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### Glow with the flow: Quantifying blood flow and photoluminescence signal in biological tissue

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# 1

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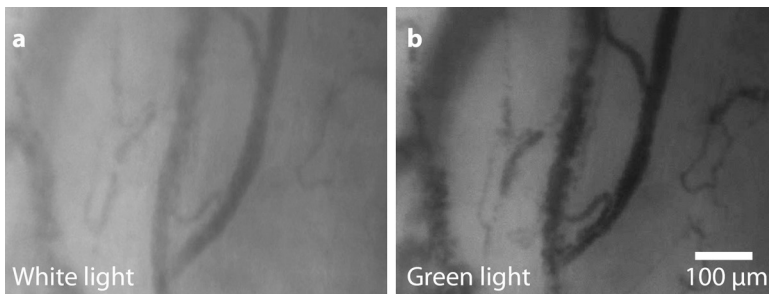
## GENERAL INTRODUCTION

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**ABSTRACT** This introductory chapter starts with an overview of the biomedical imaging scene in which this thesis is set. The area of interest for the optical imaging techniques that will be discussed is the microcirculation, therefore this chapter continues with a description of the microcirculation and the assessment of its functioning by quantifying the blood flow velocity. The microcirculation plays a role in many pathologies and a dysfunctional microcirculation can be the cause, mediator or result of other pathologies. Many tumours for example, thrive and survive due to increased microcirculatory blood supply invoked by tumour signalling molecules. However, quantifying the blood flow does not always provide adequate information, for example it does not provide adequate contrast to localize small tumour lesions. Therefore, the second part of the introduction focuses on achieving high-sensitivity molecular contrast in tumours using photoluminescent nanoparticles. Finally an overview of the thesis chapters covering quantitative flowmetry and photoluminescence techniques is given.

## 1.1 THE SCENE

Visual inspection is one of the oldest diagnostic tools used in medicine. In the early 1900's William Osler advised his students "Let not your conceptions of disease come from words heard in the lecture room or read from the book. See, and then reason and compare and control. But see first" [1]. In modern medicine, the importance of visual observation is still a critical part of teaching [2, 3]. The physician's eye and patient alone, however, do not always result in the true diagnostic outcome. Enhancing human vision has long been recognized as a valuable technique in medicine, from simple magnifying glasses and microscopes (flourishing since the 1680's) to advanced neurosurgical microscopes that allow 3-dimensional visualization through binoculars during intracranial neurovascular procedures [4-6]. Not only magnification, but also other light interactions with tissue can enhance clinical vision. For example the use of only a small part of the spectral range of light (colour) can result in a substantial increase in contrast. In Fig. 1.1 an example is shown of an image of microcirculatory blood vessels obtained using sidestream dark field (SDF) microscopy [7] (a technique that will be discussed in Chapters 2 - 4) using respectively unfiltered (white) and filtered (green) light for illumination. The large contrast in Fig. 1.1 b is due to a large absorption difference between blood and surrounding tissue for green light, whereas this absorption difference is reduced when the whole visible spectrum is used (Fig. 1.1 a).



**Figure 1.1 Spectral imaging** | Sidestream dark field (SDF) microscopy image of human sublingual microcirculation using white (a) and green (b) illumination light.

The addition of magnifying and contrast enhancing tools to visual inspection allowed advancement of the area of optical biomedical imaging. Although the most common clinical imaging modalities (20th century inventions like X-ray, CT, ultrasound imaging and MRI) are non-optical, optical imaging is currently emerging due to the following advantages: optical radiation is nonionizing and safe; optical instruments are generally cheap and portable; and optical tissue interactions provide both structural and physiological information, usually at a higher resolution compared to the aforementioned modalities [8].

In the biomedical imaging field the goal is to obtain functional information about the tissue of interest to aid in a true diagnostic outcome, to monitor therapy or more generally to improve the understanding of life in health and disease. Tissue constituents like cells, organelles, proteins and biomolecules interact with light. Tissue can absorb, scatter and depolarize light, fluoresce or phosphoresce, or all at the same time. Interpreting the result of this complex, multiscale interaction can reveal information about the tissue of interest. Diseased tissue, or samples from a diseased organism (blood, urine, biopsies, etc.) can have

a different structure or composition compared to healthy tissue. The task of biomedical imaging is to use the different optical signatures of healthy and diseased tissue to improve the differentiation between them.

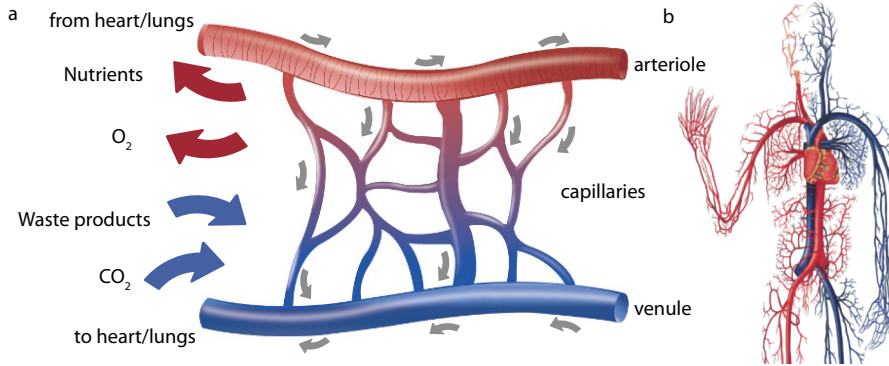
Using light-tissue interactions for diagnostics is not limited to imaging. Biomedical imaging is a subfield of the larger research area called biophotonics, covering all techniques that study light-tissue interactions in aid of the differentiation between (or understanding of) healthy and diseased tissues. To complete the biophotonics landscape, a last but certainly not least application is the use of light for clinical interventions and therapy, for example laser surgery [9] or photodynamic therapy for the treatment of cancer [10]. An upcoming research area is 'theranostics', where therapy and diagnostics are integrated, often in the field of nanophotonics [11]. Ongoing research efforts to understand the complex interactions of light with tissue will result in more effective optics-based therapies.

The challenges in biophotonics arise from the complex interactions of light with tissue. In particular the highly scattering nature of tissue at optical wavelengths requires sophisticated algorithms to obtain functional information or reconstruct images. In addition, scattering reduces the penetration depth of light in tissue to maximally a few centimetres in the near infrared region. The identification of potential application niches for optical techniques and the optimization of illumination, detection and signal processing methods are the driver behind the expansion of biophotonics in the biomedical field.

The versatile nature of tissue interactions with light logically results in a versatile field of biophotonics. Many excellent applications have already made their way to the clinic, such as the relatively simple pulse-oximeter [12] or advanced retinal imaging systems based on optical coherence tomography [13]. The advantages of adopting optics in the clinical setting are the potential for minimally invasive techniques, point-of-care diagnostics, and safe and affordable devices. In addition, the enhanced functional information (structural and physiological) that optical techniques can provide is arguably the most important added value to the clinic. However, before functional, robust and practical devices can be made, they have been preceded by in-depth, thorough and comprehensive research. This thesis is set in this particular scene where the quantification and interpretation of optical interactions with tissue are key concerns.

## 1.2 MICROCIRCULATION: WHERE PERFUSION MEETS METABOLISM

The particular biotissue of interest in this thesis is the microcirculation: a network of blood vessels less than 100  $\mu\text{m}$  in diameter. The microcirculation is the central region in the circulation system where the total vessel surface area is at its largest, and the blood flow slows down to allow exchange of gases, nutrients and metabolites providing the environment necessary for cells to function. Adequate tissue perfusion by microcirculatory blood flow is essential for healthy tissues and organs. Due to its function to meet the metabolic needs of tissues the microcirculation is found in almost all parts of the body, thus also close to the surface of tissues such as internal organs, muscles and skin (see Fig. 1.2). As light has a limited penetration depth in tissue the microcirculation is an excellent target for optical assessment with considerable clinical relevance.



**Figure 1.2 Microcirculation** | (a) Simple representation of metabolism in the microcirculation. (b) The microcirculation is everywhere (adapted from blog of Dr. S. Venkatesan, MD).

Adequate tissue perfusion by the microcirculation is essential for organ health, so inadequate perfusion by the microcirculation can result in diseases and eventual organ failure. In the metabolic disease diabetes, common complications such as retinopathy and renal disease are the results of impaired microcirculation. Microangiopathy is caused by increased microvascular pressure and flow, but the exact cellular mechanisms causing this are unknown. However, microangiopathy appears to precede cardiovascular diseases in diabetic patients, and in turn, functional microvascular changes precede microangiopathy [14, 15]. Assessing microvascular functionality thus aids in early diagnosis of diabetes and its related complications and can also function as an indicator for effectiveness of treatment [16].

Another relevant area where microcirculation assessment can aid in both diagnosis and therapy monitoring is at the intensive care unit (ICU). Sepsis is defined as the clinical syndrome characterized by the presence of infection and a systemic inflammatory response of the body, and septic shock refers to acute circulatory failure characterized by persistent arterial hypotension unexplained by other causes [17]. One of the major features of septic shock is a heterogeneous distribution of microcirculatory blood flow and in more advanced states a dysfunctional microcirculation leading to oxygen extraction deficit, severe functional shunting and organ failure [18, 19]. The pathological causes for this are not yet fully understood but suggestions includes triggers by inflammatory mediators, endothelial alterations, loss of neural control, altered leukocyte adhesion, altered red blood cell deformability and the presence of micro thrombi [20-22]. Regardless of cause, the indicators of septic shock are apparent in the microcirculation early on in disease development [23]; microcirculatory changes are more severe in nonsurvivors [24]; and an improved microcirculation functionality is associated with successful therapy and patient survival [25, 26]. In addition, during septic shock microvascular changes are independent of systemic haemodynamic variables [26, 27]. Other critical diseases where the microcirculation has been reported to play a role are cardiac arrests, heart failure and cardiogenic shock [28, 29]. In circulatory failure blood flow is diverted from the less vital tissues (skin, muscle, gastrointestinal tract) to the vital organs (heart, brain, kidneys). Consequently, monitoring the less vital tissues can be an early marker for hypoperfusion of vital organs with the advantage that the peripheral circulation is more accessible [30]. Microcirculatory function and capil-

lary recruitment have therefore been proposed as end-points for therapy in sepsis and as potential parameters in other critical diseases [29, 31].

The microcirculation does not only provide adequate tissue perfusion to ensure healthy organs, it also provides oxygen and nutrients to unwanted tissue such as tumours. Without blood vessels tumours cannot grow beyond a critical size due to the limited diffusion distance of oxygen (100 - 200  $\mu\text{m}$ ) [32]. At the beginning of their development, tumours are usually not angiogenic (triggering blood vessel growth) due to a balance between pro- and anti-angiogenic molecules. Various processes occurring around tumours (metabolic or mechanical stress, immune or inflammatory response, genetic mutations) trigger the cells to secrete pro-angiogenic signalling molecules to derail this balance and turn the angiogenic 'switch' to 'on' [32, 33]. The subsequent growth of blood vessels around the tumour allows the tumour to expand and facilitates transport of tumour cells to create metastasis elsewhere in the body. On the other hand, tumour microcirculation also enables anti-cancer drugs to be delivered to the tumour [32-34].

The microcirculation's ability or inability to accommodate the tissue's metabolic needs is thus an important pathological marker for various diseases, including those outlined above. The causes for microcirculatory dysfunction, pro- and anti-angiogenic behaviour and microangiopathy are complex, broad and warrant further investigations. However there is strong evidence that the microcirculation functionality and cellular processes are interdependent [32, 35], and hence the study of microcirculation can elucidate the related cellular processes and clinical physiology and can help to understand causes of diseases and mechanisms of diagnosis and therapy.

### 1.3 FUNCTIONAL MICROCIRCULATION IMAGING

An important perception is that assessing a single capillary is not sufficient to verify (in) adequate supply of oxygen to tissue. The functional parameters that have been identified to be relevant in septic shock are a measure of vessel density and geometry; assessment of capillary perfusion and the heterogeneity of microvascular perfusion [36]. This method of microcirculation scoring was developed due to the availability of a new-generation microcirculation microscopes that allowed capturing videos of the microcirculation of approximately 1 mm<sup>2</sup> field of view, usually containing 20 or more capillaries, venules and/or arterioles. The first imager was based on orthogonal polarization spectroscopy (OPS) [37] and the improved version of sidestream dark field (SDF) imaging [7]. In both versions the contrast is obtained by illumination with green light (higher absorption by blood compared to surrounding tissue, see Fig. 1.1) and they share the common property that the specularly reflected light is rejected from the imaging path, resulting in detailed videos of subsurface microcirculatory vessels with flowing red blood cells (RBCs). Importantly, the techniques can be implemented in hand-held and small instruments, which can be used at the ICU as opposed to the bulky nailfold capillaroscopy microscopes that are historically used for microcirculation assessment [38-40]. In addition, these devices can be placed sublingually where the mucosal tissue overlaying the capillaries is quite transparent and the images contain much more detail compared to the nailfold. SDF and OPS devices have extensively been used for research on microcirculatory function during critical diseases [18, 19, 22-29,

41-44]. The non-invasiveness of SDF and OPS imaging is favourable for patients in critical circumstances especially for vulnerable patients such as on the paediatric intensive care unit [45]. SDF microscopy was for example used to monitor the microcirculation in relation to illness severity and therapy response in children with meningococcal disease [46], hypothermia [47], and hypotension [48]. The quantitative parameters proportion of perfused small vessels ( $< 20 \mu\text{m}$ ) [24, 25, 27, 29, 46], microvascular flow index [26, 28, 46, 47, 49] and flow velocity [23, 44, 46] are found to be decreased in critically ill patients, while the microvascular heterogeneity increased [23]. Not all critical diseases lead to a change in functional microcirculatory parameters [42, 43], however this does not negate clinical relevance. On the other hand, concern has been raised about the requirement for time consuming off-line data analysis [42] and the difficult interpretation of microcirculation images [50], which could also be the reason for absence of significant microcirculatory changes in the data.

Quantification of the SDF or OPS images is currently semi-automatic using commercially available software (AVA3.0 Microvision Medical, Amsterdam, The Netherlands). The algorithm for flow assessment to determine the important microvascular parameter vessel perfusion (and the proportion and heterogeneity thereof) is based on tracking of RBCs and plasma gaps in a space-time diagram [51]. However, the maximal flow velocity measurement is limited by the frame rate and exposure time of the camera, which is around 1 - 2 mm/s in the current system. In addition, the analysis needs to be performed off-line and involves considerable user-interaction limiting its use at the bedside. An alternative range of techniques to visualize microcirculatory flow is based on the temporal statistics of coherent light backscattered from dynamic structures, referred to as Dynamic Light Scattering (DLS) techniques [52, 53]. In this case, the contrast is not based on absorption but on scattering by flowing RBCs. Techniques such as Laser Doppler Flowmetry (LDF) [54], Laser Speckle Contrast Imaging (LSCI) [55] and Diffusing Wave Spectroscopy (DWS, also called diffuse correlation spectroscopy) [56] fall in this category. These techniques are sensitive to a wide flow range, but generally lack a quantitative representation of flow velocity. Often their measurement quantities can be determined automatically with simple algorithms, but are expressed in quantities such as 'flux' or 'relative blood flow' or 'perfusion units' [57-59], related to the product of the number of moving RBCs and their average velocity. These techniques can therefore be used to visualize blood flow or quantify relative flow metrics, but are not a measure of absolute blood flow velocity. The techniques are discussed in more detail in Chapter 2 and their key differences in technology, temporal and spatial resolution, field of view and probing depth are listed. DLS techniques often use wavelengths within the so-called 'tissue transparency window' (approx. range 650 - 1300 nm) [60] and therefore probe deeper, but with reduced spatial resolution compared to SDF video-microscopy [61-63]. The handheld SDF microscope uses green light for high contrast but with limited penetration depth in tissue due to the high scattering and absorption properties of tissue for green light. The maximum focus depth of the device is 0.4 mm. SDF microscopy can only acquire microcirculation images at locations where the vessels are superficial and in optically transparent tissues, such as mucosal tissue (sublingual, conjunctiva, nail-fold) [7, 64], organ surfaces [65] or neonatal skin [47]. LDF, DWS and LSCI have the ability to image the microcirculation through e.g. skin or skull [58, 59, 66].

Besides diagnosis and monitoring of critical diseases, imaging techniques that quantify microvascular parameters have also been used e.g. to assess neurovascular changes to gain insight in brain metabolism under normal or pathological conditions [67]; skin grafts and burn wounds [68, 69] and microvascular disorders in metabolic [15], cardiovascular [70] or oncologic diseases. Microcirculatory parameters such as vessel geometry, vascular pattern and blood flow were assessed using OPS imaging on oral squamous cell carcinoma [71] and brain tumours [72]. Dilated and chaotic vessels or vessels with low flow compared to healthy areas were found to characterize cancerous regions [71, 72]. This suggests that microcirculatory imaging can be a useful tool in the detection of cancer-related vascular aberrations, the effects of therapy on the tumour microvasculature or for guidance during tumour neurosurgery. Ideally, a blood flow imager should be objective, non-invasive, and capable of performing instantaneous and reproducible readings (for some diseases continuously) [30, 73]. Therefore, a substantial part of this thesis is devoted to optimizing optical technologies to comply with these requirements.

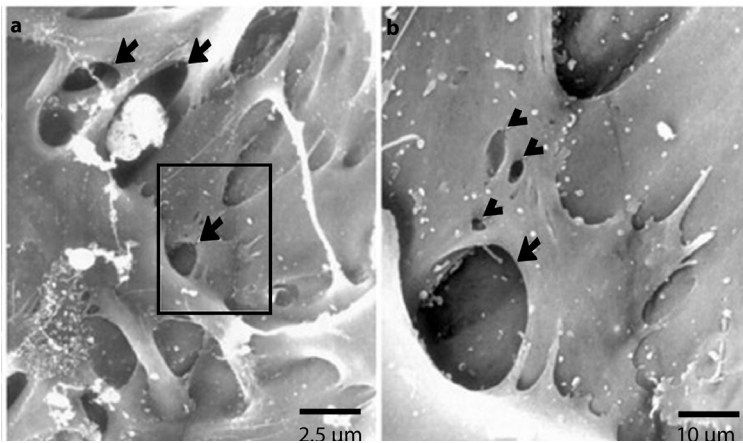
#### 1.4 MICROCIRCULATORY DELIVERY IN TUMOURS

The observation that tumour angiogenesis occurs around tumours and that the vessels display 'chaotic irregularity' was made more than 100 years ago [74]. The imbalance of angiogenic regulators and the vigorous nature of tumour and blood vessel growth cause tumour vessels to be structurally and functionally abnormal. In contrast to healthy vessels, tumour vessels are dilated, tortuous, have uneven diameters, and show excessive branching and shunting [32]. As elucidated in the previous section, the appearance of the microcirculation can be an indicator for the presence of tumours making direct visualization an important non-invasive clinical tool. At the same time, it is expected that cancerous angiogenic regions are visible in microcirculation images only when the tumour has grown to a reasonable size and is superficial enough for vessel image details to be retained in the presence of scattering. In addition, inflamed areas can also induce changes in microcirculatory perfusion similar to malignancies, as measured by LDF [75]. However, for the detection of small or deep tumours the microcirculation can still be useful as a delivery system for contrast enhancing particles to enable detection by advanced optical imaging techniques [76].

The delivery of macromolecular complexes to tumours is mediated by the enhanced permeability and retention (EPR) effect [77]. Not only do tumour vessels look distorted on a  $\mu\text{m}$  to mm scale (dilated, tortuous, irregular, etc.) the composition of the tumour vessel wall also differs from normal on a nm to  $\mu\text{m}$  scale. The pathological angiogenesis process during tumour growth as a result of dysregulation of pro- and anti-angiogenic signalling molecules can result in a defective cellular lining of tumour vessels composed of disorganized, loosely connected, branched, overlapping or sprouting endothelial cells [78]. The resulting vessel wall is 'leaky' and exhibits an enhanced permeability to macromolecules. In normal tissue such macromolecules would be cleared by the lymphatic system, however, other characteristics of tumour tissue are compressed and damaged lymphatic vessels, possibly due to the high interstitial fluid pressure built up by the growing tumour mass [32, 79]. The absence of lymphatic drainage results in macromolecules retaining in the tumour for prolonged periods. This passive targeting of macromolecules to tumours through the EPR effect

results in a higher concentration inside the tumour as compared with the surrounding tissue [80, 81]. As a result, when the macromolecular complexes have photoluminescent properties, the tumour imaging contrast can be enhanced [76, 82, 83].

The macromolecular complexes need to exhibit certain physical and biochemical properties to take full advantage of the EPR effect. Obviously the size is important since particles that are too large cannot diffuse through the permeable vessel wall, while small particles quickly diffuse through the tumour and are not retained [84]. Endothelial intercellular openings in the range 0.3 - 4.5  $\mu\text{m}$  and transcellular holes of 0.6  $\mu\text{m}$  in diameter were measured using scanning electron microscopy images of mouse mammary tumour, as shown in Fig. 1.3 [78]. In a human colon tumour xenograft a cut-off size of 400 - 600 nm was found by observing transvascular transport of liposomes [85]. Optimal sizes of macromolecules for the EPR effect around 60 - 100 nm are reported [84, 86]. In addition, these nanoscale particles must remain in the circulation long enough for accumulation in the tumour, escaping clearance by the immune system, liver and spleen and without interaction with components in the blood vessels or vessel walls [80]. Engineering nanoparticles using biocompatible molecules or coating nanoparticles with biocompatible surface groups increases their circulation time [87]. Other properties such as surface charge, hydrophobicity and shape also play a role [80, 81]. In addition, co-administration of nanoparticles with factors that increase the permeability of tumour vessels has been shown to enhance the EPR effect [80, 83], for example by increasing the blood pressure and widening the endothelial cell-cell gaps [88].



**Figure 1.3 Tumour vessel leakiness** | Scanning electron microscopy image of a mouse mammary tumour blood vessel wall. (a) Multiple intercellular openings (arrows) in endothelial cell lining of a tumour vessel. Region in box in (a) is shown at higher magnification in (b), where the arrowheads (<) point at three transcellular holes, much smaller than the intercellular hole (arrow). Figure adapted from ref. [78].

The EPR effect essentially links the microcirculation to the cellular processes leading to tumour angiogenesis, enabling a passive way of enhancing molecular contrast in tissue. An active approach to enhancing molecular contrast is to conjugate the nanoparticles with tumour-specific recognition molecules (antibodies, ligands) that actively target tumour cells [89]. Targeted nanoparticles are recognised by tumour cell surface receptors, followed by

nanoparticle binding and receptor-mediated endocytosis. Pre-clinical data on targeted nanoparticle delivery to tumours has shown an increased cellular uptake compared to non-targeted nanoparticles [90].

Drug delivery is another attractive application to use nanoparticles. The important advantage of engineering drugs as nanoparticles instead of the free drug, is the increased efficiency of delivery (either passively or targeted) to the tumour compared to the healthy tissues, as healthy vessels are not permeable for nanoparticles. This reduces side effects for patients and lowers the drug dosage necessary for tumour response (or detection). Currently, there are at least six clinically approved nanoparticle-based cancer therapies and another few hundred companies are developing novel nanoscale therapeutics [81]. The first approved form of loading drugs in a nanoparticle was the encapsulation of doxorubicin (standard chemotherapy for e.g. breast and ovarian cancer) in liposomes of around 100 nm. In comparison with conventional doxorubicin, equi-effective (and lower) doses of liposomal doxorubicin show a decreased rate of cardiotoxicity, impaired bone marrow activity, hair loss and nausea/vomiting [91].

The utility of targeted and non-targeted contrast enhancing agents aiming for a higher therapy success has been demonstrated in humans during surgical procedures. The success of surgery, measured as thorough removal of all tumour tissue while leaving as much healthy tissue as possible, can be increased by enhancing intraoperative tumour visibility for the surgeon, exemplified by the realization of tumour-specific targeted fluorescence-guided ovarian cancer surgery in humans [92]. And recently, surgeons equipped with fluorescence goggles saw an enhanced tumour-to-healthy tissue contrast while resecting liver carcinomas during fluorescence-guided surgery [93]. These studies were accomplished using clinically approved fluorescent dyes. The design and approval of new nanotechnology based delivery of contrast enhancing agents can further enhance the specificity and sensitivity of visualizing tumour regions.

In spite of the above drug delivery and surgery prospects, the clinical effects of tumour therapy are confounded. This is illustrated by the withdrawal of the drug bevacizumab for patients with metastatic breast cancer, where impressive tumour reductions were seen but there was no improvement in overall survival [94]. This reveals the limitations and challenges in understanding tumour development where structural, cellular and molecular imaging can help to improve cancer treatments and guide the adaptation of nanotechnology based drug delivery approaches. The heterogeneity of angiogenesis in tumours, both spatially and temporally, is one of the problems resulting in unreachable areas of the tumours for nanoparticles. The high interstitial fluid pressure, which is on the one hand responsible for reduced lymphatic drainage and retention, on the other hand forms a barrier for transvascular transport [95]. In addition, the tumour size, type and location, immune system activity, co-medication and other patient specific characteristics all influence the EPR effect, nanoparticle delivery and the tumour response to therapy [32, 94]. Understanding and predicting EPR effects, assessing angiogenesis in primary and metastatic tumours and correlating EPR activity to clinical response are key considerations to improve nanomedicine in oncology [32, 33, 94]. Quantitative imaging of the microcirculation perfusion, geometry, permeability and delivery of nanoparticles can contribute to this quest.

## 1.5 GLOW WITH THE FLOW: QUANTIFYING BLOOD FLOW AND PHOTOLUMINESCENCE SIGNAL IN BIOLOGICAL TISSUE

Understanding the complexity of microcirculation pathology during the course of diseases is aided by minimally invasive techniques that do not interfere with the physiology and bias results (ranging from changing the blood flow to interfering with the immune system). Optical techniques have advantageous characteristics that allow repeated monitoring, obtain functional information or allow sensitive detection using photoluminescent nanoparticles. The complex interactions of light with tissue represent important challenges in biophotonics. Understanding the complexity of these interactions can be the basis for novel biomedical imaging techniques. Designated niches for optical techniques are just below the surface of tissues. Since the microcirculation is in virtually all parts of the body, probing this part of the circulation is feasible and allows minimally invasive monitoring, even at the cellular and molecular level.

### 1.5.1 Thesis aim

This thesis aims to assess 2 optical imaging techniques for clinical applications in order to: 1. quantify microcirculatory flow velocities using laser speckle contrast imaging and 2. quantify photoluminescence signal from luminescent nanoparticles in biological tissues. The combination of these techniques can be especially useful in the context of tumour therapy by providing information on tumour angiogenesis, enabling molecular contrast and delivering nanoparticle-based drugs.

### 1.5.2 Thesis overview

The first part of this thesis addresses the problem of obtaining quantitative information on blood flow velocity, while maintaining technical simplicity. Using a hand-held microscope, a laser, LEDs and a software-controlled camera, two complementary flowmetry techniques were integrated into one device. SDF flowmetry, based on light absorption by RBCs, is excellent for visualizing blood vessels and quantifying low flows, while LSCI, based on dynamic light scattering by RBCs, is sensitive to a wide range of flow dynamics. Combining the two techniques potentially reduces their individual shortcomings: processing time and flow range (SDF), and quantification (LSCI). Building upon existing theoretical frameworks [54, 56, 96, 97] and recent advances in the understanding of optical scattering by blood [98] the quantitative abilities of LSCI are reassured in the first part of this thesis. The theoretical framework is described in **Chapter 2**, as well as the specific challenges and gaps for quantitative flowmetry. **Chapter 3** describes the validation of integrated SDF-LSCI using a flow phantom and a first *in vivo* calibration of the technique in humans. **Chapter 4** further explores the effects of light scattering with flowing particles of different sizes and concentrations to enable quantitative blood flow measurements *in vivo* for variable vessel diameters and blood volume fractions. LSCI can be used continuously, non-invasively and automated algorithms can be developed. The results of chapters 2 - 4 allow the quantitative visualization of blood flow, flow velocity and perfusion of microcirculatory beds, without being limited to relative flow measures. This enables SDF-LSCI to study microcirculation within and between organs and organisms, during the course of disease and therapy and resulting from different metabolic requirements.

Assessing the EPR effect and (targeted) delivery of macromolecules into tumours, whether it is for contrast-enhanced diagnosis, therapy, or both, is aided by highly sensitive and high contrast imaging. Therefore, macromolecules are often designed to enhance optical contrast due to their specific fluorescent, photoluminescent, scattering or absorption properties. High contrast is achieved when the macromolecules exhibit optical properties distinct from biological tissue. Since biological tissues can absorb, scatter and emit fluorescent light (autofluorescence), a novel type of luminophore capable of nonlinear ‘upconversion’ of light might be an excellent candidate for contrast-enhanced imaging *in vivo* [99, 100], given that tissues do not exhibit this property. The upconversion process and the unique optical properties of upconversion nanoparticles (UCNPs) are discussed in **Chapter 5**. To assess their utility for biomedical imaging a thorough quantification of the optical properties in the context of tissue scattering and absorption is a necessity, which is experimentally realized in **Chapter 6**. Their unique properties are exploited to achieve single UCNP imaging through an absorbing layer of haemolysed blood. This result is extended towards *in vivo* applications by theoretically modelling the ability to detect a single UCNP at different depths in skin. In **Chapter 7** we further advance this approach to include biological complexity by preparing UCNPs equipped with tumour-specific mini-antibodies and assessing their targeting abilities to human breast cancer cells *in vitro*. To model the *in vivo* detection limits of a cluster of breast cancer cells, we covered the labelled cancer cells with breast tissue simulating phantom layers and quantified the detectable upconversion signal at increasing thickness. This novel class of nanoparticles holds promise for future clinical tumour detection and therapy, especially in the context of the growing abilities of nanoscale engineering and technology. Quantitative experimental and theoretical modelling of optical upconversion signals in biomedical context is important to identify the opportunities and limitations of this unique class of photoluminescent nanomaterials, and identify feasible application niches. Chapters 5 - 7 are aimed at the quantification of optical upconversion signals in the context of biological tissue scattering, absorption and labelling performance of UCNPs. Other biologically relevant parameters of UCNPs (biological toxicity of UCNPs, blood circulation times and microvascular delivery of UCNPs to tumours in a chick embryo model) are presented in the Discussion and Conclusion **Chapter 8**. Chapter 8 also discusses additional results on quantitative laser speckle flowmetry and recommendations to further improve the LSCI technique. **Chapter 9** presents a short summary and outlook on the results presented in this thesis, in particular the potential combination of photoluminescence and perfusion imaging in the context of the challenges in diagnosing and treating tumours, and the role of the microcirculation herein.

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