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 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.”

“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. “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.

1. **System verification**
   1. Use the calibration box supplied. Remove the lid from the calibration box and shake it to avoid sedimentation in the colloidal suspension.
   2. Leave the lid off for 30 seconds to avoid bubbles.
   3. Put the lid back on the calibration box.
   4. Click *Advanced/ Verification/ Verify instrument*.
   5. Select *Routine verification* and click *Next*.
   6. Turn the head 90 degrees, fasten the calibration box using the integrated magnets and click *Next*.
   7. Enter the room temperature in the text box, select °C and click *Start*.
   8. Wait while the wizard completes the verification procedure.
   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).

1. “**Microcirculation image measurement**
   1. In the *File* menu, select and click on *New recording*. A new Image Window opens and the Setup panel is displayed.
   2. Under *Recording Setup*, select the following parameters:
      1. Select a Project: vestibulum (name of the project)
      2. Select a Site: tooth 14 (site examined in the oral cavity)
      3. Select a Subject or create a new Subject by opening the *Subject* drop-down list. The *Select subject* dialog appears.
         1. Click *New*
         2. Enter the name of the patient in the *New subject* dialog and click *OK*.
      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
      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
      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
      7. Select a point density (resolution): normal
      8. Under *Image Capture Setup*, select the number of images per second to record from the *Frame rate* drop-down list: 16 images/s
      9. In the *Duration* drop-down list, select *Time* and specify the duration of the recording: 0:30
      10. Select *Record with no averaging*
      11. Select the capture rate of the color photo: one per second
   3. Ask patient to open his mouth.
   4. Retract lips gently by two dental mirrors **(Fig. 1)**.
   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.
   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.
   7. Instruct the subject to remain still for the duration of the measurement.
   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.
   9. Remove dental mirrors and allow the patient to close his mouth and swallow.
   10. Switch back to the live image by pressing the *Resume recording* button.
   11. Repeat the steps from 4.3 to 4.10 twice.
   12. Press the *Stop recording* button.
   13. Close the file. The data are saved automatically.
2. **Offline analysis**
   1. Analyze the LSCI images using the built-in software. Go to Image or Split view

**(Fig. 2)**.

* 1. 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).
  2. Select the desired ROI shape within the ROI tools palette on the right.
  3. Select the *Apply* option in the ROI tools palette, which applies ROI operations to all images of the recording.
  4. 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. Repeat steps from 5.3 to 5.5 as many times as the number of ROIs you want **(Fig. 3)**.
  6. Define time periods of interest (TOI). Note: This allows for averaging perfusion in a ROI over a definite period of time **(Fig. 2)**.
  7. Go to Graph or Split view.
  8. Select the TOI tool.
  9. 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.
  10. Export data from the mean value table for further processing.
  11. 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 11th 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 refence 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 µm/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

1. **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).”

1. **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.”

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