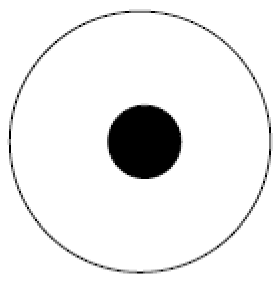

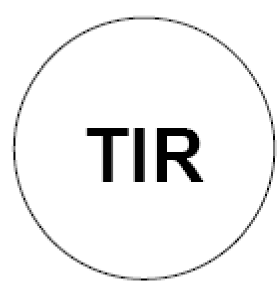

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IMAGING AND MODERN BIOPHYSICAL APPROACHES

Andjus, R., Pavle

Abstract— ***In addition to molecular biology, biophysics has a growing influence on the development of modern biology. With respect to societal aspects the concept of biomedical networking and large investments in multidisciplinary collaborations is the general worldwide trend. We present several intermingled modern biophysical approaches to the tackling of biomedical problems in neurosciences and beyond. All of these techniques (magnetic resonance imaging, automated and confocal microscopy, patch-clamp electrophysiology) can contribute to the development of a new concept of cell profiling towards high-throughput drug design, diagnostics and discovery in molecular physiology and medicine.***

Index Terms—*biophysics, neuroscience, MRI, confocal microscopy, electrophysiology.*

1. *INTRODUCTION*

BIOPHYSICS is the branch of interdisciplinary knowledge that applies principles of physics and chemistry and the methods of mathematical analysis and computer modeling to understand how the mechanisms of biological systems work. At the basic, molecular level it seeks to explain biological function in terms of the molecular structures and properties of specific molecules. These molecules, the sole building blocks of living organisms, assemble into cells, tissues, and whole organisms by forming complex individual structures with dimensions of 10 - 10,000 nm and larger. Proteins and ribonucleic acids assemble into ribosomes, the machinery for building proteins; lipids and proteins assemble into cell membranes, the external barriers and internal surfaces of cells with proteins forming permeation pathways; proteins and DNA wind up into chromosomes, and so on. Consequently, much effort in biophysics is directed to determining the structure of specific biological molecules and of the larger structures into which they assemble. Some of this effort involves inventing new methods and in building new instruments for viewing these structures. Many of the exciting new developments in biological microscopy and imaging, are part of this effort.

The biological questions with which biophysics is concerned are as diverse as the organisms of biology. Biophysics seeks to answer these questions using an eclectic approach. The specific molecules involved in a biological process are identified using the techniques of chemical and biochemical analysis. Their molecular structures and interactions are determined using the spectroscopic techniques of physics and chemistry. And the relationship between biological function and molecular structure is investigated using highly precise and exquisitely sensitive physical instruments and techniques that are able to monitor the properties or the movement of specific groups of molecules, or in exciting new developments, are able to view and manipulate single molecules and to measure their behavior. Biophysics explains biological functions in terms of molecular mechanisms: precise physical descriptions of how individual molecules work together like tiny machines to produce specific biological functions. These biophysical mechanisms are explained through detailed molecular and molecular dynamics models.

The interdisciplinary approach of biophysics is pertinent to the development of modern biology. On the other hand, there are indicators that the large-scale bioscience made of big multidisciplinary networks with large investments has benefited more than traditional, investigator-initiated research. At the same time it is questioned if this phenomenon of “Big biology” undermines the foundations of individual researcher’s discovery-based science.

We have chosen to present this foreseen development of modern biology through general worldwide societal implications and strategies and by emphasizing several examples of interdisciplinary, mainly biophysical approaches towards future biomedical discoveries and applications.

2. WORLDWIDE SOCIETAL IMPLICATIONS

Today’s developed countries and their science funding agencies are spending vast resources on

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multidisciplinary centers aimed at bringing biologists together with chemists, physicists, mathematicians and others, in the hope that new interactions will bring fresh insights to biological problems. This approach is inherent to the scientific discipline of biophysics which is thus well suited as a scientific approach for the discipline networking for new solutions in biology.

Scientific leaders agree that collaborative projects can produce results that would be impossible for specialized individuals working alone to achieve. This may, however, undermine the foundations of discovery-based science. The team approach promises to advance the work of basic scientists, but researchers need the freedom to pursue new ideas about biology — even if it's not clear how they will have an immediate impact on patients' lives.

Biophysics again can offer to address the applicative as well as basic biomedical problems. In fact, the biophysical societies of the world are organized within the International Union for Pure and Applied Biophysics (IUPAB) where both aspects of the discipline are clearly emphasized in the name of the Union.

The interdisciplinarity principle also builds the research strategy towards bigger projects and larger networks. This trend has been developed in United States and has been growingly also applied by European Commission research directorate. The worldwide commitment to "Big biology" [6] stems partly from the success of the Human Genome Project, which spurred the growth of large sequencing centers. Namely, biology is now asking questions about complex networks of cellular signals that require groups of specialists taking a team approach. A number of large research multimillion centers have been built for this purpose in USA. The genome project also helped to convince lawmakers in USA that science was proceeding in new ways. This has lead to the growth of National Institutes of Health (NIH) funding since late 90's to more than a doubled figure in the first decade of the 21st century. However, in spite of the rising budget there is a falling success rate for funding of individual projects at the NIH. It is believed at this world leading biomedical institution that the research in medicine and biology is at an important turning point that may require new strategies. There are also indications that the large-scale science has benefited more than traditional, investigator-initiated research. Peer-review criteria are already adjusted to accommodate interdisciplinary, translational and clinical projects. A trend is also recognized in NIH to shift focus from basic research to projects that target specific diseases. Many basic biologists are supportive of the shift towards team science, and agree that researchers should think more about medicine.

In China "mega projects" costing between hundreds of millions to billions of dollars are planned to be funded for the next 15-20 years. But there may be a problem in a still too intermingled relation of science with politics leading to investment in bad projects. Chinese scientists around the world are warning that the government should first build up its scientific talent and resources by investing in more small-scale research first, before committing to huge, top-down projects.

While in Europe there is a trend to build networks so that large investments can be shared – only a few can afford large initiatives. Instead - a sense of retreat from big biology is noticed. On the other hand, it is realized that there is no way around interdisciplinary work.

3. Technical and scientific implications

In the second half of this contribution we will elaborate on examples of modern biophysical techniques and approaches that have already been established and can be further developed in the academic and scientific environment of the University of Belgrade. In a multidisciplinary international network program these techniques can eventually contribute to the buildup of "big biology". The scientific field will mainly be presented by examples from neurosciences. In fact, as accepted by many scientific experts, while the second half of the 20th century in research was remembered as the "Century of the DNA" it is predicted that the 21st century will be marked as the "Century of the Brain". In his address to the Nobel Foundation Erik Kandel, who won the Nobel prize in 2000 for his studies on the molecular biology of brain memory storage [10], stated: "The biology of the mind has now captured the imagination of the scientific community of the 21st century, much as the biology of the gene fascinated the scientists of the 20th century."

Neurobiophysics of magnetic resonance neuroimaging, advanced microscopy and electrophysiology bring special interdisciplinary contribution to the development of the biomedical field of neuroscience. These scientific approaches will be further elaborated below.

A. Magnetic resonance imaging

Over the last two decades, microscopic resolution *in vivo* magnetic resonance imaging (MRI) techniques have been developed and extensively used in the study of animal models of human diseases. MRI is a relatively new technique that evolved from nuclear magnetic resonance (NMR) spectroscopy and its measurements of relaxation times of the net magnetization vector of the studied nucleus.

Small rodents are also the most commonly used species as animal models of neurological diseases and thus MRI techniques need to provide microscopic resolution and high signal-to-

noise ratio images in relatively short time. This is achieved by high field-strength magnets. In addition to standard MRI techniques, several new applications have been implemented in experimental animals, including diffusion and perfusion studies, MR angiography, functional MRI studies, MRI tractography, proton and phosphorous spectroscopy, cellular and molecular imaging using novel contrast methods. MRI has the capability of studying live organisms without exposing them to potentially harmful ionizing radiation (such as in x-rays, CT, PET, and SPECT scans). Depending on the technique used, slice as well as true 3D image sets can be obtained.

Correlation of MRI findings in humans with disease models can now be done in laboratories equipped with small animal imaging facilities. The multidisciplinarity of the technique is evident not just by the features of data output but also by the structure of the working team. Thus, most facilities employ at least one physicist, one engineer, and several technicians ranging with skills in computer operation and programming, MRI operation, data processing, image analysis, and animal handling.

Using the new generation of contrasting agents (small or ultra small super paramagnetic iron oxide particles or [U]SPIO-s), cell and even biomolecule distributions can be imaged in small animal models such as rodents [2,14]. The most basic application is to generate 3D anatomical atlases of rodent brains. This than offers the biophysical grounds for the *in vivo* nerve tract tracing by utilizing paramagnetic Mn^{2+} ions after their topical application. Functional MRI (fMRI) studies based on the blood oxygen level-dependent principle, can also be conducted on small rodents. Blood flow and oxy/deoxy-hemoglobin related changes in activated areas of the cortex produce mild hypointensity on MRI images. Images obtained in the activated and non-activated state can be used to generate activity maps. Just recently it was confirmed that fMRI images relate to brain-cell activity from animals to man. In animal models of neurodegenerative diseases lesion formations can be monitored *in vivo* and in early phase (“diffusion-weighted imaging”). Blood-brain barrier integrity can also be studied with specific contrasting agents such as gadolinium. By means of SPIO/USPIO contrasting agents cell-specific and molecular imaging studies are performed on several disease models [8]. In the near future, a surge of these techniques is expected, as the correlation between conventional histology and MRI methods is best achieved by the use of these tools. Such studies could lead to a much better understanding of, egs. immune-mediated diseases by allowing for real time tissue monitoring of inflammatory infiltration, replacing tedious and costly tissue manipulation techniques.

In addition to relaxation time – based MRI, MR spectroscopy (MRS) studies are also frequently used in the study of neurodegenerative diseases, mainly to assess axonal pathology by studying the NAA (N-acetyl aspartate) peak. Phosphorous MRS studies can also be used to study energy metabolites. Experimental stroke models in small rodents are frequently studied with MRI methods. The NAA peak may show decrease in completed strokes, but may remain normal if the animals can still recover from the ischemic event. It is important to understand that while none of the MRS visible metabolites are specific for one particular disease, MR spectra can provide a unique insight into the biochemical microenvironment of the studied voxel (3D-pixel) of interest. This opens a new field of biophysical analysis of cell image profiles (*see* bellow). The orientation of biomedicine toward *in vivo* dynamic imaging may also result in a reduction in the use of conventional histology-based techniques, which in turn may reduce the number of animals necessary for experiments. The increased versatility that MRI is beginning to provide will lead to new insights into the processes that determine health and disease and should result in the development of new diagnostic and treatment approaches.

B. Cell profiling by means of automated microscopy and confocal microscopy.

Recent technical developments in molecular biology and biochemistry, such as PCR or microarray technology have paved the road to high-throughput profiling of cellular markers. However, quantification of cellular changes caused by external perturbations is generally limited to population-level transcription and proteomic profiling techniques. However, complex cellular processes may not be tackled by such one-dimensional readouts at constant drug concentrations. Accordingly, a method of high-throughput cell profiling was introduced that enables a far wider range of phenotypic responses in cells to be quantified and analyzed [13]. Rather than grinding up cells to get population averages, this multidimensional approach allows the investigator to measure changes in individual cells. This approach again requires a multidisciplinary team and a broad collaboration that combines immunocytochemistry, microscopy, image processing and custom data analysis techniques in order to create an automated microscopy platform that can quantify changes in tens of millions of individual cells in one study. Cells are labeled with fluorescent antibodies for image processing with a software that identifies different cellular regions. A set of descriptors is then defined to track changes in protein expression and cell morphology. The data processing forms color-coded graphical representations – profiles,

of the response of each descriptor to external perturbations. By simply examining these color-coded profiles it is possible to recognize drugs according to their targets, regardless of their structure. In context of drug discovery and identification this method successfully categorizes unknown drugs and points to targets for drugs of uncertain mechanism. Such a multivariate single-cell analysis leads towards identifying drug effects and relationships at systems level and toward phenotypic profiling at the single-cell level. It proves to be useful for discovering the mechanism and predicting the toxicity of new drugs.

An important throughput in light and fluorescence microscopy has also been achieved in 1980s with the introduction of confocal microscopy with improved computers, lasers and optics. Confocal microscopy is applied in many areas of biomedical research since it provides clearer images of cells and tissue structures stained with the fluorochromes. The precise laser beam direction enables the light intensity attenuation in front of and behind the focal plane. The z-filter i.e. 'pinhole' for the emitted light determines the thickness of the optical section (in contrast to histological sections obtained by preparative methods) from which the light signal originates. Combining of the laser light source, controlled optics and z-filter enables illumination and recording of emitted light from a single point determined precisely in any part of the specimen volume. Optical sections are formed by means of laser beam scanning of sample exploration points followed by computer processing. Through computer image analysis series of optical sections can be transformed into three-dimensional images of the specimen (3D reconstruction). In addition, the background blurriness hampering epiflourescent microscopy is reduced and a high-resolution image is obtained.

The popularity of the confocal microscope is based on the simplicity of obtaining extremely high quality images from samples prepared for conventional microscopy and on wide possibilities of applications in many areas of contemporary biomedical and biotechnological research. Moreover, multiflouorescence gained by confocal microscopy is ideal for the direct visualization of the relationship and interaction between molecules and other cellular components in their natural context. For this application it is also possible to make acquisitions in the 'fourth dimension' i.e. time, for monitoring the kinetics of biochemical and physiological processes *in vivo*. Custom made instruments already exist for confocal *in vivo* measurements in living animals. More economical technical designs such as the spinning disk microscope are already on the market. Having all this in mind it is likely that this technique will further develop and become one of the main imaging methods in biology. It is also fair to assume that this microscopy technique will bring further development to the aforementioned cell profiling approach. Recent breakthroughs in spatial resolution have also been witnessed with the stimulated emission depletion (STED) [11] and structured illumination [9] microscopy.

C. Patch-clamp electrophysiology of channels in cell membranes – interfacing molecular biology with biophysics.

The technique of patch-clamp made a breakthrough in the late 80s by allowing scientists to probe molecular entities of cell membrane transport and excitability – ion channels. The Nobel prize awarded (1963) for the conceptual model of nerve impulse generation ("action potential") by Hodgkin and Huxley gained crucial confirmation through this technique of measuring pA currents through single ion channels. Moreover, this experimental approach allowed the researchers to study the role of single ion channel molecular structure function relationship underlining open, closed and inactivated conformational states, all under the same patch-clamp electrode. With the help of molecular genetics and cloning techniques this has also lead to the discovery of molecular basis of many neurological disorders, some examples being epilepsy, migraine, headache, deafness, episodic ataxia, periodic paralysis, malignant hyperthermia and generalized myotonia all given a mutual term – "channelopaties" [7]. The development of electrophysiological techniques was always closely related to the techniques of advanced microscopy in order to identify and monitor even the finest objects of electrical exploration such as dendritic processes [12]. Moreover, the technique is often merged with imaging of ion sensitive fluorophores that complement the recordings of transmembrane ion currents [15] (in fact, in 2008 Osamu Shimomura, Martin Chalfie and Roger Tsien won jointly the Nobel prize for the discovery and development of the geen florescent protein and the expansion of the techniques of intracellular ion fluorescence imaging that followed).

Mainly due to patch-clamping and additional sophisticated imaging techniques many ion channels have been discovered and their functioning explained from general to specific terms.

Ion channels form pores that allow ions to move rapidly through cell membranes down their electrochemical gradients. Channels transport ions at rates of 1,000,000 to 100,000,000 ions per sec. This flow of ions creates electrical currents on the order of 10^{-12} to 10^{-10} amperes per channel. Such currents are large enough to produce rapid changes in the membrane potential, the electrical potential difference between the cell interior and exterior. Because calcium and sodium ions are at higher concentration extracellularly than intracellularly, openings of calcium and sodium channels cause

these cations to enter the cell and depolarize the membrane potential. For analogous reasons, when potassium leaves or chloride enters the cell through open channels, the cell interior becomes more negative, or hyperpolarized.

Most ion channels are gated - capable of making transitions between conducting and non-conducting conformations. Extracellular ligands, intracellular second messengers and metabolites, protein–protein interactions, phosphorylation, and other factors can induce channel gating. In addition, many ion channels are gated by another regulatory signal – the membrane potential itself [5]. Voltage-gated ion channels respond to and modify the changes in membrane potential produced by the binding of neurotransmitters to ligand-gated ion channels at synapses. Ion channels co-localized in discrete subcellular compartments function together as signaling elements in excitable cells. These elements amplify weak signals, determine thresholds, propagate signals to other regions of the cell, and generate membrane potential oscillations, among other functions. By means of patch clamping physiologists can study the channels of tiny neuronal subcellular regions, such as dendrites, axons, and presynaptic terminals.

The discovery of ion channels and their molecular mechanisms is continuing and brings many exciting discoveries. Moreover, the population of membrane channels, a diverse class of membrane transporters, has been widened long time after Hodgkin and Huxley by completely new members such as the water channels – aquaporins (Nobel prize, awarded to Peter Agre in 2003) [1] and the protein translocating channels in the nuclear membrane [3,4]. Studying of ion channels in automated patch clamp systems on cell models is nowadays used for pharmacological studies and drug testing which also brings this technique close to the concept of cell and drug profiling and buildup of “Big biology”.

3. *Conclusion*

We have herewith presented one view of the development of contemporary biology through biophysics towards “Big biology”. Societal as well as scientific and technological aspects were implicated. Regarding the former, the concept of biomedical networking and large investments in multidisciplinary collaborations would be the general worldwide trend. This however, has to take into consideration the importance of freedom of individual researchers to pursue new ideas through discovery-based biology. In addition to molecular biology, biophysics should have a growing influence on the development of modern biology. We presented several intermingled modern biophysical approaches to the tackling of biomedical problems in neurosciences and beyond. All of these techniques (MRI, automated and confocal microscopy, patch-clamp electrophysiology) can contribute to the development of a new concept of cell profiling towards high-throughput drug design, diagnostics and discovery in molecular physiology and medicine.

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A laser system for construction of dot matrix holograms

Zarkov, G., Boban; Pantelić, V., Dejan; and Jelenković, M., Branislav

Abstract — ***Holography is the technique that enables permanent recording of 3D objects. Due to sub-micron structure, holograms are remarkable safety elements which are very difficult to counterfeit. Dot-matrix technology is one of the commonly used methods for document security. This kind of holograms is mainly used for the purpose of protection against forgery (checks, cards, passports) but it can be used for decoration, advertizing or art. This paper describes the device for generation of holograms by using the dot-matrix technique.***

Index Terms — ***anti-counterfeiting, dot-matrix hologram, security hologram***

1. *INTRODUCTION*

A dot matrix hologram is composed of large number of micron sized diffraction grating dots. The fringe period and orientation of each diffraction grating can be controlled by the computer [1]. These diffraction dots are recorded onto a photosensitive material capable of forming a surface relief. The resulting hologram (so called master) is used as a first step in the process of mass production (with embossing technique). Today, anti-counterfeiting industry is based on this technology and provides very high resolution systems of up to 24000 dpi [2].

Because of its high efficiency, large visual angle and kinetic visual effects, dot matrix holograms are widely used in printing, packing and decoration. Also, the dot matrix holograms are widely used in security printing and anti-counterfeiting. They provide higher degree of security than conventional holograms and can be easily combined with conventional holograms. In this paper we present experiential results of our research.

We have developed the device for generation of dot matrix holograms with colored images. This system (shown in Fig. 1) consists of mechanical, electrical and optical components. The whole process was controlled via software, written using Microsoft Visual Studio, Express edition. The source of coherent light was diode pumped solid state laser (DPSS) at 473 nm wavelength with 50 mW output power.

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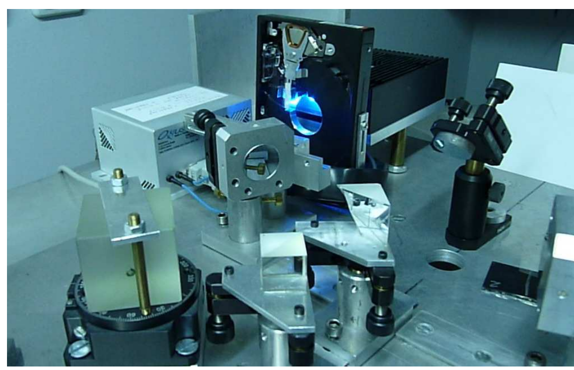

Fig. 1 Apparatus for generation of dot matrix holograms

The motorized XY table was used for positioning photosensitive material with spatial resolution up to 50 nm and reproducibility of 2 μm. Interference pattern of laser beams was formed on the photosensitive material, placed in focus of a microscope objective (50x, 0.55 NA). Diffraction gratings obtained this way were 15 μm in diameter and 1 μm periodicity. The hologram was recorded dot by dot, using the software for hologram calculation.

2. *GENERATION OF DOT-MATRIX HOLOGRAMS*

As it is shown in Fig. 2 [3], a dot matrix hologram is composed of small diffraction grating dots. Each dot is a grating whose period and orientation can be modified (under computer control).

Grating dots are formed by two-beam interference on the photosensitive material (producing sinusoidal profile grating pattern). Standard two-beam writing system includes translation and rotation stages to control period and orientation of grating dots. The photosensitive plate is placed in the focal plane of a microscope objective and the plate is recorded dot by dot. A relief pattern of dot gratings is obtained after proper exposure and chemical development. The final hologram is illuminated with a light beam. The resultant visual effect depends on the diffraction behaviors of all the dots. A well-designed dot matrix hologram will give a wonderful visual effect. It is colored with dynamical effects due to the change of observation direction.

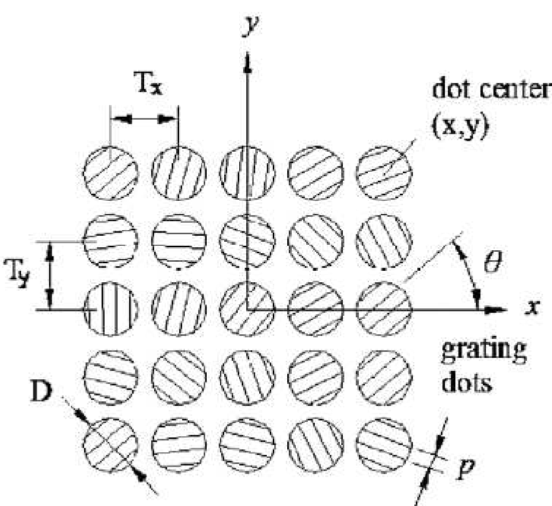

Fig. 2 Grating dots on a dot-matrix hologram with five variable parameters, coordinates Tx and Ty, the grating pitch p, the grating orientation θ, and the dot size D

3. EXPERIMENTAL SET UP

Figure 3 shows schematic of the system developed for fabrication of dot matrix holograms. The device consists of a laser and a beam splitter used to generate two light beams of equal intensity.

Beams are directed through the plane parallel plate (mounted on a rotation stage) in order to change their mutual distance. In this way we were able to control the diffraction grating period. Two beams pass through the dove prism (mounted on another rotation stage), which is used to change the diffraction grating orientation. Finally, beams are focused by a microscope objective onto photo sensitive material, mounted on a computer controlled XY table.

Holograms are recorded on a Shipley 1813 photo-resist plate mounted on the XY translation stage. The substrate was 20 x 20 mm glass slide with thickness of 1 mm. The photo resist layer coated on the slide was about 2 µm thick. The exposure time for every grating dot was 500 ms. After exposure the plate was developed in Shipley 303 developer. The developing time was 15 s. Software (developed in Microsoft Visual Studio) reads an image file and sends control data to the programmable controllers which coordinate translation stage, two rotation stages and the shutter. The hologram was recorded dot by dot using the program which determines parameters for the hologram from the image file. Diffraction gratings obtained this way had 15 µm diameter and 1 µm period, as shown in Fig. 4.

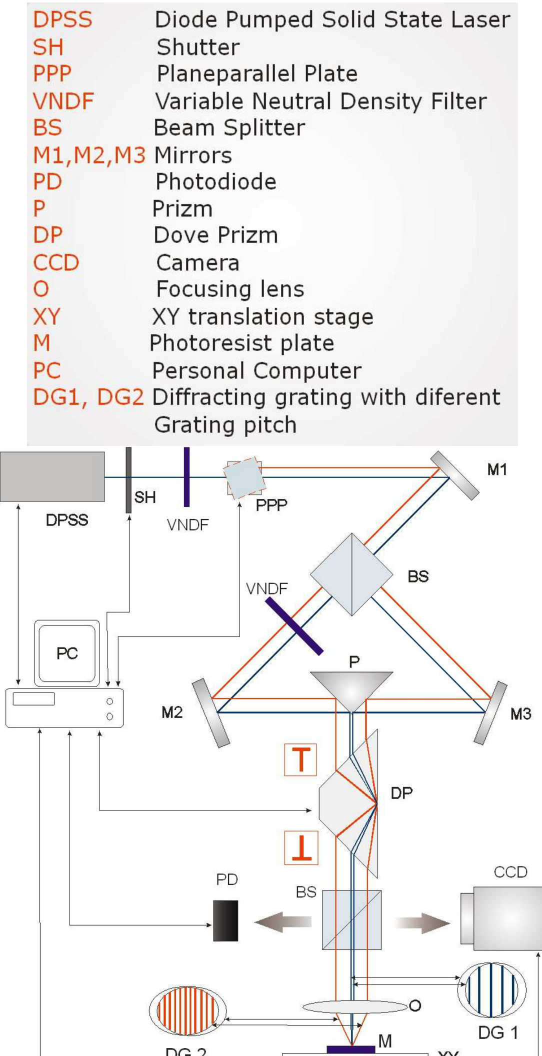

Fig. 3 The device setup used for fabrication of dot matrix holograms

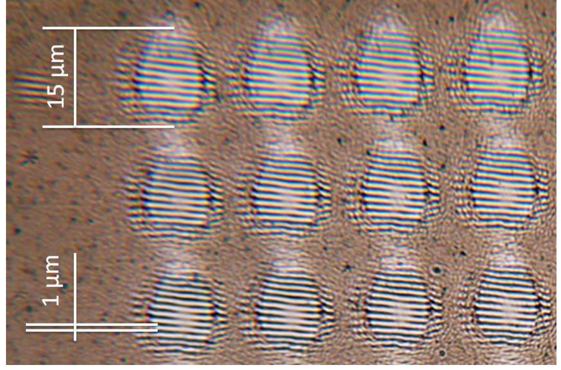

Fig. 4 Recorded grating dots on the hologram

By controlling all five parameters of grating dots we are able to create true colors holograms with kinematic movements, animation effects, hidden text, and resolution up to 1650 dpi.

4. Conclusion

In this work we have present device for fabrication of computer generated holograms using dot-matrix technique. The system consists of mechanical, electrical and optical components which were driven via control software. By using mirrors and prisms, we were able to introduce two parallel laser beams onto the front side of an objective and focus them onto the photosensitive material. Tiny interference pattern is produced and recorded on the material. Diffraction grating obtained this way had 15 μm diameter and 1 μm period. The hologram was recorded dot by dot by using the software for hologram calculation.

Acknowledgment

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High Relevance Combined with High Resolution: Advantages of *in vivo* Two-Photon Microscopy for Drug Discovery

Khiroug, L. and Pryazhnikov, E.

Abstract — *Despite breath-taking technological progress, modern pharmaceutical industry suffers from high attrition rates. In fact, only one in twenty lead compounds identified in non-clinical development passes through clinical trials and to market. The unacceptably low predictive power of many preclinical models stems, in part, from the lack of relevance and/or poor resolution of imaging techniques employed in drug development. Thus, in vivo imaging typically does not yield sufficient spatial resolution, whereas in vitro microscopy methods lack pathophysiological relevance. At present, only the in vivo two-photon microscopy (IV2PM) combines the crucial advantages of both imaging and microscopy, as it introduces the nanometer-scale spatial resolution of in vitro microscopy into the highly relevant context of in vivo imaging. In the present concise review, we discuss the most exciting applications of this game-changing technique in academic research of the past decade, and provide an outlook on the future role of IV2PM in drug discovery and development.*

Index Terms — *animal models, clinical trials, drug discovery, lead optimization, target validation, two-photon excitation microscopy.*

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1. *WAVELENGTH: RESOLUTION versus DEPTH OF PENETRATION*

"AN image is worth a thousand words". Whether it is fundamental research or bedside diagnostics, imaging is used with steadily increasing frequency for elucidating physiological pathways, deciphering disease mechanisms and validating on-target action of lead drug candidates. Visualization of organs, cells, organelles and even single molecules can be done using a whole spectrum of irradiation wavelengths spanning from electron beams through visible light to microwaves and radio frequencies.

The shorter the irradiation wavelength used to obtain the image, the greater its spatial resolution, which can be defined for a given visualization technique as the minimal distance at which this technique is able to distinguish between (i.e., resolve) two point-like sources of irradiation (Fig. 1). Thus, to distinguish individual cells from each other, a researcher would need the spatial resolution of at least 5 micrometer; visualizing cellular organelles (such as mitochondria, vesicles or endoplasmic reticulum) necessitates the resolution of at least 0.5 micrometer. Electron and light microscopy techniques, which rely on relatively short wavelengths (1 to 500 nm) are excellent tools to study cellular structures and subcellular signaling mechanisms *in vitro* and *ex vivo* (i.e., in isolated cells or fixed tissue).

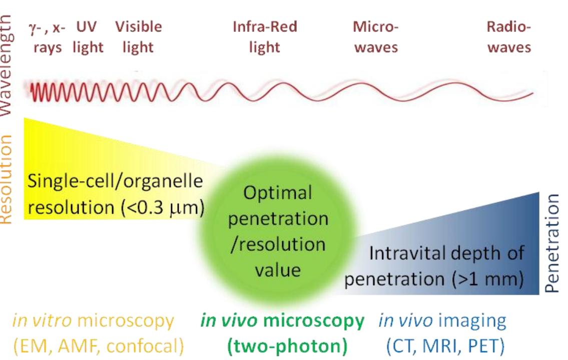


Figure 1. Wavelength of the irradiation used in an imaging/microscopy technique correlates with the spatial resolution and penetration depth of this technique.

Unfortunately, the shorter the wavelength used in a visualization method (and the greater its spatial resolution), the lower is the depth at which a given irradiation can penetrate into a living tissue. Therefore, in contrast to *in vitro* microscopy, *in vivo* imaging studies (i.e., imaging in living animals and human beings) are usually performed using techniques that are based on much longer wavelengths (1000 nm to 10 m). Even when visible light is used (wavelength of approx. 0.5 mm as, e.g., in optical imaging) the light-scattering nature of living tissue causes significant blurring of images, which degrades the spatial resolution to hundreds or thousands of micrometers.

The unfortunate necessity to choose between high spatial resolution and high penetration depth has created a gap between microscopy (which use is limited primarily to *in vitro* studies) and imaging (used *in vivo*). It is, in fact, a classical "either-or" choice: one can obtain either high resolution or high relevance, but not both at once.

Although at the first sight *in vitro* microscopy offers valuable insights into cellular mechanisms thanks to its excellent resolution, the physiological relevance of these data appears questionable considering that cells or tissue slices are cut out from the animal's body and often subjected to harsh chemical fixation prior to microscopic analysis. Conversely, the data acquired from intact animals using *in vivo* imaging bear maximal physiological relevance, but the image resolution is too low for drawing any mechanistic conclusions (Fig.2).

Fortunately, there is a unique technology that steps in to fill the gap. The *in vivo* two-photon microscopy (IV2PM) offers both high resolution and high relevance, an optimal combination for studying cellular mechanisms and subcellular signaling pathways within a living organism, i.e. with maximal (patho)physiological relevance.

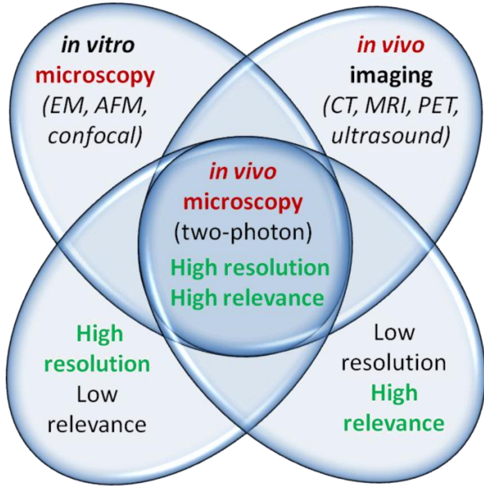
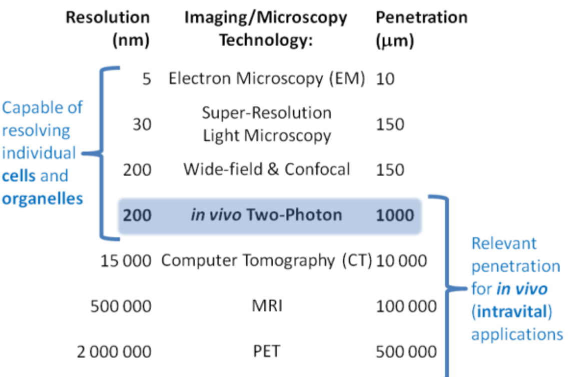


Figure 2. A diagram illustrating how the IV2PM uniquely brings together the high resolution of microscopy with the high relevance of *in vivo* imaging.

2. TWO-PHOTON MICROSCOPY: A PRIMER

In a nut shell, two-photon microscopy (also known as Multiphoton Excitation Laser Scanning Microscopy) allows making high resolution images of living cells by using the low-energy infra-red laser light that penetrates deeper than visible light (Fig. 3) into a living animal's brain, skin or other organs without damaging them (Denk et al., 1994). This technology reveals exquisite details by visualizing individual cells and even sub-cellular organelles in their natural undisturbed environment (Hillman 2007). Since IV2PM is non-invasive, it allows re-examining one and the same experimental animal (and precisely the same cellular ensemble) multiple times at days' or weeks' intervals. This yields an unmatched wealth of information on disease progression and drug action dynamics, and greatly increases statistical significance of the results.

In more technical terms, two-photon microscopy is based on the effect of simultaneous absorption of two infra-red photons by a fluorophore (most commonly, a fluorescent protein, synthetic dye or autofluorescent components of the tissue). Non-linear summation of the energy of two infra-red photons results in excitation of the fluorophore, which emits a photon in the visible light spectrum. Since the likelihood of coincident absorption of two photons is strictly limited in space, visible photons are emitted only by those fluorescent molecules that are located exactly in the focal point. The emitted photons are then collected by sensitive photomultipliers as the pulsed infra-red laser scans point-by-point through the plane of interest (and then plane-by-plane through the three-dimensional tissue, stacking two-dimensional XY images into a Z-stack by shifting focus in the vertical direction). After the dedicated computer software combines the data into a sharply focused 2D image, a 3D volumetric image can be reconstructed.



Resolution (nm)	Imaging/Microscopy Technology:	Penetration (µm)
5	Electron Microscopy (EM)	10
30	Super-Resolution Light Microscopy	150
200	Wide-field & Confocal	150
200	*in vivo* Two-Photon	1000
15 000	Computer Tomography (CT)	10 000
500 000	MRI	100 000
2 000 000	PET	500 000

Figure 3. Comparative table showing maximal spatial resolution and penetration depth for a number of imaging techniques ranging from EM through IV2PM to PET.

3. DISEASE-RELATED APPLICATIONS

3.1. Immunology

The minimally invasive in-depth tissue imaging with IV2PM has enriched many biological fields. It was especially helpful in immunology where the imaging of cell to cell interaction and migration is crucial for understanding the mechanisms of immunological response. It is not surprising, therefore, that immunologists were among pioneers of scientific applications of IV2PM. Both *ex vivo* explants (mostly for imaging of lymph nodes) and truly *in vivo* preparations have been used for immunological IV2PM studies since early 2000s (Bajenoff and Germain, 2007). While explants are easier to handle and access, they lack the *in vivo* environment of flowing vasculature and active neuronal networks. Conversely, working on *in vivo* preparations requires special care to minimize tissue damage during surgery and to stabilize imaging field against shifts resulting from muscle contractions, breathing or heart beat.

In 2002 Miller et al. (2002) for the first time used two-photon microscopy to observe the dynamics of T- and B-cell motility in isolated lymph nodes. These investigators characterized rapid movements of lymphocytes in different regions of the lymphoid tissue. Soon after, this group started to use *in vivo* preparations with preserved neuronal and vascular networks, namely the anesthetized animals with surgically facilitated access to lymph nodes (Miller et al., 2003; Cahalan et al., 2003; Mempel et al., 2004).

In the following years, immune responses were successfully visualized *in vivo* in different organs and systems including small bowel (Chieppa et al., 2006), thymus (Bhakta et al., 2005), and bone marrow (Cavanagh et al., 2005).

Recently, IV2PM was applied to dynamic real-time imaging of the brain immune microglial cells (Wake et al., 2009; Tremblay et al., 2010; Eichhoff et al., 2010). Microglial cells are implicated in the pathogenesis of chronic neuroinflammation, which is at the heart of such neurological conditions as multiple sclerosis and Alzheimer's disease.

3.2 Infectious diseases

Closely related to immunology are the studies of host-pathogen interaction in infectious diseases (see Konjufca and Miller, 2009 for review). Examples of bacterial infections investigated include studies of mycobacterial infection of the liver with macrophages expressing green fluorescent protein GFP (Egen et al., 2008); uropathogenic *Escherichia coli* infection in the kidneys of rats (Mansson et al., 2007) and the *Listeria* infection in skin (Zinselmeyer et al., 2008). In the latter paper, the authors have developed a non-invasive IV2PM approach to study trafficking of leucocytes after subcutaneous injection of bacteria. In the field of protozoan infections, the response of immune cells to *Leishmania major* was studied *in vivo* in anesthetized animals (Bajenoff et al., 2006).

Despite inherent difficulties in fluorescent labeling of viruses, several groups have successfully employed IV2PM for studying immune responses to viral infections. For example, Junt et al. (2007) investigated penetration of fluorescently labeled vesicular stomatitis virus particles from the lymph into the immune cells. In a more recent work, Kim and coworkers described the mechanisms of fatal meningitis in mice induced by murine pathogen lymphocytic choriomeningitis virus (Kim et al., 2009). In the paper, virus-specific T cells were imaged in the meninges through a thinned-skull window.

3.3 Dermatology

While skin is a superficial organ, its opaque and light-scattering nature prevents the use of classical microscopic techniques in studies of intact skin structure. Thus, for histological analysis, skin samples have to be excised, physically sectioned and fixed. Recently, IV2PM emerged as a promising high-resolution non-invasive imaging method with great potential for dermatological applications (Lin et al., 2007; Tsai et al., 2009).

In addition to two-photon excitation, the non-linear polarization effect of second harmonic generation (SHG) is of great value in dermatological imaging. Researchers have demonstrated that SHG microscopy can be used to image collagen fibers, as well as a variety of other biological structures such as muscle fibers and microtubules (Zoumi A et al., 2002; Campagnola P and Loew L, 2003).

Since human skin was first imaged *in vivo* with IV2PM in 1997 (Masters et al., 1997), many pathological processes have been studied and a variety of conditions has been characterized with this imaging modality. Examples of such pathophysiological applications include characterization of melanoma (König et al., 2003), and local scleroderma (Lu et al., 2009). Moreover, IV2PM has been used for assessing human skin ageing (Koehler et al., 2006) and for assessing the effects of cosmetic substances on skin (Bazin et al., 2010).

3.4 Cancer Biology

Cancer biology is another area into which IV2PM has introduced a new level of cellular resolution. Due to complex, chronic nature of most tumors as well as their location deep inside organs, IV2PM offers unique advantages to studies of cancer progression and effectiveness of anti-cancer therapies.

Imaging of tumors through chronic optical window preparations, such as dorsal skin chamber or cranial window, have been used in cancer research already several decades ago (for references see Jain et al., 2002). However, it was not until 2001 that IV2PM was successfully applied for anatomical and functional imaging of tumors *in vivo* (Brown et al., 2001). In their pioneering study, the authors monitored the *de novo* formation of blood vessels in tumors. Angiogenesis is one of the key factors of the tumor development and growth. Therefore, the ability to obtain high resolution three-dimensional images of vessels deep inside the cancerous tissue makes IV2PM an unsurpassed tool for cancer biology.

Metastasis is the leading cause of the death from cancer. Due to the ability of the IV2PM to penetrate deep and provide single cell resolution, progression of tumors through metastases formation can now be followed with unprecedented precision (Sahai, 2005). Recently,

detailed steps of brain metastasis were also imaged using IV2PM in mice with implanted cranial windows (Kienast et al., 2010).

3.5 Neuroscience

Physical dimensions and complexity of the brain tissue have long prevented cellular and subcellular resolution imaging of a living brain. In 1997, David Tank and coworkers have shown for the first time that IV2PM can be used to optically penetrate the highly scattering tissue of the intact brain (Svoboda et al., 1997). The Tank group used this method in combination with cranial window to measure sensory stimulus-induced dendritic Ca^{2+} dynamics of layer 2/3 pyramidal neurons. Five years later, Yoder and Kleinfeld utilized intact mouse skull preparation (thinned skull) to visualize the brain structures (Yoder and Kleinfeld, 2002).

Since these ground-breaking studies, neuroscientists have collected a wealth of information about physiological and pathological functioning of central nervous system using IV2PM. Valuable physiological insights were obtained, for example, on structural mechanisms of the memory storage in the brain (Hofer et al., 2009) or blood supply regulation mechanisms in the cortex (Nishimura et al., 2007). The range of pathological conditions visualized with IV2PM includes stroke (Li and Murphy, 2008), Alzheimer's disease (Takano et al., 2007), brain glioma (Winkler et al., 2009) and spinal cord injury (Dray et al., 2009).

4. Conclusion

In the spectrum of preclinical visualization tools, IV2PM has effectively bridged the gap between *in vitro* microscopy and *in vivo* imaging, as it has happily "married" the excellent resolution of the former to the ultimate relevance of the latter. The IV2PM technology has established itself firmly in the academic biomedical community, and its benefits both for fundamental and for clinically-oriented research are rapidly gaining recognition. It will not be long before IV2PM will make its way into the business realm of Pharmaceutical and Biotechnological industries. This is sure to increase the predictive power of many preclinical animal models, enabling companies to bring better researched and developed drugs to market quicker and in a more cost-efficient manner than is currently possible.

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Monitoring cellular metabolic interactions of nanoparticles in an ALS experimental model using SECARS microscopy

Machtoub, Lina; Bataveljić, Danijela; and Andjus, R., Pavle

Abstract—*This study highlights the novel application of recently developed emerging non-invasive nonlinear optical imaging approach based on surface-enhanced coherent anti-Stokes Raman scattering (SECARS) microscopy in investigating metabolic interactions of nanoparticles upon intravenously injected contrasts in the neurodegenerative experimental animal model of amyotrophic lateral sclerosis (ALS). The study focuses on the cellular metabolic interaction based on lipids associatied to up-taken ultra small paramagnetic iron oxide (USPIO) cross linked to anti-CD4 antibodies (CLUSPIO). Marked intensity enhancements have been observed in CLUSPIO treated ALS brain. The observed enhancement has been correlated to lipid peroxidation and degeneration observed in these regions, based on selective association of lipids to up-taken USPIO, which shows high accumulation in the brainstem and midbrain region. The obtained results were compared with MR imaging, which shows marked hyperintensities with prominent lateral ventricle and cerebral aqueduct enlargements in these regions.*

Index Terms — ***ALS, lipids, nanoparticles, neurodegenaration, USPIO***

1. *Introduction*

The explosive growth of biocompatible nanotechnologies has set the stage for an evolutionary leap in diagnostic imaging and therapy. Recently, magnetic nanoparticles based-contrast agents have been the subject of intensive pre-clinical and clinical research studies which opened up a new field in molecular imaging (1,2). They have several advantages over Gd-based agents: no long term toxicity for the biodegradable particles, higher relaxivity effects and prolonged circulation time that enables the use of high spatial resolution sequences. One of the applications that greatly benefited from the development of this class of contrast agents is cellular imaging, which relies on non-invasive tracking of magnetically labeled cells. Moreover, owing to their remarkable biocompatible and biodegradable properties, they have become very popular for their diagnostic features and therapeutic applications. The ultra small paramagnetic iron oxide particles (USPIO) have shown to be extremely strong contrast enhancers for proton MR imaging. The contrast mechanism depends on the choice of imaging modality, which itself is determined by the clinical problem and accessibility for imaging. Moreover, the physical properties of the carrier agents themselves (density and compressibility) establish the means for detection.. The molecular imaging methods are currently central in pre-clinical research and clinical routine as diagnostic tools which enable the visualization of the ongoing pathophysiological changes in living organisms, however it still remains challenging due to their relatively low spatial resolution with poor definition of anatomy. Recently, it has been shown that the combination of MRI and contrast agents can greatly enhance the possibilities to depict the vascular system, inflamed tissue as in arthritis, tumour angiogenesis, and atherosclerotic plaques and the breakdown of the blood-brain barrier related to pathologies in neurodegenerative diseases (3,4). There have been many attempts to find the best imaging modality to investigate the entire mechanism of the inflammatory processes. Intensive efforts have been made to develop a class of multimodal contrast agents, which can combine magnetic resonance imaging (MRI) and optical imaging. One of the emerging recently developed optical imaging techniques is Surface Enhanced Coherent anti-Stokes Raman Scattering (SECARS) Microscopy (5,6). Compared with other clinical imaging modalities,

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SECARS microscopy can be tuned to provide a variety of possible tissue contrast with an advantage of subcellular spatial resolution and near real-time temporal resolution compared with MRI, which is shown to be promising towards becoming a medical diagnostic tool for both *ex vivo* and *in vivo* live cell imaging applications. The potential of this imaging modality is highlighted by its ability to probe the surface enhanced signal based on the aggregations or accumulations of nanoparticles in living cells. Apart from its chemical selectivity, the main strength of this technique lies in the fact that it can image the native and unaltered state of the samples or the nanoparticles without the additional labelling or fluorescent marker. This is important since the marker may change the behaviour of the eg. metallic/ organic state of the nanoparticles in the cells. In this study we show the potential contribution of SECARS microscopy in studying the mechanism underlying iron oxide cellular metabolic interactions from an established neurodegenerative experimental animal models using CLUSPIO MRI contrast agent. This work is focused on the study of some neurodegenerative disorder systems, generally associated with the disturbances in cholesterol metabolism, the presence of the E4 isoform of the cholesterol transporter apolipoprotein E as well as hypercholesterolemia, which are important risk factors for the development of neurodegeneration. The emphasis of this study is to present and review applications using innovative molecular imaging diagnostic approaches, MR and SECARS nonlinear microscopy, the latter based on probing the surface enhanced signal correlated to the mechanism of intracellular iron accumulation and lipid oxidation and peroxidation in the ALS experimental model.

2. Materials And Methods

The experiments were performed with iron oxides nanoparticles incubated biological samples, brain tissue extracted from Sprague-Dawley rat model expressing multiple copies of mutated (G93A) human SOD-1 gene (Taconic Farms, NY), and wild type (WT-standard Sprague-Dawley rats). Commercially available antibodies against CD4+ T cells, magnetically labelled with ultra small particles of iron oxide (USPIO; MACS®, Miltenyi Biotec) cross linked to anti-CD4 antibodies (CLUSPIO) were i.v. injected into rats .The brain tissues samples were extracted from wild type rat model, CLUSPIO-treated ALS and untreated ALS rat model. SECARS microscopy was performed using NIR excitation of picosecond mode-locked Nd:YVO_4 and Ti:S laser (700-1000 nm) combined with a tunable OPO that covers the frequency range (200-3600 cm^{-1}). The beams were scanned over the sample using specially designed laser scanning microscope and focused by water immersion objective lens with 1.2 numerical apertures. Both beams have a power of several tens of milliwatts at the sample. The SECARS signal is collected in the backward direction using standard confocal microscope connected with a photomultiplier tube. Microscopic Raman mapping images were recorded using an alpha 300R instrument WITec, Inc. (Ulm, Germany) equipped with a back illuminated deep-depletion CCD camera. The samples were irradiated by a He-Ne laser at 632.8 nm, coupled into a confocal Raman microscope through a wavelength specific single mode optical fiber, and focused by a 40×0.65 NA microscope objective lens. The animal experiments were surveyed by the Committee for Animal Experimentation treated in accordance with the European Community Council Directive (Ref. Nr. 86/609/EEC) and the NIH Guidelines, with approval of the Ethical Committee of the Faculty of Biology University of Belgrade.

3. Results and Discussion

Based on earlier findings, it has been propped that the neurodegenerative cascade in ALS involves an early increase in levels of oxidative stress, induced by genetic and/or environmental factors which causes a disturbance in membrane lipid metabolism resulting in the accumulation of ceramides and cholesterolesters (7). Previous studies on cultured motor neurons and Cu/Zn-SOD mutant mice and nonneuronal cells, have shown that ceramide can mediate cell death induced by disturbances in plasma membrane redox systems, which can suggest a pivotal role for disturbances of membrane lipid metabolism in the pathogenesis of ALS (8). The crucial role of lipid oxidation and peroxidation through reactive oxygen species (ROS) in tissue pathophysiology has been further demonstrated in many neurological disorders, including bipolar disorder and schizophrenia, neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD), multiple sclerosis (MS), Niemann–Pick C (NPC), and Huntington's (HD) diseases, Friedreich's ataxia, infantile neuroaxonal degeneration (INAD), neurodegeneration with brain iron accumulation (NBIA), and Zellweger syndrome that involve deregulated lipid metabolism (9).

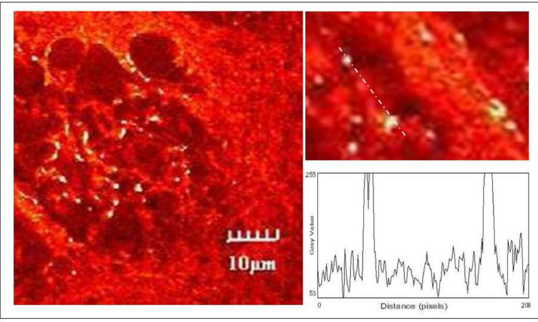

Fig.1. SECARS image of brain tissue from CLUSPIO treated ALS rats, taken at at 2850 cm^{-1}. The corresponding cross section profile shows the intensity enhancement along the indicated line.

Our recent studies on the ALS rat model, using MRI revealed T2-weighted hyperintensities in the

brainstem, rubrospinal tract and vagus motor nuclei with prominent lateral ventricle and cerebral aqueduct enlargements (10,11). Notably, with CLUSPIO antibodies against CD4, MRI revealed infiltrations of helper T cells in the interbrain regions that are shown to be correlated with foci of neurodegeneration in these areas, not observed in the WT animals (12, 13).

In line with the MRI findings, ex vivo SECARS experiments performed on CLUSPIO i.v. injected brain sections taken from ALS transgenic rat model showed marked signal enhancement in specific pathological regions particularly in the midbrain and the brainstem known to be infiltrated by helper T cells. The prominent bands that show significant enhancement observed in the high frequency region were around 2845 and 2875 cm^{-1} (figure1) which are typical bands for fatty acids dominated by valence vibrations of $C\text{-}H_2$ groups. The bands from 2700 to 3500 cm^{-1} are correlated to cholesterol ester (cholesteryl palmitate), triacylglyceride (glyceryl palmitate), phosphatidic acid, and sphingomyelin. Significant enhancements were also observed in the fingerprint region around 1660-1675 cm^{-1} which is correlated to unsaturated fatty acids and to the steroid ring of cholesterol (figure2).

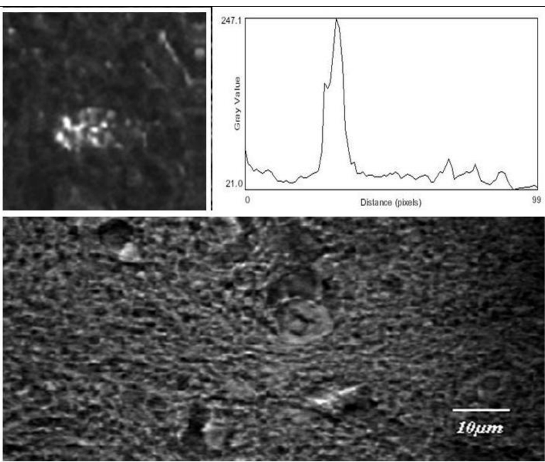

Fig.2. SECARS image of CLUSPIO- treated ALS brain tissue taken in the finger print region around 1660 cm^{-1}. The corresponding cross section profile shows the intensity enhancement from the bright regions.

In contrast experiments performed in the same tuning range on untreated samples and brain tissues extracted from the wild- type rat model have shown no significant indication of enhancement around these bands. To investigate the metabolic effect of iron particles and the possibility of iron lipid binding activity, experiments were performed on adipocytes treated with iron oxide nanoparticles and extracted lipids from biological tissues. An intensive signal enhancement could be found in the vibrational resonance wavelengths range around 2850 cm^{-1} from USPIO incubated lipids and particular regions highlighting accumulation of iron oxide nanoparticles in adipocytes (figure 3). Thus the observed SECARS enhancement in the CLUSPIO treated ALS brain can be related to lipid peroxidation and degeneration in these regions. The cellular metabolic interaction of iron oxide nanoparticles has been demonstrated in our previous studies on hepatic tissues treated with iron oxide nanoparticles (5).

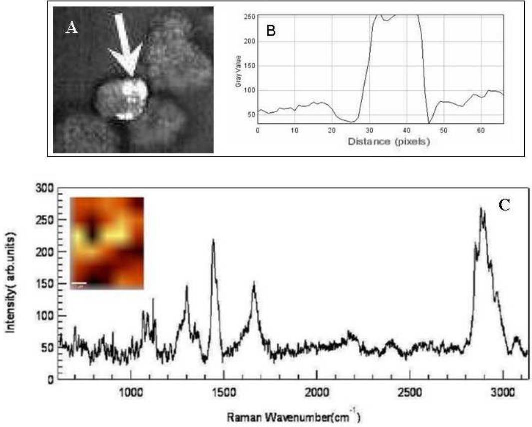

Fig.3. A) SECARS image of adipocytes treated with iron oxide nanoparticles (bright regions), taken around 2850 cm^{-1}. (B) Gray scale plot shows the intensity enhancement highlighting the accumulation of iron oxides nanoparticles indicated by arrow.(C) Raman spectrum extracted from the Raman map image (inset, scale bar 1 μm) of brain tissue from CLUSPIO treated ALS rats, in the range of 200-3100 cm^{-1}.

Control experiments performed on untreated samples showed no signal enhancements at any vibrational resonance wavelength. The signal enhancement has been further investigated on total brain tissues by Raman maps chemical imaging (Fig. 3). Clear contrast enhancement is observed in tissues from CLUSPIO-treated ALS animals as indicated by the bright regions highlighting accumulation of iron oxide nanoparticles. To support the surface-enhanced coherent Raman scattering activity of iron oxide nanoparticles, additional control microscopic Raman measurements have been performed on iron oxide nanoparticles dissolved in pyridine (C5H5N). Clear intensity enhancement could be detected from iron oxide nanoparticles in pyridine as compared to the pure solution (Fig. 4).

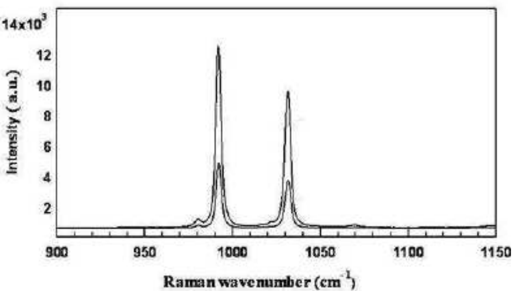

Fig 4. Raman spectra show intensity enhancement observed from the iron oxide nanoparticles in Pyridine compared with the pure solution.

Previous studies support the hypothesis that iron (Fe^{3+}) binds to membrane lipids generating free radicals at the binding site. Iron-induced lipid peroxidation has previously been demonstrated more often than for any other transition metal. For

example, an *in vivo* study has shown direct evidence of lipid peroxidation, in both mitochondrial and microsomal membrane lipids (14).The binding of Fe^{3+} to cell membranes has been investigated in a system in which lipid peroxidation was proportional to Fe^{3+} concentration; the results indicated that 95% of the Fe^{3+} was membrane bound when evaluated by labeling with $^{59}FeCl_3$ combined to measurement of nuclear magnetic resonance of water-proton relaxation times. Both spin-lattice (T1) and spin-spin (T2) relaxation times decreased with increasing Fe^{3+} concentration. This study suggested that the charge transfer to Fe^{3+} may occur at the membrane binding-site, leading to reduced Fe^{3+} effect on water-proton relaxation times. Furthermore, the iron uptake by cortex neurons has been previously demonstrated by using immunocytochemical techniques that showed the presence of transporter receptors on neurons (15). Recent studies on ceruloplasmin (CP) of brain iron homoestasis have shown that solubale CP has a role in iron uptake by iron-deficient brain neurons (16). The iron deposits in ALS have been found to be restricted to the precentral gyruses of gray matter (17). From our results we could correlate the SECARS enhancement to the lipid-iron accumulation in the inflammatory cells or from regions with perturbed sphingolipid metabolism resulting in ceramide and cholesterol ester accumulation in the ALS brain.

4. *Conclusion*

The involvement of perturbed sphingolipid metabolism resulting in ceramide and cholesterol ester accumulation in motor neurons in ALS would suggest novel approaches for future therapeutic intervention. Moreover, the surface-enhanced coherent Raman scattering - based optical properties of the MR contrast agents can be promising for the designing of future magnetic and optical probes for live cell imaging and investigation on neurodegenerative disorders.

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Shining a light on the structural dynamics of ion channels using Förster resonance energy transfer (FRET)

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Abstract— ***Förster resonance energy transfer (FRET) spectroscopy is a powerful tool for structural analysis of proteins. Over the last decade it has become one of the frequently used techniques for studying the conformational changes of membrane proteins. Here we focus on the use of FRET spectroscopy in studies of structural rearrangements involved in the gating of ion channels that function as gated membrane pores in numerous cellular processes essential for the physiology of living cells.***

Index Terms — ***BRET, electrophysiology, FLIM, fluorescence, FRET, mutagenesis.***

1. INTRODUCTION

The existence and survival of living organisms, be it microbes, plants, animals or humans, are dependent on sensing the surrounding environment and responding to changes within it. Sensations of touch, hearing, sight, taste, smell or pain are all manifestations of interactions between a living organism and the outside world, which otherwise would apparently cease to exist. The life, as we know it, is thus inherently and intricately linked to the sensory input, whose importance for the existence of life, itself, justifies the efforts made to understand its molecular origins.

All biological cells are surrounded by a plasma membrane separating the inside of a cell from the surrounding environment. This cellular boundary is the major target for physical and chemical stimuli exerted on a living cell by the outside world. Most of the receptors of environmental stimuli, be it mechanical, electrical, chemical, thermal or electromagnetic (light), are membrane ion channels functioning as molecular sensors that convert these stimuli into a cascade of intracellular signals and thus contribute to changes in electrical, chemical or osmotic activity within living cells. These membrane proteins are gated pores, which exist in two basic structural conformations, i.e. closed and open. Opening and closing of ion channels regulate the transport of ions and other solutes, which by entering or exiting living cells affect their activity.

The last three decades have been exceptionally exciting for the ion channel research field starting with early 1980's, the heyday of the patch–clamp recording technique, which revolutionized the field by enabling electrophysiologists to measure ionic currents through single ion channels [1]. By using this technique its inventors Erwin Neher and Bert Sakmann were able for the first time to demonstrate directly the existence of ionic channels in living cells [2]. Later, many other techniques have been applied to study the relation between the structure and function in ion channels, which led to a significant progress in solving molecular structure of a large number of ion channels, and greatly contributed to a better understanding of the role ion channels play in various aspects of cell physiology. Some of the milestones of this multidisciplinary approach include: (i) molecular cloning of a number of voltage-gated, ligand-gated and mechano-sensitive ion channels; (ii) site-directed mutagenesis, such as cysteine or alanine scanning mutagenesis, giving researchers the means to investigate the effect of point mutations on the function of ion channels; (iii) X-ray and electron crystallography, which have provided structural snapshots of a number of ion channel molecules at near atomic resolution, (iv) electron paramagnetic resonance (EPR) spectroscopy, and (v) fluorescence spectroscopy, both of which have made it possible to access the structural dynamics of ion channel molecules, as well as (vi) computer-assisted molecular modeling that, by using the structural and functional information obtained experimentally, has brought ion channels to life by visualizing on the computer screen the structural rearrangements underlying their function. This multidisciplinary approach has to date brought an unprecedented wealth of information and new knowledge about the structure and function of ion channels. Excellent and comprehensive reviews on these subjects are available elsewhere [3-13].

This review focuses briefly on the Förster resonance energy transfer (FRET) microscopy and spectroscopy that has, since the late 1990s, become important techniques used in studies of ion channel structure and dynamics. Interested readers can find additional information on FRET microscopy and spectroscopy in the references listed below [14-16].

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2. PRINCIPLES OF FRET SPECTROSCOPY AND MICROSCOPY

2.1 BASIC THEORY

As a technique FRET can provide information as to the proximity and orientation of molecules in relation to each other over a distance ~ 1 - 100Å [14, 16]. As the words that make up the acronym FRET suggests, the process involves the transfer of energy from one molecule to another. The molecule donating its energy is usually referred to as the *donor.* Donor molecules are chromophores which by light or chemical stimulation can go into an excited state. This excited state energy can then be transferred to a neighboring quencher molecule if certain conditions conducive to resonance transfer are met. FRET is known as a radiationless process because the energy between a donor and a quencher is transferred through dipole-dipole interactions (Fig. 1), and not by an emitted photon. If the quencher in this regard is itself a fluorescent molecule, FRET will cause it to become excited, and it can then emit its own photon, albeit of a lesser energy at a longer wavelength. Because FRET quenchers can also be emitters, a more general term of *acceptor* is often used to describe them. As a consequence of this type of energy exchange between a donor and acceptor, FRET efficiency (E), or the fraction of energy transferred per donor excitation event, depends on the donor-to-acceptor separation distance *r* with an inverse 6th power relation:

$$E = 1/[1+ (r/R_0)^6] \quad (1)$$

where R_0 is the Förster distance of the donor-acceptor pair which corresponds to the distance at which the energy transfer efficiency is 50%. The separation distance *r* at which the energy can be transferred from a donor to an acceptor is typically ≤10 nm. R_0 depends on the overlap

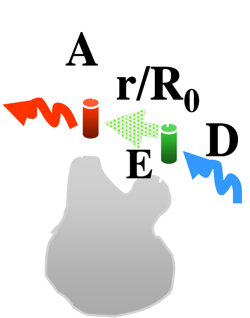

Fig. 1. Basic principle of FRET spectroscopy. A donor fluorophore (D) is excited and transfers this energy in a distance- and orientation-dependent manner (r/R_0) to an acceptor molecule (A). The energy transfer between an excited donor to an acceptor occurs through non-radiative dipole-dipole coupling (E). This process is called "Förster resonance energy transfer".

integral $J(\lambda)$ of the donor emission spectrum with the acceptor absorption spectrum and their mutual molecular orientation, i.e. the dipole orientation factor κ^2, as follows:

$$R_0^6 = \frac{9Q_0 \ln(10)\, \kappa^2\, J(\lambda)}{128\, \pi^5\, n^4\, N_A} \quad (2)$$

where Q_0 is the fluorescence quantum yield in the absence of the acceptor, n is the refractive index of the medium in which fluorescence is measured, and N_A is Avogadro's number. $\kappa^2 = 2/3$ is the usually assumed value for the orientation factor because this value applies to a freely rotating pair of dyes, which are isotropically oriented during the lifetime of the excited state. The spectral overlap integral $J(\lambda)$ can be calculated from the following expression:

$$J(\lambda) = \int f_D(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4\, d\lambda \quad (3)$$

where $f_D(\lambda)$ is the normalized donor emission spectrum, and $\varepsilon_A(\lambda)$ is the acceptor molar extinction coefficient, and λ is the wavelength. Calculating the FRET efficiency (E) is typically accomplished by measuring the donor fluorescence in the presence of the acceptor and in the absence of the acceptor. This is a frequently used method because FRET efficiency can be relatively easily determined from the following expression:

$$E = 1- (I_{DA}/I_D) \quad (4)$$

where I_{DA} and I_D is the fluorescence intensity of the donor in the presence and the absence of the acceptor. When the donor and acceptor are both fluorophores, the quickest and easiest method to measure FRET is to use the 2 colour ratiometric method. Here, the fluorescence is collected in select bands of the donor and acceptor regions of the spectrum upon excitation of the donor, and by some simple mathematics the FRET efficiency can be calculated. The problem with this method can be significant spectral bleed through of the donor fluorescence into the acceptor band, so accuracy is compromised. A preferred method, that eliminates spectral bleed through, is instead to measure fluorescence in the donor channel emission band only, before and after photobleaching or destroying the acceptor.

Once a FRET efficiency value is obtained, the distance between the donor and acceptor, can be calculated by a variation of Eq (1):

$$r = [(1/E) -1]^{1/6} R_0 \quad (5)$$

Because of the difficulties in establishing the Förster distance (R_0) and accurately measuring the FRET efficiency, FRET is most often used as a tool for comparing intra- and inter-molecular distances, as distinct from actually measuring them. If accuracy is the key though, then determining FRET efficiencies using fluorescence measured in the time-domain is often preferred. A fluorophore's fluorescence lifetime is independent of its concentration, the illumination strength, and optical scattering. Fluorescence lifetimes are only dependent on the intrinsic properties of the fluorophore and its interaction with its environment. For these reasons,

measuring FRET using fluorescent lifetime imaging (FLIM) and spectroscopy techniques are often preferable to steady state fluorescence imaging and spectroscopy methods, even though they require more complicated instrumentation and analysis. Fluorescent lifetimes are defined as the average time a fluorophore's stimulated electron will spend in the excited state before returning to the ground state. For most fluorophores this period is typically in the nanosecond range. The quenching of a donor fluorophore's fluorescence due to FRET will lead to a decrease in the fluorescence lifetime. This being the case, the relationship between donor fluorescence and FRET efficiency, as described in Equation 4 above, can simply be re-written in terms of fluorescence lifetimes (τ):

$$E = 1- (\tau_{DA}/ \tau_{D}) \qquad (6)$$

where τ_{DA} and τ_{D} are the donor fluorescence lifetimes in the presence and absence of the acceptor respectively [14, 16, 17].

Another closely related spectroscopic technique, a variant of FRET, is Bioluminescence Resonance Energy Transfer (BRET), which is predominantly used for studies of protein-protein interactions [18]. The advantage of BRET over FRET in the usage of a bioluminescent luciferase from the sea pansy *Renilla reniformis* as a donor. Because it is bioluminescent, no light excitation source is necessary to promote emission, only the addition of a substrate. This results in reduction of the background noise due to direct excitation of the acceptor, as well as a reduction of photobleaching due to the absence of external illumination.

2.2 PREREQUISITES AND METHODS

The methods of labeling of biomolecules for FRET studies need considerable deliberation. There are a number of ways in which a FRET pair can be inserted or added into a protein molecule. One requires genetic engineering such that the DNAs of 2 fluorescent proteins are themselves inserted into the host protein nucleotide sequence. When this chimera is expressed in cells the result is a fusion protein consisting of the host protein and the fluorescent proteins.

Another method of labeling is by attaching organic fluorescent dyes to the host protein by chemical modification of reactive amino acid residues like cysteine. If needs be, these reactive residues can also be introduced into the protein by mutagenesis. This method has an advantage in that it allows attachment of fluorescent dyes at individual sites of interest within the host protein. If a host protein contains native reactive residues, these can typically be replaced by another non-reactive amino acid (such as serine), to avoid unwanted labeling.

Many proteins contain amino acid residues that are themselves fluorescent. These amino acids, such as tryptophan, can then be used as the donor fluorophore in FRET pair. Artificial fluorescent amino acids can also be incorporated into proteins that are able to function as acceptors. This means proteins and peptides can be synthesized to incorporate their own FRET pair, which is especially useful in studying protein conformational changes [19-21].

In past times, one of the most popular FRET donor-acceptor pair for studies of biological molecules was cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). CFP and YFP are both color variants of the green fluorescent protein (GFP) [22-24]. However, as YFP has a significant pH and Cl^- dependence, and displays UV induced photochromism, many researchers have turned to other fluorescent proteins such as *venus* that are more dependable over a range of environmental conditions. Care must also be taken that one's DNA constructs are designed in such a way, and that the choice of fluorescent protein mutants is such, that dimerization between the fluorescent proteins cannot occur [25]. Fluorescent proteins can be readily attached to the host protein by genetic engineering as mentioned above.

A typical FRET pair of organic fluorescent dyes is the AlexaFluor maleimide pair AF488 and AF568, where 488 and 568 stand for the excitation wavelengths (in nanometres) for donor and acceptor respectively. The maleimide group enables the fluorophore to chemically bind to designated cysteine residues in the protein. For monitoring protein structural rearrangements, the target protein is either labeled with a donor and an acceptor at two loci, or at one locus only provided that the target protein is multimeric and consists of identical subunits, which is frequently the case with ion channels. FRET is observed when the distance and/or relative orientation of the donor, relative to acceptor, changes due to conformational changes in the protein. Hence the change in distance and/or orientation of the two fluorophores bound to unique amino acid residues in the protein can be measured. This in turn provides information about conformational changes of the host protein. One of the advantages of fluorescent dye labelling is their relatively small size. This enables labelling of ion channels at a greater variety of loci whilst maintaining overall channel function, which would otherwise be impossible with the incorporation of fluorescent proteins.

Using a lanthanide metal such as europium or terbium as the donor fluorophore has a couple of advantages. Firstly, their fluorescence decays are in the millisecond range (0.5-3ms). This long-

lifetime means that if fluorescence intensities are measured only after small lag period following a pulsed excitation, any interfering background fluorescence, which is typically in the order of nanoseconds, can be eliminated, so improving the accuracy FRET efficiency measurements. Secondly, lanthanide emissions are isotropic. This means that the value for the orientation factor, κ^2 in equation (2) above, can more accurately be assigned a value of 2/3 which might not ordinarily be the case using other FRET pairs. The main drawback with lanthanides is that they are very poorly excited, and so require the presence of a chelator with a similar absorption spectrum in order to increase their signal [14, 16, 26].

3. *Unraveling molecular dynamics of ion channels by FRET spectroscopy*

To date FRET has been used to detect conformational changes associated with gating in a number of ion-channel types. Here, we describe several examples illustrating the power of FRET spectroscopy in unraveling the details of structural dynamics of ion channels that would be difficult, if not impossible, to study using other methods.

3.1 *Shaker K^+ channel*

Among the first ion channels examined by FRET was the Shaker potassium channel, a voltage-gated potassium-selective channel sensitive to the cellular membrane potential [27-30]. These channels play a crucial role in returning the cell membrane potential from a depolarized state to a resting state. This channel was studied using the acceptor photobleaching method [29] and by measuring fluorescence lifetimes [27-30]. In latter case, because they used a lanthanide ion terbium as the donor, fluorescence lifetimes were of the order of milliseconds instead of the usual nanoseconds. The FRET analysis on the channel ensemble revealed microscopic movements of the S4 voltage-sensing transmembrane region, and showed a 2 Å vertical displacement of S4 during the channel voltage-dependent gating [31]. A more recent study has shown there is little translocation of the S4 region through the membrane as a result of an induced electromagnetic field. This conclusion was based on the fact that donor fluorophores attached to various loci of the S4 region did not exhibit any transient change in fluorescence when the channels were open in the presence of non-fluorescent lipophilic acceptor molecules distributed in the lipid bilayer [32].

3.2. *Mechanosensitive channels*

Mechanosensitive channels act as molecular transducers of mechanical force exerted on the membrane of living cells. These channels open in response to membrane bilayer deformations that occur in numerous physiological processes including touch, hearing, micturition, blood pressure regulation as well as muscle and bone development. In bacteria these channels regulate cellular turgor by preventing bacterial cell death upon sudden change in environmental osmolarity (i.e. hypo-osmotic shock) [11].

3.2.1 MscL

FRET spectroscopy has been used to determine the open channel structure of MscL, the bacterial mechanosensitive channel of large conductance [33, 34]. As in the case of the Shaker potassium channel, ensemble analysis was also used to examine MscL transition from the closed to the open conformation. This was done by analyzing the intensity of light emitted by Alexa-Fluor-labeled cysteine mutants of MscL reconstituted

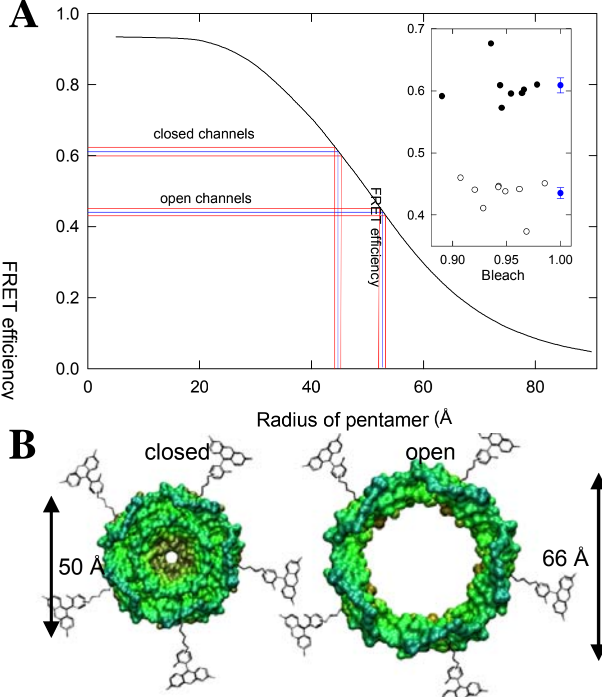

Fig. 2. Determination of the MscL open channel structure using FRET. (A) FRET efficiency for individual liposomes in closed channels (solid circles) and open channels (open circles), and average values (blue) are shown in the inset. The curve relating pentamer size to transfer efficiency is plotted with the data from the experiments shown by blue lines with error margins in red. **(B)** Schematic diagram indicating the scale of conformational change involved in channel gating. (Reproduced from [25], with permission).

into artificial liposomes. This allowed determination of the structural rearrangements of the protein in its natural lipid environment by modifying the lateral pressure distribution via the lipid bilayer. FRET efficiency was measured using the FRET acceptor photobleaching method. The diameter of the protein was found to increase by ~16 Å upon channel opening using this method (Fig. 2) [33, 34].

3.2.2 MscS

MscS, the mechanosensitive channel of small conductance [35, 36], is another mechanosensitive channel that was investigated by FRET. The channel has a characteristic large cytoplasmic (CP) domain, which is essential for its stability and activity [37]. FRET was used to determine the structural changes that occur in the CP domain during the channel opening [38]. The change in the diameter of the CP domain was monitored by FRET at several positions along the domain. This allowed for a 3D computational reconstruction of the structural changes that occurred during channel opening. The 3D view shows that the CP domain of MscS swells during channel opening. The swelling of the CP domain is presumably mediated by the electrostatic interaction between the TM and CP domains.

3.3 GRAMICIDIN

An important advancement in the usage of FRET was made by extending FRET to the single molecule level [39, 40]. The advantage of single-molecule FRET for studies of ion channels consists in detecting motions within a single channel, rather than motions which are not correlated between different ion channels in an ensemble. A very recent example of LeuT, a prokaryotic homologue of neurotransmitter/Na^+ symporters (NSSs), shows how single-molecule FRET can give very detailed structural and dynamic information about the functioning of a membrane protein [41]. While this study is on a transporter rather than an ion channel, it demonstrates the potential of this technique and how it can be applied in future ion channels studies.

A simultaneous fluorescence imaging and electrical recording of single ion channels in planar bilayer membranes was carried out on fluorescently labeled derivatives of gramicidin A (gA) [42]. gA is a hydrophobic peptide whose individual monomers reside in each monolayer of the lipid membrane, and form cationic channels when dimerisation links them into a membrane spanning protein. [43]. gA has proven to be a popular model for studying the effects of protein inclusions on lipid acyl chain order and dynamics. In this study, one peptide was labeled with a donor Cy3 dye, and the other with an acceptor Cy5 dye. Two different gramicidin peptides were used. One of them forms channels of low conductance and the other of high conductance. Electrical recordings detected gramicidin homodimer (Cy3/Cy3, Cy5/Cy5) and heterodimer (Cy3/Cy5) channels, characterized by an intermediate conductance. Given that gramicidin heterodimers could be simultaneously identified structurally and functionally by single molecule FRET spectroscopy and electro-physiology, this indicates that the combined optical and electrical approach could be used to uncover gating mechanisms in a wide variety of ion channels. This is demonstrated in the following example of TRPV ion channels.

3.4 *TRPV CHANNELS*

TRPV1 through TRPV4, members of the TRPV subfamily of the superfamily of TRP-type tetrameric cation channels, form the major cellular sensors for detecting temperature increases [44, 45]. Homomeric TRPV1-4 channels exhibit distinct temperature thresholds, with TRPV4 being activated by temperatures above 27°C, TRPV3 by temperatures within 31°C-39°C range, TRPV1 by temperatures above 43°C, and TRPV2 by noxious temperatures above 52°C. Single molecule FRET combined with the patch-clamp recording from single TRPV channels was used to investigate the co-assembly of TRPV channels [46]. Fluorescently labeled subunits of TRPV1, TRPV2, TRPV3 and TRPV4 were co-expressed in HEK293 cells. Interestingly, FRET was measured most frequently between different subunits rather than the ones of the same type, which indicated that the substantial fraction of TRPV channels in the membrane of co-transfected HEK293 cells were heterotetramers made of, for example, TRPV3 and TRPV2 or TRPV1 and TRPV4 monomers. Similar to gramicidin, the heteromeric TRPV channels exhibited in single channel experiments intermediate conductance levels and gating kinetic properties. Since TRPV1-4 monomers are widely co-expressed in different cells and tissues including sensory neurons, the heart and the brain, formation of heteromeric TRPV channels might contribute to their greater functional diversity.

4. CONCLUSION

The use of FRET to gain detailed structural information for multimeric membrane proteins such as ion channels, presents a major advancement in studies of protein structural dynamics. Although it has required different approaches to protein labeling, control of the protein state, as well as careful analysis of the orientations, geometries, and number of fluorescent probes, the FRET methodology described here provides an excellent tool for studying the structures of membrane proteins in *in vitro* and *in vivo*.

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Miracle Tale in Japan and Mathematics
- Our own will and imagination in education -

Motoya, Yoshiko

Abstract—*URASHIMA TARO is a Japanese old tale handed down from the past about 1300 years ago. This tale ends by an impressive scene, that, when Taro came back to the hometown from the happy days of Ryugujo (a Palace laid at the bottom of the sea), he found that his hometown had quite changed. 300 years had already passed. Disappointed he opened the Tamatebako (a present box from the queen), then at an instant he changed to an old man with white beard.*

Index Word— *center core axis rotation, domain drawing, geometry, topology*

1. *THE THEORY OF RELATIVITY OF SPACE-TIME IN 1905 BY ALBERT EINSTEIN & "URASHIMA TARO"*

At the beginning of the 20th century, Albert Einstein found the theory of relativity of space-time. We find that from the old ages people have had the dream of space-time, and transmitted various tales from generation to generation. This fact deserves to be treasured in our culture and education. People have held out their dream about the space-time in their imaginations and memories.

We will pay attention to the story Urashimako in "Tango Hudoki" and "Kojiki" (Shogakukan 1973) Mizunoe Urashimako has lived in a palace under the sea, and didn't come back for 700 years to his native village. When he returned to his hometown, he was told this fact. Embarrassed he forgot his promise to the Princess and opened the lid of the little comb box, given by the Princess Kamehime (in the country of eternity). Then, a sort of apparition like an orchid flower flew at an instant across the sky. The promise had been broken, and it meant for him never to meet the Princess… Since "Tango Hudoki", various Japanese poems of 'Urashima Taro' and 'Kamehime' were created in all ages.

We will deal with the series of the stories about 'Urashima Taro', recorded in "Otogizoshi" from 1390 to 1740.(Shogakukan 1973)The general story tells the following:

One day Urashima caught a turtle when he was fishing in the sea. Feeling pity, he returned the turtle into the sea. At the next day, a beautiful woman came to his ship and asked if he would accept her invitation to come to her own country.

Her country was called Ryugujo, a most splendid palace. There he was welcomed warmly, married 'Kamehime' and enjoyed a great deal of hospitality for three years. Finally, he offered her to let him return to his hometown to meet his parents. At the day of parting, when he was presented a box for keepsake, she told him "Please do not open this Tamatebako in any way",.

Having returned to his hometown, Urashima was astonished by the change of the appearance of his hometown. 700 years had already passed. He was blanked by the surprise, and opened the box, though he was told never to open the box. Suddenly, a purple cloud rose from the box, and he became an old man. And then, he was metamorphosed into a crane, and flied to the origin of the turtle in Mt. Horai.

In Japan and China they say that in Mt. Horai a turtle lives a myriad of years and the crane lives thousand years, and we than find an interesting connection with the tale.

2. *AUTHORS INTENTIONS IN THE GOVERNMENT-DESIGNATED TEXTBOOK*

Today we have various picture publications about "Urashima Taro" for children. There are over 70 kinds of illustrated books for children.(fig.1 & 2)

There are many large or small differences between the texts. Why do such differences come out in the illustrated books? We think the reason lays in the authors intention to educate children from the standpoint of the adult. They followed the idea of the government-designated textbook, and changed the tale into moral lessons (fig.3).

The second stage (1910-1917) was presented as the period of "Normal elementary school reader". The tale was quite changed as the moral instruction followed the intention of the state. It is sickening when we eat a good treat every day. When an interesting play is ideologically arranged, it will be spoiled and become dangerous. Thus,

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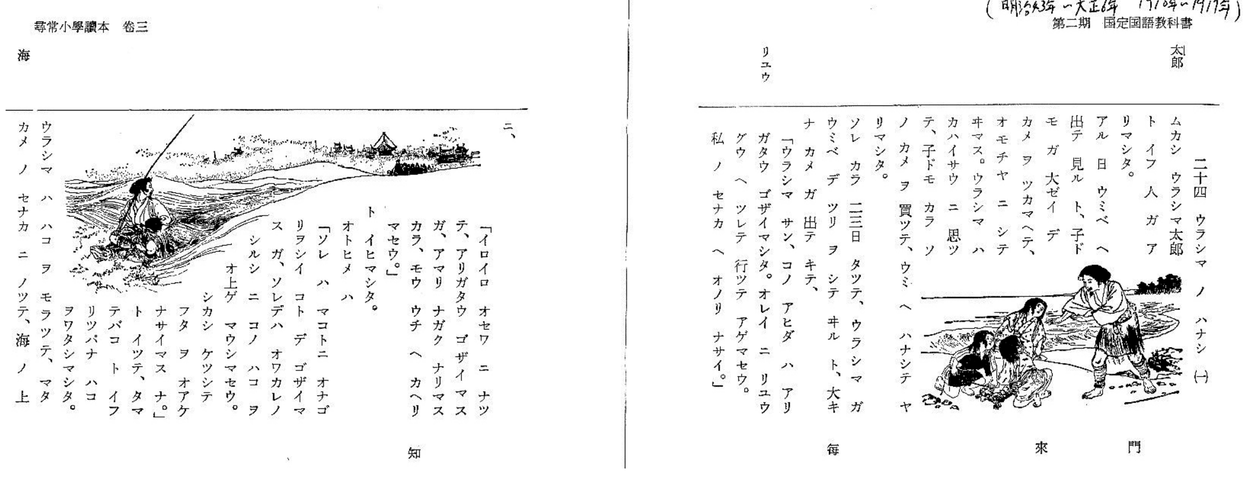
1	2	4	5
Der Fischersohn Urashima	URASHIMA TARO	URASHIMA TARO AND THEPALACE OF THE DRAGON	Urashimataro
comp.Yoichi Kanari instruction Koshio Miyao trans.U. Groth and Hedwig Schipplock	comp.Sayumi Kawaguchi, trans.Ralph F.McCarthy CLASSIC BILINCUAL PICTURE BOOKS 2	text Kerry Muler and R.Arai and Y.Inui, illustration Ryoji Arai	apanese Folk Tales Book2, trans.Kazuhisa Shimomura and Benjamin Woodward,Japan:Japan Times,
Japan:Dogakusya	Japan:Kodansya International	Japan:AsunaroSyobo	Japan:Japan Times
1982	2000	2004	2005
<the motive for visited the Dragon Palace> "mochte ich heimkehren und meinen Vater und meine Mutter, meine Bruder und meine Schwestern wiederesehen."	<The motive for visited the Dragon Palace> mischievous boys were poking a baby sea turtle. Taro carried it to the seasore. Several years had now passed.Taro saw a large sea turtle that Taro had rescued. "To show my gratitude, I'd like to take you to the Dragon Palace.	<the motive for visited the Dragon Palace> He gave the children some money and took the turtle back to the sea.	<the motive for visited the Dragon Palace> He picked up one of the tree branches and took a few threatening steps towards the children.
<reason for leaving the Dragon Palace> "Du musst aber diesen Kastern mit dir nehmen und dich huten, ihn zu offnen, Offnest du ihn, so wirstdu nie zuruckkehren konnen. "	<reason for leaving the Dragon Palace> Taro's heart began to yearn for his mother back home and their little house in the village.	<reason for leaving the Dragon Palace> he knew it was time to return home	<reason for leaving the Dragon Palace> He began to wish for the simple pleasures of his old life.
<meanig of "Tamatebako"box> "Vielleicht, wird es mir moglich, den Weg zu ihr zu finden, wenn ich den Kasten offne, welchen meine Frau mir gegeben hat." als eine weisse Wolke, die uber das Meer hinwegzog. Sein Haar wurde plotzlich weiss, wie Schnee, sein Gesicht bekam tiefe Runzeln, und sein Rucken wurde gebeugt wie der eines sehr altern Mannes.	<meanig of "Tamatebako"box> "Please take this with you. When you've returned to land ,if you find yourself feeling confused... that is when you sould open this box.	<meanig of "Tamatebako"box> the most important thing is inside farewell gift If you ever want to return to Ryugu, you must never open this box.	<meanig of "Tamatebako"box> Beautiful little box as a remembrance of their friendship. "But you must never open it not for any reason," Otohime warned him,
<time> d3 Jahre→400 Jahr	<time> some days → hundreds of years	<time> 3 years→300 years	<time> 8 days→100 years
<in the end> "Vielleicht, wird es mir moglich, den Weg zu ihr zu finden, wenn ich den Kasten offne, welchen meine Frau mir gegeben hat." als eine weisse Wolke, die uber das Meer hinwegzog. Sein Haar wurde plotzlich weiss, wie Schnee, sein Gesicht bekam tiefe Runzeln, und sein Rucken wurde gebeugt wie der eines sehr altern Mannes.	<in the end> He saw no one he knew, nor any houses he recognized. Taro hewildered "If I open it, I'll understand… " Taro timidly lifed the beautiful world at the bottom of the sea had beenonly a dream. Or was he dreaming now? Young Taro became an old man with long white beard.	<in the end> Urashima felt very sad, butnthen he remembered his present, the tamatebako from Otohime. "It might help me," he thought to himself. Purple cloud rose up and touched his face. At the moment, Urashima's hair turned white and he changed into an old man.	<in the end> as though its contents would explain everything. "Just a little look inside," he thought to himself. All of a sudden smoke came pouring out, surrounding him and making him cough. The villagers found a mad old man sitting intthe sand at his trembling hands.

fig.1 Investigaton on the difference between “Urashima Taro” picture books over 70 kinds.

fig.2 “Urashima Taro” picture books



日本教科書大系・近代編・第7巻・国語三　昭和38年　講談社
（明治43年〜大正6年　1910年〜1917年）
第二期　国定国語教科書

二十四　ウラシマ　ノ　ハナシ　㈠

ムカシ　ウラシマ太郎　ト　イフ　人　ガ　アリマシタ。

アル　日　ウミベ　ヘ　出テ　見ル　ト、子ドモ　ガ　大ゼイ　デ　カメ　ヲ　ツカマヘテ、オモチヤ　ニ　シテ　ヰマス。ウラシマ　ハ　カハイサウ　ニ　思ツテ、子ドモ　カラ　ソノ　カメ　ヲ　買ツテ、ウミ　ヘ　ハナシテ　ヤリマシタ。

ソレ　カラ　二三日　タツテ、ウラシマ　ガ　ウミベ　デ　ツリ　ヲ　シテ　ヰル　ト、大キナ　カメ　ガ　出テ　キテ、

「ウラシマ　サン、コノ　アヒダ　ハ　アリガタウ　ゴザイマシタ。オレイ　ニ　リユウグウ　ヘ　ツレテ　行ツテ　アゲマセウ。私　ノ　セナカ　ヘ　オノリ　ナサイ。」

太郎　門　來　毎　リユウ

尋常小學讀本　卷三

二、

「イロイロ　オセワ　ニ　ナツテ、アリガタウ　ゴザイマスガ、アマリ　ナガク　ナリマスカラ、モウ　ウチ　ヘ　カヘリマセウ。」

ト　イヒマシタ。

オトヒメ　ハ

「ソレ　ハ　マコトニ　オナゴリヲシイ　コト　デ　ゴザイマス　ガ、ソレデハ　オワカレノ　シルシ　ニ　コノ　ハコ　ヲ　オ上ゲ　マウシマセウ。シカシ　ケツシテ　フタ　ヲ　オアケ　ナサイマス　ナ。」

ト　イツテ、タマテバコ　ト　イフ　リツパナ　ハコ　ヲワタシマシタ。

ウラシマ　ハ　ハコ　ヲ　モラツテ、マタ　カメ　ノ　セナカ　ニ　ノツテ、海　ノ　上

知　海

fig.3 The government-designated text book

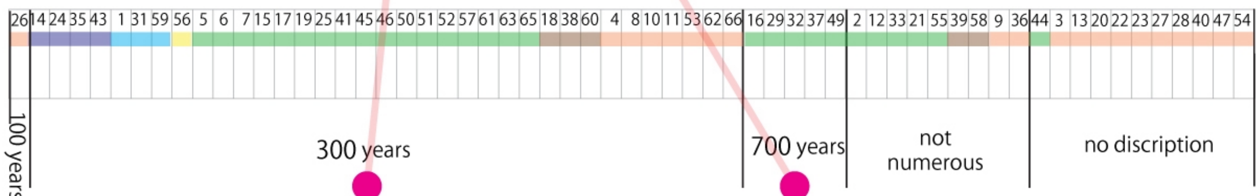


fig.4 The concept of the relativity of time
The red pointed marks are "Tango Hudoki" & "Otogi Zoshi".Each number shows the title of picture books.

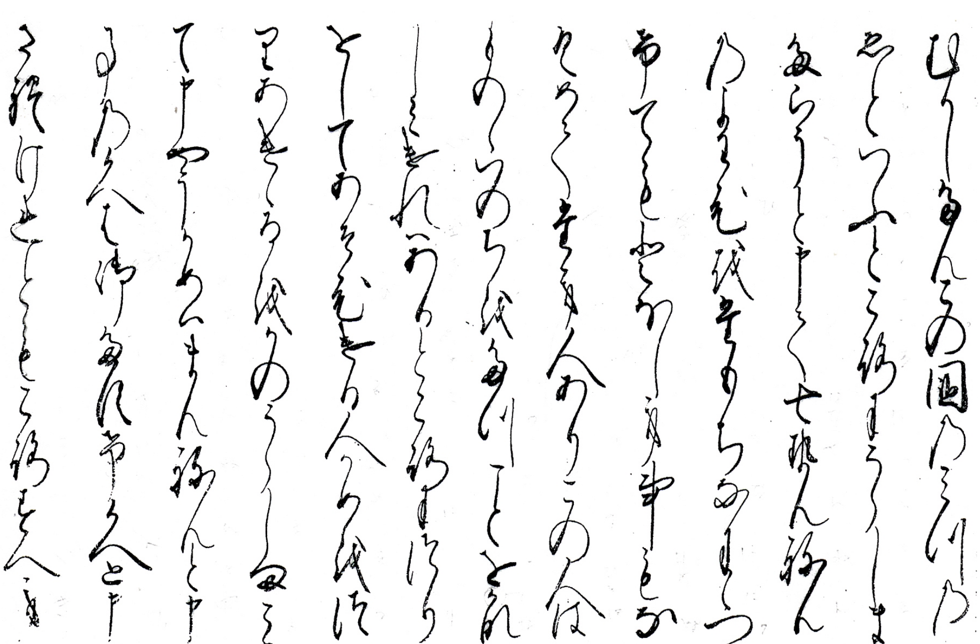


fig.5 A part of Nara picture books about “Urashima”

the children will show no interest in the tale. In the textbook, years in the palace of the sea goddess, where he spent his happy life, were cut out. Fantastic plot lines were intentionally neglected as irrationality.

Morality corresponds with the determinism of authority, such as that of Euclidean geometry. So is the case of the tale "Urashima Taro", and the authors of the textbook made use of the tale for the authority of moral. The world of tales should be protected from any authority and determinism, and assured by the principle of "the theory of relativity". The tale "Urashima Taro" is indeed the theory of relativity of time in ancient days.

It seemed that the influence of the idea of the government-designated textbook was great, and a lot of illustarted books seemed to be bound by the intention of authorities. However, " Urashima Taro" succeeded for a long time in impressing people's hearts. People have accepted the world of fantasy and the creative relativity. This deserves to be recognised as a most marvelous fact.

Especially we must pay attention to the concept of the relativity of time (fig.4). The time that Urashima had spent in the palace of the sea goddess castle was different from the time on the ground. People loved in another time and another world in all ages and countries. To be relative, and not to be deterministic is in any way important, and people have handed down what should be told. And today, a concept goes between "Urashima" researchers to point out the uniqueness and originality of the story as the national legend.

How should we tell children the tale "Urashima" ? We tried to introduce them the logic of relativity of the tale, showing various sources of "Urashima". And we showed that relativity of values is connected with the problem of history, peace, and environment. It is important that each pupil learns from his standpoint from early ages to seniority and beyond generations.

3. *Our approach*

Books of "Urashima" are various from each other. We started from reading and comparing many kinds of illustarted books "Urashima". Editions of "Urashima" reach 70 kinds of illustrated books and sources. There are four kinds of audio-visual material, video etc. of "Urashima". In addition, we tried to interview grandparents about "Urashima " that remained in the memory of their childhood. Questons asked were as, 'Are there a crane in the tale?', or 'Why 700 years?'.

4. *Studies of original "Urashima"*

To investigate the cause of various differences of "Urashima", the following two points were taken up from the original two texts.

Urashimako, "Tangohudoki", "Kojiki" , "Joudaikayou" (720), and

Urashima , "Otogizoshi" (1390-1740)

By examining the government-designated textbooks we get to understand how various differences stemmed out from the intention of adults for children. An interesting fact is that before and after the year 1905 (the year Einstein discovered the theory of relativity) the intents of the tale were somewhat different.

We tried a comparative study of several kinds of Nara illustrated books about "Urashima" (from the Muromachi period; fig.5). We also tried a translation of this text into the modern Japanese. And as the result of this work, we find that "Urashima" is not only a legend or tale for children but also the ideal way of life of people resembling an utopia, and demonstarting that over the ages people loved the miracle of time and space. We also tried a comparative study of today's "Urashima", which were written by many contemporary writers. Above all, we pay attention to Musyanokoji Saneatsu's "Urashima and Otohime", a poems "Departure from Urashima", "Urashima's monologue", and "New village". He tried to watch the present-day lives, and described the ideal way of people.

We tried another practical approach. We told children to draw a scene of "Urashima" and moreover to draw a "Kids' Guernica" (fig.6)!

Kids' Guernica is an international children's drawing art project to create "peace" on a " Picasso's Guernica" size canvas (3.5m x 7.8m) . Pablo Picasso created "Guernica" to protest against the brutality of bombing during the Spanish civil war in 1937. Kids' Guernica is an artwork on a global canvas, expressing the spirit of peace and solidarity of people. More than 160 peace paintings were already created in 40 countries of the world. Kids' Guernica project was initiated by Art Japan Network in the year of the 50th anniversary of World War II (1995). The first workshop was held in Tallahassee, Florida, U.S.A.

fig.6 We told children to draw a scene of "Urashima" .and moreover to draw "Kids' Guernica"!

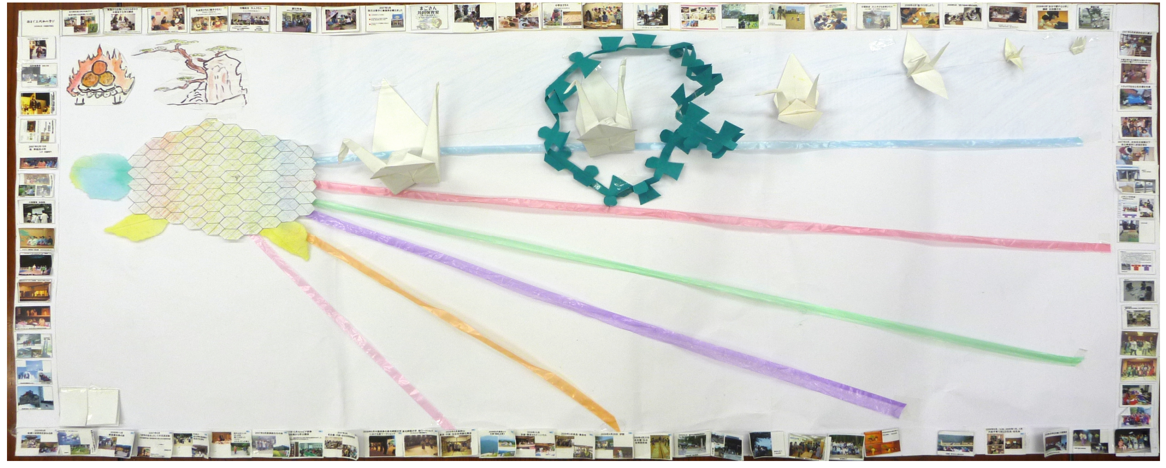


fig.7 The Frame of our Kids' Guernica

fig.8 Our wish

5. Frame of our Kids' Guernica

Our theme of Kids' Guernica was "Today's Urashima" who will live in this world. (fig.7)

To draw today's "Urashima", and to express people's desire for peace.

Not to think of self interest but to think about other people's standpoint, and to think about opposite poles of things (contrary standpoint).

To learn non-Euclidean geometry, as an exercise of free mathematical way of thinking. Euclidean geometry teaches in a way of necessity and determinism. On the contrary, projective geometry and topological geometry cultivate their imaginative power and creative thinking.

To try a Video Conference –Exchange with 4th grade-schooler in the United States through Skype.

6. Conclusion

We derived two treasures in the direction from "Urashima Tarou" the legend of Japan.
The first is that this story cultivated the starting point of Japanese hope and imagination.
Furthermore, we clarified that this story was falsified as the material of one-sided use for the nation and power.

In addition, geometrical, spatiotemporal secret was found being buried under this story. Topological and Perspective geometrical education that made the relativity of time a theme

was especially introduced through the Kid's Guernica.

Our trial is still at a start and we wish to elucidate an original method to combine Japanese classical culture and a new humanized science, especially using a new mathematical way of thinking (fig.8-15).

fig.9 Several pieces of paper crane (Origami) stand for the idea of projectuve geometry

fig.10 The biggest and the smallest paper crane (Origami)

fig.11 Thinking about today's Kids' Guernica

fig.12 Moebius' band

fig.13 Workshop with American 4th grade schooler through Skype

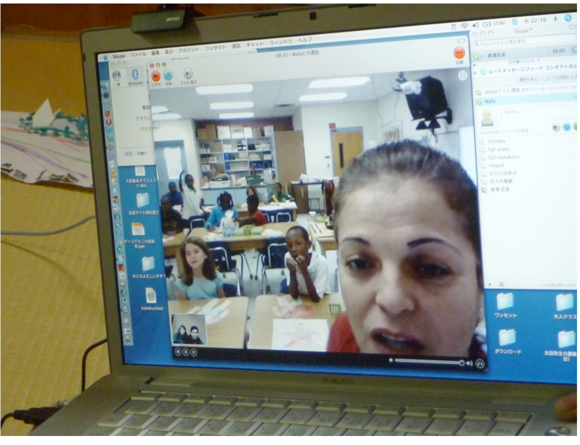


fig.14 Workshop with American 4th grade schooler through Skype

fig.15 The parts of our Kids' Guernica

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Biography

Yoshiko MOTOYA is a professor of Osaka Women's Junior College and an emeritus of Miyagi University of Education. She has investigated the status and structure of learner's conception and cognition through conflict, confusion and selection by learners, especially on mathematics education. One of her main interest is to discover a topological consideration from young children's drawings, handiworks and playing behavior Isuch as dance.

She emphasizes the bipolar thinking. In fact she also is a leading actress of Nho drama which is the most traditional performing arts in Japan.

In 2005 she established a Lifelong Learning Project called "MAGO3" for pupils from infants to elderly adults. She has been trying to organize the zest for life of her own and the members of the project with a daring and exquisite care.

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