Published in final edited form as:

J Biophotonics. 2008 December ; 1(6): 451–462. doi:10.1002/jbio.200810045.

Non-invasive multiphoton imaging of extracellular matrix structures

Katja Schenke-Layland

Cardiovascular Research Laboratory, David Geffen School of Medicine at University of California Los Angeles (UCLA), Los Angeles/CA 90095, USA

Abstract

Multiphoton microscopy has become a powerful method for the artifact-free, nondestructive evaluation of deep-tissue cells and extracellular matrix (ECM) structures in their native environment. By interacting with highly non-centrosymmetric molecular assemblies such as fibrillar collagen, the non-linear process called second harmonic generation (SHG) has also proven to be an important diagnostic tool for the visualization of ECM compartments *in situ* with submicron resolution without the need for tissue processing. This review reports on applications of multiphoton-induced autofluorescence and SHG microscopy to identify collagen and elastic fiber orientation in native, tissue-engineered and processed, as well as healthy and diseased, tissues and organs. SHG signal profiling was used to quantify ECM damage in various cardiovascular and exocrine tissues, as well as cartilage. These novel imaging modalities open the general possibility of high-resolution *in situ* and more important *in vivo* imaging of ECM structures, cells and intracellular organelles in living intact tissues.

Heart valve leaflets have a unique extracellular matrix that is organized in distinct layers, which can be non-destructively visualized by multiphoton imaging.

Keywords

second harmonic generation; cardiovascular tissue engineering; heart valve; stem cells; lacrimal gland; cartilage; collagen; elastin

1. Two-photon imaging

Two-photon microscopy has become a powerful method to image living cells with high submicron resolution [1]. Two-photon microendoscope prototypes are able to trace fluorescent proteins in living animals [2]. Most interesting, the first commercial multiphoton tomograph *DermaInspect* allows *in vivo* imaging studies with subcellular resolution in human skin [3]. As previously reviewed, major applications of this novel imaging tool include early cancer diagnostics, the study of the interaction of tissue-engineered products with human tissue, and *in situ* drug screening [4, 5].

In the case of two-photon excited fluorescence, two near-infrared (NIR) photons in the spectral range of 700 – 1200 nm (the spectral range corresponds with the wavelength range of tunable titanium:sapphire lasers) are absorbed simultaneously [5]. Each photon provides half of the energy, which is normally required to excite the fluorophore into a higher electronic state. Therefore, the blue, green, yellow and red emission of fluorophores can be induced with low-energy NIR photons (Figure 1). Multiphoton processes require a high concentration of photons in space and time, and high NIR intensities of at least 100 MW/cm², respectively [4, 5]. Although 100 mW of continuous wave laser radiation in combination with high numerical aperture objectives is sufficient to realize the required high intensity within a tiny focal volume [5, 6], the use of femtosecond laser pulses is the best choice. Mean femtosecond laser powers of less than 2 mW are sufficient when using lasers with high MHz repetition rate. They provide transient GW/cm² intensities for ultra-short time intervals and avoid trapping and heating effects. Typically, 80/90 MHz titanium:sapphire lasers with a pulse width of approximately 100 fs are employed in two-photon scanning microscopes. Using fast x, y galvoscaners and piezo-driven objectives for modification of the z-position, the intra-target location of the multiphoton excitation volume can be changed in all three directions with minimum steps in the submicron range, enabling optical sectioning (three-dimensional (3D) microscopy) with subcellular resolution without the use of confocal detection units (pinholes) [4, 5]. Out-of-focus damage to the sample and bleaching of the fluorophore can be avoided [7]. Also, there is no in-focus damage when using the right laser exposure parameters [8]. The low absorption coefficient is the reason that transient NIR intensities, even as high as GW/cm², can be applied without thermal damage [9].

Major applications of two-photon microscopes include visualization of cells that were transfected with fluorescent proteins [7, 10], 3D microscopy of excised tissue samples such as brain biopsies [11, 12], and detection of intracellular calcium dynamics [13, 14]. Furthermore, two-photon excitation enables the stimulation of a variety of endogenous fluorophores (Table 1). A major fluorophore is the reduced coenzyme NAD(P)H, which emits in the blue/green spectral range with a maximum around 460 nm; therefore unstained cells can be clearly imaged by two-photon excitation [4, 5]. The two-photon-induced autofluorescence images show characteristic fluorescence patterns within the mitochondria and flavoproteins, whereas cell membranes and the nucleus are not fluorescent. Moreover, NIR-induced signals from deep-tissue collagen-rich areas can be obtained by both, autofluorescence and a very special luminescence called second harmonic generation (SHG). SHG occurs only in certain non-centrosymmetric molecules including collagen,

myosin and tubulin when excited with intense ultra-short laser pulses, and it results in the formation of light at exactly half the incident laser wavelength [4, 5, 16, 17].

Both, two-photon-excited autofluorescence and SHG are the major processes behind the principle of multiphoton tomography that do not require tissue processing such as fixation or staining. Deep-tissue imaging of autofluorescent and SHG-active intra-tissue biomolecules with subcellular resolution can be realized non-invasively. Cell division, abnormal cellular and tissue morphology, as well as the elastin and collagen network, can be visualized without taking biopsies [4, 5, 18]. In contrast to routine histopathological techniques, non-invasive 3D high-resolution multiphoton imaging has the advantage of providing immediate access to information on tissue state and structure without tissue removal. Moreover, multiphoton imaging allows the analysis of cells, tissues and organs under natural physiological *in vivo* conditions, and permits long-term studies of the same tissue area, which is of particular interest for *in vivo* drug screening and monitoring of therapeutic effects or of developing *in vitro*-generated constructs [4, 5].

2. Extracellular matrix

The ECM is the substance that underlies all epithelial and endothelial cells (=basement membrane), and is present between various tissue- and organ-specific cells (=interstitial matrix). Gels of polysaccharides and fibrous proteins fill the interstitial space and act as a compression buffer against stress placed on the ECM. The principle fibers are composed mainly of collagens or elastin, while the soluble molecules include proteoglycans and glycoproteins. The structure and function of any tissue and organ depends on the relative proportion of these constituent molecules (Figures 2 and 3). Those tissues that have to withstand large tensional forces tend to be particularly rich in fibrillar collagens and elastin, while tissues that are exposed to compressive forces contain usually high levels of proteoglycans [19]. Collagens are the most abundant and ubiquitous ECM proteins in mammals, which are not only critically important for the biomechanical stability of tissues, but are also intimately involved in cell adhesion and migration during growth, differentiation, morphogenesis and wound healing [20]. Although both fibril- and nonfibril-forming collagens are found in mammalian bodies, the fibril-forming collagens (types I, II, III, V, and XI, XXIV and XXVII) account for the majority of the collagen mass [19, 20].

Structural changes within the ECM fiber network can occur during the course of disease and have been associated with aging. For example, it has been reported that the type I collagen content in tissues increases with age, whereas the type III collagen content is decreased [21, 22]. The proteoglycan content also appears to decrease due to disease or with age [23]. Fragmentation of elastic tissues has been reported for example in aging skin and cardiovascular tissues [24, 25].

Understanding the spatial distribution of ECM components within tissues and organs often provides insight to their function and basic mechanisms of action. Interactions of cells with the ECM are the basis of many fundamental biological processes including the formation and organization of tissue structures (morphogenesis), as well as the remodeling and turnover of the preexisting tissue matrix due to disease or aging processes. A detailed

analysis of the miscellaneous elements of the ECM is of great value in gaining structural and diagnostic information, particularly with regard to tissue engineering efforts.

3. Tissue engineering

The major goal of the multidisciplinary field of tissue engineering is the restoration of tissues and organs damaged by trauma, neoplasm, or congenital deformity through implantation of tissues grown outside the body [26]. The principal idea is the use of either acellular scaffolds made of biopolymers and/or proteins that are formed in the shape of the organ structures to be replaced or decellularized xenogeneic materials [27]. These scaffolds are seeded with preferably autologous cells. Once the cells are attached to the 3D scaffold it is hoped that they produce a cellular and extracellular matrix compound, and the newly generated construct can be used to replace diseased or damaged body parts (e.g. heart valve, blood vessel or skin) [4, 27, 28]. Today the concept of tissue engineering is being applied to a variety of tissues; however, only a few constructs have actually entered clinical trials, including bladder, blood vessels, cartilage and skin [4]. For a successful transition of tissue engineering concepts into clinical applications, further advances in genetics, immunobiology, developmental and stem cell biology, extracellular matrix biochemistry, biomaterials, and diagnostic tools will be required.

3.1. Electrospun scaffolds for tissue engineering

Design and fabrication of scaffolds that mimic the native ECM are key but are also limiting steps for the creation of functional engineered tissues, especially when focused on potential clinical applications. As summarized above, the native ECM is composed of a complex network of structural and regulatory proteins that are arrayed into a fibrous matrix. Electrospinning has been used as an effective method to fabricate biomimetic non-woven scaffolds that are comprised of a large network of interconnected fibers and pores, which allow the efficient exchange of nutrients and metabolic waste between the scaffold and its environment, and provide a high surface area for local and sustained delivery of biochemical signals to the seeded cells [29, 30].

Electrospinning utilizes a high voltage source to inject charge of a certain polarity into a biopolymer and/or protein solution, which is then accelerated toward a collector (target) of opposite polarity. As the electrostatic attraction between the oppositely charged liquid and collector and the electrostatic repulsions between like charges in the liquid becomes stronger, the leading edge of the solution changes from a rounded meniscus to a cone. Eventually, a fiber jet is ejected from the cone as the electric field strength exceeds the surface tension of the liquid. The fiber jet travels through the atmosphere allowing the solvent to evaporate, thus leading to the deposition of solid polymer and/or protein fibers onto a grounded target (Figure 4). Depending on the application, a number of target configurations can be used, including a stationary plate or rotating mandrel. Typically the use of a stationary collector will result in the formation of a randomly oriented fiber mat, whereas a rotating collector can be used to generate mats with aligned fibers. Electrospun fibers typically have diameters on the order of a few micrometers down to the tens of nanometers. The capacity to easily produce materials at this biological size scale has created a renewed interest in electrospinning for applications in tissue engineering and drug delivery

[31]. To date, electrospinning has been used for the fabrication of scaffolds from numerous biodegradable polymers, such as poly(ϵ -caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA) and polyurethane (PU); natural proteins have been used as well including collagen, elastin and gelatin [29–36].

3.2. Heart valve tissue engineering

Transplantation of donor valves derived from humans (allografts) or xenogeneic tissues (xenografts; in most cases glutaraldehyde-fixed porcine or bovine material), or the use of artificial heart valve implants is clinically well established. Both concepts have made a considerable contribution towards decreasing morbidity and mortality, improving the overall quality of life. However, the transplantation of allogeneic valves is strongly restricted by donor shortage and the use of mechanical or biological valve prostheses is flawed by problems including calcification, degeneration and the need for lifelong anticoagulation therapy. None of the currently used replacement concepts possesses growth, repair or remodeling abilities. The interdisciplinary approach of cardiovascular tissue engineering might offer an attractive pathway to overcome these shortcomings and will help to develop viable heart valves identical to natural valves. Characteristics of an ideal valve include the potential to repair and remodel, with no need for long-term anticoagulation therapy, and no immunological response. It is hoped that these biological heart valves continue to grow and remodel inside the body of the patient. Especially in the area of pediatric heart care, this process would imply a significant improvement, as the transplants would no longer have to be replaced repeatedly [4, 27, 28]. Two major concepts of heart valve tissue engineering have been pursued over the past 15–20 years: (i) the first approach involves the implantation of a resorbable acellular matrix, which is expected to repopulate and remodel *in vivo* after implantation; (ii) the second strategy focuses on the implantation of an *in vitro* pre-seeded construct to induce tissue regeneration. Despite a number of very encouraging *in vitro* results, neither of the two approaches led to a valvular construct sufficient for clinical application [4, 27, 28, 37–39].

4. Multiphoton imaging of ECM structures

4.1. Imaging of ECM in embryonic stem cell-derived embryoid bodies

There are many cell types that produce the various ECM proteins found in the mammalian body; on the other hand, due to its diverse nature and composition, the ECM can also regulate the dynamic behavior of cells [40]. During embryonic development, the ECM plays an essential role in cell migration, proliferation, as well as stem cell differentiation into different lineages. The ECM interacts with cells via cell surface receptors such as integrins, serves as a reservoir for growth factors and provides a substrate for cell attachment and spreading [41]. With the establishment of cultures of embryonic stem cells (ESC) that were derived from the inner cell mass of mammalian blastocysts, it became possible to study the behavior of these pluripotent cells *in vitro*, in particular their interplay with exogenous and endogenous ECM proteins and the effect of ECM on ESC self-renewal and differentiation [40–43]. To study cell-cell and cell-matrix interactions during early stages of development, ESC are differentiated into 3D colonies, called embryoid bodies (EBs) because of their ability to mimick post-implantation embryonic tissues [43, 44]. Confocal laser scanning

microscopy has been a very useful tool in obtaining detailed information of matrix protein composition within the developing EBs; however, this imaging technology requires sample processing such as fixation and staining with specific ECM-depicting antibodies, making long-term studies of developing EB cultures impossible (Figure 5). In contrast, non-invasive two-photon-induced autofluorescence and SHG imaging offer the unique possibility to observe stem cell growth, proliferation, differentiation and matrix protein secretion [45]. Based on the excitation of intrinsic fluorophores, long-term monitoring of ESC-derived EB-induced ECM production and turnover is therefore possible under physiological conditions, without the need for staining (Figure 6).

4.2. ECM in healthy and diseased exocrine tissues

Sjögren's syndrome (SS) represents one of the three most common chronic autoimmune disorders and is characterized by three major symptoms: arthritis, dry mouth and dry eye, as originally described by Sjögren in 1933 [46]. So far it has been shown that lymphocytes infiltrate lacrimal glands (LGs) and salivary glands (SGs), and that this infiltration is associated with atrophy and ultimately destruction of the glands [47, 48]. Because the LGs are seriously affected, patients cannot produce reflex tears, resulting in severe damage to the ocular surface; however, the precise mechanisms which trigger initiation of disease and that result in inflammatory infiltration of the LGs are still unknown, although various studies have speculated that alterations in intracellular signaling processes and/or alterations in intracellular trafficking processes are potential culprits [4, 49, 50].

Much of the fundamental architecture and functional properties of exocrine glands, including LGs and SGs, are dictated by the underlying ECM. The ECM provides a substrate for attachment of the acinar epithelial cells within the gland, while providing signaling cues that trigger selective development and differentiation in response to pressures such as cell loss due to necrosis or apoptosis. In fact, the degradation of major ECM proteins including collagens, elastin, various proteoglycans and laminin [47, 51], and the elevated expression of MMPs in LGs [47] and SGs [52, 53] have been implicated as possible contributors to the altered glandular function that gives rise to SS in these organs. In addition to routine histology and immunohistochemistry, tissue state and quality of unprocessed, fresh LG ECM structures were assessed in healthy and diseased LGs using nondestructive two-photon-induced autofluorescence microscopy and SHG imaging [47]. Using these powerful technologies LG collagen bundles and elastin-containing fibers were analyzed while maintaining the structural integrity of the assayed samples in their native state (Figure 7); therefore the possibility of detecting artifacts due to tissue processing (tissue damage caused by fixation or sectioning etc.) can be excluded.

4.3. Imaging of cartilage ECM

Articular cartilage, a specialized form of hyaline cartilage, is formed and maintained by chondrocytes that populate cavities, so called lacunae, within their ECM [54]. The ECM of articular cartilage consists principally of a collagenous framework with interspersed glycoproteins including proteoglycans and water [54, 55]. Many different types of collagen are expressed in articular cartilage including collagen types II, III, IX and XI; however,

collagen type II is the dominant ECM component in healthy mammalian adult articular cartilage [56].

Cartilage has previously been studied using conventional histological and biochemical methods, as well as novel visualization techniques including magnetic resonance imaging (MRI), contrast-enhanced microcomputed tomography (μ CT), Fourier transform infrared imaging spectroscopy (FT-IRIS), and multiphoton microscopy [57–60]. Histology and biochemical assays have been traditionally used to either show the spatial distribution of ECM or to quantify the amount and type of matrix macromolecules in cartilage; however, those methods are rather time-consuming and subject to distortion artifacts and tissue damage. MRI is the most widely-used non-invasive method for the detection of pathologic changes in cartilage that are associated with osteoarthritis. MR-derived parameters including proton relaxation times, water diffusion coefficient, magnetization transfer contrast and fixed charged density can provide insights into tissue property changes including tissue swelling and altered proteoglycan distribution patterns [57, 58]; however, only with high-field strength magnets can detailed information on compositional changes be identified in each individual cartilage zone. Contrast-enhanced μ CT is an x-ray-based imaging modality capable of producing 3D images of the articular surface of cartilage *in situ* and quantitative assessment of relative changes in the sulfated glycosaminoglycan content [60]. However, to compensate for the poor radiopacity of soft tissues such as cartilage, the tissue samples have to be equilibrated in a solution containing an ionic computed tomography contrast agent before μ CT scanning, yielding an equilibrium distribution of the ionic contrast agent that is inversely related to the density of the negatively charged sulfated glycosaminoglycans. This causes, due to an overlap of the x-ray attenuation of bone and the equilibrated cartilage, difficulties in segmenting bone from cartilage in the 3D data sets. Therefore, *in vivo* imaging of ECM changes employing μ CT will be technically highly challenging, if not impossible. FT-IRIS has been used to study changes in the spatial distribution of proteoglycans and collagen within osteoarthritic cartilage [58]. However, in order to semi-quantitatively assess the local proteoglycan content, and to obtain information on collagen fibril orientation, FT-IRIS still requires tissue sectioning and staining, and can therefore only be considered as a valuable complementary imaging technique. Multiphoton-induced autofluorescence and SHG microscopy overcome these limitations, representing true non-invasive high-resolution *in vitro*, *in situ* as well as *in vivo* imaging tools for obtaining a comprehensive picture of cellular and extracellular changes in diseased or damaged cartilage [55, 59]. For example, Mansfield et al. [59] demonstrated the distribution of collagen fibers in *ex vivo* equine articular cartilage and documented the presence of splits in the extracellular matrix, which could be early signs of osteoarthritis. In addition, they imaged pure solutions of proteoglycans, including hyaluronan, chondroitin sulfate and aggrecan; however, further work is needed to identify proteoglycans within intact tissues. Further, two-photon-excited autofluorescence microscopy and quantitative SHG signal profiling were employed to determine whether or not the collagen fiber structure of full thickness porcine articular cartilage was affected by cryopreservation and whether the level of collagen damage could be determined quantitatively in non-fixed, non-sliced, non-stained tissues [55]. Autofluorescence imaging revealed the presence of chondrocytes, as well as collagenous structures in fresh and frozen cryopreserved cartilage samples. SHG microscopy of the

frozen cryopreserved specimens showed a dramatic loss of mean gray value intensities (GVI) when compared to fresh tissues, indicating structural changes of the ECM, in particular the deformation and destruction of the collagen fibers in the analyzed articular cartilage (Figure 8).

4.4. Non-invasive imaging of heart valves

Until recently, most studies in the field of imaging of cardiovascular ECM were performed using technologies which were either unable to give insight into the 3D tissue structure or demand efforts that are often prohibitive to observations within living systems, such as routine histology, immunochemistry or electron microscopy. Two-photon imaging now permits the non-destructive high-resolution detection of molecular interactions of cells with ECM, as well as the qualitative visualization of ECM structures, providing novel insights into the mechanisms of tissue organization and remodeling; making it the technology of choice in particular to probe the architecture, degradation state, and biosafety of engineered tissues [4, 18, 38, 61–64]. The possibility to study the interaction between scaffolds and seeded cells *in vitro* prior to implantation, as well as to monitor the interplay between tissue-engineered construct with the surrounding native tissue *in vivo* is of substantial interest.

In heart valves, the interstitial cells are surrounded by primary ECM components, which perform many essential functions, including mechanical support and physical strength. The valvular ECM is uniquely arranged in leaflet layers to withstand considerable tensile and flexion forces. The ventricular layer (ventricularis; inflow side) faces the ventricular chamber and is comprised predominantly of radially aligned elastic and a few collagenous fibers, covered by an endothelial lining. The inner leaflet layer (spongiosa) is composed of loosely arranged collagen and abundant proteoglycans and glycosaminoglycans. The fibrosa, a thick fibrous layer that provides structural integrity and mechanical stability, is predominantly composed of circumferentially aligned, densely packed collagen bundles and a few elastic fibers. The arterialis is a thin layer that consists of a few collagen fibers lined by endothelial cells along the aortic surface (outflow side) of the leaflet (Figure 2). In order to understand the function of healthy and diseased valve tissues, a complete understanding of the arrangement and modification of the major structural elements, such as elastic and collagenous fibers, is crucial. Accordingly, recent studies focused specifically on the 3D-resolved visualization of native and tissue-engineered valvular matrix components using conventional imaging methods such as histology and immunohistochemical staining, as well as novel technologies such as multiphoton imaging [4, 18, 38, 61, 62]. To distinguish between the different ECM structures two laser excitation wavelengths of 760 nm and 840 nm were employed (Figure 9). By using a wavelength of 840 nm, collagenous fibers were selectively imaged by detection of SHG. In contrast, when using the short pass filter E700SP, which has no transmittance in the UV, and a wavelength of 760 nm, autofluorescent elastic fiber structures were observable [18]. Moreover, using similar parameters (two laser excitation wavelengths of 760 nm and 840 nm), non-invasive multiphoton imaging has been successfully applied for heart valve tissue state diagnosis in tissue-engineered constructs, which revealed a strong impact of varying culture conditions on cellular metabolism and elastin network formation [18]. These findings were virtually impossible to obtain by routine histological staining. Further studies addressed the impact of

cryopreservation on ECM structures of heart valve leaflets [65, 66]. The results of these studies showed that conventional cryopreservation of heart valves is accompanied by serious alterations and destruction of leaflet ECM structures. In contrast to fresh leaflet tissues, no wavy autofluorescent structures became visible by exposure of the cryopreserved tissues to laser pulses at 840 nm, and SHG signals were not inducible. Moreover, multiphoton imaging revealed an alteration of the elastic fiber system within cryopreserved specimens, which was not detectable using routine histology or immunohistochemistry.

A major limitation of multiphoton-induced autofluorescence and SHG imaging exists with regard to the visualization of heart valve ECM structures in chemically fixed tissues such as glutaraldehyde-fixed bioprosthetic valve conduits. Glutaraldehyde cross-links protein molecules and transforms the tissue in an immunological inert material. It reacts with amines and proteins to generate fluorescent products. Due to this fairly uniform and bright fixative-induced fluorescence, inducible using laser excitation wavelengths ranging from 740 nm to 860 nm, the natural fluorescence of collagen or elastic fibers can no longer be determined and distinguished by switching excitation wavelengths.

5. Conclusion

Multiphoton imaging technologies permit non-invasive high-resolution *in vitro*, *in situ* and more important *in vivo* imaging of ECM structures and cells in living intact native and tissue-engineered tissues; however, a number of challenges still need to be met. The time that is needed to acquire single images using a multiphoton system is, when compared to routine fluorescence microscopes, rather lengthy. In addition, where measurements are required *in vivo*, problems may be encountered with patients due to natural tissue movement (pulse, etc.), which may reduce the quality of the obtained images. In this situation, one must decide between the quality of the diagnostic image and the interferences met in clinical settings. This problem might be resolved by using more flexible hand-held devices. Moreover, due to the use of high-numerical aperture objectives with high magnifications, the scanned area is relatively small and single images have to be assembled in order to obtain overviews of larger samples. In order to address these problems, intense research efforts are focused on the development of smaller and more powerful turnkey laser systems. Furthermore, multiphoton mini-endoscopes are under development, which can be applied to internal organs and intra-articular spaces, rather than examining excised tissues using bulky nonlinear optical microscopes. These novel tools will revolutionize early diagnosis of tissue disorders, open the possibility of *in situ* quality assessment of grafts prior implantation and enable the *in vivo* analysis of implanted tissue-engineered constructs.

Acknowledgments

The author would like to thank Shannon Layland for his helpful suggestions, as well as Dr. Ekaterini Angelis, Dr. Kelvin G.M. Brockbank, Yekaterina Butylkova, Ilka Degenkolbe, Jens Geiling, Prof. Dr. Sarah Hamm-Alvarez, Dr. Sepideh Heydarkhan-Hagvall, Sabine Hitschke, Prof. Dr. Karsten König, Dr. W. Robb MacLellan, Dr. Hanna K. Mikkola, Dr. Iris Riemann, PD Dr. Ulrich A. Stock and Dr. Jiansong Xie for their contributions and thoughts. The author is grateful for the financial support by the Deutsche Forschungsgemeinschaft (DFG, grant numbers: Sche701/2-1 and STO359/7-1) and the National Institutes of Health (NIH, grant number: 5T32HL007895-10). Confocal laser scanning microscopy was performed at the CNSI Advanced Light Microscopy/Spectroscopy Shared Resource Facility at UCLA, supported with funding from NIH-NCRR shared resources grant (CJX1-443835-WS-29646) and NSF Major Research Instrumentation grant (CHE-0722519).

References

1. Denk W, Strickler JH, Webb WW. Science. 1990; 248:73–76. [PubMed: 2321027]
2. Flusberg BA, Jung JC, Cocker ED, Anderson EP, Schnitzer MJ. Opt Lett. 2005; 30(17):2272–2274. [PubMed: 16190441]
3. König K, Riemann I. J Biomed Opt. 2003; 8:432–439. [PubMed: 12880349]
4. Schenke-Layland K, Riemann I, Damour O, Stock UA, König K. Adv Drug Deliv Rev. 2006; 58(7): 878–896. [PubMed: 17011064]
5. König K. J Biophoton. 2008; 1:13–23.
6. König K, Liang H, Berns MW, Tromberg BJ. Nature. 1995; 377:20–21. [PubMed: 7659153]
7. König K. J Microsc. 2000; 200:83–104. [PubMed: 11106949]
8. König K, So P, Mantulin WW, Tromberg BJ, Gratton E. Opt Lett. 1997; 22:135. [PubMed: 18183127]
9. Squirrell JM, Wokosin DL, White JG, Bavister BD. Nature Biotechnol. 1999; 17(8):763–767. [PubMed: 10429240]
10. Kawano H, Kogure T, Abe Y, Mizuno H, Miyawaki A. Nature Methods. 2008; 5(5):373–374. [PubMed: 18446153]
11. Levene MJ, Dombeck DA, Kasischke KA, Molloy RP, Webb WW. J Neurophysiol. 2004; 91:1908–1912. [PubMed: 14668300]
12. Helmchen F, Fee MS, Tank DW, Denk W. Neuron. 2001; 31:903–912. [PubMed: 11580892]
13. Stosiek C, Garaschuk O, Holthoff K, Konnerth A. PNAS. 2003; 100(12):7319–7324. [PubMed: 12777621]
14. Schenke-Layland K, Rhodes KE, Angelis E, Butylkova Y, Heydarkhan-Hagvall S, Gekas C, Zhang R, Goldhaber JI, Mikkola HK, Plath K, MacLellan WR. Stem Cells. 2008; 26(6):1537–1546. [PubMed: 18450826]
15. Vishwasrao HD, Heikal AA, Kasischke KA, Webb WW. J Biol Chem. 2005; 280(26):25119–25126. [PubMed: 15863500]
16. Zoumi A, Yeh A, Tromberg BJ. Proc Natl Acad Sci U S A. 2002; 99(17):11014–11019. [PubMed: 12177437]
17. Zipfel WR, Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW. Proc Natl Acad Sci U S A. 2003; 100(12):7075–7080. [PubMed: 12756303]
18. König K, Schenke-Layland K, Riemann I, Stock UA. Biomaterials. 2005; 26(5):495–500. [PubMed: 15276357]
19. Silver FH, DeVore D, Siperko LM. J Appl Physiol. 2003; 95:2134–2141. [PubMed: 14555674]
20. Leitinger B, Hohenester E. Matrix Biology. 2007; 26(3):146–155. [PubMed: 17141492]
21. Mays PK, Bishop JE, Laurent GJ. Mech Ageing Dev. 1988; 45:203–212. [PubMed: 3266279]
22. Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, Schoen FJ. Circulation. 2006; 113:1344–1352. [PubMed: 16534030]
23. Schuckett R, Malesud CJ. J Rheumatol. 1989; 17:357–363.
24. Lavker RM, Zheng P, Dong G. J Invest Dermatol. 1987; 88:44s–51s. [PubMed: 3546515]
25. Haas G. Arch Pathol. 1943; 35:29–45.
26. Vacanti JP, Langer R. Lancet. 1999; 354:SI32–SI34. [PubMed: 10437854]
27. Schmidt D, Stock UA, Hoerstrup SP. Philos Trans R Soc Lond B Biol Sci. 2007; 362(1484):1505–1512. [PubMed: 17588875]
28. Stock UA, Vacanti JP Jr, Mayer JE, Wahlers T. Thorac Cardiovasc Surg. 2002; 50(3):184–193. [PubMed: 12077696]
29. Heydarkhan-Hagvall S, Schenke-Layland K, Dhanasopon AP, Rofail F, Smith H, Wu BM, Shemin R, Beygui RE, MacLellan WR. Biomaterials. 2008; 29(19):2907–2914. [PubMed: 18403012]
30. Venugopal J, Low S, Choon AT, Ramakrishna S. J Biomed Mater Res B Appl Biomater. 2008; 84(1):34–48. [PubMed: 17477388]
31. Sill TJ, von Recum HA. Biomaterials. 2008; 29(13):1989–2006. [PubMed: 18281090]

32. Boland ED, Matthews JA, Pawlowski KP, Simpson DG, Wnek GE, Bowlin GL. *Frontiers in Bioscience*. 2004; 9:1422–1432. [PubMed: 14977557]
33. Zhang Y, Ouyang H, Lim CT, Ramakrishna S, Huang ZM. *J Biomed Mater Res B Appl Biomater*. 2005; 72(1):156–165. [PubMed: 15389493]
34. Bhattarai SR, Bhattarai N, Yi HK, Hwang PH, Cha DI, Kim HY. *Biomaterials*. 2004; 25:2595. [PubMed: 14751745]
35. Heydarkhan-Hagvall S, Schenke-Layland K, Yang JQ, Heydarkhan S, Xu Y, Zuk PA, MacLellan WR, Beygui RE. *Cells Tissues Organs*. 2008; 187(4):263–274. [PubMed: 18196894]
36. Del Gaudio C, Grigioni M, Bianco A, De Angelis G. *Int J Artif Organs*. 2008; 31(1):68–75. [PubMed: 18286457]
37. Leyh RG, Wilhelmi M, Rebe P, Fischer S, Kofidis T, Haverich A, Mertsching H. *Ann Thorac Surg*. 2003; 75:1457–1463. [PubMed: 12735562]
38. Schenke-Layland K, Opitz F, Gross M, Döring C, Halbhuber KJ, Schirrmeister F, Wahlers T, Stock UA. *Cardiovasc Res*. 2003; 60(3):497–509. [PubMed: 14659795]
39. Vesely I. *Circ Res*. 2005; 97(8):743–755. [PubMed: 16224074]
40. Flaim CJ, Chien S, Bhatia SN. *Nat Methods*. 2005; 2:119–125. [PubMed: 15782209]
41. Chen SS, Fitzgerald W, Zimmerberg J, Kleinman HK, Margolis L. *Stem Cells*. 2007; 25(3):553–561. [PubMed: 17332514]
42. Rossant J. *Cell*. 2008; 132(4):527–531. [PubMed: 18295568]
43. Schenke-Layland K, Angelis E, Rhodes KE, Heydarkhan-Hagvall S, Mikkola HK, MacLellan WR. *Stem Cells*. 2007; 25(6):1529–1538. [PubMed: 17363553]
44. Keller G. *Genes Dev*. 2005; 19:1129–1155. [PubMed: 15905405]
45. Uchugonova A, König K. *J Biomed Opt*. 2008 in press.
46. Sjögren H. *Acta Ophthalmol*. 1933; 2:1–151.
47. Schenke-Layland K, Xie J, Angelis E, Starcher B, Wu K, Riemann I, MacLellan WR, Hamm-Alvarez SF. *Matrix Biol*. 2008; 27(1):53–66. [PubMed: 17689946]
48. Matsumoto I, Tsubota K, Satake Y, Kita Y, Matsumura R, Murata H, Namekawa T, Nishioka K, Iwamoto I, Saitoh Y, Sumida T. *J Clin Invest*. 1996; 97(8):1969–1977. [PubMed: 8621782]
49. Tsubota K, Hirai S, King LS, Agre P, Ishida N. *Lancet*. 2001; 357(9257):2054–2055.
50. da Costa SR, Wu K, Pidgeon M, Ding C, Schechter JE, Hamm-Alvarez SF. *Exp Eye Res*. 2006; 82(1):33–45. [PubMed: 16005870]
51. Molina C, Allende C, Aguilera S, Kwon YJ, Leyton L, Martinez B, Leyton C, Perez P, Gonzalez MJ. *Ann Rheum Dis*. 2006; 65:178–183. [PubMed: 16014676]
52. Konttinen YT, Halinen S, Hanemaaijer R, Sorsa T, Hietanen J, Ceponis A, Xu JW, Manthorpe R, Whittington J, Larsson A, Sali T, Kjeltzen L, Stenman UH, Eisen AZ. *Matrix Biol*. 1998; 17:335–347. [PubMed: 9822200]
53. Perez P, Goicovich E, Allende C, Aguilera S, Leyton C, Molina C, Pinto R, Romo R, Martinez B, Gonzalez MJ. *Arthritis Rheum*. 2000; 43(12):2807–2817. [PubMed: 11145040]
54. Williams GM, Klisch SM, Sah RL. *Pediatr Res*. 2008; 63(5):527–534. [PubMed: 18427298]
55. Brockbank KG, MacLellan WR, Xie J, Hamm-Alvarez SF, Chen ZZ, Schenke-Layland K. *Cell Tissue Bank*. 2008 in press.
56. Eyre DR, Weis MA, Wu JJ. *Eur Cell Mater*. 2006; 12:57–63. [PubMed: 17083085]
57. Gray ML, Burstein D, Kim YJ, Maroudas A. *J Orthop Res*. 2008; 26(3):281–291. [PubMed: 17876836]
58. Bi X, Yang X, Bostrom MP, Bartusik D, Ramaswamy S, Fishbein KW, Spencer RG, Camacho NP. *Anal Bioanal Chem*. 2007; 387(5):1601–1612. [PubMed: 17143596]
59. Mansfield, JC.; Winlove, CP.; Knapp, K.; Matcher, SJ. *Multiphoton Microscopy in the Biomedical Sciences VI*. In: Periasamy, A.; So, PTC., editors. *Proc of SPIE*. Vol. 6089. 2006. p. 608910
60. Palmer AW, Guldberg RE, Levenston ME. *Proc Natl Acad Sci USA*. 2006; 103(51):19255–19260. [PubMed: 17158799]
61. Schenke-Layland K, Riemann I, Opitz F, König K, Halbhuber KJ, Stock UA. *Matrix Biol*. 2004; 23(2):113–125. [PubMed: 15246110]

62. Schenke-Layland K, Riemann I, Stock UA, König K. J Biomed Opt. 2005; 10(2):024017. [PubMed: 15910091]
63. Zoumi A, Lu X, Kassab GS, Tromberg BJ. Biophys J. 2004; 87(4):2778–2786. [PubMed: 15454469]
64. Douma K, Megens RT, Reitsma S, Prinzen L, Slaaf DW, van Zandvoort MA. Micros Res Tech. 2007; 70(5):467–475.
65. Schenke-Layland K, Madershahian N, Riemann I, Starcher B, Halbhuber KJ, König K, Stock UA. Ann Thorac Surg. 2006; 81(3):918–926. [PubMed: 16488695]
66. Schenke-Layland K, Xie J, Heydarkhan-Hagvall S, Hamm-Alvarez SF, Stock UA, Brockbank KG, MacLellan WR. Ann Thorac Surg. 2007; 83(5):1641–1650. [PubMed: 17462373]

Biography

Curriculum Vitae

Katja Schenke-Layland received a master of science (M. Sc.) degree in biology, psychology and sociology in 2001; and her doctorate degree (Dr. rer. nat.) in biology in 2004 from the Friedrich Schiller University (FSU) Jena, Germany. She worked at the Departments of Anatomy and Cardiothoracic Surgery at the FSU Jena, the Saban Research Institute at Children's Hospital Los Angeles (CHLA), and is currently appointed at the David Geffen School of Medicine at University of California Los Angeles (UCLA). Her research interests include stem cell, extracellular matrix and developmental biology, cardiovascular research, biomaterial science, tissue engineering, and biomedical imaging. She has received several honors and prizes, including the Prize of the Klee Family Foundation awarded by the German Association of Biomedical Engineering (DGBMT), and awards from the International Society for Optical Engineering (SPIE), International Society of Heart Valve Disease, and the National Institute of Biomedical Imaging and Bioengineering (NIBIB).

Figure 1.

Principle of two-photon excitation. If the energy densities in the focal volume of a laser beam are high enough, a two-photon process can be generated. Instead of one-photon absorption with UV/blue light, two photons of less energy, but higher wavelength, can be absorbed simultaneously by a fluorophore. The state of electrons change to a higher level, which leads to emission of fluorescence in the visible (VIS) when the higher state is reduced back to normal. In addition, SHG can be generated at half the incident laser wavelength ($\lambda/2$).

Figure 2.

Heart valve leaflets have a unique extracellular matrix that is composed of fibrillar and non-fibrillar proteins as well as proteoglycans, which are organized within distinct layers. The leaflet matrix structures can be non-destructively visualized by multiphoton imaging using excitation wavelengths of 760 nm (cells and elastic fibers; green) and 840 nm (collagenous structures; red). Scale bar equals 20 μm.

Figure 3.

Extracellular matrix distribution in human cornea. **(A)** Movat-pentachrome staining reveals the presence of collagen (yellow), elastic fibers (black), glycosaminoglycans (blue to green), and corneal epithelium (purple to black). Scale bar equals 200 μm . **(B)** Corneal epithelial cells and extracellular matrix structures can be displayed by multiphoton-induced autofluorescence imaging using excitation wavelengths of 740 nm (cells and elastic fibers; green) and 840 nm (collagenous structures; red). Scale bar equals 20 μm .

Figure 4.

(A) Schematic of a typical electrospinning system. (B) Fiber composition and quality in electrospun collagen/elastin-based hybrid scaffolds can be non-invasively monitored using multiphoton-induced autofluorescence microscopy. Using this high-resolution *in situ* imaging tool, comprehensive pictures can be obtained, revealing scaffold integrity (a–d) or damage (e–h). Excitation wavelengths of 740 nm depict elastin-containing fibers (green); whereas wavelengths of 840 nm show collagen-containing structures (red). DIC = transmitted light-differential interference-contrast. Scale bar shown applies to all images and equals 50 µm.

Figure 5. Confocal laser scanning microscopic image of an embryoid body (EB) after 3 days in culture, labeled with antibodies against the proteoglycan decorin (green) and F-actin (red). Cell nuclei are stained with DAPI (blue). Scale bar equals 50 μm.

Figure 6.

Transmitted light-differential interference-contrast (DIC) and autofluorescence images show undifferentiated ESC and differentiating ESC-derived embryoid bodies (EBs) at day 3 and 6 of *in vitro* culture. Excitation wavelengths of 760 nm induce cellular autofluorescence (green). Exposure of the 6-day-EB to wavelengths of 840 nm reveals the beginning secretion of (pro)collagen (red). Note a diminished autofluorescence in the more differentiated cells (EBs day 6) when compared to undifferentiated ESC and cells within early EBs at day 3 of culture. Scale bar equals 40 µm.

Figure 7. Multiphoton-induced autofluorescence images of freshly dissected, untreated lacrimal glands (LGs) of 18 week old (A) healthy BALB/c and (B) diseased NOD mice using two excitation wavelengths of 740 nm (elastin and acinar cells; green) and 860 nm (collagen; red). (C) Schematic view of the human lacrimal apparatus. Scale bar equals 20 μm.

Figure 8.

(A) Multiphoton-induced autofluorescence images showing chondrocytes in lacunae (740 nm) and collagen structures (840 nm) within fresh and frozen cryopreserved porcine articular cartilage. Scale bar equals 50 μm . (B) SHG signal profiling of fresh and frozen cryopreserved cartilage specimens. The graphs represent peak SHG intensities, expressed as gray value intensities (GVI), of collagenous structures (ROI 1, red), as well as peak autofluorescence intensities of cells (ROI 2, green) in the corresponding lambda stack overlay image, denoted by the red (ROI 1) or the green (ROI 2) cross. Note that sufficient autofluorescence and SHG signals were only inducible in fresh tissue structures.

Figure 9. Heart valve leaflet ECM structures of the collagen-rich outflow and the predominantly elastin-containing inflow side can be visualized using two-photon-induced autofluorescence imaging. Elastic fibers and cells are visualized using an excitation wavelength of 760 nm, whereas collagen-containing fibers are imaged with a wavelength of 840 nm.

Table 1

Overview of excitation, emission and fluorescence maxima of a variety of fluorescent biomolecules.

Fluorophore	Excitation λ (nm)	Emission λ (nm)	Fluorescence Lifetime τ (ns)
Keratin		525	
NAD(P)H	340	450 – 470	0.3–0.6*
Flavins	370, 450	530	5.2
Melanin	UV/VIS	440, 520, 575	0.2/1.9/7.9
Elastin	300 – 340	420 – 460	0.2 – 0.4/0.4 – 2.5
Collagen	300 – 340	420 – 460	0.2 – 0.4/0.4 – 2.5
SHG	720 – 960	360 – 480	0
Lipofuscin	UV/VIS	570 – 590	
PPIX	405 ... 630	635, 710	11

* The lifetime of NAP(P)H is complex. The components cover the range 0.3–0.6 ns depending on its conformation and whether it is free or bound [15].