the choroidal circulation. Thus, as a result of increased oxygen delivery from the choroid, DO2 is expected to decrease as the retinal circulation autoregulates. Furthermore, due to the increase in oxygen availability from the choroid and presumably less retinal tissue, inner retinal MO2 is also

![FIGURE 2. Mean retinal arterial (A) and venous (B) diameter measurements before and during light flicker in each DR stage group.](image)

![FIGURE 3. Mean retinal arterial (A) and venous (B) oxygen saturation measurements before and during light flicker in each DR stage group.](image)

![FIGURE 4. Mean inner retinal oxygen extraction fraction measurements before and during light flicker in each DR stage group.](image)
decreased. Therefore, the finding of similar OEF between PDR and NC subjects is consistent with decreases to MO_2 and DO_2.

**Effect of Light Flicker Stimulation: Intergroup Comparison**

Increased D_3 and D_V in response to light flicker is in agreement with previous studies that demonstrated retinal vasodilation in NC. Reduced D_3 and D_V in response to light flicker was reported in diabetic retinopathy (DR) and PRP subjects. Arterial SO_2 did not respond to light flicker in all DR stage groups, whereas SO_2V significantly increased in NC, NDR, and PDR subjects, and tended to increase in NPDR subjects. These results are in agreement with previous studies that found light flicker did not change SO_2A and increased SO_2V in NC and NPDR subjects. In the current study, OEF decreased with light flicker in all DR stage groups, consistent with our previous study. A decrease in OEF indicates that the light flicker-induced change in MO_2 was greater than the respective light flicker-induced change in MO_2. This finding is in agreement with a recent study by Palkovits et al. which demonstrated that light flicker-induced increases in BF (55%) exceeded that of oxygen extraction (35%) in NC subjects.

**Effect of Light Flicker Stimulation: Intragroup Comparison**

The light flicker-induced ratio of D_3 tended to be lower in NDR compared to NC subjects, in agreement with previous studies using the Dynamic Vessel Analyzer. The light flicker-induced ratio of D_V tended to be lower in NPDR compared to NC subjects. The vasodilatory findings in the current study are not consistent with previous studies that reported a progressive reduction in D_3 and D_V with DR stage. Differences in findings may be attributed to the smaller sample size in the current study and differences in covariate corrections.

The light flicker-induced ratio of SO_2A was not different among DR stage groups, while SO_2V was lower in NPDR compared to NC and NDR subjects, in agreement with a previous study. One possible explanation for this observation is based on reduced availability of oxygen to the retinal tissue in NPDR. In the nonnecrotic state, the retinal tissue receives sufficient oxygen and, thus, during light flicker-induced augmentations of DO_2, abundant amounts of oxygen are delivered that exceed the change in metabolic demand of the tissue. This results in an increase in SO_2V with light flicker and an SO_2V greater than unity. In contrast, tissue that receives insufficient oxygen will extract more when it is made available during the light flicker-induced augmentation of DO_2. This causes a diminished increase in SO_2V and a decreased SO_2V compared to normal tissue. Indeed, the vascular pathologies of NPDR, such as capillary shunting and nonperfusion on fluorescein angiography, indicate decreased DO_2. In contrast, SO_2V was not significantly different between NC, NDR, and PDR subjects. Subjects with NDR likely had normal retinal oxygenation, supported in part by the lack of visible vascular pathologies. All PDR subjects had received PRP treatment which likely improved their inner retinal oxygenation such that abundant amounts of oxygen were available during light flicker, resulting in an SO_2V similar to that of NC subjects.

The light flicker-induced ratio of OEF quantifies the ratio of light flicker-induced responses in MO_2 to DO_2 without directly quantifying either response. The light flicker-induced ratio of OEF was not significantly different between NC and NDR subjects, despite an observed trend of impaired vasodilatory response, suggesting a diminished light flicker-induced response in DO_2. Together, these results suggested a diminished light flicker-induced response in MO_2 before the development of NPDR. The light flicker-induced ratio of OEF was higher in NPDR compared to NC and NDR, which is likely due to a diminished light flicker-induced response in DO_2, consistent with the observed trend of impaired vasodilatory response. However, a change in the light flicker-induced response of MO_2 cannot be excluded. Interestingly, OEF and the vasodilatory responses to light flicker were not significantly different between NC and PDR subjects. This result suggests that PRP treatment may promote the restoration of light flicker-induced responses in DO_2 and MO_2.

There were several limitations in the current study. First, OEF quantifies the ratio of MO_2 to DO_2 and cannot directly quantify either quantity due to a lack of BF measurements. Future studies that simultaneously measure MO_2 and DO_2 in stages of DR are needed to elucidate the underlying reason for a reduced OEF and its flicker-induced response in DR. Second, since data were acquired by optical imaging techniques, image quality may have affected measurements. However, the system was validated previously and shown to be capable of detecting light flicker-induced changes. Third, a fixed calibration factor was used to calculate vessel diameters and, thus, did not account for variations in refractive error among subjects. However, subjects with high refractive error, greater than 6 diopeters, were excluded from the study. Furthermore, the use of a constant calibration did not affect diameter measurements compared within subjects or flicker-induced diameter ratios compared between subjects. Fourth, there were variations in the clinical history and status of DR subjects. Future studies with a larger sample size that can account for clinical confounding factors are needed to substantiate the current findings and reveal differences not discernable with this sample size.

In conclusion, vessel diameters were larger at stages of DR and the flicker-induced changes tended to be decreased. Oxygen saturation of vessels increased at stages of DR and the flicker-induced changes in SO_2V were different in NPDR. Correspondingly, OEF and OEFR were decreased in NPDR, suggesting impairment of the MO_2 and DO_2 and their responses to light flicker in DR. These findings of alterations in D, SO_2, and OEF and their light flicker-induced responses may help to elucidate the pathophysiology of DR.

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