Keratocyte Density in the Human Cornea After Photorefractive Keratectomy

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Objective: To perform a quantitative analysis of keratocyte density in human corneas after photorefractive keratectomy (PRK).

Methods: In a prospective comparative trial, 24 eyes of 14 patients received PRK to correct refractive errors of between –1.25 diopters (D) and –5.75 D. Corneas were examined by using confocal microscopy before and 1 day, 5 days, and 1, 3, 6, 12, 24, and 36 months after PRK. Keratocyte nuclei were counted in 5 stromal layers in 3 to 6 scans per eye per visit. Keratocyte density in each layer post-PRK was compared with the density in the corresponding layer of the pre-PRK full stroma (included stroma that would later be photoablated) and the pre-PRK future unablated stroma (thickness adjusted by omitting the future ablation depth) (Bonferroni-adjusted paired t test).

Results: Keratocyte density in the anterior 10% of the post-PRK stroma decreased by 25% (P = .002), 41% (P < .001), 40% (P < .001), 43% (P < .001), and 45% (P < .001) at 3, 6, 12, 24, and 36 months compared with the anterior 10% of the pre-PRK full stroma and was reduced by 15% at 36 months (P = .02) compared with the anterior 10% of the pre-PRK future unablated stroma.

Conclusion: After PRK, keratocyte density in the anterior stroma is not restored to the high-density levels found in the preoperative stroma.

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PHOTOREFRACTIVE keratectomy (PRK) uses an excimer laser to precisely photoablate graded amounts of anterior corneal stroma to induce a change in corneal refraction. Although clinical results are good, variable predictability of refractive and visual outcomes, regression of the initial refractive effect, and subepithelial haze remain clinical concerns. These adverse effects have, in part, been attributed to qualitative or quantitative variations in corneal keratocytes and their subsequent effect on corneal wound healing.

An early loss of keratocytes after PRK followed by keratocyte repopulation has been demonstrated in rabbit histologic studies. Primate and human histologic studies suggest an early increase in anterior stromal keratocytes that may persist for as long as 6 months postoperatively. However, histologic studies are invasive and cannot be used to study keratocyte density in vivo. An objective quantification of changes in keratocyte density after PRK may enhance our understanding of corneal wound healing.

The clinical confocal microscope provides a means of repeated noninvasive examination of the cornea at the cellular level. Confocal microscopy has been used to quantify keratocyte density in normal human and rabbit corneas in vivo, and these measurements have been validated by comparing cell density measured by confocal microscopy with density estimated from histologic sections of the same tissues. After PRK, corneal keratocyte density has previously been subjectively and quantitatively measured using confocal images. In the current study, sequential changes in keratocyte density and variation of density with depth in the central human cornea were measured for 3 years after PRK by using confocal microscopy.

METHODS

SUBJECTS

Twenty-four eyes of 14 subjects (3 men and 11 women) aged 22 to 53 years (mean ± SD age, 40 ± 7 years) were enrolled prospectively in a nonrandomized fashion from July through October 1998. All participants were patients of the Mayo Clinic, Rochester, Minn. The mean ± SD preoperative spherocylindrical refractive error was –3.73 ± 1.30 D (range, –1.25 to –5.75 D). All
subjects were white. None of the subjects had a history of an-
terior segment disease, ocular trauma or surgery, diabetes mellit-
tus, or the use of ocular medications. Systemic medications were
permitted, unless they were known to affect the cornea or the
anterior segment. Contact lens wear was discontinued within
2 weeks (soft lenses) or 3 weeks (rigid gas-permeable lenses)
of enrollment in the study. All eyes had normal anterior seg-
ments, intraocular pressures (≤22 mm Hg), and fundi. Our in-
stitutional review board approved this study, and all subjects
gave informed consent after the nature and possible conse-
quences of the study were explained to them.

PHOTOREFRACTIVE KERATECTOMY PROCEDURE
AND POSTOPERATIVE REGIMEN

Photorefractive keratectomy was performed using the VIXS
STAR laser (VIXS Inc, Santa Clara, Calif) with a wavelength of
193 nm, a fixed pulse rate of 6 to 8 Hz, and a radiant exposure
of 160 mJ/cm². The epithelium was removed by using the laser-
scrape technique (43 µm epithelial ablation followed by manual
scrape of the remaining epithelial cells with a blunt spatula).
The mean±SD planned ablation depth was 46±18 µm (range,
13-90 µm). Emmetropia was attempted in all cases. Immedi-
ately after ablation, the cornea was cooled for 30 seconds by
irrigation with cold balanced salt solution.

After the PRK procedure, patients wore a bandage soft con-
tact lens (SoFLens 66; Bausch & Lomb Inc, Rochester, NYC) un-
til the cornea epithelialized (2-3 days). Topical medica-
tions consisted of 0.3% preservative-free ketorolac tromethamine (Acular
PF; Allergan Inc, Irvine, Calif) for 4 doses over 2 days, 0.3%
ofloxacin (Ocuflox; Allergan Inc) 4 times daily until epitheli-
alization was complete, and 0.1% fluorometholone (FML; Al-
lergan Inc) 4 times daily with a taper over 3 months.

A bandage soft contact lens was already in place on all eyes at
the 1-day examination. To protect against possible corneal
abrasion, a bandage soft contact lens was placed for approxi-
mately 15 minutes on all eyes at the 5-day examination and on
4 eyes at the 1-month examination. A bandage contact lens was
not used thereafter.

CONFOCAL MICROSCOPY IN VIVO

Corneas were examined by using a tandem scanning confocal
microscope (Tandem Scanning, Reston, Va) before and at 1 day,
3 days, and 1, 3, 6, 12, 24, and 36 months after PRK. The method
of examination was described in an earlier article. 16 Briefly, 0.3%
proparacaine hydrochloride (Bausch & Lomb Pharmaceuticals,
Inc, Tampa, Fla) was instilled into the eye to be exam-
ined. One drop of 2.5% hydroxypropyl methylcellulose (CIBA
Vision Ophthalmics, Atlanta, Ga) optical coupling medium
was placed on the tip of the objective lens. The objective was aligned
to the visual axis of the eye and manually advanced until the
medium contacted the central cornea. The position of the ob-
jective was adjusted to provide an en face view of the central
cornea to confirm correct alignment. The patient self-fixed on
a bright target with the contralateral eye to minimize eye
movements.

A full-thickness scan, consisting of a series of confocal im-
ages, was recorded as the local plane was advanced at approxi-
ately 78 µm/s from anterior to the epithelium to the endo-
thelium. Digital images were stored on a computer workstation (Indy;
Silicon Graphics Inc, Mountain View, Calif). Each image repre-
sented a coronal section (x-axis×y-axis) of 475 µm×350 µm
and a depth of field (z-axis thickness) of 9 µm. Each image was
separated from the adjacent image by an average of 2.6 µm. 18 Im-
ages were acquired by either setting the camera in a fixed-gain
mode, with a constant gain, voltage, and black level, or in auto-
matic-gain mode, with these parameters automatically adjusted
by the camera throughout image acquisition. The cornea was scanned through its full thickness 4 to 8 times per visit.

THICKNESS MEASUREMENTS

An intensity profile of back-scattered light from selected con-
focal images was obtained as described previously. 16-18 Images
were acquired with the camera operating in its fixed-gain mode.
Intensity was estimated from the mean grayscale value in a
300×300 pixel area in the center of each image. Peaks in in-
tensity corresponded to the superficial epithelium, the sub-
basal nerve plexus, the most anterior keratocytes, and the en-
dotheium. The video image corresponding to each intensity
peak was displayed, and the first focused video image of each
corneal region was identified and used in the determination of
thickness. In profiles generated from corneas after PRK, a peak
that corresponded to anterior stromal haze was often present, as
confirmed by the presence of increased reflectivity of kerato-
cocytes in the corresponding video image.

Corneal thickness was the distance between the first fo-
cused image of the superficial epithelium and the endo-
thelium. Epithelial thickness was the distance between the first
focused image of the superficial epithelium and the sub-basal
nerve plexus. 18 When sub-basal nerves were not visible in im-
ages after PRK, we determined epithelial thickness as the dis-
tance between the first focused image of the superficial epithe-
lium and the first focused image of anterior keratocytes. The
Bowman layer was the distance between the first focused im-
age of the subbasal nerve plexus and the most anterior kera-
tocytes. Stromal thickness was the distance between the first
focused image of the most anterior keratocytes and the last fo-
cused image of the posterior keratocytes having a reflected light
intensity similar to other images of the stroma but without im-
ages of endothelial cells. We corrected depth measurements for
the nonlinear separation of video images by counting the num-
ber of images between the image of the objective surface and the
surface of the epithelium. 18

An estimate of the stromal thickness destined to be ab-
lated, or the actual photoablation depth, was obtained as the sur-
gically induced stromal thinning (Sthin) between the pre-PRK (Spre)
and 1 month post-PRK (Spost) measured stromal thickness:
Sthin=Spre−Spost.

The pre- and post-PRK stroma was subdivided by depth
into 5 layers: 0% to 10% (anterior), 11% to 33%, 34% to 66%
(middle), 67% to 90%, and 91% to 100% (posterior). In the post-
PRK cornea, the boundaries of the stromal layers were deter-
mined relative to the most anterior keratocyte layer. The post-
PRK stromal thickness was compared with 2 pre-PRK stromal
thicknesses: the pre-PRK full stroma and the pre-PRK future
unablated stroma (Figure 1). The pre-PRK full stroma in-
cluded the anterior stroma that would later be photoablated.
In this case, the boundaries of the pre-PRK stromal layers were
determined relative to the most anterior keratocytes immedi-
ately posterior to the Bowman layer. In the pre-PRK future un-
ablated stroma, the anterior stroma equal to the actual photo-
ablation depth (as measured at 1 month after PRK) was omitted
from the analysis. In this case, the boundaries of the pre-PRK
stromal layers were determined relative to the most anterior
keratocytes that would remain after the future PRK ablation.
This allowed direct comparison of the same tissue layers in the
pre- and post-PRK stroma.

KERATOCYTE DENSITY MEASUREMENT

Keratocyte density was measured from images of one confocal
scan of the central cornea with the camera in its automatic-
gain mode. All scans were reviewed, and the single scan with
the least anterior-posterior and lateral ocular movement was selected for analysis.

Two images with well-defined bright objects from each layer were selected for analysis. In the anterior 10% of the stroma, 1 of the 2 selected images was the most anterior countable image. The images were presented in random order to one observer (J.C.E.) who was masked to the subject and the examination visit. The same observer manually counted keratocyte nuclei (bright objects) in each of the 10 selected images per scan per examination by using an interactive computer program. The number of cells in a predetermined area was used to determine keratocyte density (cells/mm³). The mean cell density in each layer after PRK was compared with the mean cell density in (1) the corresponding layer of the pre-PRK full stroma and (2) the same tissue layer of the pre-PRK future unablated stroma.

DATA ANALYSIS

Groups were compared using a paired t test if data were distributed normally or the Wilcoxon signed-rank test if they were not. P values were Bonferroni-adjusted for multiple comparisons. P<.05 was considered statistically significant. The general estimating equation model was investigated to account for the potential correlation between 2 eyes of the same individual. In all cases, the conclusions were the same as the results of the standard statistical tests, so only the standard results are reported. In eyes requiring a reoperation, all data after the reoperation were excluded from analysis.

RESULTS

No complications were encountered during PRK or postoperatively. Five (21%) of 24 eyes underwent a reoperation. One eye required a reoperation at 7 months after PRK to treat an initial undercorrection, and 4 eyes required a reoperation at 13 months after PRK to treat myopic regression.

KERATOCYTE DENSITY

Central keratocyte density in the pre-PRK full stroma, the pre-PRK future unablated stroma, and the post-PRK stroma is shown in Table 1 and Figure 3.

Pre-PRK Full Stroma vs Post-PRK Stroma

In the pre-PRK full stroma, the full-thickness stroma was included in the analysis (Figure 1). Keratocyte density in the anterior 10% of the post-PRK stroma never returned to the high levels of the anterior 10% of the pre-PRK full stroma; density decreased by 30% (P<.001), 25% (P=.002), 41% (P<.001), 40% (P<.001), 43% (P<.001), and 45% (P<.001) at 1, 3, 6, 12, 24, and 36 months after PRK (Figure 4).

Pre-PRK Future Unablated Stroma vs Post-PRK Stroma

In the pre-PRK future unablated stroma, the anterior stroma equal to the depth of ablation, as measured at 1 month after PRK, was omitted from the analysis (Figure 1). At 5 days after PRK, keratocyte density in the most anterior keratocyte layer (17,462±3,710 cells/mm³) was significantly less than the cell density in the next deeper analyzed image at 5% to 10% stromal depth (23,299±3,352 cells/mm³; P=.01). No difference in cell density between

Figure 1. The 5 layers of the post–photorefractive keratectomy (PRK) stroma (B) were compared with the corresponding 5 layers of the pre-PRK full stroma (A; stromal thickness included the anterior stroma that would later be photoablated) and the same 5 layers of the pre-PRK future unablated stroma (C; direct comparison of the same pre- and post-PRK tissue layers was possible by omitting the thickness of the anterior stroma equivalent to the future depth of ablation).

Figure 2. Keratocyte density was determined by manually counting keratocyte nuclei (X’s) in selected confocal images using an interactive computer program. Objects overlapping the edges of the bounding box were counted on only 2 sides of the box (left and lower sides).
the most anterior keratocyte layer and the next deeper analyzed image (5%-10% stromal depth) was observed at 1 day after PRK (P = .64) or any time thereafter.

At 3 months after PRK, the full-thickness keratocyte density had maximally increased 20% when compared with the preoperative density (P < .001), the result of keratocyte density being 14% to 23% higher in all of the post-PRK stromal layers (P < .001; Table 1). By 6 months after PRK, the full-thickness keratocyte density had returned to preoperative levels (P > .99); the minimum detectable difference (α = .05; β = .20) was 1023 cells/mm² and remained unchanged at 36 months after PRK.

Keratocyte density in the anterior 10% of the post-PRK stroma decreased by 5% per year between 12 and 36 months after PRK (Table 1). At 36 months after PRK, keratocyte density in the anterior 10% of the stroma (17,720 ± 4,308 cells/mm²) was 15% less than the density in the same tissue layer of the pre-PRK future unablated stroma (20,930 ± 2,819 cells/mm²; P = .02) (Figure 5).

**KERATOCYTE MORPHOLOGY**

Before PRK, keratocyte nuclei appeared as bright oval or bean-shaped objects. Cellular processes were not evident. The preoperative anterior keratocytes were smaller, more numerous, and more tightly packed than the posterior keratocytes.10,24-26

At 1 day after PRK, keratocyte nuclei were the same in size, shape, and reflectivity when compared with quiescent keratocytes in the same tissue layer of the preoperated image (5%-10% stromal depth) was observed at 1 day after PRK (P = .64) or any time thereafter.

### Table 1. Central Keratocyte Density Before and After Photorefractive Keratectomy (PRK)*

<table>
<thead>
<tr>
<th>Keratocyte Density, Mean ± SD, cells/mm²</th>
<th>Full Thickness</th>
<th>Regional Densities</th>
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<tbody>
<tr>
<td><strong>Pre-PRK</strong></td>
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<tr>
<td>Full stroma (n = 24)</td>
<td>19,608 ± 2,236</td>
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<tr>
<td>Future unablated stroma (n = 24)</td>
<td>18,110 ± 1,947</td>
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<tr>
<td><strong>Post-PRK</strong></td>
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<tr>
<td>1 d (n = 24)</td>
<td>18,835 ± 3,207</td>
<td></td>
</tr>
<tr>
<td>5 d (n = 24)</td>
<td>18,718 ± 2,653</td>
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</tr>
<tr>
<td>1 mo (n = 24)</td>
<td>19,861 ± 2,447</td>
<td></td>
</tr>
<tr>
<td>3 mo (n = 24)</td>
<td>22,758 ± 3,275</td>
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</tr>
<tr>
<td>6 mo (n = 24)</td>
<td>18,612 ± 2,406</td>
<td></td>
</tr>
<tr>
<td>12 mo (n = 23)</td>
<td>17,304 ± 2,572</td>
<td></td>
</tr>
<tr>
<td>24 mo (n = 19)</td>
<td>17,686 ± 2,609</td>
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<tr>
<td>36 mo (n = 19)</td>
<td>17,372 ± 2,443</td>
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*Paired comparisons were performed with a t test. P values were Bonferroni-adjusted for 8 comparisons (adjusted P = unadjusted P × 8). Only P values for significant differences are reported.

†Data from eyes requiring a reoperation were excluded.

‡P < .01 vs pre-PRK future unablated stroma keratocyte density.

§P < .05 vs pre-PRK full stroma keratocyte density.

||P < .001 vs pre-PRK future unablated stroma keratocyte density.

Figure 3. Comparison of pre- and post–photorefractive keratectomy (PRK) keratocyte density; PRK photoablation removes anterior stroma (dotted lines) containing a high keratocyte population, and this high keratocyte density is not reconstituted in the post-PRK stroma.

Figure 4. Comparison of keratocyte density in the pre–photorefractive keratectomy (PRK) full stroma with density in the post-PRK stroma at 1, 2, and 3 years after PRK. The pre-PRK full stroma includes the anterior stroma that would later be photoablated. P values are for post-PRK vs pre-PRK keratocyte density. NS indicates not significant.
erative stroma. At 5 days after PRK, keratocytes in the anterior stroma showed bright nuclei and visible cell processes in some eyes. These highly visible cells have been interpreted to represent activated keratocytes or repair fibrocytes. The number of eyes with activated keratocytes peaked at 3 months after PRK. By 12 months, keratocyte morphology was similar to that before PRK (Table 2). When present, activated keratocytes appeared in the most anterior keratocyte layer and extended posteriorly to depths ranging from 10 to 57 µm (Table 2).

**COMMENT**

Keratocytes are distributed nonuniformly throughout the anterior-posterior stroma of the human cornea and density is highest in the most anterior stroma. The first part of the current study compared the keratocyte density in post-PRK stroma with cell density in pre-PRK full stroma (preoperative stromal thickness not adjusted for ablation depth; Figure 1). The data showed that the dense keratocyte population found in the preoperative anterior stroma was partially or completely removed during PRK photoablation, and this high keratocyte density was not reconstituted in the post-PRK anterior stroma. Specifically, keratocyte density in the anterior 10% of the post-PRK stroma decreased by 41%, 40%, 43%, and 45% at 6, 12, 24, and 36 months after PRK when compared with the anterior 10% of the pre-PRK full stroma. Consequently, the distribution of keratocytes after PRK also changed; keratocytes were now distributed uniformly, rather than nonuniformly, throughout the anterior-posterior stroma.

What a depleted anterior stromal keratocyte population after PRK means for the long-term health of the human cornea is unknown. There may be no need to replace keratocytes to reach the high density found in the preoperative anterior stroma. Specifically, keratocyte density in the posterior 90% of the post-PRK stroma did not differ significantly from that in the preoperative posterior stroma. Moreover, keratocyte density in the posteriormost keratocyte layer did not differ at any time point compared with the layer below the preoperative posterior stroma. However, keratocyte density in the most anterior keratocyte layer decreased by 41%, 40%, 43%, and 45% at 6, 12, 24, and 36 months after PRK when compared with the most anterior keratocyte layer in the preoperative anterior stroma. Consequently, the distribution of keratocytes after PRK also changed; keratocytes were now distributed uniformly, rather than nonuniformly, throughout the anterior-posterior stroma.

![Figure 5](image5.png)

**Figure 5.** Comparison of keratocyte density in the pre–photorefractive keratectomy (PRK) future unablated stroma to density in the post-PRK stroma at 1, 2, and 3 years after PRK. The pre-PRK future unablated stroma omitted the thickness of anterior stroma equivalent to the future depth of ablation (as measured at 1 month after PRK) from analysis to allow comparison of the same tissue layers. *P* values are for post-PRK vs pre-PRK keratocyte density. NS indicates not significant.

![Figure 6](image6.png)

**Figure 6.** Confocal images of the most anterior keratocyte layer of the same eye after photorefractive keratectomy (PRK). At 1 day (A), keratocytes were similar in appearance to quiescent keratocytes before PRK. At 1 month (B), keratocytes had bright nuclei and visible cell processes (activated keratocytes). At 1 year (C), keratocytes were similar in appearance to those before PRK.

Apoptotic keratocyte loss immediately after PRK or epithelial scrape injury resulting in an acellular region of anterior stroma has been well documented in rabbit studies. Additionally, reduced keratocyte density in the anterior stroma has been measured in patients after laser-assisted in situ keratomileusis (LASIK) and in patients with keratoconus who wear contact lenses. Apoptotic keratocyte loss immediately after PRK or epithelial scrape injury resulting in an acellular region of anterior stroma has been well documented in rabbit studies. Similarly, Balestrazzi et al measured a 5- to 8-µm region of acellular anterior stroma at 3 months after PRK in a human histologic study. Recently, Ambrosio et al confirmed that human keratocytes undergo apoptosis in response to epithelial debridement in a manner similar to rabbits. The tandem scanning confocal microscope is not capable of detecting a completely acel-
stroma. Our data confirm previous primate9-11 and hu-
sity in the same tissue layers of the pre- and post-PRK
destined to be photoablated was omitted from the analy-
ture unablated stroma, the thickness of the anterior stroma
pre-PRK future unablated stroma. In the pre-PRK fu-
PRK stroma was compared with keratocyte density in the
part of the current study, keratocyte density in the post-
cytes is repopulated within a few days postoperatively.33

In the highly reactive rabbit, the volume void of kerato-
sus layer of the preoperative stroma. A new observation
in anterior kerocyte density was statistically signifi-
cant at 36 months after PRK. By contrast, keratocyte den-
sity in the middle and posterior stroma remained un-
changed between 1 and 3 years after PRK. Fini16 hypothesized that this decline in keratocyte density af-
PRK represents apoptotic cell loss. Alternatively, Ve-
saluoma et al37 suggested that denervation may play a role
in the diminished density of kerocytes. Mitooka et al39
noted a similarity between reinervation of the cornea
and recovery of keratocyte density after LASIK. After PRK,
however, Erie et al38 found no association between cor-
neal reinnervation and keratocyte density.

The current study provides no confocal micros-
copy morphologic evidence of activated keratocytes or
repair fibrocytes at 1 day after PRK. Activated kerato-
cytes were first identified at 5 days after PRK in some eyes
and, similar to previous human studies,3,4,13-15,21,34 were
identified up to 6 months after PRK. By contrast, acti-
vated keratocytes are present for less than 1 month after
LASIK when viewed by confocal microscopy.29

Confocal microscopy estimates of keratocyte den-
sity early after PRK have limitations. First, stromal edema
at 1 and 5 days after PRK could falsely decrease kerato-
cyte density. However, Muller et al39 showed that the stro-
mal interweave architecture of the anterior stroma is re-
sistant to swelling, which is confined mainly to the
posterior stroma. Therefore, any differences in cell den-
sity between the most anterior confocal image and the
next deeper image (5%-10% stromal depth) because of
differential swelling should be minimal. Stromal swell-
ing, however, would decrease the measured cell density
in the middle and posterior layers and should be con-
"considered when interpreting cell densities early after PRK.
Also, inflammatory cells have been identified in histo-
logic studies during the first week after PRK in the rab-
bit1 and primate cornea. Some of the cell nuclei that were
counted during the first week after PRK may have been
clusters of inflammatory cells, and this would artifi-
cially increase the measured keratocyte density.

In summary, we used a noninvasive method to quan-
tify long-term changes in keratocyte density in the hu-
man cornea after PRK. The clinical importance of the mea-
sured reduction in anterior stromal keratocyte density
after PRK is unknown and may be appreciated only af-
ter longer follow-up.

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ceived October 25, 2001; accepted February 12, 2003.

<table>
<thead>
<tr>
<th>Table 2. Number of Eyes With Activated Keratocytes and Their Anterior Stromal Depth After Photorefractive Keratectomy (PRK)</th>
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<tr>
<td>Measurement</td>
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<td>No. (%) of eyes with activated keratocytes</td>
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<td>Stromal depth,† mean ± SD, µm</td>
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</table>

*Data from eyes requiring a reoperation were excluded from analysis.
†Posterior extension from the subbasal nerve plexus or the first anterior keratocytes.

Abbreviation: NA, not applicable.
REFERENCES


