Int J Burn Trauma 2018;8(2):17-25
www.IJBT.org /ISSN:2160-2026/IJBT0075736

Review Article

Application of laser scanning confocal microscopy in the soft tissue exquisite structure for 3D scan

Zhaoqiang Zhang[1], Mohamed Ibrahim[2], Yang Fu[3], Xujia Wu[3], Fei Ren[4], Lei Chen[5]

[1]Department of Oral and Maxillofacial Surgery, Stomatological Hospital, Southern Medical University, No. 366, South of Jiangnan Road, Guangzhou 510280, Guangdong, P. R. China; [2]Division of Plastic and Reconstructive Surgery, Department of Surgery, Duke University Medical Center, Box 3181, Durham 27710, North Carolina, USA; [3]Department of Clinical Medicine, Zhongshan School of Medicine, Sun Yat-sen University, 74 2nd Zhongshan Road, Guangzhou 510080, Guangdong, P. R. China; [4]Department of Oral Medicine, Stomatological Hospital, Southern Medical University at Haizhu Square, No. 180, Taikang Road, Guangzhou 510280, Guangdong, P. R. China; [5]Department of Burns, The 1st Affiliated Hospital of Sun Yat-sen University, 58 2nd Zhongshan Road, Guangzhou 510080, Guangdong, P. R. China

Received December 25, 2017; Accepted March 23, 2018; Epub April 5, 2018; Published April 15, 2018

Abstract: Three-dimensional (3D) printing is a new developing technology for printing individualized materials swiftly and precisely in the field of biological medicine (especially tissue-engineered materials). Prior to printing, it is necessary to scan the structure of the natural biological tissue, then construct the 3D printing digital model through optimizing the scanned data. By searching the literatures, magazines at home and abroad, this article reviewed the current status, main processes and matters needing attention of confocal laser scanning microscope (LSCM) in the application of soft tissue fine structure 3D scanning, empathizing the significance of LSCM in this field.

Keywords: LSCM, 3D printing, tissue engineering

Introduction

As an important component of tissue engineering research [1-3], the structure and composition of scaffold material have important influences on growth and function of seeded cells, which is one of the key factors of the success of tissue-engineered material. What an ideal scaffold material is it should be biocompatible, biodegradable and mechanically appropriate. However, the present synthetic degradable scaffold materials such as polylactic acid and polyglycolic acid fails to provide a good microenvironment with functions such as growth, proliferation, interchange of material and signaling stimuli for cell repair like a natural scaffold material (acellular matrix tissue). Besides, with the advancement of organ transplantation, the source of allogeneic natural scaffold material is limited, carried with a risk of disease transmission (such as AIDS). That's why heterogeneous acellular matrix tissue has attracted more attention.

The sources of allogeneic biologic scaffold material are usually swine and bovine [4-8], because of the similarity of the tissue structure to that of human, which are of most concern to people. However, due to the limitation of workmanship, swine and bovine acellular matrices have certain immunogenicity with poor nutrition, penetration of material, slow cellularization and vascularization as well as low survival rates, hence can only be used for provisional wound covering. As a result, researchers started to shift their focus to collagen [9, 10], an essential structural protein in dermal matrix's connective tissue. Taking an advantage of the flexible properties of collagen, they hoped to reconstruct a scaffold material that encourages cell growth and rapid vascularization with the help of technologies such as freeze-drying.

It is challenging for traditional preparation methods of scaffolds for tissue engineering to overcome problems such as material cell support and vascularization. Also there are no personalized characteristics of the structure obtained by these traditional methods. Thanks to the emergence of 3D printing, it has introduced a new hope to solve this issue. With3D printing technology, an optimized data model

can be constructed in accordance with characteristics of patients' injured parts. Hence, tissue engineer scaffolds can be prepared rapidly and accurately based on 3D printing with the advantages of realizing close and "seamless" connection with an injured part on the external structure and matching with defects on the fine (microcosmic) structure to obtain the best remediation effect.

The main purpose of the 3D printing in tissue-engineered material is to obtain accurate 3D image data of the fine structure of human tissue [11, 12]. On one hand, currently, 3D imaging of hard tissues (e.g. bones [13, 14]) have made progress rapidly because of existing digital medical technology such as computed topography (CT). On the other hand, fine structure imaging of soft tissues (e.g. skin, nerves and vessels) is still limited to 2D. No breakthrough is noticed until now for 3D imaging of soft tissues like skin [15-18].

In 1957, confocal microscopy technology was proposed [19]. In 1980's, as an epoch-making high-tech product developed [20-23], LSCM was a combined outcome of microscope making, photoelectricity, computer technology and a modern optical microscope. LSCM 3D stereo imaging technology is similar to 3D CT [24, 25], which evenly scans tissues of a specified height in certain periods of time, and then overlaps images of each scan to build a 3D image. Its advantages are that it can really reflect distribution relations of scaffolds and seed cells by 3D images, especially topological structure in scaffolds and cell distribution that can hardly be matched by an ordinary optical or electron microscope [26-28].

Status

History

In 1957, Marvin Minsky firstly founded the confocal principle. Later in 1967, Egger and Petran developed the confocal microscopy successfully and got an optical section by scanning. In 1977, Sheppard and Wilson put forward Raman spectroscopy for the laser scanner, illuminating the non-linear relationship between light and atoms of illuminated objects. After confocal microscopes were developed by Biorad (Model SOM-100) and were commercially sold in 1984, Model MRC-500 applied light beam scanning combined with a bio fluorescent microscope hereafter in 1986.

Status worldwide

In 1985, first research contribution about LSCM biological application by Wijnaendts Van Resandt was noted [29, 30]. Afterwards, the confocal microscope has been widely used in biology and has become a powerful research tool in research of cytobiology, morphology, pharmacology and neurosciences, etc. With advancements in science, the confocal microscopy is now not only restricted to the LSCM. In recent years, some foreign scientific research institutions have invented confocal microscopes with more perfect functions, such as pinhole array disc LSCMs and two-photon LSCMs successively.

Pinhole array disc LSCM was proposed to solve problems of confocal detection in the course of quick change [31], which was invented by Yokogawa Electric of Japan. This company introduced twin disc patented technology, including synchronous rotation of a micro-lens array disc and a pinhole array disc. As compared to the conventional laser confocal method that needs a stage to scan, the pinhole array disc LSCM applies CCD as a detector. It is an important tool for in vivo fluorescent imaging of living cells that only needs synchronous rotation of a micro-lens array disc and a pinhole array disc to carry out fast confocal detection on objects with a maximal full-width collection frame rate of 1000 frame/s [32, 33].

Two-photon LSCM is a new generation of confocal microscopes introduced to solve problems of the photo-bleaching phenomenon of sample dye labeling in bioinstrumentation [34]. A single photon needs high energy to stimulate fluorescent molecules. However, under a high photon density, two low-energy photons enable to stimulate fluorescence. After get stimulated, the fluorescent molecule will emit photons of short wavelength and bring same effects. At the same time, low-energy photons reduce damage (phototoxicity) on samples. The pulse width emitted by this laser is only 100fs with a cycle of 80 to 100 MHz, high peak energy and low average energy. Also as two-photon excitation only occurs on lens focus, the fluorescence detection efficiency is able to enhance greatly without a confocal pinhole [35].

Status in China

As the trend of using confocal microscopes abroad has increased, China also followed the

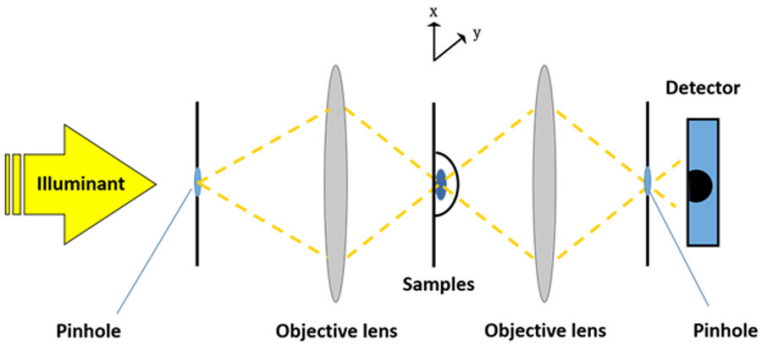

Figure 1. After the illuminant passes through the pinhole, only punctiform illuminant emits and focuses on an object via the lens so as to reach a precise point-to-point effect to scan on plane x-y forming an image of the plane. Deeper scanning can result in more images and the 3D structure drawing can be constructed by computer processing.

pace and introduced the first batch of confocal microscopes. So far, many domestic biological research institutions have brought in the confocal microscope because of its strong observation ability in the biology field. The Neurology Department of the First Affiliated Hospital of Sun Yat-Sen University has introduced the two-photon microscope for multiple biological researches. Moreover, Zeiss LSM 710/780 serves in the Public Experiment Platform of the Technology Building of the Zhongshan Medical College were also a noteworthy addition [28, 36-39].

Principle of confocal microscopy and its pros and cons

Compared with the traditional optical microscope, confocal microscope is capable to provide extremely precise 3D imaging and measure subcellular structure and dynamic processes correctly [28, 40]. LSCM is the most widely used confocal microscope. This manuscript summarized the application of LSCM in 3D scan of deep tissues. Based on the traditional fluorescence microscope, the confocal microscope has been modified, and then a laser scanning mechanism has been added with the application of confocal principle to construct fine 3D images by computer processing [41, 42]. Now it has been extensively used in fields such as biomedicine and even engineering materials.

Principle

Traditionally, scattered illuminant of the microscope leads to poor image definition and high background noise. However, to make scattered illuminant pass a small hole or to use a laser will form pointolite which will focus on a point of an object, namely the focus. And the plane contains the focus known as the focal plane. According to the principle, a clear 3D image can be obtained by moving the lens in directions of x-y-z (**Figure 1**).

Advantages

As compared to the fluorescence microscopy, following are the advantages of this microscope: It can conduct scanning of deep tissue and can deal with thick samples; It can remove background interference effectively to make the image clear and precise; It can collect a series of optical sections; It can carry out dynamic, real-time and non-invasive testing.

Disadvantages

However, some disadvantages are also seen in this microscope: LSCM still has certain phototoxicity that can damage samples. Though it can be used for observation of living cells, certain impacts still exist; Penetrability and distinguishability of LSCM also needs further improvement 3. Processes of confocal 3D scanning of tissue and its precautions.

Sample preparation

It's the requirement that a section experiment specimen should be composed of a single layer (the thinner the better). In addition, it should attach to sample cell very well. Currently, researchers used agents to attach specimen to sample include polylysine, albumen, agar-gelatin cell-Tak, concanavalin, vectabond, etc.

During cell culture, in order to get better effects, thin bottom culture flasks attached by the equipment can be used.

When observation samples are processed, the natural state of biomaterial needs to be maintained. In order to avoid transformation, artifact and distortion, biomaterial needs fixing first. During the process of creating sections, the sample should be thin and transparent. So

material needs to be cut into thin pieces or lightly pressed for material dispersion. In addition, other methods should be made so that the material can be transparent and stained by colors for observation of details. Dehydration and sealing need to be conducted on sections which require long-term storage.

Selection of fluorescent probes

Fluorescence emitted by the fluorescent probe is critical to the imaging mode of LSCM. Different experiments need different molecular probes that give out different fluorescence. Therefore, choosing a proper probe should be a priority. Selection of fluorescent probe mainly considers the following respects:

Lasers were applied in current equipment. For example, LSCM (FV-1000) applied an argon ion laser that could stimulate multiple fluorescent probes with excitation wavelengths of 351~364 nm, 488 nm or 514 nm; and the LSM710/780 system developed by Carl Zeiss AG could access at most 8 lasers among which 458 nm, 488/514 nm, 543/561 nm, 594 nm, and 633 nm are for visible lights and 405 nm, 445 nm and 680-1064 nm of continuous infrared lasers are for invisible lights. Moreover, the present settings tend to be more simple and convenient. For instance, DAPI etc. can be emitted directly by a shortcut key.

Photostability and photobleaching of fluorescent probes

During fluorescent quantization and monitoring of dynamic fluorescence, if the stability of a fluorescent probe is not enough, the fluorescence will be quenched gradually, making impact on the experimental results. In order to reduce photobleaching impacts, a probe with high stability should be selected or laser intensity or scanning times should be reduced. However, there is always a possibility of exceptions. For example, during testing of membrane fluidity or intercellular communication, it's required that a fluorescent probe should have both photostability and photobleaching. That is to say that it should depend on the concrete experiments [43, 44].

Qualitative or quantitative analysis of fluorescence

During mere qualitative analysis of fluorescence or mere observation of dynamic fluorescence change, a working curve is not needed, so a probe of single wavelength excitation can be chosen. However, in the course of quantitative analysis, a ratiometric probe of double wavelength excitation should be selected for making working curves [45].

Specificity and virulence of fluorescent probes

In principle, a probe with high specificity and low virulence should be selected as far as possible. For instance, researchers found that GFP can be expressed in multiple animals and mammals without any cytotoxicity, so it can be used as a probe. Nowadays, with the advancement in science, recombinant DNA technology allows fluorescent proteins to fuse with any other microcellular protein. The fusion proteins has no cytotoxicity and can produce fluorescence with no need for any other motivating factor [46].

Proper pH fluorescent probes

Firstly, growth and survival of cells demand a proper pH value. Secondly, the pH value of dye liquor will affect combination of fluorescent probes and intracellular specific molecules. And thirdly, many fluorescent probes are charged and hydrophilic, so it is hard or impossible for them to pass through a lipid bilayer. However, to combine a fluorescent probe with acetoxymethyl, a lipophilic compound can be generated without charges so that it can enter into cells via the lipid bilayer [47, 48].

Scanning and 3D reestablishment

Fluorescence-marked cells can be observed with LSCM and a related pattern of fluorescent activation. In the process of 3D mode, the maximal precision of LSCM can reach 0.18 μm. Many optical sections obtained within certain time can roughly reflect 3D images with a certain resolution through 3D reestablishment for utilization in multiple directions of biology [49, 50].

Application of LSCM

Application in scaffold material

Observation of situations such as cell proliferation and adhesion: Based on the current research on tissue engineering, it was found that traditional plane scaffold material could not generate a satisfying effect of growth, prolifera-

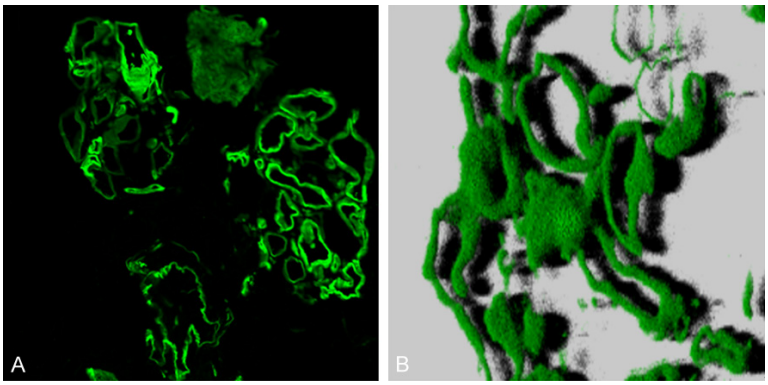

Figure 2. We used GFP to label type I collagen and observed forms of type I collagen in tissue sections via 3D reconstruction. A. Is an image under a 200× field where the green fluorescence indicated existence of collagen fibers. B. Is a 3D image reconstructed by ZEN software under a 200× field after 27 optical sections by scanning.

tion, differentiation and adhesion of cells. Therefore, in order to simulate in vivo tissue, people started to construct various 3D scaffold networks and then implanted cells into those materials. After a period of culture or transplanting them in animals, various biological shapes of cells were observed [51-53]. A valuable role in cell testing was played by LSCM. There are two testing methods in practice these days. One is to only carry out flat scanning after labeling some protein in cells with immunofluorescence. If some phosphor dots become obscure, this means cells can grow in 3D scaffold material so that fluorescence of cells does not focus on a focal plane and cell density in an image can reflect its condition of cell proliferation. Some researchers in China made an HAP-PADM (hydroxyapatite-coated swine acellular dermal matrix) scaffold and planted periodontal ligament stem cells in it during cell growth condition monitoring [54].

The other is to make 3D views directly using computer, which can reflect cell growth conditions better in a 3D scaffold. Vivian Lee et al. prepared artificial skin with 3D biological printing technology who reestablished a 3D view with computers reflecting growth and adhesion conditions of cells in scaffold material very well [55].

Observation of scaffold material

In addition to observation of cells, a confocal microscope can be also used for observing no biological materials where these materials need to be labeled with fluorescence. In the process of research on material simulating extracellular matrix, Teresa A. Martin et al. utilized photoetching technology to make various patterns on collagen-GAG material [56].

Observation of vascularization

Vascularization gained importance in research on scaffold material [57-60]. Only complete vascularization can provide nutrient substances for tissue and carry away waste products better, being a basis of an idealized biomaterial. A confocal microscope can also be applied for observation of vascularization. Actin filament can be labeled with red, vascular endothelial cells with blue, and β-catenin can be labeled with green fluorescence so that microvessels is able to be manifested clearly [61]. E. Leslie-Barbick et al. carried out immunofluorescent labeling on multiple proteins, such as laminin and fibronectin, in vascular endothelial cells and extracellular matrix during research on promoting effects of VEGF and RGDS on vascularization. All reflected changes in vascularization greatly [62].

Positioning, qualitative and quantitative analysis for sub-cellular components/specific molecules

Specific to label, a biomolecule with immunofluorescence can determine position of the molecule or the distribution state of subcellular structure to carry out qualitative research, which is widely used in the biomedical field. We used GFP to label type I collagen and observed forms of type I collagen in tissue sections via 3D reconstruction (**Figure 2**). For example, by observing nerve fibers with LSCMs by fluorescence labeling of NFP, Yibing Wang et al. constructed 3D images to show direct and clear change of skin nerve fibers [63]. For Young-Cheol Lim et al., they obtained clear and reliable results with confocal microscopes when they studied effects of C peptide on inhibiting vascular permeability change induced by VEGF in ROS-mediated cells [64].

In addition, fluorescent signals can also be collected with the help of computers which can measure quantificational content and distribution of a molecule by analysis of each optical section. As layer upon layer analysis is conducted, improvement in accuracy and reliability has been noted. Prathamesh M. Kulkarni et al. carried out quantitative analysis on cellular location, cell counting and cell distribution and space along with forms of cell processes with more than 95% accuracy by multiple fluorescence labeling of GFAP for astrocyte and observation with confocal microscopes [65]. However, the current fluorescence images requires proper density of tested molecules. If molecules are too dense, the present resolution of a microscope resulting in overlapping or spatial distribution of intracellular structure will get affected, in which will cause reduction of accuracy and reliability. At the same time, background interference is also a problem. Now the protein-protein index (PPI) method is beneficial to reduce defects mentioned above to a certain extent and improves reliability of data [66]. In addition, as the longitudinal resolution of scan images of LSCMs is less than their transverse resolution, there will be information loss between longitudinal layers. As a result, only discrete data can be obtained. Therefore, how to conduct slice interpolation better in the process of 3D reconstruction is another key in accuracy improvement.

Prospects

Widely used as LSCMs are, they also have certain limitations. With the development of science and technology, the two-photon microscope has been available with stronger penetrability, less phototoxicity and wider application. During research on multilayer collagen apatite scaffold material, Z. Xia et al. applied two-photon microscopes to display the inner structure of scaffolds [67]. Also more advanced multi-photon microscopy is under research and development. It is predicted that in future confocal microscopes will be developed better with an increasingly broad market. In addition, due to advanced technology and mature products, LSCMs are still vital tools in biomedical research these days. It's believed that further technological advancements in microscopy will accelerate the development of fields such as biomedicine.

Acknowledgements

This study was supported by the Scientific Research Foundation of Southern Medical University (Youth cultivation project, NO: PY-2017N036), Fundamental Research Fund for the Central Universities, Sun Yat-sen University (15ykjc10c) and the National Natural Science Foundation of China (NSFC: 81601703).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lei Chen, Department of Burns, The 1st Affiliated Hospital of Sun Yat-sen University, 58 2nd Zhongshan Road, Guangzhou 510080, Guangdong, P. R. China. Tel: 020-87755766-8265; E-mail: chenleigzburns@163.com

References

[1] Carletti E, Motta A, Migliaresi C. Scaffolds for tissue engineering and 3D cell culture. 3D Cell Culture 2011; 695: 17-39.

[2] Eitan Y, Sarig U, Dahan N, Machluf M. Acellular cardiac extracellular matrix as a scaffold for tissue engineering: in vitro cell support, remodeling, and biocompatibility. Tissue Eng Part C Methods 2010; 16: 671-683.

[3] Allen AB, Priddy LB, Li MT and Guldberg RE. Functional augmentation of naturally-derived materials for tissue regeneration. Ann Biomed Eng 2015; 43: 555-567.

[4] Liu Y, Bharadwaj S, Lee SJ, Atala A, Zhang Y. Optimization of a natural collagen scaffold to aid cell-matrix penetration for urologic tissue engineering. Biomaterials 2009; 30: 3865-3873.

[5] Wagner DE, Bonenfant NR, Sokocevic D, DeSarno MJ, Borg ZD, Parsons CS, Brooks EM, Platz JJ, Khalpey ZI, Hoganson DM, Deng B, Lam YW, Oldinski RA, Ashikaga T, Weiss DJ. Three-dimensional scaffolds of acellular human and porcine lungs for high throughput studies of lung disease and regeneration. Biomaterials 2014; 35: 2664-2679.

[6] Lee JH, Kim HG, Lee WJ. Characterization and tissue incorporation of cross-linked human acellular dermal matrix. Biomaterials 2015; 44: 195-205.

[7] Zhao H, Qu M, Wang Y, Wang Z, Shi W. Xenogeneic acellular conjunctiva matrix as a scaffold of tissue-engineered corneal epithelium. PLoS One 2014; 9: e111846.

[8] Jang YJ, Chun SY, Kim GN, Kim JR, Oh SH, Lee JH, Kim BS, Song PH, Yoo ES, Kwon TG. Char-

acterization of a novel composite scaffold consisting of acellular bladder submucosa matrix, polycaprolactone and Pluronic F127 as a substance for bladder reconstruction. Acta Biomater 2014; 10: 3117-3125.
[9] Zhang W, Chen J, Tao J, Jiang Y, Hu C, Huang L, Ji J, Ouyang HW. The use of type 1 collagen scaffold containing stromal cell-derived factor-1 to create a matrix environment conducive to partial-thickness cartilage defects repair. Biomaterials 2013; 34: 713-723.
[10] Bell A, Kofron M, Nistor V. Multiphoton crosslinking for biocompatible 3D printing of type I collagen. Biofabrication 2015; 7: 035007.
[11] Rengier F, Mehndiratta A, von Tengg-Kobligk H, Zechmann CM, Unterhinninghofen R, Kauczor HU, Giesel FL. 3D printing based on imaging data: review of medical applications. Int J Comput Assist Radiol Surg 2010; 5: 335-341.
[12] Horn TJ, Harrysson OL. Overview of current additive manufacturing technologies and selected applications. Sci Prog 2012; 95: 255-282.
[13] Vrtovec T, Likar B, Pernus F. Quantitative analysis of spinal curvature in 3D: application to CT images of normal spine. Phys Med Biol 2008; 53: 1895-908.
[14] Wu AM, Shao ZX, Wang JS, Yang XD, Weng WQ, Wang XY, Xu HZ, Chi YL, Lin ZK. The accuracy of a method for printing three-dimensional spinal models. PLoS One 2015; 10: e0124291.
[15] Holme MN, Schulz G, Deyhle H, Weitkamp T, Beckmann F, Lobrinus JA, Rikhtegar F, Kurtcuoglu V, Zanette I, Saxer T, Müller B. Complementary X-ray tomography techniques for histology-validated 3D imaging of soft and hard tissues using plaque-containing blood vessels as examples. Nat Protoc 2014; 9: 1401-1415.
[16] Kuijpers MA, Chiu YT, Nada RM, Carels CE, Fudalej PS. Three-dimensional imaging methods for quantitative analysis of facial soft tissues and skeletal morphology in patients with orofacial clefts: a systematic review. PLoS One 2014; 9: e93442.
[17] Appel AA, Larson JC, Jiang B, Zhong Z, Anastasio MA, Brey EM. X-ray phase contrast allows three dimensional, quantitative imaging of hydrogel implants. Ann Biomed Eng 2016; 44: 773-781.
[18] Naveh GR, Brumfeld V, Dean M, Shahar R, Weiner S. Direct microCT imaging of non-mineralized connective tissues at high resolution. Connect Tissue Res 2014; 55: 52-60.
[19] Wu Y, Zinchuk V, Grossenbacher-Zinchuk O, Stefani E. Critical evaluation of quantitative colocalization analysis in confocal fluorescence microscopy. Interdiscip Sci 2012; 4: 27-37.
[20] Kymionis GD, Diakonis VF, Shehadeh MM, Pallikaris AI, Pallikaris IG. Anterior segment applications of in vivo confocal microscopy. Semin Ophthalmol 2015; 30: 243-251.
[21] Nicklaus M, Nauenheim C, Krayev A, Gavrilyuk V, Belyaev A, Ruediger A. Note: tip enhanced Raman spectroscopy with objective scanner on opaque samples. Rev Sci Instrum 2012; 83: 066102.
[22] Yamabayashi S, Suzuki H, Tsukahara S. Application of a laser scanning microscope to the morphological study of corneal epithelium of rabbits. Ophthalmic Res 1991; 23: 115-120.
[23] Meadows DL, Paugh JR. Use of confocal microscopy to determine matrix and surface protein deposition profiles in hydrogel contact lenses. CLAO J 1994; 20: 237-241.
[24] Masters BR. Confocal microscopy and three-dimensional reconstruction of thick, transparent, vital tissue. Scanning Microsc Suppl 1992; 671-679.
[25] Masters B. Three-dimensional confocal microscopy of the living in situ rabbit cornea. Opt Express 1998; 3: 351-355.
[26] Paddock SW. Principles and practices of laser scanning confocal microscopy. Mol Biotechnol 2000; 16: 127-149.
[27] Xiao W, Chen X, Li W, Ye S, Wang W, Luo L, Liu Y. Quantitative analysis of injury-induced anterior subcapsular cataract in the mouse: a model of lens epithelial cells proliferation and epithelial-mesenchymal transition. Sci Rep 2015; 5: 8362.
[28] Song W, Liu W, Niu X, Wang Q, Sun L, Liu M, Fan Y. Three-dimensional morphometric comparison of normal and apoptotic endothelial cells based on laser scanning confocal microscopy observation. Microsc Res Tech 2013; 76: 1154-1162.
[29] Greulich KO, Wijnaendts van Resandt RW, Kneale GG. Time resolved fluorescence of bacteriophage Pfl DNA binding protein and its complex with DNA. Eur Biophys J 1985; 11: 195-201.
[30] Kneale GG, Wijnaendts van Resandt RW. Time-resolved fluorescence of bacteriophage Pf1 DNA-binding protein. Determination of oligonucleotide and polynucleotide binding parameters. Eur J Biochem 1985; 149: 85-93.
[31] Nelson M, Ledoux J, Taylor M, Bonev A, Hannah R, Solodushko V, Shui B, Tallini Y, Kotlikoff M. Spinning disk confocal Microscopy of calcium signalling in blood vessel walls. Microsc Anal 2010; 24: 5-8.
[32] Enoki R, Ono D, Hasan MT, Honma S, Honma K. Single-cell resolution fluorescence imaging of circadian rhythms detected with a Nipkow spinning disk confocal system. J Neurosci Methods 2012; 207: 72-79.
[33] Takahashi N, Oba S, Yukinawa N, Ujita S, Mizunuma M, Matsuki N, Ishii S, Ikegaya Y. High-speed multineuron calcium imaging using Nipkow-type confocal microscopy. Curr Protoc Neurosci 2011; Chapter 2: Unit 2.14.

[34] Yong CL, Zou YX, Lee JW, Chan SC. Time-interleaved analog-to-digital converter (TIADC) compensation using multichannel filters. IEEE Trans Circuits Syst I Regul Pap 2009; 56: 2234-2247.
[35] Lee JH, Lee S, Gho YS, Song IS, Tchah H, Kim MJ, Kim KH. Comparison of confocal microscopy and two-photon microscopy in mouse cornea in vivo. Exp Eye Res 2015; 132: 101-108.
[36] Morris DC, Zhang Z, Davies K, Fenstermacher J, Chopp M. High resolution quantitation of microvascular plasma perfusion in non-ischemic and ischemic rat brain by laser-scanning confocal microscopy. Brain Res Brain Res Protoc 1999; 4: 185-191.
[37] Arribas SM, Daly CJ, McGrath IC. Measurements of vascular remodeling by confocal microscopy. Methods Enzymol 1999; 307: 246-273.
[38] Arribas SM, Gonzalez JM, Briones AM, Somoza B, Daly CJ, Vila E, Gonzalez MC, McGrath JC. Confocal myography for the study of hypertensive vascular remodelling. Clin Hemorheol Microcirc 2007; 37: 205-210.
[39] Khattab A, Wertheim D, Petford N, Kirk R, Wijesinghe L, Ali I. Application of confocal microscopy for 3D assessment of carotid plaque structure: implications for carotid blood flow and stroke research. J Vasc Interv Neurol 2011; 4: 1-4.
[40] Pozarowski P, Holden E, Darzynkiewicz Z. Laser scanning cytometry: principles and applications-an update. Methods Mol Biol 2013; 931: 187-212.
[41] Hurley NF, Zhang T, Zhao W, Xu G. Method to build 3D digital models of porous media using transmitted laser scanning confocal mircoscopy and multi-point statistics: US, US20-110004447[P] 2011.
[42] Huang A, Nielson GM, Razdan A, Farin GE, Baluch DP, Capco DG. Thin structure segmentation and visualization in three-dimensional biomedical images: a shape-based approach. IEEE Trans Vis Comput Graph 2006; 12: 93-102.
[43] Heim R, Prasher DC, Tsien RY. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc Natl Acad Sci U S A 1994; 91: 12501-12504.
[44] Heim R, Tsien RY. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr Biol 1996; 6: 178-182.
[45] Tsien RY. The green fluorescent protein. Annu Rev Biochem 1998; 67: 509-544.
[46] Pines J. Green fluorescent protein. Methods in enzymology. FEBS Lett 1999; 459: 467-472.
[47] Wachter RM, Elsliger MA, Kallio K, Hanson GT, Remington SJ. Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein. Structure 1998; 6: 1267-1277.
[48] Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA. Fluorescent proteins from nonbioluminescent anthozoa species. Nat Biotechnol 1999; 17: 969-973.
[49] Wang DM, Wang XC, Zhang WC. Revealing the F_actin networks in interphase nuclei of garlic clove cells by confocal fluorescence Microscopy. Acta Bot Sin 2000; 42: 1167-1171.
[50] Xiu-Lin Y, Edward Y, Shi-Xiong X, Zee S Y, Sui-Hai T, Shiu-Hoi T. Confocal microscopic observations on microtubular cytoskeleton changes during megasporogenesis in Phaius tankervi-Uiae (Alton) B1. Acta Bot Sin 1996; 38: 667-685.
[51] O'Brien CM, Holmes B, Faucett S, Zhang LG. Three-dimensional printing of nanomaterial scaffolds for complex tissue regeneration. Tissue Eng Part B Reviews 2015; 21: 103-114.
[52] Valiente-Alandi I, Schafer AE, Blaxall BC. Extracellular matrix-mediated cellular communication in the heart. J Mol Cell Cardiol 2016; 91: 228-237.
[53] Murphy CM, O'Brien FJ, Little DG, Schindeler A. Cell-scaffold interactions in the bone tissue engineering triad. Eur Cell Mater 2013; 26: 120-132.
[54] Ge S, Zhao N, Wang L, Liu H, Yang P. Effects of hydroxyapatite nanostructure on channel surface of porcine acellular dermal matrix scaffold on cell viability and osteogenic differentiation of human periodontal ligament stem cells. Int J Nanomedicine 2013; 81: 887-895.
[55] Lee V, Singh G, Trasatti JP, Bjornsson C, Xu X, Tran TN, Yoo SS, Dai G, Karande P. Design and fabrication of human skin by three-dimensional bioprinting. Tissue Eng Part C Methods 2014; 20: 473-484.
[56] Martin TA, Caliari SR, Williford PD, Harley BA, Bailey RC. The generation of biomolecular patterns in highly porous collagen-GAG scaffolds using direct photolithography. Biomaterials 2011; 32: 3949-3957.
[57] Johnson PC, Mikos AG, Fisher JP, Jansen JA. Strategic directions in tissue engineering. Tissue Eng 2007; 13: 2827-2837.
[58] Bramfeldt H, Sabra G, Centis V, Vermette P. Scaffold vascularization: a challenge for three-dimensional tissue engineering. Curr Med Chem 2010; 17: 3944-3967.
[59] Wang MO, Vorwald CE, Dreher ML, Mott EJ, Cheng MH, Cinar A, Mehdizadeh H, Somo S, Dean D, Brey EM, Fisher JP. Evaluating 3D-printed biomaterials as scaffolds for vascularized bone tissue engineering. Adv Materials 2015; 27: 138-144.

[60] Laschke MW, Menger MD. Prevascularization in tissue engineering: current concepts and future directions. Biotechnol Adv 2016; 34: 112-121.
[61] Nguyen LH, Annabi N, Nikkhah M, Bae H, Binan L, Park S, Kang Y, Yang Y, Khademhosseini A. Vascularized bone tissue engineering: approaches for potential improvement. Tissue Eng Part B Reviews 2012; 18: 363-382.
[62] Leslie-Barbick JE, Shen C, Chen C, West JL. Micron-scale spatially patterned, covalently immobilized vascular endothelial growth factor on hydrogels accelerates endothelial tubulogenesis and increases cellular angiogenic responses. Tissue Eng Part A 2011; 17: 221-229.
[63] Wang YB, He JT, Feng YQ, Xia LI, Zhang R, Zhang YD. Observation of morphology of nerve fibers with a laser scanning confocal microscope after burns. Journal of Shandong University 2010.
[64] Lim YC, Bhatt MP, Kwon MH, Park D, Lee S, Choe J, Hwang J, Kim YM, Ha KS. Prevention of VEGF-mediated microvascular permeability by C-peptide in diabetic mice. Cardiovasc Res 2014; 101: 155-1564.
[65] Kulkarni PM, Barton E, Savelonas M, Padmanabhan R, Lu Y, Trett K, Shain W, Leasure JL, Roysam B. Quantitative 3-D analysis of GFAP labeled astrocytes from fluorescence confocal images. J Neurosci Methods 2015; 24: 638-651.
[66] Yong W, Zinchuk V. Grossenbacher-Zinchuk O, Stefani E. Critical evaluation of quantitative colocalization analysis in confocal fluorescence microscopy.Interdiscip Sci 2012; 4: 27-37.
[67] Xia Z, Villa MM, Wei M. A Biomimetic collagen-apatite scaffold with a multi-level lamellar structure for bone tissue engineering. J Mater Chemistry B 2014; 2: 1998-2007.