

# Journal of Visualized Experiments

## A novel approach to monitoring graft neovascularization in the human gingiva

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58535R1
Full Title:	A novel approach to monitoring graft neovascularization in the human gingiva
Keywords:	Oral Surgical Procedures, Vestibuloplasty, Gingiva, Oral Mucosa, Regional blood flow, Microcirculation, Wound Healing
Corresponding Author:	Reka Fazekas Semmelweis University Budapest, HUNGARY
Corresponding Author's Institution:	Semmelweis University
Corresponding Author E-Mail:	fazekas@medaker.hu
Order of Authors:	Reka Fazekas Eszter Molnár Barbara Mikecs Zsolt Lohinai János Vág
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Szentkirályi u 47., Budapest 1088, Hungary

**TITLE:**

A Novel Approach to Monitoring Graft Neovascularization in the Human Gingiva

**AUTHORS & AFFILIATIONS:**

Réka Fazekas, Eszter Molnár, Barbara Mikecs, Zsolt Lohinai, János Vág

*Department of Conservative Dentistry, Faculty of Dentistry, Semmelweis University, Budapest, Hungary*

**Corresponding Author:**

Dr Réka Fazekas

Email Address: [fazekas@medaker.hu](mailto:fazekas@medaker.hu)

**Email Addresses of the Co-Authors:**

[drvagjanos@gmail.com](mailto:drvagjanos@gmail.com)

[molnr.eszter@gmail.com](mailto:molnr.eszter@gmail.com)

[mikecsbarbara@gmail.com](mailto:mikecsbarbara@gmail.com)

[lohinai.zsolt@dent.semmelweis-univ.hu](mailto:lohinai.zsolt@dent.semmelweis-univ.hu)

**KEYWORDS:**

Laser Speckle Contrast Imaging, microcirculation, blood flow, oral mucosa, gingiva, periodontal surgery, vestibuloplasty, xenogenic graft, incorporation, wound healing

**SHORT ABSTRACT:**

This study introduces a protocol for measuring microcirculation in human oral mucosa by laser speckle contrast imaging. The monitoring of wound healing after vestibuloplasty combined with a xenogenic collagen graft is presented on a clinical case.

**LONG ABSTRACT:**

Laser speckle contrast imaging (LSCI) is a novel method for measuring superficial blood perfusion over large areas. Since it is non-invasive and avoids direct contact with the measured area, it is suitable for monitoring blood flow changes during wound healing in human patients. Vestibuloplasty is periodontal surgery to the oral vestibule, aiming to restore vestibular depth with simultaneous enlargement of the keratinized gingiva. In this special clinical case, a split thickness flap was elevated at the first upper premolar and a xenogenic collagen matrix was adapted to the resulting recipient bed. LSCI was used to monitor the re- and neovascularization of the graft and the surrounding mucosa for one year. A protocol is introduced for the correct adjustment of microcirculation measurement in the oral mucosa, highlighting difficulties and possible failures.

The clinical case study presented demonstrated that – following the appropriate protocol – LSCI is a suitable and reliable method for following up microcirculation in a healing wound in the human oral mucosa and gives useful information on graft integration.

## INTRODUCTION:

Monitoring long-term changes of human gingival microcirculation in a clinical situation is a hot topic in oral and periodontal surgery. However, reliable assessment of perfusion can be difficult. There are only a few methods that do not invasively measure changes in the blood circulation of the human mucosa. Two of these employ a laser beam<sup>1-4</sup>, but in a different way. Laser doppler flowmetry (LDF) makes use of the Doppler shift in a laser beam<sup>5,6</sup>, while the laser speckle contrast imaging (LSCI) method relies on the speckle pattern of the backscattered laser light to measure the velocity of red blood cells<sup>7</sup>.

LDF measures only in a single point, and reproducible standardization of the sensors' position is a desirable yet difficult task. Another problem is that the probe of the LDF is small in diameter (1 mm<sup>2</sup>). Measuring at predetermined points before surgery is too specific, and may be blind to postoperative circulatory changes, while edema, tissue removal, tissue movement or the implanted graft cause significant changes in the postoperative geometry of the affected soft tissue. The measuring distance of LDF is less than 1 mm which prohibits use of a dental splint with a predetermined hole for the probe in case of volumetric change of the tissue. LSCI does not require any special tools for localization and can measure in areas of several cm<sup>2</sup>. As a result, wound healing can be followed throughout the surgical site. In addition, LSCI can display blood perfusion in color-coded images at a fraction of a second, with a resolution of up to 20 µm.

The LSCI device presented in this paper is used mostly for animal research applications where high resolution in small measurement areas is desired. However, since the structure and histology of the human oral mucosa are different from area to area (attached gingiva, marginal gingiva, vestibular mucosa), blood circulation is also heterogeneous<sup>8</sup>. Therefore, high-resolution LSCI has a great advantage over normal-resolution LSCI which is usually used in human testing.

The LSCI instrument employs an invisible laser (wavelength 785 nm). The beam is diverged to illuminate the measurement area, creating a speckle pattern. A CCD camera images the speckle pattern in the illuminated area. The CCD camera used in this system has an active imaging area of 1386 x 1034 pixels and its resolution is between 20-60 µm/pixel depending on the size of the measurement area and on the setting of the software (low, medium, high). It can take images at a speed of 16 frames per second, or even more, up to 100 frames per second, if the image size is reduced. Blood perfusion is calculated by the built-in software. It analyzes variations in the speckle pattern and quantifies the contrast. The resulting flux is color coded to produce a perfusion image. According to our previous results, LSCI assesses the blood perfusion of the gingiva with good repeatability and reproducibility<sup>9</sup>. This implies that it is a reliable tool for monitoring changes in the microcirculation of the oral mucosa not only in short-term experiments, but also during long-term studies to track disease progression or wound healing<sup>10</sup>.

In this paper, we present a clinical case report to demonstrate that the high spatial resolution of LSCI makes it possible to reveal the neovascularization pattern of a xenogenic collagen graft. Furthermore, this case indicates that LSCI, owing to its high reliability, could sensitively detect individual variation. This is important as significant local anatomical variation and a different

systemic background between the cases makes it difficult to standardize the surgical intervention in clinical trials of periodontal surgery.

## **PROTOCOL:**

The reported method was employed in a clinical trial which was granted ethical approval from the Hungarian Committee of the Health Registration and Training Center (approval number: 034310/2014/OTIG).

### **1. LSCI Setup**

1.1. Switch on the computer and any peripherals.

1.2. Switch on the LSCI instrument to be used with the switch on the rear panel.

1.3. Allow the instrument to warm up for at least 5 minutes. The instrument is ready for measurement when both LEDs on the rear panel have stopped flashing.

1.4. Start the software by double-clicking on the software icon on the desktop or *via* the Start menu.

1.5. Wait until both the yellow and the green LEDs on the rear panel have stopped flashing, which indicates that the laser is warm and initialization is finished.

Note: When starting up the system, one will occasionally be prompted to perform the verification procedure for the system.

### **2. System Verification**

2.1. Use the calibration box supplied. Remove the lid from the calibration box and shake it to avoid sedimentation in the colloidal suspension.

2.2. Leave the lid off for 30 seconds to avoid bubbles.

2.3. Put the lid back on the calibration box.

2.4. Click **Advanced | Verification | Verify instrument**.

2.5. Select **Routine verification | Next**.

2.6. Turn the head 90 degrees, fasten the calibration box using the integrated magnets and click **Next**.

2.7. Enter the room temperature in the text box, select °C and click **Start**.

2.8. Wait while the wizard completes the verification procedure.

2.9. After a successful verification procedure close the wizard by clicking **Finish**.

### 3. Participant Preparation

3.1. Make sure that measurement is performed in a temperature-controlled room (26 °C).

3.2. Place the patient in a comfortable supine position in a dental chair and use a vacuum pillow for fixing his head in a position appropriate to the area under investigation (**Figure 1**).

3.3. Leave the patient undisturbed for 15 minutes before any measurements are taken.

3.4. Measure blood pressure as well as pulse before and after the LSCI measurements.

### 4. Microcirculation Image Measurement

4.1. In the **File** menu, select and click on **New recording**. A new Image Window opens and the Setup panel is displayed.

4.2. Under **Recording Setup**, select the following parameters.

4.2.1. Select a Project: vestibulum (name of the project).

4.2.2. Select a Site: tooth 14 (site examined in the oral cavity).

4.2.3. Select a Subject or create a new Subject by opening the *Subject* drop-down list. The **Select subject** dialog appears.

4.2.3.1. Click **New**. Enter the name of the patient in the **New subject** dialog and click **OK**.

4.2.4. Enter a name for the recording in the **Rec Name** field: *e.g.*, day 1 (days elapsed after the operation) Enter the name of the operator in the **Operator** field.

4.2.5. Under **Image Setup**, the current working distance is displayed. Adjust the working distance by moving the instrument in relation to the tissue. Fix the working distance at 10.0 cm.

4.2.6. Set the size of the measurement area by entering the desired width and height in the corresponding text boxes: height: 2 cm; width: 3 cm.

4.2.7. Select a point density (resolution): normal.

176 4.2.8. Under **Image Capture Setup**, select the number of images per second to record from the  
177 *Frame rate* drop-down list: 16 images/s.

178  
179 4.2.9. In the **Duration** drop-down list, select **Time** and specify the duration of the recording:  
180 0:30.

181  
182 4.2.10. Select **Record with no averaging**.

183  
184 4.2.11. Select the capture rate of the color photo: one per second.

185  
186 4.3. Ask patient to open his mouth.

187  
188 4.4. Retract lips gently by two dental mirrors (**Figure 1**).

189  
190 4.5. Adjust the instrument's head parallel to the measured area of the gingiva. A built-in  
191 visible (650 nm) indicator laser facilitates the positioning of the imager relative to the subject.

192  
193 4.6. Adjust the distance to 10 cm. The distance is measured continuously by the LSCI device  
194 and it is displayed by the software as working distance/measured value.

195  
196 4.7. Instruct the subject to remain still for the duration of the measurement.

197  
198 4.8. Click on the **Record** button to start recording. The color of the Image Window now  
199 changes to red, indicating that recording is in progress. The Setup panel is replaced by the  
200 Recording panel. Recording stops automatically after 30 s. When recording is finished, the color  
201 of the Image Window changes to blue and the Recording panel is replaced by the Review panel.

202  
203 4.9. Remove dental mirrors and allow the patient to close his mouth and swallow.

204  
205 4.10. Switch back to the live image by pressing the **Resume recording** button.

206  
207 4.11. Repeat the steps from 4.3 to 4.10 twice.

208  
209 4.12. Press the **Stop recording** button. Close the file. The data are saved automatically.

## 210 211 5. Offline analysis

212  
213 5.1. Analyze the LSCI images using the built-in software. Go to Image or Split view  
214 (**Figure 2**).

215  
216 5.2. Define regions of interest (ROI). Average the perfusion values of pixels within a ROI and  
217 define as the blood flow value of the ROI, expressed in an arbitrary value called Laser Speckle  
218 Perfusion Unit (LSPU).

219

220 5.3. Select the desired ROI shape within the ROI tools palette on the right.

221  
222 5.4. Select the **Apply** option in the ROI tools palette, which applies ROI operations to all  
223 images of the recording.

224  
225 5.5. Draw the ROI by clicking and holding the mouse button in the intensity image, dragging  
226 the ROI out to the desired size, and releasing the mouse button (click and double-click for free-  
227 form ROIs). Adjust the position of the ROI, resize or rotate it, if needed.

228  
229 5.6. Repeat steps from 5.3. to 5.5 as many times as the desired number of ROIs (**Figure 3**).

230  
231 5.7. Define time periods of interest (TOI). This allows for averaging perfusion in a ROI over a  
232 definite period of time (**Figure 2**).

233  
234 5.8. Go to Graph or Split view. Select the TOI tool.

235  
236 5.9. Click and hold on the graph at the position where you want the TOI to begin and drag  
237 the cursor to the desired end position. Then release the mouse button.

238  
239 5.10. Export data from the mean value table for further processing.

240  
241 5.11. Construct blood flow curves by a suitable software used for statistical analysis.

## 242 243 **REPRESENTATIVE RESULTS:**

244 Vestibuloplasty is periodontal surgery to the oral vestibule, aiming to increase vestibular depth,  
245 the zone of keratinized gingiva and soft tissue thickness for enhanced aesthetics and function.  
246 The apically repositioned split thickness flap combined with a collagen matrix is a frequently  
247 used vestibuloplasty procedure. Xenogenic collagen matrix is a viable alternative to autogenous  
248 gingival graft for increasing the amount of keratinized gingiva<sup>11-13</sup>; however, no data are  
249 available on the direction of graft revascularization and on how it affects the microcirculation of  
250 the surrounding tissues. Understanding these mechanisms may facilitate proper flap and  
251 incision design in periodontal surgery.

252  
253 A 17-year-old male patient with an inadequate width of keratinized gingiva at the first premolar  
254 in the maxilla was treated by vestibuloplasty, using an apically repositioned split thickness flap  
255 combined with a collagen matrix. Intraoral photographs (taken by a photo camera) and blood  
256 flow (BF) measurements by LSCI were taken before the vestibuloplasty (baseline) as well as 1, 2,  
257 3, 4, 5, 7, 9, 11, 14, 21, 27 days and 2, 3, 4, 5, 6 and 12 months postoperatively. Blood pressure  
258 and pulse were evaluated before and after each measurement.

259  
260 During offline analysis, multiple ROIs were determined in the area of the augmented mucosa;  
261 some in the graft region and others in the surrounding mucosa, defined as 'peri' regions. As  
262 shown in **Figure 3**, the 'peri' and graft regions were further split into zones depending on  
263 distance from the center of the implanted graft, marked as zone F in the picture. Zones A and B

were defined in the 'peri' region and zones C, D and E in the graft region. Each of these zones was delimited separately at all four sides of the graft (mesial, distal, apical and coronal). Each 30-second shot was identified as a TOI (**Figure 2**). Data at each ROI and TOI were exported into a spreadsheet program. Blood flow curves were constructed by a suitable software used for statistical analysis.

There was no significant change in mean arterial pressure (MAP) during the one-year experiment, either in MAP before or after the blood flow measurements per session. **Figure 4** shows a color photo, an intensity image and a perfusion image of the operated gingiva on the representative days of our study. During the first postoperative week complete flap closure, a thick layer of fibrin on the grafted area, and mild erythema and edema in the surrounding gingiva were visible. Blood perfusion images showed ischemia in the operated region and hyperemia in the 'peri' regions. From day 14, the grafted area was clinically erythematous, in parallel with severe hyperemia observed on blood perfusion images. By the third month after graft incorporation, the wound healed, and gingival perfusion was close to preoperative circulation levels.

**Figure 5** shows a blurred intensity image and the perfusion graph of the entire image. The sudden peak on the graph indicates movement by the patient. The measurement was repeated immediately, after making sure that the patient is in a comfortable position. Changes in BF in the different zones within the graft and in the 'peri' regions are shown in **Figure 6**. It is common in all curves that from the fourth month, blood flow did not change any further until the end of the investigation. The average blood flow for this period could be used as a resting blood flow value for the new tissue and the random variation between time points allowed us to calculate the time-based variance component for each ROI respectively, using a linear mixed model. The minimum detectable difference could be calculated then to identify real change (with 95% confidence) between time points during the healing period (before the fourth month) in order to determine the hyperemic and the ischemic phase. The basic characteristics of curves were similar in all ROIs within the graft, starting with an ischemic phase followed by a hyperemic phase. However, the length of these two phases was different (**Table 1**). Ischemia was longest (7-9 days) in the central and in all coronal zones, with late hyperemia starting between day 11 and 27. In other zones of the graft, ischemia lasted only 4-7 days and hyperemia started earlier, between day 7 and 21.

The BF curves of the zones at the different sides of the graft had unique characteristics (**Figure 5**). At the apical side, all four zones had similar blood flow curves. At the coronal side, perfusion was regained in the outer zone later than in the inner zones, contrary to the mesial and distal side. At both lateral sides, BF increased first in zone C, then in zone D, followed by BF increase in zone E and finally in the central zone F. In the zones of the surrounding mucosa (zone A and B) no significant ischemia was observed. Instead, hyperemia of a different magnitude and extent was observed at the different sides.

There were two time points when the BF value did not tally with the overall characteristics of the BF curve. On day 9, there was a sudden drop in most zones and mainly in the 'peri' zones of



the apical and distal side. It cannot be stated with certainty that this was a measurement error, as no measurements were taken on the previous and on the following day. However, according to a note in the measurement report, the buccal fold was retracted with too much pressure by the operator, resulting in a drop in BF. This makes sense considering that mainly the circulation of the distal and apical sides could have been affected by pulling the cheek. On day 182 (6 months later), due to longer intervals between measurement times, the patient has forgotten to keep to the agreed restrictions before the measurement. The bleeding of the marginal gingiva on the colored photograph (**Figure 5**) indicates harsh tooth brushing prior to the measurement. In the meantime, the patient underwent orthodontic treatment, too, and he used intermaxillary elastics. Both factors could greatly increase BF<sup>14,15</sup>, so the measurement was repeated at a later time under more carefully controlled circumstances.

#### **FIGURE AND TABLE LEGENDS:**

**Figure 1: Experimental LSCI setup and patient preparation for blood flow measurement in the operated area.** The lips are retracted by dental mirrors.

**Figure 2: Split view (combination of the Images view and the Graph view) of a typical recording of gingival blood flow in the treated area.** Perfusion image (upper right sub-view) is a color-coded representation of blood perfusion in the gingiva. Areas of high perfusion are shown in red while areas of low perfusion are blue. The color range of perfusion images corresponds to 0-450 LSPU; smoothing was set to 10. An intensity image (lower right sub-view) is created by the total backscattered laser light. It corresponds exactly with the perfusion image and is useful for orientation and for identifying details in the perfusion image. Regions of interest (ROI) are always defined in the intensity image. The graph (upper left panel) shows real-time blood perfusion traces for each ROI in the recording. Check boxes to the left can be used to select which traces to show. Three consecutive measurements are shown on the graph. Each 30-second shot was identified as a TOI. A mean value table showing mean perfusion values in each ROI and TOI is also displayed in Split view (lower left panel).

**Figure 3: Regions of interest (ROI) defined within the examined gingival area in the intensity image.** Zone A and B are in the 'peri' region, while zone C, D and E are in the graft at decreasing distances from the center of the graft, marked as zone F. Zone A is situated on the vestibular surface of the lips.

**Figure 4: Representative photographs (upper line), LSCI intensity image (middle line) and LSCI perfusion image (lower line) of the operated gingiva.** The images represent the preoperative state and perfusion, and wound healing and perfusion 1, 4, 7, 14, 21, 27 and 98 days postoperatively.

**Figure 5: Split view of a sub-optimal recording.** Blurred intensity image and outlying peaks on the graph as a result of incorrect setting.

**Figure 6: Scatter plot of BF over time at the coronal (a), mesial (b), distal (c) and apical (d) side of the graft.** The central part of the graft (zone F) was depicted in all graphs to serve as a reference for more external zones.

**Table 1. Time frame of the ischemic and hyperemic phase in the various zones in the graft, in days**

## **DISCUSSION:**

The aim of this study was to introduce a novel technique for monitoring the neovascularization of a graft in the human gingiva. According to our previous results, LSCI assesses the blood perfusion of the gingiva with good repeatability and reproducibility<sup>9</sup>, when strict implementation of each step of the planned protocol as a critical requirement is met. LSCI is regarded as a semi-quantitative technique that requires calibration periodically to ensure accuracy and stability. During verification, the room temperature must be measured as accurately as possible, because this value is used by the verification algorithm to calculate perfusion.

The LSCI method is highly sensitive to the working distance setting and movement artifacts as well. In this study, working distance was fixed at 10 cm. The measurement area was 2.7 cm x 2 cm, which corresponds to an approximately three teeth wide gingival area. The effective frame rate was 16 images/s and 0.06 s/image as the arterial pulse induces pulsatile changes in gingival microcirculation<sup>9</sup>, which has to be averaged out from the recording. Rapid imaging reduced the risk of movement artefacts, too. However, in case of incorrect settings or patient movements, the recording should be stopped and repeated under optimum conditions.

Two operators took part in every measurement: one adjusted the LSCI head and controlled the computer while the other retracted the lips of the patient. In this study, three repeated measurements were performed in each session, each taking 30 seconds. Since measurements always involve some kind of irritation to the soft tissue due to the inevitable retraction of the lips and cheeks, which disturbs the microcirculation of the gingiva, an increase in random error occurs. Such inter-day variation, however, can be minimized by repeating the entire measurement process, *i.e.*, by re-opening the mouth, retracting the soft tissue again, re-setting the camera's position and re-selecting ROIs in the software<sup>9</sup>.

Gingival microcirculation showed high regional variation<sup>8</sup>. Therefore, a method such as LSCI which measures blood flow in a wide area has a great advantage over single point measurement techniques like LDF. In this study, the measurement area covered the whole surgical field. The wide measurement area allowed us to compare re- or neovascularization in various regions within the wounded area in our study. Contrary to LDF, where the probe is fixed by stents fabricated before surgery, in the case of the LSCI method, there is no need to define the region to be examined in advance. For the primary aim of monitoring wound healing in personalized medicine is to recognize unexpected patterns anywhere around the wound or the flap. In addition, postoperative changes in tissue geometry and edema caused by soft or hard tissue augmentation would make the pre-fabricated stents useless after surgery. To help visual

evaluation, smoothing was turned on during recording and the smoothing value was set to 10. This means that perfusion was averaged over ten images for a smoother appearance of the perfusion image and in order to decrease background noise. However, smoothing is only a visual effect and does not influence actual recorded perfusion values.

Gingival blood flow has a high temporal variation as well. This may be related to many physiological factors which accompany everyday life, such as gingival inflammation<sup>16-18</sup>, circadian rhythm<sup>19</sup>, blood pressure<sup>20</sup>, temperature<sup>16,21</sup>, mechanical pressure<sup>8,22-24</sup>, tooth brushing<sup>14,17,25</sup> or orthodontic force<sup>15</sup>. Therefore, the standardization and stabilization of these factors is obligatory for successful follow-up measurements.

The methods used earlier for investigating graft vascularization are highly invasive, which meant a major restriction on measurement time points during healing, especially in human studies<sup>26-32</sup>. They also have limitations in terms of measuring regional differences quantitatively. Our previous studies<sup>9,10</sup> have already proved the high reliability of LSCI in clinical trials and it was found to be useful to determine the time of soft tissue healing of an individual after tooth extraction in order to optimize implant placement<sup>33</sup>. In this study, the wound area covered by a xenogenic collagen graft showed excellent neovascularization, as on the 11<sup>th</sup> postoperative day all zones within the graft achieved the maximum blood flow level. However, it could be presumed that the collagen graft sloughed off or was resorbed by day 11 and we actually measured the revascularization of the recipient bed. In addition to its non-invasive feature, another special attribute of LSCI is a capability to characterize reperfusion curves at various regions of a graft during incorporation at individual level. The centripetal characteristics of graft neovascularization are similar to previous histology observations<sup>30</sup>. This suggests that graft revascularization not only occurs from the periosteal vascular plexus but also from the wound margin.

The experiment presented shows that the revascularization of a graft can be clearly followed up if every step is followed strictly. However, on day 182, non-compliant patient preparation and instruction resulted in a significant increase in BF.

LSCI is extensively used for full-field imaging of vascular structure and associated blood flow in other tissues, like in the retina<sup>34,35</sup>, the skin<sup>7,36</sup> and the brain<sup>37,38</sup>. The most promising clinical applications of LSCI are burn wound assessment<sup>39,40</sup>, evaluation of flaps<sup>41</sup> and intraoperative cerebral blood flow monitoring<sup>42</sup>. Apparently, there are serious limitations to widespread gingival measurements by LSCI in human subjects. This tool is very robust and heavy. The main difficulties arise in connection with the documentation camera, which has low resolution and is located a few centimeters away from the measurement camera. These features make it difficult to identify regions of interest directly on color photos. The size of the LSCI machine head prevents shooting inside the oral cavity. Therefore, areas which are not visible directly may not be measured. We have demonstrated earlier that using an indirect approach with a photographic mirror may serve as an alternative method<sup>9</sup>. However, using a mirror involves more movement artifacts which LSCI is sensitive to, makes it more difficult to capture a perpendicular image and decreases the focal distance. When the measured area cannot be

captured perpendicularly, the blood flow value may be correct<sup>9,39</sup>, but region identification on the image remains complicated because of 3D torsion.

## ACKNOWLEDGMENTS

This work was carried out in part from support by the Hungarian Scientific Research Fund under Grant Number K112364, by the Hungarian Ministry of Human Capacities, Higher Education Excellence Program to Semmelweis University, Therapy Research Module and by the National Research, Development and Innovation Office KFI\_16-1-2017-0409.

## DISCLOSURES

The authors have nothing to disclose.

## REFERENCES

- 1 Nakamoto, T. *et al.* Two-Dimensional Real-Time Blood Flow and Temperature of Soft Tissue Around Maxillary Anterior Implants. *Implant Dentistry*. **21** (6), 522-527 (2012).
- 2 Kajiwar, N. *et al.* Soft tissue biological response to zirconia and metal implant abutments compared with natural tooth: microcirculation monitoring as a novel bioindicator. *Implant Dentistry*. **24** (1), 37-41 (2015).
- 3 Kemppainen, P., Forster, C. & Handwerker, H. O. The importance of stimulus site and intensity in differences of pain-induced vascular reflexes in human orofacial regions. *Pain*. **91** (3), 331-338 (2001).
- 4 Kemppainen, P., Avellan, N. L., Handwerker, H. O. & Forster, C. Differences between tooth stimulation and capsaicin-induced neurogenic vasodilatation in human gingiva. *Journal of Dental Research*. **82** (4), 303-307 (2003).
- 5 Riva, C., Ross, B. & Benedek, G. B. Laser Doppler measurements of blood flow in capillary tubes and retinal arteries. *Investigative ophthalmology*. **11** (11), 936-944 (1972).
- 6 Humeau, A., Steenbergen, W., Nilsson, H. & Stromberg, T. Laser Doppler perfusion monitoring and imaging: novel approaches. *Medical & Biological Engineering & Computing*. **45** (5), 421-435 (2007).
- 7 Briers, J. D. & Webster, S. Laser speckle contrast analysis (LASCA): a nonscanning, full-field technique for monitoring capillary blood flow. *Journal of Biomedical Optics*. **1** (2), 174-179 (1996).
- 8 Fazekas, R. *et al.* Functional characterization of collaterals in the human gingiva by laser speckle contrast imaging. *Microcirculation*. **25** (3), e12446 (2018).
- 9 Molnar, E., Fazekas, R., Lohinai, Z., Toth, Z. & Vag, J. Assessment of the test-retest reliability of human gingival blood flow measurements by Laser Speckle Contrast Imaging in a healthy cohort. *Microcirculation*. **25** (2) (2018).
- 10 Molnar, E. *et al.* Evaluation of Laser Speckle Contrast Imaging for the Assessment of Oral Mucosal Blood Flow following Periodontal Plastic Surgery: An Exploratory Study. *BioMed Research International*. **2017** 4042902 (2017).
- 11 Sanz, M., Lorenzo, R., Aranda, J. J., Martin, C. & Orsini, M. Clinical evaluation of a new collagen matrix (Mucograft prototype) to enhance the width of keratinized tissue in patients with fixed prosthetic restorations: a randomized prospective clinical trial. *Journal of Clinical Periodontology*. **36** (10), 868-876 (2009).

483 12 Nevins, M., Nevins, M. L., Kim, S. W., Schupbach, P. & Kim, D. M. The use of mucograft  
484 collagen matrix to augment the zone of keratinized tissue around teeth: a pilot study. *The*  
485 *International Journal of Periodontics and Restorative Dentistry*. **31** (4), 367-373 (2011).

486 13 Lorenzo, R., Garcia, V., Orsini, M., Martin, C. & Sanz, M. Clinical efficacy of a xenogeneic  
487 collagen matrix in augmenting keratinized mucosa around implants: a randomized controlled  
488 prospective clinical trial. *Clinical Oral Implants Research*. **23** (3), 316-324 (2012).

489 14 Perry, D. A., McDowell, J. & Goodis, H. E. Gingival microcirculation response to tooth  
490 brushing measured by laser Doppler flowmetry. *Journal of Periodontology*. **68** (10), 990-995  
491 (1997).

492 15 Yamaguchi, K., Nanda, R. S. & Kawata, T. Effect of orthodontic forces on blood flow in  
493 human gingiva. *Angle Orthodontist*. **61** (3), 193-203; discussion 203-194 (1991).

494 16 Molnár, E. *et al.* Assessment of heat provocation tests on the human gingiva: the effect  
495 of periodontal disease and smoking. *Acta Physiologica Hungarica*. **102** (2), 176-188 (2015).

496 17 Gleissner, C., Kempster, O., Peylo, S., Glatzel, J. H. & Willershausen, B. Local gingival blood  
497 flow at healthy and inflamed sites measured by laser Doppler flowmetry. *Journal of*  
498 *Periodontology*. **77** (10), 1762-1771 (2006).

499 18 Hinrichs, J. E., Jarzembinski, C., Hardie, N. & Aeppli, D. Intracuticular laser Doppler  
500 readings before and after root planing. *Journal of Clinical Periodontology*. **22** (11), 817-823  
501 (1995).

502 19 Svalstad, J., Hellem, S., Vaagbo, G., Irgens, A. & Thorsen, E. Reproducibility of  
503 transcutaneous oximetry and laser Doppler flowmetry in facial skin and gingival tissue.  
504 *Microvascular Research*. **79** (1), 29-33 (2010).

505 20 Sasano, T., Kuriwada, S. & Sanjo, D. Arterial blood pressure regulation of pulpal blood  
506 flow as determined by laser Doppler. *Journal of Dental Research*. **68** (5), 791-795 (1989).

507 21 Ikawa, M., Ikawa, K. & Horiuchi, H. The effects of thermal and mechanical stimulation on  
508 blood flow in healthy and inflamed gingiva in man. *Archives of Oral Biology*. **43** (2), 127-132  
509 (1998).

510 22 Baab, D. A., Oberg, P. A. & Holloway, G. A. Gingival blood flow measured with a laser  
511 Doppler flowmeter. *Journal of Periodontal Research*. **21** (1), 73-85 (1986).

512 23 Fazekas, A., Csempeš, F., Csabai, Z. & Vág, J. Effects of pre-soaked retraction cords on  
513 the microcirculation of the human gingival margin. *Operative Dentistry*. **27** (4), 343-348 (2002).

514 24 Csillag, M., Nyiri, G., Vag, J. & Fazekas, A. Dose-related effects of epinephrine on human  
515 gingival blood flow and crevicular fluid production used as a soaking solution for chemo-  
516 mechanical tissue retraction. *Journal of Prosthetic Dentistry*. **97** (1), 6-11 (2007).

517 25 Tanaka, M., Hanioka, T., Kishimoto, M. & Shizukuishi, S. Effect of mechanical toothbrush  
518 stimulation on gingival microcirculatory functions in inflamed gingiva of dogs. *Journal of Clinical*  
519 *Periodontology*. **25** (7), 561-565 (1998).

520 26 Rothamel, D. *et al.* Biodegradation pattern and tissue integration of native and cross-  
521 linked porcine collagen soft tissue augmentation matrices - an experimental study in the rat.  
522 *Head & Face Medicine*. **10** 10 (2014).

523 27 Schwarz, F., Rothamel, D., Herten, M., Sager, M. & Becker, J. Angiogenesis pattern of  
524 native and cross-linked collagen membranes: an immunohistochemical study in the rat. *Clinical*  
525 *Oral Implants Research*. **17** (4), 403-409 (2006).

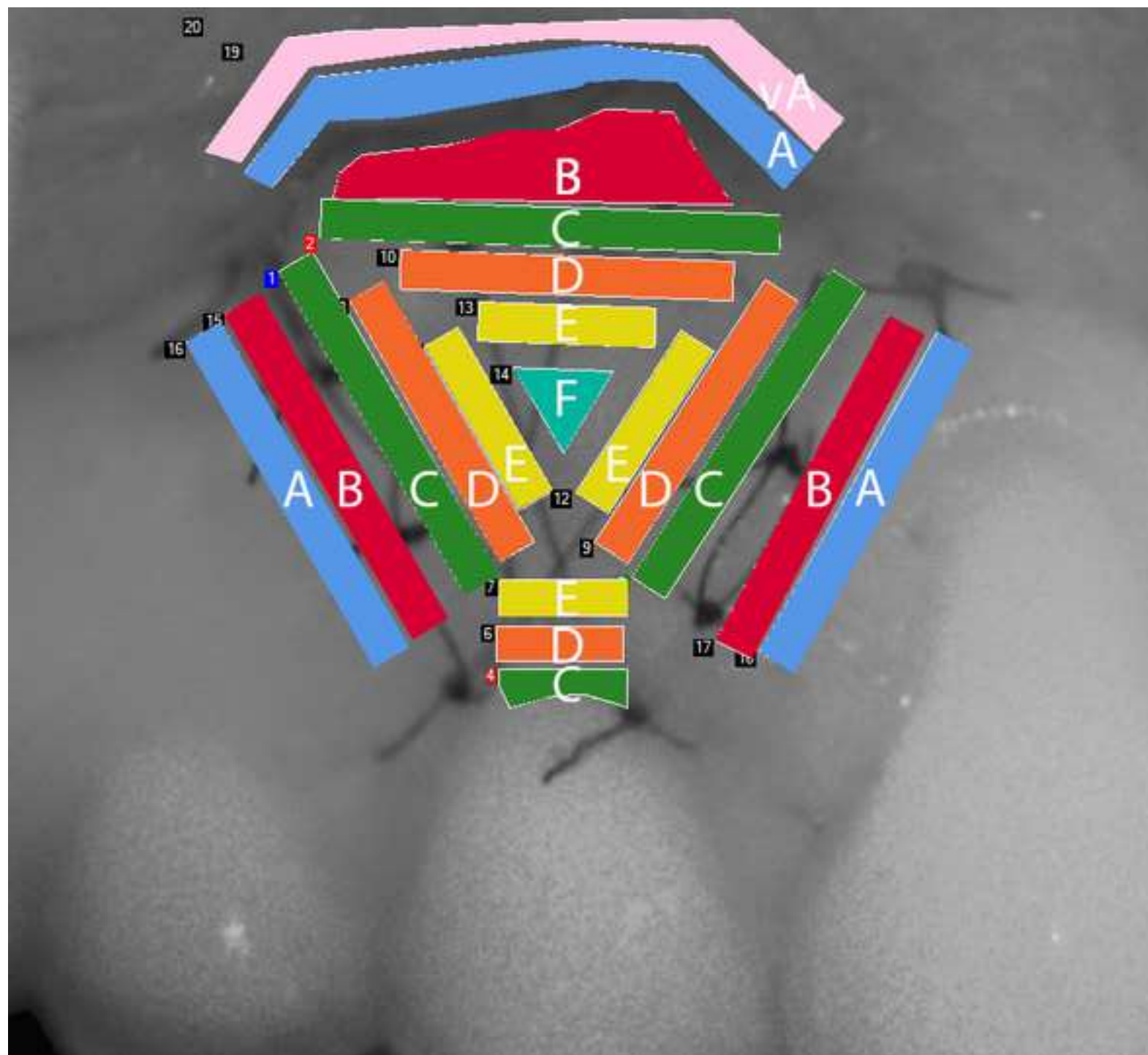
- 28 Vergara, J. A., Quinones, C. R., Nasjleti, C. E. & Caffesse, R. G. Vascular response to guided tissue regeneration procedures using nonresorbable and bioabsorbable membranes in dogs. *Journal of Periodontology*. **68** (3), 217-224 (1997).
- 29 Oliver, R. C., Loe, H. & Karring, T. Microscopic evaluation of the healing and revascularization of free gingival grafts. *Journal of Periodontal Research*. **3** (2), 84-95 (1968).
- 30 Janson, W. A., Ruben, M. P., Kramer, G. M., Bloom, A. A. & Turner, H. Development of the blood supply to split-thickness free gingival autografts. *Journal of Periodontology*. **40** (12), 707-716 (1969).
- 31 Mormann, W., Bernimoulin, J. P. & Schmid, M. O. Fluorescein angiography of free gingival autografts. *Journal of Clinical Periodontology*. **2** (4), 177-189 (1975).
- 32 Busschop, J., de Boever, J. & Schautteet, H. Revascularization of gingival autografts placed on different receptor beds. A fluoroangiographic study. *Journal of Clinical Periodontology*. **10** (3), 327-332 (1983).
- 33 Fazekas, R. *et al.* A proposed method for assessing the appropriate timing of early implant placements: a case report. *Journal of Oral Implantology*. 10.1563/aaid-joi-D-17-00295 (2018).
- 34 Briers, J. D. & Fercher, A. F. Retinal blood-flow visualization by means of laser speckle photography. *Investigative Ophthalmology & Visual Science*. **22** (2), 255-259 (1982).
- 35 Srienc, A. I., Kurth-Nelson, Z. L. & Newman, E. A. Imaging retinal blood flow with laser speckle flowmetry. *Front Neuroenergetics*. **2** (2010).
- 36 Choi, B., Kang, N. M. & Nelson, J. S. Laser speckle imaging for monitoring blood flow dynamics in the *in vivo* rodent dorsal skin fold model. *Microvascular Research*. **68** (2), 143-146 (2004).
- 37 Ayata, C. *et al.* Laser speckle flowmetry for the study of cerebrovascular physiology in normal and ischemic mouse cortex. *Journal of Cerebral Blood Flow & Metabolism*. **24** (7), 744-755 (2004).
- 38 Armitage, G. A., Todd, K. G., Shuaib, A. & Winship, I. R. Laser speckle contrast imaging of collateral blood flow during acute ischemic stroke. *Journal of Cerebral Blood Flow & Metabolism*. **30** (8), 1432-1436 (2010).
- 39 Lindahl, F., Tesselaar, E. & Sjoberg, F. Assessing paediatric scald injuries using Laser Speckle Contrast Imaging. *Burns*. **39** (4), 662-666 (2013).
- 40 Mirdell, R., Iredahl, F., Sjoberg, F., Farnebo, S. & Tesselaar, E. Microvascular blood flow in scalds in children and its relation to duration of wound healing: A study using laser speckle contrast imaging. *Burns*. 10.1016/j.burns.2015.12.005 (2016).
- 41 Zotterman, J., Bergkvist, M., Iredahl, F., Tesselaar, E. & Farnebo, S. Monitoring of partial and full venous outflow obstruction in a porcine flap model using laser speckle contrast imaging. *Journal of Plastic, Reconstructive & Aesthetic Surgery*. **69** (7), 936-943 (2016).
- 42 Hecht, N., Woitzik, J., Dreier, J. P. & Vajkoczy, P. Intraoperative monitoring of cerebral blood flow by laser speckle contrast analysis. *Neurosurgical Focus*. **27** (4), E11 (2009).

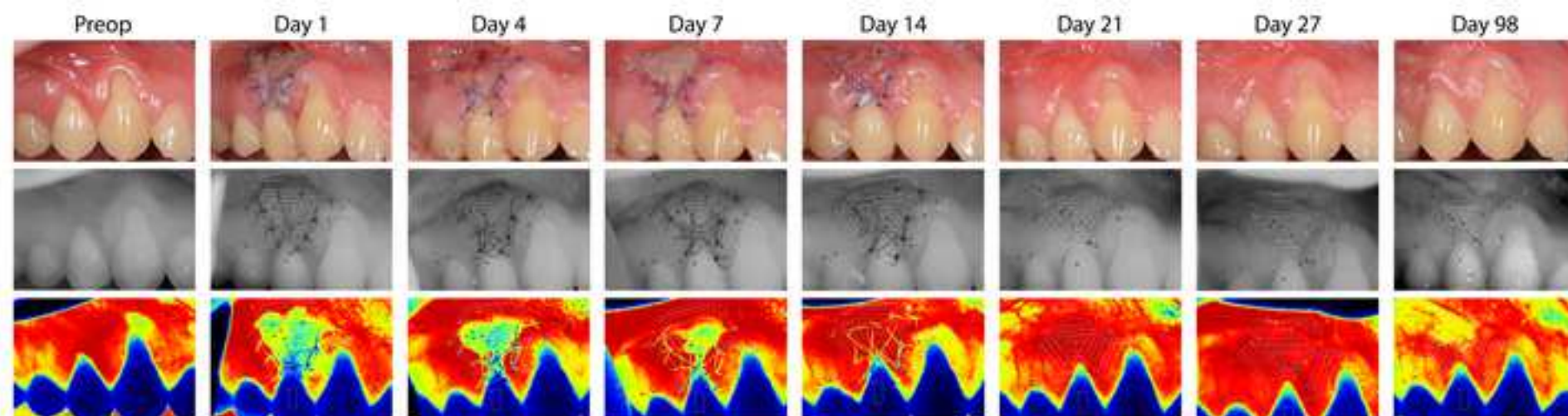


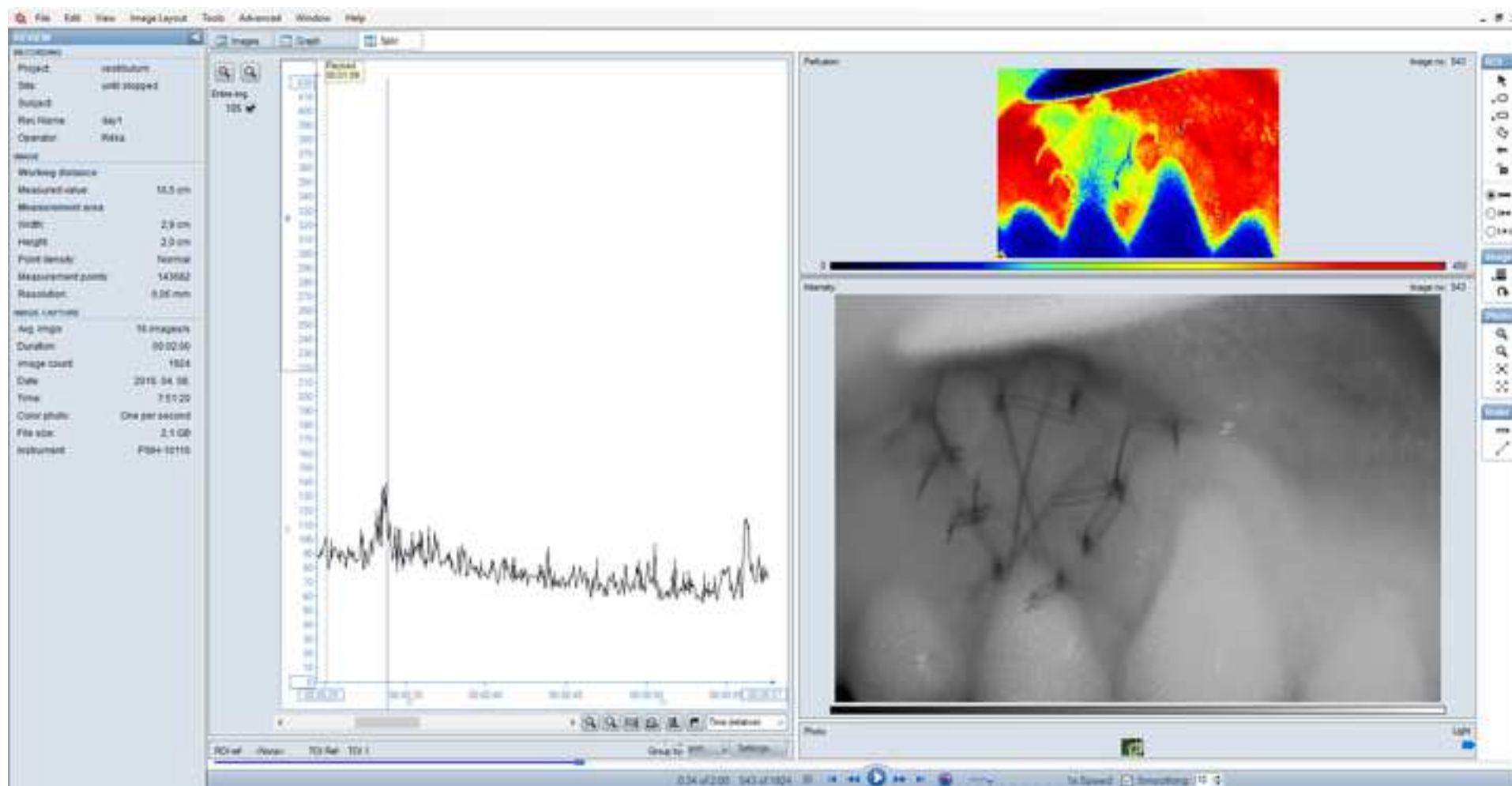


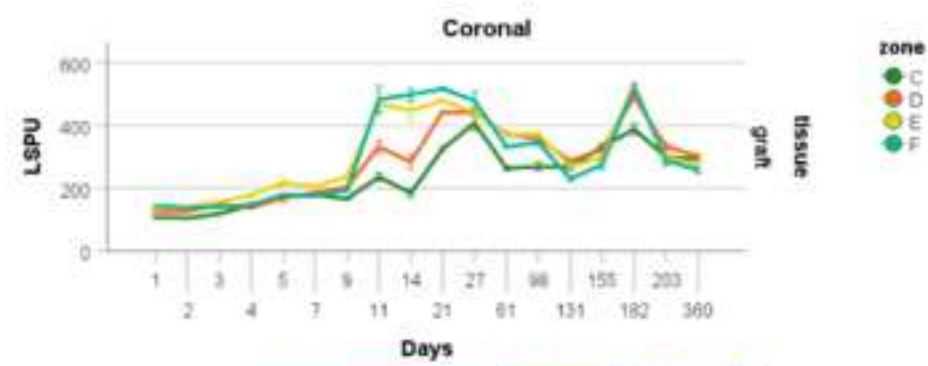
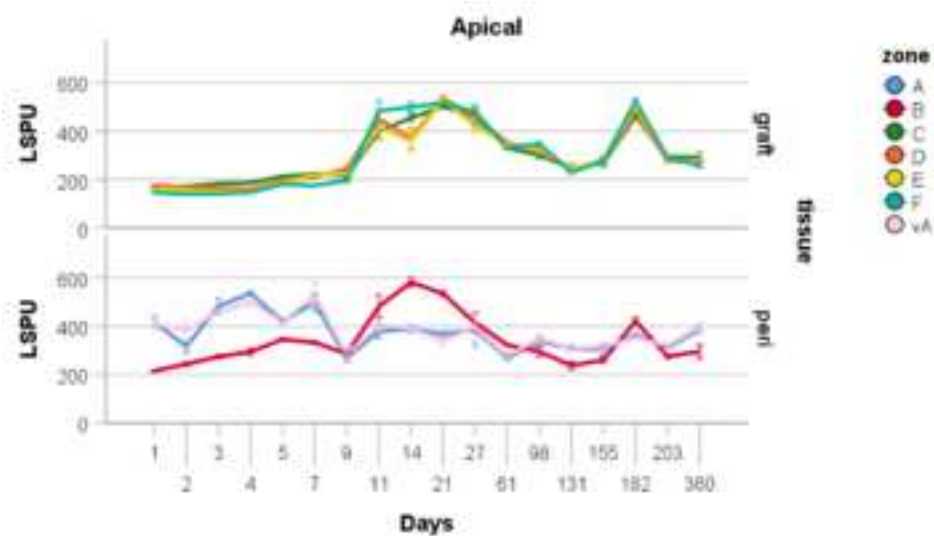




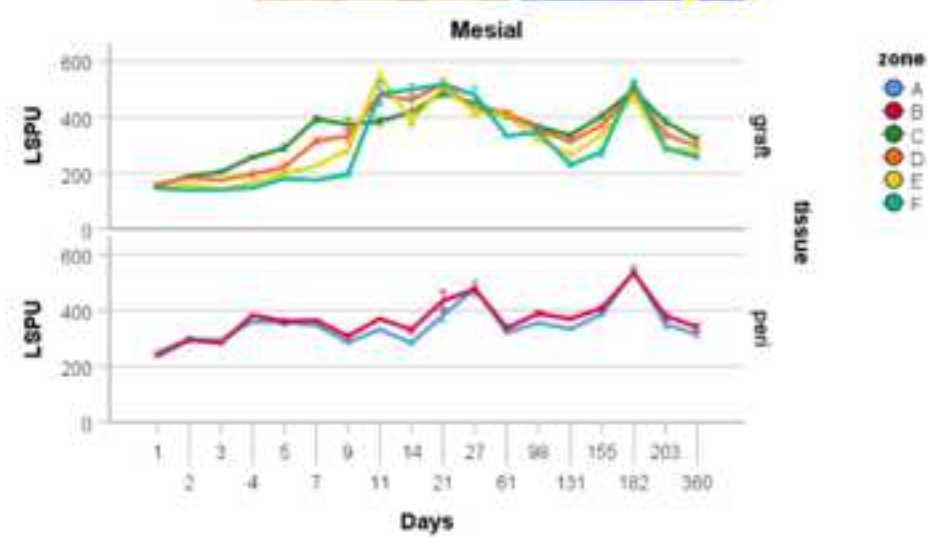
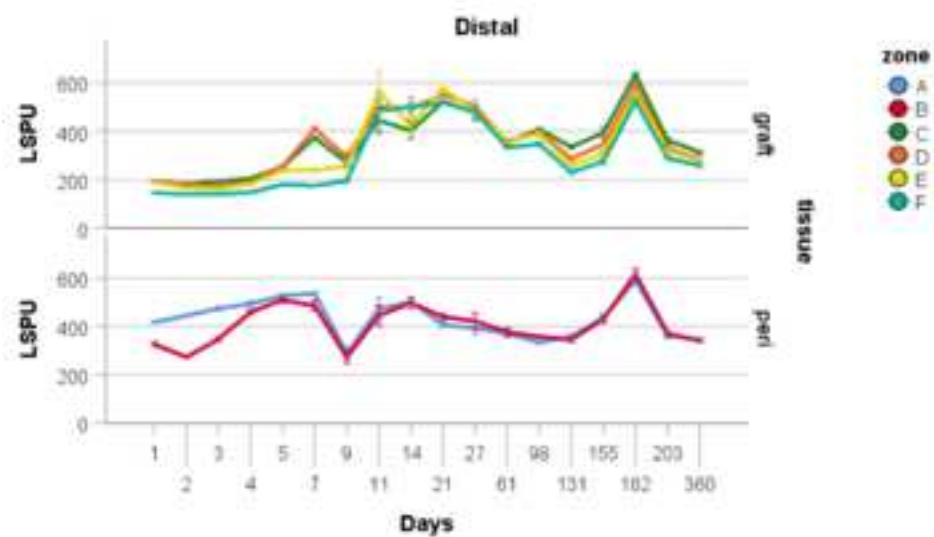
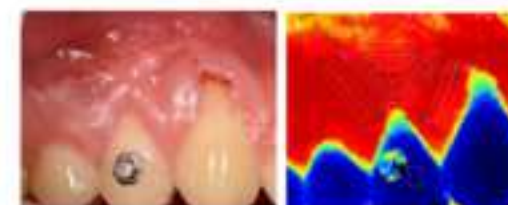








Day 182



Side	Zone	Ischemia end
coronal	c	9
coronal	d	9
coronal	e	7
central	f	9
mesial	c	5
mesial	d	5
mesial	e	7
distal	c	5
distal	d	4
distal	e	4
apical	c	4
apical	d	5
apical	e	5

Hyperemia start	Hyperemia end
27	27
21	27
11	98
11	98
21	27
11	61
11	61
11	27
7	98
11	98
11	27
11	61
11	61

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
PeriCam PSI-HR	Perimed AB, Stockholm, Sweden		The PeriCam PSI System is an imaging sys
PIMSoft	Perimed AB, Stockholm, Sweden		PIMSoft is a data acquisition and analysis
Geistlich Mucograft	Geistlich, Switzerland		It's a unique 3D collagne matrix design
Omron M4,	Omron Healthcare Inc., Kyoto, Japan		Blood pressure monitor, which gives a
<b>Nikon D5200</b>	Nikon Corporation, Tokyo, Japan		Taking intra oral photos
MS Excel	Microsoft Corporation, Redmond, Washington, USA		The software used for data managem
IBM SPSS Statistics 25	IBM Corp., Armonk, NY, USA		The software used for statistical analy

tem based on LASCA technology (LAser Speckle Contrast Analysis). The system measures superficial blood perfusion over large areas at fast capture rate software, intended for use together with the PeriCam PSI System and the PeriScan PIM 3 System, for measurement and imaging of superficial blood perfusion specifically for soft tissue regeneration. It's indicated for the gain of keratinized tissue and recession coverage.

accurate readings.

ent  
sis



ates. This makes it ideal for investigations of both the spatial and temporal dynamics of microcirculation in almost any tissue.  
perfusion.



1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Laser Speckle Contrast Imaging as a method of monitoring graft neovascularization in the human gingiva  
Author(s): Réka Fazekas, Eszter Molnár, Barbara Mikecs, Zsolt Lohinai, János Vág

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.  
☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.  
☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.



## ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have



## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name: Réka Fazekas  
Department: Department of Conservative Dentistry  
Institution: Semmelweis University  
Article Title: Laser Speckle Contrast Imaging as a method of monitoring graft neovascularization in the human gingiva  
Signature: [Signature] Date: 29th May, 2018

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

19 August 2018, Budapest

Dear Editor,

We are indebted for the further suggestions made to improve our manuscript (Manuscript ID: JoVE58535). We have carefully studied the valuable criticism and comments of the Editor and the Reviewers. The paper was rewritten to answer the questions and to adopt the suggestions made. Additional data were included and changes were highlighted by using the track changes feature of MS Word. Please note that in addition to these modifications, the title of the paper has also been changed from “Laser Speckle Contrast Imaging as a method of monitoring graft neovascularization in the human gingiva” to “A novel approach to monitoring graft neovascularization in the human gingiva”. Thank you very much for your suggestions.

I hope our manuscript is now in a form acceptable for publication.

Yours sincerely,

Dr Réka Fazekas

## **Response to the editorial comments**

Changes to be made by the Author(s) regarding the written manuscript:

### **1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

We have carefully proofread the manuscript.

### **2. Please revise lines 71-74 and 78-80 to avoid previously published text.**

We revised the lines concerned as follows:

“The LSCI instrument employs an invisible laser (wavelength 785 nm). The beam is diverged to illuminate the measurement area, creating a speckle pattern. A CCD camera images the speckle pattern in the illuminated area. The CCD camera used in this system has an active imaging area of 1386 x 1034 pixels and its resolution is between 20-60  $\mu\text{m}/\text{pixel}$  depending on the size of the measurement area and on the setting of the software (low, medium, high). It can take images at a speed of 16 frames per second, or even more, up to 100 frames per second, if the image size is reduced.”

“According to our previous results, LSCI assesses the blood perfusion of the gingiva with good repeatability and reproducibility (Molnar, Fazekas et al. 2018). This implies that it is a reliable tool for monitoring blood flow changes in the oral mucosa not only in short-term experiments, but also during long-term clinical studies to track disease progression or wound healing (Molnar, Molnar et al. 2017).”

### **3. Please revise the title to be more concise.**

The title of the paper has been changed to “A novel approach to monitoring graft neovascularization in the human gingiva”.

### **4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Geistlich Mucograft, Nikon D5200, IBM Corp., PeriCam PSI, Omron M4, etc.**

All commercial language has been removed from the manuscript.

### **5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Please move the discussion about the protocol to the Discussion. Please revise lines 111-121 and 197-206 accordingly.**

The Protocol has been simplified as requested. Lines 108-121 and 197-206 have been moved to the Representative Results.

### **6. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”**

The Protocol has been revised as requested.

### **7. 2.1: Please describe how to calibrate the PeriCam at 26 °C.**

A paragraph describing system verification has been added to the Protocol (line 96-113 with track changes enabled).

- 1.5. "Wait until both the yellow and the green LEDs on the rear panel have stopped flashing, which indicates that the laser is warm and initialization is finished.

Note: When starting up the system, you will occasionally be prompted to perform the verification procedure for the system.

## 2. System verification

- 2.1. Use the calibration box supplied. Remove the lid from the calibration box and shake it to avoid sedimentation in the colloidal suspension.
- 2.2. Leave the lid off for 30 seconds to avoid bubbles.
- 2.3. Put the lid back on the calibration box.
- 2.4. Click *Advanced/ Verification/ Verify instrument*.
- 2.5. Select *Routine verification* and click *Next*.
- 2.6. Turn the head 90 degrees, fasten the calibration box using the integrated magnets and click *Next*.
- 2.7. Enter the room temperature in the text box, select °C and click *Start*.
- 2.8. Wait while the wizard completes the verification procedure.
- 2.9. After a successful verification procedure close the wizard by clicking *Finish*."

**8. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

Specific details have been added to the protocol steps (line 123-188 with track changes enabled).

## 4. "Microcirculation image measurement

- 4.1. In the *File* menu, select and click on *New recording*. A new Image Window opens and the Setup panel is displayed.
- 4.2. Under *Recording Setup*, select the following parameters:
  - 4.2.1. Select a Project: vestibulum (name of the project)
  - 4.2.2. Select a Site: tooth 14 (site examined in the oral cavity)
  - 4.2.3. Select a Subject or create a new Subject by opening the *Subject* drop-down list. The *Select subject* dialog appears.
    - 4.2.3.1. Click *New*
    - 4.2.3.2. Enter the name of the patient in the *New subject* dialog and click *OK*.
  - 4.2.4. Enter a name for the recording in the *Rec Name* field: e.g. day 1 (days elapsed after the operation). Enter the name of the operator in the *Operator* field
  - 4.2.5. Under *Image Setup*, the current working distance is displayed. Adjust the working distance by moving the instrument in relation to the tissue. Note: Working distance must be fixed at 10.0 cm
  - 4.2.6. Set the size of the measurement area by entering the desired width and height in the corresponding text boxes: height: 2 cm; width: 3 cm
  - 4.2.7. Select a point density (resolution): normal
  - 4.2.8. Under *Image Capture Setup*, select the number of images per second to record from the *Frame rate* drop-down list: 16 images/s
  - 4.2.9. In the *Duration* drop-down list, select *Time* and specify the duration of the recording: 0:30
  - 4.2.10. Select *Record with no averaging*
  - 4.2.11. Select the capture rate of the color photo: one per second
- 4.3. Ask patient to open his mouth.

- 4.4. Retract lips gently by two dental mirrors (**Fig. 1**).
- 4.5. Adjust the instrument's head parallel to the measured area of the gingiva. Note: A built-in visible (650 nm) indicator laser facilitates the positioning of the imager relative to the subject.
- 4.6. Adjust the distance to 10 cm. Note: The distance is measured continuously by the LSCI device and it is displayed by the software as working distance/measured value.
- 4.7. Instruct the subject to remain still for the duration of the measurement.
- 4.8. Click on the *Record* button to start recording. The color of the Image Window now changes to red, indicating that recording is in progress. The Setup panel is replaced by the Recording panel. Recording stops automatically after 30 s. When recording is finished, the color of the Image Window changes to blue and the Recording panel is replaced by the Review panel.
- 4.9. Remove dental mirrors and allow the patient to close his mouth and swallow.
- 4.10. Switch back to the live image by pressing the *Resume recording* button.
- 4.11. Repeat the steps from 4.3 to 4.10 twice.
- 4.12. Press the *Stop recording* button.
- 4.13. Close the file. The data are saved automatically.

## 5. Offline analysis

- 5.1. Analyze the LSCI images using the built-in software. Go to Image or Split view (**Fig. 2**).
- 5.2. Define regions of interest (ROI). Note: The perfusion values of pixels within a ROI are averaged and defined as the blood flow value of the ROI, expressed in an arbitrary value called Laser Speckle Perfusion Unit (LSPU).
- 5.3. Select the desired ROI shape within the ROI tools palette on the right.
- 5.4. Select the *Apply* option in the ROI tools palette, which applies ROI operations to all images of the recording.
- 5.5. Draw the ROI by clicking and holding the mouse button in the intensity image, dragging the ROI out to the desired size, and releasing the mouse button (click and double-click for free form ROIs). Adjust the position of the ROI, resize or rotate it if needed.
- 5.6. Repeat steps from 5.3 to 5.5 as many times as the number of ROIs you want (**Fig. 3**).
- 5.7. Define time periods of interest (TOI). Note: This allows for averaging perfusion in a ROI over a definite period of time (**Fig. 2**).
- 5.8. Go to Graph or Split view.
- 5.9. Select the TOI tool.
- 5.10. Click and hold on the graph at the position where you want the TOI to begin and drag the cursor to the desired end position. Then release the mouse button.
- 5.11. Export data from the mean value table for further processing.
- 5.12. Construct blood flow curves by a suitable software used for statistical analysis."

## 9. Please include single-line spaces between all paragraphs, headings, steps, etc.

The requested correction has been made.

**10.** After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Essential steps of the protocol for the video have been highlighted in yellow.

**11.** Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the



**outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.**

Representative Results has been changed in order to meet your requirements. One more figure (Fig. 5) and a paragraph has been added to it (line 226-228 with track changes enabled).

“Figure 5 shows a blurred intensity image and the perfusion graph of the entire image. The sudden peak on the graph indicates movement by the patient. The measurement was repeated immediately, after making sure that the patient is in a comfortable position.”

**12. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.**

Some additional information has been added to the Discussion section (line 297-319 and 337-353 with track changes enabled).

“The aim of this study was to introduce a novel technique for monitoring the neovascularization of a graft in the human gingiva. According to our previous results, LSCI assesses the blood perfusion of the gingiva with good repeatability and reproducibility (Molnar, Fazekas et al. 2018), when strict implementation of each step of the planned protocol as a critical requirement is met. LSCI is regarded as a semi-quantitative technique that requires calibration periodically to ensure accuracy and stability. During verification, the room temperature must be measured as accurately as possible, because this value is used by the verification algorithm to calculate perfusion.

The LSCI method is highly sensitive to the working distance setting and movement artifacts as well. In this study, working distance was fixed at 10 cm. The measurement area was 2.7 x 2 cm, which corresponds to an approximately three teeth wide gingival area. The effective frame rate was 16 images/s and 0.06 s/image as the arterial pulse induces pulsatile changes in gingival microcirculation (Molnar, Fazekas et al. 2018), which has to be averaged out from the recording. Rapid imaging reduced the risk of movement artefacts, too. However, in case of incorrect settings or patient movements, the recording should be stopped and repeated under optimum conditions.

Two operators took part in every measurement: one adjusted the LSCI head and controlled the computer while the other retracted the lips of the patient. In this study, three repeated measurements were performed in each session, each taking 30 seconds. Since measurements always involve some kind of irritation to the soft tissue due to the inevitable retraction of the lips and cheeks, which disturbs the microcirculation of the gingiva, an increase in random error occurs. Such inter-day variation, however, can be minimized by repeating the entire measurement process, i.e. by re-opening the mouth, retracting the soft tissue again, re-setting the camera's position and re-selecting ROIs in the software.”

“The methods used earlier for investigating graft vascularization are highly invasive, which meant a major restriction on measurement time points during healing, especially in human studies (Oliver, Loe et al. 1968, Janson, Ruben et al. 1969, Mormann, Bernimoulin et al. 1975, Busschop, de Boever et al. 1983, Vergara, Quinones et al. 1997, Schwarz, Rothamel et al. 2006, Rothamel, Benner et al. 2014). They also have limitations in terms of measuring regional differences quantitatively. Our previous studies (Molnar, Molnar et al. 2017, Molnar, Fazekas et al. 2018) have already proved the high reliability of LSCI in clinical trials and it was found to be useful to determine the time of soft tissue healing of an individual after tooth extraction in order to optimize implant placement (Fazekas, Molnar et al. 2018). In this study, the wound area covered by a xenogenic collagen graft showed excellent neovascularization, as on the 11<sup>th</sup> postoperative day all zones within the graft achieved the maximum blood flow level. However, it could be presumed that the collagen graft sloughed off or was resorbed by day 11 and we actually measured the revascularization of the recipient bed. In addition to its non-invasive feature, another special attribute of LSCI is a capability to characterize reperfusion curves at

various regions of a graft during incorporation at individual level. The centripetal characteristics of graft neovascularization are similar to previous histology observations (Janson, Ruben et al. 1969). This suggests that graft revascularization not only occurs from the periosteal vascular plexus but also from the wound margin.

The experiment presented shows that the revascularization of a graft can be clearly followed up if every step is followed strictly. However, on day 182, non-compliant patient preparation and instruction resulted in a significant increase in BF.”

### **13. References: Please do not abbreviate journal titles.**

Reference style has been modified. We used the available reference style file: jove.ens.

### **Response to Reviewer 1**

Authors present Laser Speckle Contrast Imaging (LSCI) as a novel method for monitoring blood flow changes and graft integration in a healing wound in the human oral mucosa. The paper is well written; title and abstract are appropriate for this methods article, and almost all used materials and equipment are listed. The protocol is well detailed: steps listed in the procedure are explained, critical steps are highlighted, and limitations of LSCI with regard to this application are discussed.

The following points could be discussed by the authors:

#### **\* L73: Specify the size of the pixels of the CCD camera**

The size of the pixels of the CCD camera has been added to the description (line 68-70 with track changes enabled):

“The CCD camera used in this system has an active imaging area of 1386 x 1034 pixels and its resolution is between 20-60  $\mu\text{m}$ /pixel depending on the size of the measurement area and on the setting of the software (low, medium, high).”

#### **\* The reported method was employed for a 17-year-old male patient. Since older bodies need more time to repair, would LSCI method be as sensitive and efficient for monitoring graft integration for older patients?**

LSCI is a sensitive and efficient technique in all ages. However, there are well-known age-related anatomical and functional changes in microcirculation (Bentov and Reed 2015), and delayed healing might need a few more days to measure. Our team has experience with monitoring wound healing after periodontal plastic surgery in older patients (Molnár, Molnár et al. 2017).

#### **\* In the section related to microcirculation image measurement:**

##### **- L157: explain why the framerate was set equal to 16 images/s**

A paragraph explaining LSCI setup has been added to the Discussion (line 306-309 with track changes enabled):

“The effective frame rate was 16 images/s and 0.06 s/image as the arterial pulse induces pulsatile changes in gingival microcirculation (Molnar, Fazekas et al. 2018), which has to be averaged out from the recording. Rapid imaging reduced the risk of movement artefacts, too.”

##### **- L159: indicate the unit for the time**

The unit for the time has been indicated.

**- L169: give more information about the indicator laser and include it in the table.**

Additional information has been added to paragraph 4.5 (line 151-153 with track changes enabled):

“Adjust the instrument’s head parallel to the measured area of the gingiva. Note: A built-in visible (650 nm) indicator laser facilitates the positioning of the imager relative to the subject.”

We cannot include the indicator laser in the table as it is not a separate instrument.

**\* In the legend of figure 4, L276: it is stated that smoothing value was set to 10.**

**- Authors should justify this value. They should also add this step to the protocol.**

**- Does smoothing value change from one patient to another? If yes, according to which factor?**

Additional information about smoothing has been added to the Discussion (line 328-331 with track changes enabled):

“To help visual evaluation, smoothing was turned on during recording and the smoothing value was set to 10. This means that perfusion was averaged over ten images for a smoother appearance of the perfusion image and in order to decrease background noise. However, smoothing is only a visual effect and does not influence actual recorded perfusion values.” The smoothing value does not change from one patient to another.

**\* LSCI is well-known to be a powerful tool for full-field imaging of blood flow in other medical fields (e.g. neuroscience, dermatology and ophthalmology). Additional information about the extent of the reported technique to these fields should be added in the discussion.**

A paragraph describing the significance of LSCI in other medical fields has been added to the Discussion (line 354-357 with track changes enabled):

“LSCI is extensively used for full-field imaging of vascular structure and associated blood flow in other tissues, like in the retina (Briers and Fercher 1982, Srienc, Kurth-Nelson et al. 2010), the skin (Briers and Webster 1996, Choi, Kang et al. 2004) and the brain (Ayata, Dunn et al. 2004) (Armitage, Todd et al. 2010). The most promising clinical applications of LSCI are burn wound assessment (Lindahl, Tesselaar et al. 2013) (Mirdell, Iredahl et al. 2016), evaluation of flaps (Zotterman, Bergkvist et al. 2016) and intraoperative cerebral blood flow monitoring (Hecht, Woitzik et al. 2009).”

**There are shortcomings that must be corrected:**

**\* L59: replace "prohibit" by "prohibits"**

**\* L223: replace "as" by "us"**

Corrections in grammar and typewriting have been made as the Reviewer suggested.

## **Response to Reviewer 2**

In this manuscript, the authors demonstrated that the high spatial resolution of Laser Speckle Contrast Imaging (LSCI) has possibility to reveal microcirculation in a healing wound in the human oral mucosa and gives useful information on graft integration. They show the neovascularization pattern of a xenogenic collagen graft with a clinical case. They provided detailed protocol and highlighted difficulties and possible failures during experimental process. The work in this manuscript deserves the publication in Journal of Visualized Experiments after a few minor revisions.

**1. References. Please recheck the reference format.**

Reference format has been modified. We used the available reference style file: jove.ens

**2. Please clearly explain each sub-figure in figure 2.**

Each sub-figure in Figure 2 has been explained in detail.

**“Figure 2: Split view (combination of the Images view and the Graph view) of a typical recording of gingival blood flow in the treated area.** Perfusion image (upper right sub-view) is a color-coded representation of blood perfusion in the gingiva. Areas of high perfusion are shown in red while areas of low perfusion are blue. The color range of perfusion images corresponds to 0-450 LSPU; smoothing was set to 10. An intensity image (lower right sub-view) is created by the total backscattered laser light. It corresponds exactly with the perfusion image and is useful for orientation and for identifying details in the perfusion image. Regions of interest (ROI) are always defined in the intensity image. The graph (upper left panel) shows real-time blood perfusion traces for each ROI in the recording. Check boxes to the left can be used to select which traces to show. Three consecutive measurements are shown on the graph. Each 30-second shot was identified as a TOI. A mean value table showing mean perfusion values in each ROI and TOI is also displayed in Split view (lower left panel).”

**3. It would be better if the author can add a title before each line in figure 4.**

Thank you for your recommendation. Since we would like to avoid further reduction in the size of the photos, we rewrote the legend of Figure 4 to clarify the order of the images.

**“Figure 4: Representative photographs (upper line), LSCI intensity image (middle line) and LSCI perfusion image (lower line) of the operated gingiva.** The images represent the preoperative state and perfusion, and wound healing and perfusion 1, 4, 7, 14, 21, 27 and 98 days postoperatively.”

## References

- Armitage, G. A., K. G. Todd, A. Shuaib and I. R. Winship (2010). "Laser speckle contrast imaging of collateral blood flow during acute ischemic stroke." *J Cereb Blood Flow Metab* **30**(8): 1432-1436.
- Ayata, C., A. K. Dunn, O. Y. Gursoy, Z. Huang, D. A. Boas and M. A. Moskowitz (2004). "Laser speckle flowmetry for the study of cerebrovascular physiology in normal and ischemic mouse cortex." *J Cereb Blood Flow Metab* **24**(7): 744-755.
- Briers, J. D. and A. F. Fercher (1982). "Retinal blood-flow visualization by means of laser speckle photography." *Invest Ophthalmol Vis Sci* **22**(2): 255-259.
- Briers, J. D. and S. Webster (1996). "Laser speckle contrast analysis (LASCA): a non-scanning, full-field technique for monitoring capillary blood flow." *J Biomed Opt* **1**(2): 174-179.
- Busschop, J., J. de Boever and H. Schautteet (1983). "Revascularization of gingival autografts placed on different receptor beds. A fluoroangiographic study." *J Clin Periodontol* **10**(3): 327-332.
- Choi, B., N. M. Kang and J. S. Nelson (2004). "Laser speckle imaging for monitoring blood flow dynamics in the in vivo rodent dorsal skin fold model." *Microvasc Res* **68**(2): 143-146.
- Fazekas, R., E. Molnar, P. Nagy, B. Mikecs, P. Windisch and J. Vag (2018). "A proposed method for assessing the appropriate timing of early implant placements: a case report." *J Oral Implantol*.
- Hecht, N., J. Woitzik, J. P. Dreier and P. Vajkoczy (2009). "Intraoperative monitoring of cerebral blood flow by laser speckle contrast analysis." *Neurosurg Focus* **27**(4): E11.
- Janson, W. A., M. P. Ruben, G. M. Kramer, A. A. Bloom and H. Turner (1969). "Development of the blood supply to split-thickness free gingival autografts." *J Periodontol* **40**(12): 707-716.

Lindahl, F., E. Tesselaar and F. Sjöberg (2013). "Assessing paediatric scald injuries using Laser Speckle Contrast Imaging." Burns **39**(4): 662-666.

Mirdell, R., F. Iredahl, F. Sjöberg, S. Farnebo and E. Tesselaar (2016). "Microvascular blood flow in scalds in children and its relation to duration of wound healing: A study using laser speckle contrast imaging." Burns.

Molnar, E., R. Fazekas, Z. Lohinai, Z. Toth and J. Vag (2018). "Assessment of the test-retest reliability of human gingival blood flow measurements by Laser Speckle Contrast Imaging in a healthy cohort." Microcirculation **25**(2).

Molnar, E., B. Molnar, Z. Lohinai, Z. Toth, Z. Benyo, L. Hricisak, P. Windisch and J. Vag (2017). "Evaluation of Laser Speckle Contrast Imaging for the Assessment of Oral Mucosal Blood Flow following Periodontal Plastic Surgery: An Exploratory Study." Biomed Res Int **2017**: 4042902.

Mormann, W., J. P. Bernimoulin and M. O. Schmid (1975). "Fluorescein angiography of free gingival autografts." J Clin Periodontol **2**(4): 177-189.

Oliver, R. C., H. Loe and T. Karring (1968). "Microscopic evaluation of the healing and revascularization of free gingival grafts." J Periodontal Res **3**(2): 84-95.

Rothamel, D., M. Benner, T. Fienitz, A. Happe, M. Kreppel, H. J. Nickenig and J. E. Zoller (2014). "Biodegradation pattern and tissue integration of native and cross-linked porcine collagen soft tissue augmentation matrices - an experimental study in the rat." Head Face Med **10**: 10.

Schwarz, F., D. Rothamel, M. Herten, M. Sager and J. Becker (2006). "Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat." Clin Oral Implants Res **17**(4): 403-409.

Srienc, A. I., Z. L. Kurth-Nelson and E. A. Newman (2010). "Imaging retinal blood flow with laser speckle flowmetry." Front Neuroenergetics **2**.

Vergara, J. A., C. R. Quinones, C. E. Nasjleti and R. G. Caffesse (1997). "Vascular response to guided tissue regeneration procedures using nonresorbable and bioabsorbable membranes in dogs." J Periodontol **68**(3): 217-224.

Zotterman, J., M. Bergkvist, F. Iredahl, E. Tesselaar and S. Farnebo (2016). "Monitoring of partial and full venous outflow obstruction in a porcine flap model using laser speckle contrast imaging." J Plast Reconstr Aesthet Surg **69**(7): 936-943.