Femtosecond imaging-mode laser-induced breakdown spectroscopy

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Dipl.-Phys. Fedor Mayorov

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Erster Gutachter: Univ.-Prof. Dr. rer. nat. Thomas Baumert
Zweiter Gutachter: Univ.-Prof. Dr. rer. nat. Frank Träger
# Contents

## 1. INTRODUCTION

1.1. GENERATION OF A LASER-INDUCED PLASMA
   1.1.1. Fundamental parameter for plasma description
   1.1.2. Ionization and breakdown
   1.1.3. Plasma expansion
   1.1.4. Laser-mediated material removal - ablation

1.2. LIBS SPECTRAL ANALYSIS
   1.2.1. Plasma radiation
   1.2.2. Energy balance during laser-induced breakdown
   1.2.3. Laser-plasma interaction

1.3 LIBS AND APPLICATIONS

1.4 CONCLUSION

## 2. MICROSCOPY TECHNIQUES

2.1. STANDARD LIGHT MICROSCOPY
   2.1.1. Description of the basic scheme
   2.1.2. Resolution and point spread function
   2.1.3. Techniques and applications

2.2. CONFOCAL MICROSCOPY
   2.2.1. Description of the basic scheme
   2.2.2. Resolution
   2.2.3. Applications

2.3. NONLINEAR MICROSCOPY TECHNIQUES
   2.3.1. Description of the basic schemes
   2.3.2. Resolution

2.4 IMAGING-MODE LIBS MICROPROBING

2.5. CONCLUSION

## 3. LIBS - BASIC CONSIDERATIONS

3.1. GENERATION OF A LASER-INDUCED PLASMA
   3.1.1. Fundamental parameter for plasma description
   3.1.2. Ionization and breakdown
   3.1.3. Plasma expansion
   3.1.4. Laser-mediated material removal - ablation

3.2. LIBS SPECTRAL ANALYSIS
   3.2.1. Plasma radiation
   3.2.2. Energy balance during laser-induced breakdown
   3.2.3. Laser-plasma interaction

3.3 LIBS AND APPLICATIONS

3.4 CONCLUSION

## 4. NANOSECOND LIBS EXPERIMENTS AND RESULTS

4.1. EXPERIMENTAL SETUP FOR PRELIMINARY EXPERIMENTS
   4.1.1. Nanosecond laser setup for lines identification
   4.1.2. Triggering and data acquisition
   4.1.3. Ca $^{2+}$ ion lines identification

4.2. NANOSECOND LASER SETUP WITH ICCD CAMERA DETECTION
   4.2.1. Setup description
   4.2.2. Triggering system
   4.2.3. Nanosecond plasma dynamics measurement

4.3. CONCLUSION

## 5. FEMTOSECOND LIBS EXPERIMENTS AND RESULTS

5.1. PRELIMINARY EXPERIMENTS
   5.1.1. Laser system description
## Contents

5.1.2. Setup for the preliminary experiments ................................................................. 64
5.1.3. Femtosecond laser induced plasma ................................................................. 65
5.1.4. Plasma microscope setup description ................................................................. 66
5.1.5. Triggering schema ................................................................................................. 67
5.1.6. Adjustment procedure .......................................................................................... 68
5.2. FEMTOSECOND LIBS MEASUREMENTS ON WATER MODEL .......................... 72
5.2.1. Sensitivity calibration .......................................................................................... 72
5.2.2. Time-resolved plasma luminescence measurements .......................................... 73
5.2.3. Plasma luminescence measurement .................................................................... 75
5.3. STUDY OF THE ABLATION PROPERTIES OF THE PLASMA MICROSCOPE ............ 78
5.4. CONCLUSION ............................................................................................................ 81
5. LIBS STUDIES OF THE SUNFLOWER SEEDLINGS .................................................. 82
6. INTRODUCTION ........................................................................................................... 82
6.1. SUNFLOWER SEEDLING ............................................................................................ 83
6.2. NANOSECOND LIBS STUDY OF THE SUNFLOWER SEEDLING ................................. 86
6.3. FEMTOSECOND LIBS STUDY OF THE SUNFLOWER SEEDLING .............................. 90
6.4. PLASMA MICROSCOPE INVESTIGATION OF THE SUNFLOWER SEEDLING ............ 96
6.5. CONCLUSION ............................................................................................................ 97
7. IMPROVEMENTS TO THE PLASMA MICROSCOPE ........................................... 98
7. INTRODUCTION ........................................................................................................... 98
7.1. PRELIMINARY IONIZATION ...................................................................................... 99
7.2. PLASMA HEATING .................................................................................................. 101
7.3. PULSE SHAPING AND EVOLUTIONARY ALGORITHMS ....................................... 102
7.4. CONCLUSION ......................................................................................................... 103
8. SUMMARY .................................................................................................................. 104
APPENDIX I .................................................................................................................. 106
APPENDIX II ............................................................................................................... 109
APPENDIX III .............................................................................................................. 110
APPENDIX IV .............................................................................................................. 111
ACKNOWLEDGEMENTS .............................................................................................. 112
REFERENCES .............................................................................................................. 113
1. Introduction

Optical microscopy techniques are widely applied in biology, medicine and industry. Modern science offers a great variety of the optical microscopy techniques meeting the demands of a researcher.

Resolution is an important parameter of a microscope. The confocal microscope offers the best resolution among the far field light microscopes. 150 nm lateral resolution and 500 nm axial resolution could be achieved [1]. An improvement of the resolution and selectivity are the main objectives in the microscopy techniques development. Invention of new methods of imaging and analysis by means of laser-assisted microscopy is a challenging part of modern science and technology. A number of laser microscopy techniques, based on linear: fluorescence and non-linear: two-photon fluorescence (TPF), third harmonic generation (THG), coherent anti-Stokes Raman scattering (CARS) phenomena are spreading widely nowadays [2-4]. The highest resolution $\lambda/23$ is offered by stimulated emission depletion (STED) microscopy [5].

In this work, a new nonlinear microscopy technique is proposed. The objective of this work is the development of a minimal invasive analytical method for spectrochemical in situ investigations of biological samples with high axial resolution. In order to achieve this goal microscopy, laser-induced breakdown spectroscopy (LIBS) and femtosecond laser material processing techniques are combined. The imaging-mode LIBS microprobing setup – plasma microscope is developed. The first application of the plasma microscope - the analysis of the bound Ca$^{2+}$ ion distribution in the peripheral cell wall of a sunflower (Helianthus annuus L.) seedling stem is presented. It is demonstrated, that a spectrochemical in situ analysis of the Calcium ion distribution in the peripheral cell wall is possible with a femto- to attoliter probe volume.

Several decades ago, LIBS was established as a spectrochemical analysis method, in which a laser-induced plasma (LIP) is used as a spectrochemical source. The fundamental advantages of this approach can be summarized as follows: no sample preparations are necessary, remote sensing measurements are possible and samples of any state of aggregation can be analysed. The method is sensitive to the detection of different chemical elements (Appendix I). Moreover, multi-element analysis can be performed. Sensitivity limits in the ppm range can be achieved and spatial
information can be obtained [6-9]. Mainly nanosecond lasers are used in order to perform LIBS studies.

Femtosecond laser material processing is a new, fast spreading technology. Ultrashort pulsed lasers offer a number of the attractive advantages in comparison to the nanosecond laser pulsed sources. Orders of magnitude lower breakdown threshold, low thermal damage and high precision are the advantages in the material processing of the dielectrics [10,11]. Therefore, combination of LIBS and femtosecond material processing could be a prospective minimal invasive analytical method for the element-specific \textit{in situ} analysis of the biological samples with high spatial resolution.

In Chapter 2 the basics of the standard light microscopy, confocal microscopy, nonlinear microscopy techniques and imaging-mode LIBS microprobing are presented and discussed.

In Chapter 3 the processes related to LIBS: ionization, plasma formation and expansion, ablation and spectral analysis of the plasma are described. LIBS applications presented in the literature are also discussed.

In Chapter 4 the nanosecond laser-induced breakdown processes are investigated. Water is used in this series of the studies, because water can be considered as a model system of biological objects [12-16]. Therefore this experiments are considered as preliminary investigations in order to perform bound Calcium analysis in biological samples. Calcium lines identification is done and plasma dynamics on the nanosecond time scale is measured.

The development of the plasma microscope is described in Chapter 5. The developed adjustment procedure is presented. The results of the femtosecond LIBS experiments on Calcium lines, and plasma background emission analysis on water model are described. The sensitivity of the plasma microscope to detection of the $\text{Ca}^{2+}$ ion is determined.

In Chapter 6, LIBS application to the analysis of the bound $\text{Ca}^{2+}$ ion distribution in the peripheral cell wall is presented. The main analytical requirements for this investigation are discussed, basing on the information about the biological structure of the sunflower seedling stem. Nanosecond LIBS analysis of the Calcium ion distribution along the stem and the images of ablation craters are presented. Femtosecond laser experiments, demonstrating precise ablation without collateral damage, are shown and compared with the nanosecond laser ablation. The Calcium ion distribution analysis is done with additional axial sectioning at each point. The plasma microscope image of the
Ca\textsuperscript{2+} ion distribution in the peripheral cell wall of the sunflower seedling is presented in Chapter 6.

In Chapter 7 the improvements to the plasma microscope setup are proposed. Possible ways to improve the analytical performance of the setup, are presented.

In the conclusion, the main results are presented and discussed. The advantages of precise femtosecond laser ablation for the plasma microscopy of the biological samples are shown and the applications of the plasma microscopy to the study of the biological samples are discussed.
2. Microscopy techniques

**Introduction**

Modern far field optical microscopy techniques are discussed in this chapter in terms of the plasma microscope development. Modern laser-assisted microscope techniques are discussed and compared to imaging-mode LIBS microprobing - plasma microscopy.

Standard optical microscopy is the basic for the variety of laser microscopy techniques. Confocal microscopy is presented, as it is the first technique, which offers a high axial resolution. There are several up-to-date microscopy techniques, which involve modern ultrashort laser sources and are based on high-intensity nonlinear phenomena. The following types of the nonlinear microscopy are overviewed: two-photon microscopy (TPM), third-harmonic generation (THG) microscopy, coherent anti-Stokes Raman scattering (CARS) microscopy and stimulated emission depletion (STED). The descriptions of basic schemes and principles are presented. A comparison of different techniques is provided by considering corresponding point-spread functions (PSF). The PSF is used to quantify the resolution performance of an imaging system and thus could be used as a comparative parameter for different microscopy techniques. The imaging-mode LIBS microprobing technique is presented and the resolution of this technique is discussed.
2. Microscopy techniques

2.1. Standard light microscopy

2.1.1. Description of the basic scheme

Many modern microscopy techniques are based on the principle of a standard optical microscope. A simplified form of a standard optical reflection microscope is presented on Figure 2.1. In this instrument, the sample is uniformly illuminated through the objective lens by a filament lamp or other bright light source such as a Mercury vapor lamp. The objective lens forms a real inverted image of the object at the intermediate image plane of the microscope. The image is viewed through the eyepiece, which provides additional magnification. The eyepiece forms a virtual image of the object at a comfortable viewing distance from the eye, normally 250 mm for a „standard observer“. Numerical aperture (N.A.) is an important parameter of the microscope objective, which is defined as $N.A. = n \sin \theta_0$, where $n$ is the refraction index and $\theta$ is the half-focusing angle (Figure 2.1. – insert).

One important parameter of a standard optical microscope is the magnification. It determines the size of the image at the detector. In the case of a lens, the magnification in transverse direction $M_T$ is given by the negative ratio of the image size $d_i$ to the object size $d_o$: $M_T = -d_i/d_o$. The negative sign accounts for the inversion of the image by a lens. The eyepiece magnification $M_E$ is defined as the ratio of the size of the retinal image as seen by the unaided eye at a normal viewing distance of 250 mm. It is calculated from the formula $M_E = 250 \ mm/f$, where $f$ is the focal length of the eyepiece lens. There is no
negative sign in this formula, because the eyepiece produces a virtual image. Common values of eyepiece magnification are 2.5-10.0X. Thus, as the magnification of the objective can vary from 1.5 to 200X, a wide range of total magnification $M_r=M_TM_E$ can be obtained. Magnification is a unity not related to resolution. The resolution and PSF are considered in the following chapter.

### 2.1.2. Resolution and point spread function

The performance of an imaging system can be quantified by calculating its point spread function (PSF). The amplitude PSF, $h(x,y)$, of a lens is defined as the transverse spatial variation of the amplitude of the image received at the detector plane when the lens is illuminated by a perfect point source. Diffraction coupled with aberrations in the lens will cause the image of a perfect point to be smeared out into a blur spot occupying a finite area of the image plane. In the same way, the intensity PSF, $I_h = |h(x,y)|^2$, of an objective is defined as the spatial variation of the intensity of the image received at the detector plane when the lens is illuminated by a perfect point source. Another approximation is appropriate for the consideration of the laser assisted microscopy techniques where the objective lens with low N.A. is considered to be homogeneously illuminated [1]. In an optically matched system, the lens is assumed to accept a perfectly planar incoming wavefront and produce a perfectly spherical outgoing wavefront that produces an unaberrated point-spread function (PSF) at the focus point. The PSF is presented in the paraxial approximation by Fourier transform of the pupil function of the lens. Here, a beam of unit amplitude passing through the objective lens is considered, which is focused to a point $P_0$ at $(0, 0, z_0)$ on the axis of the lens, as it is shown on Figure 2.2.

![Figure 2.2. Scheme of the variables used in calculation of PSF in a standard optical microscope.](image-url)
The pupil function of the objective $P(x',y')$ is defined as the amplitude attenuation of the beam passing through the lens at a point $(x',y',0)$, on the plane $D_2$ just in front of the lens. All the rays reaching the point $P_0$ will be in phase if the lens introduces a phase delay $\phi_0 = A - knR_0$ at each point $(x',y',0)$, where $k = \frac{2\pi}{\lambda}$. In these expressions, $\lambda$ is a free-space wavelength, $n$ the refractive index of the medium between the lens and the point $P_0$, and $A$ is a constant. In this case, the phase change along the ray of length $R$ is $\phi = knR$.

Rayleigh-Sommerfeld diffraction theory [17] can be used to calculate the scalar potential $h(x,y)$ of the beam at the point $(x',y',z_0)$:

$$h(x,y) = B \iiint P(x',y') e^{-j\phi(x',y') \over R} dx' dy', \quad (2.1)$$

where $B$ is a constant.

To express equation (2.1) as a Fourier transform relation, the phase term is expanded to the first order in $x/z_0$, $y/z_0$, $x'/z_0$, and $y'/z_0$ using the paraxial approximation, $z_0 >> x'$, $z_0 >> y'$, $z_0 >> x$, $z_0 >> y$. Setting $R \approx z_0$ in the denominator of the integrand, the equation becomes

$$h(x,y) = D e^{-j(kx^2 + y^2)/2z_0} \iiint P(x',y') e^{jkn(x'x + y'y) / z_0} dx' dy'. \quad (2.2)$$

In this equation $D$ is a normalization coefficient.

Assuming that the spot size is small, the exponential term in front of the integral is close to unity so that

$$h(x,y) = D \iiint P(x',y') e^{jkn(x'x + y'y) / z_0} dx' dy'. \quad (2.3)$$

From this equation it follows, that the amplitude PSF of a lens at the focus is proportional to the Fourier transform of the pupil function.

The properties of the PSF include the principle that for a linear spatially invariant imaging system, the image can be calculated by convolving a function, characterizing the response (transmission or reflectivity) of the sample with PSF of the system. It could be presented in the following manner:

$$I \propto |h|^2 \otimes |\rho|^2, \quad (2.4)$$

where $h$ is a PSF of the system and $\rho$ is a sample response function, what, in case of homogeneous sample, results in the homogeneous intensity distribution [18]. In other words, the PSF describes the way, each individual point is blurred by the optical system.
In order to estimate the resolution, the function $h(r)$ is used to express the radial variation of the amplitude PSF of a circularly symmetric aberration-free lens. Here $r$ is the distance from the center point of the image to the lens: $r=\sqrt{x^2+y^2}$. If the pupil function is uniform, it can be shown from (2.3) in the paraxial approximation that $h(r)$ has the form of Airy function.

$$h(r) = \frac{2J_1(\nu)}{\nu}. \quad (2.5)$$

In this equation, the normalized distance from the optical axis of the system is defined as $\nu = kr n \sin \theta_0 = kr (N.A.)$, where $k=2\pi/\lambda$ is the wave number, $\lambda$ is the free-space wavelength and $J_1(\nu)$ is a Bessel function of the first order and the first kind. The amplitude and the intensity of the Airy function are plotted on Figure 2.3. The amplitude has a maximum at $\nu=0$ and there are subsidiary minima and maxima or sidelobes. The first zero of the response is located at $\nu=3.832$ or $r=0.61 \lambda/n \sin \theta_0$. The first sidelobe or maximum in the amplitude response is at $\nu=5.136$ or $r=0.82 \lambda/n \sin \theta_0$ and is reduced in amplitude by 0.132 or $-17.6$ dBs from the amplitude at the center of the main lobe. The amplitude PSF is related directly to the electric field at the sample, whereas the intensity PSF is related to the power per unit area of the square or the electric field.

![Figure 2.3. The amplitude variation (dotted line) and intensity (solid line) for PSF of a lens.](image)

The width between the half-power points (Figure 2.3) of the main lobe $d_r$ (3 dB), in the intensity response is known as the full width at half-maximum (FWHM) or 3-dB width and is given by the formula

$$d_r(3dB) = \frac{0.51\lambda}{n \sin \theta_0} = \frac{0.51\lambda}{N.A.}. \quad (2.6)$$
This formula for the width of the image of a point object is also called the single point resolution of the standard optical microscope.

The resolution conception of the standard optical microscope is connected to the Rayleigh criterion and two-point definition. In an imaging system: the amplitudes of signals from different parts of the image added, and the result is then squared to form the intensity image. So the use of the simple definition of resolution based on the half-power width is not always adequate. It is, therefore, common to employ the Rayleigh criterion, which states that two closely space illuminated points are distinguishable from each other if the maximum response to point A is located at the zero of the response to the point B.

For the standard optical microscope, the intensity $I(\nu)$ is given by the relation:

$$I(\nu) = \left[ \frac{J_1(\nu - 1.91)}{\nu - 1.91} \right]^2 + \left[ \frac{J_1(\nu + 1.91)}{\nu + 1.91} \right]^2,$$  \hspace{1cm} (2.7)

The plot of the expression is presented on Figure 2.4. The intensity at $\nu=0$ is 73.5% of it’s maximum value.

Figure 2.4. Image intensity for two incoherent point sources separated by the Rayleigh distance.

The Rayleigh definition is therefore often stated in the form: two points of equal brightness can be distinguished if there is a 26.5% drop in the intensity between them. This definition corresponds to the two points being separated by a distance $d_R$ (Rayleigh), defined as:

$$d_R = \frac{0.61\lambda}{N.A.}$$ \hspace{1cm} (2.8)

An alternative definition of two-point resolution is the Sparrow criterion, which states, that two points of equal brightness can be distinguished if the first and second order deviations of the intensity function at the saddle point are equal to zero. Such a criterion is much more general in application than simply placing one point at the zero
response to the other point. It can apply equally well to coherent imaging or, for example, to a Gaussian beam which has no sharp spatial zero in response. The distance between two neighboring points which are just distinguishable using the Sparrow criterion in an imaging system is:

\[ d_s(\text{Sparrow}) = \frac{0.51\lambda}{N.A.}. \]  

(2.9)

This criterion is widely used as well as Rayleigh criterion for determination of the effective resolution of the microscopes. In our study, the Zeiss objective \textit{LD Epiplan} \((N.A.=0.4)\) is used. The resolution for this objective is estimated by Rayleigh criterion as 1.22 \(\mu\text{m}\) at the wavelength \(\lambda=800\ \text{nm}\).

The depth resolution of the microscope is commonly defined \([1]\) as the distance between half-power points (3-dB points) of the intensity response given by the approximate formula

\[ d_z(3\text{dB}) = \frac{0.45\lambda}{n(1-\cos\theta_0)}. \]  

(2.10)

In the paraxial approximation with \(n=1\) this equation reduces to the relationship

\[ d_z(3\text{dB}) \approx \frac{0.89\lambda}{\sin^2\theta_0} \approx \frac{0.89\lambda}{(N.A.)^2}. \]  

(2.11)

2.1.3. Techniques and applications

Conventional farfield microscopy has the widest application field in all modern areas of science, technology and medicine. It is an important imaging tool at all areas, working with small distance scales. Conventional microscopes are used for aiming during the work at millimeter and micrometer ranges, for studying of the cell-sized objects in biology and medicine, for material study as well as for educational purposes. Several techniques are described, below which are used in the current study.

There are several imaging techniques used in standard optical microscopy, which should be mentioned, as they are used in this work. The most common technique is known as brightfield imaging, where images are produced uniformly illuminating the entire sample so that the specimen appears as a dark image against a brightly lit background. Brightfield imaging is used as a general imaging technique for observation and inspection of the samples.

An alternative technique, known as darkfield imaging is a useful method of visualizing small particles and fine lines in the microscope. In its most common
implementation, the sample is illuminated with a hollow cone of light, which is larger than the acceptance angle of the objective. In this case specular reflectors do not reflect light into the objective and only the light which is scattered into the objective by particles or the edges of the sample is imaged. In a darkfield image, structures appear as bright lines against a dark background. Darkfield microscopy is used in this work in order to image the laser ablation craters on the surface of the sunflower seedling stem.

The application and instrumental range of the conventional microscopy techniques is wide. Nevertheless it is limited due to the low axial resolution. In our study the standard light microscopes are used for aiming and investigation purposes.
2. Microscopy techniques

2.2. Confocal microscopy.

2.2.1. Description of the basic scheme.

Confocal microscopy was first developed by Minsky et. al. in the 50’s for incoherent light sources. This instrument differs from the standard light microscope in its shallow depth of focus and hence it is capable of accurate height and thickness measurements and of obtaining cross-sectional images.

The basic principle of the confocal microscopy is illustrated on Figure 2.5. The main concept of the confocal arrangement is to illuminate only one spot on the sample at a time through a pinhole. The light, reflected from the sample is imaged by the objective back to the pinhole.

By scanning the spot or the sample in a raster pattern a complete image can be formed. If the sample moves out from the focus, the reflected light is defocused at the pinhole and hence does not pass through it to a detector located on the other side. Thus, the axial or depth resolution of the microscope depends on the pinhole size.

The microscope is called „confocal“ because the objective lens is used twice, both to illuminate and to image the sample. Since only one point is illuminated at a time, speckle is eliminated, but the sample or illumination beam must be raster scanned and the image must be built up pixel by pixel like a television picture. For these reasons the instrument is called a confocal scanning optical microscope (CSOM). Scanning time per frame strongly depends on the speed of the information transfer from the detector to the
computer. The majority of the commercial CSOM employs beam scanning methods, which are typically much faster than sample scanning [18].

During the early 1970s, the confocal laser scanning microscope (CLSM) was developed. In this microscope, the parallel laser beam is used for the illumination of the sample. The scheme of the CLSM is presented on Figure 2.6.

![Figure 2.6. The scheme of a confocal laser scanning microscope (CLSM).](image)

In CLSM, the parallel laser beam is transmitted through the beamsplitter and focused by the infinity-corrected objective. The scattered light or fluorescence of the sample is collected by the same objective and directed on the pinhole by a beam splitter. The pinhole discriminates the light from the focal position from the out-of-focus radiation.

### 2.2.2. Resolution

The major advantage of the CSOM over a standard optical microscope is that defocused image disappears in the CSOM, whereas it becomes blurred in a standard microscope. This property of CSOM results in a 3-dimensional image modeling. The range resolution of the CSOM makes it possible to measure quantitative the profiles of features in the sample. The lateral resolution of the confocal microscope is defined the same way, as for the conventional microscope (2.9) considered in Chapter 2.1.2.

The amplitude of the point object image field at the detector is given by the Airy function (Chapter 2.1.2.) \( h(r) \). In an ideal instrument the intensity PSF of the microscope is equal to the square of the amplitude response of the objective lens.
2. Microscopy techniques

\[ I_c(r) = |h_{cf}(r)|^2 = |h^2(r)|^2, \quad (2.12) \]

where \( h_{cf} \) is the PSF of the confocal microscope. The amplitude of the illuminating field at the sample, is imaged by the objective onto a point detector so that the amplitude PSF of this microscope is given by \( A_c(r) = h^2(r) \), while the image intensity of a point is \( I_c(r) = |h^2(r)|^2 \), where the script \( c \) denotes a confocal microscope. For objects such as edges and points, the CSOM intensity image is square of the intensity image produced by a standard microscope. The single-point lateral resolution of the confocal microscope, defined as the width at the half-power points of the image of a point object, is [1]:

\[ d_C(3 \, dB) = \frac{0.37 \lambda}{n \sin \theta_0} = \frac{0.37 \lambda}{N.A.}. \quad (2.13) \]

This width is 73% of the single-point resolution of the standard optical microscope.

The \( z \)-response \( I(z) \) and the edge response \( I_{\text{edge}}(z) \) are important to estimate the axial resolution and could be presented as:

\[ I(z) = \int \int h_{cf}^2(x, y, z) dx dy \quad (2.14) \]

\[ I_{\text{edge}}(z) = \int_{z=\infty}^{z=z_0} I(z') dz'. \quad (2.15) \]

The receiving pinhole can be thought of as sampling a magnified reproduction of the fields on the axis of the objective lens. To measure the depth response, a mirror is moved axially through the focal plane of the lens. When the sample moves a distance \( z \) from the focal plane, the image of the illuminating pinhole moves a distance \( 2z \) away from the focal plane. It can be shown from equation (2.3) with \( z \neq z_0 \), \( x=y=0 \), that the electric field amplitude varies along the axis of the lens approximately as \( (\sin u/4)/(u/4) \), where \( u=4nkz \sin^2(\theta_0/2) = 2nkz(1-\cos \theta_0) \) [17]. Thus, the amplitude variation, \( V(z) \), of the light passing through an infinite small pinhole on the axis is described as:

\[ V(z) = \frac{\sin(u/2)}{u/2} = \frac{\sin[nkz(1-\cos \theta_0)]}{nkz(1-\cos \theta_0)}. \quad (2.16) \]

The measured and „ideal“ signal output of the depth response of a confocal microscope for 50X0.5 N.A. objective in air at \( \lambda = 633 \, \text{nm} \) is shown on Figure 2.7.
2. Microscopy techniques

2.2.3. Applications

One of the most popular CSOM techniques nowadays is a fluorescent confocal microscopy. The confocal imaging geometry provides a dramatic optical advantage for fluorescent microscopy by discriminating out-of-focus background with minimal loss of image-forming signal. Significant enhancement of both, axial and lateral imaging resolution is also available but only with substantial signal loss due to the decrease of the light current. Because of these advantages, the CSOM with laser illumination - CLSM can image thin optical sections within thick fluorescence-labeled living specimens. A stack of optical sections is easily combined to reveal three-dimensional fluorescent marker distributions with diffraction-limited spatial resolution. When bright stable fluorosphores are available, cellular dynamics can be measured by recording a time series of CLSM images. A number of sensitive, selective fluorescent indicators is
available to make this technology possible. Fluorescent markers can be selectively bound to particular cellular components using monoclonal antibodies, specific ligand affinities, or covalent bonds. Fluorescent indicators, that are sensitive to intracellular free ion distributions (e.g., Ca$^{2+}$, H$^+$, Na$^+$), membrane potential enzymatic activity and other physiochemical parameters indicative of cellular activity are also available. The accuracy, sensitivity, precision, and speed of fluorescence distribution measurements are limited by fluorescence detection and collection efficiencies and by fluorosphere photochemistry. The main disadvantage of this technique is the photobleaching, which limits the exposition time, laser fluence and thus the sensitivity of the method.
2.3. Nonlinear microscopy techniques.

2.3.1. Description of the basic schemes.

Recent decades, a progress was achieved in the development of laser sources, generating femtosecond pulses. This led towards microscopy techniques based on the high-intensity nonlinear optical phenomena, as a contrast for functional imaging.

In 1990 Webb et. al. [2] presented the first nonlinear microscopy technique - a two-photon microscope (TPM). In the next decade several nonlinear processes were identified to be applicable for nonlinear laser microscopy. Third harmonic generation (THG), coherent anti-stokes Raman scattering (CARS) and stimulated emission depletion (STED) are applied for the microscopy purposes nowadays. The application of these optical phenomena to the microscopy will be further discussed in details.

In two photon fluorescence microscopy the molecular excitation is caused by the simultaneous absorption of two photons. The excitation of fluorosphores having single-photon absorption in the ultraviolet with a stream of strongly focused subpicosecond pulses of red laser light has made possible fluorescence imaging of living cells and other microscopic objects. In this case, the fluorescence emission increased quadratically with the excitation intensity so that fluorescence and photo-bleaching are confined to the vicinity of the focal plane as expected for cooperative two-photon excitation. The scheme of the setup would not be considered here, as it is mainly similar to one discussed in Chapter 2.1. The laser light, passing through a dichroic mirror is focused by the objective on the sample. Dichroic mirror separates the initial laser light from the sample response radiation. The scanning techniques are used in analogy to CSOM for pixel-by-pixel image restoration.

A third-harmonic generation (THG) microscopy is another modern microscopy technique, intended for high-resolution imaging of various microscopic samples. In THG microscopes, the third harmonic light is generated at the focal point of a tightly focused short-pulse laser beam. When the medium at the focal point is homogenous, the third harmonic waves generated before and after the focal point interfere destructively, resulting in zero net THG [20]. However, when there are inhomogeneous areas near the focal point, such as an interface between two media, the symmetry along the optical axis breaks and measurable amount of third harmonic is generated. Due to its nonlinear nature, the third harmonic light is generated only in proximity close to the focal point.
Therefore, high lateral resolution can be obtained, allowing THG microscopy to perform sectioning and to construct three-dimensional images of transparent samples, since all materials have non-vanishing third-order susceptibilities. Nevertheless, the detection of the THG signal in this microscopy technique is possible only in case if the laser beam is perpendicular to the measured surface.

Next up-to-date technique is a three-dimensional vibrational imaging by coherent anti-Stokes Raman scattering (CARS) [4]. It can be used as a contrast mechanism technique, based on vibrational properties of the molecules. This type of microscopy technique is based on the principle of CARS spectroscopy. A pump laser and a Stokes laser beam, with centered frequencies of $\nu_p$ and $\nu_s$, respectively, are spatially overlapped. The CARS signal at $2\nu_p - \nu_s$ is generated in a direction determined by the phase-matching conditions. When the frequency difference $\nu_p - \nu_s$ coincides with the frequency of a molecular vibration of the sample, the CARS signal $\nu_a$ is strongly enhanced due to the interferences. The term schema is presented on Figure 2.8.

![Figure 2.8. The term schema of CARS microscopy.](image)

As CARS is a four-wave-mixing process, the signal intensity depends non-linearly on the incident intensities: $I_{CARS} \sim I_p^2 I_s$. Similar to other nonlinear processes, high peak powers are necessary for the efficient generation of a CARS signal. For CARS microscopy a lens with a high numerical aperture is used to focus the beams tightly. With the tight focus, the phase-matching conditions are relaxed because of the large cone of wave vectors and the short interaction length. The nonlinear intensity dependence restricts the excitation to a small volume at the laser focus, similar to a multiphoton fluorescence microscopy.

A concept, which overcomes the diffraction limit by fluorescence is stimulated emission depletion (STED) microscopy [5]. Its concept is to suppress the spontaneous emission at the periphery of the diffraction-limited fluorescence spot of a scanning confocal microscope by stimulated emission. The suppression occurs in such a way, that fluorescence is allowed at the focal point but not in its proximity. The fluorescent
sample is placed in the common focus of two opposing lenses, but excitation and
detection are performed through a single lens only. For this purpose a train of 250 fs
pulses of 554 nm wavelength is directed via mirror, beam-splitter, and the dichroic
mirror. The lenses, which are alternatively pairs of water or oil immersion lenses, feature
the numerical aperture, 1.2 and 1.4, respectively, thereby establishing a tight excitation
intensity PSF. The fluorescence is imaged onto a confocal point detector. Immediately
after the excitation, a pulse of $\lambda=745$-760 nm and 13 ps duration, denoted by STED
pulse, enters the focal region. Special phase masks are applied to the STED signal for
the spatial restriction of the fluorescence signal.

These photons primarily act on the excited state $S_1$, inducing stimulated emission down
to a vibrational sublevel of the ground state $S_0^{\text{vib}}$, as it is shown on Figure 2.9.
Subpicosecond vibrational decay empties $S_0^{\text{vib}}$, so that repumping into $S_1$ is largely
ineffective.

**2.3.2. Resolution.**

The imaging principles of the nonlinear microscopy techniques are based on the
nonlinear optical response of the media, which is possible only at a high fluence. The
nonlinear optical phenomena is detected only in the beam waist of the gaussian beam
intensity distribution, where the fluence is higher, than the detection threshold. This area
size is smaller, then the actual size of the beam waist at position $z=0$. The size of the
area depends on the nonlinearity of the optical process. The higher is the order of the
process, the less is the size of the area, where nonlinear optical phenomena could be
detected. The scheme of the principle is presented on Figure 2.10.
Figure 2.10. Principal scheme of nonlinear microscopy process in gaussian pulse.

On Figure 2.10 the tightly focused gaussian beam is presented. On the left figure the fluence distribution and on the right figure, the distribution along the z-axis are shown. The nonlinear process takes place in the beam waist and the detection threshold spatially restricts the emission area.

Considering the two photon process, the resulting intensity is proportional to the square of the basic intensity: $I_{2\omega} \sim \sigma t^2$. The same holds true for the second harmonic generation. In two photon microscope [19], the intensity PSF $h_{2\nu}^2$ has a form of:

$$h_{2\nu}^2 = h_{\text{ill}}^2 h_{\text{det}}^2$$  \hspace{1cm} (2.20)

and in two-photon confocal microscope, in analogy to conventional confocal microscope (2.15) the intensity PSF $h_{c2\nu}^2$ is presented as:

$$h_{c2\nu}^2 = h_{\text{ill}}^2 h_{\text{det}}^2$$  \hspace{1cm} (2.21)

In analogy to expressions (2.16) and (2.17) we can define $h_{\text{ill}}^2$ and $h_{\text{det}}^2$:

$$h_{\text{ill}}^2(z) \propto [1+(z/z_{R_{\text{exc}}})^2]^{-1} \hspace{1cm} (2.22)$$

$$z_{R_{\text{exc}}} = 1.169(n\lambda_{\text{exc}}/\text{N.A.}^2) \hspace{1cm} (2.23)$$

$$h_{\text{det}}^2(z) \propto [1+(z/z_{R_{\text{em}}})^2]^{-1} \hspace{1cm} (2.24)$$

$$z_{R_{\text{em}}} = 1.169(n\lambda_{\text{em}}/\text{N.A.}^2). \hspace{1cm} (2.25)$$

Here, $z_{R_{\text{exc}}}$ and $z_{R_{\text{em}}}$ are Rayleigh lengths of the excitation and emission.

The illumination process in a confocal two-photon microscope therefore defines a volume in a manner similar to the combined illumination and detection processes in a confocal single-photon fluorescence microscope. In contrast to a confocal single-photon fluorescence microscope, a detection pinhole is actually not required.

In CARS microscopy, the resulting intensity of the process is defined as $I_{\text{CARS}} \sim I_{P}^2 I_{S}$, where $I_{P}$ is a pump laser and $I_{S}$ is a Stockes laser intensity. In THG
microscopy the resulting intensity is dependent on the basic intensity as: $I_{3\omega} \sim \sigma \sigma_p^3$. As we can see, the discrimination factors for these microscopy techniques are also high. Detailed information about CARS microscopy technique and applications is given by several authors [4, 21-23]. Third harmonic microscopy is a subject of detailed studies, presented in following works [20, 24, 25].
2.4 Imaging-mode LIBS microprobing.

Imaging mode LIBS microprobing is a minimal-invasive technique, where the resolution is defined by the ablation volume only. Thus it could be related to the nonlinear microscopy techniques.

Romero and Laserna [26] presented the idea of the chemical imaging by means of LIBS. They used pulsed N₂ laser (λ=337nm) to supply a chemical mapping of the aluminum and silver on the technological samples with lateral resolution around 50 µm.

In imaging-mode LIBS the focusing element is moving across the sample with the steps, equal to the ablated square on the surface of the target, to cover the whole area of interest with the homogeneous field of the sampled points. The plasma emission after each laser shot is analyzed on the specific element lines. Each measured spectra is related to the coordinates, where it was measured. Thus, spatially resolved distribution of the specific element could be obtained. The spatial resolution of this method depends on the ablated volume and its configuration. The principle of the imaging-mode LIBS microprobing is presented on Figure 2.11.

![Figure 2.11. Scheme of imaging-mode LIBS. The same objective is used to focus the laser radiation and to collect plasma emission.](image)

On Figure 2.11 it is shown, that the microscope objective, is used both, for focusing of the laser light on the target and collection of the plasma luminescence.

The approach, described in this work, consists in the combination of the imaging mode LIBS microanalysis with the femtosecond laser techniques. This allows to provide a single femtoliter spatial resolution and extremely high reproducibility of the sampling volume. These are the advantages of the femtosecond material processing techniques,
that are known for the precision and extremely low thermal damage of the sample. That is important if the biological applications of the imaging-mode LIBS technique are considered.

Spatially resolved LIBS microprobing requires a different approach to the resolution and PSF consideration. PSF concept in case of imaging-mode LIBS is just a conditional characteristic, as no real image is transmitted. Nevertheless it is important for the comparison with other microscopy techniques.

In order to generate a plasma which can be spectroscopically analyzed, the laser fluence has to overcome the optical breakdown threshold of the sample. Therefore the spatial resolution can be estimated by the extent of the laser fluence in the focus, which exceeds the optical breakdown threshold (above threshold zone). The laser fluence in the vicinity of the focus is proportional to the PSF of the objective. If one assumes homogeneous illumination of the aperture of a low N.A. objective, the PSF can be calculated as described in [27].

Figure 2.12. (a) Lateral cross section through the point spread function (PSF) in the focal plain for an N.A. = 0.4 objective. The lateral above-threshold radius is shown for \( c = 0.5 \) (blank-blank) and \( c = 0.9 \) (dotted line). 

(b) PSF in the z, r plane and contour lines confining the above threshold zone for different c values (\( c = 0.1 \) blank-dot-dot-blank, \( c = 0.2 \) dotted, \( c = 0.5 \) blank-blank, \( c = 0.9 \) blank).
On Figure 2.12, the PSF for a N.A. = 0.4 objective is shown. The ratio \( c = \Phi_{\text{threshold}} / \Phi_{\text{max}} \) of the optical breakdown threshold fluence \( \Phi_{\text{threshold}} \) and the maximum laser fluence \( \Phi_{\text{max}} \) determines the above threshold zone.

The PSF can be expressed for both lateral and axial resolution as [27]:

\[
h(r, z) \propto \int_0^1 r' J_0(r' \nu) e^{-0.5iu r'^2} dr'.
\]  

(2.23)

Here, \( J_0(r' \nu) \) is the Bessel function of first kind and zero order and \( r' \) is the normalized radius coordinate in the lens plane. Function \( u(r, z) \) represents optical setup. The optical coordinates \( u, \nu \) are introduced:

\[
u = \frac{2\pi a^2}{\lambda f} \left( \frac{1}{f} - \frac{1}{z_f} \right) \quad \text{and} \quad \nu = \frac{2\pi a r_f}{\lambda f},
\]

where \( a \) is the lens radius, \( f \) is the focal length, \( r_f = (x^2 + y^2)^{1/2} \) is the radius coordinate in the focal plane and \( z_f \) is the distance of the object plane from the lens plane.

This shows that using LIBS at a fluence close to the threshold limit one can obtain a higher lateral resolution in comparison to conventional or confocal microscopy techniques. Due to the relation between the lateral and the axial distribution of the PSF in respect to the above threshold zone, the same holds true for the axial resolution. The cigar-like contour lines shrink with \( \Phi_{\text{max}} \) approaching \( \Phi_{\text{threshold}} \) (see Fig. 2.12 b).

However, for this imaging mode, the demanded axial resolution of around 100 nm cannot be achieved, since unrealistic \( c \) values of around 0.9999 would be necessary. Small amplitude fluctuations of the laser or minimal changes in the threshold values would preclude reproducible results.

In order to achieve high axial resolution a different approach with lower \( c \) values can be employed, since the plasma formation is accompanied by ablation. The axial zone where a plasma is formed, is determined by the ablation depth. For example, if a structured thin layer has to be analyzed, the ablation depth of each laser shot has to be smaller than the layer thickness.

Assuming nearly constant plasma generation and ablation rate conditions, a precise spectrochemical measurement within each layer is possible. The axial resolution can be in the order of a few tens nm or less for technical materials [26]. In order to obtain a constant ablation volume for each laser pulse the above threshold area along the axial direction must not change significantly inside the layer. This focusing condition can be achieved by employing a laser fluence a few times higher than the threshold fluence. As illustrated on Fig. 2.12. b, for \( c = 0.2 \) the cigar-like contour line...
shows no dependence on the lateral coordinate in the axial range of \( \pm 2 \mu m \). Hence, the spatial resolution of the imaging-mode LIBS microprobing could be completely described by the ablated volume of the target material.

For \( \lambda=790 \text{ nm} \), N.A.:=0.4 and \( c=0.9 \), a threshold radius of \( r_{\text{threshold}}=200 \text{ nm} \) is obtained (see Figure 2.12 b). The Rayleigh criterion for a standard microscope or a confocal microscope results in a lateral resolution of 1.2 \( \mu m \) and 1.1 \( \mu m \), respectively [1, 18]. This comparison shows, that with the use of LIBS at fluences close to the threshold limit, a higher lateral resolution compared to conventional microscope techniques can be obtained.
2. Microscopy techniques

2.5. Conclusion

The basic principles of the plasma microscopy were discussed in this chapter in terms of the PSF function and spatial resolution. It was compared with the modern nonlinear microscopy techniques, based on the ultrashort pulse laser sources.

As it was discussed in this chapter, there is a large variety of microscopy techniques, designed for specific applications in dependence on the demands of the researchers. Conventional optical microscopes are used for general purposes, for magnification of the small-sized objects, also in combination with phase-contrast, polarization and fluorescence techniques. These are the most wide-spread and accessible microscopes, that are widely applied in modern science, medicine and industry.

Confocal microscopy with laser sources was a breakthrough in terms of the higher axial resolution. A 3-dimentional sectioning becomes possible. Nowadays, confocal microscopy is an important technique in the spatially resolved studies of the biological and technical samples. Raster sequence of the imaging could give a video-rated information. In combination with laser illumination, autofluorescence, or fluorescent labeling, confocal microscopy becomes an important tool for the study of the intra-cellular processes. The selective labeling of the important biochemical units allows to trace the single biomolecules within a living cell. This attracts a large number of researches to fluorescent confocal scanning microscopes.

There are several disadvantages of the fluorescence microscopy, which are ought to be mentioned. Selective structures staining is still problematic. Even though there are many dyes available, often they do not meet specific demands. Also photobleaching is significant in case of laser illumination. It takes place due to the high photon flux and low stability of the native dyes and stained structures.

The development of the ultrashort pulse lasers results in an increasing number of microscopy techniques, based on nonlinear optical phenomena. Lateral and axial resolution of the nonlinear microscopy techniques is not diffraction-limited. Nevertheless, high photon flux could cause a photobleaching and destruction of the targeting objects or dyes.

The approaches, in which the resolution does not depend on the illumination intensity, such as STED, are a subject of sharp interest in modern science. In this chapter it was shown, that imaging-mode LIBS microprobing is a technique, in which the
resolution is defined by the ablation volume only. This is the reason, why plasma microscopy can be related to the nonlinear microscopy techniques. The main advantage of this technique is a labeling-free possibility of the *in situ* spatially resolved trace element analysis without sample preparation.
3. LIBS - basic considerations

Introduction

Since their invention, lasers are used as an excitation sources for plasma generation in laser-induced breakdown spectroscopy [6-8]. In this chapter, the basic processes of the laser-induced breakdown spectroscopy are discussed in respect to our experiments with nanosecond and femtosecond lasers. LIBS can be considered as a sequence of the following phenomena: laser ionization of the target which leads to the breakdown, further laser-induced plasma (LIP) formation and evolution, and the spectral analysis of the plasma luminescence. The target is ionized by a tightly focused laser radiation through avalanche, multiphoton and tunnel ionization, or all three mechanisms. After overcoming the electron density threshold level, breakdown occurs and plasma formation takes place. Ablation of the target material is considered to be the direct consequence of the breakdown and LIP expansion. Target material particles, trapped by the plasma, are ionized and thus their ionic radiation can be spectrally analyzed.

In case of the UV nanosecond laser interaction with matter, linear absorption plays an important role. For ultrashort laser pulses, the linear absorption length is much larger, than the observed energy deposition zone during ablation. Under these conditions, the tissue can be treated as a transparent dielectric (important properties are ionization energy and electron scattering time) [13-16]. For this reason, transparent dielectrics and water can be used to represent biological material in basic studies, since they are well-characterized materials [12]. In our studies, water is used to investigate LIP processes and to find meantime parameters of the femtosecond and nanosecond laser induced plasma dynamics. The calculated breakdown thresholds for the avalanche and multiphoton ionization of the water are presented in this Chapter.

The ablation of the biological and technical samples by femtosecond laser in comparison with nanosecond laser is presented. The difference in the ablation parameters is demonstrated, basing on the studies, described in the literature.
3. LIBS – basic considerations

3.1. Generation of a laser-induced plasma

3.1.1. Fundamental parameter for plasma description.

In analogy to a gas, the basic parameters that are necessary to describe a plasma state are those concerning particle densities and particle motion. Compared with gas, however, plasma is a much more complex medium, where various important characteristic lengths and characteristic frequencies exist. The basic theory of plasma physics and plasma engineering is described in several sources [28-30]. The basic plasma parameters are described as follows:

**Density.** The density of each species in a plasma is the first important parameter, and among these species, the electron and ion densities are the most important. The number density \( n_j (j=e, i) \) rather than the mass density \( \rho (\equiv m_j n_j) \) is more commonly used, and this quantity is frequently called simply the density. The difference between ion and electron densities gives the space charge \( \sigma = e (n_i - n_e) \) (the ions are assumed to be singly ionized). Another important parameter is the plasma current density \( j = (n_i v_i - n_e v_e) \), where \( v_i \) and \( v_e \) are the average ion and electron velocities respectively. An additional important property is density of neutral particles. The plasma is generated and maintained by collisions of electrons with neutral species in the background gas. In these collisions, ionization and dissociation can lead to the formation of new particle species. Unstable neutral particles, called radicals, can be produced by these collisions, and the radical species often are extremely important in plasma processing applications. That is the reason for considering plasma as many-body system in the description of its properties.

**Temperature and velocity (energy) distribution function.** The most important parameter related to the particle transport and motion is the temperature. It should be noted, that there are many plasmas in which there is a large difference between the temperatures of the electrons and ions, and a large difference in their response to electromagnetic fields. This is due to the fact that the ions and electrons are not in energy equilibrium in many plasma conditions, especially for laser generated plasmas. In addition, although the rate of energy transfer is large for the case of electron-electron collisions and ion-ion collisions, the energy exchange in an electron-ion collision is of the order of \( m_e/m_i \). Because of this, there are many plasmas in which the electron and the ion groups are at different thermal equilibrium. In this case, two temperatures, called the electron temperature and the ion temperature are defined separately. If the number of collisions
inside the electron or ion groups is insufficient for thermal equilibrium, the velocity
distribution function will differ from a Maxwellian distribution [28].

As the laser-induced plasma is a non-equilibrium media [31], its dynamical
description is meeting serious complications. The local thermal equilibrium (LTE)
conception is used for the simplification of the model. It could be considered, that due to
the inelastic scattering processes of the electron gas with ions and atoms a so-called
local thermal equilibrium is reached. The time scale when after the LIP formation the
LTE is reached, depend on the laser pulse duration and intensity. Under LTE
conditions, a single temperature describes the various energy distribution functions:
electron velocity distribution, degree of ionization and the distribution of excited states
[32]. This holds true for the radiation distribution, which is described by a blackbody
relationship. In spite of the large spatial and temporal variation in the laser plasma, the
electron density and temperature are often high enough that fast electron collision rates
dominate over slower radiative rates to keep atoms and ions in a LTE condition. When
LTE is valid, then the emission from a particular atomic or ionic line is determined by
three parameters: the elemental concentration, the electron density, and the
temperature.

3.1.2 Ionization and breakdown

The initial stages of the laser-induced plasma generation are ionization and
subsequent breakdown. In this chapter the nanosecond and femtosecond laser pulse
induced ionization and breakdown are considered.

The case of tightly focused laser beam interaction with the media is a subject of
the research in many works [33-38]. At an irradiance above approximately $10^{10}$ W/cm$^2$
for nanosecond laser radiation and $10^{12}$ W/cm$^2$ for femtosecond laser radiation, plasma
formation occurs even in nominally transparent media [15, 39]. The ionization process is
called „optical breakdown“ when a critical free electron density $\rho_{cr} = 10^{18}$ cm$^{-3}$-$10^{21}$ cm$^{-3}$
is exceeded during the laser pulse [15]. There are two main mechanisms, which can
lead to plasma formation: avalanche ionization via inverse bremsstrahlung absorption or
direct ionization of the medium by multiphoton absorption [40, 41]. The balance
between avalanche ionization $I_a$ and multiphoton and tunnel ionization $I_m$ thresholds
strongly depends on the laser pulse duration $\tau$. It can be generalized for three main
cases as follows [16]:
„Long pulses“ – \( I_a < I_m \): Avalanche (cascade) ionization is the dominating mechanism. Avalanche ionization requires a few free electrons to be present in the focal volume at the beginning of the laser pulse. These „seed electrons“ for the ionization cascade can be generated either by heating of linearly absorbing impurities or by multiphoton ionization. In highly pure media, however, multiphoton ionization is needed for the creation of initial electrons, and the measured threshold \( I_{th} \) coincides with \( I_m \).

„Short pulses“ – \( I_a > I_m \): Multiphoton ionization contributes considerably to the creation of free electrons throughout the whole process of plasma formation. The measured threshold is an intermediate value between \( I_a \) and \( I_m \). The multiphoton ionization rate is proportional to \( I \), where \( I \) is the intensity of the laser beam and \( k \) is the number of photons required for ionization [33]. The value of the proportionality constant decreases with increasing \( k \), i.e., with increasing wavelength when more photons are needed to provide the energy necessary for ionization.

„Ultrashort pulses“ – \( I_a \gg I_m \): Multiphoton ionization is the dominating mechanism, because at the high irradiance values required for breakdown, the multiphoton ionization rate is much higher than cascade ionization rate. The measured threshold is given by the irradiance \( I_m \) rendering the critical electron density \( \rho_{cr} \) at the end of the laser pulse.

In our studies, second harmonic of the nanosecond Nd:YAG laser (\( \lambda = 532 \) nm) and infrared femtosecond laser (\( \lambda = 790 \) nm) are considered. At \( \lambda = 532 \) nm, 6 photons and at \( \lambda = 790 \) nm, 9 photons are required to ionize single water molecules having ionization energy of 12.6 eV, and the probability for the multiphoton ionization is therefore very small. Sacchi argued that one should not consider the ionization energy of single molecules, but treat liquid water as an amorphous semiconductor [42, 43] and consider the energy required for the excitation of electrons from the \( 1b_1 \) molecular orbital to an excitation band: 6.5 eV [44]. This approach yields a lower value of \( k \) (\( k = 5 \) for \( \lambda = 790 \) nm and \( k = 3 \) for \( \lambda = 532 \) nm), and thus a higher probability for multiphoton processes than formerly assumed. It has been supported by the results of Kennedy et. al. [45].

In 1995, Kennedy [46] extended Shen’s theory of avalanche ionization [47]. This leads to an expression for avalanche ionization irradiance \( I_a \):

\[
I_a = \left( \frac{mcn_0e_0E_{ion}}{e^2} \left( 1 + \frac{4\pi^2v^2}{\tau} \right) \right) \left[ g + \frac{2}{t_L} \ln \left( \frac{\rho_{cr}}{\rho_0} \right) \right] + \frac{m^2E_{ion}4\pi^2v^2cn_0e_0}{e^2M} . \tag{3.1}
\]
The three terms in this equation represent, in order, diffusion losses, carrier build-up through cascade ionization, and collisional energy losses. $E_{\text{ion}}=6.5$ eV is the ionization energy, $m$ and $e$ are the electron mass and charge, respectively, $\rho_0$ is the initial electron density provided by multiphoton ionization, $\tau$ is the mean free time between inelastic collisions between electrons and heavy particles. $M$ is the mass of a water molecule, $t_L$ is the laser pulse duration, $\nu$ is the frequency of the laser light, $n_0$ is the index of refraction of the medium at frequency $\nu$, $\varepsilon_0$ is the permeability of free space, $c$ is the vacuum velocity of light, and $g$ is the rate of electron losses due to recombination, trapping in solvated states, and diffusion out of the focal volume of the beam. The losses are small in condensed media for pulses of $10^{-8}$ s or less and at fields close to the breakdown threshold [47]. For breakdown, caused by pulses, longer than few tens of picoseconds, the source of the initial conduction-band electrons that seeds the avalanche ionization is very important. Avalanche ionization is very efficient for such pulses because of the long pulse duration, that allows more time for exponential growth of the electron density. Therefore, the laser intensity required to produce damage is not high enough for direct ionization of the electrons, so either thermally excited electrons or impurity and defect states provide the initial seed electrons for the avalanche. A high concentration of easily ionized impurity electrons lowers the threshold for optical damage, compared to that of the pure material, making determination of the breakdown threshold difficult [48].

A LIP development is strongly linked to the electron-neutral recombination rate. In our approximation we used the recombination rate value, obtained by Doccio [49], $2\times10^{-9}$ cm$^3$/s.

In work of Noak [50], the decrease of the electron density in the focal volume by electron diffusion is estimated by approximating the focal volume by a cylinder with radius $r_0$ and Rayleigh length $z_R$. This leads to the following expression for the diffusion rate per electron [46]:

$$g = \frac{x E_{\text{ion}}}{3m} \left[ \left( \frac{2.4}{r_0} \right)^2 + \left( \frac{1}{z_R} \right) \right].$$  \hspace{1cm} (3.2)

The calculated avalanche breakdown threshold for the nanosecond laser-induced breakdown in water is $7.9 \times 10^9$ W/cm$^2$ ($\lambda=532$ nm, $\tau=6$ ns; detailed calculations are presented in Appendix II).
For pulses, shorter than few picoseconds, the mechanism for optical damage is simpler than for longer laser pulses. Absorption occurs on a time scale that is short compared to the time scale for energy transfer to the lattice, decoupling the absorption and lattice heating processes [10]. Electrons in the conduction band are heated by the laser pulse much faster than they can relax. So with decreasing pulse duration, the irradiance $I$ must increase for the critical electron density to be reached during the shorter pulse duration. The avalanche ionization rate $\eta_a$ is proportional to the irradiance $I$ when electron losses are neglected: $\eta_a \propto I$ [47]. Since the multiphoton ionization rate has the much stronger irradiance dependence $\eta_{mp} \propto I^k$, multiphoton processes become even more important with decreasing pulse duration. It was pointed out [16,46], that the details of the interplay between cascade and multiphoton processes depend on the ratio of the threshold $I_a$ for the completion of the ionization cascade during the laser pulse and the threshold $I_m$ for the creation of an initial electron density $\rho_0$ by multiphoton ionization.

Basing on the work of Keldysh [33], Kennedy obtained the equation for multiphoton and tunnel ionization irradiance $I_m$ [46]:

$$I_m = \frac{2}{B} \left( \frac{\rho_{omin}}{\Delta t A} \right)^{1/k},$$

(3.3)

where $\rho_{omin}$ is the minimal initial density of free electrons required to start an ionization cascade that leads to $\rho_{cr}$ at the end of the pulse, and $\Delta t$ is the time required for the generation of $\rho_{omin}$. $A$ and $B$ are constants which depend on the breakdown medium and the laser wavelength. Calculated breakdown threshold for multiphoton ionization by femtosecond laser radiation for our experimental conditions is $1.5 \times 10^{13}$ W/cm$^2$ (detailed calculations are presented in Appendix III).

The initial stage of the plasma formation is a subject of several experimental studies and theoretical simulations [12, 15, 16, 51, 52], in which the detailed descriptions are given for different pulse durations and laser pulse parameters.

The contribution of the multiphoton and avalanche ionization pathways to the electron density evolution during the ultrashort laser pulse duration is presented on Figure 3.1 [10]. It was found, that multiphoton and avalanche ionization, both take place during femtosecond laser induced plasma formation.
3. LIBS – basic considerations

Figure 3.1. The evolution of the electron density through multiphoton and cascade ionization during ultrashort laser pulse [10].

In the case presented on Figure 3.1, there is a strong contribution of the avalanche ionization to overall electron density. According to Kaiser et. al., the contribution of the avalanche processes in the subpicosecond pulse ionization is strongly overestimated. It was found that multiphoton and tunnel ionization are the main sources of free electrons in the femtosecond laser-induced breakdown formation [52].

3.1.3. Plasma expansion

After the ionization rate exceeds the limiting value and breakdown occurs, plasma starts to form. Laser-induced plasma evolution is a widely studied topic. There is a number of review works, devoted to study of laser-induced plasmas evolution with application of modern laser sources [53, 54]. Ablation of the target is considered here, as a consequence of the breakdown, plasma formation and expansion.

Nanosecond LIP evolution is considered in several works [31, 32, 49, 55-61]. The evolution of the femtosecond laser induced plasma is discussed in the following works [49, 60, 62-65]. For modeling of the ultrashort laser pulse ablation of solid aluminum and the subsequent plasma expansion in ambient air, Vidal et. al. [66] used a self-consistent one-dimensional Cartesian Lagrangian fluid code [67]. Saha-Boltzman and Thomas-Fermi models are implemented to this code in order to describe the electron temperature and density during the early stage of the LIP formation.

The electron temperature and density in the plasma are considered to be sufficient to create LTE plasma. This is a plasma in which Saha and Boltzman equations hold on for the number densities of the plasma constituents. The amount of ionized particles in LTE could be then described by Saha equation as:
3. LIBS – basic considerations

\[ \frac{n_i}{n_n} \approx 2.4 \times 10^{21} \frac{T^{3/2}}{n_i} e^{-U_i/kT}, \quad (3.4) \]

where \( n_i \) and \( n_n \) are, respectively, the density of ionized atoms and neutral atoms, \( T \) is the gas temperature, \( K \) is Boltzman’s constant, and \( U_i \) is the ionization energy of the media. This model is called Saha-Boltzman model. In greater details its application for plasma analysis is considered by Yalcin [32].

The second model, used for the electron dynamics description is Thomas-Fermi model. It requires Fermi-Dirac distribution:

\[ f(u) = \frac{1}{1 + e^{\beta(e-\mu)}}, \quad (3.5) \]

where \( \mu \) is a chemical potential. The energy \( \varepsilon \) is related to the number of electrons in elementary \( \Delta N \) cell through the formula:

\[ \Delta N = \frac{8\pi}{3} \left( \frac{2m_\varepsilon}{\hbar^2} \right)^{3/2} l^4 \varepsilon^{3/2}, \quad (3.6) \]

where \( l \) is the side of the elementary cell, \( \varepsilon \) – Fermi energy and \( m_\varepsilon \) – electron mass.

![Figure 3.2. An average plasma density as a function of time for a laser fluence 10 J/cm², pulse duration 100 fs, \( \lambda = 800 \) nm. Simulation and experiment [66]](image)

The description of the electron density evolution during LIP expansion by means of Saha-Boltzman and Thomas-Fermi models was done by Vidal et al [66]. The graphical plot of the electron density evolution within time is presented on Figure 3.2. The experimental measurement of the electron density was done by the analysis of the emission lines ( Al II at 281.62 nm and Mg I at 285. 21 nm) employing the Stark broadening formula (3.8). This figure shows, that the best coincidence of the numerical simulation with experiment is on the early stage of the plasma expansion, when the electron densities are high. This is related to the fact, that particles density is fast
relaxed by plasma spatial expansion and recombination. Higher electron densities in the beginning of the process are due to the fact, that model calculations are considering the total laser pulse energy transfer to the lattice. Other studies [68, 69] are showing, that plasma reflection of the initial laser pulse plays an important role in the energy distribution during the plasma formation, even considering femtosecond laser pulses. This will be discussed in more details in Chapter 3.2.2.

In a number of works, LIP expansion dynamics in different ambient media is studied in greater details [32, 55, 70-72]. This works present the main dynamic conformities of the LIP expansion and show the significant influence of the ambient media parameters on the plasma relaxation process.

3.1.4. Laser-mediated material removal - ablation

The process of the material removal by means of the tightly focused intense laser beam is named ablation. The material must undergo a change of the fundamental state of aggregation and transform into some volatile phase, e.g., a gas or a plasma. After that material is removed from the target by a gas flow or plasma expansion.

Recent years, a number of studies, devoted to the phase transitions during the ablation with laser pulses of the nanosecond and femtosecond duration were performed [66, 73-76]. The transition from the solid phase to the gas phase can occur in a stepwise fashion by the melting of the solid and evaporation of the liquid. Boiling occurs when the vapor pressure of the liquid phase exceeds the ambient pressure. Boiling rates become very high when the temperature of the liquid approaches to the critical temperature. The latter case is sometimes referred to as “phase explosion” [77]. Under certain conditions, sublimation may occur, i.e., a direct transition between the solid phase and the gas phase. Otherwise, a direct transition to a liquid phase can be accomplished by very rapid heating of solid matter to temperatures higher than the critical point. Finally, laser radiation of sufficiently high intensity leads to ionization and transforms solid material into dense plasma [78]. When the laser fluence is kept constant while the pulse duration is decreased, the optical electric field strength will, at some point, exceed the breakdown threshold of the material. Thus, plasma formation is expected to become the dominant mode of laser ablation when the pulse duration is made shorter and shorter. Nevertheless, several works show, that ablation takes place earlier, then the onset of plasma formation [79, 80]. This is supported by several theoretical works [81-83], which are showing, that defragmentation and ablation of the material by ultrashort laser pulses start within 10th of picoseconds after the breakdown.
Also it is shown that firstly the lighter particles – ions are ejected and later – heavier neutrals and later molecules and clusters.

As it was discussed before, the laser induced ionization mechanisms vary for different laser pulse durations. Pulse durations between nanosecond, picosecond and subpicosecond pulses are considered [84-86]. This separation is related to different mechanisms of the laser pulse energy transfer to the lattice. For pulse durations longer than a few tens of picoseconds, energy is transferred from the laser-excited electrons to the lattice within the pulse duration. According to Stuart et. al. [10], energy is deposited into the material by the laser pulse and is transported out of the irradiated region by thermal diffusion, thus it is the relative rate of energy deposition and thermal diffusion that determines the damage threshold.

For the pulses with duration of the single picoseconds and shorter, the energy deposition mechanism is different. The ultrashort laser pulse energy is deposited into the sample more efficiently [12], hence, less energy is required for the ablation and the whole process is faster. Thus, the thermal damage could be neglected, what is very important for the processing of the biological samples.

The comparison of the ablation studies of the human dental enamel is presented on Figure 3.3 by Serbin [88]. The left picture represents the ablation by the 400 µJ, 800 nm laser with 700 fs pulse duration. On the right picture the ablation by 1.6 mJ Er:YAG laser with wavelength 2940 nm and pulse duration 50 ns is shown. In case of nanosecond laser ablation, the crater size is larger. It shows, that a significant amount of the laser pulse energy is converted into mechanical energy of the shock wave. The conical form of the nanosecond ablation crater indicates, that the energy deposition is not precise. This effect is not observed in case of the femtosecond laser ablation, when the crater has a cylindrical form.

Figure 3.3. The ablation of the dental enamel. Scanning electron microscope pictures [87]: 400 µJ, 800 nm, 700 fs Ti:Sa laser (left picture); 1.6 mJ, 2940 nm, 50 ns Er:YAG laser (right picture).
Figure 3.4. represents pictures of the glass sample ablation by 5 mJ femtosecond laser pulses (wavelength 800 nm, pulse duration 200 fs) and 15 mJ nanosecond laser (wavelength 532 nm, pulse duration 6 ns) [88]. Femtosecond laser produces an oval ablation crater. Melting products spreading around the ablation zone are forming the white dotted field. Nanosecond laser produces no particular ablation crater on the glass surface. Significant cracking structure indicates large damage of the sample.

The precise ablation rates and micromachining with ultrashort pulses are a subjects of multiple studies [11, 89-94]. Comparison of the ablation by femtosecond and nanosecond lasers shows, that femtosecond laser offers more precise ablation with significantly less mechanical damage of the sample. This makes femtosecond lasers very attractive for imaging-mode LIBS applications, as they offer a reproducible ablation volume.
3.2. LIP Spectral analysis

3.2.1. Plasma radiation

In dependence on the radiation mechanism, plasma radiation could be subdivided into discrete and continuous recombinational and bremsstrahlung radiations. Discrete radiation is the result of the transition of electron to the atom from one energy level to another. Recombinational radiation is emitted as a result of the trapping of a free electron by an ion with charge $Z$, with formation of the lower charged ion. Bremsstrahlung radiation has a continuous spectrum. In a plasma with temperature $T$, a spectral line corresponding to a transition from energy level $l$ to energy level $m$ has intensity, $I_{lm}$, which is given by [28]:

$$I_{lm} = \frac{A_{lm}}{U g_i} h \nu_{lm} n \exp\left(-\frac{E_i}{KT}\right).$$

(3.7)

Here, $A_{lm}$ is the transition probability, $g_i$ is the degeneracy of the upper level, $U$ is the internal partition function for the emitting species, $\nu_{lm}$ is the frequency of the emitted radiation, $n$ is the density of the emitting species and $E_i$ is the energy of the upper level. Using this formula the relative temperature changes during the plasma evolution could be estimated.

More specific information could be obtained, analyzing the recombination lines. The Stark broadening of the lines could be analyzed using the formula:

$$\Delta \lambda_{1/2} = 2W n_e / 10^{16}$$

(3.8)

where $\Delta \lambda_{1/2}$ is a full width at half maximum of the spectral line (FWHM) in angstroms, $n_e$ is an electron density in cm$^{-3}$ and $W$ – electron impact parameter in angstroms [63, 95].

3.2.2. Energy balance during laser-induced breakdown

During the optical breakdown and plasma development, the energy delivered to the sample is transmitted, scattered, absorbed or reflected. The dependence of partition of the laser energy on the laser pulse duration is discussed in several studies [51, 96]. Pathways for the division of the absorbed energy are: the evaporation of the focal volume, the plasma radiation, and the mechanical effect such as shock wave emission and cavitation bubble formation. The experimental study of the energy distribution pathway is done by Vogel et. al. [38], where different energy pathways are considered.
for the laser-induced breakdown in the water sample. The result of the measurement is represented on Figure 3.5.

Figure 3.5. Energy balance for selected laser parameters. \( \lambda = 1064 \text{ nm}, \tau = 6 \text{ ns} \) (a, b) and \( \tau = 30 \text{ ps} \) (c, d). \( R, S, T \) and \( A \) denote plasma reflection, scattering, transmission, and absorption. The absorbed energy is divided into shock wave energy \( E_{S} \), bubble energy \( E_{B} \), evaporation energy \( E_{V} \) and energy of the plasma radiation \( E_{R} \). The difference of the complete energy balance to 100% is denoted by “?”.

The energy balances of the laser-induced breakdown in water for 6 ns laser pulses with energies 1 and 10 mJ are presented on Figure 3.5 a) and b) respectively. The energy balances of the LIB in water for 6 ps laser pulses with 50 µJ and 1 mJ energies are presented on Figure 5.3 c) and d) respectively. The conversion of light energy into mechanical energy by means of optical breakdown is larger than with any other laser-material interaction. It reaches up to 90% at 6 ns pulse duration (Figure 3.5, a, b). The effective conversion of light energy into mechanical energy is the cause of the disruptive character of laser ablation. Due to high fluences, reached at large focusing angles, short and highly absorbing plasmas are achieved. This allow a well-localized energy deposition at a low breakdown threshold. Large focusing angles are, however, also associated with high conversion efficiency into mechanical energy and therefore with a large potential for mechanically induced side effects. The mechanical effects can be
3. LIBS – basic considerations

dramatically diminished by shortening the laser pulse duration. A reduction of the pulse
duration from 6 ns to 100 fs is accompanied by a decrease of \((E_{\text{mech}}/E_v)\) ratio from 12:1
to 1:2 (where \(E_{\text{mech}}\) is a mechanical energy and \(E_v\) is evaporation energy). At the same
time, the efficiency of energy deposition decreases from >90% to ~50%, but this
decrease imposes no severe practical limitations on the applicability of femtosecond
pulses.

3.2.3. Laser-plasma interaction

The tightly focused laser light, which causes the breakdown and plasma formation is
interacting with plasma. As the plasma is forming and the electron density increases,
the laser light is reflected and scattered back from the focal area. On the later stage of
the plasma formation, with increasing of the electron density, the reflection, absorption
and scattering of the entire laser radiation play an increasing role [61].

Plasma shielding effect during nanosecond LIBS of the metallic samples in
greater details was studied by Aguilera et. al. [68]. In the nanosecond LIP a part of the
laser pulse energy is absorbed by plasma. It allows to obtain better results in material
processing as well as to increase the recombination lines intensity [32,62]. For the
shorter pulse durations, the second pulse and further pulse trains could be used to
increase the plasma and to decrease the breakdown threshold [97-101].
3.3 LIBS and applications

Laser-induced breakdown spectroscopy is a method, known since decades [6-9]. With the invention of the first laser sources LIBS is applied for spectrochemical analysis. The principle of the method is presented on Figure 3.6. A tightly focused laser light causes a breakdown on the surface of the target. The sparkling light is collected by a lens or objective and spectrally analyzed. The position of the spectral line gives an information about the type of the element; and its intensity - about the concentration of this element.

![Figure 3.6. Principles of laser-induced breakdown spectroscopy technique.](image)

This method is widely applied in different areas of science. Several works are devoted to the environmental applications of the LIBS technique [102-104]. A certain amount of works is devoted to the application of LIBS for the alloys and coatings quality analysis [105-106]. Recent time laser-induced breakdown spectroscopy becomes a tool for online control of chemical reactions and technological processes [107-110]. Medical applications are mainly presented by the teeth carious determination on early stage and trace elements analysis [111-112]. The new application presented in the literature is LIBS controlled marble and ancient manuscripts cleaning [113-114].
3.4 Conclusion

The basic processes related to the laser-induced breakdown spectroscopy are discussed in this chapter: ionization with the following breakdown, plasma formation and expansion, ablation and plasma emission spectral analysis.

The breakdown formation is a widely studied process, which strongly depends on the initial laser pulse duration. There are three main mechanisms distinguished: linear avalanche ionization, nonlinear multiphoton and tunnel ionization. The first mechanism is dominating at the laser pulse durations of hundreds of picoseconds and longer. For the shorter pulses the multiphoton and tunnel ionization become dominating mechanisms. The energy distribution during the breakdown is important for the laser material processing and applications of biological materials processing.

The plasma formation and expansion are complicated processes, which could be approximately described by hydrodynamic mathematical codes. Plasma expansion and ablation involve a certain amount of particles of the target and it is possible to observe the spectral lines of the ionized species. Proper spectral analysis of the recombination lines becomes possible on the later stages of plasma formation. In dependence on the laser fluence, plasma luminescence lifetime is typically hundreds of nanoseconds for nanosecond laser and tens of nanoseconds for femtosecond LIP.

The spectral analysis of the LIP sparking gives information about the element components of the analyzed sample. The position of the spectral lines gives information about the energy levels of the species and their intensity – about the elements’ concentrations. The analysis of the line width can be used to estimate the electron density.

Comparison of the nanosecond and femtosecond laser ablation, based on the literature study is done in this chapter. Human dentine and glass samples are considered. It is demonstrated, that femtosecond laser ablation offers a high precision and low mechanical damage of the sample in comparison to nanosecond laser ablation. This makes femtosecond laser an attractive tool for the application to LIBS investigations of biological samples.
4. Nanosecond LIBS experiments and results

Introduction

The investigation of plasma formation and Ca$^{2+}$ ion analysis in water by means of nanosecond LIBS is presented in this chapter. Water is used, because it can be considered as a model system of biological samples. Ca$^{2+}$ ion concentration in the water stream could be easily changed by varying the CaCl$_2$ salt concentration, which is important for calibration purposes. It is simple, inexpensive and thus, favorable solution for the constantly iterated source of the Ca$^{2+}$ ions.

The experimental study, presented in this chapter, is done in order to identify the specific Ca$^+$ recombination lines and to choose a preferable spectral region for further studies. The basic parameters of the nanosecond LIP, such as plasma luminescence and recombination lines lifetimes, sensitivity and calibration curves are measured. The camera gate detection delay for the optimal signal/background ratio measurement is determined.
4. Nanosecond LIBS

4.1. Experimental setup for preliminary experiments

4.1.1. Nanosecond laser setup for lines identification

Nd:YAG laser with internal second harmonic conversion (Quanta Ray INDI, Spectra Physics, USA) is used in this experiment. The laser pulse duration is 6 ns and wavelength - 532 nm. Its repetition rate is 20 Hz with external triggering and single shot modes option. The nanosecond laser radiation is guided to the focusing lens L1 (Quartz glass, biconvex, d=19 mm, f= 50 mm Linos Photonics GmbH, Germany) by the prism P1 (Quartz glass, Linos Photonics GmbH, Germany), which directs the radiation to the interaction zone. The scheme of the experimental setup is presented on Figure 4.1.

![Figure 4.1. The scheme of the nanosecond LIBS setup, used for Ca$^{2+}$ spectral lines identification.](image)

Figure 4.1. The scheme of the nanosecond LIBS setup, used for Ca$^{2+}$ spectral lines identification.

A water stream WS is used as a target. The defined CaCl$_2$ salt concentration in water is used for Ca$^{2+}$ ion spectral lines identification and detection system calibration. The water from 1 liter tank is pumped with a speed of 0.8 liter/minute through a nozzle with 1 mm$^2$ square to supply a stream. Plasma luminescence is imaged on the entrance slit of the monochromator by a telescope, consisting of two lenses L2 (d=60 mm, f=100 mm) and L3 (Quartz glass, d=60 mm F=200 mm). The monochromator (Type LTI Serie 01-002, Amko, Germany; 1200 g/mm grating, blazed at 250 nm; 125 µm slit; resolution 0.55 nm) has a photomultiplier (PMT) detection by Photon Count Detector Amko 2TI Mod. 08-15 with PMT Hamamatsu R928P (Hamamatsu Inc, Japan).
4.1.2. Triggering and data acquisition

The nanosecond LIBS exhibits a dynamic in the nanosecond time region and the whole event lasts up to several microseconds. For the detailed study of the plasma dynamics, the time resolved spectroscopy is important. For this purpose, triggering system is developed, as presented on Figure 4.2. The electronic signal, used to start the system is obtained from the photodiode, shown on the Figure 4.1 as PD. This photodiode is generates the start signal for the triggering system. The photodiode signal is coupled to the trigger input of the digital Oscilloscope (LeCroy 9372M 1GHz). The oscilloscope generates a TTL signal on the Extemal output. This signal is used as a trigger for the delay generator (SRS DG 535, Stanford Research, USA).

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![Figure 4.2. The scheme of the triggering and data acquisition for the line identification experiment.](image)

The delay generator produces an adjustable output trigger delay between the laser pulse and the moment, when the data processing starts. The PMT signal acquisition is provided with the Boxcar-Integrator SRS-250 (Stanford research, USA). The width of the boxcar gate is set manually. To supply an additional delay of 130 ns, the 30 m cable is plugged between PMT and the boxcar as a delay line. The delay generator is controlled by a computer via GPIB. The signal and the gate outputs of the boxcar are connected to the oscilloscope for the monitoring.

The typical signal outlay on the oscilloscope is presented on Figure 4.3. On this figure, the nanosecond laser pulse profile is presented, as it is measured by the photodiode and PMT. Also, the 180 ns boxcar gate, used to sample the plasma luminescence is presented. The plot shows, that right after the laser pulse, measured by a photodiode, strong plasma emission is detected by PMT. Local peak is seen on the PMT signal at a delay of 250 ns. It is related to the noise of the PMT data acquisition electronics.
4. Nanosecond LIBS

The LIBS signal for the spectrochemical analysis of the plasma is studied at a delay of approximately 250 ns, as it will be shown later. At this time delay spectral lines are detectable.

4.1.3. Ca$^{2+}$ ion lines identification

The identification of the plasma and recombination lines luminescence in nanosecond LIP is the goal of this investigations stage. Ca$^{2+}$ ion recombination lines are studied on the model - CaCl$_2$ salt solution in water. To estimate the possible background signal, the reference LIBS emission spectra of the distilled water is measured. Then the salt solution is loaded into the pump system of the water stream in the concentration, equal to one expected in the peripheral cell wall – the biological objects to be studied later. This value is in the range of 10-100 mmol/l. The comparison of the measured spectra with the reference spectra gives the characteristic lines of the dissolved substance.

For this experiment, the laser energy is set to 60 mJ at $\lambda$=532 nm. The 150 ns boxcar gate is set 150 ns after the laser pulse. The spectral area around 532 nm is not measured, to protect the PMT from the scattered laser light. The salt concentration in the water stream system is 25 mmol/l. The measurement is done in the spectral area from 300 to 900 nm with steps of 1 nm. The PMT current is 950 V. The integration is done over 10 pulses per point. Resulting spectra are presented on Figure 4.4.
Peaks at 820 nm, 745 nm and 482 nm are related to the emission of N and the peak at 775 nm is related to N⁺. Line at 654 nm is related to O⁺. The lines identification is done by comparison of the obtained spectral lines with the atomic spectra database (National Institute of Standards and Technology - NIST) [115]. In the spectral area around 400 nm the difference between two spectra is detected. This region of interest is studied with a higher spectral resolution for the detailed lines identification. Spectral range from 385 nm to 410 nm is studied with 0.1 nm steps resolution, at 30 mJ pulse energy and 700 V PMT current. The result is presented on Figure 4.5.

On the plasma emission spectra of the CaCl₂ water solution, three peaks are located at 391.9 nm, 395.5 nm and 398.3 nm. 398.3 nm peak is reproduced in the plasma
emission measurement of distilled water. The spectra database [115] shows transitions of Calcium and Nitrogen in this area:

<table>
<thead>
<tr>
<th>Measured wavelength [nm]</th>
<th>NIST data [nm]</th>
<th>Transition</th>
<th>J_i-J_k</th>
</tr>
</thead>
<tbody>
<tr>
<td>391.9</td>
<td>Ca⁺: 393.3663</td>
<td>4s-4p</td>
<td>1/2-3/2</td>
</tr>
<tr>
<td>395.5</td>
<td>Ca⁺: 396.8469</td>
<td>4s-4p</td>
<td>1/2-1/2</td>
</tr>
<tr>
<td>398.3</td>
<td>N⁺: 399.4997</td>
<td>3s-3p</td>
<td>1-2</td>
</tr>
<tr>
<td>Ca: 422.6728</td>
<td>4s²-4s4p</td>
<td>0-1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.6. The lines identification according to NIST spectra database [115].

The atomic transition of Ca is detected in the later experiments.

There is a 1.3 nm difference between the measured wavelengths of the transitions and the NIST database spectra, which is explained by the different position of the calibration Hg-Ne lamp and plasma sparkling. Due to the long basis of the collection telescope, even the small misallocation of the point light source in the focal position causes significant shift of the image on the entrance slit of the spectrometer.

Figure 4.7. The transition scheme of the Ca ion and atom lines, used in our study.

Calcium transition scheme is presented on Figure 4.7. It shows two ion transitions at 393.4 nm and 396.8 nm and an atomic transition at 422.7 nm, which is not detected in the preliminary experiment, but is measured in the following experiments with nanosecond and particularly with femtosecond lasers.
4.2. Nanosecond laser setup with ICCD camera detection

4.2.1. Setup description

The LIBS investigation of the biological samples requires a single-shot operation mode of the LIBS setup. For this purpose, the setup with ICCD detection represented on Figure 4.8, is developed. The spectral resolution of this setup is estimated as 0.5 nm [116]. With this setup it is possible to measure the whole spectral region of interest within one laser shot.

![Figure 4.8. Nanosecond LIBS with ICCD detection for detailed study of the nanosecond LIP properties.](image)

The same Nd:YAG laser is used, as in previous setup, described in Chapter 4.1. The laser light is directed to the interaction area by the dielectric mirror M1 (Linos Photonics DLHS $\lambda=532$ nm $d=25$ mm) and is focused by lens L1 (semispherical lens $f=20$ mm, $d=15$ mm) on the water stream. The emitted plasma luminescence is collected by the same lens and transmitted through the mirror M1 (transmission around 395 nm $>70\%$). The zoom objective (Nikon UV-NIKKOR 105 mm 1:4.5, Nikon Corp, Japan) collects and focuses the plasma radiation on the entrance slit (280 µm) of the spectrometer (L.O.T. Multispec with grating Nr. 77411; 1200 L/mm, blazed on 350 nm, LOT-Oriel, USA). The spectrometer transmits the spectral area from 350 nm to 450 nm on the ICCD chip. The spectra is recorded by ICCD camera (La Vision Flame-Star, La Vision, Germany). The nitrogen gas jet is used to protect the focusing lens from the water droplets, occurring due to the breakdown. The spectral calibration of the system is provided with the Hg-Ne lamp (LOT Oriel Pen-Ray Mercury-Neon, LOT-Oriel, USA).

The spectral data, represented later are deconvoluted with the transmission data of the dichroic mirror M1 (Appendix IV).
4.2.2 Triggering system

The triggering scheme of the setup is presented on Figure 4.9. The photodiode PD is used for the energy measurement. It is connected to the boxcar integrator (SRS 250). Its output signal is proportional to the laser pulse energy. Using data acquisition card (NI AT-MIO16XE-50, National Instruments, USA) the analog signal is converted to digital form and processed by the software [117]. The Nd:YAG laser is switched to the external trigger mode. Flash lamps and Q-switch are driven from delay generator (SRS DG 535), controlled by the computer via GPIB connection.

The output $T_0$ of the delay generator is used to trigger the laser. Output A of the delay generator is connected to the External Q-switch input of the laser and output B of the delay generator is connected to the Trigger input of the intensifier controller of the ICCD camera. The laser trigger (output $T_0$) is additionally connected to the camera control computer. The computer requires several hundreds microseconds to produce a pretrigger for the activation of the intensifier. The Q-switch delay of 230 µs is found to be optimal.

4.2.3. Nanosecond plasma dynamics measurement

This measurements are performed in order to identify the time delay after the plasma sparkling, when the plasma background is low, but the spectral lines are still high enough to be detected i.e. a time delay with the best signal/background ratio. The water stream system is loaded with CaCl$_2$ salt in concentration of 115 mmol/l. Original Lab View software [117] is used to scan the delay of delay generator (SRS DG 535) and to acquire the spectral data. The wavelength calibration of the ICCD system is done with the Hg-Ne lamp. The delay generator is programmed for the internal triggering rate of 10 Hz. The delay of 230 µs is supplied from an output A to a Q-switch trigger and 230
μs + t from an output B to the camera. The delay t is automatically scanned from 0 to 1200 ns with the 50 ns steps. 20 spectra are taken for each delay position. The photodiode is used to control the laser energy and the boxcar integrator is used to acquire the photodiode current. The experiment is performed with different laser energies and time resolution, to find the optimal parameters for this study. This data are measured with the 17 mJ pulse energy at a range of t=−100 to 850 ns. The laser pulse generates the plasma at time t=0. The gate of the camera intensifier is 50 ns. 20 spectral profiles are taken for each delay and the 5 most intense spectra are chosen and averaged. The integration of the spectral range of 392 to 397 nm is used by a software for this selection. This is done due to the unstable water stream surface, which causes the spatial instabilities of the plasma. Lines are shifted on 1.5 nm to the violet side, comparing to NIST data [115]. On Figure 4.10 the 3D plot of the temporal and spectral behavior of the plasma is presented.

![Figure 4.10. Spectrally resolved temporal dynamics of the nanosecond LIP.](image)

Figure 4.10 shows strong thermal radiation in the beginning of the process, almost uniformly distributed over the spectral range. 100 ns after the t=0 the Calcium lines at 393.4 nm and 396.8 nm are detectable. Also the nitrogen line at 399.5 nm, coming from the ambient air, is seen. On Figure 4.10 it is seen, that spectral lines intensity is decreasing, but spectrally broad plasma background radiation damps faster. After approximately 200 ns the weak signal of the Ca atomic line at 422.7 nm is detected.

The integration within a spectral region is used for the analysis of the plots. The spectral window for the background radiation is chosen in area from 404 nm to 407 nm.
4. Nanosecond LIBS

There are no spectral lines in this region (s. Figure 4.10.). The analysis of the spectral lines and background temporal behavior is presented on Figure 4.11. Here, the Ca\(^+\) ion line at 393.4 nm is analyzed in respect to the background. The second Ca\(^+\) ion line demonstrates the same temporal behavior and is not presented on the plot. The error bars represent the standard deviation from the average value.

![Figure 4.11. Plasma (squares) and calcium (circles) luminescence decay plot.](image)

Within a 6 ns pulse duration the plasma appears and starts to expand. At 80 ns the spectral line of the Ca\(^{2+}\) ion appears. It decays slower in comparison to the plasma background. Only after 800 ns the luminescence intensities of the Calcium line and plasma background become equal.

The analysis of the signal/background ratio is presented on Figure 4.12. It is done by the division of the signal and background values. These values are obtained by integration of the respective spectral regions, as it is described above. The maximum values of signal/background ratio are located at a time period after the plasma luminescence decays. For 17 mJ energy, the maximum signal/background ratio is located at 425 ns and equals 24. The signal/background ratio decreases to a value around 1 at 850 ns.
The dependence of the optimal signal/background ratio from the laser pulse energy is presented on Figure 4.13. It is shown, that with the increase of the pulse energy, the plasma luminescence lifetime increases. This results in the later position of the point with maximal signal/background ratio. The maximal signal/background ratio varies from 100 ns at 6 mJ up to 1000 ns at 45 mJ. At the laser pulse energy of 45 mJ, the calcium lines are still observed even at 1500 ns.

In order to present the plasma emission dynamics, the decay of the plasma luminescence is analyzed according to the following formulas:

\[ \ln(P - P_i) = \beta_i t + \alpha_i \]  \hspace{1cm} (4.1) \\
\[ \bar{P} = e^{\beta_i + \alpha_i} \]  \hspace{1cm} (4.2)

Where \( P \) is an integrated plasma luminescence signal and \( P_0 \) is a constant offset. The lifetime of the plasma luminescence \( \tau \) can be then calculated as:
The same analysis is performed for the Ca$^{2+}$ ion line signal. The Calcium line luminescence signal is treated in analogy to the plasma background emission signal. The constant value $L_0$ is subtracted from the line radiation intensity $L$. The data are normalized in order to compare the measurements with different signal intensities.

$$\ln(L - L_0 - \gamma) = \beta_2 t + \alpha_2$$  \hspace{1cm} (4.4)

$$\tau_2 = -\frac{\ln 2}{\beta_2}$$  \hspace{1cm} (4.5)

Figure 4.14 (a, b, c) represents the Ca$^+$ ion line (391.9 nm - 394.9 nm) and plasma background (404.0 nm - 407.0 nm) luminescence as a function of time for different laser pulse energies. The error bars are showing the standard deviation from the average meaning over 10 measurements. Plasma luminescence lifetime, as well as Ca$^+$ ion emission lines lifetime grow with increase of laser pulse energy.

On Figure 4.14 a it is shown, that at 40 mJ laser pulse energy, the plasma background luminescence decays at 300 ns, whereas Calcium line luminescence is detectable even after 950 ns. At 17 mJ laser pulse energy (Figure 4.14. b), the plasma luminescence time is almost the same, as in previous case, but Calcium line emission is detected only up to 800 ns. At 6 mJ laser pulse energy, the plasma luminescence completely decays at 150 ns and the Calcium line emission is detected up to 450 ns.

Some oscillations of the Ca$^+$ ion lines intensity could be observed at a 150 ns time delay for 6 mJ pulses and 400 ns for 40 mJ pulses. This intensity oscillations could be related to the temperature and density instabilities. These oscillations depend on laser pulse energy and their nature is not determined in the literature [66]. Other studies relate this kind of oscillations to the non-uniform plasma expansion [59, 118].
Figure 4.14. Temporal behavior of calcium lines and plasma background luminescence: circles – Calcium signal (391.9-394.9 nm), squares – plasma background (404.0-407.0 nm).
4.3. Conclusion

The investigation of the nanosecond laser-induced breakdown on the water sample was described in this chapter. This study was done in order to check the possibility of the LIBS detection of the Ca$^{2+}$ ions in water and to determine the important parameters of the detection system.

Ca$^{2+}$ ion emission lines were identified in the measured LIBS spectra. The spectral area between 380 nm and 430 nm was determined for the Calcium detection. Time resolved spectra of the plasma luminescence were obtained for several laser pulse energies. The Calcium lines emission was detected after the thermalization of the plasma. Analyzing these data, the decay parameters of plasma and Calcium lines luminescence were obtained. The plasma luminescence decays within several hundreds of nanoseconds, whereas at higher laser pulse energies, Ca$^+$ ion signal can be detected after a microsecond. The dependence of the maximal signal/background ratio from the laser pulse energy was presented. Optimal detection time with maximal signal/background ratio was defined after the decay of the plasma background emission. The maximal signal/background ratio delay varies from 100 ns at 6 mJ up to 1000 ns at 45 mJ. A maximal signal/background ratio equals 25 for the 17 mJ pulse energy.
5. Femtosecond LIBS experiments and results

Introduction

In this chapter, femtosecond LIBS studies on the model system – water with Calcium salt solution are described. These experiments are carried out in order to check the possibility of the Ca\(^{2+}\) ion detection in water by means of LIBS. Time-resolved spectra are measured in order to study the laser-induced breakdown with femtosecond laser excitation. Basing on the obtained data, imaging-mode LIBS setup – plasma microscope is developed. Its adjustment procedure and measurement of the sensitivity are described. The time-resolved LIP spectra are obtained and analyzed in order to define optimal detection parameters for the experiments on the biological objects. The sensitivity of the Ca\(^{2+}\) ion detection by means of plasma microscope is determined. The ablation studies on the glass samples are presented and discussed.
5. Femtosecond LIBS experiments and results

5.1. Preliminary experiments

5.1.1. Laser system description

The femtosecond Ti:Sapphire oscillator (Femtosource Scientific Pro, Femtolasers, Austria) is pumped by a diode-pumped solid-state frequency doubled Nd:YVO$_4$ laser (Verdi V-5 Coherent Inc, USA), $\lambda$=532 nm. The oscillator is producing 5 nJ pulses with 75 MHz repetition rate, 11 fs pulse duration and 800 nm central wavelength. The laser pulses are amplified with a Chirped-Pulse Multipass Amplifier (Femtopower Pro, Femtolasers, Austria). The amplifier is optically pumped with a Q-switched frequency-doubled Nd:YLF laser (YLF 621 D, B.M. Industries, France). The Nd:YLF laser produces 3 to 18 W power with 1 kHz repetition rate, 500 ns pulse duration and 527 nm wavelength. The amplifier generates 1 mJ pulses with 25 fs pulse duration and 1 kHz repetition rate with 790 nm central wavelength. The scheme of the femtosecond laser system is shown on Figure 5.1. The 1:2 beam splitter on the output of the amplifier is used to separate the amplified laser beam in two directions. By means of the dielectric mirrors, the laser radiation is coupled into the imaging-mode LIBS setup.

![Figure 5.1. The scheme of the femtosecond laser system.](image)

The spectrum and second order autocorrelation function (ACF) of the laser radiation from the output of the amplifier are presented on Figure 5.2. The full width at half maximum is 36.24 nm. The full width at half maximum of the ACF is 25 fs.
Femtosecond laser pulse accumulates a group velocity dispersion (GVD), while passing through optical elements. This leads to an increase of the pulse duration.

![Figure 5.2. The spectra and ACF of the amplified femtosecond laser radiation.](image)

The prism compressor of the amplifier can be used to compensate GVD in order to supply a minimum pulse duration (s. Figure 5.1).

### 5.1.2. Setup for the preliminary experiments

For preliminary femtosecond LIBS experiments a similar setup with spectrometer detection is used, as described in Chapter 4, Figure 4.2. The scheme of the setup is presented on Figure 5.3. The incoming laser radiation can be continuously attenuated by the circular gradient attenuator A2 (Edmund Industrie Optik GmbH, Germany).

![Figure 5.3. The setup for preliminary femtosecond LIBS experiments with spectrometer detection part.](image)

The silver mirrors M5-M2 (Linos Photonics GmbH, Germany) are used for the coupling of the laser beam into the microscope-like setup. Iris aperture I1 is used for the adjustment purposes. The neutral density filter A1 (Edmund Industrie Optik GmbH, Germany) is used for precise attenuation of the femtosecond laser radiation. The photodiode, placed after beamsplitter BS2 is used to control the pulse energy. The
microscope objective *LD Epiplan 20X0.4* is used to focus the laser light and to collect the plasma luminescence radiation. The beamsplitter BS1 (HR 400 nm, HT 800 nm, Light Conversion, Lithuania; s. Appendix IV) is used for the separation of the initial laser radiation and plasma emission luminescence. The filter F1 (BG 40) blocks the laser light before the spectrometer. The lens L1 is used to focus the plasma emission radiation on the entrance slit of the monochromator (Amko Type LTI Serie 01-002 with PMT detection by Photon Count Detector Amko 2TI Mod. 08-15 with PMT Hamamatsu R928P). The data are acquired the same way, as it is described in Chapter 4.

### 5.1.3. Femtosecond laser induced plasma

The time-resolved LIP spectra measurement is done in order to investigate Ca$^{2+}$ analysis in water with femtosecond LIBS. The luminescence decay parameters of the femtosecond laser-induced plasma are determined in this experiment.

The laser pulse energy is 90 µJ. The detection window of 30 ns is scanned from 20 ns to 150 ns. The laser pulse generates the plasma at $65 \pm 2$ ns and the start position of the detection window is set 35 ns before the laser pulse. The measurement gate starts to acquire signal already at 35 ns delay. Thus the time profile of the plasma luminescence is extended by the large gate. Smaller gate do not supply sufficient sensitivity, as its for is not perfectly rectangular. The CaN$_2$O$_6$ salt is dissolved in concentration of 21 mmol/l. The resulting plot is presented on Figure 5.4.

![Figure 5.4. Time-resolved spectra of the plasma luminescence, performed with AMKO spectrometer and PMT detection.](image)
The Ca$^{2+}$ ion lines at 393.6 nm and 397.2 nm are observed right in the beginning of the plasma formation process. The Ca atomic line at 422.9 nm has the intensity comparable to the intensities of the Ca$^+$ recombination lines. In the nanosecond laser time-resolved LIP luminescence spectra the atomic Ca line is almost not detectable. The luminescence lifetime is shorter than to nanosecond LIP luminescence. Nevertheless, femtosecond LIBS offers a possibility of the time gating over the whole plasma event, which is not possible in nanosecond LIBS [119]. Basing on this data, we conclude, that femtosecond LIBS could be used for the analysis of the Ca$^{2+}$ ion lines in water. In the next part, the development of the imaging-mode LIBS setup is described.

5.1.4. Plasma microscope setup description

The plasma microscope is based on the modified confocal laser-scanning microscope (Leica CLSM-DIAPLAN, Leica, Germany). The setup contains a tube system with light microscope (not presented on the scheme), an objective revolver and a sample table. The system could be switched between the light microscope setup and imaging-mode LIBS setup by exchanging the slider between the 90° mirror for the laser light and a focusing lens of the binocular system. The plasma microscope scheme is presented on Figure 5.5.

![Figure 5.5. The scheme of the plasma microscope.](image)

The laser beam is passing through an optical delay line (5.6 m) and is attenuated by a gradient attenuator A2. The beam splitter BS2 is used to guide a part of the beam on the photodiode PD2, which is used for the energy control. The attenuator A1 is used for a precise energy adjustment. The laser beam is coupled into the plasma microscope setup by means of the mirrors M2, M3 and M4. Beam splitter BS1 (HR 400 nm, HT 800 nm Light Conversion, Lithuania) is used to separate the entire laser beam from plasma luminescence light. The Zeiss microscope objective LD Epiplan 20X0.4 is used to focus
5. Femtosecond LIBS experiments and results

the femtosecond laser beam on the sample and to collect the plasma emission radiation. The zoom objective (Nikon UV-NIKKOR 105 mm 1:4.5, Nikon Corp, Japan) collects and focuses the plasma radiation on the entrance slit (280 µm) of the spectrometer (L.O.T. Multispec with grating Nr. 77411; 1200 L/mm, blazed on 350 nm, LOT-Oriel, USA). The spectrometer transmits the spectral area from 350 nm to 450 nm on the ICCD chip. The spectra is registered by ICCD camera (La Vision Flame-Star, La Vision, Germany). The sample is positioned by a 3-coordinate piezo-nanopositioning table (P-527.3CD with E-710.3CD controller, Physik Instrumente, Germany). The iris apertures I1-I3 are used to fix the laser beam position. The view of the setup on the optical table is presented on Figure 5.6.

Figure 5.6. Picture of the plasma microscope setup.

5.1.5. Triggering schema
The triggering schema of the setup it presented on Figure 5.7. The monitor output signal of the pockels cell driver delivers the initial system trigger. The monitor output signal is generated shortly before the high voltage is applied to the pockels cell to select an oscillator pulse for the amplification and 105 ns before the laser pulse reaches the LIBS
5. Femtosecond LIBS experiments and results

setup microscope objective. The monitor output of the controller triggers the digital delay generator (SRSDG 535). After a software-controlled delay from the output AB, the intensifier of the ICCD camera is switched on. The internal camera delay is 34 ns and the internal delay of the triggering system is 93 ns. This results in common delay of 127 ns. Therefore, the plasma appears 22 ns before the camera could be switched on and the plasma dynamics cannot be measured under this condition.

This problem can be solved by the reduction of the delay generator trigger level to 0.1 V. Than, the preliminary trigger signal of the pockels cell, which appears 49 ns earlier, could be used. It is then possible to start the camera 29 ns before the plasma appears. With this trigger signal, a camera window of 20 ns, which is typically used, could be placed before the plasma event. Additional 19 ns are introduced by an optical delay line, which is 2X280 cm long (s. Figure 5.5).

5.1.6. Adjustment procedure

The adjustment procedure of the plasma microscope is performed in four steps: adjustment of the optical setup with a He-Ne laser, coupling of the femtosecond laser, measurement of the pulse duration and adjustment of the pulse compressor of the amplifier to compensate the group velocity dispersion.

Preliminary adjustment of the setup with He-Ne laser is done by coupling of the He-Ne laser by means of the flopping mirror before M7 mirror. The precision of the adjustment is proved by a dummy-objective tool – a 50 mm long cylinder with 1 mm inner diameter, placed instead of the objective. The aluminum mirror is placed on the
optical table under the dummy-objective (distance between the objective and the table is 35 cm). The setup is adjusted in such a way, that the laser light, reflected from the mirror is imaged in the center of the zoom objective of the ICCD camera.

The precision of the plasma microscope adjustment is checked by the confocal microscope setup, presented on Figure 5.8. He-Ne laser radiation (\(\lambda=630\ \text{nm}\)) is attenuated by an attenuator A1 and enters the telescope, formed by lenses L1 and L2. Then, the expanded beam is directed to the plasma microscope setup by means of mirrors M1-M5. Gradient attenuator A2 is used for smooth adjustment of the laser energy and iris apertures I1-I3 are used to fix the beam position. Beamsplitter BS1 is used to separate the incoming and reflected laser light. The laser light is guided to the microscope objective and focused on the sample - mirror, placed on the 3D piezo-table. The reflected light is guided by the beamsplitter BS1, mirror M6 and aperture I4 to the detection part. The beamsplitter BS3 is reflecting part of the light through iris aperture I5 and a telescope L3-L4 to the webcam (Toucam Pro, Phillips, Netherlands) which is used to image the beam profile.

![Figure 5.8. Confocal microscope setup, used for adjustment check.](image)

The PMT, located after the mirror M7, lens L5 and pinhole (100 µm) PH, is used to measure the signal intensity after the pinhole. Typical confocal response picture, measured with this setup is presented on Figure 2.6. in Chapter 2.

After the preliminary adjustment of the setup, the femtosecond laser is guided through the same pathway with dummy-objective tool. After that, the objective LD Epiplan 20X0.4 is mounted.

In order to determine the pulse duration of the femtosecond laser pulses in the focal plain of the objective, autocorrelation measurement is performed. The Mach - Zehnder interferometer is mounted after the laser amplifier and the two-photon photodiode (RS Components AEPX-65, rise time 1 ns) is used to measure the second order autocorrelation function. Firstly, the laser pulse duration is measured after the
microscope setup without the objective. After the objective is mounted, the two photon photodiode is placed in the focal plane of the objective and the measurement of the uncompressed laser pulse duration is performed. To keep the diode safe from the destruction, the laser radiation is attenuated approximately by a factor of $10^4$. Then, the prism compressor of the amplifier is adjusted to the maximum output value of the two-photon photodiode, located in the focal plane of the microscope objective in order to compensate the GVD, introduced by the optical elements of the plasma microscope.

Figure 5.9. Measured ACF of the laser amplifier after passing through the system in the different conditions is presented. (a) shows the measurement of the typical ACF of the amplified femtosecond laser pulse, passed through the whole optical system, but with the microscope objective removed. (b) is the ACF after the Zeiss LD Epiplan 20x/0.4 objective without compensation. (c) shows the ACF after the adjustment of the prism compressor on the maximum output of the two-photon photodiode on the method, described above [120].
The autocorrelation measurement is done after this adjustment [120]. The results of the second order autocorrelation measurements of the laser pulse durations are presented on Figure 5.9. The initial femtosecond laser pulse (a) is significantly broadened in time domain due to GVD in the objective (b). By adjustment of the prism-compressor, the GVD could be partially compensated (c). In the routine measurements, the two-photon photodiode connected to the LeCroy oscilloscope, is used to get a shortest pulse by adjusting the prism compressor of the amplifier to the maximum signal of the photodiode. The initial femtosecond laser pulse, coming to the objective has a 30 fs duration. After passing through the objective, the pulse duration increases up to 72 fs. By adjusting the prism compressor of the optical amplifier, the GVD is precompensated and pulse duration becomes 42 fs - just 30% longer, comparing to the initial pulse of 30 fs.

After this adjustment procedures, the plasma microscope is used for the routine LIBS measurements.
5. Femtosecond LIBS experiments and results

5.2. Femtosecond LIBS measurements on water model

5.2.1. Sensitivity calibration

In the biological applications, where the distribution of the relative Ca\textsuperscript{2+} concentrations should be given, it is very important to know the sensitivity limit of the setup. The signal and background areas are defined, as it is shown on Figure 5.10. Two profiles from Figure 5.4. are presented at 64 ns and 77 ns delays. Two 2 nm spectral windows are chosen for the concentration calibration measurement. For each measurement point, the spectral data are integrated within 392.4 – 394.4 nm spectral region for the Ca\textsuperscript{+} line and for the plasma background, the signal is integrated within a spectral region 404 - 406 nm.

![Figure 5.10. Spectral windows for Ca signal and plasma background detection.](image)

The same water stream system, as described in Chapter 4, is used for the sensitivity calibration. The energy of the femtosecond laser pulses is 280 µJ and the repetition rate is 1 kHz.

For the concentration calibration measurement, the camera gate of 200 ns is set 10 ns after the laser pulse. Initially, a distilled water is used (1000 ml). Then, 50 ml of the 0.5 mol/l CaCl\textsubscript{2} solution is added to the water tank. In order to change the concentration, a defined volume of the solution is replaced by a distilled water. By means of this procedure, the concentration is constantly decreased. This method allows to avoid a multiplied error in concentration definition. 50 spectral profiles are measured at each concentration. A 1000 times on-chip averaging for each profile is done. After the averaging, the experimental point and statistical deviation are obtained. The calibration curve is presented on Figure 5.11.
5. Femtosecond LIBS experiments and results

Figure 5.11. Ca\(^{2+}\) concentration calibration. Plasma background signal is presented as the reference.

Figure 5.11 shows, that Ca\(^+\) ion signal is increasing linearly with increasing of the Ca\(^{2+}\) concentration, whereas the plasma background remains constant. The Ca\(^{2+}\) could be detected in water even at single millimole/liter concentrations. This means, that described setup could be used for the measurement of the absolute Calcium concentrations. The sensitivity limit of 0.56 mmole/l (10 ppm; 2.1 \(10^{-6}\) g/l) is found at laser pulse energy 280 µJ.

5.2.2. Time-resolved plasma luminescence measurements

In order to define the meantime parameters of the femtosecond LIP, time-resolved measurements of the femtosecond laser-induced plasma emission spectra are performed with different pulse energies. The water stream setup, as described in Chapter 5.1.3 is used for this series of experiments. Each of the presented profiles of the plot is a result of the on-chip ICCD averaging over 2000 pulses. The laser system is running at 1 kHz repetition rate. The laser pulse generates plasma at 38±2 ns. The ICCD camera gate width is 15 ns. This means that plasma is measured by the ICCD detection gate at 23 ns and could be measured up to 61 ns. It shows, that the plasma luminescence is significantly shorter in comparison to the detection gate. The result of the measurement is presented on Figure 5.12.
Figure 5.12 shows transient plasma luminescence spectra recorded for different laser pulse energies. The spectral lines appear in the very beginning of the plasma formation process. The Calcium recombination lines emission could not be detected right after the decay of the broad plasma luminescence. The Calcium atomic line could be detected even at 15 ns after the plasma luminescence decays. This data are used in the following chapter for the analysis of the plasma luminescence decay process.

Comparison of this data to the nanosecond time-resolved plasma measurement shows, that femtosecond laser-induced plasma is termalized faster, as the spectral lines could be detected from the beginning of the plasma luminescence. Lower thermal background indicates, that femtosecond LIP has lower temperature in comparison to nanosecond LIP.
5.2.3. Plasma luminescence measurement

The analysis of the transient spectra, measured at different laser pulse energies (s. Chapter 5.2.2.) is presented in this part. The plasma luminescence decay time is analyzed, for the Ca$^+$ signal in the spectral area from 392.4 nm to 394.4 nm and plasma luminescence signal in the area of 404 nm to 406 nm, as it is described in previous chapter. For each point of the plot, the signals are integrated within mentioned spectral areas. The time scale in the analysis is normalized to start from the zero point. The first two points are not presented on the plot due to their high intensity, nevertheless they are laying on the fitting curve. The analysis of the plasma decay at 280 µJ laser pulse energy is presented on Figure 5.13.

![Figure 5.13. The plasma luminescence decay time is presented of plasma (squares) and Ca lines (circles).](image)

To determine the lifetime of the luminescence decay process, the measurement results are fitted by exponential functions. The algorithm, which is used for the nanosecond LIP spectral data analysis, described in Chapter 4.2.3 is applied in this analysis. Figure 5.13. shows that both, the decay of plasma luminescence and the Ca$^+$ line emission, exhibit an exponential behavior with different decay constants. From the point of view of practical applications, it is clear, that better signal/background ratio could be obtained with the detection at times later then 20 ns, when plasma background luminescence decays completely. Moreover, the detection without time gating could be used for in the femtosecond LIP analysis, as proposed by several authors [62, 119].

The dependence of plasma and Calcium spectral line emission decay time on the femtosecond laser pulse energy is presented on Figure 5.14. The minimum plasma emission lifetime is 0.4 ns for laser pulses with an energy of 20 µJ. The Ca$^+$ line
luminescence lifetime is 1.8 ns. For the laser pulse energies of 310 µJ, the plasma luminescence lifetime increases up to 2.2 ns and Ca$^+$ line luminescence lifetime at this energy is 3.8 ns. As in the case of the nanosecond laser experiments, the plasma luminescence and the Ca$^+$ line emission lifetimes depend on the laser pulse energy.

![Figure 5.14. Femtosecond laser-induced plasma and Ca spectral line lifetime dependence on laser energy.](image)

The plasma luminescence and the Ca$^+$ line emission lifetimes as a functions of the laser energy, are presented on Figure 5.14. The lifetimes show a linear dependence from the laser energy. At low energies, the ratio of the plasma background and Ca$^+$ line emission lifetimes is 3:1 – exactly as in the case of the nanosecond LIP luminescence decay. With growth of the energy, the ratio becomes 2:1. The averaged parameters of the plasma luminescence ($\tau_1$) and Ca$^+$ line emission at 394 nm ($\tau_2$) and their ratio are presented in the Table 5.15.

<table>
<thead>
<tr>
<th>$E$/µJ</th>
<th>$\tau_1$/ns</th>
<th>$\tau_2$/ns</th>
<th>$\tau_1/\tau_2$</th>
</tr>
</thead>
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<tr>
<td>20</td>
<td>0.87</td>
<td>2.9</td>
<td>3.3</td>
</tr>
<tr>
<td>30</td>
<td>0.55</td>
<td>1.7</td>
<td>3.1</td>
</tr>
<tr>
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<td>0.64</td>
<td>2.1</td>
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</tr>
<tr>
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<td>1.4</td>
<td>3.1</td>
<td>2.2</td>
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<td>3.4</td>
<td>2.1</td>
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<tr>
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<tr>
<td>310</td>
<td>2.5</td>
<td>4.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 5.15. Averaged parameters in dependence on the laser pulse energy:
lifetimes of $\tau_1$ - plasma luminescence, $\tau_2$ – Ca line emission at 394 nm and $\tau_1/\tau_2$ ratio.
The lifetime values, listed in Table 5.15, show that with the growth of the energy, the lifetimes of the plasma background emission and Ca$^{2+}$ line luminescence increase, whereas their ratio decreases. The signal/background ratio dependence from the energy is presented on Figure 5.16. The result of this analysis shows, that the plasma background is not influencing on the Calcium line measurement result even at high energies. For the investigation of the femtosecond LIP parameters, important for the detection settings, the dependence of the Ca$^+$ ion line and plasma signals on the laser pulse energy is studied. The result is presented on Figure 5.16.

![Figure 5.16. The dependence of the plasma background and Ca$^+$ emission line intensity on the laser pulse energy.](image)

The measurement of the plasma luminescence and Ca$^+$ line emission intensities are presented 5 ns after the laser pulse. At the energies from 50 µJ to 270 µJ, both intensities behave almost linear. At the energies over 230 µJ, the plasma luminescence intensity is higher than Ca$^+$ ion line emission intensity and they could not be distinguished from each other at this time delay.
5.3. Study of the ablation properties of the plasma microscope

Femtosecond laser-induced breakdown plasma under the 20X0.4 LD Epiplan objective is presented on Figure 5.17. For the definition of the area with the above threshold fluence, where the plasma generation is possible, z-field experiment is done. This experiment is important in order to define the optimal z-axis offsets for the ablation of the target.

![Figure 5.17. Femtosecond laser-induced plasma sparkling in air at 200 µJ energy.](Image)

The laser focal position is scanned through the surface of the sample in xy-direction line by line. For each line, different z-offset is used. The result of the experiment is a rectangular field. Each line of this field corresponds to different z-offset and contains the holes of the respective diameter. The scheme of the experiment is presented on Figure 5.18.
The experiment is done on the surface of the sample cover glass (100 µm thick, 10X10 mm). Using the z-field scanning technique, the dimensions of the above threshold fluence area are defined at each specific energy, which means the z-range, where the laser can introduce surface damage. In case of the 20X0.4 objective, the height of the above threshold area exceeds 20 µm even at breakdown threshold energies. This is the z-direction limit of the piezo-table, used in this setup. Thus, the objective with the higher numerical aperture is used. For the 50X0.5 LD Epiplan objective and 1.5 µJ pulse energy, the 5 µm height of the above threshold fluence area is measured. In this experiment, each point is separated from the others with 1 µm distance. Z position is increased for each vertical row from the left side to the right with 0.2 µm steps. The scanning electron microscope image of the experimental field is presented on Figure 5.19.

Figure 5.19 A z-field scan SEM image. The single shots with energy of 1.5 µJ.

The coordinates for this scan (x/y/z)=1/1/0.2 µm.
From this measurement it can be concluded, that if the roughness of the surface has comparable size with the above threshold fluence area height, the additional positioning system is required. The constant position of the target in respect to the focus of the objective should be provided, to support the constant ablation volumes and thus the correct measurement conditions.

Definition and identification of small experimental fields under the optical and scanning electron microscopes meets serious complications. For the improvement of the experimental field localization on the sample, marking of the field is done, as it is shown on Figure 5.20.

Figure 5.20. The experimental field (small square frame with arrow) is surrounded with a rectangular field 55X34 µm² and signed with 14 µm large letters. The lines are formed by a single laser shots in 2 µm distance. This marking significantly improves the search and identification process. In case, when several experimental fields are studied, each of them could be marked separately. In addition, brief notes (time, energy) could be located in the frame.
5.4. Conclusion

The development of the plasma microscope was described in this chapter and the measurements of its main parameters were presented. Femtosecond laser-induced plasma on the water sample was studied in order to define the sensitivity of the setup. Ca$^+$ ion lines were identified in the femtosecond laser-induced plasma. Then, the time parameters of the plasma luminescence decay were measured on the nanosecond time scale. Optimal delays, required for the measurement with maximal signal/background ratio were determined. The time-resolved spectra measurement of the femtosecond LIP in water were repeated by means of the plasma microscope. The results of these experiments showed, that signal/noise ratio is better for the femtosecond LIP spectral measurement in comparison with the nanosecond LIP measurement: the plasma thermalization is faster and plasma temperature is lower.

The ablation properties of the plasma microscope were measured. The height of the above threshold fluence area in the focus of the objective was measured through a z-scan experiment on the glass sample. In case of the 20X0.4 objective, the above threshold area height is more than 20 µm in axial direction. This exceeds the limit of the piezo-table used in this setup. Thus, the objective with the higher numerical aperture was used. For the 50X0.5 objective and the 1.5 µJ pulse energy, the above threshold fluence area height of 5 µm was measured. It was found, that in the case when the roughnesses of the sample have comparable size with the above threshold fluence area height, the additional focusing system should be used to keep the focusing conditions correct.
6. LIBS studies of the sunflower seedlings

Introduction

The plasma microscope application to the investigation of the bound Ca\(^{2+}\) ion distribution in the sunflower seedling is presented in this chapter. The biological object – peripheral cell wall of the sunflower seedling stem is introduced in the beginning of the chapter. Essential information about the biological object is presented. Basing on this data, the analytical requirements are stated in order to study the Ca\(^{2+}\) distribution in the peripheral cell wall.

The nanosecond LIBS experiment result - scanning along the sunflower seedling stem is presented and discussed in the second part of this chapter. During this experiment Ca\(^{2+}\) ion distribution along the stem is measured. Microscopy images of the nanosecond laser ablation of the sunflower seedling stem surface are presented and discussed.

The femtosecond ablation and LIBS studies are presented in the third part of this chapter. The optimal pulse energy is defined for the ablation of the peripheral cell wall. The study of the ablation parameters is done by means of the light microscope and white light interferometer microscope. The ablation volume is defined through analysis of the microscope images. Femtosecond LIBS study of the Ca\(^{2+}\) ion distribution in the peripheral cell wall of the sunflower seedling stem is presented. During one measurement, both – lateral and axial bound Ca\(^{2+}\) ion distribution is measured. The results are compared with the results of the nanosecond LIBS study.

The plasma microscope image of the sunflower seedling stem surface is presented and discussed in the fourth part of this chapter.
6.1. Sunflower seedling

In this work, the plasma microscope, described in Chapter 5, is used for the spectrochemical studies of the sunflower seedling (*Helianthus annuus* L.) stem. Sunflower seedling is presented on Figure 6.1. The microscopy image of the stem section is presented in the middle of the figure. The magnified image of the outer cell wall is presented on the right side of the figure. The peripheral cell wall is 1.5-2 µm thick and it is located on the surface of the plant, as it is shown on Figure 6.1.

![Image of peripheral cell walls of the epithelial cell layer.](image)

The relative local concentrations of the Ca$^{2+}$ ions in the peripheral cell wall of the sunflower seedling stem and spatial distribution of bound Ca ions are subjects of interest. The Ca$^{2+}$ ions are mainly localized in the peripheral cell walls of the epithelial cell layer. The expected concentrations is 10-100 mmole/l. Calcium concentration in the underlying cells is expected to be lower in comparison with the outer cell wall. Bound Ca$^{2+}$ is participating in the formation of the cellulose structures of the peripheral cell wall, which are responsible for the rigidity of the plant stem [121]. The relationship between plant growth and increase of the cell-wall material (wall synthesis) investigated in hypocotyls is a biological question of this study. The problem of the plant growth study in greater details is discussed by Kutschera [122]. Cell walls are also responsible for keeping the inner hydrostatic pressure (Turgor) of approx. 0.55 MPa [123]. As soon
as the epithelial cell wall is damaged, the cell liquid is floating out and the correct analysis is no more possible. This study is superimposing the following limitations on the research methods:

1. In situ research of the plant.
3. Detection of the Ca ions in concentration range <100 mmol/l.
4. Axial resolution in order of 100 nm.

In situ study of the plant is important as any preparation of the sample could destroy the original Calcium distribution picture. Minimal invasion conditions are important, as the soft biological tissue of the sample could be easily destroyed and the inner hydrostatic pressure would immediately distort the experimental area. The topology of the sunflower seedling stem is studied in order to estimate the roughness of the surface, as it is important for the proper method development. Thermomicroscope CP-Research (Veeco Instruments, USA) with 100 µm closed loop scanner is used for this purpose in atomic force microscope (AFM) mode. The scanning cantilever has no constant contact with the object surface. Otherwise the surface could be damaged and the cantilever tip could get stuck in the soft tissue.

On Figure 6.2 the long relief structures are seen with 10 µm period. The height of the structures is in order of 2 µm. This result coincides well with the results of the microscopy studies of the sunflower seedling stem sections, presented on Figure 6.3 [123].
On Figure 6.3 it is seen, that the relief structures on the surface of the stem could be related to the cells and cell rows, oriented along the stem. This superimposes additional requirement for the method, as the relief of the stem could cause complications during the measurements.

The plants, for our experiments, are grown under the controlled conditions. The seeds are washed for 1 hour in the distilled water and seeded in wet Vermiculite (water absorbing Magnesium-Aluminum-Silicate mineral). Plants are growing 6 days at 12 hours light and 12 hours darkness cycle in closed plastic containers at 25°C.
6. LIBS studies of the sunflower seedlings


This first test experiment on the sunflower seedling is provided on the 6-days-old plants. The experimental setup, described in Chapter 4.2 is used for this experiment. Setup and laser system are modified for the third harmonic of the Nd:YAG laser – 355 nm. This is done in order to minimize the ablation depth, due to the higher linear absorption of the UV light, compare to visible second harmonic light with 532 nm wavelength.

Plant samples are mounted on the 3D mechanical mount with manual adjustment of the position. The plant surface is placed in the focus of the L1 lens (Figure 4.8). The Nd:YAG laser is running in the single shot mode. Shot to shot energy is controlled by a photodiode PD. The plant is scanned in a vertical direction from top (hypocotyls) to the root. The scan step is 2 mm. The stem length, measured from root to hypocotyls is 25 mm and stem diameter 1.5 mm. Scanning parameters are the following: 30 mJ pulse energy, 300 ns delay between the laser sparking and the measurement gate, 530 ns gate width. The laser fluence is 360 J/cm² in order to supply signal/background ratio of 10. The typical results of the scans are presented on Figure 6.4. On these plots, the abscissa represents the relative Ca²⁺ concentration and the ordinate represents the position in mm along the stem, measured from the root to the top.

The full spectrum is taken for each analysis point and is later analyzed. The signal area is chosen between 392 nm and 398 nm and background area is between 406 nm and 412 nm, according to Figure 4.10.
6. LIBS studies of the sunflower seedlings

Figure 6.4. Nanosecond spatially-resolved LIBS analysis of the sunflower seedling stem along Z axis.

The general tendency shows, that Calcium concentration is increasing to the bottom of the plant. It could be explained by a fact, that Ca$^{2+}$ ions are responsible for the rigidity of the peripheral cell wall structures in the plant, as it was discussed in previous part of this chapter. The bottom part of the plant has a higher mechanical rigidity, as it is carrying a holding function and a top part is more flexible to resist the ambient media movements.

The ablation crater study is done with the light microscope (Zeiss III RS with DP10 camera, Zeiss, Germany). The laser fluence in this experiment is 175 J/cm$^2$. The N.A. of the lens is 0.3. Typical ablation crater images on the plant stem surface are presented on Figure 6.5.
6. LIBS studies of the sunflower seedlings

The cell wall is destroyed completely with fluencies even much lower then this value. Moreover, as it is displayed on Figure 6.5, the shape of the ablation area changes from shot to shot. The ablation area resembles the subjacent cell structure and cannot be attributed to the laser beam profile. This results in significant variation of the ablation volume for each laser shot. In this experiments, ablation depth exceeds the resolution possibilities of the light microscope. From this study it is clear, that even at the laser fluence required to generate a plasma, the peripheral cell wall is completely destroyed. The ablation craters have random form and volume. The whole cell wall is destroyed within one laser shot. The depth resolution is low in this case – the underlying cell layers are also ablated. Thus, the Ca$^{2+}$ ion signal is averaged not only over the peripheral cell wall, but also over the underlying cell layers. Basing on this results, the conclusion is done, that UV nanosecond laser is not well applicable for the imaging-mode LIBS of the biological samples.

Figure 6.5. Nanosecond ablation crater. Nd:YAG laser, 3d harmonic $\lambda=355$ nm.
The literature study shows that UV nanosecond lasers are successfully applied for the imaging-mode LIBS investigation of the technical samples [26]. Our study shows, that in case of soft biological samples, such as peripheral cell wall of the sunflower seedling stem, the nanosecond laser is not a suitable laser source.
6.3. Femtosecond LIBS study of the sunflower seedling

The investigation of the $\text{Ca}^{2+}$ local concentrations in the sunflower seedling stem by means of the plasma microscope setup is presented in this chapter.

The first experiment is performed in order to define the optimal energy for the study of the $\text{Ca}^{2+}$ ion distribution in the peripheral cell wall. The information about the depth distribution of the bound $\text{Ca}^{2+}$ ions could be obtained from each point of the stem surface. The sunflower seedling is fixed in the focal plane of the objective. 200 shots are done on one position of the stem with energy 25 µJ (120 J/cm$^2$). The $\text{Ca}^{+}$ signal is measured at a spectral area from 392.4 nm to 394.4 nm. The result of the depth scan is presented on Figure 6.6.

![Figure 6.6. The sunflower seedling stem depth scan at single position with fluence 120 J/cm$^2$.](image)

As it is observed on Figure 6.6, $\text{Ca}^+$ signal is significantly decreased after three shots. This could be interpreted as if the peripheral cell wall is completely drilled within 3 shots. The rest of the laser pulses are monitoring the $\text{Ca}^+$ ion signal from the inner cell liquid, which is coming out from the drilled crater. Hence, the consequence for the following experiments is to decrease the laser pulse energy in order to improve the axial resolution.

The optimal ablation energy of 3.5 µJ (55 J/cm$^2$) is defined in this serie of the experiments. Depth scan with this laser pulse energy allows to resolve smaller structures within the peripheral cell wall, as it is shown on Figure 6.7.
Figure 6.7. The sunflower seedling stem depth scan. Measurement at single position with fluence 55 J/cm². On Figure 6.7, the defined structure is seen, which is resolved within 14 shots. The fast growth of the signal up to 5th shot and then its decrease up to 14th shot is seen. If the average thickness of the peripheral cell wall is 2 µm, the axial resolution of 140 nm is achieved in this experiment with 14 laser shots, considering the constant ablation volume for each shot.

The study of the ablation volume is a question of primary interest, as it defines the spatial resolution of our method. 5 shots of the 42 fs laser with 3.5 µJ energy are done on the surface of the 5-days-old sunflower seedling stem. The lateral damage is studied with a Zeiss microscope (Zeiss III RS with DP10 camera). The microscope image is presented on the Figure 6.8.

Figure 6.8. The result of the femtosecond ablation of the sunflower seedling stem surface. This figure presents well defined circle form ablation craters. The diameter of the holes is 3.5 µm. Their shape and diameter are constant from crater to crater. Nevertheless, the axial damage could not be measured by this microscopy technique. For this reason,
the white light interferometer microscope (New View 5000, Zygot, Germany) is used to define a complete spatially resolved picture of the femtosecond laser ablation crater. The result of the femtosecond laser ablation by 5 laser pulses with 3.5 µJ energy is presented on Figure 6.9.

| a) White light interferometer microscope image. | b) Profile of the ablation crater. |

**Figure 6.9.** White light interferometer microscope measurement results of the ablation crater.

The profile measurement shows, that axial resolution of 140 nm is achieved in this experiment at lateral resolution of 3 µm. This measurement result is reproduced at 5 positions. The lateral resolution is determined as 3,5±0.2 µm and axial resolution as 140±25 nm. Thus, the analytical volume of 1 femtoliter is assumed. This demonstrates, that femtosecond laser gives reproducible ablation results not only on technical samples, as it is discussed in Chapter 3.1.4, but also on soft biological tissues.

Now the integrating scan along the plant with the additional depth scan in each point is done with laser pulse energy 3.5 µJ. For this scan, the plant is fixed on the sample table of the plasma microscope in special holder. The sample table is moved manually with 2 mm steps. Focusing at each position is done separately, employing the light microscope, integrated in the plasma microscope setup. The scheme of the experiment is presented on Figure 6.10.
Figure 6.10. Scheme of the experiment - the study of the Ca\(^{2+}\) distribution along the stem with depth resolution.

Two depth scans from the points at 18 (a) and 4 mm (b) of the plant stem are presented on Figure 6.11.

Figure 6.11. Depth scans on two positions of the plant stem.

Figure 6.11 shows, that at higher position, closer to hypocotyl, Calcium signal is lower, than at the bottom of the plant, near the root area.
A double-peak Ca\textsuperscript{2+} signal distribution is shown on Figure 6.12. This picture draws particular interest during the study. Calcium signal does not decrease until 15 laser shots. If the constant ablation depth of 140 nm per pulse is considered, the double-peak structure shows a minimum around 700 nm followed by a second maximum at around 1.4 µm. This structure is reproduced in other measurements at different positions along the plant stem – Figure 6.13.

Figure 6.13. Double peak structures, observed during the measurements in the peripheral cell wall.
6. LIBS studies of the sunflower seedlings

The integration result in respect to the positions along the sunflower seedling stem is presented on Figure 6.14. Each point of this plot is the result of the integration over the depth scan, presented on Figure 6.11. and Figure 6.12. The Y-axis represents the distance in millimeters, measured from the root to the hypocotyl, according to Figure 6.10.

![Figure 6.14. The integrating scan along the sunflower seedling stem.](image)

It is reproducing the results of the preliminary study, described in Chapter 6.2. Thus, employing the femtosecond laser material processing in combination with imaging-mode LIBS, high spatial resolution could be obtained. In our measurements, we assume a single femtoliter analytical volumes.
6.4. Plasma microscope investigation of the sunflower seedling

High speed of the spectral data processing is important for the imaging-mode LIBS studies. Therefore, the ICCD camera is exchanged with a faster one – ICCD PI-MAX-1024-E (Roper Scientifc Instruments, USA). Several modifications are done in the triggering of the setup in order to improve the scanning speed. The Epiplan 50X0.5 objective is used in this experiment. 3-days-old plants are studied, in order to have a smoother surface of the sunflower seedling stem. The scan is done by a 10 µJ laser pulses and the detection gate is 60 ns and distance between two measurement points is 4 µm. The piezo-scanning table offers a 200X200 µm range. Thus, the resulting image consists of 50X50 points. Each of the points contains the full spectrum, measured within a shot. The image is obtained by integrating the signal within the spectral window from 392 nm to 398 nm. The resulting image is represented on Figure 6.15.

![Figure 6.15. Plasma microscope image of the sunflower seedling stem.](image)

The structures, parallel to Y-axis could be seen on Figure 6.15, between 50 and 190 µm. This structures are related to the topology of the sunflower seedling stem surface. This could be the cell rows, which were observed during the AFM study of the sample topology, presented on Figure 6.2. The period of the structures is in agreement with the size of the epithelial cells, presented on Figure 6.3. This means, that the above threshold area height is significantly smaller in comparison to the roughness of the surface. Thus, the additional focusing system should be implemented to the microscope setup in order to keep the constant focusing conditions.
6.5. Conclusion

The application of the plasma microscope to the analysis of the bound Ca\(^{2+}\) ion distribution in the peripheral cell wall was presented in this chapter. The biological object of this study – peripheral cell wall of the sunflower seedling stem was described in the beginning of this chapter.

Nanosecond LIBS study of the sunflower seedling stem is described. The result of this experiment - the Ca\(^{2+}\) distribution along the sunflower seedling stem was presented. The analysis of the microscopy images of the ablation craters on the surface of the sunflower seedling stem showed, that UV nanosecond laser pulses completely destroy the peripheral cell wall within one shot. The craters have a random shape and significant depth. This makes the UV nanosecond laser an unsuitable excitation source for the LIBS studies of the soft biological tissues.

Femtosecond plasma microscope application to the investigation of the bound Ca\(^{2+}\) ion distribution in the peripheral cell wall was described in the third part of this chapter. The optimal fluence was determined to fit the stated requirements on low ablation depth and plasma luminescence, sufficient for Ca\(^+\) ion analysis. Integrating scan along the stem with the additional depth sectioning at each measurement point was performed. The integrating results showed, that the Calcium concentration increases from the top of the plant to the root.

The plasma microscope image of the peripheral cell wall was presented in the fourth part of this chapter. This investigation showed, that additional focusing system should be implemented into the plasma microscope setup. Thus, the topology of the sample should be taken into account in order to provide equal focusing conditions during the scan.

The analytical performance of the setup was found to be sufficient for the in situ analysis of the bound Ca\(^{2+}\) ion distribution in peripheral cell wall. The lateral resolution of 3.5±0.2 \(\mu m\) and axial resolution of 140±25 nm was achieved. This showed, that analytical volume of the plasma microscope is 1 femtoliter.
7. Improvements to the plasma microscope.

Introduction

The improvement to the developed technique – femtosecond imaging-mode LIBS microprobing is a subject of interest in this chapter. Literature study shows, that there are several methods, which can be used to improve the analytical performance of the plasma microscopy technique [62, 97-101, 119, 125]. The improvement proposals are overviewed in this chapter. Our recent studies show that the ablation craters with diameter in order of 100 nm could be obtained at glass samples, as shown on Figure 7.1. Epiplan 50X0.5 objective is used in this experiment.

![Figure 7.1. 90X125-nm oval ablation crater on the surface of the glass sample.](image)

The improvement of the lateral resolution of the plasma microscope is one of the goals of the future studies.

Preliminary ionization by the below-threshold femtosecond laser pulse, foregoing the main ablation pulse is discussed in the first part. In the second part, the plasma heating and additional neutral particles and clusters ionization by the laser pulse, coming after the ablating laser pulse are proposed. In the third part, the femtosecond laser pulse shaping technique in combination with feedback loop and evolutionary algorithm pulse shape development is discussed. The combination of all three techniques is possible in order to optimize the analytical performance.
7. Improvements to the plasma microscope

7.1. Preliminary ionization

The study of the femtosecond laser-induced breakdown shows, that preliminary pulse, foregoing the ablation laser pulse, is decreasing the breakdown threshold [97, 99, 124].

In the experiment of Li et. al., 2 femtosecond pulses are used in order to define the optical breakdown dependence on the delay between the pulses [125]. The energy ratio is 1:1.3 for the first preliminary pulse and second ablation probe pulse. Both pulse energies are below the breakdown threshold. The pulse duration is 25 fs for both pulses. The measurement is done on the Barium-Borosilicate (BBS) and fused silica (FS). The result of the measurement is presented on Figure 7.2.

![Figure 7.2](image_url)

Figure 7.2. Experimental breakdown threshold vs. delay for single pulse (SP) and probe pulse in the double pulse experiment (DP) with their rescaled theoretical fit [125].

This figure presents two times lower breakdown thresholds for the double pulse ablation at delays less than 100 fs. Petite et. al. explain this effect by self-trapping of electron hole pairs under the form of transient point defects, known as self-trapped excitons [126]. Basing on this data it could be stated, that the breakdown threshold for the second pulse is lower, than for the first pulse. The concept of the idea is presented on Figure 7.3.
Figure 7.3. The concept of the preliminary pulse ionization. Continuous line – breakdown threshold.

On Figure 7.3. it is shown, that the first preliminary pulse intensity is lower, than single pulse breakdown threshold (continuous line). After the first pulse, the breakdown is decreased and second ablating pulse could have even lower intensity, than the first pulse. According to Petite et. al., the delay for this pulses should be in order of 100 fs [126].

Decrease of the breakdown threshold results in lower fluence, important for the plasma formation. Thus, side effects of the breakdown and plasma formation, discussed in Chapter 3.2.2 would be decreased. The spatial resolution of the imaging-mode LIBS could be improved by this method.
7.2. Plasma heating

As it is discussed in Chapters 3.1.3 and 3.1.4 of this work, laser-induced plasma contains not only ionized particles and electrons, but also neutrals and clusters of the ablated material. In several works it is demonstrated, that application of the second laser pulse after the ablating pulse improves signal/background ratio in the LIBS studies [100, 119]. The concept is presented on Figure 7.4. It is shown, that heating pulse could have even higher intensity, than ablating pulse.

![Figure 7.4. Concept of the plasma heating by a second laser pulse.](image)

In this experiments it should be considered, that on the early stage of plasma development – if the delay between two pulses is short, the plasma density is high and the effective energy coupling into the plasma is not possible due to high plasma reflectivity [69]. If the delay is long the effective energy coupling would be also not possible due to low density of the plasma. Also the ablated sample should not be influenced by the heating pulse due to longer lifetime and higher temperature of the plasma.
7.3. **Pulse shaping and evolutionary algorithms**

Ultrashort laser pulse shaping technique is widely spreading recent years. As it is shown by Stoian et. al. [98], this technique could be successfully applied for the material processing. It is shown, that shape of the ablation craters could be improved and shock wave effects could be reduced by application of pulse shaping technique to the femtosecond laser material processing. General idea of the pulse shaping with evolutionary algorithms is presented on Figure 7.5. Feedback signal is coupled to the computer, generating evolutionary algorithms. The computer is generating a mask, applied to the LCD matrix of the pulse shaper. The femtosecond laser pulse, formed by this mask ablates the material. LIBS signal could be used as a feedback for the optimization [127-128]. The feedback signal is analyzed by a computer and new generation of masks is applied to the pulse shaper.

![Figure 7.5. Pulse shaping technique with evolutionary algorithm.](image)

The scheme of the simple and flexible pulse shaper setup is described by Präkelt [129]. The description of the evolutionary algorithms application to the pulse shaping is described by Lami and Santoro for control of the complex multilevel system [130]. The described technique could be used on its own or in combination with other discussed techniques.

The concept of the LIBS microscope with ultrashort laser pulses and its combination with the pulse shaping techniques are protected by the patent, developed in our group: **Nr. 102 50 013.4** of the German Patent Agency.
7.4. Conclusion

In the current chapter the upgrade proposals were done in order to improve the analytical performance of femtosecond plasma microscope setup. The improvement in the spatial resolution leads to decrease of the analytical volume, which reduces the spectral signal and thus, the sensitivity of the method. The schemes of the sensitivity improvement are offered for the future development.

Sub-threshold preliminary pulse allows to reduce the breakdown threshold for the ablating pulse, coming after the defined delay. Hence, the side effects of the breakdown and plasma creation and development could be reduced and the spatial resolution could be improved. Decreased plasma volume could be enlarged by a heating pulse, transferring additional energy to the plasma. This could lead to additional ionization of the neutrals and clusters in the plasma. Thus, additional signal could be gained from the smaller analytical volume. Pulse shaping technique is proposed, for the further improvement of the discussed methods.
8. Summary

The goal of this work was the development of a minimal-invasive analytical method for spectrochemical \textit{in situ} investigations of biological samples with high spatial resolution. In order to achieve this goal, imaging mode LIBS setup – \textit{plasma microscope} is developed. It is based on the combination of microscopy techniques, LIBS and femtosecond material processing. A first application of the plasma microscope – the investigation of the local concentrations of the bound Ca\textsuperscript{2+} ions in the peripheral cell wall of the sunflower seedling stem is presented.

Comprehensive experiments on the water model were done in order to investigate plasma formation with nanosecond and femtosecond lasers. Water was used as a model of biological object. It is found, that thermalization of the femtosecond LIP is faster, than of the nanosecond LIP. This results in better signal/background ratio for the femtosecond LIBS right at the beginning of the plasma formation process. Basing on these experiments, the plasma microscope was developed and minimal-invasive element specific \textit{in situ} analysis experiments on the biological samples were demonstrated.

In Chapter 2 it was shown, that the resolution of the plasma microscope could be estimated by the ablation volume, and in Chapter 3 the advantages of the femtosecond laser ablation were shown in terms of the higher precision and lower mechanical and thermal damage.

The results of the nanosecond LIP study on the water model were presented in Chapter 4. Ca\textsuperscript{+} ion lines were identified in the LIP spectra in the area between 380 nm and 430 nm. The best signal/background ratio of 25 at a laser fluence 3 times above the threshold could be obtained. The measurement parameters for the LIBS analysis of the Ca\textsuperscript{2+} ion concentrations in the peripheral cell wall of the sunflower seedling stem were determined.

In Chapter 5, the results of the transient femtosecond LIP spectra measurements were presented: the thermalization of the plasma was faster, comparing to nanosecond LIP. A signal/background ratio of 20 was found for a laser fluence 3 times above the threshold. The Calcium detection sensitivity limit is 0.56 mmole/l.

In Chapter 6 the analytical requirements for the investigation of the sunflower seedling stem were worked out. The measurement of the averaged Ca\textsuperscript{2+} distribution in the peripheral cell wall was performed by means of nanosecond LIBS. The ablation
studies of the plant cell revealed, that the cell wall is completely destroyed by a nanosecond laser pulses even at fluence, not sufficient for the Ca\(^{+}\) ion analysis.

The plasma microscope in combination with the femtosecond laser was employed for the investigation of the local Ca\(^{2+}\) ion concentrations in peripheral cell wall. Using 3.5 \(\mu\)J femtosecond pulses, the peripheral cell wall was analyzed within 20 laser shots. The study of the ablation craters showed the lateral resolution of 3.5\(\pm\)0.2 \(\mu\)m and axial resolution of 140\(\pm\)25 nm, which corresponds to analytical volume of 1 femtoliter. The results of the femtosecond LIBS study of the Ca\(^{2+}\) distribution along the stem were presented and the correlation with the results of the nanosecond LIBS study was found. The first experimental results towards the imaging of the Calcium distribution in the peripheral cell wall were presented.

The double-pulse approaches and pulse shaping techniques applications to the improvement of the plasma microscopy technique, were discussed in Chapter 7.
Appendix I.

The element lines, measured with laser-induced breakdown spectroscopy

<table>
<thead>
<tr>
<th>Element</th>
<th>Line [nm]</th>
<th>Media</th>
<th>Laser Source</th>
<th>Source</th>
</tr>
</thead>
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<td>Ti:Sapphire, 800 nm; 100 fs; 50mJ</td>
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<td></td>
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</tr>
<tr>
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<td>steel</td>
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</tr>
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### Appendix I

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Appendix II

Calculation of the avalanche breakdown threshold for the nanosecond laser-induced breakdown in water (\( \lambda = 532 \) nm; pulse duration 6 ns). According to Kennedy [45]:

\[
I_a = \left( \frac{mcne_0E_{ion}}{e^2} \right) \left[ \frac{l + 4\pi^2v^2\tau^2}{\tau} \right] \left[ \frac{1}{t_L} \ln \left( \frac{\rho_{cr}}{\rho_0} \right) \right] + \frac{m^2E_{ion}4\pi^2v^2e_0E_0}{e^2M}.
\]

Here:

- \( t_L = 6 \) ns
- \( \nu \) – optical frequency: \( \nu = 5.6391 \times 10^{14} \) Hz;
- \( m, e \) – electron mass and charge, \( m = 9.1 \times 10^{-31} \) kg, \( e = 1.602 \times 10^{-19} \) C;
- \( E_{ion} = 6.5 \) eV = \( 10,413 \times 10^{-19} \) J;
- \( c \) – speed of light, \( c = 3 \times 10^8 \) m/s;
- \( \varepsilon_0 \) – permittivity of free space \( 8.854 \times 10^{-12} \) F/m;
- \( n_0 \) – refraction index at frequency \( \nu \), \( n_0 = 1.32 \);
- \( \tau \) – mean free time between inelastic collisions between free electron and heavy particles - 1 fs [50];
- \( g \) – rate of the electron losses due to recombination, trapping and diffusion out of the focal volume.

\[
g = \frac{\alpha E_{ion}}{3m} \left[ \frac{2.4}{r_0} \right]^2 + \left( \frac{l}{z_R} \right) = 1.884 \times 10^{-3}
\]

- \( r_0 \) – beam radius in the beam waist, \( z_R \) – Rayleigh range;
- \( \rho_{cr} = 10^{20} \) cm\(^{-3} \);
- \( \rho_0 = 3.9 \times 10^9 \) cm\(^{-3} \) [50];
- \( M \) – molecular mass of the water;

Our calculation result for the avalanche ionization: \( I_a = 1.656 \times 10^9 \) W/cm\(^2\). This value is in agreement with the value calculated by Vogel et. al. (6.47 \( \times 10^9 \) W/cm\(^2\)) – Table A1.

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Table A1. Laser parameters, investigated by Vogel et. al. in [38], corresponding spot sizes and breakdown thresholds.
Appendix III
Calculation of the multiphoton breakdown threshold for the femtosecond laser-induced breakdown in water. According to Kennedy [45]:

\[ I_m = \frac{2}{B} \left( \frac{\rho_{\text{min}}}{\Delta \rho} \right)^{1/k} \]

\[ A = \left( \frac{2}{9\pi} \right) \omega \left( \frac{m' \omega}{\hbar} \right)^{3/2} \exp\left[2k \Phi(\varphi) \left( \frac{1}{16} \right)^k \right] \]

\[ B = e^2 / m' \Delta \omega^2 c \varepsilon_0 \rho_0 \]

Here:
\( \lambda = 790 \text{ nm}; \tau = 42 \text{ fs} \)
\( \omega \) – optical frequency, \( \omega = 2.38 \times 10^{17} \text{ rad/s} \);
\( m' \) - exciton reduced mass; according to Fan et al. [130] it could be considered as \( m' = 4.554 \times 10^{-31} \text{ kg} \);
\( k \) – number of photons, required for ionization; \( k = 5 \);
\( \Delta \) – ionization energy across the bandgap, equal to ionization energy \( E_{\text{ion}} = 6.5 \text{ eV} = 10.413 \times 10^{-19} \text{ J} \);
\( e \) – electron charge \( e = 1.602 \times 10^{-19} \text{ C} \)
\( \rho_{\text{min}} \) - minimal electron density, sufficient for the breakdown: for the pure multiphoton ionization is \( 10^{18} \text{ cm}^{-3} \) [16];
\( \Delta t \) – time for the overcoming of the critical electron density: \( \Delta t = \tau \) [45];
\( h \) - Plank’s constant \( h = 1.05459 \times 10^{-34} \text{ J s} \);
\( z \approx \left[ 2k - \frac{2 \Delta}{h \omega} \right]^{1/2} ; \ z = 3.1491 \)

\( \Phi(z) = \exp(-z^2) \int_0^z \exp(y^2) dy \) represents Dawson’s integral;

numerical solution returns: \( \Phi(z) = 0.4834 \); \( \varepsilon_0 = 8.85419 \times 10^{-12} \text{ F/m} \)

Numerical calculations return:
A = 5.6324 \times 10^{40} \text{ [s}^{-1}\text{m}^{-3}], B = 2.7253 \times 10^{-18} \text{ [m}^2\text{W}] \)

\( I_m = 1.5517 \times 10^{13} \text{ W/cm}^2 \). The value, measured in our experiments is \( 2 \times 10^{13} \text{ W/cm}^2 \). This is close to the value, measured by Hammer et. al. [50] for 100 fs, 580 nm pulses (5.77 \times 10^{12} \text{ W/cm}^2) and to the measurements of Vogel et. al. presented in Table A1.
Appendix IV

Transmission spectra of the dichroic mirror Linos Photonics DLHS $\lambda=532$ nm $d=25$ mm (Linos Photonics, Germany), measured with a white light source. This spectra coincides with the spectra, obtained from the supplier (Linos Photonics, Germany).

Reflection and transmission curves of the beamsplitter (Light Conversion, Lithuania). The spectra obtained from the supplier (Light Conversion, Lithuania).
Acknowledgements

This work is a result of the coherent team-work of many people from the research groups of the Kassel University.

First of all I want to thank Prof. Dr. T. Baumert for the offered possibility of work in this team, for his support and numerous ideas and advices.

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I want to thank my family and my wife Natalia for the support during this work.
References


References


References


References


References


References


References


