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TECHNICAL NOTE

Confocal laser scanning microscopy. Using new technology to answer old questions in forensic investigations

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Abstract Confocal laser scanning microscopy (CLSM) is a relatively new technique for microscopic imaging. It has found a wide field of application in the general sphere of biological sciences. It has completely changed the study of cells and tissues by allowing greater resolution, optical sectioning of the sample and three-dimensional sanoke reconstruction. Confocal microscopy represents a valid, precious and useful tool capable of providing data (images) of unrivalled clearness and definition. This review discusses the possible applications of confocal microscopy in specific fields of forensic investigation, with specific regard to ballistics, forensic histopathology and toxicological pathology.

Keywords Confocal laser scanning microscopy · Forensic investigation · Terminal ballistic · Toxicologic pathology · Sudden cardiac death · Neonatal death

Introduction

Confocal laser scanning microscopy (CLSM) is a relatively new imaging technique, introduced in 1980 by M. Petran and A. Boyde. It has found wide applications in the biological sciences [1, 2]. At no other time have so many technologies and methodologies been available to forensic scientists, but, unfortunately, they are very rarely applied in daily judicial practice [3]. One of the most obvious ways to improve the quality of forensic investigations is by utilizing the most recent innovations in the field of microscopy. The existence of CLSM has radically transformed the field of biology in general and forensic pathology in particular. While most new techniques with forensic applications are still undergoing development and testing, the value of CLSM has already been demonstrated, although this technique is not yet available to most forensic scientists [4]. High-speed and high-resolution confocal and multiphoton microscopes allow researchers to obtain three- (3D) and four-dimensional (4D) information [5]. This development has prompted us to study and evaluate their possible applications in the specific field of forensic sciences.

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Confocal microscopy: how does it work?

CLSM has been widely applied in the general sphere of biological sciences, and it has completely changed the study of cells and tissues. It allows greater resolution, optical sectioning of the sample and three-dimensional reconstruction of the same sample [1, 2, 6–11]. Objects may now be viewed in a four-dimensional space (4D microscopy, xyzt) via a spatial analysis, repeated in time, and then combined with the spectral characterization of luminous signals coming from samples under examination (five-dimensional, 5D microscopy: xyztλ). By combining additional technologies such as fluorescent lifetime imaging (FLIM)-based analysis, the capabilities of CLSM have been further extended so that now, in some cases, it is possible to speak of six-dimensional microscopy [12]. The great possibilities provided by CLSM stem basically from two peculiar characteristics:

1. The sample is illuminated by the projection of a punctiform light source on the focal plane of the objective.

2. The collection of the emitted signal is limited by the introduction of a pinhole disk of variable aperture placed before the detector. Therefore the light coming from regions external to the focal plane is blocked, thus eliminating the contribution of the image coming from the overhanging and beneath planes.

The unquestionable value of confocal microscopy consists in the creation of optical sections: the laser beam sections the sample so that it is no longer necessary to prepare sections. The non-invasive nature of optical sectioning, which may be realized both on living and fixed cells, allows scientists to obtain images with an optimal spatial resolution but without any dramatic alteration of their "architecture", in an environment which is as close as possible to the biological reality. CLSM provides precious information regarding the distribution of a substance in the entire volume of the sample (e.g. fragment of tissue or cultured cells) and of structural and non-structural components that can emit a luminous signal, either by themselves or by means of specific stain, which will emit light when they are hit by a laser beam. CLSM also provides information regarding the various focal planes of the sample (information about depth). Because of these obvious advantages, we are applying CLSM (True Confocal Scanner, Leica TCS SP2, Cambridge, UK) to routine cases at the Department of Forensic Pathology of the University of Foggia. In order to demonstrate the usefulness of confocal microscopy in forensic medicine, we present here selected cases where investigation presents particular diagnostic difficulties:

- Sudden cardiac death (myocytes morphology, role of calcium)
- Terminal ballistics (bullet entrance wound study)
- Neonatal hypoxic–ischaemic lesions (timing)
- Electrocutions and explosions (incongruous material on the skin)
- Intoxications and poisonings (intracellular deposits)

Sudden cardiac death

Forensic investigation in cases of sudden cardiac death represents one of the most promising fields of application for confocal microscopy. The ability to detect the fine structures of myocytes both in normal conditions (distribution of structural proteins, Z disks, etc.) and especially in the presence of pathological alterations (e.g. contraction band necrosis) provides the forensic pathologist with imaging possibilities that were unthinkable until a few years ago [13–15]. In recent years, clinical and medico-legal attention has focused on the genetic causes of sudden death [16, 17]. Our knowledge about these disorders has deepened and widened thanks to the incessant progresses in the field of cardiovascular genomics. CLSM is a sophisticated research tool that may be diagnostic in some types of channelopathies, particularly those where a causal role of calcium-channel alterations have been implicated. It is well established that intracellular sodium homeostasis has a relevant role in myocellular function [18]; sodium channels and transporters, which couple sodium influx to either co- or counter-transport of other ions and solutes [18], may influence the regulation of intracellular pH and calcium homeostasis, thus impairing excitation–contraction coupling and energy production mechanisms [19]. In these cases, the absence of histological signs of either ischemia or inflammation and the exclusion of systemic hypercalcemia suggest that the presence of intracellular calcium deposits might be specifically related to the underlying molecular defect. The ability to visualize intracellular Ca^{2+} deposits with confocal microscopy provides the forensic pathologist with a set of diagnostic tools capable of screening for molecular abnormalities (Fig. 1).

Terminal ballistics

The differentiation between entrance and exit wounds and the determination of the shooting distance are basic requirements of any forensic investigation. Traditional histologic methodologies (haematoxylin–eosin staining, etc.) have become more refined, and it is possible to detect gunshot residues (GSR; barium, antimony, tin, lead, etc.) by means of specific stains (Na^+ rhodizonate). GSR particles from the primer, propellant, metals contained in the bullet, bullet jacket, cartridge case and gun barrel are all produced when a gun is fired [20]. Detection and identification of GSR can be accomplished by several different methods; X-ray fluorescence analysis, scanning electron microscopy (SEM) with energy-dispersive X-ray analysis and with wavelength-dispersive X-ray analysis, or with energy-dispersive spectros-

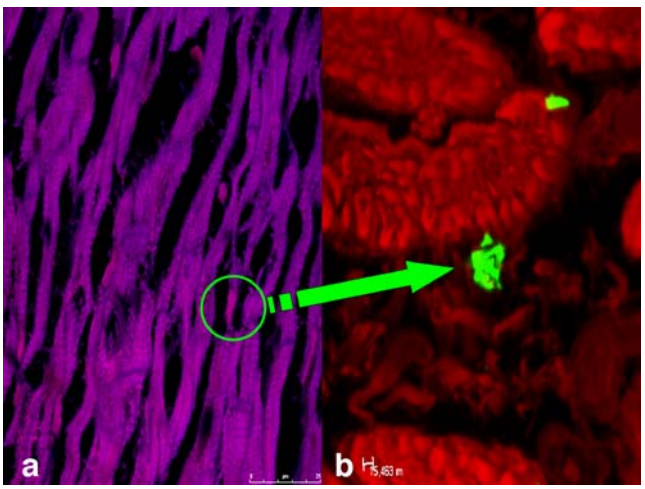

Fig. 1 CLSM micrographs of contraction band necrosis in the heart (**a**) with a myocyte (**b**) showing calcium deposits composed of needle-like crystals (*arrow*)

copy, have all been used [21, 22]. Although these methods are all relatively sensitive and specific, some are costly and require highly specialized equipment. The combined utilization of morphometric analysis and confocal microscopy using rhodizonate staining provides a simple and inexpensive technique for the analysis and the quantification of GSR deposits on skin. Confocal microscopy allows the observer to eliminate unwanted background noise in thick sections. Removal of this out-of-focus haze is achieved by optically cutting a section from the specimen. A sequence of these sections is then collected along the *z*-axis and can be used to render and quantitatively analyze the structure three-dimensionally (Fig. 2). Moreover, this method has the potential to provide quantifiable and comparable evidence for estimation of the shooting distances in the reconstruction of events in gunshot fatalities [20].

Neonatal hypoxic–ischaemic lesions

The correct forensic approach to cases of neonatal cerebral palsy is complex and requires detailed study (clinical file examination, X-ray, computed tomography and magnetic resonance imaging studies before autopsy, accurate post-mortem examination), in addition to an exhaustive histological examination by means of advanced laboratory techniques. In October 1999, the International Cerebral Palsy Task Force published a consensus statement for defining a causal relationship between acute intrapartum events and cerebral palsy [23, 24]. Their aim was 'to define an objective template of evidence to better identify cases of cerebral palsy where the neuropathology began or became established around labour and birth'. The authors defined three 'essential criteria', which should be met before attributing cerebral palsy to an intrapartum hypoxic event, and also enumerated a number of 'criteria which, when take together, suggest an intrapartum event, though they are non-specific'. The essential criteria do not depend on the traditional clinical signs of 'birth asphyxia', i.e. fetal heart rate changes and a low Apgar score at birth, but focus on the presence of severe fetal or neonatal acidosis, neonatal neurological state and type of cerebral palsy [24]. The timing of hypoxic–ischaemic damage is the central element in any medico-legal evaluation of obstetric professional liability for cerebral palsy [25]. In particular, the histopathological timing of the hypoxic–ischaemic damage using qualified immunohistochemical and microscopic investigations becomes the central moment of the forensic practice in cases of foetal hypoxic–ischaemic sufferance [26, 27]. The pattern of hypoxic–ischaemic damage in neonates varies with gestational age, and in premature infants the cerebral white matter is particularly susceptible. Manifestations of injury range from relatively selective apoptotic loss of oligodendrocytes in telencephalic leukomalacia to focal or multifocal coagulative necrosis in periventricular leukomalacia. Ischaemic degeneration of neurons may also occur, mainly in the basal ganglia, thalamus, cerebellar granule cell layer and brain stem, and apoptosis is usually prominent. In full-term infants, hypoxic–ischaemic damage is most prominent in the grey matter and manifests itself with

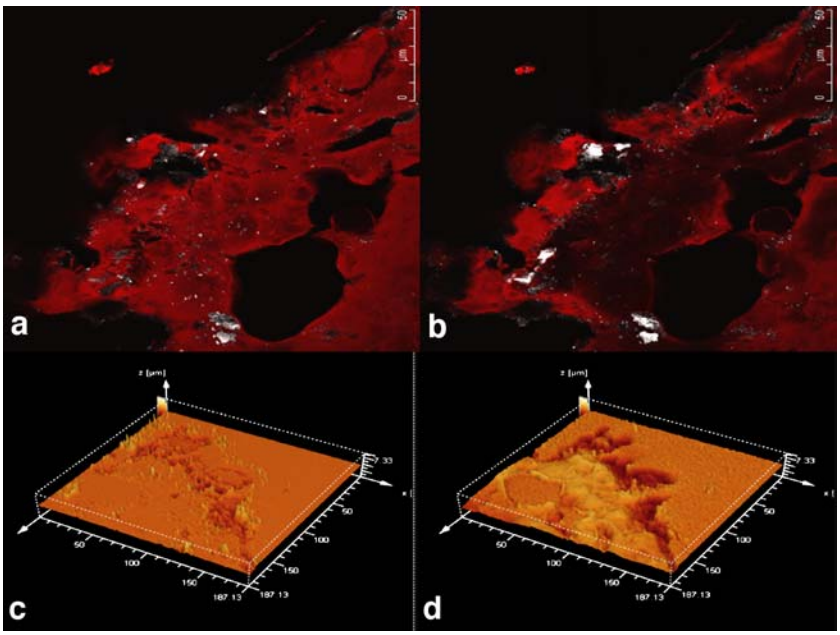

Fig. 2 Intermediate-range wound (approximately 30 cm). Confocal microscopy, 3D visualization of the gunpowder residues (birefringent bodies) on epidermal surface (**a**, **b**). 3D view of topology reproducing the superficial structure of the skin specimen in an entrance wound. The darker colour (*brown*) corresponds to the deeper layer of the specimen, the lighter colour (*orange*) refers to the epidermal layer (**c**, **d**)

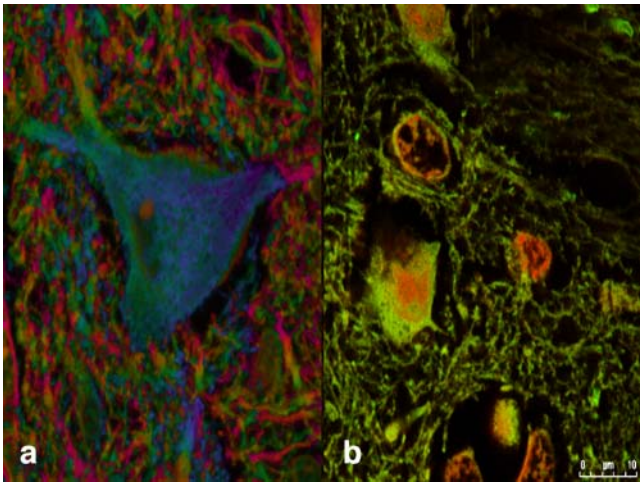

Fig. 3 3D view of neurons: micro-vacuolar degenerations (**a**) and typical morphological features of apoptosis associated with marked condensation of chromatin and its fragmentation into discrete bodies (**b**)

various combinations of neuronal apoptosis, selective neuronal necrosis and clear infarction that can involve the cerebral cortex, basal ganglia, thalamus, cerebellar cortex, cerebellar dentate nucleus and brain stem [28]. The application of confocal microscopy to these investigations allows the acquisition of images with an exceptional definition and resolution (Fig. 3).

Miscellaneous

We illustrate here some examples from our laboratory in which, besides the traditional methods of observation, we studied the different biological samples with confocal microscopy.

– Explosion injuries: skin lesions with demonstration of stratified incongruous material on the skin (Fig. 4)

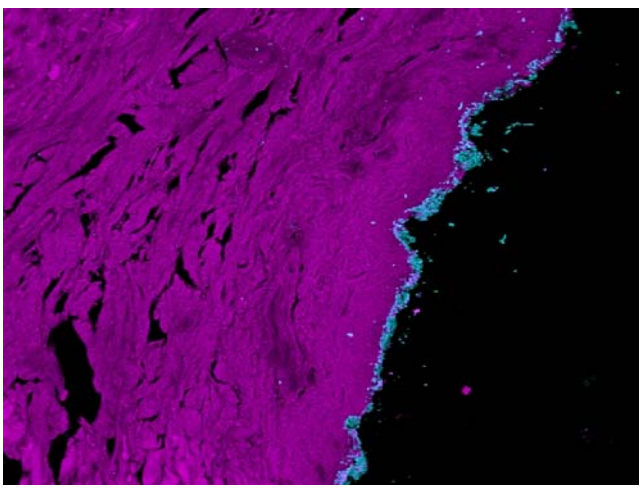

Fig. 4 CLSM using auto-fluorescence emission of skin: birefringent material stratified on epidermal surface and fixed in lower layers of cutis (*blue and green bodies*)

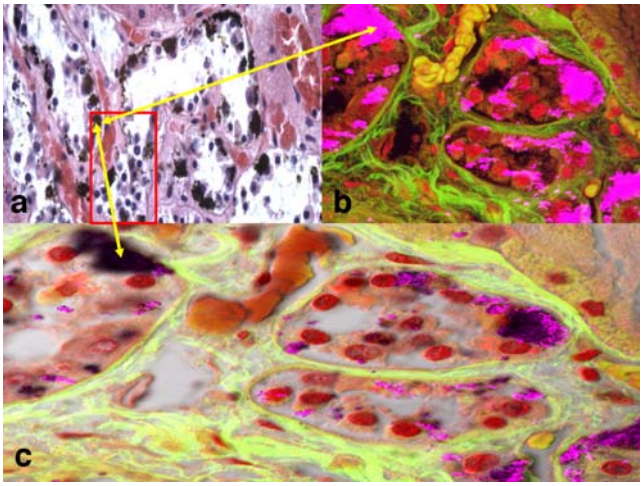

Fig. 5 **a** Kidney: acute tubular necrosis with calcium oxalate crystal (*arrows*) depositions (*black bodies*) H&E; 3D visualization of the same image, in *purple*, the calcium oxalate crystal depositions (**b**). Definitive evidence of calcium oxalate crystals within the renal tubules (in *purple*); interstitial connective (in *yellow*), erythrocytes (in *brown*) and epithelial nuclei (in *red*) (**c**)

– Intoxications and poisonings: demonstration of intracellular deposits of extraneous material (for example calcium oxalate crystals precipitated in renal tubules in ethylene glycol intoxication; Fig. 5) [29]
– Electrical injuries: electrical skin marks (Fig. 6)

Conclusions

The primary value of CLSM to the forensic scientist is its ability to produce optical sections through a 3D specimen, e.g. an entire cell or a piece of tissue that, to a good approximation, contains information from only one focal plane. By moving the focal plane of the instrument step by step through the

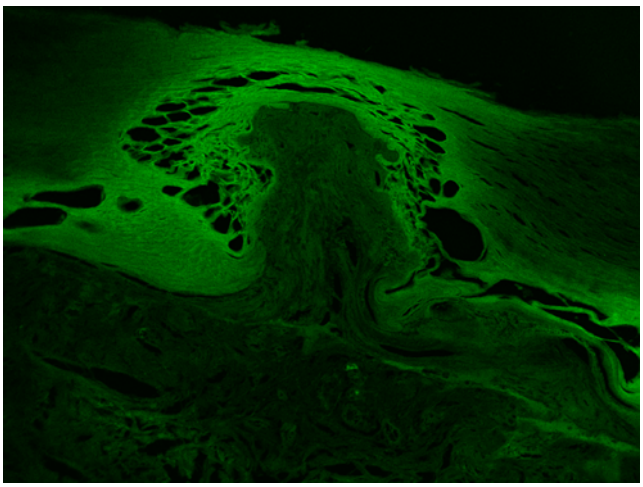

Fig. 6 Cutis: 3D visualization of the electrical mark

depth of the specimen, a series of optical sections can be recorded [7]. This property of the CLSM is fundamental for solving 3D biological problems where information from regions distant from the plane of focus can obscure the image (thick objects). As a valuable by-product, the computer-controlled CLSM produces digital images amenable to image analysis and processing that can also be used to compute surface or volume-rendered 3D reconstructions of the specimen. Thick and opaque specimens that can barely be observed under a conventional light microscope are easily visualized with CLSM. For example, 20–25 mm thick sections of bone, skin or muscle are ideally suited for 3D imaging in the CLSM. Independent of the thickness and surface quality of such tissue sections, individual confocal planes readily reveal a lateral resolution of 0.3 mm.

The ability to acquire, to keep, to work with images such as those obtained with confocal microscopy, to perform 3D reconstruction (and even more with some particular applications of confocal microscopy), to produce optical sections of the same sample, and the excellent definition of the image, all provide knowledge and ideas for further research which surely can allow for additional forensic innovations.

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