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## **Reproducibility of high-resolution laser speckle contrast imaging approaches to assess cutaneous microcirculation for wound healing monitoring in mice**

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## Introduction

The cutaneous microcirculation has a key role in wound healing, through oxygen and nutrient supply to the skin tissue. Regulation of blood flow depends of several vasodilating and vasoconstricting mechanisms mediated by substances that are released by the endothelium to surrounding vascular smooth muscle cells, mediating vascular tone. In several diseases in which regulation of the cutaneous microcirculation is impaired, patients develop ulcers (Cracowski and Roustit, 2020). Monitoring of skin microvascular reactivity is therefore of interest when studying wound healing in these diseases.

Historical assessment of in vivo skin microvascular function relies on laser Doppler flowmetry (LDF) or laser Doppler imaging (LDI). They both use the doppler effect to estimate blood flow. Yet, they have limitations, i.e. low spatial resolution (LDF) or low temporal resolution (LDI) (Roustit and Cracowski, 2013). A recent different technology, laser speckle contrast imaging (LSCI) combines an excellent spatial resolution while providing high frame rate (Draijer et al., 2009).

LSCI exhibits an excellent temporal and spatial reproducibility in humans (Puissant et al., 2013; Roustit et al., 2010), and has since become commonly used to assess human cutaneous microvascular reactivity (Loader et al., 2017; Roustit et al., 2018; Ruaro et al., 2018; Zhao et al., 2019). LSCI was initially used in animals to quantify cerebral blood flux (Wang et al., 2010). It has also been used in rodents on other organs such as the intestine (Zhu et al., 2007) kidneys (Mottard et al., 2020), skin, eyes and ears. (Du et al., 2015; Li and Wang, 2017; Remer et al., 2019). Recently, the use of LSCI on mouse skin has gained interest as a tool to assess wound healing (Hedhli et al., 2020; Pan et al., 2018; Tang et al., 2018). However, its reproducibility and performance against a reference technique (i.e. laser Doppler) have never been assessed.

Recent development has led to the marketing of enhanced LSCI devices, with high spatial resolution (up to 20µm/pixel resolution; LSCI-HR) in addition to their high temporal resolution. In addition, new imaging systems integrate reflectance spectrometry allowing the mapping of haemoglobin oxygenation (LSCI-OX) through the use of RGB LED and additional visible light sensor (Steimers et al., 2013).

The objective of this study was to evaluate the reproducibility of LSCI-HR and LSCI-OX, and to compare them to LDI in the assessment of cutaneous blood flux during wound healing monitoring (WHM) of excisional wounds in mice.

## Material & methods

### *Animals*

Two protocols were performed on male C57Bl/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) aged 10 weeks old. Animals were housed in controlled conditions with ambient temperature of 22°C, 12h/12h light-dark cycle and with food and water *ad libitum*. Mice were anesthetized with isoflurane and depilating on the back with clipper and depilatory cream before experimentation. All the experiments were approved by the Grenoble Animal Ethics Committee.

### *Protocol 1: Inter-day reproducibility of blood flux measurement*

Sixteen mice per group were included (i.e. 48 total). Eight were removed after depilation (and before experimentation) because of pigmented skin (Plikus and Chuong, 2008). Mice were anesthetized with isoflurane (1.5% isoflurane concentration with a 50/50 mix of ambient air and pure oxygen at 1l/min). Skin temperature was kept constant with a target at 34.5°C, using a thermoregulated enclosure. After an acclimatization period, cutaneous blood flow was recorded for 3 min. These measurements were done first at day 0 and were repeated 2 days later. The three devices were tested across three separate sessions, one device per testing session.

### *Protocol 2: Inter-day reproducibility and daily assessment of wound perfusion*

In this protocol, 28 mice underwent preoperative anaesthesia 30 min before ulcer creation, using subcutaneous buprenorphine (0.1mg/kg). Each mouse received two 40mm<sup>2</sup> wounds on the back

using a 6-mm biopsy punch, on each side of the spine and approximately 3 cm away from the tail. Wounds were covered using a transparent dressing (Tegaderm®, 3M Healthcare, St Paul, MN, USA). Skin perfusion was assessed the day after ulcer creation, and was subsequently assessed daily over 8 days, using LDI and LSCI-HR, consecutively (n=14), or LSCI-OX (n=14).

During the measurement of skin perfusion, the mice were anesthetized with isoflurane at same concentrations than in protocol 1 and kept warm using a heating pad set to 37.5°C, to maintain skin temperature at 34.5°C. A thin pad was placed between the skin and the dressing to avoid erythema when the latter was removed. After a 5-min acclimatization period, skin blood flow was recorded.

### *Blood flow measurement*

We used LDI (PeriScan PIM 3, Perimed AB, Järfälla, Sweden) with a laser wavelength was 650-690 nm. The laser head was placed 22 cm above the skin and the image size was 2.8 x 2.1 cm. The resolution was 1 mm/pixel for protocol 1 and 0.66 mm/pixel for protocol 2. Image acquisition rate was 1 every 30 sec.

For LSCI-HR (PeriCam PSI HR, Perimed AB, Järfälla, Sweden), the laser wavelength was 785 nm. The laser head was placed 10 cm above the skin and the image size was 2.8 x 2.1 cm. The resolution was set at 60 µm/pixel (medium resolution) for protocol 1 and 20 µm/pixel (high resolution) for protocol 2. Image acquisition rate was 1 sec<sup>-1</sup> for both protocols.

For LSCI-OX (MoorO2Flow, Moor Instruments, Devon, UK). The laser head was placed 28 cm above the skin for protocol 1 and 25 cm away from the skin for protocol 2. For protocol 1 resolution was 33 µm/pixel and image size was 2.5 x 1.8 cm; and for protocol 2 resolution was 20 µm/pixel and size was 1.7 x 1.3 cm. Image acquisition rate was 1 sec<sup>-1</sup> for both protocols.

### *Data analysis*

Data were analysed using the manufacturer's software for each instrument (PIMsoft v1.5 and MoorO2Flo Review v1.0). For protocol 1, the cutaneous blood flux was expressed as arbitrary perfusion units and averaged over a standardized 80 mm<sup>2</sup> circular region of interest (ROI), and three duration of recordings (also named time of interest (TOI) by manufacturers) to explore temporal variability of the devices (Rousseau et al., 2011): 30 sec, 90 sec and 180 sec. For protocol 2, measurements were performed on 2 different areas for each wound: the ulcer area, and a 2-mm wide periulcer area. For both protocols, haemoglobin oxygenation was expressed as relative change of oxyhaemoglobin and deoxyhaemoglobin concentration measured relative to the start of the measurement.

### *Statistical analysis*

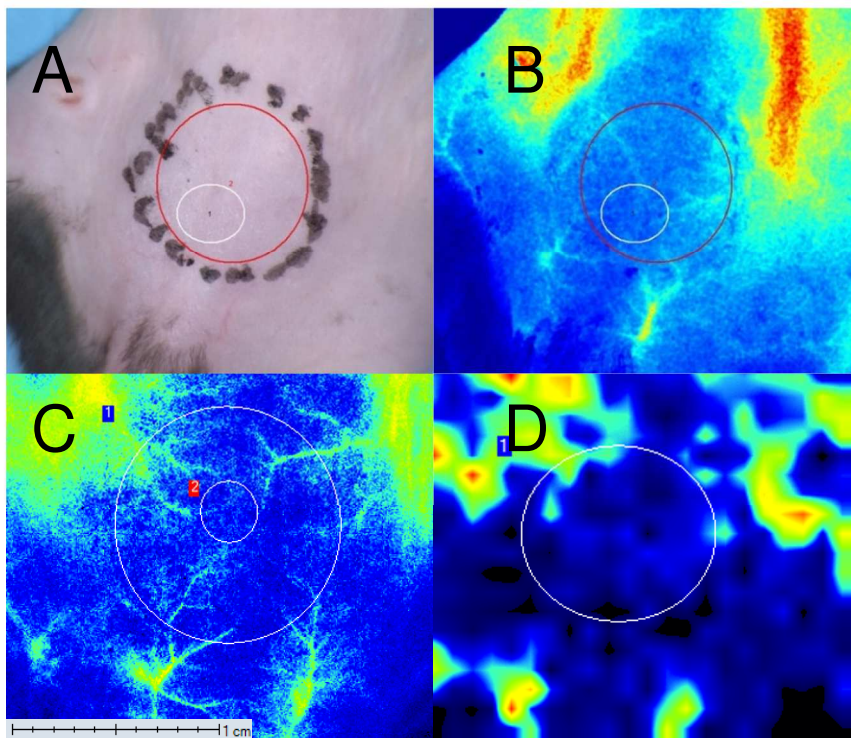
Quantitative data are expressed as mean and standard deviation. Reproducibility was expressed as within-subject coefficient of variation (CV) (Bland, 2000; Roustit et al., 2010). A CV of less than 35% was considered acceptable (Harris et al., 2007). CVs were compared using a method adapted from Levene's test (Gautier-Veyret et al., 2019). For protocol 2, the correlation between LDI and LSCI was performed only for the LSCI-HR, since measurements could be done on the same animals. Correlations were assessed using Spearman correlation coefficient. We then calculated an average correlation using Fisher Z transformation (Corey et al., 1998). Post Hoc tests were corrected for multiplicity using bonferroni correction. Two-sided significance tests were used throughout. P values of <0.05 were considered significant. Statistical analyses were performed using SPSS 21.0 for Windows (IBM, SPSS, Chicago, IL).

## **Results**

### *Protocol 1: Inter-day reproducibility of blood flux measurement*

Mean skin temperature at D0 was 34.77°C (0.76), 34.48°C (0.98), 31.70°C (1.30) for LSCI-HR, LSCI-OX and LDI respectively. Mean skin temperature at D2 was 35.47°C (0.65), 34.51°C (0.75), 31.98°C (1.22) for LSCI-HR, LSCI-OX and LDI respectively. Inter-day reproducibility of blood flux measurement was very good (CVs between 12 and 17%) for both LSCI-HR and LSCI-OX irrespective of the duration of the recording. However, reproducibility was poorer for LDI, with CVs

ranging between 26 and 29%, although the difference did not reach statistical significance (Table 1). Since the high resolution of both LSCI devices allowed visualisation of the vessels, we performed a post hoc analysis on a smaller ROI of 8 mm<sup>2</sup>, on an area without visible vessels (Figure 1). Reproducibility results were similar on this smaller area (Table 1). On the other hand, the reproducibility of haemoglobin oxygenation was poor (CVs>200%).



**Figure 1.** Recording data from laser speckle contrast imaging and Laser Doppler Imaging. A. Color Photo, B. LSCI-OX, C. LSCI-HR, D. LDI. The *post hoc* analysis on the smaller area without visible vessels was conducted for LDI, since the vessels were not visible.

#### *Protocol 2: Inter-day reproducibility and daily assessment of wound perfusion*

We observed a good positive correlation (average  $r=0.77\pm0.24$ ) between perfusion measured with LDI and LSCI-HR on the periulcer area. However, the correlation was weaker within the ulcer area ( $r=0.36 \pm 0.24$ ) (Table S1).

CVs were higher in LDI and LSCI-OX in both ulcer and periulcer ROIs (Table 2). Paired comparisons show a better reproducibility in the ulcer area for LSCI-HR compared to LDI, but there was no significant difference between LSCI-HR and LSCI-OX, or between LSCI-OX and LDI (Table 2). In the periulcer area, we observed a trend but no statistically significant difference between the three measurement methods. Haemoglobin oxygenation could not be recorded within the wound and was poorly reproducible in the periulcer area (CVs > 160%).

**Table 1.** Reproducibility of blood flux measurement assessed with PeriCam PSI High Resolution, MoorO2FLO and PeriScan PIM3 System on the back between day 0 (D0) and day 2 (D2).

	Duration of recordings (s)	Region of interest surface (mm <sup>2</sup> )	D0	D2	CV (%)
LSCI-HR (n=15)	30	80	93.41 ± 18.15	96.38 ± 13.29	13.64*
		8	88.63 ± 17.14	90.99 ± 12.83	14.78

	90	80	93.51 ± 17.32	94.48 ± 13.41	13.54 <sup>#</sup>
		8	88.83 ± 16.98	88.95 ± 12.66	15.08
	180	80	92.24 ± 18.60	92.24 ± 13.27	12.68 <sup>\$</sup>
		8	87.71 ± 17.69	86.74 ± 12.60	14.18
LSCI-OX (n=13)	30	80	263.05 ± 73.70	245.82 ± 57.81	16.45 <sup>*</sup>
		8	248.75 ± 77.68	241.01 ± 58.23	14.76
	90	80	249.95 ± 32.41	249.01 ± 58.24	13.96 <sup>#</sup>
		8	235.16 ± 37.57	242.59 ± 58.04	13.72
	180	80	246.14 ± 24.84	249.25 ± 58.34	14.65 <sup>\$</sup>
		8	231.74 ± 31.83	243.06 ± 58.12	14.90
LDI (n=12)	30	80	106.35 ± 42.04	134.43 ± 49.05	26.36 <sup>*</sup>
	90	80	64.27 ± 55.19	135.47 ± 54.01	27.53 <sup>#</sup>
	180	80	73.88 ± 56.83	138.03 ± 58.06	28.57 <sup>\$</sup>

Skin blood flux recorded at day 0 (D0) and day 2 (D2) are expressed as mean (± standard deviation) arbitrary perfusion units, and reproducibility is expressed as the within-subject coefficient of variation. \*P=0.077; #P=0.105; \$P=0.081.

**Table 2.** Reproducibility of measurements between left and right ulcer for each day expressed.

ROI	Method	D1	D2	D3	D4	D5	D6	D7	D8	Mean CV ± SD
Ulcer	LDI	26.7	27.0	30.1	23.9	24.2	19.7	27.2	39.0	26.8 ± 6.3
	LSCI-HR	12.8	14.6	9.2	15.4	10.4	11.4	9.8	12.4	12.0 ± 2.2
	LSCI-OX	16.4	17.1	21.2	16.2	21.5	19.1	17.3	29.1	19.7 ± 4.3
P-value		0.015* <sup>\$</sup>	0.319	0.012 <sup>*</sup>	0.166	0.042 <sup>*</sup>	0.088	0.012 <sup>*</sup>	0.003 <sup>*</sup>	
Periculer	LDI	23.2	20.5	18.9	24.6	23.9	24.9	22.6	28.0	23.3 ± 2.8
	LSCI-HR	11.3	12.1	9.5	14.4	11.6	13.8	12.9	14.2	12.5 ± 1.7
	LSCI-OX	18.6	19.8	24.5	21.7	27.8	20.5	20.8	35.2	23.6 ± 5.5
P-value		0.092	0.030	0.055	0.208	0.043	0.034 <sup>*</sup>	0.092	0.054	

Data are expressed as within subject coefficients of correlation (CV). P-values were computed with adapted Levene's test. Significant post hoc p-value: \* LDI vs LSCI-HR; \$ LDI vs LSCI-OX. P-values for all post hoc comparisons are provided in table S2.

## Discussion

In the present study, we show that two recently developed laser speckle imagers exhibit very good inter-day reproducibility of blood flux measurement in healthy mouse skin, better than LDI, consistent with results in humans (Roustit et al., 2010). The two imagers used in the present study (i.e. LSCI-HR and LSCI-OX) had very similar CVs, and those did not change according to the duration of recording or the surface area recorded, as has been observed in humans (Rousseau et al., 2011). Similar results were found on wounded skin, where LSCI-HR and LSCI-OX showed higher intra-individual reproducibility in comparison with LDI, especially on the ulcer area. At the wound site, the increased variability due to small haemorrhages may interfere with the signal. In addition, subcutaneous vascular structures differ between the two wounds (as seen on Figure S2) and between each animal. This results in greater variability and a weaker correlation between techniques on the ulcer area.

Differences in reproducibility between LSCI devices and LDI may be explained by the higher spatial and temporal resolutions of LSCI over LDI. Indeed, while these full-field imaging techniques scan skin blood flux over wide areas, the sampling frequency of LDI is much slower than that of LSCI, which results in rougher averaging of skin blood flux. In addition, the depth of skin perfusion measurement is different (0.5-1 mm for LDI and 0.3mm for LSCI) (O'Doherty et al., 2009).

Robust and reproducible methods to assess skin blood flux in rodents are needed to conduct studies on wound healing, or to assess the effect of vasoactive drugs for example. These results thus provide valuable data to estimate the variability of imaging techniques assessing skin blood flow in mice, which, like in human studies, is needed for sample size calculation or estimation of study power. Although the reproducibility of LSCI has been widely studied in humans, there was, to our knowledge, limited data in rodents. Yet, the reproducibility of these techniques cannot be extrapolated from results in humans. Indeed, skin structure is different in mice, with an epidermal thickness <25 µm, and dermal thickness of 200-300µm depending on the age, strain and hair growth stage of the animal (Khavari, 2006; Lee et al., 2016; Monteiro-Riviere et al., 1991; Wei et al., 2017). Furthermore, murine hypoderm has two layers, dermal adipose tissue and subcutaneous adipose tissue (Driskell et al., 2014), which develop independently (Wojciechowicz et al., 2013). These two fatty tissues are separated by a thin layer of muscle tissue called *Panniculus carnosus*.

There were several technical limitations in this study. First, the lower resolution of LDI made the distinction between the ulcer and the periulcer areas less accurate than with LSCI. In addition, skin temperature was lower for LDI. This was due to an interference between the laser beam and the Plexiglas of the thermoregulated enclosure, which had to be kept open during the experiments. Although this may have affected the correlation between LDI and LSCI-HR, it is unlikely to have altered the reproducibility of LDI, since temperature was stable between measurements. In addition, the setting was simplified to maintain skin temperature at 34.5°C in protocol 2, since the use of the thermoregulated enclosure is time-consuming when measurements are repeated daily. However, we estimated that changes in perfusion related to wound healing might be less sensitive to small variations in skin temperature. Instead, we favored to have a higher spatial resolution for ulcer evaluation (protocol 2), in order to be able to study the spatial variability of the peri-ulcer area as finely as possible. Another issue was met with the use of LSCI-OX to assess wounds, due to the reflection of RGB lights used for the oximetry measurement on the exposed wound fascia. These reflections interfered with blood flow measurements and prevented oximetry from being recorded on the ulcer area.

In conclusion, two recent high resolution LSCI devices are well suited and reproducible to assess skin perfusion in mice. However, the reproducibility of haemoglobin oxygenation assessment with reflectance spectroscopy is poor. When studying wound healing, LSCI and reflectance spectroscopy interfered, suggesting that LSCI-OX is less suited in this specific indication. Further studies on microvascular reactivity tests (e.g. pharmacological), for which such interference is less likely, should be conducted.

## **Conflict of interest**

None to disclose.

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