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Full-field optical coherence microscopy for histology-like of stromal features in corneal grafts

--Manuscript Draft--

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Alisha DSouza, Ph.D.
Review Editor
JoVE

Dear Dr. DSouza:

Please find enclosed the revised manuscript entitled "Full-field optical coherence microscopy for histology-like analysis of stromal features in corneal grafts," authored by myself, Kate Grieve, Marie Borderie, Djida Ghoubay, Cristina Georgeon, and Vincent Borderie.

Two versions are attached, one with all changes tracked and another one with all changes accepted.

Each of the editorial and peer reviewer comments and recommendations has been fully considered in the revised manuscript. Please see detailed responses (in red) to editorial and reviewer comments in the attached file "Responses to editorial and reviewer comments."

We respectfully re-submit this manuscript for consideration of publication in your journal.

Thank you for your time and consideration.

Sincerely,

Kristina Irsch, Ph.D.

1 **TITLE:**
2 Full-field Optical Coherence Microscopy for Histology-like Analysis of Stromal Features in Corneal
3 Grafts
4
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28 **KEYWORDS:**
29 Imaging, cornea, keratoconus, optical coherence tomography, microscopy, donor tissue,
30 keratocyte, stroma, corneal transplantation, corneal storage
31
32 **SHORT ABSTRACT:**
33 We describe use of full-field optical coherence microscopy as a method for high quality
34 assessment of corneal donor stroma. This protocol can be used to identify features indicative of
35 health or disease, and is aimed at improving the screening and selection of donor tissues, and
36 hence the results of keratoplasty.
37
38 **LONG ABSTRACT:**
39 The quality of donor corneal stroma, which makes up about 90% of total corneal thickness, is
40 likely to be one of the main, if not the major, limiting factor(s) for success of deep anterior
41 lamellar and penetrating keratoplasty. These are surgical procedures that involve replacing part
42 or all of the diseased corneal layers, respectively, by donated tissue, the graft, taken from a
43 recently deceased individual. However, means to evaluate stromal quality of corneal grafts in eye
44 banks are limited and lack the capability of high-resolution quantitative assessment of disease

45 indicators. Full-field optical coherence microscopy (FF-OCM), permitting high-resolution 3D
46 imaging of fresh or fixed *ex vivo* biological tissue samples, is a non-invasive technique well suited
47 for donor cornea assessment. Here we describe a method for the qualitative and quantitative
48 analysis of corneal stroma using FF-OCM. The protocol has been successfully applied to normal
49 donor corneas and pathological corneal buttons, and can be used to identify healthy and
50 pathologic features on both the macroscopic and microscopic level, thereby facilitating the
51 detection of stromal disorders that could compromise the outcome of keratoplasty. By improving
52 the graft quality control, this protocol has the potential to result in better selection (and
53 rejection) of donor tissues and hence decreased graft failure.
54
55 **INTRODUCTION:**
56 Corneal diseases are amongst the major causes of blindness worldwide[1]. Some diseases can only
57 be treated surgically, often involving the replacement of part (*i.e.*, lamellar keratoplasty) or the
58 entire (*i.e.*, penetrating keratoplasty) diseased cornea, by donated tissue, the graft, taken from a
59 recently deceased individual. For corneal diseases that do not affect the endothelium (*e.g.*,
60 keratoconus, stromal scars after infectious keratitis, trauma, and stromal dystrophies), deep
61 anterior lamellar keratoplasty (DALK) is currently considered as the surgical technique of choice[2-
62 5]. This technique makes possible the preservation of the recipient's corneal endothelium, by
63 replacing only the central corneal epithelium and stroma, which is associated with a lower
64 incidence of graft rejection, absence of endothelial rejection, lower endothelial cell loss, and
65 favorable cost-effectiveness ratio[6-11]. DALK further allows corneas with less than optimal
66 endothelium quality to be used as grafts, as this compromised layer will not be transplanted[12].
67 Conversely, quality of donor corneal stroma is likely to be the major limiting factor for graft
68 success and vision recovery because the stroma is the only donor corneal layer to remain, while
69 the donor epithelium will be replaced by recipient epithelium. Unfortunately, means to assess
70 donor corneal stroma in eye banks are limited. They usually include slit-lamp examination of the
71 donor eyeball when tissue retrieval is made by enucleation and light microscope examination of
72 the donor stroma[13]. Some eye banks have started supplementing such standard procedures using
73 Fourier-domain optical coherence tomography (FD-OCT)[14].
74
75 Ophthalmic optical coherence tomography (OCT), an optical analogue to ultrasound imaging[15],
76 uses interference of broadband or tunable light to generate optical sections of the retina[16] and
77 anterior segment[17]. In time-domain OCT, the basis of early clinical systems, the position of a
78 reference mirror is altered, so that interference patterns show up whenever the reference beam
79 has traveled almost the same amount of time as the beam reflected at the various tissue
80 interfaces, with A-scans being generated as a function of time. In FD-OCT (also called spectral- or
81 frequency-domain OCT), the basis of most modern clinical systems, the reference mirror is fixed
82 at one position and an individual A-scan, with all interference patterns mixed together, is
83 acquired at a time, and dissected apart via Fourier analysis.
84
85 While clinical (time or spectral-domain) OCT systems permit cross-sectional views of the cornea
86 and the detection of stromal opacities at a higher axial resolution than slit-lamp biomicroscopy,
87 their lateral resolution is limited. Confocal microscopy[18] allows examination of the cornea at a
88 lateral resolution approaching histological detail, but is limited axially.

89

90 Full-field optical coherence tomographic microscopy (FF-OCT or FF-OCM)[19,20] combines elements
91 of both confocal microscopy and OCT, achieving a lateral resolution comparable to the axial
92 resolution of about 1 μm. More specifically, FF-OCM uses incoherent broadband light sources
93 (*e.g.*, a halogen lamp) and high numerical aperture optics to acquire *en face* 2D tomographic
94 images without lateral scanning. By scanning in the depth direction, FF-OCM enables non-
95 invasive 3D imaging of fresh or fixed *ex vivo* biological tissue samples. It has been used to image
96 the cornea[21-23]. By providing both high-resolution *en face* and cross-sectional views, FF-OCM
97 provides information on both the histological structure and cellular details of the cornea. In fact,
98 FF-OCM has been shown to provide structural information superior to histology and was able to
99 identify more disease indicators as was possible with the combination of spectral-domain OCT
100 and confocal microscopy[24,25].

101

102 Here we describe a protocol for the qualitative and quantitative assessment of corneal donor
103 stroma using FF-OCM. The method is based on the histology-like analysis of macroscopic and
104 microscopic features indicative of stromal condition, including three quantitative stromal
105 parameters (*i.e.*, Bowman's layer thickness and its variability, and stromal reflectivity). The
106 described protocol is therefore applied to normal and abnormal cornea tissues and permits
107 differentiation of diseased from normal human corneal tissues.

108

109 **PROTOCOL:**
110 All methods described here were carried out according to the tenets of the Declaration of Helsinki
111 and followed international ethical requirements for human tissues. This was a prospective
112 observational case control study. Informed consent was obtained from patients. No
113 modifications to French standards of treatment or follow-up were made. Institutional Review
114 Board (IRB) approval was obtained from the Patient Protection Committee, Ile-de-France V
115 (14944).

116

117 **1. Tissue Selection and Preparation**

118

119 **1.1. Select donor corneas.**

120

121 1.1.1. Transfer donor corneas stored in organ culture medium[26] to dextran-supplemented organ
122 culture medium for 3 days to allow deturgescence[27] prior to FF-OCM imaging[28] (see **Table of**
123 **Materials**).

124

125 **1.2. Prepare the samples.**

126

127 1.2.1. Position the cornea, immersed in storage medium, in the sample holder with the
128 epithelium facing up.

129

130 1.2.2. Place a clean silica coverslip (provided with the sample holder) on top of the cornea and
131 close the holder by gently turning its base until the sample is slightly flattened and immobilized
132 under the coverslip providing a relatively even imaging surface. Take precautions to avoid any air

bubbles.

1.2.3. Apply a thick layer of ophthalmic or optical gel on the coverslip as the immersion medium.

2. FF-OCM Initialization, Setup, and Image Acquisition

2.1. Initialize the device.

2.1.1. Turn on the device by operating the power switch on the back of the device; illumination of a green LED on the front of the device indicates that the power is on.

2.1.2. Turn on the dedicated computer and halogen light source by operating the power switch on the front.

2.1.3. Launch the acquisition software (see **Table of Materials**) by double-clicking on the desktop shortcut.

2.1.4. Ensure that the imaging stage is clear except for the movable tray. Then click "OK" to initialize the motors at the prompt.

2.1.5. Pull out the tray and insert the sample holder in the dedicated container, then gently push back the tray.

2.2. Setup the device.

2.2.1. Enter a "sample identifier" in the designated and mandatory field; optionally enter a "sample description" and/or "study description".

2.2.2. Click "Acquire macro image" when ready, to create a snapshot of the sample that can be used for lateral positioning and navigation purposes later; once satisfied, validate the image at the prompt by clicking "Yes", after which the device moves the sample tray underneath the objective and performs an auto-adjustment.

2.2.3. Ensure that the microscope objective is well immersed in the optical gel before proceeding further.

2.3. Acquire the stacks.

2.3.1. Select the "Explore" tab to prepare an acquisition.

2.3.1.1. Before acquiring a stack of images, navigate to the center of the cornea, via translation of the joystick or manual selection on the screen (that is, by clicking and dragging the red square on the acquired macro image to the desired location).

177 2.3.1.2. Vary the imaging depth via rotation of the joystick, adjustment of the slider, or manual
178 keyboard input in the graphical user interface (GUI), and adjust the averaging value (an averaging
179 of 40 is generally recommended for optimal corneal imaging).
180
181 Note: This is done to determine the thickness of the corneal sample and the averaging necessary
182 to image through the entire corneal thickness while noting the first and last image location in
183 depth. The bottom surface of the coverslip creates parallel interference fringes that are seen on
184 the tomographic image (on the right-hand side of the GUI), which facilitate locating the corneal
185 surface.
186
187 2.3.1.3. Enter the corneal surface value or first image location in depth into the "Depth" field.
188
189 **2.3.2. Select the "Acquire tab", to acquire images.**
190
191 2.3.2.1. Select the "Slice spacing" (the default and recommended setting is 1 μm, matching the
192 axial resolution of the device) and enter the corneal thickness value accordingly, under "Number
193 of slices".
194
195 2.3.2.2. Review parameters and acquisition time, and when satisfied press "OK" to launch the
196 acquisition.
197
198 2.3.2.3. During the acquisition, avoid any contact with the table on which the FF-OCM is
199 positioned.
200
201 **3. Management of Acquired Images**
202
203 **3.1. View and export images.**
204
205 3.1.1. Launch the FF-OCM viewing software (see **Table of Materials**) by double-clicking on the
206 desktop shortcut; acquired images (identified by the sample ID) appear in the "Local Studies" list.
207
208 3.1.2. Select the study with corresponding sample ID, which contains both the macro image and
209 acquired image stack, and "export" the latter by right-clicking on the series of images and
210 selecting the "DICOM" format to retain the raw pixel data and metadata for further analysis.
211
212 3.1.3. Display the 3D image stacks in *en face* and cross-sectional views using the multiplanar
213 reconstruction (MPR) mode, by double-clicking on the series of images; navigate through the
214 images (*e.g.*, via mouse-wheel scroll or slider adjustment) and select representative *en face* and
215 cross-sectional views of each stack.
216
217 3.1.4. Export the selected views by clicking on the icon at the bottom right of the window, using
218 the "DICOM" format.
219
220 **3.2. Import images.**

221

222 3.2.1. Open the image processing software (see **Table of Materials**) by double-clicking on the
223 desktop shortcut, and navigate to the "Bio-Formats" "Importer" under "Plugins" to import
224 DICOM images; make sure that "Group files with similar names" is selected in the "Bio-Formats
225 Import Options" window.

226

227 **4. Image Analysis: Qualitatively and Quantitatively Assessment of Stromal Morphology and**
228 **Features**

229

230 **4.1. Assess stromal and Bowman's layer thickness.**

231

232 4.1.1. Measure distances manually on corneal cross-sections, for example at five equally spaced
233 points across the cross-section[25].

234

235 4.1.1.1. Draw a line between two points of known distance (such as, from left to right of the
236 entire image, that is according to the default field-of-view, 1,024 pixels or 780 μm), go to
237 "Analyze" and select "Set Scale", and enter the "Known distance" and "Unit of length" in the
238 appropriate fields and click "OK".

239

240 4.1.1.2. Draw a line between two points of unknown distance; read the length or distance
241 measured directly from the status bar.

242

243 4.1.2. Record the mean and coefficient of variation (COV). Bowman's layer thickness less than
244 6.5 μm and a COV greater than 18.6% have been associated with abnormal corneal stroma[25].

245

246 **4.2. Determine the keratocyte density.**

247

248 4.2.1. Following convention of confocal microscopy: sum stromal *en face* images in groups of 7
249 to produce slices of comparable thickness. For this, go to the "Image" tab and select "Reslice Z"
250 under "Stacks".

251

252 4.2.2. Take into account the differing (decreasing) cell density on progression through the
253 stroma[24,25]. For this, the stroma may be considered as being composed of 4 regions according to
254 depth: (1) the very anterior stroma below Bowman's layer, that is 2% of entire stromal thickness;
255 the remaining stroma (that is, 98% of entire stromal thickness), with three zones of identical
256 thickness: (2) anterior stroma, (3) mid stroma, and (4) posterior stroma.

257

258 4.2.3. For further analysis, include all available *en face* slices for the very anterior stroma, 15
259 images for the anterior stroma, 5 images for the mid stroma, as well as 5 images for the posterior
260 stroma (where the number of images per region required for a reliable count was determined by
261 stepwise analysis[29]).

262

263 4.2.4. On each *en face* image, select a 300 μm x 300 μm region of interest. To enhance nucleus
264 visualization, invert the image, using "Invert" under the "Edit" tab, and adjust contrast and

265 <mark>brightness. For the latter, go to "Image" and navigate to "Brightness/Contrast" under "Analyze".</mark>
266

267 <mark>4.2.5. Manually count cell nuclei, using for example the "cell counter" function[25]. For this, go to</mark>
268 <mark>"Plugins" and navigate to "Cell Counter" under "Analyze". Select a counter type and then press</mark>
269 <mark>"Initialize". Then start counting the cell nuclei by clicking on dark oval features in the inverted</mark>
270 <mark>image, considering those landing on an image border only for two of the four sides of the image[25].</mark>
271

272 4.2.6. Record the cell density in terms of area density, that is in cells/mm² (multiply the number
273 of cells counted by 0.09, to convert from cells/90,000 µm² to cells/mm²), following confocal
274 microscopy convention where keratocyte densities have been shown to be lower in patients (*e.g.*,
275 with keratoconus) than in healthy subjects and to be correlated with disease severity[25].
276

277 Note: Further clinical studies are necessary to determine whether a minimal threshold of
278 keratocyte density is required to obtain a perfectly clear corneal graft after transplantation.
279

280 **4.3. Assess the stromal reflectivity.**
281

282 4.3.1. Generate mean intensity depth profiles of stromal image stacks, using for example the "Z-
283 axis profile" function and/or custom software[30,25].
284

285 4.3.1.1. Calculate the mean grey level for each *en face* stack.
286

287 4.3.1.2. Subtract the minimum grey value and normalize by the maximum grey value.
288

289 4.3.1.3. Display in log scale as a function of stromal depth (% of the stromal thickness).
290

291 4.3.1.4. Approximate the resulting logarithmic profile by a linear regression line, which minimizes
292 the sum of the squared residuals (least-squares fit).
293

294 4.3.1.5. Record the R-square value (R^2) as a measure of linearity. Values below 0.94 could be an
295 indication of corneal pathology[25].
296

297 **4.4. Assess the visibility of other stromal features and disease indicators.**
298

299 4.4.1. Check for the presence of scars, fibrotic tissue, lakes, or Vogt striae.
300

301 4.4.2. Assess mean thickness of nerves in the stroma, if sufficiently visible for measurement[24].
302

303 4.4.2.1. Select one *en face* image where the stromal nerve is most visible (generally in the mid-
304 stromal region).
305

306 4.4.2.2. Measure the thickness of the nerve, for example at five points[24], then calculate the mean
307 and standard deviation. Nerve thicknesses above 9 µm may be an additional indicator for
308 pathology, such as keratoconus[24].

309
310   **REPRESENTATIVE RESULTS:**
311   The FF-OCM device (see **Table of Materials**)[28] and general setup used in this manuscript is shown
312   in **Figure 1**. **Figure 2** shows a swelled human donor cornea after storage in organ culture medium,
313   giving a pathophysiological model of edematous cornea and preventing FF-OCM image
314   acquisition through the entire corneal thickness due to limited penetration depth. Transfer to
315   dextran-supplemented organ culture medium causes deswelling and results in donor corneas of
316   normal thickness, as represented in **Figure 3**. Diseased corneas can be recognized by
317   morphological changes and typical stromal features, including a decreased and variable thickness
318   of the stroma (**Figure 4**) and/or Bowman's layer (**Figure 5**). Assessment of the keratocyte density
319   (**Figure 6**) and stromal reflectivity (**Figure 7**) may further aid in the histology-like analysis and
320   differentiation of normal from pathologic corneal tissues with FF-OCM, beyond the capabilities
321   of clinical OCT systems (**Figure 8**).
322
323   **FIGURE LEGENDS:**
324
325   **Figure 1: Schematic and photographs of the general setup and FF-OCM device used in this work**.
326   (**A**) Photograph of the FF-OCM device (see **Table of Materials**)[28]. (**B**) Schematic and optical setup
327   of the device. (**C**) Photograph of a human donor cornea in the sample holder. (**D**) Zoom on the
328   immersion objective and the sample holder.
329
330   **Figure 2: Organ-cultured normal human cornea before deturgescence**. Cross-sectional (**A**) and
331   *en face* view (**B**, slice through the stroma) of swelled ("edematous") cornea caused by storage in
332   organ culture medium, where lakes can be seen as dark areas (as indicated by arrows). Corneal
333   thickness has doubled to over 1,100 µm, preventing image acquisition through the entire depth.
334
335   **Figure 3: Organ-cultured normal human cornea after deturgescence**. Cross-sectional view
336   through the entire cornea (**A**) and *en face* stromal view (**B**) of de-swelled cornea immersed in
337   dextran-supplemented medium, showing regularly distributed hyper-reflective (white)
338   keratocytes.
339
340   **Figure 4: Overall assessment of stromal morphology and features, including stromal thickness.**
341   Compared with normal human cornea (**A**), corneas with keratoconus (**B**) feature decreased and
342   variable stromal thickness, along with numerous, parallel Vogt striae that are observable as dark
343   vertical bands in cross-sectional views, and thick stromal nerves that may be found in mid-
344   stromal *en face* slices (**C**).
345
346   **Figure 5: Assessment of Bowman's layer thickness, which is delineated anteriorly by the hyper-
347   reflective epithelial basement membrane and posteriorly by the hyper-reflective keratocytes
348   in the very anterior stroma**. Compared with a normal human cornea (**A**), pathologic cornea (*e.g.*,
349   keratoconus) (**B**) features decreased and variable Bowman's layer thickness due to interruption
350   and scarring.
351
352   **Figure 6: Assessment of keratocyte density.** (**A**) Keratocyte nuclei are manually counted on

353   enhanced and inverted *en face* images after selection of a 300 µm x 300 µm region of interest.
354   (**B**) Counted nuclei are indicated by arrows.

355

356   **Figure 7: Assessment of stromal reflectivity.** The intensity of backscattered light decreases
357   (exponentially) with depth in the stroma of normal donor corneas (see **Figure 3** and **Figure 4A**),
358   resulting in linear logarithmic depth profiles, represented by R-square values close to 1 (green
359   trace). This may not hold true for pathological corneas featuring stromal regions of increased
360   light backscatter (see **Figure 4B**). The presence of such macroscopic features, as in the example
361   of a cornea with stromal scar after infectious keratitis (depicted in the inset), will thus create non-
362   linear logarithmic intensity depth profiles, represented by an R-square value lower than 0.94[25]
363   (red trace).

364

365   **Figure 8: Demonstration of benefits of FF-OCM over histology and spectral-domain OCT.** (**A**)
366   Histology and (**B**) corresponding spectral-domain-OCT image acquired on the same cornea *in vivo*
367   prior to keratoplasty. (**C**) Corresponding FF-OCM cross section of the *ex vivo* corneal button post-
368   keratoplasty, illustrating superior resolution compared to the clinical spectral-domain OCT
369   images. The fibrosis is also more clearly visible in FF-OCM images than in the histology. The high
370   density of keratocytes in the upper stroma can clearly be seen in both the (**C**) cross-sectional and
371   (**D**) *en face* views.

372

373   **DISCUSSION:**
374   The protocol described here for the qualitative and quantitative assessment of corneal donor
375   stroma using FF-OCM is based on the histology-like analysis of macroscopic and microscopic
376   features indicative of stromal condition, beyond the capabilities of spectral-domain OCT and
377   confocal microscopy[21,24,25], and enables differentiation of diseased from normal human tissues.

378

379   Aside from an excellent endothelial quality assessment of human donor corneas by means of
380   specular microscopy, assessment of stromal quality is challenging in eye banks, and generally
381   limited to a gross observation with slit-lamp biomicroscopy and/or light microscopy in current
382   protocols. Lack of fine resolution with existing methods not only means that corneas with some
383   stromal disease may be selected that compromise the result of keratoplasty, but also that
384   corneas may be rejected for stromal opacities that are in fact constraint to anterior stroma or
385   epithelial regions and could still be used for endothelial keratoplasty procedures[14].

386

387   The current eye-bank protocol could be supplemented by the addition of FF-OCM, which due to
388   its superior resolution, constitutes a powerful and non-invasive tool to complete the quality
389   assessment of the cornea, especially the stroma (including Bowman's layer). Unlike during slit-
390   lamp examination, the graft remains immersed in a closed chamber filled with storage medium
391   throughout FF-OCM image acquisition, decreasing any potential risk of contamination.

392

393   For successful image acquisition with FF-OCM (see **Table of Materials**), it is important for the
394   microscope objective to be well immersed in the optical gel that is applied on top of the coverslip
395   of the sample holder (step 2.2.3). It is further recommended to regularly check the calibration of
396   the device, a procedure also to be performed after unsuccessful auto-adjustment (step 2.2.2) and

accessed via "Tools and options" in the acquisition software (see **Table of Materials**). The procedure, which involves the utilization of a calibration mirror in the sample holder, is the same as the usual sample preparation (see step 1.2) except that the optical gel should be applied on the mirror before positioning of the coverslip.

A series of donor corneal grafts, considered to have normal stroma as per existing eye-bank procedures, were used to describe the protocol in this manuscript and specifically demonstrate the suitability of FF-OCM for precise and reliable assessment of donor stromal quality. These normal donor corneas were compared with pathological corneas immersed in storage medium, showing that the histology-like analysis made possible with FF-OCM of several stromal features (illustrated in **Figures 2–8**) in corneal grafts allows distinguishing diseased from normal human corneal tissues.

Aside from morphological changes, such as the presence of scars (**Figure 5** and **Figure 7**), fibrotic tissue (**Figure 8**), lakes (**Figure 2**), Vogt striae (**Figure 4**), or increased stromal nerve diameter (**Figure 4**), typical stromal features are present in diseased corneas. Stromal parameters particularly relevant in the stromal quality assessment appear to be Bowman's layer thickness and its variability, and stromal reflectivity. Critical steps within the protocol are thus steps 4.1 and 4.3.

While being secreted during human corneal development, Bowman's layer, in particular, becomes distinct by 19 weeks of gestation and never repairs after birth[31]. Damage to Bowman's layer is thus irreversible and serves as an ideal indicator of previous stromal damage in donor corneal tissue, including damage caused by refractive surgery, infectious keratitis, keratoconus. Such corneal diseases, which constitute contraindications for donor corneal usage, are associated with decreased and variable Bowman's layer thickness due to interruption and scarring (**Figure 5**), and are likely to be missed by current eye-bank protocols when the donor history is not precisely known.

Although corneal transparency is impaired after donor death due to *post mortem* corneal edema, the intensity of backscattered light, or stromal reflectivity is expected to decrease exponentially with depth in the stroma (see **Figure 3** and **Figure 4A**); as a result, the logarithm of normalized stromal reflectivity will be a linear function of stromal depth in normal donor corneas, represented by R-square values close to 1. Conversely, the presence of macroscopic features is associated with non-linear logarithmic intensity depth profiles and indicative of stromal disease (**Figure 4B** and **Figure 7**)[25].

Since keratocyte density is responsible for stromal collagen fibril and extracellular matrix synthesis and renewal, it appears reasonable to assume that keratocyte density is another relevant parameter for assessing donor stromal quality, and that tissues exhibiting very low keratocyte counts should not be transplanted. The protocol therefore includes a precise and reliable method to measure keratocyte density from FF-OCM images that can be easily used in eye banks[25] and follows the convention of confocal microscopy. Note that with FF-OCM, keratocyte density may also be determined by counting keratocytes directly in the cross-sectional

441 view[32], a potential advantage over confocal microscopy, which requires keratocytes to be
442 counted on multiple *en face* slices. However, unlike in living patients, where keratocyte densities
443 have been demonstrated to be lower in disease patients than in normal controls[33-36] and to
444 correlate with disease severity[33,37], this was not the case in human *ex vivo* tissue samples[25], and
445 further studies are necessary to determine whether a minimal number of keratocytes is required
446 in donor corneas to result in good visual recovery after transplantation. Low keratocyte density
447 in donor tissue as in pathological tissue could be explained by aging, *post mortem* loss of cells
448 induced by ischemia, and/or storage of donor tissue[27,38-40]. It should also be pointed out that the
449 normal donor corneas that were obtained and imaged in this protocol were either stored and
450 edematous or de-swelled, or had been discarded by the eye bank before transplantation because
451 of poor endothelial quality according to the standards of the EU Eye Bank Association. Were FF-
452 OCM imaging along with the described protocol to be included in the eye bank setting, the
453 corneas would typically be assessed in a fresher state than was possible here, which may affect
454 the keratocyte densities.

456 The protocol described here for the stromal quality analysis could be extended for assessment of
457 Descemet's membrane, which can also be resolved with FF-OCM in terms of thickness and
458 structure[21,24]. This may prove useful for selection of tissues for Descemet's membrane
459 endothelial keratoplasty, where thin Descemet's membranes may be more difficult to separate
460 from the stroma.

462 In conclusion, FF-OCM enables precise and reliable assessment of human donor corneal stroma
463 during storage. By improving the graft quality, the addition of this protocol to current eye-
464 banking procedures has the potential to improve the screening and selecting of donor tissues,
465 and hence the results of keratoplasty. Real-life integration of the FF-OCM device into the eye-
466 bank routine should be facilitated by recent technological updates, including faster image
467 acquisition and larger field of view thanks to the development of a custom CMOS camera, and
468 the design of custom sterile disposable cassettes for cornea storage and handling during imaging.

477 **DISCLOSURES:**
478 The authors have nothing to disclose.

480 **REFERENCES:**
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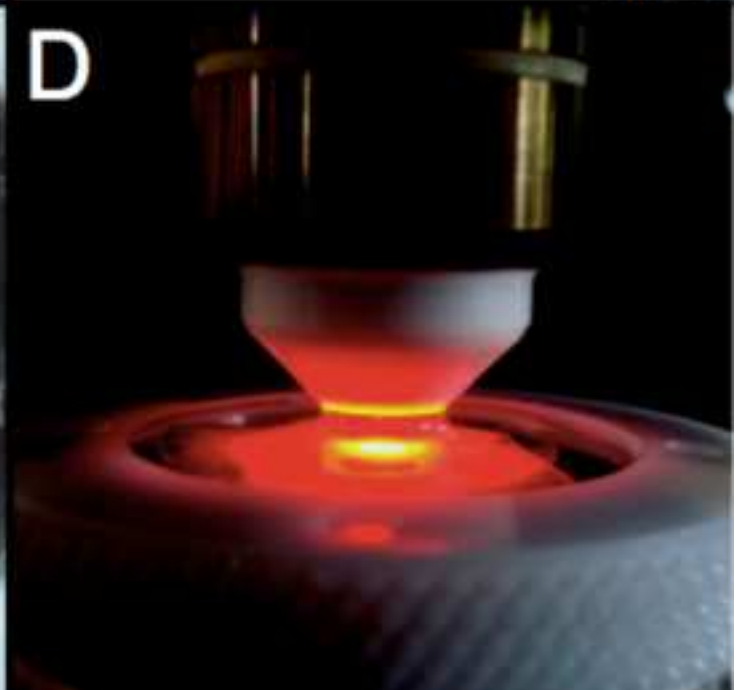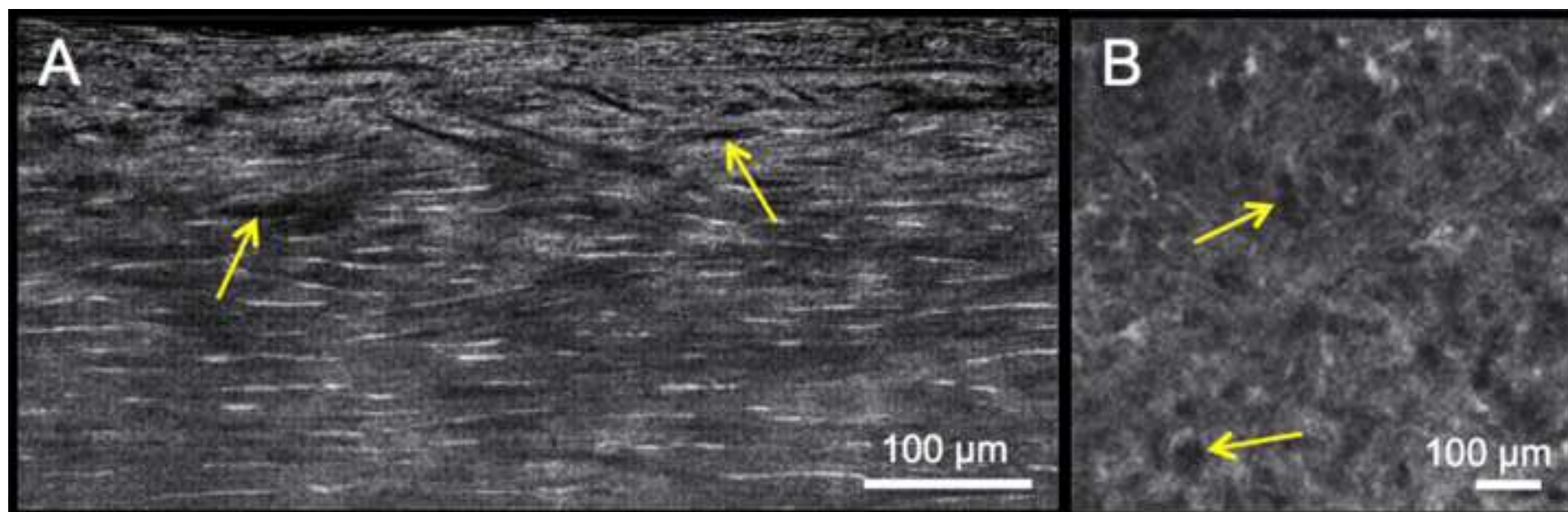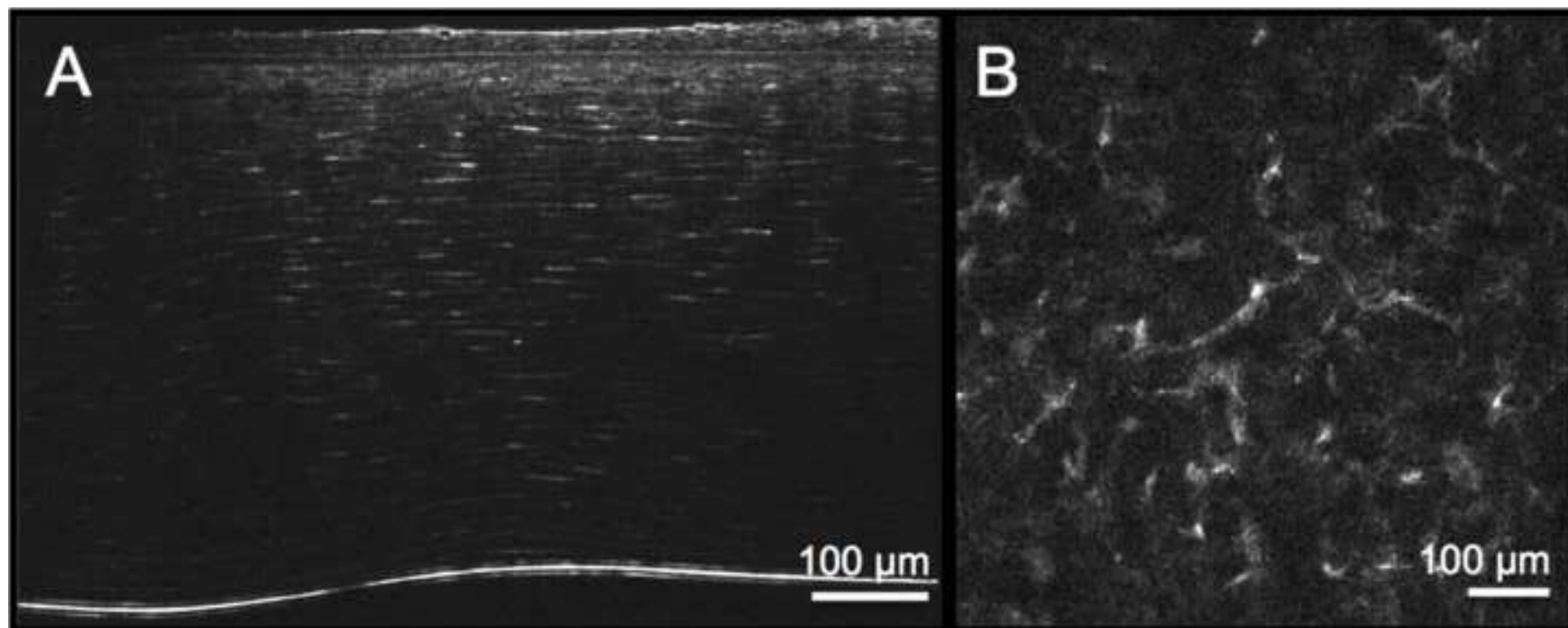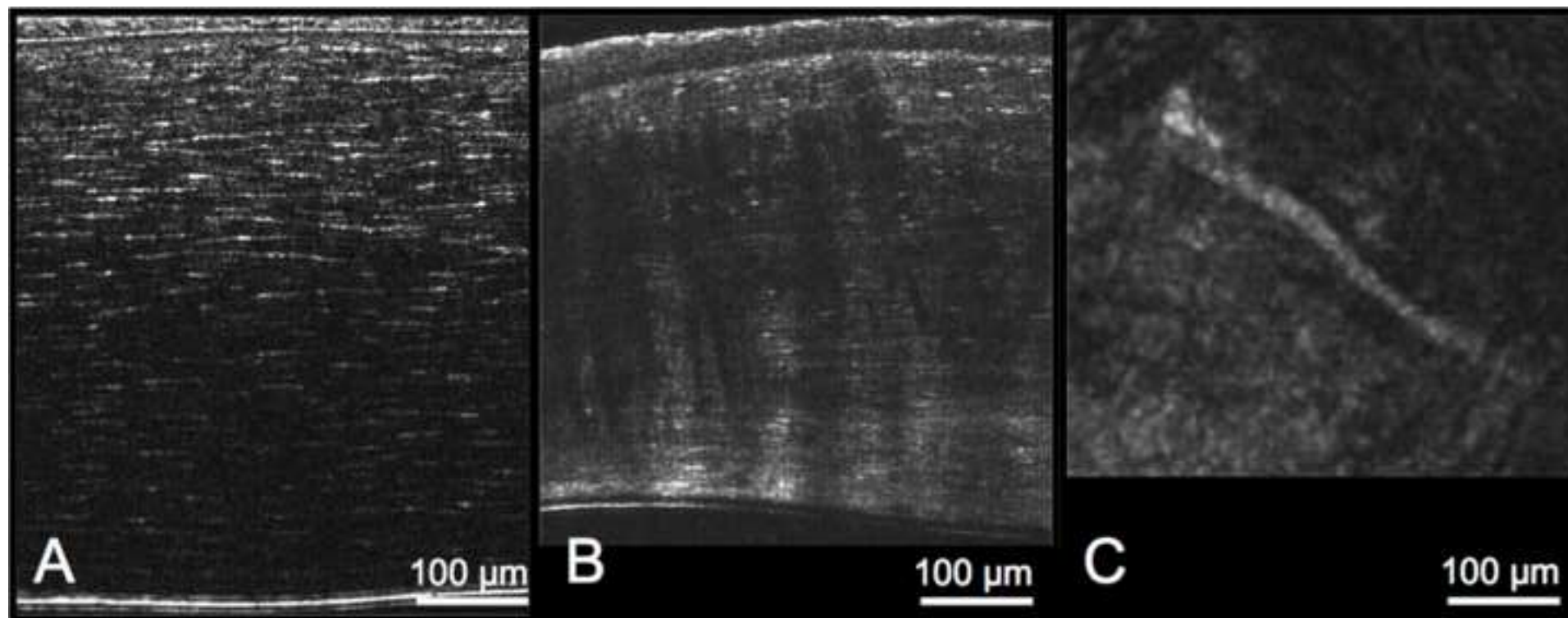579   **253**, 267 – 275 (2015)

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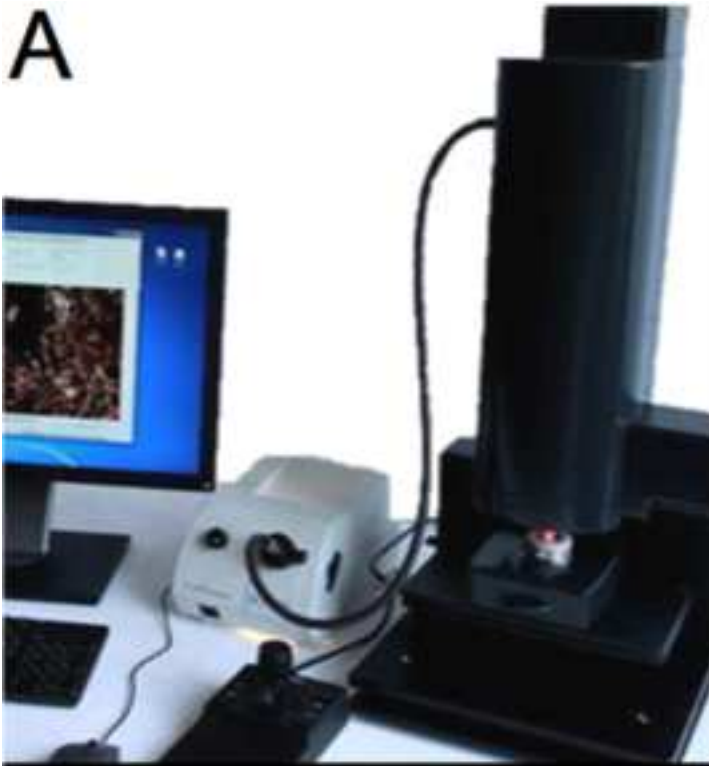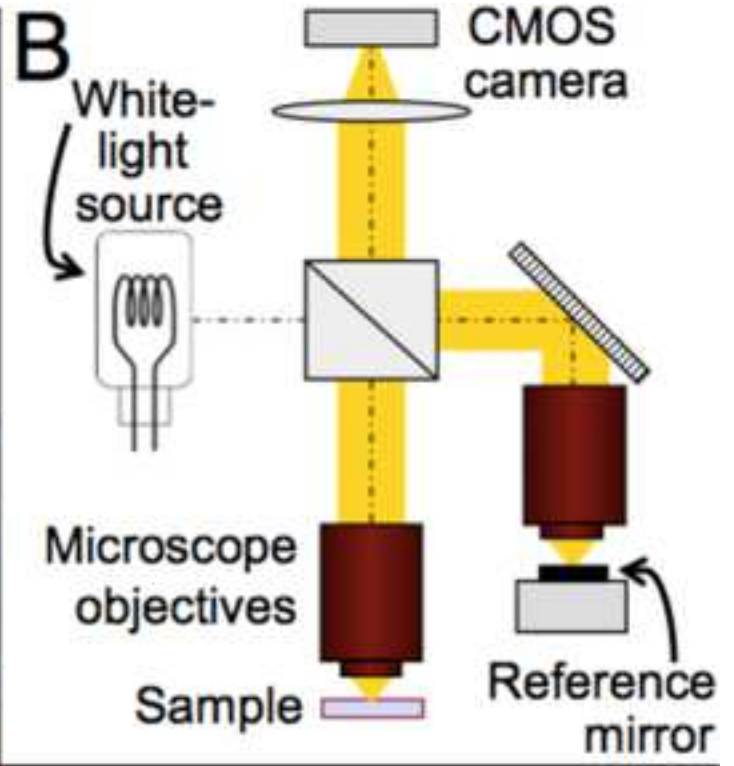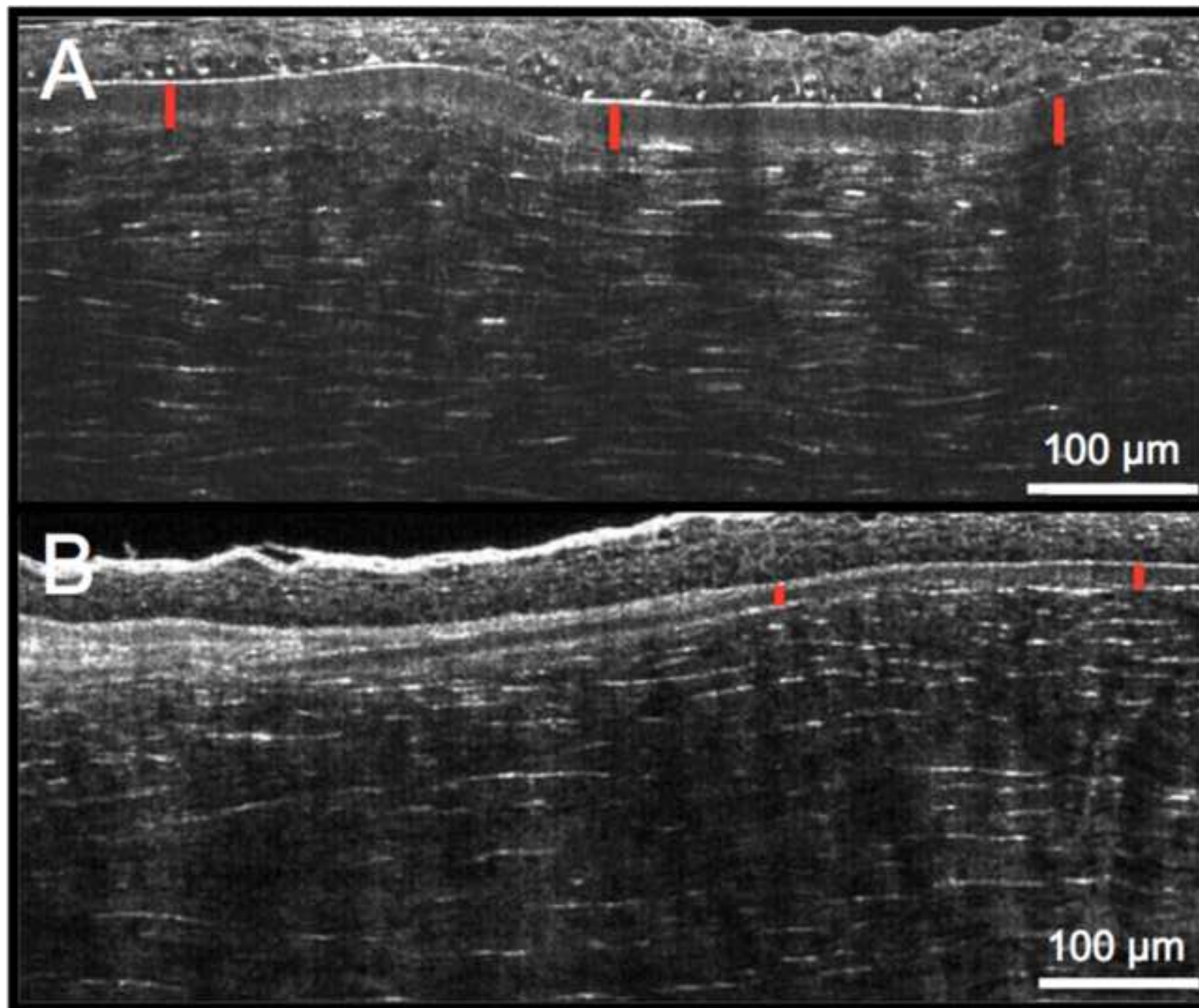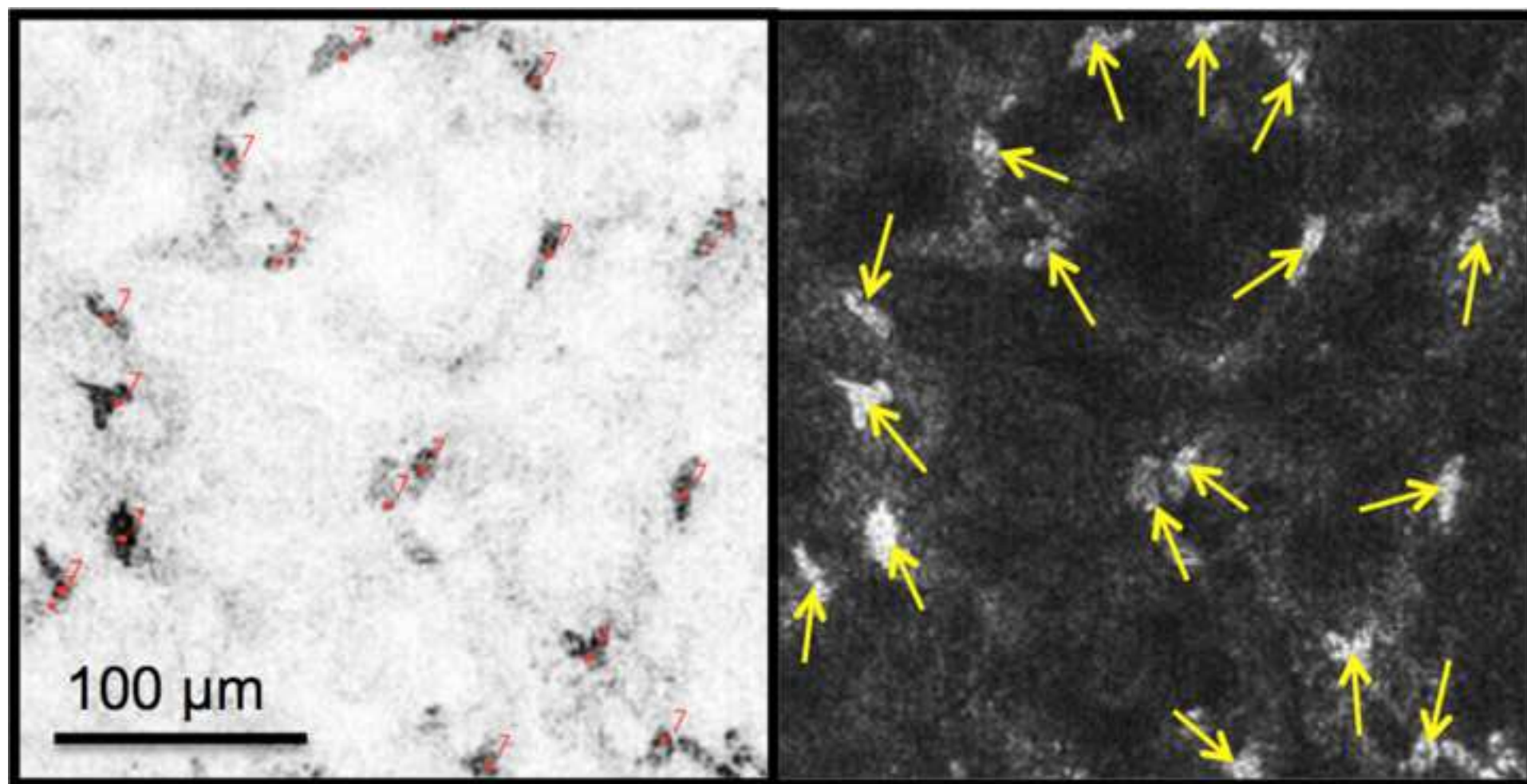
Figure 7

Figure 8
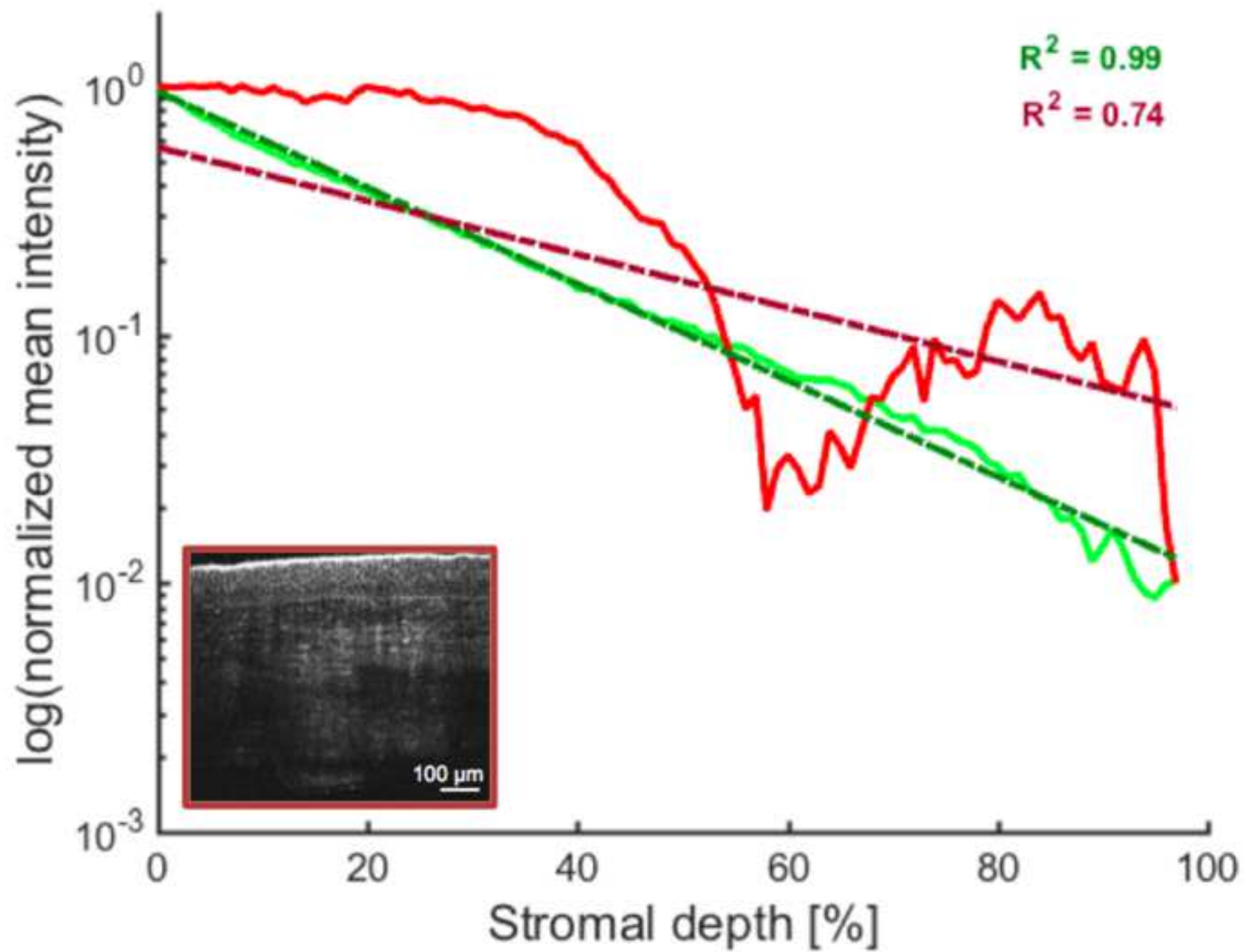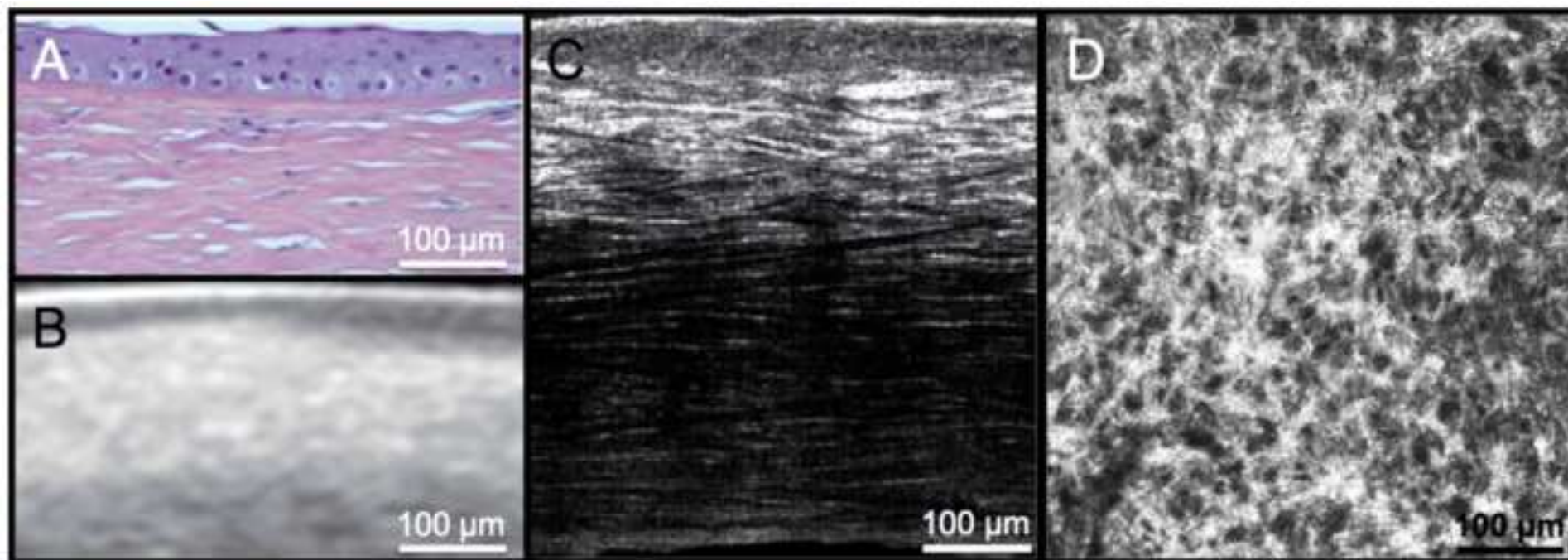
Name of Material/ Equipment	Company
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Comments/Description

FF-OCM device used in this manuscript for imaging

Organ culture medium in which donor corneas are stored

Dextran-supplemented organ culture medium used for deturgescence

Open source image processing software
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o The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

o Please bear in mind that software steps without a graphical user interface/calculations (e.g. 3.2.3) cannot be filmed.

The protocol steps to be visualized have been highlighted ==in yellow== and follow the guidelines elaborated above.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. The Discussion has been updated to also cover "troubleshooting" and "critical steps within the protocol," see lines 388 through 395, as well as 409 and 410.

• **Figures:**
1) Fig 1A: Please ensure that appropriate reprint permissions are obtained. We will provide appropriate permissions once obtained.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are LightCT, Matlab, LLTech, (CorneaMax, Eurobio,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific.

All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. All commercial sounding language has been removed or replaced by generic, non-company-specific terms followed by "(see table of materials)."

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. The table of materials has been revised to include all requested information.

• Please define all abbreviations at first use. Yes, thank you.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit. Yes, thank you.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are reusing figures from a previous publication, you must obtain explicit permission to reuse the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to republish the figure). Please upload the text of the reprint permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]." While none of the figures has been previously published in the form presented here, we are checking journal policies for reusing modified versions of previous figures.

Comments from <u>**Peer-Reviewers**</u>**:**

Reviewer #1:
Manuscript Summary:
Clearly written, good structured, no major concerns.

Major Concerns:
none.
Thank you!

Minor Concerns:
line 138: how can this be better assured? Can cornea be marked? This can be better assured by the visual feedback provided by acquisition of a "macro image," that is, a snapshot of the sample that can be used for lateral positioning and navigation purposes in preparation of an acquisition, as outlined in 2.2.2. of the revised text (see line 152).
line 152: is depth resolution of 1micron standard? Why 1 micron? Yes, "the default and recommended setting is 1 µm, matching the axial resolution of the device" as explained now in step 2.3.2.1. (see line 181).
line 163: please provide some more detail about how and why. More details have been added in the revised text (see line 223 and step 4.1.1.1.).
line 193: this might be clear during the experiment, but: is there a better definition of "clear oval features"? The text has been revised, with more detail added, to describe the counting of keratocyte nuclei, such as in step "4.1.2.5.1. Go to "Plugins" and navigate to "Cell Counter" under "Analyze". Select a counter type and then press "Initialize" and start counting the cell nuclei by clicking on dark oval features in the inverted image, considering those landing on an image border only for two of the four sides of the image[25]" (see line 261).
figures: not required, but arrows for might help to point out some of the structures (for example the "lakes" in figure 2, one could mark one "lake" as example. We have added arrows to mark two lakes in Figure 2 as example.

Reviewer #2:

Manuscript Summary:

Authors described innovative method of imagining corneal stroma using full-field optical coherence microscopy (FFOCM).

This technique allows qualitative and quantitative analysis of corneal stroma. This procedure could be very useful in detection of corneal stromal diseases. It may have positive impact on better outcome of corneal transplantation.

Major Concerns:

N/A

Thank you!

Minor Concerns:

N/A

Thank you!