Confocal and Two-Photon Microscopy: Image Enhancement

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Abstract

Confocal and two-photon microscopy have become essential tools in biological research and today many investigations are not possible without their help. The valuable advantage that these two techniques offer is the ability of optical sectioning. Optical sectioning makes it possible to obtain 3D visualization of the structures, and hence, valuable information of the structural relationships, the geometrical, and the morphological aspects of the specimen.

The achievable lateral and axial resolutions by confocal and two-photon microscopy, similar to other optical imaging systems, are both defined by the diffraction theorem. Any aberration and imperfection present during the imaging results in broadening of the calculated theoretical resolution, blurring, geometrical distortions in the acquired images that interfere with the analysis of the structures, and lower the collected fluorescence from the specimen. The aberrations may have different causes and they can be classified by their sources such as specimen-induced aberrations, optics-induced aberrations, illumination aberrations, and misalignment aberrations.

This thesis presents an investigation and study of image enhancement. The goal of this thesis was approached in two different directions. Initially, we investigated the sources of the imperfections. We propose methods to eliminate or minimize aberrations introduced during the image acquisition by optimizing the acquisition conditions. The impact on the resolution as a result of using a coverslip the thickness of which is mismatched with the one that the objective lens is designed for was shown and a novel technique was introduced in order to define the proper value on the correction collar of the lens. The amount of spherical aberration with regard to the numerical aperture of the objective lens was investigated and it was shown that, based on the purpose of our imaging tasks, different numerical apertures
Abstract

must be used. The deformed beam cross section of the single-photon excitation source was corrected and the enhancement of the resolution and image quality was shown. Furthermore, the dependency of the scattered light on the excitation wavelength was shown empirically.

In the second part, we continued the study of the image enhancement process by deconvolution techniques. Although deconvolution algorithms are used widely to improve the quality of the images, how well a deconvolution algorithm responds highly depends on the point spread function (PSF) of the imaging system applied to the algorithm and the level of its accuracy. We investigated approaches that can be done in order to obtain more precise PSF. Novel methods to improve the pattern of the PSF and reduce the noise are proposed. Furthermore, multiple sources to extract the PSFs of the imaging system are introduced and the empirical deconvolution results by using each of these PSFs are compared together. The results confirm that a greater improvement attained by applying the in situ PSF during the deconvolution process.
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Chapter 1

Introduction

1.1 Confocal Microscopy

The word "confocal" consisting of the prefix "con" meaning together and the word "focal" describes "the commonality of the optical path from the light source to the sample and the optical path from the sample to the pinhole" [18]. Although it took more than thirty years to present the first practical confocal microscope, the invention of the system is accredited to Marvin Minsky in 1955 [38].

The major difference between confocal laser scanning microscopy (CLSM) and wide-field light microscopy is that in wide-field microscopy, the light from the source illuminates the specimen uniformly while in CLSM, the excitation light is focused and illuminates one voxel\(^1\) of the specimen at a time and therefore, image construction in CLSM is point by point or voxel by voxel.

Figure 1.1 shows a typical layout of a CLS Microscope. Image formation in CLSM is achieved as follows. Light from the laser passes the scanner unit and is focused by the objective lens into the focal plane inside the specimen. By the galvanometer scanning mirrors, embedded inside the scanning unit, the focused beam scans the focal plane point by point. The focused light not only illuminates the focal point but also illuminates the area around it and produces a conical volume of illumination which is shown in Figure 1.2.

After the fluorophores in the specimens are excited, they emit light, which enters the objective lens and travels backward along the same path as the exciting beam through the scanner. The collected fluorescence comes from the focal point and the conic illumination,

\(^{1}\)Voxel is a combination of the words VOlumetric and piXEL which represents a volumetric pixel.
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Figure 1.1: Typical layout of CLSM [2].

Figure 1.2: Conic illumination pattern in CLSM [49].
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and hence, it contains background fluorescence. By employing a pinhole in the emission path in front of the detector such as Photomultiplier Tube (PMT), most of the out-of-focus emissions are eliminated, and therefore, the detector only receives the emission from the focal plane. Such a design makes it possible to obtain optically sectioned images by moving the focal point through the sample in the Z direction and only detecting the fluorescence at the focal plane. The term “descanned detection” is used for confocal microscopy since the emitted light is fed back to the scanner and through the pinhole [2].

After passing through the pinhole, more manipulations can be done. The fluorescence could be a mixture of light emitted by different molecules in the specimen with different emission spectra. To separate the different components of the fluorescence, a longpass dichroic mirror can be placed after the pinhole aperture and before the detectors to split the light into two parts: one part is transferred through the mirror and the other part is reflected; each part is sent to an individual photon detector. Depending on the spectrum of the dichroic mirror used in the scanner, different transmitted and reflected fluorescence spectra are obtained. More manipulation can be done to detect a narrower wavelength range by using optical bandpass filters in each detection channel between the dichroic mirror and the pinhole. For more elaborate imaging tasks, the emitted fluorescence may be detected in more than two channels. In such cases, multi-wavelength detectors are employed. In these detectors, the fluorescence is divided into multiple wavelengths. These types of detectors are commercially available, such as the one offered by Becker and Hickle GmbH and called PML-16-C, which detects photons simultaneously in 16 channels of a multi-anode PMT [1]. After detection, the voltage obtained in the PMT is converted, digitized, and the image is displayed on the computer screen via specific imaging software.

The light source selection in confocal microscopy depends on the absorption spectrum of the sample. Most fluorophores fluoresce in the green region (around 550 nm) [2, 23]. Since confocal microscopy uses the one-photon absorption process, which is the absorption of a photon by the fluorophore and re-emission at a longer wavelength, the light sources are mostly selected to be in the blue or UV region.
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Selection of the light source can be problematic for confocal microscopy systems. The number of the laser sources in the desirable wavelength region of the spectrum is limited. Gas (e.g. Argon or Kr/Ar) and semiconductor (e.g. Diode) lasers in the UV, blue and red regions and a very few sources in the green are used but these sources have their own limitations; for example, gas failure, costs for cooling maintenance, short life time, and limited output power could affect the performance of gas lasers. And also, temperature-dependent wavelength variation and beam cross section deformations are the most common drawbacks in semiconductor laser such as diode lasers [23]; however, the most commonly used single-wavelength lasers for CLSM in the blue and red regions of the spectrum are diode lasers, due to their affordable price and ease of operation.

Some recent works have searched for ways to overcome light source shortcomings and also to fill the gap of the spectrum between the UV and red regions. Vertical External Cavity Surface Emitting Lasers (VECSELs) is a new technology which presents a solution to this issue. This technology is an external cavity-designed type of semiconductor lasers which is based on electronically or optically pumping a semiconductor gain medium.

The external cavity design, which is the advantage of such types of lasers, allows further manipulations for different applications; for instance, as the emitted light from the laser is usually in the infrared region, one can insert a frequency-doubling crystal in the cavity and obtain blue light [23]. VECSELs have different advantages such as: high power, a circular and symmetric output beam, and the possibility of designing the gain medium for a desired wavelength [31]. These lasers are now commercially available from several companies (e.g. Coherent).

Another work reported by Dunsby et al. 2004 [19], presents a promising solution to the source light of confocal microscopy by the use of photonic crystal fiber PCF. They report "the first continuously electronically tuneable (435-1150 nm) visible source for confocal microscopy and other imaging modalities based on supercontinuum generation in PCF followed

\^PCF is an optical fibre which its waveguide composition is an arrangement of small and closely spaced air holes through the whole length of the fibre.
by continuously electronically tuneable spectral selection” [19]. Despite the noise introduced to the system by the use of PCF, they claim beneficial performance of the system for imaging purposes. Their method is complementary to the works by Birk and Storz in 2001 [3] and McConnell 2004 [34]. Figure 1.3 illustrates the experimental set-up of this technique.

Figure 1.3: Experimental set-up of electronically tuneable visible source using PCF [19].

One of the other alternatives for the excitation source in CLSM is the frequency-doubling technique in combination with an infra-red laser. Using a specially designed nonlinear optical crystal, a frequency-doubled beam with exactly half of the wavelength of the fundamental beam is produced. If the fundamental laser beam is tuneable over a certain range, it is possible to also obtain a tuneable frequency-doubled beam. Depending on the type of the crystal, a specific wavelength range is achievable. Commercial frequency-doubling crystals are available with a specific cut and mounted on the holder with a specific pre-calculated angle in order to produce the most efficient and phased-matched frequency-doubled beam.

3PBS1-3 represents polarizing beam splitters. FR stands for Faraday rotator, λ/2 is half-wave plates, and BD means beam dump.
Due to the spectrum broadening, if a femtosecond pulsed laser is used, the predicted output
pulse width of the beam emerging from the frequency-doubling unit is on the order of 10 ps.
The output power is in mW range which is enough to excite a typical biological sample that
requires about 50 $\mu$W of power [2].

Other than the light source, special considerations are needed for the light detector
sensitivity, proper optics alignment, selection of objective lenses, excitation intensity, sample
preparation, and staining (if needed).

In confocal microscopy the conic illumination patterns causes the loss of intensity in the
focal plane. This factor along with the scattering nature of the excitation light are two major
factors that limit the penetration depth in confocal microscopy.

Although the invention of confocal microscopy led to the advantages of optical sectioning,
higher resolution, and elimination of some of the common aberrations in optical microscopy,
the desire to image deeper into the specimens to search for more detail without significant
signal and resolution loss demanded an optical imaging technique which could compensate
for the shortcomings of the confocal microscopy. This led to the introduction of two-photon
microscopy to the optical imaging field in 1990.

1.2 Two-Photon Microscopy

A theoretical prediction of the Physicist Göppert-Mayer (1931) in the field of quantum
mechanics opened a new door to the optical imaging world [13]. She predicted that if multiple
photons with a total energy equal to that of a single photon are absorbed simultaneously, they
will lead to the same quantum event that occurs with single photon absorption; therefore,
for the two-photon absorption process we have:

$$\lambda_{1p} = \left(\frac{1}{\lambda_1} + \frac{1}{\lambda_2}\right)^{-1}$$  \hspace{1cm} (1.1)

where $\lambda_{1p}$ refers to the wavelength of the single photon and $\lambda_1$ and $\lambda_2$ are the wavelengths of
the two photons absorbed simultaneously. The implication of “simultaneously” is calculated
in the temporal gap of $10^{-16}$ s. Figure 1.4 shows the energy transition diagram for the single and two-photon absorption case.

![Energy transition diagram](image)

Figure 1.4: Energy transitions for single and two-photon absorption cases. $S$, $K$, and $S^*$ are the ground state, virtual state, and the excited state respectively [49].

The first application of her prediction in two-photon microscopy (TPM) was reported by Dr. W. W. Webb's group in Cornell University in 1990. Since then, a wide range of investigations and research has been done by different groups all over the world to improve the technique.

The experimental set-up for TPM is very similar to CLSM. A typical TPM configuration is shown in Figure 1.5. However, due to the illumination pattern in TPM, no pinhole is needed in the detection path of TPM. The excitation beam is highly localized and focused in the focal point, in contrast to single-photon imaging, in which a conic illumination pattern is shaped in the focal region causing excitation not only of the focal region, but also the region around it. In two-photon excitation process the wavelength range approximately twice the wavelength provided by the excitation source in single-photon imaging is required. Furthermore, since two individual events (i.e. photon absorption) must happen simultaneously, high density of excitation photons must be provided. Therefore, the most common excitation sources in TPM are infra-red ultra-short pulsed lasers.
To describe a two-photon transition in a fluorescent molecule quantum mechanically, a perturbation Hamiltonian theory is used. For a detailed mathematical discussion on the fundamentals of quantum mechanics of two-photon microscopy refer to [41]. The results obtained from this study indicate:

1. The perturbation Hamiltonian describing the photon is proportional to the amplitude of the optical field.

2. The transition probability from the virtual state, which results in two-photon absorption, is proportional to the second order of the intensity of the optical field.

3. Through the first order perturbation, transitions are allowed between wave functions with different parities.

4. Through the second order perturbation, which corresponds to two-photon absorption, transitions are allowed between wave functions with the same parity.

\(^4\)Electro-optic modulator (EOM) is used for controlling the laser intensity.
From part 2, it can be concluded that, since the highest excitation intensity is present at the focal point, this is where the highest probability rate of absorption events happens. The probability drops nonlinearly moving away from the focal point as opposed to the linear drop in one-photon excitation case [49, 44]. The nonlinear dependence is the cause of highly localized illumination pattern in two-photon excitation. Figure 1.6 shows a comparison of single-photon and two-photon absorption. The dependence of the fluorescence intensity on area is shown for both cases confirming the highly localized illumination pattern in TPM. If we employ the term “affected area” for the illuminated areas in the $xy$ planes through the conic illuminated shape in one-photon case, the constant line in Figure 1.6 for one-photon absorption implies that the total intensity over each affected area is equal to the others.

From part 3 and 4, it can be concluded the parity of the wave function is not changed in two-photon excitation, while in one-photon absorption the wave function parity changes. Due to parity selection rules, which modify the transition probability, the wavelength required for two-photon excitation is not exactly twice the one used in single-photon case. The parity selection rules enables a selected excitation beam with a certain wavelength to excite the spectrally different fluorophores simultaneously [30]. The optimal wavelength for TPM also
depends on the structure of the fluorophores [61].

For simplicity of the experimental set-up, one source is used instead of two sources of light to provide the two long-wavelength beams. Since the probability of two-photon absorption depends quadratically on the incident intensity, excitation power on the order of kW is required to obtain adequate fluorescence signals. If continuous-wave lasers are used as the excitation source with the power of kW, the biological specimen degrades dramatically. This problem is more pronounced for in vivo imaging. To overcome this issue, ultrashort pulsed lasers in the infrared wavelength region are employed as the excitation source. Typically the TPM light source produces ultrashort pulses (which is achievable by the mode-locking technique) on the order of femtoseconds with a high repetition rate (~ 80 MHz) to make the simultaneously two-photon absorption possible while maintaining high peak power (~ kW) and a modest average power (a few mW) for the produced pulse train. The most commonly used mode-locked infra-red laser in TPM is the ubiquitous Ti-Sapphire laser. Motorized and computer controlled Ti-sapphire lasers are now available and have the great advantage of minimal user intervention. One of the shortcomings presented in some of Ti-Sapphire lasers is that mode-locking is not possible over the entire wavelength range. One of the other drawbacks in using ultrashort pulsed lasers is related to the pulse dispersion which can lengthen the pulse duration. This problem can be overcome by using PCFs [35].

Other than the advantage of exciting a localized region in TPM, one should also consider that by applying the excitation wavelength almost twice the wavelength of single-photon excitation, the Rayleigh scattering decreases by a factor of 1/16 since Rayleigh scattering is proportional to the inverse of the fourth power of the excitation wavelength [41, 20]:

\[
I = \frac{a}{\lambda^4} + c
\]

where \( a \) corresponds to the intensity of the incident light, number of scatterers, the polarization, and the distance between the scatterer and an observation point and \( c \) is related to the instrumentation of the imaging system.
Due to the high localization of the excitation beam and less scattering rate in TPM, there is no need for confocal detection in the emission path. However, it has been suggested theoretically that improvement in the TPM resolution is possible by using confocal detection [41, 26]. This theoretical prediction has been tested experimentally and the improvement has been shown by using a finite-sized pinhole in the emission path of the TPM [22]. However, in the case of imaging a highly scattering specimen, confocal detection for TPM may not be beneficial since the scattered signals introduced to the pinhole plane may originate from the focal plane of the specimen [61].

Neither CLSM or TPM is aberration-free. Since optical aberrations lead to poorer resolution and weaker signals, attempts to correct the aberrations present a further challenge in the optical imaging field.

1.3 Spherical Aberration

In practice, no optical imaging that is free of aberrations can be carried out. Although in CLSM and TPM some of the optical aberrations are not observed any more (e.g. Coma, which occurs on objects off of the optical axis), some of the aberrations are inherent for any type of optical imaging system and need to be considered. Images obtained in the presence of the aberrations suffer from degraded resolution, low signal to noise ratio or geometrical distortion. One example of a common aberration is the chromatic aberration which results from the dependency of the refractive index of the objective lens on the wavelength (the effect called dispersion). The effect of this aberration is a shift in focal plane, or in other words, the excitation and collection happen in the different planes; thus, the objective lens does not function at its maximum numerical aperture and signal-loss happens [13]. Figure 1.7 shows the excitation and emission light paths in the objective lens with chromatic aberration.

Chromatic-corrected lenses (achromatic lenses) are available from different manufactures; however, these lenses are corrected for the range of 400-700 nm of excitation wavelength. Although, achromatic lenses do not compensate for the chromatic aberration in TPM, since
usually wavelengths longer than 700 nm are used, the fully-opened detection aperture in TPM helps to keep the signal-loss minimal and therefore, the effect of chromatic aberration can be neglected in TPM.

Another common and challenging aberration in optical imaging is spherical aberration. The term "spherical aberration" refers to the fact that an optical interface refracts the spherical wavefront, and hence, affects the shape of the wavefront and prevents the formation of a localized defraction-limited focal spot [56]. The illumination happens inside a broader region in the specimen, thus significant loss of signal and resolution occurs. In practice, the resolution of an imaging unit is defined by its point spread function PSF\(^5\). The distribution of electromagnetic field of a point source on the image plane represents the amplitude PSF. Since amplitude fluctuations are not observable directly, the intensity PSF, which is the squared of amplitude in the image plane, is used. To obtain the intensity PSF empirically, it can be simply measured by recording the images of subresolution objects such as fluorescent beads in both axial and lateral image planes. The effect of spherical aberration on the resolution can be described as broadening the axial and lateral PSF. Figure 1.8 shows the

---

\(^5\)Further details on PSF will be given in the next section
light traces in a mismatched image path and the resulting focal point and PSF broadening.

Figure 1.8: Spherical aberration. Incident rays with different entrance angles are focused at different positions which are distributed on the optical axis. The focal shift increases with the increase of incident angle [56].

In general, spherical aberration arises from the mismatch of refractive indices of the mediums through which the excitation beam needs to travel to reach the object (biological sample). The biological specimen itself could worsen the case when varying refractive indices are present in it. Typically, the refractive index in a biological specimen can vary in the range 1.2–1.7. Spherical aberration is exacerbated by imaging deeper into the specimen; serious signal loss can occur and geometric distortion may interfere with morphological analysis of the biological specimen.

A few static corrections for spherical aberration have been done [47, 28, 52]. These correction methods can be applied either on a specific specimen or for a specific imaging depth. Due to the varying nature of the refractive index of a biological specimen and also the need for deep imaging in most imaging tasks, a dynamic aberration correction method is required. Before introducing a dynamic correction method, it is helpful to discuss the aberration mathematically.

Consider a point source \( P_0 \) that is imaged by a lens to the point \( P'_1 \) (Figure 1.9). \( D_0 \) and \( D'_1 \) are the transverse distances of the object and its image from the optical axis
respectively. By considering an observation point, \( P \), in the image space, we can create the reference spherical wavefront with respect to \( C \), the center of the exit pupil. \( Q \) and \( \bar{Q} \) are the intersection points of the line drawn from the point \( P \) to the reference and aberrated wavefront respectively.

The aberration function, \( \Phi \), is then the distance \( QQ' \). In general, \( \Phi \) is considered to be the superposition of a series. A complete set of Zernike’s circle polynomials over a unit circle is used to model the aberration function \( \Phi \) [25, 7]:

\[
\Phi(D_1', \rho, \theta) = \sum_l \sum_n \sum_m a_{lmn} D_1^{2l+m} R_n^m(\rho) \cos(n\theta) \tag{1.3}
\]

where \( \rho \) is radial variable of the point \( Q \) normalized by the radius of exit pupil, \( \theta \) is the angular variable of the point \( Q \). \( a \)'s are the constants. \( l, m, n \) are non-negative integers when \( n \geq m \) and \( n - m \) is even.

For a given position of the point source, \( D_1' \) is constant and, therefore, equation 1.3 is reduced to double-index Zernike’s polynomials:

\[
\Phi(\rho, \theta) = \sum_n \sum_m A_{nm} Z_n^m(\rho, \theta). \tag{1.4}
\]

If \( m = 0 \), the function \( \Phi \) is radially symmetric. In this case, we have:
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\[ \Phi(\rho) = \sum_n A_{n0} P_n^0(\rho). \] (1.5)

Equation 1.5 expresses spherical aberration where \( n \) represents the order of spherical aberration and \( A_{n0} \), the Zernike coefficients, represent the amplitude of the aberration for the different modes. These coefficients have the unit of phase, thus they can also be scaled by the wavelength of the illumination beam. Figure 1.10 shows a series of the double-index Zernike polynomials patterned over a unit circle for different orders corresponded to different type of aberrations.

![Diagram of Zernike polynomials](image)

Figure 1.10: Double-index Zernike polynomials [51].

Since the aberrations—characterized with Zernike polynomials—deform the shape of the wavefront, one could compensate for the aberrations by measuring the changes and altering the shape of the wavefront to the correct spherical wavefront with regard to the measured changes: this method is so-called adaptive optical correction.
The traditional adaptive optical approach for correction, which was first used in astronomy in 1991, is based on the wavefront sensing/wavefront correcting system; a wavefront sensor measures the wavefront errors in the emitted light path from the sample; the calculated errors are used as the feedback for the wavefront corrector which compensates for the aberration. Figure 1.11 displays a schematic layout for this approach.

Figure 1.11: Combination of wavefront sensor and wavefront reconstructor in a typical adaptive optics set-up.

However, the main issue in employing this technique in fluorescence microscopy is that the emitted photons from the sample do not form an imaging beam, and therefore, it is difficult to measure the aberration by wavefront sensing [60, 4]. Due to this fact, an alternative method must be used to provide the feedback data for the wavefront corrector.

In one approach, the fluorescence intensity can be used as the feedback data. This technique (so-called sensor-less adaptive optics) is based on employing a single deformable mirror (Figure 1.12) in conjunction with a specific algorithm. This algorithm is based on the fact that the averaged intensity difference between two images acquired from an incident beam with preset positive bias aberration \(bZ_n\) introduced by the mirror and the other one with preset negative bias aberration \((-bZ_n)\) is approximately proportional to the present \(Z_n\). Therefore, the new state of the mirror in order to compensate for \(Z_n\) is defined by a calculation based on this averaged intensity difference. A significant aberration correction can be obtained by the correction of the higher orders of the Zernike modes. For a significant improvement regardless of the type of the specimen, correction up to \(n = 8\) (so-called spherical aberration of the third order) is needed [5].
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1. Introduction

Control electrodes

Al-coated membrane

Spacer

Si chip

Substrate PCB

Bias voltage

V1 V2 V3 Vn

Control voltages

Figure 1.12: Structure of a deformable mirror. The shape of the mirror is controlled by the voltages of the electrodes [42].

Different research groups [48, 60, 4] have applied this approach in both confocal and two-photon microscopy and their results confirm the higher resolution and deeper imaging range. The adaptive optics technique is practical and used widely, since it has the great advantage of being simply assembled to the microscope without changing the architecture of the microscope and also low power loss due to using a single deformable mirror. Figure 1.13 displays an experimental layout for a microscope with an adaptive optics unit.

Figure 1.13: Adaptive optics two-photon microscopy and four main components [60].

Spherical aberration, arising from the presence of multiple interfaces in the excitation path, can also be described by an alternative calculation expressed by the following equation
\[ \Phi(\theta_1) = -h_1 n_1 \cos \theta_1 + h_{N-1} n_N \cos \theta_N + \sum_{j=2}^{N-1} (h_{j-1} - h) n_j \cos \theta_j \]  

(1.6)

where \( h \) is the horizontal distance of each interface from the point of the focus, \( n \) is the refractive index of the medium, and \( \theta \) is the beam angle at the interface.

One of the static spherical aberrations in the imaging process results from the coverslip. In this instance, equation 1.6 is reduced to the case with only two interfaces:

\[ \Phi(\theta_1) = -h_1 n_1 \cos \theta_1 + h_2 n_3 \cos \theta_3 + (h_1 - h_2) n_2 \cos \theta_2 \]  

(1.7)

where \( n_1, n_2, \) and \( n_3 \) are the refractive indices of the objective immersion medium, the coverslip, and the specimen respectively.

In the case that \( n_1 = n_3 \) (e.g. imaging an aqueous-cultured specimen using the water-immersion lens) equation 1.7 is reduced to:

\[ \Phi(\theta_1) = d_c (n_2 \cos \theta_2 - n_1 \cos \theta_1) \]  

(1.8)

where \( d_c = (h_1 - h_2) \) is approximately the thickness of the cover glass, and \( \theta_1 \) and \( \theta_2 \) are defined by Snell’s law [25].

The aberration function in equation 1.8 is independent of the focal depth. Therefore, the aberration can be compensated for by introducing the opposite amount of the aberration to the imaging system. This fact has resulted in a new generation of objective lens designs. In such designs, the objective lens has been corrected for a specific thickness of the coverslip; hence, a coverslip with the thickness value given on the body of the objective lens must be used. For more convenience, lenses with multiple coverslip corrections have also been designed and are commercially available. In this design, the objective lens is corrected for different thicknesses of the coverslip. The adjustment for the desired thickness is done by using a collar ring on the objective lens.
A new alternative solution for spherical aberration has been offered by the use of afocal variator technology. The motorized version of the device using this technology sold by Infinity is called a Motorized Infocus Device (MID). Combination of this device with controlling software offered by Intelligent Imaging Innovation (3i) provides a solution to spherical aberration called NTSAC (New Technology Spherical Aberration Correction) which is applicable to any type of lens and any kind of imaging sample [40].

Beside the effect of spherical aberration resulting from mismatched refractive indices in the excitation path, which can be described by broadening and stretching lateral and axial PSFs, mismatched refractive indices also results in geometrical distortion in the obtained image as a shift in the focal point.

In the data analysis, when determining the axial distance geometry of a region of interest inside the specimen, the focal shift between the actual and nominal focal points (AFP and NFP) alters the data and may cause the meaningful geometrical distortion. In order to know the AFP in the sample, a theoretical correction is needed after imaging. This correction which is a combination of ray-tracing and Snell's law is described as [27]:

\[ NFP = \frac{\tan \alpha_1}{\tan \alpha_2} AFP = \frac{\tan \left[ \sin^{-1} \left( \frac{NA}{n_1} \right) \right]}{\tan \left[ \sin^{-1} \left( \frac{NA}{n_2} \right) \right]} AFP \]  \hspace{1cm} (1.9)

where \( \alpha_1 \) and \( \alpha_2 \) are the incident and refracted angles respectively. \( NA \) is the numerical aperture of the objective lens. \( n_1 \) and \( n_2 \) are the refractive indices of the first and second mediums.

With some modification, equation 1.9 can also be expressed as:

\[ NFP = AFP \sqrt{\frac{(n_2^2 - NA^2)}{(n_1^2 - NA^2)}} \]  \hspace{1cm} (1.10)

It could be noticed for a low \( NA \), equations 1.9 and 1.10 are reduced to:

\[ AFP = NFP \frac{n_1}{n_2}. \]  \hspace{1cm} (1.11)
Such correction can simply be applied to the acquired data in order to define the actual focal distance, however, one should consider that in this correction the focal distortion caused by spherical aberration is not taken into account; therefore, the result obtained by employing this correction does not lead to the exact focal distance, especially in deeper imaging when spherical aberration is more severe.

As stated earlier, spherical aberration causes signal and resolution loss in both confocal and two-photon microscopy. Since in two-photon microscopy the fluorescence signal has a quadratic dependence on incident intensity, it could be predicted that the signal drop in the presence of spherical aberration is higher when compared to confocal microscopy. However, it has been proven theoretically and experimentally that the amount of spherical aberration is higher in the confocal microscopy case [21]. One justification for this observation could be due to the fact that higher incident intensity localization occurs in two-photon excitation process.

More investigations on the effects of spherical aberration in both two-photon and confocal imaging systems have been done in this thesis and the results have been presented.

1.4 Point Spread Function and Deconvolution

In order to study the performance of the microscope, one can take advantage of the concept of the PSF of the microscope. The electromagnetic distribution of the optical field of a point source is referred to as amplitude PSF. To obtain an observable measurement of the PSF, the intensity distribution of a subresolution point-like object, resembling the point source, is used. We refer to such a PSF as the intensity PSF. A complete theoretical calculation of the amplitude and intensity PSF has been done by Born and Wolf, 1999 [7].

Different diffraction theories can be employed to calculate the diffraction pattern resulting from an objective lens; when dealing with a high numerical aperture lens (which is the common case in microscopy), the Debye approximation diffraction integral is adopted [25, 7]. The amplitude distribution at the point $P$ (an arbitrary point in the focal region of the point
source) by using Debye approximation is defined as:

\[
U(P) = -\frac{2\pi i a^2 n A}{\lambda f^2} e^{i\left(\frac{\pi}{2}\right)^2 u} \int_0^1 J_0 (v \rho) e^{-\frac{1}{2} i u \rho^2} \rho d\rho
\]

(1.12)

where \( A \) is the area of illumination or detection aperture. \( J_0 \) is the zero-order Bessel function. \( \lambda \) is the wavelength of the source in vacuum, \( n \) is the refractive index of the medium, \( f \) is the radius of the wavefront emerging from the circular aperture of the lens, \( a \) is the radius of the aperture of the lens, \( \rho \) is the radial variable of an arbitrary point on the wavefront normalized by \( a \), and \( v \) and \( u \) are the normalized variables that define the position of the point \( P \) in the focal region such that:

\[
v = \frac{2\pi}{\lambda} \left(\frac{a}{f}\right) r = \frac{2\pi a}{\lambda f} \sqrt{x^2 + y^2}, \quad u = \frac{2\pi}{\lambda} \left(\frac{a}{f}\right)^2 z.
\]

In equation 1.12, \( \lambda \) refers to either excitation or emission depending on the calculation of illumination or detection PSF. The intensity PSF is then calculated by \(|U|^2 = I\). For more accurate calculation of PSF, vectorial Debye approximation should be used where vectorial nature of electromagnetic field and incident light polarization are taken to the account [33].

In confocal microscopy, the PSF is a product of the illumination PSF and detection PSF, since a fluorophore can be excited and detected only if it is in the shared area of illumination and detection PSFs [13], therefore:

\[
I_{\text{confocal}} = I_{\text{ill}} I_{\text{det}}
\]

(1.13)

where \( I_{\text{ill}} \) and \( I_{\text{det}} \) are the intensity PSFs of illumination and detection respectively.

As stated earlier, two individual events must happen the in two-photon excitation case. Moreover, there is no need for a pinhole for the detection of the signal in this case. Therefore, the final PSF in two-photon excitation is the product of the squared illumination intensity PSF:

\[
I_{2p} = I_{\text{ill}}^2.
\]

(1.14)
To experimentally measure the PSF of a microscope, a subresolution fluorescent point-like object is required to be imaged (further discussion on the concept of subresolution will be given in chapter 4). The acquired image represents the intensity distribution of the microscope in the focal plane. The intensity distribution versus position gives a plot of the PSF. If an analysis of the microscope PSFs is required, the obtained PSF plots should be fit to a proper function because of the limitations (e.g., noise) in acquiring PSF profiles. In most of the cases, for simplicity, the Gaussian approximation is used [59]. The full width at half maximum (FWHM) of the PSF function is related to the resolution and is used as the measured resolution of the imaging system.

1.4.1 Resolution

Based on equation 1.12, the intensity distribution in focal plane \((u = 0)\) is calculated to be:

\[
I(0, v) = \left[ \frac{2J_1(v)}{v} \right]^2 I_0
\]

where \(I_0 = \left( \frac{n_0 a^2 |A|}{\lambda f^2} \right)^2\) and \(J_1\) is the first-order Bessel function.

According to equation 1.15, the intensity pattern on the lateral focal plane forms a ring-shaped or Airy pattern. Therefore, the Rayleigh criterion can be used for the definition of the resolution on the lateral plane. According to the Rayleigh criterion, the minimum separation distance between two point-like objects such that they are still differentiable is equal to the distance from the central maximum to the first minimum to the Airy disk [13]. Based on equation 1.15, the first zero intensity is at \(v = 3.832\), hence:

\[
r = \frac{1.22\lambda_{em}}{2a} \]

where \(\lambda_{em}\) is the emission wavelength and \(\frac{n_0}{f}\) gives the expression of the numerical aperture of the lens (NA). Therefore, the lateral resolution is expressed as:

\[
r_{lateral} = \frac{0.61\lambda_{em}}{NA}.
\]

Since the intensity distribution pattern is also periodic on the optical axis \((u = 0)\), the same approach is applied to define the axial resolution; therefore, we have:
\[ r_{axial} = \frac{2\lambda_{em} n}{NA^2}. \]  

(1.17)

Supposing an ideal imaging system, equation 1.16 and 1.17 gives us the expressions for the lateral and axial resolution of a conventional fluorescence microscope.

As mentioned, the resolution is defined by the FWHM values of the lateral and axial PSFs. In a confocal microscope under ideal conditions (i.e. very small pinhole and no aberration), FWHM extents are 30% smaller than those of a conventional microscope [13]; hence, the lateral and axial resolutions of the confocal microscope are given by:

\[ r_{lateral} \approx \frac{0.4\lambda_{em}}{NA}, \]  

(1.18)

\[ r_{axial} \approx \frac{1.4\lambda_{em} n}{NA^2}. \]  

(1.19)

In case of TPM, the FWHM extents of the lateral and axial PSFs are both broader than those of the conventional fluorescence microscope by about 15%; therefore, the lateral and axial resolutions in TPM are given by:

\[ r_{lateral} \approx \frac{0.7\lambda_{em}}{NA}. \]  

(1.20)

\[ r_{axial} \approx \frac{2.3\lambda_{em} n}{NA^2}. \]  

(1.21)

One can conclude that the resolution in confocal microscopy is higher than TPM; however, due to the higher scattering rate and lower excitation intensity in the focal plane as a result of the conic illumination pattern, the achievable imaging depth is more limited in confocal microscopy; hence the preference on selection of the imaging method is given to TPM when imaging a thick specimen.
1.4.2 Deconvolution

In microscopy, PSF affects the imaging process and presents the blurring effect on the final acquired images. In other words, the image is considered as the convolution of the object and PSF [58]. This concept is mathematically expressed by the following equation:

\[ g(\vec{r}) = f(\vec{r}) \otimes h_o(\vec{r}) \]  

where \( g(\vec{r}) \), \( f(\vec{r}) \), \( h_o(\vec{r}) \) are the image, the object, and the original PSF of the microscope respectively and \( \otimes \) is the convolution operator.

A real biological specimen can be considered as a superposition of many point-like objects; thus, each point in the final image \( (\vec{r}) \) is the superposition of the intensities of the other points \( (\vec{r'}) \) weighted by the PSF. Therefore, we have:

\[ g(\vec{r}) = f(\vec{r}) \otimes h(\vec{r}) = \int \int \int f(\vec{r}) \cdot h(\vec{r} - \vec{r'}) \, d\vec{r}'. \]  

(1.23)

Figure 1.14 shows a schematic diagram of equation 1.23.

![Figure 1.14: Convolution of a PSF and an object [54].](image)

In microscopy, it is desirable to have the acquired image as close to the ideal image as possible (i.e. without the blurring effect). The process for restoring an image to its original
de-blurred version is then called "deconvolution" which tries to negate the effect of the convolution and, consequently, construct a sharper image close to the real object. There are a few approaches to accomplish deconvolution which will be explained later on.

Although the main purpose of applying deconvolution technique on the images is to obtain higher resolution by removing the captured aberrations, the optimal results are achievable when the microscope is optimized for all the possible aberrations prior the collecting the PSF which will be used in deconvolution algorithm. As it was discussed in section 1.3, different aberrations are presented in the imaging tasks. In the best case, the alignment of the system is optimal, no malfunctioning is present at any part of the system, the objective lenses are corrected for the chromatic aberration, coverslip corrected lenses are used having the thickness of the coverslip match the correction value, and the refractive index difference of the lens medium and the specimen is minimal; however, the presence of the aberration, even minimal, is unavoidable and, in such case, the best possible result is obtained by performing a deconvolution technique.
Chapter 2

Methods

2.1 Experimental Setup

All parts of the experiment were carried out with the use of inverted Olympus IX71 microscope in the fluorescence mode with four different objective lenses. The objective lenses used for the experiment are Olympus UplanAPO/IR 60x/NA = 1.2 Water co/0.13-0.21 which is equipped with coverslip correction from the thickness of 0.13 mm to 0.21 mm, and Olympus Japan LUM Plan Fl 40x/NA = 0.8 Water oc/0 which is a long working distance lens ~ 3.3 mm and no coverslip is applicable.

The excitation source for the microscope in the case of single-photon imaging is provided by a diode laser (PicoQuan GmbH) with the single wavelength of 407 nm. The diode laser delivers pulses with the duration of several hundred picoseconds. Due to the age of the diode laser and efficiency loss, in order to have the adequate intensity, the pulse repetition rate was set on the minimum.

For TPM, the excitation source is a solid state mode-locked Ti-Sapphire laser (Tsunami, Spectra-Physics). Although the laser is designed to be tuned from 700 nm to 1080 nm, the practical obtainable tuning range is from 690 nm to 820 nm. The laser is designed to deliver pulses at both picoseconds and femtosecond pulse width; however, for two-photon imaging, the laser is needed to be operated at femtosecond pulse width which varies in a range of 80 to 200 fs with 80 MHz repetition rate [2]. The Tsunami laser is pumped with a CW Millennia-Pro laser from Spectra Physics which emits in the green (532 nm) with output power > 5 W. To obtain a longer tuning range, and also, to increase the imaging depth into the specimen, the power of the pumping laser is needed to be set to a higher setting.
Other than the dispersion problem which was mentioned before, instability of the laser can be counted as one of the other drawbacks in the experiment, which arises after extended duration of operation, and also, almost each time that the laser is run different output power value and band width are observed.

The scanning unit (Fluoview Olympus) consists of the optics, filters, steering mirrors, scanning mirrors, and the detectors (i.e. PMTs) placed in the rear part of the box. Figure 2.1 displays the scanner unit with its components.

![Fluoview 300 scanning unit](image)

Figure 2.1: Fluoview 300 scanning unit [43].

While in two-photon imaging there is no need to use the pinhole, for confocal imaging it is essential to use the aperture as it was explained earlier. It should be noted that different sizes of the pinhole must be used for different numerical aperture of the objectives which have different emission collection rate. The ideal pinhole size for confocal imaging can be obtained during the imaging task by adjusting different pinhole sizes, but it is suggested to use the larger apertures with lower numerical aperture lenses since they have lower emitted fluorescence collection rates.

The scanning mirrors inside the scanning units are two high-speed oscillating mirrors driven by galvanometer motors, which pivot on mutually perpendicular axes and are placed very close to each other. One mirror scans along X axis and the other one along Y axis; the
coordination of these two mirrors produces the raster scanned image on the XY plane.

Two detection channels are achievable by utilizing a dichromatic mirror with a combination of filters for the certain range of wavelength detection. The band pass filters (Chroma) with the width of 50 nm are used in our equipment.

The fluorescence after filtering through the channels enters the detectors. The data from the detectors is digitalized and displayed via software (Fluoview v 5.0 Olympus). Multiple features are available in the software in order to analyze the acquired data and perform more manipulations on the images.

One of the challenges in aligning the imaging system is to steer the excitation beam to the center of the back aperture of the objective lens. As described in chapter 1, the beam from the excitation source (in our case, diode laser and Tsunami) should enter the scanner unit, strike the scanning mirrors, and then enter the objective lens. Any misalignment in guidance of the beam results in significant signal loss, or even, no collected signal. In order to ensure that the excitation beam is steered on to the center of the back aperture, a protocol is required.

In steering the beam, two considerations are needed: 1) the beam must lie on the beam path through the scanner and 2) the excitation beam must be non-angled. To do so, two flat mirrors, mounted on the kinematic mirror mount with three adjusters (Thorlabs), were used. It is important to note that one of the mirrors was used for adjusting the position of the beam so that the beam lay on the beam path through the scanner and the other mirror was used for angular adjustments. If only one mirror is used, the position adjustment must be done by raising or lowering the beam source (e.g. diode laser) and by changing the location of the mirror holder. In most cases, it is not possible to displace the laser (e.g. Tsunami laser in our case) and even if it is possible, it is inconvenient and very challenging to level the height of the beam by changing the laser height accurately. By using two mirrors, the alignment of a laser beam is then a simple iterative process of sequential adjustments of both mirrors. Figure 2.2 shows the alignment set-up for the diode laser. To create a non-angled levelled beam (i.e. parallel to the optical table), two small diaphragms (same height), separated
by some distance on the optical table, were placed in front of the mirror which faced the scanner entrance. Afterwards, the laser beam was directed through the diaphragms using the two mirrors. While still keeping the diaphragms in place, next step was to ensure that the beam is centered at the back aperture of the lens; one of the lenses was removed from the microscope lens stage. The system was put on the scanning mode. The excitation beam illuminated the top part of the microscope unit which is a diaphragm for the microscope normal lamp. Using a webcam facing up the lamp diaphragm, the illumination spot was shown on the computer screen and by adjusting the mirrors, the beam was positioned exactly on the center of the lamp diaphragm.

Figure 2.2: Excitation source lineup for single-photon. Figure shows the diode laser, mounted anamorphic pair of prisms, and two facing mirrors.
2.2 Sample preparation

2.2.1 Bead Recording

Fluorescent beads solution (Tetraspeck 0.2 μm, Molecular Probe) was used. The beads fluoresce at four different emission peaks which are shown in Figure 2.3.

![Excitation spectra for Tetraspeck bead](image)

Figure 2.3: Excitation spectra for Tetraspeck bead [39].

For “depth-independent bead sample”, 5 μl of the bead solution was pipetted on the microscope slide and dried completely. The slide was heated on the heater at the low temperature (close to body-temperature) in order to prevent the floatation of the beads. 5 μl of deionized water was then pipetted on the slide over the beads spot; next, the coverslip (#1 Fisherbrand) was placed on the water drop and it was sealed with nail polish. The same steps were applied to prepare the beads in antifade reagent solution (Slowfade Gold, Molecular Probe) only with replacing the deionized water with the antifade solution.

For “depth-dependent bead sample”, the Tetraspeck beads were embedded in agarose gel (Agarose II. Mandel). 1% agarose gel (0.2 g / 20 ml water) was boiled. To prevent the lens-effect and obtain an even imaging surface, the sample was prepared on the coverslip. A metal filter holder resembling a large pinhole with the depth of about 4 mm, was used as a frame and it was placed on the coverslip. To simply define the 0 μm depth during the image acquisition, first 5 μl of the vortexed bead solution was pipetted on the coverslip through
the frame. Next, 0.5 ml of the aqueous gel was poured on the center of the frame slowly. 20 µl of the bead solution was then pipetted into the center of the gel in the frame. In the end, using the pipette tip, the solution was mixed vertically at the center and it was left to be solidified in the room temperature; then the gel was slowly detached from the frame. Instead of detaching the gel from the frame, the coverslip was detached from the bottom of the gel when preparing the sample for 40x lens with which no coverslip is applicable. Afterwards, the gel attached to the frame was placed on the microscope slide holder.

For the in situ bead recording, two types of leaves were used: Arabidopsis and Fern. For the arabidopsis leaf, the solution of 10 µl of the beads diluted (factor 2) by deionized water, was carefully microinjected by a microsyringe (10 µl volume) with 30 gauge needle (Hamilton co.) into the petiole of a fresh leaf. The leaf was washed with water in order to remove the residue of the solution from the surface of the leaf. For the fern leaf, the petiole part of a fresh leaf was placed into the bead solution (solution:water = 1:4). Approximately after 30 minutes, the leaf was washed.

2.2.2 Fluorescent Agarose Gel

30 µl of a red fluorescent dye (emission peak at 640 nm) was mixed with 1 ml of 0.5% agarose gel in a microcentrifuge tube on the vortex and it was poured into the filter holder frame placed on the coverslip until it solidified and then, it was detached from the frame.

2.2.3 Microchopped Leaf Tissue

A fresh leaf was washed. A flat section of the leaf was cut and it was placed in a tissue chopper container already half-filled with paraffin. The container was then completely filled with paraffin. A sectioning value on the tissue chopper machine was set on 60 µm. A cut piece of 60 µm thickness was placed between two coverslips and sealed.
2.3 Software

Three major software packages were used for data analysis and the preparation of the results and the images. Mathematical operations and calculations and also presentation of the plots were done by MATLAB V7.4. Specifically, it was used to perform a number of simple data averaging tasks and some noise reduction filters on the images; mostly, it was used to calculate plots and display them.

PeakFit V4.12 was used to fit the Gaussian functions to the PSF intensity profiles recorded from the beads. The FWHM values provided by the software as the numeric data of the Gaussian functions were used in our data analysis.

We also used a free image processing software which is called ImageJ. This software has the ability to be extended in functionality using external plug-ins. Moreover, the software itself has various useful image manipulation functions such as filters, mathematical functions, editing tools, and scripting. We used this software to perform simple image editing actions and more importantly, used some plug-ins to align the center of the images and do deconvolution algorithm which is a main part of this thesis. ImageJ is available online and can be found in [53]. Figure 2.4 shows a snapshot of the main window of the ImageJ software.

![ImageJ Software](image.png)

Figure 2.4: A snapshot of ImageJ software [53].
Chapter 3

Image Acquisition Enhancement

3.1 Optimization of the Lens Coverslip Collar

"In practice, no imaging system is aberration-free" [25]. Minimization, or if possible, elimination of the potential sources of the aberrations during the image acquisition will result in higher signal and resolution. If further improvement is desired, deconvolution and image processing techniques can be applied.

One of the important parameters that impact the resolution and quality of the images is spherical aberration. Hence, a specific concern should be paid to eliminate this factor as much as possible.

As explained earlier in section 1.3, by introducing the opposite amount of spherical aberration (originated from the coverslip interface) to the lens, correction of such aberration is possible. For a more versatile application, a specific design of the objective lens comprises the correction for the multiple thicknesses of the coverslip. A collar ring is embedded on the body of the lens indicates the values of the different thicknesses of the coverslip (Figure 3.1). For each coverslip used in the imaging task, the collar ring should be set on the thickness value of the coverslip.

Selecting a mismatched value on the collar has a noticeable influence on the resolution, especially on the axial resolution, and collected signal. Therefore, it is essential for the user to have the knowledge of the thickness of the coverslip in sample preparation prior to the imaging process. This value is indicated on each coverslip box. For instance, the standard thicknesses of the coverslip sold by the Warner Instrument brand are: #1: .15 mm, #2: .17 mm and #3: .22 mm, or for the Fisherbrand are: #1: .13 mm to .17 mm and #2: .17 mm
Chapter 3. Image Acquisition Enhancement

Figure 3.1: Olympus UplaApo 60x/W objective lens. The array shows the correction collar ring [43].

...to .25 mm.

In order to investigate the effect of a mismatched coverslip, we used a homogenous fluorescent sample. In the selection of the sample, it is important to note that the sample must have the same refractive index (which practically is impossible) as that of the lens immersion medium or have a small refractive index difference: the reason for such selection is due to the fact that mismatched refractive indices is the source of spherical aberration. Therefore, to focus on the effect of the mismatched collar value, a proper homogenous sample must be utilized. Hence, fluorescent agarose gel was used with assumption that it has the small refractive index difference with water (which was proven to be right). The reason that the fluorescent dye itself was not used as the homogenous sample was due to the movement of the liquid during the data acquisition.

In our case, the experiment was performed for the Olympus UplanAPO/IR 60x/NA = 1.2 co/0.13-0.21 which is the water immersion lens and has the coverslip correction from the thickness of .13 mm to .21 mm with working distance of ~ 280 µm. Fisherbrand coverslip with #1 thickness was used. The axial fluoresence response was obtained. The test was run for both single-photon and two-photon. The excitation wavelength for two-photon source was selected at ~ 775 nm and for one-photon at 407 nm. For both cases, 680 nm filter was used in the scanner unit. For each collar value, stack acquisitions in the Z direction were obtained with the Z step of 1 µm up to the depth of 280 µm. At each acquisition, the
imaging parameters were kept the same. Each time, the collected intensity along the optical axis (Z direction) was acquired. Figure 3.2 shows the results of the test. The difference in the intensity signal acquired using different collar values in Figure 3.2 is apparent, leading us to select the value of .15 on the collar for the coverslip.

Beside the significant amount of signal loss by using an mismatched collar value, the loss of the resolution is also significant. To show this effect the depth-independent bead sample was imaged using both excitation sources. The results (Figure 3.3) confirm the resolution degradation, significantly, in axial direction with an mismatched value on the collar ring.

The higher the difference between the collar number and the actual size of the coverslip is, the higher the resolution degradation and the lower the signal would be. Therefore, one can conclude that the choice of the coverslip (for the objective that are corrected for a standard coverslip) has a significant impact on the image quality.

### 3.2 Amount of Spherical Aberration

As described earlier, one of the major factors that affects the resolution and fluorescence signal is spherical aberration. It was shown in equation 1.8, for the three media case (two interfaces), when the third medium has the same refractive index as that of the immersion medium of the lens, spherical aberration resulting from the coverslip interface can be corrected by introducing the opposite amount of the aberration in the lens structure. In the previous section a method to optimize the collar adjustment was explained. Although using the appropriate coverslip (for the objectives that corrected for one standard coverslip thickness) or collar value (in the case of employing a lens with the coverslip correction collar) improves the signal, the quality of the images is still under the influence of spherical aberration, since the refractive index of the biological specimens, in most of the cases, are different from the medium of the objective lens. Any mismatch results in spherical aberration; the situation is worsened when a variation of the refractive indices are present in the region of interest inside the specimen.
Figure 3.2: Axial fluorescence response obtained from the fluorescent agarose gel with different collar values at two excitation sources. a) Two-photon. b) Single-photon.
Figure 3.3: Effect of using mismatched coverslip thickness on PSF. a) Lateral and axial PSF variation for two-photon excitation. b) Lateral and axial PSF variation for single-photon excitation. c) Lateral PSF intensity distribution for two-photon. d) Axial PSF intensity distribution for two-photon. e) Lateral PSF intensity distribution for single-photon. f) Axial PSF intensity distribution for single-photon.
Referring to spherical aberration equation (1.6), the parameters that affect the amount of the aberration are numerical aperture of the lens, imaging depth and involved refractive indices of media. We studied spherical aberration with regard to the parameters mentioned above. The experiment required the analysis of the imaging system PSFs acquired under different conditions. Therefore, it was necessary to image the subresolution beads through the different penetration depths. This test was first examined using gelatin mixed with the bead solution. Sample was kept in the low temperature, and after it solidified, was imaged. However, due to the low melting temperature of gelatin, the test failed and agarose gel with high melting temperature was examined.

Depth-dependent bead sample was prepared. Two water immersion objective lenses with numerical aperture of 1.2 for 60x lens with correction collar ring, and 0.8 for 40x lens with long working distance (3.3 mm). Since no coverslip is applicable for 40x/0.8 W lens, in preparation of the depth-dependent bead sample intended to image with this lens, instead of detaching the gel from the frame, the coverslip was detached from the frame (refer to 2.2), and the frame with the attached gel was placed directly on the slide stage of the microscope. The sample was imaged with two-photon source at \( \sim 770 \text{ nm} \) excitation wavelength and the emission peak of the beads was detected at 580 nm. Optical zoom factors of 6 and 9 were used for acquisition with 60x and 40x lenses respectively in order to achieve same pixel size for both lenses (0.076726 \( \mu \text{m} \)). Digital display window of 512x512 pixels on Fluoview software was chosen. In order to reduce the random noise during the data acquisition, slow scanning with factor 3 of the Kalman averaging (further details on Kalman averaging will be presented in chapter 4) was applied. Due to the dehydration of the sample during the data acquisition, several samples were prepared and new one was replaced in the case of dehydration.

For the lateral PSFs, between 2 to 5 beads from the image were visually selected and cropped at each acquisition depth. For each cropped bead, the intensity distribution over \( x \) direction was plotted; the FWHM extents of the PSFs at each acquisition depth were averaged over 2 to 5 acquired beads at the same depth and the mean value was calculated.
For the axial PSFs calculation, 6 μm-long stack of the slides with the step size of 0.075 μm were acquired at the different depths. FWHM extents were obtained and averaged over 2 to 5 beads. Although the refractive index difference between the prepared agarose gel and water was small, the effect of spherical aberration on FWHM extents of both axial and lateral PSFs (i.e. resolution) is observable. Figure 3.4 shows the result for both objective lenses.

From the results, it is concluded that at low penetration depths, no significant changes in the FWHM extents of the lateral PSF for our 1.2 numerical aperture lens is apparent, while at higher penetration depth (> 90 μm), broadening of the FWHM is observed. The results indicate the investigated lens is more suitable for the low penetration imaging tasks. However, it is noticeable that the axial FWHM extent varies correspondingly with the penetration depth.

To confirm the results on an actual biological sample, 60 μm-microchopped Ficus Benjamin Variegata leaf was imaged with water immersion 60x/1.2 lens. The sample was imaged with two-photon excitation source at wavelength of ~ 775 nm, slow scanning mode with 3x Kalman averaging, pixel resolution of 0.456 μm, and detection filter of 680 nm. A spot on the sample surface was imaged at depth 10 μm; since the thickness of the sample was 60 μm, the sample was inverted and the same spot was found and imaged at the depth of 50 μm. A significant difference in the fluorescence between these two acquired images was observed (Figure 3.5).

The experiment was also carried out using 40x/0.8 lens with the exact same image acquisition parameters as was used with 60x/1.2. The results show the FWHM extents of the lateral PSFs do not vary with depth appreciably before reaching the penetration depth of ~ 300 μm. Although the lateral FWHM extent at low penetration depths for 40x/0.8 lens is higher than 60x/1.2 lens, which is in conformity with the resolution equation, 60x/1.2 proves to be more sensitive toward the refractive index variance especially at relatively high pen-
Figure 3.4: Variation of FWHM extents of lateral and axial PSFs with regard to the acquisition depth. a) Lateral FWHM extents using water immersion 60x/1.2 lens. b) Axial FWHM extents using water immersion 60x/1.2 lens. c) Lateral FWHM extents using water immersion 40x/0.8 lens. d) Axial FWHM extents using water immersion 40x/0.8 lens.
Figure 3.5: Effect of deep imaging with high numerical aperture. a) Image of a spot in a Ficus Benjamina Vagriegata leaf acquired at 10 μm by the 1x zoom factor. b) Image of the same spot in (a) acquired at 50 μm by the 1x zoom factor.
traction depths (> 90 μm). However, it is important to note even with significant broadening in the axial FWHM extent of 60x/1.2 objective up to the depth of 100 μm, it is yet smaller than the axial extent of 40x/0.8 lens.

To investigate more on the role of the numerical aperture of the objective lens, a homogenous sample of the red fluorescent glass block was imaged with the both lenses. The result shown in Figure 3.6, also confirms the higher amount of the aberration resulting from using 60x/1.2 than 40x/0.8. The results can also be compared to the results obtained using fluorescent agarose gel which has less refractive index difference with water than fluorescent glass (Figure 3.6(b)).

![Graphs showing fluorescence response](image)

Figure 3.6: Amount of the introduced spherical aberration with regard to different numerical apertures of the objective lens. a) Collected fluorescence response of a fluorescent glass block. b) Collected fluorescence response of a fluorescent agarose gel.

The investigation confirmed the presence of spherical aberration even for very small refractive index mismatch between utilized media. From this study, it is concluded that the selection of the objective lens for imaging task has a great impact on the image quality. A higher numerical aperture introduces more amount of the aberration in the deep penetration, while higher axial and lateral resolution in surface imaging compared to a lower numerical aperture. This fact makes them more practical for surface imaging and low numerical aperture lens are preferred for deep imaging. Figure 3.7 displays images of an area in the fern leaf at depth ~ 20 μm imaged with 60x/1.2 lens and 40x/0.8 with exact same image acquisition.
parameters for both. Higher image quality and signal are observed in the image with 60x/1.2 W.

### 3.3 Beam Shaping

Deformed shape of the excitation beam cross section represents a drawback in microscopy. As mentioned earlier, one of the conventional excitation sources of confocal microscopy is a diode laser. Among the diode lasers, UV is the common source of excitation for the fluorophores in the range of the green or even higher (in some of the cases, they excite the fluorophores in the red spectrum as well).

Most of the diode lasers, including the 407 nm-diode laser of our confocal source, suffer from aberration of the beam shape which results in a non-circular, and in general, elliptical output cross section.

To correct the beam shape of the diode laser, several methods are available including a pair of anamorphic correcting prisms, cylindrical lens, and optical fiber. It should be noted that any of these methods presents output power loss [32].

Because of convenient assembling process, lower price, and lower power loss comparing to fiber [32], an anamorphic pair of prisms was selected as the correction method for the diode laser (407 nm) used in our experimental set-up.

The anamorphic prisms have the ability to compress/extend the beam cross section in only a specific direction with a defined magnification. The manufactured pair of prisms has a predetermined wavelength range. Output beam exits at the same direction as the incident beam. A typical layout of the anamorphic pair of prisms can be seen in Figure 3.8.

The beam cross section of the diode laser in our experimental set-up was significantly expanded in the vertical direction. Therefore, a pair of prisms was applied in order to compress the beam cross section in vertical direction. The mounted pair of anamorphic prisms (Melles Griot), with 2x magnification, was placed between the diode laser and the first mirror; it was then rotated until the finest beam cross section was achieved. Two-mirror
Figure 3.7: Low penetration depth imaging by two different numerical apertures. a) Image of the fern leaf by water immersion 60x/1.2 with pixel size of 0.076726 μm. b) Image of the fern leaf with water immersion 40x/0.8 with pixel size of 0.076726 μm.
iterative beam adjustment method was performed again since the prism raised the output beam by 5 mm. The laser output had a power loss of about 22% as a result of using the corrector prisms.

We investigated the effect of beam correction on the image quality and resolution. The intensity axial and lateral PSFs in both cases (corrected and uncorrected beam) were compared together. Table 3.1 illustrates the reduction of the FWHM extents of the PSFs, especially those of the axial PSF.

<table>
<thead>
<tr>
<th>Beam Cross Section</th>
<th>Lateral Resolution (µm)</th>
<th>Axial Resolution (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected</td>
<td>0.34 (Wid. Std. Err. ±0.06)</td>
<td>0.86 (Wid. Std. Err. ±0.09)</td>
</tr>
<tr>
<td>Uncorrected</td>
<td>0.37 (Wid. Std. Err. ±0.08)</td>
<td>2.29 (Wid. Std. Err. ±0.03)</td>
</tr>
</tbody>
</table>

Table 3.1: Comparison of the lateral and axial PSFs obtained with corrected and uncorrected diode laser beam cross section.

More spherical aberration rate was also observed during the imaging by uncorrected cross section. A homogenous sample (red fluorescent glass) with the refractive index of ~1.52 was imaged through the z direction by 60x/1.2 W objective lens in both cases (Figure 3.9).

Finally, the effect of the beam correction was examined on an actual biological sample. A 60 µm-microchopped leaf (name unknown) was imaged by 60x/1.2 W objective lens in
Figure 3.9: Comparison of the induced spherical aberration using corrected and uncorrected diode laser beam cross section. The fluorescence response from a thick fluorescent glass block imaged by the corrected and uncorrected beam was collected along \( z \) axis. The curve representing the uncorrected beam has been enlarged by a factor of 2 for the ease of visual comparison. The original curves are shown at the bottom right corner.

both cases (Figure 3.10). Significant higher resolution and less blurring were observed in the image acquired by the corrected beam. Note the details on the surface of the green part of the image in Figure 3.10(a) which are blurred in Figure 3.10(b). Different intensity distribution patterns are also noticable in two images.

3.4 Excitation Wavelength

In fluorescence microscopy, the detected signal is consisted of three elements: fluorescence, scattered light, and stray light. The desired element is the fluorescence; therefore, the scattered and stray light elements should be eliminated. While it is possible to diminish the stray light by improving the ambient conditions, a deliberate approach is needed for reducing the scattering rate of the light.

The excitation spectra of most of the biological fluorophores usually have a wide band. Based on the Rayleigh scattering (equation 1.2), the scattering rate will be reduced significantly by increasing the excitation wavelength since they have an inverse biquadratic
Figure 3.10: Effect of circularizing the cross section of the excitation beam on image quality. a) Image of a leaf acquired by corrected diode laser at the depth of 10 μm b) Image of the same spot in part (a) acquired by uncorrected diode laser at the depth of 10 μm.
relation. Therefore, when imaging a biological specimen, it is important to choose a proper excitation wavelength. The excitation wavelength of the fluorophores should be chosen such that the trade-off between the minimal scattering rate and enough fluorescence is optimized.

To investigate the fact further, a fern leaf was imaged using two-photon excitation. The 680 nm (band width of 50 nm) emission filter was used for detection of the fluorescence. The excitation wavelength was changed over the range of 700 nm to 810 nm and the red fluorescence was detected. Figure 3.11 shows the acquired images of a same position of the leaf with the exact same imaging parameters except the wavelengths of 800 nm in (a) and 770 nm in (b). A considerable amount of scattering is observed in the image acquired by 770 nm excitation wavelength comparing to image acquired by 800 nm excitation wavelength.
Figure 3.11: Effect of excitation wavelength on image quality. a) Image of the chloroplast and the background of a fern leaf acquired at wavelength of 800 nm with two-photon excitation at zoom one. b) Image of same spot in part (a) with 770 nm excitation wavelength.
3.5 Summary

Several parameters and their effects on the image quality were studied in this chapter. It was observed that using a coverslip with a thickness other than the value for which the objective lens is designed causes a noticeable degradation on the image quality.

Furthermore, the influence of the numerical aperture of the objective lens on spherical aberration was studied. Two water immersion objective lenses with different numerical apertures were experimented; obtained results suggested that a lower numerical aperture should be used in deep penetration imaging and vice versa.

The discussion was followed by investigating the effect of the deformed beam cross section the resolution. The non-circular cross section of the diode laser in our experimental set-up was corrected and shaped with an anamorphic pair of prisms and the improvement on the resolution and collected fluorescence was shown. The chapter was ended by introducing the concept of the excitation wavelength on the image quality. Higher amount of the scattering rate, and consequently, more blurring was observed in the images acquired by higher excitation wavelengths.

Beside the parameters that we investigated in this work, many other already studied parameters are also efficacious in the quality of the images. For instance, the fluorophore molecule saturation results in broadening the FWHM extents of the PSFs hence the lower resolution [11].

Optimization of the image acquisition parameters results in a major improvement on the image quality. However, as it was stated, the improvement is restricted due to unavoidable aberrations and scattering nature of the optical imaging. Further improvement can be done by the help of image processing techniques such as deconvolution. The next chapter will discuss this subject in details and experimental results are presented.
Chapter 4

Deconvolution

4.1 Fundamentals

As stated earlier, the acquired images in microscopy always are blurred and some kind of restoration is needed for the enhancement. The blurring of these images is modeled by the mathematical convolution of the microscope PSF with the original object image using the following equation [54, 57]:

\[
g(\vec{r}) = f(\vec{r}) \otimes h(\vec{r}) = \iiint f(\vec{r}) \cdot h(\vec{r} - \vec{r}') d\vec{r}'.
\] (4.1)

In the above equation, \(g\), \(f\), and \(h\) represent the acquired image, the ideal image (no blurring) and the PSF respectively. They are defined in 3D coordination such that \(f(\vec{r}) = f(r_x, r_y, r_z)\) but can similarly be defined and used in 2D. The goal of deconvolution is to calculate the unknown \(f\).

The very basic idea of deconvolution is to reverse the convolution given by equation 4.1. In order to facilitate the inversing, every component is transformed to the frequency domain in which the convolution equation is also transformed to a much simpler shape of multiplication as shown in equation 4.2 [54, 57]:

\[
G = F \cdot H
\] (4.2)

Supposing that the PSF is available, \(G\) and \(H\) are calculated by transforming acquired image and PSF to the frequency domain. This means \(F\) is simply gained by dividing \(G\) by \(H\) [54]:
Finally, $f$ can be found by transferring $F$ back to the spatial domain by the inverse Fourier transform. However, the process is not that simple. The division cannot be performed because of two facts: First, some components of $H$ are zero and therefore a division is not possible. Second, the acquired image always carries noise which has not been accounted for in the above model [36, 54].

Since the PSF is an image, $h$ will be a 2D or 3D matrix. Using the Fourier transform to compute $H$, which is also a matrix, causes in some zero components and it makes the division troublesome. Even if zero components are exchanged with small values, the final deconvolved image will contain high value spikes which are artifacts, especially if the acquired image is noisy [36, 54].

Noise is always a limiting factor of the image quality. There are various methods to reduce noise, specifically if the noise source and its type is known. However, that is not always the case. Moreover, we are not always able to recover from different kinds of noise even if its type is known to us. Therefore, the formula presented in the convolution equation is in fact [54]:

$$g = f \otimes h + \varepsilon$$

These are the reasons that simple linear methods such as division by $H$ will not be practical all the time. Consequently, researchers tried to accomplish the task by other means. One of the most successful approach is to use iterative methods to estimate $f$ and try to improve the estimation gradually. The next section describes how iterative methods work and presents one example. It is also notable that there are many deconvolution methods which may or may not be iterative in nature such as statistical image restoration and blind deconvolution where the former is based on statistical characteristic of an image and the latter tries to estimate the PSF ($h$) as well as the image ($f$) [36, 57].
Chapter 4. Deconvolution

4.2 Algorithm

Instead of deriving the deconvolved image directly from the acquired image, the iterative algorithms use a build-up approach by eventually estimating the deconvolved image. This means they try to start from an initial estimation \( f_0 \) and then, compute an improved version \( f_i \) called the deconvolved estimation by using a recursive function. Then, they perform a convolution operation on this estimate (using the PSF) to compute the convolved estimate \( g_i \). The closer the convolved estimate is to the acquired image \( g \), the better the actual object \( f \) is estimated by the deconvolved estimation \( f_i \). Finally, if certain convergence conditions are met (i.e. the deconvolved estimate is close enough), the algorithm stops. Otherwise, another iteration of estimation is performed until the convergence conditions are satisfied [57]. Figure 4.1 shows the general iterative deconvolution algorithm.

\[
\begin{align*}
1. & \text{ Choose an initial estimate } f_0. \\
2. & \text{ Compute the next estimation } f_i \text{ using a recursive function: } f_i = RecFcn(f_{i-1}, g, h) \\
3. & \text{ Compute the convolved estimation } g_i \text{ using the given PSF } h: \quad g_i = f_i \circ h \\
4. & \text{ Find the score of estimation using an objective function: } \text{Score} = \text{ObjFcn}(g_i, g) \\
5. & \text{ If the convergence conditions are met return } f_i \text{ as the final result otherwise go to step 2.}
\end{align*}
\]

Figure 4.1: General algorithm of deconvolution

The above algorithm is a general template and there are three concepts which can be defined differently to form different variations. In fact, most of the iterative deconvolution algorithms can be mapped to such template introduced above.

The first concept is the recursive function \( RecFcn \) which determines how the next estimation is improved. This function can be designed to minimize the error or optimize some statistical characteristic of the estimation. Either way, it is the responsibility of the designer to show that if enough iteration are performed, the recursive function will finally result in the deconvolved image \( f_{est} \) to be equal to the actual object image \( f \).

Next is the objective function which determines how much improvement is obtained. This function could come from a variety of choices. A simple subtraction of \( g \) and \( g_i \), their
mean score error, or a more complex statistical measure can be selected as the objective function. Usually the objective function is in accordance with recursive function because the convergence proof is mostly based on the same objective function.

Finally, the convergence conditions are often one or a combination of three popular methods. The easiest way is to set a maximum number of iteration as the limit of the algorithm. This method is mostly included in final condition as an "OR" disjunction to ensure that the algorithm stops eventually. The next method is to stop when the score of objective functions reaches a threshold. This means the algorithm stops when a desired accuracy is achieved. This method is usually the best way to set the end of the algorithm because if the threshold is chosen wisely, it can ensure that algorithm would stop in a reasonable time frame and also acquires a proper accuracy. The final method is to check whether change of current estimation compared to the previous one is considerable. If the change is subtle, the algorithm stops. This method is not usually used alone since it may take a long time or even infinite time to happen. However, combining it with the objective function method is a good idea since it is possible for an algorithm to keep computing similar estimations which would not meet the objective function threshold. Therefore, if the change is subtle and the improvement is not considerable either, it is better to stop the algorithm. In general, if all three conditions are available, a disjunction of all of them is the best way to define the convergence condition (i.e. the algorithm stops if any of these conditions are met).

The version of deconvolution algorithm used in this thesis is a free code available on the Internet as a plug-in for ImageJ software. The code is written by Robert Dougherty and is called "Iterative Deconvolution" which can be found in [15]. Figure 4.2 shows a snapshot of the software. There is also another version called "Iterative Deconvolve 3D" which works with stacks of 2D images to perform deconvolution in three dimensions. Author also recommends the use of two other plug-ins: "Diffraction PSF 3D" to create theoretical PSFs and "Convolve 3D" to perform 3D convolution.

These deconvolution algorithms are based, in part, on DAMAS2 and DAMAS3 algorithms
[9, 10] of NASA-Langley Research Center. The related web pages of these algorithms [16, 17] provide instructions on how to use them. A simple explanation of the algorithm in terms of general template stated above (Figure 4.1) is given here.

The convergence condition is simply the first method which is a specific number of iterations set by the user. Therefore, no objective function is required. The recursive function is computed for the number of iterations specified and the result will be displayed. The recursive function used here is simply:

\[ f_{r+1} = f_r + g - \frac{f_r \otimes h}{hSum} \]  \hspace{1cm} (4.5)

where \( f_r \) is the estimate and \( g \) and \( h \) are the acquired image and the PSF respectively. Finally, \( hSum \) is the sum of the values of all the pixels of the PSF image, \( h \) (equation 4.6).

It is notable that \( f, g, \) and \( h \) are all two-dimensional images and therefore, are functions of \( x \) and \( y \).

\[ hSum = \sum_x \sum_y h(x, y) \]  \hspace{1cm} (4.6)

Using the above configuration, if the algorithm runs until the complete convergence,
(suppose the number of iteration is set to infinity), the improvement should become zero meaning \( f_i \) would be equal to \( f_{i+1} \). Therefore, we would have:

\[
\begin{align*}
  f_{i+1} &= f_i + g - \frac{f_{i} \otimes h}{hSum} \quad f_i \otimes h = \frac{g \otimes h}{0} = g - \frac{f_{i} \otimes h}{hSum} \\
  f_i \otimes h &= g \cdot hSum \quad f_i \otimes h = (f \otimes h) \cdot hSum \\
  f_i \otimes h &= (f \cdot hSum) \otimes h \\
  f_i &= f \cdot hSum = f_{est}
\end{align*}
\]

Therefore, the final estimate will be \( hSum \) times of the ideal image \( f \). To compute the final deconvolved result, the final estimation \( f_{est} \) will be divided by \( hSum \).

\[
f_{deconvolved} = \frac{f_{est}}{hSum} \quad (4.7)
\]

The reason why the above configuration is used is explained in the provided references above and is not the intention of this thesis to delve into. We are more interested in knowing how deconvolution affects the image quality and how the PSF acquisition helps to acquire better results. The next section will discuss the PSF acquisition and the factors which affect its accuracy.

### 4.3 PSF Acquisition

One major component of any iterative deconvolution algorithm is, in fact, the PSF. It is a measure of accuracy of an imaging system and a tool to recover images from distortions as well. Since the PSF is a main part of deconvolution process, it should be obtained from the imaging system as accurately as possible. Failing to capture a precise PSF will lead to loss of the details and/or misleading geometrical distortion in the deconvolved images. Therefore, in this thesis, we gave special emphasis to finding the methods which improve the accuracy of the PSF acquisition. A very crucial question about using a PSF in deconvolution
algorithms is the way it is acquired. The very obvious answer to this question is to say it should be acquired using exactly the same conditions that the image itself is acquired. This is usually a good and reasonable approach. However, there are recommendations which might result in some improvement over PSF acquisition and final deconvolution. One of these recommendations involves the resolution with which a PSF is captured.

4.3.1 Resolution

The sampling rate which defines the resolution of an image is the number of samples acquired per one length unit. In scanning microscopy, it would be described as the number of pixels acquired from a specific spatial distance. For example, scanning 200 pixels over 100 nm will result in 2 pixel/nm sampling rate. This means that the size of a pixel (i.e. sampling distance) would be 0.5 nm. Therefore, scanning over a field of the size of 500 nm * 500 nm will have a resolution of 1000 * 1000 pixel².

A very famous Nyquist-Shannon theorem states that the sampling rate should be equal or larger than two times of the bandwidth of the signal to make it possible to create back the original signal using the sampled one [46]. In scanning microscopy, this translates to the necessity of having the sampling distance (or pixel size) to be at least half of the spatial resolution (the mathematical discussion on spatial resolution is provided in section 1.4.1). Once the sampling distance is calculated, one can attain this value by selecting different sizes of the display screen (e.g. 512 * 512, 1024 * 1024, etc.) and/or different zoom factors. There are also online tools [55] which can calculate minimum sampling rate based on the Nyquist theorem. The user should provide a number of parameters namely microscope type, numerical aperture, excitation wavelength, emission wavelength, and the number of excitation photons for the tool to make it possible to calculate the right value.

In microscopy, the resolution increases when the optical zoom is increased and depending on the lens used, a range of different resolutions is attainable. While lower resolutions (lower zoom factors) might be used when acquiring an image of a biological specimen, there are some restrictions on the resolution with which a PSF is obtained.
A bead used as a subresolution point-like object to determine the PSF is usually so small that is considered virtually as a single point. Imaging such object with low resolution might result in loss of crucial data and, consequently, inability to perform a proper deconvolution process. On the other hand, the PSF is needed to be of the same resolution as the image for a deconvolution algorithm. To resolve this issue the concept of oversampling is introduced.

Capturing an image (or generally a digital signal) on a higher sampling rate than what is needed is called oversampling. Basically, except the larger memory, the longer time needed to capture an image with a higher resolution (oversampled), and possibly bleaching effect as the result of higher zoom factor, it is not harmful to obtain more data than needed. However, failing to capture enough data might result in a phenomenon called undersampling which potentially makes deconvolution useless. Not having enough samples when digitizing an analog signal might result in loss of data so that the original signal cannot be re-generated any longer. In the imaging terms, undersampling causes deconvolution algorithm to create artifacts due to the lack of important data.

After the PSF is acquired with enough pixel width (higher resolution), it should be shrunk back to the same resolution the image was acquired. Shrinking the PSF to the lower resolution should be performed in a way that meaningful information is preserved. To explain how acquiring a PSF with higher resolution and then shrinking it back can preserve meaningful data and how it differs from acquiring the PSF with lower resolution directly, we should first consider how the scanner works.

Suppose a scanner have the maximum resolution of “n” dot per inch or similarly in microscopy, “n” pixels per micron. This means that at best, the scanner can move \( \frac{1}{n} \) of a micron. Now, if we choose a lower resolution, the scanner should move longer distances. For example, let us say for a resolution of 1 pixel/micron, such scanner should move “n” times longer than its best resolution. Figure 4.3 shows how these two different resolutions are acquired having \( n = 5 \).

When the image is captured using lower resolution, the data between the jumps are lost. For instance, in example of Figure 4.3, using the lower resolution of 1 pixel per micron causes
the loss of every other 4 pixels in between meaning only pixels 1, 6, 11... are stored and pixels 2-5, 7-10 are lost. In other words, pixel 1 becomes the representative of the next 4 pixels and so on.

However, if the image is captured using a higher resolution, the data for other pixels are preserved and a better representative could be chosen. Since we need to shrink the PSF back to the lower resolution, we have to find a representative that preserves the data obtained using the higher resolution imaging. A very easy way to choose the representative is to average every “n” pixels which counts as shrinking the PSF by a factor of “n”. In the above example, instead of choosing first pixel of every 5 pixel (Pixel 1, Pixel 6, Pixel 11,...), the average of every 5 pixels would be chosen ((Pixel 1 + Pixel 2 + Pixel 3 + Pixel 4 + Pixel 5)/5,...). It is obvious that the result is more meaningful and much closer to the reality using the average.

As it was stated before, it is essential that the PSF and the image to be deconvolved
(raw image) have the same resolution. That is why the PSF is shrunk back to the lower resolution to match the raw image resolution. An example below shows that how using different resolutions for acquiring the PSF affects deconvolution results. An image of a fern leaf taken at the depth of 10 μm is used as the sample to be deconvolved. The image is taken by a two-photon excitation source, excitation wavelength of 800 nm, Kalman factor of 3, slow mode scanning, 60x lens, window size of 512 × 512 pixels, and zoom factor of 1x (pixel size = 0.456 μm). The image is then deconvolved using the PSFs with different resolutions. Figure 4.4 depicts deconvolution results using PSFs obtained by zoom factors of 10x, 5x, and 1x. PSFs are collected from depth-independent bead sample with the exact same imaging parameters used for imaging the fern leaf except different zoom factors. Since the raw image is acquired with zoom factor of 1x, last deconvolved image has a matching PSF resolution. It can be observed that the deconvolved images with higher zoom factor PSFs are even more distorted than the raw image: in the 10x image, surfaces are faded away and only high intense edges remained, structures melted together, and general illumination is decreased.

Another way to ensure that the correct deconvolved image is the one with matching zoom factor is the fact that the deconvolved image and the raw image should almost look alike for zoom factor of 1x. The reason is that the PSF in zoom factor of 1x, in our configuration, is almost equal to 1 pixel. Mathematically, convolving an image with such PSF (1 pixel) is equal to the image itself. This means the best possible deconvolution would be equal to the raw image (improvement is not attainable). As shown in the figure, the deconvolved image with zoom factor 1x is the same as the raw image which confirms that the resolution of the raw image and the PSF should match.

4.3.2 Detection Limitation

Another factor in forming a good PSF is how well a PSF is detected. In microscopy, when an image of a bead is recorded using photon excitation, it is common that not all the light from the head is recorded and therefore, some pixels of the image of the bead are wrongfully low
Figure 4.4: Deconvolution by the mismatched PSF sizes. a) Raw image of a fern leaf acquired by two-photon excitation at 800 nm wavelength with zoom factor 1x. b) Deconvolved image using the PSF captured with zoom factor of 10x. c) Deconvolved image using the PSF captured with zoom factor of 5x. d) Deconvolved image using the PSF captured with zoom factor of 1x.
in intensity. To correct this error, one method is using averaging. When an image of a bead sample is taken, there are usually a number of beads in the region. Since the low intensity pixels of the different beads occur in random places and since different beads are treated as the same, the erroneous pixels can be recovered by centering and averaging different beads. Figure 4.5 shows a typical recorder image of a bead sample.

![Typical recorded bead image for the purpose of PSF extraction.](image)

After a number of the beads are selected and cut out of the recorded image, they should be accurately aligned so that their centers correspond to the same place in their image frame coordination. To achieve a good alignment, another free plug-in of ImageJ was used. This plug-in which is called “TurboReg” and can be found in [50] has the ability to get a number of images and automatically rotate and move them so that they all match a target image meaning the mean square error between the source images and target image would be minimal. This software is used to correct the dislocations that happen while imaging the same spot over and over again in any kind of imaging system. We took advantage of it and since all the beads are supposed to have the same shape, used it to align the bead images. Moreover, the software makes all the source images the same size as the target image.

Afterwards, by averaging all the cropped bead images, a smooth PSF was achieved. Figure 4.6 shows 10 single beads and their averaged one (bottom-right) magnified for easier comparison. The figure clearly shows that the averaged bead is more symmetrical and
smooth while others contain random noise, disfiguration, and dissimilarities in diameter. An interesting advantage of averaging beads is the removal of noise both on the bead and in the background. Noise reduction is an important enhancement issue in all imaging systems. The next section will cover more about how to reduce noise using various methods.

![Averaging result over 10 cropped beads captured by two-photon excitation at zoom 6x.](image)

**Figure 4.6:** Averaging result over 10 cropped beads captured by two-photon excitation at zoom 6x.

### 4.3.3 Noise Reduction

As stated earlier, noise is always a part of imaging systems and an important challenge of researchers was always to find methods to reduce or remove noise. The presence of noise always brings some difficulties in every aspect. One of the difficulties mentioned earlier
in this chapter is the complication opposed on deconvolution process. In this section, we introduce a common noise reduction method called Kalman Averaging which is available in most scanning imaging software. We will then discuss the drawbacks of this method and propose two new methods to acquire noise-free images.

Kalman Averaging

As it can be seen from previous section, averaging can help smooth out the noise. In fact, averaging, which is a specific form of low pass filters\(^1\) in image processing, is one of the famous methods of noise reduction \([24]\). However, the smoothing effect of averaging is not always desirable since it blurs the rest of the image (dulls the sharpnesses and specially, image edges). Kalman averaging method is a way to reduce noise without blurring the image itself. Instead of average the pixels in spatial axes, each pixel is averaged with itself in a period of time. This means multiple images of the same place of the object are averaged over a period of time.

The justification of Kalman averaging is that since noise follows a random distribution, different pixels will be affected with noise in different imaging attempts. Therefore, it is not highly probable that the same pixel in different images would be affected with noise many times. Consequently, averaging pixels with themselves over many instances of the same image will be closer to the real value of the pixel. Figure 4.7 shows an example of a bead imaged once (4.7(a)) and also averaged 5 times (4.7(b)). The noise is clearly reduced in the averaged image. Note that this method can be applied on any image and is not limited to imaging beads.

Figure 4.7 suggests that the Kalman averaging method seems to work properly. However, there are three major potential drawbacks in using this method:

- Movement of the structure under imaging: if the structure moves, averaging over consecutive frames will result in deformation, motion blur, and/or artifacts. Moreover,

\[^1\]A low pass filter is a kind of filter in signal processing which preserves the low frequency components of a signal and eliminates the high frequency components resulting in smoothness of a signal.
in the case of having movement, when Kalman averaging is used during the optical sectioning, the projection of the Z slices will not result in a correct outcome since the slices are misaligned.

- Bleaching: In the case that the bleaching is observed during the imaging process, consecutive scans when applying Kalman averaging will result in greater degree of bleaching which causes the image to fade away gradually.

- High noise rate: If the noise rate is too high, the probability of the occurrence of noise on each pixel increases which makes the averaging useless.

These drawbacks limit the use of Kalman averaging and call for more elaborate methods.

**Anti-Fade Solution**

In order to eliminate the bleaching effect when an experimental PSF is being recorded from the depth-independent bead sample, we simply suggest replacing water with an anti-fade
solution (Slowfade Gold, Molecular Probes). It should be noted that although the refractive index of this solution (1.42) differs from that of water and mismatch aberration could happen, we only record the beads on the surface of the coverslip (0 $\mu$m). Therefore, it is safe to assume that the effect of the aberration is not present. Figure 4.8 depicts three beads recorded with and without the anti-fade reagent. The bead on the left is recorded without the anti-fade solution using the Kalman averaging of 5. The one in the middle is captured using the anti-fade solution with the Kalman averaging of 5 and the one on the right is taken using the anti-fade solution and the Kalman averaging of 15 times. The bead without anti-fade has lost its circular shape and is very low in intensity. However, the one with anti-fade is round and strong. It is interesting to note, even after applying Kalman averaging 15 times, the result is still better than the bead recorded without the anti-fade solution.

![Figure 4.8: Effect of employing the anti-fade solution in bead recording. a) A bead imaged with 5 times Kalman averaging with two-photon excitation. b) Image of the bead captured from sample made with the anti-fade solution and same image acquisition parameters as part (a). c) Image of the bead captured from sample made with the anti-fade solution and same image acquisition parameters as part (a) except 15 times Kalman averaging.](image)

The anti-fade reagent might solve the bleaching problem for recording the beads from a depth-independent bead sample; however, it is not applicable to most of the biological specimens (e.g. living tissues). Moreover, we have not addressed the movement problem yet since the anti-fade reagent only solves the bleaching problem and we still need to perform Kalman averaging. Therefore, when imaging a biological specimen for which the anti-fade
solution is not applicable and Kalman averaging could not be applied either. A general approach should be used to reduce the noise. The method that we are suggesting here is an image processing noise reduction filter that matches the type of the noise observed in the images acquired with CLSM or TPM. The noise is called salt-and-pepper and the filter is called the Median filter [24].

**Salt-and-Pepper Noise and the Median Filter**

The reason this kind of noise is called salt-and-pepper is because of how the noise looks. This type of noise causes random pixels to suddenly jump to a high intensity or drop to a low intensity. Based on the color map used to display the images, low and high intensities are shown in black and white dots. Usually high intensities are shown with brighter pixels and low intensities with darker ones. However, the images are sometimes inverted in this thesis for a better visual depiction.

The filter to remove these kind of noise is called a Median filter. The filter works as follow: First, a grid window should be defined for the filter. This window has a of size $m \times m$ (often $3 \times 3$) pixels in which “m” should be an odd number. Therefore, there is always a center for the window. The window starts sliding on the image from left to right and top to bottom. Each pixel that has the center of window on it, is then replaced with the median of its $m \times m$ neighbour pixels (inside the window). After all pixels are processed, the filter stops. The result will be salt-and-pepper noise free. It is because finding median needs sorting the data and low and high intensity pixels will be sorted at the beginning and the end of the list; therefore, noise pixels will never be the medians and a neighbour pixel with an intensity in the middle of the sorted list will replace the noise pixel. Figure 4.9 shows how the Median filter works in details.

The curved arrow determines the path that the filter window (here $3 \times 3$) will slide. The window is shown on an arbitrary pixel. We assumed that pixels with higher intensities are darker (inverted for the sake of presentation). Suppose the pixel in the center of the window is a pepper noise (black). If we apply the Median filter, the pixel value changes from 230
to the median of all the neighbours specified by the window (25, 83, 83, 83, 120, 120, 180, 180, 230) which is 120. The implementation of the Median filter is done by a function in Matlab called “medfilt2”. To apply the filter on image A and save it in image B with a window of 3 by 3, the command will be \( B = \text{medfilt2}(A,[33]) \). The same action can be done using ImageJ software and “Process>Filters>Median” option. For more information on salt-and-pepper noise and the Median filter refer to [24].

To show the effect of the Median filter on a noisy image, an example is given in Figure 4.10 which shows the same sample as Figure 4.4. The image is inverted so that the noise would become visible. The image on the left shows the sample before applying the Median filter and the one on the right shows the same image afterwards. The Median filter was able to remove the noise without major blurring\(^2\). The structures remained intact and intensity levels were preserved. Note that the Median filter can be applied on the recorded beads for the PSF extraction purpose similarly.

After acquiring a noise-free averaged PSF with proper resolution, it can be used to perform deconvolution process on the images recorded from the specimens. Next section will

\(^2\)Note that the Median filter is classified as a low pass filter and its multiple use over the same image and/or using a large window will result in major blurring of the image.
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4.4 PSF Type Effect on Deconvolution

In the previous sections, we discussed the parameters that affect the acquisition of an appropriate PSF. We showed that a certain resolution should be used to capture a bead, many beads should be aligned and averaged in order to compensate the "quantum-limited nature of photon detection" [44], and finally noise must be filtered out as much as possible. However, an important question is left unanswered: What is the perfect source of a PSF for different kinds of imaging tasks?

Some deconvolution programs (e.g., SVI Huygens and Diffraction PSF 3D plug-in for ImageJ) come with an extra tool which can generate a noise-free PSF based on the parameters of the imaging system including numerical aperture of the objective lens, excitation wavelength, emission wavelength, refractive indices of lens medium and specimen medium, microscope type, and number of excitation photons. If we are lucky, this tool possesses

Figure 4.10: Effect of applying the Median filter on an image with "salt-and-pepper" noise. a) Raw image. b) Image after applying the Median filter.

present a number of deconvolution results with various parameters.
The PSF can be obtained from various sources, each with its own advantages and limitations. For example, the PSF can be calculated based on the diffraction pattern of a point source, which is the theoretical PSF. However, since the PSF is calculated based on a limited number of typical parameters, it may not perfectly match the PSF of different microscopes of the same type, even if they are of the same type. Physical line up and set-up of each commercial microscope and the assembled objective lenses affect the final resolution of the image. Therefore, no two microscopes can be found that have the exact same PSF practically and match the theoretical PSF perfectly. For instance, a subtle deviation in the light source beam can cause the shape of PSF to change dramatically. This calls for a more specific way to acquire a PSF which represents the performance of the microscope more precisely unlike the theoretical PSF which is general in nature.

As described in section 1.4, the computable PSF is the intensity PSF which is the product of the square of the amplitude PSF (the amplitude PSF is obtained from the diffraction pattern of a point source). To resemble a point source, a point-like object can be used. Based on equation 4.1, if the profile of the point-like object is known, the original PSF can be restored. Figure 4.11 shows the images (i.e. measured PSFs) and their restored PSFs from three fluorescent beads with different diameters.

In another alternative and simple approach, the image of a subresolution point-like object can be used itself as the PSF. However, it is very crucial to choose a real subresolution subject. As it can be seen in Figure 4.11, beads with different diameters produce different measured PSFs. While the difference between the measured and restored PSF for the size of 170 nm is very subtle, it will be very significant for larger sizes. The data suggest the smaller the object is, the better the measured PSF would be. However, due to the lack of signal and more bleaching effect in imaging a very small object, there is a trade-off in defining the subresolution subjects.

It is suggested that the diameter of the bead used in PSF extraction should be less than 30% of the diameter of the first dark ring in Airy pattern [36, 45]. In other words:

\[ d_{\text{bead}} < 0.366 \frac{\lambda_m}{N.A} \]  \hspace{1cm} (4.8)
Although sometime large beads (e.g. 500 nm) are used for deconvolution task [8], beads with diameter larger than 200 nm are not recommended. Otherwise, the shape of the PSF will mostly reflect the shape of the bead instead of the real PSF of the microscope [14]. Due to the above protocol, we used 200 nm fluorescent beads to measure the PSF.

To extract the PSF from these beads, the solution of the beads (which is commercially available) is spread between a slide and the cover glass (details on the preparation of this sample is given in “Methods” chapter under the name of “Depth-Independent Bead Sample”). The beads are recorded with the same image acquisition parameters as the sample to be deconvolved. We refer to such PSFs as “Depth-Independent Experimental PSFs”.

Figure 4.11: Effect of the size of the bead on the PSF. The right column shows the profile of the different beads. The middle column shows the image of the beads and the left column shows the restored PSFs [58].
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It is important to note that since these kinds of beads are recorded from the surface of the coverslip, they do not count for spherical aberration present in the raw images which are mostly recorded at high penetration depth (usually more than 5 μm. However, if the biological sample is imaged at low penetration depth (between 0 μm and 5 μm) and the refractive index of specimen medium is close to the one of lens medium, these PSFs can be used for deconvolution. Figure 4.12 shows an image acquired with single photon excitation at depth ~ 3 μm and its deconvolved image using a depth-independent experimental PSF. The upper part is the deconvolved image and it clearly is sharper than the lower part which is the raw image. Note that in comparison, how deconvolved image depicts the amount of blurring that raw image was affected with.

![Image of deconvolution result](image)

Figure 4.12: Deconvolution using depth-independent PSF for an image acquired at low depth. The sample has been imaged with single photon excitation source at zoom 4x with the same acquisition parameters with which depth-independent bead sample is imaged.

Many of biological samples are recorded at high penetration depths; also in most of the cases a variation of refractive indices exists inside the biological sample [6]. As stated,
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PSFs should encompass all the aberrations of the biological sample image. Therefore, a more accurate PSF is required. According to our results shown in section 3.2, the PSFs extracted from the agarose gel include spherical aberration especially at high penetration depths. Therefore, we suggest to use these PSFs acquired at the same depth as the sample image for deconvolution purpose. We refer to this PSF as “Depth-Dependent Experimental PSF”.

Although the depth-dependent PSFs are recorded at the same depth as the biological sample, the most accurate PSF is not achievable unless it is extracted from specimen itself. To obtain such PSF, we need to somehow embed the beads inside the biological sample. For instance, if the sample is a tissue, it can be simply soaked in the solution of the beads. The PSF recorded from these kinds of beads counts for the aberrations, background fluorescence, and the scattered fluorescence. These PSFs are called in situ PSFs.

Not all the in situ PSFs are, however, useful. Since background noise and scattering of the light varies in different positions of the image, it is better to select a PSF which has an even background and is not superposed on any part of the sample structure. Table 4.1 is a comparison of the lateral and axial PSFs in all 4 different methods of PSF extraction. The extracted PSF from the agarose gel was at depth of 15 μm. The depth acquisition parameter in the theoretical PSF calculator (provided by SVI Imaging) was also set at 15 μm and the refractive index of the specimen was chosen to be 1.45. For the in situ PSF, injected Arabidopsis leaf was used and the PSF was obtained at the same depth.

<table>
<thead>
<tr>
<th>Type of PSF</th>
<th>Theoretical</th>
<th>Depth-Independent</th>
<th>Depth-Dependent</th>
<th>In Situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Resolution(μm)</td>
<td>0.3222</td>
<td>0.2929 (±0.0002)</td>
<td>0.318 (±0.005)</td>
<td>0.42 (±0.02)</td>
</tr>
<tr>
<td>Axial Resolution(μm)</td>
<td>1.0566</td>
<td>0.87 (±0.01)</td>
<td>0.95 (±0.01)</td>
<td>3.1 (±0.3)</td>
</tr>
</tbody>
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Table 4.1: Comparison of PSF profiles extracted from different sources. The depth used in spherical-aberration-corrected theoretical PSF, depth-dependent PSF, and in situ PSF is 15 μm.

To illustrate the effect of the type of the PSF on deconvolution performance, the following examples are presented. The raw image is obtained using the fern leaf at the depth of 5 μm by two-photon excitation at the wavelength of 775 nm, 60x/1.2 W. an optical zoom factor
of 6 (pixel size = 0.076726 μm) and filter detection of 680 nm. Figure 4.13 shows the raw image. Figure 4.14 presents the deconvolved images of the raw image using the theoretical, depth-independent, depth-dependent, and in situ PSFs respectively. For ease of comparison, all figures are split in two; the right part is the same as 4.13 and left parts are deconvolution results.

Figure 4.13: A chloroplast imaged from a fern leaf by two-photon excitation source at zoom 6x. Yellow box on the image shows the cropped in situ PSF.

Theoretical PSF at depth of 5 μm was calculated by using the Huygens Deconvolution online PSF calculator tool. The deconvolved result clearly shows that the structures are faded away and the image is degraded. The deconvolved images using the depth-independent and depth-dependent (5 μm) experimental PSFs are more or less the same since the profile of the PSF extracted from the agarose gel at the depth of 5 μm did not show significant difference comparing to the depth-independent PSF. Finally, the image deconvolved using the in situ PSF show an acceptable level de-blurring and restoration while still preserving sample structures (compared to theoretical PSF).
Figure 4.14: Effect of different types of PSFs on deconvolution results. Raw image shown in Figure 4.13 is deconvolved with a) Theoretical PSF. b) Depth-independent PSF. c) Depth-dependent PSF. d) In situ PSF.
This investigation confirms that the best improvement is achieved by using the in situ PSF in deconvolution process. However, recording an in situ PSF is not always feasible due to the following limitations.

- Injecting or embedding the beads is not possible for all types of the specimens.
- Due to the high complexity of the structure of some of the biological specimens recognizing the beads is impossible.
- Because of high background fluorescence, especially at high depths, identifying one single bead is challenging and a cluster of the beads may be mistaken as a single bead.

In one solution to these problems it has been suggested to extract the PSF from the point-like objects inside the structure of the specimen [12]. These approach may be convenient, but the accuracy of the obtained PSF is highly doubtful since the size of the point-like object in most of the cases is unknown and it might not satisfy equation 4.8.

In the cases which acquiring the in situ PSF is not feasible and the sample is recorded at relatively low penetration depths, either of the depth-dependent (with a slight preference) or independent experimental PSFs can be used.

4.5 Summary

This chapter introduced the idea of deconvolution. A general template algorithm for all iterative deconvolution algorithms was presented and a specific implementation of such algorithms was discussed.

Since the PSF is the basic building block of deconvolution algorithm, the parameters which affect the acquisition of a proper PSF were introduced. These parameters were the resolution of the PSF, limitation on detection, and amount of noise present in the image. The methods of handling the challenges for each of these parameters were discussed. These concepts introduced during the discussion were Nyquist-Shannon theorem which specifies
the correct resolution to capture a PSF, oversampling effect and its supremacy over undersampling, averaging bead images in order to correct ill-captured single beads, and noise reduction techniques: Kalman averaging method, anti-fade solution, and the Median filter to correct "salt-and-pepper" noise.

Afterwards different types of the PSFs (theoretical, depth-independent, depth-dependent, \textit{in situ}) were described and their applications and limitations were discussed. Finally, the results of using these different PSFs in deconvolution algorithm were compared.
Chapter 5

Conclusion

Resolution in optical imaging systems is limited by diffraction and is restricted by optical aberrations. The case is even worse for the axial resolution based on the diffraction law [29]. However, by having a comprehensive knowledge about the nature and the sources of the aberrations, reduction or even elimination of the errors is possible. Therefore, we presented important fundamentals and physical backgrounds of CLSM and TPM and optical explanation of some of the important aberrations.

In this thesis, we proposed and demonstrated some of the approaches that can be taken during the image acquisition process in order to achieve a higher resolution level. The results of these approaches were presented and the improvement was significant.

Afterwards, we put our emphasis on increasing the level of accuracy of the PSF in order to obtain more accurate deconvolution results: to do so, we proposed different methods, such as applying the Median filter along with several other methods, to improve the acquisition of PSF; obtained results showed the effectiveness and practicalities of the methods.

CLSM and TPM are becoming one of the basic tools in biological research and different research groups all over the world have studied the barriers imposed to these techniques. So many solutions in the forms of different architecture designs of the microscopes, adaptive optics, or image processing methods have been offered and improvements are noticeable. However, demands for higher resolution, better image quality, and more features for more applications require further work and investigation in the future.
Bibliography


