Integrated photoacoustic ultrasound imaging

Applications & new techniques

Pim van den Berg

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INTEGRATED PHOTOACOUSTIC/ULTRASOUND IMAGING: APPLICATIONS AND NEW TECHNIQUES

PROEFSCHRIFT

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Chapter 1. Introduction

1.1 Why photoacoustic imaging?

1.1.1 A view on biomedical imaging

The human body, at least by eye, is rather opaque. Good luck trying to distinguish someone's heart through all the intermediate tissue. Still, we can at times see when someone has a problem: we can see someone's red eye, their bruises or if they have burns. However, we have often trouble determining how bad an injury is – and we would not perceive most deeper problems at all. So it is for the times when the eye starts to misjudge things that are wrong with us that we need better tools to sort things out.

Biomedical imaging is one of those tools. The eye, its direct competitor, is only sensitive to a very tiny range of colors and besides: is it is not the most objective observer ever. The first way biomedical imaging can be used to improve our judgment is by using the same light the eyes use, but in a more objective way. Hyperspectral imaging does this [1]: it accurately tracks exactly which colors reflect from the skin, which may in the future be used to grade the severity of burns for instance [2]. But what are you looking for in this grading? Turns out, a lot of our problems have something to do with blood: blood tells you something about how well our bodies are functioning, and provides so-called 'functional information' [3]. An imaging technique named LASCA (laser speckle contrast analysis)[4] uses light to determine the blood perfusion close to the skin and how well the blood supply is working – or lacking and burned away [5]. However, these two techniques have in common that they only work on the superficial layer of the body. To go deeper, you have to consider the full range of light available in nature, not just those used by the eye. X-rays especially, travel particularly readily through tissue, a property extensively used in medical radiography [6]. Also on the other side of the spectrum, near-infrared light enables diffuse optical imaging [7]. We might also forgo light all together, as is done in ultrasound imaging ('echography') [8]. Another option is to use magnetic fields to investigate properties of tissue, as is used in magnetic resonance imaging (MRI) [6]. So choice aplenty, but there may still be room for improvement.

1.1.1 It's in your blood

As was the case with superficial imaging, deeper in our bodies many problems also have something to do with blood. Either there is too little of it (heart attack or stroke) or more than common (tumor, inflammation). The big advantage of optical techniques (defined as either using visible light or near-infrared) is that they are directly sensitive to the hemoglobin molecule and therefore to blood [9]. However, a problem with most optical techniques is light scattering [10]. This problem is the reason we cannot resolve most structures inside tissue:

Photoacoustic imaging

when light passes through tissue it becomes diffuse, like sunlight on a cloudy day. Other (non-optical) techniques like x-ray or ultrasound are more than sufficient to provide structural information, for instance of bone damage. However, they require additional tricks to get in touch with blood, mostly indirectly, by injection of contrast agents [11], or by looking for movement of red blood cells [12], which is not an easy thing to do.

There may still be a use for ‘regular’ light, with all its benefits from its sensitivity to hemoglobin. The solution is to convert the light energy into something that is not affected by optical scattering. Photoacoustic imaging (PAI) does this converting light into sound waves via something called the thermo-elastic effect [13-15]. PAI gives a measure for the ‘darkness’ of a material. If a material is dark to our eyes then that means the light does not reflect from

it, but is absorbed instead. This absorbed light in PAI slightly heats up the structure (for instance the blood in a blood vessel), increasing the local pressure. If this all happens fast enough then this pressure is relaxed in the form of sound waves [16] (see panel “Photoacoustic imaging”). PAI systems often use light pulses, and the effect is similar to hitting a bell with a hammer. The sound waves that are created can be detected much in the same way as echography detects back-reflected ultrasound waves [17,18]. So while the light becomes diffuse and without detail, a blood vessel absorbing it is sharply defined and will provide an equally well defined ultrasound source.

1.1.2 Photoacoustic setups

Photoacoustic imaging as shown in the “photoacoustic imaging” panel is a type of reflection-mode imaging, where a medium is imaged with illumination and detection on a single side. It is good to realize that this is by far not the only approach in the field of photoacoustics. Also called photoacoustic or optoacoustic tomography, it is rather a broad field of research due to the range of approaches, with each its own name and acronym. Broadly speaking, there is photoacoustic microscopy (PAM) at one end of this range [19], and what is usually called photoacoustic computed tomography (PACT) on the other [20]. The difference between the two extremes is one of resolution versus imaging depth [21].

In PAM the resolution is high, especially when using the optical resolution of a focused laser beam to generate PA signals. This high resolution comes at the cost of imaging depth however, because the optical focus deteriorates rapidly for greater depths [21]. This makes PAM an alternative to other microscopy techniques – but rather without the need for fluorescent labels. In the other categories, PACT is the more typical photoacoustic approaches: light is diffuse and not focused, and the ultrasound detection capabilities determine the resolution [18]. In PACT, the setup is designed to rotate around the sample or subject to form a single image. This makes PACT different from the otherwise very similar reflection-mode PAI, where a medium is only imaged from one side [22]. This latter reflection-mode PAI still relies on diffuse light, and is the topic of this thesis. It should be noted that this list is not complete, that other implementations like transmission mode imaging and PAM with acoustic resolution also exist, for which I recommend the reader a few of the excellent reviews on photoacoustics [13-15,21].

1.1.3 Limited view

One point of care when designing a setup is that structures are best detected when they are horizontal in the image, for instance when a detector array is parallel to a blood vessel [23]. This is also called the limited view problem in photoacoustics, and happens because PA pressure waves travel straight out from the objects that generate them, but not along the object. An analogy is text written on the sides of a bottle: you can read the text best if you hold the bottle straight up in front of you.

The above described limited view problem is the reason why PACT setups rotate around the subject or sample. This way they acquire an image from multiple directions and make sure to detect all structures, regardless of their orientation, equally well. This rotation also improves the resolution of images, as the resolution in depth is typically much better than along a detector array. Another advantage of the rotation is that any artefacts and clutter in the image, for example from indirectly detected PA signals, tend to change and disappear when rotating, and therefore fades away in PACT [24]. While this imaging technique is termed computed tomography, note that it is slightly different from x-ray CT where the rotation is required for depth information, whereas in photoacoustics depth information is already available via the ultrasound time of arrival. PACT works well in some applications, like small animal imaging and mammography, but in other applications it is hard to view an object from all sides – either because it is too thick, or because there is bone in between, and PA pressure waves like ultrasound do not travel well through it.

1.1.4 Pitch perfect

But what is the pitch, or the frequency, of the sound waves generated by PAI? In ultrasound imaging, the shape of a back-reflected signal resembles what you put in. In PAI also the structure that ‘generates’ the PA signal determines what comes out. For a homogeneous object, PAI generates a signal from both the front and the back of objects, two contributions that merge together into a bipolar signal [25,26]. The size of the object determines the distance between the two sides of signal, and this distance determines the pitch of the pressure waves and the dominant frequency. An ultrasound transducer with higher detection frequency is therefore more sensitive to smaller blood vessels and vice-versa.

But even structures like blood vessels often look homogeneous to PAI, even though they are not completely homogeneous: red blood cells (containing hemoglobin) absorb the light and generate the PA signals. The signals from individual red blood cells blend together to create a signal from both the front and the back of objects [27,28]. Similarly, a cluster of vessels such as in tumor angiogenesis will also appear as a PA signal representative of the overall tumor size and therefore lower in frequency.

In generating the PA signal, the laser pulse itself also affects the shape of the PA signal, as do the properties of the ultrasound detectors when detecting the signal. The laser pulse shape smears out the generated bipolar signal – you have pressure relaxation while you are still heating [29]. This smearing out also lowers the amplitude of the PA signal, such that if the laser pulse is especially long, then the PA signal may be hard to detect. Ultrasound detectors are only sensitive to a certain frequency range (the bandwidth), and the better the detection bandwidth matches with that of the PA response, the more homogeneous a structure appears [17]. If the detector’s bandwidth misses most of the high frequencies of PA signals from small blood vessels then the vessel’s signal is smeared out, comparable to the effect of a long laser

pulse. If a detector misses the *low* frequencies of PA signals from large blood vessels, then these show only their edges: the vessel is essentially high-pass filtered.

In essence, the pulse length and ultrasound transducer determine what structure sizes best show up in an image. This also has obvious implications for the choice of transducer and light source for a specific application. These implications furthermore exaggerate the limits of ultrasound imaging: for deep yet small vessels, the high-frequency PA pressure waves they generate will have mostly attenuated by the time they arrive at the surface. Like with ultrasound imaging, a high resolution in depth requires the detection of a wide range of ultrasound frequencies. However, high frequencies are attenuated more when they travel through tissue, limiting high resolution imaging to superficial applications. Within PACT/PAI research there are groups that try to push the imaging depth as far as possible: for instance in PA mammography, where a whole breast should be visualized [30]. Breast tumors are relatively large, such that the resolution is less critical and imaging depth can be preferred. Other approaches use instead high frequency and high bandwidth transducers to get a good resolution, for example in skin imaging, but do not need very high imaging depths [31]. As we will see later, the device used for this thesis falls somewhere in between these two approaches.

1.2 Topics of this thesis

1.2.1 Handheld

One approach to building a PA imaging system is to focus on flexibility – most PACT setups are designed only for one application. The epitome of this approach is to integrate the illumination and detection into a handheld probe. This way, any location can be imaged so long as the probe can physically be placed there. For the purpose of this thesis, the term ‘photoacoustic imaging’ and abbreviation PAI will be reserved for this handheld type imaging; inferring its syntax from ultrasound imaging. Various research groups have developed their own handheld systems [22,31-38]. These implementations have in common that they rely on multispectral PAI by using a Q-switched Nd:YAG laser in combination with an optical parametric oscillator (OPO) that tunes the optical wavelength. This in turn allows these systems to determine exactly what chromophore is present from their unique absorption spectra. They couple the light from the laser source into an optical fiber and couple this to a handheld probe. The systems mostly differ in the type and arrangement of the ultrasound transducers: while some systems employ a ~7 MHz linear array [22,32,33,37], others do not. For instance, the system developed by VisualSonics uses a high-frequency linear array for high resolution superficial imaging [35], a system by iThera a curved array to limit the limited view problem [34], the probe developed by Razansky’s group features a 2D array for volumetric imaging, and Kim et al. developed a system that can use various ultrasound probes with linear or curved arrays [38]. Some of these systems are also capable

of combining PAI with ultrasound imaging. While the handheld probe of these devices is compact and a Nd:YAG laser is relatively powerful, these lasers are also bulky and require thick optical fiber bundles that limit the flexibility. Using an OPO adds to the price of using an Nd:YAG laser. One exception to the above systems is the one developed by PreXion who developed pulsed LEDs for PAI, and arranged them in strips that can be attached to their ultrasound probes.

The unique feature of the imaging device used in this thesis is to use compact and cost-effective diode lasers instead of the Nd:YAG and OPO combination. The device was developed as part of the European Fullphase project, to realize “Fully integrated real time multi-wavelength photoacoustics for early disease detection” as the punch line goes. The big advantage of using pulsed diode lasers is that they are small enough to integrate into the probe housing without affecting its footprint too much. It is even possible to use multiple stacks of diode lasers with each a different wavelength as an alternative to wavelength switching with an OPO. Moreover, the efficiency of diode lasers is much higher than that of LEDs, resulting in lower power use and heat generation. A consortium worked together on designing, testing and applying the system, and consisted of universities (Ruhr Universität Bochum, Universität Bern, Technische Universiteit Eindhoven, and Universiteit Twente), companies (ESAOTE Europe, Brightloop, OSRAM Opto Semiconductors, Quantel, Silios Technologies and TP21) and the Ziekenhuisgroep Twente hospital.

While others have also used pulsed diode lasers for photoacoustics [39-46], this is the first system where it is fully integrated in the probe, and where pulse energies are high enough to enable *in-vivo* imaging. Over the years four prototypes have been developed, each with increasing pulse energy ($0.1 \Rightarrow 0.5 \Rightarrow 1$ mJ) and the final also with four optical wavelengths. The diode lasers feature rows of small laser cavities and, for larger pulse energies, more rows are stacked on top of each other. This is far from trivial. Pulsing a diode laser requires building up quite high voltages, something that is not so easy to begin with, but can also cause significant heat and electrical interference within the probe. More detail about the actual prototypes will be given in the following chapters, starting with **chapter 2** on the 0.5 mJ prototype. The chapter will go into more detail on the probe’s internals, both optical and ultrasonic. It also includes an investigation of resolution and imaging depth and concludes with an example of *in-vivo* imaging of healthy human finger joints. The other chapters in this thesis show results with the 1 mJ (single wavelength) prototype for chronological reasons: the 1 mJ prototype was developed later on in the project.

After chapter 1 the thesis will be split in two parts. **Part I** of this thesis will investigate PA imaging of disease state. This part will start with a pre-clinical study in **chapter 3**, an investigation into a mouse model of liver fibrosis and whether the 1 mJ prototype is able to detect this. Liver fibrosis refers to a large amount of liver scarring as a result of repeated liver damage and accompanying inflammation. Next, **chapter 4** continues with a clinical study.

This chapter investigates whether the 1 mJ prototype can be used for imaging finger joints of rheumatoid arthritis (RA) patients, and whether the probe can detect clinically evident synovitis: an inflammation of capsules around the joints, and the prime issue in RA.

Biomedical imaging in this thesis thus focusses on two inflammatory diseases, liver fibrosis and rheumatoid arthritis. Inflammation is an immune response by the body which requires a large amount of oxygen to function. As a result, the oxygen saturation of blood therefore drops around the inflamed area, in response to which the body sends out markers that signal for more blood vessels to grow and for existing vessels to widen – both to increase the blood flow for more oxygen supply. This increase in vessels is a giveaway of inflammation, something that PAI can potentially detect.

1.2.2 Imaging flow

Nevertheless, there are more ways to detect inflammation, for instance the drop in oxygen saturation can potentially be imaged using multispectral PAI. The other way, besides multispectral, is PA flow imaging. Flow imaging creates a map of how fast things are moving in tissue, and can for instance determine the velocity of blood flow. Therefore, PA flow imaging may be an extra diagnostic tool for detecting inflammation, visualizing the increase in blood flow toward a site of inflammation. Because of this potential, **Part II** of this thesis focusses on PA flow imaging. First off, **chapter 5** reviews the flow imaging techniques that have been applied to photoacoustics and explains the benefits and challenges of the field relative to other imaging modalities. Then, **chapter 6** focusses on one specific flow imaging technique and investigating its application using the second probe prototype. This technique is pulsed PA flow imaging, which aims to track a fingerprint signal from particles or cells as they flow through a medium. This is a different approach from other techniques that either use continuous-wave excitation, contrast agents or local heating; because pulsed PA flow imaging relies on tracking of a PA fingerprint, it benefits specifically from imaging with a diode laser due to its high pulse repetition frequency, which enables high frame-rate tracking.

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Chapter 2. Handheld integrated probe for ultrasound/photoacoustic dual modality imaging

Abstract

Ultrasound and photoacoustics can be utilized as complementary imaging techniques to improve clinical diagnoses. Photoacoustics provides optical contrast and functional information while ultrasound provides structural and anatomical information. As of yet, photoacoustic imaging uses large and expensive systems, which limits their clinical application and makes the combination costly and impracticable. In this work we present and evaluate a compact and ergonomically designed handheld probe, connected to a portable ultrasound system for inexpensive, real-time dual-modality ultrasound/photoacoustic imaging. The probe integrates an ultrasound transducer array and a highly efficient diode stack laser emitting 130 ns pulses at 805 nm wavelength and a pulse energy of 0.56 mJ, with a high pulse repetition frequency of up to 10 kHz. The diodes are driven by a customized laser driver, which can be triggered externally with a high temporal stability necessary to synchronize the ultrasound detection and laser pulsing. The emitted beam is collimated with cylindrical micro-lenses and shaped using a diffractive optical element, delivering a homogenized rectangular light intensity distribution. The system performance was tested in vitro and in vivo by imaging a human finger joint.¹

2.1 Introduction

During last decades, the field of biomedical imaging has witnessed an emerging technique called photoacoustics [1-3] that is making its way towards clinical applications. In photoacoustic imaging, a short pulse of light is absorbed by tissue chromophores, which induces a local instantaneous temperature rise. The volume containing the chromophore instantaneously expands and consequentially builds up a pressure, which leads to the emission of an ultrasound wave. This wave can be detected with an ultrasound transducer array at the tissue surface. Then by knowing the sound propagation characteristics, the origin of the pressure wave can be traced back [4]. The spatial resolution is governed by the laser pulse duration and the bandwidth of the ultrasound probe, and the amplitude of the pressure wave is proportional to the absorbed energy density.

The excitement around photoacoustics is due to several important reasons. While commonly used optical techniques, such as diffuse optical tomography [5] and optical coherence tomography [6], suffer either from poor resolution or limited penetration depth due to the

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scattering nature of human tissues, photoacoustics combines high penetration depth and sub-millimeter ultrasound resolution, thanks to the weak ultrasonic scattering in biological soft tissues. Photoacoustics can be used to identify different functional activities of tissues by visualizing the presence of small blood vessels, the content of hemoglobin and its degree of oxygenation. Moreover, the ultrasound transducer arrays used in photoacoustic detection have also facilitated its combination with ultrasound pulse echo for dual modality imaging [7-9]. This association allowed to achieve valuable results, since ultrasound can provide complementary information, such as anatomy and structures, deep inside the interrogated medium with a likewise sub-millimeter resolution [10]. Until now, bulky lasers were used, making the proposed dual modality imaging systems large, costly, and with low frame rate imaging, complicating their clinical integration [11]. There is a growing need to develop photoacoustic imaging systems (PAI) that are compact, affordable and offering real-time imaging which will contribute to make photoacoustics a standard technique for clinical applications such as breast tumor, melanoma imaging and rheumatoid arthritis.

In this work we present a cost-effective portable PAI system combining ultrasound and photoacoustic imaging modalities. The PAI system takes advantage of the continuing development of efficient and cost-effective pulsed diode lasers and their use as a source for photoacoustics [12,13]. In close collaboration with industrial partners (ESAOTE, Quantel and Silios) we developed a compact photoacoustic and ultrasound (PA/US) handheld probe, integrating an ultrasound transducer and a pulsed laser diode. The probe is used with a modified commercial portable ultrasound system for dual-modality imaging. The ultimate aim of the collaboration is to provide a portable real-time dual-modality imaging system which may help establishing photoacoustics as a routine instrument and offer more versatile tissue information. In the following sections we describe the dual-modality handheld probe and we evaluate the performance and feasibility of in-vivo measurements.

2.2 System development

The main breakthrough of the proposed system is the miniaturization of the illumination system and its integration in the handheld probe. Figure 2.1 shows the developed imaging system combining photoacoustic and ultrasound imaging modalities. The system consists of two principal components: the commercial ultrasound scanner and the handheld probe integrating ultrasound detector, optical excitation and beam shaping systems.

2.2.1 Laser pulsing and Beam shaping

Figure 2.2 shows a schematic representation of the handheld probe. The probe was designed to fully integrate the ultrasound and illumination modules without exceeding a practical ultrasound probe size for the end user. We take advantage of the recent developments of diode laser technology, which has witnessed a tremendous improvement in efficiency and cost effectiveness. In this project we used a laser module specifically developed by Quantel

Figure 2.1. Portable imaging scanner combining photoacoustics and ultrasound, left is the ultrasound scanner system, right is the picture of the probe integrating laser module and ultrasound transducer array.

(Paris, France). The emitting source consists of highly efficient diode arrays (Osram, Regensburg, Germany) mounted in a stack and emitting at 805 nm wavelength. The diodes are driven by a customized laser driver (Brightloop, France), allowing a pulse width of 130 ns at half maximum and a maximum 10 kHz pulse repetition rate. The driver can be triggered by an external trigger with high temporal stability, which is of utmost importance to synchronize the ultrasound detection and laser pulsing. The total delivered energy is around 0.56 mJ per pulse. The efficiency of the driver is around 60% and the total heat dissipation in the probe is about 36%. To limit the heat increase an aluminum rim is added to the design to provide cooling of the diode array and driver via air or operator's hand.

Light emitting from typical diode lasers suffers from bad beam quality with divergence angle much bigger than that of conventional lasers. The divergence is very pronounced, with angles of up to 40 degrees in the axis perpendicular to the diode arrays ('fast axis') and 10 degrees in the parallel axis ('slow axis') at Full Width Half Maximum (FWHM). It results in a rapidly expanding elliptical cone beam. Therefore, it is important to collimate and shape the laser beam to minimize energy loss and to illuminate the region of interest with a desired beam profile. There are different ways to reshape diode beams, and we opted for combination of cylindrical lenses and diffractive optical elements (DOE) provided by SILIOS Technologies (Peynier-Rousset, France). The DOEs can realize almost the same phase functions as refractive optics such as lenses, prisms or aspheres. They can also provide optical functions that are not achievable with conventional refractive optics because the phase function cannot be manufactured in a refractive way. Moreover, as the optical function is coded on the surface and not in the bulk part of the substrate, they are much smaller and lighter which is important for the miniaturization. In our system, the beam is first collimated by means of cylindrical

Figure 2.2. A schematic of the handheld probe. US: ultrasound array transducer, P: deflecting prism, DOE: diffractive optical elements, DS: diode stack, MCL: micro-cylindrical lenses, CR: Aluminum cooling rim.

micro-lenses placed in front of the diodes to minimize the divergence of the beam in the fast axis and allow beam shaping. Using a fused silica DOE composed of $400\text{ }\mu\text{m}$ diffractive cells and 8 discrete phase levels, the beam is homogenized in the fast axis and reshaped in rectangular form onto the skin. The DOEs designed for our shaping system shows an efficiency of 80%. The optical system is mounted in front of the diode stack (see Figure 2.2).

To ensure overlap between deep illuminated tissue volume and the ultrasound detection plane, the medium will be illuminated under an angle. Monte Carlo simulations have shown that at targeted depths 5 to 10 mm, the fluence decreases by increasing the distance between the injection point and the ultrasound detection plane while the angle of illumination has less effect due to scattering. To optimize the fluence deep in tissue we need to minimize the distance between the injection point and ultrasound imaging plane by either minimizing the distance ultrasound-laser modules or by deflecting light with a large angle. The maximum angle of 51° , imposed by dimension limitations, allows an injection point at 2 mm from the ultrasound imaging plane when the skin is at 2 mm away from the probe. This angle will also allow the user to fine-tune the injection point by slightly varying the probe-skin distance. Around 5 mm distance the laser beam and ultrasound imaging plane are overlapping. In order to obtain the required beam deflection, a prism was designed and integrated into the probe (Figure 2.2). By deflecting the light under an angle through a glass prism we also prevent hazardous light emission when the probe is in contact with the air, thanks to the total internal reflection at the glass-air interface.

Figure 2.3. Beam intensity distribution obtained 5 mm after the front-end of the handheld probe.

2.2.2 Ultrasound detection and image reconstruction

The ultrasound detection is performed with an ultrasound pulse/receiver array (based on the commercial Esaote SL3323 ultrasound probe) composed of 128 elements, each with a length of 5 mm and a pitch of 0.245 mm. The array has a central frequency of 7.5 MHz and a measured -6 dB bandwidth of around 100%. The array incorporates an acoustic lens to focus the ultrasound in the elevation plane at about 20 mm distance. The ultrasound module is separated from the laser system using electromagnetic shielding to prevent EM noise, which may be generated by the laser driver, from interfering with US detection. The probe is connected to a modified portable ultrasound scanner MyLab_One (ESAOTE Europe B.V, Maastricht, the Netherlands).

For the purpose of the project, the scanner underwent certain modifications to allow photoacoustic imaging: 1) providing an external signal to trigger the laser driver in order to synchronize between the detection and illumination, and 2) providing the possibility to block US transmission during photoacoustic measurements to allow switching between ultrasound and photoacoustic imaging, 3) modification of the ultrasound beam forming method. The commercial ultrasound scanner uses a standard line-by-line detection approach in combination with dynamic beam focusing and steering. Each pulse is used to build a line of the image and a full frame is generated from 128 pulses. Thus, to maintain real-time imaging of for example 25 images/sec without averaging, one will need a 4.2 kHz pulse repetition

frequency (PRF). This PRF is possible since the ultrasound imaging system has a PRF of 12.8 kHz giving a maximum frame rate of 80 frames/sec. On the other hand photoacoustics uses laser pulses to interrogate the medium and the maximum permissible exposure (MPE), depending on the laser energy per pulse and laser PRF, restricts the illumination features. Therefore having 4200 pulses per second to obtain real time imaging will limit the maximum permissible energy to 0.01 mJ/cm^2 . Hence, imaging through tissue will be a challenging task. Using advanced beam-forming of all elements and subsequent reconstruction instead of the line-by-line technique would allow high frame rate imaging with fewer pulses and therefore maintaining high pulse energy. The advanced beam-forming and reconstruction algorithm is not yet implemented onboard the MyLab_One in the current version of the system. Instead the scanner is connected to a laptop where RF data of all individual ultrasound elements are saved after being simultaneously acquired by the MyLab_One scanner with 50 MHz sampling frequency and digitized with a dynamic range of 12 bits. Afterwards, the data is reconstructed off-line using a Fourier reconstruction algorithm [14].

2.3 System performance

2.3.1 Beam characteristics

Figure 2.3 shows the laser beam intensity distribution at the front end of the probe, obtained by imaging the emitted beam at normal incidence 5 mm away from the probe using a CCD-based beam profiler with imaging optics. The image shows a rectangular beam shape spot with size at $1/e^2$ of 17.6 mm horizontal to the ultrasound detector and 2.2 mm transversal to the probe. The profiles in both directions show a presence of a few peaks which will be of small effect in scattering media. The intensity variations were around $\pm 10\%$ in a direction perpendicular to the beam, with approximately 8 lines/mm.

The energy delivered by the probe was measured using an integrating sphere and calorimeter. It was around 0.56 mJ per laser pulse corresponding to a total fluence of about 1.3 mJ/cm^2 of the angled beam on the skin. The stability of the laser energy was tested by measuring the pulse-to-pulse energy variation over a long period. A maximum variation of about 0.7% was found. The presence of the laser module in the handheld probe may cause an increase in local temperature of the probe. To evaluate this increase, direct measurements of temperature at different PRF were performed using a thermocouple positioned at the probe surface. The probe was in the air without any skin contact and measurements were done at laser side at different locations of the probe. Over ten minutes of laser firing at 2 kHz we only measured a slow increase of 3 degrees Celsius in the polymer casing and 7 degrees in the aluminum rim which will be drained out by the hand of the user. The temperature rise increases with pulse repetition frequency, and at 10 kHz we measured a rapid and more pronounced increase of 5-to-10 degrees in about 2 minutes of firing. However, the laser module will not be used at such PRF continuously due to the safety limitations related to the MPE.

Figure 2.4. Resolution estimation experiment, (a) schematic of the experimental set up, (b) (unreconstructed) time trace a human hair, (c) resolution of reconstructed hair in lateral and axial axis at different depths, (d) axial and lateral resolution at different lateral positions for three depths (7 mm, 17 mm and 22 mm).

2.3.2 Photoacoustic resolution

The resolution of the system was determined by measuring the PA point spread function using a black human hair of 80-85 μm diameter. The hair was placed in a bath containing water mixed with Intralipid to obtain a scattering medium of $\mu_s' = 3 / \text{cm}$ (see Figure 2.4a). The hair was translated in different directions to evaluate the variation of the resolution as a function of the absorber position. Figure 2.4(b) displays an example of the obtained time traces of the hair. To estimate the resolution RF-data of PA signals at each position of the scan were recorded, filtered, reconstructed and Hilbert transformed and subsequently fitted with a 2D Gaussian function. The size of the Gaussian was expressed as the Full Width Half Maximum (FWHM) which defined the resolution of the system. Figure 2.4(c) shows the lateral and axial resolution obtained by placing the hair at the center of the probe and changing the depth. The lateral resolution obtained deteriorates with axial location, from a FWHM of around 0.4 mm at 3 mm away, to a FWHM of 0.6 mm at 30 mm distance. Figure 2.4(d) is obtained by scanning the hair parallel to the probe (off axis) at three different depths

Figure 2.5. (a) Schematic of the phantom used for the maximum depth experiments and (b) the contrast of the signal amplitude to the mean noise background as a function of depth and frame rate.

(7 mm, 17 mm and 22 mm). Close to the transducer the lateral resolution also degrades from 0.4 mm to 0.6 mm when moving off-axis. Both trends are likely caused by the reduction of numerical aperture of the probe while moving deep or away from the probe center. The axial resolution in both scans is fairly invariant to location with 0.28 mm on average.

2.3.3 Penetration depth

The maximum imaging depth of the system was measured in phantom experiments. The phantom used for these experiments consists of a bulk of Agarose gel with a mixture of Intralipid^{20%} and Ecoline black in water, leading to a tissue mimicking reduced scattering coefficient of 10 /cm and absorption coefficient of 0.03 /cm. Polyethylene tubing of 0.58 mm inner diameter was embedded at eight different depths (see Figure 2.5a). Before measurements, the imaged tube is centered in the US field of view and filled with a 0.5% Ecoline black solution mimicking the absorption coefficient of blood: 4.2 /cm at 805 nm while the other tubes are filled with water. 4000 photoacoustic frames (RF signals of all elements) are acquired at a PRF of 2 kHz and saved for offline processing. Before moving to the next tube, the current tube is flushed with water again.

Out of the 4000 photoacoustic frames, all, 500, 200, 43 or 10 are averaged, filtered and reconstructed, leading to image frame rates f of 0.43 Hz, 1 Hz, 5 Hz and 20 Hz respectively. From these resulting images the contrast as a function of depth and frame rate is determined by considering the maximum peak at $1.5 \times 1.5 \text{ mm}^2$ region around the tube to be the signal

amplitude S and defining the mean noise level N in a $1.5 \times 1.5 \text{ mm}^2$ region of interest 5 mm to the side of the tube. The contrast C as a function of depth d and frames f is then given by:

$$C(d, f) = \frac{S(d)}{N(d, f)}.$$

The contrast converted to decibel is shown in Figure 2.5b. Lines of twice (6 dB) and three times (9 dB) the mean noise level were indicated in the graph as well: contrast values above these levels indicate where a tube is barely recognizable or is well visible respectively. Figure 2.5(b) shows how frame rate and imaging depth are interchangeable: by increasing the averaging at fixed PRF we improve the imaging depth but we decrease the imaging rate to remain under MPE. For real-time imaging (20 Hz, 10 averages) the imaging depth is fairly limited at 4-5 mm, while by lowering the frame rate to 5 Hz the depth can be increased to 7-8 mm. Moving to low frame rates like 1 Hz and 0.43 Hz penetration depths of 10 mm and 15 mm can be achieved respectively.

2.3.4 In-vivo imaging

The combined ultrasound and photoacoustic imaging was applied to one of the authors' index finger's proximal interphalangeal (PIP) joints to demonstrate the usability of the probe for in-vivo imaging. The Mylab_One was used with line by line mode for ultrasound detection and parallel mode for photoacoustic detection, such that each laser pulse provides a complete 2D image of the finger while 128 ultrasound pulses are used to reconstruct the ultrasound images. During the measurement, photoacoustic and subsequently ultrasound imaging modes were used with a corresponding acquisition of RF data. The switching between the two modes was done by changing the detection software in the MyLab FPGA. This caused a delay of 5 minutes between the acquisition of the US and PA images, where the subject tried to minimize positioning differences between the US and PA image. In the next version of the system both modes will be implemented together with reconstruction of parallel acquired signals within a GPU-based computation framework.

The photoacoustic images are reconstructed from RF data that was averaged over 20 laser pulses to improve the signal to noise ratio. The MPE for the maximum possible fluence, 1.5 mJ/cm^2 when the laser beam is orthogonal to the skin, would allow for a continuous PRF of 210 Hz, giving a maximum imaging rate of 10 Hz. In these measurements a PRF of 1 kHz was used to reduce motion blur. Appropriate pauses were kept between the photoacoustic acquisitions to keep the average exposure below the MPE for non-uniform temporal exposure.

The index finger was positioned on an ergonomic support and submerged in water to facilitate easy translation of the probe and precise selection of the imaging plane. The measurements were performed with 4-5 mm distance between the probe and the skin for

Figure 2.6. Photoacoustic/ultrasound images of a human proximal interphalangeal joint in sagittal (a) and transverse (b) planes, with the upper part of the image corresponding to the dorsal side of the finger. On the right side, (b) and (c) show corresponding ultrasound only with anatomical structures indicated.

optimal illumination and to evade initial EM interferences which are present at the laser firing corresponding at 2 first mm in the image. At acquisition delay times corresponding to the > 3 mm imaging depths the initial interference was no longer present.

Combined PA/US images of the sagittal and transverse plane of the PIP joint are shown in Figure 2.6. In these images the grayscale pixels correspond to US data whereas the heat-colored pixels correspond to PA data. The sagittal ultrasound image shows the skin and the underlying bone and joint gap. The transverse slice is located near the joint gap, showing bone, the skin and subcutaneous blood vessels. The photoacoustic images show the skin and blood vessels running parallel to the finger. The deeper photoacoustic signals correspond to the reflections of the PA signals on the bone.

2.4 Discussion and conclusion

We presented a fully integrated handheld probe, combining an ultrasound array transducer and a diode laser module in a compact and ergonomic design. The first prototype presented in this work can achieve a laser fluence of up to 1.3 mJ/cm^2 on the skin with an illumination spot size of $18.2 \times 2.3 \text{ mm}^2$. This was obtained by using stacked arrays of highly efficient diode lasers in combination with cylindrical micro-lenses and diffractive optical elements for beam shaping. The probe was used with a commercially available portable ultrasound scanner that was modified regarding the synchronization and detection to allow for photoacoustic imaging.

Phantom measurements showed a possible imaging depth of 10 to 15 mm for a frame rate of 0.5 Hz, while this depth is limited to 4 mm in real time imaging of 20 frames per second. This shallow imaging at high frame rate is dictated by the MPE regulations, which limit the PRF

in relation to laser fluence. A possible measurement strategy would be to use a high frame rate PA imaging for selection of a field of view, after which the operator could acquire a single image with high imaging depth. Additionally we performed phantom experiments to estimate the axial and lateral resolution of the photoacoustic system at different locations. Results showed a lateral resolution of 0.4 mm which degrades to 0.6 mm with depth and the position off axis due to the limited numerical aperture, whereas the axial resolution in both scans was around 0.28 mm on average with negligible variation. The measured axial resolution agrees within 0.05 mm to the theoretical band limited resolution.

This resolution can be improved by reducing the pulse length which was estimated around 130 ns for the current prototype. In fact, in photoacoustic imaging, the resulting pressure wave can be seen as the photoacoustic signal (for delta heating) convolved by the pulse shape. Thus, the frequency content of the signal is directly related to the temporal width of the pulse. For longer pulses, higher frequencies are not generated which will reduce the sensitivity of the system to smaller blood vessels. We are currently investigating the possibility to decrease this pulse length in order to improve the image resolution.

The designed probe was tested by performing in-vivo measurements on a healthy human finger joint. A co-registration of photoacoustic and ultrasound images was obtained. The ultrasound scanner system was used with parallel detection for photoacoustic imaging, giving a maximum achievable imaging rate of 10 images per second, while averaging over 20 frames per image. On the other hand ultrasound images were acquired using a standard line by line mode which can achieve 80 frames per second.

The system was used with two detection modes, line by line for US imaging and parallel detection mode for PA imaging. In the current study this was done consecutively by changing the scanner firmware. In the next version both modalities will be simultaneously available.

Concluding, a compact and handheld hybrid photoacoustic and ultrasound system has been presented. It has been successfully tested on a simple phantom and compared to beam profile data to confirm proper functioning. The study on a more complex phantom showed a penetration depth up to 15 mm for 0.5 Hz frame rate imaging, an axial resolution of 0.28 mm and lateral resolution ranging from 0.4 mm till 0.6 mm depending on the lateral position and depth. The hybrid probe has also been applied in vivo on a proximal interphalangeal joint of a healthy human subject, showing a detailed absorption distribution alongside the anatomical structure of the finger joint. The authors believe that the described system is a first step towards an affordable portable combined US-PA imaging system.

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Part I: Applications

Investigating integrated PA imaging of disease state

Chapter 3. Preclinical detection of liver fibrosis using dual-modality photoacoustic/ultrasound system

Abstract

Liver fibrosis is a major cause for increasing mortality worldwide. Preclinical research using animal models is required for the discovery of new anti-fibrotic therapies, but currently relies on endpoint liver histology. In this study, we investigated a cost-effective and portable photoacoustic/ultrasound (PA/US) imaging system as a potential non-invasive alternative. Fibrosis was induced in mice using CCl₄ followed by liver imaging and histological analysis. Imaging showed significantly increased PA features with higher frequency signals in fibrotic livers versus healthy livers. This corresponds to more heterogeneous liver structure resulting from collagen deposition and angiogenesis. Importantly, PA response and its frequency were highly correlated with histological parameters. These results demonstrate the preclinical feasibility of the PA imaging approach and applicability of dual PA/US system.²

3.1 Introduction

Liver fibrosis is scarring of the liver caused by recurring injury, and is a clinically silent and slow disease developing over 20-40 years. The process is most often the result of either chronic hepatitis B or C infection (viral hepatitis), alcohol over-consumption (alcoholic liver disease) or obesity (fatty liver disease/non-alcoholic steatosis) [1,2]. These factors cause repeated damage to primary liver cells (hepatocytes) and invoke the infiltration of inflammatory cells and the deposition of extracellular matrix (ECM) proteins. If the damage is acute or self-limited, these changes are temporary and liver architecture is restored to its normal composition [1,2]. However, if the liver cells are damaged repeatedly, chronic inflammation and excessive accumulation of ECM proteins results in major scarring of the liver, accompanied by significant angiogenesis, and leading to portal hypertension [2,3]. This progressive fibrosis culminates into cirrhosis and end-stage liver failure leading to high morbidity and mortality worldwide [1,3,4]. The disease is mostly asymptomatic such that patients, when first visiting a clinic, are mostly in an advanced state of liver fibrosis.

Currently, there is no clinically effective treatment available for liver fibrosis; development of anti-fibrotic therapies is ongoing research. A large number of preclinical studies are being performed with experimental fibrotic animal models to evaluate the therapeutic efficacy of promising anti-fibrotic drugs. However, these preclinical studies rely on endpoint fibrotic

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analysis due to lack of a non-invasive imaging technique for the evaluation/monitoring of efficacy of anti-fibrotic drugs in animal models. Rather than just performing endpoint measurements, non-invasive imaging techniques would allow longitudinal monitoring of individual animals. Photoacoustic imaging (PA imaging, or PAI) is an imaging technique that can be of great benefit to preclinical imaging due to its high resolution, great imaging depth and its ability to provide functional information without the use of contrast agents [5,6]. Importantly, PAI has been applied in a wide range of preclinical applications including tumor models [7-9], cerebral imaging [10,11], burn wounds [12,13], pharmacokinetics [14], cardiovascular dynamics [15], rheumatology [16,17] and recently in preclinical differentiation of liver fibrosis and steatosis [18,19].

PAI relies on pulsed or intensity modulated light which – when absorbed by tissue chromophores such as hemoglobin – causes a small pressure build-up that is relaxed in the form of pressure waves. Harmless light is used, often near infrared which can 'diffuse' up to several centimeters in living tissues. While this light diffusion typically prevents high-resolution imaging in optical imaging techniques, this is not the case with PA imaging. PAI can use the strong optical absorption contrast between vasculature and the surrounding tissues for high contrast imaging, and suffers little from background speckle [20]. In addition, by tuning the optical wavelength, spectroscopy is possible, which allows for instance the estimation of oxygen saturation of hemoglobin within vasculature.

Typical PA systems that are used in preclinical research, for example recently published by Xu et al. [19], rely on sizable and expensive lasers. Here we report a cost-effective and portable system, which is unique since it integrates a pulsed diode laser within the housing of a handheld imaging probe. Because of the integrated diode laser, no external laser and fiber optics are required, vastly reducing footprint and cost of the system. Traditionally, while affordable and small in size compared to traditional Q-switched lasers, pulsed laser diodes are not as powerful and have comparatively longer pulse lengths. The PA/US system described here minimizes this problem with the use of a powerful diode laser newly developed for PAI. Moreover, the custom diode laser is housed together with ultrasonic detection and is used in combination with a small-form-factor ultrasound scanner, making this cost-effective and low footprint system also flexible and portable. With the ultrasonic detection performed by an array from a commercial echography probe, PA/US system is also capable of high-quality ultrasound (US) imaging. Both modalities are thus combined in dual modality PA/US imaging, which enables robust non-invasive identification of the liver during experiments and accurate ROI (region-of-interest) selection.

In this study, we use the PA/US system to image the tissue remodeling that takes place during liver fibrogenesis in mice. This tissue remodeling is a highly heterogeneous sub-millimeter process, involving both collagen accumulation and angiogenesis. We initially quantified the results based on the extent of the PA response by calculating the number of reconstructed PA

Figure 3.1. Experimental setup: **(a)** experimental setups for CCl₄-induced liver fibrosis mouse model. CCl₄ was administered to mice for 6 weeks to induce liver fibrosis. Control mice are healthy mice that received olive oil. **(b)** bimodal photoacoustic and ultrasound hand held imaging probe, **(c)** photoacoustic and ultrasound imaging setup and **(d)** experimental imaging setup, and scanning plane and direction in mice.

pixels above detection threshold. Next, we analyzed the relative changes in the PA frequency spectrum of liver signals, as raw PA response could also be affected by a change in the optical properties of the skin. We investigate whether single-wavelength PA frequency analysis can be used to assess the structural heterogeneity. Finally, the obtained results were correlated with histological assessment, both to collagen I (reflecting ECM deposition/scar formation) and CD31 staining (angiogenesis).

3.2 Materials and Methods

3.2.1 CCl₄-induced liver fibrosis mouse model

All the animals (male mice, 6-8 weeks old) were obtained from Harlan (Zeist, The Netherlands). Animals received a normal diet and 12h light and 12h dark cycle. All the animal experiments in this study were performed in strict accordance with the guidelines and regulations for the Care and Use of Laboratory Animals, Utrecht University, The Netherlands. The experimental protocols were approved by the Institutional Animal Ethics Committee of the University of Twente, The Netherlands.

Liver fibrosis was induced in male Balb/c mice by administration of increasing concentrations of carbon tetrachloride (CCl₄, prepared in olive oil) for 6 weeks (n=6), while control mice received olive oil (n=5). After 6 weeks, mice were euthanized, imaged immediately using photoacoustic/ultrasound dual probe, and livers were collected for the subsequent histological analysis (see Figure 3.1).

3.2.2 Photoacoustic/ultrasound imaging system

A compact and cost-effective imaging system was used – capable of dual modality imaging with both photoacoustics and ultrasound. The system, developed as part of European project FULLPHASE, is composed of a MylabOne portable ultrasound scanner (ESAOTE Europe, Maastricht, the Netherlands), and a PA/US probe with a pulse energy of 1 mJ in 130 ns; up from 0.5 mJ in an earlier prototype [21]. As shown in Figure 3.1b, the probe houses a diode laser module with beam-shaping optics and an output prism for delivery of nanosecond light pulses at 808 nm. The laser module is designed and manufactured for the PA/US probe by Quantel (Paris, France). The module is comprised of four stacks of diode lasers by OSRAM (Regensburg, Germany) and is powered by a custom laser driver by Brightloop (Paris, France). A diffractive optical element by Silios (Peynier-Rousset, France) in combination with two cylindrical lenses shape the laser output into a rectangular shape of 2.2 mm by 17.6 mm ($1/e^2$). Ultrasonic detection is performed by an array of 128 piezoelectric transducers, which register photoacoustic pressure waves and emit and detect pulse-echo ultrasound. The ultrasound transducer is based on an ESAOTE SL3323 echography probe, with a 7.5 MHz center frequency with a 100% one-way bandwidth from 2.5 MHz until 10 MHz.

The imaging probe was mounted on a translation stage for the purpose of this study. The animals were shaved and sacrificed immediately before imaging. Imaging was done on the sacrificed mice to exclude the possibility of encountering motion artefacts in this study. Mice were covered with a thin plastic foil, which had its edges raised to allow a layer of water for the transmission of PA and US pressure waves (see Figure 3.1c). Mice were scanned in a 15 mm range with 16 positions 1 mm apart, (5 min average scan time), which enabled volumetric rendering of the liver. At each step, photoacoustic data was recorded by accumulating pressure transients over 500 laser pulses at 2 kHz. In addition, B-mode ultrasound scans were acquired at each step. Ultrasound frames were recorded in slices of 30 mm wide lateral by 20 mm deep, and photoacoustic frames in slices of 15 mm lateral by 20 mm deep. A Fourier domain algorithm was used to reconstruct the initial PA image [22]. This reconstructed PA image was compressed logarithmically to increase the dynamic range and overlaid on the grayscale US image for 2D visualization of each step. The dynamic range and image gain were kept constant over all images. A Matlab algorithm was developed to select the liver region of interest (ROI) and was used to select the relevant data for 3D visualization using VolVIEW. The Matlab algorithm was further used to quantify the fibrosis from PA images, and determine from the liver ROI the number of PA pixels above the detection threshold. The quantification metric was computed per scan position per mouse and results were averaged per mouse over the scan positions where the liver was visible, yielding one value per metric per mouse.

Figure 3.2. Non-invasive photoacoustic (PA) imaging and quantitation in control and CCl₄-induced liver fibrosis mouse models. **(a)** Representative PA/US overlay scanned images of liver from healthy (control) mice (n=5) and CCl₄-treated fibrotic mice (n=6). The confined blue lines denote the region of interest (ROI) registered during PA imaging and quantitation for the PA images. **(b)** Cross-view representation of the 3-dimensional volumetric reconstruction of PA imaging in the control livers and fibrotic mouse livers based on the ROI as shown in overlay images (see online visualizations 1 and 2). **(c)** The graph depicts the number of PA pixels (calculated in ROI within scanned livers, detailed in methods) in control livers and 6 weeks fibrotic livers. Each symbol represents an individual mouse. Data represent mean $\pm$ SEM. ***p<0.001 denotes statistical significance.

3.2.3 Immunohistochemistry

After imaging, liver tissues were harvested and transferred to Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA), and snap-frozen in 2-methyl butane chilled in dry ice. Cryosections (4 μ m) were cut using a Leica CM 3050 cryostat (Leica Microsystems, Nussloch, Germany). The sections were air-dried and fixed with acetone for 10 min and were rehydrated with PBS and incubated with the primary antibody in appropriate dilution (refer to appendix table 1) for 1h. Endogenous peroxidase activity was blocked by 3% H₂O₂ prepared in methanol. Sections were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1h, washed and incubated with HRP-conjugated tertiary antibody for 1h. Thereafter, peroxidase activity was developed using AEC (3-amino-9-ethyl carbazole) substrate kit (Life Technologies) for 20 min and nuclei were counterstained with hematoxylin (Fluka Chemie, Buchs, Switzerland).

Sections were mounted with Aquatex mounting medium (Merck, Darmstadt, Germany) and slides were scanned using Hamamatsu NanoZoomer Digital slide scanner 2.0HT (Hamamatsu Photonics, Bridgewater NJ). High resolution scans were viewed using NanoZoomer Digital Pathology (NDP2.0) viewer software (Hamamatsu Photonics). About 20 images (100x) of entire section (from NDP) were imported into NIH ImageJ software (NIH, Bethesda, MD) and were analyzed quantitatively at a fixed threshold.

Figure 3.3. Photoacoustic frequency analysis. **(a)** Example of a control PA spectrum versus the spectrum of a fibrotic liver. Notice the difference in spectral content, especially the shift in the spectral peak from 0.5 MHz (control, $n=5$) towards 1.5 MHz (fibrotic, $n=6$). **(b)** Contrast in PA spectral amplitude between 1.5 MHz and 0.5 MHz as quantified in a heterogeneity index. In **(b)**, each circle represents an individual mouse. *** $p < 0.0001$ denotes statistical significance.

3.2.4 Statistical analyses

All the data are presented as mean $\pm$ standard error of the mean (SEM). The graphs and statistical analyses were performed using GraphPad Prism software version 5.02 (GraphPad Prism Software, Inc., La Jolla, CA, USA). The statistical comparison between the control and fibrotic groups was performed using an unpaired students' t-test. Correlations between the histological parameters and photoacoustic or ultrasound imaging were assessed using Pearson's correlative analysis. The dot plots for all the correlations were generated using the GraphPad Prism. Statistical significance was defined as $p < 0.05$.

3.3 Results

3.3.1 Photoacoustic/ultrasound imaging and quantification

The PA/US probe's imaging plane was positioned transverse to the mice as shown in Figure 3.1d. Then, the liver was scanned from posterior to anterior using the probe. At each imaging position ultrasound and photoacoustic images were co-registered resulting in PA/US overlaid images such as those shown in Figure 3.2a. These overlay images showed the ultrasound backscatter in gray scale: with on top the mouse skin on the ventral side, with below in bright echoic tissue and a dark hypoechoic liver. The red-and-yellow structures were the reconstructed photoacoustic sources overlaid on the ultrasound image, showing cross-sections of superficial blood vessels and the liver is visible below, showing varying amounts of PA response. The signal appearing above the livers in the fibrotic mouse was due to some interference which couldn't be completely reduced as for the healthy livers.

Figure 3.4. Histological analysis of fibrotic parameters in control and CCl₄-induced liver fibrosis mouse model. Representative photomicrographs of **(a)** Collagen I and **(c)** CD31 from healthy (control, n=5), and 6 weeks (n=6) CCl₄-treated fibrotic mice. Quantitative histological analysis of **(b)** Collagen I and **(d)** CD31 immuno-histochemical stainings were performed in livers of the individual groups. Each symbol represents an individual mouse. Data represent mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$ denotes statistical significance.

The blue curve in Figure 3.2a indicates the liver region-of-interest (ROI) which was drawn manually based on the ultrasound image, in which the liver is visible as hypoechoic. This ROI was used for a 3D volumetric reconstruction of the adjacent PA slices recorded at each scan position (Figure 3.2b, see also video online). Notably, after 6 weeks of CCl₄, mice livers showed an increased photoacoustic response compared to the healthy livers. This increase was quantified by the number of photoacoustic pixels that were above the noise floor within the liver ROI in the overlay images. This allowed the quantification of the physical extent of the PA response, which showed a significant increase in 6 weeks CCl₄-treated fibrotic mice as compared to the healthy control mice (Figure 3.2c).

3.3.2 Frequency analysis of photoacoustic liver signals

We further investigated the relationship between the tissue remodeling caused by fibrosis and the photoacoustic response. In the CCl₄-induced liver fibrosis mouse model, the hepatic scar formation creates fibrotic nodules resulting in a highly heterogeneous liver. In addition, angiogenesis has also been described as a heterogeneous process during liver fibrosis. When irradiated by pulsed light, heterogeneous structures generate relatively high-frequency PA signals due to the smaller size of the structure components [23]. The frequency bandwidth of the system demonstrated in this study makes it most sensitive to absorbing structures from 600 μ m and smaller in size. Hence, it is expected that it is the heterogeneous tissue remodeling that causes the enhanced PA response in fibrotic livers. To verify this, a frequency analysis of the PA signals from the reconstructed liver was carried out: using the ROIs drawn previously, axial pre-enveloped PA signals were Fourier transformed, averaged over the liver and compensated for the detectors' frequency sensitivity. The resulting frequency spectra are shown in Figure 3.3a, with spectra corresponding to the same positions as in Figure 3.2a. The spectra showed a large difference in content: for instance, the peak in the low frequency range

was shifted from 0.5 towards 1.5 MHz. In addition, extra spectral peaks appear at 2-5 MHz for fibrotic mouse livers, albeit smaller in amplitude. The peaks at 0.5 MHz and 1.5 MHz would correspond to structures of 2 mm in size (likely the light penetration distance in the liver) and 600 μm respectively [24] (i.e. fibrotic nodules).

To confirm these findings, the relative magnitudes of the spectral peaks (0.5 vs 1.5 MHz) were investigated for the fibrotic and control mice. For this, the contrast between the two peaks was calculated, and a 'heterogeneity index' *HI* is defined as:

$$HI = \frac{|Y(1.5 \text{ MHz})| - |Y(0.5 \text{ MHz})|}{|Y(1.5 \text{ MHz})| + |Y(0.5 \text{ MHz})|},$$

where $|Y(f)|$ is the spectral amplitude at frequency f . The heterogeneity index was below zero for control mice (lower signal at 1.5 MHz), whereas it was found to be above zero for fibrotic (larger signal at 1.5 MHz, significant at $p < 0.0001$) as shown in Figure 3.3b. This confirms that the spectral peak shifted from 0.5 MHz in controls towards 1.5 MHz in fibrotic mice systematically, providing evidence for fibrotic nodule formation. This fibrotic process involves both deposition of collagen and angiogenesis, as will be shown with histology.

3.3.3 Histopathological analysis of liver fibrosis

Exposure to CCl_4 for 6 weeks resulted in increased accumulation of the major extracellular matrix protein with an increased degree of fibrosis as evidenced by increased collagen bridges in CCl_4 -treated mice as compared to healthy control mice (Figure 3.4a, b). Liver fibrosis induces progressive distortion of the intrahepatic vasculature and is accompanied by significant angiogenesis concurring due to portal hypertension. We therefore also evaluated angiogenesis using CD31 marker staining, and found that increased fibrosis was associated with enhanced angiogenesis as depicted in Figure 3.4c, d.

3.3.4 Correlation of photoacoustic quantification with histological fibrotic parameters

To further validate PA imaging, we performed a correlative analysis between photoacoustic imaging and the histological analyses i.e. collagen expression as a major fibrotic parameter. We found a significant correlation between the number of PA pixels in the liver ROI and collagen I intensity as shown in Figure 3.5a ($R^2=0.8558$, $p<0.0001$). Furthermore, modest correlation was observed between angiogenesis marker (CD31) and the number of PA pixels (Figure 3.5b) ($R^2=0.5161$, $p=0.0128$). It should be noted that CD31 and collagen I both result from the same fibrotic process, and that they are therefore also significantly correlated (Figure 3.5c, $R^2 = 0.6607$, $p = 0.0024$). These correlations suggest the existence of a significant link between PA imaging outcome and the fibrotic process.

Correlation of PA and histology

Correlation of HI and histology

Figure 3.5. Correlative analysis of number of photoacoustic pixels (PA) and heterogeneity index (HI) with histological parameters. Panels **a** and **b** depict correlations between PA pixels and % intensity of Collagen I and CD31 staining respectively. In addition, panel **c** shows the correlation between collagen I and CD31. Panels **d** and **e** show the correlation between the heterogeneity index (HI) and collagen I and CD31 staining respectively. Finally, panel **f** depicts the correlation between number of PA pixels and HI. Correlations were assessed using Pearson's correlative analysis. The dot plots for all the correlations were generated using the GraphPad Prism software. 'R²' denotes Pearson correlation coefficient and 'p' denotes statistical significance.

We also computed the correlations between the heterogeneity index (HI) and histological stainings to verify the significance of liver heterogeneity. Figure 3.5d,e show indeed this is the case: with $R^2 = 0.5863$ and $p = 0.006$ for HI versus collagen I, and modest correlation between HI versus CD31 as $R^2 = 0.3009$ and $p = 0.0806$. As expected, HI is furthermore significantly correlated with the number of PA pixels (Figure 3.5e).

3.4 Discussion

In this study, we have demonstrated the applicability of the dual modality PA/US system in assessing the liver heterogeneity as caused by the experimental liver fibrosis. We have observed that, based on the PA frequency analysis, the increase in PA response in fibrotic livers due to the fibrotic nodules is linked to the sensitivity of the system to sub-millimeter structures. The fibrotic nodules are of a size of around 600 μm , whose PA signal would then

fall within the detection bandwidth of the probe. Conversely, the liver without nodules acts as a single large absorber and – including the light penetration – would not provide a detectable PA response in its healthy state.

It should be noted, however, that the PA/US system is highly sensitive to the presence of nodules but not to its exact composition. While hemoglobin is a well-known absorber at 808 nm, our optical transmission measurements indicate that collagen I also absorbs significantly at this wavelength, especially when in large concentrations around the fibrotic nodules (see appendix figure 1). These nodules may therefore act as a shell-like absorber. Unfortunately, the system cannot confirm this exact absorption structure, as the primary frequency response does not change whether a spherical absorber is homogeneous or shell-like in nature [25]. It is also unclear to what extent angiogenesis plays a role in the absorption of the nodules, as the corresponding staining appears to be less sensitive to fibrosis than collagen (Figure 3.4d).

Some reserve should be placed on the relative amplitudes of the spectra in Figure 3.3a, as potential inaccuracies in the estimated PA impulse response, that is used to correct the spectra, may influence the amplitudes for 0.5-2 MHz. Nevertheless, the peak locations at 0.5 MHz and 1.5 MHz are expected to be quite accurate, as these spectra are the average over many PA responses from the liver, thereby lowering the potentially erroneous contributions from for instance unrelated blood vessels. For fibrotic mice, the spectral content for frequencies *above* 2 MHz is likely related to smaller fibrotic nodules in combination with harmonics of the larger nodules. They are present in quite varying degrees for the fibrotic livers, and are therefore assumed not to be a fundamental indicator of fibrosis.

It should also be noted that the light has to propagate further through tissue to reach the liver in case of the control mice as shown in Figure 3.2a (about 1 mm extra). It could be possible that this would lower PA response compared to fibrotic livers. Note that this would only affect the number of PA pixels, not the heterogeneity index as spectral differences are normalized by their mean amplitude. The ultrasound attenuation in tissue, is on a larger length scale than optical attenuation making it highly unlikely to affect the PA frequency spectra and therefore the heterogeneity index. In addition, the skin's PA response is also higher in case of the fibrotic mice. While no evidence of skin inflammation with CCl₄ fibrosis models has been reported, this could nevertheless explain the enhanced response due to increased perfusion or angiogenesis. Alternatively, the enhancement can be explained by the modification of the livers' optical properties during fibrogenesis: an increase in scattering relative to absorption would lead to more reflectance and therefore increase the fluence within the skin.

One drawback of the current system is its lack of optical wavelength tuning, a technique available to the large laboratory lasers commonly used in PAI, via an optical parametric oscillator (OPO). While an OPO adds to the cost and size of a laser system, it allows the

interrogation of the optical absorption spectrum of tissue chromophores, for instance hemoglobin and collagen. Because both hemoglobin and collagen absorb the 808 nm light pulses from the diode laser, both are expected to contribute to the photoacoustic signal generation. To resolve these contributions, a multi-wavelength PA/US probe – based on multiple diode lasers with different optical wavelengths, housed within the probe – is under development. Such a system could then also be used to interrogate the oxygenation of vasculature in mouse livers, which may provide quantification of liver hypoxia for improved staging of liver fibrosis, since hypoxia is an effect of inflammation and fibrosis. In addition, targeted exogenous probes, labeled with PA contrast agents can further expand the range of diagnostic imaging techniques in liver fibrosis, and would allow research into targeted therapeutics. When finished, a multi-wavelength diode laser system would remove the need for an OPO, further reducing the cost and footprint of a multi-wavelength PA system.

PAI is also a promising modality for the assessment of fibrosis in liver biopsies. Liver biopsy has been considered as the “gold standard” for the diagnosis and staging of liver fibrosis [26]. However, liver biopsies, *ex-vivo* excised tissues or human liver slices undergo extensive pathological processing: fixation, slicing and staining’s for microscopic analysis. The process is highly time-consuming accompanied by distortion of the sample. PA imaging will provide fibrosis assessment of a sample non-invasively, imaging microscopic morphological features without the need for sample modification.

Altogether, we have shown that a single-wavelength PA frequency spectrum can be used to quantify the structural changes within the liver. US imaging was beneficial for selection of a relevant liver region of interest and must be regarded as an essential feature for PA imaging. Furthermore, PA/US imaging does not require exogenous contrast agents for assessing liver fibrosis as shown in this study.

Therefore, this study provides the important evidence for the feasibility of PA/US dual modality imaging as a non-invasive approach for the detection of liver fibrosis in mice. Furthermore, this device holds a great promise for real-time imaging, and longitudinal animal studies to investigate therapy responses and disease progression.

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Author Contributions

RB, PVB and JP conceived and designed the experiments. RB, PVB and KD performed the experiments. RB, PVB and KD analyzed the data. RB and PVB wrote the paper. WS, JP and KD interpreted the results and critically revised the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Appendix

Appendix table 1: Antibodies used for the immunohistochemistry		
Primary Antibody	Source	Dilution
Polyclonal goat anti-collagen I	Southern Biotech	1:100
Monoclonal rat anti-mouse CD31	Southern Biotech	1:100
Secondary Antibody	Source	Dilution
Polyclonal rabbit anti-goat IgG	DAKO	1:100
Polyclonal goat anti-rabbit IgG	DAKO	1:100
Polyclonal rabbit anti-rat IgG	DAKO	1:100

Appendix Figure 1. Absorption measurements for collagen I. (a) Absorption spectra measured using a Shimadzu UV-2501 spectrophotometer of collagen I / PBS solution in cuvettes. The concentrations were taken as per the average of collagen I concentration in typical fibrotic livers. (b) Plotting of the absorption at 808 nm for the range of concentrations shows proper linear behavior. Assuming 2% of the liver shows collagen scarring and that the total amount of collagen I is concentrated only in these scars, then the approximate absorption coefficient within scars is (0.025 / cm) / 2% = 5 / cm. This is of the same order as the absorption of hemoglobin in functioning blood vessels.

Chapter 4. Feasibility of photoacoustic/ultrasound imaging of synovitis in finger joints

Abstract

Objective To determine whether photoacoustic imaging (PAI) can be used to detect clinically evident synovitis. Current imaging modalities in rheumatoid arthritis (RA) can be used to predict disease progression, even in subclinical synovitis. However, these imaging standards still have their specific limitations. PAI has a unique combination of features that may aid future assessment of synovitis. We study a compact photoacoustic/ultrasound imaging probe for possible use in this assessment.

Methods 10 patients with RA and 7 healthy subjects were included. Inflamed and non-inflamed proximal interphalangeal joints of patients were examined and compared with joints from healthy volunteers. Examinations with a PAI scan, ultrasound power Doppler (US-PD), and clinical examination by an experienced rheumatologist were performed. We quantified amount of PA signal and its spatial extent in the PAI scans using a region of interest (ROI) drawn over the hypertrophic joint space. The US-PD scans were scored by two experienced rheumatologists.

Results The amount of PAI signals was significantly increased when comparing the inflamed with contralateral non-inflamed joints and with joints from healthy volunteers ($p < 0.001$ for both). However, when comparing inflamed and non-inflamed joints with US-PD, significance was $p < 0.01$ and only $p < 0.05$ when only considering the size of the hypertrophic area. US-PD and PAI were strongly correlated with Spearman's $\rho = 0.64$ and $p < 0.001$.

Conclusion This feasibility study shows that PAI with our compact probe is capable of detecting clinically evident synovitis. Further studies are required to compare the predictive value of PAI, including multispectral PAI, with US-PD and/or MRI.³

4.1 Introduction

In rheumatoid arthritis (RA), imaging of synovitis can predict disease progression. Indeed, ultrasound power Doppler (US-PD) and magnetic resonance imaging (MRI) are able to predict bone erosion [1-3]. In clinical remission, detection of subclinical synovitis indicates disease progression and increases the risk of disease flare [4-7]. US-PD has gained a place in the clinical workflow based on these qualities. However, US-PD has inherently high operator dependency and suboptimal reproducibility [8,9]. MRI is rather costly, specificity is modest and it requires contrast agents [10]. In optical imaging, optical spectral transmission (OST) has shown fair performance at detecting synovitis while presumably low in cost [11-

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13]. However, sensitivity and specificity are modest and the low spatial resolution limits differentiation between synovitis and tenosynovitis. Fluorescence optical imaging [14-17] appears to have higher performance than OST, but also has low resolution and in addition requires costly contrast agents.

Photoacoustic imaging (PAI), a hybrid optical-and-ultrasound imaging technique, could find a good balance in features: combining the sensitivity to hemoglobin of optical techniques with the resolution of clinical ultrasound [18-21]. To form a PA image, short laser pulses are shone on the skin and subsequently enter the tissue, where the light is scattered by cells and becomes diffuse. The light pulse is then absorbed by dark tissue constituents such as hemoglobin and melanin. The absorption slightly heats structures containing these constituents which leads to a small pressure buildup. This pressure buildup causes these structures to generate sound waves that can be picked up by ultrasound transducers. PAI is therefore similar to sonography, except that the ultrasound is generated within tissue, instead of reflected ('backscattered') by it.

PAI differs significantly from US-PD in two aspects. First, movement of erythrocytes is not required for signal generation, since the generation of PA signals relies only on the presence of hemoglobin (or other chromophores) [19]. Second, there is a larger concentration of hemoglobin within vasculature than in surrounding tissue, leading to more signal generation, whereas in US-PD, erythrocytes reflect comparatively *less* signal than the surrounding tissue [22,23]. A wall filter is therefore not required in PAI, and 'flash' artefacts or motion clutter are not present. These properties imply that slow blood flow in synovial microvasculature poses no problem to PAI. As a result, we expect PAI to be particularly sensitive to subclinical synovitis. We also expect PAI to be less prone to observer variability since no 'Doppler angle' is required.

PAI has been investigated in pre-clinical studies of synovitis [24-27], and several setups have been proposed for human finger joints [28-33]. In addition, a few early feasibility studies have been performed with RA patients [34,35], but the amount of clinical data so far is limited. PAI has also been investigated in other medical areas involving angiogenesis, for instance in clinical studies into mammography [36-39].

A unique handheld PA/US probe was developed [29], which in this study is investigated for possible use in assessing synovitis. The objective of this study is to investigate whether the PA/US probe can be used to detect clinically evident synovitis.

Figure 4.1: The PA/US probe (**left**) with view of the front end showing the light delivery window (dark aperture) and acoustic lens in medium gray. The patient's hand is submerged in water (**right**) where it rests on a series of supports. The probe is mounted on a 2-axis motorized stage and positioned above the joint.

4.2 Methods

4.2.1 Patient inclusion

Patients undergoing care in the Ziekenhuisgroep Twente hospital were asked by their rheumatologist to participate in this study. Healthy volunteers were recruited in person or via flyers at the University of Twente.

Patients aged over 18 years with rheumatoid arthritis fulfilling 6 or more ACR/EULAR criteria were included. Specific inclusion criteria were: swelling of at least one PIP 2, 3 or 4 joint with at least grade 1 power-Doppler signal on US examination. Test subjects (healthy or patient) were excluded from participation if they had clinically significant bone deformation and/or osteoarthritis in the joint of interest. All subjects received written information and gave informed consent, resulting in a delay of 3 to 8 days between the inclusion by a rheumatologist and time of measurement.

4.2.2 Imaging system

The imaging study is performed using a dual modality photoacoustic/ultrasound system. The system relies on a probe that houses both a small diode laser together with ultrasound transducers (see Figure 4.1). The probe is a second generation prototype developed from the probe described earlier in detail [29]. The diode laser is pulsed to generate photoacoustic waves, which are then detected by the ultrasound transducers. These transducers are also used to transmit ultrasound in order to generate high-quality b-mode ultrasound images.

The diode laser fires 1 mJ pulses of 120 ns duration, and can be operated up to a pulse repetition frequency (PRF) of 10 kHz. The pulses are formed into a rectangular shape of 2.2 mm by 17.6 mm ($1/e^2$) by a diffractive optical element, after which the light exits the probe via a prism. The laser emission is at 808 nm, which corresponds to the isosbestic point of

Table 1: Subject characteristics

Characteristic	Healthy volunteers	RA patients
	(N=7)	(N=10)
Age: mean (range)	56 (49-62)	63 (49-80)
Gender (% female)	43%	50%

Values are the subject's mean (standard deviation, SD) or (range).

hemoglobin and oxy-hemoglobin, which leads to PA signal amplitudes independent of the blood oxygenation.

The ultrasound detection is based on an ESAOTE SL3323 probe. Transducers are placed in an array of 128 elements with a pitch of 0.245 mm. Each element has a bandwidth from 2.5 MHz to 10 MHz with a 7.5 MHz center frequency. An acoustic lens (focal length: 24 mm) is placed in front of the transducers to moderately focus the detection in the elevational plane.

The probe is connected to a MylabOne ultrasound scanner (ESAOTE Europe), which can be used in two modes. In the first it transfers the collected time-pressure data from the middle 64 elements directly to a laptop. This mode is used to acquire photoacoustic data, but is also used to pulse these elements for plane-wave ultrasound. In the second mode the scanner operates regularly and is used to acquire b-mode ultrasound using all 128 elements in a line-by-line transmission and acquisition scheme.

The US-PD examination is done using an identical MylabOne scanner (in the second mode as described above) in combination with a 14 MHz center frequency linear array (SL3116, ESAOTE). The PRF was set at 750 Hz, and the wall filter at its lowest and the sensitivity at its highest setting.

4.2.3 Scan protocol

Per subject examination, a minimum of two PIP joints were scanned: one clinically inflamed joint and an uninflamed joint – preferably the same joint contra-lateral. A complete examination of one subject included a series of longitudinal images using power Doppler ultrasound for each applicable joint and another series using the PA/US system. Both examinations took place with the subject's arm placed in a water bath fitted with supports for the arm, hand and the finger to be scanned (see Figure 4.1). The water temperature was controlled to 29-31 °C during the examination, which took place between 10:30 and 14:00 hours. During measurements there was no contact of the PA/US and US-PD probes with the skin in order to avoid pressure artefacts. In addition, the PA/US probe was placed 4-5 mm from the skin such that the laser beam intersects with the ultrasound elevational plane.

For the PA/US examination the PA/US probe was placed on a motorized stage for better control of the measurement. The probe was aligned longitudinal to the finger and on the

dorsal side. The stage was moved orthogonal to the finger in 0.5 mm steps for over 6 mm. At each step, a PA image was accumulated over 500 laser pulses for 0.25 s. In addition, 100 frames of plane wave ultrasound (one fixed angle) were recorded each step. Each scan was repeated with the same probe and at identical steps, but then with high-quality line-by-line b-mode ultrasound. One scan yielded therefore 13 PA, 13 plane wave and 13 b-mode images at identical locations. In our scan protocol there was approximately 1 minute between a PA and plane-wave US acquisition, and the subsequent b-mode US image.

US-PD examination was either performed by an experienced rheumatologist or by placing the US-PD probe in the motorized stage. For each joint, 3-5 images are recorded.

4.2.4 Scoring of US-PD images

Representative US-PD images were digitally stored and anonymized. They were graded (0-3) according to the EULAR classification by two rheumatologists who were blinded to the allocation of the images. Discrepant results were reviewed to reach consensus resulting in a final PD-score for each individual joint.

4.2.5 Data analysis

The PA channel data – the pressure as a function of time as measured by the transducers – is converted into a map of the original pressure distribution using a Fourier domain reconstruction algorithm [40]. All data analysis is automated using Matlab (Massachusetts, USA). To account for the light attenuation within tissue, a depth-dependent correction ('gain') is introduced to account for this. Since the finger in the longitudinal orientation is fairly flat, a basic exponential gain of $1/\exp(-\mu_{\text{eff}} z)$ is used with $\mu_{\text{eff}} = 1/\text{mm}$ the effective attenuation coefficient and z the depth in tissue [41,42]. A different $z = 0$ is set for every axial line in the PA image, such that the fluence correction starts at the skin level. Determining the position of the skin surface was done visually using the PA response from the melanin layer in the skin.

For image formation, the PA data is compressed logarithmically at a dynamic range of 40 dB or 18 dB, with the same minimum and maximum amplitude for inflamed and non-inflamed images. These dynamic ranges were selected based on the noise level (-40 dB) and the amplitude of healthy joint's background PA signals (-18 dB) respectively. Pixels within the dynamic range are color coded in Matlab's red-and-yellow color map 'hot' and finally overlaid on a b-mode ultrasound image.

For each joint scan, a region-of-interest (ROI) is drawn to select the hypertrophic joint area. The ROI is drawn on the b-mode ultrasound image, where the hypertrophic area is defined as to include any pixels between the tendon and the bone surface. The ROI is then transferred to the PA image, from which the number of PA pixels is calculated that fall within the 18 dB

Figure 4.2: PA/US and US/PD images of an inflamed (upper row) and non-inflamed contra-lateral joint (bottom row) of an RA patient. PA/US images in **(a)** show a difference in color between inflamed and non-inflamed corresponding to an increase in amplitude levels. When discarding low PA amplitudes in **(b)**, only features in the inflamed joint are visible. Corresponding US-PD images are shown in **(c)**. The blue line in the PA/US images indicates the ROI used for quantification of PA features in the synovial space. The 0 dB level is the maximum PA amplitude from the inflamed joint.

dynamic range. A secondary quantification metric is provided by the mean amplitude of non-compressed PA signals within the ROI. In case of healthy joints there is no hypertrophic area and the ROI selection will include more tissues than just the synovial space.

4.2.6 Statistical analysis

Mann-Whitney U-test (left-sided) is used for comparing the control group (either joints from healthy volunteers or non-inflamed joint from the same subject) with inflamed joints. Spearman's rank correlation is used when comparing the PD grading with PA quantification.

4.3 Results

4.3.1 Subject characteristics

7 healthy volunteers and 10 RA patients were included in the study. All subjects had Caucasian skin. The characteristics of these subjects are shown in table 1. The RA patients had a mean disease duration of 117 months (range 5-133), all were positive for rheumatoid factors and 7 were positive for anti-CCP, mean CRP levels prior to the measurement were 6.3 (SD 5.6).

4.3.2 Photoacoustic/ultrasound imaging

Figure 4.2 depicts examples of fluence corrected PA/US and US-PD images for an inflamed joint and the contra-lateral non-inflamed joint of an RA patient. The reconstructed PA signals are shown ranging from dark red (low signal amplitudes, starting at -40 dB) to light yellow (high/abnormal signal amplitudes, up to 0 dB); the data is overlaid on the grayscale US b-mode image. The PA images in Figure 4.2a show a superficial blood vessel in both the inflamed and non-inflamed joint, with underneath, above the bone surface additional PA features. Larger amplitudes and more confluent features are recorded for the inflamed joint, as can be further observed in Figure 4.2b where only high amplitudes (18 dB dynamic range) are plotted. With this threshold, almost no PA features are visible for the non-inflamed joint.

4.3.3 Quantification of PA and US-PD imaging

The numbers of high amplitude PA pixels (such as those visible in Figure 4.2b) were computed for inflamed and non-inflamed joints, and of joints from healthy volunteers. The result (Figure 4.3a) indicates a larger number of high amplitude PA pixels for inflamed joints, compared to healthy and non-inflamed joints. In addition, an alternative quantification method for PAI also shows a larger value for inflamed joints: the mean (non-compressed) pressure amplitude of PA features (Table 2). Both quantification methods provide significance with $p < 0.001$ when comparing inflamed joints with those from control groups. Significance drops slightly when comparing non-inflamed with inflamed joints using PD scores ($p < 0.01$). When comparing joints based on the sizes of the ROIs (corresponding to the hypertrophic area), significance is only $p < 0.05$. Grading of US-PD images shows a strong agreement ($\rho = 0.64$, $p < 0.001$) of the PA pixel count with the consensus PD score assigned to the images by two rheumatologists (Figure 4.3b and Table 3).

4.4 Discussion

We found that PAI was sensitive to clinically evident synovitis as demonstrated by the significant difference in PA features between inflamed and control joints. In addition, the PA quantification agreed well with the corresponding semi-quantitative PD score. PA findings seem to discriminate better between inflamed and non-inflamed joints of patients than US-PD or the hypertrophic area when comparing inflamed and non-inflamed joints of patients.

Hyper-vascularization and angiogenesis are hallmarks of rheumatoid arthritis and are markers for imaging with US-PD, as the increase in blood flow is detectable using ultrasound flow imaging. In joints that are close to the skin, the increase in vascularity is an attractive target for PAI. It should be realized, that US-PD and PAI do not provide an identical representation of vascularity, synovial or otherwise. On one hand, US-PD is expected to highlight larger feeding vessels and, theoretically, the movement of other structures such as villous synovial folds within the hypertrophic region. On the other hand, PAI is expected to

Figure 4.3: PA quantification with **(A)** comparing the number of high PA pixels for each joint group and **(B)** comparing the same quantification for discrete PD score (0, 1, 2 or 3, offset on the x-axis is to visualize individual markers); Spearman's $\rho = 0.64$, $p < 0.001$. One triangle represents one joint and horizontal bar is median of one group.

Table 2: PD score, PA quantification and hypertrophic size

Parameter	Healthy (N=12)	Non-inflamed (N=11)	Inflamed (N=11)
PD score	0.1 (0.3)***	0.5 (0.7)**	1.7 (0.9)
Number of high PA pixels	225 (299)***	444 (694)***	2792 (1742)
Mean PA amplitude	13.2 (4.4)***	14.9 (11.7)***	56.7 (36.0)
ROI size (pixels)	4540 (1318)***	7900 (3690)*	12468 (4554)

Quantification values: mean (standard deviation). Rank test p-values for testing inflamed joints versus either of the control groups (healthy or non-inflamed): *** $p < 0.001$, ** $p < 0.01$ or * $p < 0.05$.

Table 3: PD score versus other parameters

Parameter	PD-0 (N=19)	PD-1 (N=7)	PD-2 (N=6)	PD-3 (N=2)
Number of high PA pixels	252 (367)	2368 (2494)	1909 (1219)	2741 (472)
Mean PA amplitude	12.2 (4.1)	43.8 (39.8)	50.1 (38.8)	53.6 (6.5)
ROI size (pixels)	5263 (2115)	11075 (5265)	12162 (3868)	14013 (4445)

Quantification values: mean (1σ)

be particularly sensitive to increased blood volume in smaller vasculature within the synovial membrane. PAI typically works best for small vessels, networked mostly parallel to the probe; US-PD rather visualizes large vessels, angled to the probe. The unique photoacoustic probe that we used in this study is sensitive to vessels from 0.2 mm in size and larger. Interestingly, the appearance of synovitis in PAI is quite similar for all the clinically inflamed joints that were imaged in this study – unlike that of the US-PD representation, which varied considerably.

These fundamental differences between PAI and US-PD may help explain the variation between the PD score and the PAI quantification (Figure 4.3b). Even so, there are a few data points that fall outside the ‘natural’ spread. More specifically, Figure 4.3b shows two grade 1 joints with a very high photoacoustic signal and three grade 1-2 joints that hardly show a PAI signal. The former (“too high PAI signal”) might be due to a PA signal originating from a different source, as the shape of these corresponding structures was decidedly different from the regularly seen representation of the synovium in PAI. The latter offsets (“too low PAI signal”) may in fact be due to PD scoring that were too high as a result of artefacts: notes from one of the two blinded examiners confirm this possibility.

While this work provides evidence of PAI detecting synovitis, there exist a few methodological limitations to this study. For one, the selection of patients took place approximately a week before the PA examination. This might explain also the variance in the PA quantification of inflamed joints (Figure 4.3a), as some patients’ synovitis subsided after selection, but were still included in the inflamed group. In addition, US-PD is hard to standardize, which may have caused the PD artefacts explained earlier. Also, the researcher in charge of drawing the ROIs was not blinded to the joint inflammation, which may have biased the interpretation. This issue was moderated however, since the ROI was drawn only on the US image without showing the PA overlay. A technical limitation of the system was the inability to co-acquire high-quality b-mode and PA images – and as a consequence there was a time delay between both – but this will be solved in the future. Despite these limitations this study shows positive and highly significant findings in PAI.

This is the first clinical study with a compact and fully integrated PA/US imaging probe. This means a big step up from existing PAI systems, where sizable and costly external lasers are used. Furthermore, our system relies on a near infrared (NIR) light source at 808 nm, in contrast to previous studies, which used visible light of 580 nm [35]. While hemoglobin absorbs less NIR light than it does in the visible range, light attenuation in the surrounding tissue is also lower. This means that with NIR light the PA outcome depends less on the exact tissue composition. In addition, absorption by superficial structures would be much more pronounced with visible light, for instance in the melanin layer and of regular vessels. Absorption like this is known to cause pronounced clutter when these PA signals also travel down and reflect on lower structures.

Previous studies showed that linear array-based systems such as used in this study are susceptible to clutter and reflection artefacts [43]. Future studies should therefore include clutter reduction and artefact removal [44,45]. We were able to reject the possibility of most types of artefacts by moving the illumination position in relation to the finger – for most types of artefacts the appearance of PA features would change in relationship to the US image [46], but this did not happen in the cases investigated here. However, clutter may have caused the baseline PA signal as can be seen in Figure 4.2, and also some of the outlying data points in Figure 4.3.

Future applications of PAI to synovitis can take advantage of its multi-spectral imaging capabilities, allowing in principle the estimation of the oxygenation saturation (sO_2) of the synovium. Multi-spectral PAI is expected to improve the specificity of the technique. Targeted PA contrast agents with specific spectral signature linked to molecular markers also deserve investigation, as they could provide information about inflammation similar to for example positron emission tomography. A new prototype of our system, which includes diode lasers of various wavelengths, is currently under testing. This prototype merits further use for investigating subclinical synovitis in a larger patient population, and its predictive value for a disease flare. In addition, comparison with MRI angiography is needed, as this would allow a closer look at which of the vascular structures are depicted by PAI.

4.5 Conclusion

PAI is a unique modality due to its optical imaging contrast in combination with ultrasound-based resolution. We have shown PAI capable of detecting clinically evident synovitis, which is a first step toward the application of PAI for monitoring disease activity. These results provide a basis for further research to investigate the detection of subclinical synovitis and the benefit of PAI over other modalities.

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Contributions

All authors took part in the conception and design of the study. PJB and HJBM performed the measurements. PJB processed and analyzed the data and wrote the manuscript draft. HJBM took part in grading the US-PD images. Each author took part in editing the manuscript, read and approved its final version.

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Competing interests

None.

Patient consent

Written informed consent was obtained prior to inclusion.

Ethics approval

The ethical committee METC Twente gave its approval of the study protocol.

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Part II: New techniques

Investigating PA flow imaging

Chapter 5. Review of photoacoustic flow imaging: its current state and its promises

Abstract

Flow imaging is an important method for quantification in many medical imaging modalities, with applications ranging from estimating wall shear rate to detecting angiogenesis. Modalities like ultrasound and optical coherence tomography both offer flow imaging capabilities, but suffer from low contrast to red blood cells and are sensitive to clutter artefacts. Photoacoustic imaging (PAI) is a relatively new field, with a recent interest in flow imaging. The recent enthusiasm for PA flow imaging is due to its intrinsic contrast to haemoglobin, which offers a new spin on existing methods of flow imaging, and some unique approaches in addition. This review article will delve into the research on photoacoustic flow imaging, explain the principles behind the many techniques and comment on their individual advantages and disadvantages.⁴

5.1 Introduction

Blood flow in arteries, veins and smaller capillaries is an important aspect in the diagnosis of a wide range of pathologies and diseases. Blood flow can be estimated with either flow imaging or perfusion imaging, and both provide distinct and valuable information. Flow imaging is the process of mapping where functional vascularity is, or what the flow profile is within an artery or vein. The latter can be used for estimating, for example, the wall shear rate [1] or detecting where turbulent blood flow occurs [2,3]. Imaging vascularity is important to reveal for example angiogenesis [4], the process during which rapid growth of new vasculature occurs. The resulting vasculature, irregularly and haphazardly shaped, is the pathological result of for instance tumour growth [5] and inflammatory diseases such as rheumatoid arthritis [6].

Perfusion imaging is related to flow imaging, but provides a more global picture of vascularity, that describes how much blood reaches organs, muscles or skin over time [7]. The amount of skin perfusion determines for instance the chance of a burn healing [8], and in cerebral ischemia, malperfusion of parts of the brain leads potentially to stroke [9]. The perfusion can be visualised as the integration of flow speed over the total cross sectional area of the feeding vasculature [10]. Perfusion and the amount of flow imaged are therefore closely

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related in some clinical applications, however, computing the perfusion from imaged vascularity is often challenging [11].

Photoacoustic imaging has the potential to do both: perfusion imaging and flow imaging. In this review we will only focus on the latter. A wide variety of methods investigated by different research groups, inspired by other imaging modalities, in addition to a few approaches that utilise unique aspects of photoacoustic imaging, make the review a worthwhile investigation. We will first briefly discuss existing imaging modalities that are capable of both perfusion imaging and flow imaging before describing the different photoacoustic approaches developed so far.

5.1.1 Current modalities

Magnetic resonance imaging (MRI) is a widely used modality for imaging perfusion and flow, using a range of methods [12,13]. For instance, imaging flow can be performed using phase contrast MRI [14], while perfusion imaging can be done with arterial spin labelling [15] or dynamic contrast enhanced imaging [16]. MRI can provide blood flow information of the whole body, with high sensitivity and resolution [17], which makes it unique in that aspect. Moreover, it can be combined with blood-oxygenation-level dependent (BOLD) MRI [18]. However, MRI is very expensive in both the initial investment and the upkeep, and the extensive pulsing schemes in MRI also make imaging a slow process [19].

Dynamic contrast enhanced computed tomography (CT) is a more affordable modality for perfusion imaging, unlike MRI. It can also be combined with positron emission tomography (PET) for quantification of e.g. glucose consumption for estimation of the complete metabolic activity [20]. Like MRI, CT is also capable of imaging complete organs or even the whole body, but with worse resolution and it relies on ionizing radiation, making it unpractical for monitoring [19].

Ultrasound (US) imaging is another widely used technique for both flow and perfusion imaging. Over several decades, the ultrasound community has developed many techniques. For instance, flow imaging can be performed using continuous wave excitation in spectral Doppler US [21], by imaging phase change of reflected US pulses in colour flow imaging [22] or by transverse speckle tracking [23]. Perfusion imaging is performed using dynamic contrast enhanced US, for example by using a flash-replenishment technique [24]. US imaging is very scalable through the inverse relationship of penetration depth and resolution, which can be tuned using the ultrasound emission frequency and bandwidth [25]. Furthermore, US imaging is both affordable and portable [26]. While imaging depth is larger than optical coherence tomography, the resolution is poorer and it suffers from clutter [27].

Several modalities are being developed to estimate flow and perfusion using visible or infrared light, which makes these techniques both harmless and affordable. For example, laser

Doppler perfusion imaging (LDPI) and laser speckle imaging (LSI) are two modalities based on tracking diffusely reflected light over time and can be used for perfusion imaging of the skin [28], with the advantages of portability, low cost and real-time imaging [29,30]. On the other hand, LDPI and LSI are limited to superficial imaging and low resolution [28] and do not feature depth resolution. While LDPI has the objective underlying principle of velocity related Doppler shifts, quantifying perfusion in an absolute manner remains challenging in both LDPI and LSI because of the unknown optical properties of the tissue [31]. Another optical technique, orthogonal polarization spectral imaging (OPS), can be used for high-resolution imaging of micro-vasculature [32]. Resolution is high and penetration depth is fair, but flow quantification is very challenging.

Optical coherence tomography (OCT) is an optical technique which can be used for flow imaging as a function of depth [33]. Flow can be quantified, for instance, by tracking the phase change of the reflected light over time, or by computing the speckle variance [34]. OCT has high resolution, is depth resolved and can be portable, but suffers from limited penetration depth and is hindered by clutter [35].

In this regard PAI is comparable to fluorescence microscopy approaches like confocal microscopy [36] or the two-photon variant [37]. In both microscopy techniques flow imaging is performed by laser scanning along the flow direction. However, confocal and two-photon require fluorescent markers that are susceptible to photobleaching; are only useable in superficial applications; and the laser scanning makes it only suitable for imaging a few blood vessels at a time [38]. As we will see, photoacoustics can be used to overcome these problems in flow imaging. Photoacoustic flow imaging, uses endogenous contrast, and allows approaches not limited to specific targeting of blood vessels.

5.1.2 The case for PA flow imaging

Photoacoustic imaging (PAI) is an optical modality that relies on light pulses to generate ultrasound at locations of high optical absorption [39]. Nanosecond light pulses are directed onto the skin, where they diffuse through tissue, down to several centimetres for near-infrared light. The light is locally absorbed by tissue chromophores, and is converted into heat, causing a pressure build-up. This build-up is released in the form of pressure waves: sound waves very similar to those emitted in pulse-echo ultrasound.

The main tissue chromophores in the visible and near infrared wavelengths (NIR) are haemoglobin and melanin (<1000 nm); at NIR wavelengths (>930 nm) lipids also exhibit absorption peaks that can be utilized [40]. PAI can therefore be used spectroscopically and, using spectral unmixing, the relative concentrations of chromophores can be extracted [41]. In this way, the oxygenation of blood vessels can be determined by exploiting the oxygenation-dependent absorption spectrum of haemoglobin [42,43].

Like ultrasound imaging, PAI is relatively low cost, can be used in portable devices [44], and the resolution can be increased relatively easily though at the cost of imaging depth. PAI is used in microscopy, tomography and linear array systems, with varying resolutions and imaging depths [45]. An important distinction in systems can be made, namely between acoustic and optical resolution setups. In optical resolution mode, laser light is focussed onto a sample; in acoustic resolution mode, generated sound waves are focussed in detection with a large numerical aperture – either physically or in computed reconstruction. The focal size determines the lateral resolution. The optical focus therefore gives a higher resolution, but at the cost of penetration depth: optical scattering will broaden the optical focus considerably. It should be noted that in both cases the axial resolution – along the ultrasound transducer axis – remains governed by the acoustic bandwidth of the transducer.

The promise of using photoacoustics for imaging flow is due to its reliance on absorption for contrast, as opposed to backscattered waves in US and OCT. Moreover, because of the relatively low concentration of haemoglobin and other chromophores in tissue, there is a high contrast in PAI between vasculature and the surrounding tissue [46]. In addition, PAI – unlike ultrasound – is relatively speckle free [47,48]. The lack of these two properties is the main reason it is challenging to detect flow in small blood vessels and near vessel walls with OCT and US flow imaging [49-51].

In the following review, we present a summary of the research performed on photoacoustic flow imaging. We describe the similarities between some flow imaging methods using PA with other modalities, and conclude with the key advantages and unique features of PA flow imaging. The review is partly written as a tutorial on the various flow imaging techniques, but it will also go into detail on performance characteristics like the minimum and maximum measureable velocities.

5.2 Photoacoustic flow imaging methods

5.2.1 Doppler shift

5.2.1.1 *Continuous-wave photoacoustic Doppler*

Fang et al. proposed a technique where the Doppler shift of a PA modulation was estimated [52], similar to what is done with spectral Doppler US. The authors used a diode laser that was intensity modulated with a frequency f_0 :

$$I(t) = \frac{I_0}{2} [1 + \cos(2\pi f_0 t)]$$

with t the time within one acquisition, I_0 and $I(t)$ the maximum and time-variant light intensity respectively. A modulated laser will generate a PA response that is also oscillating

Figure 5.1. In continuous-wave photoacoustic Doppler, sinusoidal modulation of the excitation light source causes a photoacoustic response, which is Doppler shifted if absorbing particles or cells are flowing.

with frequency f_0 (see Figure 5.1). If excited red blood cells (RBCs) are flowing at v_{flow} , then the particles will emit a PA response that is Doppler shifted approximated by:

$$\Delta f = f_0 \frac{v_{flow}}{v_s} \cos \theta$$

with θ the angle between the flow direction and the detector's viewing line, and v_s the speed of sound. They implemented the technique on an acoustic resolution setup with a ~ 2.5 MHz

focussed mono-element transducer and a lock-in amplifier for detection; the modulation was matched to this frequency. Fang et al. measured a distribution of Doppler shifts – not a single value – due to spectral broadening, caused by the opening angle of the ultrasonic transducer and the presence of a distribution of velocities in the tubing.

The authors demonstrated the technique on flowing carbon particles, which served as a model for RBCs. In their first paper they measured velocities of flowing carbon particles through tubing, which ranged from 0.055 to 8.8 mm/s. With their technique, the minimum measureable velocity was fundamentally limited by the frequency resolution ($=1/\text{acquisition time}$) of the system, whereas the maximum measureable velocity was limited by the system's SNR: for greater flow velocities the distribution of Doppler shifts broadened, and therefore the amplitude decreased.

In a second study they used a more realistic phantom where they included also scattering ($\mu'_s = 0.4/\text{cm}$, path length 3 cm) and increased the density of carbon particles from 15% to 40% (v/v) [53]. The maximum measureable velocity dropped to 1 mm/s due to the scattering, which reduced the SNR; but measurements seemed unaffected by the increased particle concentration. The authors indicated blood measurements were successful, but only in water, without any scattering.

Sheinfeld et al. followed up on this work and improved the axial resolution using short bursts of modulated light [54,55]. They used rectangular bursts with intensity

$$I(t) = \frac{I_0}{2} [1 + \cos(2\pi f_0 t)] \sum_{n=1}^N \text{rect}\left(\frac{t - nT_{\text{rep}}}{T_{\text{on}}}\right)$$

where T_{rep} and T_{on} are the repetition interval and cycle length respectively, of burst number n .

They used an acoustic resolution setup with a 1 MHz transducer to detect the Doppler shift from the PA responses of flowing carbon particles (in their case in a 10% (v/v) suspension). They applied a time gate to the time traces and estimated the Doppler shift in a 3 μs window for a range of positions. The size of time gate determined the axial resolution, which therefore was 4.5 mm. Their 1 s acquisition length was long enough to detect multiple bursts, so the time gate was repeated every $T_{\text{rep}} = 70 \mu\text{s}$ to increase the spectral resolution.

The authors measured velocities from 3.5 to 203 mm/s; in their case the maximum speed was limited by the syringe pump, the minimum – as before – by the spectral resolution.

For both Sheinfeld et al. and Fang et al., the lateral resolution was determined by the ultrasound transducer's beam width. Speculating on the maximum penetration depth; it is expected to be fairly limited. In fact, Fang et al., found imaging through scattering ($\mu'_s = 0.4/\text{cm}$ with a 3 cm path length) challenging in spite of using highly absorbing carbon particles and much lower optical scattering than in tissue ($\mu'_s \approx 8/\text{cm}$). For an overview of the technique's key attributes, see Table 4 at the end of the review article. In this table, the flow imaging techniques yet to be discussed will be listed also.

5.2.1.2 Structured-illumination photoacoustic Doppler

Zhang et al. implemented photoacoustic modulation in the spatial domain instead of time domain by creating illumination fringes with a nanosecond pulsed laser [56]. The modulation frequency corresponds to $f_0 = v_s/d$, with a fringe pitch of d ; and the Doppler shift of the PA modulation as before:

$$\Delta f = f_0 \frac{v_{\text{flow}}}{v_s} \cos \theta$$

Since their detection geometry was parallel to the modulation – as can be seen in Figure 5.2 – the Doppler angle was $\theta = 0^\circ$.

They estimated not only the Doppler shift, but also related quantities, namely the phase shift, the change in arrival time and time compression of the photoacoustic response. In their experiments they used an ink solution with flow speeds from 20 mm/s to 1400 mm/s. When

Figure 5.2. Using spatial laser modulation in structured-illumination photoacoustic Doppler causes a modulation of the PA response similarly to the time-domain version, and the modulation is likewise Doppler shifted under flow.

the authors added optical scattering ($\mu'_s = 6.2/\text{cm}$) they measured up to ~ 600 mm/s. Zhang et al. found the time compression to give the best accuracy compared to the Doppler shift (Root-mean-square error, RMSE of 57 mm/s instead of 89 mm/s), especially with optical scattering (RMSE=66 mm/s instead of 120 mm/s). Note that time compression is inversely related to bandwidth broadening: it seems likely that their flow setup shows a wide distribution of flow speeds, and therefore high amount of broadening.

The minimum and maximum measureable velocities were determined, as before, by the frequency resolution and the SNR respectively. The authors achieved a much higher SNR by using a pulsed laser – compared to CW modulation – which explains the high maximum measureable velocity.

Optical scattering decreases the modulation depth and therefore decreases the SNR of the Doppler shift. This makes spatial modulation more suited for flow estimation with superficial applications like photoacoustic microscopy. The lateral resolution would then be determined by the length of the fringe pattern; the resolution in depth would be very limited because the ultrasound transducer is used to determine flow component along the transducer time-axis.

Yao et al. presented a method using a microscopy setup with spatial light modulation to estimate the *transverse* flow component – which is perpendicular to the transducer's symmetry axis – instead of the axial component [57]. Modulation was performed using a laser fringe pattern:

$$I(x) \propto 1 + a \cos\left(\frac{2\pi x}{d}\right)$$

with a the modulation depth and x the transverse axis. They recorded $p(n T_{\text{rep}})$, the maximum of each photoacoustic response sampled at each excitation pulse n , also called the

Figure 5.3. In the transverse structured-illumination method, the PA response as the cells or particles move through the fringes gets modulated with a frequency based on their flow speed.

‘slow-time response’. It is termed such to distinguish it from the fast-time response that is the result of an individual laser pulse, of which Zhang et al computed the Doppler shift. Yao et al.’s fast-time response was a representation of the particle density distribution that flowed through the fringe pattern, and the amplitude of any photoacoustic response was proportional to the number of particles within one fringe. This density distribution ‘finger print’ is deterministic and changes slowly over time. As the particle distribution flowed from fringe to fringe, an amplitude modulation was created in the slow-time response (see Figure 5.3), which could be observed as side bands at $\pm 2\pi v_x/d$. They assumed the particles to have no correlation length and therefore a flat spatial frequency spectrum.

Yao et al. first demonstrated the method by flowing bovine blood through tubing and for various ($< 10 \mu\text{m}$) particle sizes with flow speeds ranging from 5 to 20 mm/s. The authors also showed the radial flow profile of the flow in the tubing. They also demonstrated their method in the superficial vasculature of a mouse ear, where they measured a flow speed of approximately 1.9 mm/s.

The theoretical minimum measureable speed in the system was 0.01 mm/s, limited by the total acquisition time of 1 s. Their maximum measureable speed was ~ 25 mm/s, limited in their case by the combination of fringe spacing and laser pulse repetition frequency (PRF), since the density distribution requires adequate sampling in time. The lateral resolution was limited by the size of the acoustic focus, about $71 \mu\text{m}$ and the axial resolution at $\sim 15 \mu\text{m}$ by the bandwidth of the ultrasound transducer.

Note that both spatial-modulation techniques only measure one lateral flow component at a time, and that no directionality is included; the latter might be possible by scanning the system back and forth. Also, a second scan could be made to determine the other lateral flow component.

Figure 5.4. In time-domain cross-correlation flow imaging, the cells' or particles' density distribution is interrogated with two consecutive laser pulses. The PA response of these two pulses is shifted in time due to flow.

5.2.2 Density tracking

5.2.2.1 Cross-correlation flow imaging in the time domain

Brunker and Beard used two pulsed lasers to excite particles with a small time delay in between, such that the time-of-flight could be extracted [58]. They use the fact that the density distribution of particles or RBCs can also be sampled along the transducer axis (the 'fast-time response'). If the transducer is aligned along the particle flow then the distribution will move toward or away from the transducer – depending on the flow direction (see Figure 5.4). This also holds when the transducer is aligned at an angle θ ($\neq 90^\circ$) to the flow: the particles within the measurement volume will shift along the detection-time axis. During the two laser pulses the density distribution shifted along the detection time by Δt in the fast-time response. This time shift could be estimated using a cross-correlation, and the axial velocity could then be estimated with:

$$v_{axial} = \frac{v_s \Delta t}{T_p \cos(\theta)}$$

with T_p the time between the two laser pulses. The method is similar to how ultrasonic colour flow imaging works (CFI, also termed 'Colour Doppler'), where the back scattering amplitude of ultrasound is linked to the particle density. In CFI the flow estimation is often performed with a two-dimensional autocorrelation, since the imaging is at a constant PRF (the reader is referred to [22] for more information).

Brunker and Beard demonstrated the method on a rotating Perspex disk with an acetate sheet imprinted with a random dotted pattern. The rotation of these dots would simulate the flow of red blood cells. The authors initially used two individually triggered 10 Hz pulsed lasers to

Figure 5.5. *In-vivo* example of flow mapping using a scanned photoacoustic microscope. Adapted with permission from [60]. Copyright (2012) by Sage Publications.

interrogate the random dotted pattern on the rotating disk. They measured velocities up to 1 m/s, with a systematic error of 1-3% and a random error of 0.02-0.05 m/s.

Brunker et al. later used a double-pulse laser, which provided 2 pulses per 10 Hz, and applied the method successfully to flowing particles and whole blood [59]. Here, they used time gating on the sampled density distribution to estimate the maximum velocity in the tubing. With whole blood the maximum measureable velocity was 20 mm/s and the random error ~ 5 mm/s.

The minimum measureable velocity was limited by the precision of the time shift estimation. While this estimation can be performed sub-sample – interpolating or fitting the cross-correlation with a Gaussian function – it is fundamentally limited by the SNR. In addition, the degree of correlation between two subsequent PA responses and the transducer bandwidth also affect the precision. The fundamental lowest possible random error in delay estimation is described in ultrasound colour flow imaging by the Cramer Rao lower bound [61]:

$$\sigma_{\Delta t} \geq \sqrt{\frac{3}{2f_0^3\pi^2T(B^3 + 12B)} \left(\frac{1}{\rho^2} \left(1 + \frac{1}{\text{SNR}^2} \right)^2 - 1 \right)}$$

with T the window length of the time gate, ρ the correlation coefficient of the two photoacoustic responses, f_0 the centre frequency and B the fractional bandwidth.

The maximum measureable velocity in their rotating disk experiments was limited by the size of the transducer focus and T_p , because the dots had to be in the detection area for both laser pulses. On blood, their measurements at higher flow velocities were limited by decorrelation of the density distribution between the two laser pulses. In theory also aliasing might play a role, but this was not observed with the rotating disk, and it is expected at higher velocities than the authors could measure with blood. The axial resolution was determined by the size of the time gate; the lateral resolution by the size of the transducer focus.

Figure 5.6. Principle behind spatial-domain cross-correlation flow imaging [62,63]. The density distribution is interrogated at two locations over with alternating laser pulses. The time-shift between the two slow-time responses is determined by the flow velocity.

Their setup would be limited in penetration depth to < 1 mm due to the use of 532 nm excitation (limited light penetration in tissue and blood), and the use of a 30 MHz transducer (high ultrasound attenuation in tissue). Nevertheless, there is no fundamental reason which prevents the technique from being used in a tomography or linear array system. This would also enable the estimation of the angle θ , which in its current form would require scanning the mono-element transducer.

Yao et al. implemented a similar method using an optical resolution photoacoustic microscope (OR-PAM) [60]. Instead of computing the cross-correlation of two consecutive photoacoustic time traces, they estimated the phase shift $\Delta\phi$ to determine the axial flow velocity:

$$v_{axial} = \frac{1}{2\pi} \frac{v_s \Delta\phi}{f_0}$$

They validated the technique on blood flow in tubing, where they could measure flow velocities from 0.1 mm/s to 8 mm/s. Their theoretical maximum measureable velocity was 20 mm/s, limited by the width of their optical focus.

They combined the axial velocity estimation with a transverse method based on bandwidth broadening (see section 5.2.3.2). In this way they could estimate the total flow velocity and determine the Doppler angle; then scanning the optical focus allowed them to perform flow mapping as shown in Figure 5.5.

Song et al. applied this method with a laser scanning photoacoustic microscope (LS-PAM) [64]. In LS-PAM, an unfocussed ultrasound transducer is kept static, while the optical focus is scanned within the field of view of the transducer. From a scan line along tubing they used the depth information to estimate the flow angle θ .

5.2.2.2 Cross-correlation flow imaging in the spatial domain

Liang et al. implemented cross-correlation flow imaging in the spatial-domain by projecting an image of a digital micro-mirror device (DMD) onto a sample using an objective [62]. The DMD was programmed to provide photoacoustic excitation at two locations, a certain distance d apart. By switching the DMD pattern, the excitation locations could be alternated, thereby sampling the density distributions at either location separately (see Figure 5.6). The time-shift ΔT between the two slow-time responses over successive laser pulses could be used to estimate the transverse flow velocity:

$$v_{\text{trans}} = \frac{d}{\Delta T \sin(\theta)}$$

They demonstrated the technique using various particle sizes (5, 10, 15 μm) and they showed the flow measuring performance is fairly independent of these particle sizes. Measureable flow velocities were from 1.13 to 13.20 mm/s when 300 μm of chicken breast was placed on top of the tubing. The systematic error in their latter experiments was ~ 0.29 mm/s and their random error ~ 0.19 mm/s. The same group demonstrated the technique with blood-flow in a different publication [63]. They showed measureable velocities of bovine blood flowing through tubing from 0.45 mm/s to 18 mm/s. Several examples of flow estimation *in-vivo* with mouse ear vasculature were also shown.

In theory the maximum measureable velocity was limited through $|v_{\text{max}}| = d/T_{\text{rep}}$, but this limit was not reached. It seems likely that their measurements were limited by decorrelation of the density distribution, comparable to what Brunker et al. observed. As was the case with other methods, the minimum measureable velocity was limited by the accuracy of the errors in estimating the time-shift ΔT . Implemented as a ‘point’ evaluation, the measurements did not take long (100 ms), but when scanned in 2D the technique would perhaps be on the slow side.

The axial resolution was limited as before by the transducer bandwidth; the lateral resolution along the flow direction was determined by the distance between the excitation spots; the lateral resolution across the flow direction was determined by the spot size.

Wang et al. developed a related technique [65]. They used PA microscopy with repeating line scanning along a vessel in a mouse ear to image individual RBCs in the vessel. By repeatedly scanning the vessel they could image the movement of these cells over time and could use this to quantify the flow velocity. They combined the flow velocity with the oxygenation to model the rate of oxygen release. The technique was also used by Yao et al. to determine the effect of sensory input on blood flow in mouse brains [68].

Figure 5.7. Transit-time flow imaging principle for single particles [66,67]: The time during which a cell or a particle is visible is determined by the speed at which it transits the optical focus.

5.2.3 Transit time

5.2.3.1 Transit-time flow imaging of single-particles

Fang and Wang used a PAM, but in optical resolution mode to estimate the transit time of particles flowing through the optical focus [66]. This transit time, when combined with the size of the focus provided an estimation for the lateral flow velocity as can be seen in Figure 5.7. Since the slow-time response as the particle transits the focus was a convolution of the optical intensity profile and the particle size, it needed compensation for the size of the particles. They approximated the response by a Gaussian function with a waist equal to the sum of the focal and particle sizes, and fitted the measured data using this approximation.

They showed a parabolic flow profile of particles flowing in tubing, and measured speeds ranging from ~ 0.35 mm/s to ~ 1 mm/s. Note that only the magnitude of the flow could be determined, not its directionality in the lateral plane. The minimum and maximum measureable flow speeds were not investigated by the authors, but the maximum was likely determined by the optical focus size and the PRF: the particle must be in the focus during at least 3 laser pulses. The minimum was likely limited by the length of the total acquisition. The axial resolution was, as before, mainly determined by the transducer bandwidth, whereas the lateral resolution was determined simply by the optical focus.

Sarimallaoglu et al. applied the technique to estimate the flow speed of melanoma cells injected into mice [67]. They also used it *in-vitro* to determine whether gold nanorods (GNRs) were bound to breast cancer cells. They noticed a slower speed when GNRs were bound, arguing that unbound GNRs would flow in the centre of the tubing and these heavier cells would not. Besides investigating this transit time technique, Sarimallaoglu et al. also used

Figure 5.8. Transit-time principle for particle ensembles [69-72]. As cells transit the focus with a greater speed, features of the density distribution will show up with sharper peaks.

a method similar to those in section 5.2.2.2, instead using three beams to record the time it takes melanoma cells to flow between these beams.

The method employed by both authors is limited to the detection of single particles or single cells: in an application on whole blood it is likely difficult to distinguish individual RBCs. Nevertheless, it might find an application in the detection of single contrast particles or circulating tumour cells in whole blood.

5.2.3.2 *Transit-time flow imaging of particle ensembles*

Yao & Wang and Chen et al. proposed a solution to make the single-particle method more suitable for use on whole blood [69-72]. What holds for single particles also holds for a density distribution of particles moving through the focus. The faster the distribution moves through the optical focus, the sharper its features (the ‘peaks’) become (see Figure 5.8). While it is ineffective to fit every single peak with a Gaussian function, it is relatively easy to compute either the autocorrelation or the bandwidth of the total photoacoustic acquisition: both determine the average peak width. For faster flow the autocorrelation coefficient becomes smaller, whereas the bandwidth increases, since the bandwidth is a Fourier transform pair with the autocorrelation function.

Starting with Yao and Wang, they used the slow-time bandwidth broadening for transverse flow velocity imaging [72]. As particles flow quicker through the focus, the transit time becomes shorter and the bandwidth, which is its inverse, broadens by:

$$B_d \approx f_0 \frac{v_{\text{trans}}}{v_s} \frac{w}{R} \sin(\theta)$$

where w is the optical focus width, R the focal length and f_0 the centre frequency of the US transducer. The authors found their inspiration for this technique in OCT [73]. Yao and

Figure 5.9. *In-vivo* example of flow mapping of the speed (a) and where the directionality is included (b). Adapted with permission from [71]. Copyright (2010) by OSA Publishing.

Wang applied the technique using an optical resolution PAM. They scanned the PAM back and forth, causing a change in broadening: an increase when moving against the flow and vice-versa. They used this change to determine the flow velocity via $v_{\text{trans}} = \sqrt{v_{t+}^2 + v_{t-}^2 - 2v_m^2}$, where v_{t+} and v_{t-} are speeds measured in both directions while scanning, and v_m is the motor speed. This scanning approach can be viewed as a combination of laser scanning confocal [36] and OCT [73].

They demonstrated the technique using particles ($\varnothing$ 6 μm) flowing through tubing, with measureable flow velocities from ~ 0.1 mm/s up to 2.5 mm/s. They also mapped the parabolic flow profile in the tubing. Yao et al., in a different article, subsequently applied the technique on blood-flow *in-vitro* and *in-vivo* [71]. *In-vitro*, they showed measureable flow velocities up to 5 mm/s, and used this to calibrate their *in-vivo* measurements. They finally applied their method to blood flow on vasculature in a chicken embryo (see Figure 5.9), and were able to measure the flow-speed variations caused by the heart beating.

The theoretical maximum measureable flow velocity of this technique was limited by the optical focus and the PRF of the laser illumination, at about 7.4 mm/s. The minimum, indicated to be about 0.1 mm/s, was limited by SNR, and presumably acquisition time.

Chen et al. used the autocorrelation $G(\tau) = \langle \delta P(t) \delta P(t + \tau) \rangle / \langle P(t) \rangle^2$ to estimate the flow speed [69]. The autocorrelation function, when assuming a Gaussian optical focus, could be approximated by:

$$\frac{G(\tau)}{G(0)} = \exp\left(-\left(\frac{\tau}{\tau_0}\right)^2\right)$$

with $\tau_0 = w/v_{\text{trans}}$. Instead of scanning the system, they limited themselves to estimating flow at a few locations, and therefore were not able to determine the flow direction. They demonstrated the method in their first article [69] using particles flowing at speeds from 14 $\mu\text{m/s}$ to 200 $\mu\text{m/s}$. In their second article [70] they applied the method on chicken embryo

vasculature, measuring flow speeds at individual locations 56-77 $\mu\text{m/s}$ – based on an *in-vitro* calibration using particles.

Ning et al. used the autocorrelation approach to investigate blood flow in the ear of a nude mouse [74]. Using the diameter from the PA image, they were able to determine the volumetric flow rate of the vasculature, and the inflow and outflow at bifurcations. They calibrated their flow estimation and verified the computation of the volumetric flow rate both *in-vitro*.

Although not investigated by the authors of either approach, universal quantification of flow speed or velocity will remain difficult. The reason: quantification relies on knowledge of the optical focus and presumably also cell sizes. Although both can be either measured independently or otherwise calibrated for, the optical focus may increase through scattering, and RBCs can form clusters at small flow speeds.

Tay et al. proposed a technique to reduce the effect that optical scattering has on the optical focus [75]. They implemented wavefront shaping using the photoacoustic amplitude as a feedback mechanism. Wavefront shaping can be used to compensate for scattering by iteratively guessing the very complex phase-and-amplitude transformations that the input beam experiences. The ‘goodness’ of the guesswork was observed here using the photoacoustic amplitude, which therefore served as the feedback. This resulted in a bright optical spot at the location of the ultrasound focus. Tay et al. implemented the iterative process with a DMD projecting a series of Hadamard patterns on the sample. The optimal pattern could be found by multiplying the photoacoustic response of the m -th Hadamard pattern (vector I_m) by the inverse Hadamard matrix.

to quantify the flow velocity. They combined the flow velocity with the oxygenation to model In this way, Tay et al. could measure the flow speed of particles behind a ground diffusor from ~ 0.4 mm/s up to ~ 2 mm/s. The random error of their measurements was – at ~ 0.5 mm/s – quite high, which they attributed to particle size variations. The axial and lateral resolutions were governed here by the ultrasound transducer.

The optimization of the DMD pattern took 2 hours, making their setup unsuitable for tissue imaging as the authors note. To speed up the process, a faster DMD and laser would be required to beat the tissue dynamics, which are on a millisecond-timescale. A current limitation to their method is the inability to use wavefront shaping to focus on a spot *inside* and not *behind* a scattering medium. In theory the autocorrelation/bandwidth broadening technique likely also works based on just the acoustic focus, assuming the width and numerical aperture of the US transducer provide sufficient SNR without the optical focus.

Figure 5.10. In the photoconversion and replenishment technique with constant energy [76], at every laser pulse, a fraction of the gold nanorods is converted into spheres. After every laser pulse, the rods replenish partly, until an equilibrium forms.

5.2.4 Amplitude encoding

5.2.4.1 Photoconversion and replenishment

Li et al. estimated flow using a shape transition of gold nanorods [76]. Exciting the rods at their peak-absorption wavelength converts them to nanospheres, which have a different peak-absorption wavelength. Therefore, pulsing light on a volume of nanorods effectively photo-destroys the rods. After a light pulse, new nanorods will flow into the volume; a recovery that is related to the flow speed (see Figure 5.10). Over time, repeated pulsing will bring the photoacoustic amplitude $P(k)$ down to a constant level P_{const} .

The drop in amplitude could be approximated by:

$$P(k) = P_{const} + (1 + P_{const}) r^k \exp\left(-u \frac{k}{PRF}\right),$$

$$P_{const} = \frac{1 - \exp(-u/PRF)}{1 - r \exp(-u/PRF)}$$

with k the k -th laser pulse, u a parameter proportional to the flow speed, and r a constant related to the laser power.

They estimated the flow speed of a suspension of gold nanorods flowing through tubing. They measured speeds from 0.35 mm/s up to 2.83 mm/s with a systematic error of ~ 0.2 mm/s and random error of $\sim 30\%$ the measured speed.

Wei et al. proposed an alternate method [77], using one initial high-energy laser pulse to convert a large fraction of the nanorods, and subsequently using lower-energy laser pulses to

Figure 5.11. Flow measurement based on photoconversion using a flash/replenishment approach [77]. A single intense laser pulse converts most of the nanorods, after which low intensity pulses are used to observe the rods' replenishment.

track the in-flow of new nanorods, as can also be seen in Figure 5.11. The recovery of the photoacoustic amplitude could then be approximated by:

$$P(t) = 1 - c \exp(-\lambda v_{\text{flow}} t),$$

with c and λ constants. This method is very similar to the ultrasound contrast flash-replenishment type methods, where a high-intensity burst of ultrasound is used to destruct micro-bubbles.

The authors measured flow speeds of a GNR solution from 0.35 to 2.83 mm/s as before. They compared the photoacoustic flash-replenishment method with the constant-energy method from Li et al., but found the constant-energy method to provide the better performance. A reason for this might be, the authors write, their limited measurement time that made the measurements inaccurate.

Liao et al. implemented the constant-energy method with a linear array, providing B-mode flow imaging [78]. They also tested the performance under 5 mm of chicken breast, and could measure flow speeds from 0.125 to 2 mm/s with an average error of 30%. However, they had to recalibrate their measurements because the beam width had changed due to optical scattering. The axial and lateral resolutions were determined by the ROI selection in the B-mode image of the tubing, 0.6 mm and 1.2 mm respectively. The theoretical lower limits of these resolutions were determined by the point spread function of the system. The time resolution was determined by the total measurement time, 5-20 s depending on the settings.

Although not investigated by the authors, the maximum measureable speed – when there is only a relatively small drop in PA amplitude – was likely determined by the laser fluence at the GNRs and the measurement time. A larger fluence would cause a larger drop in amplitude and a longer measurement time would provide a more accurate determination of P_{const} . The

Figure 5.12. Flow measurement using photothermal modulation [79]. A low-frequency heating laser modulates the Grueneisen coefficient, which can be observed using a modulated PA laser.

minimum measureable speed likely depended on whether slow flow could be distinguished from no flow, which would be mainly determined by the SNR. Note that the authors did not mix the GNRs with blood, which might deteriorate the contrast further.

5.2.4.2 Heat encoding and convection

Sheinfeld and Eyal proposed the use of photothermal modulation for amplitude encoding [79]. They used two sine modulated laser diodes of different frequency to create an amplitude modulated photoacoustic signal: the heating of the medium increases its Grueneisen parameter and causes the photoacoustic response to increase (see Figure 5.12).

Modulating the heating process caused an amplitude modulation of the photoacoustic signal, of which the modulation frequency response was given by:

$$P(\omega_{PT}) \propto \frac{t_{\text{eff}}}{1 + j\omega_{PT}t_{\text{eff}}}$$

with $\omega_{PT} = 2\pi f_{PT}$ the photothermal modulation frequency and $t_{\text{eff}} = (t_{\text{conduction}}^{-1} + t_{\text{convection}}^{-1})^{-1}$ a combination of time constants of conduction and flow-dependent convection. Sheinfeld and Eyal used a constant 0.5 MHz photoacoustic modulation and varied the PT modulation ω_{PT} from 0.1 Hz to 20 Hz. They fitted the observed $P(\omega_{PT})$ with the above function to determine t_{eff} . The authors obtained the contribution of conduction to t_{eff} by measuring the response for zero flow, but found the contribution to be relatively small. They calibrated the relationship between t_{eff} and the flow speed.

The authors applied the PT modulation on sheep blood flowing through tubing, heating the blood less than 2 °C. They measured flows in a range of 1 to 21 mm/s. Their maximum error was 0.3 mm/s, which, when ignoring the contribution of conduction increased slightly to

Figure 5.13. Flow measurement based on convection of an US-generated temperature increase [81]. High-frequency focussed ultrasound is emitted in bursts to heat a part of the flowing medium. The flow of this spot can be tracked using a photoacoustic tomography system.

0.75 mm/s. Although the authors did not investigate the speed limits, the minimum measureable speed was likely determined simply by whether flow and no flow can be distinguished, which might be dependent on whether the conduction can be modelled accurately. The maximum measureable speed was probably linked to the maximum modulation frequency, since for high flow speeds the modulation response changes only at higher frequencies. The axial resolution was likely very limited, whereas the lateral resolution was probably determined by the size of the heating beam. As with photoconversion, deep flow measurements with this technique are likely very challenging because optical scattering changes the size of the heating beam.

Wang et al. proposed to use ultrasound to heat the flowing medium [80] to solve the optical scattering issues with the above methods. They heated during a period of 300 ms, causing a 4 to 7 °C temperature increase, and measured the decrease in photoacoustic amplitude as the heated section travelled out of the ultrasound detection area. They found this decrease could be approximated by:

$$P(t) = c_1 + c_2 e^{-\lambda t}$$

with c_1 and c_2 constants and $\lambda = \lambda(v_{flow})$ a flow-dependent decay constant. They implemented the method on an AR-PAM system to quantify bovine blood flowing through tubing under 1.5 mm chicken breast. After calibration they could measure flow speeds from 2.97 mm/s up to 41 mm/s.

Wang et al., in a separate paper, showed ultrasound-heating-based PA flowmetry using a tomography setup [81]. Instead of monitoring at a single point the photoacoustic response after heating, they used the tomography setup to reconstruct a 2D image of the tubing, in which they could track the movement of the heated spot (see Figure 5.13). The setup was based on a HIFU transducer, which heated the medium with a 7.5 MHz waveform, which

was modulated with a 0.0625 Hz sine to generate the heating in cycles. The amount heated was approximately 1 °C. The velocity could then be calculated from the rate at which the heating travels along the vessel at a certain radial position. This technique has some parallels with arterial spin labelling from MRI [15], although the spin labelling is used for perfusion imaging, and not for estimation of flow velocities because it allows longer labelling times but not a very rapid imaging rate.

Wang et al. measured blood flow in tubing – without scattering – with velocities from 0.24 mm/s to 11.8 mm/s and a RMSE of 2.7%. They also showed a measurement under a 5 mm thick chicken breast layer with a single flow velocity, which they measured at 1.6 ± 0.2 mm/s. To achieve this they averaged over additional heating cycles (35 instead of 7).

Wang et al.'s minimum measureable velocity was determined by whether the flow was fast enough to ensure that the heated region was not yet smoothed by thermal conduction, and that the heated part would not be visible above the noise level. Their maximum measureable flow velocity was determined by the size of the field of view and the 2D imaging rate, since the heated spot needs to be within the field of view in at least a few images.

An advantage of this technique is its capability of imaging blood flow at diffuse optical depths, which is made possible due to the use of ultrasound instead of light for heating. Therefore, the maximum imaging depth will likely depend only on the SNR of the base photoacoustic response and on the acoustic attenuation by tissue of the HIFU cycles. The resolution in this case was likely optimal along the transducer axis and across the flow profile, however, along the flow direction it was limited by the distance the heated region was required to move. Zhang et al. also applied this technique, but with optical CW heating instead of HIFU [82]. They implemented the technique on an OR-PAM system and scanned the microscope for 2D imaging instead of relying on a tomography system. Their analysis is identical to that of Wang et al. [81]. They could measure comparable *in-vitro* velocities (0.23 to 11 mm/s with a RMSE of 2%). Finally, they also successfully applied their technique to blood flow in a mouse ear.

5.2.5 Discussion and conclusion

In this review we investigated all the approaches that have been developed for photoacoustic blood flow imaging. Table 4 gives an overview of the key elements of each method. Our overview shows that photoacoustic flow imaging can overcome limitations of other modalities such as ultrasound and optical techniques. Photoacoustics allows a direct translation of flow imaging techniques from ultrasound pulse-echo, like the time-domain cross-correlation, or bandwidth broadening from OCT. There are also some similarities between heat encoding in PA and arterial spin labelling in MRI. But some approaches are unique for photoacoustics, like the spatial-domain Doppler flow imaging. All in all, a wide range of flow imaging methods is available, of which many warrant further investigation.

As described in the above review, not all methods are equally advanced in its development, and many have been applied only *in-vitro* with moderate results. Methods in the most advanced stage of development are the implementations using PA microscopy, having often been demonstrated *in-vivo* such as in a mouse ear or a chicken embryo. PAM allows high-frequency ultrasound detection and focussed optical excitation for an optimal SNR and interrogation of the densely packed red blood cells in blood – though at the cost of penetration depth.

Acoustic resolution setups also show promise, and a number of techniques, such as the tomography implemented heat encoding, have been shown to work under optical-scattering conditions. Signal tracking methods, such as the time-domain cross-correlation, in theory are also promising for tomography setups, as they are based only on the use of a pulsed laser. The methods do require some optimization of detection frequency and bandwidth however, for it seems that the same principle that makes PAI speckle-free at ~5 MHz, will also smooth density variations in red blood cells. On the other hand, at higher frequencies it appears PAI might surpass ultrasound in the visibility of RBCs [47,48].

Up to now, PA flow imaging seems not yet applied clinically or pre-clinically. Although it will not likely compete with modalities like MRI or CT, it might find applications in areas where US, two-photon microscopy or OCT flow imaging are active. In the future, photoacoustic tomography and microscopy may provide flow imaging capabilities – exploiting its high contrast to haemoglobin – to applications ranging from rheumatoid arthritis diagnosis, to burn-wound assessment.

Table 4. Summary of the attributes of photoacoustic flow imaging techniques.						
	Method	Calibration required?	Meas. quantity	Flow axis	Resolution limit	
					Lateral	Axial
Doppler shift						
Continuous-wave (Sec. 2.1.1)	Fourier transform	No	Velocity spectrum	Axial	Acoustic*	#Cycles
Structured-illumination (Sec. 2.1.2)		No		Either	#Fringes	Acoustic
Density tracking						
Time-domain (Sec. 2.2.1)	Cross correlation	No	Velocity	Axial	Acoustic or optical	Acoustic
Spatial domain (Sec. 2.2.2)		No		Lateral	Distance between foci	Acoustic
Transit time						
Single-particles (Sec. 2.3.1)	FWHM	Yes	Speed	Lateral	Optical*	Acoustic
Particle ensembles (Sec. 2.3.2)	Autocorrelation or bandwidth	Yes	Velocity	Lateral	Optical*	Acoustic
Amplitude encoding						
Contrast based (Sec. 2.4.1)	Contrast inflow	Yes	Speed	Any	Acoustic*	Acoustic
Heat encoding (Sec. 2.4.2)	Heated spot	No	Velocity	Any	Heating spot	Acoustic

* indicates alternative resolution (acoustic/optical) might be usable, but has not been demonstrated yet.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter 6. Pulsed photoacoustic flow imaging with a handheld system

Abstract

Flow imaging is an important technique in a range of disease areas, but estimating low flow speeds, especially near the wall of blood vessels remains challenging. Pulsed photoacoustic flow imaging can be an alternative since there is little signal contamination from background tissue with photoacoustic imaging. We propose flow imaging using a clinical photoacoustic system, that is both handheld and portable. The system integrates a linear array with 7.5 MHz central frequency in combination with a high-repetition-rate diode laser to allow high-speed photoacoustic imaging – ideal for this application. This work shows the flow imaging performance of the system *in vitro*, using micro-particles. Both 2D flow images and quantitative flow velocities from 12 mm/s to 75 mm/s were obtained. In a transparent bulk medium, flow estimation showed standard errors of ~7% the estimated speed; in the presence of tissue-realistic optical scattering the error increased to 40% due to limited signal to noise ratio. In the future, photoacoustic flow imaging can potentially be performed *in vivo* using fluorophore-filled vesicles, or with an improved setup on whole blood.⁵

6.1 Introduction

Flow imaging is a broad and immensely varied field of research. For instance ultrasound (US) flow imaging is a widely used modality, and can be used for relatively low cost *in-vivo* imaging of arterial functioning [1] and of microvasculature [2,3]. Although successfully used in many applications, US flow imaging suffers from poor performance at low flow speeds, especially near vessel walls and in small vasculature [4,5]. The relatively weak ultrasound backscatter of red blood cells (RBCs) in these scenarios is hard to distinguish from the overwhelming tissue backscatter, making flow estimation challenging.

Magnetic resonance imaging offers flow imaging using phase contrast, and is under investigation for cardiovascular, cerebral and hepatic flow imaging [6]. While MRI phase contrast offers a high penetration depth it remains expensive and time consuming. Purely optical flow imaging methods such as orthogonal polarization spectral imaging and optical coherence tomography can provide more affordable flow imaging [7,8]. These techniques allow flow imaging of micro-vasculature for ophthalmology and dermatology. Although they benefit from high resolution (1-10 μm), allowing imaging small capillaries, penetration depth is very limited (1-2 mm).

Photoacoustic (PA) imaging is a more recent imaging modality, and relies on tissue chromophores such as haemoglobin for providing image contrast. In this technique, pressure

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Figure 6.1. Flow imaging setup. **(a)** Photograph of the clinical photoacoustic probe that houses a diode laser on top and ultrasonic detection below. **(b)** Schematic of the alignment to tubing, where θ is the flow angle. The probe is aligned to have the imaging plane cross the laser illumination. The tubing is looped such that it crosses the imaging plane thrice.

waves are generated by pulsed or modulated light. This light is absorbed by endogenous or exogenous chromophores, after which a small pressure builds up, that is then ‘released’ as sound waves [9]. The high optical absorption of red blood cells, due to haemoglobin, compared to surroundings makes PA imaging ideal for imaging vascularity, and can even be used to extract the oxygenation level. Due to their optical absorption, RBCs act as individual PA sources and can consequently be tracked over time, and PA imaging can therefore be used for the estimation of blood flow. The advantage of using PA over US for flow imaging is due to low signal amplitudes from surrounding tissues: there is little to no clutter that can affect the flow estimation.

Several groups have published work on the use of photoacoustics for the estimation of blood flow, see [11] for a recent review. Fang et al. used the Doppler shift of CW-modulated photoacoustics to extract velocity information from particle flow [10,11]. In a similar approach, Sheinfeld et al. used tone-burst excitation – making a trade-off between spectral and spatial resolution – on particle flow [12]. Other techniques include heat-based PA flow estimation, which has been used with blood flow *in vitro* [13-16]; as well as bandwidth broadening with photoacoustic microscopy *in vivo* [17-19].

A different method, pulsed photoacoustic flow imaging (pulsed PFI), has a potential advantage over the alternatives in terms of spatial and temporal resolution. Pulsed PFI, like its ultrasound counterpart, aims to follow the motion of red blood cells (RBCs) over time by tracking an acoustic fingerprint [20]. Previous work on pulsed photoacoustic flowmetry was performed with high-frequency detection, such as by Brunner and Beard to estimate particle flow [20] and later also for estimation of blood flow velocities [21] *in vitro*. The technique has been used in PA microscopy, both *in vitro* [22] and *in vivo* [23]. In addition to using high-frequency detectors, these microscopy studies rely on focussed optical excitation. Both optical

focussing and high-frequency detection serve to maximize the detectability of the finger print signal, however, they also limit the penetration depth, and optical focussing also affects the scanning time of the technique. Both limit clinical applications.

High-frequency detection is used for detecting the photoacoustic fingerprint of RBCs, since in photoacoustic imaging, small absorbers like RBCs – or density variations on a small length scale – generate very high frequency signals [24,25]. This limits the detected fingerprint pressure amplitude even with typical high-frequency transducers used in microscopy. Additionally, it has been shown that speckle in PA imaging is best visible at high frequencies; at low ultrasonic frequencies speckle is overshadowed by a ‘constructive’ boundary pressure wave [26], for instance in a blood vessel. The latter is one of the reasons PA tomography is devoid of speckle; the fingerprint signal can be regarded as a form of speckle.

Nevertheless, the photoacoustic frequency response from small absorbing features is also very wide-band, which means the requirement for high-frequency detection is not absolute. In this work we investigate the feasibility of low-frequency detection for pulsed PFI. To this end we use a portable and handheld clinical photoacoustic system, which integrates a rapid pulsing laser diode and low frequency array transducer allowing to move towards clinical imaging. As such, we approached PFI from the low-frequency detection side, instead lowering the density of absorbers, and increasing their size to improve the speckle visibility. We demonstrate the technique using a blood-mimicking phantom using sub-resolution polyethylene micro-particles, and show the effects of optical scattering on the performance of the flow estimation. We will conclude with a discussion on the steps required for estimating blood flow using the photoacoustic system *in-vivo*.

6.2 Materials and methods

6.2.1 Clinical photoacoustic system

The handheld clinical photoacoustic system (Figure 6.1a) is an integrated photoacoustic/ultrasound (PA/US) probe developed as part of an European Union-funded project named FULLPHASE. Based on a design from ref [27], the PA/US probe integrates both a 7.5 MHz linear array and a 805 nm pulsed laser module within the housing of a hand held probe. The linear array is adapted from an ESAOTE SL3323 echography probe and is used for the photoacoustic detection and ultrasound pulse-echo. This array consists of 128 elements with a 7.5 MHz center frequency and a 100% bandwidth.

Data acquisition is performed by a MylabOne clinical ultrasound scanner (ESAOTE Europe, Maastricht) at 50 MHz sampling frequency, which transfers pre-beamformed data to a laptop. The unprocessed data from the ultrasound scanner is then band-pass filtered, and reconstructed offline using a Fourier domain algorithm [28]. The axial resolution of the system is ~0.3 mm, the lateral resolution ~0.5 mm [27]. The elevational width ranges from

~3 mm, close to the probe, to 1 mm wide at the acoustic focus, at an axial distance of ~24 mm from the acoustic lens. The relevant elevational thickness for the experiments is 3 mm wide, 5 mm away from the acoustic lens. Laser pulses of 1 mJ and 130 ns are generated by a stack of diode lasers (Osram Opto Semiconductors GmbH, Regensburg, Germany; Quantel, Paris, France). There are four bars stacked vertically, each bar with 64 individual emitters. The diodes are powered by a custom laser driver (Brightloop, Paris, France), that can be triggered by the MylabOne. This one laser driver, if triggered, fires all bars simultaneously. The raw beam from the stack is, after collimation, beam-shaped using a diffractive optical element (SILIOS Technologies, Peynier-Rousset, France). A prism is used to change the angle of the beam at the front-end of the probe; from which a rectangular beam of 2.2 mm by 17.6 mm ($1/e^2$) exits the device under an angle of 51 degrees.

6.2.2 Flow phantom

To validate the setup and the flow estimation, black polyethylene micro-particles were used (BKPMS, Cospheric). At 53-63 μm in diameter, these particles were smaller than the probe's resolution, but larger and more absorbing than red blood cells – which are about 10 μm in their longest dimension – ensuring good detection of individual particles while still demonstrating the principle. Likewise the concentration was kept to 4% v/v, lower than the 45% v/v of whole blood. The particles were suspended in a 50% v/v glycerol solution to prevent the particles from sedimenting.

Polyethylene tubing, with 0.58 mm and 0.96 mm inner and outer diameters respectively, was used to model a small artery or vein. The particle suspension was pumped through the tubing using a syringe pump. The expected average flow velocity [mm/s] was computed from the flow rate [mL/min] set on the syringe pump. The measurements were started 2 minutes after initiating the flow, as the particle suspension was viscous it required some time before the flow was fully developed.

The PA probe was placed at an angle θ to the particle flow (see Figure 6.1b). An angle of approximately 50° was used – reducing the internal reflections of PA sound waves within the tubing while still obtaining an as long as possible shift in distance for a certain pulse repetition frequency. The exact flow angle θ was measured by moving the probe a distance x_h horizontally using a translation stage. The tubing was then observed moving a distance x_t away from the transducer, from which the flow angle was estimated as $\theta = \sin^{-1}(x_t/x_h)$. Note that the flow direction determines the sign of the flow velocity: positive and negative for flow towards and away from the transducer respectively. The probe was positioned 5 mm away such that the illumination from the prism crosses the ultrasound detection plane. The polyethylene tubing was looped such that it is visible three times as it crosses the detection plane (see Figure 6.1c). Initially, the tubing was placed in a tank filled with water.

Figure 6.2. Principle of flow estimation. **(a)** Side view geometry of estimation principle. Because flow is angled to the transducer axis, any flow will cause a change in arrival time of PA responses from cells or particles. **(b)** 1 dimensional PA measurements of particles for successive laser pulses. **(c)** 2D reconstruction of micro-particles in tubing, showing the geometry for flow estimation. Flow estimation is performed on axial lines w within the reconstruction using a sliding window at positions τ . See online for a time-lapse movie (PRF: 1 kHz, frame rate: 50 Hz, MPEG-4, 6 MB). **(d)** Mean interpolated cross-correlation for the window indicated in **(c)**, showing a shift in the peak location due to the particle flow.

Later, Intralipid (20% stock) was added to the tank in order to mimic tissue optical scattering ($\mu'_s = 5.2/\text{cm}$) and as such improve the realism of the phantom.

6.2.3 Flow estimation

The tracking of the photoacoustic fingerprint can be performed along the detection time axis by placing the transducer off-normal to a blood vessel of interest. This way, the particles appear to be flowing toward or away from the transducer, therefore the axial velocity component can be determined by estimating the change in arrival time as shown in Figure 6.2(a). A 1D example of a fingerprint signal for the micro-particles is shown in Figure 6.2(b), where the amplitude is encoded in grayscale along depth. The movement of the particles is visualized by the shift in depth of the fingerprint for subsequent laser pulses.

A complete cross-correlation [29] was used to estimate flow from axial lines w for each lateral position in the photoacoustic image (see Figure 6.2c). Cross-correlations were performed in

a window within w , where for each window position τ the cross-correlation function $R_{k,\tau}$ was computed between lines w_k and w_{k+1} from subsequent pulses k and $k + 1$:

$$R_{k,\tau}(m) = \begin{cases} \frac{1}{L - |m|} \sum_{n=0}^{L-m-1} w_k(\tau + n + m) w_{k+1}^*(\tau + n) & m \geq 0 \\ R_k^*(-m) & m < 0 \end{cases}, \quad (1)$$

where L is the window length, $*$ denotes the complex conjugate and m is the time-shift index of the cross-correlation. These cross-correlations were averaged:

$$R_{av,\tau}(m) = \frac{1}{K} \sum_{k=1}^K R_{k,\tau}(m), \quad (2)$$

the result of which was spline interpolated at 5 GHz sampling to increase the accuracy of the peak location estimate $\Delta t_{est}(\tau)$ as shown in Figure 6.2(d). The axial flow speed then is $v_{axial}(\tau) = \text{PRF} \Delta t_{est}(\tau) v_{sos}$, where PRF is the pulse repetition frequency of the laser, $\Delta t_{est}(\tau)$ the estimated peak location of the cross-correlation for each position of the window and v_{sos} the speed of sound of the bulk medium. The full flow velocity can be calculated using $v_{flow}(\tau) = v_{axial}(\tau) / \cos \theta$.

PA responses of 500 light pulses were collected with a pulse repetition frequency (PRF) of 1 kHz, from which the flow was estimated in a 300 μm window of 10 samples. This window was positioned at depths τ from 3 to 8 mm for each recorded sample. The pixel size is at 50 MHz equal to 20 ns, corresponding to $\sim 30 \mu\text{m}$ at water's speed of sound.

6.3 Flow imaging results

6.3.1 Validation using particles with water bulk

The clinical PA system is first used for flow imaging with a transparent bulk medium to optimize the signal-to-noise ratio and as such validate the technique. The results are presented in Figure 6.3, which shows flow images for increasing mean set speeds. Enveloped versions of the photoacoustic reconstruction were used to show only flow at regions with a PA amplitude larger than $1/e^2$ of the maximum (defined here as 'flow pixels'). The images show the cross-sections of tubing with flows going up (in plane) for number 2, and down for 1 and 3. This is detected as flow toward and away from the transducer respectively, and expressed as positive and negative flows.

Figure 6.3. Flow imaging of micro-particles in a transparent bulk medium for a range of speeds. The tubing crosses the imaging plane thrice, flowing up (cross-section 2) and down (1 and 3) through the plane. Each image is acquired at a specific pump speed as mentioned in left column. The ellipse drawn in cross-section 1 at 75.7 mm/s represents the physical size of the tubing's cross-section – elliptical due to the imaging angle of 50°.

Figure 6.4. Flow quantification performance for a transparent bulk medium. **(a)** The mean flow speed per cross-section is compared with the mean pump speed. **(b)** The per-pixel error of the cross-sections expressed as a percentage of the mean estimated flow speed, plotted versus pump speed.

Note that the axial positions (the ‘height’ in the image) is not equal for all three sections. This is a true representation of the flow phantom, in which tubing 2 was somewhat closer to the probe. The absolute mean flow speed per section of tubing is calculated from the relevant flow pixels. The result in Figure 6.4(a) shows a good ($< 5\%$) agreement with the speed set on the syringe pump. In addition, all three tubing sections’ flow speeds are estimated within $< 4\%$ of each other. Each image shown in Figure 6.3 is the average over 10 individual flow images to reduce any random fluctuations in the flow pixels’ velocity. The influence of these fluctuations is determined separately. For each flow pixel, its fluctuations over the 10 individual flow images is computed with the standard error. This results in an ‘error’ image. The ‘error’ pixels corresponding to photoacoustic pixels are averaged for each section of tubing. This metric therefore shows the accuracy of a single pixel of one individual flow image. The result is displayed in Figure 6.4(b) as the per-pixel standard error as a percentage of the measured flow speed. The error starts off relatively high at $\sim 30\%$ for low speeds of ~ 6 mm/s, dropping down to $\sim 7\%$ at higher speeds.

6.3.2 Flow imaging in a scattering medium

Next, flow imaging is performed in a scenario where realistic optical scattering ($\mu'_s = 5.2/\text{cm}$) reduces the SNR of the detected PA signals. As before, the mean flow speed per tubing section is determined. Figure 6.5(a) gives the comparison with the set speed, showing a better than 15% agreement between the measurement and the set speed. Rather, the effect of the reduced SNR is mainly visible through the standard error in the flow estimation, as shown in Figure 6.5(b). Although it shows a per-pixel standard error that is worse than under non-scattering conditions by a factor of ~ 5 , errors do drop down to $\sim 40\%$ at higher flow speeds.

Figure 6.5. Flow quantification performance under realistic optical scattering. For each flow speed the mean estimated flow speed (a) and the per-pixel error (b) per cross-section is evaluated. Compared to optimal (non-scattering) conditions, the mean flow estimation remains accurate, but the standard error is increased due to reduced SNR.

6.4 Discussion

6.4.1 Flow imaging performance

Pulsed photoacoustic flow imaging was successful at both imaging and quantifying flow of 53–63 μm particles. This was true for both a transparent bulk medium and one with realistic optical scattering. Arguably however, the quantification is more accurate than the imaging resolution, since the axial resolution is somewhat poor (see Figure 6.3). While the tubing cross-section at a 50° intersection angle is 0.76 mm in inner diameter, the imaged axial tubing size is more than twice that value. In part this is caused by the bandwidth of the transducer, combined with the flow-estimation window size. On the other part, the elevation detection width plays a role, which at ~ 3 mm wide increases range of axial positions at which the tubing is imaged. The limited resolution likely also prevents imaging of a laminar flow profile, if applicable. The axial resolution can be improved by reducing the flow estimation window size. However, a smaller window size will be at the cost of flow estimation precision. In the flow images with optical scattering, on the other hand, the lateral size is underestimated (see Figure 6.5(a), inset), likely because of significantly reduced SNR near the edges of the cross-section. In both cases – with and without scattering – there is a small flow gradient visible in the images, with higher flow speeds closer to the probe than farther away. The reason is likely due to a slightly higher density of particles at bigger depth, which would have caused additional friction and slowed down the particles. This higher density of particles would have been caused by a horizontal section of tubing, which supplies the vertical sections seen in the flow images. In this horizontal section, particles would have sedimented slightly, resulting in a somewhat higher density.

In the flow estimation, the minimum measureable speed is where the relative error becomes 100%. At optimal SNR – without scattering – this is ~ 2 mm/s. With optical scattering the

minimum is ~ 15 mm/s. The SNR can be improved by using a higher laser power or more sensitive detectors. The relative error can potentially also be reduced through better flow estimators: in ultrasound flow imaging this can be achieved with Bayesian speckle tracking [30] for example.

The maximum measureable speed is determined by decorrelation of the particle distribution over subsequent laser pulses. This decorrelation can be limited by increasing the PRF of the diode laser, which has an upper pulse rate limit of 10 kHz. The maximum flow speed we measured was 75 mm/s, at a PRF of 1 kHz. Therefore using the probe at its highest PRF will allow flow measurements at speeds higher than 750 mm/s.

6.4.2 Limitations and outlook

The experiments from this article are performed using micro-particles instead of red blood cells. We will shortly discuss what would be required – based on this research – for flow imaging of whole blood. RBCs are smaller than the micro-particles used here (~ 6 μm versus ~ 60 μm) and are denser in whole blood ($\sim 50\%$ versus 4% v/v), but this will not pose a fundamental problem. However, while the overall photoacoustic signal might increase with density, the fingerprint visibility will decrease, negatively affecting the SNR of the flow estimation. The increase in density can be addressed by using detectors with narrower elevational focusing, in combination with a higher detector bandwidth. Compared with the current system, using high-frequency transducer with a 10 MHz bandwidth should sufficiently improve the axial resolution, while not suffering too much from frequency-dependent ultrasound attenuation in tissue. Likewise, a 0.5 mm elevational focus should further improve the fingerprint visibility while not limiting the transducer's depth of field too much. The smaller size of RBCs can be dealt with by increasing the center frequency of the detectors and reducing the pulse length of the laser. In addition, the pulse energy can be increased by adding more laser cavities to the diode laser or by selecting a different laser for further improving the SNR. These changes will serve to improve the fingerprint visibility. Preliminary research shows that flow imaging of whole blood is achievable when implementing most of these improvements. Initial experiments using a 15 MHz transducer (up from 7.5 MHz) and a 4 mJ diode-pumped solid state laser (up from 1 mJ) with 10 ns long pulses at 500 Hz PRF, indicate that flow imaging of whole blood is practically feasible.

In an *in-vivo* application of the current clinical photoacoustic system, contrast agents could be used to perform the flow imaging, for example by using fluorophore-filled vesicles. Also with contrast agents it holds that photoacoustic-based flow imaging would suffer less from sources of clutter than is the case when using ultrasound.

In general, a realistic comparison of PA and US flow imaging capabilities should be made to test the hypothesis that PA-based flow estimation works better with imaging small vasculature and estimating flow near vessel walls. The flow phantom presented in this article

is – when including optical scattering – mainly realistic for PFI, not so much for ultrasound flow imaging. Therefore, a specific US/PA flow phantom would have to be developed in order to do the comparison.

6.5 Conclusion

Results were presented of successful pulsed photoacoustic flow imaging (PFI) using a clinical handheld photoacoustic system. Experiments were performed using micro-particles, which were pumped in suspension through tubing, of which cross-sectional flow images were created. The system allowed 2D imaging of multiple tubing sections with opposite flow directions. Quantification performance was evaluated both with the tubing in a transparent medium, and with optical scattering that was introduced such to mimic tissue ($\mu'_s = 5.2/\text{cm}$). In both cases, flow imaging up to 75 mm/s was shown. With optical scattering the minimum measured speed was 15 mm/s; without scattering speeds down to 6.3 mm/s can be measured.

The estimated mean flow speed agreed within 5% to the pump speed in the transparent medium, and within 15% when optical scattering was introduced. The standard error of the flow estimation suffered somewhat more when reducing the SNR, and increased from approximately 10% to 40% (at 40 mm/s) due to optical scattering.

In the future, PFI could be used with vesicle-based contrast agents for *in-vivo* flow imaging. Future research should also be directed into flow imaging of whole blood by relying on the availability of high-frequency linear arrays in the 10-20 MHz range. In addition, flow estimators developed specifically for PFI could further improve the flow quantification. Flow estimators that rely on specific spectral content or shape of the photoacoustic fingerprint, for instance custom wavelet-based estimators, may improve the estimation considerably.

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Chapter 7. Epilogue: pulsed photoacoustic flow imaging of whole blood

As explained in chapter 6, PA flow imaging (PFI) of whole blood with the Fullphase probe proved difficult. The probe may not have been ideal for this purpose due to the pulse length and detection bandwidth. It is important to assess what system is optimal for PFI and whether imaging with a detector array is feasible. To investigate these two questions we performed a study together with University College London (UCL)⁶. The cooperation with UCL meant that we were able to use a 15 MHz ultrasound probe and ESAOTE Mylab_One scanner, in combination with an external laser which had a double-cavity: effectively two Nd:YAG lasers that can be used for particle image velocimetry (PIV). Even more so than with PAI regularly, PFI requires proper synchronization of laser firing with the ultrasound acquisition, where the delay between the laser pulse and acquisition should remain the same within nanoseconds. The PIV laser had the proper triggering options, such that the synchronization with acquisition, and tracking of the flow between subsequent PA frames were precise.

The Q-switches of the PIV laser (one for each cavity) can be triggered separately, which enabled accurate synchronization between the Mylab's start of a frame acquisition and the corresponding laser pulse. However, this synchronization required some additional tools. The reason behind this that, to fully fire one cavity requires two triggers. The first trigger is used to activate the flash lamp, in doing so building up power in the cavity. The second trigger should arrive later, after sufficient energy is built up, to release this energy using the Q-switch. Nevertheless, the most important timing is the pause between the two Q-switch triggers: this determines the time between the two laser pulses that interrogate the flow. There should be a low jitter in this time (random electronic variability in the exact timing) in order to reduce errors in flow estimation. First a timing circuit was developed to split the continuous triggering from the Mylab_One into separate triggers for each of the two laser cavities (see Figure 7.1a). Due to technical limitations, the Mylab_One acquired 20 frames at 1 kHz, not just two frames. The other 18 were therefore empty and ignored in further processing. Next, a single function generator was used to control both Q-switch triggers, and since both triggers are generated by one device, there was low jitter between the two laser pulses (see Figure

¹Experiments in this chapter were performed together with Thore Bucking of UCL. Design and interpretation of the experiments was done in further cooperation with Joanna Bruncker, Paul Beard (both of UCL) and Wiendelt Steenbergen.

Figure 7.1. Setup for flow imaging. **(a)** Use of a timing circuit to create separate triggers for the double laser cavities. **(b)** Triggering scheme for the acquisition of two PA frames. **(c)** Alignment of the US probe in relation to the flow channel (tubing) and the laser illumination. **(d)** Example acquisition of two PA frames (dashed ellipse indicates the approximate cross section of the tubing).

7.1b). An additional delay generator was used to delay one of the flash lamp triggers because the corresponding Q-switch delay was different from the other cavity.

Polyethylene tubing (0.58 mm inner diameter) was vertically placed in a water bath, the ultrasound probe at a 60° angle to this (see Figure 7.1c). The PIV laser (a Litron Nano L) was aligned to match the intersection of the imaging plane and the tubing. The harmonic crystal was removed from the laser, such that its output was 1064 nm light and not the frequency-doubled 532 nm. Hemoglobin has lower optical absorption at this wavelength, but while this lowers the SNR, the optical penetration in whole blood increases from $\sim 100\ \mu\text{m}$ up to a few millimeters. The laser fires $<10\ \text{ns}$ pulses, and each cavity has a PRF of up to 15 Hz. The ultrasound probe (ESAOTE SL3116) has 64 elements that can be used for photoacoustics, with a pitch of $100\ \mu\text{m}$. The bandwidth ranges from 11 to 17 MHz (at -6 dB).

In total 20 laser-pulse pairs were used to acquire a corresponding number of photoacoustic frames. These frames were high-pass filtered and subsequently reconstructed (see Figure 7.1d). In these frames, static signals are filtered out using DC-removal. This filtering ensures that a part of the PA response, which does not move with the flow and remains the same over all frames, does not interfere with the flow estimation. Data analysis is very similar as with

Figure 7.2. Flow imaging results. **(a)** Example of five flow maps for a flow of 12 mm/s. The green rectangle represents the ROI that was used for calculations of the average flow speed. **(b)** Estimated flow speed within ROI with flow of 40% RBCs in PBS and **(c)** whole blood. The data points are the average of five flow maps, the error bars their standard deviation, and the diagonal line ($x=y$) the ideal flow estimation values.

chapter 6. Flow is calculated pixel by pixel from the reconstructed image, where the PA signals in a window around each pixel are compared between two subsequent laser pulses, where the shift (down or up) is estimated using a cross-correlation between the two windows. This process is repeated for all 20 image pairs, providing 20 cross-correlations that are averaged and from which a single shift value is calculated. This shift is converted into a flow velocity by using the time between laser pulses and the angle between the flow and the probe. The window is one element wide and 16 samples in length and thus only axial flow velocities are calculated, i.e. flow away or towards the transducer as is also common in clinical ultrasound color Doppler imaging.

A syringe pump provided for velocities ranging from 3 mm/s up to 25 mm/s. For each flow velocity, five repeated flow maps were created (Figure 7.2a). Within each flow map, the median flow speed was calculated within a region of interest (ROI) and plotted against the syringe pump setting. Flow imaging was performed on two types of human donor blood: on 40% (v/v) RBCs in phosphate-buffered saline, and on whole blood including plasma (Figure 7.2b and c respectively). Both graphs show comparable flow estimation performance, with relative errors ranging from ~50% down to 15%. On average there is a low bias within the 6-25 mm/s velocity range. The minimum measureable flow speed is 6 mm/s, as the measurement at 3 mm/s appears to be biased in both cases. The maximum velocity likely falls outside the measured range.

The exact composition of the blood currently does not provide measureable differences, while it was expected to matter via enhanced clustering of RBCs in whole blood by the interaction with proteins in the plasma. The main source of error appears to be overwhelming any differences between the two blood compositions: floating particles in the water tank seem to have played a substantial role in creating fluke flow estimation by the algorithms. This might have been dust or bits of calcification in the water tank, because it was worsened by attempts to clean the tank. Figure 7.2a shows these floating particles as flow at places around the tubing, predominantly in the bottom left figure. This was also the reason for calculating the median flow speed (not the mean) to limit the effect of outliers. In a sense this is good news though: if the debris is causing the large errors then the most precise measurements are hopefully the ‘real’ performance measures for the flow estimation.

While it still has its limitations, this work does show the feasibility of PFI when using a clinical ultrasound probe for imaging of blood flow. It shows that on one hand PFI of dense RBCs is possible with relatively low frequency detection, compared to microscopy transducers (with >25 MHz detection). On the other hand, it shows the reconstruction of the fingerprint signal from the dense RBCs retains their phase information correctly.

The measurements should probably be repeated with a cleaner water tank, and other implementation issues can then also be fixed. First, the synchronization of the detection and laser systems should be simplified. Now the Mylab_One triggers the laser, but it would be simpler to replace the ultrasound scanner with one that can be triggered itself (the Mylab cannot be externally triggered). This will reduce the complex timing issues that were now encountered. The second issue that should be solved is the setup instability. Vibrations in the setup, possibly due to tremors in the building, meant that the loosely suspended tubing oscillated fairly easily. A solution would be to use a bench top vibration isolation platform to improve the damping of the table it rests on. In addition, using a wall-less phantom (i.e. a gel block with a cylindrical channel) would likely also be mechanically more stable than tubing. Differences in beam profiles between the two cavities is a further issue, as this may reduce the coherence between PA fingerprint pairs. Adding optical scattering to the bulk medium would hopefully smooth out the beam profiles and make flow estimation more accurate.

Chapter 8. Conclusion

8.1 Findings

How can an integrated photoacoustic/ultrasound (PA/US) imaging probe be used? The system has proven remarkably useful in a range of applications and very few concessions had to be made in terms of performance. It also shows how powerful single-wavelength PAI is – of course multispectral imaging may provide extra diagnostic information like oxygen saturation – but it emerges that ‘absorption imaging’ is an imaging modality of its own. The focus of this thesis on single-wavelength PAI means that the work underlines how a PA system affects the appearance of biological structures. A few notable examples:

1. Imaging depth of the PA/US imaging probe is flexible and can be extended at the cost of frame rate (chapter 2).
2. Structure heterogeneity in liver fibrosis plays a large role in the strength and frequency content of detected PA responses (chapter 3).
3. Synovitis in rheumatoid arthritis results in significantly more and larger PA image features in inflamed joints (chapter 4).
4. Sub-resolution PA tracking and imaging of particles is feasible, in turn enabling flow imaging with good resolution (chapter 6).
5. Even in dense media like whole blood, PAI detects a fingerprint response from sub-resolution cells that can be successfully used for flow imaging (chapter 7).

Single-wavelength PAI was able to visualize the morphology of absorbing structures in a range of applications, and assessing these structures in terms of overall size, heterogeneity, or flow velocity. As a result, PAI is a unique imaging tool for these applications, owing to its diverse options for quantification.

8.2 Discussion

8.2.1 Handheld imaging

The previous section highlighted the successes of the thesis, but it is equally important to take note of the current limitations. One of the main drawbacks of the current system is the lack of a good contact mechanism between the skin and the probe. The light only exits the probe when there is good contact with a medium of refractive index higher than air (i.e. water, gel or stand-off) and no total internal reflection takes place at the glass window. Imperfect contact with the coupling medium means low light emission and poor image quality, which is a serious risk with the large probe front surface when imaging small or highly curved structures like finger joints. In imaging studies shown in this thesis, the contact problem was

therefore tackled by using a water layer, for instance by submerging of the subject's finger joint. The water layer is not ideal for (pre-) clinical use, however.

The contact problem can be solved by modifying the front of the probe and designing the illumination for use with a specific US stand-off pad. Currently the front surface is too irregular to be used with typical stand-off pads, and the thickness of the pads is not ideal either. Second, a custom stand-off can also be made for the probe, matching perfectly with the surface design. In both cases, a mechanism should be in place to fix the stand-off pad to the probe, or possibly integrate it completely with the probe. A third alternative option is to design a cavity in front of the probe to fill with US gel (or water) that can be covered with a film layer, but while easy to fabricate, this may be too flimsy or labor intensive for clinical use. Finally, modifying the glass window to remove total internal reflection can ensure stable light output and good image quality, but other safety measures must be put in place to prevent accidental eye damage due to laser irradiation. Whichever approach to take, it will be important to test it prior to fabrication, for instance using rapid prototyping of the front design, possibly in combination with an existing echography probe.

Even with good probe contact, the imaging depth is still limited compared to other systems. The pulse energy of the diode laser is lower than typical Q-switched lasers, which means that the SNR is practically worse (all other things equal) in our system. Because of exposure limitations, the average intensity $I = \text{PRF} \times \phi$ (pulse repetition frequency times fluence ϕ on the skin) should be kept below a certain value. Although a lower pulse energy allows a higher PRF to be used, averaging over multiple laser pulses is always less efficient than increasing the pulse energy. This reasoning assumes a constant imaging rate with averaging over a number of laser pulses to form one image. The approach taken in this thesis is to reduce the imaging rate to improve the SNR and increase the imaging depth. This was facilitated by allowances in the exposure limitations to temporarily increase the exposure, which allowed a burst of laser pulses at a higher PRF than normal – limiting motion artefacts. After acquiring a high SNR image this way, a pause before resuming PA imaging ensured that the exposure limitations were kept. This meant that the imaging rate was not improved in any way, such that this technique is not suitable for real-time imaging.

One method to improve the SNR – regardless of burst acquisition – is to better match the diode pulse length with the spectral detection sensitivity. Now the relatively long pulse smears out the PA response in such a way that it reduces the amplitude of PA pressure waves at frequencies where the 7.5 MHz transducers are most sensitive. On one side, the diode pulse length can be shortened, but this has the disadvantage of also affecting the output energy of the diodes. The alternative is to lower the detection frequency to for instance 5 MHz to better match with the pulse length. This has the added benefit of detecting pressure waves with lower tissue attenuation, further improving the imaging depth. One disadvantage of this approach is lowering the resolution of the system, but likely not by much, as the original

effective bandwidth would have been limited anyhow by the long pulse length. Even if there is a significant decrease in resolution, it would not harm applications like detecting synovitis, as the synovial membrane is sufficiently far away from other PA sources to prevent overlap – and more angiogenesis would mean a higher signal regardless of the resolution.

A second more straightforward way to improve the SNR is to amplify the analogue PA signals more. The traditional amount of amplification for ultrasound in the Mylab_One scanner appears to be somewhat underwhelming, as current PA signals are often in the single bit range of the analog-digital (AD) converters. Additional low-noise amplification of the PA signals – much more than just to counter the bit rate error – may improve SNR even further.

The better the SNR, the more important it becomes to limit imaging artefacts as well, as these can then start to affect the image quality more than noise. To reduce imaging artefacts, optical absorption by the acoustic lens (in front of the ultrasound detectors) should be prevented. The pulsed light from the diode laser is partly backscattered from the skin onto the lens, and if this light is in any way absorbed, this will generate a PA signal at the lens surface, traveling down toward the skin and reflecting back again to mix with the real PA signals – unwanted ultrasound planewave imaging basically. The acoustic lens is currently dark gray, which is not ideal for preventing absorption. To counter this absorption in the lens, a layer of gold film was therefore attached to the acoustic lens to reflect the laser light away again from the lens. In the future, a lens made from white material would be better than the extra film, but the lens material should be tested to make sure the near infrared absorption is sufficiently low, not only the visible absorption, and the light scattering is high enough that no light reaches the ultrasound detector below.

8.2.2 PA flow imaging

PA flow imaging in chapter 7 mentioned an interesting property of PA signal generation. The photoacoustic response from a vessel with red blood cells (RBCs) is composed of two separate signals: first the overall response related to the joined summation of the individual PA responses from RBCs, due to which the vessel appears as homogeneous, and a second signal, the PA fingerprint that is the random speckle-like superposition of the RBCs. The homogeneous response is well known via the diameter-dependent bipolar signal [1]. This homogeneous response is static over time, whereas the RBC fingerprint moves with the blood flow. The homogeneous response overlaps with the fingerprint signal, and is more apparent when the transducer's bandwidth matches closer to the homogeneous response's frequency as tied to the vessel diameter. In typical PAI applications, this homogeneous response overshadows the fingerprint signal [2]. The main challenge with pulsed PA flow imaging (PFI), where the goal is to track this fingerprint, is therefore to distinguish the fingerprint from the homogeneous signal. This appears to limit the potential imaging depth of the technique due to the need for high-frequency transducers to reduce the detection of the homogeneous signal and highlight the high-frequency signals from the RBCs.

However, Brunker and Beard (2016) observed a transition between the homogeneous and the fingerprint responses depending on the density of the absorbers [3]. During this transition it appears, as seen in chapter 7, that it is possible to distinguish between the two responses. The method to do so, as implemented in chapter 7, is to reject any static signals: implemented in the chapter using a type of DC-removal to filter out this homogeneous response. This approach means that high-frequency detection is less critical and imaging depth may be improved. However, this DC-removal may be less suitable for *in-vivo* imaging, as there can be movement of the probe, or motion of the vessel walls. In effect the problem transforms into something similar as in ultrasound flow imaging: filtering away the signal from the vessel wall. In US flow imaging this is far from easy due to movement of the vessel wall and other motion clutter. What then, is the benefit of using PFI over ultrasound flow imaging? Likely the wall filtering will be significantly easier in PFI, as the homogeneous signal is well defined and should remain well correlated over time. A ‘double cross-correlation’ approach can first overlap the homogeneous signals, then apply wall-filtering and finally estimate the shift in the fingerprint signal.

One disadvantage remains that the laser and high-frequency ultrasound probe that were used in chapter 7 for PFI are fairly non-standard for general photoacoustic imaging. However, there may be a second benefit to using PFI, and not just for estimating the flow velocity. This advantage is in tackling the limited view problem, the issue in PAI where structures are only visible when parallel to the detector surface. The limited view problem affects only the homogeneous signal however, and is likely not so for the RBC fingerprint. PFI may then be used similarly to power Doppler ultrasound: estimating the magnitude of the fingerprint signal, which can furthermore be done with multispectral excitation. This would then allow imaging the rate at which oxygen is delivered, by combining the flow velocity with measurements of the oxygen saturation and vessel diameter. This PA ‘power flow’ imaging would be an improvement on similar work by Gateau et al., who used coherent light to create an optical speckle illumination inside the medium to generate PA signals from bright speckles [4]. While this also allowed them to circumvent the limited view problem, it did require multiple speckle orientations to generate a complete image, and the small speckle size generated even higher frequency signals than the comparably larger RBCs. Another benefit of PA power flow imaging would be that clutter or reflection artefacts originating from the skin will be removed from the resulting power flow image, as there is no flow present in the melanin layer, and capillary flow right beneath the skin is likely unresolvable by PFI. As such, PFI may be able to solve two major issues that now affect PAI.

8.3 Outlook

The next step in handheld imaging will be validation of the multispectral imaging capabilities of the latest prototype. It will be important to develop real-time algorithms that can correct for the wavelength-dependent light attenuation in tissue. This is especially critical for

applications in rheumatoid arthritis (RA). The apparent oxygenation of blood would be different from the true value when imaging through a layer of tissue, as light is scattered and absorbed by perfused tissue, which affects some wavelengths more than others. Because light has to travel further in swollen joints, the blood oxygenation may appear lower in swollen than in healthy joints without actually being different.

Proper attenuation-correction algorithms will hopefully allow the multispectral prototype realized in the Fullphase project to image oxygen saturation in synovitis. The system would be an excellent research platform for investigating how hypoxia influences the disease state, and whether hypoxia is relevant for drug development. More processes may be imaged using PA contrast agents which can be detected by their absorption spectrum. These contrast agents may be linked to markers related to RA to further advance knowledge on the disease pathways and drug takeup. Using PAI instead of PET imaging for this type of functional imaging would allow greater flexibility, as PAI can be applied more freely, lacking a patient burden due to radiation dose. Using PAI as a research tool first would allow the technique to be developed further, as PAI - for maximal clinical usefulness - would benefit from larger imaging depth in order to include more joints in clinical assessment.

Finally, PFI may in fact be easier to implement *in vivo* than in the phantoms used in chapters 6 and 7, since many issues like beam overlap between the two cavities will not affect the measurements due to light diffusion, and mechanical vibrations will be lessened as well. Pre-clinical imaging of liver fibrosis may be a very good starting point for PFI: this would allow combined imaging of liver hypoxia and perfusion, and portal hypertension using a single technique. Preclinical imaging is furthermore very suited for high-frequency array transducers, and would allow high resolution ultrasound imaging for accompanying structural information. Finally, for accurate flow measurement, more flow vectors than just the axial component should be measured. Lateral tracking of the PA fingerprint in the reconstructed image can be used for the lateral component, as is scanning of the linear array for the elevational component.

8.4 A personal reflection on photoacoustics

The first chapter introduced the Fullphase project and the consortium that worked on (and with) the integrated probe. This last section is a personal reflection on my experience with the project, from which I hope to distill a few things that can make PAI a success. First a small reflection on the Fullphase project: why was it successful? In PAI, all the components – laser, detectors, electronics – need to be perfectly organized for good measurements: accurately synchronized, low noise and perfectly aligned. The probe design was therefore not a small feat, and the expertise of companies in designing the components and then integrating them was invaluable. The integrated nature of the system meant that, once everything was developed, the user does not have to take overly much consideration into the probe internals.

The highly integrated probe meant that the system is relatively low in complexity from a user's perspective and meant that division of labor was clear in the Fullphase project: it allowed each partner in the consortium their own tasks and expertise – after all, a jack-of-all-trades is master of none.

While a single project can be successful, for the overall success of PAI it is important to acknowledge the existence of reflection and clutter artefacts and deal with these appropriately. Experiments should be designed in such a way that artefacts can be discounted or distinguished from actual vasculature. While in some cases these are obvious, for example: signals arriving from below a bone surface are obvious artefacts from the bone surface, others are not. A few methods to recognize artefacts were used in this thesis. For example in finger imaging, the bone reflections were a feature: if there is a structure with a mirror image below the bone then the original is likely real because an artefact does not cause a mirror reflection. But more can be done. In general, moving the detector in relation to the object, the illumination in relation to the detector, and compressing the object, would each modify the appearance of artefacts more than it does real structures. Nevertheless, the more elegant solution is to remove the artefacts altogether. Work by Kuniyil Ajith Singh & Steenbergen showcases reduction of reflection artefacts by cleverly interpreting ultrasound data[5], and more approaches exist [6-11]. It would benefit the field of PAI greatly if such artefact removal techniques are freely available to all, for example as an open source toolbox.

Even when the perfect PA images are made, (pre-)clinical acceptance of PAI will in my personal opinion also depend on a single name. Currently too many different flavors exist: is it 'multispectral' or 'multi-wavelength'; is it 'photoacoustic' or 'optoacoustic'; is it 'imaging' or 'tomography'? The etymology should in the end be less important than choosing one and sticking to it.

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Chapter 9. Summary

How can an integrated photoacoustic/ultrasound (PA/US) imaging probe be used? This thesis presented various examples of this. PAI is a unique combination of optical sensitivity to hemoglobin and other tissue chromophores and ultrasonic resolution, as we saw in **chapter 1**.

Next, we went into more depth on the probe's design considerations and its performance in **chapter 2**. The combination of PA and US imaging modalities is a very suitable one. Both generate pressure waves in the ultrasound regime and can as such be detected using the same type of ultrasound transducers. They image complementary information as well: PA imaging (PAI) visualizes vasculature and functional information, whereas US imaging provides structural information and provides clinicians landmarks with which to interpret the PA data. However, for the best translation into the clinic, a PA system should be practical and cost-effective, something that cannot be said about current imaging systems. This can change with the imaging probe proposed in this chapter. It relies on a compact pulsed diode laser, which is integrated into the probe's housing together with additional optics and the ultrasound detectors. Using a diffractive optical element that shapes the otherwise uneven light from the diode laser, the probe outputs a rectangular beam with a pulse energy of 0.56 mJ. The laser can be fired for short bursts of up to 10 kHz, which is important to minimize motion artefacts. The actual imaging rate is lower as multiple laser pulses are combined to improve the signal-to-noise ratio of the image. We achieved for instance an imaging rate of 10 PA images per second, while averaging over 20 laser pulses per image. In experiments on a tissue mimicking phantom we found the imaging depth can be improved from 5 down to 15 mm, but with a lower imaging rate. The resolution was determined, in phantom experiments on a sub-resolution hair, to be 0.28 mm axially (in depth) and between 0.4-0.6 mm laterally (sideways).

In **part I** of this thesis we focused on the imaging applications of the integrated PA/US imaging. First, in **chapter 3** we moved to a more powerful prototype that provides 1.3 mJ pulses. The chapter is a pre-clinical study on liver fibrosis in mice, a disease involving scar formation as a result of repeated liver damage. As no cure exists, currently animal studies are used in order to find one. In these studies, PA/US imaging can be a non-invasive alternative to histopathology, allowing to follow an animal over a longer period of time without sacrificing it at a certain stage of disease progression. In this study, CCl₄ was administered repeatedly to the mice to induce fibrosis. After 6 weeks the mice (n = 6) were sacrificed to perform histology and compare this with PA/US imaging. We saw significantly more PA image features ($p < 0.001$) in the livers of fibrotic mice than a group of healthy mice (n = 5). A frequency analysis was performed on the radiofrequency PA signals. This showed that PA

signals in fibrotic mice have a higher frequency content, and that this can be linked to fibrotic nodule formation. Histology confirmed fibrosis in the respective mice livers, showing significant increases in collagen I deposition and angiogenesis ($p < 0.001$ and $p < 0.05$ respectively). These histological markers of fibrosis furthermore correlated well with the amount of PA response from the livers, as well as with a quantification of the PA frequency content. This work shows that PAI using an integrated system is capable of assessing a fibrotic state, and may therefore have an important role in longitudinal animal studies.

Continuing with a clinical application in **chapter 4**, we studied detection of synovitis in finger joints of rheumatoid arthritis (RA) patients. Synovitis is an inflammation of the membrane around joints, and imaging of this inflammation can be used to predict how RA progresses. However, current modalities like MRI and ultrasound power Doppler (US-PD) have their drawbacks, mainly high cost and low reproducibility respectively. PAI may be a solution, with its high sensitivity to hemoglobin and its excellent ultrasound-based resolution. The goal of this feasibility study was to determine whether PAI is capable of detecting synovitis in joints with clinically evident inflammation. We made PA scans of clinically inflamed proximal interphalangeal joints of 10 patients, which were compared to non-inflamed joints from the same patients. In addition, joint scans were recorded of 7 healthy subjects, providing an added control group. When comparing these joint types, we found a significant increase in PA image features, on average 10 times more, near the joint membrane for the inflamed joints ($p < 0.001$). Each joint was also scanned using US-PD, with resulting images semi-quantitatively scored (0-3) by experienced rheumatologists who were blinded to the joint type. The US-PD score and the PA quantification agreed well (Spearman's $\rho = 0.64$ and $p < 0.001$). This shows that we are capable of detecting clinically evident synovitis, but future research is required to investigate if PAI also has extra value over clinical examination. PAI may for example be capable of predicting how the disease would progress.

In **part II** of this thesis we focused on flow imaging using photoacoustics, a tool that may help future assessment of inflammatory, as well as cardiovascular diseases. **Chapter 5** reviews first the state of the PA flow imaging field, which is quite broad due to the large number of ways to implement it. The main advantage of photoacoustics for flow imaging is that its sensitivity to hemoglobin in red blood cells (RBCs) allows a direct measurement of their flow with low tissue background amplitude. PA flow imaging techniques include those based on the Doppler effect, using a modulated continuous-wave laser and estimating the frequency shift in the returning PA response. When using pulsed light instead, RBCs generate a PA fingerprint that can be directly imaged. The 'flow' of the fingerprint can then be tracked to determine the velocity. Another approach is labelling of a part of the flow and tracking its subsequent motion is another good option, for instance by heating with high-intensity focused ultrasound it to increase its PA amplitude.

Chapter 6 investigates one of the flow imaging approaches, pulsed PA flow imaging, using the PA/US prototype probe, with the high pulse repetition frequency (PRF) of the integrated diode laser principally allowing continuous tracking of the PA fingerprint. The linear array of the system presents furthermore the opportunity to create a 2D map of flow velocities. The fingerprint signal was tracked over time using a cross-correlation to estimate the shift between laser pulses; this was done in a sliding window to obtain a flow estimation for each image pixel. PA flow imaging was studied in phantoms, both transparent and with tissue mimicking properties, with sub-resolution micro-particles flowing through tubing embedded in the phantoms. Results show flow imaging of velocities from 12 to 75 mm/s, with errors of 7% and 40% in transparent and realistic phantoms respectively. However, imaging of blood directly proved difficult due to the long pulse length of the diode laser, which limited the amplitude higher frequency PA signals.

Chapter 7 then goes on to show that PA flow imaging using the PA fingerprint of blood is feasible, though with a different PA system. To improve the detection of the PA response from RBCs, a high-frequency US probe is used: a 15 MHz linear array. The array was combined with a PIV-laser, which emits two timed pulses that are used to acquire PA image pairs a specified time apart. The shift in fingerprint signal during this specified time is computed using a windowed cross-correlation similar to chapter 6. Flow imaging was performed on both 40% RBCs in phosphate-buffered saline and on whole blood, with flow speeds ranging from 3 mm/s up to 25 mm/s. The relative errors were below 50%, down to 15%. The precision can likely be improved significantly by improving the setup, ensuring less distortions from floating particles, vibrations in the setup and differences in beam profile between laser pulse pairs.

In conclusion, **chapter 8** summarizes the main findings of the thesis and the strength of the integrated imaging approach. It also highlights how single-wavelength PAI provides a treasure of information. The chapter also discusses the limitations of the work in the thesis, and how this can be improved in the future. For handheld imaging the contact mechanism between the probe and the skin should be improved and the imaging depth should be increased. PA flow imaging should demonstrate its advantage over ultrasound, ideally by combining it with multispectral PAI for estimating the rate of oxygen delivery.

Chapter 10. Samenvatting voor niet-ingewijden

Hoe scherp kun je zien wat er in iemand omgaat? En dan heb ik het niet over gedachten, maar over het bloed dat in je aderen stroomt. Dit is verrassend lastig – normaal gesproken zijn alleen de oppervlakkige bloedvaten met het oog zichtbaar. En dat terwijl het heel belangrijk kan zijn om wel diep naar het doen en laten van je bloed te kijken: in veel ziektebeelden is er iets aan de hand met je vaten. Bij ontsteking heb je bijvoorbeeld een ongebreidelde groei aan bloedvaten ('angiogenese'), zo ook bij tumoren. Aan de andere kant, bij een beroerte of een hartaanval blokkeert een propje een bloedvat, waardoor je lichaam te weinig bloedtoevoer heeft ('ischemie').

Veel bestaande medische beeldvorming kan angiogenese of ischemie wel in kaart brengen, maar moet dat via omwegen doen, bijvoorbeeld door het toedienen van een (meestal duur) contrastmiddel. Een van de weinige directe technieken die direct bloedvaten kunnen afbeelden, is fotoakoestiek, een techniek waar nog volop onderzoek naar wordt gedaan. Bij fotoakoestiek wordt licht, meestal in korte pulsen, via de huid het lichaam ingestuurd. Daar wordt dit licht geabsorbeerd door kleurende moleculen zoals het melanine dat de huidskleur bepaalt en, belangrijker, het hemoglobine dat ons bloed rood doet kleuren. Als de lichtpuls kort genoeg is, dan warmt deze de ruimte rond de moleculen op, waardoor er een kleine drukopbouw plaatsvindt op een plek waar bijvoorbeeld veel hemoglobine is, zoals in een bloedvat. Die drukopbouw verspreidt zich vervolgens als een geluidsgolf, waarvan de toonhoogte in hetzelfde bereik valt als echografie ('ultrasound'). Een rij van detectoren kan de geluidsgolf opvangen en op die manier bepalen waar het oorspronkelijk vandaan kwam. Dit maakt fotoakoestiek uitermate geschikt om een interne ontsteking af te beelden: meer bloedvaten betekent uitgebreidere absorptie van licht en een geluidsignaal van meer plaatsen dan gebruikelijk.

Het liefst willen we dat een fotoakoestisch systeem zo veelzijdig mogelijk is. *De manier om dat te doen is door belichting en detectie te combineren in een meetkop die je kunt vasthouden, vergelijkbaar met echografie.* Maar de meeste fotoakoestische systemen zijn betrekkelijk fors omdat er een grote laser bij nodig is, en er stugge optische fibers nodig zijn om de laser met de meetkop te verbinden. Afgezien van het formaat van de externe laser, beperkt de noodzaak van een optische fiber de flexibiliteit van een hanteerbare meetkop. De oplossing is om de laser te integreren in de meetkop, iets wat mogelijk is met diode-lasers: miniatuurlasers die op zich niet heel sterk zijn, maar als je er genoeg bij elkaar zet behoorlijke puls-energieën kunnen leveren. Diodelasers zijn in het verleden vaker gebruikt voor fotoakoestiek, maar het integreren van de laser in een hanteerbare meetkop is een tamelijk

unieke prestatie, iets wat mogelijk is gemaakt door het Fullphase-project en waaruit dit proefschrift is voortgekomen. In dit project werkten universiteiten met kennis van fotoakoestiek, samen met bedrijven die het diode-systeem ontwierpen.

In dit proefschrift staat het hanteerbare systeem dat is ontwikkeld binnen het Fullphase-project centraal. Als eerste is het systeem uitvoerig getest in termen van de scherpheid van de beelden en maximaal haalbare meetdiepte (**hoofdstuk 2**). Dit laatste was extra belangrijk, omdat de diode-lasers minder energierijk zijn dan een grote externe laser, en het dus van belang was om te kijken wat de uiteindelijke doordringdiepte was. De meetdiepte bleek respectabel, maar een grotere meetdiepte betekende wel dat minder beelden per seconde opgenomen konden worden. In metingen voor dit proefschrift is ervoor gekozen de meetdiepte zo groot mogelijk te houden, ongeveer 15 mm, om er zeker van te zijn dat ook dieperliggende structuren zichtbaar zijn in de beelden. Die 15 mm lijkt misschien weinig, maar met licht kom je normaal gesproken nog niet dieper dan 1 mm.

Het Fullphase-systeem is voor verschillende doeleinden getest. De eerste toepassing van het Fullphase-systeem was om leverfibrose in muizen te ontdekken, waarbij we hebben samengewerkt met de *Targeted Therapeutics* groep van de Universiteit Twente. Leverfibrose wordt veroorzaakt door herhaalde leverschade en daar bestaat nog geen geneesmiddel voor. Momenteel worden daarom dierproeven gedaan om een geneesmiddel te ontwikkelen. Om het aantal dieren bij zulk soort proeven te verminderen, kan fotoakoestiek in de toekomst mogelijk gebruikt worden om muizen voor langere tijd te volgen, in plaats van dat er een nieuw dier nodig is bij elk meetpunt. Een eerste stap daartoe, het onderscheiden van gezonde muizen van muizen met leverfibrose bleek goed mogelijk: fibrotische levers lieten op meer plekken fotoakoestiek signaal zien, vermoedelijk veroorzaakt door littekenweefsel (**hoofdstuk 3**). Tevens lijkt fotoakoestiek het ontstaan van kleine compartimenten, gevormd door die littekens, te kunnen afbeelden. Hoewel het aantonen van fibrose iets anders is dan de voortgang van fibrose voor langere tijd te volgen, is het een belangrijke eerste stap in die richting.

Ten tweede is het systeem gebruikt bij mensen met reumatoïde artritis ('reuma'), een ziekte waarbij het kapsel rond de gewrichten ontstoken raakt, ook wel synovitis genaamd. Fotoakoestiek heeft de potentie om beter te kunnen inschatten hoe de ontsteking ervoor staat dan huidige technieken. In dit proefschrift hebben we eerst gekeken of fotoakoestiek überhaupt onderscheid kan maken tussen gezonde en ontstoken vingergewrichten, iets dat we hebben gedaan in samenwerking met het ZGT-ziekenhuis (**hoofdstuk 4**). Dit onderscheid maken bleek inderdaad zeer goed mogelijk: nagenoeg zonder uitzondering bleek rondom de ontstoken gewrichten aanzienlijk meer fotoakoestiek-signalen zichtbaar: gemiddeld tien keer zo veel. In de toekomst zal gekeken kunnen worden of de hoeveelheid fotoakoestisch signaal ook het verdere verloop van de ziekte kan aangeven, zodat de medicijnen daarop kunnen worden aangepast.

In dit proefschrift is alleen gemeten met fotoakoestiek op basis van een enkele kleur licht, wat een maat geeft voor de hoeveelheid bloedvaten. In de toekomst kan ook gemeten worden met een reeks kleuren om zo de zuurstofgraad van het bloed te bepalen, een graad die meestal verlaagd is bij ontstekingen. Bloed heeft een andere kleur, afhankelijk van de zuurstofgraad, wat fotoakoestiek kan gebruiken om die graad in te schatten; de verschillende kleuren laserlicht veroorzaken nu niet meer dezelfde signaalsterkte.

Bij een ontsteking zijn echter niet alleen de hoeveelheid bloedvaten en zuurstofgraad abnormaal, maar ook de toestroomsnelheid van het bloed zelf. De stroomsnelheid neemt bij een ontsteking toe, wat gecombineerd met de toename aan bloedvaten de gelegenheid biedt aan huidige technieken om ontstekingen af te beelden. Bij echografie reflecteren rode bloedcellen maar een beetje ultrageluid; dit is toch genoeg om die reflectie te gebruiken voor het meten van de hoeveelheid aan stromend bloed. Bij MRI en bij het nemen van een röntgenopname worden hiervoor veelal contrastmiddel gebruikt. Bij fotoakoestiek zijn het juist de rode bloedcellen die hoofdzakelijk signaal geven. Deze gevoeligheid voor rode bloedcellen maakt dat fotoakoestiek potentieel erg geschikt voor het in kaart brengen van hoeveel bloed er met welke snelheid aan het stromen is, ook wel *flow imaging* genoemd. In het proefschrift komt een groot scala aan methoden langs om fotoakoestiek te gebruiken voor het in kaart brengen van stroomsnelheid, die elk worden bediscussieerd in **hoofdstuk 5**.

Bij een van die technieken voor *flow imaging* wordt in opeenvolgende beelden gekeken hoever een patroon van cellen verschuift, wat omgerekend kan worden naar een snelheid. Die techniek heeft profijt van de diode-laser in het Fullphase-systeem. Die laser is in staat om in hoog tempo lichtpulsen te produceren, waardoor veel beelden met korte tussenpozen kunnen worden genomen, wat gunstig is voor de snelheidsbepaling. In dit proefschrift hebben we gekeken naar een basale opstelling met drie slangetjes om bloedvaten na te bootsen, waar we kleine zwartgekleurde plastic deeltjes doorheen lieten stromen (**hoofdstuk 6**). In de fotoakoestische afbeelding zijn die deeltjes zichtbaar door hun verdeling in een specifiek patroon, een soort vingerafdruk. Door de meetkop onder een schuine (niet-loodrechte) hoek te zetten ten opzichte van de slangetjes, lijkt het alsof de deeltjes en het bijbehorende patroon naar- of van de meetkop stromen; dat gebruikten we om er een stroomsnelheid aan te koppelen. Dit bleek goed te werken. Maar ondanks dat de deeltjes vrij klein waren, waren ze acht keer groter dan een rode bloedcel. Op bloed was dus iets te hoog gegrepen: bloed is verder te dichtgepakt met cellen om er nog een duidelijk patroon in te kunnen herkennen.

Om toch de stroomsnelheid van bloed in kaart te kunnen brengen, waren nauwkeurigere detectoren nodig dan die in het Fullphase-systeem verwerkt waren. Zulke detectoren zouden scherpere beelden moeten kunnen maken van het dichte bloed, en ook waren kortere laserpulsen nodig om scherpere fotoakoestiek-signalen te geven. Het combineren van geschiktere detectoren – als onderdeel van een precisie-echografiemeetkop – en een betere laser is uiteindelijk gerealiseerd door samen te werken met een vakgroep van het *University*

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Appendices

About the author

List of publications and presentations

Acknowledgements/Dankwoord

About the author

Pim van den Berg was born in October 1986 in Utrecht, The Netherlands. He studied for and attained bachelor and master degrees in Applied Physics (in 2010 and 2012 respectively), at the University of Twente, also in The Netherlands. He specialized in Optics and Biophysics, with a master's project in super-resolution fluorescence microscopy at the Nanobiophysics group. The goal of the project was to circumvent the diffraction limit in regular microscopy, for which Pim designed the optical setup for a new microscope and used it to image protein clustering related to Parkinson's disease.

Pim's PhD research (2012-2016) was part of the European Fullphase project and focused on the development of an integrated photoacoustic/ultrasound system. The research was performed at the Biomedical Photonic Imaging group at the University of Twente under supervision of prof. dr. ir. W. Steenbergen. Pim's focus was largely on two aspects: fundamental development of photoacoustic flow imaging, and using photoacoustic image quantification for clinical and pre-clinical applications.

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*Contributed equally to this work

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