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INVITED REVIEW

# Methods for the determination of skeletal muscle blood flow: development, strengths and limitations

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## Abstract

Since the first measurements of limb blood flow at rest and during nerve stimulation were conducted in the late 1800s, a number of methods have been developed for the determination of limb and skeletal muscle blood flow in humans. The methods, which have been applied in the study of aspects such as blood flow regulation, oxygen uptake and metabolism, differ in terms of strengths and degree of limitations but most have advantages for specific settings. The purpose of this review is to describe the origin and the basic principles of the methods, important aspects and requirements of the procedures. One of the earliest methods, venous occlusion plethysmography, is a noninvasive method which still is extensively used and which provides similar values as other more direct blood flow methods such as ultrasound Doppler. The constant infusion thermodilution method remains the most appropriate for the determination of blood flow during maximal exercise. For resting blood flow and light-to-moderate exercise, the non-invasive ultrasound Doppler methodology, if handled by a skilled operator, is recommendable. Positron emission tomography with radiolabeled water is an advanced method which requires highly sophisticated equipment and allows for the determination of muscle-specific blood flow, regional blood flows and estimate of blood flow heterogeneity within a muscle. Finally, the contrast-enhanced ultrasound method holds promise for assessment of muscle-specific blood flow, but the interpretation of the data obtained remains uncertain. Currently lacking is high-resolution methods for continuous visualization and monitoring of the skeletal muscle microcirculation in humans.

**Keywords** Exercise hyperemia · Xenon clearance · Dye dilution

## Abbreviations

| | |
|---|---|
| $A$ | Cross-sectional area of the artery |
| ICG | Indo-cyanine green |
| $C_{con}$ | The concentration of ICG in the non-experimental limb |
| $C_{infused}$ | Concentration of ICG infused |
| $C_{sample}$ | Concentration of ICG measured downstream (artery or vein) |
| $C_I$ and $C_B$ | Mass of the infusate (4.17) and blood (3.51), respectively. |
| CEUS | Contrast-enhanced ultrasound |
| HCT | Hematocrit |
| MI | Mechanical index |
| PET | Positron emission tomography |
| $S_I$ and $S_B$ | Specific gravity of the infusate (1.005) and blood (1.045), respectively |
| $T_B$ | Blood temperature before infusion |
| $T_I$ | Temperature of the infusate |
| $T_M$ | Mean blood temperature during the steady state of infusion |
| $V$ | Infusion rate |
| $v_{mean}$ | Time and space averaged, angle corrected and amplitude weighted mean blood velocity |

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## Introduction

Skeletal muscle contractile work requires energy production from oxygen-dependent and independent energy sources with the largest amount of energy derived from oxygen requiring processes in the mitochondria. Skeletal muscle is therefore highly dependent on adequate oxygen delivery by the cardiovascular system and thereby also precise

regulation of blood flow. In going from rest to maximal exercise, muscle-specific blood flow has been shown to increase as much as 100-fold (Rådegran 1999). During small muscle mass exercise, skeletal muscle blood flow increases rapidly and in direct proportion to workload from rest to maximal exercise (Andersen and Saltin 1985), but this linear relationship is compromised during maximal whole-body exercise as cardiac output reaches its upper limit (Mortensen et al. 2005).

Blood flow measurements in combination with determination of arterial and venous blood oxygen content can be used for precise determinations of oxygen delivery and oxygen uptake in an isolated limb. These measurements are useful for the determination of aspects such as basal blood flow regulation, limitations in oxygen delivery in aging and disease and estimations of aerobic and anaerobic energy contributions. Similarly, measurements of blood flow in combination with assessment of nutrients or radiolabeled compounds can provide valuable information on substrate uptake and metabolism. For such applications and general studies of blood flow regulation, accurate measurements of flow to skeletal muscle both at rest and during exercise is clearly of great importance.

Some of the earliest observations of increased skeletal muscle blood flow during muscle contraction were made in the late 1800s by measurements of venous outflow in animal models (Sadler 1869; Gaskell 1877). Gaskell first interpreted his findings to be related to nervous regulation of the blood vessels, but he subsequently also proposed a vasodilator role of waste products from the muscle (Gaskell 1880), thereby providing some of the first evidence for metabolically coupled vasodilation. These first studies on blood flow changes with muscle activity were followed by measurements using venous occlusion plethysmography, xenon clearance and thermodilution (Fig. 1). Today the most frequently used methods are venous occlusion plethysmography, ultrasound Doppler, and, to some extent, dye dilution, but more advanced methods such as positron emission tomography, are also applied and allow for determination of blood flow in specific regions of the muscle as well as flow heterogeneity.

The current review covers some of the methods for skeletal muscle blood flow determination in human skeletal muscle. Each method includes a brief description of the origin and the basic principles of the method, a description of important aspects of the procedure, and for some of the methods, proposed requirements for successful/correct assessments. It should, however, be noted that several variations may exist for each given method and that the method of choice depends on the specific situation. Each section will also include the strength and weaknesses of the method including applications.

## Venous occlusion plethysmography

### The first methods for measuring limb blood flow by venous occlusion plethysmography

The basic principles of venous occlusion plethysmography are fairly simple; when obstructing venous outflow from a limb, without affecting arterial inflow, changes in limb volume over time are directly proportional to arterial inflow rate (Greenfield et al. 1963). Brodie was the first to describe the volume changes in an organ by sudden occlusion of its efferent vein and measurement of the volume by an air-oncometer (Brodie 1902). At first, arterial inflow is unaltered, but will be attenuated when capillary and venous pressures increase. As a consequence, the organ swells rapidly at first, then progressively more slowly and the earliest portion of the volume curve represents arterial blood flow rate. Measurements of changes in limb volume were first described by Hewlett and van Zwaluwenburg who used a Brodie volume recorder connected to a plethysmograph (Hewlet and van Zwaluwenburg 1909). This was done by inflating a cuff to a supra-venous but sub-diastolic pressure on the proximal portion of the limb while measuring changes in limb volume. Measurement of changes in limb volume initially required cumbersome equipment. Various forms of the oncometer, an air- or water-filled jacket or sleeve connected to a volume recorder by an

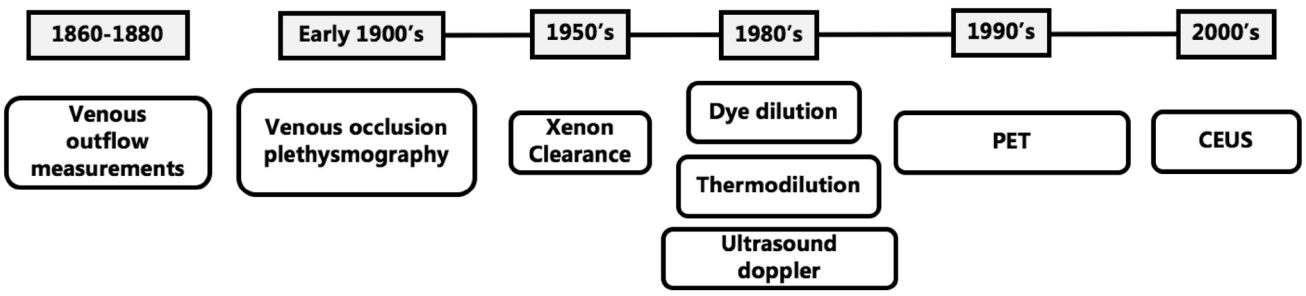


**Fig. 1** Timeline for use of different methods for limb blood flow in humans after the initial assessment of changes in limb blood flow with nerve stimulation by Sadler (1869) and Gaskell (1880) by venous outflow measurements

air- or water line (Whitney 1953; Dahn 1964), were used until the introduction of mercury-in-silastic strain gauges in 1953 (Whitney 1953). The principle of the mercury-in-silastic strain gauges was that the electrical resistance of a mercury-filled rubber tube would change linearly with changes in the rubber tube length (Whitney 1953). This new method was validated against the classical air and water filled oncometer models and proven equally precise and considerably easier to use (Dahn and Hallböök 1970; Eickhoff et al. 1980). The mercury-in-silastic strain gauges have since been the preferred method but a few attempts have been made to create a setup that could eliminate the need for toxic mercury (Stenow and Oberg 1993; Christ et al. 2000). For comprehensive details of the method, the reader is directed to Whitney (1953) and Wilkinson and Webb (2001). Venous occlusion plethysmography was among the first methods applied to study skeletal muscle blood flow during exercise (Williams and Lind 1979; Bernink et al. 1982) and has contributed significantly to our understanding of blood flow regulation.

### Important aspects of the procedure

The basic methodology has changed little since the origin of the method and the typical protocol consists of automated rapid inflation of the cuff to 40 mmHg for intervals of 10 s interspersed by 5 s of deflation with the limb positioned at the level of the heart (Chang et al. 1988; Wilkinson and Webb 2001). This protocol allows for venous emptying with minor altering of arterial inflow (Wilkins and Bradley 1946). Both the arm and leg can be used as experimental limb, but since arterial access is easier on the forearm, this is the preferred limb. Under resting conditions, blood flow to the forearm is distributed ~ 70/30 to the skeletal muscle and skin, respectively (Cooper et al. 1955). Moreover, blood flow measured with venous occlusion plethysmography is non-linear when the hand or foot is included and it is therefore standard procedure to inflate a cuff at the wrist or ankle to a supra-systolic pressure (Whitney 1953).

### Strengths

One of the major strengths of the method is that it is possible to assess the local response to intra-arterial infusion of a vasoactive compound on peripheral resistance vessels without affecting the systemic circulation (Allen et al. 1946; Benjamin et al. 1989). In addition, the method is fairly simple and has high validity and reproducibility (Roberts et al. 1986; Petrie et al. 1998; Walker et al. 2001). The applicability of the method includes arterial infusion of vasoactive compounds (Duff et al. 1953).

### Limitations

The method is an indirect measure of blood flow to a region and thus is limited in terms of absolute numbers. Since the subject needs to be completely still during the measurements, investigation of blood flow during active exercise is not feasible. Instead, investigators have used short exercise breaks where blood flow in the initial recovery phase has been studied (Williams and Lind 1979; Bernink et al. 1982). It should be considered that cuff inflation does limit arterial inflow while venous pressure is increased, leading to reduced perfusion pressure and this also makes collection of mixed venous blood difficult wherefore oxygen uptake cannot be calculated. In addition, it is difficult to deduct what percentage of a given intervention that relates to the vascular bed of the skeletal muscle versus the skin (Cooper et al. 1955). Standard procedure includes complete occlusion of the hand or foot this segment gets ischemic and thus the measuring period is limited.

## Xenon clearance

### The basic principle of limb blood flow measurements by xenon clearance

Xenon is an inert, highly diffusible lipophilic gas which can cross cell membranes. The principle of the xenon clearance method is that a small amount of a radioisotope of xenon ($^{133}$xenon) is injected directly into the skeletal muscle of interest and the clearance of the isotope is monitored by use of sodium iodide crystal probes mounted on the limb. The assumption is that clearance of an indicator such as xenon is directly related to blood flow, i.e., that the higher the blood flow the greater the clearance rate (Kety 1951). This relationship depends, however, on several assumptions, including the main assumption that the xenon tracer is indeed freely diffusible. In a study by Lassen et al. (Lassen 1964) clearance of $^{133}$xenon in skeletal muscle was compared to the previously used (Kety 1949, 1951), but less diffusible hydrophilic indicator/tracer $^{24}$Na. Clearance of $^{133}$xenon was markedly more rapid than that of $^{24}$Na (Lassen 1964) supporting the importance of high diffusibility. For specific details of the method, we suggest Lassen et al. (1964) and Grimby et al. (1967).

In the paper from 1964 (Lassen et al. 1964), Lassen and co-workers compared their values from the $^{133}$xenon clearance method to published values obtained by venous occlusion plethysmography (Allwood et al. 1958; Hillestad 1963). The two methods were reported to correspond well in healthy patients at rest; 2.1 mL 100 $g^{-1}$ $min^{-1}$ with xenon clearance versus 1.9 and 3.6 mL 100 $g^{-1}$ $min^{-1}$, for the two plethysmography studies, respectively (Lassen et al.

1964). The methods were also reported to be comparable during ischemia and plantar flexion combined; with peak flows reaching 44 and 53 mL 100 $g^{-1}$ $min^{-1}$ for the $^{133}$xenon and plethysmography methods, respectively (Lassen et al. 1964). These peak flows also corresponded well with those determined a few years later with $^{133}$xenon during maximal exercise in trained and untrained individuals (Grimby et al. 1967). Nevertheless, later studies showed that the xenon clearance method underestimates skeletal muscle flow several-fold compared to plethysmography and microsphere trapping (Cerretelli et al. 1984) and thermodilution (Saltin 1985).

### Strengths

The method can be used for the determination of changes in limb blood flow with interventions.

### Limitations

A drawback of the method is the need for injection of a radioactive isotope into the muscle tissue, albeit in relatively low amounts. The most important limitation is clearly the apparent inaccuracy of the method in skeletal muscle, with estimated blood flow values being approximately half of what is found with methods such as thermodilution and ultrasound Doppler (Saltin 1985; Rådegran et al. 1999).

## Dye-dilution technique: indo-cyanine green (ICG)

### The basic principle of limb blood flow measurements by dye dilution

Dye dilution is based on the principle that the blood concentration of the injected indicator is dependent on blood flow. The principle was first introduced for measurements of cardiac output (Stewart 1897, 1921), but was later applied for determination of organ blood flow (Colacino et al. 1981) and the first assessments of blood flow to exercising skeletal muscle were conducted in the early 1970s (Jorfeldt and Wahren 1971; Wahren and Jorfeldt 1973). Practically, a known amount of ICG is injected either as a continuous infusion into an artery feeding the region of interest, or as a bolus. When ICG is infused, it rapidly binds to plasma proteins and thereby is confined to the vascular space (Cherrick et al. 1960). The concentration of dye is then measured in a large vein draining the limb (Jorfeldt and Wahren 1971). Alternatively, dye can be injected in the distal part of a vein draining the limb and the concentration of dye measured in the vein proximal to the infusion site (Wahren and Jorfeldt 1973). The method has been described in detail by Wahren and Jorfeldt (Jorfeldt and Wahren 1971; Wahren and Jorfeldt 1973).

### Important aspects of the procedure

With constant-rate ICG infusion, the infusion is normally continued for several minutes to allow for equilibration of dye and blood in the entire vascular system (> 3 min at rest, > 1–2 min during exercise). Venous blood is drawn continuously through a photo-densitometer (10–20 mL/min) or blood samples are drawn periodically for later determination of ICG concentration. ICG is extracted by the liver and has a half-life of 3–4 min in individuals with normal liver function. To correct for recirculation of dye, the ICG concentration has to be determined simultaneously in samples obtained from the non-experimental limb with similar blood flow (e.g., during cycling). Blood flow is calculated as

$$\text{Blood flow} = \frac{\text{ICG infusion rate}(C_{\text{infused}} - C_{\text{sample}})}{(C_{\text{sample}} - C_{\text{con}})(1 - \text{HCT})},$$

where $C_{\text{infused}}$ is the concentration of ICG infused, $C_{\text{sample}}$ is the concentration of ICG measured downstream (artery or vein), $C_{\text{con}}$ is the concentration of ICG in the non-experimental limb and HCT is hematocrit.

With bolus ICG infusion, ICG is rapidly injected into the feeding artery immediately followed by flush of saline (~ 5 mL). Venous blood is continuously drawn (10–20 mL/min) through a linear photodensitometer. To correct for recirculation of dye, the ICG curve has to be extrapolated on the downslope (Fig. 2). Blood flow is calculated as the ratio of dye injected to the average arterial ICG concentration over the time interval of the ICG curve.

With both approaches, an ICG calibration curve is created at the end of the experiment by measuring the voltage deflection from samples of blood with different concentrations of ICG added (usually 3–4). A small amount of ICG should always be given to the subjects before starting the procedure to test for allergic reactions.

### Strength

ICG provides reliable measurements (especially constant infusion ICG) and can be performed during dynamic exercise, including intense whole-body exercise. ICG can be used to determine the mean transit time with bolus injections (Lassen et al. 2011). In combination with near-infrared spectroscopy, venous ICG has been used to determine regional blood flow (Boushel et al. 2000).

**Fig. 2** ICG curve obtained after bolus injection of ICG during 2-legged cycling. Blood flow is calculated from the area under the curve (yellow). Note that due to recirculation of ICG (green line), the last part of the curve is extrapolated from the first part of the downslope (red line)

### Limitations

Resolution is low and there has to be sufficient time between measurements as clearance of ICG from the circulation is slow. The method is invasive, but this can be reduced by using venous instead of arterial infusion of ICG. If blood is drawn continuously for ICG, blood has to be reinjected as the blood volume will otherwise decrease during the experiment. The dye used for the procedure is relatively expensive. Allergic reactions to the dye occur.

## Constant infusion thermodilution

### The basic principle of limb blood flow measurements by thermodilution

Thermodilution is an indicator technique, similar to that of the dye-dilution method, but instead of dye the indicator is ice-cold saline. The method was originally developed for measurements of cardiac output (Fegler 1954). Measurements of peripheral blood flow with bolus injections was first applied in resting animals (Fronek and Ganz 1960) and later in resting and exercising in humans (Ganz et al. 1964). The method was since modified to use a constant infusion of saline (Andersen and Saltin 1985) similar to thermodilution measurements of cardiac output (Pavek et al. 1964). Please see Andersen and Saltin (1985) and Gonzalez-Alonso et al. (2000) for further details of the method. Thermodilution was used to demonstrate that skeletal muscle perfusion can reach ~ 2.5 L/min/kg (Andersen and Saltin 1985) and that leg blood flow does not increase linearly from rest to maximal cycling (Rosenmeier et al. 2004; Mortensen et al. 2005), indicating that the heart cannot optimally perfuse skeletal muscle during maximal whole-body exercise.

### Important aspects of the procedure

Ice-cold saline is infused into a vein draining the limb at interest at a constant rate (30–200 mL/min) with the aim to reduce blood temperature by ~ 1 °C and blood temperature is measured downstream (Andersen and Saltin 1985). Infusion should preferably be through a catheter with side holes to secure instant mixing of saline with blood. Due to the marked effect of muscle contractions and the heart cycle on muscle blood flow, the measurement should be averaged for several cycles. At low blood flows, tissue cooling may occur with constant infusion which affects the measurements, therefore bolus injections (5–10 mL) are more appropriate during resting conditions. Measurements have to be kept short (< 20 s) and there should be sufficient time between measurements to avoid cooling of the surrounding tissue. The accuracy of the measurement is increased if the temperature of the infusate is monitored (Gonzalez-Alonso et al. 2000).

Blood flow is calculated as

$$\text{Blood flow} = \left(\frac{V_1 \times (S_1 \times C_1)}{(S_B \times C_B)}\right)\left(\frac{(T_B \times T_I)}{(T_B \times T_M)}\right) - 1,$$

where $V$ is the infusion rate, $S_I$ and $S_B$ are the specific gravity of the infusate (1.005) and blood (1.045), respectively, $C_I$ and $C_B$ are the mass of the infusate (4.17 kJ/g/°C) and blood (3.51 kJ/g/°C), respectively, $T_B$ is the blood temperature before infusion, $T_I$ is the temperature of the infusate, $T_M$ is the mean blood temperature during the steady state of infusion.

### Strength

The major advantage of thermodilution is that it is independent of body movements and therefore can be used during whole-body exercise and maximal exercise. It provides reliable measurements compared to ultrasound Doppler (Fig. 3). Because the indicator is saline, the measurements are not complicated by recirculation or toxicity as ICG. The technique is fairly easy to learn.

### Limitations

A primary limitation is the relatively low temporal resolution. The method is invasive and requires venous

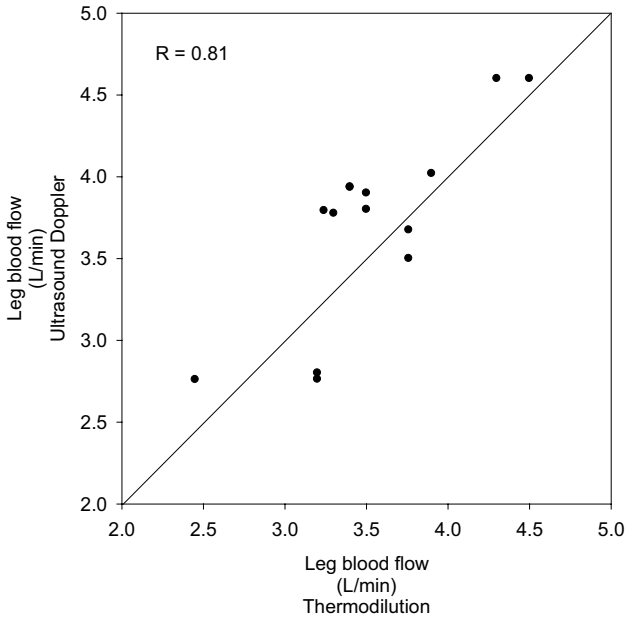


**Fig. 3** Leg blood flow simultaneously determined by ultrasound Doppler (x-axis) and constant infusion thermodilution (y-axis) during arterial ATP infusion (0.03 µmol/min/kg leg mass) in young healthy male subjects. For femoral arterial blood flow determined by ultrasound Doppler, blood velocity was averaged over 16 heart cycles. For femoral venous blood flow determined by constant infusion thermodilution, blood temperature was averaged over 8–10 s during infusion of ice-cold saline. $R = 0.81$, $P < 0.05$. Previously unpublished data

catheterization. Equipment for thermodilution is not readily available. Many measurements in an experiment can lead to hemodilution.

## Ultrasound Doppler

### The first use of ultrasound Doppler for measuring arterial blood flow

The ultrasound Doppler was first introduced as a novel method for studying living tissue in the clinical setting (Wild and Neal 1951; Howry and Bliss 1952) and for studies of heart wall and valve function (Satomura and Matsubara 1956; Satomura 1957). Improvements to hardware and software made possible accurate estimates of skeletal muscle blood flow at rest (Lewis et al. 1986) and during active exercise in the 1980s (Wesche 1986; Walloe and Wesche 1988). One hallmark of the methodological development was the introduction of duplex Doppler ultrasound (Barber et al. 1974). This system allows for continuous concomitant vessel imaging (knows as B-mode imaging) and Doppler tracing. Duplex ultrasound was intended for improving the diagnostic validity of atherosclerotic vessels where plaque, that in B-mode appears like blood, could be recognized as not following arterial blood flow (Barber et al. 1974). The principles of ultrasound Doppler flow measurement are based on the Doppler frequency shift that occurs when sound waves are reflected by a moving object. In the vasculature, transmitted ultrasound is reflected by moving erythrocytes which causes a shift in the ultrasound frequency which is sensed by the receiver (Gill 1979). Knowledge of the angle by which the ultrasound beam meets the moving erythrocytes (insonation angle) and the difference in transmitted and received ultrasound frequency, allows for calculation of the speed at which the erythrocyte is moving (Fig. 4). Combined with measurement of the vessel cross-sectional area obtained in a perpendicular B-mode image blood, flow rates can be calculated (Gill 1979, 1985). Blood flow rate (L/min) $= 6 \times 10^4 \times v_{mean} \times A$, where $v_{mean}$ is the time and space averaged, angle corrected and amplitude weighted mean blood velocity and $A$ is the cross-sectional area of the artery (Gill 1985). For further details of the method the reader is directed to (Rådegran 1997). The ultrasound Doppler method has been a widely used method to study skeletal muscle blood flow and the method was, together with thermodilution, central to the first records of muscle blood flow during rhythmic muscle contraction (Walloe and Wesche 1988), to advancements in our knowledge about mechanisms of blood flow regulation (Rådegran and Saltin 1999; Rådegran and Calbet 2001; Schrage et al. 2004) and to elucidating blood flow kinetics at the onset of exercise (Rådegran and Saltin 1998).

### Important aspects of the procedure

To obtain accurate blood flow, the probe should be positioned directly above a straight segment of the vessel and away from bifurcations to avoid turbulence. Correct probe orientation and operation is key to valid measures. Sample volume should be maximized to the width of the vessel and the lowest possible insonation angle should be used (always below 60°) by tilting the probe. Low-velocity filter can be applied to reject noise from turbulence at the vessel wall. Mean blood flow velocities during steady state conditions should be measured continuously over a minimum of 30 s in duplex mode (2D and Doppler), during simultaneously 2D vessel visualization and audiovisual blood velocity feedback. Vessel diameter used for calculation of blood flow should be obtained in 2D mode with the probe parallel to the vessel and, if not tracked continually during the heart cycle, be measured during the systolic phase.

### Strengths

The ultrasound Doppler technique has some great advantages over some of the other blood flow measuring methods. For starters, it is non-invasive which puts it on par

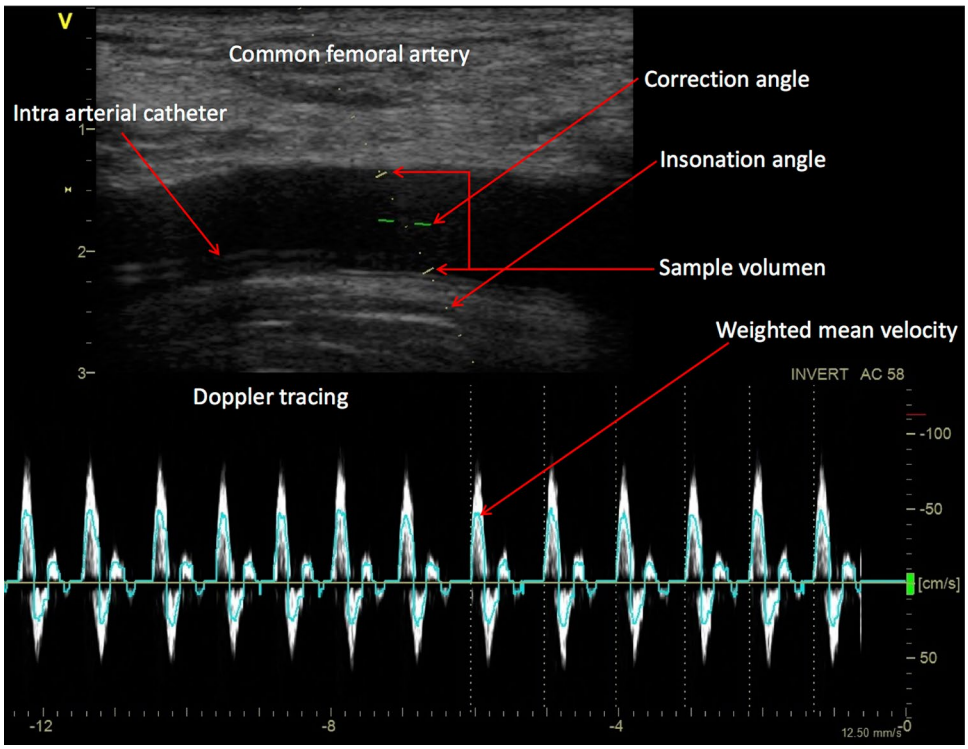


**Fig. 4** Image of ultrasound Doppler tracings from the common femoral artery. Note the arterial catheter for drug infusion and/or arterial blood pressure measurements. Insonation angle, correction angle and sample volume are of great importance for a valid determination of arterial flow. The blue line in the Doppler tracings is weighted mean blood flow velocities, i.e., a weighted average of the sample volume

with methods like venous occlusion plethysmography and MRI. However, ultrasound Doppler is capable of measuring blood flow continuously during active exercise which gives the method a great advantage over the other methods when studying exercise physiology. The one-leg knee-extensor model is one of the preferred setups for measurements during exercise (Andersen et al. 1985). In particular, the high temporal resolution allows for accurate measures of changes in blood flow under conditions like onset of exercise or drug infusion (Rådegran 1997). With a trained operator, ultrasound Doppler is a very accurate method, which correlates well with invasive techniques like thermodilution and venous occlusion plethysmography (Figs. 3, 5) (Rådegran 1997) and which has a low day-to-day variability (Shoemaker et al. 1996).

## Limitations

Mastering the technical aspects of ultrasound Doppler measurements requires a substantial training period to learn consistent handling of the ultrasound probe and software settings, allowing for valid and reliable measures. Since the angle of the probe is vital, exercise modalities are required to be arranged such that the vessel of interest is in a fixed position. In addition, ultrasound Doppler machines that are capable of measuring blood flow rates within a broad physiological range are expensive which can limit the application of this model in some research facilities. During arterial drug infusion, depending on the catheter position, noise from the infusion catheter during arterial infusion can interfere with the Doppler signal and result in inaccurate measures.

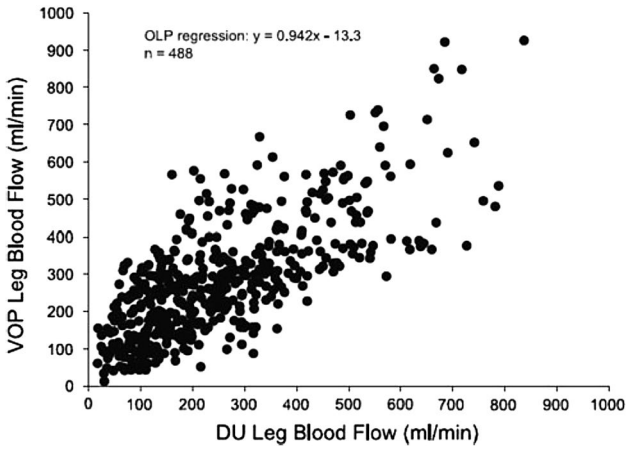


**Fig. 5** Scatterplot of paired venous occlusion plethysmography (VOP) and Doppler ultrasound (DU) estimates of leg blood flow during the relaxation period after 50 duty cycles (15 s intervals) of intermittent contractions (50–400 N) in 11 subjects. Pearson correlation $r = 0.91$. Venous occlusion plethysmography measures are consistently ~ 13 mL/min lower compared to values obtained with ultrasound Doppler as indicated by the *y*-intercept. Reproduced from Green et al. 2011

## Positron emission tomography

Positron emission tomography (PET) is a nuclear functional imaging technique utilizing radionuclides which decay by positron emission. For analysis of skeletal muscle blood flow by PET, radiolabeled water $^{15}O–H_2O$, with a half-life of about 2 min has commonly been used (Nuutila et al. 1996; Ruotsalainen et al. 1997). The procedure involves intravenous injection of a small amount of the tracer and the radioactivity emitted by the tracer is followed over time by a PET scanner consisting of gamma ray detectors positioned in a circular formation around the object to be measured. The kinetics of the tracer is then used in a mathematical model to assess the magnitude of blood flow. As with the xenon clearance method, the PET method assumes free diffusibility of the tracer used.

Historically, a technical development key to the PET methodology was the ability to artificially produce short-lived radionuclides in sufficiently large quantities by use of a cyclotron. One of the first cyclotron used for this purpose was developed by Ernest O. Lawrence in the 1930s. These radionuclides were subsequently coupled to relevant freely diffusible biological compounds such as glucose and water and one of the earliest reported applications of radionuclides in man was the use of $^{11}C$-labeled carbon monoxide in 1945 (Tobias and Lawrence 1945). The first measurements of radionuclides were primarily conducted with Geiger Muller counters and in the 1970s the first computerized transaxial tomograph was built based on important ground work by a number of researchers from different research institutions (Rich 1997). The first measurements of human skeletal muscle blood flow with the use of radiolabeled water were conducted in the 1990s (Nuutila et al. 1996; Ruotsalainen et al. 1997; Ament et al. 1998). See Ruotsalainen et al. (1997) and Laaksonen et al. (2013) for methodological details. The method has been important for the field of exercise physiology by contributing to our understanding of regional differences in muscle flow and how aspects such as age and training status impact on blood flow heterogeneity (Kalliokoski et al. 2001b; Laaksonen et al. 2003, 2013; Rudroff et al. 2014).

### Strengths

A major advantage of the PET methodology is that it can provide quantitation of blood flow in specific regions of the muscle (Fig. 6), allowing for determination of regional differences of flow in the muscles (Laaksonen et al. 2013; Heinonen et al. 2017). The method also allows for determination of blood flow heterogeneity (Kalliokoski et al. 2000, 2001a) which is a useful complementary parameter to understand changes in muscle perfusion with aging, training, etc. Another advantage is that blood flow assessment can be combined with the determination of metabolism by also infusing nutrient tracers such as glucose (Kemppainen et al. 2003). Furthermore, the short half-life of the tracers used limits exposure to radiation and allow for measurements to be repeated within a limited time-frame.

### Limitations

The method requires a PET scanner which are very expensive and rarely found in research settings. The short-lived radioisotopes have to be generated on-site in a special particle accelerator device and the tracers used are therefore also rather expensive. Measurements are sensitive to movement of the measurement site and the subject has to be in a fixed supine position in the scanner. Therefore, measurements during exercise only possible with e.g. specifically designed single leg knee extensor device (Kalliokoski et al. 2000; Heinonen et al. 2007).

## Contrast-enhanced ultrasound (CEUS)

### The basic principle of CEUS for determination of microcirculatory blood flow

Contrast-enhanced ultrasound (CEUS) with the use of second generation (gas filled) microbubbles as contrast agent was originally introduced as a method to determine myocardial blood flow (Wei et al. 1998) and later adapted for determination of skeletal muscle perfusion (Coggins et al. 2001; Krix et al. 2005). At low insonation power, the microbubbles will oscillate enhancing the ultrasound signal. During low mechanical index imaging (MI; a measure for the output power), the signal intensity (measured in decibel) indicates the volume of blood in the region but cannot be used directly to estimate perfusion (blood flow per tissue unit) in the region. The microbubbles can be destroyed by use of high-energy ultrasound pulses. Usually microbubbles are destroyed at a mechanical index (MI; a measure for the output power) $> 0.5$, but usually a MI at $> 1$ is used. After destruction of the microbubbles, muscle perfusion can be assessed by analyzing the replenishment kinetics in the measured area (Krix et al. 2005; Hudson et al. 2009). The method is described in details here (Coggins et al. 2001; Krix et al. 2005).

### Important aspects of the procedure

Microbubbles for determination of perfusion are most commonly administered as a continuous intravenous infusion

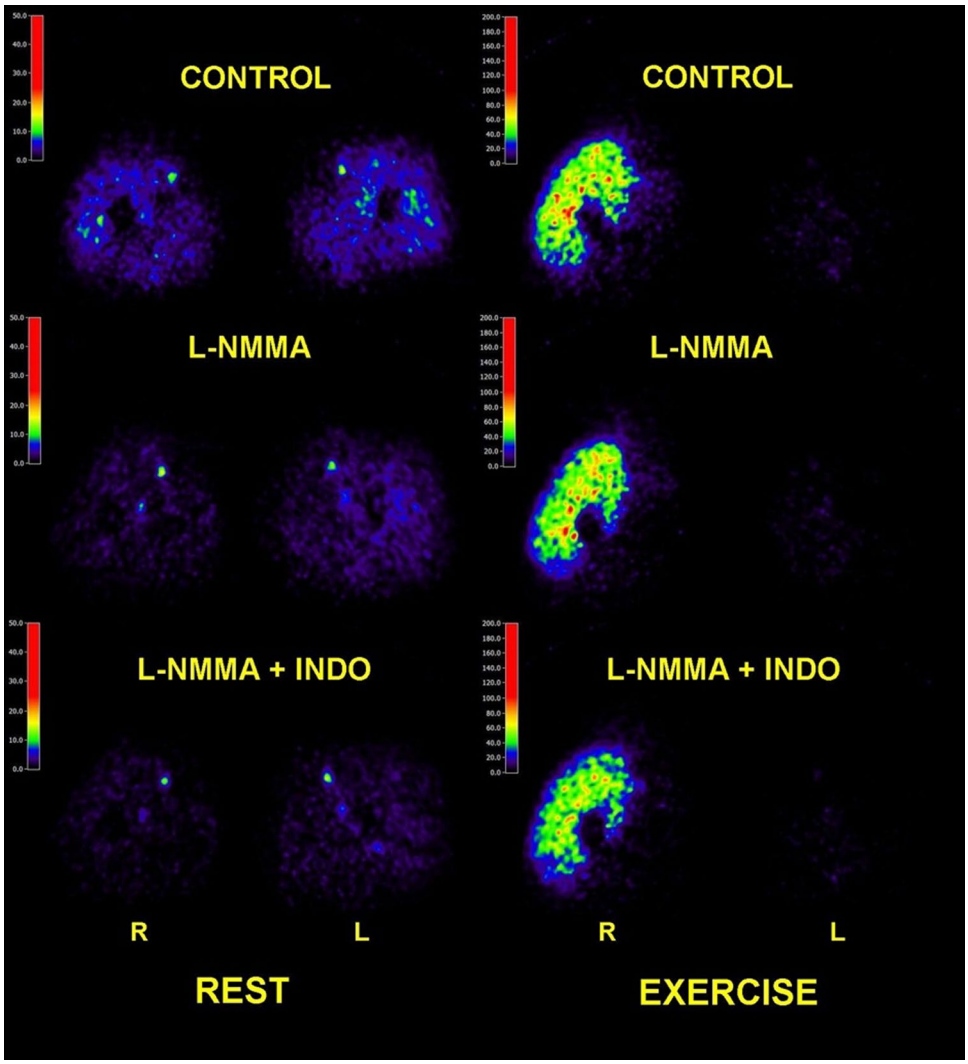


**Fig. 6** Images of cross-sectional positron emission tomography (PET) blood flow with $H_2O^{15}$ in the middle thigh region at rest and during knee extensor exercise with the right leg. The images show representative graphs during control condition and with infusion of an inhibitor (NG-monomethyl L-arginine: L-NMMA) of nitric oxide synthase (NOS) alone and in combination with an inhibitor of prostaglandin formation (indomethacin). L-NMMA and L-NMMA + indomethacin lowered muscle blood flow at rest whereas during exercise flow was reduced with the combination of L-NMMA + indomethacin. *R* right, *L* left. Figure reproduced from Heinonen et al. 2011

(Wei et al. 1998; Krix et al. 2009), but bolus injections have also been used (Krix et al. 2005; Mulder et al. 2008). If a continuous infusion is applied, a special infusion pump, that insures continuous mixing of microbubbles, should be used. The region of interest should be chosen so that it represents a region in which no large arteries are included and with the measured signal primarily representing the microvasculature.

## Strengths

Strength of CEUS is that it is a measure of local tissue blood flow and that it is minimally invasive.

## Limitations

An important limitation is that quantification of blood flow is difficult as the exact measuring area is difficult to determine (e.g., ultrasound beam thickness and shape). Measurements during dynamic exercise are, furthermore, questionable as the measuring site is fixed, whereas the muscle tissue moves during contractions. During dynamic exercise, capillary blood flow is impeded due to the contractions which influences the quantitation of microbubble disruption as microcirculatory blood flow is blocked by the muscle contraction.

The CEUS signal, influenced by acoustic parameters and the ultrasound settings (e.g., depth and measurement area), therefore have to be kept constant when comparing different measurements. Time resolution is limited as measurements with continuous infusion require steady state conditions and $>5$ min is therefore needed to obtain a measurement. Both the required ultrasound equipment and the commercially available microbubbles are expensive.

Microbubbles vary in size, but are generally of similar size or smaller than erythrocytes. The in vivo kinetics of microbubbles have been reported to resemble those of erythrocytes, but with some variability and a tendency towards a faster velocity of microbubbles than erythrocytes

at higher blood flows (Jayaweera et al. 1994). In the microcirculation, erythrocyte flow is higher than plasma flow (Fahraeus–Lindqvist effect). Thus, it is important to take into account that CEUS resembles erythrocyte flow rather than plasma flow.

## Summary of strengths and applications of the muscle blood flow methods

The choice of method for determination of limb blood flow relies on a complex interplay between a number of factors such as the experimental setup, the subject characteristics, research economy and expertise of the investigators. Here we summarize our view of the suitability of the different methods (see also Table 1).

### Resting condition

The simplest measure of limb blood flow is in the resting condition where the tissue is not moving and blood flow is in a steady state. In this situation, all of the abovementioned methods for blood flow measurements can be applied, but ultrasound Doppler and plethysmography are the preferred methods in a non-invasive setup. Thermodilution and dye dilution are associated with variability at rest due to the low flows. Xenon clearance is likely to underestimate flow.

### Drug infusion

During arterial drug infusion, blood flow to a limb of interest is manipulated with vasoactive substances while the limb is in a resting condition. In this setup, venous occlusion plethysmography and ultrasound Doppler are ideal methods for determination of changes in limb blood flow given their non-invasive nature and high precision. While both methods are applicable, the ultrasound Doppler has the advantage that it is capable of measuring changes in blood flow during each heart cycle, whereas plethysmography has an inherent lower temporal resolution.

### Exercise modalities

When investigating blood flow kinetics in the transition from rest to exercise and flow during low-to-moderate intensity exercise, ultrasound Doppler is the method with highest temporal resolution and combined with beat-by-beat changes in perfusion pressure provides the most accurate assessment of changes in blood flow.

Venous occlusion plethysmography can be used for measuring blood flow in association with exercise, but as it requires exercise to be paused during the actual measurement it reflects recovery blood flow more than actual exercise blood flow. Nevertheless, comparisons between ultrasound Doppler and venous occlusion plethysmography show a good correlation for flows during exercise at low and moderate intensities.

Since ultrasound Doppler requires an experimental setup where the site of blood flow measurement is not moving, measurements during high-intensity exercise are difficult even for very experienced operators and flowrates can exceed the detection range of the ultrasound system. The preferred methods for blood flows during intense exercise are instead the thermo- and dye-dilution techniques. Likewise, during whole-body exercise and exercise modalities that require activation of several muscle groups, like running, cycling or cross-county skiing, dilution techniques are the only real options.

### Regional blood flow distribution at rest

For determination of muscle-specific flow and distribution of flow within a muscle or between muscle groups, PET is the only method available. PET also has the advantage that it can be combined with parallel regional metabolic assessments. CEUS does allow for determination of regional of blood flow, but is limited to a relatively small site where the probe is situated and uncertainties with regard to interpretation of the data remain.

## Perspective

Over the years, several methods for the determination of skeletal muscle blood flow in humans have been developed and refined, allowing not only for the assessment of global flow to limbs, but also for determination of more local muscle specific flows. However, still lacking is a high-resolution method for the continuous, precise determination of muscle blood flow at the microcirculatory level in humans, in line with the outstanding information provided in animals with intravital microscopy. Such a method would provide much needed detail and bring us closer to an understanding of the specifics of muscle blood flow regulation and oxygen delivery within human muscle in health and disease. Although CEUS in principle has the potential to provide an estimate of overall microcirculatory flow, the method is unlikely to allow for the necessary resolution. Furthermore, improved methods to precisely assess muscle power output and metabolism in parallel with microcirculatory blood flow measurements are warranted.

**Table 1** Summary of strengths and applications of the muscle blood flow methods

| | Rest | Drug infusion | Limb exercise, low intensity | Limb exercise, high intensity | Whole-body exercise | Special requirements |
|---|---|---|---|---|---|---|
| Venous occlusion plethysmography | **Good** | **Good** | *Limited, *requires exercise to be paused* | *Limited, *requires exercise to be paused* | N/A | ***Requires mercury-filled silastic tubes that are being phased out*** |
| Ultrasound Doppler | **Good** | **Good** | **Good, *requires skilled operator** | *Limited, *requires skilled operator* | N/A | ***Requires substantial training of operator. Equipment is expensive*** |
| Dye dilution | *Limited, *low flow rates give high variation* | **Good, *limited temporal resolution** | **Good, *limited temporal resolution** | **Good, *limited temporal resolution** | **Good** | ***Requires invasive procedures*** |
| Thermodilution | *Limited, *low flow rates give high variation* | **Good, *limited temporal resolution** | **Good, *limited temporal resolution** | **Good, *limited temporal resolution** | **Good** | ***Requires invasive procedures*** |
| Positron emission tomography | **Good** | **Good** | *Limited, *requires exercise to be paused* | *Limited, *requires exercise to be paused* | N/A | ***Requires PET scanners and facilities for isotope production. Normally limited to clinical settings*** |
| Contrast-enhanced ultrasound | *Limited, *uncertainties in data interpretation* | *Limited, *uncertainties in data interpretation* | N/A | N/A | N/A | ***Requires invasive procedures. Equipment is expensive*** |

Xenon clearance method has not been included in the above table as it is not considered a sufficiently accurate measurement for determination of skeletal muscle blood flow during exercise

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