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THE LONG ROAD TO THE USE OF MICROSCOPE IN CLINICAL MEDICINE IN VIVO: FROM EARLY PIONEERING PROPOSALS TO THE MODERN PERSPECTIVES OF OPTICAL BIOPSY

DUGI PUT PREMA UPOTREBI MIKROSKOPA U KLINIČKOJ MEDICINI IN VIVO: OD PRVIH PIONIRSKIH PRIJEDLOGA DO MODERNIH PERSPEKTIVA OPTIČKE BIOPSIJE

Giovanni Ponti,[1] **Umberto Muscatello**[2]

Summary

For a long period the scientists did not recognized the potentialities of the compound microscope in medicine. Only few scientists recognized the potentialities of the microscope for the medicine; among them G. Campani who proposed the utilization of his microscope to investigate the skin lesions directly on the patient. The proposal was illustrated in a letter Acta Eruditorum of 1686. The recent development of optical techniques, capable of providing in-focus images of an object from different planes with high spatial resolution, significantly increased the diagnostic potential of the microscope directly on the patient.

Key words: *clinical use of microscope; Campani's mocroscope; optical biopsy in vivo.*

[1] Surgical, Medical and Dental Department of Morphological Sciences related to Transplant, Oncology and Regenerative Medicine; University of Modena and Reggio Emilia; Italy.

[2] National Research Center S3 , CNR-NANO, c/o Department of Physics, University of Modena and Reggio Emilia, Modena, Italy.

Corresponding author: Dr Giovanni Ponti, Department of Clinical-Diagnostic Medicine and Public Health, University Hospital of Modena and Reggio Emilia.
Electronic address: giovanni.ponti@unimore.it

Nowadays the collective imaging regards the microscope as the icon of medical research and practice. However, for a long period after its availability, the idea of using the microscope to solve medical problems was hindered by a number of factors, both fashionable and conceptual. The widespread use of the compound microscope during the whole 17th century, was largely due to the curiosity of observing the marvels of the invisible word revealed by this instrument [1]. This exciting curiosity was a potent motivation to make use of the microscope not only by the upper classes, but also by several scientists including the greatest ones of them. Galileo was delighted by observing the way the flies can walk upside-down: "I have seen with great contentment how the flies and other small animals are capable of walking on the mirrors and even upside-down"[2]. Robert Hook was captivated by the microscopic anatomy of insects, in particular that of the flea, *Pulex irritans* deeply fascinated by the mechanics of its jumping [3,4]. Van Leeuwenhoek, untiringly looking for the presence of microorganisms in water, blood, infusions, sperm, was aware of exploring a great secret of nature: hence he entitled his seminal observations "Arcana Naturae detecta"[5,6]. However, a number of scientists, enthusiastic followers of Galileian and Cartesian method, maintained a different point of view as to the possible role of this instrument. They proposed a new approach to the study of living organisms inspired by an atomistic and democritean view of nature. According to this view, the living organisms were conceived as an assembly of minute machines. Their evidence was essential to explain the biological functions in mechanistic terms [7-9]. In this perspective, the microscope appeared to be the indispensable instrument to reveal the existence and structure of these micromachines. Their impairment or malfunctioning would result in diseases [10,11]. Undoubtedly this approach produced a number of relevant observations of microscopic anatomy; however, at the same time it gave rise in medicine to the so-called iatro-physics, or iatro-mechanics, a theory asserting that the mechanisms of diseases should be explained only on the basis of natural laws of physics and chemistry. However, the over-simplified and arbitrary explanations advanced by iatro-physicists generated medical systems devoid of any utility [12]. Hence the rejection of this approach by Thomas Sydenham and his proposal of a fully clinical interpretation of disease [13].

During the same 17th century and even well later only a few scientists clearly perceived the potential utility of the microscope in medicine. This potentiality was generically asserted by the French scholar Pierre Borel (Petrus Borelli); however the examples he reports are rather irrelevant and

devoid of any general significance [14]. The utility of microscope to solve particular medical problems was clearly intuited and expressed by the Jesuit Athanasius Kirchner. In fact, in his book *Ars magna lucis et umbrae,* published in 1646, Kirchner affirmed that "many things might be discovered with the microscope in the blood of patients with fever" [15]. Coherently, he examined the blood of victims of pestilence in Rome of 1656 and claimed to have observed the presence of *minima animalia* in the corpses of individuals dead of pest [16]. Although the limited resolving power of the microscope used by Kirchner almost certainly prevented him from observing Yersinia pestis, his suggestions are undoubtedly seminal for his time. The first unambiguous assertion of the potential value of microscope as a diagnostic tool in clinical medicine is due a Giuseppe Campani, a scientist widely recognised as the pre-eminent lens-maker of his time. The innovative characteristics of the microscope constructed by Campani and his proposal of using the microscope to examine lesions eventually found in a patient were reported by Mons. Emanuel Schelstratenus, Prefect of the Vatican Library, in a letter to Acta Eruditorum of Lipsia of June 1686 [17]. The letter is a significant document for the history of microscopy in medicine because of the remarkable precision of the text, the extraordinary intelligibility of figures and the seminal proposals about the use of microscope in clinical medicine. The letter first underlines the substantial improvements made by Campani to ameliorate the performance of the microscope. His lenses, perfectly polished and geometrically innovative, provide a very large field-amplitude and a magnifying power much higher than that of any other microscope at that time. The instrument is designed as to permit a much greater precision of adjustment. It is underlined that "the microscope allows to observe any kind of objects" included "the animacula present in the sperm of all living organisms already discovered by Van Leeuwenhoek". In the latter case it is suggested to keep the samples between two glasses. Finally it is emphasized an important characteristics of the Campani's microscope, that is the possibility of removing the tube, that holds the lenses, from its support. In this way the hand-held tube may be conveniently oriented and the lenses can be focused also on objects that, because of their large dimensions, cannot be accommodated between the tube and the support. This key feature characteristics permits to observe by this instrument wounds, plaques and scars directly on living patients. The proposals are beautifully illustrated by figures included to the text. The figure n.3 is particularly interesting since it shows a patient lying

on a bed while a doctor observes a skin injury on the patient's leg and an assistant concentrates the light on the surface of the skin.

However the awareness of the utility of microscope in pathology and in medicine, so clearly intuited by Athanasius Kirchner and by Giuseppe Campani, was in fact hindered by a long-lasting lack of a disease theory that would require investigation at microscopic level. The Sydenham's clinical approach to the study of diseases undoubtedly casts-off the idea of using the microscope in medicine. So for a long period the University Doctors and Practitioners considered ridiculous the use of microscope and completely disregarded the potentialities of this instrument in medical studies. Even the founders and masters of pathological anatomy entirely ignored the use of microscope in their studies. The first Atlas of Pathology, published by Mettew Baillie in 1799, contains more than 100 illustrations none of which is elaborated by the aid of microscope [18]. The skepticism toward the utility of microscope to solve medical problems, was suddenly surmounted when Rudolph Virchow advanced his revolutionary theory of cellular pathology according to which the basis of all diseases are to be found at the cell level [19]. This implied inevitably the need of using the microscope. During the same years, the replacement of miasma theory of infectious diseases by the germ theory, consequent to the work of Pasteur and Koch, resulted in a further formidable impulse to use the microscope in diagnostic medicine [20]. The increased demand for more suitable instruments stimulated technical and theoretical researches to ameliorate the performance of compound microscopes [21] and to develop adequate methods of fixation and staining. A further relevant advance in using the microscope in cellular studies was realised during the first decades of 20th century by the development of phase-contrast microscopy, an optical technique that does not require any preparative treatment and the living cells can be studied in their natural milieu and during their dynamic evolution [22].

The idea of using the microscope to analyse tissue injuries *in situ* and to follow their evolution directly on the patient, foreseen by Campani, has been recently revitalised by the development of new optical techniques. These techniques, that allow the formation of in focus-images of an object from different planes (optical tomography), are named reflectance scanning confocal microscopy and scanning two-photon fluorescence microscopy. The former optical technique was originally designed in transmission mode to increase the contrast of an image by eliminating the out-of-focus light contributions in specimens that are thicker than the focal plane. This allows high depth

sensitivity, substantial reduction of background noise and capacity of collecting serial optical sections from thick specimens for a three-dimensional reconstruction of the structures from the images [23,24]. In most biological studies, the samples are treated with fluorescent dyes in view of their high sensitivity coupled with their ability to specifically target structural components and dynamic processes. A further evolution of scanning confocal microscopy is the reflectance scanning confocal microscopy in which the image is formed by photons scattered from a surface. The light reflected reveals the structure of either the outer surface of biological tissues or the inner interfaces where there are significant changes in the refraction index. This technique allows therefore to investigate the physiological or pathological state of cells located not only at the surface of tissues but also at the interfaces of layers of different types of cells in stratified tissues. This optical technique is applied with success particularly in ophthalmology where the majority of localized defects in retinal nerve fiber layers can be detected and differentiated in reflectance scanning tomograms [25]. The optical coherence tomography is also applied to evaluate changes in function and morphology of normal human skin [26].

Very promising diagnostic perspectives are implicit in clinical applications of the two-photons excitation microscopy, an optical technique capable of imaging planes of living tissues at different depth down to 1 mm [27]. The working principle of this optical technique is based on the following concept: there is a definite probability that two photons, the energy of either the two is lower than that necessary to excite one single atom, may excite the same single atom in a quantum event with consequent emission of a photon. The energy of this photon is higher than that of either of the two excitatory photons [28, 29]. The probability of a quasi-simultaneous absorption of the two photons is extremely low and increases with the square of the excitation intensity. Hence the need of using an excitation beam of very high intensity tightly focused on an extremely restricted region. Under these conditions, the photon density outside the focal region is insufficient to excite any atom, so that null signal can arise from regions out of focus: the fluorescence is therefore restricted to a very small volume whereas the regions out-of-focus are not fluorescent at all. The possibility of obtaining an extremely punctate fluorescence that can be made to vary along the Z axis, allows the optical sectioning of a living tissue, i.e. the acquisition of in-focus images of tissue planes at different depth. Significant advances in this field have been obtained by using infrared light (730 nm wavelength) as excitation beam. In

fact, the lower energy photons of infrared light causes minor damages to the cells outside the focal region. Moreover the infrared radiation penetrates into tissue more efficiently than radiations of shorter wavelength. A difficulty encountered in all forms of optical sectioning of living samples derives from the fact that samples may shift very rapidly: this implies that the optical sections have to be acquired at a rate high enough to resolve the individual phase of the process to be studied.

The two-photons excitation microscopy opens interesting perspectives in clinical medicine, especially in the case of skin lesions [26]. Two series of advantages may be expected by the use of this technique: the imaging of structural details with sub-micron spatial resolution of living tissues at different depth without excision and with very low out-of-focus damage, and the quantitative determination of the autofluorescence of the cells at different planes. Structural analysis of the skin at different planes may provide information about the physiological evolution of skin tissues and their organization as a function of aging and about the presence of specific lesions in different types of cells. The non-invasive visualisation of the deep layers of a wound, may allow observation of granulation tissue formation and the evaluation of the effects of tissue repair under specific treatment (30).

Great advantages may be expected by the use of two-photons microscopy for quantitative determination of auto-fluorescence of cells at different planes. In fact, the major source of auto-fluorescence in human skin are NADPH and Flavo-proteins (FAD and FMN). NADPH is a coenzyme required in a number of anabolic pathways and in the **oxidation-reduction** system against the toxicity of **reactive oxygen species. Moreover, it is involved in generating** free radicals in immune cells to destroy pathogens. Therefore, its quantitative determination may be considered an intrinsic probe of the overall skin cell metabolism and of the capability of the skin to react against toxic and microbic agents [31]. Flavoproteins play a relevant role in the process of oxidative phosphorylation: they are therefore intrinsic probe of aerobic metabolism. Quantitative determination of auto-fluorescence of the cells at different planes may therefore provide relevant information as to the vital capacity of skin transplantation or the efficiency of the cells involved in wound repair, since both processes rely on mitochondrial oxidative metabolism [31,32].

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Sažetak

Dugo vremena znanstvenici nisu prepoznavali potencijale optičkog mikroskopa u medicini. Samo je nekoliko znanstvenika prepoznalo potencijale mikroskopa za medicinu. Među njima je G. Campani koji je predložio korištenje mikroskopa u istraživanju kožne lezije izravno na pacijentu. Prijedlog je ilustrirao u pismu Acta Eruditorum iz 1686. Nedavni razvoj optičke tehnike, kojom je moguće proizvoditi fokusirane slike objekta iz različitih uglova s visokom prostornom rezolucijom, značajno povećava dijagnostički potencijal upotrebe mikroskopa izravno na pacijentu.

Ključne riječi: *klinička primjena mikroskopa; Campanijev mikroskop; optička biopsija in vivo.*