Studies on Polarised Light Spectroscopy

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Abstract

This thesis project focuses on measurements of dermal microcirculation during vascular provocations with polarised light spectroscopy. This is done with a non-invasive method commercially available as Tissue viability imaging (TiVi) which measures concentration and oxygenation of red blood cells in the papillary dermis. Three studies were done with human subjects and one with an animal model, to validate and compare the TiVi technique with laser Doppler flowmetry, which is an established method of measuring dermal microcirculation.

The TiVi consists of a digital camera with polarisation filters in front of the flash and lens, with software for analysis of the picture. When taking a picture with the TiVi, the polarised light that is reflected on the skin surface is absorbed by the second filter over the lens (which is perpendicular to the first filter) but a portion of light penetrates the surface of the skin and is scattered when it is reflected on tissue components. This makes the light depolarised, passes the second filter, and produces a picture for analysis. The red blood cell (RBC) has a distinct absorption pattern that differs between red and green colour compared to melanin and other components of tissue. This difference is used by the software that calculates differences in each picture element and produces a measure of output which is proportional to the concentration of red blood cells. The oxygenation of RBC can also be calculated, as there is a difference in absorption depending on oxygen state.

The first paper takes up possible sources of error such as ambient light, and the angle and distance of the camera. The main experiment was to investigate how the local heating reaction is detected with TiVi compared to LDF.

In the second paper arterial and venous stasis are examined in healthy subjects with TiVi.

The Third paper is an animal study where skin flaps were raised on pigs, and the vascular pedicle is isolated to enable control of inflow and outflow of blood. The measurements were made during partial venous, total venous, and total arterial occlusion. The TiVi recorded changes in the concentration of RBC, oxygenation and heterogeneity and the results were compared with those of laser Doppler flowmetry.

In the fourth paper oxygenation and deoxygenation of RBC: s was studied. Studies were made on the forearms of healthy subjects who were exposed to arterial and venous occlusion. Simultaneous measurements were made with TiVi and Enhanced perfusion and oxygen saturation or EPOS, which is a new device that combines laser Doppler flowmetry and diffuse reflectance spectroscopy in one probe.

With TiVi, one can measure RBC concentration and oxygenation in the area of an entire picture or in one or multiple user defined regions of interest (ROI). Methods such as laser Doppler flowmetry makes single point measurements, which is a potential source of error both because of the heterogeneity of the microcirculation, and that

the circulation be insufficient in the margins of the investigated area. TiVi has been able to measure venous stasis more accurately than laser Doppler flowmetry, and venous stasis is the more common reason for flaps to fail.

The TiVi is an accurate way to measure the concentration of RBC and trends in oxygenation of the dermal microcirculation. It has interesting possible applications for microvascular and dermatological research, monitoring of flaps, and diagnosis of peripheral vascular disease. Future clinical studies are needed as well as development of the user interface.

Populärvetenskaplig sammanfattning

Fokus i detta avhandlingsarbete är inriktat på experimentella mätningar i läderhudens mikrocirkulation. På försökspersoner och i en djurmodell. Mätningarna görs med ett instrument som benämns Tissue viability imaging (TiVi), vilken kan mäta koncentrationen av röda blodkroppar samt dess syremättnad i läder huden över en större yta. Utrustningen består av en vanlig systemkamera med polarisationsfilter på lins och blyxt samt en dator med programvara för bildanalys.

Mätmetoden utnyttjar att ljus kan vara polariserat ("ordnat") eller depolariserat ("oordnat") samt att röda blodkroppar har ett absorptionspektrum som avviker mot annan vävnad i läder huden såsom kollagen.

Vid fotografering passerar blyxtljuset genom ett polarisationsfilter och blir polariserat. Ljuset som studsar på hudytan är fortsatt polariserat och absorberas av polarisationsfiltret framför linsen. Det ljus som tränger igenom hudytan reflekteras i vävnaden och blir depolariserat innan det passerar linsens filter och når bilddetektorn. Röda blodkroppar har ett annorlunda absorptionsmönster, det vill säga ljuset tas upp, respektive reflekteras på ett annat sätt jämfört med omgivande vävnad. Det avvikande absorptionsmönstret utnyttjas av systemets programvara, vilken beräknar ett koncentrationmått på röda blodkroppar i bilden. Det finns dessutom en skillnad i absorptionsmönstret mellan oxygenerade (syresatta) och deoxygenerade (icke syresatta) blodkroppar, därför går det att beräkna förändringen i syresättningsgrad mellan dessa mönster.

TiVi -tekniken mäter således koncentration av röda blodkroppar i en större hudyta, vilket skiljer sig från den mer etablerade tekniken Laser Doppler Flowmetry (LDF) vilken mäter perfusion endast i en punkt. Perfusion kan definieras som en produkt av hastighet och koncentration av rörliga blodkroppar.

I det första delarbetet studerades först faktorer som kan påverka mätvärdet såsom avstånd mellan kamera och hudyta, bakgrundsljus och vinkel mellan kamera och hudyta. Vidare så jämfördes TiVi och LDF vid mätning av hur cirkulationen förändras vid värmning av huden. Lokal värme är ett sätt att studera avvikelser i mikrocirkulationen. Det gick att visa att TiVi- liksom den mer etablerade LDF-tekniken kan registrera reaktionen på lokal värme.

I delarbete II studerades arteriell och venös stas hos friska försökspersoner och TiVi -tekniken jämfördes mot LDF-tekniken. Detta gjordes genom att medelst blodtrycksmanschett och Esmarchlinda först trycka ut blodet i armen, därefter stoppa flödet med uppblåst blodtrycksmanschett över överarmen och sedan mäta under arteriell stas. Därefter utfördes mätserier efter att blodtrycksmanschetten tömts för att observera hudrodnaden (post-ocklusiv hyperemi) som uppstår. Vidare studerades hur TiVi och LDF förmår att registrera venös stas där blodtrycksmanschetten blåsts upp så att blod kan pumpas in i armen men ej tillbaka. I ett delexperiment utfördes stegvis uppblås-

ning av blodtrycksmanschetten för att studera metodens känslighet för små förändringar i kärlocklusion. Först ökades trycket för att sedan stegvis tömmas.

I delarbete III användes en djurmodell för att studera blodkoncentration och oxygenering i en hudlambå. En hudlambå är i detta fall en bit av grishud och underhuds-fett som är fripreparerad så att den endast har kontakt med grisens kropp via en artär och ven. Genom att stänga av dessa helt eller delvis kan man styra blodflödet till och från lambån. I detta arbete studerades hur TiVi och LDF kunde registrera förändringar i mikrocirkulationen vid partiell och total avstängning av venöst återflöde samt total avstängning av tillförande blod i artären. Även oxygeneringsgrad av röda blodkroppar studerades. Den tredje parametern som studerades var så kallad heterogenitet vilket är ett mått på fördelningen av blod i huden. Vid venös stas kan ökad flammighet eller heterogenitet observeras i huden.

I delarbete IV studerades oxygenering och deoxygenering mer i detalj. I en experimentell modell på friska försökspersoners underarmar studerades oxygenering av röda blodkroppar vid arteriell och venös stas. Mätmetoden TiVi jämfördes mot en teknik som kombinerar LDF och diffus reflektansspektroskopi.

Användandet av Tissue viability imaging ger möjlighet att på ett enkelt noninvasivt sätt kunna undersöka blodkoncentration, oxygenering och heterogenitet i ett större hudavsnitt. TiVi tekniken medger en förbättrad möjlighet att detektera venös stas än LDF samt ökar kunskapen om fysiologin och patofysiologin i hud när cirkulationen är påverkad.

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List of papers

This thesis is a summary and results of the following articles. In the text they will be referred to by their roman numerals.

Paper I

Polarized light spectroscopy for measurement of the microvascular response to local heating at multiple skin sites.

Tesselaar E, Bergkvist M, Sjöberg F och Farnebo S.

Microcirculation, 19: 705-713 Nov 2012 PMID: 22716906

Paper II

Assessment of microcirculation of the skin using tissue viability imaging: A promising technique for detecting venous stasis in the skin

Bergkvist M, Henricson J, Iredahl F, Tesselaar E, Sjöberg F, Farnebo S.

Microvascular Research. 2015 Sep; 101:20-5. doi: 10.1016/j.mvr.2015.06.002 PMID: 26092681

Paper III

Vascular Occlusion in a Porcine Flap Model: Effects on Blood Cell Concentration and Oxygenation.

Bergkvist M, Zötterman J, Henricson J, Iredahl F, Tesselaar E, and Farnebo S

Plastic and Reconstructive Surgery Global Open. 2017 Nov 17; 5(11):e1531. doi: 10.1097/GOX.0000000000001531. eCollection 2017 Nov PMID: 29263951

Paper IV

Assessment of tissue oxygenation and red blood cell heterogeneity enables new means for detecting vascular events in the skin

Bergkvist M, Henricson J, Bergstrand S, Strömberg T, Tesselaar E and Farnebo S

Manuscript

Abbreviations

ABI Ankle brachial index

AU Arbitrary units

BP Blood pressure

°C degrees Celsius

cGAP Cranial Gluteal Artery Perforator flap

CRBC Concentration of red blood cells

CRBC_{TiVi} Concentration of red blood cells as measured with TiVi

CMBC Concentration of moving blood cells

CV Coefficient of variation

DIEP Deep inferior epigastric artery perforator flap

DRS Diffuse reflectance spectroscopy

EPOS Enhanced perfusion and oxygen saturation

FCD Functional capillary density

Hb Haemoglobin

ICC Intraclass correlation coefficient

IQR interquartile range

LDF Laser Doppler flowmetry

LDPI Laser Doppler perfusion imaging

LSCI Laser Speckle Contrast Imaging

MAP Mean artery pressure

NO Nitric oxide

OPS Orthogonal polarization spectral imaging.

PORH Post Occlusive Reactive Hyperaemia

PU Perfusion units

RBC Red blood cell

RBC_{deoxy} Deoxygenated red blood cell

RBC_{oxy} Oxygenated red blood cell

ROI Region of interest

s Second

SDF Sidestream dark field imaging.

SEM Standard error of the mean

SvO₂ Mixed venous oxygen saturation

TiVi Tissue viability imaging

VOT Vascular occlusion test

ΔCoh Change in oxygenated haemoglobin

ΔC_{doh} Change in deoxygenated haemoglobin

ΔC_{tissue} Change in tissue concentration
ΔCoh_{TiVi} Change in oxygenated haemoglobin using TiVi

$\Delta C_{doh_{TiVi}}$ Change in deoxygenated haemoglobin using $TiVi$
 ϵ_{oh} Extinction coefficient oxygenated haemoglobin
 ϵ_{oh} Extinction coefficient deoxygenated haemoglobin
 ϵ_{tissue} Extinction coefficient tissue

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1 Introduction

1.1 Human skin

The skin is the largest organ of the human body with a surface of about 1.8 m² that comprises 15-20% of the total body mass. Its most important function is to act as a mechanical barrier between the internal organs and the exterior environment. It is virtually impermeable to fluids, prevents fluid loss, and prevents microbes such as bacteria, viruses and fungi from entering the body. Through hyperaemia, piloerection and sweating it is also important in the regulation of body heat. It is also involved in the production of vitamin D and protects against ultraviolet radiation.

The three main layers of the skin are the epidermis, the dermis, and the subcutis. The epidermis is a stratified squamous epithelium that varies in thickness between 0.1 and 1.6 mm depending on its location. In the epidermis there are living dividing cells only in the basal layers. As new cells are formed they move up towards the surface and gradually form the layer of keratin (stratum corneum) of the skin surface, which consists of keratinised “dead” cells that are continuously exchanged through shedding. The epidermis has no blood vessels and gets oxygen and nutrients by diffusion from the underlying dermis.

The dermis starts below the basal lamina of the epidermis. It is not a flat surface as there are dermal papillae that protrude into the epidermis. In the soles of the hands and foot there are dermal ridges which forms the “fingerprint skin” of these surfaces. The dermis consists mainly of connective tissue and the vascular network which nourishes both the dermis and epidermis. It has two structurally distinct layers, the papillary and reticular layers.

The papillary dermis is the superficial layer that consists mainly of loose connective tissue with collagen and elastin. It is the substance of the dermal papillae and ridges, and contains the superficial vascular plexus that nourishes the epithelium, as well as numerous nerve endings and sensory bodies of cutaneous nerves.

The reticular dermis is the deeper layer, and has a thicker and less cellular connective tissue which is more organised than the papillary layer.

The subcutis consists mostly of subcutaneous fat and loose connective tissue. It serves as isolation, protection and as a reserve of energy (1).

Figure 1. Schematic illustration of human skin

1. Epidermis 2. Papillary dermis 3. Reticular dermis 4. Subcutis 5. Subpapillary plexus 6. Vascular loops 7. Lower horizontal plexus 8. Cutaneous nerves

1.2 Human dermal microcirculation

The general definition of microcirculation is the circulation in the smallest vessels within organs and tissues that are less than 100-150 μm in diameter. These will include terminal arterioles, capillaries, and venules (2, 3).

The microcirculation of the human skin consists of a lower horizontal plexus on the boundary between the dermis and subcutaneous fat, which is fed by perforating vessels from the subcutis. From this deep plexus there are multiple vascular loops up into the upper horizontal or subpapillary plexus and, from there, there are multiple capillary loops into the papillary dermis. It is thought that it is the upper horizontal plexus that has the main source of nutrition to the skin, where it branches into capillaries responsible for gas and nutritional exchange. The lower horizontal plexus is thought to have a more important function in regulating body temperature (4).

Basal skin blood flow is around 250 ml/min but can reach up to 6-8 L/min in severe hyperthermia comprising 60% of cardiac output (5).

Skin blood flow is regulated mainly by sympathetic vasoconstrictive and vasodilatory nerves. The sympathetic vasoconstriction is regulated by fibres that release noradrenaline, and an increased or decreased release of noradrenaline balances the basal vascular tone in the arterioles. In the parts of the skin not covered by hair (glabrous skin) there are vasodilatory fibres and multiple arteriovenous anastomoses for thermoregulation (6). In addition, vascular tone is also regulated by local factors that are released by the vascular endothelium such as nitric oxide (NO) and prostaglandins that cause vasodilatation (7, 8). In the periphery, as the arterioles branch into terminal arterioles and capillaries, the vascular tone of the arterioles is thought to govern the local need for perfusion and adaptation to oxygen. The smooth muscles of the arterioles are present down to the 15 μm diameter arterioles, whereas the terminal arterioles and capillaries are surrounded by pericytes that have contractile properties (9).

At a capillary level, gas is exchanged with the tissue cells by diffusion, which means that no living cells are farther away from a capillary than can be reached by diffusion. This is referred to as the Krogh length (10). This proposed model for the nutritive function of capillary “bundles” plays an important part when the microcirculation is challenged, as is seen in various diseases (11).

1.3 Studying the dermal microcirculation

The easiest way of measuring the microcirculation of the skin is by observation of the skin and to assess colour, skin temperature, turgor, and capillary refill time. A red colour suggests hyperaemia or stasis, whereas a pale colour indicates vasoconstriction or arterial occlusion. These measures are commonly used, but are imprecise and investigator-dependent. One reason for this is that the nutritional need is low compared with total skin blood flow. The skin can be sufficiently perfused despite being pale and ischaemia can be severe despite being erythematous. A method of measurement that can assess the nutritive capillaries will provide a more accurate picture of the tissue’s viability.

Several techniques have been developed to investigate dermal microcirculation, which can be divided into invasive, minimally invasive, and noninvasive.

The invasive methods are in one way considered to be the gold standard, but because they are invasive they are usually reserved for animal experiments. Examples are experimental models in which a limb or flap is cannulated or exposed to occlusion of a vessel. In those instances, blood flow can be acquired in ml/s. In addition detectable substances such as radionucleotides, microspheres, or fluorescent substances can be injected into and traced through the circulatory tree. Thereby a relatively accurate assessment of blood velocity and perfusion in the tissue compartments can be assessed. Cannulating a flap and injecting microspheres are, for obvious reasons, not for routine clinical use.

Important research has been done with a combination of the above techniques to examine blood flow during arterial and venous occlusion (12, 13).

The quantification of blood flow and concentration of blood cells within the dermal microcirculation may be used as an indirect way to assess the viability of the skin. This may be particularly useful in conditions where a challenged microcirculation is part of the pathological mechanisms behind the tissue morbidity. Techniques for monitoring alterations in microcirculation of the skin have therefore, in recent years, been playing an increasingly important part in burn care (14); postoperative monitoring of free flaps (15) (16-19); intensive care (20); and diagnosis of peripheral vascular (21) -and skin diseases (22). In some cases it is the viability of the skin itself that is of interest, whereas in other cases, the microcirculation of the skin can be used as a target for assessing the state of the underlying tissue, or even the whole body. For example, the response to iontophoresis of acetylcholine has been shown to be impaired

in patients with type I diabetes (23), and in patients with increased risk of coronary heart disease (24, 25). Abnormal responses to postocclusive reactive hyperaemia (PORH) and local heating have both been found to be related to systemic endothelial dysfunction (26, 27).

Microdialysis is an example of a minimally-invasive technique, which allows for measurement of metabolite concentrations in the interstitium, and gives an indirect picture of the state of the tissue, for instance, if it is ischaemic. It can enable visualisation of local tissue blood flow in the skin, by assessing clearance of substances that were added to the perfusate, for example, urea clearance (28).

Iontophoresis is a minimally invasive technique by which a drug is delivered transdermally through one anode and one cathode. The drug, which is positively or negatively charged, is instilled by electrophoresis or electro-osmosis. In this way vaso-active drugs can be instilled locally to study microvascular reactivity. Iontophoresis is commonly used in conjunction with laser Doppler flowmetry.

Noninvasive methods have been developed and some of these are popular within research and also in clinical use for instance, in dermatology, diagnostics and monitoring of flaps.

1.4 Experimental models in microcirculatory research

Assessment of the blood flow in human skin is difficult in the resting state, as the blood flow in the basal state is low. Different provocations are therefore used to examine physiological and pathophysiological responses.

1.4.1 Local heating

Local heating is a common way to examine the dermal microcirculation. In such an experiment, the skin site is heated gently to 43°C. A normal heat response is a biphasic reaction. First, there is an increase in perfusion that is caused by activation of afferent local sensory nerves. This is followed by a local release of nitric oxide, which relaxes smooth muscle cells within the vessel walls and causes vasodilatation, (5, 29) (30), and reaches a plateau after 20-30 minutes. In states of endothelial dysfunction such as diabetes mellitus, the reaction is typically attenuated (26).

1.4.2 Exsanguination, and occlusion of arterial and venous blood flow

Occlusion of blood flow in an extremity enables the researcher to examine alterations in microcirculatory blood flow.

Local occlusion of an extremity is easily done by applying a blood pressure cuff and inflating it well above the systolic pressure (usually above 200 mmHg). Most subjects can tolerate about 15-20 minutes before the ischaemia becomes too painful, but five minutes is usually sufficient. (Operations in the upper extremity under general

anaesthesia are routinely done in a “bloodless field”, which can be safely maintained for two hours).

The occlusion experiment can be done with or without previous exsanguination. To exsanguinate the extremity as much as possible one can raise it and wind a latex Esmarch bandage round it to press out the blood before inflating the cuff (31). By doing this the amount of blood in the extremity is reduced to a minimum, which is a way to simulate the “biological zero”.

To simulate venous stasis or occlusion but maintained arterial inflow to an arm the same blood pressure cuff is used, but now the pressure is lower to allow for arterial flow. The venous pressure in the upper extremity is quite low (10 mmHg) but in order to occlude the deeper vessels, it is advisable to have a higher pressure, usually about 50-80 mmHg.

1.4.3 Post occlusive reactive hyperaemic response

The response after release of the cuff can be evaluated in a similar manner. After a period of arterial occlusion of an extremity with a tourniquet (usually 3-5 minutes), the cuff is rapidly released and a physiological hyperaemic period distal to the cuff ensues. This is known as the “post occlusive reactive hyperaemia” response (PORH). The first phase of the hyperaemia response is called the myogenic phase, and is likely to be endothelium dependent. The second phase is the metabolic phase, which is mediated by local release of prostaglandin and NO that cause smooth muscle relaxation (7, 32).

The hyperaemic response is altered in some diseases, such as diabetes mellitus (33) or peripheral vascular disease. In healthy subjects the hyperaemic curve is steeper, and the recovery time shorter (27).

1.4.4 Drug-induced alterations to the microcirculation

Different methods of local application of drugs to the skin have been developed to block neural, endocrine, or paracrine signal. Most commonly, drugs have been delivered topically, by iontophoresis, or by microdialysis. For example, topical application of the nicotinic acid methyl nicotinate induces local cutaneous erythema when it is applied to the skin (32).

1.5 Noninvasive methods used to study dermal microcirculation

This dissertation mainly revolves around one particular method, Tissue viability imaging (TiVi), which will be described below, but other methods investigated will also be dealt with.

1.5.1 Laser speckle contrast imaging

Laser speckle imaging is a technique that combines a camera with a divergent laser beam to illuminate an area of skin. The reflection forms a speckled or blurred pattern

that is detectable with the camera. The algorithm calculates the so-called “speckle contrast” which correlates with the movement of red blood cells and is therefore a measure of perfusion that can be calculated in perfusion units. The camera typically measures an area 15x15cm. The temporal resolution is up to 44 images/sec with a spatial resolution of 500 μm and a measurement depth of roughly 0.2-0.3 mm. It is a much faster technique than the laser Doppler imager that was previously used (34) (35).

1.5.2 Laser Doppler perfusion imaging

The laser Doppler imager (LDPI) is a development of laser Doppler flowmetry (36), in which a laser beam scans the surface of the skin and measures multiple points (65000). The back scattered light that has interacted with moving blood cells undergoes a Doppler shift and in the same manner as laser Doppler flowmetry perfusion can be calculated. Measurements are done in each point and can be presented as a colour coded map. This method has the advantage of being able to measure a larger area but has a low temporal resolution as the scanning is time consuming. Perfusion imaging has largely been replaced by Laser speckle contrast imaging.

1.5.3 Techniques of microscopy

There are several techniques based on dermal microscopy. The purpose of which is to visualise the microcirculation and measure capillary density and heterogeneity. The original device was transillumination videomicroscopy which can be used to measure capillary density, heterogeneity, and microvascular blood flow. It is limited in that it can be used only where the body part can be transilluminated. The nail fold and fingertip can be studied, but one important limitation is peripheral vasoconstriction during haemorrhage and sepsis. Developments of the technique are orthogonal polarisation spectral imaging (OPS) (37) (38) and sidestream darkfield imaging (SDF) (39). OPS is a technique with similarities to TiVi in that polarised light that penetrates the skin or epithelium is scattered and reflected as depolarised light and that haemoglobin has a different absorption pattern than the surrounding tissue. SDF has multiple diode flashes that encircle the optics of the camera. Superficial reflections do not reach the central optics, but light that is scattered against deeper layers such as capillaries does. Both these techniques are used to assess vascular density, heterogeneity of perfusion, and microvascular blood flow. They have both been used to investigate microcirculatory heterogeneity by applying them to the sublingual area in septic patients as this is not affected by peripheral vasoconstriction.

1.6 Techniques of measurement used in this dissertation

1.6.1 Laser Doppler Flowmetry

Laser Doppler flowmetry (LDF) of the skin can be considered to be the gold standard in noninvasive microvascular monitoring and was developed in the early 1980s (40) (41) (42).

The measurement probe is a low power diode laser that is attached directly to the skin. The laser beam that is emitted is scattered as it enters the skin tissue and is partially absorbed. The laser light that hits static objects (such as tissue) retains the same frequency but light that hits moving objects (such as moving red blood cells) undergoes a Doppler shift. Through the Doppler shift the laser light changes its wavelength as it ricochets back through the skin. The returning laser light is collected by a receiving probe that is paired with the emitting laser. The change and magnitude in frequency are directly proportional to the number and velocity of red blood cells in a small area, and this is called the measurement volume.

The signal is interpreted as velocity and concentration of moving blood cells (CMBC) by dedicated software, and the product of these two is the perfusion value, which is defined in arbitrary perfusion units (PU).

The measurement depth of the signal depends largely on three factors: the wavelength of the laser beam, fibre separation (meaning distance between the laser beam and the receiver), and tissue properties such as the structure and density of capillaries in the organ being investigated.

A high capillary density reduces the measurement depth and vice versa.

With the commonly used 780 nm wavelength laser, and a fibre separation of 0.25 mm, the measurement depth in human skin is between 0.5-1 mm (42).

The measurement area is approximately 1 mm². The measurement volume is therefore the area multiplied by the measurement depth which is in the region of 0.5-1 mm³. The temporal resolution (number of observations/ unit of time) is usually 33/s.

One drawback with the laser Doppler-technique, compared to camera or scanning-based laser techniques, is that laser Doppler does not allow measurements over larger areas and so has no spatial resolution. This is an important limitation, particularly clinically, as there may be regional differences within a flap or body part that is monitored for signs of morbidity.

1.6.2 Ultrasonic flowmeter

In paper III, an ultrasonic flowmeter was used to measure blood flow in the artery and vein of a pedicled flap. The ultrasonic flowmeter is an instrument that uses ultrasound to measure both blood flow and velocity and this is done with a time transit flowmeter. This means that a probe is held around a vessel, which sends ultrasonic signals slightly upstream and downstream. The difference in transit time is recorded and from these

measurements we can calculate volume and velocity, and blood flow in ml/min. This technology is used in cardiovascular surgery to measure the patency of grafted vessels.

1.6.3 Enhanced perfusion and oxygen saturation (EPOS)

The EPOS device has been developed by a research group at IMT Linköping University in cooperation with Perimed instruments (43) (44). The measurement probe combines a Laser Doppler flowmeter probe and a diffuse reflectance spectroscopy (DRS)-probe. The output variables are haemoglobin oxygen saturation (%), red blood cell tissue fraction (%), and speed-resolved RBC perfusion in the intervals 0-1 mm/s, 1-10 mm/s, and >10 mm/s. Conventional perfusion (PU) can also be recorded.

The speed resolved perfusion is considered to be an aid in finding out which type of vessel is involved in the physiological response. The nutritive blood flow is mainly in the superficial plexus, and has lower velocity than the deeper horizontal plexus.

The Red blood tissue fraction means the fraction of red cells in the sampling volume. This is dependent of the haematocrit which varies in the vessel tree (45).

1.7 TiVi - Tissue viability imaging

Tissue viability imaging is based on digital photography and the principle of polarised light spectroscopy. The technology was initially developed by Gert E. Nilsson and scientifically described by O' Doherty in 2007 (46). It measures the concentration of red blood cells in the reticular dermis and can also provide trends in oxygenation and deoxygenation of haemoglobin.

White light emitted from a light source (in this context a camera flash or LED illuminator ring) is shone on the skin, and a polarisation filter is placed in front of the flash to polarise the shining light.

A portion of this polarised light is directly reflected on the skin surface as glare (4%), or reflected in the superficial layers of skin (3%), and this light retains its state of polarisation. The remaining light is partly absorbed by the tissue, and partly backscattered from the dermal layer, when it loses its original state of polarisation. The light that is directly reflected is absorbed (blocked) by a second polarisation filter, which is in front of the camera lens and placed orthogonally with respect to the first filter. (This effect can also be achieved,

Light and polarisation

Light is regarded as an electromagnetic wave within the visible spectra, namely wavelengths between 400-700 nm. Sometimes it has been explained as a stream of zero-mass particles called photons. The particle theory can help the reader to understand the theory but in this text light will be explained with the wave theory.

Light from a light source contains waves that oscillate in different directions. So they are regarded as unpolarised. When they pass through a linear polarisation filter only the waves that are perpendicular to the filter will pass through and the other waves are absorbed. If a second filter is placed with its axis perpendicular to the first, the polarised light will be completely absorbed.

when one puts two Polaroid® sunglass lenses in front of each other and turns one of them 90°.) Only depolarised light that has scattered inside the dermis, therefore, reaches the detector array of the camera. This produces a slightly blurred image without any glare or sharp reflections, originating from a depth of approximately 0.5-1 mm.

Polarisation filters are used in cameras to avoid glare as well as in sunglasses as they filter out surface glare. It is then possible to “see through”, for instance, the surface of water (see below).

Figure 2. Schematic figure of the TiVi principle

Figure 3. Use of polarisation filters. Published with the permission of Wiley X EMEA, LLC

1.7.1 Concentration of red blood cells (RBC)

The image is analysed by an algorithm that is based on a model of the absorption of the main chromophores in the skin, haemoglobin and melanin. Haemoglobin has a high absorption coefficient which is dependent on the wavelength, as light is absorbed less in the red wavelength region than in the green (46). The algorithm suppresses tissue components that have a similar spectral signature in both the red and green colour planes, and enhances RBC, which has a different spectral signature in the red and green wavelengths. In this way, a measure is obtained of the concentration of red blood cells (TiVi-index), originating from the reticular dermis of the skin and thereby an indicator of the viability of the skin.

Chromophores

Chromophores are molecules or compounds that absorb light in the visible range. In human skin there are two main chromophores, melanin and hemoglobin (Hb). The latter can also be divided into oxygenated and deoxygenated Hb. Melanin is produced by the melanocytes and its purpose is to absorb and scatter ultra violet rays, thereby protecting the body from harmful ultraviolet radiation. The amount and type of melanin gives the colour tone of the skin. The absorption spectra of Hb differ from that of melanin and the surrounding tissue in that they differ in the red and green colour spectra. It is also a difference in absorption between oxygenated and deoxygenated Hb. These basic relations and the different chromophores absorption patterns form the theoretical basis of the TiVi algorithm and its different toolboxes (see

Absorber	Absorption coefficient	Absorption coefficient
	Red (600-700nm)	Green (500-600nm)
Tissue	2,7	3
RBC _{oxy}	3,5	177
RBC _{deoxy}	25	201
Epidermis	35	40

Table 1. Absorption coefficients (cm^{-1}) for components in the epidermis and dermis. Note the difference between red blood cells and surrounding tissue as well as between oxy- and deoxygenated haemoglobin. Table adapted from O'Doherty et al 2007 (46).

1.7.2 Oxygenation and deoxygenation of haemoglobin

The oxygen carrying molecule in red blood cells is haemoglobin. As already stated, the haemoglobin molecule has a special absorption pattern. Light in the green wavelength region is absorbed to a greater extent than in the red wavelength region. The surrounding tissue absorbs less light and is not as wavelength dependent. The haemoglobin molecule itself has a significantly different absorption pattern that is dependent on its oxygen saturation. It will therefore change when the haemoglobin molecule is

oxygenated or deoxygenated. This effect can be measured by a specific algorithm within the tissue viability imager system to analyse trends in oxygenation and deoxygenation of haemoglobin molecules.

The measurement technique is as follows: The camera detector array records local alterations in diffusely backscattered light in the area of the red and green wavelengths (ΔOD (red) and ΔOD (green)). ΔC_{oh} , ΔC_{doh} and ΔC_{tissue} are the unknown variables and because the ΔC_{tissue} can be set to zero (it is assumed not to change in the short term perspective) an equation system can be set up where ΔC_{oh} , ΔC_{doh} can be calculated as the extinction coefficient of oxygenated and deoxygenated haemoglobin and tissue are known variables (ϵ_{oh} , ϵ_{doh} , ϵ_{tissue}). The measurement output is change in oxygenation and deoxygenation of haemoglobin. The trend curve displayed is set in respect to a reference region of interest. This can be the first picture in a series of pictures.

1.7.3 Previous research in tissue viability imaging

There are currently 20 publications about TiVi listed in PubMed, the first of which is by O' Doherty et al who first described the device in 2007 (46). Initial studies have dealt with methodological issues and papers in which new applications have been tested. One important initial study was done by the inventor, Nilsson. It is a performance study in which the short and long term stability and image uniformity were tested as were the influence of distance, image size, ambient light influence, and curvature of the object. (47) These tests showed that the performance of the device was stable in relation to the stability and spatial variation of the image obtained. Distance from camera and size of image had no influence on sensitivity, but ambient light was a minor source of error since it reduced the TiVi-index slightly. Measurements done on curved objects showed minor distortion near the edges (<10%). An algorithm which reduces the effect of rotation and torsion of a picture has been added to the system (48). TiVi was initially developed for still photography but was later adapted for use with a video camera, which made it possible to monitor rapid alterations in skin blood flow (49). TiVi has been compared with other techniques, such as laser Doppler perfusion imaging, Laser speckle contrast imaging (50) and colorimetry (51). In several studies the aim is to evaluate the TiVi techniques ability to accurately measure changes in dermal circulation during various provocations. Some research workers have used substances that induce vasodilation and vasoconstriction, that include topical application (22), intradermal injection (52), iontophoresis (53), and microdialysis (28). In other studies the focus has been on the response to arterial (54) or venous occlusion (Paper II), or both, and post-occlusive reactive hyperaemia (22, 55).

	TiVi	LDF	EPOS
Measurement principle	Polarisation spectroscopy	Doppler effect	Doppler effect, white light spectroscopy
Measurement variable	Red blood cell concentration, oxygenation	Perfusion (red blood cell concentration and velocity)	Perfusion (red blood cell concentration and velocity) and oxygenation
Spatial resolution	50µm	*	*
Measurement area	20x20cm ²	≈1mm ²	≈1mm ²
Temporal resolution	Up to 44 images/s	33 observations/s	120 observations/s
Measurement depth	≈0,5mm	≈0,5mm	≈0,5mm

Table 2 Technical characteristics of the TiVi, LDF and EPOS systems.

* As LDF and EPOS measures perfusion in a single point, the spatial resolution (distance between adjacent measurement points) is not defined.

2 Aims

The overall aim of this thesis was to study microcirculatory changes in the skin using polarised light spectroscopy. The studies included focus on the usability of the TiVi technique in microcirculatory provocation models.

Specific aims for the ingoing studies I-IV

1. To investigate surrounding factors such as the use of a transparent glass heater, distance between camera and skin surface and camera angle as possible sources of error in measurement.
To evaluate TiVi as a way to measure the local heating response with a heating glass in healthy volunteers. To test the site-to-site and day-to-day reproducibility of the TiVi technique.
2. To investigate and validate TiVi as a tool to detect venous and arterial occlusion in the dermal microcirculation of healthy young volunteers.
3. To validate TiVi during vascular provocations in a pedicled flap model. To describe how the heterogeneity of concentration of RBC can be used to differ between arterial and venous stasis, and to assess changes in oxygenation and deoxygenation with the built-in oxygen mapper application.
4. To validate the oxygen mapper application that measures trends in oxygenation and deoxygenation of haemoglobin in healthy subjects during arterial and venous occlusion.

3 Material and Methods

Studies I, II and IV involved healthy young volunteers, and study III was an animal study done on pigs.

3.1 Subjects and environment during experiments study I, II, IV

For all human studies, healthy young volunteers (mean age 27.4 range 18-38) were included after they had given written informed consent. No regular use of medications except for oral contraceptives was permitted and nicotine users were excluded. Medical reasons for exclusion were cardiovascular disease, diabetes, skin disease and blood pressure above 150 mmHg systolic and above 90 mmHg diastolic. Each subject's blood pressure (BP) was measured before and after each study protocol. Subjects were asked not to drink anything containing caffeine, tea or alcohol on the day of the experiment (in study I, two hours before the experiment)

All human studies conformed to the principles of the Declaration of Helsinki and were approved by the ethics review board at Linköping University, Sweden.

In all experiments room lighting was kept as constant as possible. Ambient light was kept to a minimum by turning off ceiling lights and closing window blinds. The remaining light came mostly from the TiVi laptop which gave enough light for reading and orientation. Room temperature was kept at a constant level of 22 (0.5) °C.

Subjects were positioned supine on a bed, with the right arm resting on a pillow on level with the heart. The volar side of the forearm was facing upwards. A blood pressure cuff (pneumatic tourniquet) was placed on the upper arm. Subjects were asked to keep the arm still during the entire experiment. Blood pressure was measured before and after each experiment using an automatic sphygmomanometer on the contralateral arm.

In study II the arm was exsanguinated by raising it for one minute, applying an Esmarch bandage, and inflating the pressure cuff to 250 mmHg. The bandage was then removed and the arm re-positioned so that it was level with the heart. In the other experiments in study II and IV the purpose was either to examine stepwise occlusion (study II), arterial occlusion without exsanguination (study IV), and venous occlusion (study II and IV). In these experiments the arm rested level with the heart when the cuff was inflated, and no Esmarch bandage was applied beforehand.

3.2 Subjects and environment during study III

In this study we used an animal model. The study required five healthy Swedish landrace pigs (mean age 4 months, mean weight 45 kg) The pigs were bred for use in the centre for teaching and research in disaster medicine and traumatology in Linköping and the study took place in their operating theatre.

The use of research animals was approved by the regional animal research committee at Linköping University and conformed to the principles of the Declaration of Helsinki.

All experiments on pigs were done under general anaesthesia. During operations vital measurements including body temperature and blood pressure were closely monitored and kept at constant by giving Ringer's acetate and covering the animals with blankets. Total experimental time was around 8 h for each pig, after which the animals were euthanized at the end of the experiment protocol without regaining consciousness.

On each pig, two 12 x 15 cm pedicled flaps (one on each side) were raised based on the cranial gluteal artery perforator (cGAP) (56, 57). When experiments had been completed on one side, the animal was turned over and the procedure repeated on the other. Two flaps were lost as a result of unintentional thrombosis of the pedicle.

The vascular pedicle was stripped so that the only vascular supply to and from the flap was the cranial gluteal artery and one vena comitans. This experimental model simulates a free flap before detachment of the vascular pedicle. Two (TS 420) perivascular flowmeters with two ultrasonic flowprobes (MA-2PSB, Transonic Systems Inc. Ithaca, N.Y.) were attached to the artery and vein. An inflatable vascular occluder was placed around the vein distal to the flow probe. The vascular occluder was inflated to reduce venous outflow with 50% or 100%. To enable complete arterial occlusion the artery was clamped with a microvascular clamp. A thermostatic LDF probe was placed in the middle of the flap surface.

As in the other studies the ambient light was kept at a minimum. The operating theatre had no windows and ceiling lights were turned off. The only light source was from the laptop and electronic devices.

3.3 Ultrasonic flowmeter

To monitor blood flow in the vascular pedicle in paper III an ultrasonic flowmeter was used to measure blood flow in the artery and vein simultaneously. The equipment consisted of two (TS 420) perivascular flowmeters with two ultrasonic flowprobes (MA-2PSB, Transonic Systems Inc. Ithaca, N.Y., USA) These flow probes are developed for use in laboratory animals and are suitable for the diameter of the vessel in question. (1-2mm) The measure of output ml/min.

3.4 Vascular occluder

To obtain a partial and total vascular occlusion a purpose made "vascular occluder" (Norfolk medical products Inc. Skokie, Ill, USA.) was used, which consisted of a cushion shaped cuff that was placed around the vessel and inflated with saline to make a

partial or full occlusion. Both the vascular occluder and the ultrasonic flowprobes are illustrated in Fig. 12.

3.5 Tissue viability imaging

In papers I-III a TiVi 600 camera system standing on a camera tripod was used. In paper IV the upgraded TiVi 700 system was used (for a detailed description see section 1.7).

TiVi pictures were analysed with the dedicated analysis software (TiVi version 2,1 Wheelsbridge AB, Linköping, Sweden) and the customised software (Matlab R2007b, The Mathworks Inc. Natick, MA) The TiVi pictures covered a large area such as the forearm but the mean concentration of RBC was obtained from one or several ROI defined by the TiVi-software. In papers III-IV pictures were analysed with the updated TiVi 700 analyser and the TiVi 106 oxygen mapper to plot trends in oxygenation and deoxygenation.

In studies I, II and IV the camera was placed on a tripod and placed so that the camera light hit the surface at a 90° angle. In studies II, III, and IV the camera was placed 30 cm above the skin surface of the volar forearm and, in study I, at 20 cm and with a slightly oblique angle to avoid direct reflections from the glass.

3.6 Heating glass

In study I a heating glass was used to obtain a local heating response. The transparent heating glass was to induce a local heating of the skin site and at the same time be able to make microcirculatory measurements with an optical instrument.

The heating glass was a 2 mm glass slide (as used for microscopy slides) with a transparent heating foil (Minco SA, Aston, France) attached to the top surface. The foil was attached to a thermal controller unit (CT198 Minco SA, Aston, France), which made it possible to adjust the temperature of the heater. The glass was attached to the volar aspect of the forearm with double-adhesive tape at the edges and creating a tape-free area in the middle. The top surface was covered with a sheet of paper with two holes cut out for measurements with TiVi. In each image two circular ROI were placed within the cut-out holes. The TiVi software has an application called rigid image recording. With this the ROI stays in the same paper free area regardless of movements in the picture. The paper had reference markers for analysis of the pictures. A thermistor attached to a digital thermometer was placed under the glass but not in the cut-out holes to measure the temperature and adjust it with the thermal controller unit.

The heater's ability to maintain the temperature set by the thermal controller unit was tested by measuring the temperature with the thermistor at different locations under the glass. The variation in temperature was found to be less than 0.5 °C.

3.7 Laser Doppler flowmetry

In studies I-III we used a Periflux 5000 laser Doppler perfusion monitoring unit (Perimed AB, Järfälla Sweden). And in those studies a thermostatic laser Doppler probe (probe 457 Perimed AB) was used to measure perfusion, CMBC, and skin temperature. A thermostatic probe can be set to a specific temperature and heat up the measurement area to examine the local heating reaction.

Laser Doppler data were recorded continuously with a sample rate of 33 recordings/ second, and compressed into 10 s intervals. Perfusion data were analysed using PeriSoft for Windows; version 2.5.5 (Perimed AB).

The probe had a bandwidth of 15 kHz. The separation of fibres was 0.25 mm, which enabled a measurement depth of about 0.5 mm (42).

Laser Doppler probes were calibrated before each experiment according to the manufacturers' guidelines.

3.8 Enhanced perfusion and oxygen saturation (EPOS)

In study IV a new device called a PeriFlux 6000 EPOS system (enhanced perfusion and oxygen saturation; Perimed AB, Järfälla, Stockholm, Sweden) was used. It integrates diffuse reflectance spectroscopy (DRS) and laser Doppler flowmetry in one fibre-optic probe. The system consists of a PF 6010 laser Doppler unit, a PF 6060 spectroscopy unit, a broadband source of white light (Avalight-HAL-S, Avantes BV, The Netherlands) and a custom-made fibre-optic thermostatic heating probe. The PF 6010 unit contains a laser light source at 785 nm and a thermostatic heating controller. The PF 6060 unit has two spectrometers (AvaSpec-ULS2048L, Avantes BV) and an optical notch filter before the spectrometer to suppress wavelengths 790 (20) nm and ensure minimal influence from the PF 6010 laser light source on the DRS spectra. The fibre-optic probe consisted of two central emitting fibres and three detecting fibres. The emitting and detection fibres for the LDF laser light source and one detecting fibre at a distance of 0.8 mm had a diameter of 125 μm . Two detecting fibres were placed at a distance of 0.4 and 1.2 mm from the white light source and were connected to one spectrometer each. Those fibres had a diameter of 200 μm and all fibres had a numerical aperture of 0.37 and were made of fused silica. During the measurements, the probe was fixed to the skin using rings of double-sided adhesive tape, that did not cover the ends of the fibres. The output measures were oxygen saturation (SO%), red blood cell fraction (%), and perfusion (PU). The latter were the product of concentration of moving blood cells (CMBC) and their velocity.

3.9 Statistical analysis

Statistical analysis for paper I was made with Microsoft Excel 2007 and GraphPad Prism (version 5.0 for Mac OS X, GraphPad Software, San Diego, CA, USA), and for

papers II-IV with Microsoft Excel 2008 and GraphPad Prism (version 6.0 for Mac OS X GraphPad Software, San Diego, CA, USA)

Paper I

For each image, we calculated the mean RBC concentration in both ROI. These were saved for data analysis. In four subjects, the spatial heterogeneity of concentrations of RBC at baseline and after 40 minutes of heating was calculated. Heterogeneity was expressed as the coefficient of variation of TiVi indices within the ROI. All data were presented as mean (SD). One-way analyses of variance (ANOVA) for repeated measures with Dunnett's multiple comparisons test were used to test whether changes from baseline were significant. Site-to-site and day-to-day reproducibility was expressed as percentage, coefficients of variation (CV), and intraclass correlation coefficient (ICC).

For all analyses, probabilities of less than 0.05 were accepted as significant.

Paper II

Data from TiVi and LDF at the end of provocations were found not to be normally distributed after inspection of the histograms and by testing for normality using the d'Agostino and Pearson omnibus test. K2 values ranged from 7.1 (TiVi index) to 23.7 (CMBC). The significance of differences in changes in the TiVi index, perfusion and CMBC from baseline were therefore analysed with the Wilcoxon matched pair signed rank tests and data in text are presented as median (range). Data in figures were shown as median with error bars indicating the interquartile range (IQR).

For all analyses, probabilities of less than 0.05 were accepted as significant.

Paper III

In each image, eight ROI were selected, having been chosen in relation to their proximity to the pedicle, ROI 1 being closest and ROI 8 farthest away. For each image and each ROI, the mean concentration of RBC, heterogeneity, and changes in oxy-haemoglobin and deoxy-haemoglobin were calculated. The heterogeneity for the whole flap was calculated as the mean value in ROI 1-8.

D'Agostino & Pearson omnibus normality tests indicated that all data were normally distributed. Two-way ANOVA for repeated measures were used to analyse changes in concentration of RBC and oxygenation compared with baseline, for different positions in the flap (ROI 1-8 or proximal compared with distal). Data from the last five minutes of each phase were used for the analyses. With all ANOVA, Dunnett's test was used to correct for multiple comparisons. All data in text and tables are presented as mean (SD). For all analyses, probabilities of less than 0.05 were accepted as significant.

Paper IV

Maximum responses for all measurements at the end of the provocations were normally distributed after inspection of the histograms and by testing for normality using the D'Agostino and Pearson omnibus test. The significance of differences in changes in concentrations of RBC, change in oxygenated Hb (ΔCoh), PU and $\text{SO}_2\%$ were therefore analysed using repeated measures ANOVA, with baseline defined as the mean of the last minute of baseline period, and occlusion response defined as the mean of the last minute of the occlusion period in each subject. Data in text are presented as mean (SD). Data in figures are shown as mean, with error bars representing 1 SD. For all analyses, probabilities of less than 0.05 were accepted as significant.

4 Review of the studies and main results

4.1 Paper I - “Polarized light spectroscopy for measurement of the microvascular response to local heating at multiple skin sites”

Purpose of the study

The first experiment was to find out whether surrounding factors could be significant sources of error for the TiVi system. The following were tested: absorption and reflection of the heating glass, effects of distance between the camera and surface of the skin, the effects of the incident angle, and the influence of background light.

In the second experiment the heating glass described in the Materials and Methods section was used to induce a local heating response in healthy subjects. We assessed whether the heating response in the skin could be reliably detected with the TiVi technique. LDF with a heating probe was used as a control method. Site-to-site and day to day reproducibility between TiVi and LDF were also investigated.

Figure 4. Experiment design in paper one

Experiment 1

In eight subjects the possible technical sources of error were examined. First, absorption and reflection of the heating glass were tested by measuring the pixel intensity in a circular ROI with and without the heater in place. In the same experiment measurements were made with different distances between the camera and surface of the skin (15, 20, 25 and 30 cm). The effect of the angle of the camera was tested by placing the camera at a 90° angle to the skin surface (normal procedure), or tilting it in a 45° angle. The effect of background light was tested by taking pictures with ceiling lights on and off.

Experiment 2

The heating glass was used to produce local hyperaemia. Ten subjects were examined on two separate days (1-7 days). The test subjects were placed semisupine with the volar forearm on a pillow at the level of the heart. The heater was attached to the forearm and a high sensitive thermometer placed under the glass. The thermostatic LDF probe was attached distal to the heating glass. The TiVi camera was placed 20 cm above the arm and tilted to a slightly oblique angle to avoid direct reflections of the glass.

Three TiVi pictures were taken every 10 s at the end of a 30 minute acclimatisation period to obtain baseline values. Thereafter the heater was turned on and adjusted to 42 °C, which was reached within three minutes. The test period was 40 minutes and the TiVi camera took pictures every 10 s. At the same time the thermostatic LDF probe was set to 42 °C and the equipment recorded continuously during the same period.

As measurements were done on two different days and on different sites, day-to-day and site-to-site reproducibility could be calculated and compared with LDF.

Figure 5. Experiment protocol for experiment 2. Three pictures every 10s at the end of the baseline period. One picture per 10s taken during the heating period.

Main results

Experiment 1

The heating glass reduced CRBC to 87% of the value obtained without the heater in place. Increasing the distance between camera and skin meant increasing the TiVi-value significantly (4.4% between 15 and 30 cm $p=0.016$). The incident angle (90° compared with 45°) did not significantly affect the TiVi value.

Light sources such as ceiling lights and sunlight affected the results significantly. In subsequent experiments, therefore, ambient light was kept at a minimum, and as constant as possible.

Figure 6. Diagram of TiVi values within a circular ROI, 1cm in diameter. The picture illustrates the spatial heterogeneity in concentrations of RBC within a small area of skin.

Experiment 2

The main result of experiment 2 is that changes in concentrations of RBC can be measured reliably by the TiVi system when the skin is heated and the measurements are made with a transparent heating glass.

It also shows that the measurements are more reliable in terms of site-site and day-day variability, when compared with LDF.

Site-to-site reproducibility when measuring concentrations of RBC with TiVi was high during both baseline (CV 6.7%, ICC 0.98), and after 40 minutes of heating (CV 6.4%, ICC 0.96). The day-to-day reproducibility with TiVi at baseline between different days was high (CV 8.6%, ICC 0.89), and moderate (CV 16% ICC 0.26) after 40 minutes of heating. The day-to-day reproducibility for LDF was moderate both during baseline (CV 18%, ICC 0.26) and after 40 minutes of heating (CV 23%, ICC 0.52).

It is thought that this difference in reliability between the two techniques can be explained by the spatial heterogeneity of the microcirculation of the skin, as can be appreciated by the use of a picture-based technique. We assessed the heterogeneity within a circular ROI 1 cm in diameter that plots the different TiVi values within the area. The diagram shows how much the concentration of RBC varies within a measured area. The LDF-probe has a measurement area of one mm² and therefore the obtained value could differ significantly depending on placement, even within an area of 1 cm².

Figure 7. TiVi index during local heating with the transparent heater. Figure from Paper I.

Conclusions

- TiVi can be a useful way of measuring the microvascular response local heating in human skin
- The reproducibility between different sites on the skin when measuring local heating response is high.
- Environmental background light may affect measurements and should therefore be kept to a minimum during measurements by TiVi, and the distance between camera and skin should be kept constant.
- Under controlled conditions (low lighting, fixed distance), the day-to-day and site-to-site reproducibility when measuring the response to local heating is high.

4.2 Paper II - “Assessment of microcirculation of the skin using tissue viability imaging: A promising technique for detecting venous stasis in the skin”

Purpose of the study

We examined how well the TiVi system was able to detect changes in concentration of RBC, concentration of moving blood cells (CMBC) and perfusion during exsanguination and arterial occlusion, post-occlusive reactive hyperaemia, and venous occlusion in the forearm of healthy subjects.

Experiment 1

The subjects lay semisupine with the right arm on a pillow on level with the heart. A pneumatic tourniquet was placed on the upper arm, and an LDF probe attached to the volar aspect of the forearm and recording continuously. A TiVi camera took pictures of the volar forearm from a fixed distance of 30cm.

First we had an acclimatisation period for 20 minutes with one picture/minute. Then the arm was elevated for one minute. An Esmarch bandage was wound tightly around the arm to squeeze the venous blood out. The blood pressure cuff was inflated to 250 mmHg and the arm repositioned. The LDF-probe removed before the Esmarch bandage was applied and replaced in the same site after exsanguination (the position of the probe was marked in ink). A five minute period of arterial occlusion followed with pictures taken every 10 s. The pressure cuff was released and the post-occlusive hyperaemic response observed with a picture rate of 1 picture/s for the following 15 minutes. This also served as a recovery period. After 15 minutes of PORH and recovery the pressure cuff was inflated to 30 mmHg above diastolic pressure to obtain venous occlusion of the forearm. The tourniquet was kept inflated for five minutes and TiVi pictures were taken every 10 s.

Figure 8.
Experiment design in paper II

Experiment 2

We then assessed stepwise venous occlusion of the blood flow. The tourniquet was inflated and deflated in steps of 10 mmHg over 60 minutes.

Subjects and equipment were placed in the same manner as in experiment 1. After 15 minutes of acclimatisation, the LDF recording was started. One minute of LDF recording and six TiVi pictures (one every 10s) were obtained to establish baseline values.

Directly afterwards, the stepwise occlusion experiment started. LDF kept on recording while the TiVi camera took one picture/20 s, which gave nine pictures/pressure step.

The cuff was inflated in steps of 10 mmHg up to 80 mmHg after which it was deflated in steps of 10 mmHg until the cuff pressure was 0 mmHg. Each step lasted three minutes.

Figure 9. Experiment protocol for experiment 1 and 2 in paper II.

Main results

Experiment 1

During exsanguination and arterial occlusion the TiVi index as well as PU and CMBC decreased significantly to a constant level. After release of the cuff there was a rapid and significant increase in all measurements that indicated a post occlusive hyperaemic response. The values then gradually decreased towards baseline.

During venous occlusion there was a consistent gradual increase in TiVi index. This effect was not seen in PU, which showed no significant change from the end of the reperfusion period nor in CMBC, which had continued to decrease significantly.

Figure 10. Typical example of the changes in; TiVi-index (solid line), perfusion (dashed line), and CMBC (dotted line), during baseline, exsanguination, reperfusion (arrow) and venous occlusion.

Note that the time scale is different for the different provocations.

Experiment 2

When the forearm was occluded in a stepwise manner, there was a gradual 46% increase in median TiVi index from 107 (70-129) AU at 0 mmHg to 158 (129-183) AU at 80 mmHg.

As cuff pressure rose to 20 mmHg and above the increase in concentration of RBC became significant from baseline. As pressure decreased TiVi index returned to the normal range when pressure went below 20 mmHg.

Perfusion decreased gradually with 54% as the cuff pressure increased from 0-80 mmHg ($p=0,008$). There was a significant reduction in PU at a cuff pressure above 10 mmHg. Perfusion returned gradually in the same manner as for TiVi. Below 20 mmHg changes were no longer significant.

Figure 11. Change in TiVi-index and perfusion during stepwise occlusion. Figure from paper II.

Conclusions

- TiVi can measure changes in RBC reliably during total and stepwise increased arterial and venous occlusion.
- Laser Doppler flowmetry was less consistent in detecting venous stasis than TiVi.

4.3 Paper III: Vascular Occlusion in a Porcine Flap Model: Effects on Blood Cell Concentration and Oxygenation

Purpose of the study

This study was designed to assess the ability of TiVi to measure changes in the concentration of RBC, oxygenation, and heterogeneity during vascular provocations in a porcine fasciocutaneous flap model.

Experiment

The pigs in the experiment were under general anaesthesia, the CGAP-flap raised and equipment prepared as described in the “Materials and Methods” section. The TiVi camera was mounted 30 cm above the surface of the flap. During the experiment the TiVi system acquired images at a rate of 1/min. The laser Doppler flowmeter recorded

tissue perfusion and CMBC continuously. Arterial and venous flow was recorded every five minutes by the ultrasonic probes. Vital variables including temperature of the flap, blood-pressure, heart rate and body temperature, were registered every five minutes. The sequence of the experiment is shown in the figure below, and involves periods of arterial and venous occlusion.

Figure 12. Experimental design in paper III. CGAP flap with vascular pedicle, flowmeters, and pressure cuff.

Figure 13. Experimental sequence in paper III

Main results

During the experimental phases there was a significant increase in the TiVi index from baseline in all ROI during 50% and 100% venous occlusion. During recovery the TiVi index returned to baseline. During arterial occlusion the TiVi index decreased significantly in all regions except in ROI 2, and afterwards (during final recovery) concentration of RBC returned to a level that did not differ significantly from baseline. When we looked at a different ROI we found that the mean concentration of RBC was lowest in the central ROI and increased towards the proximal and distal regions. During both 50% and 100% venous occlusion the largest increases were distal parts of the flap.

Heterogeneity increased significantly both during 50% and 100% venous occlusion. It decreased gradually during recovery, and it continued during arterial occlusion. During the later phases heterogeneity did not differ significantly from baseline.

Tissue oxygenation

During 50% and 100% venous stasis there was a significant increase in oxygenation in the proximal part of the flap. This was also seen in the distal part but was less pronounced and not significant ($p=0.11$). During the first recovery period (after venous occlusion) oxygenation in the proximal ROI returned to baseline. Oxygenation in the distal ROI decreased even further to -33 (93) AU. During the complete arterial occlusion that followed oxygenation both in the proximal and distal ROI decreased further and stabilised at -47 (75) AU ($P=0.25$) and -66 (81) AU ($P=0.02$). After release of the arterial cuff a minor “overshoot” or postocclusive hyperoxygenation was seen in the proximal ROI. This effect was less clear in the distal ROI.

Figure 14. CRBC (TiVi-index) and Heterogeneity during the experiment in paper III

Conclusions

- TiVi can measure regional changes in red blood cell concentration (CRBC) during venous and arterial occlusion in a fasciocutaneous flap.
- The oxygen mapper application enables the TiVi system to measure differences in oxygenation of red blood cells.
- Heterogeneity of the skin, a measure derived from TiVi- data, increased significantly during venous occlusion, and could be used as an additional indicator for venous occlusion.

4.4 Paper IV: Assessment of tissue oxygenation with polarised light spectroscopy enables new means for detecting vascular events in the skin

Purpose of the study

This is a comparative study where the forearm model is used to compare the oxygen mapper application with diffuse reflectance spectroscopy and laser Doppler flowmetry combined in the EPOS device.

Experiment 1

A previously described laboratory bench model was used for this experiment (46).

Thin latex tubes were laid out in single layer loops, simulating blood vessels on top of a skin-like material (Duoderm, Convatec, Princeton, NJ, USA) that was painted with a colour that resembled “bloodless skin”. The TiVi camera was placed 30 cm above the tubing model. Ambient light was kept at minimum and care was taken not to disturb the equipment.

A venous blood sample was obtained from one of the research workers. Venous blood 5 ml was infused undiluted through the tubes to fill them with blood. When the tubes were filled, 5 consecutive images were taken with the TiVi camera. The pictures were cropped to a constant ROI over the tubes and background. After the images had been acquired, the tubes were thoroughly rinsed with saline and air to remove blood and air bubbles. To simulate complete desaturation and saturation of oxygen the experiment was repeated, first with a blood sample bubbled with nitrogen for five minutes and then a further sample bubbled with oxygen, also for five minutes. The experiment was repeated the following day.

Figure 15. Experiment design during paper IV experiment 2

Experiment 2

In vivo model

This protocol is similar to that of study II but in this study the main focus was oxygenation and deoxygenation of haemoglobin. And we used the EPOS was used to compare with the TiVi. The EPOS probe was placed on the volar side of the forearm and rec-

ordered continuously during the experiment, and TiVi images were taken at 10-second intervals throughout.

The experimental sequence can be seen in the figure below.

Figure 16. Experimental sequence in paper 4 experiment 2. One picture per 10s during the entire experiment

Results

Experiment 1

During the first experiment one could see a clear difference in oxygenation between blood bubbled in nitrogen, venous blood and oxygenated blood.

Experiment 2

During arterial occlusion without previous exsanguination there was no significant difference in $CRBC_{TiVi}$ from baseline. After release of the cuff $CRBC_{TiVi}$ rapidly rose to 39.2 (18.8) AU ($p < 0.001$). A post occlusive reactive hyperaemia could be seen. After which the $CRBC_{TiVi}$ gradually returned towards baseline.

During the venous occlusion phase there was a gradual increase in $CRBC_{TiVi}$ with 28.9 (11.2) AU ($p < 0.001$), and after release of the cuff it gradually returned to baseline.

Oxygenation and deoxygenation during arterial occlusion

There was a gradual decrease in oxygenation($\Delta C_{oh_{TiVi}}$) by 44.2 (10.4) AU compared to baseline ($p = 0.04$). This corresponded to an increase in deoxygenation that mirrored oxygenation. After release of the cuff, oxygenated Hb ($\Delta C_{oh_{TiVi}}$) rapidly returned and further increased to 38.5 (24.5) AU ($p < 0.001$). Deoxygenated Hb ($\Delta C_{doh_{TiVi}}$) showed a corresponding decrease below baseline. Two minutes after occlusion ended, both $\Delta C_{oh_{TiVi}}$ and $\Delta C_{doh_{TiVi}}$ returned to baseline.

Oxygenation and deoxygenation during venous occlusion

When exposed to venous occlusion the $\Delta C_{oh_{TiVi}}$ decreased gradually to -20.3 (27.7) AU ($p = 0.003$). $\Delta C_{doh_{TiVi}}$ showed an inverse pattern with a gradual and somewhat steeper increase. On release of the cuff the $\Delta C_{oh_{TiVi}}$ $\Delta C_{doh_{TiVi}}$ rapidly returned to baseline levels. There was a notable difference between the arterial and venous occlusions. During arterial occlusion the oxygenated- and deoxygenated values mirrored

each other, while during venous occlusion the deoxygenation curve rose more than the oxygenation curve sank.

Conclusions

This study showed that TiVi can measure trends in oxygenation and deoxygenation during arterial and venous stasis in healthy subjects. This method is a complement to the measurement of CRBC as it takes the oxygen demand and metabolism into account rather than only the concentration and blood flow.

5 Discussion

5.1 General comments

The main focus in this thesis has been to investigate and validate an optical noninvasive method that uses polarisation light spectroscopy, TiVi, to measure concentration and oxygenation of red blood cells in the dermal microcirculation.

The technique has been compared with other noninvasive methods such as LDF and EPOS for the measurement of different aspects of the dermal microcirculation using different well-defined provocation models. These will be discussed further.

Studying dermal microcirculation is difficult for several reasons. As already mentioned, the dermal blood flow may vary greatly depending on heat stress and level of exercise to maintain homeostasis(6). It is therefore important to allow research subjects to lie still during an acclimatisation period and to keep ambient temperature comfortable and even to prevent shivering or sweating. Another challenge is that the intrasubject variation within a resting state is large, because of the heterogeneity of the dermal microcirculation (4, 58). The main reason for this is the random location of ascending arterioles and venules, this means that some areas are dominated by arterial inflow, and some by venous flow while some are void of vascular elements. This is why single point measurements may show greater site-to-site variability than optical techniques that typically have an inherent larger spatial resolution.

Colour of skin and epidermal thickness are other important sources of variability when analysing microcirculation with optical methods such as TiVi. A thicker epidermal layer provides a longer light pathway, and possibly more scattering events before the light hits the red blood cells in the microcirculation. The other main chromophore of the skin, melanin (depending on type and content), also absorbs both visible light and ultra violet rays, which may affect the results. To minimize these sources of error as much as possible, we only used research subjects with Fitzpatrick skin type 1-2 in our studies. The experiments were also made on the volar aspect of the forearm, which is less pigmented. The pigs in paper 3 were all fair coloured, with a complexion similar to Fitzpatrick skin type 1.

The two dermal plexuses have different functions, the upper one having the main nutritive function while the lower one is more involved with thermoregulation. The estimated depth of measurement of both TiVi and LDF are supposed to measure CRBC and perfusion within the upper dermal plexus, but given the sources of variation stated above it is possible that circulatory data are obtained in the lower plexus (42). The user should therefore be aware that alterations in the depth of measurement will affect the outcome data, and interpretation of the results may have to be adjusted to include these differences.

Optical methods display results not in absolute units, such as ml/min, but in AU or arbitrary units. This, combined with the variability in baseline values, makes it difficult to calculate and use threshold values. With optical methods, accuracy and comparison between individual measures ought to be done as comparisons of changes from baseline recordings, such as before and after a provocation or analysis of a trend (46).

When comparing different methods, one must take spatial and temporal resolution into account. Spatial resolution in this context is the precision of the optical equipment and differs if it is a single point or optical measurement. Temporal resolution is the resolution with time. Scanning techniques such as laser Doppler imaging have a low temporal resolution but the camera based TiVi is high (44 pictures/second) and is in a way limited only by speed of the shutter. Movement artefacts are a bigger problem when the temporal resolution is low. With a single point measurement there is a risk of false high or low results depending on where the probe is placed. With an optical technique the output is a mean over the measurement area or a defined region of interest. As can be seen in Fig. 6 the TiVi index is highly heterogeneous even within an area of 1 cm³.

5.2 Aspects of methods of assessment of the microcirculation of the skin

I have focused mainly on how the TiVi system reacts after vascular provocations of the dermal microcirculation. These provocations include heat, forearm occlusion with and without exsanguination, and vascular (venous and arterial) occlusions in pedicled flaps.

5.2.1 Local heating

Local heating has been extensively used for the evaluation of local skin hyperaemic responses (59-61). These are typically local, and the hyperaemia is not seen outside the heater or area of stimulus. The vasodilatory response to heating is caused by axon reflexes that relax vascular smooth muscle and lead to endothelial release of NO, which is a potent vasodilator (62). The physiological purpose of the resulting hyperaemia is thought to increase local blood flow, and thereby cool the area to prevent skin damage and maintain homeostasis. If the stimulus is prolonged and the nerve reflexes are intact, there is a die away effect. (30)

Measurement of local heating with optical methods is difficult as the device that is used to induce the heat is likely to interfere with the one that is used to monitor the reaction. Heat pads or thermal conductors will function in conjunction with single point measurements, such as the LDF probe, but it will not permit objective measurements with a system such as TiVi. Heat fans or fluid chambers filled with heated water have been tested. Measurements with these methods are, however, distorted as there is a rapid cooling effect when the heater is removed.(63). We therefore tested a new ap-

proach whereby a transparent heating glass was used for the experiments. The ROI studied can be altered by using larger or smaller pieces of glass that are prepared with a transparent heating foil that is attached to the top surface.

In paper I we got a similar but slightly different local heating response from TiVi than from LDF. The early peak in perfusion that is well described with this method was not seen with the TiVi technique. The heating glass, however, meant that we could provoke the skin with the same accuracy in temperature as the LDF probe, while still enabling spatial CRBC measurements through the glass. From the first experiments in paper I it was calculated that the heating glass reduced TiVi values to 87% of the value without the heater. The site-to-site variability was better with TiVi than with LDF and day-to-day reproducibility was moderate, but with similar results to LDF.

Figure 6 illustrates that even within a circular ROI measuring 1 cm there is considerable variability in CRBC. With an LDF-probe the measurement area is only 1 mm³. Measuring a local heating response within an area of skin provides better site to site reproducibility than single point measurements. The heating glass and TiVi are therefore a good to heat a skin area instead of just a single point.

The results from paper I confirm that heating provocation of the skin can be studied with TiVi and heating glass but the reaction looks somewhat different in that the initial peak seen with LDF is not seen with TiVi. This difference in response is mainly attributed to the fact that LDF measures the product of velocity and concentration whereas TiVi only measures concentration. The initial peak depends on increased velocity after occlusion. An improvement to the technique would be to use an algorithm that takes the 13% change in TiVi index into account when the heating glass used.

5.2.2 Forearm model – post occlusive reactive hyperaemia

The human forearm is a good test site for microvascular research. It is easy accessible and, arterial and venous stasis can easily be simulated by using a blood pressure cuff. The volar side also provides a relatively flat surface and one can study possible distortion phenomena from the curvature of the arm.

Factors that make results more difficult to generalise are that the cutaneous microcirculation is likely to differ depending on the region of the body. The thickness of the subcutaneous fat, skin, and muscle will also affect measurements. It is therefore important to be consistent in placing the ROI: s in the same site during different provocations.

During vascular provocation of the forearm one must take into account the likelihood of discomfort for the subject. Occluding the vessels of the arm with a blood pressure cuff causes increasing pain with increasing duration of occlusion and reperfusion during the PORH phase can be painful. Short operative procedures (around 15 minutes) such as release of a carpal tunnel are routinely done with a tourniquet under local anaesthesia, and occlusion times of less than 15 minutes are therefore regarded as

acceptable. Prolonged operations on an extremity also require a bloodless field (up to two hours) but requires general or regional anaesthesia.

With the short periods of ischaemia and stasis used in these experiments, damage to extremities and more than mild discomfort are unlikely. A previous study that examined occlusion times showed that the PORH response increased with prolonged occlusion time, but that a five-minute occlusion was sufficient for measurements to be made, and was better tolerated than 10 minutes by subjects. (55)

5.2.3 Flap model

In clinical practice, particularly in reconstructive microvascular surgery, the most feared complication is pedicle thrombosis, either in the anastomosed artery or vein(64). Venous thrombosis is more common, but more difficult to detect (65). A method for flap monitoring that can accurately detect both arterial and venous thrombosis would be of great interest to clinicians. During reconstructive microvascular surgery it is also of vital importance to raise a flap that is sufficient in relation to the vascular pedicle. A flap that is too large in relation to the nutritive vessels will not be properly perfused peripherally with possible partial necrosis no matter how patent the pedicle is.

The fasciocutaneous flap model, used in paper III, is used to obtain exact control of inflow- and outflow of blood. This makes it behave almost exactly like a fasciocutaneous free flap where one pedicle perfuses and drains the entire flap and there are no autonomic nerves to affect the microcirculation. By altering arterial and venous flow one can observe the spatial changes in CRBC in different areas of the flap. At the same time trends in oxygenation and deoxygenation can be measured thanks to the oxygenation toolbox. In paper III we found a spatial difference in CRBC. The appearance of venous stasis was best seen in the most distal part (ROI 1-3) as well as in the most proximal ROI (ROI 8). This is explained by the route by which the pedicle penetrates the flap below ROI 5-7 and not in the proximal rim. The venous drainage diminishes gradually in the periphery of the flap and at one point the flap will not be able to maintain sufficient arterial perfusion or venous drainage, or both. Observations of spatial differences of CRBC and oxygenation in flaps can be useful in calculating the adequate size and shape of a flap to prevent complications peripheral healing. In this dissertation a fasciocutaneous flap model was used. An interesting future pursuit would be to study myocutaneous flaps. In such flaps the muscular tissue might act as a vascular reserve and be able to distribute the blood differently.

Just as in a free flap any nerves are cut, which means that the function of vasoconstrictive and vasodilatory nerves is obliterated. This makes it similar to a free flap but it indicates an important difference from the forearm model, which has an intact autonomic nerve function and also an underlying muscle layer.

5.2.4 Measurements of concentration of red blood cells

It has been found that assessment of CRBC, using the TiVi technique, have been more sensitive than perfusion-based methods (such as LDF) in detecting alterations in blood flow when the microvascular bed is impaired by vasoconstriction, for example, after local distribution of noradrenaline (66), or as a result of decreased inflow, for instance after stasis and exsanguination using a tourniquet (Paper II). It is thought, therefore, that there may be advantages to measuring concentration of RBC than perfusion under these specific circumstances.

On the other hand, the TiVi technique and values of RBC concentration have displayed larger variability between individual subjects than perfusion values provided by LDF. The use of absolute values may give skewed results with the TiVi technique, so with this type of device one has to measure responses in relation to a given provocation instead of absolute values.

The most important reason for variability in absolute values is the variation in thickness of the skin and the melanin content of the epidermis which reduces transmission of light (46). The melanin content and skin pigmentation can be considered stable in the short term. One improvement for the future would be to use the system to subtract the melanin content of the reflected light and let an algorithm subtract the melanin content from the TiVi index. The TiVi system provides a “Pigment compensation” which works in a similar way. This was not used in our studies since the functionality has not been validated. A compensatory function for pigmentation would potentially avoid a measurement error and allow for future use of absolute values.

Comparison of the results from paper III and IV (Fig. 3, paper IV, Fig. 4, paper III) show some major differences in CRBC in the forearm and flap model that need to be addressed. First, as seen in Fig. 4, there is instability in CRBC during the baseline period. This effect could have been caused by the fact that half the study population was randomised to a venous occlusion before the arterial occlusion, which resulted in an incomplete recovery. Second, CRBC increases similarly during venous stasis in both models. However, after occlusion ended and the tissue was allowed to recover, we found that CRBC had a somewhat biphasic return to normal values in the forearm model, whereas a negative overshoot in the flap model. The biphasic return to normal in the forearm model may be explained by a rapid depletion of congested blood in the initial phase, followed by a slower decrease of tissue oedema paralleled by a further decrease in CRBC in the tissue.

The effect of tissue oedema and how blood is distributed between skin and deeper tissue (such as muscle) has to our knowledge been little studied. It is speculated that the oedema acts as a diffusion barrier for delivery of oxygen to the cells. This would be present particularly in states of venous occlusion when lymphatic and blood drainage are impaired. The priority of muscle over skin during vascular compromise is logical, because muscle is more sensitive to ischaemia and has a larger tissue mass. This

cannot be studied with optical methods but interesting studies have been done. In two related studies on venous and arterial insufficiency in flaps in pigs (12, 13) the investigators found a higher degree of arteriovenous shunting in venous insufficiency flaps and, most importantly, that the oxygen tension was lower during recovery from venous insufficiency than from arterial insufficiency. One reason is thought to be because the tissue oedema is a diffusion barrier that also causes a slower recovery with reduced oxygen tension during the study's three-hour recovery period. The authors observed a shift in capillary blood flow from subcutaneous tissue to muscle. This finding was not considered "striking" but is interesting, as it indicates that muscle is prioritised over subcutaneous tissue and skin.

5.2.5 Heterogeneity

It is well known that the microvasculature in the human skin is characterised by high spatial heterogeneity (58) (67) which is primarily the result of variations in the location and orientation of arterioles and capillaries in the upper dermal plexus. Studies using laser Doppler flowmetry have shown that ascending arterioles, accompanied by descending postcapillary venules, form "vascular units" in the dermis. Each arteriole-venule pair divides into four or five branches to form the upper horizontal plexus in the skin (4). These units are spaced randomly at intervals of a few mm. The heterogeneity of the concentration of red blood cells has been evaluated in both papers I and III. In paper I, we found pronounced variations in CRBC within a 1 cm diameter circular skin area. The ability to measure the mean concentration red blood cells over a larger area of skin is therefore an advantage of the technique, as it decreases the risk of erroneous measurements, as may be the case when single point measurement techniques are used.

In paper III, we found that the heterogeneity of the concentration of red blood cells itself changes during different kinds of provocations. The heterogeneity, which was defined as the SD of the concentration of red blood cell within a ROI, increased during venous occlusion. This increased heterogeneity was also visible on the skin as a blotchy pattern on the skin of the flap. On the other hand, the heterogeneity was not increased during arterial occlusion, but was comparable to the heterogeneity in the basal state. Because venous and arterial occlusions produce differences in the heterogeneity, the measure can possibly be used in addition to CRBC to separate arterial occlusion from venous stasis. It may also be speculated that hyperaemia and stasis, that both will cause an increase in CRBC, will result in them having different heterogeneity. Algorithms that take the increased heterogeneity and CRBC increase into account may therefore be valuable.

The results of study III do not give a direct explanation for the increase in heterogeneity during venous occlusion. As described by Braverman (4), there are intervening avascular spaces in between the vascular units that are connected to each other at their periphery. It can be expected that during venous occlusion, the vascular units in

the upper plexus are filled and expanded, whereas the intervening avascular spaces remain pale. A redistribution of blood from capillaries into arteriovenous shunts can also be expected, possibly as a secondary effect of the formation of oedema in the tissue. (12)

The TiVi system does not in the current software provide a toolbox for RBC heterogeneity. However, our data suggest that this may an important role in future improvements of the technique.

The RBC heterogeneity of RBC has a possible clinical use. In states of septic as opposed to haemorrhagic shock there is increased heterogeneity in the microvascular bed (2). In the normal microvasculature there is a balance between demand and consumption of oxygen. In haemorrhage the microvascular blood flow is low but homogeneous, which leads to increased extraction of oxygen. In sepsis the blood flow is heterogeneous since the microvascular bed is partly shut down. The SvO₂ is therefore increased, meaning that the VO₂ or oxygen extraction is low. The reason for this partial shutdown and increased heterogeneity of capillaries is not known, but it increases when subjects are exposed to endotoxin. An increased heterogeneity in the microvascular bed is associated with increased morbidity and mortality, even if the systemic medial arterial pressure is restored. It may therefore be speculated that increased heterogeneity will lead to an increased diffusion distance in the microvascular bed with hypoxia of the tissues and progression of organ dysfunction.

5.2.6 Oxygenation

Tissue oxygenation was assessed in both paper III and IV, using the built-in software application Oxygen Mapper. Experimental arterial and venous occlusion was used in both a porcine fasciocutaneous flap model in paper III, and in a forearm occlusion model in healthy subjects. Interestingly we found important differences between the models. In the porcine model we found that changes in the concentration of oxygenated Hb ($\Delta\text{Coh}_{\text{TiVi}}$) decreased during arterial occlusion but increased during venous partial and full occlusion. In the human model, on the other hand, $\Delta\text{Coh}_{\text{TiVi}}$ decreased during both arterial and venous stasis.

As discussed in paper IV, several factors may explain this difference between models. The upper extremity is a large mass of skin, muscle, and bone with a large metabolic demand and an intact autonomic nervous system. The tissue of a fasciocutaneous flap has a much smaller mass and mainly consists of skin, fat, and connective tissue. The skin has a relatively low basal metabolic need but the variability in skin perfusion is large. In an extremity the blood can be shunted between superficial and deeper tissues to direct the oxygenated blood through perforators to underlying muscles where it is most needed. The metabolic need of the skin is much less than that of muscle. The oxygen extraction and consumption are therefore likely to decrease when the venous drainage is impaired in a fasciocutaneous flap. In the dermal microcirculation, the upper plexus also provides the nutritive blood flow while the deeper plexus

has more thermoregulatory functions and could be seen as the vascular reserve for the skin's nutritive blood flow. In the porcine flap model a relative oedema that develops in the skeletonised flap is also thought to impair extraction of oxygen from the tissue.

5.3 Concerns about, and limitations of, TiVi

Although we found good reproducibility between skin sites in the same subject, we found that the absolute concentration of RBC at baseline varied widely among subjects, much more than with LDF. This probably results from variations in skin colour related to different amounts of melanin, even though the absorption of melanin is taken into account in the calculation of concentration of RBC. Considering these variations in the absolute TiVi index, the use of relative responses may result in more consistent results when comparing responses between subjects.

5.3.1 TiVi and nutritive blood flow in the skin

Under normal circumstances the blood flow of the skin is considerably larger than is needed for its basal needs, and it is estimated that the nutritive blood flow is around 20% of the total blood flow. Skin blood flow can vary between 0.3 L/min at rest, and 8 L/min during strenuous exercise in a hot environment. How the nutritive capacity is affected when the dynamics of the microcirculation is altered is not clear, however, it is hard to speculate how much of the measured perfusion detected by the TiVi system that is part of the nutritive flow. The difficulties in defining nutritive flow in the skin are the result of the fact that there is a lack of clear and precise definitions of nutritive and non-nutritive flow in the skin. We know, however, that the measured depth of the TiVi system coincides with the papillary dermis, which is why we think that the measurements seen through the TiVi system may be of nutritive capacity. Measurement techniques with larger depths, or those that will measure at only one point are however likely to be less precise in this.

Our studies have focused mainly on how the TiVi system functions in healthy subjects. Further studies on groups of patients with challenged microcirculation will help us to find out if the TiVi changes we see in CRBC, oxygenation and heterogeneity can predict tissue morbidity in these instances as well. One challenge is that the TiVi technique has proved to be most reliable and well-functioning when measurements are related to baseline recordings. The large variability in individual dermal perfusion therefore poses a problem if the technique were to be used for snapshots in ill patients, as no baseline recordings are available and no threshold values defined to assess compromised tissue. Further validations in these patient groups are therefore needed.

The use of a technique that can effectively assess the nutritive flow of skin is therefore likely to be clinically attractive. For example, it has been shown that the microcirculation of the skin is highly affected during disease states such as sepsis and

haemorrhage. Clinically, much focus in these patients has been about restoring the systemic circulation, but even with a restored systemic circulation there can still be an established organ failure and poor outcome with large tissue morbidity.

This can be exemplified in intensive care patients with sepsis where the capillary density of the microvascular bed of the skin has been shown to decrease in a random heterogeneous pattern. Some vessels are closed or have little perfusion, whereas others are well perfused. This leads to an increased diffusion distance to the tissue cells, and local hypoxia.

In this condition the microvascular bed cannot respond to increased local demands for oxygen. Even with restoration of global homeostasis with a MAP of >65 , a microvascular ischaemia is associated with a poor outcome.

In haemorrhagic shock there is a more homogenic decrease in perfusion and a larger portion of the capillary bed will remain open. This will lead to a high extraction of oxygen from the capillaries and low mixed venous oxygen saturation (SvO_2). In heterogeneously decreased blood flow, fewer capillaries will be open and able to extract oxygen, and the SvO_2 will be higher. This also occurs in reperfusion after cardiac arrest. It does not matter if the systemic circulation is maintained at a physiological level because, as a portion of the capillary bed is closed, there will be ischaemia in the microcirculation. A camera based method such as TiVi can be used in a vascular occlusion test (VOT) or directly to assess heterogeneity, but one must consider the risk of peripheral vasoconstriction when using vasoactive drugs.

5.4 Limitations

There are several limitations to these studies that need to be addressed. Most of the studies (I, II and IV) have been done on healthy young subjects. Studies on groups of patients have not been within the scope of this project but are of course an interesting possibility for the future.

Although we found a good reproducibility between skin sites in the same subject, we found that the absolute concentration of RBC at baseline varied widely between subjects, much more than with LDF. This was probably the result of variations in the colour of the skin related to different concentrations of melanin, even though the absorption of melanin is taken into account in the calculation of concentration of RBC (paper II). Considering these variations in the absolute TiVi index, the use of relative responses may result in more consistent results when comparing responses between subjects.

Ambient light or light pollution is a cause of erroneous results but its importance is not completely clear. Previous studies have concluded that ambient light has a low impact, but in paper I the conclusion was that ambient light did affect results and should be diminished as much as possible to avoid distortion and obtain consistent results. The angle of the camera to the object seems to be of less importance, which is

good as the research object is rarely completely flat. On the other hand, distance between camera and skin is a factor that must be consistent during a study as was shown in paper I. The use of an advanced device such as a system camera with lots of settings connected to an external device (laptop) with multiple settings can in itself be the cause of multiple errors. A more simplified type of camera, such as a smartphone, compact camera or tablet with integrated software where the user can see results directly is easier for both researchers and less experienced staff to use and interpret results. A possibility to measure and compensate for ambient light and distance between lens and object would be desirable to diminish possible sources of error.

In paper III, 50% venous occlusion was not absolute, the actual occlusion was probably more. In a previous study the researchers used software with a feedback loop that obtained partial occlusion with seemingly high precision.(68) When planning the experiment this advanced setting was not considered a priority but measuring a difference between partial and full venous occlusion was considered enough. With the cuff and flowmeters a near 50% occlusion could easily be obtained.

The TiVi technique has not been previously validated in porcine skin. Porcine skin is most likely thicker than human but structurally it is similar and reacts similarly to human skin in the provocation experiments.

6 Conclusions

The studies in this thesis have led to the following conclusions.

Paper I

This study showed that local heating can be used to examine the local heating response in forearm skin with a transparent heating glass. The heating response differs in that the initial peak is absent compared with the LDF but the plateau phase is the same. Technical factors were studied, and we concluded that the camera needed to be at a constant distance throughout the measurements. Ambient light should be kept as low as possible. The angle of the camera angle was of less importance. The heterogeneity of the dermal microcirculation can be studied with TiVi.

Paper II

TiVi can measure rapid changes in CRBC during arterial and venous occlusion of the forearms of healthy subjects. It can be used to detect changes both during stepwise and total occlusion, and has a higher sensitivity than LDF in measuring venous occlusion. This implies that it can be used to monitor free flaps, as venous occlusion is a common cause of failure of a flap but difficult to detect.

Paper III

TiVi can be used to measure regional changes in CRBC and oxygenation of haemoglobin in a porcine fasciocutaneous pedicled flap. Changes were detected during both venous and arterial occlusion. A measure of heterogeneity can be assessed during vascular occlusion.

Paper IV

TiVi can measure trends in oxygenation and deoxygenation during arterial and venous stasis in healthy subjects. The method is a complement to the measurement of CRBC as this takes the oxygen demand and metabolism into account, rather than concentration and blood flow alone.

6.1 Future perspectives

This project has focused on studies to evaluate Tissue Viability imaging as a way to measure concentration and oxygenation of red blood cells in the dermal microcirculation. In addition to the scientific evidence some aspects of its use need to be dealt with. First, it is desirable if the technique is easy to use and not too expensive. Easy use means that the equipment should not have too many settings (and therefore possibili-

ties for error). Interpreting the output data should be as easy as possible. If the equipment is light and can easily be moved its usefulness increases. From a technical point of view, the device must provide accurate and reproducible data, and allow for easy processing of data points that can assist correct clinical judgement. The technique must be robust in the sense that it is not affected by external or environmental factors such as ambient light, movement artefacts, or the colour or, temperature of skin.

The TiVi technique is not limited to the present-day system camera. As long as polarisation filters can be fitted perpendicularly to the lens and flash, and the pictures can be processed by the software, the TiVi instrument can be a pocket camera, tablet, or smartphone. In the two latter cases a dedicated app can simplify interpretation of the picture.

The results of the papers in this dissertation have shown that the technique is reliable and reproducible and the natural next step is studies on groups of patients.

The areas where TiVi could be further investigated are monitoring of free flaps, peripheral vascular disease, and microcirculation in patients with sepsis and shock.

When monitoring free microvascular flaps during and after operation the monitoring device must be able to detect accurately both arterial and venous thrombosis, and be easy to use by the staff in a postoperative ward. The TiVi has a possible application since it is able to detect spatial changes in concentration of RBC and can detect arterial and venous obstruction. Intraoral and buried flaps are impossible to use with the standard TiVi equipment used in this thesis so it is limited to skin flaps such as deep inferior epigastric perforator flaps for breast reconstruction. a smaller TiVi camera for use intraorally has been developed but has not yet been scientifically tested.

In patients with peripheral vascular disease, as well as those in intensive care, there can be a substantial difference between the macrocirculation and microcirculation. In a diabetic patient, ankle brachial index can be falsely high, despite severe impairment of the peripheral circulation of the foot, which makes the method potentially misleading.

In patients with septic shock there is an increasing interest in the microcirculatory changes. The microcirculation becomes heterogeneous because many small vessels close and this can aggravate organ dysfunction despite a good systemic blood pressure.

Studies should be aimed to detect microcirculatory derangements in septic shock and how to direct the treatment to reestablish good systemic and microcirculatory homeostasis.

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Papers

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