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Effect of anti-VEGF on retinal blood flow in diabetic mice using laser speckle flowgraphy

Zheng Zhe Steven Su[1,2,3] | **Bryan Chin Hou Ang**[1,4,5] | **Praveen Kumar Balne**[6] | **Sai Bo Bo Tun**[7] | **Hla Myint Htoon**[7] | **Leopold Schmetterer**[2,7,8,9,10,11,12,13,14] | **Veluchamy Amutha Barathi**[7,8,9] | **Rupesh Agrawal**[1,2,7,9]

[1]National Healthcare Group Eye Institute, Tan Tock Seng Hospital, Singapore, Singapore
[2]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore
[3]Ministry of Health Holdings, Singapore, Singapore
[4]National Healthcare Group Eye Institute, Woodlands Health, Singapore, Singapore
[5]Ophthalmology, Mayo Clinic, Jacksonville, Florida, USA
[6]College of Veterinary Medicine, University of Missouri, Columbia, Missouri, USA
[7]Singapore Eye Research Institute, Singapore National Eye Centre, Singapore, Singapore
[8]Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
[9]Ophthalmology and Visual Sciences Academic Clinical Program, Duke-NUS Medical School, National University of Singapore, Singapore, Singapore
[10]School of Chemistry, Chemical Engineering and Biotechnology, Nanyang Technological University, Singapore, Singapore
[11]Department of Clinical Pharmacology, Medical University Vienna, Vienna, Austria
[12]Center for Medical Physics and Biomedical Engineering, Medical University Vienna, Vienna, Austria
[13]SERI-NTU Advanced Ocular Engineering (STANCE), Singapore, Singapore
[14]Institute of Molecular and Clinical Ophthalmology, Basel, Switzerland

Correspondence
Rupesh Agrawal, National Healthcare Group Eye Institute, Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433, Singapore.
Email: rupesh_agrawal@ttsh.com.sg

Veluchamy Amutha Barathi, Singapore Eye Research Institute, The Academia, 20 College Road, Singapore 169856, Singapore.
Email: amutha.b.veluchamy@seri.com.sg

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Abstract

Purpose: To assess intra- (repeatability) and inter-observer (reproducibility) variability of laser speckle flowgraphy (LSFG) for retinal blood flow (RBF) measurement in 20 eyes of wild type (C57BL/6J) mice and effect of intravitreal Aflibercept on RBF in optic nerve head (ONH) region of 10 eyes of Ins2 (Akita) diabetic mice.

Methods: 'Mean blur rate (MBR)' was measured for all quadrants of tissue area (MT), vessel (MV) and total area (MA) of ONH region. Changes in MT were analysed at each timepoint. Repeatability was evaluated by measuring MBR variability without changing mouse head position, and reproducibility after re-setting mouse head position by another operator. Coefficient of repeatability (CR) through Bland–Altman plot method coefficient of variation (COV) and Intraclass correlation coefficient (ICC) was calculated. Intravitreal Aflibercept (1 μg) was administered to Akita eyes and intraocular pressure (IOP) was measured using a tonometer at baseline, day 7, 14, 21 and 28 post-injection. Hurvich and Tsai's criterion was used.

Results: Coefficient of repeatability values of repeatability and reproducibility for all quadrants were within limits of agreement. Reliability was excellent (ICC 0.98–0.99) and reproducibility was moderate to excellent (ICC 0.64–0.96). There was a non-significant IOP increase in all Akita eyes at Day 28 ($p > 0.05$), and significant increase in MT in all quadrants at Day 21 and superior, inferior and temporal quadrants at Day 28 ($p < 0.05$).

Conclusion: Laser speckle flowgraphy demonstrates excellent repeatability and moderate to excellent reproducibility in measuring RBF. Intravitreal

Aflibercept injection results in a significant increase in MT up to 28 days post-injection without significant increase in IOP.

KEYWORDS
anti-VEGF, intraocular pressure, laser speckle flowgraphy, reliability, reproducibility, retinal blood flow, wild type mouse

1 | INTRODUCTION

Retinal disease, including diabetic retinopathy (DR) and age-related macular degeneration (AMD), remains one of the leading causes of blindness in the world (Flaxman et al., 2017). Retinal blood flow (RBF) is often decreased in these conditions (Bek, 2017; Harris et al., 1999; Pemp & Schmetterer, 2008; Wei et al., 2018), accompanied by an increase in vascular endothelial growth factor (VEGF), which is released by retinal cells in response to tissue damage and ischemia (Gupta et al., 2013). VEGF activates endothelial cells, promotes cell proliferation and migration and increases vascular permeability (Bates, 2010). In recent years, anti-VEGF agents have been used to reduce VEGF levels, which in turn improves retinal blood flow and reduces both retinal and choroidal ischemia (Cheung et al., 2014; Tah et al., 2015; Yorston, 2014). They have been useful in treating retinal diseases such as AMD and Vogt-Koyanagi-Harada (VKH) disease (Takahashi et al., 2021; Yamaguchi et al., 2022).

Several techniques have been employed in the study of RBF, including bidirectional Laser Doppler Velocimetry (LDV), Doppler Fourier-domain Optical Coherence Tomography (D-OCT), Laser Doppler Flowmetry (LDF) and Laser Speckle Flowgraphy (LSFG) (Doblhoff-Dier et al., 2014; Garhofer et al., 2012; Haindl et al., 2016; Luft et al., 2016; Nakazawa, 2016; Pechauer et al., 2018; Riva et al., 2010; Sugiyama et al., 2010; Werkmeister, Dragostinoff, et al., 2012; Werkmeister, Palkovits, et al., 2012).

Laser speckle flowgraphy (LSFG) is an emerging non-invasive imaging technique, which uses laser speckle phenomenon to measure RBF and has been successfully applied in both humans and animals to study blood flow in different ocular diseases. Images of the speckle pattern in the retina produced by random laser interference are analysed, with the variation in these patterns corresponding to the blood flow in the retina. Flow rate is assessed as mean blur rate (MBR), with the MBR of tissue area (MT) corresponding to blood flow in the retinal tissue (Kunikata & Nakazawa, 2016).

The temporal retina is nourished by the short posterior ciliary artery, and its blood flow was reported to be usable for interindividual and intergroup comparisons (Aizawa et al., 2014). Furthermore, in a normal population, MV was reported to be affected by age and sex, while MT was not (Aizawa et al., 2016). This makes MT an important measurement that is stable and independent of age or sex in assessing repeatability and reproducibility of LSFG for RBF.

The first studies of using LSFG to measure RBF were conducted in the early 1980s by imaging the retina on film and using high-pass spatial filtering optical techniques to obtain speckle contrast images (Briers & Fercher, 1982; Fercher & Briers, 1981). More recently, LSFG has been used to assess with the aid of digital cameras and computational techniques to calculate speckle contrast images (Srienc et al., 2010).

Currently, the gold standard for measuring retinal blood flow is fluorescein angiography (FA), which requires intravenous administration of fluorescein dye and taking images of the retina at timed intervals. This investigation has been reported in literature from as early as the 1930s (Spaide et al., 2015). Other available methods include intraocular probes, laser doppler flowmetry, retinal oximetry and an adaptation of optical coherence tomography (OCT) called OCT angiography (OCTA). While FA remains the gold standard, it has its limitations, including its inability to visualize all the retinal capillaries and its inability to differentiate fluorescence from overlapping structures (Madhusudhan & Beare, 2014).

Laser speckle flowgraphy has the unique advantage of having particularly high reproducibility. Even after drug intervention, the same site and eye can be monitored over time (Aizawa et al., 2011). LSFG also allows for non-contrasted and non-invasive quantification of the microcirculation in the optic disk, choroid and retinal vessels (Kunikata & Nakazawa, 2016). Furthermore, it is patient friendly and allows real-time measurements of RBF. Lastly, it can evaluate the MBR of the optic disc locally in the superior (S), temporal (T), inferior (I) and nasal (N) quadrants and in both the vascular and tissue area.

This study aims to:

1. Evaluate the repeatability and reproducibility of LSFG for RBF measurements in the wild-type (C57BL/6J) mouse model.
2. Evaluate the effect of anti-VEGF on RBF in Ins2 (Akita) diabetic mice using LSFG on the optic nerve head (ONH) region.

2 | MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of SingHealth, Singapore. All experiments were conducted according to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in research.

Twenty eyes of ten wild-type (C57BL/6J) mice (Charles River Laboratory, Wilmington, MAJackson Laboratory, USA) were used for repeatability and

reproducibility arms of the study. Repeatability and reproducibility were assessed on the same wild type and disease mice model. This was done on few batches for reproducibility.

Wild-type C57BL/6J is a common strain of laboratory mice and is often used as a wild-type control in experiments. It has been extensively characterized, and its genome is well-sequenced. Researchers often use C57BL/6J as a baseline for comparing the effects of genetic modifications or experimental treatments. Hence, it was used as a wild-type control in our experiments.

Ins2 Akita is a mouse model for type 1 diabetes. The Ins2 Akita mouse carries a mutation in the insulin 2 gene, leading to the development of diabetes. Hence, it was selected to investigate the influence of hyperglycaemia on ocular vascular changes and blood flow measurements. It is crucial for understanding the disease mechanisms and testing potential treatments.

Ten eyes of five Akita mice aged 12–19 weeks were included for evaluating the effect of anti-VEGF on the RBF in the ONH region using LSFG. Both wild-type (C57BL/6J) and Ins2 (Akita) diabetic mice were housed in standard mouse cages, with 3–4 mice per cage, at 25°C on a schedule of 12 h dark: 12 h light cycle, with mouse pellet and 23°C water available ad libitum. All the mice were anaesthetised using a combination of Ketamine Hydrochloride (50 mg/kg) and Xylazine Hydrochloride (0.5 mg/kg) (Troy Laboratories PTY. Limited, Australia) according to schedule 1, under ARVO guidelines (National Research Council, 2011). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride solution. A few minutes after the induction of anaesthesia, the mouse was positioned with one eye facing downwards onto the stand of the LSFG. Vidisic gel was applied onto the eye prior to placing the cover glass and images were acquired over a 4 s period and then averaged to produce a composite map of ocular blood flow.

One microlitre of intravitreal anti-VEGF Aflibercept (Eylea 1 μg/eye) was administered to each eye of Ins2 (Akita) diabetic mice and the IOP and RBF were measured in each eye at baseline (pre-injection), day 7, 14, 21 and 28 post-injection. The IOP was measured in each eye of each animal with a handheld tonometer (TonolabTV02; M.E. Technica, Tokyo, Japan). A pairwise *t*-test was used to analyse longitudinal changes in IOP. A 95% confidence interval was used for statistical significance. For retinal blood flow, the relative blood flow velocity 'mean blur rate (MBR)' was measured in arbitrary units (AU) for the S, T, I and N quadrants of the ONH region and the changes in mean blur rate of tissue (MT) were analysed at each time point using a linear mixed model to adjust for the bilateral eyes and repeated measurements over time. This was done with the IBM SPSS ver.24 statistics software (Released 2016. IBM SPSS Statistics for Windows. Armonk, NY: IBM Corp. USA). Hurvich and Tsai's criterion (AICC) was chosen as the information criterion due to our small sample size. First-order autoregressive, compound symmetry, diagonal and unstructured covariance structures were compared to obtain the better information criterion for the estimated marginal means (Chan, 2004).

The repeatability of LSFG was evaluated by RBF variability in each eye without changing the Wild-type (C57BL/6J) mouse's head position, performed by same operator (intra-observer variability), and reproducibility was evaluated by another operator measuring the RBF variability in each eye after resetting the mouse's head position (inter-observer variability). Coefficient of repeatability (CR) through the Bland–Altman plot method (Bland & Altman, 1986) coefficient of variation (COV) and Intraclass correlation coefficient (ICC) were calculated to evaluate the repeatability and reproducibility of LSFG using MedCalc Statistical Software version 16.8 (MedCalc Software bvba, Ostend, Belgium) (Bland & Altman, 1986; Portney & Watkins, 2000). The ICC criteria used for reliability were based on the 95% confidence interval of the ICC estimate. Values less than 0.5, between 0.5 and 0.75, between 0.75 and 0.9 and greater than 0.90 were taken as indicative of poor, moderate, good and excellent reliability, respectively.

3 | RESULTS

3.1 | Repeatability and reproducibility

Twenty eyes of ten wild-type (C57BL/6J) mice were used to assess the repeatability and reproducibility of RBF measurements using LSFG. The mean IOP of the mice was 10.8 ± 3.0 mmHg. In the Bland and Altman method, the coefficient of repeatability (CR) was defined as 1.96× within subject standard deviation (Bland & Altman, 1986). The CR values of intra- and inter-observer variation for the MT, MV and MA in the superior, inferior, nasal and temporal quadrants of the ONH region in both eyes of wild-type (C57BL/6J) mice were within the limits of agreement (LoA) (Figures 1–3). These are detailed in Tables 1 and 2, respectively. The COV of intra-observer variability was 4.4%–6.59%, 6.1%–6.51% and 4.14%–4.65% in MT, MV and MA, respectively (Table 1). The COV of inter-observer variability was 16.26%–23.05%, 11.82%–16.58% and 13.27%–41.07% in MT, MV and MA, respectively (Table 2). The level of reliability was excellent for intra-observer variability (ICC values 0.98–0.99) (Table 1) and moderate to excellent for inter-observer variability (ICC values 0.64–0.96) (Table 2).

3.2 | Effect of anti-VEGF on blood flow

Ten eyes of five Ins2 (Akita) diabetic mice were used to assess the longitudinal effects of anti-VEGF on RBF. Table 3 shows the estimated marginal means of MT values following treatment. Based on estimated marginal means[a], Table 4 shows the statistical significance of differences in mean MT values after treatment across different timepoints.

The longitudinal changes of retinal blood flow in ONH region are shown in Figure 1. Figure 2 shows the longitudinal changes in mean MT values across 28 days of treatment. There was a statistically significant

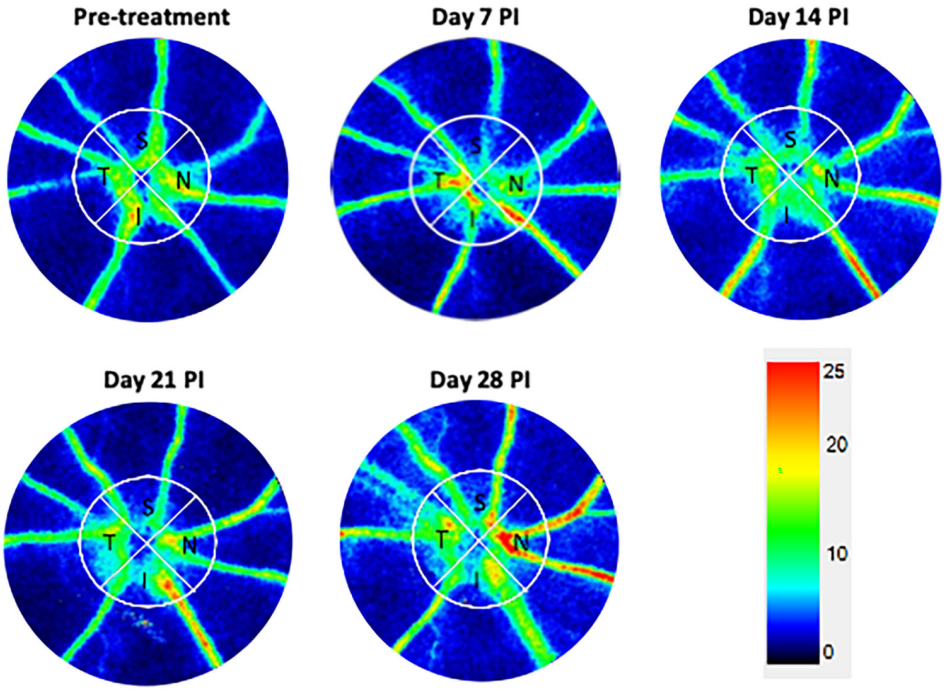

FIGURE 1 Representative LSFG images showing the longitudinal changes of retinal blood flow in optic nerve head region in the same Akita mouse at pre-treatment, day 7, 14, 21 and 28 days post-treatment. I, inferior quadrant; N, nasal quadrant; S, superior quadrant; T, temporal quadrant. The colour scale is shown at the bottom right.

TABLE 1 Repeatability of mean blur rate in the optic nerve head region of wild type (C57BL/6J) mice.

	MT			MV			MA		
Region of measurement	**COV %**	**CR**	**ICC**	**COV %**	**CR**	**ICC**	**COV %**	**CR**	**ICC**
Superior	6.59	1.27	0.98	6.51	3.26	0.99	4.65	1.3	0.99
Inferior	5.81	1.08	0.99	6.45	3.54	0.99	4.31	1.2	0.99
Nasal	5.97	1.29	0.99	6.32	3.65	0.99	5.24	1.85	0.99
Temporal	4.4	0.8	0.99	6.1	3.07	0.99	4.14	1.03	0.99

Abbreviations: COV, coefficient of variation; CR, Coefficient of repeatability; ICC, Interclass correlation coefficient.

TABLE 2 Reproducibility of mean blur rate in the optic nerve head region of wild type (C57BL/6J) mice.

	MT			MV			MA		
Region of measurement	**COV %**	**CR**	**ICC**	**COV %**	**CR**	**ICC**	**COV %**	**CR**	**ICC**
Superior	18.2	1.66	0.87	16.58	4.68	0.89	41.07	5.81	0.64
Inferior	14.35	1.34	0.95	14.74	4.57	0.84	14.16	2.19	0.9
Nasal	23.05	2.39	0.9	11.82	3.78	0.93	13.27	2.44	0.96
Temporal	16.26	1.69	0.94	14.21	4.53	0.87	13.75	2.61	0.95

Abbreviations: COV, coefficient of variation; CR, Coefficient of repeatability; ICC, Interclass correlation coefficient.

increase in RBF in all quadrants of MT region of ONH at day 21 post-injection compared to pre-injection ($p<0.05$). On the other hand, no statistically significant differences in IOP were observed longitudinally ($p>0.05$). Table 5 shows the mean IOP values of mice in right and left eyes throughout the period of treatment. Figure 3 represents the mean IOP changes throughout treatment. Although increased IOP was observed in both eyes of each animal at day 28 post-injection compared to pre-injection (Figure 3), the difference from the baseline IOP was not statistically significant ($p>0.05$) at all timepoints.

4 | DISCUSSION

Laser speckle flowgraphy appears to be a precise method of measuring RBF across various types of tissue (MT, MV, MA) and regions (superior, inferior, nasal, temporal) of ONH. The level of reliability was found to be excellent for repeatability measurements (ICC values 0.98–0.99). Similar findings were observed by Luft et al. (2016), where measurements of RBF via LSFG were successfully obtainable, reproducible and not influenced by pharmacological pupil dilation in 20 eyes of 20 healthy Caucasian subjects. Wada et al. (2016) also

TABLE 3 Table showing the estimated marginal means of MT values in diabetic mice at pre-treatment, day 7, 14, 21 and 28 days post-treatment.

	MTS (AU)		MTN (AU)		MTI (AU)		MTT (AU)	
	Mean (AU)	Std. error	Mean (AU)	Std. error	Mean (AU)	Std. error	Mean (AU)	Std. error
Baseline	4.541	0.588	4.943	0.618	4.905	0.549	4.692	0.643
Day 7	4.724	0.576	4.899	0.603	4.974	0.536	5.030	0.630
Day 14	5.914	0.606	6.675	0.639	6.316	0.566	6.527	0.662
Day 21	6.629	0.620	7.298	0.656	7.351	0.581	7.401	0.677
Day 28	6.922	0.606	6.722	0.639	6.700	0.566	7.012	0.662

Note: Adjustment for multiple comparisons: Bonferroni.

TABLE 4 Table showing statistical significance of the differences in mean MT values in diabetic mice at pre-treatment, day 7, 14, 21 and 28 days post-treatment.

	Pairwise comparisons[a]							Univariate analysis[b]
					95% confidence interval for difference			
ONH region	Timeline		Mean difference	Std. error	Lower bound	Upper bound	*p* value[c]	*p* value
Akita MT-S (AU)	Baseline	Day 7	−0.183	0.606	−1.957	1.591	1.000	0.001
		Day 14	−1.373	0.635	−3.230	0.484	0.350	
		Day 21	−2.087*	0.650	−3.989	−0.186	0.022	
		Day 28	−2.381*	0.635	−4.237	−0.524	0.004	
Akita MT-N (AU)	Baseline	Day 7	0.043	0.691	−1.979	2.066	1.000	0.003
		Day 14	−1.733	0.723	−3.845	0.379	0.199	
		Day 21	−2.355*	0.740	−4.518	−0.192	0.024	
		Day 28	−1.779	0.723	−3.891	0.333	0.170	
Akita MT-I (AU)	Baseline	Day 7	−0.069	0.591	−1.799	1.662	1.000	0.001
		Day 14	−1.411	0.619	−3.220	0.398	0.265	
		Day 21	−2.446*	0.634	−4.299	−0.594	0.003	
		Day 28	−1.795	0.619	−3.604	0.014	0.053	
Akita MT-T (AU)	Baseline	Day 7	-0.339	0.654	−2.251	1.574	1.000	0.000
		Day 14	−1.835	0.685	−3.837	0.167	0.097	
		Day 21	−2.709*	0.701	−4.759	−0.659	0.003	
		Day 28	−2.320*	0.685	−4.322	−0.318	0.013	

Note: Day 7, 14, 21 and 28 are post injection readings.Abbreviations: Baseline, pre-treatment; I, inferior quadrant; MT, mean blur rate of tissue; N, nasal quadrant; ONH, optic nerve head region; S, superior quadrant; T, temporal quadrant.

[a]This test is based on the linearly independent pairwise comparisons among the estimated marginal means (Bland & Altman, 1986).

[b]This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

[c]Adjustment for multiple comparisons: Bonferroni.

showed good reliability of LSFG in measuring longitudinal changes in optic nerve head blood flow in 9 rats over 60 weeks. LSFG appears to be able to produce consistent results in RBF measurements during similar experimental conditions.

Laser speckle flowgraphy appears also to consistently produce similar RBF measurements across independent operators. Our study revealed moderate to excellent reliability for reproducibility measurements (ICC values 0.64–0.96). Shiga et al. (2014) showed that LSFG is a fast and reproducible method of measuring ocular blood flow in 65 eyes of 65 patients with normal tension glaucoma (NTG) and 22 eyes of healthy control subjects. Reproducibility analysis demonstrated that the COV for RBF was 5.9%±3.6%. Excellent reproducibility of LSFG was also reported by Calzetti et al. (2018), where ICC for choroidal vessel diameter (CVD) and relative flow volume (RFV) in choroidal vessels of 31 eyes of 31 healthy, non-smoking subjects was greater than 0.8.

There were minimal differences in measurements when examining for both repeatability of MV (ICC values 0.99) and MT (ICC values 0.98–0.99), as well as reproducibility of MV (ICC values 0.84–0.93) and MT (ICC values 0.87–0.95). However, the reproducibility of MBR in the superior optic nerve head region appears to be lowest (ICC value 0.64) compared to other regions. Tomita et al. (2020) reported that the MBR was significantly greater in the superior retina than the inferior retina. A greater retina flow may result in greater variability in results upon resetting the position of mice's head as LSFG is dependent on average flow velocity, which is increased with greater flow (Sugiyama et al., 2010).

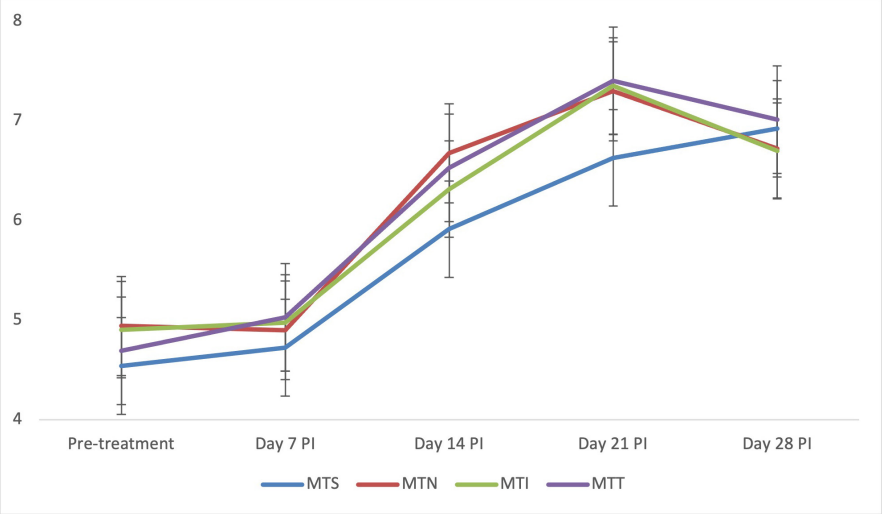

FIGURE 2 Representative diagram showing longitudinal changes of mean MT values in diabetic mice at pre-treatment, day 7, 14, 21 and 28 days post-treatment. I, inferior quadrant; N, nasal quadrant; PI, Post injection; S, superior quadrant; T, temporal quadrant.

TABLE 5 Table showing mean IOP values of mice at pre-treatment, day 7, 14, 21 and 28 days post-treatment.

	Baseline	Day 7	Day 14	Day 21	Day 28
IOP OD (mmHg)	11.2	10.6	11.3	12.0	11.6
IOP OS (mmHg)	10.5	9.9	10.4	10.5	12.2

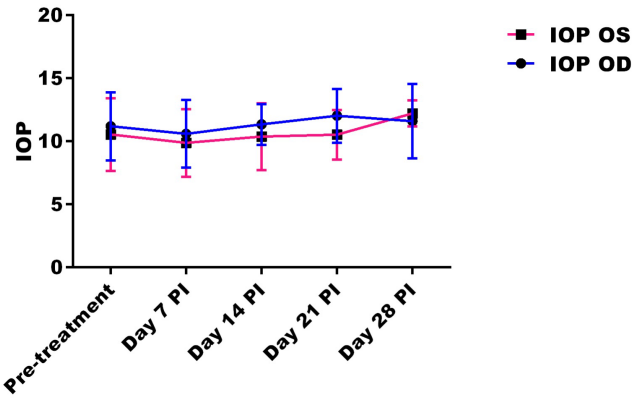

FIGURE 3 Longitudinal changes in mean IOP in Akita mice (n = 8) pre-treatment and post-treatment. PI, post injection.

Vascular endothelial growth factor increases the expression of intercellular adhesion molecule-1 (ICAM-1) on capillary endothelial cells (Lu et al., 1999). This causes the binding of leukocytes to retinal vessels, triggering apoptosis, cell death (Ferrara & Davis-Smyth, 1997) and resulting in retinal ischaemia. VEGF also increases vascular permeability (Ku et al., 1993), causes endothelial cell migration and vasodilation, and reduces pericyte function, resulting in a leakage of fluid and protein from the vasculature (Greenberg et al., 2008).

Anti-VEGF therapy inhibits the above pathological processes, limits leukocyte recruitment and improves retinal ischemia. Our study demonstrates a significant increase in RBF at days 21 and 28 post-injection of intravitreal anti-VEGF. The rapid efficacy of anti-VEGF 1 month post injection is similarly noted in the RESOLVE, RESTORE, RISE and RIDE studies for diabetic macular oedema where a single-agent ranibizumab gained the best BCVA when compared to laser treatment alone or in association with laser treatment itself (Kimoto & Kubota, 2012; Massin et al., 2010; Nguyen et al., 2009, 2012; Simha et al., 2020). Patients treated with aflibercept were also noted to show improvements in BCVA from 1 month post injection based on the VISTADME and VIVIDDME trials (Korobelnik et al., 2014). Although different markers and types of anti-VEGF may be used, these studies are in keeping with ours of the rapid efficacy of intravitreal anti-VEGF therapy.

In our study, the superior quadrant of the retina demonstrated the lowest RBF compared to all other quadrants. This is in contrast to a study by Tomita et al. (2020), where blood flow was measured in sitting position of 68 healthy subjects. Retina flow volume was noted to be greater in the superior retina than in the inferior retina. The results may be explained by the wide difference in duration of measurements – 30 min total in Tomita et al.'s (2020) study, compared to 28 days in our study. There may also be blood flow differences in humans compared to diabetic Akita mice. For example, tissue oxygenation was noted to be decreased by 40% after approximately 6 months of diabetes in mice (Bakri et al., 2008), suggesting that most of the decrease in RBF in these mice may be attributed largely or solely to diabetes.

While there was an increase in the IOP after anti-VEGF therapy, this increase was not statistically significant when observed longitudinally. The mechanism for the initial increase in IOP after anti-VEGF therapy is not yet well understood. Direct damage of trabecular meshwork cells by anti-VEGF has been postulated (Bakri et al., 2008) but could not be demonstrated in

cultured human cells (Kernt et al., 2007). Another postulation suggests that inflammation caused by anti-VEGF treatment in the form of trabeculitis (Adelman et al., 2010) causes damage to the trabecular meshwork and increases IOP. Recent studies have shown that the risk of developing sustained high IOPs after anti-VEF therapy is uncommon (Atchison et al., 2018; Nariani et al., 2016). In a study by Nariani et al. (2016) regarding the long-term effect of anti-VEGF on IOP, the average pre-injection IOP for patients with DME was 15.7 and 14.4 mmHg after 7–9 injections. Similarly, in an analysis of the American Academy of Ophthalmology Intelligent Research in Sight Registry (IRIS) by Atchison et al. (2018), patients receiving bevacizumab, aflibercept and ranibizumab injections showed a mean decrease of 0.9 mmHg compared to 0.2 mmHg in untreated eyes. Our study results support prior real-life data, which suggests that anti-VEGF therapy may not have a sustainable effect on IOP.

The good reliability and repeatability of LSFG in RBF measurements have implications for its potential for screening, diagnosis, monitoring and management of various retinal diseases. An investigative tool with minimal inter- and intra-observer variability may have further potential for screening and diagnosis as it allows for more consistent results while reducing observer bias (Popović & Thomas, 2017). This study is not without limitations. Firstly, the anaesthesia used for the mice (ketamine hydrochloride and xylazine hydrochloride) in this study may cause blood pressure reductions, which in turn reduces RBF, and this may affect the accuracy of our results (Moult et al., 2017). The mean heart rate of both types of mice measured while awake were 550–669 beats per minute and the mean systolic blood pressure was 100–110 mmHg. However, these values dropped to 370–450 beats per minute and 70–80 mmHg after the mice were anaesthetised. Secondly, although the ocular beds are autoregulated (Iester et al., 2007; Pournaras et al., 2008; Schmidl et al., 2011, Venkataraman et al., 2010), blood pressure may fall outside the autoregulatory range. Autoregulation is achieved by the adaptation of the vascular tone of the resistance vessels (arterioles, capillaries) to changes in the perfusion pressure or metabolic needs of the tissue. It attempts to keep the retinal blood flow constant despite changes in ocular perfusion pressure within a set range of blood pressure changes. Autoregulation may fail to work outside of this autoregulatory blood pressure range, and this may affect retinal perfusion. Finally, while pupil dilatation determines the image quality obtained by LSFG, it is difficult to ensure that the pupil width remains the same for each operator despite administration of topical tropicamide. No study has systematically explored the relationship between pupil size and NBR (Sugiyama et al., 2010). Tropicamide itself has also been shown to reduce retinal capillary blood flow (Harazny et al., 2013), and this may further contribute to the variability of blood flow in various regions of the eye.

Our experiments for repeatability and reproducibility were conducted in wild-type mice with normal RBF. However, inter- and intra-observer variability in RBF measurements may be influenced by pathological characteristics in ocular disease. For example, Pechauer et al.'s (2018) study showed that total RBF was significantly reduced in more severe DR compared with control or early NPDR, consistent with previous reports of RBF in proliferative DR using Doppler OCT. However, laser Doppler studies have reported both decreased and increased retinal blood flow in PDR. These conflicting results show that DR is not a linear disease with predictable clinical manifestations. The haemodynamics of vessels and genetic and cellular factors may affect retinal blood flow measurements. Experimental models have also shown that bolus hyperglycaemia can increase retinal blood flow (Sullivan et al., 1990). Further studies involving the use of LSFG in diseased or genetically modified mice with retinal pathologies may be necessary to assess the repeatability of LSFG in RBF measurements. Secondly, the mice in this study were anaesthetised, so any movement was artificially removed. Involuntary movements from the subjects were completely negated and hence did not affect the reliability and repeatability measurements of LSFG. While in real-life application of the LSFG patient movements may be minimized by standardizing the distance of patient's eyes to the LSFG machine and with other mechanical restraints, it will not be possible to entirely replicate these experimental conditions.

5 | CONCLUSIONS

In conclusion, LSFG is a reliable non-invasive method of determining RBF in wild-type C57BL/6J mouse with excellent repeatability and good reproducibility. These findings may aid in diagnosing diseases associated with RBF changes, as well as in monitoring progression and response to therapy. This study is one of the first of its kind to examine the effect of anti-VEGF on RBF in diabetic mice using LSFG. Early RBF data may be particularly beneficial in patients with high visual requirements or with an only-seeing eye, where a change in treatment regimen or drug may be required early to prevent further damage to the eye. Because of LSFG's high reproducibility even after drug intervention, the same site and eye may be monitored non-invasively without contrast over time. It was also noted that intravitreal anti-VEGF injection causes a significant increase in RBF (MT values) at ONH region in Ins2 (Akita) diabetic mice, without demonstrating a sustainable increase in IOP. Anti-VEGF therapy may prevent retinal ischaemia and improve blood circulation without increased risk of developing glaucoma. These findings may play a crucial role in retinal cell therapy in patients with DR.

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ORCID

Leopold Schmetterer https://orcid.org/0000-0002-7189-1707
Rupesh Agrawal https://orcid.org/0000-0002-6662-5850

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