# Diabetic Ocular Surface Has Defects in Oxygen Uptake Revealed by Optic Fiber Microsensor

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**Purpose.** Diabetes mellitus causes diabetic keratopathy (DK). This and other ocular surface disorders are underdiagnosed and problematic for affected patients as well as recipients of diabetic donor corneas. Thus, it is important to find noninvasive means to facilitate determination of the potentially vision-threatening DK. It has been reported that diabetic corneas uptake significantly less oxygen ($O_2$) than healthy controls. However, an integral assessment of the ocular surface is missing.

**Methods.** Using an optic-fiber $O_2$ micro-sensor (optrode) we demonstrated recently that the healthy ocular surface displays a unique spatiotemporal map of $O_2$ consumption. We hypothesize that diabetes impairs the spatiotemporal profile of $O_2$ uptake at the ocular surface.

**Results.** Using streptozotocin (STZ)-induced diabetic mice, we found diminished $O_2$ uptake and loss of the unique pattern across the ocular surface. A diabetic cornea consumes significantly less $O_2$ at the bulbar conjunctiva and limbus, but not the central and peripheral cornea, compared to controls. Further, we show that, contrary to the healthy cornea, the diabetic cornea does not increase the $O_2$ consumption at the limbus in the evening as the normal control.

**Conclusions.** Altogether, our measurements reveal a previously unknown impairment in $O_2$ uptake at the diabetic cornea, making it a potential tool to diagnose ocular surface abnormalities and suggesting a new etiology mechanism.

Keywords: cornea, diabetes, oxygen, microsensor

Diabetes mellitus (DM) has been cited as the main cause of blindness in the world's working-age population.[1,2] Additionally, the incidence of this deadly metabolic disease has been projected to double from the years 2015 to 2030.[2–4] These projections predict an urgent and anticipated increased health and financial burden worldwide.[4] DM is a chronic metabolic disorder characterized by elevated blood glucose levels. The two most common forms are type 1, in which insulin deficiency is caused by the loss of insulin-producing pancreatic $\beta$-cells, and type 2, which is caused by insulin resistance.[4] Both forms of DM are linked to impairments in the body's vasculature.[3,5] Some microvascular complications that have been observed are retinopathy, nephropathy, and neuropathy.[3–5] Additionally, ocular surface disorders frequently arise in patients with DM, afflicting up to 70% of those examined.[1] Although the incidence is high, these disorders are underdiagnosed. In part, this is because the diabetic cornea has significantly reduced sensitivity. But largely this is because, until an injury is sustained, the diabetic cornea resembles that of a healthy patient.[1,6] Once damage occurs, the corneal epithelium has a significantly delayed rate of healing and epithelial disorders are prolonged.[6]

Epithelial complications arising from DM are referred to as diabetic keratopathy (DK), highlighting their significant effect on the cornea epithelium.[1] DK has been estimated to affect 47% to 64% of patients with DM.[4] Clinical manifestations of DK include non-healing ulcers, epithelial fragility, edema, increased tendency to injury, slow or incomplete wound healing, and superficial punctate keratitis.[1] As of 2017, 18% of the transplanted corneas in the United States were sourced from diabetic donors.[1] This may pose an issue for recipients as these corneas likely carry deleterious epigenetic changes.[1] To help patients impacted by DM, and






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recipients of affected corneas, it is important that we understand how these tissues differ from healthy corneas as well as to have a simple means of diagnosing DK before injury occurs. One well-known characteristic that can easily be assessed is the metabolic rate of patients with DM, which is abnormal, marked by a reduction in $O_2$ consumption.[7] Cornea oxygen uptake (COU) measurements are indicative of the metabolic state of the cornea.[8] A decades-old study found that there was a definite decline in whole cornea oxygen consumption in diabetic humans and rats compared to controls.[7] Another early study corroborated these findings, and found that patients with diabetic retinopathy also had significantly lower COU rates compared to age-matched controls.[8] It is not known whether the defective COU in the diabetic cornea affected the whole ocular surface, or some specific regions more than the others, and whether the defects also affect temporal fluctuation at the limbus.

Very recently, we generated a comprehensive and distinct spatiotemporal map of $O_2$ uptake at the ocular surface of healthy mouse, rat, and rhesus monkey, using a noninvasive optical fiber microsensor, which revealed characteristically higher COU at the limbus across species. We found a spatial centripetal gradient with significantly higher $O_2$ uptake at the limbus and conjunctiva which decreased toward the center of the cornea. Further, we found that, temporally, there was a significant increase in the $O_2$ uptake at the limbus in the evening (5 PM).[9]

In the present study, we aim to reproduce these experiments in a diabetic model to compare the tissue's discrete metabolism to that in healthy conditions. Because it has been shown that diabetic corneas uptake less $O_2$ in general, we hypothesize that the unique spatiotemporal COU previously described will be absent. Generating a diabetic, spatiotemporal COU map, will give us further insights into the pathophysiology of the diabetic ocular surface and may serve as a means of DK diagnosis prior to injury.

Unsurprisingly, the development of methods to measure physiological oxygen has a long history. The earliest $O_2$ probes were the polarographic (Clark-type) electrodes developed in 1953 to measure the oxygen concentration ($pO_2$) in blood.[10] These electrodes depend on a chemical reaction consuming oxygen and generating an electric current proportional to the $O_2$ concentration. The disadvantages of this type include chemical disturbance due to the probe consuming oxygen, signal drift, and low sensitivity in reduced $O_2$ environments. Because $O_2$ is one of the best fluorescence quenchers,[11] this led to the development of optical-based $O_2$ probes, first based on fluorescence intensity,[12] and then on fluorescence decay time.[13] To combat the signal drift inherent in single (or multiple) static microelectrodes, self-referencing was next introduced, whereby a single probe moves by computer control at a relatively low frequency (approximately 0.1 hertz [Hz]) between two positions near the sample.[14,15] If a difference in $pO_2$ is detected, the oxygen flux into or out of the sample can be calculated. When it comes to determination of very low $O_2$ concentrations associated with oceanic oxygen minimum zones (OMZs), these probes above have been proven to be unreliable. This led to the development of the switchable trace oxygen (STOX) sensor, developed specifically for analysis of OMZ regions.[16] The STOX sensor can be used to detect and quantify $O_2$ concentrations as low as 10 nM.

In this study, the self-referencing $O_2$-specific microoptrode (SMOT) quantifies the oxygen concentration by quenching of the fluorescence emitted by an $O_2$-sensitive fluorophore excited with blue–green light, whereas the automated scanning electrode technique (ASET) software simultaneously calculates the $O_2$ flux based on Fick's first law of diffusion.[17]

## Methods

### Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California – Davis and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Eight-week-old c57/bl6 streptozotocin (STZ; 5 mg/mL)-induced diabetic male mice and age-matched control mice with vehicle injection were purchased from Jackson Laboratory. All male mice were used for consistency of data. A 2011 study found almost identical $O_2$ levels in the eyes of human male patients versus female patients ($t$-test, $P = 0.79$).[18] Mice were kept in a standard 12 hour light / 12 hour dark regime. Group numbers were six STZ diabetics and five controls. The blood glucose levels were measured from the tail vein using a glucometer (Accu-Chek Aviva Plus Glucometer; Roche Diagnostics) before mice shipped, after they arrived, and after all experiments finished, and also sometimes in between, to confirm diabetic status. In a previous study,[19] mice developed stable diabetes on day 5 after administration of STZ. In this study, the time between the first STZ injections and shipping was 21 days. Upon arrival, mice were acclimated to the new environment for 1 week. At the time of the experiments, the mice had therefore been diabetic for at least 3 weeks. Glucose levels of STZ-induced mice were all above 320 mg/dL, and glucose levels of control mice were between 150 and 215 mg/dL (see the Table). For experiments, mice were anesthetized with a mixture of ketamine hydrochloride (Zetamine, VetOne; 10 mg/mL) and dexmedetomidine (Dexdomitor, Zoetis; 0.1 mg/mL). We found that, under these anesthesia conditions, the eyes stayed naturally open, so no ophthalmic speculum was required. Only one eye per animal was tested as per the IACUC protocol. Mice were placed under a microscope and a well of hydrophobic silicone grease (Dow Corning) was made around the test eye and filled with artificial tear solution (BSS+ saline; Alcon Laboratory) as measuring medium. The untreated control

Table. Blood Glucose Readings in Control and Diabetic Mice Before and After Experiments

| | | Glucose Reading (mg/dL) | |
|---|---|---|---|
| | ID # | Before Experiment | After Experiment |
| Control mice | 6961 | 195 | 189 |
| | 6962 | 204 | 186 |
| | 6963 | 211 | 169 |
| | 6964 | 213 | 196 |
| | 6965 | 190 | 158 |
| Diabetic mice | 6966 | 517 | 492 |
| | 6967 | 436 | 488 |
| | 6969 | 323 | 470 |
| | 9771 | 362 | 485 |
| | 9772 | 458 | 507 |
| | 9773 | 322 | 463 |

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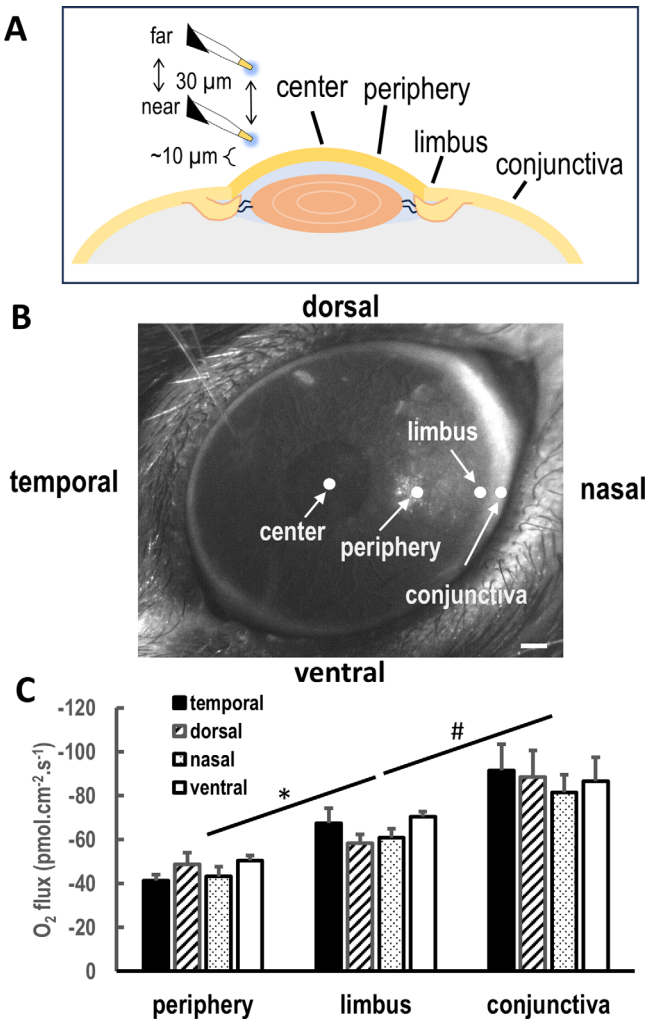


**Figure 1.** Micro-optrode for measuring $O_2$ flux at cornea in vivo. (**A**) Diagram showing a side view of the eye and the optrode (not to scale) in measuring position. After manually placing the optrode close to the ocular surface, the ASET software takes over and automatically moves the probe 30 µm between the "near" and "far" positions, measuring the $pO_2$ and simultaneously calculating the $O_2$ flux. Measurements were taken at four positions across the ocular surface as shown. (**B**) Measurements were also made at the four quadrants shown. The optrode is indicated by the *red arrow*. *White dots* indicate the nasal measurement positions. Scale bar 200 µm. (**C**) Increasing oxygen flux from periphery outward to limbus and conjunctiva in healthy cornea. Data are mean ± SEM (*$P < 0.001$; #$P < 0.05$).

eye received Soothe sterile lubricant eye ointment (Bausch & Lamb) to prevent cornea drying. After adding the artificial tear solution to the test eye, the eye was allowed to equilibrate for 5 minutes before measurements began. All measurements were done in anesthetized mice in vivo. Measurement of oxygen flux at the four positions of the eye (see Fig. 1) takes about 20 minutes. At the end of the study, all mice were humanely euthanized by carbon dioxide inhalation and cervical dislocation as per the IACUC protocol.

### Scanning Micro-Optrode Technique

The SMOT and ASET interface software (version LV4), purchased from Applicable Electronics, have been recently described in detail[9,17] and will be described here only briefly. The SMOT is composed of a fiber-optic microsensor probe (also known as micro-optrode or optode), amplifier, and 3D micro-positioner with computer control. The fiber-optic probe is tipped with an $O_2$-sensitive fluorophore. A fluorescent light source excites the fluoro-phore (through the fiber-optic cable) with blue green light ($\lambda = 505$ nm). Upon collision with $O_2$, the excited fluorophore is quenched, therefore affecting emission. The rate of fluorescence quenching is therefore proportional to the $O_2$ concentration ($pO_2$). Calibration of the SMOT system in saline with 0% $O_2$ and air saturated (20.95%) $O_2$ was performed as previously described.[9,17] The $pO_2$ and $O_2$ flux were calculated in real time by the ASET software during cornea measurements and calibrations.

### Cornea Measurements

With the anesthetized mouse under the microscope with the eye-well containing saline in place, the cornea surface was brought into focus and the micro-optrode placed into the measuring solution and moved close to the cornea manually using the 3D micro-positioner. Then, using the ASET software to control the movement, the optrode was carefully moved to about 10 µm from the cornea surface (see Fig. 1A). After waiting a few minutes for the optrode signal to stabilize, the recording began, with the ASET software automatically moving the optrode 30 µm between the "near" and "far" positions at a frequency of 0.1 Hz, while simultaneously recording the $pO_2$ at the 2 positions and automatically calculating the $O_2$ flux. Before and after measurements at the cornea surface, reference readings were taken more than 1 mm away from the specimen for about 2 minutes to confirm zero $O_2$ flux (for example, see Figs. 2A, 2B). Oxygen flux was measured at the following cornea positions: center, periphery, limbus, conjunctiva, and also at these orientations: the dorsal, ventral, temporal, nasal (see Fig. 1B).

### Data Analysis

Data are shown as mean ± standard error of the mean (SEM). Differences between samples are compared using the Student's *t*-test, and statistical significance accepted at 95% confidence limits ($P < 0.05$). All data analysis, graphs, and statistics were made using Excel (Microsoft).

## Results

### Diabetes Impairs the Spatial Pattern of $O_2$ Uptake Present in the Healthy Ocular Surface

In our previous study, we found a unique spatial profile for $O_2$ uptake across the healthy mammalian ocular surface.[9] Specifically, we detected a centripetal gradient with the highest uptake in the conjunctiva and cornea limbus, decreasing toward the center of the ocular surface. Based on this map, we wondered whether the metabolic disease diabetes impairs this unique profile of $O_2$ uptake.

First, we reproduced our previous results by measuring the in vivo $O_2$ flux dynamics at the ocular surface of healthy mice, measuring at the cornea center, periphery, and limbus, as well as the conjunctiva (see Fig. 1C). We found that the outer segments of the eye uptake significantly more $O_2$ than the central locations. The limbus and conjunctiva uptake significantly more $O_2$ when compared

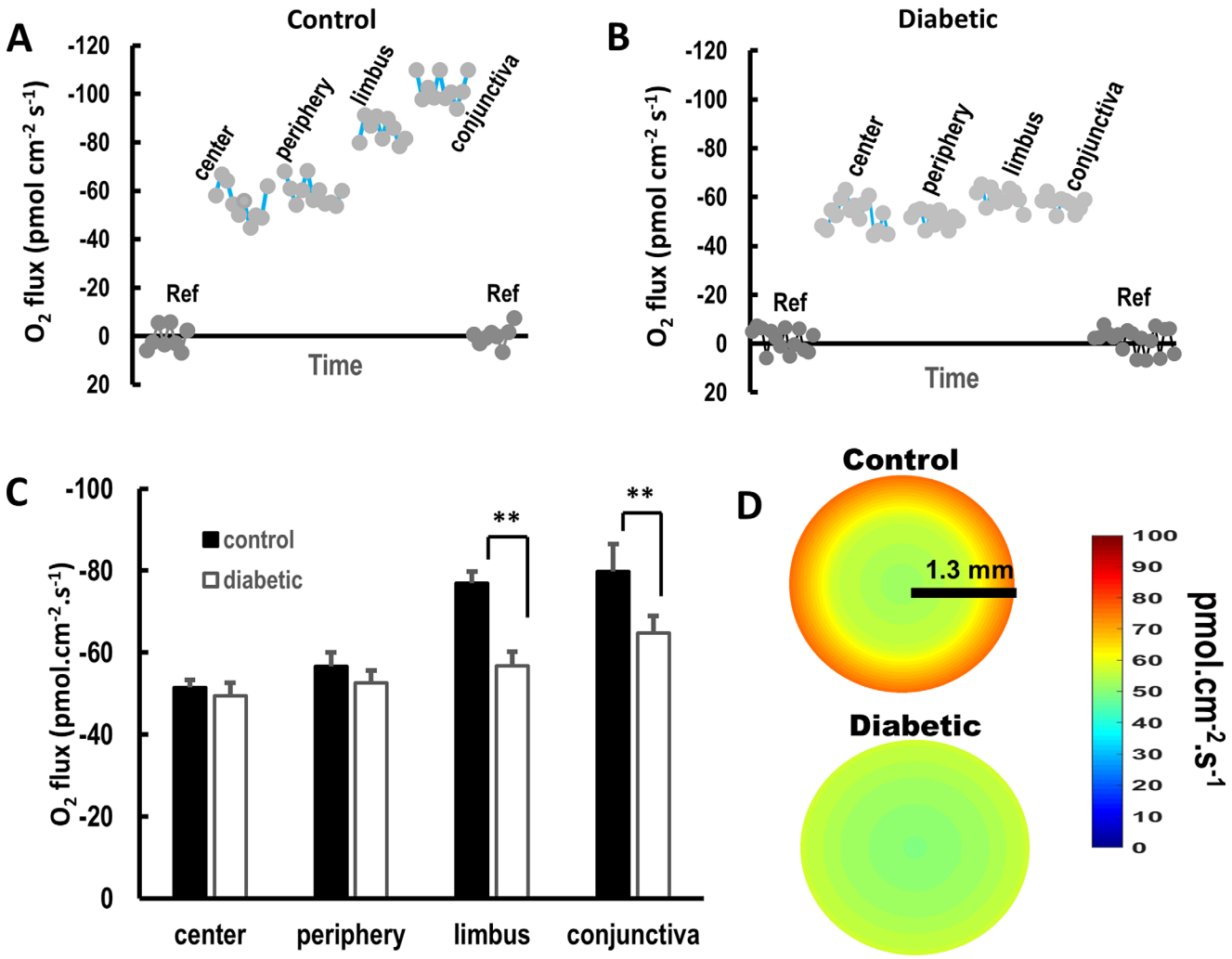


**Figure 2.** Diabetic cornea lost the characteristics high $O_2$ uptake at the limbus. (**A**, **B**) Typical $O_2$ uptake measurements at the ocular surface of non-diabetic age-match control mice (**A**) and diabetic mice (**B**). Ocular surface is a $pO_2$ sink, where $O_2$ is taken up by the cornea in relation to reference position away from the cornea ("Ref."). $O_2$ uptake is at approximately the same level at the four positions at the diabetic ocular surface (**B**), in contrast to the high $O_2$ uptake at the control limbus and conjunctiva (**A**). Negative values indicate that the oxygen flux direction is inwards (influx). (**C**) $O_2$ uptake at the limbus and conjunctiva are significantly lower in diabetic animals compared to healthy age- and sex-matched control animals. Data are mean ± SEM (**$P < 0.001$). (**D**) *Pseudo color* images to visually compare $O_2$ uptake across the cornea, showing decrease in $O_2$ uptake specifically at the diabetic limbus, and losing the typical centripetal gradient of $O_2$ uptake increasing from the center outward in healthy cornea.

to the cornea center and periphery ($P < 0.001$, $n = 5$). Additionally, the cornea limbus uptakes significantly more $O_2$ than the periphery ($P < 0.001$, $n = 5$) and likewise the conjunctiva uptakes significantly more $O_2$ that the cornea limbus ($P < 0.05$, $n = 5$). The healthy ocular surface therefore presents a significant and spatially changing sink of $O_2$, with limbus and the conjunctiva consuming significantly more $O_2$ than the center and periphery of the cornea. These results are consistent with the findings of our previous study.[9]

To test whether diabetes affects this robust centripetal gradient, we measured the in vivo $O_2$ uptake at the ocular surface of STZ-induced diabetic mice. Placing the micro-optrode in the same spatial positions of the healthy mice allowed us to detect only mild variations in $O_2$ uptake across the ocular surface of the diabetic mice (see Fig. 2B). There was no significant difference between the cornea periphery and limbus ($P = 0.354$, $n = 5$) or the limbus and sclera conjunctiva ($P = 0.213$, $n = 5$) as was observed in healthy age-matched control mice (see Fig. 2A). Taken together, these data show that diabetes has effectively impaired the centripetal oxygen gradient at the ocular surface.

## Diabetic Cornea Has Significantly Decreased $O_2$ Uptake at the Limbus and Conjunctiva

After revealing the impaired centripetal gradient of $O_2$ uptake in diabetic mice, we tested whether the uptake at the different positions was significantly different from healthy controls. Measuring healthy and diabetic corneas in the same regions with the micro-optrode showed a slight decrease in the $O_2$ uptake, although not statistically significant at the cornea center ($P = 0.573$, $n = 5$) and periphery ($P = 0.322$, $n = 5$; Fig. 2C). However, as we moved away from the periphery, we found a substantial deficiency in $O_2$

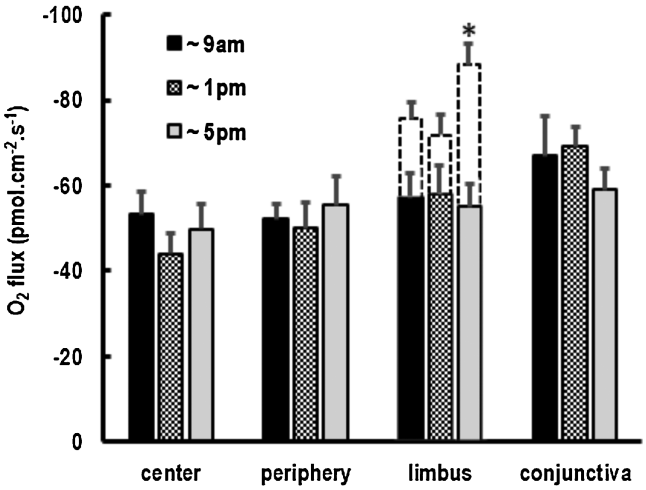


FIGURE 3. Diabetic cornea lost temporal dynamics of limbal $O_2$ uptake. Corneas in diabetic animals did not show the evening rise in $O_2$ uptake that happened in non-diabetic corneas. $O_2$ uptake at the limbus in the evening has a significant increase only in healthy control animals (*bars with dashed lines* at the limbus; data from Ref. 9). Such changes were not detected at the cornea of diabetic animals (*bars with solid lines*). Data are mean ± SEM (*$P < 0.001$ for limbus at 5 PM).

uptake at the limbus and conjunctiva of the diabetic cornea. When compared to healthy controls, the diabetic $O_2$ uptake was significantly lower at the limbus ($P < 0.001$, $n = 5$) and conjunctiva ($P = 0.001$, $n = 5$), comprising only 74% ($-56.78 \pm 3.33$ pmol $cm^{-2}$ $s^{-1}$) and 81% ($-64.69 \pm 4.18$ pmol $cm^{-2}$ $s^{-1}$) of the non-diabetic control amount, respectively (see Fig. 2C). These large drops are responsible for the flattening of the spatial gradient (see Fig. 2B), making the ocular surface more uniform in terms of $O_2$ uptake. To help visualize the differences between healthy and diabetic corneas, we overlaid the $O_2$ uptake profiles onto the ocular surface. The resulting heatmap simulation demonstrated a drastically different spatial mapping of $O_2$ uptake between healthy control and diabetic mice. Healthy cornea displayed a strong centripetal gradient with much higher $O_2$ uptake at the marginal ocular surface compared with the center (Fig. 2D). In contrast, the $O_2$ uptake in diabetic cornea was similar across the whole surface.

### Diabetic Cornea Lost Significant Temporal $O_2$ Uptick at the Limbus

In our recent study, we found that the $O_2$ uptake at the mouse ocular surface changed during the day. Specifically, we observed a significant 20% increase in the limbus $O_2$ uptake in the evening when compared against the morning and midday measurements.[9] Based on this time-dependency, we sought to understand whether the diabetic cornea would experience similar diurnal/temporal variations. For this, we measured the same spatial $O_2$ uptake of diabetic mice at different time points throughout the day, recapitulating the time points of our previous study (9 AM, 1 PM, and 5 PM), to yield a spatiotemporal profile. Interestingly, although there is some variation in the measurements, there was no significant difference in $O_2$ consumption between any time points measured ($P > 0.05$ for all comparisons; Fig. 3). The cornea limbus of the diabetic eyes completely lost the $O_2$ uptick that we previously observed in healthy cornea late in the day and was significantly lower than the controls ($P < 0.001$ comparing diabetic and control limbus at 5 PM; control data from Ref. 9). Therefore, the diabetic cornea does not show signs of increased diurnal metabolic activity at the limbus as seen in healthy cornea.

Altogether, our data demonstrates a drastic impairment and homogenization of the spatiotemporal profile of $O_2$ uptake in diabetic corneas. This disease-specific uniform profile may become a diagnostic tool to help assess patients with diabetes.

## Discussion

In this study, we examined the in vivo $O_2$ uptake at the ocular surface of healthy and STZ-induced diabetic mice using a fiber-optic microsensor. We confirmed the findings of our previous study[9] in which the healthy ocular surface uptakes $O_2$ with a distinct centripetal gradient, the conjunctiva and limbus consuming significantly more $O_2$ than the periphery and center. Because $O_2$ is a well-established indicator of metabolic activity,[7,8] we can affirm that the outer segments of the ocular surface are more metabolically active than those more central. As we postulated previously, this is likely due to the population of limbal epithelial stem cells (LSCs) that reside at the intersection of the conjunctiva and the limbus.[1] This finding also confirms that the SMOT system is a reliable and consistent means of noninvasively measuring $O_2$ uptake in discrete structures.

In diabetic eyes, we found that the unique spatial COU profile and centripetal gradient was significantly decreased. Although there were significant differences between the central and outer segments of the ocular surface, the limbus and conjunctiva took up significantly lower levels of $O_2$ when compared to healthy control corneas. This means that in diabetic eyes there is a marked loss of metabolic function at the critical limbus and conjunctiva intersection where LSCs should be active. Although this is alarming, it is not surprising. Previous studies have found that in patients with diabetes LSC activity is dysfunctional as demonstrated by a significant reduction in putative markers.[1] This loss of function may be attributed to the vascular complications that arise in diabetic tissue. Although the cornea is virtually avascular and thus sources its $O_2$ almost exclusively by atmospheric diffusion through the tear film, the sclera conjunctiva is vascularized.[20] In diabetic eyes, conjunctival blood vessels show dilation, uneven distribution, and lost capillaries with increased tortuosity.[1,21,22] This apparent loss in vasculature to the conjunctiva likely negatively affects the viability of this tissue and its ability to support LSCs. Furthermore, there has also been observed a loss in cornea nerve structures, known as diabetic neuropathy (DN).[1,4] Interestingly, the healthy cornea is the most densely innervated tissue in the body.[4] The intricate nerve architecture supplies the cornea with important trophic support, secreting neuromodulators, maintaining healthy tissue, and promoting wound healing.[4] DN cornea nerve abnormalities include changes in nerve fiber density and thickness, increased tortuosity, and overall degeneration and apoptosis.[1,4] This may put even more demand on the already underperforming LSCs as the center and periphery segments of the cornea need to be renewed more rapidly due to the loss of neurotrophic support. Loss of sensi-

tivity in the diabetic eye is also to blame on the loss of neurons, leading patients to be less aware when an injury occurs.[1,2,4,8]

Although it has been known for decades that diabetic mammals have a reduced oxygen uptake in cornea and other tissues (e.g. the retina) and that patients with diabetes have a systemic decrease in oxygen saturation, the elucidation of the underlying mechanisms still warrants further research.[7,8,23,24] These studies, and others, have argued that general metabolic constraints, insulin dysregulation, corneal neuropathy, dysfunctional vasculature, as well as loss of corneal sensitivity associated with diabetes, are the potential causes for the decreased oxygen uptake, which in turn would exacerbate the pathology.[1,4,25] However, direct experimental testing of these hypothetical causes (either in isolation or in combination) remains to be thoroughly conducted. A recent study showed that chronic hyperglycemia-induced mitochondrial dysfunction decreases respiration in corneal epithelial cells in vitro.[26] This could be another factor contributing to reduced oxygen consumption in diabetes. Finally, given our data, we speculate that the limbal stem cells may, at least partially, explain the decreased oxygen uptake. This is because, in diabetes, these cells have dysfunctional activity that could be further underperforming due to the neural and vascular pathophysiology in the vicinity of those cells.[1,4]

Temporally, we found that the significant uptick in $O_2$ consumption at the limbus in the evening was missing in diabetic eyes. This again points to the decreased functionality of diabetic LSCs. Although we are not sure as to why healthy limbus shows higher metabolic activity around 5 PM in mice, these activities are not present in the diabetic model. Mice are nocturnal animals that have systemic and tissue level increases in oxygen consumption during the dark period (active phase).[27–29] As such, part of the increased corneal uptake may be explained by the circadian rhythms. We believe that the differences at the limbal area are additionally explained by local physiology. It has been shown that cell proliferation in the cornea follows the circadian rhythms and, importantly, the mitotic index is higher around the limbal area,[30] which may lead to localized increase in oxygen uptake. Further, the cornea epithelial thickness significantly increases in the evening as mice enter the active phase.[31] In our previous study,[9] we speculated that an upregulation in limbal epithelial stem cells activity could lead to this epithelial thickening owing to circadian-dependent localized proliferation. Taken together, these would increase the need for oxygen and explain our observations in the limbus.

This new spatiotemporal $O_2$ uptake profile of the diabetic ocular surface provides a more informative look into the metabolism of the tissue. The tissue is likely overtaxed due to loss in nutrient supply and is therefore less metabolically active. It is likely that the loss of vascular support at the conjunctiva as well as the neuropathy occurring at the cornea pose too much stress on the LSC population. This is likely why, when a wound occurs in diabetic corneas, wound healing is significantly delayed.[1,2,4,6,22,32] In addition to further insight, this diabetic COU profile may also serve as a new means for early DK detection and diagnosis. Because the diabetic cornea appears healthy prior to injury, currently, there is no method of prior diagnosis. Because the SMOT system is noninvasive and relatively easy to use, this could be a promising new avenue for diagnosis, although these experiments would first need to be recapitulated in human tissue.

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