Optical methods for the assessment of microvascular perfusion and oxygenation
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VALIDATION OF LASER SPECKLE IMAGING FOR ASSESSING MICROVASCULAR (RE)PERFUSION BY COMPARISON TO SIDESTREAM DARK FIELD IMAGING

Adapted from

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The present study was conducted to compare laser speckle imaging (LSI) with sidestream dark field (SDF) imaging (i.e., capillary microscopy) so as to validate the use of LSI for assessing microvascular (re)perfusion. For this purpose, LSI and SDF measurements were performed on the human nail fold during gradual occlusion of the upperarm circulation to modify nail fold perfusion under controlled circumstances. Additionally, a vascular occlusion test was performed to test the ability of LSI to detect rapid changes in tissue perfusion during reactive hyperemia and a hyperthermic challenge was performed to measure LSI perfusion at maximum functional capillary density. Normalized LSI measurements (i.e., normalized to baseline is 100%) were shown to correlate positively with normalized SDF measurements (Pearson’s r=0.92). This was supported by linear regression analysis (slope of 1.01, R²=0.85, p<0.001). During the vascular occlusion test, LSI perfusion decreased from 307±90 AU (baseline) to 42±8 AU (ischemia). Peak perfusion during reperfusion was 651±93 AU (212% of baseline), which had returned to baseline after 2 minutes. Hyperthermia increased LSI perfusion from 332±90 AU to 1067±256 AU (321% of baseline). The main finding was that changes in perfusion as measured by LSI correlated well with changes in capillary red blood cell velocities as measured by SDF imaging during controlled reduction of the (micro)vascular perfusion. It was further shown that LSI is capable of measuring tissue perfusion at high temporal and spatial resolution. In conclusion, LSI can be employed to accurately quantify microvascular reactivity following ischemic and hyperthermic challenges.
INTRODUCTION

Microvascular function is considered an important parameter in many clinical scenarios ranging from vascular surgery to critical care medicine [e.g., Klijn et al., 2008; Den Uil et al., 2008; Le Dorze et al., 2009]. A typical test for assessment of microvascular function, defined as the ability to increase microvascular flow after an ischemic challenge, is the vascular occlusion test, where the arterial and venous flow are transiently stopped and post-ischemic reactive hyperemia is monitored. Other tests for assessing microvascular function include thermal challenges, as applied in patients with systemic sclerosis and primary Raynaud’s phenomenon [Murray et al., 2009], and fluid and vasoactive drug challenges, as applied in intensive care patients [Büchele et al., 2007]. To monitor the effects of these tests several techniques can be used such as sidestream dark field (SDF) imaging [Goedhart et al., 2007], laser Doppler velocimetry (LDV) [O’Doherty et al., 2009], near-infrared spectroscopy (NIRS) [Skarda et al., 2007; Doerschug et al., 2007; Bezemer et al., 2009], and thermography [Hassan and Togowa, 2001]. However, each of these measurement techniques has specific shortcomings, such as low spatial and/or temporal resolution, required tissue contact, and small sampling area/volume, limiting their clinical applicability.

A technique potentially overcoming these shortcomings is laser speckle imaging (LSI) [O’Doherty et al., 2009; Briers, 2001]. The main advantage of LSI with respect to clinical applicability is the ability to measure tissue perfusion in large areas (e.g., skin grafts, burn wounds, complete organ surfaces) in a non-contact and thus non-perturbing manner at high spatial and temporal resolution using a relatively simple setup including a laser diode for illumination and a grayscale CCD camera for imaging [Briers, 2001, Wang et al., 2007]. An additional advantage of using a conventional CCD camera for imaging is that it allows a normal video mode for morphological identification of organ surfaces at high resolution, complementing the quantitative LSI data with anatomical and morphological detail.

To date, LSI has been used mainly in experimental research [e.g., Dunn et al., 2001; Cheng et al., 2003; Choi et al., 2004; Kharlamov et al., 2004] and only scarcely in patients [e.g., Steward et al., 2005; Dusch et al., 2007; O’Doherty et al., 2009], which is unfortunate inasmuch as LSI may circumvent the shortcomings associated with the currently used techniques [O’Doherty et al., 2009]. However, whether LSI is indeed sensitive to changes in capillary perfusion has not been validated before. Although percentual flow changes determined by LSI have been compared to the changes measured by LDV, LSI has never been juxtaposed to a quantitative technique capable of measuring tissue perfusion at the capillary level [Goedhart et al., 2007]. In this respect, intravital capillary microscopy constitutes a suitable quantitative technique for the assessment of the cutaneous microcirculation under resting conditions and after different provocation tests [Park et al., 2008; Wasik et al., 2009]. Hence,
validation of LSI by intravital capillary microscopy would provide strong support for the employment of LSI in settings that require information on (changes in) microcirculatory perfusion.

Sidestream dark field (SDF) imaging is a validated intravital microscopic imaging technique for imaging capillaries on organ surfaces and for measuring red blood cell velocities in individual capillaries [Goedhart et al., 2007]. In contrast to LDV, SDF therefore constitutes a useful tool for validating LSI responses to alterations in capillary perfusion. Consequently, the present study was conducted to compare LSI to SDF imaging so as to validate the use of LSI for assessing microvascular (re)perfusion. For this purpose, LSI and SDF measurements were performed on the human nail fold during gradual occlusion of the upperarm circulation to modify nail fold perfusion under controlled conditions. To determine the ability of LSI to detect rapid changes in tissue perfusion during reactive hyperemia a vascular occlusion test was performed. Additionally, a hyperthermic challenge was applied to measure tissue perfusion using LSI under conditions of maximal functional capillary density.

METHODS

Subjects
The study was approved by the medical ethics research board and informed consent was obtained for each subject. Ten healthy, non-smoking, male subjects receiving no medication voluntarily participated in the validation experiments. The subject population had a mean±SD age of 24±3 years and weight of 73±5 kg. Heart rate was 65±6 beats/min and systolic and diastolic blood pressures were 118±5 and 65±7 mmHg, respectively.

Laser speckle imaging setup
For LSI measurements, a commercially available system was used (Moor Instruments, Devon, UK). A 785-nm class 1 laser diode was employed for illumination of the tissue up to a depth of approximately 1 mm. Directly reflected light by the tissue surface was blocked by a tunable polarization filter placed in front of the lens system since it was not scattered by flowing red blood cells and therefore contained no information on tissue perfusion. Laser speckle images were acquired using a 576x768-pixel grayscale CCD camera at a frame rate of 25 Hz (exposure time of 4 ms) and converted to pseudo-color images, where the level of perfusion was scaled from blue (low perfusion) to red (high perfusion) [Cheng et al., 2003; Choi et al., 2004]. The lens system allowed a variable zoom, ranging from 0.6x0.8 cm (corresponding to 10 µm/pixel) to 9x12 cm at a working distance of 15-45 cm, and focus optimization. Using a 5x5 pixel window to calculate speckle contrast, the maximal image resolution was 50 µm/pixel.
Sidestream dark field imaging setup
For SDF imaging, a MicroScan video microscope (MicroVision Medical, Amsterdam, the Netherlands) was employed, which was fitted with a 5x magnifying objective lens system. The illumination intensity was optimized during imaging to obtain clear microcirculatory images. Focusing was achieved by axial translation of a 576×768-pixel grayscale CCD camera (frame rate of 25 Hz) with respect to the fixed lens system in the tip of the SDF probe. Image resolution was ~1.4 µm/pixel. The device was mounted in a specially engineered holder (Department of Instrumentation, Academic Medical Center, University of Amsterdam, the Netherlands) for accurate positioning and stabilization of the probe. Video output was visualized on a monitor and connected to a computer via a signal converter (ADVC110, Canopus Corporation, Kobe, Japan) for digital recording of the images onto a hard drive. From the SDF images, all in-focus capillaries were selected for further off-line analysis. Image analysis software developed for the SDF video images (Automated Vascular Analysis, MicroVision Medical) was used to generate space-time diagrams of single capillaries for quantitative measurement of red blood cell velocities [Goedhart et al., 2007; Dobbe et al., 2008]. The use of space time diagrams to determine red blood cell velocities is described extensively elsewhere [Dobbe et al., 2008]. In brief, a space time diagram is generated by plotting a selected capillary center line in time. Moving cells and plasma gaps in the selected capillary cause a pattern to appear in the space-time diagram and the orientation of this pattern is indicative for the capillary red blood cell velocity and is converted to an actual velocity value (velocity = distance / Δtime).

Validation protocol
The nail fold microcirculation was selected as the site for validation of LSI since it is easily accessible for both techniques and its perfusion can be systematically reduced using a pneumatic cuff placed around the upper arm. The cuff was inflated to 30, 60, 90, 120, 150, and 180 mmHg. Between each measurement, the cuff was deflated for >5 minutes to allow stabilization of the nail fold microcirculatory perfusion.

Vascular occlusion test and hyperthermic challenge
Following the validation protocol, a 3-minute VOT was performed with an occlusion pressure of 210 mmHg for 3 minutes. Subsequently, the occlusion pressure was released and reperfusion was recorded until the LSI perfusion value had restored to baseline. Five minutes after return to baseline, local hyperthermia was created by flowing heated air onto the hand. Skin temperature was measured using a skin temperature sensor (Smiths Medical ASD, Rockland, MA) and kept between 41 and 43°C by adjusting the distance between the heat source and the hand.
**Statistical analysis**

All data was analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). All data are presented as mean±SD. Comparison of LSI with SDF imaging was done by linear regression analysis and Pearson’s correlation analysis. For the SDF and LSI measurements, the inter-individual coefficient of variability (CV) was calculated as $CV = SD/\text{mean}$. Comparative analysis of tissue perfusion measured with LSI during normothermia and hyperthermia was performed using a paired Student’s t-test. Differences were considered statistically significant at $p<0.05$ and denoted with (*).

**RESULTS**

LSI perfusion maps for each step of occlusion pressure and the corresponding video image of the nail fold are shown in Figure 1.

**Validation of laser speckle imaging**

Perfusion measurements by LSI and SDF imaging for each occlusion pressure are shown in Figure 2. Baseline LSI perfusion was $321\pm186$ AU (CV=0.58) and baseline capillary red blood cell velocity as measured by SDF imaging was $791\pm332$ μm/s (CV=0.42). The reduction in perfusion following gradual occlusion of the upperarm circulation was similarly measured by LSI and SDF imaging as shown in Figure 2. Furthermore, Figure 2 shows that there is considerable spread in the absolute velocity values measured by SDF imaging, which reflects the normal physiological variability between the subjects.

![Figure 1](image1.png)

**Figure 1:** Representative laser speckle imaging perfusion maps for 0 (baseline), 30, 60, 90, 120, 150, and 180 mmHg occlusion pressure and the corresponding video image. Blue indicates low perfusion and red indicates high perfusion.
Overall, normalized LSI measurements (i.e., normalized to baseline equals 100%) were shown to correlate positively with normalized SDF measurements (Pearson’s $r=0.92$). This was supported by linear regression analysis as shown in Figure 3 (slope of 1.01, $R^2=0.85$, $p<0.001$).

**Vascular occlusion test and hyperthermic challenge**

A typical LSI trace during the VOT is presented in the left panel of Figure 4. In the right panel of Figure 4 it is shown that baseline (BSLN) LSI perfusion was $307\pm90$ AU (CV=0.29) which was reduced to $42\pm8$ AU (CV=0.19) during ischemia (ISCH). Peak perfusion (PEAK) during reperfusion after 3 minutes of ischemia was $651\pm93\pm\sigma$ AU (CV=0.14) (212% of baseline) which decreased to $422\pm107$ AU (CV=0.25) after 1 minute of reperfusion (1 min) and was fully restored to baseline level, $309\pm92$ AU (CV=0.30), after 2 minutes of reperfusion (2 min).

**Figure 2:** Perfusion data for the laser speckle imaging (LSI) and sidestream dark field (SDF) measurements for each occlusion pressure (0-180 mmHg). LSI data are plotted against the left y-axis and SDF data are plotted against the right y-axis allowing optimal visual comparison of the perfusion data obtained using each technique as a function of occlusion pressure.

**Figure 3:** Linear regression analysis on normalized laser speckle imaging (LSI) data versus normalized sidestream dark field (SDF) imaging data. Data were normalized so that baseline = no occlusion pressure = 100%. Pearson’s $r = 0.92$, slope = 1.01, $R^2 = 0.85$, $p < 0.001$. 


AU (CV=0.14) (212% of baseline) which decreased to 422±107 AU (CV=0.25) after 1 minute of reperfusion (1 min) and was fully restored to baseline level, 309±92 AU (CV=0.30), after 2 minutes of reperfusion (2 min).

A typical LSI trace during increasing skin temperature is presented in the left panel of Figure 5. In the right panel of Figure 5 it is shown that tissue perfusion as measured using LSI increased from 332±90 AU (CV = 0.27) during normothermia to 1067±256 AU (CV = 0.24) (321% of baseline) during hyperthermia. The LSI perfusion during hyperthermia is significantly higher than the LSI peak perfusion during reactive hyperemia (p<0.05). No statistical differences were identified for the baseline LSI perfusion during the validation experiment, the vascular occlusion test, and the hyperthermic challenge (p>0.05).

**Figure 4**: Left: typical laser speckle imaging (LSI) perfusion trace during the vascular occlusion test. Right: perfusion changes as measured using laser speckle imaging (LSI) during the vascular occlusion test. BSLN = baseline, ISCH = ischemia, PEAK = peak, 1 min = 1 minute of reperfusion, 2 min = 2 minutes of reperfusion. *p<0.05 vs. BSLN and †p<0.05 vs. previous time point.

**Figure 5**: Left: typical laser speckle imaging (LSI) perfusion trace during heating of the skin, starting at 30 s. Right: perfusion changes as measured using laser speckle imaging (LSI) during heating of the skin. *p<0.05 vs. normothermia.
DISCUSSION AND CONCLUSIONS

The aim of the study was to validate near-infrared LSI for assessing microvascular (re)perfusion by comparison to a technique capable of quantitatively measuring capillary red blood cell velocities, i.e., SDF imaging. The main finding was that changes in perfusion as measured using LSI correlated well with changes in capillary red blood cell velocities as measured using SDF imaging during controlled reduction of the (micro)vascular perfusion. We have furthermore shown that LSI is capable of measuring tissue perfusion in high temporal and spatial resolution and that this technology can be used to assess microvascular reactivity to ischemic and hyperthermic challenges.

This is the first study to validate the use of LSI for the assessment of tissue perfusion at the microcirculatory level by comparison to capillary red blood cell velocities measured quantitatively with SDF imaging. Since SDF imaging allows direct visual observation of red blood cells flowing through individual capillaries, this technique serves as a gold standard for measuring microcirculatory perfusion. Hence, in contrast to LDV, comparison of LSI to SDF measurements constitutes a reliable means to validate LSI responses to alterations in capillary perfusion. During controlled gradual reduction of the nail fold capillary perfusion, LSI and SDF imaging were shown to be in good agreement (correlation and linear regression analysis), indicating that LSI perfusion is a sensitive parameter for the detection of changes in tissue perfusion at the microcirculatory level.

In the validation protocol, the SDF measurements demonstrated a large variability in baseline capillary red blood cell velocities between each volunteer (CV=0.42), which may have been caused by the native perfusion variability between the individuals. Contrasting, baseline variability in LSI measurements (CV=0.58), although of similar extent as to the variability in SDF-derived velocities (Figure 2), is difficult to interpret due to the fact that LSI measures movement of cells in tissue irrespective of the direction in which they move. The measurement is, among other factors, influenced by the position of the camera with respect to the target tissue. Hence, application of LSI is more appropriate for intra-individual comparison of perfusion (e.g., perfusion at different time points) rather than inter-individual comparison (e.g., perfusion in different patient groups).

It must be noted that both laser Doppler and laser speckle techniques essentially measure average velocity rather than flow or perfusion. As the distribution of underlying velocities is unknown, it is safer to use these techniques for relative rather than absolute measurements [Briers, 2001; Draijer et al., 2009]. Indeed, Cheng and Duong have recently shown that the assumption of a Lorentzian velocity distribution is valid for calculating relative blood flow (or perfusion) changes rather than absolute values [Cheng and Duong, 2007].

Using LSI in clinical research would overcome some limitations of other commonly used techniques used to measure microcirculatory (re)perfusion, such as SDF imaging, LDV, NIRS, and thermography. Although LSI and SDF imaging
were shown to correlate well during controlled reduction of capillary blood flow velocity during the validation protocol, SDF imaging could not be used to quantitatively determine red blood cell velocities during post-occlusive reactive hyperemia. High red blood cell velocities will result in a vertically rather than diagonally oriented pattern in the space-time diagrams, making it impossible to determine red blood cell velocities (velocity = distance / ∆time). On the other hand, LDV as a fiber-based modality is able to measure (micro)vascular perfusion at high speed, but only in a small volume of tissue and is therefore very sensitive to measurement site. As an imaging modality (i.e., scanning LDV), LDV overcomes this shortcoming. However, LDV imaging is a relatively slow technique (typically 10-60 s/image) and therefore not able to detect the rapid perfusion changes during reactive hyperemia [O’Doherty et al., 2009]. NIRS is a spectroscopic technique for measuring microcirculatory oxygenation and supra-baseline levels of oxygenation following a period of ischemia (i.e., reactive hyperemia) [Bezemer et al., 2009]. However, since NIRS is measuring oxygenation, this technique provides only limited information on the actual microvascular flow alterations. Thermography, in contrast, is indeed capable of generating images of tissue perfusion at a high frame rate. However, thermography is based on changes in tissue temperature following changes in tissue perfusion and is therefore integrative in both the time- and space-domain. Due to the thermal capacity of the tissue, rapid changes in tissue perfusion do not immediately induce rapid changes in tissue temperature and, furthermore, thermal diffusion limits the spatial resolution of the technique. In this paper we have shown that LSI overcomes these issues and that it is capable of measuring tissue perfusion at both high spatial and temporal resolution at rest and during different provocation tests.

To date, LSI has been applied scarcely in a clinical setting [e.g., Stewart et al., 2005; Murray et al., 2009; Dusch et al., 2007]. In a surgical setting a macroscopic, non-contact technique such as LSI would be preferable over SDF imaging and LDV because the latter techniques are limited by the small measuring surfaces and require physical contact with the tissue surface. Compared to the available macroscopic non-contact imaging techniques such as video thermography and scanning laser Doppler flowmetry, LSI has a better spatial and temporal resolution [Murray et al., 2009; O’Doherty et al., 2009]. The high spatial resolution allows for the matching of local perfusion to anatomical characteristics, making it a potentially useful technique for e.g., neuro-, vascular-, and transplantation surgery. Furthermore the high temporal resolution enables the possibility to study responses of the microvasculature to pharmacological interventions during intensive care and surgery.

In conclusion, we have shown that LSI responds linearly to changes in red blood cell velocity in the nail fold of healthy volunteers during gradual occlusion. Therefore LSI is a valid modality for measuring microcirculatory perfusion both at rest and during different provocation tests. Additionally, LSI is a macroscopic
non-contact imaging technique with a high spatial and temporal resolution, rendering it especially suitable for clinical and experimental scenarios where surface contact is undesirable and microcirculatory perfusion in large areas (e.g., skin grafts, burn wounds, organs) requires online monitoring.