Summary of the Ph.D. Thesis

Differential polarization laser scanning microscopy. Technical development and biological applications

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2012

Szeged

Introduction

Hierarchically organized complex molecular macro-assemblies are widespread among biological samples: e.g. cellulose fibers, stacked membranes, ordered macromolecular fibrils, self-assembled aggregates. In the examination of this type of structures, the interaction between the sample and the polarized light provides unique information by revealing the anisotropic molecular architecture of the sample.

Earlier, in the Biological Research Centre, differential polarization laser scanning microscopy (DP-LSM) has been developed, which unites the following advantages: the simplicity of sample preparation for light microscopy techniques, the quality of fluorescent confocal imaging and access to structural information provided by dichrographs. Although, the spectral measurements cannot be carried out in the DP-LSM, the measurements are restricted to discrete wavelengths, polarization parameters obtained via this way provide similar information as measurements in dichrographs but on a microscopic scale and with the possibility of 3D mapping of the anisotropy parameters.

The aim of my thesis is to demonstrate the usefulness of DP-LSM through examination of various highly organized structures in biological samples, and to develop this imaging technique to broaden the field of its application. Some preliminary measurements were carried out earlier, e.g. on the O-ring canal that connects the oocytes and nurse cells in *Drosophyla melanogaster*. It has been shown that imaging the anisotropy of the O-rings in wild type and mutants provided important information on the molecular organization of the actin-based canals [Gorjanácz et al., 2006].

The DP-LSM – beyond structural investigations – can also be applied to examine the microenvironment of fluorescent molecules. Lipid rafts in the membrane of human lymphocytes were successfully identified with the help of mapping the changes in the degree of polarization of the fluorescence emission of fluorescent dyes labeling the lipid membranes [Gombos et al., 2008].

At the beginning of my work I also took part in the elucidation of the three dimensional topology of granal thylakoid membranes, a research topic which had been debated for decades in the literature [cf. Mustárdy and Garab, 2003]. Recently, the so-called helical model of granum-stroma membrane assembly has been questioned and an alternative three

dimensional structure proposed [Shimoni et al., 2005]. I participated in the work of high resolution electronmicroscopic tomography of granal thylakoid membranes [Mustárdy et al., 2008], and also investigated these structures by DP-LSM. Our DP (linear birefringence) imaging of isolated plant chloroplasts revealed a highly organized photosynthetic membrane system, which exhibited very strong local birefringence in the granal regions [Steinbach et al., 2005], a feature consistent with the semicrystalline organization of the complexes in these membranes [Dekker and Boekema, 2005]. This property can be also used for micromanipulation of chloroplast and subchloroplast particles [Garab et al., 2005a].

Throughout my Ph.D. research work I took part in various biological applications of DP-LSM and also in projects to improve this imaging technique. The chapters are presented in the order of the realization of the different investigations: results of experiments carried out on plant cell wall (*Convallaria majalis*) are presented first, followed by the project on the macroorganization of isolated human amyloid fibrils and on the anisotropic features of self-assembling synthetic porphyrin nanorods. In these works, I used the DP-LSM based on Zeiss LSM410 of the Biological Research Centre in Szeged. Thanks to the publications and conference materials, the possibility arose to attach a DP-unit to the Olympus FV500 microscope at the Immunology Department of the Eötvös Loránd University, Budapest. I also took part in the construction of a high frequency DP-LSM in Szeged.

My Ph.D. thesis is based on the following publications: Steinbach et al., 2008; Steinbach et al., 2009; Steinbach, 2011a; Steinbach et al.; Chappaz-Gillot et al. Concerning the technical part of my work no papers have been published yet; a patent is pending about the high frequency modulator.

Aims

The major aim of my Ph.D. research work was to test the DP-LSM for various applications. The technical development and adaptation of DP imaging technique to new generation microscopes were also part of my project in order to widen the applicability of this technique.

I. Biological applications:

1. linear dichroism measurements on stained cell wall samples to map the anisotropy of cellulose fibers
2. determining the supramolecular architecture of amyloid fibrils and building a 3D model of macroaggregates
3. elucidating the anisotropic organization of self-assembling synthetic Zn-ketol porphyrins, nanorods designed for light harvesting (‘artificial chromosomes’)

II. Technical developments:

1. corrections of DP images, based on Mueller-matrix analyses of the dichroic mirrors of DP-LSM
2. adaptation of the DP-attachment to new generation confocal microscopes (the harmonization of the hardware and software units, test measurements)
3. building the prototype of a high frequency DP-LSM (HFR DP-LSM)

Materials and methods

Cell wall of Convallaria

The specimen used in this study was a 15-µm section of the root (rhizome) of *Convallaria majalis* plant, which was stained with Acridin Orange and embedded in Eukitt [Schaffer and Bauch, 2004]. It was purchased from Carl Zeiss Jena (Jena, Germany), and was produced by Johannes Lieder GmbH & Co. KG Laboratorium.

Amyloid filaments

After the preparation, the sections were stained with freshly prepared 0.1% aqueous Congo Red solution according to Romhányi [1979], for 10 min and, after rinsing in running water (30 min), were mounted with diluted gum arabic solution [Romhányi, 1958]. The samples were provided for the measurements by József Makovitzky (Department of Neuropathology, University of Heidelberg, Heidelberg, Germany).

Zn-ketol porphyrins

The self-organizing bacteriochlorophyll molecules are forming chlorosomes and it is an efficient antenna to transfer the harvested light energy. Using the blue-print of chlorosomes it became possible to shyntetize artificial porphyrin molecules, that can self-assemble into nanorods (‘artificial chlorosomes’). The final goal is to design nonpolluting, nontoxic, robust molecular devices converting photons to current.

For my DP-LSM measurements the Zn-ketol porphyrins were prepared by Silviu Balaban and his co-workers [Balaban et al., 2009]; contribution of Anita Župčanová (Biological Centre, Academy of Sciences of the Czech Republic, Ceské Budejovice, Czech Republic) was also essential.

Mueller-matrices

Polarimetric investigation was performed on the Wollam M-2000F ellipsometer of the Department of Optics and Quantum Electronics, at the University of Szeged.

Confocal microscopy

The measurements on the biological samples were performed on a Zeiss LSM410 confocal microscope. It is based on an Axiovert 135 fluorescent microscope and a 3 channel scanning unit with internal HeNe lasers @543 nm and @633 nm and external Ar-ion laser @488 nm and @514 nm. This LSM was the basis of the high frequency (HFR DP-LSM) project as well.

The DP-attachment for the new generation microscope was connected to the Olympus FV500 of the Department of Immunology at Eötvös Loránd University. This LSM contained a scanning unit with 3+1 channels. The HeNe and the multiline Ar-ion lasers were fiber coupled.

Measuring by modulation/demodulation

The high speed differential polarization measurements are based on photoelastic modulators (PEM). The fused silica in the PEM upon mechanical stress becomes birefringent: periodically, phase shifts can be obtained via a piezoelectric transducer and by this way the polarization state of the light can be changed periodically between orthogonally polarized states, at a frequency of 50 kHz for circularly polarized, and 100 kHz for linearly polarized light. This device can also be used for the analysis of the polarization content of the fluorescence emission or the reflected beam.

I have used three different types of modulators. The HINDS Instruments PEM-90 was used in the DP-LSM of the BRC, the HINDS Instruments PEM-100 was built in into the Olympus FV500 microscope, and I applied the special version of Envitech Ltd. for the HFR DP-LSM.

To record the different intensities belonging to the orthogonal states I used frequency selective amplifier combined with phase sensitive detector card for PC's ISA system (constructed by Pawel Kamasa, Wigner RCP, Budapest) and a SIGNAL Recovery 7280 lock-in amplifier.

The DP-LSM based on Zeiss LSM410

The differential polarization laser scanning microscope developed in the Biological Research Centre allows the modulation of the polarized light both in the excitation and emission lightpaths during the imaging, so the most commonly used DP-parameters can be measured and mapped [Pomozi, 2002; Garab et al., 2005b; Garab et al., 2007]:

	Modulation of the laser	Analysis of the fluorescence
Linear polarization	Linear dichroism (LD) Fluorescence detected linear dichroism (FDLD) Linear birefringence (LB)	Degree of polarization of fluorescence emission (P) Anisotropy of fluorescence emission after depolarized excitation (r)
Circular polarization	Circular dichroism (CD) Fluorescence detected circular dichroism (FDCD)	Circularly polarized luminescence (CPL)

Summary of my results

The elucidation of the molecular architecture of complex, highly organized molecular macro-assemblies is an important task for basic biology. Differential polarization measurements with dichrographs are widely used to gain information about ordered structures (e.g. CD spectra of proteins and photosynthetic membrane-complexes, LD spectra on macroscopically ordered membranes and complexes and supercomplexes). Dichrographs provide data averaged for the whole sample. For inhomogeneous samples – e.g. cells and tissues – measurements on macroscopic scale are not satisfactory, and in some cases not feasible and thus microscopic techniques must be applied. The microscopic DP-imaging technique allows the mapping of anisotropy (using its different parameters) in 2D and 3D.

The main results of my PhD work – related to projects to improve the technique of DP-imaging and to biological applications using the DP-LSM – are summarized as follows:

1st thesis. DP measurements have some difficulties because of the confocal microscope's fluorescent modes: the dichroic mirrors in the fluorescent lightpath can alter the polarization states of light. In order to have reliable DP-values, it was necessary to perform Mueller-matrix measurements on these mirrors; these data were then used for the correction of DP-images in a real-time regime.

The data processing using corrections based on Mueller-matrix analyses, enables us to perform quantitative real time measurements on the DP-LSM.

2nd thesis. By using a cell wall sample of *Convollaria* stained with Acridine Orange I have demonstrated the advantages of FDLD measurements in confocal regime compared to the measurement performed in the non-confocal transmission mode LD imaging. The optical slicing avoided the smearing of the DP-image by the layers above each other, containing dipoles in different orientations, and it provides an excellent image quality in 3D.

The DP-LSM technique is suitable for quantitative measurements on cell walls in 3D.

3rd thesis. In neuroscience, the structure of amyloid filaments and their plaques are currently are of substantial interest. These filaments, insoluble, highly ordered protein aggregates are in connection with many neurodegenerative diseases. Isolated human amyloid fibrils stained with Congo Red was mapped using FDLD measurements. The DP-images revealed anisotropic structures and periodic patterns at the supermolecular organization level, whereas the fluorescence images appeared to be homogenous.

Amyloid filaments exhibited periodic polarization patterns in DP images. The periodicity was 1.6 µm, evidently originating from a previously unknown suprahelical structure of the filaments, probably formed via supercoiling of a lower level helical structure identified earlier.

4th thesis. Although, the level of solar energy exceeds by about four orders of magnitude the current need of mankind, its density is low. Photosynthetic organisms have developed light harvesting antennae to supply efficiently their photochemical reaction centres by excitation energy. This type of structures have served the basis for designing similar, artificial systems, such as the self-assembling 'artificial chlorosome' composed of a Zn-ketol porphyrin molecule synthetisized by Silviu Balaban and his coworkers. Using FDLD and r images, I provided evidence for the highly anisotropic molecular organization of these biomimetic structures; DP data, combined with macroscopic LD and fluorescence polarization measurements could be used to determine the orientation of the different transition dipoles of the Zn-ketol porphyrin, leading to the construction of a 3D model of the rods. Strong anisotropy, similar to that in the chlorosomes, together with the possibilities to manipulate these nanorods in magnetic and electric fields make these structures of high interest for solar energy technology.

Synthetic Zn-ketol porphyrins self-assemble into homogenous, magnetically orientable highly anisotropic nanorods, the 3D model of which could be in large part based on DP imaging data.

5th thesis. Since DP-measurements can be efficiently used in characterizing highly organized molecular structures, even in living tissues, it is desirable to adapt this technique to new generation microscopes. Because of their compact design, however, this required special considerations and technical solutions. I constructed a new generation DP-LSM based on Olympus FV500, via designing a DP-attachment suitable for most new generation microscopes, and special optical pathways for the given model, as well as using electronic units similar to the one in the DP-LSM based on Zeiss LSM410.

The DP-LSM technique can be adapted to new generation microscopes, by using a DP-unit that is readily attachable to new generation microscopes essentially without modifying their internal structures. By this means, LSMs can be equipped with a DP unit, lending the capability to measure additional physical parameters.

6th thesis. The essential unit of the DP-measurement is the photoelastic modulator, which limits the scanning rate for several cycles of its periodicity - for commercially available modulators this is 100 kHz for imaging using linearly polarized light. In order to accelerate the scanning rate, a 400 kHz PEM from Envitech Ltd. was employed. To this end, mechanical and optical difficulties had to be overcome when I tested this unit and assembled into the LSM; also, I wrote a user interface program for DP measurements.

The prototype of the new DP-LSM equipped with a high frequency modulator (HFR-PEM) provide four times quicker measurements. This fact improves considerably its applicability both for optical slicing and measurements on living objects.

In summary, In my PhD work, I have demonstrated the applicability and usefulness of DP-imagings in different biological systems, improved the technical performance of DP-LSM, and constructed a DP attachment for new-generation LSMs as well as the prototype of a HFR DP-LSM. These works are thought to bring differential polarization laser scanning microscopy closer to the everyday practice in biology and material sciences and to aid researchers to unravel novel, anisotropic features in highly organized molecular macroassemblies.

References

Balaban T. S., Bhise A. D., Bringmann G., Bürck J., Chappaz-Gillot C., Eichhöfer A., Fenske D., Götz D. C. G., Knauer M., Mizoguchi T., Mössinger D., Rösner H., Roussel C., Schraut M., Tamiaki H., Vanthuyne N. (**2009**) Mimics of the self-assembling chlorosomal bacteriochlorophylls: regio- and stereoselective synthesis and stereoanalysis of acyl(1-hydroxyalkyl)porphyrins. J Am Chem Soc 131:14480–14492.

Bognár K., Czitrovszky A., Illyés Á., Nagy A., Garab Gy., Steinbach G. (**2012***)Magas frekvenciás fotoelasztikus fénymodulátor. *Benyújtott szabadalom.

Chappaz-Gillot C, Marek P. L., Blaive B. J., Canard G., Bürck J., Garab G., Hahn H., Jávorfi T., Kelemen L., Krupke R., Mössinger D., Ormos P., Malla Reddy C., Roussel C., Steinbach G., Szabó M., Ulrich A. S., Vanthuyne N., Vijayaraghavan A., Zupcanova A., Balaban T. S. (**2012**) Anisotropic organization and microscopic manipulation of self-assembling synthetic porphyrin microrods that mimic chlorosomes: bacterial light-harvesting systems. J Am Chem Soc 134:944-954.

Dekker J. P., Boekema E. J. (**2005**). Supramolecular organization of thylakoid membrane proteins in green plants. Biochim Biophys Acta 1706: 12–39.

Garab G., Galajda P., Pomozi I., Finzi L., Praznovszky T., Ormos P., van Amerongen H. (**2005a**) Alignment of biological microparticles by a polarized laser beam. Eur Biophys J 34:335-43.

Garab G., Pomozi I., Jörgens R., Weiss G. (**2005b**) Method and apparatus for determining the polarization properties of light emitted, reflected or transmitted by a material using a laser scanning microscope. US Patent 6,856,391.

Garab G., Pomozi I., Jörgens R., Weiss G. (**2007**) Method and apparatus for determining the polarization properties of light emitted, reflected or transmitted by a material using a laser scanning microscope. EP 1 334 339 (01 996 722.3) Patent

Gombos I., Steinbach G., Pomozi I., Balogh A., Vámosi G., Gansen A., László G., Garab G., Matkó J. (**2008**) Some new faces of membrane microdomains: a complex confocal fluorescence, differential polarization and FCS imaging study on live immune cells Cytometry Part A 73A:220–229.

Gorjanacz M., Török I., Pomozi I., Garab G., Szlanka T., Kiss I., Mechler B.M. (**2006**) Domains of importin-α2 required for ring canal assembly during Drosophila oogenesis. J Struct Biol 154:27–41.

Mustárdy L., Garab G. (**2003**) Granum revisited. A three-dimensional model – where things fall into place. Trends Plant Sci 8:117–122.

Mustárdy L., Buttle K., Steinbach G., Garab G. (**2008**) The three-dimensional network of the thylakoid membranes in plants: quasihelical model of the granum-stroma assembly. Plant Cell 20:2552–2557.

Pomozi I. (**2002**): Polarizációs mintázatok nagy és kis látószögű képalkotó polarimetriai mérése légköri optikai és biológiai vonatkozásokkal. PhD értekezés, ELTE-TTK

Romhányi G. (**1979**) Selective demonstration of amyloid deposits and methodical possibilities of analysis of their ultrastructural differences / Selektive Darstellung sowie methodologische Möglichkeiten der Analyse ultrastruktureller Unterschiede von Amyloidablagerungen. Zentralbl Allg Pathol 123:9–16.

Schaffer J, Bauch H. (**2004**) Test preparation for microscopes. US Patent. Application number: 20040180384. www.freepatentsonline.com/20040180384.html (a letöltés dátuma: 2012. 03. 12.)

Shimoni E., Rav-Hon O., Ohad I., Brumfeld V., Reich Z. (**2005**) Three dimensional organization of higher-plant chloroplast thylakoid membranes revealed by electron tomography. Plant Cell 17:2580–2586.

Steinbach G., Besson F., Pomozi I., Garab G. (**2005**) Differential polarization laser scanning microscopy: biological applications. Proc SPIE 5969:566–575.

Steinbach G., Pomozi I., Zsiros O., Pay A., Horváth G. V., Garab G. (**2008**) Imaging fluorescence detected linear dichroism of plant cell walls in laser scanning confocal microscope. Cytometry A 73A:202–208

Steinbach G., Pomozi I., Zsiros O., Menczel L., Garab G. (**2009**) Imaging anisotropy using differential polarization laser scanning confocal microscopy. Acta Histochem. 111:316–325.

Steinbach G. (**2011a**) Differenciál-polarizációs mikroszkópos technikák fejlesztése és amiloid filamentumok finomszerkezeti vizsgálata differenciál-polarizációs lézerpásztázó mikroszkópia felhasználásával. egészségügyi mérnök szakdolgozat, BMGE-VIK

Steinbach G., Pomozi I., Jánosa D. P., Makovitzky J., Garab G. (**2011b**) Confocal fluorescence detected linear dichroism imaging of isolated human amyloid fibrils. Role of supercoiling. J Fluoresc 21:983–989.

Publications *Cumulative Impact Factor: 28,033*

Steinbach G., Besson F., Pomozi I., Garab G. (**2005**) Differential polarization laser scanning microscopy: biological applications. Proc SPIE 5969:566–575.

***Steinbach G.**, Pomozi I., Zsiros O., Páy A., Horváth G. V., Garab G. (**2008**) Imaging fluorescence detected linear dichroism of plant cell walls in laser scanning confocal microscope. Cytometry A, 73A: 202-208. IF: 3,259

Mustárdy L., Buttle K., **Steinbach G.**, Garab G. (**2008**) The Three-Dimensional Network of the Thylakoid Membranes in Plants: Quasihelical Model of the Granum-Stroma Assembly. Plant Cell 20:2552-2557. IF: 9,296

Mustárdy L., Buttle K., **Steinbach G**., Garab G. (**2008**) Three-dimensional architecture of the granum-stroma thylakoid membrane system revealed by electron tomography. In: Allen J. F., Gantt E., Golbeck J. H., Osmond B. eds. Photosynthesis. Energy from the Sun: 14th International Congress on Photosynthesis.: Springer, Heidelberg. pp. 771-774.

Gombos I., **Steinbach G.**, Pomozi I., Balogh A., Vámosi G., Gansen A., László G., Garab G., Matkó J. (**2008**) Some new faces of membrane microdomains: a complex confocal fluorescence, differential polarization and FCS imaging study on live immune cells. Cytometry A 73A:220-229. IF: 3,259

***Steinbach G.**, Pomozi I., Zsiros O., Menczel L., Garab G. (**2009**) Imaging anisotropy using differential polarization laser scanning confocal microscope. Acta Histochem 111:317-326. IF: 1,234

***Steinbach G**., Pomozi I., Jánosa D. P., Makovitzky J., Garab G. (**2011**) Confocal Fluorescence Detected Linear Dichroism Imaging of Isolated Human Amyloid Fibrils. Role of Supercoiling. J Fluoresc 21:983-989. IF: 1,966**

*Chappaz-Gillot C., Marek P. L., Blaive B. J., Canard G., Bürck J., Garab G., Hahn H., Jávorfi T., Kelemen L., Krupke R., Mössinger D., Ormos P., Malla Reddy C., Roussel C., **Steinbach G.**, Szabó M., Ulrich A. S., Vanthuyne N., Vijayaraghavan A., Zupcanova A., Balaban T. S. (**2012**) Anisotropic organization and microscopic manipulation of self-assembling synthetic porphyrin microrods that mimic chlorosomes: bacterial light-harvesting systems. J Am Chem Soc 134:944-954. IF: 9,019**

Bognár K., Czitrovszky A., Illyés Á., Nagy A., Garab Gy., **Steinbach G**. Magas frekvenciás fotoelasztikus fénymodulátor. Patent pending.

*: publications related to the thesis – *Cumulative Impact Factor of them: 15,478*

**: expected impact factor