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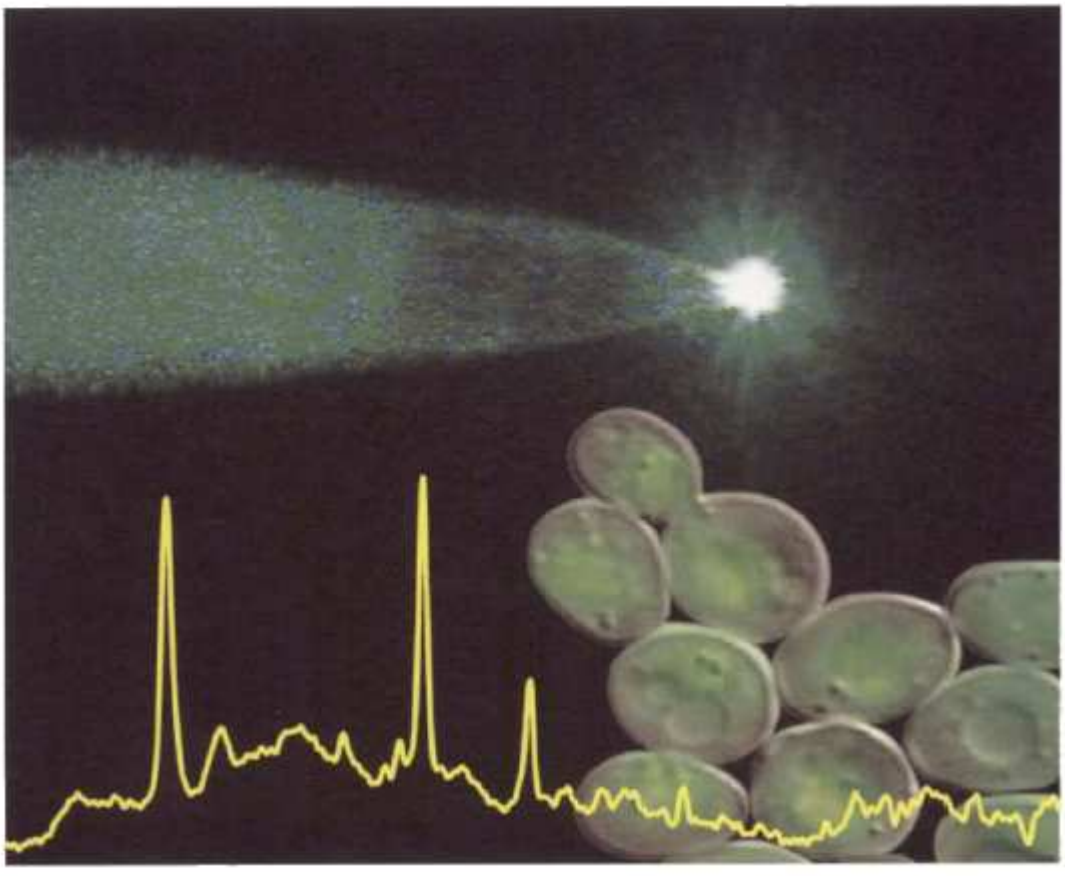
Biophotonics

Visions for Better Health Care



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Regenerative Surgery (MeMo)

Volker Andresen, Heinrich Spiecker, Jörg Martini, Katja Tönsing, Dario Anselmetti, Ronald Schade, Steffi Grohmann, Gerhard Hildebrand, Klaus Liefeith[1]

7.1
Regenerative Surgery and Tissue Engineering: Medical and Biological Background

Owing to the tremendous advances in health care and the demographic changes that began in the 20th century, there is an increased awareness in the medical profession of the need to address the high demand for the replacement or repair of organs. While there has been great progress in the pharmacological treatment of patients for a variety of conditions since the 1970s, tissue engineering has initiated a new era in the field of regenerative medicine. In contrast to the repair/healing (i.e., *reparatio*) its focus is on the *restitutio ad integrum* – the restitution of the organ with the complete resumption of the organ's function. Regenerative medicine benefits from the prospering research in the fields of tissue engineering, biomaterial science, bio- and nanotechnology, stem-cell biology/cloning, proteomics and genomics.

The triad of trauma, aging and disease accounts for the either partial or complete loss of tissues and organs. Most types of tissue possess a limited regenerative potential, thus a spontaneous healing process is possible. However, when the degree of damage passes a certain level, or if the tissue does not exhibit a regenerative potential (for example nerve or cartilage cells), the damaged tissue has to be replaced.

Tissue engineering provides a new approach for regenerative medicine by using autologous tissue, while overcoming the fatal donor site morbidity. In general, autologous cells, isolated from only a small biopsy taken from the patient, are expanded *ex vitro* and are then re-implanted into the cleaned de-

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fective site. Considering the fact that tissue engineering can be regarded as the regeneration of biological tissues through the use of cells with the aid of supporting structures (scaffolds) and appropriate bioreactors great efforts were made to extend the potential applications of tissue engineering to nearly all types of tissues:

- Skin substitutes
- Cardiovascular substitutes
- Substitutes of the peripheral nervous system
- Soft tissue substitutes (e.g. breast implants)
- Organs (like kidney, liver and lung)
- Orthopaedic cartilage and bone replacement

Cartilage is a highly specialized tissue with unique properties regarding stiffness, elasticity and friction. This tissue guarantees the movements of the skeleton apparatus with low friction forces at joints. Since 95% of the articular cartilage is composed of extracellular matrix (ECM) its biofunctional properties are mainly determined by the chemical composition of the ECM: water (60-80%), collagenes (collagen type II: 10-20% and minor parts of collagenes type V, VI, XI, X, XI), proteoglycans (aggrecan including keratan and chondroitin sulfate: 5-7%), non-collagenous proteins (for example link proteins and fibronectin) and other components like hyaluronic acid, lipids and glycoproteins [1]. The chondrocytes are embedded in a three-dimensional network of collagen fibrils which is highly organised (Fig. 1.1) and forms the hyaline cartilage representing healthy and biofunctional cartilage. A more detailed review is given in [2]. Thereby the biochemical structure ensures the biophysical tasks of cartilage. Changes in the cartilage composition due to aging or pathological processes (arthrosis) and injuries lead to the loss of biofunctionality being one of the largest medical problems and afflictions especially of the elderly [3]. Unfortunately the regeneration of cartilage tissue in humans is restricted due to the failure of proliferation and a reduced capacity of adult chondrocytes for a turnover of matrix components. The conventional treatment of cartilage damages involves bone marrow stimulating techniques (microfracturing, pridie-drilling and abrasion plastic), the transplantation of autologous osteochondral cylinders (OCT), and eventually the replacement by an artificial joint. These techniques did not enable physicians to treat arthrosis and osteoarthritis satisfyingly and in many cases additional treatment was necessary. Thus, therapies require new methods like tissue engineering approaches.

Applying the tissue engineering approach expanded autologous chondrocytes are either directly injected into the defective site [4] or they are cultured

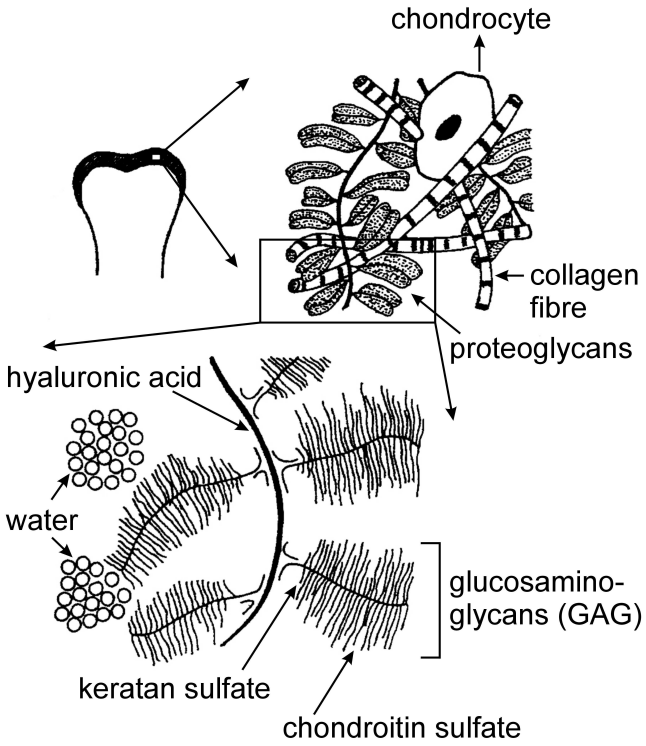

Fig. 1.1 Organisation of the extracellular matrix (ECM) within cartilage tissues (modified from [8]).

on three-dimensional carrier systems (i.e. scaffolds) and/or stimulated before re-implantation into the joint (also see 8.3) [5, 6, 7]. The quality of engineered cartilage is determined by the cells ability to synthesize the extracellular matrix (ECM). There has to be a well balanced production of collagens and proteoglycans in order to withstand mechanical loading in the transplantation site. From the medical point of view it is crucial to provide implantable chondrocytes which are able to rebuild new functional cartilage tissue at the defect site. Since many tissues are mechanically challenged, in principal tissue engineered constructs have to guarantee the same. Therefore functional requirements have to be taken into account in culture to be able to engineer cartilage tissue with an optimised biological, chemical, and morphological performance and above all with an appropriate stress-strain behaviour that can tolerate expected in vivo loads. To avoid the re-implantation of ineffective chondrocyte populations into the defect site it would be helpful to determine the needed cell number and cartilage specific differentiation of in vitro expanded chondrocytes before re-implantation in terms of a quality control.

Current techniques for a quality control solely comprise dead-end procedures like (immuno) histology, gene expression profiling by PCR and biochemical analyses. Hitherto the laser scanning microscopy has not been taken into account as a promising controlling tool but there is no doubt that this non-invasive optical technique possesses a tremendous potential in comparative validation of cartilage specific components and cell populations. Therefore it is worthwhile to discuss the enormous impact of optical technologies and especially of the Two Photon Laser Scanning Microscopy (TPLSM) as a new alternative and a promising approach for the minimally-invasive and online quality control of 3D-tissue engineered constructs.

1.2 State-of-the-art and markets

Statistical surveys have shown, that approximately 20 million people were treated with implanted medical devices. The associated costs for prostheses and organ replacement therapies exceed 300 billion U.S. dollars per year, corresponding to nearly 8% of the total healthcare spending worldwide [9]. Over the past decades the proportion of the world's population formed by the elderly has risen dramatically. The discovery of antiseptics, penicillin, improved hygiene, and vaccination on the one hand and the introduction of new immunosuppressant regimes including improvements in post-surgical care on the other hand, have established transplantation as the "golden standard" to successfully replace tissues often as a life-saving procedure for patients with severe organ failure. Traditionally organ transplantation is employed for the replacement of diseased tissue. There are four available sources of tissue for transplantation:

1. autologous (same body)
2. allogenic (intra species)
3. xenogenous (inter species)
4. artificial organs (for example implants, kidney, heart)

Autologous material is the most preverable source but it is only available in very limited amounts. Additionally the donor site morbidity is a negative side-effect, causing pain or even malfunction of the donor tissue. Though the availability of allogenic material is less limited, there are still long waiting periods for suitable donor organs. Furthermore the restricted survival time of donor tissues exacerbates the supply of donor organs for patients around the world. According to the United Network of Organ Sharing (UNOS), 3,216

possible donor tissues could not be transplanted in 2004 due to the short time window for a functional transport from the donor to the recipient [10]. Owing to the risk of rejection and infection, the disadvantages of an immunosuppressive treatment have to be well balanced with the estimated effort of the transplantation. While the availability of xenogenous material is almost unlimited its actual application is unfavourable for ethical reasons, difficulties with its compatibility, risk of rejection and transmission of infection and/or disease to the patient.

Though there was good progress in stem cell research their use (especially for embryonic stem cells) is still controversially discussed through out the world. Nevertheless adult and mesenchymal stem cell may be a possible cell source for regenerative medicine in the future.

Another concept is based on the implantation of artificial, man-made materials such as high technology polymeric, metallic, and ceramic materials. Medical devices or prostheses made of these materials serve the affected patients well for extended periods by alleviating the conditions for which they were implanted. Despite the fact, that the longevity and the quality of life are clearly improved for patients with prostheses /implants the long-term failure of artificial biomaterials can lead to a clinically significant event caused by adverse effects or foreign body reactions under certain very specific conditions. According to Hench [11] the inability for self-repair and the missing potential of artificial biomaterials to respond to environmental factors such as mechanical stimuli were identified as main drawbacks associated with a certain ratio of benefit to risk.

Taking into consideration these developments tissue engineering offers a compelling new approach to the still existing major problems. Basically, tissue engineering can be defined as the application of engineering principles to biology, for the purpose to construct three-dimensional functional tissues. A more detailed definition was given by Skalak and Fox [12] as “the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure - function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain, or improve tissue function”. In contrast to the hitherto discussed approaches tissue engineering is directed to the complete regeneration of natural tissues. According to the particular case this process comprises in vitro or combined in vitro and in vivo approaches leading to the implantation of biological substitutes at the diseased site to achieve full functionality. Obviously this comprises the restoration of structure, function, metabolic and biochemical behaviour as well as the restoration of the biomechanical performance. Thus, there can be no doubt that the basic concept of “regenerative medicine” will envisage a completely new form of therapy with the potential to change the medical practice significantly.

It's of value to point out, that there are two major scientific challenges to reach this goal. First the development of biomaterials that enhance the body's own reparative potential and secondly the availability of a technical system which allows the ex vivo cultivation of cell seeded scaffold materials under conditions that mimic as closely as possible the natural process of tissue formation. Such systems, usually referred to as bioreactors, offer in principle the possibility to perform a static or alternatively a dynamic cell cultivation process under controlled biochemical and biomechanical conditions. Despite the fact that bioreactors possess, according to nature, a certain potential to enable a real large-scale expansion of cells because they provide excellent possibilities to guarantee an uniform mixing and precise control over mass transfer rates, pH-values and oxygen consumption rates and the maintenance of optimal nutrient levels, it should be accepted that the whole process of tissue formation and regeneration remains unexplored up to certain extent [13, 14].

Still existing difficulties include the insufficiently detailed understanding of cell-molecular control processes like the regulation of matrix formation by highly specific signalling pathways, pattern formation, and tissue/organ morphogenesis.

Therefore it can be concluded that innovative non-invasive detection methods are necessary to enable a deeper understanding of all subcellular (molecular), cellular and supracellular processes which may occur during a three-dimensional tissue formation. Indeed, optical methods like the TPLSM comply with the most important demands and provide the necessary spatial and time resolution to be able to detect metabolic pathways of newly formed tissues on a molecular level. The second scientific challenge mentioned above is the use of biomaterials as scaffolds and carrier of cells, proteins, genes and growth factors. Due to the fact that the new formed tissue compartments should gradually replace the scaffold material to enable a nearly complete tissue regeneration biodegradable or bioresorbable materials will be employed. Examples are

- polymeric scaffold materials (natural polymers [for example collagen, hyaluronic acid], synthetic polymers [like poly(-hydroxy acid, polyphosphazenes]),
- ceramic scaffold materials (CaP materials [like hydroxyapatite, glass-ceramics, tricalcium phosphate, octacalcium phosphate]),
- metallic scaffold materials (like metallic foams based on Mg-alloys)

and different composites made off the various materials. It should be mentioned that also non-biodegradable materials are used especially in load-bearing situations due to their superior mechanical properties.

However, recently published studies [15] have shown that an appropriate scaffold material alone is not able to provide an engineered tissue construct with a physiological relevant architecture and composition. To enable this often external stimuli are needed like electrical or mechanical stimuli, which can be applied by means of specifically designed bioreactors. A very comprehensive and illustrative overview about the current status of bioreactor development and related biochemical and biophysical stimulation techniques is given by Müller [2].

Summarizing the state-of-the-art as described above it can be estimated that a significant progress in regenerative medicine and tissue engineering is based mainly on three major issues:

1. the detailed knowledge of molecular and cellular events during tissue formation and morphogenesis including the effect of external stimuli,
2. the availability of specific scaffold materials with an appropriate architecture and composition to enable a three-dimensional tissue formation and
3. the availability of advanced tissue bioreactors to provide cultivation conditions according to the physiological environment at the recipient site.

Keeping in mind the discussed facts, it is self-evident that none of the still existing problems can really be solved without using non-invasive and image generating measuring techniques. Therefore the TPLSM or generally spoken laser microscopy seems to be a powerful tool to solve the problems at least up to a certain extent due to the unique spatial and time resolution and the possibility to provide three-dimensional data describing the cellular environment with subcellular resolution.

Cartilage pathologies of traumatic and/or degenerative origin, among which osteoarthritis is by far most common, are a major concern in public health care. These joint ailments lead to severe articular pain for millions of individuals and, because of the lack of satisfactory repair capacity, often reach an end stage in which the affected individual is severely incapacitated with artificial joint replacement as the only possible eventual outcome.

The world-wide scope of this problem can be clearly discerned from a few self-explanatory figures: 40 million people in USA and also in Europe suffer from osteoarthritis. More than 500,000 arthroscopic procedures and total joint replacements are performed each year in the United States. Every year in Europe 150,000 injured knee joints with cartilage defects requiring treatment are diagnosed (see also Tab. 1.1). Therapeutic approaches relying on bone marrow stimulation (like drilling and microfractures) lead to a fibrocartilaginous tissues type with a limited load-bearing capacity. Likewise mosaicplasty has

not fulfilled the expectations because this procedure obviously requires an invasive and technically demanding surgery [19].

In contrast the autologous chondrocyte implantation (ACI) described by Bentley [19] and Zheng [20] represents a promising method for restoring defects of hyaline cartilage in the majority of cases. However, the conventional autologous chondrocyte implantation suffers from some disadvantages like the risk of leaking out if sealing is insufficient and above all a relatively strong cell dedifferentiation which may possibly be due to the lack of a suitable scaffold structure [21]. To overcome these problems a new therapeutic option was developed based on a Matrix-induced Autologous Chondrocyte Implantation (MACI®, Verigen AG, Leverkusen, Germany) [21, 5]. The MACI® technique requires the use of a three-dimensional type I-III collagen membrane seeded with chondrocytes to improve the structural and the biological performance of the graft. Corresponding to the physiological environment within the natural joint cartilage tissue it is understandable that such an environment is favourable for the proliferation and differentiation of chondrocytes.

Nevertheless the promising clinical results should not obscure the fact that any further progress in therapeutic research and clinical treatment depends strongly on a deeper understanding of the underlying biomolecular mechanisms.

1.3 Cell and tissue culture technologies

The cultivation techniques for cells and tissue engineering constructs are diverse and a wide range of culture approaches for many clinical applications were developed. From a methodical point of view they can be distinguished generally into two different methods: static and dynamic culture techniques.

The classical technique for static cultivation of cells is the monolayer technique established in plastic dishes. In 1994 Brittberg et al. [4] published the first approach for tissue engineering of cartilage - the autologous chondrocyte transplantation (ACT). Autologous chondrocytes of a healthy cartilage biopsy are expanded in vitro as a monolayer and are then re-injected into the defective site. In order to minimize donor site morbidity only a small autologous specimen can be sacrificed and a cell expansion is inevitable. To ensure an effective cell expansion cultivation chambers were developed to minimize the needed amount of medium (and so the costs for expensive medium supplements) and to enlarge the available growth surface. However the extensive proliferation of autologous cells in vitro is correlated with a progressive dedifferentiation (Fig. 1.2) and may eventually result in the formation of fibro-cartilage with minor mechanical properties [22, 23, 24]. According to

the requirements of functional tissue engineering, the scientific focus is now directed on the physiological relevant stimulation of these cells to redifferentiate into a phenotype typical for native chondrocytes.

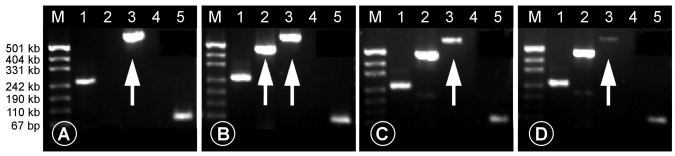

Fig. 1.2 Dedifferentiation during the monolayer cultivation indicated by starting of nonspecific collagen type I synthesis coupled with decreasing expression of collagen type II (RT-PCR; GAPDH (1), collagen type I (2), collagen type II (3), collagen type X (4), aggrecan (5); A: fresh isolated chondrocytes, chondrocytes without subcultivation (B) and after one (C) and two (D) subcultivations).

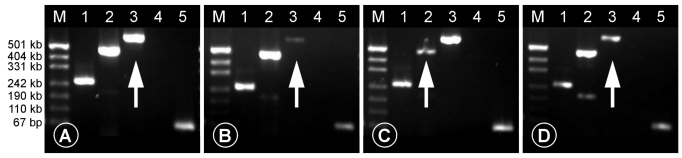

Fig. 1.3 Delayed dedifferentiation of chondrocytes depending on the substrate. On 3D scaffolds (C, D) the gene expression pattern indicates a prolonged synthesis of cartilage specific ECM components (RT-PCR; GAPDH (1), collagen type I (2), collagen type II (3), collagen type X (4), aggrecan (5); chondrocytes in a monolayer without (A) and after two subcultivations (B), chondrocytes on a 3D scaffold without (C) and after two (D) subcultivations).

As proven by many scientists and our own results (Fig. 1.3) 3D culture technologies can enhance the cell response with respect to the differentiation status compared to 2D culture techniques [25]. Different approaches of static 3D techniques utilize agarose, alginate and hydrogels as scaffolds [23, 26, 27]. Currently biomaterials, based on either synthetic or natural polymers, are designed which might be able to mimic the native extra cellular matrix. In addition to the chemical composition the microstructure (for example pore size, porosity, interconnecting pores, elasticity, stability) of a scaffold material may stimulate the inherent cells, as well. Thus, the choice of a suitable substrate is a crucial issue [28].

The MACI® is a promising 3D cultivation technique derived from the classical ACT technique. A porcine type-I/III collagen bilayer seeded with cultured chondrocytes is subsequently glued (suture free) into the debrided defective site. The regenerated cartilage appears hyaline to hyaline-like and shows satisfying biomechnical properties [5, 6, 7].

A common disadvantage imposed by the static culture conditions are the occurring diffusion gradients of oxygen, nutrients and metabolites especially

within the deeper layers of a 3D scaffold construct. Some culture systems like the wave bioreactor [29] implement a mixing of the media through the wave motion of the whole system to avoid any such gradients. Systems providing dynamic culture conditions enable the researcher to monitor critical components in the influx and efflux media and are thus more preferable. For instance it was found that the supply with a low-oxygen gas mixture can enhance the synthesis of ECM-specific cartilage components up to a certain extent [30, 31]. Another important aspect is the simulation of natural conditions of cells in tissues to induce cellular interactions using co-cultures of different cell types and their cross talk by emitting signalling molecules. This cross talk can be supported by adding of appropriate molecules like vitamins, growth factors, hormones and beta-glycerophosphate [32, 33] originating from a better understanding of the metabolic processes, genomics and gene regulation. However, the progress in tissue engineering in the last decade is not only deduced from the increased knowledge about the biological aspects of functional tissues but also from the embedding of biotechnological procedures and devices into the in vitro cultivation process. A broad spectrum of different dynamic cultivation systems was developed and adapted to the requirements of cartilage tissue engineering ranging from simple flow chambers up to more elaborated culture systems like the hollow fibre reactor [34], the flat membrane reactor [35], gradient containers [36, 37, 38] and bioreactors with rotating components and air-liquid phases , [39, 40, 41]. The rotating wall vessel bioreactor is one example allowing the harbouring of cells or cell seeded scaffolds and assure dynamic controllable culture conditions [42]. A comprehensive description of the different approaches and their applications is given in [2].

Especially for load bearing tissues like cartilage the aforementioned techniques are not sufficient for the in vitro construction of completely redifferentiated and functional tissues. Since 1998 a new discipline called functional tissue engineering (FTE) seeks to combine biomechanical considerations with the tissue engineering techniques [43]. Biomechanical stresses, strains and strain rates have to be imposed on the cells during cultivation in order to stimulate redifferentiation. The aim is the stimulation of redifferentiation indicated by an increased synthesis of typical tissue specific ECM components like proteoglycans and collagen type II. Currently bioreactors equipped with diverse (bio)mechanical loading tools are designed and successfully applied [44, 45]. The generation of stress/strain can generally be achieved by hydrostatic and hydrodynamic forces, mechanical loading and magnetically imposed stress with different frequencies and amplitudes as well as with and without rest intervals. Thereby the combination of reduced oxygen tension and intermittent hydrostatic pressure can enhance the response of chondrocytes [30]. However, the frequency and amplitude necessary to stimulate an in vitro tissue engineering construct in an optimal way is not yet validated.

Interestingly, the results reveal that a static compression over a long period is contra-productive compared to intermittent mechanical loading [44, 46, 47]. These facts illustrate the complex spectrum of scientific challenges which has to be taken into consideration in cartilage tissue engineering.

All of the described approaches were designed to generate implantable tissue constructs. But the structure and organisation of these constructs range from expanded but dedifferentiated cells to redifferentiated cell populations and structural reconstructed cartilage tissues with various qualities depending particularly on the patients own cells. So it would be useful to detect a) the quality of biopsies (including the number of cells) before the expanding phase and b) the effects of culture conditions on the cell response. However, it has to be admitted that clinical applicable approaches as a special kind of quality control are currently not available. In this context the embedding of non-invasive optical online-techniques to detect corresponding cell parameters is a crucial issue with increasing importance. The most important points are:

- cell cultivation under reproducible and contollable culture conditions
- options to stimulate the cell populations during the cultivation process
- coupling of optical laser scanning techniques with the tissue engineering process
- applicability of the optical method for the detection of autofluorescent signals in thick and strongly scattering samples
- distinct analysis of fluorescent signals of tissue typical marker components

The coupling of biotechnological devices including bioreactors, flow chambers and mechanical manipulation ports with microscopic laser scanning techniques is a very promising approach to meet the requirements of online analytical systems in regenerative medicine. Though the here described system uses an adapted tissue culture chamber with an optical window to enable the direct access to the TPLSM as shown in figure 1.4.

1.4 Controlled tissue cultivation through laser optical on-line monitoring

To control the cultivation of tissue in order to generate better and patient-specific tissue engineering products first of all parameters that are relevant for their quality must be identified. In case of cartilage repair tissues according to the MACI« technique two aspects are of special interest: the number and

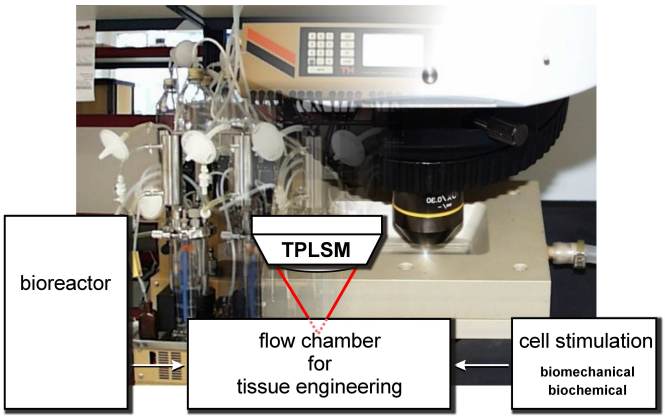

Fig. 1.4 Scheme of a tissue engineering flow chamber coupled with online-TPLSM.

morphology of chondrocytes on scaffolds in the preimplantative cultivation stage and the capability of chondrocytes to synthesize extracellular matrix components indicating the differentiation status which depends strongly on extrinsic stimulations corresponding to the local functional environment. A set of suitable non-invasive measurement techniques and adequate detection methods has to be defined to evaluate those parameters during the growth process. At present the only measurement technique that allows the investigation of optical dense tissues with sub-cellular resolution and a low damaging potential is the two-photon microscopy. Since very weak autofluorescence signals must be recorded arising from endogenous fluorophores as well as from rather fast responses of cartilage to mechanical stimulations the scanning process must be parallelised to enhance the resulting fluorescence signal. At the same time period the time needed to acquire a 3D image stack of the sample can be reduced. Due to the complex tissue architecture and composition the detection method must be able to distinguish between the different endogenous fluorophores like NAD(P)H, flavine, elastin and ECM-specific collagens as well as chondrocytes. Using spectral-resolved detection the fluorescence from the endogenous fluorophores, the ECM and the chondrocytes can be separated. However, the fluorescence of several ECM-components and the collagen membrane frequently show a large spectral overlap so that further contrast modes have to be applied. For this purpose Fluorescence Lifetime Imaging Microscopy (FLIM) can be used that provides more specific results. Additionally Second Harmonic Generation Imaging Microscopy (SHIM) is used in single beam scan mode to highlight non-centrosymmetric structures. To sum up it can be concluded that the combination of optical laser scanning measurements with highly specialized tissue-bioreactors and perfusion cham-

bers adapted for tissue engineering provides excellent conditions to monitor the cultivation process continuously. In addition flow or perfusion chambers commonly offer the possibility to apply mechanical stress or strain onto the cell seeded scaffolds. It seems to be clear that the influence of the most important cultivation parameters on the resulting tissue composition and morphology can be directly observed during the cultivation process under real-time conditions. Using this feedback specific cultivation protocols can be developed that enhance the quality of the final tissue engineering product and potentially adapt it to the needs of the patient.

1.5 Characterization and evaluation of tissues by innovative biophotonic technologies

In biophotonics [48, 49, 50] organic and biological materials consisting of molecules, cells and tissue are imaged, analysed and manipulated utilising photons.

There are many different biomedical (in vivo) imaging methods for thick tissue sections that differ significantly in terms of spatial resolution and maximum achievable imaging depth. Techniques that feature a resolution in the order of several millimeters include X-ray imaging, magnetic resonance tomography and positron emission and ultrasound imaging. Other techniques like scanning electron and atomic force microscopy allow for a higher resolution (down to 0.1 nm). However, imaging is restricted to the surface and therefore mechanical slicing of the sample is mandatory. Light imaging methods are very promising alternatives because of their high spatial resolution, large penetration depth and non-invasive nature. Consequently these techniques are more frequently used to image, analyse and manipulate the structure and the function of molecules, cells and tissues in biomedical applications.

Several endogenous fluorophores contained in tissues like NAD(P)H, flavine, elastin and collagen show autofluorescence allowing the direct visualization of morphology, cell metabolism, and disease states (e.g. Alzheimer's disease, cancer). Thereby no fixation or staining procedures are required. As these components are best excited with light between 260-400 nm up to now mostly UV light sources were used for investigation. Thereby two considerable drawbacks are the very low penetration depth due to strong scattering and absorption and the sample destruction and heating caused by this type of light.

A new approach to study tissues and the inherent endogenous fluorescent species is the use of non-linear microscopic methods like the TPLSM [51] and Second Harmonic Generation Imaging Microscopy (SHIM) [52]. TPLSM has already been successfully used to perform optical sectioning of various biological tissues like brain, lung or intestine slices [51, 53, 54, 55]. Beside the intrin-

sic high spatial resolution scattering and absorption is clearly reduced due to the longer near-infrared (NIR) excitation wavelengths in the spectral range of 700 nm to 1,100 nm enhancing the penetration depth significantly. As a result autofluorescence of biomedical tissue sections (collagen, cells) can be excited and detected down to a depth of 1,000 μm. SHIM is based on the homonymous non-linear optical effect [56, 57] in reference to a frequency doubling of the incident light. Similar to TPLSM, the amplitude of second harmonic generation (SHG) is proportional to the square of the incident light intensity. SHIM therefore also offers intrinsic three-dimensional sectioning. The application of SHIM is restricted to the imaging of highly non-centrosymmetric molecular assemblies like cellular membranes or collagen fibrils.

In conclusion, the two non-linear imaging methods TPLSM and SHIM provide enhanced in-depth information at a high spatial resolution in contrast to common imaging techniques including confocal laser microscopy. TPLSM has clearly demonstrated the potential of this technique for both scientific investigation and clinical diagnosis and is becoming an indispensable tool for non-invasive observation of tissue features in situ.

In addition to tissue morphology imaging, further parameters like emission wavelength, fluorescence lifetime, and the emitted lights polarization provide complementary and essential information for tissue characterization. Therefore they represent a powerful method of identifying endogenous fluorescence species. The relative occurrence of these species is related to tissue physiological and pathological states. Fluorescence spectroscopy has also been used to characterize different tissue types such as cartilage and skin [53, 57].

1.5.1 Microscopy Basics and Techniques

1.5.1.1 Excitation, Fluorescence and Second Harmonic Generation

Fluorescence techniques are important tools to study a large variety of applications in biology and medicine. In particular this is due to recent advances in the development of more selective, specific, stable, efficient and over all easy to use fluorescent probes (e.g. cyanine dyes, GFP, RFP, quantum dots). The principal physical mechanism of excitation and fluorescence is illustrated in the Jablonski diagram below.

A fluorophore (i.e. atom, molecule or fluorescent probe) in its energy ground state E_0 is excited by a photon to a higher energy state E_n (Fig. 1.5A). This photon holds the energy difference $E_d = E_n - E_0$, which is connected to its frequency or wavelength. The fluorophore first relaxes by non-radiative transitions to a lower energy state via inter- or intra molecular collisions. From this energy state the molecule returns into its ground state, emitting a photon.

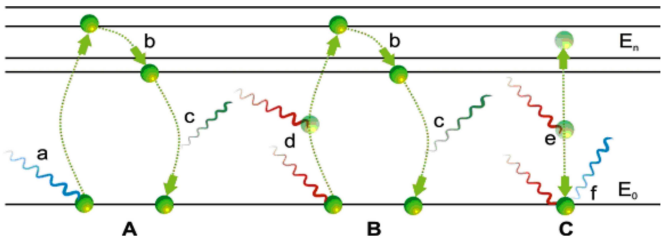

Fig. 1.5 A) fluorescence emission after 1-photon excitation; B) fluorescence emission after 2-photon excitation; C) second harmonic generation.

As there are many unoccupied energy states in molecular fluorophores the absorption (range of wavelengths suitable for excitation) as well as the emission spectrum of these molecules are rather broad ($\sim$100 nm).

The average time after a molecule relaxes from the excited state E_n to the ground state E_0 is called fluorescence lifetime (typically 1-5 ns). In addition to its emission spectrum, the fluorescence lifetime of a molecule is an important parameter since it does not only carry information about the molecule itself but also about its local chemical environment and its bonding conditions.

In case of 2-photon excitation the energy transfer is performed by two photons, each carrying half of the required energy E_d and therefore twice the required wavelength (Fig. 1.5B). According to Heisenberg's uncertainty principle this absorption takes place within approximately 10^{-16} s. Therefore 2-photon excitation is an extremely improbable process. From the excited state E_n the fluorophore then thermally relaxes and emits fluorescence light in the visible spectrum just like in the 1-photon excited case. It has to be mentioned that due to thermal relaxation the emission wavelength λ_{em} is always larger than the excitation wavelength λ_{ex} (Stokes shift) in the 1-photon excitation case.

The conversion of two photons with wavelength λ_{ex} into a single one with wavelength $\lambda_{ex}/2$ is called second harmonic generation (Fig. 1.5C). This effect happens in the vicinity of highly organized, crystal like specimens that exhibit a local polarisation. The strong electrical fields of intense light waves (i.e. laser light) induce an oscillation of the electrons in the sample. As these electrons are influenced by the non-harmonic potential of their nuclei, their oscillation generates electromagnetic waves not only with the incoming (light) wavelength but also with half (quarter, eighth,...) of this wavelength (non-vanishing Fourier-Terms of higher order harmonics). From the physical point of view the process of SHG is more comparable to the effect of Raman scattering than to the effect of fluorescence, as it has neither a lifetime that underlies Heisenberg's time uncertainty nor does SHG require free energy states of a molecule.

1.5.1.2 **Conventional Microscopy**

In conventional brightfield or epifluorescence microscopy the sample is illuminated with a homogenous light source. Therefore a combination of stray and fluorescence light from the complete sample is collected by the microscopes objective lens resulting in a microscopic image that consists of the focal plane and blurred off-focus optical planes. This means that for example all depths of a cell contribute to its microscopic image, creating a blurred projection of the complete cell. Therefore it is impossible to distinguish between different objects that lie in series on the optical axis within the sample without applying deconvolution image processing.

1.5.1.3 **Three-Dimensional Laser Scanning Microscopy**

The first light microscope that offered a true three-dimensional resolution was introduced by M. Minsky [58] and is called confocal laser scanning microscope (CLSM). The basic idea of the confocal principle is to place an aperture with a very small diameter (10-50 μm) in front of the detector and focus the fluorescence light on it. Solely light from the focal volume of the objective lens is able to pass whereas scattered light and light arising from out-of-focus planes is almost completely blocked. With this simple but ingenious setup it is possible to get an image of a point-like object within the sample.

In order to generate a 3D image of the sample it is necessary to scan the focus of the objective lens point by point across the focal plane. Thereby the size of the focus defines the resolution of the measurement. The fluorescence intensity of a sample is detected point by point by scanning the focused laser beam across a cartesian coordinate system - point-by-point in x-direction, line-by-line in y-direction, and section-by-section in z-direction through the region of interest. Thus a series of x-y-planes at different z-positions is acquired to represent a 3D-fluorescence map of the sample. There are two commonly used scanning methods. In a stage scanning microscope the complete object under investigation is moved in all three spatial directions. This method allows for very large scan fields in the order of several square centimeters. The most important drawback is the slow scan speed and the disturbance of the sample by the inherent acceleration of the stage. In a beam scanning microscope the exciting laser beam is moved by two galvanometric scanning mirrors or acousto-optic modulators across the sample. To generate a 3D image the detected fluorescence intensity at each time point has to be correlated with the position of the exciting laser beam in the sample (i.e. the position of the scan mirrors). The maximum size of the field of view of this type of scanning is limited to the field of view of the objective lens. To overcome the disadvantage of a relatively small scanning area, beam scanning systems are often combined with mechanical scanning stages. The great advantage of beam scanning setups is the high scanning frequency (approx. up to 7 kHz for resonant scan-

ners), which allows for the detection of fast (like intracellular) processes and does not disturb the sample.

The variation of the imaging depth inside the sample is usually done by moving the objective lens along its optical axis. This can be performed either through the microscopes mechanical focus drive or a piezoelectric focus drive. The 3D scanning concept is identical for both, confocal and two-photon laser scanning microscopy.

1.5.1.4 Two-Photon Laser Scanning and Second Harmonic Generation Imaging Microscopy

The simultaneous absorption of two photons was first predicted in 1931 by Maria Göppert-Mayer in her doctoral thesis. As this effect requires extremely high photon densities, it is no surprise that the experimental proof in 1961 [59] had to involve a laser, which was developed only one year before by Theodore H. Maiman at Hughes Research Laboratories. Also in 1961 the first second harmonic generation experiment was reported by P. A. Franken et al. [60], again using a ruby laser to generate the necessary excitation power.

Denk et al. [61] presented an impressive example of the improbability of a 2-photon excitation process for an excellent 1- and 2-photon absorber molecule of rhodamine B in bright sunlight. Such a molecule is excited about once every second by a 1-photon process but only once every 10 million years by a 2-photon process. This calculation illustrates the fact that 2-photon excitation requires power densities in the range of GW/cm^2 to achieve sufficient fluorescence. This photon density can be achieved by focussing a pulsed Ti:Sa Laser with a high NA objective lens. 2-photon excited fluorescence is then generated only in the attoliter focal volume of the objective lens. Using this principle, filtering the exciting laser light, detecting the fluorescence and scanning the focus through the sample, Denk et al. [51] introduced the first 2-photon laser scanning microscope in 1990, allowing the generation of fluorescence images from deep inside the specimen with a high 3D resolution.

Most 2-photon laser scanning microscopes use a pulsed femtosecond Titanium:Sapphire (Ti:Sa) Laser with a (tunable) wavelength range between 710 nm and 1,050 nm, thus in the near infrared (NIR) spectrum. This prevalent choice is justified by several advantages of this type of laser source. First of all, modelocked Ti:Sa lasers are commercially available and nowadays reliable turn key systems. They deliver output pulses with a pulse duration of typically 120 fs at a repetition rate of 80 MHz and the time averaged laser power ranges in between 1 and 2 W. Furthermore IR laser light is well capable of 2-photon excitation of a great variety of fluorescent probes and native biological fluorophores such as NAD(P)H, flavine, elastin and collagen [56]. As biological tissue has an absorption window [62] in the NIR spectral range, such light has indeed a higher penetration depth and a lower (out of focus) photodam-

age potential than the visible excitation light used in confocal laser scanning microscopy.

The probability n for a fluorophore to absorb two photons simultaneously during one laser pulse is given by Denk [51] and Diaspro [63]:

$$n = \frac{s_2 \cdot P_{ave}^2}{\tau \cdot f_p^2} \left(\frac{NA^2}{2 \cdot \hbar \cdot c \cdot \lambda} \right)^2$$

(s_2: 2-photon cross section of fluorophore, P_{ave}: average laser power, τ: laser pulse duration, f_p: laser repetition frequency, NA: numerical aperture of the objective lens, c: speed of light, λ: wavelength, $h = 2\hbar\pi$: Planck's constant)

Through this formula it becomes apparent that not only laser characteristics (wavelength, repetition frequency and pulse duration) play an important role for the excitation probability but also the characteristics of the objective lens used to focus the laser beam to a very tiny spot. This is due to the fact, that the 2-photon excitation probability is proportional to the laser intensity squared and therefore dependent on the focal volume that the incident laser light is confined to. The focal volume is also connected to the resolution of a TPLSM in that it essentially states the capability of separating two point objects next to each other. Many aspects of the experimental setup [64] contribute to a detailed description of a fluorescence microscopes resolution but a good estimation is [63]:

$$r_{lat} = 0.7 \cdot \frac{\lambda_{em}}{NA} \qquad r_{ax} = 2.3 \cdot \frac{\lambda_{em} \cdot n}{NA^2}$$

(r_{lat}: lateral resolution, r_{ax}: axial resolution, λ_{em}: emission wavelength, NA: numerical aperture of the objective lens, n: refractive index)

The resolution of high numerical aperture objective lenses can be calculated more precisely according to Born and Wolf [65].

For a GFP-expressing cell (emission peak at 508 nm, n_{water}=1.33) the resolution is calculated to be r_{lat}=0.25 μm and r_{ax}=0.79 μm when using a 1.4 NA objective lens and an excitation wavelength of 800 nm. It has to be mentioned that 2-photon cross sections usually have broader absorption spectra than 1-photon cross sections so that in many cases fluorescence of different fluorophores is induced simultaneously upon 2-photon excitation. The main difference between these two microscopy methods is, that in the confocal case only light from the focal volume is detected, whereas in the 2-photon case light is only generated in the focal volume (Fig. 1.6). Therefore photobleaching and photodamage is restricted to this small volume.

SHIM is based on the homonymous non-linear optical effect [57, 66] in reference to a frequency doubling of the incident light. Similar to TPLSM, the

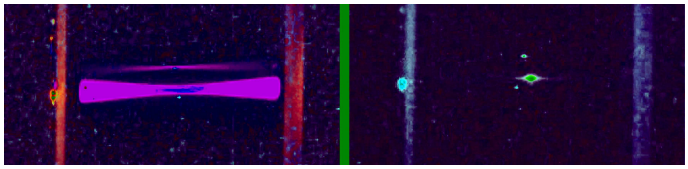

Fig. 1.6 Excitation of samples: Left: one-photon excitation of Pyridin 2 @ 560 nm, Right: two-photon excitation of Coumarin @ 770nm.

amplitude of SHG is proportional to the square of the incident light intensity. SHIM therefore also offers intrinsic three-dimensional optical sectioning without the need for a confocal aperture. Since SHG is based on photon scattering and does not involve excitation of molecules, out-of-plane photobleaching and phototoxicity is significantly reduced, which often limits the usefulness of fluorescence microscopy for imaging of living specimen. However, it differs from other non-linear microscopy modes, since SHG is restricted to specimens that are highly non-centrosymmetric molecular assemblies like cellular membranes or collagen fibrils. In practice SHIM can be performed with TPLSM instruments by using detection filters that transmit half of the exciting laser wavelength and therefore use the advantage of low NIR scattering losses and high penetration depths in thick tissue samples. Recent studies of the three-dimensional in vivo morphology of native and unstained, well-ordered protein assemblies, such as collagen, microtubules and muscle myosin, have proven the superior applicability of SHIM as a non-destructive and label-free imaging method [52, 67].

1.5.2 Multifocal Multiphoton Microscopy

The most important drawback of single beam laser scanning microscopy is the small yield of fluorescence per time unit generated by a single focus. This is particularly true for 2-photon microscopy as all dyes feature very low 2-photon absorption cross sections. Since endogenous fluorophores exhibit even weaker signals than most fluorescent dyes used for staining up to tens of seconds are required to record a single plane of a sample. As a result the observation of fast dynamics as well as the acquisition of 3D data stacks of unstained living samples is nearly impossible with a single beam laser scanning microscope. In addition sample movement or morphological changes within the sample during the acquisition can degrade the spatial resolution considerably.

To overcome these problems a common approach is to increase the excitation laser power in order to generate more fluorescence photons per time unit. But above a critical power level ($> \sim 5$ mW) the induced photodamage rises

faster with increasing laser power than the number of fluorescent molecules - thus it does not solve the problem. The only way to increase the amount of fluorescence per time unit without raising photodamage is to use more excitation beams in parallel. The simultaneous application of N excitation foci results in an N-fold increase of the excited molecules such that the sample emits more fluorescence light in the same time interval (even with a lower laser power in each focus).

About six years ago two different parallelised 2-photon microscopes were invented [68, 69]. The first one uses a microlens disk to split up the laser beam into typically 25-36 beams. As the microlenses are arranged in spirals on the disk the scanning process is accomplished simply by rotating the disc. In the second approach a single laser beam is split up into 64 beams by multiple transitions through a 50% beamsplitting substrate (see Fig. 1.9). A two-axis galvanometric scanner rasters all foci simultaneously across the object plane. This method has a very high optical throughput and offers uncompromised optical sectioning quality which is due to the exclusive use of flat optics to devide the incoming laser beam and also to the fact, that all beams arrive at slightly different points in time at the sample (no cross-talk). The latter of these two parallelised 2-photon microscopy techniques is the basis for the tissue imaging microscope that was developed in the context of this study. Recent publications on multifocal multiphoton microscopy have already shown the large potential for biomedical applications and the superior sectioning and image quality compared to confocal Nipkow Disk laser-scanning microscopes [70].

1.5.3 Detection Methods

1.5.3.1 Descanned and Non-Descanned Detection

In TPLSM and SHIM it is possible to use two fundamentally different detection arrangements to record the sample fluorescence. In the non-descanned mode, excitation and fluorescence are separated by a dichroic mirror located directly behind the objective lens (see Fig. 1.7).The dichroic mirror is tuned to transmit signal from the sample to the detector or the eyepiece of the microscope, while the excitation NIR light is reflected. The position of the fluorescent focal volume in the sample is directly imaged on the detector, resulting in a direct image of the sample if the exciting beam is scanned. Therefore it is possible to use a camera (2-dimensional detector), the eyepiece or a large field point detector like a PMT as detector. In the latter case the scan field must be scaled down by intermediate optics to fit the PMT surface. The advantage of

non-descanned detection using a PMT is its great sensitivity due to the fact that even strongly scattered fluorescence photons contribute to the signal.

In the second detection mode (descanned mode) the fluorescence from the sample is directed back via the scanning mirrors. As the average fluorescence lifetime of a molecule is much shorter than the time needed to move the scanner from one position to the next the fluorescence signal is directed back onto the excitation beam axis. A dichroic mirror (see Fig. 1.7) located in front of the scanning mirrors separates the excitation laser light and the fluorescence signal. The main difference to the non-descanned mode is the fact, that the fluorescence is always directed onto the same spot. In order to construct an image of the sample under investigation, it is necessary to correlate the time dependent fluorescence with the time dependent position of the scanning mirrors. Therefore the use of point detectors is much easier in this measurement mode and in addition it is possible to introduce a confocal pinhole to improve the resolution.

1.5.3.2 **Spectral-resolved Imaging**

As fluorophores have different emission properties, it is reasonable to provide contrast mechanisms for fluorescence detection that go beyond mere intensity registration. For measurements that involve fluorescent probes with known and distinguishable emission spectra, it is effective to use different filters adapted to these spectra for instance in epifluorescence microscopy. This enables fast measurements with a very effective use of the generated fluorescence signal. For weak native fluorescence signals, it is advantageous to use broad filters to improve the signal to noise ratio or to distinguish between fluorophores and SHG signals.

The use of spectrographs or spectrometers allows for the measurement of complete emission spectra with a much higher spectral resolution. When using a spectrometer in the non-descanned arrangement, line scans in the sample are projected onto its entrance slit and an emission spectrum for each point along the line scan is generated perpendicular to the slit axis. Therefore a two-dimensional camera image is generated that consists of the emission spectra in the x-axis along different positions in the line scan which are represented in the y-axis. As the line scan is imaged onto the spectrographs entrance slit it must remain at the same position inside the sample. Therefore it is only possible to perform beam scanning along this axis. To acquire a spectral-resolved image of a sample plane stage scanning along the direction perpendicular to the entrance slit is required. To overcome the disadvantage of sample scanning it is also possible to perform spectral-resolved measurements in the descanned mode. Through the use of a camera up to 64 spectra (corresponding to the number of foci in the sample) can then be measured simultaneously. Nevertheless this measurement mode is comparably slow as it requires a slow

scanning process because the generation of complete camera pictures takes milliseconds.

Generally spectral-resolved measurements are a trade-off of the factors resolution (locally and spectrally), time, photodamage, fluorescent yield and data volume. Different samples and the scientific questions connected to them require different techniques of spectral measurements.

1.5.3.3 Fluorescence Lifetime Measurements

The average time a molecule remains in the excited state is determined by the number of de-activation pathways and their competing rates. Using fluorescence lifetime measurements information about complex photophysical processes can be obtained to determine the rates of de-activation. Lifetime measurements are extremely sensitive to the molecular environment of fluorescent molecules.

Fluorescence lifetime measurement techniques can principally be divided into time- and frequency-domain approaches. In frequency-domain measurements [71] the fluorophores are excited with sinusoidally modulated light at high frequencies (20-80 MHz). The emitted fluorescence signal has the same frequency, but undergoes a phase shift and a decrease in amplitude (demodulation) with respect to the excitation radiation. These dynamic parameters can be related directly to the lifetime of the emission. The maximum temporal resolution is determined by the modulation frequency and is roughly 1 ns.

Far more common are time-domain measurement methods that use a short pulsed light source and detect the time-dependent fluorescence with respect to the excitation pulse. One of them uses an intensified CCD camera as a time-gated detector [72]. The lifetime image is generated by recording the intensity of the fluorescence at a series of different time points after the excitation pulse. The maximum temporal resolution is determined by the minimum gate width of the intensified CCD camera ($\sim$ 50 ps). Although a lot of photons are lost due to the use of gates this method delivers FLIM images much faster than all other methods as lifetimes are recorded simultaneously in each point of a whole sample section. A different technique is time correlated single photon counting (TCSPC) which is probably the most widespread method [73, 74]. Thereby a point detector (PMT) delivers an electrical pulse for each registered photon and a fast counting electronic measures the time difference between the excitation radiation and the detected photon. Thus the fluorescence decay curve can directly be measured with a maximum temporal resolution of $\sim$ 10 ps. The only drawback of this method is the long time required to generate a FLIM image ($\sim$ 30 s) which is due to the point by point acquisition process. An interesting new time-domain approach is a streak camera FLIM system [75] that uses a sweep electrode that deflects photoelectrons to different positions on a phosphor screen depending on their arrival time at the

detector. This method has an acceptable temporal resolution ($\sim$ 20 ps) and the potential to acquire fast FLIM images. But at present it still needs several 10 seconds for a FLIM image.

1.6 Results and Application

1.6.1 Optics

1.6.1.1 Development of a parallelised 2-photon measurement system for fast and high-resolution tissue imaging

The aspired aim to monitor tissue engineering products for cartilage regeneration during the in vitro cultivation process required the invention of a very special measurement system. The demand for sub-cellular resolution deep inside dense optical material could only be fulfilled using TPLSM. In addition weak fluorescent endogenous fluorophores contained in the ECM of cartilage should be observed as well as the response of unstained cartilage tissue to mechanical or biochemical stimulation. Therefore the 2-photon microscope had to be parallelised to acquire as much light as possible in a given time interval. The underlying principle to split up a single beam into 64 beams that are scanned simultaneously across the object plane was developed in the framework of the BMBF-project "Non-Linear Laser Raster Microscopy". Using this technique the acquisition of images or 3D volumes can be accelerated by a factor of 64.

The scheme in figure 1.7 illustrates the principle of operation of the parallelised 2-photon laser scanning microscopes which are adapted to the requirements of strongly scattering samples.
The incoming laser beam first passes an attenuator, a telescope and the prechirp arrangement. Afterwards it is split up into up to 64 beams by multiple transitions through a 50% splitting substrate (Fig. 1.8).
The splitted beams are coupled into the microscope through intermediate optics and focused onto the sample by the objective lens. A single line of foci is generated that is rastered by a galvanometric xy-scanner in the object plane. Together with the microscope z-drive this enables the three-dimensional imaging process. The fluorescence can be observed through the eyepieces, imaged onto a CCD-camera or onto a PMT in non-descanned arrangement. Furthermore it can be detected with a parallelised descanned PMT detector.

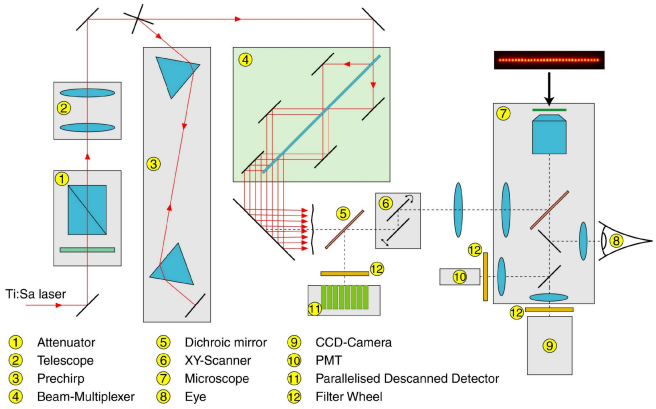

Fig. 1.7 Schematic setup of the parallelised 2-photon laser scanning microscope.

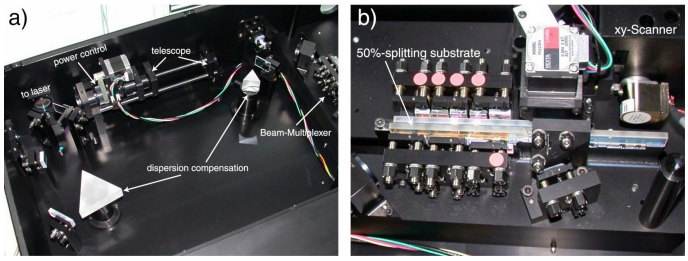

Fig. 1.8 Photographs of the inside of the scan head. a) The beam shaping and steering elements and b) the Beam-Multiplexer.

All optical elements were optimised with regard to the highest transmission achievable as this determines the maximum degree of parallelisation that in turn limits the image acquisition speed. A throughput of over 75% was achieved for the whole Ti:Sa wavelength area of 710-980 nm. Since the two-photon generated fluorescence decreases linear with increasing excitation pulse length the dispersion compensation was set to completely equalise the pulse broadening introduced by the optical elements. As a result the temporal length of the pulse is 120 fs in the sample. Special scan optics were developed that enable in combination with an innovative objective lens (20x, 0.95 NA) the observation of very large sample sections (up to 500x500 μm) at high resolution. A key feature is the possibility to reduce the degree of parallelisation from 64 to 32, 16, 8, 4 and even to a single beam (Fig. 1.9).
This is done through a stepwise exchange of the 50%/AR coated substrate with the AR/AR coated one. Both substrates are arranged in line and mounted on a motorised holder allowing a simple and software controlled conversion.

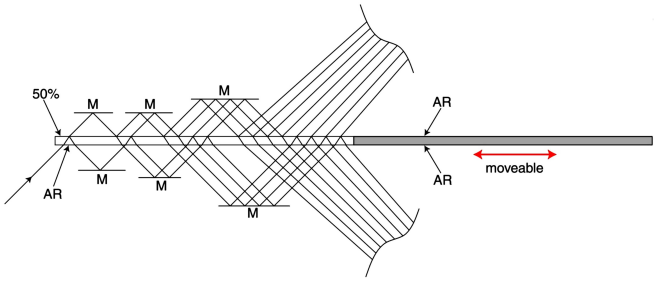

Fig. 1.9 Principle of changing the degree of parallelisation.

Each reduction of the number of beams by a factor of two increases the power in each remaining beam by the same factor yielding an overall gain in fluorescence of two. The increase of laser power per beam is crucial if deep sample planes shall be imaged. It enables roughly 20% additional imaging depth in dense tissue. By replacing the 50%/AR substrate completely with the AR/AR substrate only one beam passes the setup. It seems to be clear that this is extremely useful since very deep sample planes cannot be observed with multiple beams and a field detector like the CCD camera due to the strong scattering. Thus a single beam scan mode was realised featuring a PMT in non-descanned arrangement as detector. In addition SHIM is enabled that cannot be done in parallelised mode which is an important tool to discriminate between collagen matrix structures, chondrocytes and autofluorescence signals.

The maximum penetration depth in parallelised mode was measured to be roughly 80% of the single beam mode whereas the resolution is nearly equal during the first 60%. At the surface the resolution of both modes is 310 nm in lateral and 900 nm in axial direction when using a 1.4 NA objective lens and 800 nm light for excitation. The maximum achievable frame rate of the instrument is roughly 1,000 Hz but is nearly always limited by the small number of photons emitted by the sample.

1.6.1.2 **Control and automatisation of the system**

All components of the system like the power control, xy-scanner, shutter, parallelisation selector, z-stepper, filterwheel, xy-sample stage, CCD-camera, and the A/D-converter to readout the PMT are software controlled. To observe in vitro tissue cultivation automatically over several hours or days up to 6-dimensional data sets (x, y, z, P, t,) can be recorded whereas all measurement settings are saved together with the appendant data set.

A synchronisation module was developed that enables the exact timing of all system components that take part in a complex measurement. It assigns

a time point to each acquired image thus allowing the exact determination of the time elapsed between interesting molecular or cellular events.

To observe dynamics like the response of cartilage to mechanical stimulation with high temporal resolution a fast time lapse mode was realised that already reads out the CCD camera during the time needed to acquire a new image. For many typical applications this speeds up the imaging process by a factor of two.

1.6.1.3 Development of new measurement methods to image strongly scattering tissues

As already mentioned three-dimensional tissues often possess a very complex architecture and composition. To be able to acquire informative data from strongly scattering tissues additional optical methods have to be employed as described below:

Sequential imaging of different sample positions

Through the use of a motorised xy-sample stage it is possible to scan different regions within the sample in a single measurement each with its own settings (e.g. scan mode, emission filter). The number of recurrences and the time between two scans can be freely chosen. In addition this concept allows high-resolution imaging of very large sample sections or volumes in the order of several square or cubic millimeters. Thereby images of neighbouring sample sections are recorded sequentially and patched together by the software to the final image.

Spectral Unmixing

If emission filters are used to spectrally separate different fluorophores and endogenous species a common problem is cross-talk between these filters caused by the broadness of the emission spectra. To overcome this problem and to visualise weak contrasts a spectral unmixing mode was developed: data points in an acquired image that contain characteristic spectra are chosen and used as reference points; afterwards a software algorithm searches for these spectra in all other pixels and assigns only one spectrum to each (Fig. 1.10).

Z-Drop

A fundamental problem in 3D microscopy is the decrease of fluorescence signal as a function of penetration depth (z-drop) caused by the focus degradation and increased scattering of the photons on their way back through the sample. To compensate for these effects it is possible to automatically reduce the number of beams or/and to increase the excitation power with increasing depth. The aim is to keep the camera integration time and the recorded fluorescence intensity constant. Otherwise it is hardly possible to measure a large

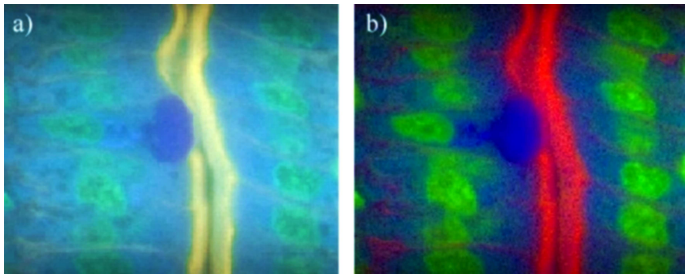

Fig. 1.10 Spectral-resolved measurement of a mouse intestine section using a filterwheel and three different detection filters. a) Overlay of the three colour channels and b) spectral-unmixed image

depth range and to perform a subsequent 3D reconstruction of the imaged volume. Using this method it was possible to measure to a depth of 1 mm into a collagen gel in a single measurement (Fig. 1.11).

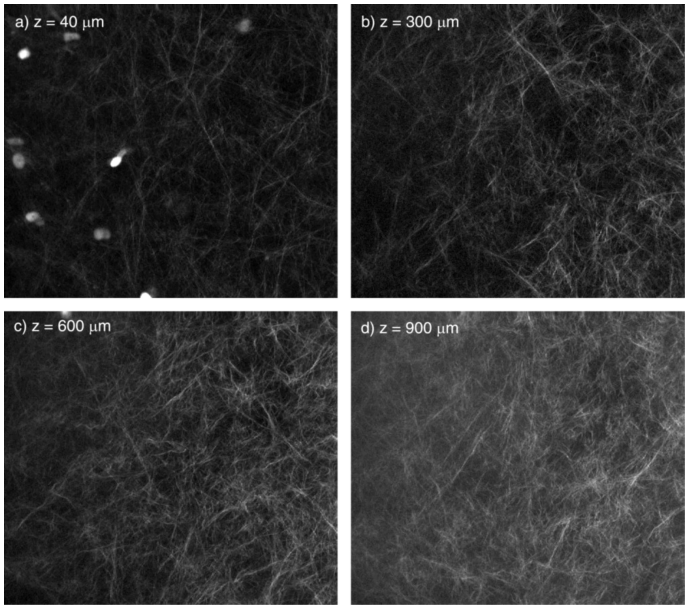

Fig. 1.11 Adaptation of the number of beams and the excitation power as a function of depth inside the sample to compensate for the z-Drop.

High throughput spectral measurements

The spectroscopical non-descanned mode of operation to determine native fluorescence spectra (see Fig. 1.16) is compromised by the fact that these spec-

tra are rather weak and broad. The total fluorescence intensity arising from one point inside the sample is not only collected on one camera pixel like in imaging measurements, but is dispersed to a complete line on the CCD chip, generating the emission spectrum of the fluorescent point. This, however, requires long acquisition times for a complete analysis. To optimize the optical throughput of this type of spectral measurement, a new spectral measurement mode was developed which uses a straight vision prism instead of a spectrograph. As a line scan in the sample works as an entrance slit for the prism no fluorescence light is blocked by a mechanical slit and all of the fluorescence from the line scan can contribute to the spectrum. Furthermore losses due to absorption in the detection pathway are minimal, as only the prism is additionally introduced. The advantages of this high throughput, easy and affordable setup and wide a detection spectrum (180 nm for 8 mm by 8 mm CCD chip) are only compromised by relatively high sensitivity to stray light.

1.6.2 Cartilage and chondrocytes

1.6.2.1 Human cartilage tissue

Due to the intrinsically dense structure of its extra cellular matrix (ECM), cartilage is a tissue that strongly scatters but does not absorb much light. Hence articular cartilage has an opaque optical appearance and is called hyaline. Consequently, penetration depth of visible light into this tissue is rather low, making a characterization with conventional brightfield microscopy difficult, as it requires staining and/or microtome cuts.

Figure 1.12 shows the 3D-autofluorescence reconstruction of unstained healthy human cartilage measured with TPLSM [76]. Measurements with a tissue penetration depth of up to 200 μm (up to 460 μm are possible for arthritic bovine cartilage) were performed at an excitation wavelength of 800 nm with 64 parallel foci at a total laser power of 260 mW, keeping laser power at 4 mW per focus to prevent photodamage [77]. The spectral discrimination between ECM and chondrocytic cells was achieved by recording two separate image stacks with fluorescence emission filtering for ECM (HQ 525/50) and for the chondrocytes (HQ 575/50). As figure 1.12 demonstrates, spectral discrimination between ECM and chondrocytes is possible. Furthermore, the complete mapping of the tissue allows a direct estimate of the corresponding chondrocyte density in the tissue region (in this case approximately 20x106 cells/cm^3). It has to be mentioned though that the chondrocyte density varies vastly for different samples, even within the same sample, depending on the relative position of the region of interest within the cartilage. We found chondrocyte densities that range from approximately 2x10^6 cells/cm^3 to 20x10^6 cells/cm^3

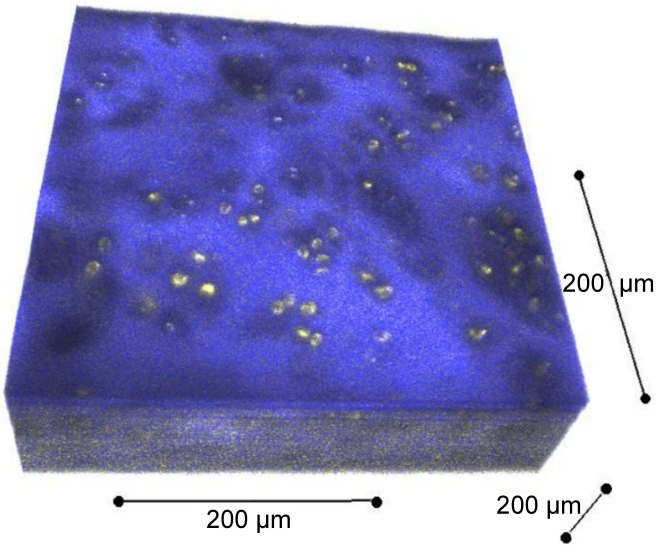

Fig. 1.12 3D-autofluorescence reconstruction of unstained healthy human cartilage tissue. The ECM (HQ 525/50) and the chondrocytic cells (HQ 575/50) are represented in blue and yellow colors. The presented plane lies 50 microns below the tissue surface of the sample.

in the same cartilage sample, which is indeed in accordance with data derived from healthy cartilage found by other research groups [78]. Furthermore in order to compare healthy and arthritic tissue samples from the same patient were investigated (Fig. 1.13).

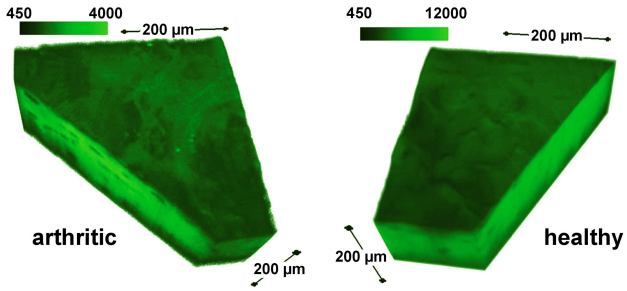

Fig. 1.13 3D-autofluorescence reconstruction of the surface of unstained healthy and arthritic human cartilage tissue from the same proband.

Both tissue samples were characterized with TPLSM at 800 nm using a laser power of 240 mW in 64-foci parallel operation mode. For mapping the sur-

face morphology only the green fluorescent emission filtering for the ECM (HQ 525/50) was recorded.

Considering the autofluorescence images two aspects that are related to the macroscopic diagnosis are quite evident (Fig. 1.13). First, healthy cartilage tissue displays a much higher autofluorescence emission from ECM than arthritic tissue, which can be interpreted as an indication of reduced tissue density in the arthritic case. Second, the two outer surface structures differ significantly in respect of smoothness and morphology. Whereas the surface of healthy tissue is smooth and isotropic, the arthritic surface is fibrous and rather rough. This change in arthritic tissue morphology to a rough, fibrous surface is consistent with an increased frictional resistance and consequently it can cause an increased wear damage between articulating cartilage surfaces.

These experiments reveal that native hyaline cartilage from a human knee joint can directly be investigated with TPLSM without using additional staining or labeling protocols. It is important to note that this technique can potentially be used in future diagnostic applications, for example for a better quantitative definition of different stages of arthritis or osteoarthritis of articular cartilage.

1.6.2.2 **Chondrocytes on collagen scaffolds**

The influence of scaffold materials and structures on the cell performance was described above. To demonstrate the capability of appropriate scaffold materials to enhance the chondrocyte response in the tissue engineering process on the one hand and to show the potential of the laser scanning microscopy associated with the ability to monitor relevant process parameters on the other hand two different collagen scaffold structures were used (Fig 1.14).

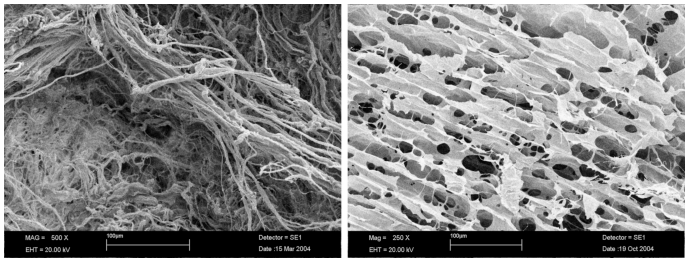

Fig. 1.14 Collagen I/III scaffolds for cultivation of chondrocytes based on porcine collagen (SEM). Left: fleece (ACI-Maix^{TM}, Matricel GmbH), Right: sponge-like structure (Matricel GmbH, Germany).

Based on native fluorescence and SHG properties impressive images could be taken from the different collagen matrices via TPLSM. Figure 1.15 shows a collagen I/III fleece. The autofluorescent signals provide a high resolution image

of the scaffold structure similar to the performed non-native SEM investigations. A distinct fibrous structure, providing a 3D scaffold for chondrocytes is apparent.

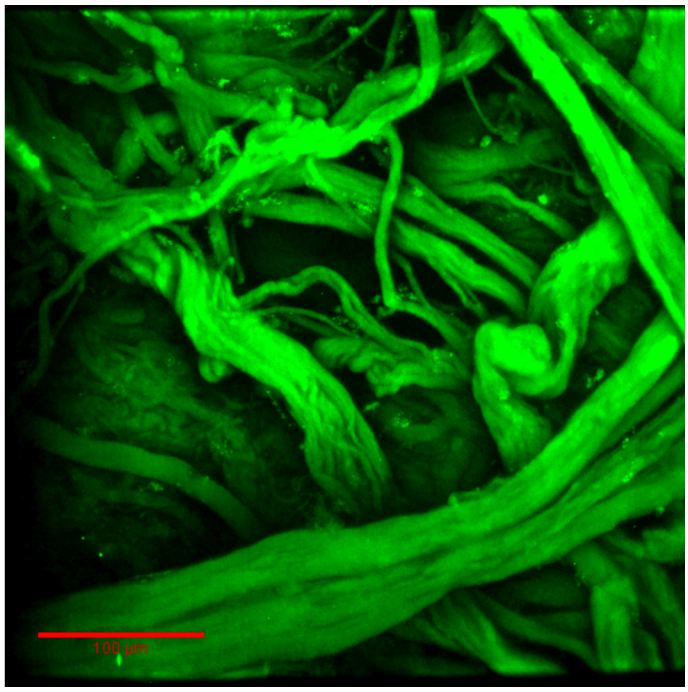

Fig. 1.15 Projected image of a 170 μm thick and unstained collagen membrane, 2-photon-excitation at 800 nm taken in parallel beam modus (64 beams; bar: 100 μm).

In figure 1.16 a sponge-like collagen scaffold is presented. According to preliminary test results in this project, the sponge-like scaffold provides higher viability for chondrocytes than fibrous membranes. This could be shown by comparative investigations on the influence of scaffold structures on the chondrocyte response.

The crucial point of an online analysis of cells and their differentiation dependent on the incubation conditions is the visualization of cells and their metabolic products within an engineered tissue construct. Investigations by means of laser scanning microscopy require signals which are specific for the distinct components. In this context implantable functional chondrocytes are a suitable example to demonstrate the potential of laser scanning microscopy as a tool for quality control. Primary bovine chondrocytes (healthy femoral knee joint) cultured on collagen I/III scaffolds were used as a model system to detect the fundamental components of cartilage tissue engineering constructs: scaffold material, cells and synthesized ECM. Figure 1.17 shows

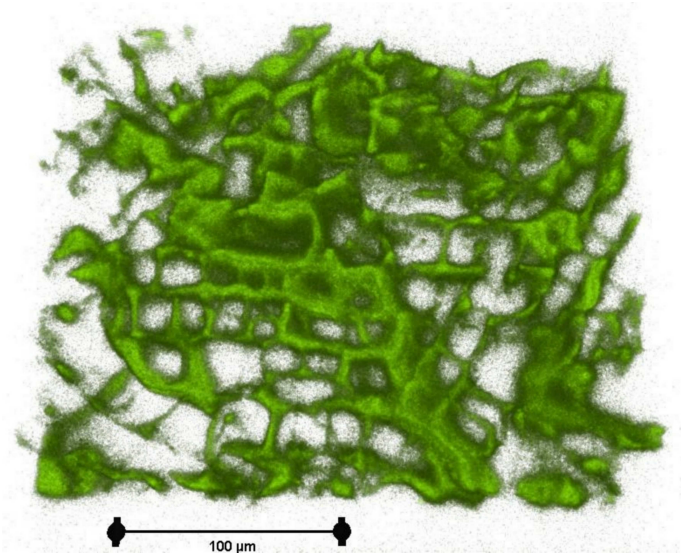

Fig. 1.16 Reconstruction of sponge like collagen membrane, two-photon-excitation at 800 nm, 64 beams, <5 mW per focus, native fluorescence with filter HQ 525/50 (bar: 100 μm).

chondrocytes on a collagen I/III-fleece cultivated for 7 days detected with a common CLSM which is applicable for thin and low-scattering samples. Autofluorescent signals of the collagen-fleece can be separated from the cells (labeled with Syto 83) and the synthesized proteoglycans (keratan sulfate, labeled with a monoclonal antibody for keratan sulfate conjugated with FITC). The cells are attached to the collagen fibres. The proteoglycans are synthesized by the chondrocytes and appear as released compounds around the cells. The quantity of synthesized ECM components around the cells can be an essential marker for cell differentiation and cell stimulation. Interestingly it could be shown that the cell response can be enhanced by cultivation on collagen I/III scaffolds with a sponge-like structure. Figures 1.18 shows the appearance of differentiation markers in dependence of the scaffold structure. In contrast to the fleece scaffold the cell morphology on the sponge-like scaffold is similar to native chondrocytes embedded in cartilage tissue. The synthesis of ECM components like chondroitin sulfate and collagen type VI appears more homogeneous and is enhanced on sponge-like scaffolds indicating an improvement of cell redifferentiation. The cell promoting effects of structured collagen scaffolds detected by laser scanning microscopy correlate with quantitative biochemical data concerning the amount of released proteoglycans (Fig. 1.19). Despite the fact that a progressive dedifferentiation during the expansion phase cannot totally be prevented the sponge-like scaffolds reveal a clear enhancement of the cartilage specific ECM synthesis. The results

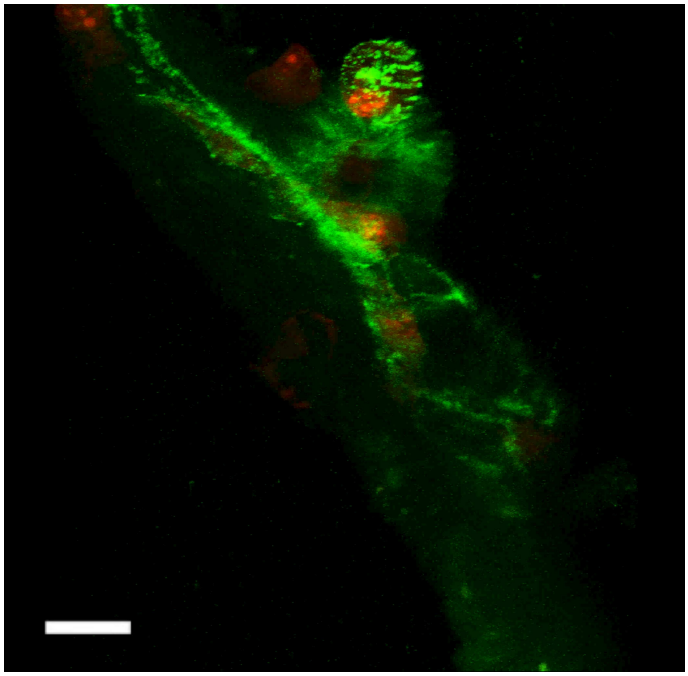

Fig. 1.17 Chondrocytes on collagen I/III-fleece synthesizing keratan sulfate (red: cells, green: keratan sulfate around the cells, the strung-out green structure represents an autofluorescent collagen fibre; CLSM, bar: 20 μm).

show the tremendous potential of laser scanning microscopy to detect cells and ECM components directly within deep tissue regions engineered under in vitro conditions. However, a real online analysis of tissue engineering constructs during the incubation process requires the visualization of the most important components (scaffold, cells, ECM marker) based on autofluorescence signals. In fact, a wide spectrum of biological samples show autofluorescent signals generated from a wide range of involved molecules. The excitation of autofluorescent molecules in biological samples is very effective using TPLSM due to the employed NIR-excitation wavelengths that enables observation in the VIS-region caused by the simultaneous absorption of two photons. The parallelised TPLSM provides a wide range of flexibility to control the excitation energy by splitting the beam into up to 64 single beams. Thus, the excitation of autofluorescence can be maximized without damaging the sample. The basic task in online analysis of tissue constructs represents the visualization of three main components described above within a sample. However, beside the laser optical conditions for a sensitive autofluorescence monitoring the selective detection of distinct components requires further analytical

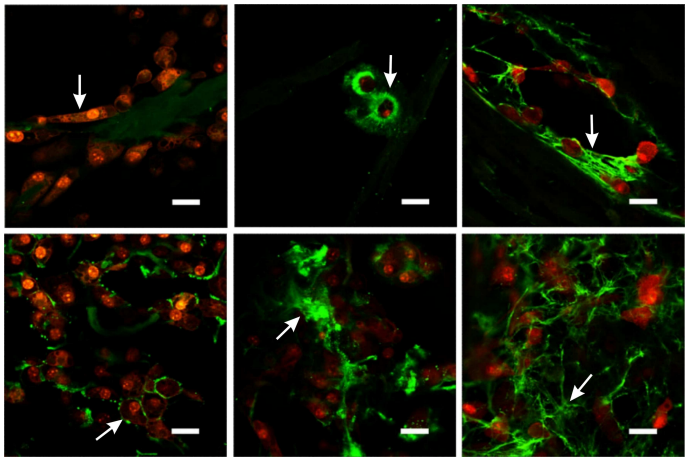

Fig. 1.18 Performance of chondrocytes (after one subcultivation) on collagen fleeces (top) and sponge-like collagen scaffolds (bottom). Left: morphology, Middle: synthesis of chondroitin sulfate, Right: synthesis of collagen type VI (Syto 83-staining of cells, FITC-labeled Anti-CS and Anti-Coll VI; CLSM; bars: 20 μm); arrows indicate the main results described in the text.

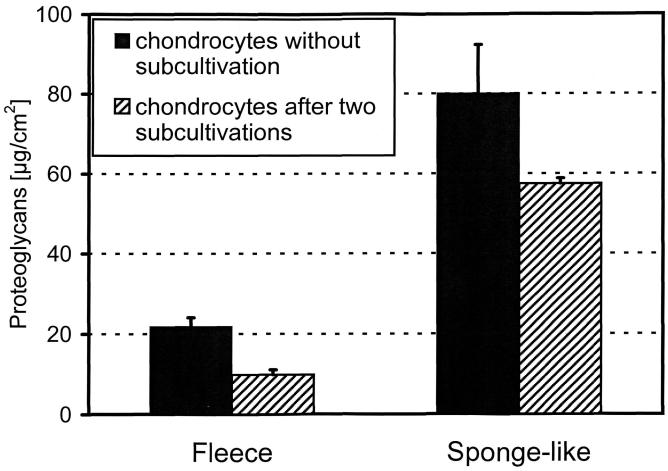

Fig. 1.19 Synthesis of proteoglycans dependent on the scaffold structure. Sponge-like collagen scaffolds promote the ECM-synthesis by chondrocytes seeded on scaffolds without and after two subcultivations in the cell expansion phase (n=6).

methods. The excitation of autofluorescence within a tissue construct is realizable using the advantages of TPLSM. Chondrocytes as well as collagen fibres can be detected (Fig. 1.20, left). SHG signals of collagen fibres (Fig. 1.20, right) allow the separation of scaffold structures from cells by spectral unmixing and enable a quantitative estimation of cell numbers. Furthermore, the ac-

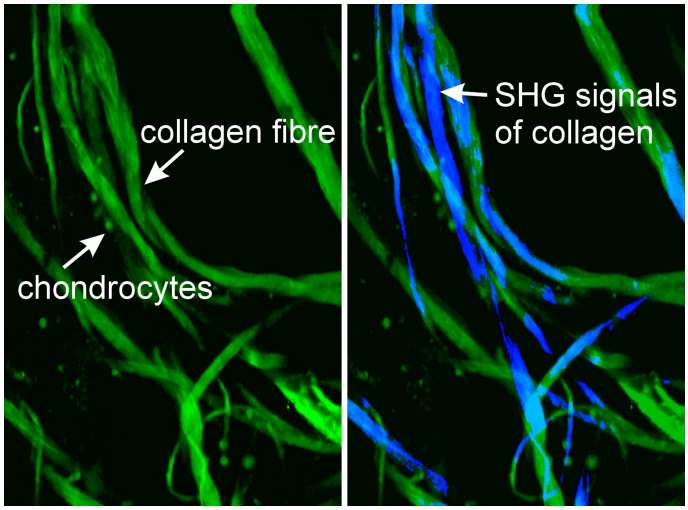

Fig. 1.20 Chondrocytes on collagen fleece: TPLSM, @ 800 nm; Left: autofluorescence, Right: autofluorescence and SHG of collagen fibres (blue, HQ 10/20; merged image).

tual number of vital chondrocytes in the tissue matrix often represents a key parameter for the optimization of tissue engineering technologies. In order to obtain primary information, a spectral discrimination of the chondrocytes from the collagen matrix and the synovial fluid and the used culture media is mandatory. Accordingly, we analyzed the spectral response of isolated chondrocytes, collagen matrix and culture medium by 2-photon excitation in the accessible wavelength range from 450 nm to 700 nm. All investigated systems exhibited spectra that were very similiar and therefore not helpful for proper spectral discrimination. However, collagen fleece scaffolds generate distinct SHG signals as displayed in the spectrum shown in figure 1.21. This property provides possibly the background to develop an effective contrast mechanism to detect the collagen scaffold and to discriminate the scaffold material from the chondrocytes.

By using 2-photon-excitation at 820 nm with a single laser beam (power <15 mW) and a set of filters, a three-color image was generated in which a single chondrocytic cell (yellow) is clearly visible on a collagen matrix (blue) (Fig. 1.22a). In figure 1.22b, the same dataset has been digitally reconstructed in a pseudo-3D representation displaying the chondrocyte in red color. The advanced separation of ECM specific autofluorescent signals provides the basis for a qualitative evaluation of the status quo of cartilage specific differentiation of cells in dependence of the cultivation conditions including the observation of the effect of a biochemical and mechanical stimulation. Furthermore, the subject of current research is the analysis of fluorescence decay times to pro-

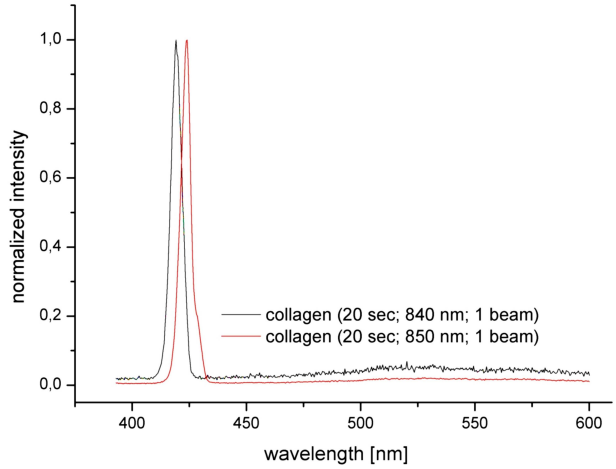

Fig. 1.21 SHG of collagen I/III fleece.

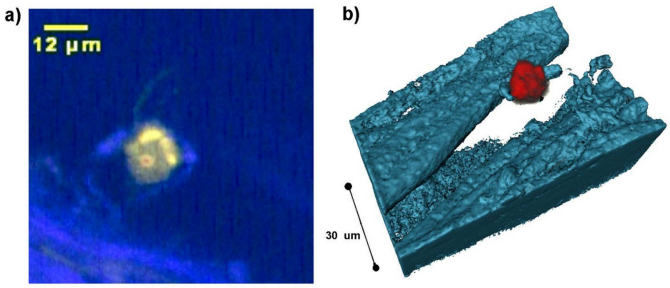

Fig. 1.22 a) Spectral discrimination of chondrocyte and collagen matrix (filter set: blue: SHG with HQ 410/20, red: native fluorescence with HQ 525/50, green: native fluorescence with HQ 575/50), b) Same dataset in digital pseudo-3D representation.

vide detailed data referring to the appearance and possible kinetic changes of synthesized ECM components within cultured tissue constructs. In this context FLIM is expected to be a powerful tool to detect and to analyse biological tissues and molecular interactions quantitatively [79, 80].

1.7 Summary and outlook

Regenerative medicine and tissue engineering are exciting new fields taking advantage from both engineering and biology. The process of creating living, physiological, three-dimensional tissues utilizes specific biomaterials as

scaffolds to guide tissue growth in vivo and in vitro. The most appropriate scaffolds are the ones that provide the intricate hierarchical structure (e.g. 3D-architecture, chemical composition) that characterize the native tissue to be replaced. In the framework of the present study two chemically equivalent but structurally different collagen scaffolds were investigated and it was found that the analysed sponge-like collagen membrane inhibits the cellular dedifferentiation of the chondrocytes after being seeded on the membrane. Obviously the sponge-like membrane offers favourable conditions for tissue formation and tissue regeneration. It's of value to point out that the whole process of a three-dimensional tissue formation is a highly orchestrated set of sub-cellular (molecular), cellular, and supra-cellular events that are far away from being well understood. That's the reason why non-invasive measuring methods with an appropriate spatial and temporal resolution are necessary.

Beside the necessity to provide suitable scaffolds consisting of advanced bioactive materials a second major challenge in tissue engineering was identified. The cultivation process itself needs a technology platform to guarantee reproducible and controllable conditions for tissue growth and cell differentiation according to the nature of the tissue engineered product. It is widely accepted that bioreactors and flow chamber systems offer a tremendous potential to ensure that all relevant aspects are fully considered. In this context growth conditions (e.g. pH-value, pO_2, temperature, nutrient supply), scale-up, and sterility issues are important factors to launch safe, clinically effective, and last but not least competitive tissue engineered products to the market. Keeping in mind the concept of functional tissue engineering it is inevitable especially in the field of cartilage repair to establish a biomechanical stimulation during the cultivation process to be able to mimic the native mechanical environment within the bioreactor.

The whole process of bioreactor design and bioreactor based tissue and cell cultivation will be like a "mission impossible"without using proper measuring methods with an appropriate spatial and temporal resolution to obtain a reliable feedback from the cultivation process.

The present study has shown that non-invasive imaging methods like laser scanning microscopy provide striking advantages over conventional fluorescence microscopy and appear to be a novel detection tool for three-dimensional resolved fluorescence imaging. Of special importance is the possibility to monitor and to control cell cultivation processes in the field of regenerative medicine to ensure a high quality of tissue engineered constructs that can be used successfully to treat the affected patients. The application of these methods benefits from the fact of being non-invasive without disturbing the cultivation process itself. A spatial resolved analysis of tissue engineering constructs will be extended by kinetic data (4D analysis) to obtain the necessary temporal resolution. In this way NIR multiphoton excitation laser scan-

ning microscopy will become a powerful tool in the area of quality control in biomedical applications. Due to the fact that the present study is focused on tissue engineering approaches the basic feature of selective imaging of thick biological samples allows in principal an equivalent application in therapeutic and diagnostic fields as well as in biofilm monitoring.

Key publications

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Glossary

MACI®/ACT (Matrix-induced Autologous Chondrocyte Implantation Autologous Chondrocyte Transplantation), Tissue engineering techniques for the regeneration of cartilage. Autologous chondrocytes are isolated, expanded in vitro and subsequently re-implanted into the defect site with and without supporting collagen scaffold materials, respectively.
TPLSM (Two Photon Laser Scanning Microscopy), In TPLSM a focused pulsed laser beam is used to excite (native) fluorophores through the simultaneous absorption of two near-infrared (NIR) photons. The 2-photon absorption process requires a high power density and therefore only takes place in the focal volume of the microscopes objective lens. This intrinsic sectioning property allows for the generation of three dimensional fluorescence images deep inside living samples.
SHG/SHIM (Second Harmonic Generation / Second Harmonic Generation Imaging Microscopy), Second harmonic generation is a nonlinear optical effect that generates one photon out of two, carrying the total energy of both incident photons. This conversion requires high optical power densities and the vicinity of polarised structures. Therefore SHG can be used to image certain materials like collagen fibers resulting in a new microscopy technique called

SHIM.

FLIM (Fluorescence Lifetime Imaging Microscopy), The average time a molecule remains in the excited state is called fluorescence lifetime. Fluorescence lifetime measurements are extremely sensitive to the molecules environment and provide information about complex photophysical processes. In FLIM typically a short pulsed light source and a detector that registers the time-dependent fluorescence with respect to the exciatation pulse is used to generate the fluorescence decay curves.

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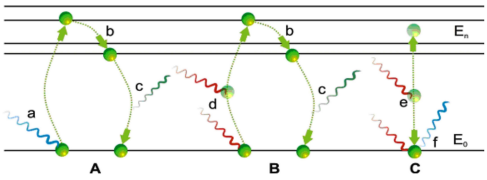
Excitation, Fluorescence and Second Harmonic Generation

Fluorescence techniques are important tools to study a large variety of applications in biology and medicine. In particular this is due to recent advances in the development of more selective, specific, stable, efficient and over all easy to use fluorescent probes (e.g. cyanine dyes, GFP, RFP, quantum dots). The principal physical mechanism of excitation and fluorescence is illustrated in the Jablonski diagram below.



A) fluorescence emission after 1-photon excitation; B) fluorescence emission after 2-photon excitation; C) second harmonic generation.

A fluorophore (i.e. atom, molecule or fluorescent probe) in its energy ground state E_0 is excited by a photon to a higher energy state E_n (see Figure A). This photon holds the energy difference $E_d = E_n - E_0$, which is connected to its frequency or wavelength. The fluorophore first relaxes by non-radiative transitions to a lower energy state via inter- or intra molecular collisions. From this energy state the molecule returns into its ground state, emitting a photon. As there are many unoccupied energy states in molecular fluorophores the absorption (range of wavelengths suitable for excitation) as well as the emission spectrum of these molecules are rather broad ($\sim$100 nm).

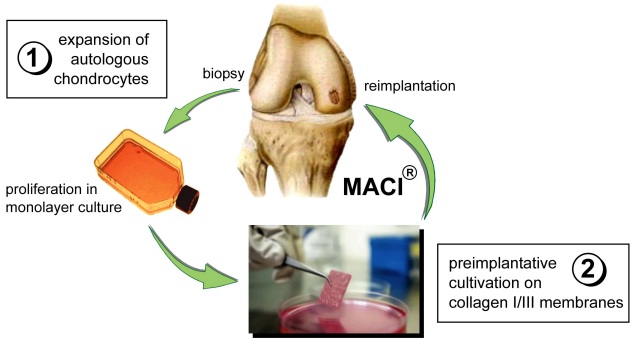
The average time after a molecule relaxes from the excited state E_n to the ground state E_0 is called fluorescence lifetime (typically 1-5 ns). In addition to its emission spectrum, the fluorescence lifetime of a molecule is an important parameter since it does not only carry information about the molecule itself but also about its local chemical environment and its bonding conditions.

In case of 2-photon excitation the energy transfer is performed by two photons, each carrying half of the required energy E_d and therefore twice the required wavelength (Fig. B). According to Heisenberg's uncertainty principle this absorption takes place within approximately 10^{-16} s. Therefore 2-photon excitation is an extremely improbable process. From the excited state E_n the fluorophore then thermally relaxes and emits fluorescence light in the visible spectrum just like in the 1-photon excited case. It has to be mentioned that due to thermal relaxation the emission wavelength λ_{em} is always larger than the excitation wavelength λ_{ex} (Stokes shift) in the 1-photon excitation case.

The conversion of two photons with wavelength λ_{ex} into a single one with wavelength $\lambda_{ex}/2$ is called second harmonic generation (Fig. C). This effect happens in the vicinity of highly organized, crystal like specimens that exhibit a local polarisation. The strong electrical fields of intense light waves (i.e. laser light) induce an oscillation of the electrons in the sample. As these electrons are influenced by the non-harmonic potential of their nuclei, their oscillation generates electromagnetic waves not only with the incoming (light) wavelength but also with half (quarter, eighth,...) of this wavelength (non-vanishing Fourier-Terms of higher order harmonics). From the physical point of view the process of SHG is more comparable to the effect of Raman scattering than to the effect of fluorescence, as it has neither a lifetime that underlies Heisenberg's time uncertainty nor does SHG require free energy states of a molecule.

Cartilage repair using autologous chondrocytes

Damages of the cartilage, like arthritis and traumatic injury, cause severe pain and restrict the motivity of millions of patients worldwide. Unfortunately the capability of the cartilage tissue to regenerate the damaged area is limited. Modern therapies for cartilage reconstruction are focussed on the support of the tissue to self repair by the transplantation of healthy autologous chondrocytes. The Autologous Chondrocyte Implantation (ACI) represents the basic technique for transplanting precultured chondrocytes into the defect site. By covering with a periosteum membrane sutured to the surrounding healthy tissue the transplanted cells are retained at the site.



A variation of this technique is the Matrix-induced Autologous Chondrocyte Implantation (MACI®) promoted by Verigen AG (Leverkusen, Germany). This technique includes two basic steps (see Figure): A biopsy of healthy cartilage is arthroscopically obtained from the patient. Subsequently the chondrocytes are released by enzymatic digestion of the tissue, expanded/grown in vitro and seeded into a collagen type I/III membrane in a cleanroom facility. After debridement of the lesion the cell seeded membrane is cut to the size and shape of the defect and glued in place with fibrin. The main difference between the MACI® treatment and the original ACI is the usage of a collagen type I/III membrane rather than an autologous periosteal flap. Since the MACI® membrane can be suture-free attached to the base of a prepared chondral defect with fibrin glue, this novel procedure imposes the following surgical advantages:

- access to the lesion can be gained through a mini-arthrotomy
- no requirement for periosteal harvesting and therefore a reduction of the number of grafts and graft sites
- no risk of leakage of chondrocytes and uneven distribution because there is no injection of a cell suspension below a membrane
- substantially reduced operating theatre time and, because it is performed by minimally invasive surgery, shortened rehabilitation period

Current research findings have revealed that the success of a therapy employing tissue engineering products can be enhanced, when specific stimuli are applied in the preimplantative cultivation phase. A chondrocytic differentiation status can be achieved through three dimensional cultivation techniques, as well as biochemical and mechanical stimulation. The MACI® technique has promising prerequisites for this purpose. The necessity to monitor the cell performance in response to the applied stimuli is not solved to a satisfactory degree yet. Non invasive optical techniques like the multiphoton microscopy are a promising approach for an online quality control tool.

Tab. 1.1 Market sizes correlated with cartilage defects/cartilage repair [16]

Region	Market size (EUR)	Year	Remarks	Source
Europe	2 billions	1999	Market value for joint implants (prosthesis costs only)	Biomet Merck
World	1.5 billions	1999	Market value for knee implants (prosthesis costs only)	Data-monitor
USA	5.2 billions	2001	annual spending for total knee replacement	[17]
World	6.5 billions	2001	market potential of surgical procedures for cartilage regeneration	[18]
World	25 billions	2011	market potential of surgical procedures for cartilage regen-	[18]