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Automated Quantification of Microvascular Perfusion

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Abstract

Objective: Changes in microvascular perfusion have been reported in many diseases, yet the functional significance of altered perfusion is often difficult to determine. This is partly because commonly used techniques for perfusion measurement often rely on either indirect or by-hand approaches.

Methods: We developed and validated a fully automated software technique to measure microvascular perfusion in videos acquired by fluorescence microscopy in the mouse gastrocnemius. Acute perfusion responses were recorded following intravenous injections with phenylephrine, sodium nitroprusside (SNP), or saline.

Results: Software-measured capillary flow velocity closely correlated with by-hand measured flow velocity ($R^2=0.91$, $p<0.0001$). Software estimates of capillary hematocrit also generally agreed with by-hand measurements ($R^2=0.64$, $p<0.0001$). Detection limits range from 0 to 2000 $\mu\text{m/s}$, as compared to an average flow velocity of 326 ± 102 $\mu\text{m/s}$ (mean $\pm$ std) at rest. SNP injection transiently increased capillary flow velocity and hematocrit and made capillary perfusion more steady and homogenous. Phenylephrine injection had the opposite effect in all metrics. Saline injection transiently decreased capillary flow velocity and hematocrit without influencing flow distribution or stability. All perfusion metrics were temporally stable without intervention.

Conclusions: These results demonstrate a novel and sensitive technique for reproducible, user-independent quantification of microvascular perfusion.

Keywords

Microvascular perfusion; intravital microscopy; computational image processing; nitric oxide; capillary recruitment

1. Introduction

The microcirculation is the site of substrate exchange between blood and peripheral tissues. As such, microvascular perfusion plays a vital role in the normal physiological functions of virtually every organ. Microcirculatory alterations have been noted in a variety of disease states, ranging from sepsis¹ to type 2 diabetes² to inflammatory bowel disease³. The ubiquity of microvascular involvement in the pathophysiology of nominally unrelated diseases is to be expected, considering the well-documented influences of inflammation on the microcirculation⁴, and the ubiquity of inflammation in disease. Given this widespread importance of the microcirculatory system in pathophysiology, it is essential to develop techniques that enable comprehensive measurements of microcirculatory function.

Clinical investigations of microcirculatory function typically rely on techniques such as laser-doppler flowmetry⁵, contrast-enhanced ultrasound⁶, MRI-based techniques⁷, PET-based techniques⁸, and near-infrared spectroscopy⁹. Although these methods allow for assessment of microvascular endpoints in humans, they cannot resolve individual capillaries and therefore make only indirect and/or aggregate measures of microvascular perfusion. Theoretical analyses suggest that capillary density, blood flow, and the distribution of blood flow among available capillaries each exert distinct influences on microvascular exchange processes¹⁰. To measure these parameters, techniques which can assess the behavior of individual capillaries must be used. However, direct observation of the microcirculation remains relatively uncommon, and comprehensive measurement of all relevant microvascular parameters even more so.

Several systems do exist that can theoretically provide simultaneous quantification of all relevant parameters in laboratory settings^{11–15}, but these techniques often involve specialized equipment (e.g. photoacoustic microscopy) that is not available to most investigators. Intravital microscopy, on the other hand, can be performed with relatively inexpensive equipment and allows direct observation of capillaries in both clinical and laboratory contexts. Thus, intravital microscopy theoretically enables simultaneous measurements of capillary density, flow velocity, and flow distribution. While numerous laboratories have utilized intravital microscopy to measure flow through microvascular networks^{14–23}, these studies are generally limited by some combination of slow image acquisition rates (thus impeding detection of the full range of capillary flow dynamics), a lack of direct capillary flow velocity measurements, a lack of automated, comprehensive quantification techniques, and limited documentation of technique-specific detection limits.

Previous computational approaches have been developed for measurement of capillary perfusion using intravital microscopy^{12, 14, 15, 24–27}. Such techniques include the cross-correlation algorithms of Ortiz et al¹⁹, the Cap-Image software¹², the computer-assisted RBC tracking techniques developed by Japee et al^{14, 15}, and You et al's technique for line detection in space-time matrices²⁴. These algorithms produce a signal corresponding to flow velocity. However, no technique yet exists that can meet the combined requirements of 1) identifying in-focus capillary segments, 2) measuring capillary flow velocity, 3) removing invalid (e.g. noisy) flow measurements, and 4) performing the above in a statistically meaningful sample of capillaries without user intervention. In part because of these

limitations, many intravital microscopy experts still rely on labor-intensive by-hand measurement techniques. For established intravital microscopy experts, improved methods for dynamic image analysis would improve the efficiency and reproducibility of detailed microcirculatory measurements; in addition, simplification of data analysis would increase accessibility to new investigators.

To address these opportunities, we set out to create a fully automated technique for quantification of capillary density, flow velocity, and flow distribution, along with estimates of capillary hematocrit using intravital fluorescence microscopy. Because previous investigations in our laboratory have focused on the delivery of glucose, insulin, and/or oxygen to skeletal muscle^{28–31}, this investigation was focused on the mouse white gastrocnemius muscle. We utilized intravital widefield microscopy to perform high frame rate imaging of fluorescently labeled plasma in the mouse gastrocnemius. This imaging modality allows for the simultaneous measurement of flow velocities in >100 individual capillaries per field of view at a frame rate of 100 fps. Drawing from consensus recommendations for assessment of the microcirculation³², we designed the software to report the proportion of vessels sustaining RBC flux in addition to measurements of capillary density, flow velocity, and flow distribution. We observed that capillary flow velocity often varies considerably over time, even within a 5-second video, so we also included an index of short-term temporal flow heterogeneity, which we termed perfusion lability. In this paper, we document the development and validation of an automated software technique for assessing each of these perfusion parameters, along with analysis of software detection limits and noise sensitivity.

2. Materials and Methods

2.1 Animal care, carotid artery catheterization, and jugular vein catheterization

C57BL/6J male mice (Jackson Laboratory) aged 9–11 weeks were fed a standard chow diet (5001 Laboratory Rodent; LabDiet) for all experiments. Mice were housed in a humidity and temperature-controlled facility with a 12-hour light/dark cycle. Prior to microvascular or blood pressure studies, mice were implanted with either an in-dwelling jugular vein catheter (imaging studies) or both a carotid artery catheter and a jugular vein catheter (blood pressure studies) as previously described³³. Mice were allowed 2–5 days (venous catheter only) or 5–8 days (both arterial and venous catheters) to recover from the catheterization surgery and were weighed daily to ensure complete restoration of body weight prior to experimentation. A total of 42 mice were used for this study. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC).

2.2 Surgical preparation and visualization of gastrocnemius muscle microcirculation

Mice were anesthetized using a Somnosuite isoflurane delivery system (Kent Scientific). A dose of 2% isoflurane at a flow rate of 200 mL/min was used for induction, and a dose of 1.5% isoflurane at a flow rate of 80 mL/min was used to maintain anesthesia. A sufficient plane of anesthesia was confirmed prior to beginning experiments by the lack of response to toe-pinch.

To expose the gastrocnemius for intravital microscopy, a preparation developed recently by our group was used³⁴. Briefly, the rear-left hindlimb was shaved and rinsed to remove hair, and then the skin and fascia were trimmed away to expose the superficial white gastrocnemius. Exposed muscle was rinsed with 0.9% saline periodically during preparation to prevent desiccation. Mice were placed on their sides with the exposed gastrocnemius flush against a glass coverslip continuously bathed in 0.9% saline. Body temperature was maintained at 37°C using a heating blanket and rectal temperature probe (Harvard Apparatus), and mice were continuously monitored. A pre-heated, partially water-filled glove was placed on top of the mouse above the heating blanket to dampen breathing motion artifacts during imaging.

The following three exclusion criteria were used to ensure consistent preparation: 1) surgical preparation lasting more than ten minutes, 2) blood on the coverslip at the end of the imaging experiment, and 3) damage to the gastrocnemius muscle during surgery. Mouse preparations meeting any of these three criteria were discarded from analysis.

2.3 Microscopy and video acquisition

Once situated on the microscope stage, mice were injected with 8 mg/kg (~100 μ L injection volume) of tetra-methyl rhodamine conjugated to 2-megadalton dextran sulfate (Rho-Dex, excitation and emission wavelengths of 570nm and 590nm, respectively, selected using a standard rhodamine filter Thermo Fisher) for fluorescent visualization of plasma in the microcirculation. After allowing 3 minutes for fluorophore recirculation and ensuring stable body temperature, a field of view was selected near the center of the exposed gastrocnemius, taking care to avoid hair or large vessels that could confound computational image processing. Subsequently, the focal plane for imaging was maintained using the Perfect Focus System on the Nikon Eclipse Ti-E (Nikon Instruments). Plasma fluorescence was excited using a Sola Light Engine LED lamp (Lumencore) and visualized with a Plan Apo 10x objective (Nikon Instruments). Images were recorded at 100 fps using a 10 ms exposure time with a Flash 4.0 sCMOS camera (Hamamatsu). High frame rate imaging was achieved by directly streaming time-lapse experiments to computer RAM using a Camlink interface (Olympus).

Videos underwent real-time 4×4 pixel binning during acquisition to improve signal/noise ratio, and image brightness was manually adjusted post-hoc in NIS-Elements (Nikon Instruments) to ensure an average pixel intensity of approximately 0.5 with a minimal extent of over/under exposed regions prior to export in a Matlab-readable format. The resulting videos were then exported in uncompressed AVI format for subsequent automated analysis. Each video comprised of 5 seconds of acquisition at 100 fps (i.e. 500 frames total) in a field of view comprising 488×488 pixels with a pixel size of 2.6 μ m/pixel. The resulting field of view size was thus 1.61 mm².

2.4 Stabilization and pre-processing of microscope videos

Prior to flow tracking, raw microscope videos were imported into Matlab 2016 (Mathworks 2016) for stabilization and pre-processing. Motion artifacts caused by breathing were evident in most videos, and these motion artifacts interfere with the quantification of

microvascular perfusion parameters. To remove these motion artifacts, 2-dimensional cross correlation (a common image registration technique) was used to detect the offset between background-subtracted frames (see Section 2.5 for details of our background subtraction technique). This technique involves cross-correlating all subsequent video frames to the first frame (see Figure 1A). The resulting maximum in the 2-dimensional cross correlation surface then allows automated localization of the offset of each frame relative to the first frame (Figure 1B). Subsequent frames are then translated and trimmed to ensure maximum agreement with the first frame. In addition, 3×3 smoothing was applied to all video frames as an additional noise-reduction strategy. 2-dimensional cross-correlation was performed using the entire FOV to maximize the influence of translational motion of the entire capillary bed (e.g. breathing motion) and minimize the influence of movement within capillaries (i.e. blood flow) on motion-correction, thus isolating motion within capillaries for flow tracking. These pre-processing steps significantly improved the number of vessels with adequate signal/noise ratio (SNR) for quantitative analysis ($p < 0.0001$, Figure 1C, see Section 2.6 for description of vessel inclusion criteria). Motion artifacts were visibly reduced by these pre-processing steps (see Supplemental Videos 1 and 2).

2.5 Automated detection of in-focus capillaries for flow tracking

Following stabilization and pre-processing, a time average of fluorescence intensity was calculated (Figure 2A). Capillaries were then identified by calculating the percent excess brightness (intensity prominence) relative to the surrounding 5-pixel (13 μm) region (Figure 2B, red channel). Next, the image gradient (Figure 2B, green channel) was used to illuminate vessel crossings or other edge-producing objects across which flow tracking would be invalid (Figure 2B, green channel). It was observed that the resulting vessel map typically achieved the strongest correlation with the raw microscope image using a threshold value of 2–3%, whereas the strongest correlation with the intensity prominence image was achieved with a threshold of 8–10% (see Supplemental Figure 1). As a compromise between these two optima, regions in which the intensity prominence was at least 5% greater than the image gradient were marked as plasma-perfused vessels. This threshold value yielded a balance between melding of adjacent capillaries and detection of out of focus capillaries with too low of a threshold vs rejection of well-resolved capillaries with too high of a threshold (see Supplemental Figure 2). Objects smaller than 10 total illuminated pixels (67.6 μm^2) were then removed to prevent flow tracking in excessively small vessel fragments. At a mean capillary diameter of $>3 \mu\text{m}$ and a frame rate of 100 fps, this minimum vessel fragment size ensures that velocities up to at least 2000 $\mu\text{m/s}$ will be detectable in all vessels in which the software attempts to track flow.

Next, vessel centerlines were identified in the resulting vessel mask using the Matlab image skeletonization command *bwmorph()* with 'skel' option (Image Processing toolbox in Matlab 2016). Capillary segments less than 8 pixels (20.8 μm) in length were removed from the skeletonized image to prevent spurious detection of vessel bifurcations/confluences (see Supplemental Figure 3) and to ensure a detection of flow velocities up to 2000 $\mu\text{m/s}$ at 100 fps. With the remaining skeletonized image, all pixels within a 2-pixel (5.2 μm) radius of remaining vessel bifurcations/confluences were removed, leaving only vessel segments uninterrupted by vessel bifurcations or confluences so that the software would not attempt to

track flow across vessel confluences/bifurcations, where flow velocities may abruptly change. These vessel segments were then used for capillary flow tracking (Section 2.6).

Because the flow tracking algorithms employed in this technique rely on detectable motion of relatively large deviations from mean pixel intensity, regions with a mean pixel intensity of greater than 0.83 or less than 0.17 were removed as over/under exposed, respectively (Figure 2C, green channel). These criteria for over and under-exposure ensure that intensity deviations of >20% (required for flow tracking as described in Section 2.6) are detectable. All videos analyzed had appropriate image exposure in >90% of the total FOV. The total length of trackable vessel segments (Figure 2C, blue channel) was normalized to the area of the appropriately exposed FOV (Figure 2C, red channel) as an index of plasma-perfused capillary density in mm/mm².

2.6 Automated flow tracking and removal of poorly resolved vessels

After identifying vessel centerlines suitable for computational flow tracking (Section 2.5), a normalized fluorescence intensity profile was calculated for each vessel segment by subtracting 1.2x the time average of plasma fluorescence intensity prominence from the current-frame intensity prominence profile (thus isolating deviations of 20% or more), eliminating values less than zero (thus isolating positive from negative deviations), and then taking the square root to enhance contrast (Eqs 1–2). A second normalized fluorescence intensity profile was calculated for each vessel segment by subtracting 1.2x the current-frame intensity prominence profile from the time average of plasma intensity, eliminating values less than zero, and taking the square root (Eqs 3–4). Thus, deviations within $\pm 20\%$ of time average intensity prominence were excluded from flow tracking to prevent image noise from producing false peaks in the normalized profiles, while the velocities of negative deviations (unusually dark objects, e.g. RBC shadows) and positive deviations (unusually bright objects, e.g. plasma gaps) were considered separately as an additional safeguard against image noise.

$$PI1 = CF - 1.2 * TA \quad (1)$$

$$P1 = \begin{cases} \sqrt{PI1} & PI1 \geq 0 \\ 0 & PI1 < 0 \end{cases} \quad (2)$$

$$PI2 = TA - 1.2 * CF \quad (3)$$

$$P2 = \begin{cases} \sqrt{PI2} & PI2 \geq 0 \\ 0 & PI2 < 0 \end{cases} \quad (4)$$

Here, CF represents the raw intensity prominence profile in the current frame, TA represents the time-averaged intensity prominence profile, PI represents an intermediate profile used to calculate the positive-deviation intensity prominence profile, $P1$ represents the positive-deviation intensity prominence profile itself, $P2$ represents an intermediate profile used to calculate the negative-deviation intensity prominence profile, and $P2$ represents the negative-deviation intensity prominence profile itself.

In well-resolved vessels, similar image features were identified along these intensity prominence deviation profiles in successive frames (Figure 3A). The positive-deviation and negative-deviation profiles in each frame for each vessel segment were used to compute a composite cross-correlation curve (Figure 3B) according to Eqs 5–9:

$$CC1(\delta x) = \int_{-\infty}^{\infty} P1(f, x) * P1(f + 1, x + \delta x) * dx \quad (5)$$

$$CC2(\delta x) = \int_{-\infty}^{\infty} P1(f + 1, x) * P1(f + 2, x + \delta x) * dx \quad (6)$$

$$CC3(\delta x) = \int_{-\infty}^{\infty} P2(f, x) * P2(f + 1, x + \delta x) * dx \quad (7)$$

$$CC4(\delta x) = \int_{-\infty}^{\infty} P2(f + 1, x) * P2(f + 2, x + \delta x) * dx \quad (8)$$

$$CC(\delta x) = CC1(\delta x) * CC2(\delta x) * CC3(\delta x) * CC4(\delta x) \quad (9)$$

Here, δx represents the axial displacement of image features between successive frames, f represents the current video frame, x represents axial position along the vessel centerline,

$CC1$ - $CC4$ represent intermediate cross-correlation curves from individual frame-frame comparisons, and CC represents the composite cross-correlation curve used for velocity estimation.

Each cross-correlation curve (both the intermediates and the composite) represents the degree of similarity between two profiles as a function of their axial offset. The intermediate cross-correlation curves used here represent cross-correlation using only unusually bright image features between the current frame and the next frame (Eq 5) and between the next frame and the one after (Eq 6), and of only unusually dark image features between the current frame and the next frame (Eq 7) and between the next frame and the one after (Eq 8). Thus, to produce a peak in the composite cross-correlation curve (Eq 9), both bright and dark objects appearing in three or more consecutive frames must yield high values of relative agreement at similar degrees of axial displacement.

For well-resolved vessel segments, the striated patterns of light and dark image features produced by plasma gaps and/or monocytes (bright) alternating with RBC shadows (dark) produce velocity estimates that converge in frame-frame comparisons with high relative agreement (Figure 3C, relative agreement calculated according to Eq 9). To reduce the influence of individual frames without trackable objects or with poor signal to noise ratio (SNR), we averaged the top 20% highest relative agreement composite cross-correlation curves within any given 1 second period to produce a single composite cross-correlation curve for each 1 second period in each vessel. Quadratic regression was used to estimate the peak of the cross-correlation curve with sub-pixel accuracy. With a pixel size of $2.6\text{ }\mu\text{m}$ and a frame rate of 100 fps, single-pixel accuracy corresponds to a measurement precision of $\pm 260\text{ }\mu\text{m/s}$ in each frame-frame observation. By averaging 20 values for each 1 second velocity estimate, the measurement precision is thus improved to $\pm 58\text{ }\mu\text{m/s}$. By averaging five 1 second measurements together, the accuracy of the time-averaged flow velocity calculated for each vessel is $\pm 29\text{ }\mu\text{m/s}$ under steady-flow conditions (i.e. when algorithm error is the sole determinant of measurement uncertainty). With sub-pixel accuracy due to quadratic regression estimation of the cross-correlation peak in each frame, this value represents an upper bound for algorithmic velocity measurement error at the single-capillary level, and composite measures drawn from multiple capillaries will achieve higher resolution still. Thus, the intrinsic spatial and temporal variability of blood flow are the dominant contributions to measurement uncertainty.

Poorly resolved vessel segments produced either no detectable features at all or else randomly-spaced image features along the vessel centerline, resulting in an average cross-correlation curve with no distinct peak (see Supplemental Figure 4). Additionally, individual-frame velocity estimates do not converge to a single value at higher levels of relative agreement in poorly resolved vessels (Supplemental Figure 4C). Measurements were most consistent and frame-frame comparisons of tracked image features most credible in cases where the 1 second average composite cross-correlation curve had a single peak at least twice as high as the second highest peak, and when the relative agreement value corresponding to this peak was at least 0.05. Thus, vessels with low or uncertain values of relative agreement were rejected as poorly resolved. These inclusion criteria were designed

to err on the side of false negatives (i.e. rejection of valid traces) rather than false positives (i.e. inclusion of invalid traces).

2.7 Automated estimation of capillary hematocrit

In each capillary segment used for flow tracking (see Section 2.6 for discussion of inclusion criteria), a space-time image of fluorescence intensity along the vessel centerline as a function of time was created, consistent with previous techniques for RBC tracking^{14, 15, 24}. Because RBCs do not take up the Rho-Dex plasma marker, they produce dark streaks in the space-time image (See Figure 4). Space-time images were adjusted so that the 5th percentile pixel value was 0 (assumes capillary hematocrit of >5%) and the 75th percentile pixel value was 1 (assumes capillary hematocrit of <75%) to enhance contrast, and then automatically segmented into RBC and plasma regions using a sliding threshold as defined in Eq 10.

$$T = 0.75 - \frac{1.5}{\left(\frac{|V|}{PS * FPS}\right) + 3} \quad (10)$$

Here, T is a capillary segment-specific threshold value for hematocrit estimation, V is the flow velocity within the capillary segment, PS is pixel size, and FPS is the video frame rate. The correction for velocity employed in Eq 10 was pragmatically tuned to mitigate blurring of RBC shadows at high flow velocities. This algorithm assumes a capillary hematocrit in the range of 0.05–0.75 to enable pre-hoc adjustment of pixel intensities for contrast enhancement and thus underestimates the dynamic range of capillary hematocrit and biases software results towards false negatives (i.e. failing to detect changes in RBC distribution) rather than false positives (i.e. spurious detection of changes in RBC distribution). Figure 4 shows contrast enhanced (left) and binarized (right) space-time images for capillaries across a representative range of velocity and hematocrit values. Note that the capillary hematocrit values returned by this algorithm concern the only fraction of the vessel centerline occupied by RBCs. Thus, this metric provides a crude estimate of discharge hematocrit, and not tube hematocrit, within each capillary segment.

2.8 By-hand measurements used for software validation

To assess the validity of software outputs, measurements of capillary flow velocity and capillary density were taken by hand by a blinded investigator as illustrated in Figure 5 and then compared to software measurements. Flow velocity was assessed by tracking a single visible object along the length of a capillary and then recording the distance travelled and time elapsed (Figure 5A). Five objects per capillary were recorded by hand for 5 capillaries in each of 12 stabilized videos used for comparison. By-hand velocity measurements were compared to software velocity measurements at the single capillary level for a total of $n=58$ capillaries (2 capillaries measured by hand were rejected by the software as poorly resolved). Capillary density was measured by hand using the intensity prominence image generated by the software (see Section 2.5, Figure 2B) by drawing three lines perpendicular to the orientation of the capillary network and recording the number of vessel intersections divided by the total length of the lines. By hand capillary density measurements were

compared to software capillary density measurements at the single-field of view (FOV) level for a total of $n=9$ videos. Finally, by-hand estimates of capillary hematocrit were obtained by manually thresholding space-time images to segment plasma from RBCs in 5 capillaries each from 4 videos for a total of $n=20$ capillaries.

2.9 Testing of software sensitivity to image noise and exposure time

To test the sensitivity of the software technique to noise, modified versions of the videos were created with added white noise with a magnitude of either 20% maximum image brightness or 80% maximum image brightness. Number of vessels with adequate SNR for flow tracking and mean capillary flow velocity were compared on a per-video basis between raw microscope videos, 20% noise-added videos, and 80% noise-added videos (each $n=16$). 20% added noise produced a slightly grainy image, while 80% added noise almost entirely obscured visualization of capillaries (Supplemental Figure 5).

We also hypothesized that the technique would be sensitive to camera exposure time. If too short an exposure time were used, signal would be reduced while noise would be maintained, thus reducing SNR. If too long an exposure time were used, cells and plasma gaps would blur together, thus preventing detection of trackable image features. To estimate the detection limits of the technique using a 10 ms exposure, simulated 20 ms exposure videos were generated from raw microscope videos by averaging each pair of successive frames and then re-running the tracking script. Maximum velocity observed in each raw (10 ms) microscope video was then compared to the bias in mean capillary flow velocity observed in the 20 ms video relative to the raw microscope video to determine if and at what velocity increased exposure time would cause measurement errors. Given that distance travelled in 20 ms is twice that in 10 ms, the maximum detectable velocity with 10 ms exposure is twice that with 20 ms exposure.

2.10 Characterization of baseline perfusion and responses to vasoactive drugs

To test the ability of the software to detect dynamic changes in muscle perfusion, we characterized changes from baseline perfusion in response to a bolus intravenous injection of 0.45 mg/kg sodium nitroprusside (SNP, $n=8$ mice), 0.35 mg/kg phenylephrine ($n=8$ mice), an equivalent volume bolus intravenous injection of 0.9% saline ($n=8$ mice) or without any injection ($n=8$ mice). An injection volume of 1.67 mL/kg was used (e.g. 50 μ L in a 30g mouse). A single FOV was selected for each mouse, and then a baseline video was recorded. The bolus injection was then infused into the jugular vein catheter and 5-second perfusion videos were recorded exactly 1 minute, 2 minutes, 3 minutes, 4 minutes, and 5 minutes post-injection, for a total of 6 videos in each FOV. In the non-injection group, a video was recorded once per minute for five minutes following baseline acquisition to assess basal stability of perfusion without injections.

2.11 Characterization of blood pressure responses to vasoactive drugs

To ensure that SNP and phenylephrine produced the expected hypotensive and hypertensive effects, respectively, and to test the hypothesis that microvascular perfusion responses are largely independent of central hemodynamics, we characterized changes from baseline blood pressure in response to a bolus injection of 0.45 mg/kg SNP ($n=6$ mice), 0.35 mg/kg

phenylephrine (n=4 mice), or 0.9% saline (n=6 mice). In addition, to ensure that central hemodynamics were stable before the start of imaging in our perfusion studies, we monitored blood pressure changes in response to injection of Rho-Dex (n=12 mice). Blood pressure was recorded through the carotid artery catheter using a Blood Pressure Analyzer (Digimed, Inc). Blood pressure readings were obtained every ten seconds starting immediately before Rho-Dex injection and finishing five minutes after drug injections.

2.12 Parameters recorded by the software

In addition to recording flow velocity on a per-capillary basis, the software provides estimates of mean flow velocity, a perfusion heterogeneity index, a perfusion lability index (variability with respect to time over short time frames), plasma-perfused capillary density, and proportion of plasma-perfused vessels supporting blood flow, along with approximations of capillary hematocrit and its variability. These metrics were based on consensus recommendations for assessment of the microcirculation in clinical contexts³², and on our own observations of microvascular perfusion.

Mean capillary flow velocity was calculated on a length-weighted basis as shown in Eq 11, where MFV is mean capillary flow velocity, L_i is the length of each individual vessel segment, and V_i is the flow velocity of each individual vessel segment. Length weighting was used to prevent single capillary segments from being counted multiple times due to fragmentation caused by vessel crossings or image noise.

$$MFV = \frac{\sum L_i * V_i}{\sum L_i} \quad (11)$$

Microvascular perfusion heterogeneity and lability indices were calculated as defined in Eq 12, where I is the perfusion index, V_{max} is the maximum observed velocity, V_{min} is the minimum observed velocity, and V_{mean} is the mean observed velocity. When the variability in question is between vessels within the same FOV, we define this index as perfusion heterogeneity index (PHI), whereas when the variability is between successive observations in the same vessel segment, we define it as perfusion lability index (PLI). Thus, PHI is calculated exclusively on a per-FOV basis, whereas PLI is calculated on a per-capillary basis, and the value reported for each FOV represents a length weighted average similar to MFV. The decision to use the extrema for our variability indices rather than a standard deviation or similar was informed by previous investigations^{32, 35} and the observation that the extrema were more sensitive to subtle changes in perfusion state.

$$I = \ln \left(\frac{V_{max} - V_{min}}{V_{mean}} \right) \quad (12)$$

Capillary density was estimated by the software using the total length of detected vessel segments per unit area of adequately exposed FOV. This metric was expressed in units of mm/mm². Proportion of vessels with a flow velocity greater than zero has been shown to predict patient outcomes in sepsis^{1, 32, 36} and is reduced in animal models of diabetes^{2, 37}. The proportion of perfused vessels (PPV) was calculated by dividing the total length of vessel segments with velocity greater than our detection limit by the total length of vessel segments for which a valid velocity estimate was obtained.

2.13 Statistical techniques and power calculations

Pearson's correlation coefficient or Spearman's correlation coefficient (both reported as R, used for normal and non-normal distributions, respectively) was used to assess correlations of by-hand velocity measurements with software velocity measurements, noise-added video results with raw video results, and 20ms exposure results with 10ms exposure results. R² and p are reported for all correlations. To assess statistical bias in software measurements, a paired t-test or a Wilcoxon matched-pairs signed rank test (for normal and non-normal distributions, respectively) was used to compare to by-hand measurements. For each treatment or control time course, a 1-way repeated measures ANOVA or Friedman test (for normal and non-normal distributions, respectively) was used, along with Tukey's post-test to assess individual timepoint differences and correct for multiple comparisons. The D'Agostino and Pearson omnibus normality test was used to check for departures from normality in all distributions. Departures from normality are noted in the appropriate results section for each distribution. Values for statistical significance are reported as p=NS, p<0.05 (denoted * in figures), p<0.001 (denoted ** in figures), or p<0.0001 (denoted *** in figures). All comparisons for which p<0.05 were reported as statistically significant. All statistical analyses were performed in Prism Graphpad (Graphpad Software, Inc).

3. Results

3.1 Comparison of software and by-hand measurements

Results of software and by-hand comparisons are shown in Figure 6. Red points indicate data affected by software detection limits. 5-second average of flow velocity is consistent between software and by-hand measurements at the single-capillary level (Figure 6A, R²=0.91, p<0.0001). This consistency is also observed for maximum and minimum flow velocity in each capillary (Figure 6B, R²=0.85 and Figure 6C, R²=0.87, respectively, both p<0.0001). Software estimates of capillary hematocrit captured a majority of the variance in capillary hematocrit as measured by hand (Figure 6D, R²=0.64, p<0.0001), although this agreement was less robust than for measures of capillary flow velocity. A slight statistical bias was observed in software velocity measurements (Figure 6E, p<0.05), which underestimated flow velocity relative to by-hand measurements by an average of 9.4%. This statistical effect was driven by two capillaries for which the maximum flow velocity recorded by hand was greater than our detection limit of 2000 $\mu\text{m/s}$ (see Section 3.2 for discussion of detection limits). For capillaries in which the maximum flow velocity recorded by hand was within software detection limits, there was no statistically significant difference between software and by-hand velocity measurements (p=NS). Capillary density as measured by software also did not significantly differ from capillary density as measured by

hand (Figure 6F, $p=NS$). Velocities at the single-capillary level were found to significantly depart from normal distribution ($p<0.01$, distribution was approximately log-normal), and so Spearman's correlation was used for analysis of relationships between by-hand and software velocity measurements, along with a Wilcoxon matched-pairs signed rank test in place of a paired t-test for assessment of statistical bias.

3.2 Sensitivity of software measurements to image noise and exposure time

Noise and exposure time sensitivity analysis results are summarized in Figure 7. Addition of white noise with an intensity of 20% maximum image brightness reduced the number of vessels with adequate SNR for flow tracking from 120 ± 30 to 114 ± 35 (Figure 7A, $p<0.0001$), while addition of white noise with an intensity of 80% dynamic range reduced the number of vessels with adequate SNR to 53 ± 18 ($p<0.0001$). Similarly, MFV as measured in videos with 20% added noise correlated strongly to MFV measured in unmodified videos (Figure 7B, $R^2=0.94$, $p<0.0001$), whereas this agreement was lost with 80% added noise ($R^2=0.05$, $p=NS$). With 20% added noise, software measurements were also largely self-consistent for PHI (Figure 7C, $R^2=0.68$, $p<0.0001$) and PLI (Figure 7D, $R^2=0.90$, $p<0.0001$). With 20% added noise, videos were visibly grainier, but capillaries were still clearly visible, whereas with 80% added noise, the capillary network was no longer readily discernible.

By comparing measurements taken in the raw microscope videos to measurements taken in videos modified to simulate a 20ms camera exposure (see Section 2.9), influences of exposure time and video frame rate on the accuracy of software measurements were determined. There was no detectable difference in MFV as measured using 10ms exposure videos and 20ms exposure videos for videos in which the highest individual-capillary flow velocity was less than $1000\text{ }\mu\text{m/s}$ (Figure 7E). Given that objects travel twice as far in 20ms as in 10ms at a consistent velocity, this would correspond to a detection limit in the range of $2000\text{ }\mu\text{m/s}$ with a 10ms exposure. In addition to introducing bias in velocity measurements, increasing exposure time to 20ms also reduced the number of vessels with adequate SNR for flow analysis from 120 ± 30 to 91 ± 34 ($p<0.0001$, Figure 7F).

3.3 Time course of microvascular perfusion response to a bolus injection of SNP

Microvascular perfusion responses to a bolus intravenous injection of 0.45 mg/kg SNP are shown in Figure 8. MFV (Figure 8A) increased from $302\pm116\text{ }\mu\text{m/s}$ to $580\pm157\text{ }\mu\text{m/s}$ (mean $\pm$ standard deviation) within the first two minutes post-injection ($p<0.0001$), and then returned to $338\pm89\text{ }\mu\text{m/s}$ by 5 minutes post-injection. This transient hyperemia corresponded to a transient decrease in PHI (Figure 8B) from 1.12 ± 0.182 at baseline to 0.807 ± 0.135 at 2 minutes post-injection ($p<0.001$), which then returned to normal values (1.02 ± 0.151) by 5 minutes post-injection. PLI (Figure 8C) also transiently decreased from -0.506 ± 0.278 at baseline to -1.04 ± 0.230 1 minute post-injection ($p<0.001$), and was still slightly lower than at baseline at 2 minutes post injection (-0.909 ± 0.261 , $p<0.05$). Although PPV started at $94.4\%\pm2.83\%$, SNP still produced a statistically significant increase in PPV to $97.2\%\pm2.22\%$ within the first minute post-injection ($p<0.05$), which returned to baseline values ($93.0\%\pm4.57\%$, $p=NS$) by the end of the 5-minute time course. Mean hematocrit (Figure 8E) also increased from $29.6\%\pm1.6\%$ to $31.7\%\pm1.5\%$ at 2 minutes post-injection, and then

returned to $29.7\% \pm 1.7\%$ at 5 minutes post-injection ($p < 0.05$, while the coefficient of variance of hematocrit (Figure 8F) decreased from 0.19 ± 0.02 to 0.16 ± 0.01 within the first minute post-injection and then returned to 0.20 ± 0.02 by the end of the 5-minute time course ($p < 0.05$).

3.4 Time course of microvascular perfusion response to a bolus injection of phenylephrine

Microvascular perfusion responses to a bolus intravenous injection of 0.35 mg/kg phenylephrine are shown in Figure 9. MFV (Figure 9A) decreased from $334 \pm 174 \mu\text{m/s}$ to $60 \pm 62 \mu\text{m/s}$ (mean $\pm$ standard deviation) within 1 minute post-injection ($p < 0.001$), and then returned to $313 \pm 63 \mu\text{m/s}$ by 5 minutes post-injection. This decrease in perfusion corresponded to an increase in PHI (Figure 9B) from 0.89 ± 0.15 to 1.98 ± 0.54 at 1 minute post-injection ($p < 0.0001$), which subsequently returned to 1.1 ± 0.08 by 5 minutes post-injection. PLI (Figure 9C) simultaneously increased from -0.46 ± 0.28 to 1.38 ± 0.55 in the first minute post-injection ($p < 0.0001$) and then returned to -0.58 ± 0.15 5 minutes post-injection. PPV also decreased from $94.5\% \pm 3.6\%$ to $20.3\% \pm 29.8\%$ at 1 minute post-injection ($p < 0.0001$), and then returned to $90.7\% \pm 4.2\%$ by 5 minutes post-injection. Meanwhile, mean hematocrit (Figure 9E) decreased from $29.0\% \pm 1.3\%$ to $23.8\% \pm 3.1\%$ in the first minute post-injection, and then returned to $29.7\% \pm 0.17\%$ by 5 minutes post-injection ($p < 0.05$), while the coefficient of variance of hematocrit (Figure 9F) increased from 0.19 ± 0.03 to 0.25 ± 0.02 and then returned to 0.21 ± 0.01 within the same time frames ($p < 0.001$).

3.5 Time course of microvascular perfusion response to a bolus injection of saline

Microvascular perfusion responses to a bolus intravenous injection of 1.67 mL/kg of 0.9% saline are summarized in Figure 10. MFV (Figure 10A) decreased from $443.8 \pm 106 \mu\text{m/s}$ to $342 \pm 164 \mu\text{m/s}$ within the first minute post-injection ($p < 0.05$), and then returned to $398 \pm 104 \mu\text{m/s}$ by the end of the five-minute time course. PHI (Figure 10B), PLI (Figure 10C), and PPV (Figure 10D) were not significantly changed in response to saline injection (all $p = \text{NS}$). Mean hematocrit (Figure 10E) underwent a transient decrease from $29.8\% \pm 1.6\%$ to $27.8\% \pm 2.4\%$ within the first two minutes post-injection ($p < 0.01$), and then returned to $29.1\% \pm 1.9\%$ by 5 minutes post-injection. Hematocrit heterogeneity (Figure 10F) was not significantly changed by saline injection ($p = \text{NS}$).

3.6 Time course of microvascular perfusion without intervention

Changes in microvascular perfusion and image quality metrics over time without intervention (neither SNP nor saline boluses) are summarized in Figure 11. MFV (Figure 11A), PHI (Figure 11B), PLI (Figure 11C), and PPV (Figure 11D), as well as capillary hematocrit and its heterogeneity (data not shown) all remained stable over time without intervention ($p = \text{NS}$). Image quality deteriorates over sufficiently long time-frames following fluorophore injection, and so changes in the number of vessels for which valid flow measurements could be obtained and the fraction of the FOV used for perfusion analysis were assessed over time during the first five minutes of imaging (the time frame used for all analyses in this study). The number of vessels with adequate SNR for inclusion in aggregate perfusion metrics declined from 136 ± 22 at baseline to 106 ± 23 by the end of the 5-minute

time course (Figure 11E, $p<0.001$). In addition, the fraction of the FOV adequately exposed for flow tracking decreased from $98.1\pm1.41\%$ at baseline to $96.1\pm1.90\%$ at the end of the experiment (Figure 11F, $p<0.01$). Fraction of the FOV adequately exposed for flow tracking was found to significantly depart from a normal distribution ($p<0.05$), and so a Friedman test was used in place of repeated measures ANOVA for this analysis.

3.7 Comparison of blood pressure responses to vasoactive drugs

Changes in blood pressure measured through a carotid artery catheter following injection with 0.45 mg/kg SNP, 0.35 mg/kg phenylephrine, or an equivalent volume of 0.9% saline are shown in Figure 12. Baseline mean blood pressure was 91.8 ± 13.1 mmHg. Mean blood pressure underwent a small transient increase following intravenous injection with fluorescent dextran (~ 3 mmHg, $p<0.001$, Figure 12A) but returned to baseline within 3 min. Following drug injections, blood pressure responses diverged (Figure 12B). Saline injection produced a slight transient decrease in blood pressure peaking at 2.5 minutes post-injection (~ 5 mmHg, $p<0.01$), whereas SNP injection caused a substantial decrease in blood pressure (~ 37 mmHg, $p<0.0001$), peaking at 1 minute post-injection and largely recovering within 5 minutes post-injection. Meanwhile, phenylephrine injection caused a substantial increase in blood pressure (~ 47 mmHg, $p<0.0001$), peaking within 30 seconds post-injection, and then slightly over-correcting by the end of the five-minute time course.

4. Discussion

This manuscript documents the development and validation of a novel software technique for quantification of microvascular perfusion parameters in intravital microscopy videos. Software outputs agreed closely with by-hand measurements at the single-capillary level and provided a much greater amount of information in much less time (~ 20 s per capillary vs ~ 10 min per capillary including pre-processing with the equipment and specifications used in this study). The microscopy techniques and image processing algorithms employed in this study allow for measurements of flow velocity, flow distribution, time variance in flow velocity, and functional capillary density, along with estimates of capillary hematocrit and its heterogeneity. These endpoints incorporate measures from an average of over 100 capillaries per FOV. This technique is robust to image noise, and software accuracy is maintained for flow velocities up to 2000 $\mu\text{m/s}$, as compared to mean flow velocities of 300–400 $\mu\text{m/s}$ at baseline.

Given that addition of white noise comprising 20% of maximum image brightness only minimally influenced software measurements, it appears that this technique is generally robust to image noise. Videos with 20% added noise were visibly grainy but capillaries were still clearly visible (see Supplemental Figure 5), thus providing a heuristic for acceptable image quality. Likewise, an MFV of 300–400 $\mu\text{m/s}$ measured at baseline is in general agreement with previous literature values for MFV in resting skeletal muscle^{18, 38–43}, although it should be noted that velocities ranging from $<100 \mu\text{m/s}$ ¹⁴ to $>1000 \mu\text{m/s}$ ⁴⁴ have previously been reported, suggesting that flow velocity varies by preparation. For the preparation used in this study, the estimated upper limit of velocity measurement allows quantification of perfusion states ranging from complete ischemia to 5–7x resting blood

flow. It is worth noting that the empirically defined upper limit of reliable detection (2000 $\mu\text{m/s}$) will influence not only MFV, but also potentially PHI and PLI (since these are calculated from the extrema of flow velocity) in conditions of high flow velocity. Thus, the software is best suited to measuring perfusion states ranging from ischemia to 5x basal flow in the mouse gastrocnemius.

It is our intention to make this software freely available to all interested investigators. However, the idiosyncratic computational requirements of alternative sample preparations, fluorescent probes, and microscopes cannot necessarily be determined a priori. Thus, investigators interested in using this software are encouraged to contact the study authors directly. This software requires a contrast agent that does not escape the vasculature to visualize capillaries, an exposure time of less than 20ms to mitigate blurring in high-flow capillaries, a video frame rate of at least 25 fps to ensure repeated observation of trackable objects in several successive frames, image magnification no greater than 20x to ensure a meaningful number of capillaries in each FOV, a pixel size smaller than 3 μm to resolve RBC shadows, and a computer capable of running Matlab 2016, which is necessary to run the software. As the algorithmic requirements of alternative approaches become apparent, the software will be released as a stand-alone executable for use by the general scientific public.

It is important to note that the microcirculatory effects recorded by the software do not necessarily track with changes in central hemodynamics. For example, blood pressure was maximally affected by SNP at 1 minute post-injection, at which time MFV was not yet significantly altered. MFV reached a maximum 2 minutes after SNP injection, during a transient increase in blood pressure. Meanwhile, saline injection caused significant changes in MFV despite negligible effects on blood pressure. Of the three treatment groups used in this study, only phenylephrine showed a similar time course for both blood pressure and local perfusion responses. These findings demonstrate that microvascular perfusion in peripheral tissues cannot necessarily be determined from central hemodynamic parameters, nor vice versa, thus necessitating techniques for direct perfusion measurement such as the software developed in this manuscript.

Furthermore, local skeletal muscle perfusion is highly variable, varies over time (changes in MFV of up to 100 $\mu\text{m/s}$ between time points were not uncommon in the untreated control group), and in validation studies for this software, was sensitive even to intravenous injection with saline, which significantly reduced capillary flow velocity for two minutes following injection. Average PHI at baseline was approximately 1, which represents a nearly 3-fold range of capillary flow velocities within a given field of view. A high degree of variability was also observed between fields of view, consistent with previous reports in the human microcirculation³², and the observation of high time variance in basal perfusion is consistent with reports of vasomotion in skeletal muscle^{45–48}. That even saline injection caused significant changes in perfusion serves to underscore the dynamism, variability, and sensitivity of microcirculatory endpoints.

We found that bolus injection of SNP (an NO donor) caused a transient hyperemic state, consistent with the well-established fact that NO increases microvascular perfusion in

skeletal muscle⁴⁹. Additionally, SNP decreased both the heterogeneity (variance between capillaries) and lability (time variance within the average capillary) of flow velocity, thus making flow steadier and more evenly distributed, along with a transient increase in mean hematocrit and a decrease in its heterogeneity. Conversely, we found that phenylephrine (an α_1 agonist vasoconstrictor) caused near-ischemia of the hindlimb, along with increased perfusion heterogeneity and lability, decreased hematocrit, and increased hematocrit heterogeneity. In light of these findings, it is perhaps unsurprising that previous reports indicate increased perfusion heterogeneity in diseases such as type 2 diabetes^{22, 50}, in which adrenergic tone is increased⁵¹ and NO bioavailability is decreased⁵².

There has been controversy as to whether recruitment of previously non-perfused capillaries occurs *in vivo*. Proponents of capillary recruitment argue that increased microbubble distribution volume as measured by contrast-enhanced ultrasound reflects capillary recruitment^{53, 54} whereas critics of the capillary recruitment model arguing that because the vast majority of capillaries are recruited at baseline, mechanisms other than recruitment must account for the increased efficiency of microvascular exchange under conditions of presumed capillary recruitment⁵⁵. The results of this study appear to lend some support to both arguments. Nearly all (90+%) capillaries were perfused at baseline, yet SNP still recruited an additional ~3%. That said, the degree of capillary recruitment observed was quite small relative to the magnitude of changes in microbubble distribution volume reported elsewhere (e.g.⁵⁶), and also quite small relative to the degree of capillary de-recruitment observed in this study with phenylephrine treatment. It is possible that changes in the contrast enhanced ultrasound signal which are usually attributed to capillary recruitment reflect distension of perfused vessels, changes in microbubble concentration within perfused vessels (perhaps through mechanisms like those regulating capillary hematocrit), or other factors that could modulate microbubble density within skeletal muscle *in vivo*.

Although our technique holds great promise for improving sensitivity and reproducibility in assessment of microvascular perfusion, there are several notable limitations. Some degree of sampling bias with respect to which capillaries are measured cannot be fully eliminated. Only superficial muscle capillaries (<50 μm from surface) can be visualized using this technique, and ~120 capillaries per FOV is a small fraction of the total number of capillaries in the muscle. However, in comparisons using averages drawn from 5 FOVs per mouse, which we recommend for unpaired study designs, the technique yields a total of ~600 capillaries per mouse, which is more a comprehensive sample than has previously been achieved using by-hand measurements or alternative software approaches.

Some caution is warranted in the interpretation of capillary hematocrit results obtained using this software. The slope of the line relating software and by-hand capillary hematocrit measures is less than unity (see Figure 6D), indicating that software estimates underestimate the dynamic range of capillary hematocrit. Although it is numerically possible to correct for this discrepancy, we decided that it would be more rigorous to use the raw measurements (which are biased towards false negative results) rather than introducing additional mathematical manipulations (which could produce false positives). For studies aiming to characterize biophysical interactions of RBCs and their flux through capillaries with maximal resolution, existing methods that directly visualize RBCs may be more

applicable^{14, 15, 23}. Our software is instead designed to provide a detailed assessment of perfusion in a maximal number of capillaries for future integration with published theories relating blood flow distribution to small-molecule exchange^{10, 57} to facilitate translational research relating microcirculatory function to the delivery of substrates and clearance of metabolic wastes.

An inherent limitation of studying anesthetized animals is that anesthesia may influence results. Commonly used anesthetics such as isoflurane and ketamine have previously been shown to alter endothelial NO metabolism^{58, 59}, and we observed in preliminary experiments that microvascular perfusion was unstable during our five-minute observation period when a ketamine/xylazine/acepromazine cocktail was used for anesthesia. Results obtained in this study must be interpreted in the context of mild anesthesia using isoflurane. That said, the fact that near-normal blood pressure and strong, consistent changes in microvascular perfusion following injections with SNP, phenylephrine, and even saline were observed collectively suggest that physiological regulation of blood flow and its distribution is largely preserved under isoflurane anesthesia.

In conclusion, we have developed an automated software technique for quantification of microvascular perfusion from intravital microscopy videos. This software is capable of accurately measuring flow velocities ranging from 0 to 2000 $\mu\text{m/s}$ in over 100 capillaries per FOV, is robust to image noise, and is sensitive enough to consistently detect subtle changes in microvascular perfusion following intravenous injection of saline. In future studies, this technique may be combined with existing techniques for quantifying transcapillary efflux of small molecules³⁴ or endothelial glycocalyx dimensions⁶⁰ for a more complete characterization of microvascular function and small molecule exchange. Although our research interests primarily concern the delivery of glucose, insulin, and/or oxygen to skeletal muscle as determinants of health or disease in obesity and type 2 diabetes^{61, 62}, this technique is imminently adaptable to other vascular beds and disease states. A more comprehensive and less labor-intensive means of characterizing microvascular perfusion will enable further clarification of the physiological and pathophysiological roles of the microcirculation in vivo.

5. Perspectives

Studies investigating microvascular perfusion have historically relied on techniques with some combination of unknown precision and significant user-dependency, or else that require specialized equipment and/or laborious analysis. In this study, we developed and validated a fully automated software platform for precise quantification of blood flow, its distribution, and its variability in over 100 capillaries per microscopic field of view. This technique holds great promise for improving sensitivity and reproducibility in future studies investigating the roles of the microcirculation in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

FOV	field of view
RBC	red blood cell
SNP	sodium nitroprusside
SNR	signal to noise ratio
PHI	perfusion heterogeneity index
PLI	perfusion lability index
PPV	proportion of perfused vessels
MFV	mean flow velocity
NO	nitric oxide

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Figure 1: Stabilization of raw microscope videos. A) Intensity prominence of the first frame (green) and a subsequent frame (red), showing a translational offset. B) 2D Cross-correlation reveals a maximum agreement at the appropriate offset between consecutive frames, allowing correction for motion artifacts. C) Following stabilization, the number of vessels with adequate signal-noise ratio for inclusion in final analysis significantly increases ($p < 0.0001$).

Figure 2:

Detection of vessel segments suitable for tracking. A) Time-average of plasma fluorescence in the stabilized video. B) Fluorescence intensity prominence (red, defined as percent brighter than 5-pixel neighborhood average intensity) and intensity gradient (green) are used to identify plasma perfused capillaries. C) Under/overexposed regions (green) are discarded, and suitable capillary segments for tracking (blue) are identified.

Figure 3:

Example of cross-correlation measurement of capillary flow velocity. A) Corrected fluorescence intensity profiles along the centerline of an example capillary segment in two consecutive frames, showing similar features with an axial offset. B) Average of the 20% highest relative agreement cross-correlation curves corresponding to the same capillary segment as in panel A, showing a clear peak at velocity = 800 $\mu\text{m/s}$. C) Individual frame-frame observations converge to an average value of velocity = 800 $\mu\text{m/s}$ for the 20% of frames with the highest signal/noise ratio in this example, but do not converge for frames

with poor signal/noise ratio. Note that the flow velocity in this example is faster than average and was used to exemplify the case during a situation requiring high rigor. It is a computationally more difficult measurement due to increased risk of blurring and aliasing.

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Figure 4:

Examples of binarized space-time images used to estimate capillary hematocrit. The left panel in each example image pair is the raw space-time image corresponding to each capillary. Plasma gaps are bright, while RBCs are dark. Thus, RBC flow produces diagonal dark streaks in the space-time image, and the slope of these streaks corresponds to flow velocity. The right panel in each example pair is the binarized version of the space-time image, where RBCs are shown as white, while plasma gaps are shown as black. The fraction of the binarized space-time image filled by RBCs is used as an estimate of capillary discharge hematocrit.

A)

Time = 9 frames (0.09 seconds)

Distance travelled = 41 pixels (106.6 μm)

Velocity = 1184 $\mu\text{m/s}$

B)

Figure 5:

By-hand measurement techniques used for software validation. A) Visible objects (either bright or dark deviations) were tracked by hand, and for each object, the total distance travelled and time elapsed were recorded. B) In the intensity prominence image produced for each video, three lines were drawn perpendicular to the prevailing orientation of the capillary network, and the average number of vessel intersections per mm was recorded. Note that the flow velocity in this example is unusually fast, and thus represents a case in

which blurring of flowing cells and plasma gaps makes velocity measurement particularly challenging.

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Figure 6:

Comparison of software and by-hand measurements. A) 5-second average of capillary flow velocity as measured by hand is consistent with 5-second average of capillary flow velocity as measured using our software technique ($R^2=0.91$, $p<0.0001$). Red indicates values affected by software detection limits. Slope of the regression line shown is slightly less than unity due to these values. B) Maximum flow velocity recorded within each vessel as measured by hand is consistent with maximum flow velocity as measured using our software technique ($R^2=0.85$, $p<0.0001$). C) Minimum flow velocity recorded within each vessel as

measured by hand is consistent with minimum flow velocity as measured using our software technique ($R^2=0.87$, $p<0.0001$). D) Capillary hematocrit as measured by hand is consistent with capillary hematocrit as estimated using our software technique ($R^2=0.64$, $p<0.0001$), although the dynamic range of hematocrit measurements is underestimated by the software, as reflected by a slope less than unity in the regression line. E) Statistically significant bias ($p<0.05$, mean difference 9.4%) was detected comparing 5-second average of capillary flow velocity as measured by hand and by software, but this bias was absent ($p=NS$) for measurements within software detection limits (see Section 3.2). F) No significant bias was found in software measurements of capillary density ($p=NS$).

Figure 7:

Sensitivity of software measurements to image noise and exposure time. A) Addition of 20% noise induces a small but statistically significant ($p < 0.0001$) decrease in number of vessels with adequate SNR, whereas addition of 80% noise induces a large, statistically significant ($p < 0.0001$) decrease in the number of vessels with adequate SNR. B) MFV as measured in the raw microscope videos correlates strongly with MFV as measured in videos with 20% added noise ($R^2 = 0.94$, $p < 0.0001$), but not in videos with 80% added noise ($R^2 = 0.05$, $p = \text{NS}$). C) PHI as measured in the raw microscope videos correlates strongly with PHI as measured

in videos with 20% added noise ($R^2=0.68$, $p<0.0001$). D) PLI as measured in the raw microscope videos correlates strongly with PLI as measured in videos with 20% added noise ($R^2=0.90$, $p<0.0001$). E) MFV as measured in 20ms exposure time videos is equivalent to that observed in 10ms exposure time videos up to a maximum capillary flow velocity of $V=1000 \mu\text{m/s}$, above which 20ms exposure time causes underestimation of MFV. F) Increasing exposure time from 10ms to 20ms significantly decreases the number of vessels with adequate SNR ($p<0.0001$).

Figure 8:

Software characterization of acute microcirculatory changes in response to an intravenous 0.45 mg/kg SNP bolus injection. Data presented as mean $\pm$ SEM. A) MFV significantly increased ($p < 0.0001$) within the first two minutes of SNP injection and then returned to baseline within 5 minutes. B) PHI significantly decreased ($p < 0.001$) within the first two minutes following SNP injection and then returned to baseline within 5 minutes. C) PLI significantly decreased for the first two minutes following SNP injection ($p < 0.0001$), and then returned to baseline within 5 minutes. D) PPV significantly increased within 1 minute

following SNP bolus injection ($p<0.05$) and then returned to baseline within 5 minutes. E) Mean capillary hematocrit significantly increased ($p<0.05$) within the first two minutes of SNP injection and then returned to baseline within 5 minutes. F) Hematocrit heterogeneity significantly decreased ($p<0.05$) within 1 minute following SNP bolus injection and then returned to baseline within 5 minutes.

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Figure 9:

Software characterization of acute microcirculatory changes in response to an intravenous 0.35 mg/kg phenylephrine bolus injection. Data presented as mean $\pm$ SEM. A) MFV significantly decreased ($p < 0.001$) during the first two minutes following phenylephrine injection and then returned to baseline within 5 minutes. B) PHI significantly rose ($p < 0.0001$) during the first two minutes following phenylephrine injection and then returned to baseline within 5 minutes. C) PLI significantly increased ($p < 0.0001$) during the first two minutes following phenylephrine injection and then returned to baseline within 5 minutes.

D) PPV significantly decreased ($p<0.001$) during the first two minutes following phenylephrine injection and then returned to baseline within 5 minutes. E) Mean capillary hematocrit significantly decreased ($p<0.05$) within 1 minute following phenylephrine injection and then returned to baseline within 5 minutes. F) Hematocrit heterogeneity significantly increased ($p<0.001$) during the first three minutes following phenylephrine injection and then returned to baseline within five minutes.

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Figure 10:

Software characterization of acute microcirculatory changes in response to an intravenous bolus 1.67 mL/kg injection of saline. Data presented as mean $\pm$ SEM. A) MFV significantly decreased for the first two minutes following injection of saline ($p < 0.05$), and then returned to baseline within 5 minutes. B) PHI did not significantly change in response to saline injection ($p = \text{NS}$). C) PLI did not significantly change in response saline injection ($p = \text{NS}$). D) PPV did not significantly change in response to saline injection ($p = \text{NS}$). E) Mean capillary hematocrit significantly decreased ($p < 0.01$) within 1 minute of saline injection and

then returned to baseline within 5 minutes. F) Hematocrit heterogeneity did not significantly change in response to saline injection (p=NS).

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Figure 11:

Software characterization of microvascular perfusion time course without intervention. Data presented as mean $\pm$ SEM. A) MFV does not significantly change over time without intervention ($p=NS$). B) PHI does not significantly change over time without intervention ($p=NS$). C) PLI does not significantly change over time without intervention ($p=NS$). D) PPV does not significantly change over time without intervention ($p=NS$). E) The number of vessels with adequate SNR for flow measurement significantly decreases over time

($p < 0.001$). The fraction of the FOV with appropriate illumination for flow tracking significantly decreases over time ($p < 0.05$).

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Figure 12:

Changes in blood pressure in response to vasoactive drugs. Data presented as mean $\pm$ SEM. A) Blood pressure underwent a small (<3 mmHg) but statistically significant ($p < 0.001$) transient increase in blood pressure between injection of fluorescent dextran (Rho-Dex) and injection with 0.45 mg/kg SNP, 0.35 mg/kg phenylephrine, or 0.9% saline (time 0 minutes through time 3 minutes in panel A). B) Saline injection produced a small, transient decrease in blood pressure (~ 5 mmHg, $p < 0.01$), whereas SNP injection caused a large, transient

decrease in blood pressure (~37 mmHg, $p<0.0001$) and phenylephrine caused a large, transient increase in blood pressure (~47 mmHg, $p<0.0001$).

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