Applications of multiphoton tomographs and femtosecond laser nanoprocessing microscopes in drug delivery research

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A B S T R A C T

Multiphoton tomography for in vivo high-resolution multidimensional imaging has been used in clinical investigations and small animal studies. The novel femtosecond laser tomographs have been employed to detect cosmetics and pharmaceutical components in situ as well as to study the interaction of drugs with intratissue cells and the extracellular matrix under physiological conditions. Applications include the intra-tissue accumulation of sunscreen nanoparticles in humans, the monitoring themetabolic status of patients with dermatitis, the biosynthesis of collagen after administration of anti-aging products, and the detection of porphyrins after application of 5-aminolevulinic acid. More than 2000 patients and volunteers in Europe, Australia, and Asia have been investigated with these unique tomographs. In addition, femtosecond laser nanoprocessing microscopes have been employed for targeted delivery and deposition in body organs, optical transfection and optical cleaning of stem cells, as well as for the optical transfer of molecular beacons to track microRNAs. These diverse applications highlight the capacity for multiphoton tomography and femtosecond laser nanoprocessing tools to advance drug delivery research.

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1. Introduction

Femtosecond laser technology has been used for more than two decades in Life Sciences. In particular, two-photon fluorescence microscopy, second harmonic generation (SHG) microscopy, and third harmonic generation (THG) microscopy are widely used tools for high-resolution in vitro imaging of cells and excised tissue samples [1–3].

A major step into the field of high-resolution imaging of in vivo and in situ human tissue was done with the introduction of the multiphoton tomographs DermaInspect and MPTflex by the German HighTech company JenLab GmbH (Fig. 1) [4–6]. These novel tissue imaging systems use an 80 MHz femtosecond near-infrared (NIR) titanium: sapphire tunable laser (“MaiTai” from Newport/Spectra Physics, USA or “Chameleon” from Coherent Inc, USA).

The tomographs are based on the multiphoton excitation of endogenous and exogenous fluorophores and of SHG active biological structures such as collagen. They are capable of three-dimensional non-confocal imaging with superior spatial resolution and single photon sensitivity based on single photon counting photon detectors [7]. Multiphoton absorption as a non-linear process occurs only in a tiny sub-femtoliter focal volume when using focusing optics of high numerical aperture (NA) and low <40 mW mean powers. This results in superior submicron lateral and 1–2 μm axial resolution in turbid tissues such as skin.

In vivo multiphoton tomographs detect the autofluorescence of endogenous fluorescent biomolecules such as flavoproteins, NAD(P)H, keratin, melanin, porphyrins, and elastin. Collagen is a natural source for SHG. In contrast to autofluorescence, SHG is an instantaneous nonlinear process where two photons interacting with a nonlinear material generate a photon at half the wavelength of the initial photons. Interestingly, most exogenous pharmaceutical and cosmeceutical substances also possess a weak fluorescence or emit a SHG signal. For example, ZnO nanoparticles used in sunscreens produce a weak blue fluorescence and may also provide SHG.

Three dimensional optical biopsies down to a depth of about 200 μm can be obtained non-invasively by x, y, z scanning using galvoscanners and piezodriven optics. The imaging depth is limited by scattering effects as well as the working distance of the focusing optics. The 200 μm working distance in clinical multiphoton tomographs avoids any dangerous situation to the operator’s eyes and to the person of investigation. For animal and ex-vivo studies, the working distance can be enhanced up to 2 mm.

When multiphoton tomography is required for deeper and intra-body tissue areas, endoscopic techniques have to be applied. A rigid miniaturized GRIN endoscope has been developed for non-invasive and minimally invasive imaging of deeper tissue areas as well as in the case of tissue morphologies not accessible by conventional bulky focusing optics. This two-photon microendoscope with an outer diameter of 1.7 mm and a length up to 2 cm can be added to the tomographs [8].

The multiphoton tomographs provide not only three-dimensional optical biopsies with subcellular resolution (Fig. 2). These novel bioimaging tools give also information on the spectral characteristics and

\[\text{Fig. 2. Multiphoton biopsies of in vivo human skin. The pseudocolored z-stack of the volar forearm contains the information on autofluorescence intensity and the occurrence of SHG-active collagen (brightness) as well as the fluorescence lifetime (color). Adapted from [68]. All images are 214×214 μm}^2\text{ and taken with 740 nm excitation light at 20 mW with a 350–450 nm band pass filter in front of the detector.}\]
the fluorescence lifetime. Spectral imaging can be performed (i) by tuning the excitation wavelength as well as (ii) by the detection of the emission spectrum at a fixed excitation wavelength (Fig. 3, [9]). For example, the reduced coenzyme NAD(P)H can be excited by two-photon absorption with laser radiation between 700 nm and 800 nm but not above 800 nm [6]. Flavins can be excited preferably above 800 nm. When taking emission spectra, a narrow line due to the SHG signal of collagen can be detected at half the laser wavelength as well as broadband emission cannot be detected in standard configurations due to the transmission properties of the internal optics. NAD(P)H has fluorescence maxima at 440 nm and 460 nm with respect to the binding status.

A further interesting contrast mechanism in high-resolution imaging can be obtained when detecting the fluorescence decay curves per pixel and by calculating the corresponding fluorescence lifetime [7,10]. The fluorescence lifetime depends on the fluorophore and the environment and not on the concentration. For example, free NAD(P)H has a fluorescence lifetime of about 200 ps. This time rises up to about 2 ns during binding to proteins. The fluorescence lifetime can be depicted as false-color coded signal within the optical section. Fluorescence lifetime imaging (FLIM) in Life Sciences, in particular for microscopic imaging of living cells and living small animals, has been introduced in the eighties by König and coworkers [10,11]. Using multiphoton tomography, FLIM can be applied to in vivo skin of human subjects. Clinical multiphoton FLIM tomography is performed by time-correlated single photon counting (TCSPC, Fig. 4) using photomultipliers with a fast rise time. The typical temporal resolution is 200–300 ps but can be improved down to 20 ps when using multichannel plates (MCP). Using MPT technology it is possible to distinguish between different melanin types [12].

Multiphoton tomography provides high-contrast optical images with single-photon sensitivity at submicron spatial resolution (3D), picosecond temporal resolution (4D), and spectral resolution (1–10 nm, 5D). It is therefore the clinical tissue imaging technology with the best resolution compared to ultrasound, photoacoustic imaging, digital photography, confocal reflectance microscopy, magnetic resonance tomography, Raman microscopy, and optical coherence tomography (Fig. 5) [6,13,14]. The major disadvantage of multiphoton tomographs is the relatively high price (~300 k€) due to the use of femtosecond laser technology.

Multiphoton tomography is a safe imaging technology. The safety was proven in two-year long studies that included DNA damage assessments. In particular, a study investigated the effects of the NIR femtosecond laser pulses of the DermaInspect to human skin in comparison to UVA exposure of standard light sources such as sunlight simulators used in test centers of the cosmetics industry. The published study conducted by Beiersdorf AG with partners from university and hospitals confirms that the DermaInspect is neither able to induce erythema nor to produce a significant amount of cyclobutane pyridine dimers (CPN) in contrast to UV exposed skin [15].

The CE certified class 1 M multiphoton tomographs are used in a variety of major hospitals such as in Paris, London, Berlin, Modena, and Brisbane as well as in the research centers of leading cosmetics companies in Asia and Europe. Long-term multiphoton studies (up to three months) have been performed on volunteers and patients. Major clinical applications include the early detection of malignant melanoma, the optimization of the treatment of patients suffering from dermatitis, actinic keratosis, side effects of chemotherapy, and chronic wounds.

In addition, animal and biopsy studies on brain tissue including glioma detection, on tissue engineered skin, blood vessels, and cardiovascular tissue as well as on ocular tissue have been performed [16–18].

This review focuses on major applications of multiphoton tomography in the field of drug delivery such as the bio-safety of intratissue nanoparticles and the measurement of the effects of anti-aging products and pharmacological products. Furthermore, the use of
NIR femtosecond laser pulses for an optical drug delivery based on multiphoton ionization and optoporation will be highlighted.

2. Applications for multiphoton tomography and femtosecond lasers

2.1. Biosafety of nanoparticles

Whilst the nanotechnology industry is expected to exceed $1 trillion in investment by 2012, a number of studies have reported nanoparticle toxicity [19–21] and the question of how to assess adverse effects is still being debated. One of the key questions in determining the biosafety of nanoparticles is whether the nanoparticles reach viable tissue. Skin is a natural nanoparticle barrier and is arguably the organ that is exposed to the most nanoparticles. Nanoparticles from a variety of sources, including cosmetics, sunscreens, antimicrobial ointments, and wound dressings, are in contact with our skin on a daily basis and the number of products is increasing [22–24]. With increasing production and use, nanoparticle biosafety is increasingly important. We have found multiphoton...
tomography an exceptional tool for defining the risk of nanoparticle exposure [25,26]. Prior to using multiphoton tomography, we used inductively coupled plasma-mass spectrometry to determine the levels of zinc from nanoparticles penetrating human skin [22]. The limitation of this study was that the zinc penetration was measured, not the nanoparticle penetration. We had to use transmission electron microscopy in order to evaluate nanoparticle penetration. This was costly, time consuming, required fixation of the tissue, and did not lead to quantitative results.

We have since monitored in vivo and under the normal physiological conditions the penetration of zinc and silver nanoparticles in healthy and compromised human skin with multiphoton tomography based on the luminescence of the metals under femtosecond NIR laser excitation [24].

In particular, multiphoton tomography in combination with FLIM [7,71]) has enabled us to differentiate between ultrafast metal nanoparticle signals and endogenous fluorescence (NAD(P)H etc.) in intact and compromised skin (Fig. 6). TCSPC FLIM data can be considered to consist of a 512 × 512 pixel array, with each pixel containing a number of detected photons with respect to the arrival time after the femtosecond laser excitation pulse. The arrival time is measured by the use of 128 time channels. Thousands of photons are measured in each pixel in order to perform fluorescence lifetime analysis. Because the system works at a high 80 MHz repetition rate, the laser beam dwell time per pixel is just some microseconds to obtain these photon numbers. To obtain fluorescence lifetimes from these data points that build up a fluorescence decay curve per pixel, the curve has to be fitted typically for a double-exponential decay profile:

\[ f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \]

with

\[ a_1 + a_2 = 1 \]

where \( \tau_1, \tau_2 \) and \( a_1, a_2 \) being the lifetimes and the amplitudes of the fast and slow decay component. In addition, \( f(t) \) is convoluted with the instrument response function IRF(t) which represents the temporal resolution of typically 200–300 ps.

For FLIM data containing SHG contribution, the fit will combine the SHG component ("SHG lifetime" = 0 ps) and possible fast fluorescence decay components in the fast decay component.

Normal epidermis autofluorescence has \( a_1 \) values of no more than 0.65. Values of above 0.95 are a clear indication that SHG by collagen or by metal nanoparticles are detected, i.e. from zinc or silver nanoparticles. Thus, multiphoton FLIM tomography is a useful tool to measure metal nanoparticle exposure to viable skin.

This application is also capable of simultaneously measuring NAD(P)H lifetime changes that are useful for evaluating metabolic changes after nanoparticle exposure [25]. The fluorescence lifetime characteristics of this endogenous fluorochrome have been defined and found to help determine metabolic flux, specifically free and protein bound NAD(P)H changes [26]. Multiphoton tomography images were used to quantify changes in NAD(P)H lifetime and contribution, an indicator of metabolic rate [27,28], after sunscreen application. Fig. 7 shows the \( a_1/a_2 \) ratio (free NAD(P)H/protein bound NAD(P)H), inversely related to the metabolic rate, for non-lesional and lesional areas. Increases in the \( a_1/a_2 \) ratio indicate more free NAD(P)H and/or less protein bound NAD(P)H suggesting an overall decrease in the metabolic rate. The \( a_1/a_2 \) ratio is shown in Fig. 72 h after zinc oxide containing sunscreen (14 mg/cm² at 60% ZnO) application. These data show no significant changes in NAD(P)H \( a_1/a_2 \) ratios after application for 2 h. These examples illustrate the usefulness of multiphoton tomography for evaluating biosafety aspects of nanoparticles in human subjects in vivo under normal physiological conditions.

2.2. Measurement of anti-aging effects

Skin is our major protective barrier from the elements, but can be affected by photoageing (Fig. 8a). The use of multiphoton tomography for optical analysis of anti-aging effects in human skin in vivo was reported by König and coworkers [29,30]. They showed that individual intratissue cells and skin structures (collagen) could be clearly visualized (Fig. 9). Second harmonic generation was used to detect collagen structures whereas two-photon autofluorescence was employed to detect the elastin network. Intracellular components and connective tissue structures could be further characterized by fluorescence excitation and emission spectra and by fluorescence lifetime imaging. It was reported that autofluorescence and SHG imaging by multiphoton tomography provided a better resolution and contrast mechanism than optical coherence tomography (OCT) and ultrasound as well as enhanced in-depth information, resolution, and the possibility to image the extracellular network compared with confocal imaging techniques based on reflectance [6,13,14].
In 2005, Lin et al reported the use of ex vivo multiphoton microscopy to evaluate photoaging by imaging collagen and autofluorescence in the excised upper dermis [31]. They defined an ageing index parameter SAAID based on the measurement of elastin autofluorescence versus collagen SHG. In this study, collagen was visualized by SHG with a 760 nm excitation source and a 380/20 bandpass emission filter whereas autofluorescence was detected with a 435 nm long pass filter, capable of collecting autofluorescence from NAD(P)H, FAD, collagen, elastin, melanin, and keratin. The study examined excised facial skin, fixed in formalin, from three patients aged 20 (−0.45+/−0.04), 40

Fig. 6. Multiphoton FLIM tomography images of nanoparticle treated study subjects with compromised skin. The images are pseudo-colored for a, 0–100% from blue to red. The stratum granulosum is shown and nanoparticles are highlighted by SHG shown in orange to red (*). The strongest nanoparticle signals are found in the furrows and in patients with psoriasis. Healthy skin cannot be penetrated unless tape stripped (removal of the stratum corneum barrier). The bar indicates 50 μm and the color scale (a1 range). All images are 214 x 214 μm² @740 nm, 20 mW.
(−0.66+/−0.11) and 70 (−0.93+/−0.12) years old (SAAID score in brackets). Five images were collected from the superficial dermal layers for analysis. The conclusion was that with aging, the SHG signal decreased and the number of AF positive pixels increased. The hypothesis was that the collagen was damaged and there was a decrease in quantity; while reciprocally, the collagen was being replaced with elastin (AF positive pixels).

We investigated some months later the relationship between age and SAAID score on volunteers under in vivo and physiological conditions[32]. A strong gender and age dependant relationship with SAAID scoring was found. We chose the inner forearm which can be defined as ‘photo-protected’ as the test site for the study subjects (all with Caucasian skin from Jena, Germany.

The dorsal side of the arm is exposed to more sunlight and is therefore more “photoaged” than the volar side, as illustrated by enhanced collagen SHG signals in volar dermis (Fig. 8). Further multiphoton studies are currently performed to study the impact of tanning studies and smoking on the SAAID index [33]. Smoking is a known factor in premature skin aging, specifically, smoking has been reported to increase levels of matrix metalloproteinase (MMP-1) [34]. The enzyme MMP-1 facilitates degradation of both collagen and elastin, resulting in macroscopic changes visible on the skin surface associated with photoageing.

Similar to smoking, UV-photodamaged skin results in premature skin aging. Ultraviolet exposure to skin reduces the levels of the most abundant structural protein in the dermis, collagen, by reduced precursor synthesis (type I procollagen) and increased degradation by MMP-1.

Therefore, studying smoking and UV related effects on photoageing progression using multiphoton tomography has the potential for quantifying collagen and elastin associated changes over time in vivo.

An Australian study of Queensland residents showed that 72% of men and 47% of women had moderate to severe photoageing[35]. Photoageing severity is significantly associated with skin cancer (p<0.05).

Treating photoageing is a multimillion dollar worldwide industry, based on topical products. A major challenge in this area is quantifying the effect of the set topical products on collagen and elastin in the dermis. A major limitation in assessing epithelial condition and product effectiveness has been the availability of appropriate methods that can directly measure in vivo morphology, biochemistry, and pathology. Currently, dermatologists use gross observations of sagging, wrinkling and skin colour to estimate photodamaged skin and to motivate patients to avoid the sun. However, the gold standard is still hematoxylin and eosin stained sections of physical taken biopsies.

Previously, we studied together with the company L’Oreal the effects of topical soy and jasmine extracts on collagen and elastin that are commonly damaged by photoageing[36]. We used the in vivo multiphoton tomograph Dermalspect to quantify changes in intensity over 12 weeks in 24 Caucasian women aged between 45 and 65.

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**Fig. 7.** FLIM measurements of NAD(P)H a1/a2 ratios are shown for lesional and non-lesional (5 psoriasis subjects and 3 atopic dermatitis subjects), either untreated or treated with ZnO nanoparticle containing sunscreen (14 mg/cm²) for 2 hours. [21] SB: stratum basale, SG: stratum granulosum, SC: stratum corneum.

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**Fig. 8.** (a) The NAD(P)H a1/a2 ratio is inversely related to the metabolic rate which is influenced by UV light and antioxidants. Also the SHG signal is influenced by photostress. The images (b) and (c) demonstrate SHG differences of the dorsal sunlight exposed site vs. the volar sunlight-protected site of a forearm.

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longer wavelengths. This is in accordance with the absorption behaviour of the coenzymes NAD(P)H and flavins. Dermal signals were found to be a combination of two-photon excited autofluorescence and SHG formation at half the excitation wavelength. The signal of backscattered SHG is stronger than the autofluorescence signal in the upper dermis. Adapted from [2].

Fig. 9. In vivo multiphoton tomography showing the dependence on excitation wavelength. The laser was tuned from 770 nm to 820 nm resulting in a decrease in fluorescence intensity at longer wavelengths. This is in accordance with the absorption behaviour of the coenzymes NAD(P)H and flavins. Dermal signals were found to be a combination of two-photon excited autofluorescence and SHG formation at half the excitation wavelength. The signal of backscattered SHG is stronger than the autofluorescence signal in the upper dermis. Adapted from [2].

The topical creams were applied two times per day on the volar forearm, upper knee and face. The subjects were imaged such that a z-stack from 0 to 200 μm was taken at 350 × 350 μm² using 760 and 820 nm excitation wavelengths. At 760 nm autofluorescence was exclusively measured since any SHG light at 380 nm was absorbed by the optics. At 820 nm however, SHG could be detected and the autofluorescence was due to the absorption characteristics of the main fluorophores strongly reduced. The integrated intensity from collagen SHG and elastin fluorescence was measured in each image. These values were used to calculate the ratio between image intensity of cream A or B treated skin. Variance analysis was used to calculate the differences between pre and post treatment for each cream at a superficial (118–130 μm) and deep dermal range (165–178 μm).

The vehicle only control (cream A) showed no significant changes over the course of the study. However, the dermis of those treated with the active ingredient containing cream B showed statistically significant increased in collagen and elastin signals after 12 weeks of treatment, but not at earlier time points. The greatest improvement in extracellular matrix intensity was found in the deeper dermis. The high-resolution images showed changes in collagen and elastin in vivo with topical treatment. This is the first time that cosmetic effects have been measured in the dermis using non-destructive and non-invasive technologies [36].

2.3. Multiphoton tomography and FLIM of dermatitis patients treated with nanoparticle containing sunscreens

Dermatitis, or inflammation of the skin, can be either acute (atopic dermatitis) or chronic (eczema). Often these terms are interchangeable. Two common causes of acute dermatitis are chemical irritation and allergen contact. Atopic dermatitis is a common cause of dermatology visits, nearly 30%, and the prevalence is increasing [37]. This disease is now recognized as having autoimmune aspects that involve Toll-like receptor 2 signalling induced by the normal bacterial flora on the skin [35,38]. This signalling leads to Th1 but not Th2 activation, resulting in skin inflammation induced epidermal thickening [39].

Imaging technologies, such as multiphoton FLIM tomography, could shed light on the progression of disease and metabolic changes associated with therapy. We have used clinical multiphoton tomography to image several patients suffering from dermatitis from the Department of Dermatology at Münster (Germany) and the Princess Alexandra Hospital Dermatology clinic. Most interestingly, we found a correlation between FLIM data and the level of the disease and FLIM data and therapeutic effects [40,41]. The application of FLIM was technically challenging due to the increased stratum corneum thickness. In Fig. 10 we show examples of three Australian volunteers with atopic dermatitis and the NAD(P)H a1/a2 ratio. We found that the lesional areas contained a spectrum of epidermal morphologies that ranged from those that were similar to non-lesional skin (Fig. 10c), intermediate disorganization (Fig. 10d), to severely disorganized epidermal morphology (Fig. 10e). Noteable morphological differences in atopic dermatitis lesions were increased inter-cellular spacing, loss of the typical hexagonal shape, condensation of the mitochondria, and enlarged nuclei.

The NAD(P)H a1/a2 ratio was consistent in the non-lesional areas, but the neighbouring lesional areas had much greater variance. The lesional coefficient of variation increased 1.6 times in the stratum granulosum, 8.1 times in the stratum spinosum, and 3.6 times in the stratum basale compared to non-lesional areas. These morphological and FLIM data show substantial differences between atopic dermatitis lesion and adjacent non-lesional areas that could be used to help define therapeutic or toxic effects of agents in subjects with atopic dermatitis. Sunscreens containing nanoparticles are an example an area where a topical products’ toxicity is hotly debated [24]. We have used multiphoton tomography-FLIM to evaluate metabolic changes in NAD(P)H on dermatitis lesions and non-lesional areas. Fig. 10g, and h shows one example of this application of multiphoton FLIM tomography. Study subjects with dermatitis lesions (n = 3) applied a formulation

![Image of stratum corneum, stratum spinosum, and dermis with wavelengths 770 nm and 820 nm](image-url)

Fig. 10.
containing 60% ZnO nanoparticles dispersed in caprylic capric triglyceride (CCT), where ZnO nanoparticles are the active ingredients in many sunscreens. The formulation was applied at 14 mg/cm² for 2 h prior to imaging. While there were some minor changes in the a₁/a₂ ratio, 3.54 to 3.37 in non-lesion to lesional areas, respectively, the metabolic rate did appear to be affected. Our conclusion was that the multiphoton-FLIM approach gave valuable insight into the cellular NAD(P)H responses, or lack thereof, to sunscreen application on dermatitis lesions.

2.4. Porphyrin detection after ALA administration

Administration of compounds such as 5-aminolevulinic acid (ALA) and methyl aminolevulinate (MAL) results in selective cellular uptake, in particular by metabolically active cells such as dividing malignant cells. ALA and MAL are pro-drugs that are metabolized within mitochondria into a photosensitive molecule, Protoporphyrin IX (PpIX). ALA in very low concentrations is also a naturally occurring component in our cells as part of the biosynthesis of heme. The concept of ALA based photodynamic therapy (PDT) is the external application of ALA to cancerous tissue followed by exposure to red light that is absorbed by PpIX, resulting in phototoxicity through apoptosis/necrosis. Another application of ALA induced PpIX is the detection and imaging of the relatively highly metabolically active cancerous cells and tumor tissue for tumor diagnostics including tumor margin detection. One-photon fluorescence microscopy has been used to image ALA/MAL induced PpIX, however this method is susceptible to photobleaching and the formation of photoproducts and limited to the low penetration depth of the UV/blue excitation light [42,43]. PpIX fluorescence in cells is relatively weak and can be masked by endogenous autofluorescence [44,45]. To overcome this problem, researchers are exploring the use of two-photon imaging of ALA/MAL induced PpIX fluorescence.

Lu, S. et al. (2008) compared in cell studies the fluorescence detection of PpIX after excitation with both one-photon excitation (SPE) and multiphoton excitation [45]. Fluorescence images were obtained using a laser-scanning microscope. Fluorescence emission was filtered using a bandpass filter from 585 to 640 nm prior to detection. A semi-conductor, argon ion and Ti:sapphire laser source was used for 405 nm, 488 nm and 800 nm excitation, respectively. Samples were prepared by seeding
hepatoma cells on glass slides and incubating directly with PpIX thirty-six hours after seeding. Lu, S. et al [45], showed variations in PpIX fluorescence between one-photon and two-photon excitation. Both 405 and 488 nm SPE resulted in PpIX fluorescence, however 405 nm excitation also resulted in autofluorescence signals of native flavins in the cells. Photobleaching of PpIX fluorescence occurred in both cases (Fig. 11). In contrast, 800 nm two-photon microscopy showed much greater sensitivity and quality of PpIX fluorescence images with less endogenous autofluorescence and photobleaching over the 80 second time frame studied.

In the case of tissue imaging, multiphoton tomography is the superior method to obtain high-resolution images of the intracellular biosynthesis and the accumulation of PpIX in deep-tissue cells.

The DermaInspect system equipped with in-vitro and in-vivo adapters provides non-invasive optical biopsies with high sub-cellular resolution in human subjects, small animals, and cells.

These benefits have been utilized for PpIX imaging within brain tumors both in in vitro cell lines and ex vivo tissue samples [17,44,46,47]. The general consensus from these studies was that multiphoton tomography is an efficient and rapid tool for the study of brain and brain tumor tissue, which allows for the detection of individual tumour cells and tumour cell clusters in native tissue biopsies. This technology provides a non-invasive optical tissue analysis that may potentially be applied to an intra-operative analysis of resection planes in tumor surgery. Multiphoton tomography, therefore, has potential to be used to more clearly define tumor margins in many tissues, in addition to skin.

Kantelhardt, S. R. et al (2008) detected and analysed ALA-induced PpIX fluorescence in both in vitro cell lines and brain tumor tissue using the DermaInspect imaging system [44]. Multiphoton excitation for detection of PpIX fluorescence at different excitation wavelengths was achieved with a red sensitive photomultiplier and an emission filter combination of short pass filters (680 and 750 nm). An orthotopic glioma mouse model was prepared by implanting human glioblastoma derived cell lines G-112 and U87 into the region corresponding to the internal capsule 0.5 mm below the fiber tracts of the corpus callosum. Four weeks post implantation 125 mg/kg ALA was orally administered. After 4 h, tumor infected brains were explanted, coronal sectioned, and imaged using multiphoton tomography. Analysis of the solid tumor fluorescence intensity was compared to normal brain parenchyma and a maximum of the tumor/brain fluorescence intensity ratio was determined. It was reported that at 800–810 nm excitation, the resolution of the intra-tumoral microstructure and a high fluorescence intensity of the tumor/brain ratio provided the highest quality diagnostic information. The use of the multiphoton tomography for detection of ALA induced PpIX fluorescence in brain tumor tissue provides a high resolution diagnostic tool for defining solid tumor margins in brain tissue.

Currently, a multiphoton study on patients with dermatological disorders who obtained topical administration of ALA ointment is performed at the Department of Dermatology at Jena.

2.5. Metabolic corneal imaging

Multiphoton tomography has been used to successfully image NAD(P)H in the cornea for over a decade [48–50]. Fluorescence microscopy has also been used to evaluate NAD(P)H and FAD, e.g. redox state, in cultured corneal epithelial cells by the Chuck laboratory [51,52]. These studies have revealed that metabolic imaging can provide clinically relevant information on corneal storage and health [50–52]. From the outside of the eye inward, the cornea is composed of an epithelium, a collagenous stroma, and a single cell layer of endothelium. This avascular tissue is optically clear, but has a structure similar to skin.

Multiphoton tomography offers the capacity for quantitative study of corneal morphology and NAD(P)H. We have used multiphoton tomography to investigate NAD(P)H in corneas from mouse, rabbit, pig, and man (Fig. 12). The human cornea work was done with approval from Queensland Health, Australia 2007/048, with technical support from Peter Madden, Queensland Eye Institute, Australia. The corneal epithelia NAD(P)H a1/a2 ratio has been shown to be inversely related to the metabolic rate [53]. Our species comparison of the a1/a2 ratio of superficial corneal epithelial cells has revealed that all species have an a1/a2 ratio between 1.0 and 2.5, with rabbit having the highest metabolic rate and man with the slowest, recognizing that the human tissue was stored at 4 °C for longer than the animal tissue. The morphological characteristics of the superficial cornea only differ slightly between species, with the cells maintaining a hexagonal shape, and are similar to the stratum granulosum in skin (Fig. 12). The mean lifetime weighted by the contribution of free and protein-bound NAD(P)H ($t = t_{a1} + t_{a2}$).
was characterized for human (1067 ps), porcine (1284 ps), rabbit (1646 ps), and mouse (1031 ps) corneas.

Eyes can be stored in a moist chamber and kept at 4 °C for up to 10 days (eye banking) before corneal transplant, or keratoplasty, depending on the media and technique. Removing the cornea from the globe, known as corneal buttons, for storage is the preferred strategy for maximizing storage time. Developing storage techniques and media for preserving corneas for an extended period of time is an active area of research [54,55]. This is one area where multiphoton tomography–FLIM of NAD(P)H could be useful for predicting graft survival. This non-invasive technique could be used to examine and quantify the metabolic status of a corneal button prior to transplant or after storage to assess viability. Ideally, both the epithelium and endothelium could be analysed. However, we have found that endothelial imaging was technically challenging, especially in aged and damaged corneas. Therefore, we focused our efforts on the epithelium. We have compared the NAD(P)H lifetime characteristics of fresh human corneas to those that were stored for 1 week and 1 month in a moist chamber (Fig. 13). These data show that the NAD(P)H \( a_1/a_2 \) ratio increases (1.8, 2.6, and 5.1) with storage time (fresh, 1 week, and 1 month), suggesting decreased metabolism with storage. There were also NAD(P)H lifetime changes associated with storage, to \( t_{10} \), 1307.8, 1012.0, and 513.6 for fresh, 1 week, and 1 month, respectively. These data show an overall decrease in NAD(P)H over storage for 1 week. The lack of epithelium in the 1 month corneal button reflects the epithelial delamination that takes place in storage. These data show that multiphoton tomography–FLIM can be used to track and investigate corneal metabolic changes.

Corneal metabolic changes are important for understanding the effects of drug delivery on the metabolic state. One of the more common topical ocular drugs is the fluoroquinolone, ofloxacin. Ofloxacin is used to treat ocular infections and is applied in an eye drop [56]. Although there are side effects, ofloxacin is widely accepted as a safe ocular drug and reaches therapeutic concentration after topical treatment. However, the metabolic effects on healthy, infected, and healing cornea are not known. Multiphoton FLIM tomography has the capacity to shed light on this area of ocular research. In experiments with human corneas, we have found that while there are some changes in the metabolic rate, \( \Delta a_1/a_2 = -0.42 \) or \(-17\%\) (Fig. 14), the corneal endothelial cell metabolism is not grossly affected by acute ofloxacin treatment. This application shows the potential for multiphoton FLIM tomography in assessing ocular drug delivery and toxicity.

2.6. Targeted transfection and optical selection of stem cells

Stem cells have gained interest because of their self-renewal properties, potential to treat diseases, and ability to generate replacement tissues [57]. In recent years, stem cells have become a significant topic in many areas of research including cell biology, medicine, pharmacology, tissue engineering and biotechnology. Many traditional transfection methods such as cationic reagents, liposomes, electroporation, viral, and microinjection are commonly used to deliver genes to large populations of living cells in an untargeted manner. These methods were proven to work effectively and efficiently on many cell types. Unfortunately, these delivery methods are refractory to stem cells given...

Fig. 12. Species differences in NAD(P)H \( a_1/a_2 \) ratios by multiphoton FLIM tomography. Fresh (a) human, (b) porcine, (c) rabbit, and (d) mouse corneas were removed from the globe and mounted into an artificial chamber prior to imaging. Superficial epithelia (214 \( \times \) 214 \( \mu \)m\(^2\)) are shown with intensity images on the left and pseudocolored \((a_1/a_2 \text{ 0--8, blue to red})\). An excitation wavelength of 740 nm was used to excite NAD(P)H and a band pass filter (350–450 nm) to collect a primarily NAD(P)H signal.
their poor efficiencies. Haleem-Smith et al. showed increased transfection efficiency of up to 90% in human mesenchymal stem cells using optimized methods but 50% of the cell population died after treatment [58]. The ability to deliver compounds or genes of interest to genetically modify stem cells, and to subsequently monitor their effects or differentiation pathways without causing damage to the cells has always been a barrier in this area of study. König and coworkers have assessed the potential of transfection using femtosecond laser pulses. Femtosecond laser-based transfection can provide a targeted and sterile delivery of foreign DNA into the cells of interest [59,60]. This method allows non-invasive delivery to cells via the creation of a transient nanohole in the cellular membrane. Through this opening, foreign DNA can diffuse into the cytoplasm before self-repair of the membrane [59]. Besides targeted transfection, femtosecond laser microscopes are also capable of monitoring stem cell differentiation [61] and cellular responses to mechanical stimuli [62], optically eliminate single undesired cells [63], and optoinject payloads [64].

In 2002, Tirlapur and König performed targeted transfection of Chinese Hamster Ovarian (CHO) and rat-kangaroo kidney epithelial (PtK2) cells with a DNA plasmid encoding GFP [63]. The study demonstrated single cell transfection without affecting adjacent cells. This was performed by exposing the edge of the membrane of targeted cells with a high intense femtosecond laser at 800 nm with a mean power of 50–100 mW for 16 ms to allow transfection to take place with an efficiency of 100%. Stem cells and other cell types react more sensitive to NIR femtosecond laser pulses than CHO cells. To increase the survival rate by keeping the transfection efficiency high, the mean laser power should be dropped. This can be realized by the use of extremely short sub-20 fs laser pulses. Unfortunately, nonlinear laser microscopy is limited by the dispersive effect that broadens the pulse and restricts the users from employing the short pulse duration emitting from the laser [64]. To overcome this limitation, we employed 12 fs laser pulses in combination with integrated highly dispersive mirrors to allow compensation of higher-order dispersion. Using this novel nanoprocesing microscope, we successfully delivered DNA plasmid into human stem cells [64]. Our study by Uchugonova et al. (2008) demonstrated that a low mean power of 0.4 mW was sufficient to induce two-photon autofluorescence in mitochondria of human salivary gland stem cells (Fig. 15a–c). Using the same system, we performed a single point illumination of the target cell membrane with a mean power as low as 5–7 mW for 50–100 ms and realized transfection of several types of stem cells with a high 70–80% transfection efficiency. All transfected cells survived the study and demonstrated normal cell division and migration activity (Fig. 15d–f) [61].

The occurrence of spontaneous differentiation in cell cultures can cause difficulties controlling the purity of a stem cell population and limit undesired cell differentiation. We have employed ultrashort near infrared femtosecond laser pulses to optically clean stem cells, i.e. to identify and eliminate undesirable single cells without stem cell clusters (Fig. 16) [63]. Two-photon autofluorescence images of the cell monolayer and 3D/spheroids were obtained for visualization and nanoprocesing of cell population. Optical cleaning or selection was performed using single point illumination or scanning of single cells of interest.

2.7. Optoinjection of molecular beacons to detect intracellular microRNA

Delivering compounds and macromolecules into living cells to understand intricate mechanisms of basic cellular function are desirable in many areas of biological and drug research. The cellular membrane is a very effective barrier that can block out charged or polar materials. Invasive techniques like microinjection function by disrupting the cellular membrane barrier. Most of the techniques, with the exception of microinjection, are applicable to broad ranges of compounds and cell types. However, these methods contribute to substantial cell toxicity. In recent years, interest in optoporation performed by near infrared femtosecond pulse lasers (also known as optoinjection) has increased.

Optoinjection, similar to targeted transfection, facilitates molecule entry into cytoplasm via transient pores that are self repaired. To investigate if optoinjection by near infrared femtosecond pulse laser is effective in the introduction of macromolecules into the cell, we used a fluorescein isothiocyanate (FITC) labelled dextran to detect uptake and 10 mM propidium iodide to determine cell viability after laser exposure [62]. From the study, no non-specific uptake of dextran into cells before optical perforation was found. After optical perforation, 7% of dextran-FITC from the medium was introduced into the cells (CHO and SK-Mel 28). We also performed experiments to show the possibility of transiently perforating single cells within spheroids of carcinoma cells.

![Fig. 13. Effect of hypothermic storage on NAD(P)H in human corneas. Corneas from three different donors were imaged (a) fresh, (b) after 1 week, and (c) after 1 month of storage in a moist chamber. Superficial epithelia are shown with intensity images (left) and pseudocolor (a1/a2 0–8, blue to red, right).](image-url)
without causing photo-induced destruction to adjacent cells. Individual cells found 90 μm deep in the T47D-spheroid were optically perforated with a laser power of 160 mW (270 fs). To optically perforate 25 micrometers deep in a smaller SK-Mel 28-spheroid, a laser power of 70 mW at 800 nm wavelength was sufficient. After optoporation, cascade blue binding was used to demonstrate the efficiency and accuracy of cell perforation by only binding to perforated cells but not to adjacent cells [65].

Using much shorter pulses of about 10 fs, nanoprocessing including nanoporation could be performed at much lower power levels. We were able to perform optoporation with 5 mW mean laser power to transfect DNA plasmid into stem cells and to observe the diffusion of ethidium bromide into the cell and nucleus [61]. Ethidium bromide is an impermeable double stranded nucleic acid dye.

Micro-RNA are small RNA molecules that have been recently discovered and are thought to provide an important post-transcriptional regulatory role for gene expression (MicroRNA expression profiles classify human cancers). We have recently reported using femtosecond laser-enabled optoinjection of molecular beacons to track microRNAs in vitro [66]. The 10-femtosecond laser microscope Femtogene (JenLab GmbH, Germany) was used for optoinjection of super-quencher molecular beacon (SQMB) probes has been reported to study trafficking of mature miRNA-122 in human liver cell lines. Looked Nucleic Acid oligonucleotides (LNAs) were added to SQMB.
probes for in situ detection of selected miRNA. The membrane, nucleus, or both membrane and nucleus of single cells were exposed to three consecutive single point illuminations at 8–10 mW for 0.5 s each time. Post optoinjection, we could identify brightly fluorescing, bound SQMB probes to miRNA-122 in the cytoplasm of Huh-7D12 cells and later in intact nucleus. All diffused probes with or without LNAs reacted and bound to miRNA-122 in the cytoplasm. Negative control cells that do not express miRNA-122 were used to verify that the fluorescence observed was induced by the binding of the optoinjected SQMB probe to miRNA-122 [66].

All optoinjection and transfection experiments were conducted with near infrared femtosecond laser pulse but different laser powers were used due to the difference in pulse width of the laser systems i.e. 170 fs, 250 fs, sub-20 fs, and sub-15 fs. These studies have included appropriate positive and negative controls, and evidence of demonstrating cell viability and effectiveness of optoinjection and transfection via fluorescent probes. These studies showed that femtosecond lasers could be used to successfully optoinject fluorescence probes and transfected cells via an optically induced transient pore without causing significant damage to the cells in the immediate microenvironment. These studies illustrate the delivery potential for near infrared femtosecond laser-mediated techniques with a wide variety of cell lines, including stem cells that do not respond well to traditional transfection methods. Not all cell lines are as robust to optical perforation, and this technique can be limited by delicate cells that often show low cell viability post optoinjection. This technique, at present, is also unable to control the amount or concentration of molecules to be delivered into cell or specific cellular organelles such as mitochondria, cytoplasm and nucleus.

The growing need and interest to deliver broad cell impermeable compounds and macromolecules have led to the development of 3D-nanoprocessing techniques by using ultrashort femtosecond laser pulse and multiphoton microscopy. This non-invasive technique is a promising technology for optical manipulation of cells in a sterile environment. It has the potential for being employed in stem cells research to treat diseases and advance the use of gene therapy, investigate different drug effects in cell lines and even in vivo investigation given their capability to selectively target single cells within tissue structures.

Some studies describe the use of the Bessel beam generated by an axicon in femtosecond laser pulses, its relatively less damaging properties makes it possible to penetrate through obstructing cell layers to reach deeper tissues. For the purpose of optoinjection of cells on culture plates, target cells located along the entire axial distance of the regenerated beam receive equal energy, thus avoiding the need for precision focussing optics [67]. Antkowiak et al. (2010) has advanced the above technique by allowing the control of the lateral and axial positioning of the laser beam using a spatial light modulator (SLM) which acts as a dynamic, diffusive optical element to overcome the problem of exact beam positioning on the cell membrane. The SLM can steer and multiplex an axicon created Bessel beam, enabling increased tolerance of axial positioning and improve transfection range. The incorporation of the SLM provides (i) full control of the focal laser spot with sub-micron precision in a three-dimensional position, enabling users to achieve to consistent and repeatable poration process; (ii) allows controlled application of a timed sequence of doses at different angles, which increases the likelihood of targeting the membrane and optoinjection efficiency from 45% to 72%; (iii) allows implementation of high throughput optoinjection and transfection at an average speed of 1 cell per second; and (iv) performs accurate and selective co-transfection with “point-and-shoot” system of targeted cells. Future advancements of targeted transfection and optoinjection should be achievable with submicron precision, conducted under high throughput configurations and allow co-transfections of multiple genes or optoinjected with multiple drugs.

### 2.8. Targeted drug delivery and disposition in organs

The use of multiphoton tomography to study the delivery of drugs to and disposition in organs is at an early stage. A major advantage of the technique is that it enables the study of dynamic events within normal and diseased organs in vivo in space (3D) so that, with time, 4D imaging is possible and can be done so without fixation. Examples of its
application to various organs include the kidney [68], liver [69] as well as the lung, brain and heart [70].

Our work has utilised the DermaInspect applied to exteriorised organs at low and high magnification. Fig. 17 shows a comparison of fluorescein disposition in rat liver in vivo at low and high magnifications at various times obtained by fluorescence imaging and by multiphoton tomography. Fluorescein moves from the sinusoidal (vascular space) into cells and from thence into bile ducts in between cells (evidenced by strong fluorescence intensity) from where it is excreted. It is evident that multiphoton tomography gives excellent resolution and comparable results with fluorescence microscopy at low magnification but provides superior resolution at higher magnification, without also requiring the fixing and freeze drying used to obtain fluorescence images at longer times. Further, in our analysis, we have been able to use the different fluorescent lifetimes for the fluorescein and its glucuronide metabolite to show that fluorescein uptake into hepatocytes is dominant at early times but that at later times, formation of the glucuronide metabolite makes a significant contribution to the fluorescence seen.

3. Conclusions

Multiphoton tomography and femtosecond laser nanoprocessing microscopes are employed in academic, clinical, and industrial areas. These tools are capable of non-invasive high-resolution in vivo optical biopsies and enable deep-tissue molecular imaging. Multiphoton tomography has been used in a wide variety of academic studies focused on such diverse fields as nanoparticle toxicity testing, dermatological diseases [71], tumor margin delineation in brain, and cornea metabolic imaging. The variety of disciplines that utilize multiphoton tomography illustrate the potential for this novel imaging technology in biomedicine and biotechnology. Of the 992 publications currently available, 75% (746/992) have been published in the last 5 years. This shows the rapid uptake of this technology by researchers and successful application of multiphoton tomography in diverse systems.

Multiphoton tomography provides better resolution than current state-of-the-art skin imaging methods. Markers are not required in contrast to one-photon confocal fluorescence detection. MPT provides also information on the collagen network by the non-linear process of SHG which cannot be generated with confocal on-photon reflectance/fluorescence systems. Multidimensional multiphoton imaging is possible by adding modules for spectral imaging and FLIM (5D-MPT).

Furthermore, MPT can be combined with other skin imaging tools such as ultrasound and OCT (multiplexing). The advantage would be to obtain in a first step wide-field images with depth information down to several hundred micrometers by ultrasound and OCT. In a
second step, MPT can be performed at certain regions of interest with subcellular resolution.

The most exciting hybrid multiphoton tool is the combination of two-photon excited fluorescence, SHG and non-linear Raman techniques like coherent anti Stokes Raman scattering (CARS, [72–74],) which would provide high-resolution morphological information with chemical specificity. Also non-fluorescent and non-SHG-generating biological specimens can be imaged with subcellular resolution. So far, CARS microscopy has only been used for in vivo studies of single cells and of small animals. Interestingly, the first clinical pilot CARS studies in combination with fluorescence/SHG have been performed at the Charite in Berlin, Germany in April 2010[75]. In particular, the lipid distribution in patients suffering from psoriasis as well as the diffusion of oil–water-immersions have been studied.

Also the femtosecond laser microscope as nanoprocessing tool has a great potential to be employed for non-contact highly precise nanosurgery, optical cleaning, optical delivery, and targeted transfection. The use of multiphoton tomography and femtosecond laser nanoprocessing is expected to continue to grow with miniaturization, cost reduction, and improved interfaces and be used extensively for drug delivery research and drug toxicity testing.

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