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Some engineering methods used in biophotonics as support in the investigation of insects

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***Abstract*— Joining efforts from different fields of research is an interesting way of easing solving problems or helping developing various applications which would be more difficult the common way. Using insects studying in other-than biology areas is a good alternative and it can also help develop various engineering applications. In the other way round, studying of insects can be boosted by implementing various engineering and biophotonics methods. Studying the structure of cocoons and other parts of insects by using laser microcopy, laser induced fluorescence and femtosecond laser interaction, is selected and described.Ovaj dokument predstavlja šablon za pripremu rada sa već definisanim stilovima u samom dokumentu za određene delove rada [naslov, tekst rada, naslovi poglavlja i potpoglavlja, označavanje tabela i slika, navođenje referenci, itd].**

Keywords—two-photon absorption, laser induced fluorescence, solitary bees, cocoons, hairs

I. Introduction

There are many definitions and approaches to the term of interdisciplinary science, but it could be understood as the research mode where theories, concepts, tools/techniques and perspectives from multiple scientific disciplines are integrated for better understanding of issues and problems in a single discipline [1]. Although it evolved in the 20th century in response to the institutionalization and segmentation of academic research and major transitions in society, it could be traced back to the endeavors in ancient Greek philosophy or Roman engineering.

Many scientific disciplines used biology as a source, mostly medicine and pharmacology, in modern times ecology and social sciences, but also very important influence is in mathematics and computer science - informatics (artificial intelligence, neural networks, genetic algorithms, evolutionary strategies, …), criminalistics (forensics), cultural heritage protection, food, civil engineering, … For instance, the earliest record of entomology use in criminal investigation is the discussion in a book The Washing Away of Wrongs (by Sung Tzu, 1295 AD), where during the investigation of a homicide flies landed on a sickle which indicated the murder weapon and resulted in a confession by the murderer [2].

In biology, concepts and techniques from other scientific disciplines are used, like mathematics, chemistry, astronomy, physics, photonics, … Here, some techniques and methods from physics, optics and photonics used in entomology research will be shown.

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II. Materials and Methods

A. Improving Microscopy by Two-photon Absorption, Confocal Laser Beam Scanning and Fluorescence

Microscopy, as one of the imaging techniques which enables seeing small objects commonly not visible by human eye, has significantly improved for last several decades by employing several physical concepts. In confocal scanning microscopy, point illumination is used and a pinhole is placed in front of the detector to eliminate the out-of focus signal, and in this way the optical resolution and contrast is increased. In order to obtain the information from all parts of the object, the illumination point is scanned across the object. Modern techniques use laser beam for the illumination. Two-photon absorption, though of low probability if compared to one-photon absorption, is a process used in microscopy with advantages like preserving objects and increasing the penetration depth. Low probability of the absorption is overcome by using high intensity illumination, like laser pulsed beams. In two-photon absorption, two photons of lower wavelength are simultaneously absorbed to excite an atom or a molecule to an excited state via a virtual energy level. This concept is used in two-photon excitation fluorescence microscopy, where the atom or molecule fluoresces after the two-photon excitation and the fluorescence wavelength is shorter than the excitation wavelength. Using lower excitation wavelengths (mainly infrared) preserves the materials of biological origin, e.g. tissues. Using confocal laser beam scanning microscopy together with two-photon excitation fluorescence the imaging is significantly improved. The advantages are optical sectioning (small volume excited), longer wavelength excitation (preserving tissues), increasing the penetration depth, no need for dyeing, while the disadvantage is the higher equipment cost, since mostly ultrafast lasers are used for illumination (excitation).

B. Nonlinear Laser Microscopy in Entomology Research

The microscopy that uses laser beam scanning, two-photon excitation fluorescence and confocal scanning would have a long and a complex term. For this reason, it is in many

occasions called nonlinear laser microscopy. It is an innovative method in entomology research, which offers the simplicity in sample preparation and enables the monitoring of tiny and hidden structures, whereas standard light microscopy poses some difficulties. Fluorescence is exploited in the sense that chitine autofluorescense enables the investigation of insects structures [3], [4], [5].

C. Setup

The apparatus used in the experiments was a standard light microscope (Carl Zeiss – Jena), modified, reconstructed and upgraded in the Institute of Physics Belgrade (see acknowledgments section), Fig. 1. The source of the illumination beam is Coherent laser system consisted of Mira 900F with femtosecond beam as an output (739 nm, pulsed, ~150 fs, 76 MHz) and Verdi V12 (532nm, CW, 9.3W) as a pump laser. A beam splitter with a detector is used to use small part of the beam for the power measurement. Laser beam scanner and a home-made telescope (beam expander) were added to the microscope body in order to scan and introduce the beam. Dichroic and fluorescence filters separate IR (illumination) part from the mid-range visible (light microscopy) and violet (photomultiplier detection).

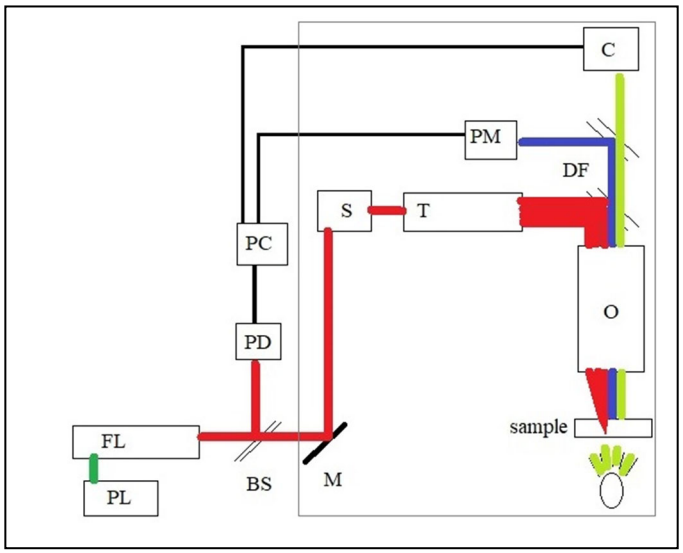

Fig. 1 A schematic representation of the setup used in the experiments. PL – pump laser, FL – femtosecond laser, BS – beam splitter, PD – photodetector, M – mirror, S – XY scanner, T – beam expander, DF – dichroic filter, O – objective, C – campera, PM – photomultiplier, PC – computer.

Added devices for observation were Canon EOS 100D camera and a photomultiplier. A PC, equipped with home-made software for the integration of the software for the control of the devices (photodetector, scanner, photomultiplier, camera), for imager analysis and for the system control, was a control part of the system.

The beam from the pump laser (green) excites the femtosecond (fs) laser to emit the NIR beam (red). One, small, part of the fs beam is used to measure the power, while the greatest part is introduced into the microscope. The scanner (S) enables the beam to scan across the surface of the sample. The beam expander (T) widens the beam in order to be more tightly focused by the objective (O). The fluorescence signal (blue), occurred due to the two-photon absorption, passes through the dichroic filter (DF) and is directed to the photo-multiplier (PM). A camera (C) is used to monitor thesample in visible light (green).

D. Solitary Bees

Solitary bees are in the family of Megachilidae, and are one of the best pollinating agents. The objects of this investigation are several examples of the genus *Osmia* (mason bees): *O. caerulescens*, *O. cornuta* and *O. bicornis*.

Characteristic of *Osmia* are: use hollow small tubular spaces for eggs, like hollow branches or common reed straws. They have common four-stage development (egg, larva, pupa, adult); larva spins cocoon around itself and enters a pupal stage. An adult matures either during autumn or winter by hibernating inside its insulatory cocoon. The anatomy shows head (three small ocelli, two large compound eyes, antennae and mouth), thorax (six legs and four wings) and abdomen (scopa for collecting pollen – females only; scopa is a cluster of hairs). Pollination is very efficient due to both the anatomy and the behavior. A bee is almost completely covered with hairs for pollen collecting [6] and it also “dances” in a flower in order to collect as much pollen as it could. Foraging occurs in early spring mainly on apples, pears, almonds, strawberries, even in bad weather.

A bee uses its hairs to collect pollen and oil, as well as for the thermo-regulation. A cocoon is made of silk (a protein polymer excreted by labial glands), and is consisted of two to three layers with the roles of protection, diffusion of gases and waterproofing. Samples (cocoons and hairs) were prepared in a standard way [7].

III. Results

The results were obtained by microscopic analyses in light as well as in nonlinear laser microscopy. In *O. cornuta* and *O. bicornis*, the cocoon is consisted of three layers, while in *O. caerulescens* of two layers, Fig. 2 [7].

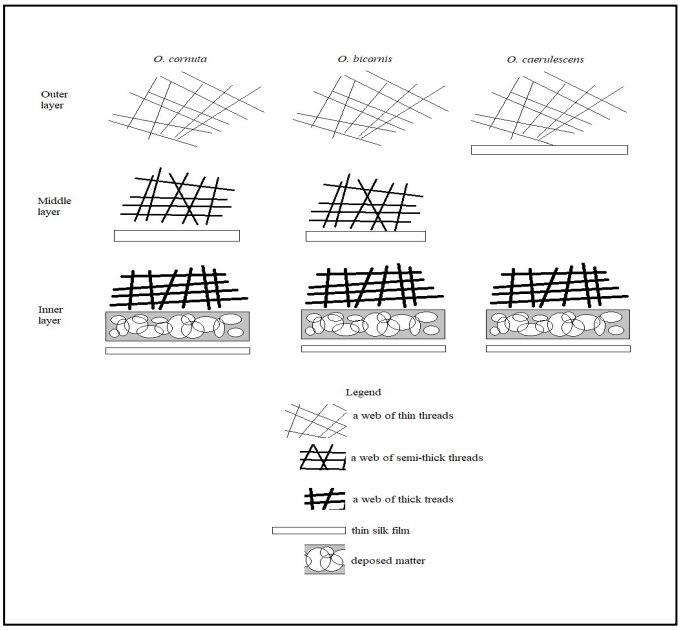

Fig. 2 Structural presentation of the cocoon wall of three *Osmia* bees. A legend shows the types of sub-layers.

Sub-structure of the cocoon layers in *O. cornuta* and *O. bicornis* shows that the outer layer is a net of silk threads, the middle layer is also a net of silk layers supported from the inner side by a thin silk film, and the inner layer is made of three sub-layers: a net of silk threads, a layer of deposited material, like pollen, and a thin silk film as the most inner sub-layer. In *O. caerulescens*, the outer layer has two sub-layers: a net of silk threads supported by a thin silk film, and the inner layer is of same structure as in *O. cornuta* and *O. bicornis*. Increasing the number of layers more serves as protection against predators than against water, since only one layer is suffcient for the defence.

The thin silk film of the outer layer in *O. caerulescens* probably originated from widening of silk threads, as indicated by red arrows in Fig. 3.

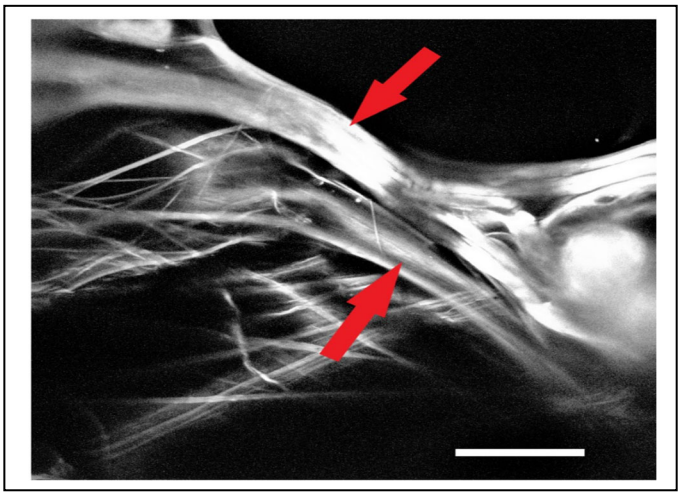

Fig. 3 Nonlinear laser microscope image of the outer layer of *O. caerulescens*. Red arrows indicate widening of the threads. White bar denotes 50 µm.

Fluorescence microscopy shows the middle layer od *O. bicornis* is consisted of silk threads supported by a thin silk film, Fig. 4. Some of the threads are widened and formed a thin film which supports the other threads. The threads of the outer layer are thinner and their color is lighter than the threads of the middle layer.

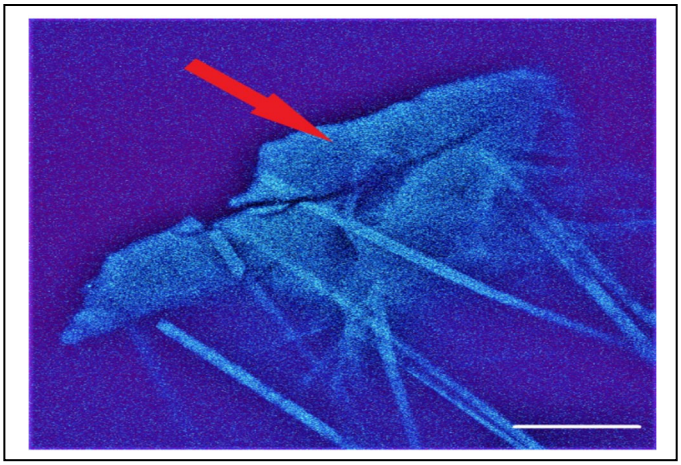

Fig. 4 Nonlinear laser microscopy image of the middle layer of *O.bicornis*. Red arrow indicates a sublayer of a thin silk film. White bar denotes 50 µm.

The advantage of using laser fluorescence microscopy over common light microscopy is the visibility of the thread structure. In Fig. 5, the middle layer noticed in both *O. cornuta* and *O. bicornis* is consisted of two sub-layers, similarly to the outer layer of *O. caerulescens*. The outer sublayer is a net of silk threads, but the net is thicker and more compact than in the outer layer and is supported by a thin silk film.

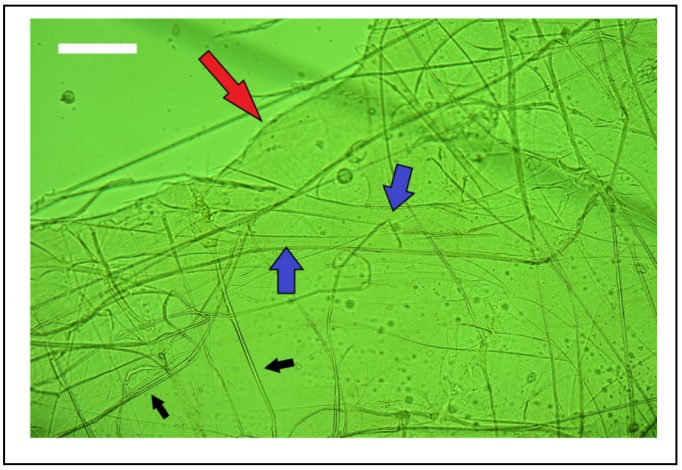

Fig. 5 Light microscopy image of the middle layer of *O.bicornis*. Red arrow indicates a sublayer of a thin silk film, blue arrows indicate widened threads which itegrate into the thin silk film, and black arrows indicate free silk threads.. White bar denotes 50 µm.

In Fig. 6, red arrows point to surface bumps in threads of *O. cornuta*, where the silk accumulated. White arrows point to elongated parts, probably tensioned. The threads of the inner layer are around 5 µm thicker than the threads of the outer layer.

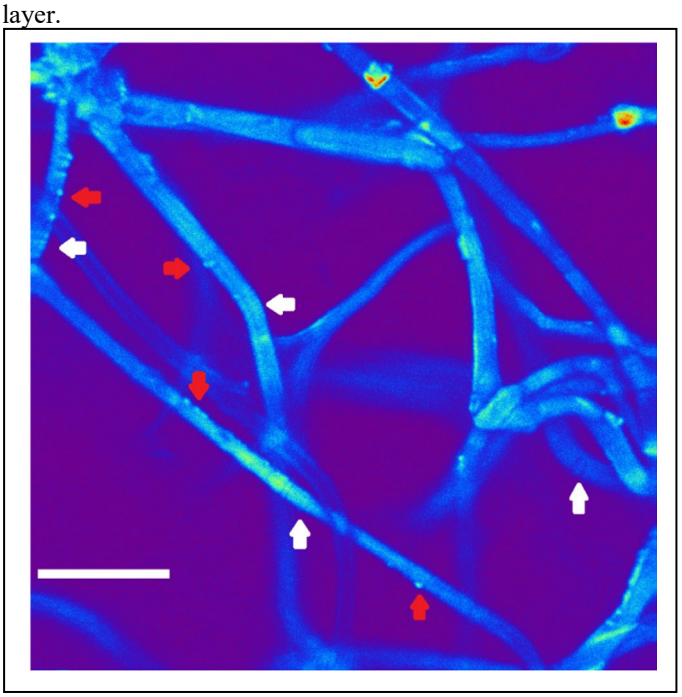

Fig. 6 Nonlinear laser microscope image of the middle layer of *O. cornuta*. White arrows indicate tensions of the threads, while red arrows indicate bumps on the threads. White bar denotes 50 µm.

The hair from the scope of *O. bicornis* is around 20 µm thick and it exhibits spiral rows on the hair surface, Fig. 7. The

presence of the rows probably improves the fetching of pollen grains on the hair surface.

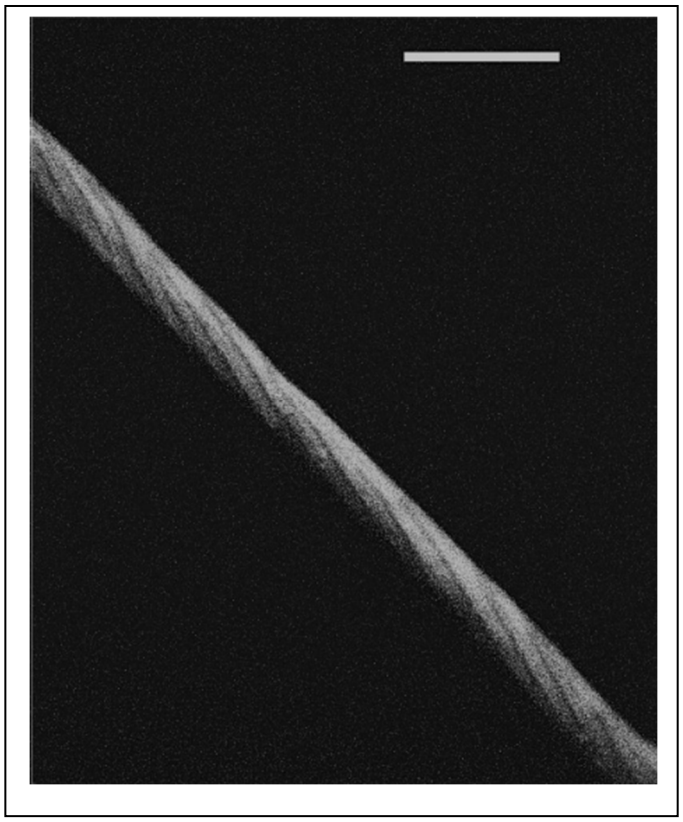

Fig. 7 Nonlinear laser microscope image of a hair from the scopa of *O. bicornis*. White bar denotes 50 μm.

IV. Conclusion

The implementation of physical concepts of two-photon absorption, confocal laser beam scanning and fluorescence, the investigation in entomology is significantly improved. Through nonlinear laser microscopy a better insight in the morphology of the cocoon and hairs of the taxa within the genus *Osmia* is given. Due to this method, the investigation and the characterization of the structures of *O. cornuta*, *O. bicornis* and *O. caerulescens* is more precise and simpler than it would be by the means of common light microscopy.

In *O. cornuta* and *O. bicornis*, three layers in the cocoon wall have been determined, while in *O. caerulescens*, the cocoon wall is built of two layers. There is a similarity in substructure of the inner layer of all three spieces. In all three species, the hairs from the scopa have spiral rows on the surface which improves pollen collecting.

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