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Selected imaging techniques applied in forensic science

Forensic science is a broad, multidisciplinary science using various techniques and research methods, which have been recognized and developed for a long time in the natural sciences. For thousands of years, a human ammased the knowledge about animated objects based on what he could see with the naked eye. In the 16th and early 17th centuries the first microscopes became available. The Dutch, Hans and Zacharias Janssenn are considerd to be the invention of the microscope. They made the first designs around 1590. At that time microscopes didn't get recognizing as a research tool, due to the weak magnification (approximately 10x). The power of microscopy was realized by Antonie van Leeuwenhoek already in the 17th century. He has refined the design of the microscope, and then developed the production of these devices. A. Leeuwenhoek first watched live cells – sperm cells, protozoa, erythrocytes, etc. The development of imaging methods contributed to the discovery of X rays by Wilhelm Röntgen. These methods allow for non-destructive examination of the interior of living organisms.

The technological progress that we see in the modern world entails changes in all areas of life, improving them, accelerating their development and opening to new possibilities. In order to optimize results and deliver their justice in the fullest and safest form, the modern forensic science has adapted new research methods, technologies and equipment. The task of the experts working in the forensic labs is to provide the fullest information on the secured material. The examination of trace amounts of a wide variety of chemical substances that make up the trace on the site of the crime and which constitute evidence is especially relevant. Thanks to the use of both destructive and non-destructive methods of research. Microtrace as evidence of the material in the case, should not be destroyed or used up during the test. It places great demands on the experts and the techniques which they use. In the structural studies of microtraces and their physical and chemical properties a variety methods and imaging techniques are used. The main objective of these methods – whatever technical solutions and tools (from the simplest magnifying glass to advanced electron microscopes) is to obtain a magnified view of small objects.

Currently, in the world many types of microscopes and joined with them other multi-functional devices are used. In order to obtain information about the shape of the analysed samples and objects, their size, chemical composition, crystal structure, mechanical, electrical, or magnetic properties. The most commonly used techniques are optical microscopy and electron microscopy, spectrometric techniques, such as infrared and Raman, and x-rays analysis.

In this article some imaging techniques such as confocal microscopy[1], electron microscopy and atomic force microscopy and micro-Raman spectrometry will be presented, which are applied in many fields of forensic science. In table 1 a comparision of basic properties of the microscopes is presented: optical microscope, scanning electron microscope, (SEM) and atomic force microscope (AFM).

Tab. 1. Basic properties of the microscopes: optical microscope, scanning electron microscope, (SEM) and atomic force microscope (AFM).[2]

Feature of the microscope	Optical microscope	SEM	AFM
Resolution x, y axis	1000 nm	5 nm	0,1 nm
Resolution z axis	–	–	0,01 nm
Magnification	do 2000	do 10^6	do 10^8
Depth of field	the average	large	small
Environment research	air, liquid, vacuum	vacuum	air, liquid, vacuum
Type restrictions	diffraction of light (CLSM – confocal microscope – diffraction of laser)	diffraction of electrons	needle size

Regardless of the type of devices used to image, one of the important parameters is called a resolving power – the shortest distance between two points, which are considered as separate on the obtained image. Diffraction of light is the cause of limited resolving power of the **optical microscope**. This makes the images adjacent on the sample details become indistinguishable, when the distance between them was close to the wavelength of light. The idea of the construction of optical microscope became the basis for electron microscopy. The beam has been replaced by electron beam for getting the image parameters (magnification, resolution) that are not available for the classical light microscopy. The technique of electron microscopy, provides images with a resolution of the order of 0.1–0.05 nm, more than 4000 times better than a typical optical microscope. It is a resolving power of about 4 000 000x better than the ability of the human eye. Optical microscopy has many advantages, features and functionality that are not available for other types of microscopy. Recent conceptual and technical solutions used in confocal scanning microscopy increase the scope this method.

[1] Specific form of the light microscopy characterized by increasing contrast and optical resolution. Capturing multiple two-dimensional images at different depths in a sample enables the reconstruction of three-dimensional structures within an object, https://pl.wikipedia.org/wiki/Mikroskopia_konfokalna [access: 29 XII 2017] (note ed.).

[2] All the tables and artwork have been developed by the author of the text (note ed.).

Optical microscope is an essential device presented in biological, medical and forensic laboratories. Due to the simple construction and low price it is still the most commonly used microscope. For more than a century, the construction of a standard optical microscope has remained unchanged. Classic light microscope consist of two lenses: lens and eyepiece, whose combined magnification is the final magnification of the image.

In studies of quality materials stereoscopic microscopes are indispensable (fig. 1). These are optical microscopes which enable spatial vision of the observed object.

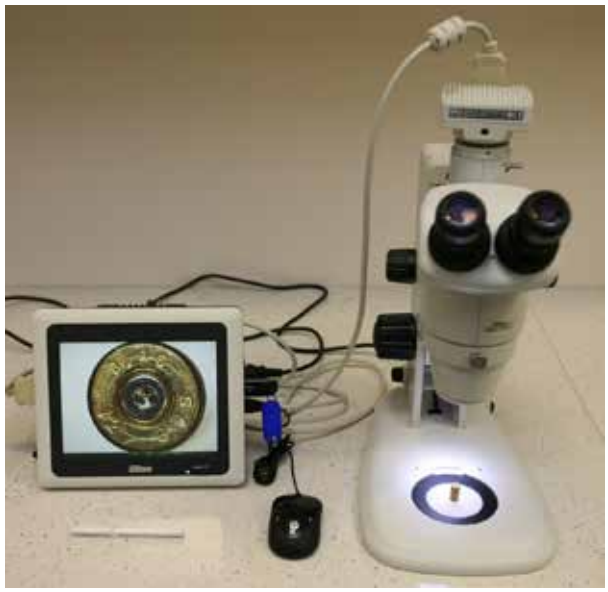
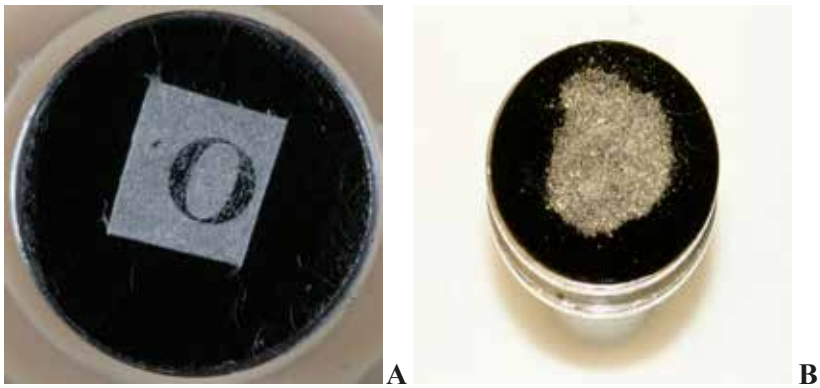


Fig. 1. The stereomicroscope integrated with microscopic digital camera and LCD screen.

Light running by the optical stereomicroscope, separately for each eye, produces the image perceived by the human mind as three-dimensional. This kind of microscopes are characterized by low magnification, a little more than 300 x. The observation is done by transmitted or reflected light. Stereomicroscopes are recommended for work related to the study of handwriting, archaeology, gemmology, botany, research quality electronic circuit boards, research quality samples for analysis of SEM/EDX, for observation of crystalline structures at the level of micrometer, for the preliminary examination of evidence (fig. 2) and any precision work.



A **B**

Fig. 2. SEM stubs with samples of printout on paper (A) and pyrotechnic mixture (B) seen in stereomicroscope.[3]

The light originated from the outside of the focal plane is one of the important limitations of classic optical microscopy. The lens of the microscope collects images not only from the focal plane, but also from the entire cross section of the sample. The image of the focal plane is sharp and clear. Images of objects lying in front of and behind the focal plane are not sharp (blurry) and much more, the further the lie from this plane. As a result, image of the visible object in the classic microscope is characterized by high background reducing the sharpness of outlines.

The most perfect type of light microscope in terms of optical is **confocal microscope**. Marvin Minsky said, that the perfect light microscope should cut the light not originated from the focus plane of the lens and scan point by point the tested material. Nowadays, the parameters of the Laser Scanning Confocal Microscope, LSCM, moved closer to the theoretical limits of magnification and image resolution. These parameters are valid for light microscopy from the 19th century (their author is a German physicist, Ernst Abbe). The construction of laser confocal microscopy (fig. 3) was based on fluorescent microscope[4], and a laser or a few lasers are a light source. Lasers generate beam with very high intensity.

[3] A. Łasińska, K. Barszcz, *Ilościowa i jakościowa analiza mieszanin pirotechnicznych SEM/EDS*, „Przegląd Bezpieczeństwa Wewnętrznego" 2014, No 11, p. 181.

[4] Fluorescence microscopy – the type of light microscopy, which is used to test of fluorescent sample (absorbing single wavelength of light and emitting different). Light of a single wavelength is used to excitattion fluorescent molecules and the light at a different wavelength, which is emitted by the particles is gathered and used to produce the image. In most cases, for example, parts of the cells or tissues that may be observed, do not exhibit fluorescence. Therefore, it must be marked before the observation of the fluorescent dye or marker.

Fig. 3. Inverted confocal microscope Nikon TiE Eclipse A1 with microselection and laser microdissection (A) optical microscope with the C1 confocal equipment Nikon Eclipse 80i (B).

The light beam is focused on the examined object. The confocal microscpopy assumes eliminating images, which are originated from outside of the focal plane. The light is focused on one point of preparation and at a predetermined depth. The fluorescence is excited at the point of focus, but also above and below the plane point. With the help a pinhol conjugated with the ortificial aperture of the path of the illumination in detector, only by size and position image of the focal plane point of sample is recorded. The depth of laser excitation of the fluorescence and the getting of the transmitted lighting is about 50 micrometers. In two-Fign confocal microscopes the range of excitation is extended to 400–500 micrometers. Automatic repeating scanning and other reallocations along the z axis enable the collection of a stack of images (called optical slices) of next layers of specimen. The resulting image layer is characterized by a weak background and sharper contours. This method allows to obtain a higher magnification tested materials and facilitates their interpretation. It creates a three-dimensional image of the object and its internal structure through a set of images. In confocal microscopes the most frequently used lasers are: argon, argon-krypton, helium-neon, semiconductor – diode, on the solid – state (femto-, nano-, pico- second - titanium-sapphire). Depending on the type of the laser the spectral lines[5] of the emitted light are different. For example, laser Ar, which is a type of ion gas laser emits light in the visible light and UV wavelengths (351, 454, 528 nm).

[5] Spectral line – dark or bright line in an otherwise uniform and continuous spectrum, resulting from emission or absorption of light in a narrow frequency range, compared with the nearby frequencies.

In modern confocal microscopes two different design solutions are applied. In both cases the cross section image object is obtained at a certain depth. However, these images are obtained in a different way. In laser scanning confocal microscope the laser beam goes into the object and excites emission of fluorochrome particles. Pinhola is placed in the optical path in order to obtain strong focus of the light beam in the focal plane. The light beam goes to the dichromatic mirror, after passing through the pinhola. Dichromatic mirror reflects the light of the excitation beam and transmits the light emitted by the fluorochrome (with longer wavelength). The excitation beam then passes through the lens, which ultimately focuses it in the form of a microscopic spot on the focal plane. The light emitted by the fluorochrome particles passes through the lens and dichromatic mirror (without reflection), and goes to the second aperture. The light from the focal plane passes through the aperture to the detector. The others are stopped at the aperture. Any change to the position of the dichromatic mirror allows to change the position of the focus beam on the focal plane. The position of the mirror is controlled by the computer system. The system records the position of the light spot and the intensity of the light, which are recorded by the detector. Information about the analyzed object is obtained by systematic scanning of the focal plane.

In **two-Fign confocal microscope** (TPCM) for the excitation of fluorochrome, near-infrared radiation with a quantum energy equal to half of the excitation energy is used. Under such conditions, excitation of the fluorochrome molecule occurs only when the molecule absorbs two quanta of radiation simultaneously. In practice, only in the place of a strong focus of the beam this phenomenon occurs. Therefore, in TPCM do not need pinhola before the detector, as emitted light comes only from the focal plain. The laser generates short pulses of radiation with high Fign density (tens of kilowatts at maximum impulse), but with low-energy pulse (nanodżul per pulse). In this case the principle of scanning object is analogous to the operation of the microscope with scanning laser. Two-Fign microscopes or multiFign microscopes have many advantages that derive from the application of the excitation beam of near-infrared range. These are:

- low energy carried by a beam that causes much less thermal and Figchemical damage to the object,
- better penetration of biological specimen than using shortwave visible light or UV radiation. This enables to work with the objects of greater thickness,
- greater sensitivity than LSCM, which allows to work with lower concentrations of fluorochromes.

The analysed data are collected in the form of luminous intensity of the point and by their nature are monochrome. After collecting the data set, the image is processed on a typical format for digital Figs. In this form it can be easily coloured. If it is stained with more than one fluorochrome, they must differ in the wavelength of the excitation light. Independent data about the object are collected for each wavelength. Typically emissions of each fluorochrome assigned a contrasting color. At the stage

of creating digital pictures data are compiled in one image. Currently, there are available confocal microscopes, which allow the excitation of light with three or more wave ranges. Confocal microscopy is becoming an increasingly available research technique that provides large amounts of detailed information. This is done with the development of the deals available fluorochrome and increase in their specificity. It is possible to obtain a single scan at the time of less than 0.05 s. This allows for recording rapidly changing processes in the living cell or tissue and observation in the form of a video.

The advantage of confocal microscopy, not present in other technologies, is a possibility to obtain a series of cross-sections of the tested specimen from different depths. The series of cross-sections can be used to reproduce the spatial structure of the object.

The technique of confocal microscopy is widely applied in the biological sciences, medicine, genetic engineering[6], as well as in forensic science. In forensic medicine this technique allow to detect and visualize pathological changes in the case of sudden death, to distinguish between input and output of the wound after the bullet, specify the distance of the shot, as well as skin lesions and injuries incurred after the explosion. Confocal microscopy gives the possibility to direct three-dimensional microscopic imaging of individual cells or pieces of tissue (fig. 4).

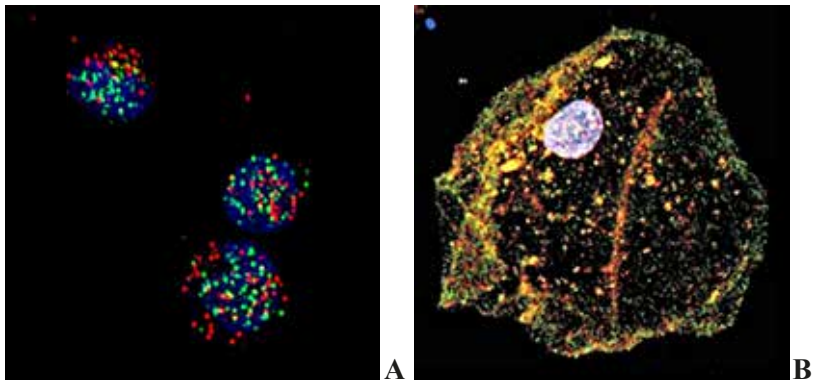


Fig. 4. 3D image of human lymphocyte cells (A) and (B) HeLa made using inverted laser confocal microscope Nikon Eclipse TiE A1.

Already in the 19th century it was noted that some of the fluorochrome have the ability to impact on some components of the cells: proteins, lipids, nucleic acids, etc. Over time, standard staining procedures were developed to allow visualization of significant elements for the cell. Fig. 4 shows the microFiggraphy of human cells stained with a mixture of three dyes. Due to the combination of dyes, both cell membranes and cell nuclei can be seen

[6] A. Raczkowska and others, *Pleiotropic effects of a Yersinia enterocolitica ompR mutation on adherent-invasive abilities and biofilm formation*, „FEMS Microbiology Letters" 2011, No 321, pp. 43–49.

simultaneously. Also some fluorescent dyes have the ability to selectively bind to the cell components. The use of fluorescent dyes has a lot of advantages and allows:

- to observe light emission on the dark background, which increases the sensitivity of the method and enhances the contrast observed details. This increase in sensitivity is so important, that in all the techniques of selective staining of specific macro--molecules fluorescent dyes are used in principle, and not the classic absorption dyes,
- to differentiate between the cells or their fragments due to the existing conditions in them. For this purpose the strength and colour of fluorescence are used. The latter depends on conditions in the environment, ie: pH, redox potential, lipophilicity, membrane potential. Fig. 4 shows the cell nuclei stained the dye DAPI. This compound fluoresces blue after intercalation[7] to DNA,
- to detect differences in the concentration of some ions, e.g.. Ca^{2+}, what determines the strength and colour fluorescence.

Currently, advanced laser microdissection systems are equipped with laser modules emitting UV or IR, integrated with high-quality fluorescence or confocal microscopes and a motorised table. The unit completed a computer system to control the process of cutting the cells by using software, camera and monitor. Developed originally for use in the field of oncology, microdissection systems are also applied in areas of the biological and forensic sciences. Confocal microscopy conjuncated with laser microdissection have created new opportunities for genetic profiling of biological traces, traditionally secured to swab and forensic tape.[8] As a result of the phenomenon of cold ablation, by the action of the laser the individual cells or fragments of tissues are separated from the rest of the mixture along with the part of the membrane. Laser mikrodisection module contains the picosecond pulse laser, on 4 mW solid state, emitting radiation in the UV spectrum (at the wavelength of 355 nm). Imaging and qualification of fragments of undergoing microdissection is done by transmitted light, using high-speed films internal digital camera. The microdissection is carried out with variable parameters and a focal length of the laser beam.

Now another method of analysis and isolation of cells is microselection. The system is controlled by computer using dedicated software. Previously the isolation process requires the adequate calibration of the system, which allows to obtain high-precision acquisition and deposition of selected cells. Isolating single cells takes place in three stages: identification, acquisition and embedding. Imaging and qualification of fragments of the specimen is done by transmitted light, using high-speed films internal digital camera, as in laser microdissection. The technique of laser confocal

[7] In chemistry, intercalation is the reversible inclusion or insertion of a molecule (or ion) into materials with layered structures. Examples are found in graphite and transition metal dichalcogenides, https://en.wikipedia.org/wiki/Intercalation_(chemistry) [access: 29 XII 2017] (note ed.).

[8] A. Łasińska and others, *Ocena przydatności folii adhezyjnych do zabezpieczania śladów biologicznych oraz izolacji komórek ludzkich metodą mikrodysekcji laserowej*, „Problemy Kryminalistyki" 2014, No 283, pp. 18–28.

microscopy is also used to identify documents printed on paper[9], and to determine the uniformity of the implementation of the document by the disclosure, whether the text was added to an already existing (fig. 5).

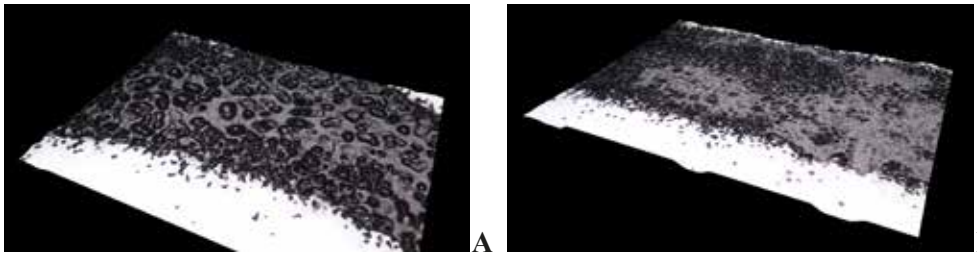


Fig. 5. 3D image of the surface of toner visible on the printout obtained from the laser printer: the document twice passed by the device (A), the document once passed through the device (B). Figgraphs in 200x of magnification, using a Nikon 80i microscope.

In order to determine the characteristics of the toner on printouts optical microscope Nikon Eclipse 80i with confocal equipment C1 was used (fig. 5B). Fragments of printouts were observed in transmitted light using a Nikon DS5NC color CCD camera with cooling, with 2560 x 1980 resolution and LU Plan Fluor 200x N.A. 0.90, WD 1.0. lens. The construction of the microscope allows to perform examination of documents of A4 format, in a non-destructive method. Microscopic observation shows that the surface of toner on the paper is strongly enhanced (fig. 6). For each printout creates a distinctive individual layout structures. Visible are two types of specific areas: more flat solid surface and granular surface, creating smaller or larger aggregations of varying density.

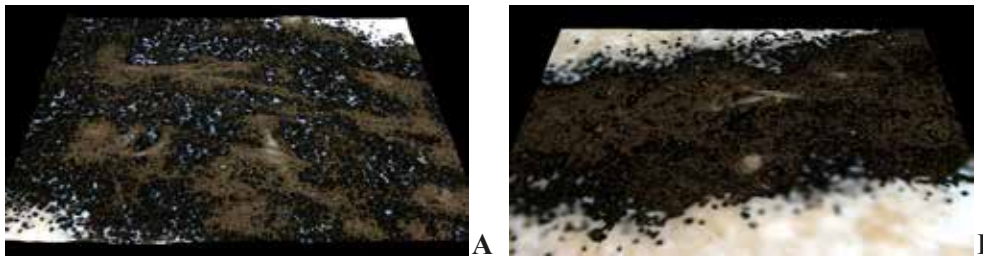


Fig. 6. 3D image of toner on printouts obtained from colour laser printer C734/C736w (A) and monochrome laser printer T650n/T652/T654n (B). Figgraphs in 200x of magnification, using a Nikon 80i microscope.

[9] S. Szczepańczyk, U. Konarowska, *Zastosowanie mikroskopii optycznej w weryfikacji dokumentów przerobionych za pomocą drukarki laserowej*, „Problemy Kryminalistyki" 2012, No 276, pp. 65–73; A. Łasińska, *Badania mikrośladów*, in: *Technika kryminalistyczna w pierwszej połowie XXI wieku. Wybrane problemy*, B. Hołyst (note ed.), Warszawa 2014, pp. 804–840; A. Łasińska, U. Konarowska, *Zastosowanie mikroskopii optycznej i elektronowej w badaniach autentyczności dokumentów*, in: *Dokumenty a prawo prawne oraz praktyczne aspekty korzystania z dokumentów i e-dokumentów*, M. Tomaszewska-Michalak, T. Tomaszewski (ed.), Warszawa 2015, pp. 105–112.

A good method of imaging is also Raman Spectroscopy. This is a spectroscopic techniques used to observ the oscillation spectra of materials, similarly to infrared absorption spectroscopy. It can be used for the anaysis of gases, liquids, and solids. As a source of excitation, in most Raman spectrometers the lasers are used. This technique is complementary to infrared spectroscopy. In practice, most of the spectrum is recorded with the Stockes maximum[10], which have a higher intensity than anti-Stockes[11], because the number of molecules in the first excited state is less than in the basic state. As the temperature increases, the number of molecules in the first excited state increases, thus the intensity of anti-Stokes lines increases. The total Raman spectrum consists of: maximum Rayleigh[12] scattering (high intensity, wavelength the same as the excitation wavelength), Stokes maxima (lower frequencies, higher wavelengths) and anti-Stokes maxima (higher frequencies, lower wavelengths). Raman shifts in the Raman spectra are identical in magnitude with the position of the absorption peaks in the infrared spectra, however, their relative intensities differ. Some maxima can be seen in one spectrum, while the others cannot. Raman scattering is related to the distortion of the electron density distribution around the bond, followed by radiation rebinding combining bonds back to the original shape. Homonuclear molecules, inactive in infrared, give Raman lines, because the polarizability of bonds (i.e. the binding force of electrons in a molecule) changes periodically and in phase with the stretching stretches. In the spectrum, the Raman band appears when polarizability changes during normal vibration.

During the vibrations, also the polarizability may change periodically. Vibration causes periodic changes in atomic structures. This diversity of selection rules of the infrared and Raman spectra causes that some infrared inertial vibrations may be active in the Raman spectrum and vice versa. For example, the vibration of a two-atom homonuclear molecule (including O_2, N_2) is inactive in infrared, however, it appears in the Raman spectrum because the polarization changes in this oscillation. In the case of NaCl, KCl molecules, we do not observe Raman spectra even in the gas phase, where they are not dissociated. This is because they are ionic molecules, their valence electrons belong to the appropriate atomic frameworks and during the vibrations they move with their atomic framework. Movement of atomic frameworks has no effect on polarizability. In fact, the bonds in molecules are not ideally ionic and therefore it is possible to obtain

[10] Stokes band – they arise when the molecule is transferred to higher oscillatory level (after interacting with radiation) and the *scattered* photon has energy lower by the energy difference of oscillation levels *hv*.

[11] Anti-Stokes band – if the molecule was on an excited oscillatory level, before interacting with radiation, then the interaction transfers it to the basic (zero) oscillatory level. The scattered photon has energy higher by the energy difference of oscillation levels hν. This band usually has a lower intensity than the Stokes band.

[12] Rayleigh band – they arise due to the interaction of photons of incident radiation with the frequency $\nu 0$, which do not correspond to the energy levels of the molecule. When the molecule returns to the same energy level, after interaction with radiation, this phenomenon is called Rayleigh's classic scattering.

the Raman band of such molecules. These bands have such a low intensity that they are practically invisible. Another example is the molecule of HCl which gives the Raman spectrum not only in a gas state, but also in a concentrated aqueous solution. The solution is so concentrated that the dissociation of acid is reversed. This shows a high proportion of covalent bond in H–Cl binding. In the case of strongly polarized ionic bonds, the electric dipole moment and its change over the time are quite large. High intensity of the bands corresponding to strongly polarized bonds in the infrared spectrum was observed, unlike in the Raman spectrum. As a result, the term appeared in the literature, called criterion spectroscopic polarity binding. It says that if the intensity of the infrared band increases, and in the Raman spectrum decreases, it means that the corresponding binding of the molecule becomes more polarized. And vice versa – if the intensity of the Raman band increases, and the intensity of the infrared band decreases, this means an increase in the covalence of the corresponding bond.

The advantage of Raman spectroscopy is the ease with which samples can be prepared. This technique does not require the use of chemically unstable KBr cuvettes. Measurements of aqueous sample solutions can be made. It is also possible to perform measurements at higher temperatures. Raman spectroscopy is particularly useful for measuring the frequency of oscillating metal-ligand complexes, which have vibration frequency is in the range of 100–700 cm^{-1}. Measurement of the infrared spectra are very difficult to carry out in this range. Another advantage of Raman spectroscopy is that the sources of excitation emit radiation in the visible range. This allows for precise tuning of the optical system of the Raman microscopes. Raman spectra can be used to identify unknown substances, detection in a molecule specific groups of atoms or types of chemical bonds, for determining the geometric structure of molecules and vibration analysis. A valuable modification of spectroscopy is **micro-Raman spectrometry** which also to apply in forensic studies. Procedures for determining the intensity of the bands is an important element of correct Raman image creation. Micro-Raman spectrometry is a combination of Raman spectrometry and optical microscopy.[13] The microscopic image (at appropriate magnification) of the tested surface preparation is visible on the screen (fig. 7).

[13] M. Bowden and others, *Thermal degradation of poly-urethane-backed poly (vinyl chloride) studied by Raman microline focus spektrometry*, „Polymer" 1994, No 35, pp. 1654–1657.

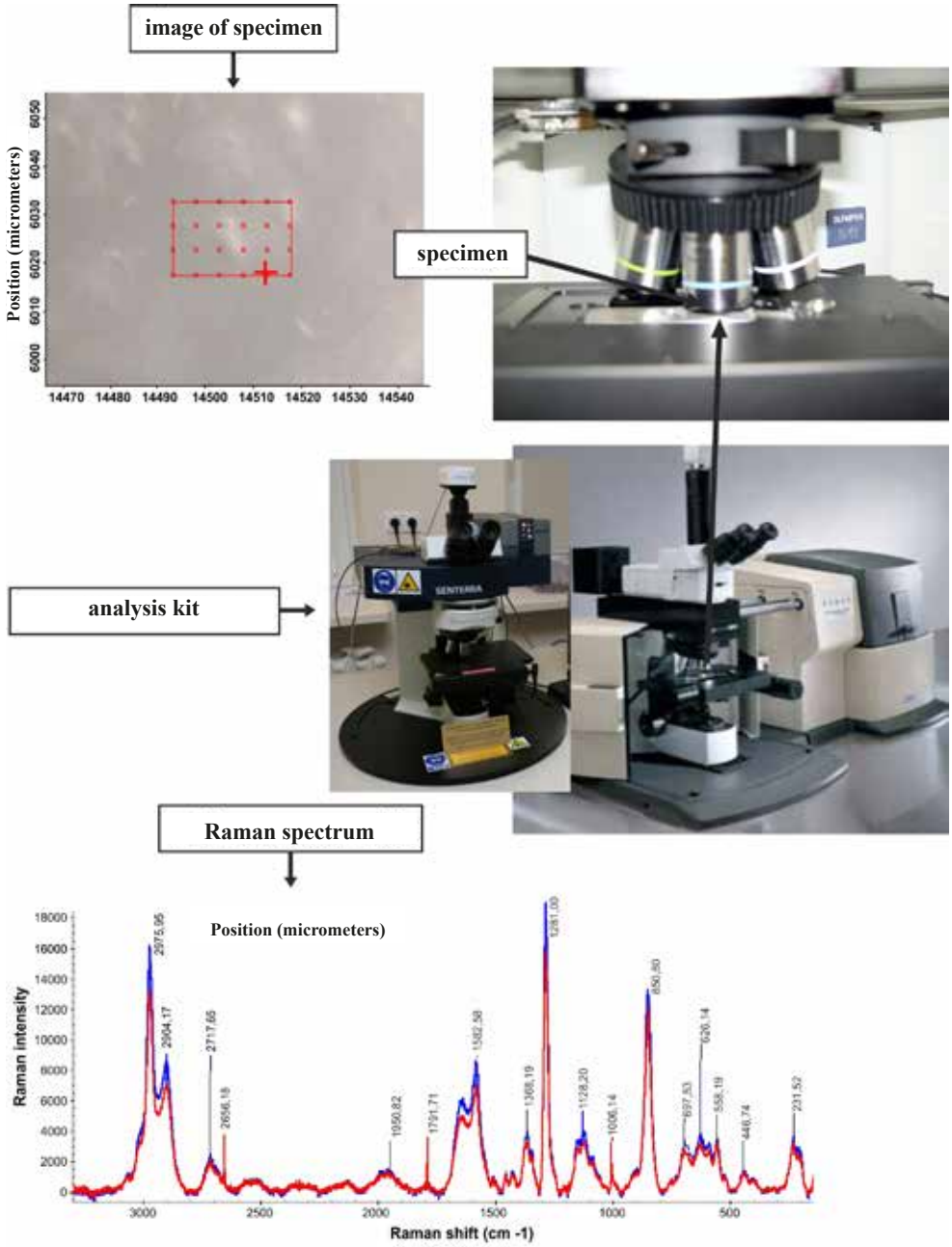


Fig. 7. Surface analysis scheme of unburned gunpowder grains by using micro-Raman spectrometry.

Motorized microscope table allows to choose the right position for Raman spectrometry analysis. Micro-Raman spectroscopy is considered as a non-destructive method. It allows to obtain information about the chemical composition of the sample

in a microscale. Research of toner cartridges carried out by Raman spectroscopy showed the presence of carbon molecular structures based on bands originated from amorphous carbon or (and) graphite, and amorphous silica. Depending on the excitation lines, the band was observed in 1574 cm^{-1} i 1332cm^{-1} (fig. 8) and 1375 cm^{-1}, 1253 $cm^{-1,}$ and 2693 cm^{-1}.

It is possible to collect spectra from several points of the upper or lower surface of the sample and to make maps based on the size, chosen for spectral comparison, by coupling the Raman spectrometer with a microscope equipped with a scanning movement. The appropriate image is presented in the form of maps with colored squares. Thus, the distribution of the discrete function is obtained. To the distribution of the continuous function approximated by the program using the appropriate polynomial.

Fig. 8. Raman spectrum of the toner sample in the form of powder taken directly from the cartridge of the Lexmark C780 device – replacement, made by excited by a 532nm laser.

Raman maps of several gunpowder have been made to examine the homogeneity of the particles of unburned or partially burnt gunpowder. Only some grains showed the presence of sharp characteristic bands. The bands corresponding to the groups of NO_2 and CH_2, nitroglycerine and nitrocellulose are the most evident in the Raman spectrum of gunshot residue. Fig. 9. show map of the intensity change of the stretching vibrations band of the NO_2 group and the band of symmetric stretching vibrations of the CH_2 groups. In the case of micro-particles originated from the residue of ammunition Fiocchi G.F.L 9×19 mm containing nitroglycerin and nitrocellulose, the intensity of the bands NO_2 group changes from 0.10 to 5.50. This change suggests that the mixture is not homogeneous. There are areas which are almost devoid of chemical compounds containing NO_2 groups.

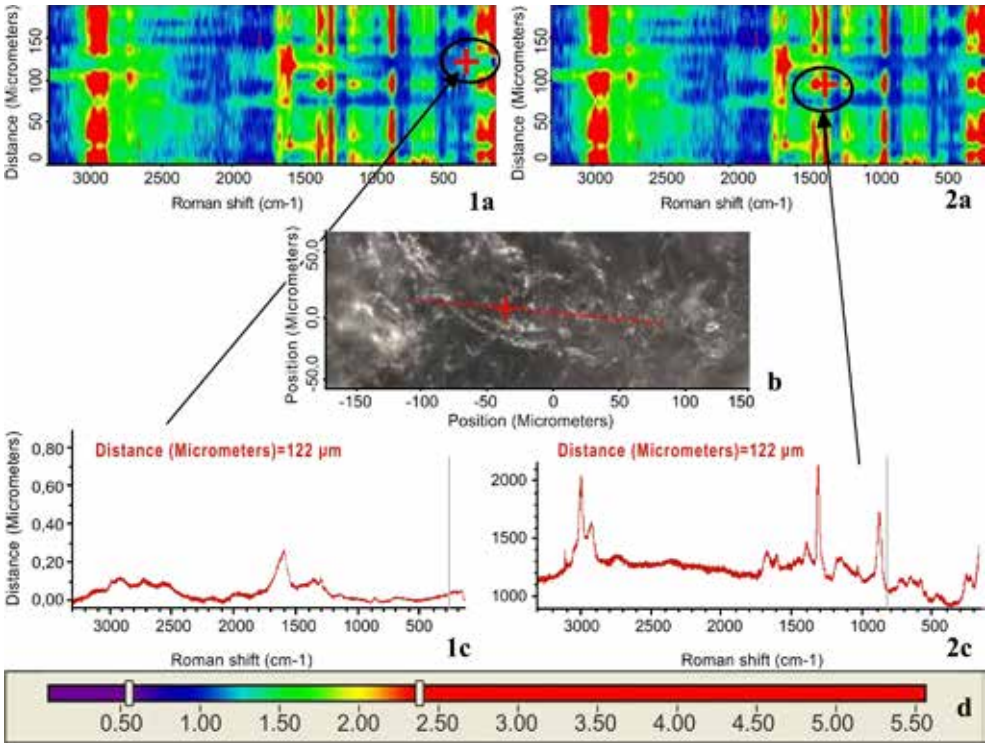


Fig. 9. Intensity change maps of stretching vibrations band of NO_2 group in unburned gunpowder originated from Fiocchi G.F.L 9 × 19 mm ammunition (1 a, 2 a); image of the analyzed area of the sample obtained from confocal microscope (b); Raman spectra in points marked with a red cross on maps, respectively (1 c, 2 c); the scale of the color assignment of the change values of the NO_2 group band intensity (d).[14]

Fig. 9 shows spectra and the nature of the micro-particles in terms of chemical composition, in one point on the map. Depending on the color distribution on the map, changes of the band intensity were observed. At the point marked on the map with a red cross in the light green area (fig. 9.1 a), the spectrum mainly shows the band corresponding to the C–C stretching vibrations (fig. 9.1 c). However, at the point marked on the map with a red cross in the red area (fig. 9.2 a) all bands associated with the presence of nitroglycerine and nitrocellulose are visible in the spectrum (fig. 9.2 c).[15]

The analytical method showed the possibility of identification of some organic compounds contained in the gunshot residue, and the possibility of imaging (mapping) changes in the band intensity of some functional groups of organic substances

[14] A. Łasińska, *Analiza nieorganicznych i organicznych pozostałości po wystrzale z broni palnej*, „Przegląd Bezpieczeństwa Wewnętrznego" 2017, No 16, pp. 157–189.

[15] Ibidem.

presented in a single grain. The morphology and inorganic components study of the typical particles of gunshot residue provide full identification evidence.

In Raman spectroscopy, many new techniques are currently used, which primarily increase the sensitivity of this method. Resonance Raman Spectroscopy, RRS is a technique in which the frequency of a monochromatic laser beam is tuned to the frequency of electron transitions of the analyzed compound. This leads to a selective increase of the intensity of Raman bands 10^2–10^6 times, in relation to classic Raman dispersion spectroscopy.

A Surface Enhanced Raman Spectroscopy, SERS, is also applied in examinations where Raman scattering of molecules adsorbed on the surface of a metal or metallic sol (mainly silver) is measured. The phenomenon of amplification is explained by two different mechanisms: chemical and electromagnetic. In the SERS method, the intensity of the Raman signal increases 10^3–10^7 times in relation to the signal obtained in the classic Raman experiment.[16] Spectra can be obtained from a very small, precisely selected sample area (about 1 μm^2), using confocal Raman microscopy. The possibilities offered by the confocal microscope, obtain optical cross-sections of the examined materials, three-dimensional images and visualization of cells and fragments of tissues, varnish coatings from various cars, gunshot residues, detection of trace amounts of substances or contaminants, documents that are difficult to forge (hard-to-forge) security documents.

Using some types of the modern light microscopy, a very high resolution images were obtained. In the past, the primary limitation of the quality of the obtained microscopic images, were a defect lenses (chromatic aberration, spherical aberration). This problem was overcome, at least, in the most expensive models, in the second half of the 19th century. However, another barier was encountered – diffraction and interference phenomenon, that cause the appropriate image sharpness can only be obtained for objects with sizes many times larger than the length of the wave used. Objects smaller than 1/2 of the wavelength are not visible at all. Objects, in a distorted form, with a wavelength order of magnitude appear in the microscope image: surrounded by colored rims. Therefore, even the best optical microscopes that work in the visible spectrum, give maximum magnification of the order of 1500x. The maximum magnification up to approx. 3500x is achieved by microscopes working in the ultraviolet range. The development of electron microscopy was determined in order to obtain large magnifications of objects, impossible to obtain in optical microscopes. If we see something very small with very high resolution, and to know the elemental composition of the tested substance, should be used the technique of **electron microscopy coupled with an X-ray microanalyzer** (fig. 10).

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[16] B. Sharma and others, *SERS: Materials,applications, and the future*, "Materials today" 2012, No 15, pp.16–25.

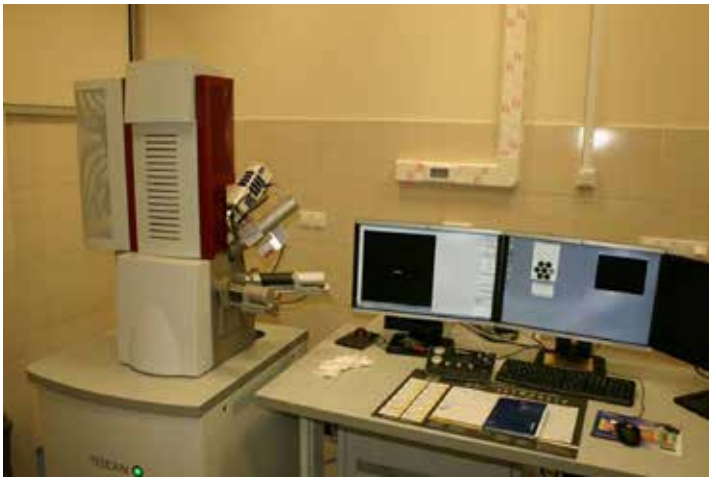
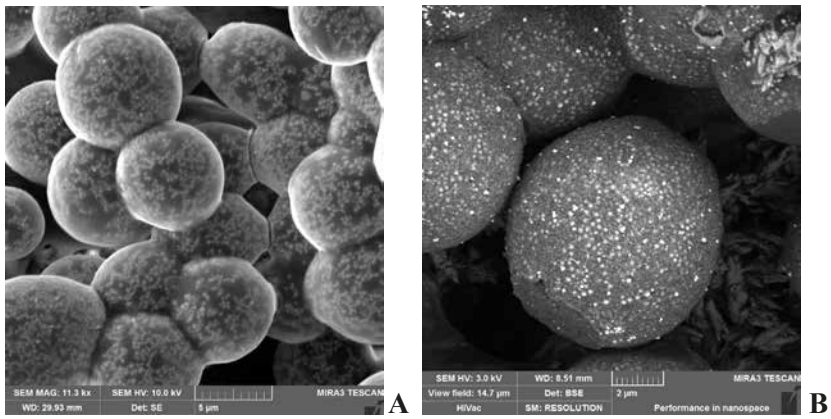


Fig.10. Scanning electron microscope with an X-ray microanalysis detector.

For 70 years the **electron microscope** has become a commonly used device in science, industry, and forensic science. Electron microscopes differ from light microscopes. They form the sample image using electron beam but not the light. Electrons have a much shorter wavelength than visible light. Electron microscopes allow to obtain images with a higher resolution than the standard images obtained in light microscopes. The electron microscope offers a wide range of research possibilities for microscopic samples of chemical substances and complex materials in the form of solids state, after the development of appropriate analytical methodology. The microscope enables simultaneous studies of their chemical composition and morphology. An additional advantage is that, in many cases, it does not require destruction of the sample, neither in the process of preparing it for analysis, nor during the measurement.

In the scanning electron microscope (SEM) the image is obtained as a result of detection of secondary and (or) reflected electrons with which the sample is bombarded. Imaging techniques such as: BSE (back-scattered electrons), SE (secondary electrons), CL (cathodoluminescence), LVSTD (in a variable vacuum) and X-ray microanalysis allow to analyze samples – except gas – both conductive and non-conductive. For the imaging of samples, secondary and backscatter electrons are commonly used. Secondary electrons are the most valuable for showing the morphology and topography of the samples. On the other hand, backscattered electrons are used to illustrate the contrast of multiphase samples. Secondary electrons are emitted from the area which is very close to the specimen surface. Therefore, the images from this detector clearly show the topography (shape) of the sample surface. They are characterized by a large depth of field. Back-scattered electrons – or reflected electrons emerge from deeper locations within the specimen and consequently the resolution of BSE images is less than SE images. BSE images are characterized by a smaller depth of field and they do not show the topography of the sample well,

but they provide information about the density of the observed surfaces. They show qualitative phase (chemical) differentiation of the sample surface. Higher or much lower number of electrons in the primary beam are backscattered, depending on the atomic number Z of the element. The number of protons in the nucleus describes the atomic number Z of the element. This means that the signal coming from heavy elements is much stronger than from the light elements. This allows the detection of metallic inclusions or heavy elements in the observed samples. The contrast characteristic of BSE imaging is proportional to the square of the mass number and instead of the topographic contrast (for SE imaging), the material contrast can be observed (fig. 11).



A **B**

Fig. 11. SE (A) and BSE (B) image using a scanning electron microscope.

Another very useful technique is EDS spectroscopy. The EDS spectrometer's functionality is based on detection of the radiation. Characteristic radiation is emitted by atoms of the samples excited by the electron beam. During the bombardment of a sample with the primary electron beam, electrons are ejected from elements present in the sample. After ejection of the electrons the empty spaces are replaced by electrons from higher orbitals. In order to compensate for energy differences between electrons during this transition, X-rays characteristic for the given element are emitted. X-rays are analysed by EDS detector, as a result the elemental composition of the tested sample is determined. In addition, a semi-quantitative test was performed and the various samples were examined not only in terms of diversity, which are visible in BSE image, but also to determine the qualitative and quantitative changes in their composition. Study of the surface of the samples also allows to perform linear analysis and map content. This research are carried out, in order to capture the subtle changes in the elemental composition, which are not visible in the BSE image. The elemental composition can be easily interpreted and converted into percentages of cells. Considering composition, in terms of atomic percentage, conclusions can be readed about the stoichiometry of compounds in the sample (fig. 12 and tab. 2).

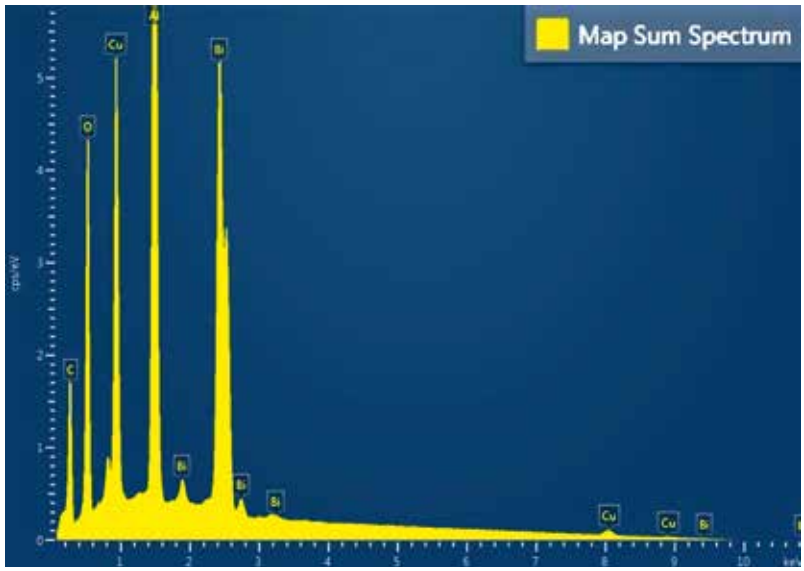


Fig. 12. EDS spectrum obtained during the SEM observation. The characteristic line K, L, M of the characteristic x-rays radiation by using symboles of the elements of the aluminium, coper, oxide and bizmud were marked.

Tab. 2. Quantitative results of x-ray microanalysis. The table of the results was generated using the Aztek program included in the SEM Mira 3XMU microscope software.[17]

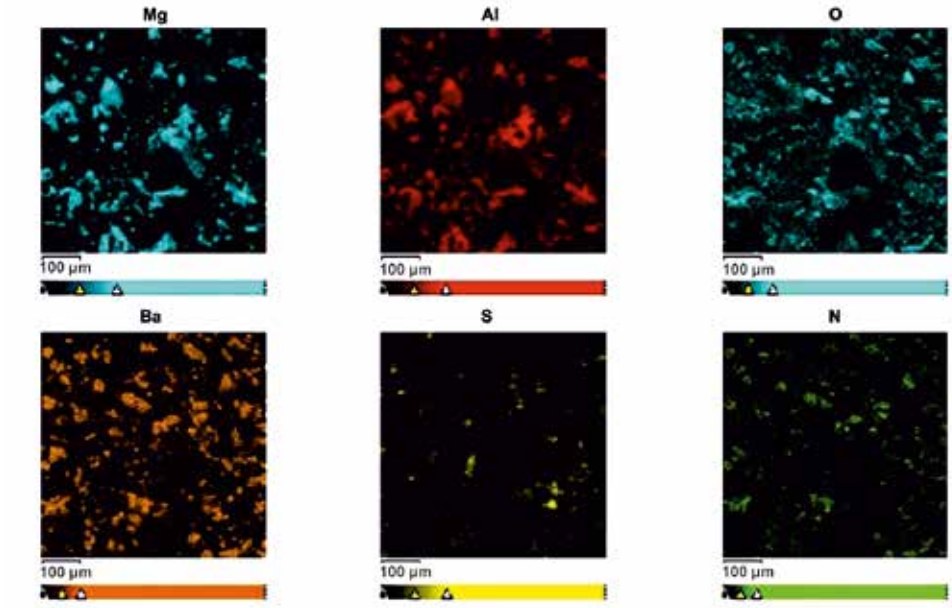
Element	Line type	k Ratio	Wt%	Wt% Sigma	Compound
O	K	0,00576	10,38	0,05	SiO_2
Al	K	0,01818	19,11	0,06	Al_2O_3
Cu	L	0,01357	18,53	0,08	Cu
Bi	M	0,04348	51,97	0,12	Bi

The results of the quantitative analysis contain the following information:
• Wt % – weight percentage of the element in the sample
• Wt% Sigma – weight percentage error
• k-Ratio – the ratio of the radiation intensity of the characteristic line of the element in the sample to the intensity of the pure element under the same conditions.

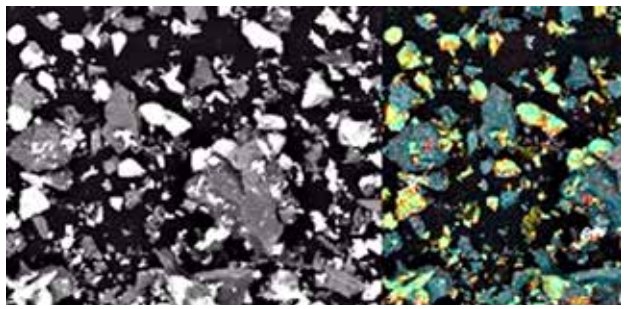
The characteristic X-rays radiation is used to obtain maps of distribution of the elements' concentrations (called mapping). Analytical beam scans the tested area point by point. The spectrometer is set to automatically record a point in tested

[17] A. Łasińska, *Badania mikrośladów*…, pp. 804–840.

area when it detects X-ray pulse with the characteristic energy for the corresponding element. In this way, a map of the elemental distribution was created. Colorful maps in appropriate shades are created by modern EDS systems, which show the most probable value of the pulse intensity at each point. However, it may require application with a sufficiently long time of the beam stop for each analytical point. During the one run of the analytical beam, distribution maps for several elements were recorded (fig. 13). The map is a bag of spots corresponding to pulses of X-rays. The degree of the spots concentration corresponds to the concentration of the element. Collection time of pulse have a significant implications for the assessment of distribution of the element concentration. However, the maps do not allow to capture small differences in concentrations and low concentrations (due to the presence of background spots – continuous radiation).



A



B

Fig. 13. Examples of elements concentration distribution maps (A); BSE image and the total image overlay maps of concentrations of elements (B). The maps were generated using the Aztek software included in the SEM Mira 3XMU microscope, based on the measured spectrum of EDS.

The chemical compounds and the percentage of these compounds in tested material are initially determined on the basis of the maps, using appropriate software. A sample map of the phases distribution and their percentage are shown in the fig. 14 and tab. 3.

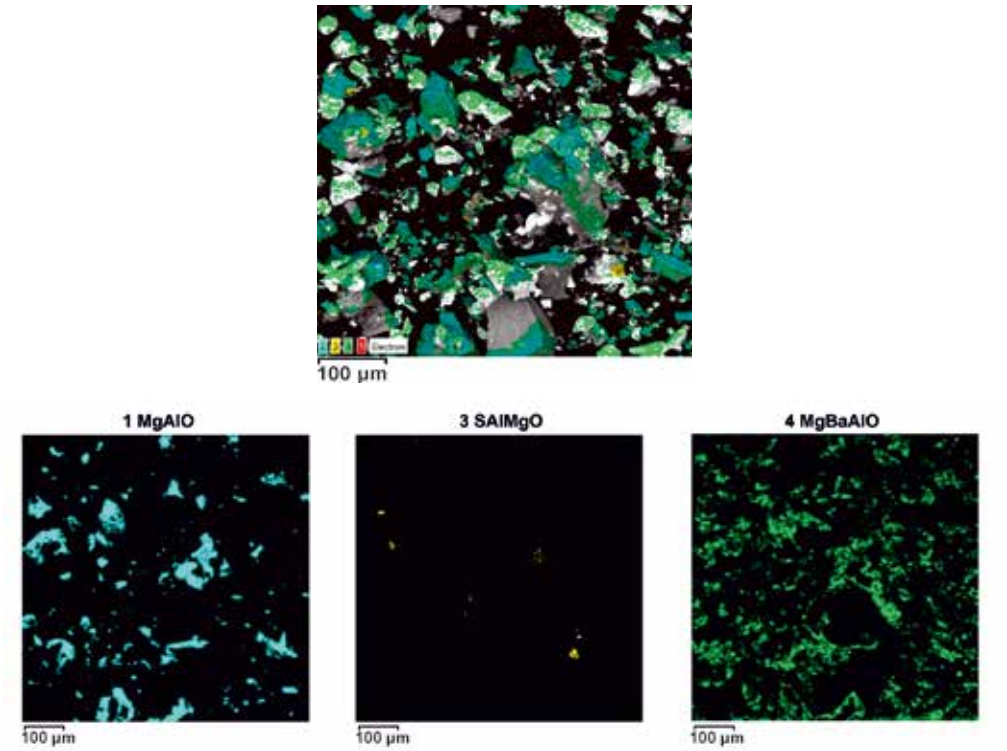


Fig. 14. Examples of maps of the distribution phase. The maps were generated using the Aztek software included in the SEM Mira 3XMU microscope.

Tab. 3. The values of the founded fractions percentage.

Compound	Fraction (%)	Number of pixels
1 MgAlO	9,1	23 849
3 SAlMgO	0,2	553
4 MgBaAlO	11,9	31 172

In the X-ray microanalysis the technique of linear analysis can also be applied. It involves scanning with an electron beam, along a defined line on the surface of the sample with simultaneous recording of changes in the intensity of the emitted X-ray radiation. A linear analysis of chemical composition shows the changes

in the concentration of defined element, along the line, selected on the surface of the tested sample. In the diagrams in fig. 15 an example of a line analysis is presented.

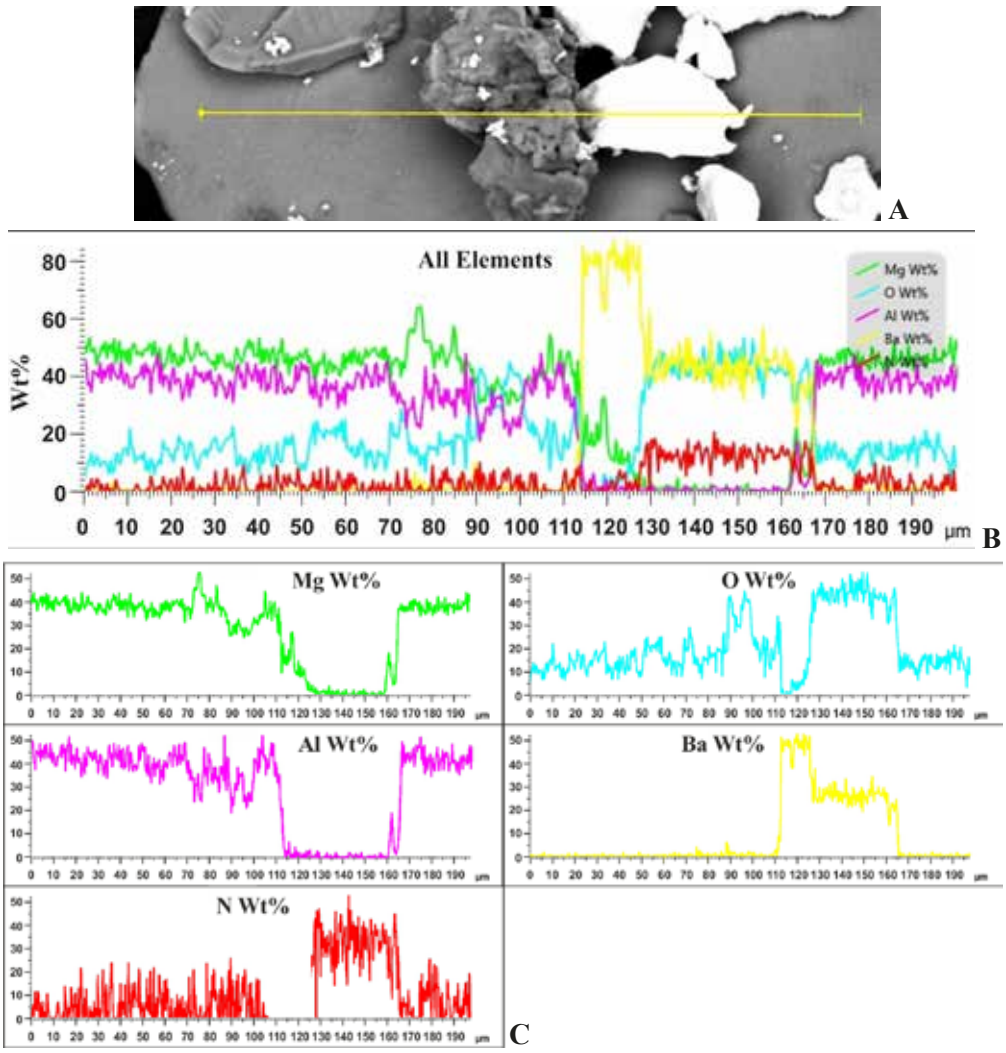


Fig. 15. BSE /SEM microFiggraphy of the grain surface with the scan line marked (A); curves: summary result of the EDX line anaysis along the line (B); EDX line analysis of the elemental distribution (Mg, O, N, Al, Ba) in single grain (C) The line analysis was generated using the Aztek software included in the SEM Mira 3XMU microscope.

One of the most attractive features in electron microscopy is its wide range of applications in various fields of forensic science. It is used for identification tests and comparative evidence, such as: glass particles and varnishes, metals, toners for printing devices, fiber morphology, pyrotechnic mixtures and their residues, pyrotechnic devices

and gunshot residues (GSR). SEM is used to analyze micro-traces of solid substances found on clothes at the micrometer level (plant pollen, soil grains), paint splashes from the crime scene, and even to determine if the car had headlights turned on at the time of the accident. Electron microscopy allows us to analyze poorly visible or invisible microscopic traces by using imaging, image comparison and X-ray microanalysis.

From the point of view of the forensic science one of the essential problems faced by analysis, among others, appears to be the identification of the implementation device. Within the framework of the present research project extended research has been conducted aimed at obtaining the individual identification of printing and copying devices. In this study, 134 printouts from laser printers from 10 manufactures were analyzed. For this purpose, toners and their replacements were used. Microscopic images obtained allowed us to allocate characteristic features of toners to specific groups of devices: monochrome laser printers, color laser printers, monochrome multifunction devices and color multifunction devices. Clear criteria were established for individual assessment, which allowed to assign and select distinctive features of toners to the printouts obtained from the respective printer devices. Based on the SEM images and established criteria the details of topography of the toner structure were revealed. Based on this study, the differences or similarities of toners on tested printouts were determined. In case of printouts obtained from different laser printing devices the conducted analysis of the toner surfaces showed that not in every case toner layer on the printouts has solid or granular form. If both appear, the difference between the two consists in ratio of one layer to the other and the form of their distribution within the area of one sign, depending on the kind of device.

In some cases, certain features such as: straight rod structures and irregular structures (*T650n/T652/T654n* Lexmark), (fig. 16), bent rod structure (X264/X363/X364 Lexmark), a single spherical structure resembling balls scattered about on the surface of a toner (X940e/X945e Lexmark), clearly the same size (MX410 Lexmark), spheres and cubes on the grain surface (Canon iRAdv C9070) or only cubes on the grain surface (OKI MB461/MB471/MB491) are individual for a specific print job. Some features may appear only in a few cases, such as: rod structures characteristic for two models of laser devices (Dell 5310n and Dell 5350dn), for one color laser multifunctional device OKI C5550MFP and for a few models of Lexmark monochrome laser devices.

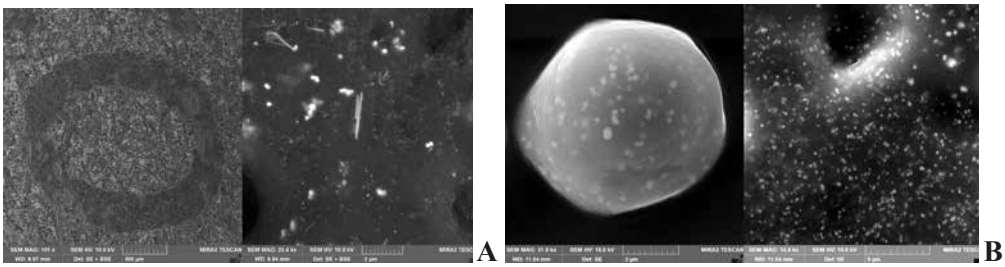


Fig. 16. SEM image of the solid surface (A), grain and grain surface (B) of toner on the printout obtained from Lexmark T650n/T652/T654n.

On the other hand, alternative features may be unique for a given type of printer device. For example, irregular or spherical structures under the grain surface and solid surface of the toner, were distinguished by different contrast in SEM image. This type of structure occurred in monochrome laser printers and monochrome laser multifunctional devices. Toners, creating text on the printouts are characterized by a number of features that allow you to distinguish among them but only within certain limits. Unfortunately, many of these features are repeated in different models, which significantly impedes their individual identification. However, these features allow you to specify the exact group of printing devices (monochrome, color).

Electron microscopes for forensic examinations can be also equipped with additional software for analysis of gunshot residue particles (GSR), with characteristic morphology and specific elemental composition. The software allows to analyze the residue found on clothing or skin of a firing person and particles originated from pyrotechnic reactions[18]. In forensic science, the analysis of gunshot residue is a frequently used method of research in order to explain the circumstances of the events with the use of firearms. Traces after firing from a firearm are routinely collected on a microscopic stub. Based on the high average atomic number (*Z*) of the GSR particles originated from the composition of the primer mixtures, the screening of such particles is carried out on the basis of their brightness on the backscattered electrons image (BSE) (fig.17).

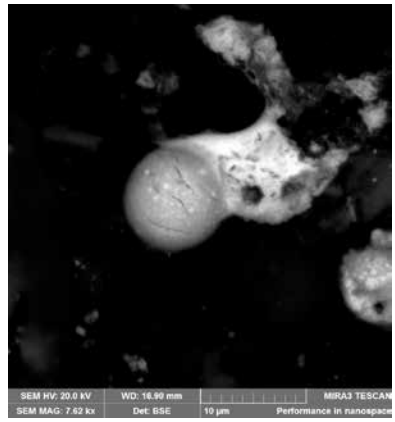


Fig. 17. SEM image of the characteristic particie of ganshot residua on the surface of microscope stub.

The elemental composition of the particles is analyzed by the EDX system, in which a common type of detector is a silicon detector doped with Si (Li) lithium in the solid state. Over 25 years ago, automated search systems were introduced, which

[18] H. Krüsemann, *SEMs and forensic science*, „Problems of Forensic Science" 2001, No 47, pp.110–121.

replaced the manual search of gunshot residue particles originated from the primer.[19] Such automatic search systems locate particles, analyze their elemental composition and identify them. Due to the small size of the GSR particles (e.g., 0.5–10 μm) in relation to the area of the sample (120–480 mm^2 depending on the size of the SEM microscope stub) and the large number of environmental particles with a high average atomic number (*Z*) usually found in the analyzed samples, the time needed to carry out the automatic search is long. The result of the GSR analysis is an automatically generated test report, which contains the number of particles, including the defined classes. The possibilities of automatic systems in the field of detection of a larger number of particles and of smaller sizes, carried out in shorter time, are increased by the continuous technological development of computer hardware and software.

Another technique of imaging is to obtain a surface image with a resolution capability in the order of dimensions of a single atom. For this purpose, atomic force microscope (AFM) is used. Among the many devices associated with nanotechnology, none is as well recognizable and simple in its construction as the atomic force microscope, also called the microscope of close interactions. Over the past few years, field of the applications of the AFM has expanded, mainly due to enormous potential, not available for other research techniques. The large resolution allows the testing of individual molecules, their groups and elements of supramolecular structures. The AFM consists of a cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface, above or below the surface of the sample. AFM uses the intermolecular forces. The cantilever bias allows to determine the intermolecular forces between the atoms of the tip and the tested surface. The force map for each surface point of the sample is computer-processed into an image. Cantilever deflection measurement is made by optical methods. The sensitivity of the cantilever it is tenths of angstrema.[20] The tip consists of several hundred atoms to even one atom. The tips are usually made of silicon and silicon nitride. In contact atomic force microscopy mode, the cantilever is held from the surface of the sample at a distance of less than a few tenths of a nanometer, and repulsive forces are present. In non-contact atomic force microscopy mode, the cantilever is held from the surface of the tested material at a distance of a few to several dozen nanometers.

In this case, the probe-sample force of attraction can be found (van der Waals interactions). The appropriate choice of the research method using the AFM is important, because the correctness of the results depends on it. The AFM control computer must be characterized by high speed and large memory capacity.

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[19] R.S. White, A.D. Owens, *Automation of gunshot residue detection and analysis by scanning electron microscopy/energy dispersive X-ray analysis (SEM/EDX)*, „Journal of Forensic Science" 1987, No 32, pp. 1595–1603; M. Germani, *Evaluation of Instrumental Parameters for Automated Scanning Electron Microscopy/Gunshot Residue Particle Analysis*, „Journal of Forensic Science" 1991, No 36, pp. 331–342.

[20] Ångström (Å) – unit of length equal to 10^{-10} m. It is used for numerical expression of values of very small lengths, comparable to the sizes of atoms.

Computer programs used to generate of sample images and to determine their properties are constantly improved. Their development requires knowledge, not only in the field of mathematics and computer science, but also on the physical and chemical properties of the tested materials. It is also important to study the type of the material, the dimensions of the tip and the effect of the AFM on the force appearing between this tip and the tested sample. The research procedures and methods of statistical calculations are also developed, which are the basis for determining the properties of the geometrical structure of the examined surfaces. Application possibilities of the AFM are very large in various fields of science, technology and forensics. First of all, they include the study of metals, ceramic and silicon structures and recently conducted biology research on an increasing scale. AFM's forensic microscopy technique is used to investigate the effects of fire, blood, hair, fibers, paper texture, depth of penetration of cotings (inks, pen pastes)[21] and to investigate gunshot residue (fig. 18), including the determination of the shape, size and average density of particles around the bullet inlet opening.[22]

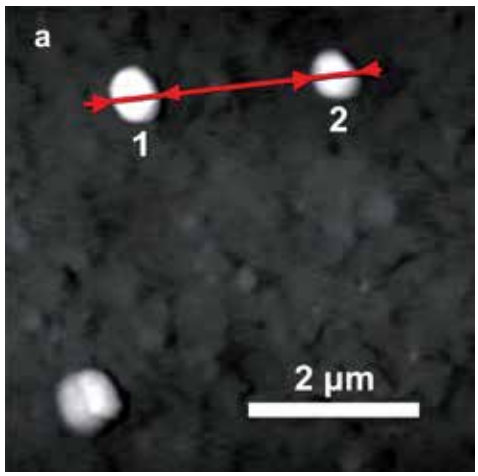

Fig. 18. AFM image of individual GSR particles.[23]

A.J. Goddard et all used the AFM technique to study imaging fingerprint ridges on polished brass substrates. For the production of ammunition, brass is a very popular material. In this case the AFM microscopy technique was used to visualize the corrosion of metals, between fingerprints and metal, after heat treatment[24] on a nanometer scale.

[21] S. Kasas, A. Khanmy-Vital, G. Dietler, *Examination of line crossings by atomic force microscopy*, „Forensic Science International" 2001, No 119, pp. 290–298.

[22] Y. Mou, J. Lakadwar, J.W. Rabalais, *Evaluation of shooting distance by AFM and FTIR/ATR analysis of GSR*, „Journal of Forensic Science" 2008, No 58, pp. 1381–1386.

[23] Ibidem.

[24] A.J. Goddard, A.R. Hillman, J.W. Bond, *High Resolution Imaging of Latent Fingerprints by Localized Corrosion on Brass Surfaces*, „Journal of Forensic Sciences" 2010, No 55, pp. 58–65.

The AFM microscope was also used for the analysis of textile fibers. AFM images were used to quantify the surface texture parameters of fabrics exposed to environmental factors (different soil types, water) as a function of time. The tests showed the difference between fiber damage due to environmental factors and mechanical damage.[25]

The AFM technique was also used to detect explosives. In order to determine the characteristics of explosives[26] more accurately, the surface morphologies of substances such as triamine-trinitro-benzene, ammonium perchlorate and PBX were analyzed. Due to the continuous work, both on the construction of the microscope and the physical basis of the method used, the possibilities of the AFM microscope are increasing. The interpretation of the results obtained by using AFM method is still a difficult and complex issue, despite continuous improvement of the atomic force microscope, the development of computer software and new achievements in the field of theoretical and experimental foundations.

Summary

The use of advanced technology to study the evidence allows extremely accurate analysis. Imaging of material in high magnification is necessary for the preparation of different samples documentation (the study of the morphology of the surface, determining the diagnostic features). The use of optical microscopy, electron microscopy or atomic force microscopy and integrated spectroscopic systems is a basic and powerful tool for the obseration of various materials. Currently, such device systems should be available in every forensic laboratory. These systems should be used as basic devices, routinely used in court expertise, despite their high market value and high exploitation prices. They allow quick, uncomplicated and non-destructive testing of material. Therefore, in a short time, it is possible to obtain information about the tested samples, which can be easily processed and visualized by making Figgraphic documentation, with regard to various construction details, examining their optical properties and determining their chemical composition. The obtained results, enable preliminary classification of evidence before being passed to further research using other highly advanced instruments.

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[25] E. Canetta, K. Montiel, A.K. Adya, *Morphological changes in textile fibres exposed to environmental stresses: atomic force microscopic examination*, „Forensic Science International" 2009, No 191, pp. 6–14.

[26] M. Tourne, *Developments in explosives characterization and detection*, „Journal of *Forensic Research"* 2013, No S12, pp. 002 (pp. 2–10); G.C. Yang, F. Nie, H. Huang, L. Zhao, *Pang preparation and charakterization of nano-TATB explosive*, „Propellants Explosives, Pyrotechnics" 2006, No 31, pp. 390–394; A. Kumari and others, *Nano-ammonium perchlorate: preparation, characterization and evaluation in composite propellant formulation*, „Journal of Energetic Materials" 2013, No 31, pp. 192–202; A.E.D.M van der Heijden and others, *Energetic materials: crystallization, characterization and insensitive plastic bonded explosives*, „Propellants Explosives, Pyrotechnics" 2008, No 33, pp. 25–32.

Abstract

The article presents issues that include various imaging techniques used in forensics. Technology development observed in the modern world, implies changes in all areas of life, improving, speeding up and opening them to new research capabilities. The use of advanced technology allows precise analysis of the evidence. The opportunity to observe different samples at high magnification is necessary in the performance of their documentation (examination of the morphology of the surface, setting the diagnostic characteristics). The application of optical microscopy, electron microscopy or atomic force microscopy, and integrated spectroscopic systems is an essential and powerful tool for implementation observations of a variety of materials.

Keywords: imaging techniques, microscopy, SEM, X-ray microanalysis, EDS, Raman spectroscopy, atomic force microscopy, AFM.

Acknowledgments

In this article some of the results of the research were presented. The study was supported by NCBiR, Nr 0023/R/ID3/2012/02 and provided in pyrotechnic and structure of the toner surface range.